

Bioconversion of Ginsenosides from Red Ginseng Extract Using *Candida allociferii* JNO301 Isolated from *Meju*

Sulhee Lee¹, Yong-Hun Lee¹, Jung-Min Park², Dong-Hoon Bai³, Jae Kweon Jang⁴ and Young-Seo Park^{1,*}

¹Department of Food Science and Biotechnology, Gachon University, Seongnam 461-701, Korea

²Korean Culture Center of Microorganisms, Seoul 120-091, Korea

³Department of Food Engineering, Dankook University, Cheonan 330-714, Korea

⁴Department of Food Science, Chungkang College of Cultural Industries, Icheon 467-744, Korea

Abstract Red ginseng (*Panax ginseng*), a Korean traditional medicinal plant, contains a variety of ginsenosides as major functional components. It is necessary to remove sugar moieties from the major ginsenosides, which have a lower absorption rate into the intestine, to obtain the aglycone form. To screen for microorganisms showing bioconversion activity for ginsenosides from red ginseng, 50 yeast strains were isolated from Korean traditional *meju* (a starter culture made with soybean and wheat flour for the fermentation of soybean paste). Twenty strains in which a black zone formed around the colony on esculin-yeast malt agar plates were screened first, and among them 5 strains having high β -glucosidase activity on *p*-nitrophenyl- β -D-glucopyranoside as a substrate were then selected. Strain JNO301 was finally chosen as a bioconverting strain in this study on the basis of its high bioconversion activity for red ginseng extract as determined by thin-layer chromatography (TLC) analysis. The selected bioconversion strain was identified as *Candida allociferii* JNO301 based on the nucleotide sequence analysis of the 18S rRNA gene. The optimum temperature and pH for the cell growth were 20~30°C and pH 5~8, respectively. TLC analysis confirmed that *C. allociferii* JNO301 converted ginsenoside Rb1 into Rd and then into F2, Rb2 into compound O, Rc into compound Mc1, and Rf into Rh1. Quantitative analysis using high-performance liquid chromatography showed that bioconversion of red ginseng extract resulted in an increase of 2.73, 3.32, 33.87, 16, and 5.48 fold in the concentration of Rd, F2, compound O, compound Mc1, and Rh1, respectively.

Keywords β -Glucosidase, Bioconversion, Ginsenosides, Red ginseng extract, Yeast

Ginseng (*Panax ginseng*) is a medicinal plant that grows in cool areas of Asia, North America, and Siberia. It is a herbaceous perennial plant belonging to the family Araliaceae and is well known as a traditional food for refreshment and immunostimulation, continuing to be focus of research. Red ginseng is prepared when ginseng roots are processed by slow heating at low temperature, and it contains many

active components such as saponins, phenols, proteins, amino acids, vitamins, and minerals. Ginsenosides, saponins of red ginseng, have a glycosidic form containing sugars and are grouped into protopanaxadiol (PPD) and protopanatriol (PPT) according to the position, number, and the type of the sugars. Currently, more than 40 types of ginsenosides are known [1]. The major ginsenosides Rb1, Rb2, Rc, Re, and Rg1 account for 80% of the total ginsenosides, among which some major ginsenosides are converted into minor ginsenosides by heat treatment [2], acid treatment [3, 4], alkali treatment [5], or enzyme treatment [6]. Minor ginsenosides such as Rg3, Rh1, Rh2, and compound K are more aglyconic than major ginsenosides. In recent studies, various therapeutic effects of minor ginsenosides such as anti-tumor activity [7], liver protection [8], anti-allergic effects [9], and neurotherapeutic effects [10] have been described. PPD-type ginsenosides such as Rb1, Rb2, and Rc can be hydrolyzed, and their glycosidic sugar component is released by β -glucosidase, resulting in conversion into Rd, F2, Rg3, Rh2, compound Mc1, O, Mc, Y, and K [11-13]. PPT-type ginsenosides such as Re and Rf can also be converted to Rg1, Rg2, and Rh1 by β -glucosidase [14, 15].

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© The Korean Society of Mycology

*Corresponding author

E-mail: ypark@gachon.ac.kr

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Previous studies mainly focused on the bioconversion of ginsenosides using microorganisms such as bacteria [16] and fungi [17] isolated from soil on ginseng fields, lactic acid bacteria from *kimchi* [18], and intestinal microorganisms from the human gut [19]. Yeasts are representative strains for the fermentation as well as good producers of β -glucosidase, of which properties are suitable for the bioconversion of ginsenosides. However, studies on bioconversion using yeast strains are limited to evaluation of the effect of the concentration of ginseng extract on the growth rate and enzyme activity of yeast strains, and few studies are available on the bioconversion of ginsenosides using specific yeast strains [20-22].

