

D-Tagatose production by *Lactococcus lactis* NZ9000 Cells Harboring *Lactobacillus plantarum* L-arabinose Isomerase

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ABSTRACT

D-tagatose is a functional sweetener present in medicine, food, and dairy products and with broad market prospects, and L-arabinose isomerase gene (*araA*) can mediate the bioconversion of D-galactose into D-tagatose. In this study, a *Lactococcus lactis* NZ9000 strain harboring exogenous L-arabinose isomerase was constructed to produce D-tagatose. *Lactobacillus plantarum* CGMCC 8198 exhibits L-arabinose isomerase activity and its genome has been sequenced. The *araA* gene of *Lactobacillus plantarum* CGMCC 8198 encoding L-arabinose isomerase was identified by sequence analysis and was successfully cloned and expressed in *Lactococcus lactis* NZ9000. The D-tagatose production by the whole cell of the recombinant strain was optimized. The optimal condition for conversion reaction was at 50°C, pH 7.0 and with 300 mmol/L Mn²⁺ and 60 g/L galactose added. The D-tagatose yield and conversion rate at the optimal condition was determined and reached 40.2 g/L and 67%, respectively.

Key words: D-tagatose, L-arabinose isomerase, *Lactococcus lactis* NZ9000, *Lactobacillus plantarum*, Galactose.

INTRODUCTION

D-tagatose, a unique and rare hexoketose monosaccharide, is a C4 epimer of D-fructose and an isomer of aldohexose D-galactose. D-tagatose naturally occurs in a small amount of *Sterculia setigera* gum¹ and dairy products,² such as hot cocoa,³ various cheeses and yogurts,⁴ ultra-high-temperature (UHT) lactose-hydrolyzed milk.⁵ The sweetness of D-tagatose is almost equivalent to sucrose but with a lower calorific value at 1.5 kcal/g for humans.⁶ Furthermore, it has many health and medical benefits, such as antioxidant, prebiotic, and tooth friendly properties. D-tagatose has the GRAS (Generally Recognized as Safe) status granted by the US Food and Drug Administration (FDA), and was currently tested as a potentially important new drug for treating type II diabetes and obesity.^{7,8,9} In recent years,

D-tagatose has been drawing great attention as a non-caloric sweetener substitute for sucrose,^{10,11,12} as well as considerable commercial use.

D-tagatose can be synthesized from D-galactose through chemical or enzymatic way. The production of D-tagatose was first achieved by chemical method, using calcium catalyst and strong acid.¹³ However, the chemical method has many drawbacks, such as byproduct, chemical waste formation and high energy-consuming.¹⁴ In the enzymatic method, L-arabinose isomerase (L-AI) is the most efficient enzyme for isomerizing D-galactose to D-tagatose.¹⁵ Generally, a major consideration in biotransformation processes is the development suitable biological catalysts.^{16,17} L-AI from various prokaryotic microbes have been identified,

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including those from *Escherichia coli*,¹⁸ *Lactobacillus plantarum*,^{19,20} *Lactobacillus fermentum*,²¹ *Arthrobacter sp.*,²² *Lactobacillus sakei*,²³ *Bifidobacterium longum*,²⁴ and *Thermus sp.* Strain.²⁵

Lactococcus lactis is one of the best-characterized GRAS microorganisms and an efficient expression host for the heterologous proteins. It is a good candidate for different biotechnological applications related to food products, such as D-tagatose production. This paper reports the expression of L-arabinose isomerase gene (*araA*) from *Lactobacillus plantarum* CGMCC 8198 in *Lactococcus lactis* NZ9000. The result shows that recombinant *L. lactis* cells, harboring the *Lactobacillus plantarum* L-AI enzyme, produce D-tagatose efficiently after the conversion condition optimization.

MATERIALS AND METHODS

Plasmid and strains

Lactobacillus plantarum CGMCC 8198, *Lactobacillus acidophilus* 1.1859, *Lactobacillus acidophilus* 1.2686, *Lactobacillus casei* 1.2435, *Lactobacillus casei* 1.539, *Lactobacillus sake* 23K, *Bifidobacterium infantis*, *Bifidobacterium bifidum* ATCC29521, were stored at laboratory and cultured anaerobically at 37°C in MRS broth. *Escherichia coli* DH5 α was used as a host for the construction of expression vectors, which was grown in LB medium and supplemented with ampicillin (100 μ g/mL) at 37°C with rotary shaking at 200 rpm or on LB medium solidified with 1.5% (w/v) agar. *L. lactis* NZ9000 was cultivated at 30°C in M17 medium with 2% (w/v) glucose. Nisin, 10 ng/mL, was used for inducing the expression of L-AI. Chloramphenicol was used at a concentration of 10 μ g/mL for the selection of the expressed vector pCYT (China Pharmaceutical University).

