Conversion of L-arabinose to L-ribose by genetically engineered 
Candida tropicalis

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Abstract
L-Ribose, a starting material for the synthesis of L-nucleoside, has attracted lots of attention since L-nucleoside is responsible for the antiviral activities of the racemic mixtures of nucleoside enantiomers. In this study, the L-ribulose-producing Candida tropicalis strain was engineered for the conversion of L-arabinose to L-ribose. For the construction of a uracil auxotroph, the URA3 gene was excised by homologous recombination. The expression cassette of codon-optimized L-ribose isomerase gene from Acinetobacter calcoaceticus DL-28 under the control of the GAPDH promoter was integrated to the uracil auxotroph. The resulting strain, K1 CoSTP2 LsaAraA AcLRI, was cultivated with the glucose/L-arabinose mixture. At 45.5 h of fermentation, 6.0 g/L of L-ribose and 3.2 g/L of L-ribulose were produced from 30 g/L of L-arabinose. The proportion between L-ribose and L-ribulose was approximately 2:1 and the conversion yield of L-arabinose to L-ribose was about 20% (w/w). The L-ribose-producing yeast strain was successfully constructed for the first time and could convert L-arabinose to L-ribose in one-pot fermentation using the mixture of glucose and L-arabinose.

Keywords Candida tropicalis · L-Arabinose · L-Ribose · L-Ribose isomerase

Introduction
Infectious diseases including avian influenza, swine flu, and coronavirus disease 2019 (COVID-19) spread rapidly throughout the world, infect many people, and even lead to death [1]. Different kinds of viral diseases are threatening human beings every year. Moreover, it is difficult to prevent or treat them when various mutations appear. Hence, the need for effective antiviral drugs is getting bigger and bigger. Promising antivirals such as lamivudine, clevudine, and telbivudine are made from nucleoside and nucleotide analogs [2]. They are interrupting viral replication by the termination of viral DNA/RNA synthesis. As L-nucleosides, the enantiomers of D-nucleosides, are proven to be responsible for the antiviral activities of racemic mixtures of the D- and L-nucleosides in general, antivirals based on L-nucleosides have gathered much attention [3].

The major starting material for the synthesis of L-nucleosides is L-ribose. Primarily, chemical reactions have been applied for the synthesis of L-ribose [4]. The synthesis of L-ribose is accomplished by multiple steps of complex reactions with various catalysts in harsh conditions (high temperature and high pressure). Also, extensive purifications are needed for the removal of unwanted by-products. Therefore, chemical production approaches are not suitable for the industrial scale. Biotechnological methods are promising alternatives as those processes are stereospecific and have simple reaction steps in moderate conditions without harmful catalysts.

The biological production of L-ribose is mainly achieved by enzymes or resting cells. Purified enzymes of mannose-6-phosphate isomerase (EC 5.3.1.8) from Geobacillus thermodenitrificans were used for the production of L-ribose from L-ribulose, and the conversion yield was 71% (w/w) [5]. The Escherichia coli cells expressing mannitol-1-dehydrogenase (EC 1.1.1.255) from Apium graveolens were investigated for the production of L-ribose from ribitol, and the yield was 55% (w/w) [6]. The E. coli cells expressing D-lyxose isomerase (EC 5.3.1.15) from Thermoflavimicrobium dichotomicum, in combination with L-arabinose isomerase (EC 5.3.1.4) from Alicyclobacillus hesperidum, converted

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l-arabinose to l-ribose with the yield of 20.9% (w/w) [7]. The l-ribose isomerase (EC 5.3.1.23) from Acinetobacter calcoaceticus DL-28 was expressed in l-ribulokinase-deficient E. coli for the conversion of l-arabinose to l-ribose and the yield was 20% (w/w) [8].

As one of the xylose-assimilating yeasts, *Candida tropicalis* is well-known for its xylitol-producing ability [9–11], and biological production of xylitol has been developed and showed a high conversion yield of over 90% [12]. *C. tropicalis* can also utilize even n-alkanes and fatty acids as carbon sources. Thus, it has been widely used to produce ω-hydroxy fatty acids and long-chain dicarboxylic acids [13–15]. However, *C. tropicalis* cannot assimilate l-arabinose, because the cells do not possess genes related to the l-arabinose metabolic pathway. Yoon et al. (2011) expressed the three genes related to the bacterial l-arabinose metabolic path, *araA* (l-arabinose isomerase), *araB* (l-ribulokinase), and *araD* (l-ribulose-5-phosphate 4-epimerase), in *C. tropicalis* and constructed an l-arabinose-assimilating strain to reduce l-arabitol accumulation as a by-product during xyitol production. As a result, the recombinant cells assimilated l-arabinose without the accumulation of l-arabitol in the medium [16].

