Mycobiology

Research Article

The Bioconversion of Red Ginseng Ethanol Extract into Compound K by *Saccharomyces cerevisiae* HJ-014

Hak Joo Choi[1], Eun A Kim[1], Dong Hee Kim[1,2] and Kwang-Soo Shin[3,*]

[1]*Traditional and Biomedical Research Center, Daejeon University, Daejeon 300-716, Korea*
[2]*Department of Pathology, College of Korean Medicine, Daejeon University, Daejeon 300-716, Korea*
[3]*Division of Life Sciences, College of Natural Science, Daejeon University, Daejeon 300-716, Korea*

Abstract A β-glucosidase producing yeast strain was isolated from Korean traditional rice wine. Based on the sequence of the *YCL008c* gene and analysis of the fatty acid composition, the isolate was identified as *Saccharomyces cerevisiae* strain HJ-014. *S. cerevisiae* HJ-014 produced ginsenoside Rd, F_2, and compound K from the ethanol extract of red ginseng. The production was increased by shaking culture, where the bioconversion efficiency was increased 2-fold compared to standing culture. The production of ginsenoside F_2 and compound K was time-dependent and thought to proceed by the transformation pathway of: red ginseng extract → Rd → F_2 → compound K. The optimum incubation time and concentration of red ginseng extract for the production of compound K was 96 hr and 4.5% (w/v), respectively.

Keywords Bioconversion, Compound K, Red ginseng extract, *Saccharomyces cerevisiae* HJ-014

Ginseng (*Panax ginseng* C. A. Meyer) belongs to the Araliaceae family of flowering plants and its' root has been used as a traditional medicine in Asian countries for over 2000 years. The major active components of ginseng are ginsenosides, which have a number of pharmacological effects, including anti-inflammatory [1], anti-cancer [2, 3], anti-aging [4], and antioxidant [5] activities. However, naturally occurring ginsenosides are poorly absorbed by the human intestinal tract [6]. As a result, recent studies have focused on the bioconversion of the ginsenosides to increase their absorption and pharmacological activity [7, 8].

Compound K is the active form of protopanaxadiol saponins and has been reported to be easily absorbed by the human digestive tract [9]. In addition compound K has been shown to exhibit hepatoprotective activity [10], inhibit tumor invasion and metastasis, and induce tumor cell apoptosis [11-13]. Since compound K does not exist in natural products, many studies have focused on the development of methods for the production of compound K. Microbial biotransformation with the crude extract of *Leuconostoc mesenteroides* DC102 [14] and *Lactobacillus paralimentarius* [15] has been used to prepare compound K from ginsenoside Rb1 via Rd and F_2. Enzymatic transformation of gypenoside LXXV by β-glucosidase from *Thermus thermophilus* [16], has also been used to prepare compound K. β-Glucosidase specifically hydrolyzes two glucose molecules at C-3 and one glucose molecules at C-20 of ginsenoside Rb1 to generate compound K. However, only a few β-glucosidase have reported to be able to produce compound K [17, 18].

So far, the majority of studies have used purified ginsenosides for the production of compound K using bioconversion. In this paper, we isolated and identified a β-glucosidase producing yeast, from the traditional Korean rice wine Makgeolli. Significantly, we have demonstrated that this novel yeast strain is capable of producing compound K from a crude ethanol extraction of red ginseng. This research provides a promising solution for the industrial production of compound K form raw ginseng preparations.

Mycobiology 2014 September, **42**(3): 256-261
http://dx.doi.org/10.5941/MYCO.2014.42.3.256
pISSN 1229-8093 • eISSN 2092-9323

***Corresponding author**
E-mail: shinks@dju.kr

Received July 12, 2014
Revised August 19, 2014
Accepted September 17, 2014

MATERIALS AND METHODS

Chemicals. The ginsenoside standards Rd, F_2, and compound K were purchased from Sigma (St. Louis, MO,

USA) and Ambo Laboratories (Daejeon, Korea). All other chemicals used were reagent grade.

Screening for microbes producing β-glucosidase. Esculin-MRS agar, containing 0.3% (w/v) esculin and 0.02% (w/v) ferric citrate in MRS agar [19], was used to isolate β-glucosidase producing microorganisms. β-Glucosidase producing-microbes hydrolyze the esculin appearing as colonies surrounded by a reddish-brown to dark brown zone. Potential β-glucosidase producing microbes were subjected to additional two-day incubation at 30°C in MRS medium.

