

L-Arabinose Isomerase and D-Xylose Isomerase from *Lactobacillus reuteri*: Characterization, Coexpression in the Food Grade Host *Lactobacillus plantarum*, and Application in the Conversion of D-Galactose and D-Glucose

Petra Staudigl, Dietmar Haltrich, and Clemens K. Peterbauer*

Food Biotechnology Laboratory, Department of Food Science and Technology, University of Natural Resources and Life Science Vienna, Muthgasse 18, 1190 Vienna, Austria

S Supporting Information

ABSTRACT: The L-arabinose isomerase (L-AI) and the D-xylose isomerase (D-XI) encoding genes from *Lactobacillus reuteri* (DSMZ 17509) were cloned and overexpressed in *Escherichia coli* BL21 (DE3). The proteins were purified to homogeneity by one-step affinity chromatography and characterized biochemically. L-AI displayed maximum activity at 65 °C and pH 6.0, whereas D-XI showed maximum activity at 65 °C and pH 5.0. Both enzymes require divalent metal ions. The genes were also ligated into the inducible lactobacillal expression vectors pSIP409 and pSIP609, the latter containing a food grade auxotrophy marker instead of an antibiotic resistance marker, and the L-AI- and D-XI-encoding sequences/genes were coexpressed in the food grade host *Lactobacillus plantarum*. The recombinant enzymes were tested for applications in carbohydrate conversion reactions of industrial relevance. The purified L-AI converted D-galactose to D-tagatose with a maximum conversion rate of 35%, and the D-XI isomerized D-glucose to D-fructose with a maximum conversion rate of 48% at 60 °C.

KEYWORDS: L-arabinose isomerase, D-xylose (glucose) isomerase, D-tagatose, D-fructose, food grade, *Lactobacillus*

INTRODUCTION

L-Arabinose isomerase (L-AI; EC 5.3.1.4) catalyzes the reversible isomerization of L-arabinose into L-ribulose. Due to the ability to convert D-galactose into D-tagatose, this enzyme is also referred to as D-galactose isomerase.

D-Tagatose, an isomer of D-galactose, has physical properties similar to those of sucrose.² This low-calorie sweetener has numerous health and medical benefits, including prevention of dental caries, regulation of intestinal flora, and reduction of symptoms of type 2 diabetes.¹ The sweetness of D-tagatose is comparable to that of sucrose, and it has received “generally recognized as safe” (GRAS) status from the U.S. Food and Drug Administration.³ D-Tagatose can be produced from D-galactose by a chemical method using a calcium catalyst, but this process has some disadvantages including complex purification steps and the formation of chemical waste and byproducts. Therefore, biological production of D-tagatose using L-AI has been studied intensively in recent years.⁴ Several enzyme sources have been reported or patented including the L-AI from *Escherichia coli*,⁵ *G. stearothermophilus*,⁶ and *L. pentosus*.^{7,8} High temperature improves the enzymatic isomerization because the reaction equilibrium is shifted toward D-tagatose. Acidic conditions have the advantage that the formation of undesirable byproducts is prevented.^{9,10}

D-Xylose isomerase (D-xylose ketol-isomerase; D-XI; EC 5.3.1.5) catalyzes the reversible isomerization of D-xylose into D-xylulose. This enzyme is also referred to as glucose isomerase (GI) due to its ability to convert D-glucose to D-fructose, a reaction of commercial importance in the production of high-fructose corn syrup (HFCS). The chemical conversion (Lobry de Bruyn-Alberda van Ekenstein transformation) is nonspecific

and leads to the formation of nonmetabolizable sugars (e.g., psicose) and other undesirable colored products. These disadvantages can be overcome by enzymatic isomerization.¹¹ Several enzyme sources have been reported and patented, including *Actinoplanes missouriensis*,¹² *Bacillus licheniformis*,¹³ and *S. rubiginosus*.^{11,14} HFCS is used in the food industry because it does not cause a crystallization problem like sucrose. Further advantages are a lower price (10–20%) and higher sweetness (1.3 times) compared to sucrose.