Meju is a Korean traditional starter culture used to ferment Korean traditional sauces such as *doenjang*, *gochujang*, or *ganjang*. It is prepared by fermenting soybean as the main material, and during the fermentation biologically active microorganisms such as bacteria, fungi, and yeasts, which show many types of enzyme activity are cultivated [23-25].

In this study, a yeast strain having high β -glucosidase activity was isolated from *Meju* and the bioconversion of ginsenosides of the red ginseng extract was characterized.

MATERIALS AND METHODS

Materials and reagents. Red ginseng extract was obtained from Wellbuy Korea (Seoul, Korea), yeast malt (YM) broth was purchased from Difco Co. (Detroit, MI, USA), and esculin and *p*-nitrophenyl- β -D-glucopyranoside were from Sigma-Aldrich Co. (St. Louis, MO, USA). The API kit was obtained from the Biomérieux Co. (Marcy l'Etoile, France). The thin-layer chromatography (TLC) Silica gel 60 F254 plate was from Merck & Co. Inc. (Whitehouse Station, NJ, USA), and chloroform, methanol, and acetonitrile for high-performance liquid chromatography (HPLC) were provided by Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Rd, Re, and Rg1 as standards for ginsenoside were from BTGin (Okcheon, Korea), and Rb1, Rg3, Rh1, and compound K were from Ambo Institute (Daejeon, Korea).

Isolation of yeast strains from conventional *Meju*.

Conventional *meju* was collected from various regions in Korea for the selection of yeast strains able to bioconvert ginsenosides. The *meju* samples were appropriately diluted into peptone water and spread onto YM agar plates. Single colonies, which were produced in the plates, were transferred to esculin agar plates [26] by using a toothpick. Yeast strains in which a black zone formed around the colony were selected as β -glucosidase-producing strains.

Assay of β -glucosidase activity. The Kohchi method [27] was modified to assay β -glucosidase activity. A single colony of each yeast strain was inoculated into YM broth and incubated at 30°C for 72 hr at 145 rpm. The culture

broth was centrifuged at 7,600 \times g for 10 min to remove the cells, and the supernatant was used as a crude enzyme solution. Then, 400 L of 50 mM phosphate buffer (pH 7.0) containing 5 mM *p*-nitrophenyl- β -D-glucopyranoside was pre-incubated at 50°C for 5 min. Then, 300 μ L of crude enzyme solution was added to start the reaction. The reaction was performed at 50°C for 10 min. After incubation, 700 μ L of 0.5 M Na₂CO₃ was added to stop the reaction, and the absorbance at 405 nm was measured. One unit of enzyme is defined as the amount of enzyme that releases 1 μ mol of *p*-nitrophenol under a given condition when *p*-nitrophenyl- β -D-glucopyranoside is used as a substrate.

Thin-layer chromatography. The yeast strain was cultured in YM broth containing red ginseng extract at a concentration of 1% (w/v) at 30°C or 36°C for 5 days at 145 rpm. The culture broth was then centrifuged at 8,000 rpm for 10 min to remove the cells. The supernatant was extracted with *n*-butanol and the extract was vacuum-dried. The dried pellet was dissolved in methanol and TLC was performed using a silica gel plate with CHCl₃:CH₃OH:H₂O (65:35:10, v/v/v) as a developing solvent. After the samples were developed, 10% H₂SO₄ was sprayed on the silica gel plate and heat-dried for 5 min.

HPLC analysis. The quantitative and qualitative analysis of ginsenoside was conducted using HPLC (Younglin Instrument, Seoul, Korea) with a SP930D detector at a wavelength of 203 nm. The sample (20 L) was injected into a C₁₈ column (4.6 \times 150 mm, ID 5 μ m). The temperature of the column oven was 40°C and the flow rate was 1.6 mL/min. The mobile phase was prepared as a gradient with solvents A (acetonitrile) and B (distilled water). The ratio of solvents A and B was as follows: 15/85 (0~5 min), 15/85 (5~55 min), 40/60 (55~75 min), 58/42 (75~90 min), 90/10 (92~97 min), and 90/10 (97~99 min).

Identification of yeast isolates. The isolated yeast strains were identified by analyzing their 18S rRNA nucleotide sequences. The chromosomal DNA was extracted using a Wizard genomic DNA purification kit (Promega, San Luis Obispo, CA, USA) according to manufacturer's instructions. The 18S rRNA gene was amplified by PCR using the universal primers NS1 (5'-GTAGTCATATGCTTGCTC-3') and NS8 (5'-TCCGCAGGTTACCTACGGA-3'). The amplified PCR product was purified using a Wizard SV Gel and PCR clean-up system (Promega), and the nucleotide sequence of purified PCR product was determined using an ABI PRISM 3700 DNA analyzer. The nucleotide sequences of the isolates were analyzed using the National Center for Biotechnology Information (NCBI) database and the programs BLASTN, Clustal X, and Mega 4 [28]. The carbohydrate utilization pattern of the strain was characterized using the API 20C kit according to the manufacturer's instructions, and the results were interpreted using apiweb (Biomérieux Co.).