Isolate identification. The genomic DNA was isolated and purified using a Genomic DNA Purification Kit (Qiagen, Hilden, Germany). PCR amplification was performed using *Saccharomyces* specific primers YCL008c-U (5′-TTCCGT-TGGATGTGCCATCG-3′) and YCL008c-L (5′-GGAGCC-ACCAAGGGATGG-3′) [20]. The resulting PCR product was sequenced by Macrogen (Seoul, Korea). Homologous sequences were identified using the BLAST, at the NCBI database. Cells were grown for 3 days at 30°C on MRS agar and fatty acid methyl esters (FAMEs) were extracted and prepared according to the standard protocol of the Microbial Identification System (MIDI; Microbial ID, Newark, DE, USA). Briefly, whole cells extracts were saponified at 100°C in 1 mL of reagent 1 (15% w/v NaOH in 50% v/v methanol). Fatty acids were then esterified at 80°C with 2 mL of reagent 2 (3.25 N HCl in 46% v/v methanol) and FAMEs were extracted in 1.25 mL of reagent 3 (hexane and methyl-*t*-butyl ether mixture, 1 : 1 v/v). Finally, the organic extract was washed with 3 mL of 1.2% (w/v) NaOH. The washed extracts were then analyzed on an Agilent 6890N-MIDI Sherlock system 6.0 (Microbial ID).

Nucleotide sequence accession number. The GenBank accession number for the *S. cerevisiae* HJ-014 sequence is KM061793.

Assay of enzyme activity. β-Glucosidase activity was determined using *p*-nitrophenyl-β-D-glucose (*p*NPG) as a substrate. The reaction mixture, containing 50 μL of 10 mM *p*NPG, 50 μL of enzyme solution in 50 mM acetate buffer (pH 5.0), was incubated at 50°C for 30 min. The reaction was stopped by adding 100 μL of NaOH-glycine buffer (0.4 M, pH 10.8) and measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmole of *p*-nitrophenol per min. Activity staining of β-glucosidase was performed as described previously [21]. After electrophoresis, the gel was incubated in 0.2 M acetate buffer containing 0.1% (w/v) esculin and 0.03% (w/v) ferric chloride for 5 min at 50°C. β-Glucosidase appeared as black bands against a transparent background.

Transformation of ethanol extracted red ginseng by *S. cerevisiae* HJ-014. The 80% ethanol extract of red ginseng was pretreated with α-herbzyme (Korea Enzyme Co., Seoul, Korea). The red ginseng extract (0.5 g) was resolved in 3% α-herbzyme (w/v) in water and incubated at 30°C with agitation (200 rpm) for 24 hr. MRS broth was inoculated with *S. cerevisiae* HJ-014 and incubated at 30°C for 24 hr, the culture broth was then mixed with an equal volume of pretreated red ginseng. The mixture was then incubated at 30°C with shaking at 200 rpm and a 1.0 mL aliquot was then taken at the indicated time and analyzed by high-performance liquid chromatography (HPLC).

HPLC analysis. The reaction mixtures were analyzed by HPLC (Shimadzu Prominence, Shimadzu, Japan), using a Zorbax Eclipse plus C18 column (4.6 × 250 mm, 5 μm) at 203 nm. The analytes were eluted via gradient elution at 40°C with acetonitrile/water from 18 : 82 (v/v) to 100 : 0 (v/v) at a flow rate of 1.0 mL/min.

RESULTS AND DISCUSSION

The isolation and identification of β-glucosidase producing microbes. The β-glucosidase producing microbes were screened, using Esculin-MRS agar medium. Black colonies demonstrating β-glucosidase activity were picked and transferred to fresh Esculin-MRS agar medium (Fig. 1A). The production of β-glucosidase from the isolate was confirmed by activity staining (Fig. 1B). The consensus sequence of the *Saccharomyces YCL008c* gene (1,514 bp) from the isolate was aligned with other *S. cerevisiae* sequences and the taxonomic relationships were analyzed. Phylogenetic trees based on the aligned sequences were constructed using MEGA ver. 5.1 [22] with neighbor-joining [23], based on 1,000 randomly chosen bootstrap replication and tree/branch style was circle (Fig. 1C). The sequence was 97% homologous to the *S. cerevisiae Stp22p* genes. The MIDI system was used to analyze the fatty acid composition of the cell membrane from the isolate. The analysis, shown in Table 1, identified palmitoleic acid (16 : 1), oleic acid (18 : 1), palmitic acid (16 : 0), trace amounts of stearic acid (18 : 0) and short chain fatty acids (10 : 0 to 14 : 1). This demonstrated that the isolate contains the same principal fatty acid chains found in *S. cerevisiae* [24, 25]. Together with the phylogenetic and chemotaxonomic analyses the isolate was identified as *S. cerevisiae* strain HJ-014.

