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Production of D-tagatose with Recombinant *Escherichia coli* Strain Secreting β-galactosidase and L-arabinose isomerase from *E.coli* K-12

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This study demonstrated the enzymatic synthesis of D-tagatose from lactose by engineered *Escherichia coli*. Recombinant vector pET-32a(+)*lacZ-araA* that contained the β -galactosidase gene (*lacZ*) and L-arabinose isomerase gene (*araA*) of *Escherichia coli* K-12 was constructed and co-expressed in *E. coli* BL21 (DE3). The recombinant strain could secreting great amount of β -galactosidase and L-arabinose isomerase simultaneously in soluble fraction. A high conversion rate (≥45 mol%) of D-tagatose from lactose was obtained under the optimized conditions: 50 °C, pH 7.0, with Mn^{2+} and at a substrate concentration of 25 g/L. This novel approach could be used to convert D-tagatose directly from lactose and reduce the production cost by approximately 90% at almost the same conversion rate compared with other biological methods.

Key words: D-Tagatose; Lactose; *Escherichia coli*; enzymatic synthesis.

D-tagatose, a rare natural hexoketose, has attracted much attention in basic research and industrial production for several years[1]. For instance, D-tagatose is an isomer of D-fructose that can be used as a potential natural sweetener because its sweetness and taste is >90% similar to those of sucrose with low calories[2,3]. It also has physiological functions and biological effects on hyperglycemia, intestinal dysbacteriosis, and cariogenicity[4,5].

Studies have established chemical and biological methods, such as calcium-catalyzed chemical hydrolysis of lactose[6] and lactose fermentation[7], for producing D-tagatose. However, the chemical methods usually have certain disadvantages, including low production ability, complex purification, byproduct or waste formation, and limited commercial applications[8]. The biological methods are more efficient when L-arabinose isomerase (*araA*) is used as the key enzyme[9-11]. In numerous biological methods, D-galactose is used as a substrate for converting into D-tagatose. However, the use of the substrate D-galactose as a raw material entails high costs[12], which possibly impede future industrial production in food industry or cosmetics. Furthermore, feasible and commercial processes have not been characterized yet[13].

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In this study, recombinant *Escherichia coli* BL21 (DE3), in which the β-galactosidase gene (*lacZ*) and *araA* could be expressed simultaneously was constructed, and a novel low-cost method for producing D-tagatose by directly converting from lactose was established. With this method, lactose was initially degraded to D-glucose and D-galactose by β-galactosidase, and then converted into D-tagatose by L-arabinose isomerase. The proposed process could be more effective and economical compared with other chemical and biological methods. This process could also reduce the production cost without decreasing the conversion rate.

MATERIALS AND METHODS

Strains, Plasmids and Reagents

E. coli DH5a and *E. coli* BL21 (DE3) were used as the recombinant plasmid host and the expression host, respectively. These hosts were stored in the State Key Laboratory of Bioreactor Engineering in East China University of Science and Technology (Shanghai, China). The *E. coli* expression vector pET-32a (+) was also obtained from this laboratory. The restriction enzymes *Xba*I and *Hind*III as well as T4 DNA ligase were purchased from Takara. 2× Taq PCR MasterMix was purchased from Tiangen Biotech and GeneClean kit for DNA purification was purchased from SH Geneway Biotech. Luria-Bertani medium (LB; 0.5% yeast extract, 1% glucose, and 0.5% NaCl) contained 50 μg/mL of kanamycin for plasmid selection.

Cells cultivation

E. coli strains with the corresponding plasmids were grown at 37 °C in the LB medium.

Amplification of *araA* and *lacZ*

AraA and *lacZ* were amplified from the *E. coli* K-12 genome by PCR. All of the primers used in this study (Table 1) were constructed on the Primer3 software (http://www.genome.wi.mit.edu/ftp/distribution/software/). The PCR conditions for *araA* were as follows: denaturation (94 °C) for 5 min, 30 cycles of denaturation (94 °C) for 30 s, annealing (65 °C) for 30 s, and extension (72 °C) for 90 s. For *lacZ*, the same PCR conditions for *araA* were used except annealing, which was performed for 60 s at 77 °C, and a final elongation step (72 °C) was performed for 5 min.