Previously, the l-ribulose-producing strain, *C. tropicalis* K1 CoSTP2 LsaAraA, was constructed with constitutive expression of the transporter protein (AtStp2) and the l-arabinose isomerase (LsaAraA) [17]. In this study, to construct an l-ribose-producing yeast strain from l-arabinose as a substrate, the l-ribose isomerase gene from *A. calcoaceticus* DL-28 was integrated into the strain. The resulting strain, K1 CoSTP2 LsaAraA AcLRI, successfully converted l-arabinose to l-ribose from a glucose/l-arabinose mixture in one-pot fermentation with a conversion yield of about 20% (w/w).

**Materials and methods**

**Strains and media**

The l-ribulose-producing strain with constitutive expression of the transporter protein (AtStp2) and the l-arabinose isomerase (LsaAraA), *C. tropicalis* K1 CoSTP2 LsaAraA, was used in this study (Fig. 1). Genotypes of *C. tropicalis* strains are listed in Table 1. K1 CoSTP2 LsaAraAp, a uracil auxotroph, was used as a host strain for transformation. The *URA3* gene originated from *C. tropicalis* ATCC 20,336 (GenBank accession no. AB006212.1) was used as a selection marker. Partial sequences of *arg* gene obtained from *Bacillus subtilis* were used for pop-out the *URA3* marker. For growth and selection of *C. tropicalis*, YM medium (3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, 20 g/L of glucose), YNB medium (6.7 g/L of yeast nitrogen base without amino acids, 20 g/L of glucose), and YNB-SFOA medium (6.7 g/L of yeast nitrogen base without amino acids, 20 g/L of glucose, 0.1 g/L of uracil, 0.1 g/L of uridine, 0.8 g/L of 5'-fluoroorotic acid) were used. *E. coli* DH5α (Enzynomics, Daejeon, Korea) was used as a host for the transformation of plasmids. Luria–Bertani medium (5 g/L of yeast extract, 10 g/L of tryptone, 10 g/L of NaCl) containing 50 μg/mL of ampicillin was used to grow the transformed *E. coli* strains. The sequences of all primers used in this study are listed in Table 2.

**Codon optimization and construction of expression cassette**

The genomic sequences of l-ribose isomerase from *A. calcoaceticus* DL-28 (GenBank accession no. AB062121.1) was used as a source for codon optimization (Supplementary Materials Table S1). Because *C. tropicalis* belongs to the CTG clade species [18, 19], some codons should be changed for appropriate translation. Based on the codon usage database [20], the codon-optimized gene with *XbaI* or *XhoI* on each terminal was synthesized (Bioneer, Daejeon, Korea) and the resulting plasmid was designated as pBHA-AcLRI.

The construction of the expression cassette was described previously [16]. To integrate the expression cassette into *XYL3* locus of *C. tropicalis* K1 CoSTP2 LsaAraAp, the pXK4 plasmid was synthesized containing the construct of a pair of 60-bp homologous fragments of *XYL3* (XK4F and XK4R) with the *NruI* sites at both ends and the *BanHI* site in the middle of XK4F and XK4R. The PTAUA fragment containing *P* _GAPDH*-T _GAPDH*-arg-*URA3*-arg with the *BglII* or *BanHI* sites on each terminal was inserted into the *BanHI* site of pXK4 and the resulting plasmid was designated as...
pXK4_PTAUA. The codon-optimized AcLRI was ligated into the XbaI and XhoI sites of pXK4_PTAUA and designated as pXK4_PTAUA-AcLRI. The constructed plasmid was linearized by digestion with NruI before the transformation.

**Transformation of yeasts**

The lithium acetate/single-stranded carrier DNA/polyethylene glycol method with slight modifications [12, 21] was used to transform *C. tropicalis*. Cells were grown in 50 mL of YM media for at least 3 h, harvested by centrifugation at 2000×g for 5 min at 25 °C, and washed and resuspended in LiOAc solution (0.1 M LiOAc, 10 mM Tris–HCl, and 1 mM EDTA, pH 8.0). Next, 30 μL of cell suspension was mixed with 30 μL of DNA fragments (30 ng/μL) and 5 μL of salmon testis single-stranded DNA (Sigma, St. Louis, MO, USA). The mixture was added to 325 μL of polyethylene glycol solution (0.1 M LiOAc, 10 mM Tris–HCl, and 1 mM EDTA in 40% (w/v) polyethylene glycol 8000) and mixed thoroughly. After incubation at 30 °C for 45 min, the mixture was placed in a 42 °C heat block for 15 min. The cells were harvested by centrifugation at 3000×g for 3 min at 25 °C. The cell suspension in YNB media was spread onto YNB plates.

