Proc. Int'l Symp. Microorganisms and Health
April 28~29, 2000, Seoul, Korea

Enzymatic Production of D-Tagatose, a Sugar-substituting Sweetener, from D-Galactose

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Abstract

D-Tagatose is a potential bulking agent in food as a non-calorific sweetener. To produce D-tagatose from cheaper resources, plasmids harboring the L-arabinose isomerase gene (*araA*) from *Escherichia coli* was constructed because L-arabinose isomerase was previously suggested as an enzyme that mediates the bioconversion of galactose to tagatose as well as that of arabinose to ribulose. In the cultures of recombinant *E.coli* with pTC101, which harboring *araA* of *E.coli*, tagatose was produced from galactose in 9.9 % yield. The enzyme extract of *E.coli* containing pTC101 also converted galactose into tagatose in 96.4 % yield. For the economic production of D-tagatose, an L-arabinose isomerase of *E.coli* was immobilized using covalent binding on agarose. While the free L-arabinose isomerase produced tagatose with the rate of 0.48 mg/U·day, the immobilized one stably converted galactose into average 7.5 g/l·day of tagatose during 7 days with higher productivity of 0.87 mg/U·day. In the scaled up immobilized enzyme system, 99.9 g/l of tagatose was produced from galactose with 20 % equilibrium in 48 hrs. The process was stably repeated additional 2 times with tagatose production of 104.1 and 103.5 g/l.

Introduction

A rare ketohexose, D-tagatose is one of the D-galactose isomers. D-tagatose is reported as having the sweetness (0.92) and taste most similar to that sucrose. In addition, D-tagatose does not show laxative effect whereas other polyols do. For those reasons, D-tagatose has received interests recently as a non-caloric sweetener substitution to sucrose[1, 2].

Several methods have been studied for the manufacture of D-tagatose. D-Galactose could be converted to D-tagatose by a calcium catalyst[3]. Although the chemical synthesis is economical process, this process also has disadvantageous high temperature and high pressure. Recently, the biological process has been of interest for an environmentally clean process. The biological production of D-tagatose as a consequence has been intensely studied in the recent years. Among such studies, D-tagatose production from galactitol using galactitol dehydrogenase is well known[4-6]. Galactitol, however, is more expensive than galactose and seems to have only little potential to commercial application. It has been suggested that L-

arabinose isomerase (AraA) could convert galactose to tagatose (Fig.1). Cheetham reported that L-arabinose isomerase of *Mycobacterium* and *Lactobacillus* catalyzed the D-galactose to D-tagatose conversion as well as the L-arabinose to L-ribulose conversion because of the similar substrate configuration[7]. *Enterobacter agglomerans* could also produce tagatose from galactose when grown on an arabinose pre-induced medium[8]. This implied that arabinose isomerase could mediate the conversion of tagatose.

Results and Discussions

PCR and Recombinant Strain Development

An L-arabinose isomerase gene (*araA*) from *Escherichia coli* was cloned using PCR-techniques. The primers were designed based on the sequence of *araA*, and containing restriction enzyme sites at the end of start and stop code for insertion into the expression vectors. The pKK223-3 vector containing *araA* from *E.coli* was named pTC101. After double digestion of PCR product and vector by the designated restriction enzyme set, the different mixtures were ligated and transformed into *E.coli* JM105. Single colonies were selected on the ampicillin-containing medium and the constructs were confirmed by restriction enzyme digests.

Tagatose Conversion by araA Expressing E.coli cells

Single colony of JM105 harboring pTC101 was inoculated into the 3 ml of LB-ampicillin medium. After 12 hrs of cultivation at 37°C (250 rpm), the cells were transferred to 50 ml of LB-medium, 1mM IPTG, for the induction of *araA*. When the biomass reached an O.D.≈ 2.0, the cells were washed with 0.8% saline solution twice and further transferred to the conversion medium. These conversions mixtures were maintained at 37°C (250 rpm) in shaking incubator for 72 hrs. Table 1 shows the results, that *E.coli* JM105 did not produce tagatose in the galactose-containing medium, whereas *E.coli* expressing L-arabinose isomerase produced tagatose from galactose. The final concentration obtained of tagatose was 0.86 g/l.

