Biotransformation of Major Ginsenoside Rb₁ to Rd by Dekkera anomala YAE-1 from Mongolian Fermented Milk (Airag)

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Introduction

In Korea, ginseng products are widely used as health functional foods and generally enjoy brisk sales. In 2018, health functional food sales totaled US$2.1 billion and red ginseng products accounted for 44%, or US$927 million in sales [1]. Biologically functional components found in most ginseng species include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids [2]. Ginsenoside Rd is the main hydrolyzed component of ginsenosides Rb₁, Rb₂, and Rc, which are components in more than 80% of ginseng roots [3, 4]. Among ginsenosides, the minor ginsenoside Rd has been reported to have various immunological and anticancer activities. Airag was collected from five different mare milk farms located near Ulaanbaatar, Mongolia. YAE-1 strains were isolated from airag to examine the hydrolytic activities of β-glucosidase on Korean Panax ginseng using an API ZYM kit. Supernatants of selected cultures having β-glucosidase activity were examined for hydrolysis of the major ginsenoside Rb₁, at 40°C, pH 5.0. The YAE-1 strain was found to be nearly identical at 99.9% homology with Dekkera anomala DB-7B, and was thus named Dekkera anomala YAE-1. This strain exerted higher β-glucosidase activity than other enzymes. Reaction mixtures from Dekkera anomala YAE-1 showed great capacity for converting ginsenoside Rb₁ to ginsenoside Rd. The β-glucosidase produced by Dekkera anomala YAE-1 was able to hydrolyze ginsenoside Rb₁, and convert it to Rd during fermentation of the ginseng. The amount of ginsenoside Rd was highly increased from 0 to 1.404 mg/ml in fermented 20% ginseng root at 7 days.

Keywords: Fermented mare’s milk, airag, ginsenoside, β-glucosidase, Dekkera anomala YAE-1

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D. anomala YAE-1 could be used to hydrolyze the ginsenoside Rb1. The objectives of this study are to separate and characterize D. anomala YAE-1 having β-glucosidase activity from airag, and to investigate the enzymatic capacity of D. anomala YAE-1 strain on the hydrolysis of a major ginsenoside (Rb1) in Korean ginseng.

**Materials and Methods**

**Collection of Airag Samples**

Airag was prepared according to the method [11]. Airag samples were collected from four different mare milk farms located in close proximity to Ulaanbaatar, Mongolia.

**Isolation of Strains with β-Glucosidase Activity from Airag**

The experimental strains were isolated from airag, a Mongolian fermented milk product. Samples were plated according to the method [12-14]. Esculin-positive colonies were inoculated in pH 4.0 Yeast Malt broth (HiMedia Laboratories, USA), and sample preparation and β-glucosidase activity were determined by the procedure [12]. Conversion of ginsenosides was checked by TLC analysis [15].

**18S Ribosomal RNA Gene Sequencing of the Strain YAE-1 with β-Glucosidase Activity**

18S rRNA gene sequencing of selected strains was conducted according to the method [12]. PCR sequences were compared with those in the NCBI database using BLAST. The primers used were ITS1: 5'-TCCGTAGGTGACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3'.

**Effect of Temperature and pH on Growth of D. anomala YAE-1**

To determine the optimum pH (3.0-7.0) and temperature (20-40°C) for growth of strain YAE-1, 3% (v/v) of the yeat strain was inoculated in YM broth for 72 h in an incubator, and the specimen was checked for viable cell count.

**Enzyme Activity by API ZYM Kit**

Enzyme activity was detected using the API ZYM Kit (BioMérieux, Mercy l’Etoile, France) in accordance with the manufacturer’s instructions. Selected colonies from the surface agar plates were suspended in medium (0.085% NaCl, Ref. 20070, BioMérieux,) to a turbidity adjusted to a McFarland No. 5.0-6.0 standard (BioMérieux). Each microcupule of the API ZYM gallery containing 19 dehydrated chromogenic enzyme substrates was inoculated with 50 μl of the suspension, and the strip was incubated 4 h at 37°C. (BioMérieux).

**Analysis of Carbohydrate Utilization**

Carbohydrate fermentation tests were performed according to the method [11]. Selected colonies from the surface agar plates were suspended in medium (0.085% NaCl) to a turbidity adjusting to a McFarland No. 2.0 standard (BioMérieux) and inoculated into API C medium. Each microcupule of the API 20C AUX kit containing 19 carbohydrates with pH detector enzyme substrates was inoculated with 100 μl of the suspension. The first microcupules were used as a negative control. The strips were incubated 48 and 72 h at 29 ± 2°C.