An enzymatic procedure for the direct conversion of lactose into D-tagatose and D-glucose was described by Jørgensen et al.¹⁵ In this reaction the enzyme β -glycosidase catalyzes the hydrolysis of lactoserum, a byproduct of the manufacture of cheese and therefore a cheap source for D-galactose production at industrial scale. The resulting D-galactose is further isomerized to D-tagatose by L-AI and the residual D-glucose has to be removed. Alternatively, the enzyme D-XI could catalyze the conversion of D-glucose to D-fructose.²

Lactic acid bacteria (LAB) are extensively used in the food industry¹⁶ and are generally harmless to human beings.^{16,17} These Gram-positive bacteria are used in dairy production, wineries, and other industrial food fermentation processes. Furthermore, they are extensively used in the animal feed industry.¹⁸

Members of the genera *Lactobacillus* and *Lactococcus* are ideal candidates as safe cell factories.^{19,20} *Lactobacillus plantarum*, a

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promising host for heterologous gene expression, was already successfully used for the production of β -galactosidase from *Lactobacillus delbrueckii*²¹ and chitinase from *Bacillus licheniformis*.²² Available inducible expression systems²³ are based on quorum-sensing mechanisms involved in the production of sakacins A and P.^{24–26} More recently, the erythromycin antibiotic resistance gene (*erm*) was replaced by the homologous alanine racemase gene (*alr*), and an alanine racemase-deficient host strain was constructed that obviates the undesirable fermentation in the presence of antibiotics and is suitable for applications in the food industry.¹⁹

In this study we present the heterologous expression of the L-AI and D-XI-encoding genes from the food grade microorganism *L. reuteri* in *E. coli*, the most commonly used organism for heterologous protein production, and the characterization of these novel enzymes.²⁷ *L. reuteri*, a heterofermentative and symbiotic species, is well adapted to colonize human and animal gastrointestinal tracts. Among probiotic microorganisms *L. reuteri* is unique due to its ability to produce and secrete the antimicrobial substance reuterin.²⁸ Furthermore, we coexpressed the genes in the food grade host *L. plantarum* using two different expression systems and performed comprehensive studies on the conversion of D-glucose and D-galactose, respectively.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals were of the highest purity available and purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Restriction endonucleases, T4 DNA ligase, and Phusion High-Fidelity DNA polymerase were obtained from Thermo Fisher Scientific Biosciences (St. Leon-Rot, Germany), whereas GoTaq DNA polymerase was from Promega (Madison, WI, USA). Phenylmethanesulfonyl fluoride (PMSF) was obtained from Fluka (Buchs, Switzerland), and chromatographic materials were obtained from GE Healthcare (Chalfont St. Giles, UK).

Bacterial Strains and Culture Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus reuteri* DSMZ 17509 (strain designation 100-23) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Lactobacillus* strains were cultivated in deMan–Rogosa–Sharpe (MRS) medium at 30 °C without agitation. *E. coli* strain NEB5 α (New England Biolabs, Ipswich, MA, USA), MB2159 (D-alanine auxotroph), and BL21 Star (DE3) (Invitrogen, Carlsbad, CA, USA) were grown in Luria–Bertani (LB) medium at 37 °C with shaking at 125 rpm. When needed, media were supplemented with ampicillin at a concentration of 100 μ g/mL and erythromycin at concentrations of 5 and 200 μ g/mL for *L. plantarum* and *E. coli*, respectively. *E. coli* MB2159 and *L. plantarum* strain TLG02 were cultivated by adding 200 μ g/mL D-alanine to the respective media. Solid media were prepared by adding 1.5% agar to the respective media.

DNA Amplification and Subcloning. DNA amplification was performed with Phusion High-Fidelity DNA Polymerase according to the manufacturer's instructions. The oligonucleotide primers were obtained from VBC Biotech (Vienna, Austria) and are listed in Table 2. PCR products and restriction enzyme digested DNA were purified with GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare) as recommended by the supplier. When needed, the PCR fragments were subcloned into the pJET1.2 vector (CloneJET PCR cloning kit, Thermo Fisher Scientific Biosciences), and *E. coli* was used as host for plasmid propagation before transformation into *Lactobacillus*. The amplified sequences were verified by a commercial sequencing service (LGC Genomics, Berlin, Germany).

The *araA* gene (NCBI reference sequence for the genome: NZ_AAPZ02000002.1) was amplified by colony-PCR using the primers *araA_LbReu_fwd1* and *araA_LbReu_rev1*. The *xylA* gene was amplified with the primer pair *xylA_LbReu_fwd1* and