Preveously, Cheetham and Wotton[7] mentioned that L-arabinose isomerase from *Mycobacterium* and *Lactobacillus* mediated the conversion of galactose to tagatose as well as of arabinose to ribulose of similar substrate configuration. The above result also indicates that AraA of *E.coli* mediates the galactose to tagatose conversion. Due to other metabolisms such as the formation of organic acid, the yield of tagatose was very low (below 10%). To improve this yield, reaction with a crude enzyme was carried out.

Tagatose Production by a Crude Extract of an E.coli Transformant harboring AraA

The crude AraA solution from *E.coli* was prepared from an IPTG (1mM) induced *E.coli* JM105 harboring pTC101 (50 ml, Abs.=2.0) harvested by centrifugation and disrupted by

sonication. The crude enzyme solution was obtained after removal of cell debris by centrifugation. A 10 ml of reaction mixture was made by 1 g/l of galactose, 1 mM $MnCl_2$, and 2 ml of crude enzyme solution in 50 mM phosphate buffer (pH 7.0). The reaction was maintained at 37°C for 5 days.

As shown in Fig. 2, tagatose (0.27 g/l) was produced by consumption of 0.28 g/l galactose after 5 days. The consumed galactose converted into tagatose in a yield of 96.4% by AraA, while in a control experiment, no tagatose was formed by using an extract of *E.coli* JM105 (data not shown). No cell growth was found because the medium contained antibiotics. Apparently AraA was not extracted efficiently because the final titer was lower than that found in a whole cell conversion experiment (0.86 g/l).

From these results, direct bioconversion of tagatose from galactose was made without any pre-induction by arabinose. In addition, tagatose could be produced with high yield even by crude AraA solution. Although the final content of tagatose was relatively low (0.27 g/l) and reaction was very slow to apply commercially, there is a potential to obtain higher amounts of tagatose by increasing the amount of AraA present by using a high-expression system and by enzyme immobilization.

Partial Purification of L-Arabinose Isomerase and Tagatose Conversion Thereby

The L-arabinose isomerase of *E.coli* was prepared from a recombinant *E.coli* JM105/pTC101. The 3-liter culture broth of $O.D._{600}$=12.4 was harvested, which yield 11.5-g cell. After procedures of disruption, ammonium sulfate precipitation between 40-60 % saturation, and dialysis, a 100 ml L-arabinose isomerase solution was obtained. The enzyme solution showed protein concentration of 1.08 mg/ml and volumetric L-arabinose isomerase activity of 43.0 U/ml. This enzyme solution was used for the further conversion experiments.

Tagatose production profile by free L-arabinose isomerase was traced from 50 ml of galactose medium (100 g/l, pH 7.0) for 7 days (Fig. 3). Free L-arabinose isomerase of 430 U converted galactose into tagatose of 19.5 g/l at first 24 hrs. The amount of tagatose production per a day, however, decreased drastically. Tagatose production was nearly stopped after the 6^{th} day. Altogether, 28.8 g/l of tagatose was produced, and the overall productivity was 0.48 mg/U·day.

Stability of the Immobilized L-Arabinose Isomerase

Stability of tagatose production by immobilized L-arabinose isomerase was investigated. L-Arabinose isomerase of 430 unit was used for immobilization Immobilized L-arabinose isomerase produced tagatose of 7.4-7.7 g/l for 7 days. Though the tagatose production rate of the first day by an immobilized enzyme was lower than free one, production was stable

at least 7 days. Table 2 represents the comparison of tagatose production between free and immobilized L-arabinose isomerase.

