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A New Thermophile Strain of *Geobacillus thermodenitrificans* Having L-Arabinose Isomerase Activity for Tagatose Production

BAEK, DAE-HEOUN, YUJIN LEE, HONG-SIG SIN, AND DEOK-KUN OH[1]*

Chebigen, 98 Gunja-dong, Gwangjin-gu, Seoul 143-747, Korea
[1]Department of Bioscience and Biotechnology, Sejong University, Seoul 143-747, Korea

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Abstract Five strains, producing bacterial thermostable L-arabinose isomerase, were isolated from Korean soil samples obtained from compost under high temperature circumstances. Among these strains, the CBG-A1 showed the highest L-arabinose isomerase activity at 60°C and was selected as a D-tagatose producing strain from D-galactose. This strain was identified as *Geobacillus thermodenitrificans* based on the 16S rRNA analysis, and biological and biochemical characteristics. The isolated strain was aerobic, rod-shaped, Gram-positive, nonmotile, and an endospore-forming bacterium. No growth was detected in culture temperature below 40°C. The maximum growth temperature and maximum temperature of enzyme activity were 75°C and 65°C, respectively. In metal ion effects, Ca^{2+} was the most effective enzyme activator with the reaction rate by 150%. In a 5-l jar fermentor with 3-l MY medium, L-arabinose isomerase activity was growth-associated and pH decreased rapidly after the initial logarithmic phase.

Key words: Thermophile, L-arabinose isomerase, *Geobacillus thermodenitrificans*, D-tagatose production

L-Arabinose occurs as a component of hemicelluloses, pectic polysaccharides, and in the cell walls of certain bacteria such as *Norcardia* species. It is metabolized by various bacteria such as *Escherichia coli*, *Salmonella*, *Bacillus*, and lactic acid bacteria [17]. L-Arabinose is also widely distributed among many heteropolysaccharides of different plant tissues such as arabinans, arabinogalactans, xylans, and arabinoxylans [13]. In *B. subtilis*, the pathway of L-arabinose utilization has been described [9]. After it enters the cell, L-arabinose is sequentially converted to L-ribulose, L-ribulose-5-phosphate, and D-xylulose-5-phosphate by the action of L-arabinose isomerase (araA, EC: 5.3.1.4), L-ribulokinase, and L-ribulose 5-phosphate 4-epimerase, respectively. D-Xylulose 5-phosphate is further catabolized through the pentose phosphate pathway.

In vitro, it has been suggested that L-arabinose isomerase catalyze the conversion of L-arabinose to L-ribulose as well as D-galactose to D-tagatose because of the similar substrate configurations [3].

D-Tagatose, a zero-energy-producing ketohexose and a stereoisomer of fructose, is structurally similar to D-fructose and showed absorption to a greater extent than most other low-energy bulk sweeteners [10]. D-Tagatose can inhibit the activity of carbohydrase in the small intestine with the possibility for further reduction of the energy value of the diet and a depression of the glycemic response [16]. Administration of D-tagatose to rodents does not affect plasma glucose levels. However, orally administered D-tagatose attenuates the rise in plasma glucose in mice with orally preadministered sucrose, but the mechanism of this effect has not been clarified [17]. In addition, D-tagatose has no caloric value or no laxative effect, whereas other sugar-substituting polyols do [12]. For these reasons, D-tagatose has been given considerable attention and has been studied intensely in recent years in the field of medicine and foodstuff.

Until recently, there have been many reports on the production of D-tagatose [3, 6, 7]. D-Tagatose is mainly produced from D-galactose by chemical synthesis or enzymatic conversion method. In chemical synthesis, D-galactose is derived from lactose hydrolysate consisted of galactose and glucose, where the galactose component is separated from the hydrolysate by column separation. The processes of chemical synthesis of D-tagatose and column separation of galactose are expensive, complicated, and inefficient. To shunt these problems, enzymatic conversion of D-tagatose from D-galactose by araA was developed by some researchers. For example, Kim *et al.* [7] used an immobilized *E. coli* araA for production of D-tagatose from D-galactose by the one-step enzyme reaction, and Cheetham and Wootton [3] suggested that an araA of *Lactobacillus* and

*Corresponding author
Phone: 82-2-3408-3767; Fax: 82-2-3408-3911;
E-mail: deokkun@sejong.ac.kr

Mycobacterium could mediate conversion of D-galactose to D-tagatose as well as L-arabinose to L-ribulose.

