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RESEARCH NOTE

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Comparative Analysis of Tagatose Productivity of Immobilized L-Arabinose Isomerase Expressed in *Escherichia coli* and *Bacillus subtilis*

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Abstract Although arabinose isomerase (E.C. 5.3.1.4), a commercial enzyme for edible tagatose bioconversion, can be expressed in an *Escherichia coli* system, this expression system might leave noxious by-products in food. To develop an eligible tagatose bioconversion with food-safe system, we compared the tagatose production activity of immobilized arabinose isomerase expressed in *Bacillus subtilis* (a host generally recognized as safe) with that of the enzyme expressed in *E. coli*. A 48% increase in tagatose production (4.3 g tagatose/L at 69.4 mg/L·hr) was found using the *B. subtilis*-expressed immobilized enzyme system, compared to the *E. coli*-expressed enzyme system (2.9 g tagatose/L). The increased productivity with safety of the *B. subtilis*-expressed arabinose isomerase suggests that it is a more eligible candidate for commercial tagatose production.

Keywords: tagatose bioconversion, food safety, expression host, *Bacillus subtilis*, *Escerichia coli*

Introduction

Tagatose, an isomer of the hexoketose monosaccharide D-galactose, is used commercially as a sweetening agent. It is rarely found in nature except in cooked milk and galactose-grown mycobacteria (1). It is 92% as sweet as sucrose, and its sweetness profile is similar to that of sucrose, but its sweetness is detected slightly sooner than that of sucrose. The unique cooling effect of polyalcohol sugar-substitutes (e.g., xylitol) is not present. The caloric value for humans is 1.5 kcal/g. Its bulk value is similar to that of sucrose, and its humectant properties are similar to those of sorbitol. It is less hygroscopic than fructose. Since tagatose is a reducing sugar, it participates in browning reactions during heat treatment (i.e., cooking). Many properties of tagatose are more similar to those of sucrose than to those of other sugar substitutes, and it has been designated as a generally recognized as safe (GRAS) material (2) and provoked industrial interest in tagatose as a low-calorie sweetener. It can be used in confectionery, beverages, health foods, and dietary products as a low-calorie, full-bulk sweetener. Tagatose might also be used as a prescription drug additive to mask unpleasant tastes, and as a sweetener for toothpaste, mouthwash, and cosmetics such as flavored lipstick (3).

Arabinose isomerase, which mediates conversion of arabinose into ribulose *in vivo*, also mediates conversion of galactose into tagatose *in vitro* (4). Many tagatose production efforts have been focused on comparing arabinose isomerases from various sources (5-7), on improving the enzymatic properties of arabinose isomerase by directed evolution (8-10), and on development of bioconversion processes for tagatose manufacture (7,11,12). All of the studies on biological tagatose production, however, have been performed using arabinose isomerases derived from an *Escherichia coli* expression system. For commercial production of tagatose for use in food, expression of arabinose isomerase in a GRAS host has been proposed as a way to avoid potential problems related to *E. coli* toxins (13-15).

The expression of the arabinose isomerase of *Geobacillus stearothermophilus* (GSAI) in *Bacillus subtilis*, a GRAS host, as well as its characterization has been recently reported by the authors (16). In the present study, the tagatose productivity of immobilized GSAI derived from a GRAS expression system vs. that of immobilized GSAI derived from an *E. coli* expression system is compared.

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Materials and Methods

Enzyme preparation The strains and plasmids used in this study are listed in Table 1. *B. subtilis* 168 strain harboring pJN02 was the GRAS expression system and *E. coli* BL21 (DE3) harboring pKK-GSAI was the control expression system (17). A single colony of the GSAI-expressing *E. coli* or *B. subtilis* strain was inoculated into a 250 mL-Erlenmeyer flask containing 50 mL of Luria-Bertani (LB) medium supplemented with antibiotics. After cultivation at 37°C for 12 hr in a rotary shaking incubator (230 rpm), the cultures (10 mL) were transferred into two 5-L jar fermenters (Kobiotech, Incheon, Korea) each containing 3 L of medium composed of 20 g/L LB, 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), and antibiotics (20 μg/mL chloramphenicol for *B. subtilis* and 50 μg/mL ampicillin for *E. coli*). The fermenters were operated at 37°C at 300 rpm with 0.5 vvm aeration until the optical density at 600 nm (OD_{600}) was 1.0 (18).