Growth condition of yeast strains. Selected yeast strains were cultivated in YM broth until the absorbance at 600 nm reached 1.0, and the culture broth was inoculated in YM broth at a final concentration of 1% (v/v). The cells were then incubated at various temperatures (20°C, 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C) for 72 hr in a shaker at 145 rpm, and the initial pH of the YM broth was respectively adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 with 1 N HCl or 1 N NaOH. The yeast cells were then incubated at 30°C for 72 hr at 145 rpm. The growth rate of yeast cells was estimated by measuring the absorbance of the broth at 600 nm. Additionally, the growth rate of the yeast cells for different concentrations of red ginseng extract was evaluated using YM broth supplemented with

red ginseng extract at concentrations of 0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% (w/v).

Bioconversion of ginsenoside with selected yeast strains. The selected yeast strains were inoculated in YM broth containing 1% (w/v) red ginseng extract at 30°C for 10 days at 145 rpm. After incubation, the culture broth was centrifuged at 8,000 rpm for 10 min, and the supernatant was extracted with water-saturated *n*-butanol. The *n*-butanol fraction was vacuum-dried, and the pellet was dissolved in methanol and then subjected to TLC and HPLC analysis.

RESULTS AND DISCUSSION

Isolation of yeast strains for bioconversion of ginsenosides. In total, 50 yeast strains were isolated from *meju*, and among them, 39 strains showed a black

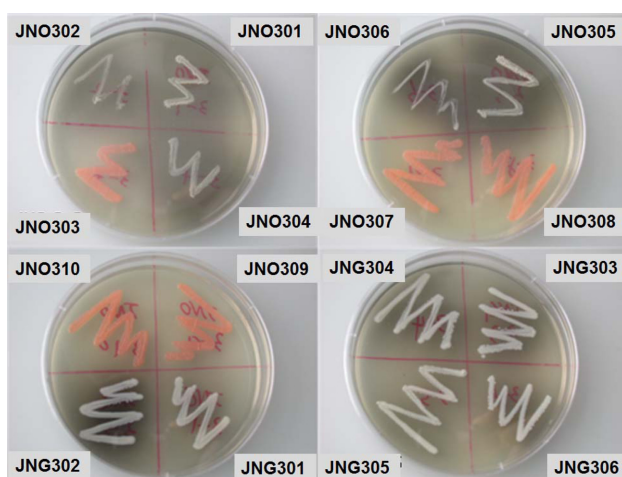


Fig. 1. Yeast strains that showed β -glucosidase activity on the esculin-yeast malt agar plate.

Table 1. β -Glucosidase activity of isolated strains showing a black zone on the esculin plate

Strain	β -Glucosidase activity (ΔA_{405})	Strain	β -Glucosidase activity (ΔA_{405})
JNO301	2.147	JNG301	0.001
JNO302	2.056	JNG302	0.029
JNO303	-0.003	JNG303	0.063
JNO304	2.042	JNG304	0.065
JNO305	1.875	JNG305	0.066
JNO306	1.005	JNG306	0.052
JNO307	-0.007	JNG307	0.048
JNO308	-0.006	JNG308	0.020
JNO309	-0.011	JNG309	0.050
JNO310	0.006	JNG310	0.032