Construction of co-expression vector

The amplified DNA fragments of *araA* were cloned into the expression vector pET-32a (+), and then transformed into *E. coli* BL21 (DE3). The expression vector was extracted from the overnight culture by using a GenClean Column-GK2002 (Generay Biotech, Shanghai, China). After the enzyme digestion (*Hind*III/*Xho*I), *lacZ* was then ligated into pET-32a (+)-*araA* by T4 DNA ligase to construct the vector that co-expressed pET-32a (+)-*lacZ*-*araA* (Fig. 1). The ribosome binding site was inserted into the upstream region of *lacZ* to ensure that *araA* and *lacZ* were expressed simultaneously. Nucleotide sequences of the target DNA fragment in the expression vector were confirmed by DNA sequencing (Shanghai Yingjun Biotech).

Induced expression of enzyme protein

A fresh clone of *E. coli* that contained the expression vector was grown in the LB medium (50 mL) that contained 50 μg/mL of Ampicillin at 37 °C. After the culture was propagated (37 °C) to an optical density (OD_{600}) of 0.6, the expression was induced by 0.5 mM IPTG, and then cultured for another 5 h at 28 °C.

Preparation of supernatant and precipitate

The cells were harvested by centrifugation (4,000 rpm; 20 min) at 4 °C and resuspended in 50 mM Tris-HCl (pH 7.6). Afterward, the cells were ultrasonically disrupted and centrifuged (12,000 rpm; 20 min) at 4 °C. The supernatant and the precipitate of the induced cells after ultrasonication were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis

SDS-PAGE was performed using 8% resolving gels. The gels were initially run at 100 V, and then at 200 V after the dye entered the resolving gel. The enzyme protein bands were visualized by Coomassie brilliant blue R250, and then analyzed using Quantity One (Bio-Rad, Hercules, CA) to evaluate the expression level of the target enzyme protein.

Determination of D-tagatose and conversion of lactose into D-tagatose

D-Tagatose was determined by the cysteine-carbazol-sulfuric acid method[14]. Lactose was hydrolyzed by the recombinant strain that co-expressed *lacZ* and *araA*, and then diluted to 0.1 mL

of the sample solution, 0.2 mL of cysteine-hydrochloride, and 6 mL of sulfuric acid solution. Carbazol ethanol solution (0.2 mL) was rapidly added to the mixture and mixed immediately. The mixture was then incubated at 60 °C in a water bath for 10 min. The pink-violet colored solution was analyzed at 560 nm. D-Tagatose in this sample solution was determined according to the following equation:

$$Y = \frac{(\Delta X \times 0.005)}{0.034 \times 0.1} \times \varepsilon$$

where Y is the D-tagatose content (mg/mL), ΔX is the OD d-value of different hydrolyzed samples at 0 h, 0.1 is the sample volume (mL), and ε is the dilution fold.

Thus, the conversion rate of D-tagatose can be expressed as follows:

$$\text{Conversion rate (\%)} = \frac{Y}{M} \times 100\%$$

where Y is the D-tagatose content (mg/mL) and M is the lactose content (g/L).

RESULTS AND DISCUSSION

Cloning and Co-expression of β-galactos idase and L-arabinose isomerase

lacZ and *araA* were successfully amplified from *E. coli* K-12 genomic DNA and the bands were obtained at 3100 bp and 1500 bp, respectively (Fig. 2). Then they were inserted into pET-32a(+) vector to construct pET-32a(+)-*lacZ-araA* (Fig. 1). To indentified the recombinant plasmid, it was digested by *Hind*III and *Xho*I. As expected, the bands were obtained at 6800 bp and 3100 bp, respectively. Besides, We also obtained the band at 9900 bp (the sum of the aforementioned bands) when pET-32a- *lacZ-araA* was only digested by *Hind*III, suggesting that the plasmid was constructed successfully (Fig. 2).

Co-expression of β-galactosidase and L-arabinose isomerase

After OD_{600} reached 0.6, the expression was induced by 0.5 mM IPTG and the sample was cultured for another 5 h at 28 °C. Fig. 3 showed that β-galactosidase and L-arabinose isomerase could be co-expressed in the recombinant strain. The molecular weights of L-arabinose isomerase and β-galactosidase were approximately 56 kDa and 116 kDa, respectively. Both target enzyme proteins were mainly soluble with only a few inclusion bodies in the recombinant strain.

To investigate whether or not the expression levels can be improved, several one-factor experiments were conducted under different expression conditions in the subsequent experiments.