In general, higher yields in the conversion of D-glucose to D-fructose have been achieved by increasing the reaction temperature [2]. In recent years, thermostable araA [8] was used as a biocatalyst for production of D-tagatose. The enzymatic reaction was operated at temperatures above 55°C to minimize the risk of contamination, improve the mass transfer, and lower the viscosity of substrates. In metal ion effects, the hyperthermostable araA was strongly activated by Co^{2+}, and it was not properly applicable to the food industry because of the incomplete removal of metal ion.

To overcome these problems, this study attempts to isolate a thermophilic bacterium producing a thermostable araA which was strongly activated by Mg^{2+} and Ca^{2+}. This paper describes the screening, identification, and characterization of a new thermophilic bacterium producing thermostable araA and its culture characteristics at high temperature conditions.

MATERIALS AND METHODS

Microorganism and Culture Conditions

To produce thermostable araA, the cells were cultured in MY medium (1.5% pancreatic digest of casein, 0.2% yeast extract, 0.2% beef extract, 0.2% glycerol, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4$, 0.0004% D-biotin, pH 6.8). For preservation, the isolate was kept at −70°C in a deep freezer. The seed cultures were prepared by incubating the organisms for 10 h at 65°C and 200 rpm with a rotary shaking incubator in a 250-ml flask containing 50 ml of MY medium. These cultures were used to inoculate a 5-l fermentor (BioTron Co. Ltd., Bucheon, Korea) containing 3-l of MY medium and the preparation was incubated for 14 h at 65°C with agitation (400 rpm) and aeration (1.0 vvm). The pH of the culture medium was not controlled and the cells were harvested after the stationary phase by centrifugation for 20 min at 14,000 ×*g* at 4°C and washed twice with 0.85% NaCl solution.

Isolation of AraA-producing Thermophile

Isolation of thermophilic bacteria was carried out using ten-fold serial dilution [21], and the diluents were inoculated on Luria-Bertani (LB) agar medium between 60°C and 80°C. Colonies were formed after 10 h and were then transferred into liquid medium with the same composition. The purity of isolates was checked microscopically, and the 200 thermophiles isolated from Korean soil were cultivated in LB medium (pH 7.0) at 75°C for about 8 h in a rotary shaking incubator. The harvested cells were disrupted by sonication at 30 kHz for 5 min, and centrifuged at 15,000 ×*g* for 30 min. The supernatant was used as crude enzyme solution and tested for araA activity.

Identification and Characterization of Isolate CBG-A1

Taxonomical and biological characteristics of strain CBG-A1 were investigated using the procedures described in bacterial handling methods [4, 5, 18–20]. Bacterial growth was monitored by measuring the OD at 600 nm (Shimadzu mini-1240, Kyoto, Japan) in liquid media. Anaerobic growth was tested by incubation of the cultures in an anaerobic jar (Oxoid Ltd., Basingstoke, England) with an anaerobic gas-pack. General biological and biochemical characteristics were examined by API kit (bioMérieux Vitek, Inc., U.S.A.), and other reactions were carried out by the methods described by MacFaddin [11].

Analytical Methods

Protein concentration was determined by the Bradford method [1] using bovine serum albumin as a standard protein. The amounts of D-tagatose and D-galactose were determined by HPLC (Shimadzu UV 1240, Kyoto, Japan) with a RI detector (RID-10A, Shimadzu). The separation was made with a C18-amine column (KR100-10NH_2, Kromasil, Bohus, Sweden) eluted isocratically using 80% (v/v) acetonitrile (35°C, 2 ml/min).