Selection of strain with high L-AI activity

8 *Lactic acid bacteria* strains were cultured for 24 h and harvested by centrifugation (10000 g for 20 min). The cells were suspended by 10 mL sodium phosphate buffer (200 mmol/L pH7.0), disrupted by sonication (150 W; working time, 4 s; interval, 4 s; cooling temperature, 4°C), and centrifuged with 10000g for 1 min at 4°C. The supernatant was collected and used as crude enzyme.

0.5 mL crude enzyme and 1.0 mL galactose (500 mmol/L) was reacted for 1 h at 60°C. And then the reaction was terminated with 0.5 mL HCl (100 mmol/L). The generated D-tagatose was determined by the cysteine-carbazol-sulfuric acid method, with the absorbance of 560 nm. The concentration of D-tagatose was calibrated on the basis of the standard linear graph prepared using

different D-tagatose concentrations. One unit of L-AI activity was defined as the amount of enzyme catalyzing the formation of D-tagatose (1 μ g/min) under optimal conditions.

Cloning of *araA* gene from *Lactobacillus plantarum* CGMCC 8198 and construction of recombinant plasmid

The genome of *Lactobacillus plantarum* CGMCC 8198 has been sequenced in our previous study, and its *araA* was identified by gene annotation. The oligonucleotides P1 (5'-CCAATGCATCAATGTTATCAGTACCTGAT-TATGAGT-3') and P2 (5'-ATCTGTCTAAGCITTT-TACTTTAAGAATGCCTTAGTC-3') were used for PCR amplification of *araA* gene. The restriction endonuclease *Nsi*I and *Hind*III sites were incorporated into the primer P1 and P2 respectively. The purified PCR product were digested with *Nsi*I and *Hind*III and inserted into pCYT. Sequentially, the resulted plasmid pCYT-*araA* was transformed into *E.coli* DH5 α for cloning and then isolated and transformed into *L. lactis* NZ9000 for expression.

Overexpression of L-AI isomerase in *L. lactis* NZ9000

The obtained recombinant strain *L. lactis* NZ9000-pCTY-*araA* was inoculated to 10 mL of GM17 medium supplemented with 10 mg/mL chloramphenicol. 10 ng/mL nisin was added to induce the protein expression when OD₆₀₀ reached 0.6. After the nisin was added, the culture was cultivated at 30°C overnight. The cells were harvested by centrifugation (10000 g for 20 min), resuspended by 10 mL 200 mM sodium phosphate buffer (pH 7.0), and disrupted by sonication (150 W; working time, 4 s; interval, 4 s; and cooling temperature, 4°C). The supernatant was obtained and treated as crude enzyme. The L-AI activity of the crude enzyme was determined as description above and the protein expression was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Effect of temperature, pH, metal ions, and substrate concentration on tagatose production

L. lactis NZ9000-pCTY-*araA* was cultured and induced as description above, and the cells were harvested and resuspended by 200 mmol/L sodium phosphate buffer (pH7.0) to OD₆₀₀ of 0.6 for D-tagatose production. The basic reaction condition was as follows: the reaction system was supplemented with 100 g/L D-galactose, 100 mmol/L Mn²⁺ and incubated at 50°C for 96 h.

The D-tagatose production was measured when the reaction proceeded for 48 h-120 h as description above. The effect of temperature was determined by measuring the D-tagatose production after the reaction buffer was incubated at different temperature (30-70°C) at

pH7.0 for 96 h. The effect of pH was determined by measuring the D-tagatose production when the reaction was at pH 6.0-9.0 using sodium phosphate buffer incubated at 50°C for 96 h. The effect of metal ion was determined by measuring the concentration of D-tagatose when the reaction system was added with 100 mmol/L of Mg^{2+} , Ca^{2+} , Cu^{2+} , Fe^{2+} , K^{+} , Li^{+} , Ni^{2+} , Zn^{2+} , Co^{2+} , Mn^{2+} respectively and incubated at pH7.0 and 50°C for 96 h. Additionally, the effect of substrate and Mn^{2+} concentration to D-tagatose yield was determined by adding galactose with various concentration of 20-100 g/L and Mn^{2+} with various concentration of 50-500 mmol/L.