Effect of different enzymes on the conversion rate of D-tagatose

Lactose (100 g/L) as the substrate was hydrolyzed in the suspension cells for 96 h at pH 7.0. The conversion rate of D-tagatose was determined by using different enzyme sources of the resuspended cells, including a mixture of fragmental cells, supernatant, and cell precipitate (Fig. 4). The highest conversion rate of D-tagatose could reach 42.6% after 96 h when the resuspended cells were used as the enzyme source.

Effect of temperature on the conversion rate of D-tagatose

To analyze the effect of temperature on the conversion of D-tagatose, in this study, the conversion rate of D-tagatose was detected under the reaction temperature setting within the range of 35 °C to 60 °C for 96 h at pH 7.0. We found that this conversion rate was enhanced in a temperature-dependent manner (Fig. 5). the most effective cooperation of these two enzymes could be got at 50 °C, and obtained the highest D-tagatose conversion rate as 42.8 %.

Table 1. Primers used in this study for the construction of expression plasmids

Primer	Sequences 5→3', restriction enzyme site (underlined)
lacZ (+)	GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGACCATGATT ACG, XbaI, rbs (dotted line)
lacZ (–)	GGAAGCTTTTGACACCAGACCAACTG, HindIII
araA (+)	CCCAAGCTTGAAGGAGATATACCATGACGATTTTTG, HindIII, rbs (dotted line)
araA (-)	CCGCTCGAGTTATTTTTAGCGACGAAACCCGTAATACACTTCGTTC, XhoI

Effect of pH on the conversion rate of D-tagatose

To determine the optimum pH for the D-tagatose biotransformation, the conversion rate of D-tagatose in different pH (5.0 to 9.0) buffer systems was analyzed when lactose (100 g/L) was hydrolyzed by the suspension cells for 96 h at 50 °C. In particular, Na_2HPO_3-KH_2PO_3 (100 mM; pH 5.0 to 6.6) and Tris-HCl (100 mM; pH 7.0, 7.6, 8.0, 8.6, and 9.0) were used as the buffer systems. As shown in Fig. 5, the conversion rate increased as pH increased from 5.6 to 7.0. The pH for the maximum D-tagatose conversion rate was 7.0 and the conversion rate could reach 41.6% after 96 h. At pH < 5.0, the conversion rate decreases to approximately 0. At pH > 7.0, the conversion rate reduced in alkaline conditions. Therefore, 100 mM Tris-HCl at pH 7.0 could be used as the optimum pH in this D-tagatose conversion system.

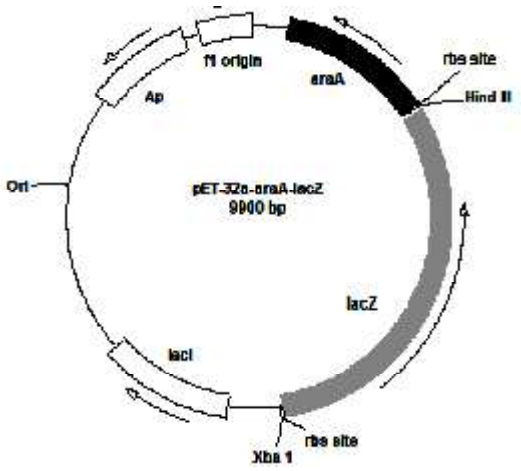

Fig. 1. Map of the tandem recombinant plasmids with *lacZ* and *araA*. *lacZ* and *araA* were successfully amplified from *Escherichia coli* K-12 genomic DNA

Effect of substrate concentration on the conversion rate of D-tagatose

Different concentrations of lactose were hydrolyzed in the suspension cells for 96 h at 50 °C and pH 7.0. The effect of the substrate concentration on the conversion rate of D-tagatose was investigated at lactose concentrations of 25 g/L to 500 g/L. The highest conversion rate of 44% was achieved at a substrate concentration of 25 g/L.

Effect of different divalent metallic ions on the conversion rate of D-tagatose

To confirm the effect of divalent metallic ions on the conversion of D-tagatose, lactose (25 g/L) was hydrolyzed in the suspension cells for 96 h at 50 °C and pH 7.0 with a concentration of 5 mM of different ions, including Mn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, and Cu^{2+}. The results was showed as Fig. 6B, Mn^{2+}, Co^{2+}, and Fe^{2+} could increase the conversion rate of D-tagatose to approximately