Microbial bioconversion of ethanol extracted red ginseng to compound K. The culture broth of *S. cerevisiae* HJ-014 was mixed with an equal volume of pretreated red ginseng extract and incubated at 30°C for 24 hr at 200 rpm. The reaction mixture was analyzed by HPLC, with standard ginsenosides including Rd, F_2, and compound K. Ginsenoside F_2 and compound K were not detected in the absence of *S. cerevisiae* HJ-014 treatment (Fig. 2A). Three ginsenosides were detected in the reaction mixture incubated with isolate and the retention times of Rd, F_2, and compound K were approximately 94, 103, and 116.5 min, respectively (Fig. 2B). It has been shown that

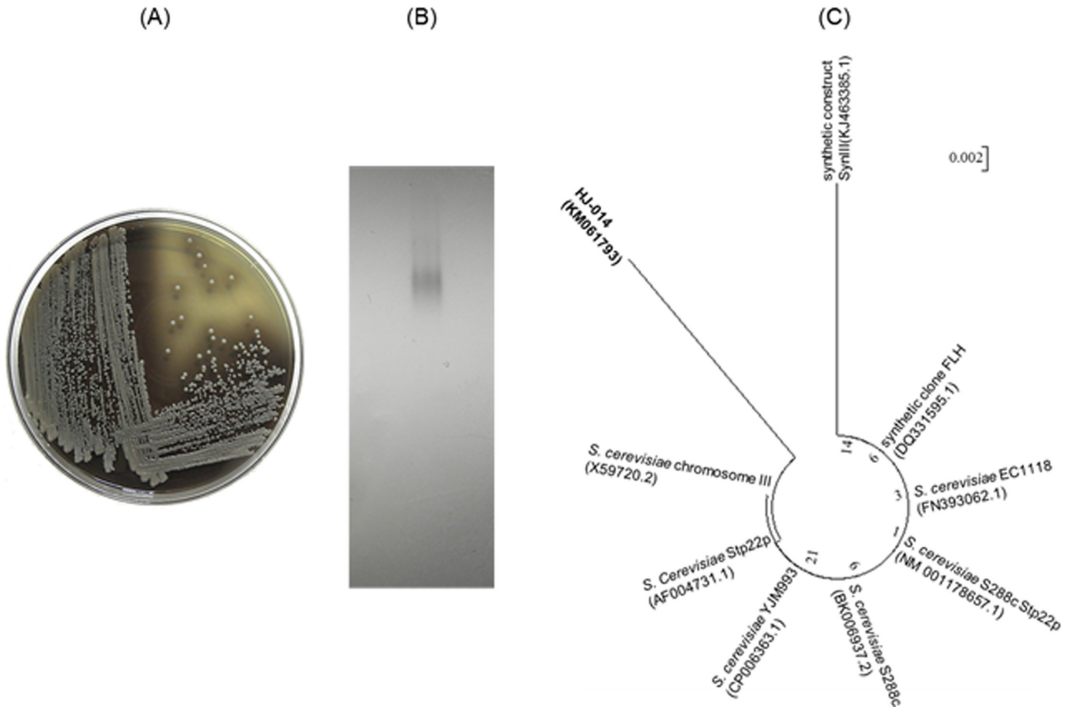

Fig. 1. A, Production of β-glucosidase from isolated yeast detected in Esculin-MRS agar medium; B, Activity staining to detect β-glucosidase; C, Phylogenetic tree based on *YCL008c* gene sequences.

Table 1. Cellular fatty acid composition (%) of *Saccharomyces cerevisiae* HJ-014

Fatty acid	Composition (%)
$C_{10:0}$	1.78
$C_{12:0}$	1.92
$C_{14:1}$ *w5c*	0.75
$C_{14:0}$	1.37
$C_{16:0}$	11.35
$C_{18:1}$ *w9c*	22.47
$C_{18:0}$	1.99
Summed feature[a]	
3	57.47
8	0.90

[a]Summed features represent groups of two or more fatty acids that could not be separated using the MIDI system. Summed feature 3 comprised $C_{16:1}$ *w7c*/$C_{16:1}$ *w6c* and/or $C_{16:1}$ *w6c*/$C_{16:1}$ *w7c* and summed feature 8 comprised $C_{18:1}$ *w7c* and/or $C_{18:1}$ *w6c*.