**β-Glucosidase Activity of D. anomala YAE-1 Supernatant**

β-Glucosidase activity of the supernatant was determined by the rate of hydrolysis of 5 mM p-nitrophenyl-β-D-glucopyranoside (PNPG, Sigma-Aldrich, Germany) at 40°C and pH 7.0 (50 mM potassium phosphate buffer). The optimum pH and temperature on β-glucosidase activity were measured over a range of pH from 3.0 to 9.0 and temperature from 25°C to 70°C, respectively. β-Glucosidase activity of the supernatant was determined according to the method [11].

**Conversion of Ginsenoside Rb1 by Supernatant of D. anomala YAE-1**

Selected colonies were inoculated in YM broth (pH 4.0) (HiMedia Laboratories) for 3 days at 30°C. Supernatant activities were tested for hydrolysis of the major ginsenoside Rb1 under optimum condition (40°C, pH 5.0) of β-glucosidase. The reaction condition was cultured at 40°C for 48 hours. Conversion of ginsenoside Rb1 was checked by TLC and HPLC analysis [12].

**Fermentation of Ginseng Root by D. anomala YAE-1**

Ginseng roots were sliced and sterilized at 75°C for 10 min. A 20% (w/v) ginseng root solution was incubated with 3% (v/v) of yeast strain YAE1 at 30°C for 7 days. The fermentation characteristics of ginseng root by the strain YAE-1 were analyzed by TLC and HPLC, and viable cell counts were performed according to the method [12].

**Analysis of Ginsenoside Hydrolysates**

Hydrolyzed ginsenosides obtained from filtrate samples showing β-glucosidase activity were analyzed using the reversed-phase HPLC system Shimadzu LC-6AD (Shimadzu, Japan) and an ACE-5-C18 column (4.6 × 250 mm) equilibrated with solvent A (H2O). HPLC analysis was conducted according to the method [12].

**Statistical Analysis**

All analyses were repeated at least 3 times and are expressed as means ± standard deviation (SD) [12].
Results and Discussion

Isolation and Screening of the Strain Exhibiting β-Glucosidase Activity

Samples of the Mongolian traditional fermented dairy product airag were collected from 5 different farms. The respective isolated strains were: Airag A (strain YAA1-YAA7), Airag B (strain YAB1-YAB8), Airag C (strain YAC1-YAC6), Airag D (strain YAD1-YAD8), Airag E (strain YAE1-YAE8), and they were examined for β-glucosidase activity. Fifteen strains showed esculin-positive reaction. The YAE-1 strain was found to be the strongest esculin positive. When the major ginsenosides Rb1, Rb2, Rd, Re, and Rg1 were hydrolyzed by the strains, strain YAE-1 can hydrolyze ginsenoside Rb1 converted to Rd in minor quantity, while no conversions occurred for ginsenosides Rb2, Rd, Re and Rg1 (Fig. 1). β-Glucosidase is mainly a hydrolyzing enzyme for conversion of protopanaxadiol ginsenosides in ginseng. Many types of microorganisms with β-glucosidase activity have been used to hydrolyze ginsenosides Rb1, Rb2, Rd, Re and Rd to minor ginsenoside F2 and compound K [15-18].

Penicillium dipodomyicola strain isolated from the soil of wild ginseng also biotransformed major ginsenosides into compound K. The optimum transforming conditions for this fungus are 40°C, medium pH of 4.0 - 6.0 and incubation time of 7 days [15]. Aspergillus usamii KCTC6954 converted ginsenoside Rb1 to compound K and the incubation time was 15 days. The optimum temperature and pH of β-glucosidase produced by this mold is 60°C and 6.0 [16]. On the other hand, Candida allociferrii JNO301 isolated from meju (fermented soybean) converted ginsenosides of red ginseng extract, and the optimum temperature and pH were 20-30°C and 5-8, respectively. C. allociferrii JNO301 converted ginsenoside Rb1 to Rd → F2, Rb2 → Compound O, Rc → Mc1, Rf → Rh1 [17]. The β-glucosidase purified from the tomato pathogen Cladosporium fulvum was hydrolyzed with Rb1 to Rd. The enzyme has an optimal pH of 5.5 and an optimal temperature of 45°C [18].

Strain YAE-1 was selected for DNA analysis. The PCR produced 18S rRNA sequences of the strain YAE-1. The sequence of the strain was compared using the NCBI database, and it was found to be 99.9% homologous with D. anomala DB-7B (Fig. 2). The isolated YAE-1 strain was thus named as D. anomala YAE-1.

![TLC analysis of ginsenoside Rb1, Rb2, Rd, Re and Rg1 fermented by D. anomala YAE-1 at 30°C for 48 h.](image)

Fig. 1. TLC analysis of ginsenoside Rb1, Rb2, Rd, Re and Rg1 fermented by D. anomala YAE-1 at 30°C for 48 h. TLC was performed on Silica gel 60 F_{254} plates. A solvent mixture of chloroform: methanol: water (65:35:10, v/v/v, lower phase) was used as the developing solvent.