Cells were harvested by centrifugation at 4°C (4,500×g for 10 min) and re-suspended in 200 mL of 50 mM Tris-HCl buffer (pH 8.0). Aliquots of re-suspended cells (30 mL) were sonicated for 5 min at 140 W on ice using a UP200S ultrasonic processor (Hielscher Ultrasonics Co., Teltow, Germany). Sonications were performed 4 times for *B. subtilis* cultures and 3 times for *E. coli* cultures. After

Table 1. Strains and plasmids used in this study

Strains and plasmid	Description	Source
Strains		
B. subtilis 168	GSAI expressing host	ATCC 27370
E. coli BL21 (DE3)	GSAI expressing host	Novagen (Madison, WI, USA)
Plasmid		
pJN02	A pHT01 (MoBiTec Co, Goettingen, Germany)-derived shuttle vector expressing GSAI with 6 × His-tag under control of *Grac* promoter allowing constitutive or IPTG induced expression in *B. subtilis*. Cm^R and Amp^R	Ref. (16)
pKK-GSAI	A pKK223-3 (GenBank M77749)-derived vector expressing GSAI without 6 × His-tag under control of *tac* promoter allowing IPTG induced expression in *E. coli*. Amp^R	Ref. (16)

centrifugation at 4°C (10,000×g for 12 min), the resulting supernatant fractions were passed through 0.2-μm filters to remove cell debris. The resulting enzyme solutions were concentrated using an ultrafiltration device with a 10,000 Da cut-off (Vivascience AG, Goettingen, Germany) and used for tagatose production.

Immobilization and reaction condition To immobilize the enzyme, the enzyme solution was mixed with 2 mg of Chitopearl (Fuji Spinning, Tokyo, Japan) and incubated at 4°C for 24 hr. The immobilized enzyme was mixed with 50 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 10 g/L galactose. The amount of Chitopearl-bound protein was estimated by subtracting the amount of protein remaining in solution from the initial amount of protein in the solution. In experiments using free (non-immobilized) enzyme, the enzyme solution was mixed with the same buffer. Tagatose was produced at 60°C in a reciprocal shaking water bath at 60 rpm for 62 hr, and the pH was maintained by addition of 1 N NaOH to the reaction mixture. Samples (0.5 mL) were removed at intervals for analysis during the enzymatic conversion (19).

Stability of the immobilized enzyme The thermal stability of the immobilized GSAI was estimated by averaging the enzyme activity of beads after incubation. The immobilized GSAI beads in a 50 mM Tris-HCl (pH 8.0) with 100 g/L galactose buffer were incubated at 60°C, and beads were removed at intervals for enzyme activity measurement. Initial tagatose production activity was analyzed by incubating a bead for 1 hr at 60°C in 200 μL of fresh buffer. The experiment was repeated 3 times (20).

Analysis Tagatose concentration was determined from the absorbance at 560 nm after color development using cysteine-sulfuric acid-carbazole (21). To determine enzyme activity, a reaction mixture (125 μL) containing 40 mM substrate (galactose or arabinose) and 100 μL of enzyme solution was incubated at 60°C for 1 hr before tagatose was quantified. One unit of enzyme activity was defined as 1 μmole of product formation per min per mg protein (1 U = 1 $\mu mole_{tagatose}/mg_{protein} \cdot min$). The protein concentration of the enzyme solution was determined using a protein assay kit (Bio-Rad, La Jolla, CA, USA) with bovine serum albumin as a standard. Enzyme expression was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining.

Results and Discussion

Actively growing *E. coli* or *B. subtilis* cultures were harvested when they reached $OD_{600} = 1.0$. The cells were re-suspended in a 50 mM Tris-HCl buffer (pH 8.0) before cell disruption. After repeated sonication, enzyme solutions were obtained from *B. subtilis* and *E. coli* with protein concentrations of 0.73 and 0.81 mg/mL, respectively. The *B. subtilis*-derived enzyme solution yielded 145.4 mg total protein with 5.83 U of specific GSAI activity; the *E. coli*-derived enzyme solution yielded 162 mg total protein with 6.50 U of specific GSAI activity.

Tagatose production was investigated using immobilized enzymes derived from the *B. subtilis* and *E. coli* expression systems (Fig. 1). Chitopearl was selected as the enzyme-supporting bead because of its stability at high temperature (>60°C). For the *B. subtilis*-derived proteins, 2 mg of Chitopearl beads bound 6.3 mg protein, whereas for the *E. coli*-derived proteins, 0.82 mg of beads bound 6.3 mg protein. Chitopearl beads containing 6.3 mg of *B. subtilis*- or *E. coli*-derived proteins produced 4.3 and 2.9 g/L tagatose, respectively, from 10 g/L galactose over 62 hr, for productivities of 69.4 and 46.8 mg/L·hr, respectively. Therefore, tagatose production from the *B. subtilis* system was 48% higher than that from the *E. coli* system.