A uracil prototroph, as a result of the transformation, was screened to allow the pop-out of the selection marker gene. The transformants were grown in 4 mL of YM media for at least 12 h, plated onto the YNB-5FOA plate for 2 days. Single colonies were picked and incubated onto YNB and YM plates for 2 days to confirm the deletion of the URA3 gene. The specific removal of the URA3 gene was examined by PCR.

**Yeast total RNA isolation and RT-PCR analysis**

A mini-prep version of yeast RNA isolation was performed with slight modifications [22]. Cells grown in YM medium were washed and resuspended in 400 μL of AE buffer (50 mM Na acetate pH 5.3, 10 mM EDTA). The resuspended cells were transferred to a microcentrifuge tube and 40 μL of 10% SDS, 440 μL of buffer-equilibrated phenol (Bioneer, Daejeon, Korea), and glass beads (Sigma) were added. The aqueous phase was extracted with 400 μL of phenol (pH 5.2):chloroform:isoamyl alcohol (25:24:1) (Bioneer) by centrifugation (13,000×g, 25 °C for 5 min). The aqueous phase...
was added to 40 μL of 0.3 M Na acetate, pH 5.3, after which 2.5 volumes of 95% ice-cold ethanol were added. The tube was incubated for 30 min at -20 °C and centrifuged at 4 °C for 5 min. The pellet was dried at 60 °C for 5 min and resuspended in 55 μL of sterile water and stored at −70 °C until use.

For cDNA synthesis from total RNAs, 400 pmol of oligo dT was incubated with extracted RNAs at 70 °C for 5 min. The mixture was then chilled rapidly on ice. Each cDNA was synthesized (AccuPower RT PreMix kit, Bioneer) and used in subsequent PCR with specific primers to amplify the protein-coding regions.

**L-Arabinose isomerase and L-ribose isomerase activity assays**

Cultured yeast cells were harvested by centrifugation and washed with 50 mM potassium phosphate buffer (pH 7.0). The cells were suspended in the same buffer and disrupted using glass beads. Cellular debris was separated by centrifugation (13,000×g, 4 °C for 10 min). Protein concentration was determined using a Quant-iT Assay Kit (Invitrogen, Carlsbad, CA, USA).

Under standard conditions, the reaction mixture contained 100 μg of crude extract, 100 mM of L-arabinose or L-ribose (substrate), and 50 mM acetate buffer (pH 4) in a final volume of 500 μL. The mixture was incubated at 30 °C for 10 min, and the reaction was terminated by adding 50 μL of trichloroacetic acid (10%). The converted L-ribose or L-ribose was detected by the cysteine carboxazole sulfuric acid method [23], and the absorbance was measured at 540 nm. One unit of L-arabinose isomerase or L-ribose isomerase activity was defined as the formation of 1 pmol keto- or aldo-sugar per min under the above-specified conditions.

**L-Ribose fermentation and analytical methods**

L-Ribose fermentation with C. tropicalis K1 CoSTP2 LsaAraA AcLRI was performed in a 250-mL Erlenmeyer flask containing 50 mL of fermentation medium at 30 °C with 200 rpm. The fermentation medium was contained 30 g/L or 50 g/L of L-arabinose, 20 g/L of glucose, 10 g/L of yeast extract, 5 g/L of KH₂PO₄, and 0.2 g/L of MgSO₄·7H₂O.

The concentrations of glucose, L-arabinose, L-ribose, and L-ribose were analyzed by high-performance liquid chromatography equipped with a refractive index detector (Waters, Milford, MA, USA). The samples were separated by a Sugar-Pak I column (Waters) with degassed DDW at a flow rate of 0.3 mL/min. Cell growth was determined at 600 nm spectrophotometrically. One A₆₀₀ was equivalent to 0.474 g (dry cell weight)/L.