Table 1. Strains and Plasmids Used in This Work^a

strain or plasmid	relevant characteristics	ref
strains		
<i>L. reuteri</i>		
DSMZ 17509	source for L-AI and D-XI encoding genes	DSMZ
<i>L. plantarum</i>		
WCFS1	wild type	49
TLG02	Δ alr, D-alanine auxotroph	19
<i>E. coli</i>		
MB2159	D-alanine auxotroph, cloning host	50
NEB5 α	cloning host	New England Biolabs
BL21 Star (DE3)	expression host	Invitrogen
plasmids		
pET-21a(+)	Amp ^r , T7 promoter, C-terminal His-Tag	Novagen
pSIP409	Erm ^r , pSIP401 derivative, P _{sppQ}	25
pSIP609	pSIP409 derivative, <i>alr</i> replaced with <i>erm</i>	19
pPW002	L-AI from <i>L. reuteri</i> in pET-21a(+)	this work
pPW004	D-XI from <i>L. reuteri</i> in pET-21a(+)	this work
pPS1	pSIP609 derivative, <i>NcoI</i> replaced with <i>NheI</i>	this work
pPW-015	pSIP409 derivative, <i>NcoI</i> replaced with <i>NheI</i>	this work
pPS2	L-AI and D-XI from <i>L. reuteri</i> in pPS1	this work
pPS3	L-AI and D-XI from <i>L. reuteri</i> in pPW-015	this work
pPW116	L-AI from <i>L. reuteri</i> in pJET1.2	this work
pPW114	D-XI from <i>L. reuteri</i> in pJET1.2	this work

^aAmp^r, ampicillin resistance; Erm^r, erythromycin resistance; *alr*, alanine racemase encoding gene; *erm*, erythromycin resistance gene.

xylA_LbReu_rev1. Each PCR product was ligated into the pJET1.2 vector as recommended by the supplier, resulting in plasmids pPW116 and pPW114, respectively. Each plasmid was transformed into chemically competent *E. coli* NEB5 α cells according to the manufacturer's instructions.

Plasmid Construction and Expression in *E. coli*. The *araA* and *xylA* genes were amplified using the primer pairs *AraReuNheI_fwd1*/*AraReuEcoRI_His6* and *XylReuNheI_fwd1*/*XylReuEcoRI_His6* as well as pPW116 and pPW114 as templates, respectively. The resulting PCR fragments were cut with the restriction enzymes *NheI* and *EcoRI* and ligated into the equally treated expression vector pET21-a(+) (Novagen, Darmstadt, Germany) in frame with the C-terminal His₆-tag encoded by the vector. The resulting plasmids pPW002 and pPW004 were transformed into electrocompetent *E. coli* BL21 Star (DE3) according to the method of Inoue et al.²⁹

Plasmid Construction and Expression in *L. plantarum*. The *NcoI* restriction site on the pSIP609 and pSIP409 vector, respectively, was replaced by a *NheI* restriction site by site-directed mutagenesis.³⁰ A PCR was performed using the primer pair pSIP_SD_*NheI_fwd1* and pSIP409_SD_*NheI_rev1*. Subsequent digestion with *DpnI* and transformation into *E. coli* resulted in the plasmids pPS1 (pSIP609 derivative) and pPW015 (pSIP409 derivative), respectively.³⁰ The fusion of the *araA* and the *xylA* genes was performed according to the overlap extension method.³¹ PCR reactions were done with the primer pairs *XylReu_OE_fwd1*/*XylReu_OE_rev1* (pPW114 as template) and *AraReuNheI_fwd1*/*AraReu_OE_rev1* (pPW116 as template). The resulting PCR products were purified and used as templates for a third reaction with the primers *AraReuNheI_fwd1* and *XylReu_OE_rev1*. The resulting amplicon was digested with *NheI* and *EcoRI* and

primer	sequence (5'–3')
araA_LbReu_fwd1	ATGGATATTAAAGAATTACGAATTTTGGTTT
araA_LbReu_rev1	CTATTC AATAAAAATCAACTTTTAACA
xylA_LbReu_fwd1	TCATCATGATAAGGATAAAGTTTTGG
xylA_LbReu_rev1	ATTGCAATCGTTGTTCATAAAGCCAATC
pSIP_SD_NheI_fwd1	TACGTCCTGTAGAAACCCCAAC
pSIP409_SD_NheI_rev1	CTACAGGACGTAGCTAGCCTAAAAATCTCCTTGT
AraReuNheI_fwd1	TTTTCGCTAGCATGGATATTAAAGAATTACG
AraReuEcoRI_His6	AACGC GAATTCTGTTTCAATAAAATCAACTT
XylReuNheI_fwd1	ATTTTGCTAGCATGGCTGATTTATGGA
XylReuEcoRI_His6	CCGTGGAATTCTGCTTAGCAAGCGTTTC
AraReu_OE_rev1	TAAAACCTCTGAAC TAGTGGTGGTGGTGGTGGTCAATAAAATCAAC
XylReu_OE_fwd1	TTCAGGAGATTTT AATGGCTGATTTATGGAATAA
XylReu OE rev1	CGAATGAATTCTTAGTGGTGGTGGTGGTGGTGGTCTAGCAAGCGTTTC

The amount of D-xylulose or D-fructose was determined either by HPLC analysis or using the cysteine–carbazole–sulfuric-acid method³⁵ with slight modifications (see above). For HPLC analysis and calculation of the reaction products D-xylose, D-glucose, D-xylulose, and D-fructose standards were included in the run. One unit of D-xylose isomerase activity is defined as the amount of enzyme needed to produce 1 μ mol of D-xylulose or D-fructose per minute under the assay conditions.

