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ORIGINAL PAPER

Identification and characterization of a novel L-arabinose isomerase from *Anoxybacillus flavithermus* useful in D-tagatose production

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Abstract D-Tagatose is a highly functional rare ketohexose and many attempts have been made to convert D-galactose into the valuable D-tagatose using L-arabinose isomerase (L-AI). In this study, a thermophilic strain possessing L-AI gene was isolated from hot spring sludge and identified as *Anoxybacillus flavithermus* based on its physio-biochemical characterization and phylogenetic analysis of its 16s rRNA gene. Furthermore, the gene encoding L-AI from *A. flavithermus* (AFAI) was cloned and expressed at a high level in *E. coli* BL21(DE3). L-AI had a molecular weight of 55,876 Da, an optimum pH of 10.5 and temperature of 95°C. The results showed that the conversion equilibrium shifted to more D-tagatose from D-galactose by raising the reaction temperatures and adding borate. A 60% conversion of D-galactose to D-tagatose was observed at an isomerization temperature of 95°C with borate. The catalytic efficiency (k_{cat}/K_m) for D-galactose with borate was 9.47 mM^{-1} min^{-1}, twice as much as that without borate. Our results indicate that AFAI is a novel hyperthermophilic and alkaliphilic isomerase with a higher catalytic efficiency for D-galactose, suggesting its great potential for producing D-tagatose.

Keywords D-tagatose · L-arabinose isomerase · *Anoxybacillus flavithermus* · Thermophilic · Alkaliphilic enzyme

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The authors Y. Li and Y. Zhu contributed equally to this work.

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Introduction

D-Tagatose is a ketohexose rarely found in nature. Recently, there has been increasing industrial interest in D-tagatose as a low-calorie sugar-substituting sweetener (1.5 kcal/g), because it has only 30% of the energy content of sucrose (Kim 2004). Physiological studies have shown that D-tagatose does not elicit any increase in blood glucose levels and is thus safe for patients suffering from diabetes (Levin 2002; Talebi et al. 2008). Since it has unique properties as a functional sweetener, D-tagatose has been approved as a "generally recognized as safe (GRAS)" material for human consumption (FDA 2003; Levin 2002).

D-Tagatose is expected to create new markets and compete with sugar-substituting polyalcohol. Currently, it has been used in confectionery, beverages and health foods (Talebi et al. 2008; Lu et al. 2008). In addition to the above uses, it could potentially be used as a prescription drug additive to mask unpleasant tastes, and as sweetener, stabilizer and humectant in cosmetics and toothpastes (Ibrahim and Spradlin 2000). Furthermore, it can be used in the Maillard reaction to improve food flavor and antioxidant activity (Brands et al. 2000).

Since D-tagatose has a great potential for use in a variety of applications, it is important to develop a method for its mass production. In the past, chemical isomerization of

D-galactose was used in the production of D-tagatose (Beadle et al. 1992). However, the complexity of the chemical production and the high costs and environmental concerns associated with it have limited the development and application of D-tagatose. The enzyme conversion method can be applied to produce D-tagatose without these problems and, not surprisingly, many research groups have attempted to develop a biocatalysis process for D-tagatose production (Izumori and Tsuzaki 1988; Freimund et al. 1996; Haltrich et al. 1998; Manzoni et al. 2001).

L-arabinose isomerase (L-AI) can be used to produce D-tagatose from D-galactose using the Izumoring strategy (Izumori 2006). Because of the structural similarity between L-arabinose and D-galactose, including the shared L-cis-hydroxyl configuration at C3–C4, L-AI can catalyze the isomerization of D-galactose into D-tagatose in addition to the interconversion of L-arabinose and L-ribulose (Cheetham and Wootton 1993; Yoon et al. 2003; Kim et al. 2002, 2003). Several groups have cloned the L-AI genes from different strains and transformed them into *E. coli*. The rate from D-galactose to D-tagatose in the conversion equilibrium is 28.8% (w/w) at 30°C using *E. coli* L-AI (Oh et al. 2001); 45% (w/w) at 60°C using the recombinant L-AI from thermophilic *Geobacillus thermodenitrificans* (Kim and Oh 2005); 50% (w/w) at 70°C and 56% (w/w) at 80°C using the recombinant L-AI from hyperthermophilic *Thermotoga maritima* (Lee et al. 2004). These results indicate that using L-AI at higher temperatures may be particularly useful for D-tagatose production. Therefore, the development of a thermostable enzyme is a key step in the development of a method for efficient bioconversion of D-tagatose. Moreover, the food grade L-AIs from *Lactobacillus* genus were characterized and proved attractive for D-tagatose production during storage of milk and yoghurts (Chouayekh et al. 2007; Rhimi et al. 2010).