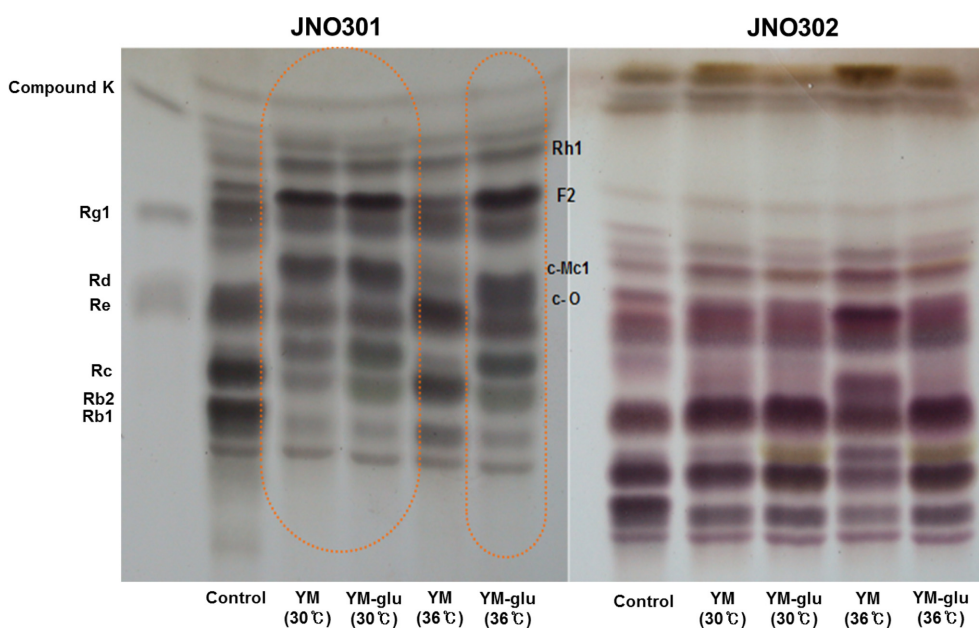


Fig. 2. Thin-layer chromatography of the culture supernatant of strains JNO301 and JNO302 extracted with water-saturated *n*-butanol, which shows the bioconversion of ginsenosides. YM, yeast malt.

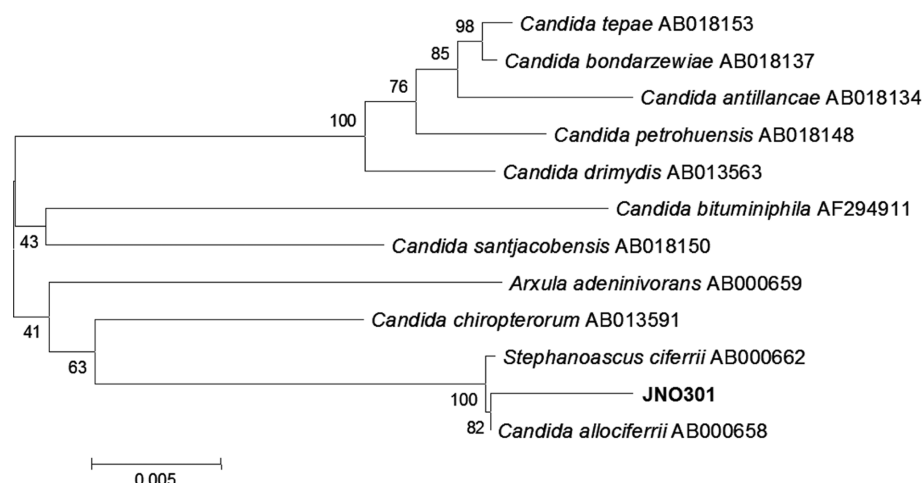


Fig. 3. Phylogenetic tree of strain JNO301 based on the 18S rRNA gene sequence.

zone around the colony on YM agar plates containing 0.1% (w/v) esculin (Fig. 1). The esculin method is used to screen strains showing β -glucosidase activity. Kim *et al.* [12] and Quan *et al.* [18] isolated lactic acid bacteria and some soil microorganisms having β -glucosidase activity using the esculin method. Among 39 strains that showed a positive reaction on the esculin plate, the β -glucosidase activities of 20 strains were measured using *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG) as a substrate. Strains JNO301, JNO302, JNO304, JNO305, and JNO306 had high β -glucosidase activity, but the other strains showed little or no β -glucosidase activity (Table 1). Some strains that showed a positive reaction on the esculine plate did not have β -glucosidase activity when *p*NPG was used as a substrate, indicating that the reaction mechanisms are different between esculin and *p*NPG. The bioconversion abilities of the selected 5 strains were analyzed by subjecting their culture supernatants to TLC. As shown in Fig. 2, strain JNO301 had the highest bioconversion activity, and strain JNO302 had the second highest activity. However, strains JNO304, JNO305, and JNO306 did not show bioconversion activity on TLC (data not shown). When strain JNO301 was compared with the control, the major ginsenosides Rb1, Rb2, and RC were not present, whereas minor ginsenosides, such as compound Mc1, F2, and Rh1, were produced. This result implies that the glucose attached to the major ginsenosides was efficiently hydrolyzed by strain JNO301 to obtain the aglycone form. Therefore, strain JNO301 was finally selected and used for further experiments. There were some differences in the bioconversion pattern according to the presence of glucose in the culture media when the bioconversion was performed at 36°C, although there was no difference in the bioconversion pattern at 30°C.