Tagatose Production

For the high production of tagatose, the galactose medium of 500 g/l was applied on the immobilized L-arabinose isomerase system of higher scale (Fig. 3). L-Arabinose isomerase of 2150 unit was used for the immobilization. In this case, the fresh medium was exchanged every 48 hrs for 3 times. In the first batch, 99.9 g/l of tagatose was produced (Fig. 4). The second and the third batch also yield 104.1 g/l and 103.5 g/l, respectively. The overall productivity was 1.19 mg/U·day.

References

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Table 1. Bioconversion of tagatose by AraA expressing cell.

Strain	[1]*Biomass Increase (Δ O.D.$_{600nm}$)*	*D-galactose consumed (g/l)*	*D-tagatose produced (g/l)*	[2]*Yield (%)*
JM105	0.47	8.8	0	0
JM105/ pTC101	0.95	8.7	0.86	9.9

[1] Difference between final and initial O.D.

[2] Produced tagatose per consumed galactose

Table 2. Effect of immobilization of L-arabinose isomerase on tagatose production

	Tagatose (g/l)							Productivity (mg/U·day)
	1st	2nd	3rd	4th	5th	6th	7th	
[1]Free enzyme	19.5	6.0	2.1	0.8	0.3	0.1	0	0.48
[2]Covalent binding	7.4	7.6	7.5	7.7	7.4	7.4	7.5	0.87

[1]The reaction mixture was sampled every 24 hrs instead change the fresh medium. Enzyme of 430 U was used. The data are expressed the amount of tagatose per a day.

[2]The fresh media were added by changes the reservoir every 24 hrs. Enzyme of 430 U was used.

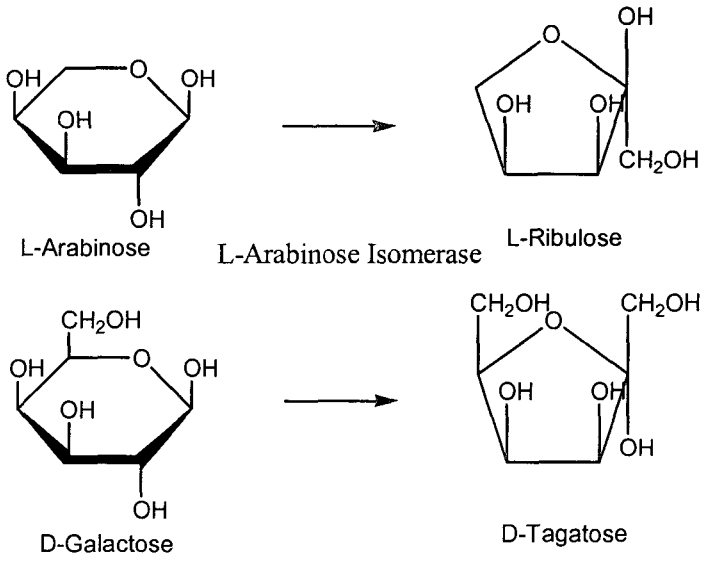

Figure 1. Isomerization of L-arabinose and D-tagatose by L-arabinose isomerase

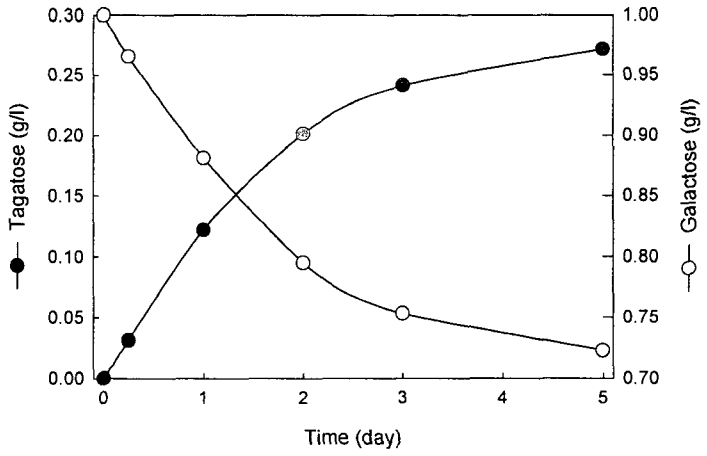

Figure 2. Bioconversion of tagatose from galactose by AraA extract.
Tagatose (●), Galactose (O)