The araA activity was analyzed by measuring D-tagatose formation using D-galactose as a substrate. A 0.9 ml portion of 50 mM Tris-HCl buffer (pH 8.0) containing 5 mg of D-galactose was mixed with 0.1 ml crude enzyme solution. After incubation for 3 h at 60°C, the reaction mixture was centrifuged and filtered through 0.25 μM membrane for HPLC analysis. To investigate the effect of metal ion, 1 mM of Mg^{2+} and Ca^{2+} was added in the reaction mixture and compared with the reactant without metal ion.

One unit of araA activity was defined as the amount of enzyme required to produce 1 μg of D-tagatose per min at the same conditions as described above.

Sequencing and Analysis of the 16S rRNA Gene

16S rRNA partial sequence analysis was entrusted to the Korean Culture Center of Microorganisms (KCCM). The 16S rRNA gene sequences for representative group of *Bacillus* sequences were obtained from the NCBI, DDBJ, and EMBL databases.

Phylogenetic Analysis

The nucleotide sequences of the 16S rRNA genes from thermophile CBG-A1 were aligned manually against representatives of the NCBI Blast database. Pairwise evolutionary distances and phylogenetic tree were constructed by MegAlign software (DNA star, U.S.A.).

Nucleotide Sequence Accession Number

The partial nucleotide sequence of the 16S rRNA reported here has been assigned to GenBank accession no. AY166589.

RESULTS

Screening of AraA from Thermophile

Primarily, thermophilic bacteria that could grow at 75°C were isolated and screened for araA-producing positive strains. The high araA-producing thermophilic bacteria were screened by measuring the production of D-tagatose from D-galactose by using araA as a biocatalyst. Among 250 isolated thermophilic bacteria, CBG-A1 showed the highest araA activity (0.2 U/mg of protein) at 70°C, hence it was isolated and further characterized.

Identification and Characterization of Isolate CBG-A1

Morphological and physiological characteristics of strain CBG-A1 were investigated and compared with the *Geobacillus* species as shown in Table 1. The vegetative cell was a nonmotile rod-shaped structure (in the exponential phase) with many sporulating cells, and refractive spores could be observed in old cultures. Endospores were located in the central area, and morphological and cultural characteristics are presented in Table 1. The phylogenetic tree in Fig. 1 shows the relationship of the strains belong to a closely-related family of bacilli. Partial sequence of 16S rRNA of strain CBG-A1 (473 bp) was determined and showed similar identity with 99.8%, 99.6%, 97.7%, and 93% to that of *Geobacillus thermodenitrificans* AF114426, *Bacillus* sp. AB063312, *Geobacillus subtereaneus* AF276306, and *Geobacillus stearothermophilus* AJ005760, respectively. From these results, the CBG-A1 was described as a *Geobacillus thermodenitrificans* CBG-A1.

Nutritional Requirements

In aerobic conditions, isolate CBG-A1 produced acid but no gas from glycerol, arabinose, ribose, xylose, glucose, fructose, cellobiose, maltose, saccharose, trehalose, manitol, mannose, or esculin. However, as described in Table 1, CBG-A1 produced weak or no acid from galactose, rafinose, and starch, unlike *G. thermodenitrificans*.

Growth and Enzyme Production of Thermophile *G. thermodenitrificans* CBG-A1

When grown at various temperatures, the isolate could grow between 45°C and 75°C, and maximum specific growth rate (μ) was observed at 65°C (data not shown). Under light microscopy, the CBG-A1 showed thin rod-shaped cells at early logarithmic phase, but thread-like vegetative cells of 0.6 to 1.2 μm by 1.6 to 3.5 μm were most abundant in the mid-logarithmic phase. As shown in Fig. 2, without controlling pH in the fermentor, the production of araA was growth-associated and maximum specific activity (0.28 U/mg) was reached at the end of the logarithmic phase (9 h) at 65°C with 400 rpm in the range of 1.0 vvm. The pH of the culture medium was decreased rapidly after the initial lag phase and there was no further pH change after the logarithmic phase in fermentor conditions.