Identification and biochemical analysis of selected strains. The finally selected strain JNO301 was identified as *Candida allociferii* based on the phylogenetic taxonomy with sequence alignment of the 18S rRNA gene. This strain had the

Table 2. Carbohydrate utilization of *Candida allociferii* JNO301 using API kit

Carbohydrate	Utilization
Glycerol	+
2-Keto-D-gluconate	+
Arabinose	+
Xylose	+
Adonitol	+
Xylitol	-
Galactose	+
Inositol	+
Sorbitol	+
α -Methyl-D-glucoside	-
<i>N</i> -Acetyl-D-glucosamine	+
Cellobiose	+
Lactose	-
Maltose	+
Saccharose	+
Trehalose	+
Melezitose	-
Raffinose	+
Hyphae	+

+, positive reaction; -, negative reaction.

highest genetic similarity to *C. allociferii* AB000658 (Fig. 3). Recently, *C. allociferii* was taxonomically branched from *Stephanoascus ciferrii* through a taxonomic identification study [29]. The characteristics of the biochemical and carbohydrate utilization of *C. allociferii* JNO301 are shown in Table 2. This strain utilized glucose, 2-keto-D-gluconate, glycerol, galactose, and arabinose among monosaccharides; xylose, adonitol, inositol, and sorbitol among sugar alcohols; and cellobiose, maltose, saccharose, and trehalose among disaccharides, which are the typical characteristics of *Candida* spp.

Effect of bioconversion temperature and initial pH on growth rate. *C. allociferii* JNO301 was incubated at different temperatures to examine the optimum growth

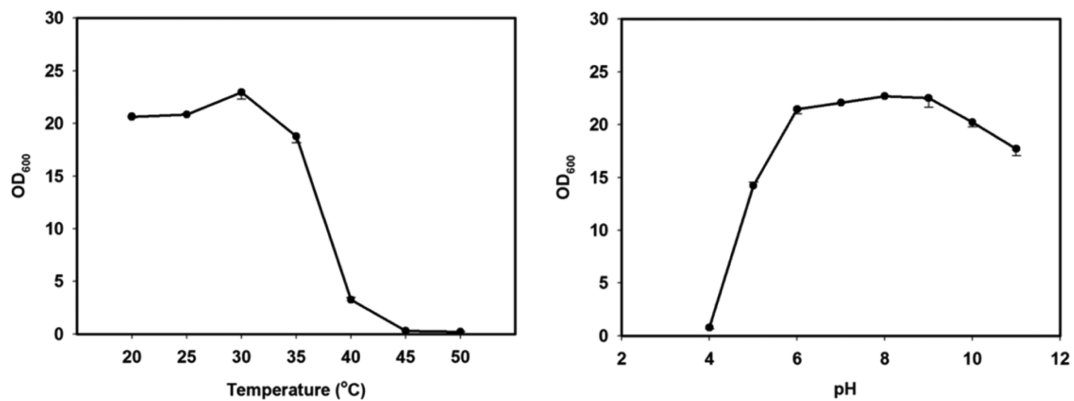


Fig. 4. Cell growth of *Candida allociferrii* JNO 301 according to temperature and pH.

temperature. The optimal temperature was 20~30°C and the growth rate rapidly decreased at temperatures higher than 35°C (Fig. 4A). This result agrees with the optimum growth temperature of most yeast, which is 25~30°C [29]. *C. allociferrii* JNO301 did not grow at pH 4, and the optimal initial pH of the culture medium was 8~9. *C. allociferrii* JNO301 was alkaline-resistant, as it grew well at pH 11.

Effect of concentration of red ginseng extract on growth rate. *C. allociferrii* JNO301 was inoculated in culture medium containing different concentrations of red ginseng extract, and its growth rate was evaluated (Fig. 5). The lag phase continued until 16 hr and the number of cells subsequently increased rapidly until 72 hr regardless of the concentration of the red ginseng extract. Generally, yeast cells enter the stationary phase before 48 hr when YM broth is used as culture medium, but the growth rate of *C. allociferrii* JNO301 was slower than that of the other yeast cells. When *C. allociferrii* JNO301 was cultured in YM medium containing 3% red ginseng extract, the A₆₀₀ value was higher than that of the control (no addition of red ginseng extract), indicating that red ginseng extract stimulated cell growth. Park and Jo [20] reported that growth rate of yeast increased on addition of ginseng extract, and

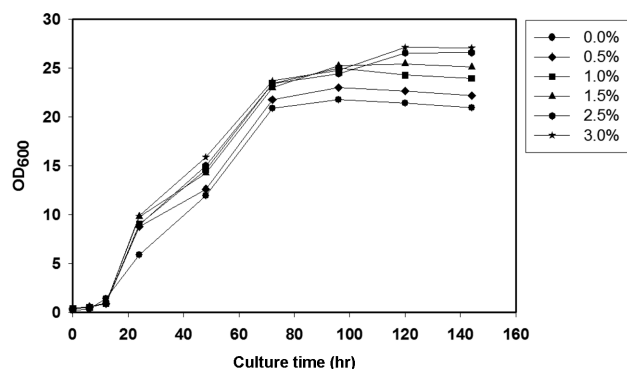


Fig. 5. Effect of the concentration of red ginseng extracts on the cell growth of *Candida allociferrii* JNO 301.