In this study, a thermophilic bacteria *Anoxybacillus flavithermus* that produces L-AI was isolated and identified from hot spring sludge. The gene encoding L-AI from *A. flavithermus* was cloned and expressed in *E. coli* BL21(DE3). The ability of the recombinant L-AI to convert D-galactose to D-tagatose at different reaction conditions was then determined. This is the first report about L-AI from *Anoxybacillus flavithermus*, and the properties of the enzyme were studied in detail.

Materials and methods

Materials

L-arabinose, D-galactose, D-tagatose, D-xylose and D-xylulose were purchased from Sigma-Aldrich Inc. Tryptone, yeast extract, agar and other chemicals and reagents used in this study were of analytical grade.

Bacterial strains, plasmid and media

Medium A was a Thermus medium (Williams and Da Costa 1991) used to isolate bacterial strains from high-temperature surroundings using L-arabinose as the sole carbon source. Medium B was used for cell growth and contained 5 g of tryptone, 1 g of yeast extract, 0.7 g of $NaNO_3$, 0.1 g of Na_2HPO_3, 0.06 g of $CaSO_4{\cdot}2H_2O$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of $CaCl_2$ and 2.2 mg of $MnSO_4{\cdot}H_2O$ per liter, at pH 7.5. The solid media of A and B contained 15 g of agar per liter.

E. coli strain BL21(DE3) and plasmid pET-21a(+) (Novagen) were used as the host cell and expression vector, respectively. The L-AI gene was transformed into *E. coli* BL21(DE3), and transformed cells were cultivated in Luria–Bertani (LB) medium.

Culture conditions and screening of bacteria

Samples were taken from a hot spring sludge in Tengchong Atami Park, Yunnan Province, China. The samples were diluted and spread on medium A agar plates. The plates were incubated at 65°C for 1–2 days until single colonies appeared. Those single colonies were incubated at 65°C in liquid medium B while being shaken at 200 rpm for 1–2 days to enrich the cultures. Cells in the cultures were disrupted by an ultrasonic process, and the suspension was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was then used for the L-AI assay.

Physiological and biochemical analysis

To find optimal growth conditions for the isolated strain, it was cultivated in medium B at different conditions (temperature and pH ranged from 20 to 90°C, and 3.0 to 10.0, respectively). The morphology of the strain was observed using a light microscope. Salinity tolerance was tested by incubation of bacterial cells in LB medium supplemented with 0–10% (w/v) NaCl. Catalase activity, motility, hydrolysis of starch, reduction of nitrate and utilization of carbon source were studied according to the "Bergey's Manual of Determinative Bacteriology, 9th edition" (John et al. 1994).

Phylogenetic determination

The sequence of the 16s rRNA gene was obtained from the total DNA of *A. flavithermus* by PCR amplification with primers 16s-27F (5′-AG(A/G)GTTTGATC(A/C)TGGCTCAG-3′) and 16s-1387R (5′-GGGCGG(A/T)GTGTACAA

GGC-3′). Phylogenic trees were constructed by alignment of the 16s rRNA gene sequence with related sequences from GenBank using the CLUSTAL X software package and the MEGA3.0 package.

Gene cloning of L-AI from *A. flavithermus*

According to the conserved sequence of L-AI amino acid residues that have been reported, two degenerate primers, AI370F (5′-AA(C/T)CA(A/G)TC(A/T/G/C)GC(T/C/G)CACGG(T/C)GA-3′) and AI1330R (5′-GT(A/G)TG(A/G)TG(A/T/C)GC(A/T)CCGCC(A/T/G)GC-3′), were designed. The partial sequence of L-AI was amplified from the chromosomal DNA of *A. flavithermus*. The full-length sequence of the L-AI was amplified by PCR using two oligonucleotide primers, AI-F (5′-CGGAATTCATGCTGTCATTACGTCCTTA-3′) and AI-R (5′-GCGTCGACCGCCCCCGCCAGAATACTTC-3′), which were designed according to the sequence of L-AI from *G. stearothermophilus* (accession number EU394214). The restriction sites *Nde* I and *Eco*R I (underlined) were added to the primer sequences to aid in the construction of the expression vector.

Enzyme expression and purification

The expression vector pET-21a(+) and PCR product of the L-AI gene were digested with *Eco*R I and *Sal* I. The gene was then ligated with the expression vector to create pET-AI. The recombinant plasmid was transformed into *E. coli* BL21(DE3).

The recombinant *E. coli* was cultivated in LB medium containing 100 μg/ml of ampicillin while being shaken at 200 rpm and at 37°C. When the OD_{600} reached 0.6, IPTG was added to the culture medium at 0.5 mM (final concentration) to induce enzyme expression, and the culture was grown at 37°C for 2–4 h.

The cells were harvested by centrifugation at 4°C for 10 min at 12,000 rpm. They were disrupted by sonication, and the suspension was centrifuged at 4°C for 10 min at 12,000 rpm. The supernatant was heated at 80°C for 5 min and centrifuged at 4°C for 10 min at 12,000 rpm to remove host proteins. The supernatant was further purified using an NTA nickel-ion column. Purification of AFAI was determined by SDS-PAGE. Protein concentration was determined using the Lowry method with BSA as a standard (Lowry et al. 1951).