Detection of D-Tagatose

After enzyme reaction, a small aliquot of reaction mixture was analyzed by HPLC and the produced D-tagatose was determined after comparison with authentic D-galactose and D-tagatose. After 3 h of conversion at 60°C, the D-tagatose peak was detected after 6 min without byproducts and

Table 1. Identification of the isolated strain CBG-A1.

Characteristics	CBG-A1	*G. stearothermophilus**	*G. thermodenitrificans**
Shape	Rod	Rod	Rod
Size (width×length, μm)	0.6-1.2×1.6-3.5	0.6-1.0×2-3.5	0.5-1.0×1.5-2.5
Motility	-	+	-
Gram stain	+	+	+
Spore	+ (central)	+	+
Catalase test	+	+	+
Production of acid from:			
Glycerol	+	+	+
Arabinose	+	D	+
Ribose	+	ND	+
Xylose	+	D	+
Cellobiose	+	-	+
Lactose	W	-	+
Starch	W	+	+
Glucose	+	+	+
Galactose	-	-	+
Anaerobic growth	-	-	-
Temperature range (°C)	45-75	40-65	45-70

+, Positive reaction; -, negative reaction; D, 11-89% of strains are positive; ND, not determined; w, weak.
*, Citation from Bergy's manual.

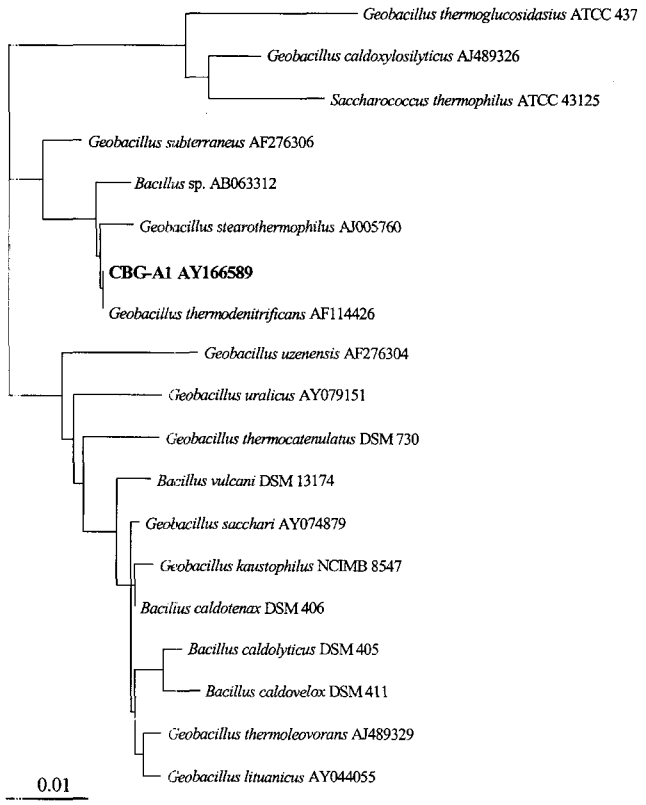

Fig. 1. Phylogenetic positions of the isolate CBG-A1 among other thermophilic members of family *Bacillaceae*.

residual D-galactose was remained and was detected around 8 min at a flow rate of 2 ml/min (Fig. 3). In bioconversion of D-galactose at 65°C by crude enzyme solution of the *G. thermodenitrificans*, the maximum amount of D-tagatose was obtained. In metal ion effects, Ca^{2+} was the most effective enzyme activator with a 15% increase in the reaction rate,