![Phylogenetic tree based on 18S rRNA sequences showing the position of yeast strain YAE-1. Scale length is 0.01.](image)

Fig. 2. Phylogenetic tree based on 18S rRNA sequences showing the position of yeast strain YAE-1. Scale length is 0.01.
Optimum Temperature and pH for Growth of *D. anomala* YAE-1

The effect of temperature and pH on growth of *D. anomala* YAE-1 is shown in Fig. 3. The number of viable cells started increasing at 1.12 × 10⁶ CFU/ml, but at 40°C, the number of viable cells rapidly decreased to 6.0 × 10⁴ CFU/ml after 48 h incubation, and 5.0 × 10¹ CFU/ml after 72 h incubation at 40°C. However, the viable cell count was highest at 4.78 × 10⁷ CFU/ml after 48 h incubation at 25-30°C. The optimum temperature for growth of *D. anomala* YAE-1 was found to be 25°C and 30°C, where its growth rate increased at these two temperatures compared to other temperature treatments during the incubation (Fig. 3A).

By changing the pH of the medium, *D. anomala* YAE-1 grew well at pH 4.0 during the beginning stage of incubation, but also grew well at pH 7.0 during 48 and 72 h incubation (Fig. 3B). Optimum growth temperature and pH of *D. anomala* generally were between 25°C to 30°C, and pH 7.0, respectively.

Enzymatic Activity and Carbohydrate Utilization of *D. anomala* YAE-1

Enzymatic activities of *D. anomala* YAE-1 strain exerted higher β–glucosidase activity (≥ 30 nmol) than α-galactosidase (≥ 5 nmol) and α–glucosidase (0 nmol). On the other hand, fungi (*P. dipodomyicola* and *A. usamii* KCTC6954), bacteria (*C. fulvum*) and yeast (*C. allociferrii* JNO301) had β–glucosidase activity [15-18]. *D. anomala* YAE-1 fully consumed carbohydrates such as glucose, D-galactose, N-acetyl-D-glucosamine, D-cellulose, D-lactose, while glycerol and raffinose were consumed 90% and 50%, respectively. *C. allociferrii* JNO301 can utilize carbohydrates such as glucose, galactose, N-acetyl-D-glucosamine, glycerol and raffinose, but lactose is not utilized [17].

Optimum Temperature and pH of β-Glucosidase Activity in Supernatant

The β-glucosidase of the supernatant exhibited maximum enzyme activity at 40°C (Fig. 4), but gradually decreased activity at 45 to 65°C, and finally showed significantly decreased activity above 65°C. The optimum pH for the activity occurred around 4.0 to 5.0 at 30°C. The enzyme activity was rapidly decreased above pH 5. Enzyme maximal activity was at pH 5.0 (Fig. 4). On the other hand, the optimum pH and temperature of β-glucosidase isolated from *C. fulvum* was 5.5 and 45°C, respectively [18]. Furthermore, β-glucosidase produced by *P. dipodomyicola* isolated from soil of wild ginseng best converted ginsenoside Rb1 to minor ginsenosides under a pH value from 4.0 to 6.0 and the best fermentation temperature was 40°C [15]. Thus, the optimum conditions for β-glucosidase activity are considered to be in the range of pH 5.0-5.5 at the temperature of 40-45°C.

Fig. 4. Optimum temperature and pH of β-glucosidase activity in supernatant of *D. anomala* YAE-1.
Biotransformation of Ginsenoside Rb1 to Rd by *D. anomala* YAE-1

*D. anomala* YAE-1 exhibiting β-glucosidase activity was incubated in YM broth (pH 4.0) for 48 h at optimum condition of 30°C. Hydrolysis of the major ginsenoside Rb1 was tested under optimum condition for β-glucosidase (40°C, pH 5.0). After 48 h, 3.095 mg/ml of ginsenoside Rb1 was fully hydrolyzed by supernatant with β-glucosidase activity, and released 3.0 mg/ml of ginsenoside Rd (Fig. 5), (Table 1A). Our results showed that Rd was the main ginsenoside in the final fermentation product of ginsenoside Rb1. Enzymatic hydrolysis made it possible to produce minor ginsenosides. The biological activities of ginsenosides increase according to their molecular mass. [19]. Many reports have been conducted on the conversion of the ginsenoside Rb1 into Rd during fermentation [13, 20, 21]. Our results on the biotransformation of ginsenoside Rb1 by *D. anomala* YAE-1 revealed that there were some similarities with the previous studies conducted with different types of strains. Also, most of the microorganisms used for the transformation of ginsenoside do not meet food-grade standards and researchers.