Enzyme activity assays

The activity of AFAI was analyzed by determining the amount of D-tagatose obtained from D-galactose using the cysteine-carbazole method (Dische and Borefreund 1951). The concentration of D-tagatose in the reaction was calibrated based on the standard linear graph prepared using different concentrations of D-tagatose. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of D-tagatose per minute.

To determine the effect of temperature on AFAI activity, AFAI was incubated in 50 mM glycine–NaOH buffer (pH 9.5) and 5 mM substrate for 20 min at different temperatures (55–100°C). The reaction was stopped by cooling the tubes in ice. The amounts of D-tagatose produced were determined using the cysteine-carbazole method. The effect of pH on AFAI activity was determined at different pH using citric acid–sodium phosphate buffer (pH 6.5–8.0) and glycine–NaOH buffer (pH 8.0–10.5).

To evaluate the effect of metal ions on AFAI activity, enzymes were used after incubation with the addition of 1 mM LiCl, NaCl, KCl, $NiCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$, $BaCl_2$, $MnCl_2$, $MgCl_2$ or $FeCl_3$ at 20°C for 1 h, and after dialysis overnight at 4°C against the glycine–NaOH buffer (pH 9.5). The activity of AFAI was defined as the value relative to its activity in the absence of added metal ions.

To determine the kinetic parameters, all enzymatic assays were performed for 30 min at 95°C in 50 mM glycine–NaOH buffer (pH 9.5) or 10 mM borate buffer (pH 9.5) at various concentrations of substrate (0.5–10.0 mM). The values of K_m, V_{max} and k_{cat} were obtained from Lineweaver–Burk plots.

Different aldoses, L-arabinose, D-xylose, D-glucose and D-allose, were used as substrates to examine the specificity of AFAI as described above. All experiments used to determine the properties of the recombinant AFAI were carried out at least in duplicate.

Analysis of isomerization product

The product of the isomerized reaction was identified by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). TLC of isomerization product in ethyl acetate–acetic acid–water (3:3:1, v/v/v) was performed using the ascending technique and 0.2 mm silica gel-coated aluminum sheets. Each plate was sprayed with 10% concentrated H_2SO_4 and then heated to visualize the spots. The product obtained from the aldose isomerization by AFAI was also estimated by HPLC with RI detector, using Sugar-Pak™ column (6.5 × 300 mm, Waters USA) and eluting at 80°C by deionized H_2O (0.5 ml/min).

Results and discussion

Isolation and identification of the strain producing L-AI

To select thermophilic bacterial strains producing L-AI more easily and efficiently, samples were coated on the

Table 1 Comparison of the phenotypic characteristics of strain TC-06 and related strains

Characteristic	Strain designations				
	Strain TC-06	*A. flavithermus* DSM 2641^{T} [a]	*A. kamchatkensis* JW/VK-KG4^{T} [b]	*A. kestanbolensis* NCIMB 13971^{T} [c]	*A. pushchinoensis* DSM 12423^{T} [d]
Motility	+	+	+	+	−
NaCl(3%,w/v)	+	−	+	+	+
NO_3^- to NO_2^-	−	+	+	+	+
Starch hydrolysis	+	+	−	+	+
Catalase	+	+	−	+	−
Mannose	+	+	−	+	ND
Arabinose	+	+	−	−	−
Galactose	+	+	+	ND	−
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	ND
Lactose	−	ND	−	−	−

+, positive; −, negative; *ND* not determined

[a] Pikuta et al. 2000, [b] Kevbrin et al. 2005, [c] Dulger et al. 2004, [d] Pikuta et al. 2000

plates of Thermus medium A containing L-arabinose as the sole carbon source and cultured at 65°C. Several strains were isolated from the hot spring sludge of Tengchong Atami Park and inoculated into liquid medium B. The lysate was used to measure the L-AI activity for isomerizing D-galactose into D-tagatose by the cysteine-carbazole method. Our initial observations showed that only the strain TC-06 could convert D-galactose to D-tagatose (data not shown).

Upon visual inspection, the colony morphology of TC-06 was circular, white and smooth with regular edges and a diameter of 1–3 mm. A close microscopic examination revealed that the strain TC-06 was typically a rod-shaped Gram-positive bacteria. The strain was cultivated at different temperature and pH conditions for 3 days, and the OD_{600} values of culture solutions were determined (data not shown). Our results showed that the strain TC-06 could be cultivated in a wide range of growth temperature (45–70°C) and pH (5.5–9.5) conditions, while its growth optimum temperature and pH values were 55°C and 8.5, respectively. Therefore, not only could the strain TC-06 be grown at higher temperatures, but it could also tolerate higher alkaline pH.