Multifactor optimization of the D-tagatose production

The condition of D-tagatose production by *L. lactis* NZ9000-pCTY-*araA* cells was optimized. The experimental design is shown in Table 1. Design Expert 8.0 software was used for constructing the experiment designs and performing the statistical analysis. The four variables used were temperature between 40 and 60°C, pH between 6.0 and 8.0, substrate concentration between 20 and 100 g/L, and Mn^{2+} concentration between 100 mmol/L and 500 mmol/L. The D-galactose yield and the conversion ratio of D-galactose to D-tagatose were calculated and all the samples were incubated for 96 h.

RESULTS

Cloning and sequence analysis of gene encoding *araA* from *Lactobacillus plantarum* CGMCC 8198

The crude L-AI activity of 8 wild Lactic acid bacteria was determined, and the result was shown in Figure 1. *Lactobacillus plantarum* CGMCC 8198 exhibits the highest activity of L-AI and was chosen as the donor of *araA* gene (Figure 1). The *araA* gene of 1450 bp encoding 483 amino acid from *L. plantarum* CGMCC 8198 was successfully cloned. The gene exhibited homology of 99% to that from *Lactobacillus plantarum* strain JBE245 (EMBL accession number CP014780) and *Lactobacillus plantarum* strain ZS2058 (EMBL accession number CP012343), 95% to that from *Lactobacillus paraplantarum* strain L-ZS9 (EMBL accession number CP013130) and 86% to that from *Lactobacillus pentosus* MP-10 (EMBL accession number FR871824). The sequence of *araA* was shown in supplementary material Text S1.

Overexpression of L-AI in *L. Lactis* NZ9000

L-AI from *L. plantarum* CGMCC 8198 was ligated with pCYT to construct the recombinant expression plasmid pCYT-*araA* (Figure 2). L-AI expression strain *L. lactis*

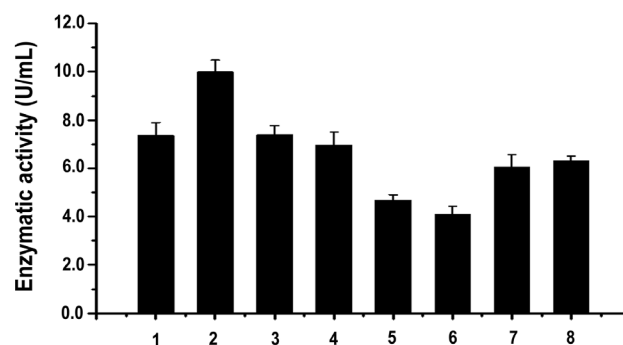


Figure 1: The L-AI activity of 8 lactic acid bacteria. 1: *Lactobacillus casei* 1.539; 2: *Lactobacillus plantarum* CGMCC 8198; 3: *Lactobacillus acidophilus* 1.2686; 4: *Lactobacillus acidophilus* 1.1859; 5: *Bifidobacterium. Infantis*; 6: *Lactobacillus sakei* 23K; 7: *Lactobacillus casei* 1.2435; 8: *Bifidobacterium bifidum* ATCC 29521.