**Table 1. Quantities of biotransformation of ginsenoside Rb1 to Rd and viable cell counts of 20% fermented ginseng by *D. anomala* YAE-1.**

| Incubation time (h) | 0  | 4   | 8   | 16  | 24  | 48  |
|---------------------|----|-----|-----|-----|-----|-----|
| **A** Rb1 (mg/ml)   | 3.095 ± 0.012a | 2.331 ± 0.022b | 1.946 ± 0.029c | 0.962 ± 0.014d | 0.698 ± 0.010e | 0.00f |
| Rd (mg/ml)          | 0.055 ± 0.007a | 0.669 ± 0.069b | 1.044 ± 0.034c | 2.038 ± 0.046d | 2.302 ± 0.014e | 3.0 ± 0.096a |

| Incubation time (day) | 0 | 1 | 3 | 7 |
|-----------------------|---|---|---|---|
| **B** Rb1 (mg/ml)     | 0.763 ± 0.026a | 1.175 ± 0.011b | 1.738 ± 0.044c | 4.807 ± 0.0873 |
| Rd (mg/ml)            | 0.003 ± 0.0011a | 0.0287 ± 0.0016b | 0.0737 ± 0.0039c | 1.4041 ± 0.03130 |
| **C** *D. anomala* YAE-1 (Log CFU/ml) | 7.2 ± 10^5 | 1| 10^7 | 10^7 | 2.5 ± 10^7 |

*Means ± SD (*n* = 3)
+a,b,c,d,e,fMeans with different superscript letters among the same row are significantly different at *p* < 0.05.

A) Quantities of biotransformation of ginsenoside Rb1 by supernatant of *D. anomala* YAE-1 during 48 h. The quantities of ginsenoside Rb1 and Rd were determined by HPLC.

B) Quantities of biotransformation of ginsenoside Rb1 and Rd in ginseng root by *D. anomala* YAE-1 during 7 days. The amount of ginsenoside Rb1 and Rd were determined by HPLC.

C) Viable cell counts of *D. anomala* YAE-1 in 20% fermented ginseng root.
were searching for microorganisms that are safe for use in various foods [22]. Most microorganisms with β-glucosidase activity cannot hydrolyze major ginsenosides and β-glucosidase hydrolyzing ginsenosides very rarely occur among foodborne microorganisms [12]. Jo et al. [17] reported that after 48 h at 60°C, β-glucosidase enzymes of 3- and 6-day cultures of A. usamii KCTC6954 were unable to convert the ginsenoside Rb1, and enzymes of 9-, 12- and 15-day cultures fully converted ginsenoside Rb1 to ginsenoside Rd, F2 and compound K. Moreover, Lunpeng et al. [15] observed that after 48 h, β-glucosidase produced by P. dipodomyicola isolated from soil of wild ginseng completely converted ginsenoside Rb1 to ginsenoside Rd, and subsequently, ginsenoside Rd completely converted to compound K after 7 days. In addition, β-glucosidase isolated from P. dipodomyicola was able to convert ginsenoside Rb2 to compound Y; ginsenoside Rc to compound Mc; and ginsenoside Rd to compound K. Characteristics of Ginseng Root Fermented by D. anomala YAE-1

D. anomala YAE-1 hydrolyzed ginsenoside Rb1 to ginsenoside Rd in 20% fermented ginseng root. The peak size of ginsenoside Rb1 was small and that of ginsenoside Rd was significantly large after 7 days, as revealed in HPLC chromatograms (Fig. 6). Furthermore, the quantity of ginsenoside Rd was elevated from 0 to 1.404 mg/ml at the same period (Table 1B). In fermented ginseng roots, ginsenoside Rb1 was converted to Rd, reducing ginsenoside Rb1 and increasing Rd. The factor that absolutely affects ginsenoside conversion is fermentation time. Rd was increased as the main ginsenoside in the final fermentation product of ginseng roots, confirming that Rb1 was decreased continuously during the 7-day fermentation. The quantity of minor ginsenoside Rd was elevated from 0 to 0.060 ± 0.011 mg/ml at 7 days in Paenibacillus sp. MBT213, which converts ginsenoside Rb1 into ginsenoside Rd from Panax ginseng [12]. The ginsenoside conversion by Paenibacillus sp. MBT213 was absolutely affected by fermentation time as was the ginsenoside conversion by D. anomala YAE-1. The initial viable cell count of D. anomala YAE-1 was 4.85 log CFU/ml, and maximum cell count was 7.39 log CFU/ml after 7 days (Table 1C). D. anomala YAE-1 has strong β-glucosidase activity, which can be used to convert major ginsenoside Rb1 to Rd during the fermentation of ginseng. It is also believed that the converted ginsenoside Rd has the potential to be used in a variety of health functional foods and pharmaceutical products.
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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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