Some phenotypic properties of strain TC-06 were analyzed and are summarized in Table 1. Compared to related strains, the characteristics of strain TC-06 were almost the same as those of *A. flavithermus DSM 2641*^{T} (Pikuta et al. 2000), but were quite different from that of other species of the *Anoxybacillus* genus (Kevbrin et al. 2005; Dulger et al. 2004; Pikuta et al. 2000). Furthermore, the 16s rRNA gene of strain TC-06 was found to share 99% identity with the

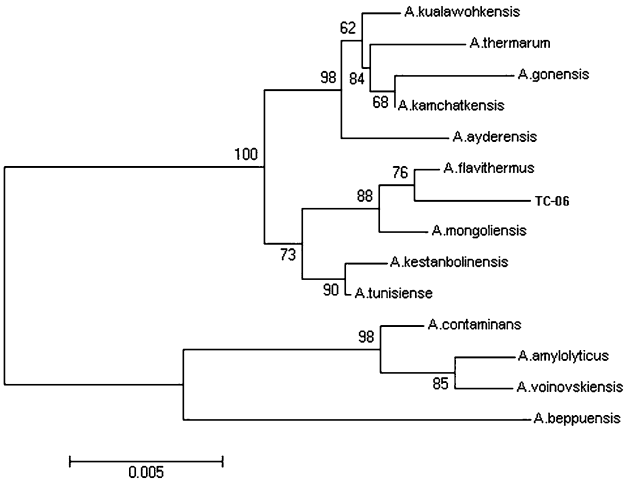

Fig. 1 Phylogenetic relationships of 16s rRNA gene of TC-06 and related species. Sequence alignment and phylogenetic tree construction were done by CLUSTAL X v1.83 and MEGA 3.0. *A. amylolyticus* AJ618979, *A. ayderensis* NR_024837, *A. beppuensis* AB243446, *A. contaminans* NR_029006, *A. flavithermus* FN666242, *A. gonensis* AY248706, *A. kamchatkensis* AM999779, *A. kestanbolinensis* AY248710, *A. kualawohkensis* DQ401072, *A. mongoliensis* EF654664, *A. thermarum* EU326496, *A. tunisiense* AM902721, *A. voinovskiensis* NR_024818, TC-06

A. flavithermus strain according to the BLAST program. In the phylogenetic tree (Fig. 1), the relationship of strain TC-06 and the related species was identified by calculating genetic distance of the 16s rRNA gene in the GenBank, which indicated that the relationship between strains TC-06 and *A. flavithermus* was very close. On the basis of phenotypic and phylogenic analysis, strain TC-06 was thus identified as a strain of *A. flavithermus.*

Gene cloning and sequence analysis

The 934 bp partial sequence of L-AI gene was obtained by PCR using a pair of degenerate primers. Sequence alignment showed that the partial nucleotide sequence shared high identity with that of *G. stearothermophilus*. Hence, the full-length gene was amplified by using primers based on *G. stearothermophilus* L-AI. A 1491-bp PCR product was obtained. Sequence analysis showed that it encoded a 496-amino-acid polypeptide with a calculated molecular weight of 55,876 Da. The pI value of AFAI was calculated to be 5.09. The nucleotide sequence of AFAI gene has been deposited in the Genbank sequence database under accession number HQ589928.

Figure 2 shows the amino acid sequence alignment of several L-AIs with the enzyme from *A. flavithermus*. Comparison of the AFAI amino acid sequence with those of other AIs showed that the enzyme has the highest identity to AIs from a closely related thermophilic bacteria *G. stearothermophilus* (96.3%). In addition, AFAI also showed high similarity and identity to other AIs from different species such as *E. coli* (59.4%), *B. subtilis* (62.6%) and *T. maritima* (60.83%). Although AFAI displayed the highest sequence identity with L-AI from *G. stearothermophilus* (GSAI), the properties of these two enzymes were a little different (Table 2). Slight differences in the amino acid sequence between the two L-AIs may account for difference in structure and function of the enzyme molecules.

Overexpression and purification of AFAI

The full-length AFAI gene was cloned into pET-21a(+) expression vector, which was transformed to *E. coli* BL21(DE3). After induction of enzyme expression by IPTG, the recombinant protein was overexpressed, which produced an apparent band in an SDS-PAGE gel (Fig. 3). SDS-PAGE gels were analyzed using Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software from Bio-Rad. Our results show that the molecular weight of the recombinant protein must be 56.3 kDa, which is close to the theoretically predicted value. Moreover, the recombinant protein comprised 20–25% of the total proteins in the cell, which indicated it was overexpressed.

The *E. coli* lysates were heated at 80°C for 5 min to remove the majority of endogenous proteins, while the recombinant AFAI remained soluble and active. The enzyme was purified using an Ni–NTA affinity column after heat treatment. The overall yield of the purified recombinant protein was 30 mg/L of bacterial culture.