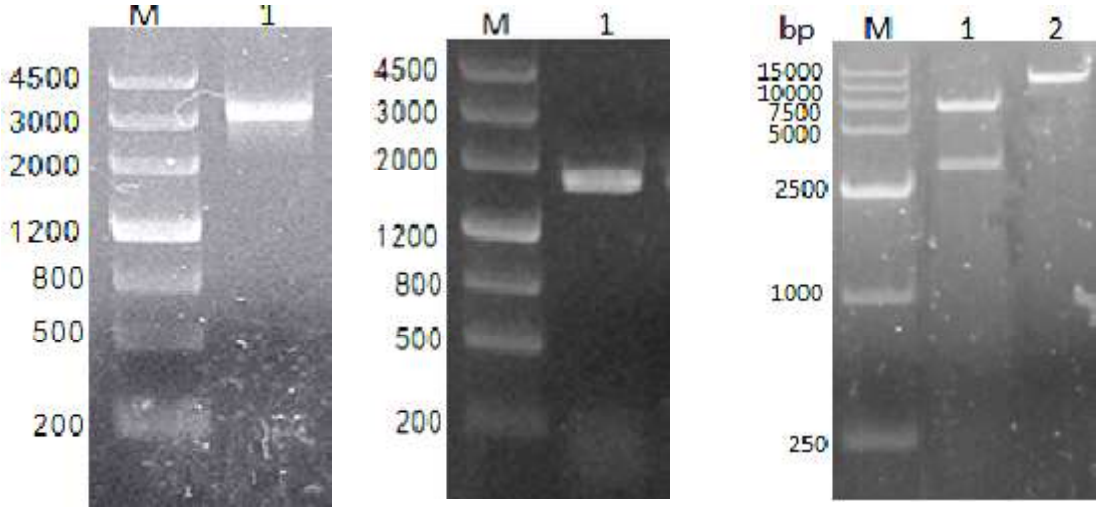

Fig. 2. PCR amplification of *lacZ* and *araA* and identification of the recombinant plasmid. (A) PCR amplification of *lacZ*; Lane M, protein marker; Lane 1, *lacZ*. (B) PCR amplification of *araA*; Lane M, protein marker; Lane 1, *araA*. (C) Identification of recombinant plasmid; Lane M, DNA Marker; Lane 1, pET-32a-*lacZ-araA* digested by the restriction enzyme *Hin*dIII and *Xho*I; Lane 2, pET-32a-*lacZ-araA* digested by the restriction enzyme *Hin*dIII

44%, while Ni^{2+}, Zn^{2+}, and Cu^{2+} evidently decreased the activity.Among the investigated ions. Mn^{2+} is the most effective one to improve the production of D-tagatose. and should be added in the conversion reaction of D-tagatose.

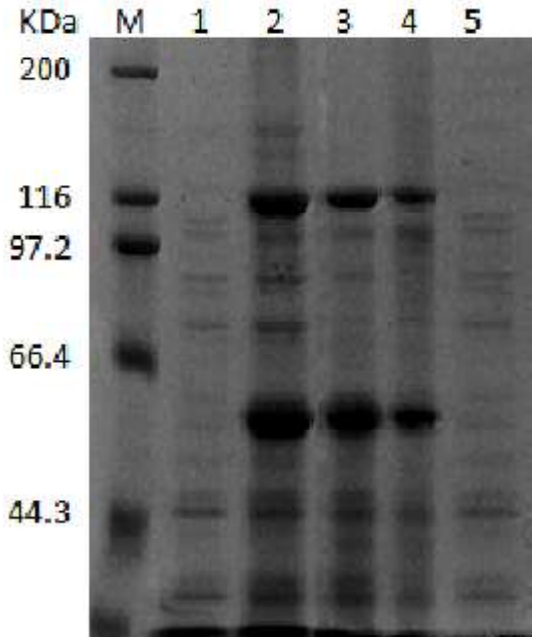

Fig. 3. SDS-PAGE analysis of the recombinant strains with tandem co-expression plasmid. Lane M, protein marker; Lane 1, negative control without induction; Lane 2, recombinant *E. coli* proteins after the sample was induced by 0.5 mM IPTG at 28 °C; Lane 3, supernatant of induced cells after sonication; Lane 4, precipitate of induced cells after sonication; Lane 5, empty vector plasmid of pET-32a induced by 0.5 mM IPTG at 37 °C

In this research, *E. coli* was selected as the expression host because of several advantages, such as well-described genetic background, ability to express high amounts of simple proteins, convenient and established experimental procedure, and other characteristics. The genes that encode L-arabinose isomerase and β-galactosidase in *E. coli* K-12 were tandem cloned into the expression vector pET-32a (+). The T7 lac promoter of pET-32a (+) functioned as an efficient expression promoter which made endogenous genes be more effectively expressed in *E. coli*. After the recombinant plasmid was transformed into *E. coli* BL21 (DE3), L-arabinose isomerase and β-galactosidase were highly expressed simultaneously in the soluble form when the bacteria was induced with 0.5 mM IPTG at 28 °C for 5 h.