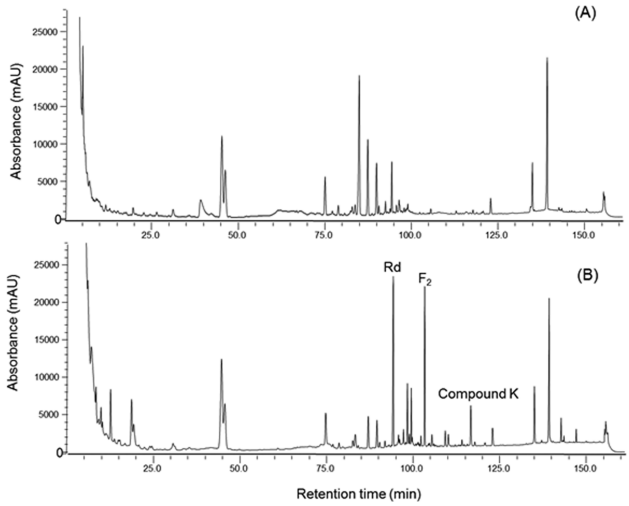

Fig. 2. Production of ginsenoside Rd, F_2 and compound K by *Saccharomyces cerevisiae* HJ-014. A, The reaction mixture was incubated at 30°C for 24 hr at 200 rpm without *S. cerevisiae* HJ-014; B, The reaction mixture was incubated with *S. cerevisiae* HJ-014 at same conditions.

Sphingomonas echinoides GP50 can completely convert ginsenoside Rb_1 to ginsenoside Rd, but it produces a limited amount of compound K [26]. In *Lactobacillus pentosus* DC101, over 97% of ginsenoside Rd is degraded and converted into compound K [27]. However, only one report has been published on the production of compound K from raw ginseng extract [28].

The effects of agitation on the production of compound K. To investigate the effect of agitation on the production of compound K, we incubated the reaction mixture either with or without agitation at 30°C for 48 hr. As shown in Fig. 3 ginsenoside Rd was the major product detected in the standing culture, while compound K was only detected in trace amounts, compared to the shaking culture. The bioconversion pathway of ginsenoside Rd by *L. pentosus* DC101 has been reported; where Rd is initially converted to F_2, followed by its conversion into compound K [27].

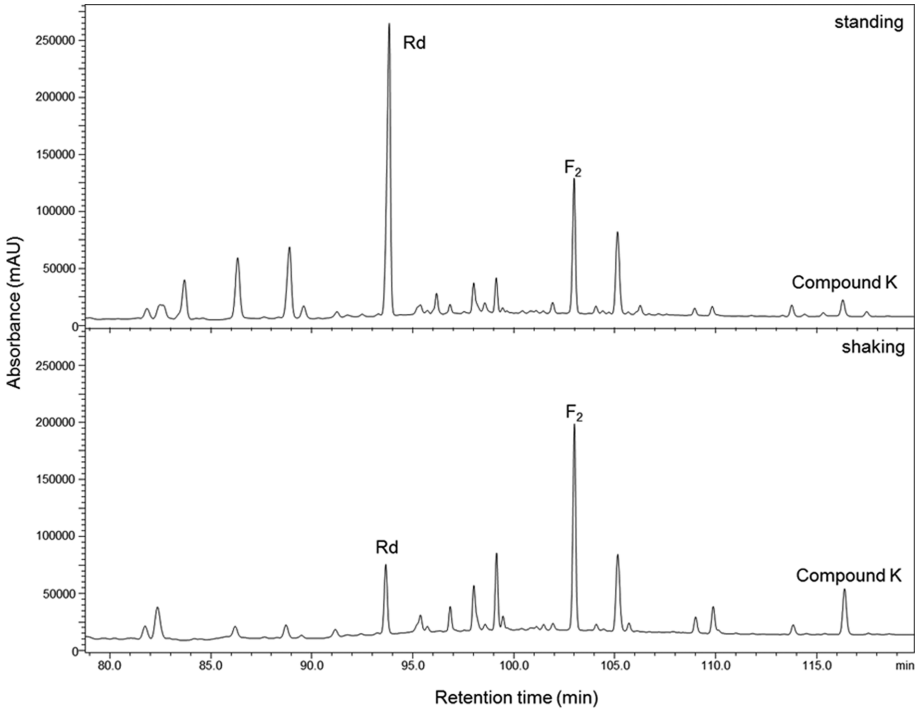

Fig. 3. The effect of agitation on the production of ginsenoside Rd, F_2 and compound K. The reaction mixture was incubated the either with (200 rpm) or without agitation at 30°C for 48 hr.