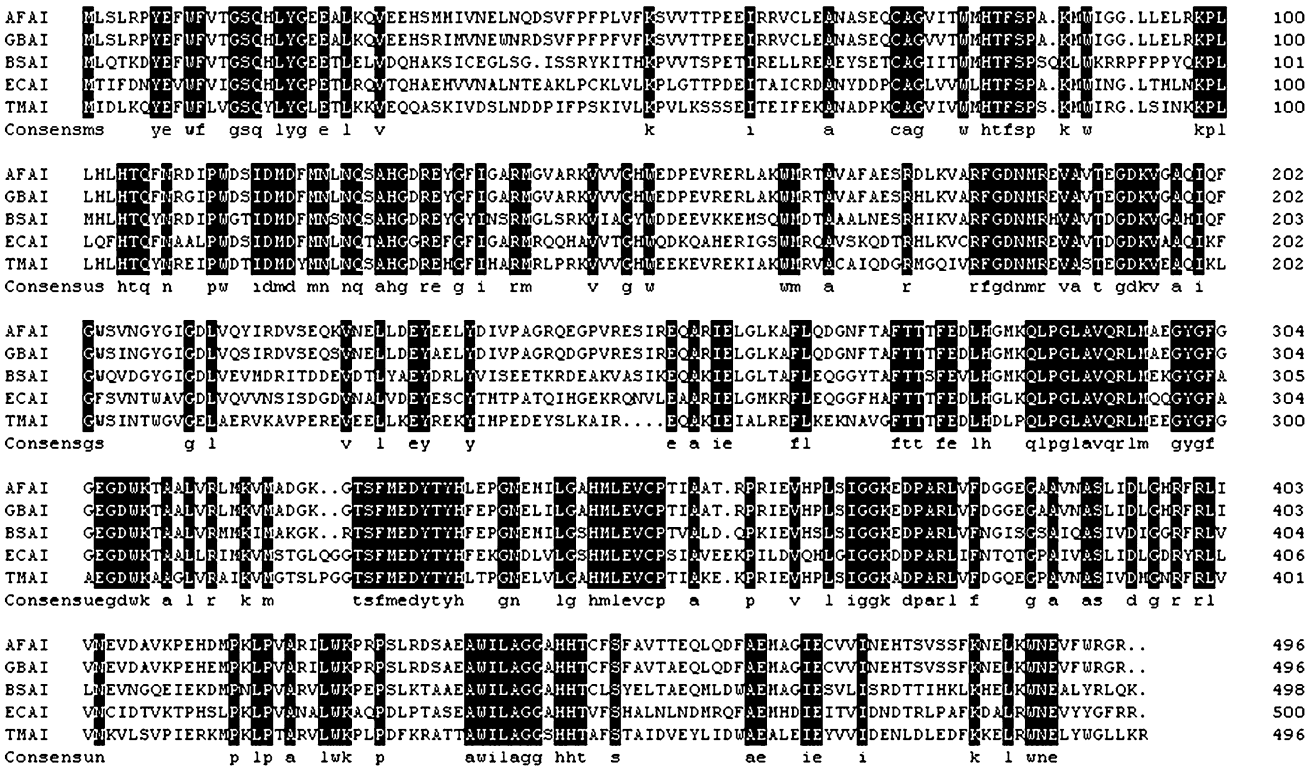

Fig. 2 Alignment of the amino acid sequences of *A. flavithermus* and other microbial L-AIs. AFAI, *A. flavithermus* L-AI(HQ589928); GSAI, *G. stearothermophilus* L-AI (EU394214); BSAI, *B. subtilis* L-AI (1769994); ECAI, *E. coli* L-AI (M15263); TMAI, *T. maritima* L-AI (AE000512)

Table 2 Biochemical of AFAI and other microbial L-AIs and apparent K_m values for L-arabinose and D-galactose

Strain for enzyme source	Temp opt. (°C)	pH opt.	Metallic ion	K_m (mM)	
				L-arabinose	D-galactose
Aerobacter aerogenes[a]	50	6.4–6.9	Mn^{2+}	33	270
G. stearothermophilus[b]	60	7.5	Mn^{2+}	67	145
Mycobacterium smegmatis[c]	45	7.0–7.5	Mn^{2+} Co^{2+} Mg^{2+}	NR	NR
Thermoanaerobacter mathranii[d]	65	8	Mn^{2+}	80	120
Thermotoga neapolitana[e]	85	7	Mn^{2+} Co^{2+}	116	250
T. maritima[f]	90	7.0–7.5	Mn^{2+} Co^{2+}	31	60
L. sakei[g]	30–40	5.0–7.0	Mn^{2+} Mg^{2+}	31.6	59
A. flavithermus	95	9.5–10.5	Ni^{2+}	78.5	25.2

NR not reported

[a] Yamanaka and Wood 1966, [b] Kim et al. 2003, [c] Izumori et al. 1978, [d] Jørgensen et al. 2004, [e] Kim et al. 2002, [f] Lee et al. 2004, [g] Rhimi et al. 2010