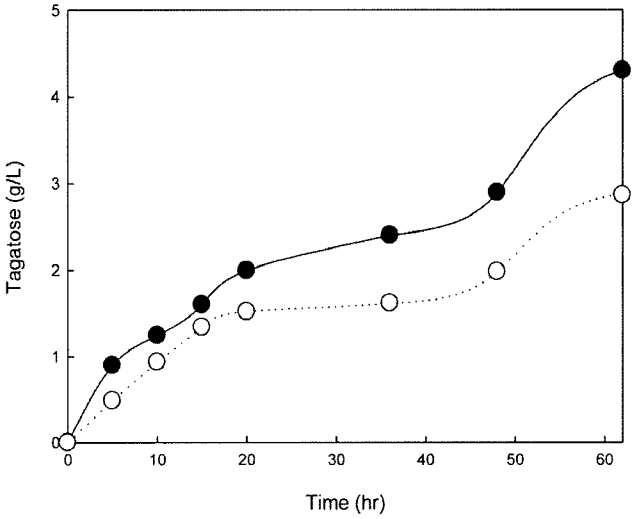

Fig. 1. Tagatose production by immobilized *Geobacillus stearothermophilus* (GSAI). ●, GSAI expressed in *B. subtilis*; ○, GSAI expressed in *E. coli*.

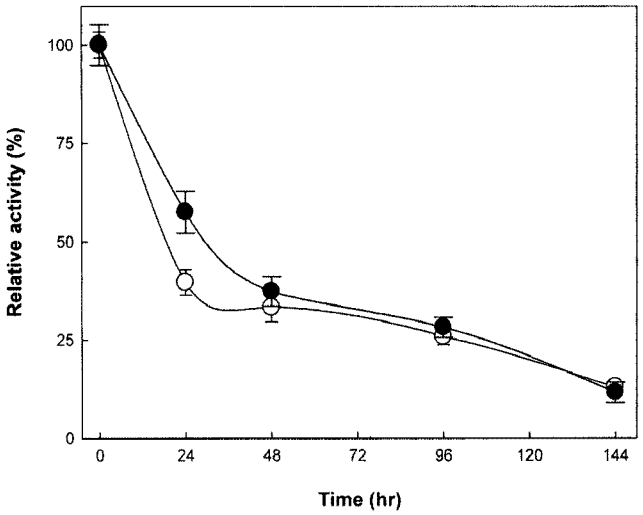

Fig. 2. Thermal stability of immobilized *Geobacillus stearothermophilus* (GSAI). A bead containing immobilized GSAI was taken from its storage buffer to measure the activity of each bead, and the remaining activity of each bead was normalized by dividing it by the amount of tagatose formation measured at 0 hr. ●, GSAI expressed in *B. subtilis*; ○, GSAI expressed in *E. coli*.

The GSAI from *B. subtilis*, but not that from *E. coli*, contained a 6 × His tag at its *N*-terminus. Since Chitopearl beads are positively charged, one might expect that the 6 × His-tagged GSAI from *B. subtilis* would bind less efficiently to the immobilized beads than would the untagged GSAI from *E. coli*, even if the protein content were the same. In light of this assumption, the 48 % increase in productivity from the *B. subtilis* expression system is even more impressive and, in fact, tagatose production assays using free enzyme supported this assumption. When the free enzyme solutions were reacted under the same conditions as the Chitopearl-bound enzyme (6.3 mg protein, 10 g/L galactose solution, 62 hr), the relative productivity of the *B. subtilis*-derived enzyme was even higher, with a 75% increase in productivity (0.56 vs. 0.32 g tagatose/L, respectively; data not shown).

Why the free enzymes were less productive than the immobilized enzymes is unknown (compare 0.32 or 0.56 to 2.9 or 4.3 g/L, respectively), though it may be due to partial loss of enzyme activity after cell disruption. The overall process of cell disruption, ultrafiltration, and immobilization took 7 days. Since the free and immobilized enzyme solutions were stored at the same conditions, free GSAI activity might have been lost more than immobilized GSAI activity during the storage.

To estimate the thermal stability of the immobilized GSAI preparations, the tagatose conversion activities of the beads were compared after incubation at 60°C (Fig. 2). For both the *B. subtilis*- and *E. coli*-derived enzymes, activity declined over time. After 6 days of incubation, the remaining GSAI activity for one bead was less than 13% of its initial activity. The 50% thermal decay times (t_{d50}) of enzymes derived from *B. subtilis* and *E. coli* were calculated to be 28.2 and 19.9 hr, respectively.

The substrate affinity and catalytic efficiency for galactose isomerization of the *B. subtilis*-expressed GSAI (K_m = 279 mM and k_{cat}/K_m = 11.4 /mM·min, respectively) were 2 and 5 times higher, respectively, than those of the *E. coli*-expressed GSAI (K_m = 578 mM and k_{cat}/K_m = 11.4/mM·min, respectively). As expected, based on its higher galactose affinity and catalytic efficiency, the *B. subtilis*-derived enzyme in both its immobilized and free forms produced more tagatose. Furthermore, as a benefit of GRAS host expression, this enzymatic process is safe. The more stability of *B. subtilis*-derived enzyme stability is also a potential of commercial process. From these results, we conclude that the GSAI expressed in *B. subtilis* is more suitable than that expressed in *E. coli* for edible tagatose production.

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