Effect of Temperature, pH, and Divalent Metal Ions on Enzyme Activity. The temperature optimum was measured by assaying the enzyme samples over the range of 30–90 °C, at pH 5 (D-XI) and pH 6 (L-AI). Three buffer systems (sodium acetate/sodium phosphate/Tris-HCl) were used for measuring the pH optimum of enzyme activity at 65 °C with D-xylose (D-XI) and L-arabinose (L-AI) as substrate. The effects of various metal ions were determined by the addition of 1 mM MgSO₄, CoCl₂, or MnCl₂ (or more than one in various concentrations) and assaying D-XI and L-AI activity, respectively, under standard conditions without 0.5 mM MnCl₂ and 0.5 mM CoCl₂ (D-XI) and without 0.5 mM MnCl₂ and 1 mM CoCl₂ (L-AI).

Determination of Kinetic Parameters. The kinetic parameters of L-AI were determined in 50 mM sodium phosphate buffer (pH 6.0) containing 0.5 mM MnCl₂ and 1 mM CoCl₂ and 5–800 mM L-arabinose (10–800 mM D-galactose). The reaction mixtures were incubated for 10 min at 65 °C. The kinetic parameters of D-XI were determined in 50 mM sodium acetate buffer (pH 5.0) containing 0.5 mM MnCl₂, 0.5 mM CoCl₂ (5 mM CoCl₂, 5 mM MnCl₂, 5 mM MgSO₄), and 1–500 mM D-xylose (25–1500 mM D-glucose). The reaction mixtures were incubated for 10 min at 65 °C (60 °C). The observed data were fitted to the Michaelis–Menten equation, and kinetic constants were calculated by nonlinear least-squares regression. Using the molecular mass, turnover numbers (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) were calculated.

Enzymatic Conversion of D-Galactose to D-Tagatose. The conversion was carried out at 60 °C with 0.8 and 1.4 U of purified enzyme (i.e., 1.0 mg of L-AI produced in *E. coli* and 0.9 mg of L-AI expressed in *L. plantarum*), respectively, in a total volume of 1 mL. The conversion medium contained 500 mM D-galactose, 1 mM CoCl₂, and 0.5 mM MnCl₂ in 50 mM sodium phosphate buffer (pH 6.0). Samples were taken regularly for the analysis of D-galactose and D-tagatose by HPLC. The conversion rate represents the ratio between the formed D-tagatose and the initial D-galactose.

Enzymatic Conversion of D-Glucose to D-Fructose. The conversion was carried out at 60 °C with 2.1 and 1.1 U of purified enzyme (i.e., 1.9 mg of D-XI produced in *E. coli* and 0.9 mg of D-XI expressed in *L. plantarum*), respectively, in a total volume of 1 mL. The conversion media contained 500 mM D-glucose, 5 mM CoCl₂, 5 mM MnCl₂, and 5 mM MgSO₄ in 50 mM sodium acetate buffer (pH 5.0). Samples were taken regularly for the analysis of D-glucose and D-fructose by HPLC. The conversion rate represents the ratio between the formed D-fructose and the initial D-glucose.

RESULTS AND DISCUSSION

Heterologous Expression of the L-AI and D-XI Encoding Gene in *E. coli*. The L-AI encoding gene from *L. reuteri* DSMZ 17509 was successfully expressed in *E. coli* BL21(DE3). The nucleotide sequence contains an open reading frame of 1422 bp encoding a polypeptide of 473 amino acids. On the basis of the DNA sequence, two modified oligonucleotide primers containing restriction sites for in-frame ligation into the pET21-a(+) were designed and used to reamplify the *araA* sequence and to construct the expression vector pPW002. The L-AI encoding sequence was fused in frame with the C-terminal His₆-tag encoded by the vector, and the construct was expressed under the control of the lactose- or IPTG-inducible T7 promoter. Approximately 137.9 U of L-AI activity/L fermentation medium was produced under the described conditions with a volumetric activity of 1.6 U/mL

and a specific activity of 0.18 U/mg using D-galactose as substrate. The D-XI encoding gene from *L. reuteri* DSM 17509 contains an open reading frame of 1350 bp encoding a polypeptide of 449 amino acids. The *xylA* sequence was fused in frame with the C-terminal His₆-tag encoded by the vector, and the heterologous expression resulted in approximately 780.5 U of D-XI activity/L fermentation medium with a volumetric activity of 8.9 U/mL and a specific activity of 0.57 U/mg using D-glucose as substrate.