Our study also showed the importance of process optimization on the bioconversion efficiency. The highest conversion rate of D-tagatose could reach when the resuspended cells were used as the enzyme source compared with fragmental cells, supernatant, and cell precipitate after sonication, suggesting that the enzymes were expressed in soluble fraction and sonication dereased the enzyme activity. Our previous study revealed that the optimum temperatures of β-galactosidase and L-arabinose isomerase were 50 °C and 60 °C, respectively, and the activity of β-galactosidase in *E. coli* would decrease when the temperature exceeded 50 °C[15-17]. In this study

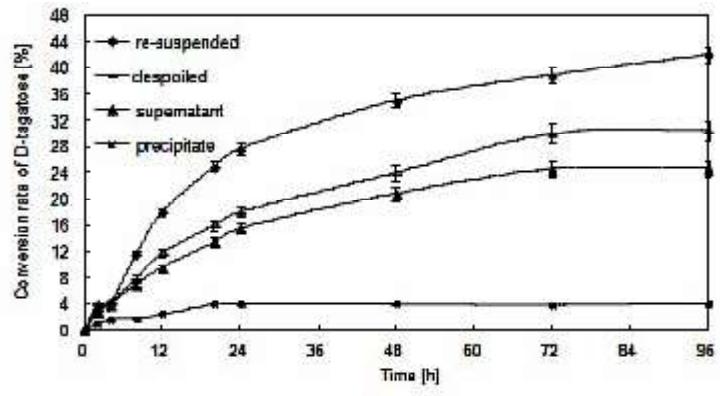

Fig. 4. Effect of different kinds of enzyme on the conversion rate of D-tagatose. Lactose (100 g/L) as the substrate was hydrolyzed in the suspension cells for 96 h at pH 7.0. Conversion rate of D-tagatose was assayed by enzyme source of re-suspended cells (◆), despoiled cells by supersonic (▲), supernatant cells by supersonic (■), and precipitate cells by supersonic (%)

the highest conversion rate of D-tagatose was achieved at 50 °C which is in accordance with the previous result. pH value had significant impact on the conversion efficiency. The optimum pH for cooperation of these two enzymes was at 7.0 which was consistent with the effect of pH on the activity of β-galactosidase or L-arabinose isomerase in the previous studies[18]. The effect of substrate concentration to the conversion showed that the degradation of lactose was limited by viscosity and the solubility of lactose. The high concentration of lactose produced a high viscosity, which resulted in a high solute transfer resistance and decreased diffusibility of lactose and D-tagatose. These changes caused the decrease in the degradation rate of lactose. The effect of divalent metallic ions on the conversion rate of D-tagatose was also investigated in this work. Mn^{2+} is the most effective metallic ion to improve the production of D-tagatose because Mn^{2+} is a co-factor for conversion of D-tagatose in certain thermophilic bacteria at a high reaction temperature of 50 °C [19]. Mn^{2+} could enhance the reaction rate of β-galactosidase or L-arabinose isomerase as observed in previous tests. Finally the highest conversion rate of 44% was achieved under the optimum conditions (50 °C, pH 7.0, with Mn^{2+} and at a substrate concentration of 25 g/L).

An industrial-grade lactose costs approximately $1800/MT, which is only one-twentieth of the price of D-galactose[20]. Thus, the direct conversion of D-tagatose from lactose by using β-galactosidase and L-arabinose isomerase engineered bacteria could significantly reduce the