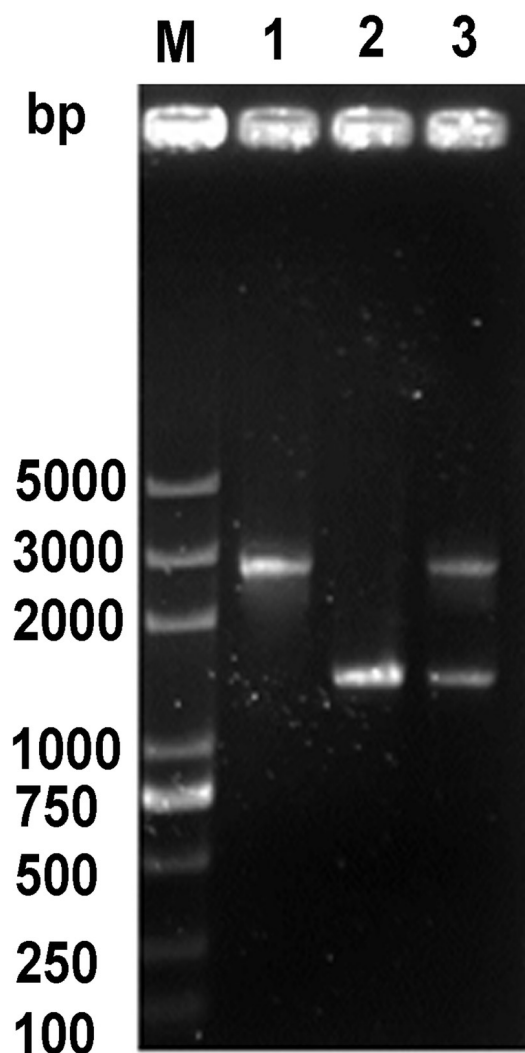


Figure 2: PCR detection of pCYT-*araA*. M: Marker; 1: pCYT digested by *NsiI* and *HindIII*; 2: *araA* amplified by PCR; 3: pCYT-*araA* digested by *NsiI* and *HindIII*.

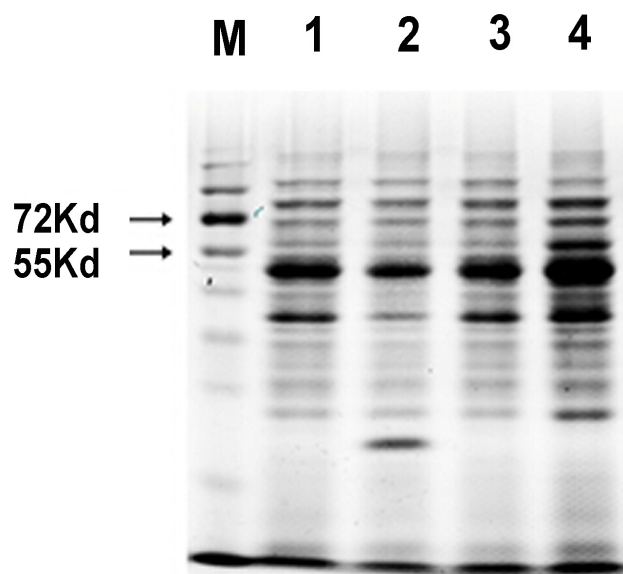


Figure 3: SDS-PAGE analysis of the expression of AraA by *L. lactis* NZ9000. M: Marker; 1: *L. lactis* NZ9000 with pCYT without induction by ninsin; 2: *L. lactis* NZ9000 with pCYT with induction by ninsin; 3: *L. lactis* NZ9000 with pCYT-araA without induction by ninsin; 4: *L. lactis* NZ9000 with pCYT-araA with induction by ninsin.

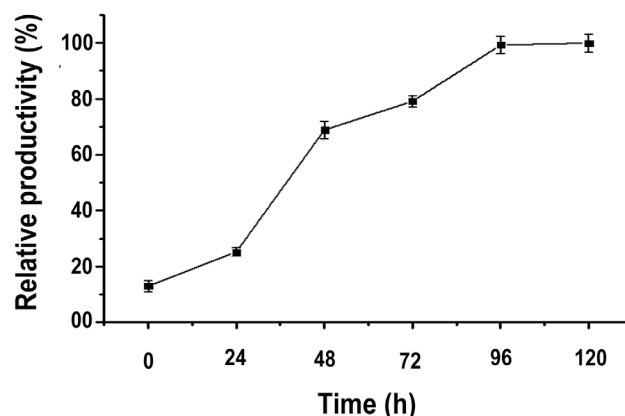


Figure 5: The relative productivity of D-tagatose at different time.

NZ9000-pCTY-araA was successfully constructed by inducing pCYT-araA harboring L-AI from *L. plantarum* CGMCC 8198 to *L. lactis* NZ9000. The expression of the protein was detected by 10% SDS-PAGE (Figure 3). As expected, the results showed that the L-AI with theoretical molecular weight of 53kD was successfully expressed in the host. The activity of L-AI was detected in the supernatant of cell lysate and reached 47 U/mL.

Effect of temperature, pH, metal ions, and substrate concentration on tagatose production

The influence of the different factors on D-tagatose production by the recombinant strain was shown in Figure 4. The optimum temperature for the D-tagatose production was 50°C (Figure 4A) and the optimum pH was 7.0 (Figure 4B). Compared with other ions, the addition of Mn^{2+} dramatically promoted the reaction (Figure 4C). The effect of Mn^{2+} concentration was also investigated, and the result showed that the highest yield was obtained when 200 mmol/L Mn^{2+} was added (Figure 4D). The substrate concentration not only affected the yield of D-tagatose but also affected the conversion rate. Figure 4E&F showed that the D-tagatose yield was improved when the addition of galactose increased from 20 g/L to 100 g/L. However, the conversion rate reached the peak when 50 g/L galactose was added. Figure 5 showed that the D-tagatose production was continuously increasing from 0 h to 96 h, and the yield at 96 h and 120 h has no remarkable difference. So, 96 h was chosen as the optimum reaction time.