Due to the use of an ampicillin resistance marker, the potential of the pET21-a(+) expression system is limited to food applications. Furthermore, the use of *E. coli* strains is not permitted in the food industry in most countries,³⁶ due to possible endotoxin production.³⁷

Coexpression of L-AI and D-XI Encoding Gene(s) in *L. plantarum*. The resulting expression vector contains the *araA* gene fused with a C-terminal His₆-tag (CAC₆) and a stop-codon (TAG). The following ribosomal binding site (RBS)³⁸ was flanked downstream (5') with TTC (three nucleotides downstream from the RBS) and upstream with the nucleotides ATTTTA (six nucleotides upstream of the RBS).^{26,39} The *xylA* gene was also fused with a His₆-tag followed by the stop-codon (TAA). Approximately 28.6 U (18.3 U) of L-AI activity/L fermentation medium with a volumetric activity of 0.8 U/mL (0.5 U/mL) and a specific activity of 0.15 U/mg (0.08 U/mg) with D-galactose as substrate was produced in *L. plantarum* using the pSIP609 (pSIP409) vector. The coexpression of the *xylA* gene allowed production of approximately 32.3 U (55.9 U) of GI activity/L fermentation medium with a volumetric activity of 0.9 U/mL (1.6 U/mL) and a specific activity of 0.17 U/mg (0.23 U/mg) using the pSIP609 (pSIP409) expression system.

A food grade LAB strain was chosen as expression host as demonstrated by Rhimi et al.⁴⁰ and Salonen et al.³ Although expression yields in *E. coli* are much higher, the combination of the (homologous) alanine racemase gene (*alr*) as selection marker and the safe cell factory *L. plantarum* has considerable potential for the production of ingredients and additives for the food industry.¹⁹ Compared to the erythromycin-dependent expression system pSIP409, the expression yield of the (first) *araA* gene was higher; in the case of the (second) *xylA* gene, the induction/expression worked less efficiently. However, the antibiotic-independent expression system pSIP609 allowed the successful coexpression of the genes *araA* and *xylA* from *L. reuteri* DSMZ 17509. The slightly higher yields with the pSIP609 system (alanine racemase as selection marker in combination with a suitable deficient host strain) are in agreement with previous data and can be explained by the avoidance of antibiotic detoxification and subsequently reduced plasmid loss.¹⁹

Purification of Recombinant L-AI and D-XI. The recombinant enzymes were purified to apparent homogeneity from cell extracts by a single-step purification protocol using immobilized metal affinity chromatography (Figure 1).

The specific activities of the purified enzymes were 0.9 U/mg for L-AI and 1.1 U/mg for D-XI with *E. coli* as expression host and D-galactose and D-glucose, respectively, as substrate. The use of *L. plantarum* as expression host resulted in 1.6 U/mg (in the case of pSIP609 and 409) for L-AI and 1.2 U/mg (pSIP609) and 1.8 (pSIP409) for D-XI after purification. Although the coexpressed genes contained the same affinity tag, the resulting proteins are separated successfully and elute in different fractions.

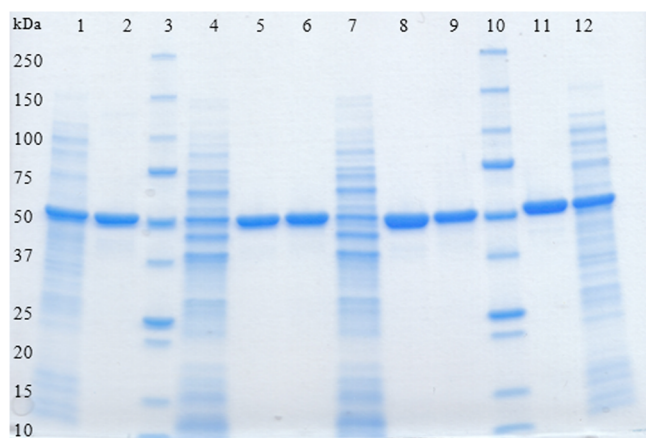


Figure 1. SDS-PAGE analysis of recombinant L-AI and D-XI from *L. reuteri*. Lanes: 1, L-AI-containing crude extract from *E. coli*; 2, purified L-AI (*E. coli*); 3, Precision Protein standard (Bio-Rad); 4, L-AI-containing crude extract from *L. plantarum* (pSIP609); 5, purified L-AI (pSIP609); 6, purified D-XI (pSIP609); 7, D-XI-containing crude extract from *L. plantarum* (pSIP409); 8, purified L-AI (pSIP409); 9, purified D-XI (pSIP409); 10, Precision Protein standard (Bio-Rad); 11, purified D-XI (*E. coli*); 12, D-XI-containing crude extract from *E. coli*.