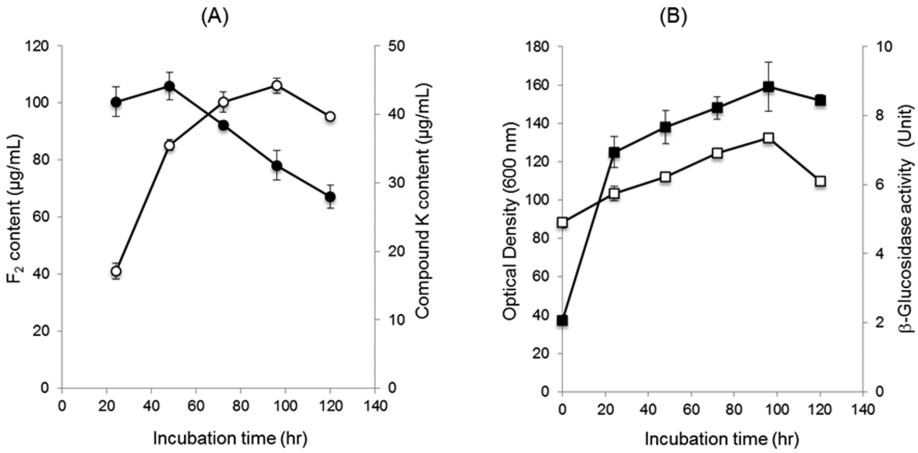

Fig. 4. The time-dependent production of ginsenoside F_2 and compound K. A, Time-dependent change in the concentrations of F_2 and compound K. The closed and open symbols represent the concentration of F_2 and compound K, respectively; B, The growth (closed square) and the production of β-glucosidase (open square) according to the incubation time.

The bioconversion efficiency of the standing culture was lower than that of the shaking culture possibly because sufficient degradation of Rd had not occurred in in the standing culture.

Time-dependent production of compound K. The production rate of F_2 and compound K was time-dependent [27]. The ginsenoside F_2 peak reached a maximum level (106 μg/mL) (Fig. 4A) after 48 hr incubation and then decreased gradually (Fig. 4A). The compound K peak also increased continuously until 96 hr, when the amount of compound K produced reached its maximum (44 μg/mL) (Fig. 4A). The growth rate and β-glucosidase activity was increased gradually up to 96 hr and then decreased (Fig. 4B). Our results show that the transformation pathway of red ginseng extract by *S. cerevisiae* HJ-014 is red ginseng extract → Rd → F_2 → compound K and β-glucosidase may play an important role in compound K production.

The effects of red ginseng extract concentration on the production of compound K. It have been demonstrated that a high concentration of ginseng extract inhibits