### Results

**Homologous excision of the URA3 gene**

In the previous study, the L-ribulose-producing C. tropicalis strain was constructed by constitutive expression of proteins which were an L-arabinose isomerase (LsaAraA) and a pentose transporter (AtStp2). The resulting C. tropicalis K1 CoSTP2 LsaAraA strain is a uracil prototroph because the strain harbors a stably integrated URA3 gene in its genome. For additional transformations to construct an engineered strain, however, the URA3 gene should be removed from the strain. The excision of the selection marker gene was done by homologous recombination between the two arg genes on either side of the URA3 gene (Fig. 2).

After incubation of the C. tropicalis K1 CoSTP2 LsaAraA strain for 12 h in YM medium, 200 μL of cell suspension was plated onto the YNB-SFOA plate for 2 days. The colonies on the plate were selected and the deletion of the URA3 gene was confirmed by examining the growth of the cells on YM and YNB plates. Also, the specific removal of the URA3 gene was examined by PCR. The resulting strain, a uracil auxotroph, was designated as K1 CoSTP2 LsaAraAp and used as a host strain for the construction of L-ribose-producing strain.

**Chromosomal integration of L-ribose isomerase gene**

Biotransformation of L-ribulose to L-ribose was done by the isomerization reaction of mannose-6-phosphate isomerase, D-lyxose isomerase, or L-ribose isomerase [5, 7, 8]. Here, L-ribose isomerase from A. calcoaceticus DL-28 was used to construct L-ribose-producing strain from L-arabinose since its optimal temperature was in the range of 30–40 °C [24], similar to the optimal temperature for the growth of C. tropicalis.

The genomic sequence of the L-ribose isomerase was codon-optimized for heterologous expression as C. tropicalis utilizes alternative yeast nuclear code which decodes CUG codon as serine, not leucine [18, 19]. The optimized sequence designated as AcLRI was used for the construction of the expression cassette to chromosomally integrate into XYL3 locus of the C. tropicalis K1 CoSTP2 LsaAraAp strain. The XYL3 gene locus was chosen not to disturb the conversion of L-arabinose to L-ribose through minimizing any unwanted reactions by xylulokinase. The XK4F and XK4R fragments of the expression cassette, homologous to XYL3, was enabled the genetic integration of the cassette by homologous recombination. The GAPDH promotor fragments were enabled the constitutive expression of AcLRI.
Uracil prototrophs, as a result of the transformation, formed colonies on YNB plates. Single colonies were selected and the specific integration of the expression cassette was confirmed by PCR with the appropriate primer sets (Fig. 3). The transcription of the AcLRI, LsaAraA, and AtStp2 was confirmed by reverse transcription PCR (Supplementary Materials Fig. S1). The stably integrated strain was designated as K1 CoSTP2 LsaAraA AcLRI and the specific activity of l-arabinose isomerase and l-ribose isomerase was determined as 81.3 ± 13.4 and 98.8 ± 16.7 U/mg, respectively.

Fermentation for the conversion of l-arabinose to l-ribose

It was expected that the newly constructed *C. tropicalis* K1 CoSTP2 LsaAraA AcLRI strain could convert l-arabinose to l-ribose. For investigating the conversion of l-arabinose to l-ribose, fermentation was performed using a medium containing l-arabinose as a substrate. And glucose was used for supporting the growth of cells.

Flask fermentation was performed with 50 mL of fermentation medium containing 20 g/L of glucose and...
l-arabinose was gradually converted to l-ribose with the growth of the cell. Upon the exhaustion of glucose, the consumption rate of l-arabinose was increased and l-arabinose was converted into l-ribulose and l-ribose. After 45.5 h of fermentation, 6.0 g/L of l-ribose and 3.2 g/L of l-ribulose were produced from the consumption of 9.2 g/L of l-arabinose. Further increase of l-ribose or l-ribulose was not observed after 32.5 h. The proportion between l-ribose and l-ribulose was approximately 2:1 and the conversion yield of l-arabinose to l-ribose was 20% (w/w).

To verify the conversion of l-arabinose to l-ribose, the culture was conducted again with the medium where the concentration of l-arabinose was elevated to 50 g/L (Fig. 4b). Similar to the previous experiment, l-arabinose was consumed after the depletion of glucose. By utilizing 14.4 g/L of l-arabinose, 9.8 g/L of l-ribose and 4.6 g/L of l-ribulose were produced after 45.5 h of fermentation. About 1.5-fold increase in the production rates of l-ribulose and l-ribose were observed (Table 3). But, the ratio of l-ribose to l-ribulose and the conversion yield of l-arabinose to l-ribose remained 2:1 and 20% (w/w), respectively.