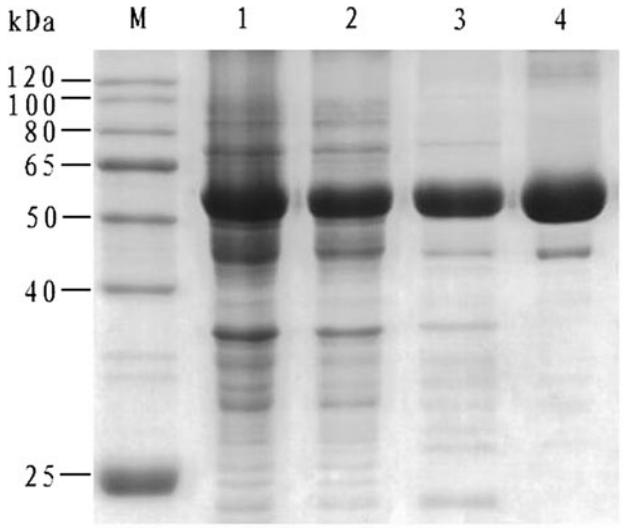

Fig. 3 SDS-PAGE analysis of AFAI. Lane M protein markers; Lane 1 total cell; Lane 2 supernatant; Lane 3 heat treatment at 80°C for 5 min; Lane 4 purified recombinant AFAI

It had more than 90% purity and a specific activity of 26.4 U/mg. These results indicated that the recombinant AFAI was successfully overexpressed and purified.

Assessment of AFAI activity in different conditions

The effects of pH and temperature on recombinant AFAI activity were examined with D-galactose as substrate. AFAI activities at the optimal pH and temperature were defined as 100%. The activity of AFAI was dependent on high pH and temperature, and the enzyme was not active below pH 7.0 and 75°C (Fig. 4). The maximal activity of AFAI was at 95°C and pH 9.5–10.5.

The effect of metallic ions on the recombinant AFAI activity is depicted in Fig. 5. The enzyme activity was inhibited by Zn^{2+}, Fe^{3+}, Mg^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+}, and was increased by Ni^{2+}. As far as we know, the majority of L-AIs were not active in the absence of metallic ions (Oh 2007). They required Co^{2+} and Mn^{2+} as cofactors to enhance their activities or thermostabilities. In our study, the recombinant AFAI had higher activity without adding metallic ions at 95°C. This result was very similar to that of previous reports (Rhimi and Bejar 2006) showing that Mn^{2+} or Co^{2+} was not necessary for the activity of AFAI, while they could inhibit its activity.

The properties of L-AIs from the different strains are summarized in Table 2. Compared to L-AIs from other strains, AFAI had the highest optimal temperature and pH, suggesting that this enzyme was hyperthermophilic and alkaliphilic. Our results showed that the relative activity was less than 50% below 85°C and pH 9.5 and that the recombinant AFAI activity was dependent on higher temperature and pH value. Also, AFAI was stored at 4°C, and the activities were examined after 5, 15, 30 and 60 days with the standard reaction conditions. The activities of AFAI stored for various days remained unchanged (data not shown).

Substrate specificity of AFAI

The specificity of AFAI was investigated in different substrates (L-arabinose, D-xylose, D-galactose, D-glucose, D-allose) by the cysteine-carbazole method. The results showed that no activity or slight activity of AFAI was found for all the tested substrates in the temperature range from 55 to 75°C (data not shown). However, when the temperature was increased to 95°C, the activity of AFAI was greatly elevated and it had an unexpectedly high preference for D-galactose. While L-arabinose and D-xylose