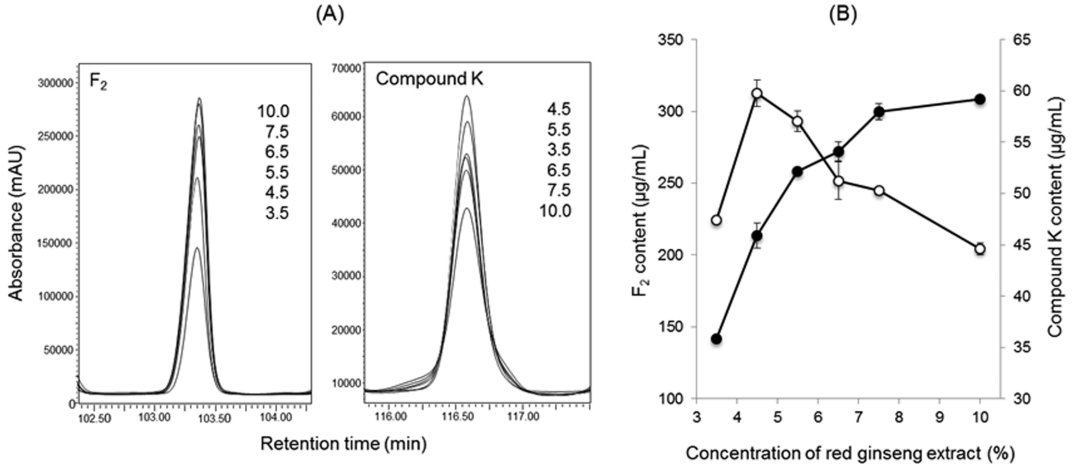

Fig. 5. The effect of red ginseng extract concentration on the production of ginsenoside F_2 and compound K. A, Enlarged HPLC chromatogram of F_2 and compound K. The numbers in the figure indicate the concentration of red ginseng extract (%, w/v); B, Effect of red ginseng extract on the production of F_2 and compound K. The closed and open symbols represent the concentration of F_2 and compound K, respectively.

microbial growth [28]. The optimum concentration of red ginseng extract was determined for the production of the ginsenoside F_2 and compound K in *S. cerevisiae* HJ-014. HPLC was used to monitor the production of F_2 and compound K produced from various concentrations of red ginseng extract. The amount of F_2 increased in proportion to the concentration of red ginseng extract treated (Fig. 5A) and reached a maximum (308 μg/mL, Fig. 5B), at 10% (w/v). However, a large amount of compound K was produced at low concentrations of red ginseng extract, the optimum concentration being 4.5% (w/v) (Fig. 5).

The bioconversion of raw red ginseng extract into compound K by *S. cerevisiae* HJ-014 has a potential application in industry. Further studies concerning the purification, characterization, and application of this strain and its enzyme system to the production of compound K is required.

ACKNOWLEDGEMENTS

This work was supported by the RIC program of MOTIE (Ministry of Trade, Industry and Energy) in Daejeon University.

REFERENCES

1. Wang L, Zhang Y, Chen J, Li S, Wang Y, Hu L, Wang L, Wu Y. Immunosuppressive effects of ginsenoside-Rd on skin allograft rejection in rats. J Surg Res 2011;176:267-74.
2. Sato K, Mochizuki M, Saiki I, Yoo YC, Samukawa K, Azuma I. Inhibition of tumor angiogenesis and metastasis by a saponin of *Panax ginseng*, ginsenoside-Rb_2. Biol Pharm Bull 1994;17:635-9.
3. Mochizuki M, Yoo YC, Matsuzawa K, Sato K, Saiki I, Tonooka S, Samukawa K, Azuma I. Inhibitory effect of tumor metastasis in mice by saponins, ginsenoside-Rb_2, 20(*R*)- and 20(*S*)-ginsenoside-Rg_3, of red ginseng. Biol Pharm Bull 1995;18:1197-202.
4. Wang BX, Cui JC, Liu AJ, Wu SK. Studies on the anti-fatigue effect of the saponins of stems and leaves of *Panax ginseng* (SSLG). J Tradit Chin Med 1983;3:89-94.
5. Cho WC, Chung WS, Lee SK, Leung AW, Cheng CH, Yue KK. Ginsenoside Re of *Panax ginseng* possesses significant antioxidant and antihyperlipidemic efficacies in streptozotocin-induced diabetic rats. Eur J Pharmacol 2006;550:173-9.
6. Odani T, Tanizawa H, Takino Y. Studies on the absorption, distribution, excretion and metabolism of ginseng saponins. III. The absorption, distribution and excretion of ginsenoside Rb_1 in the rat. Chem Pharm Bull (Tokyo) 1983;31:1059-66.
7. Park SY, Bae EA, Sung JH, Lee SK, Kim DH. Purification and characterization of ginsenoside Rb_1-metabolizing β-glucosidase from *Fusobacterium* K-60, a human intestinal anaerobic bacterium. Biosci Biotechnol Biochem 2001;65:1163-9.
8. Kim MK, Lee JW, Lee KY, Yang DC. Microbial conversion of major ginsenoside Rb_1 to pharmaceutically active minor ginsenoside Rd. J Microbiol 2005;43:456-62.
9. Akao T, Kanaoka M, Kobashi K. Appearance of compound K, a major metabolite of ginsenoside Rb_1 by intestinal bacteria, in rat plasma after oral administration - measurement of compound K by enzyme immunoassay Biol Pharm Bull 1998;21:245-9.
10. Lee HU, Bae EA, Han MJ, Kim NJ, Kim DH. Hepatoprotective effect of ginsenoside Rb_1 and compound K on tert-butyl hyroperoxide-induced liver injury Liver Int 2005;25:1069-73.
11. Hasegawa H, Uchiyama M. Antimetastatic efficacy of orally administered ginsenoside Rb_1 in dependence on intestinal bacterial hydrolyzing potential and significance of treatment with an active bacterial metabolite. Planta Med 1998;64:696-700.
12. Wakabayashi C, Murakami K, Hasegawa H, Murata J, Saiki I. An intestinal bacterial metabolite of ginseng protopanaxadiol saponins has the ability to induce apoptosis in tumor cells. Biochem Biophys Res Commun 1998;246:725-30.