Molecular masses of approximately 300 kDa (L-AI) and 190 kDa (D-XI) were determined using size exclusion chromatography. Considering the calculated molecular masses of 53.6 kDa (L-AI) and 51.04 kDa (D-XI), these data suggest that L-AI is naturally active as a homohexamer and D-XI is a tetramer. The hexameric quaternary structure of the L-AI from *L. reuteri* is also observed in the enzymes from *E. coli*⁴¹ and *L. plantarum* NC8,⁴² whereas many other L-AIs exhibit tetrameric structures.^{9,42} The tetrameric quaternary structure of the D-XI from *L. reuteri* is the most common form of D-XIs,⁴³ but dimeric and trimeric forms are also described.¹¹

Effect of Temperature, pH, and Divalent Metal Ions on Enzyme Activity. The temperature profile of purified L-AI and D-XI (expressed in *E. coli*), respectively, was determined from 30 to 90 °C using the standard assay according to Dische and Borenfreund.³⁵ L-AI exhibited maximum activity at 65 °C, with 90 and 97% activity at 60 and 70 °C, respectively (Figure 2). The enzyme D-XI exhibited the same temperature optimum (65 °C) and retained 85% of its maximum activity at 60 and 70 °C, respectively (Figure 2). For industrial production of D-tagatose and D-fructose, respectively, an isomerization at elevated temperature of 60–65 °C resulted in higher conversion yields, better sugar solubility, and lower risk of microbial contamination. Higher temperatures above 70 °C resulted in the formation of undesired byproducts and browning effects.^{1,44}

The standard assay was performed using different buffers with pH ranging from 4 to 10. The pH optimum of L-AI and D-XI was found in the slightly acidic pH range (6.0 and 5.0, respectively) (Figure 3). Acidic conditions avoid the formation of undesired byproducts, a great advantage for industrial purposes.^{3,44}

The L-AI enzyme activity was assayed at 65 °C and pH 6.0 in the presence of various cations and D-galactose as substrate (Table 3). The activity was increased by the addition of 1 mM Co^{2+} (3.7-fold) and 1 mM Mn^{2+} (1.4-fold). A mixture of 1 mM Co^{2+} and 0.5 mM Mn^{2+} increased the enzyme activity 3.9-fold. The D-XI enzyme activity was assayed at 65 °C and pH 5.0 in the presence of various cations and D-xylose as substrate (Table

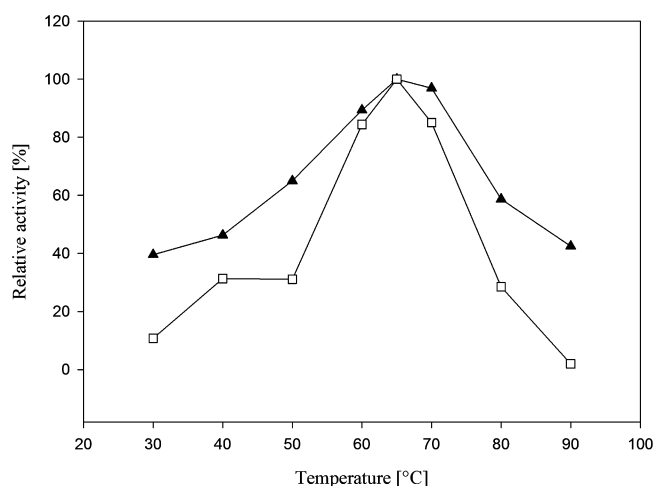


Figure 2. Effect of temperature on L-AI (solid triangles) and D-XI (open squares) activity. Relative activities are presented as functions of temperature with L-arabinose and D-xylose, respectively, as substrate. The reaction mixtures were incubated for 10 min.

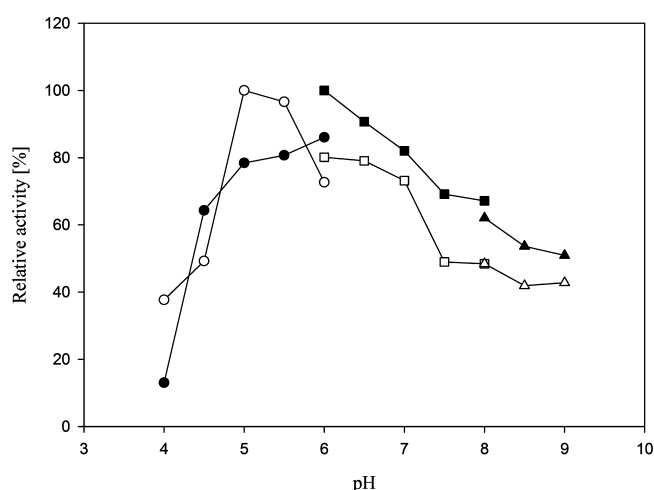


Figure 3. Effect of pH on L-AI (solid symbols) and D-XI (open symbols) activity. Relative activities are presented as functions of pH with L-arabinose and D-xylose, respectively, as substrate. The reaction mixtures were incubated for 10 min. The buffers used were acetate (circles), phosphate (squares), and tris (triangles).