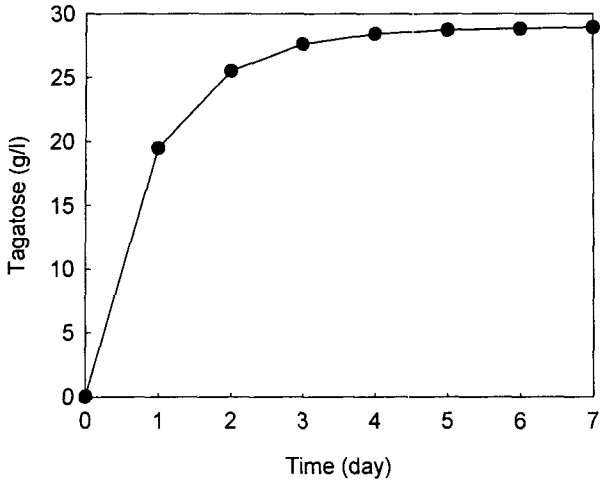

Figure 3. Bioconversion of D-galactose into D-tagatose by L-arabinose isomerase

The initial galactose concentration was 100 g/l. Temperature and pH were maintained at 30°C and 7.0, respectively. The enzyme activity of 430 U was used.

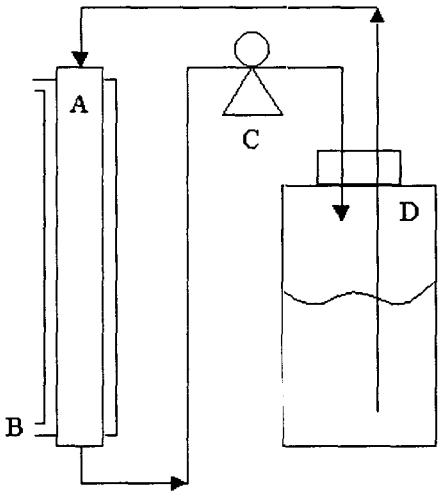

Figure 4. Schematic diagram of immobilized enzyme column for tagatose production

A: column including immobilized L-arabinose isomerase; B: water circulation jacket for temperature control; C: micropump; D: reservoir for fresh medium

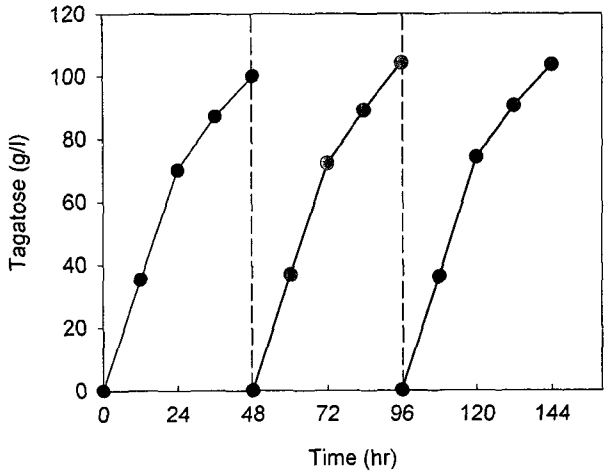

Figure 5. Production of tagatose by immobilized L-arabinose isomerase

The total activity of 2150 U was immobilized on agarose backbone by covalent bond. The initial galactose concentration was 500 g/l. The medium was eluted with rate of 0.5 ml/min, and temperature of 30°C was maintained during the reaction. The dotted lines indicates the time of fresh medium exchange.