13. Oh SH, Yin HQ, Lee BH. Role of the Fas/Fas ligand death receptor pathway in ginseng saponin metabolite-induced apoptosis in HepG2 cells. Arch Pharm Res 2004;27:402-6.
14. Quan LH, Piao JY, Min JW, Kim HB, Kim SR, Yang DU, Yang DC. Biotransformation of ginsenoside Rb_1 to prosapogenins, gypenoside XVII, ginsenoside Rd, ginsenoside F_2, and compound K by *Leuconostoc mesenteroides* DC102. J Ginseng Res 2011;35:344-51.
15. Quan LH, Kim YJ, Li GH, Choi KT, Yang DC. Microbial transformation of ginsenoside Rb_1 to compound K by *Lactobacillus paralimentarius*. World J Microbiol Biotechnol 2013;29:1001-7.
16. Shin KC, Seo MJ, Oh HJ, Oh DK. Highly selective hydrolysis for the outer glucose at the C-20 position in ginsenosides by β-glucosidase from *Thermus thermophilus* and its application to the production of ginsenoside F_2 from gypenoside XVII. Biotechnol Lett 2014;36:1287-93.
17. Hu Y, Luan H, Hao D, Xiao H, Yang S, Yang L. Purification and characterization of a novel ginsenoside-hydrolyzing β-D-glucosidase from China white jade snail (*Achatina fulica*). Enzyme Microb Technol 2007;40:1358-66.
18. Yan, Q, Zhou XW, Zhou W, Li XW, Feng MQ, Zhou P. Purification and properties of a novel β-glucosidase, hydrolyzing ginsenoside Rb1 to CK, from *Paecilomyces* Bainier. J Microbiol Biotechnol 2008;18:1081-9.
19. De Man JD, Rogosa M, Sharpe ME. A medium for the cultivation of lactobacilli. J Appl Microbiol 1960;23:130-5.
20. Casaregola S, Nguyen HV, Lapathitis G, Kotyk A, Gaillardin C. Analysis the constitution of beer yeast genome by PCR, sequencing and subtelomeric sequence hybridization. Int J Syst Evol Microbiol 2001;51(Pt 4):1607-18.
21. Kwon KS, Lee J, Kang HG, Hah YC. Detection of β-glucosidase activity in polyacrylamide gels with esculin as substrate. Appl Environ Microbiol 1994;60:4584-6.
22. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.
23. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406-25.
24. Cottrell M, Viljoen BC, Kock JL, Lategan PM. The long-chain fatty acid compositions of species representing the genera *Saccharomyces*, *Schwanniomyces* and *Lipomyces*. J Gen Microbiol 1986;132:2401-3.
25. Van der Rest ME, Kamminga AH, Nakano A, Anraku Y, Poolman B, Konings WN. The plasma membrane of *Saccharomyces cerevisiae*: structure, function, and biogenesis. Microbiol Rev 1995;59:304-22.
26. Kim MK, Im WT, Ohta H, Lee M, Lee ST. *Sphingopyxis granuli* sp. nov., a β-glucosidase-producing bacterium in the family Sphingomanadaceae in α-4 subclass of the Proteobacteria. J Microbiol 2005;43:152-7.
27. Quan LH, Cheng LQ, Kim HB, Kim JH, Son NR, Kim SY, Jin HO, Yang DC. Bioconversion of ginsenoside Rd into compound K by *Lactobacillus pentosus* DC101 isolated from Kimchi. J Ginseng Res 2010;34:288-95.
28. Doh ES, Chang JP, Lee KH, Seong NS. Ginsenoside change and antioxidation activity of fermented ginseng. Korean J Med Crop Sci 2010;18:255-65.