Table 3. Effect of Metal Ions on L-AI and D-XI Activity^a

metal ion	L-AI relative activity (%)	D-XI relative activity (%)
none	26	32
Co^{2+}	94	62
Mg^{2+}	25	36
Mn^{2+}	41	93
$\text{Co}^{2+} + \text{Mn}^{2+}$	100	100

^a CoCl_2 , MgSO_4 , and MnCl_2 were added to the enzyme reaction mixture at a final concentration of 1 mM. The mixture of CoCl_2 and MnCl_2 was added at a final concentration of 1 or 0.5 mM (in the case of L-AI) and 0.5 or 0.5 mM (in the case of D-XI), respectively.

3). The activity was increased by the addition of 1 mM Co^{2+} (1.9-fold) and 1 mM Mn^{2+} (2.9-fold). A mixture of 0.5 mM Co^{2+} and 0.5 mM Mn^{2+} increased the enzyme activity 3.2-fold. Glucose isomerase activity was assayed at 60 °C and pH 5.0 and was maximally enhanced by the addition of 5 mM Co^{2+} , 5 mM Mn^{2+} , and 5 mM Mg^{2+} (data not shown).

A number of L-arabinose and D-xylose isomerases require metal ions for (maximal) activity (and stability).^{4,11} The use of metal cofactors, especially Co^{2+} , increases downstream processing costs as they have to be removed from the final product.⁹ For industrial purposes, independence from or a very low requirement for metal ions is advantageous.³

Kinetic Parameters/Properties. The apparent K_m , V_{\max} , k_{cat} , and catalytic efficiency (k_{cat}/K_m) of L-AI using D-galactose as substrate were $647 \pm 109 \text{ mM}$, $11 \pm 1 \text{ U/mg}$, $59 \pm 5 \text{ s}^{-1}$, and $0.09 \text{ mM}^{-1} \text{ s}^{-1}$, respectively. For L-arabinose the apparent K_m , V_{\max} , k_{cat} , and catalytic efficiency (k_{cat}/K_m) were estimated to be $633 \pm 69 \text{ mM}$, $179 \pm 10 \text{ U/mg}$, $959 \pm 55 \text{ s}^{-1}$, and $1.5 \text{ mM}^{-1} \text{ s}^{-1}$, respectively.

The high K_m value for D-galactose is comparable with that of the enzyme from *Bifidobacterium longum*³ and *L. pentosus*,^{7,45} whereas enzymes from other *Lactobacillus* strains exhibit up to 10 times lower K_m values.^{1,9,42} The low affinity for L-arabinose is comparable with the enzyme from *Pediococcus pentosaceus* PC-5, which shows no activity for this substrate.¹⁰

The apparent K_m , V_{\max} , k_{cat} , and catalytic efficiency (k_{cat}/K_m) of D-XI using D-glucose as substrate were $1099 \pm 98 \text{ mM}$, $4.6 \pm 0.2 \text{ U/mg}$, $15.5 \pm 0.8 \text{ s}^{-1}$, and $0.014 \text{ mM}^{-1} \text{ s}^{-1}$, respectively. For D-xylose the apparent K_m , V_{\max} , k_{cat} , and catalytic efficiency (k_{cat}/K_m) were estimated to be $177.4 \pm 5.6 \text{ mM}$, $43.1 \pm 0.6 \text{ U/mg}$, $146.6 \pm 1.9 \text{ s}^{-1}$, and $0.83 \text{ mM}^{-1} \text{ s}^{-1}$, respectively.