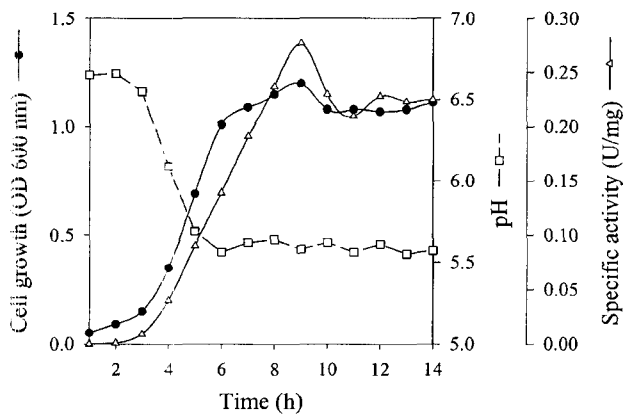

Fig. 2. Time course of thermostable L-arabinose isomerase (araA) from *G. thermodenitrificans* CBG-A1 in MY medium.
Cultivation was carried out at 65°C with 400 rpm, 1.0 vvm for 14 h with 3-l working volume in a 5-l jar fermentor. Symbols: ●, cell growth; △, L-arabinose isomerase activity; □, pH.

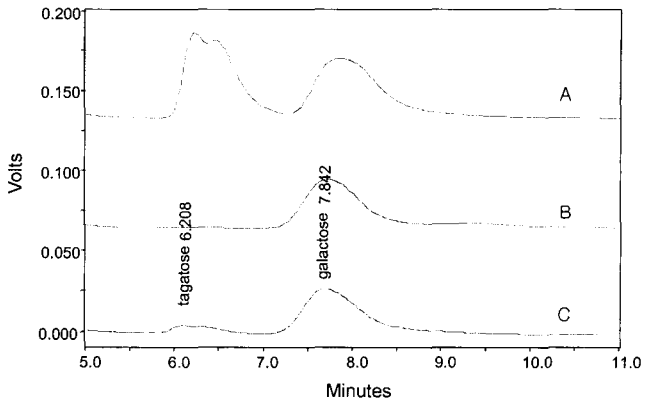

Fig. 3. HPLC analysis of D-tagatose produced from D-galactose by crude enzyme solution of *G. thermodenitrificans* CBG-A1.
The quantitative determination of D-tagatose was confirmed by means of 80% acetonitrile and amount of the D-tagatose was estimated after reacting for 3 h at 65°C in comparison with authentic sugars. A, authentic sugars; B, after 0 h reaction; C, after 3 h reaction.

and other metal ions such as Ba^{2+}, Mg^{2+}, and Co^{2+} increased the rate by 130%, 128%, and 126%, respectively.

DISCUSSION

A new thermostable L-arabinose isomerase-producing thermophile was screened and isolated from Korean soil, and the isolate was identified as *Geobacillus thermodenitrificans* based on the 16S rRNA analysis, and biological and biochemical characteristics. This strain could grow rapidly above 60°C and the maximum and minimum growth temperatures were determined to be 75°C and 40°C, respectively. In a 5-l fermentor with MY medium, the thermostable araA activity of this strain increased in accordance with cell growth without any other induction mechanisms. Cell growth and enzyme activity reached a maximum after the logarithmic phase at 65°C. Recently, there have been some mesophilic araA isolated from mesophiles [7, 14, 15], but a new thermostable araA that could be useful for industrial scale production of D-tagatose from D-galactose was not discovered except for thermostable araA from *B. stearothermophilus* [8] and for extremely thermostable araA from *Thermotoga neapolitana* [6]. The extreme thermophile was not used in foodstuff since Co^{2+}, a cofactor of araA for production of D-tagatose, was not completely removed in food manufacturing processes. For these reasons, a more appropriate thermostable araA to produce D-tagatose without Co^{2+} was needed for D-tagatose manufacturing processes on an industrial scale. Accordingly, a new thermostable araA from *G. thermodenitrificans* CBG-A1 may be a potential enzyme biocatalyst for the production of D-tagatose from D-galactose under high-temperature reaction conditions.

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