Joo and Lee [30] also reported that growth rate of yeast cells increased on addition of 0.3% ginseng extract, but the cell growth was reduced at the concentration of 1.5% because of unknown growth inhibitory effects of the red ginseng extract. *C. allociferrii* JNO301 promoted the growth of yeast cells even at the high concentration of 3.0% because this strain was tolerant to some growth-inhibiting factors in the red ginseng extract.

Bioconversion of ginsenosides using *C. allociferrii* JNO301. Bioconversion of ginsenosides in red ginseng extract using *C. allociferrii* JNO301 was analyzed by TLC,

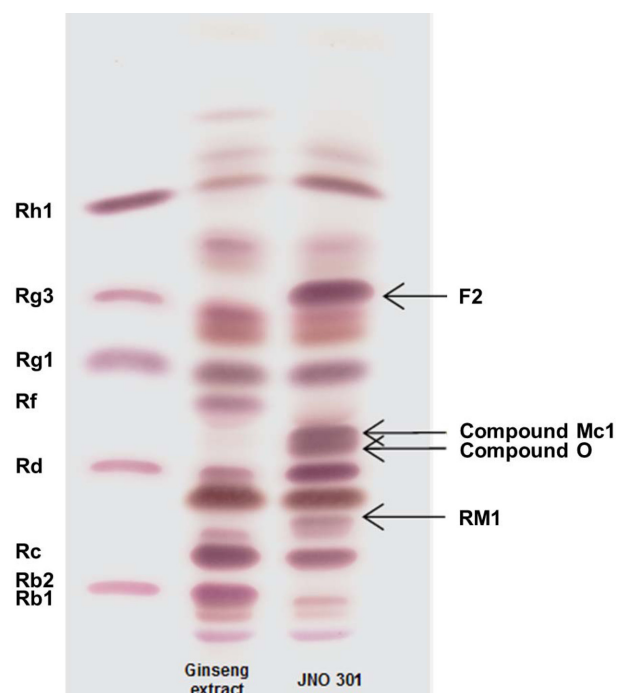


Fig. 6. Thin-layer chromatography assay of the bioconversion of ginsenosides in red ginseng extract by *Candida allociferrii* JNO301. *C. allociferrii* JNO301 was incubated into yeast malt broth supplemented with 1% (w/v) red ginseng extract at 30°C for 10 days at 145 rpm.

and noticeable changes in the ginsenoside composition were detected (Fig. 6). From the preliminary analysis and the current state of knowledge regarding the bioconversion mechanism [31, 32], it is concluded that one of the major ginsenosides, Rb1 (Rf, 0.31), was converted to Rd (Rf, 0.44) by the removal of a glucose residue (C_{20} of Glc (1-6) Glc), and that a glucose residue (C_3 of Glc (1-2) Glc) of Rd was continuously hydrolyzed, resulting in conversion into F2 (Rf, 10.7). Ginsenoside F2 was an intermediate in the bioconversion from Rb1, Rb2, Rc, and Rd and was converted into compound K and Rh2. Studies on the mechanism of reaction from Rb1 to F2 using various microorganisms have been reported. Cheng *et al.* [31] converted Rb1 into F2 using *Caulobacter* and Ma *et al.* [32] induced bioconversion of Rb1 into F2 and compound K using fungi. TLC revealed that the concentration of Rb2 (Rf, 0.35) and Rc (Rf, 0.36) decreased, and two spots appeared just above the Rd spot. These results indicate that glucose residues (C_3 of Glc (1-2) Glc) of Rb2 and Rc were removed, resulting in conversion into compound O (Rf, 0.48) and compound Mc1 (Rf, 0.49), respectively. Bioconversion of Rb2 and Rc has been studied, and the reaction mechanism of Rb2 (Rc) \rightarrow Rd \rightarrow

Rg3 (F2) \rightarrow compound K (Rh2) has been elucidated using strains with arabinosidase activity [6, 33, 34]. Recently, the biologically functional aspects of compound Y and compound Mc were elucidated [11], and the mechanism of the bioconversion of Rb2 to compound Y and that of Rc to compound Mc have been actively studied [19, 35]. *C. allociferii* JNO301 had β -glucosidase activity that can remove glucose residues from Rb1, Rb2, and Rc, and it belonged to PPD in the red ginseng extract. Rf spot (Rf, 0.52), a type of PPT, disappeared and the density of Rh1 (Rf, 0.76) increased. There are 3 glucose residues at C_{20} and C_6 of Rf, and among them 2 glucose residues were removed at position C_{20} and C_6 and thereby converted to Rh1. Generally, Re is converted into Rg1 by removal of glucose at position C_6 and Rg1 is converted into Rh1 by removal of glucose at position C_{20} [15]. It has however been reported that some microorganisms remove glucose residues of Rf to convert into Rh1 [36]. This study showed that β -glucosidase produced by *C. allociferii* JNO301 has special substrate specificity for the glucose residues at position C_6 and C_{20} because *C. allociferii* JNO301 did not affect the ginsenosides Re, Rg1, and Rg2.