### Table 3

| Substrate concentration | Production rate of l-ribulose (g/L h) | Production rate of l-ribose (g/L h) |
|------------------------|--------------------------------------|-------------------------------------|
| 30 g/L of l-arabinose   | 0.069                                | 0.132                               |
| 50 g/L of l-arabinose   | 0.102                                | 0.215                               |

30 g/L of l-arabinose (Fig. 4a). l-Arabinose was gradually converted to l-ribose with the growth of the cell. Upon the exhaustion of glucose, the consumption rate of l-arabinose was increased and l-arabinose was converted into l-ribulose and l-ribose. After 45.5 h of fermentation, 6.0 g/L of l-ribose and 3.2 g/L of l-ribulose were produced from the consumption of 9.2 g/L of l-arabinose. Further increase of l-ribose or l-ribulose was not observed after 32.5 h. The proportion between l-ribose and l-ribulose was approximately 2:1 and the conversion yield of l-arabinose to l-ribose was 20% (w/w).
Discussion

Biological production of L-ribose from L-arabinose is possible by enzymatic reactions of L-arabinose isomerase and L-ribose isomerase. In the previous study, the efficient L-ribulose-producing strain was constructed by constitutive expression of an L-arabinose isomerase (LsaAraA) and a pentose transporter (AtStp2) [17]. For establishing L-ribose-producing strain, the C. tropicalis K1 CoSTP2 LsaAraA strain was engineered to stably integrate the codon-optimized L-ribose isomerase gene from A. calcoaceticus DL-28 into its chromosome. The newly transformed strain, designated as K1 CoSTP2 LsaAraA AcLRI, were then fermented to determine the conversion of L-arabinose to L-ribose and 6.0 g/L of L-ribose and 3.2 g/L of L-ribulose were produced from the medium containing 30 g/L of L-arabinose and 20 g/L of glucose.

Purified enzymes or resting cells have been investigated for the production of L-ribose from L-arabinose, L-ribulose, or ribitol [5–8]. For the utilization of enzymes or resting cells, however, the pre-cultivation and harvest steps are necessary. This makes the production process more complicated and expensive. Besides, bacterial endotoxins and the use of antibiotics can cause health problems [25–27]. In this study, to overcome these limitations, the L-ribose-producing yeast strain was constructed with expressing L-arabinose isomerase and L-ribose isomerase under the control of the GAPDH promoter. This strain successfully converted L-arabinose into L-ribose in one-pot fermentation.

For the conversion of L-arabinose to L-ribose, L-ribose isomerization proceeds in concert with L-arabinose isomerization [28]. The equilibrium ratio among L-arabinose/L-ribulose/L-ribose was reported as 71:6:23 in the free enzymes, and 63:16:21 in resting cells [5, 7]. In this study, the ratio was calculated as 7:1:2. Although the exact values are somewhat different, it is consistent that most of the L-arabinose remains after the reaction because of the inefficient conversion from L-arabinose to L-ribulose [29].

In general, the isomerization of aldopentose (L-arabinose) to ketopentose (L-ribulose) is thermodynamically unfavorable [30, 31]. The addition of borate resulting in stable borate-ketose complexes could improve the yield of L-ribulose [32], but extra purification for borate removal is required, which makes the production process complicated. Another promising candidate for the enhanced L-ribulose production to overcome the limited conversion of isomerization is the dehydrogenation of L-arabitol by L-arabitol 2-dehydrogenase. This energetically favored reaction, however, has limitations because the enzyme is catalytically efficient in reduction reaction rather than oxidation under physiological conditions [33]. Further studies for the discovery of novel enzymes or protein engineering are needed for the oxidation of L-arabitol. When efficient L-arabitol 2-dehydrogenase is expressed in combination with the expression of L-arabinose reductase, it is expected that increased production of L-ribulose could be possible by the recombinant strain with L-arabinose as a substrate.

To the best of our knowledge, it is the first recombinant yeast strain to convert L-arabinose to L-ribose in a one-step fermentation. As a substrate for L-ribose production, L-arabinose can be obtained from agricultural wastes such as birch bark, sugarcane bagasse, corncob, and coconut shell. By applying this developed fermentation process industrially, the biological production of L-ribose will be economically feasible.

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Availability of data and materials All data generated or analyzed during this study are included in this published article and its supplementary information files.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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