The lower K_m value for D-xylose than for D-glucose is in agreement with the natural function of the D-XI to produce xylulose for the pentose phosphate or phosphoketolase pathway.⁴⁶ The high K_m value for D-glucose is comparable to that from *L. brevis*.⁴⁷

Conversion Experiments. L-AI converted D-galactose to D-tagatose at a conversion ratio of approximately 35% after 25 h of incubation at 60 °C (Figure 4). The use of L-AI (120 U) from the related organism *L. plantarum* SK-2 resulted in a conversion rate of 39% after 96 h at 35 °C,⁴⁵ whereas the

enzyme from strain NC8 showed a yield of 30% after 6 h at 60 °C. A similar equilibrium was observed by using D-AI from *L. sakei* as biocatalyst (36% after 7 h at 40 °C),⁹ whereas a higher conversion yield (55% after 96 h at 65 °C) can be obtained by using the enzyme (9.98 U) from *L. fermentum* CGMCC2921 and 50 mM substrate.¹

The conversion of D-glucose resulted in approximately 48% D-fructose after 25 h of incubation and the use of D-XI produced in the expression host *E. coli* (Figure 4). Using D-XI produced in *L. plantarum* resulted in approximately 38% D-fructose after 25 h of incubation (Figure 4) when approximately 1 unit (approximately 1 mg) of purified protein was used for the conversion experiment. After an incubation of 3 h, conversion rates of approximately 42% (*E. coli*) and 30% (*L. plantarum*) were obtained; thus, the reaction reached its equilibrium after 3 h. A reaction temperature at 40 °C for 3 h resulted in conversion rates of approximately 30 and 9% (data not shown). For industrial purposes an operating temperature of 60 °C is favorable due to the reasons mentioned above (i.e., limited color formation and byproduct formation). HPLC analysis revealed that no byproducts were formed, either in the conversion of D-galactose or in the case of D-glucose (data not shown).

In summary, this study demonstrates the successful expression of the L-arabinose isomerase and D-xylose isomerase encoding genes from *L. reuteri* in *E. coli* as well as the coexpression of both genes in the food grade microorganism *L. plantarum*. Small-scale conversion experiments demonstrate the principal suitability of both enzymes for isomerization of D-galactose and D-glucose, the hydrolysis products of lactose, under preferred industrial conditions (acidic pH and 60 °C). Neither enzyme is ideally suited for the envisaged application due to their rather unfavorable K_m values as well as their dependence on divalent metal cations. However, enzymes with more favorable properties can be screened from a large number of organisms that are considered safe, or an improvement of these properties by enzyme engineering methods⁴⁸ may be an alternative. The expression system based on the pSIP vector series is suitable for other lactic acid bacteria as well, and construction of alanine racemase-deficient strains containing no foreign DNA has been shown to be straightforward for organisms that are amenable to transformation.¹⁹ Higher yields can be achieved by expression in *E. coli* and presumably also by expression of the single genes; however, the presented coexpression of two enzymes in a food grade host–vector system offers the possibility of cost-efficient production of enzyme preparations for the conversion of mixed substrates.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*(C.K.P.) Phone: +43 1 47654 6144. Fax: +43 1 47654 6199. E-mail: clemens.peterbauer@boku.ac.at.

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Notes

The authors declare no competing financial interest.

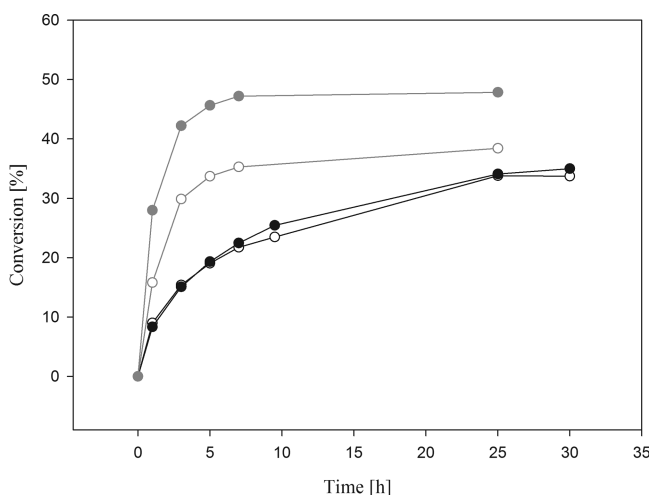


Figure 4. Time course of D-tagatose (black symbols) and D-fructose (gray symbols) production during L-AI- and D-XI-catalyzed isomerization of D-galactose and D-glucose, respectively: black, open circle) conversion curve at 60 °C performed with 0.9 mg of L-AI (expressed in pSIP609 *L. plantarum*); (black, solid circle) conversion curve at 60 °C performed with 1.0 mg of L-AI (expressed in pET21-a(+) *E. coli*); (gray, open circle) conversion curve at 60 °C performed with 0.9 mg of D-XI (expressed in pSIP609 *L. plantarum*); (gray, solid circle) conversion curve at 60 °C performed with 1.9 mg of D-XI (expressed in pET21-a(+) *E. coli*).

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ABBREVIATIONS USED

L-AI, L-arabinose isomerase; D-XI, D-xylose isomerase; GI, glucose isomerase; L., *Lactobacillus*; HCFS, high-fructose corn syrup

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