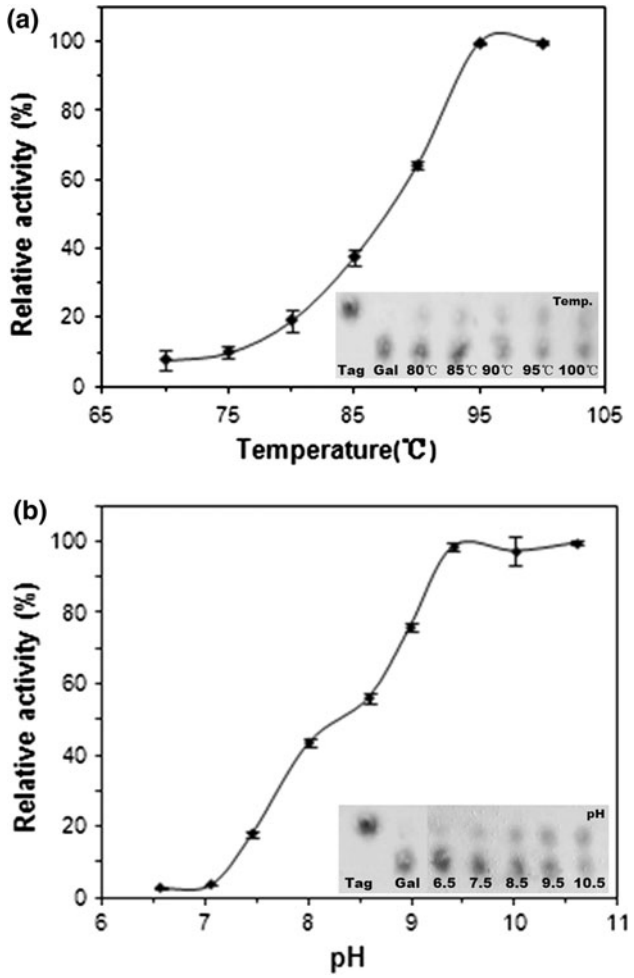

Fig. 4 Effect of temperature (**a**) and pH (**b**) on the activity of the recombinant AFAI. The AFAI activity was assayed at different pH values ranging from 6.5 to 10.5 at 95°C for 20 min and at different temperatures ranging from 70 to 100°C at pH 9.5 for 20 min using 5 mM D-galactose as substrate. Activities at the optimal pH and temperature were defined as 100%. *Inset* TLC analysis of bioconversion of D-galactose into D-tagatose at 80, 85, 90, 95 and 100°C (**a**); pH 6.5, 7.5, 8.5, 9.5 and 10.5 (**b**). Tag, D-tagatose; Gal, D-galactose

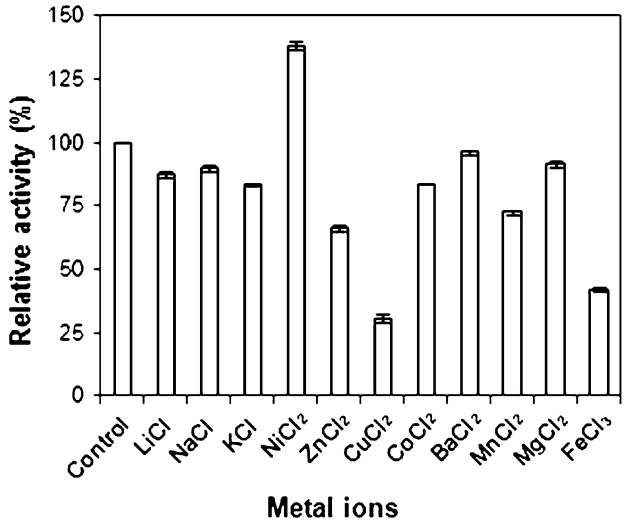

Fig. 5 Effect of metallic ions on the activity of the recombinant AFAI. Assays were carried out in the presence of 5 mM D-galactose and 1 mM various metallic ions for 20 min at 95°C. Activities of the control were defined as 100%

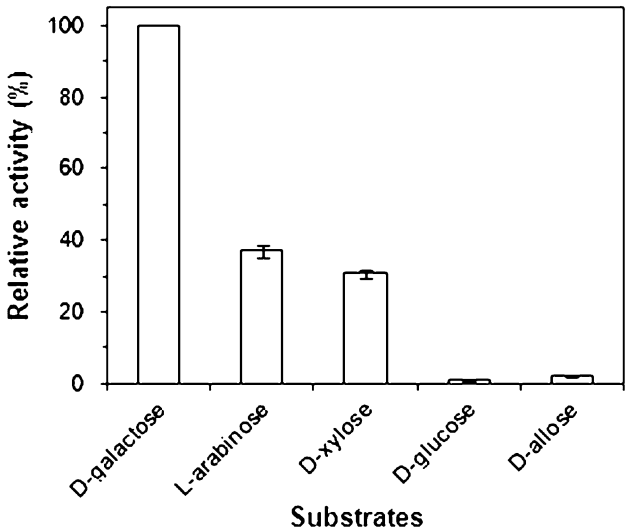

Fig. 6 Substrate specificity of AFAI

showed lower activity (40% or less, compared with D-galactose), D-glucose and D-allose could not serve as substrates for AFAI (Fig. 6).

The substrate specificities for L-AIs from many different strains are shown in Table 3. It suggested that AFAI had higher specificity toward D-galactose than L-arabinose. Other L-AIs reported normally had lower substrate activity for aldose except L-arabinose and no activity on D-xylose, so AFAI might be a new isomerase.

Kinetic parameters of AFAI

The Michaelis constant (K_m) of AFAI was calculated from Lineweaver–Burk plots. The K_m obtained for the conversion of D-galactose under standard assay conditions is shown in Table 4.