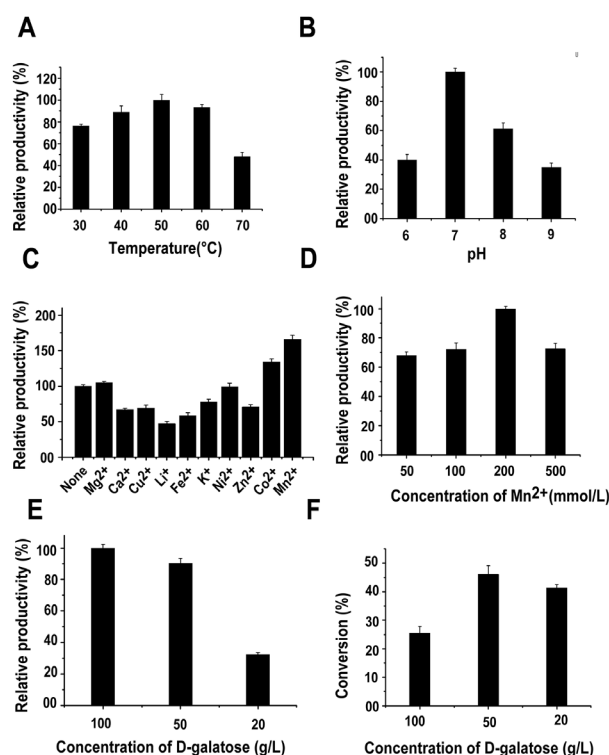


Figure 4: Effect of temperature, pH, metal ions, and substrate concentration on tagatose production. A: The relative productivity at different temperature; B: The relative productivity at different pH; C: The relative productivity with different ions added; D: The relative productivity with different concentration of Mn^{2+} added; E: The relative productivity with different concentration of D-galactose added; F: The conversion with different concentration of D-galactose added.

Multifactor optimization of the D-tagatose production

The experimental design of multifactor optimization is shown in Table 1. Finally, the D-tagatose production by *Lactococcus lactis* NZ9000 reached the highest when the bioconversion was at 50°C, pH 7.0, and with 60 g/L D-galactose and 300 mmol/L Mn^{2+} added. The highest

Table 1: Experiment design and results of multifactor optimization.

Std	Run	A:Temperature (°C)	B:pH	C: Mn ²⁺ (mmol/L)	D: Substrate concentration (g/L)	Productivity (g/L)	Conversion (%)
19	1	40.00	7.00	500.00	60.00	21.8	36.4
23	2	50.00	6.00	300.00	100.00	25.0	25.0
1	3	40.00	6.00	300.00	60.00	23.6	39.3
12	4	60.00	7.00	300.00	100.00	28.2	28.2
2	5	60.00	6.00	300.00	60.00	25.7	42.9
7	6	50.00	7.00	100.00	100.00	21.3	21.3
16	7	50.00	8.00	500.00	60.00	25.5	42.5
17	8	40.00	7.00	100.00	60.00	20.7	34.6
9	9	40.00	7.00	300.00	20.00	10.8	54.4
27	10	50.00	7.00	300.00	60.00	40.2	67.0
15	11	50.00	6.00	500.00	60.00	23.3	38.9
25	12	50.00	7.00	300.00	60.00	36.3	60.5
20	13	60.00	7.00	500.00	60.00	25.5	42.5
5	14	50.00	7.00	100.00	20.00	10.1	50.6
26	15	50.00	7.00	300.00	60.00	37.9	63.2
14	16	50.00	8.00	100.00	60.00	32.8	54.7
10	17	60.00	7.00	300.00	20.00	11.2	56.1
3	18	40.00	8.00	300.00	60.00	25.5	42.5
29	19	50.00	7.00	300.00	60.00	38.6	64.4
28	20	50.00	7.00	300.00	60.00	40.2	67.0
22	21	50.00	8.00	300.00	20.00	13.8	69.4
24	22	50.00	8.00	300.00	100.00	27.3	27.3
21	23	50.00	6.00	300.00	20.00	12.5	62.5
18	24	60.00	7.00	100.00	60.00	24.7	41.2
8	25	50.00	7.00	500.00	100.00	28.2	28.2
4	26	60.00	8.00	300.00	60.00	27.8	46.4
13	27	50.00	6.00	100.00	60.00	17.3	28.9
6	28	50.00	7.00	500.00	20.00	10.3	51.6
11	29	40.00	7.00	300.00	100.00	20.7	20.7

production was 40.2 g/L and the conversion of D-galactose to D-tagatose was 67%.