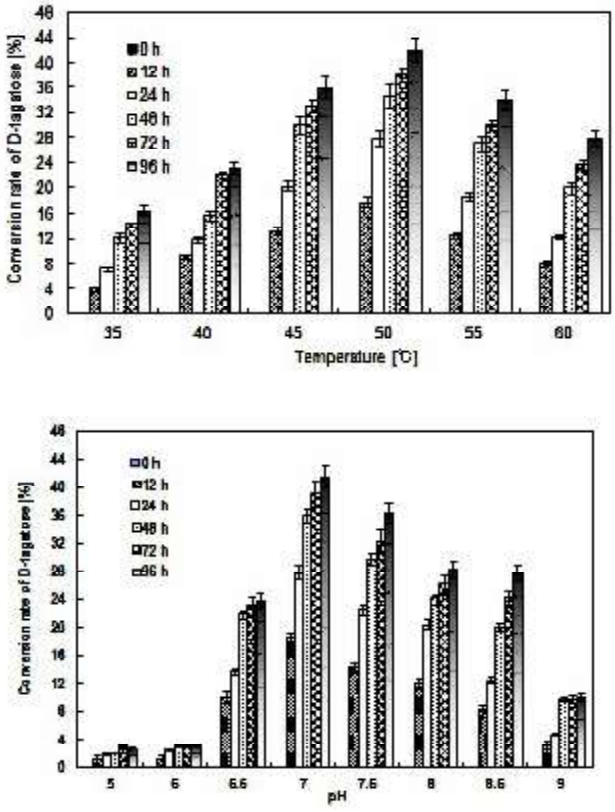

Fig. 5. Effect of different temperatures and pH on the conversion rate of D-tagatose. (A) Lactose (100 g/L) as the substrate was hydrolyzed in the suspension cells for 96 h at pH 7.0. The reaction temperature (35 °C to 60 °C) was used and the conversion rate of D-tagatose was compared. (B) Lactose (100 g/L) as the substrate was hydrolyzed in the suspension cells for 96 h at 50 °C. The conversion rate of D-tagatose was analyzed in the buffer systems at pH 5.0 to 9.0. Na_2HPO_3-KH_2PO_3 (100 mM; pH 5.0 to 6.6) and Tris-HCl (100 mM; pH 7.0, 7.6, 8.0, 8.6, and 9.0) were used as the buffers

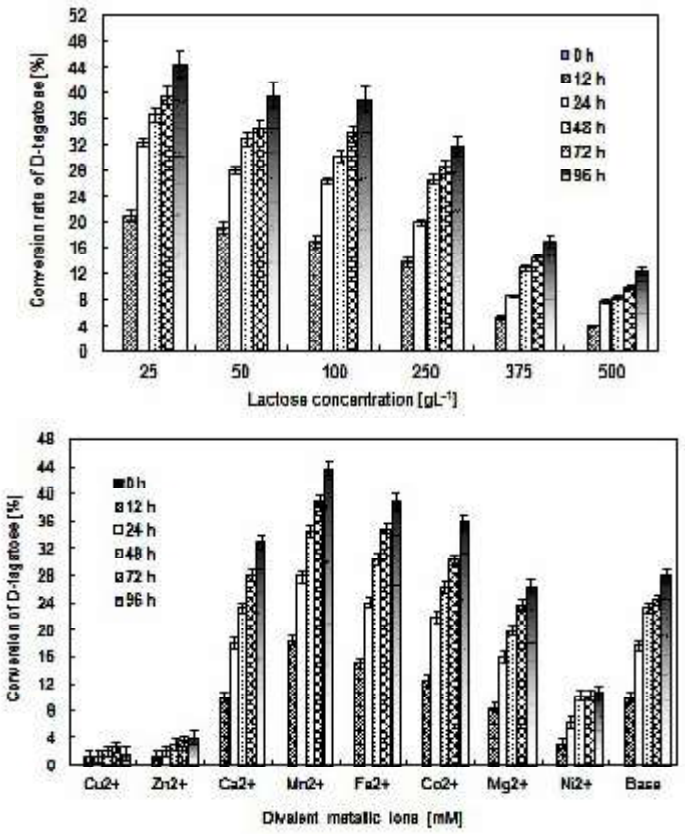

Fig. 6. Effect of different concentrations of substrate and divalent metallic ions on the conversion rate of D-tagatose. (A) Lactose as the substrate was hydrolyzed in the suspension cells for 96 h at pH 7.0 and 50°C. The effect of substrate concentration on the conversation rate of D-tagatose was investigated at lactose concentrations of 25 g/L to 500 g/L. (B) Lactose (25 g/L) as the substrate was hydrolyzed in the suspension cells for 96 h at pH 7.0 and 50 °C. The effect of metal ions on the conversion rate of D-tagatose was investigated at an ion concentration of 5 mM (Mn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, and Cu^{2+})

production cost by approximately 90% at almost the same conversion rate compared with other biological methods of D-tagatose production that uses D-galactose. The proposed method should be recommended for future commercial production of D-tagatose.

ACKNOWLEDGMENTS

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