Moreover, it was found that the addition of borate into the reaction mixture would promote D-tagatose formation (Kim et al. 2009). The K_m for D-galactose with borate was 26.81 mM, which was close to that for D-galactose without borate. These results indicated that borate could not change the affinity of AFAI for D-galactose. However, AFAI had twofold higher enzyme efficiency (k_{cat}/K_m) toward D-galactose, which indicated that the trend of reaction equilibrium was shifted to produce D-tagatose by borate. It is worth noting that it has previously been postulated that borate can change the equilibrium of any enzyme reaction

Table 3 Isomerization activities of AFAI and other microbial L-AIs

Strain for enzyme source	Relative activity (%)			
	D-galactose	L-arabinose	D-xylose	D-glucose
Geobacillus stearothermophilus[a]	100	239	17	ND
Geobacillus thermodenitrificans[b]	100	1482	7	0
Thermoanaerobacter mathranii[c]	11	100	0	0
Lactobacillus plantarum[d]	100	950	15	ND
Bacillus subtilis[e]	0	100	0	ND
Bacillus licheniformis[f]	2	100	0	0
A. flavithermus	100	40	32.3	0

ND not determined

[a] Kim et al. 2003, [b] Kim and 2005, [c] Jørgensen et al. 2004, [d] Chouayekh et al. 2007, [e] Kim et al. 2009, [f] Prabhu et al. 2008

Table 4 Kinetic parameters of AFAI

Substrate	V_{max} (μmol/L min)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
D-galactose	232.56	25.19	129.94	5.16
D-galactose with borate	454.55	26.81	253.94	9.47
L-arabinose	94.53	78.5	52.81	0.67

involving cis-diol sugars by generating sugar–borate complex (De Muynck et al. 2006; Kim et al. 2008).

Bioconversion of D-galactose and D-tagatose by AFAI

The bioconversion-product patterns of D-galactose were analyzed by TLC. The most significant result obtained from TLC analysis was that D-tagatose formation increased with increasing temperature (80–100°C) and pH (6.5–10.0). These results were consistent with the data shown in Fig. 4.

Figure 7 shows the time course of D-tagatose production from D-galactose by purified AFAI at the optimal conditions (95°C and pH 9.5) with borate. A 60% conversion of D-galactose to D-tagatose was obtained when the equilibrium was reached after 1 h. It has been reported that the rates of L-AIs in the conversion equilibrium from *B. stearothermophilus* and *T. maritima* were 48% at 70°C after 7 h, and 56% at 80°C after 6 h, respectively (Rhimi and Bejar 2006; Lee et al. 2004). Thus, the L-AI from *A. flavithermus* showed higher activity when compared to the other L-AIs under the optimum conditions (Table 2).

As indicated by these results, using AFAI to produce D-tagatose from D-galactose has more advantages than using the other L-AIs: (1) AFAI is more thermophilic and thermostable than mesophilic enzymes, which can increase the utilization efficiency of the enzyme. (2) As an alkaliphilic isomerase, AFAI is favorable for constructing a stable bioreactor by using immobilized enzyme to produce D-tagatose in the presence of borate without sacrificing enzyme activity. (3) AFAI showed the best substrate specificity for D-galactose and gave the highest yield of D-tagatose, so the enzyme is suitable for industrial production of the rare sugar. In conclusion, AFAI may be a new isomerization enzyme, which will have the potential to be used widely for efficient conversion of D-galactose to D-tagatose.

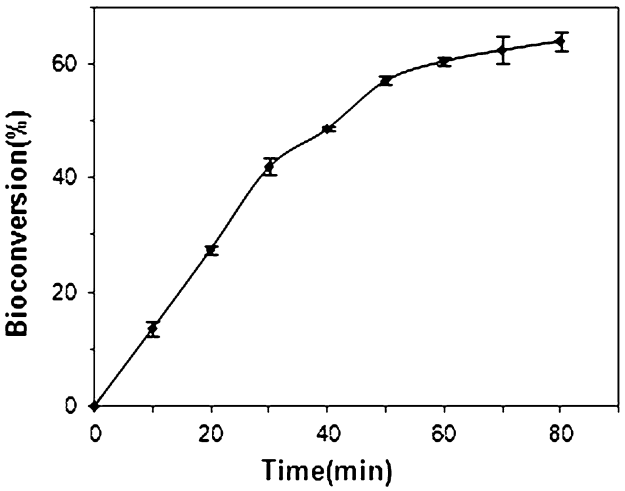

Fig. 7 Bioconversion of D-galactose into D-tagatose using the purified AFAI. The substrate solution comprising 5 mM D-galactose and 10 mM borate (pH 9.5) was prepared. AFAI was added to initiate the reaction by the enzyme/substrate ratio of 1/10 (w/w). Assays were carried out in standard conditions without addition of metal ions

In future studies, the best applicable reaction conditions will be investigated to obtain high production of D-tagatose. The structure–function relationship of the recombinant

AFAI will also be analyzed and more insights into the mechanisms of the isomerization reaction will be gained.

In summary, we have isolated, overexpressed and characterized an alkalithermophilic L-AI from *A. flavithermus*. Data obtained from TLC, quantitative cysteine-carbazole method and HPLC analysis suggest that the active AFAI obtained could efficiently catalyze D-galactose into D-tagatose.

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