DISCUSSION

D-tagatose is a functional sweetener present in food,²⁶ dairy products and medicine with broad market prospects.²⁷ The aim of this work was to investigate the possibility of producing D-tagatose from D-galactose with the recombinant *Lactococcus lactis* NZ9000 cells harboring *araA* from lactic acid bacteria. As described previously, the recombinant strain was successfully constructed and D-tagatose is produced efficiently after optimization.

The *araA* was derived from *L. plantarum* CGMCC 8198 whose genome has been sequenced and exhibiting L-AI activity. The sequence of the L-AI protein exhibited high homology to that of other L-AIs, suggesting that these genes might have evolved from a common ancestor. The mesophilichomo-fermentative bacterium *Lactococcus lactis* NZ9000 is an important industrial microorganism,²⁸ which has GRAS (generally regarded as safe) status. The availability of technology for genetic and metabolic engineering of *Lactococcus lactis* NZ9000 combined with a long history of safe usage opened opportunities for applications in modern biotechnological applications especially as a heterologous expression host.²⁹ So, *Lactococcus lactis* NZ9000 is a good candidate

to express L-AI from lactic acid bacteria to produce D-tagatose.

In this study, we use the whole cell of the recombinant strain to produce D-tagatose, which make the production process more facility. It is also the first report to produce D-tagatose by *Lactococcus lactis* NZ9000. The conversion reaction performed at high temperatures offers several advantages such as high conversion yield and fast reaction rate, and the moderate-high temperature is appropriate in industry production. The optimum temperature of the conversion reaction by the recombinant strain was 50°C, suggesting that it has potential in industrial use. The optimum pH of the conversion reaction was 7.0, which is accordant with the most L-AIs reported. The reaction converting aldoses into corresponding ketoses often uses Mn^{2+} as a cofactor and in this study Mn^{2+} was determined to indeed enhance the D-tagatose yield.

By multifactor optimization, the optimal condition of D-tagatose production was obtained, and the yield and conversion rate were determined. Also the productivity is relatively low compared with some recombinant *E. coli* strains reported, *Lactococcus lactis* NZ9000 has its own advantage. As a GRAS strain it is more suitable to produce D-tagatose which is mainly used in medicine and food area. However, in this study, the antibiotics chloramphenicol still should be added in the media in order to maintain the recombinant plasmid, and in the future we will attempt to integrate the exogenous gene into the genome of *Lactococcus lactis* NZ9000 to avoid the usage of antibiotics.

CONCLUSION

D-tagatose has broad market prospects in medicine and food industry. In this study we constructed a recombinant D-tagatose producing strain by introducing L-arabinose isomerase from *Lactobacillus plantarum* to *Lactococcus lactis* NZ9000. After multifactor optimization, the D-tagatose yield and conversion rate reached 40.2 g/L and 67% respectively. This study is the first report to produce D-tagatose by *Lactococcus lactis* NZ9000.

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CONFLICT OF INTEREST

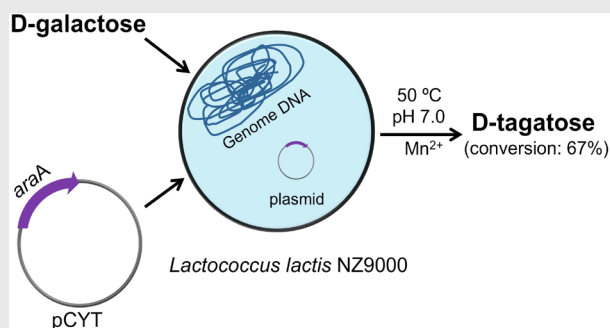
No conflict of interest are declared.

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PICTORIAL ABSTRACT



SUMMARY

- A D-tagatose producing strain was constructed by introducing L-arabinose isomerase gene from *Lactobacillus plantarum* to *Lactococcus lactis* NZ9000.
- After multifactor optimization, the D-tagatose yield and conversion rate reached 40.2 g/L and 67% respectively.
- This study is the first report to produce D-tagatose by *Lactococcus lactis* NZ9000.

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