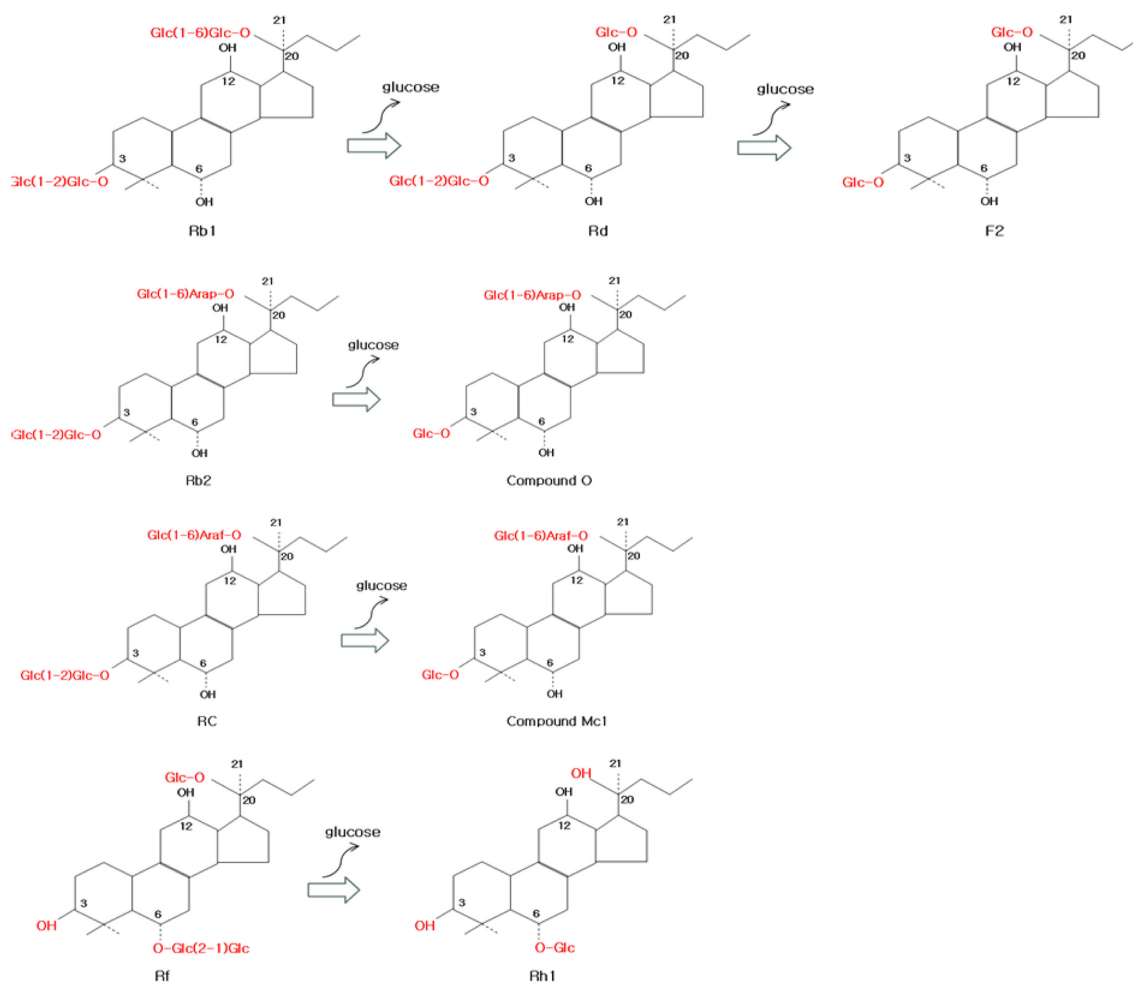


Fig. 7. Proposed reaction mechanism pathway for ginsenosides in red ginseng extract treated with *Candida allociferii* JNO301.

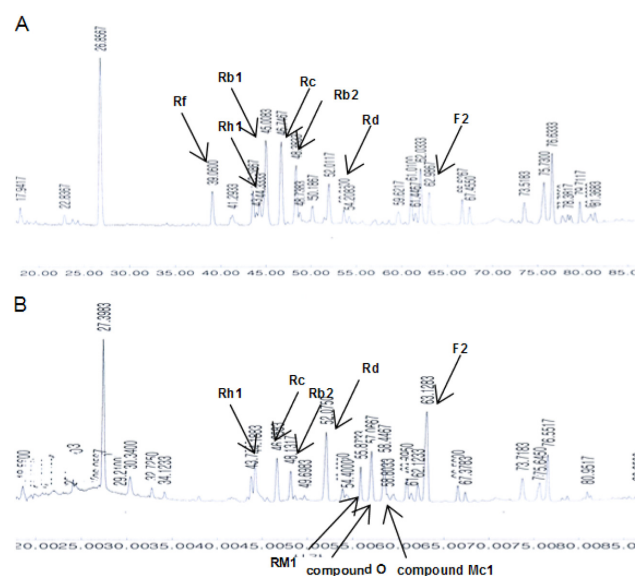


Fig. 8. High-performance liquid chromatography assay of the bioconversion of ginsenosides by *Candida allociferrii* JNO301. A, Control; B, Bioconversion of ginsenosides by *Candida allociferrii* JNO301.

An unknown spot called RM1 (Rf, 4.1) appeared between RC and Rd on the TLC plate after the reaction; this substance may have been produced from ginsenosides located below Rb1 on the TLC plate. The putative reaction mechanisms of ginsenoside bioconversion using *C. allociferrii* JNO301 are shown in Fig. 7.

HPLC analysis of the bioconversion of ginsenosides using *C. allociferrii* JNO301 was performed, and the results are shown in Fig. 8 and Table 3. The ginsenosides Rb1 (Rt, 45 min), Rb2 (Rt, 48 min), and Rc (Rt, 46 min) disappeared, whereas the levels of Rd (Rt, 52 min) and F2 (Rt, 63 min) increased rapidly. Compound O (Rt, 57 min) and compound Mc1 (Rt, 46 min) were newly produced when compared with the control. An unknown peak appeared at the retention time of 55 min, corresponding to RM1, which was detected on the TLC plate. In addition, the ginsenoside Rf (Rt, 39 min) disappeared after the reaction, and accordingly the level of Rh1 (Rt, 43 min) increased. When the quantitative changes of ginsenosides were examined, the levels of the ginsenosides Rb1, Rb2, Rc, and Rf decreased by 0.01, 0.61, 0.57, and 0.05 fold, respectively, whereas those of the ginsenosides Rd, F2, compound O, compound Mc1, and Rh1 increased by 2.73, 3.32, 33.87, 16.00, and 5.48 fold, respectively.

Korean red ginseng is a popular health functional food. Ginsenosides are well known as pharmaceutical ingredients, and their functionality has been identified through many studies. Recently, many food companies and laboratories have attempted to prepare red ginseng products with a high content of minor ginsenosides, which have a high absorption rate into the human intestine and have enhanced functional properties. Hence, in this study, bioconversion of ginsenosides

Table 3. Quantitative analysis of relative concentration of ginsenosides in red ginseng extract transformed by *Candida allociferrii* JNO301 using high-performance liquid chromatography

Ginsenoside	Control ^a	<i>C. allociferrii</i> JNO301 ^b	Fold (<i>C. allociferrii</i> JNO301/control)
Rb1	6,045.05	75.23	0.01
Rd	2,035.24	5,549.65	2.73
F2	1,752.62	5,824.24	3.32
Rb2	2,875.63	1,752.36	0.61
C-O	84.02	2,845.53	33.87
Rc	4,752.63	2,702.41	0.57
C-Mc1	153.52	2,456.36	16.00
Rf	1,824.32	86.23	0.05
Rh1	489.63	2,682.63	5.48

Values are presented as peak area.

^aNo reaction.

^b*C. allociferrii* JNO301 was incubated into yeast malt broth supplemented with 1% (w/v) red ginseng extract at 30°C for 10 days at 145 rpm.

in red ginseng extract was performed using yeast to prepare high concentrations of minor ginsenosides, whereupon Rb1, Rb2, Rc, and Rf in red ginseng were successfully converted into minor ginsenosides such as F2, compound O, compound Mc1, and Rh1. Ginsenosides F2, compound O, and compound Mc1 are considered to be intermediates of compound K, Rh2, compound Mc, and compound Y, and their functionality should be studied to determine commercial applicability. Furthermore, ginsenoside Rh1, one of the main ginsenosides converted in this study has been shown to have anticancer, antitumor, and antiallergic activity in many studies [1, 37]. The bioconversion of ginsenosides in red ginseng using yeast is expected to result in the development of a highly functional food product.

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