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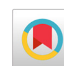

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Dihydrodaidzein production from soybean hypocotyl extract by human intestinal bacterium MRG-1

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Abstract Phytoestrogenic *S*-equol production in human gut exclusively depends on the biotransformation of daidzein to dihydrodaidzein (DHD). With a growing demand for the DHD enriched biomaterials, the commercial soybean hypocotyl extract (SHE) was chosen as a substrate for the microbial DHD production by human gut bacterium MRG-1, anaerobic DHD producer. To optimize the production of DHD, anaerobic fermentation conditions, including sterilization time, growth stage of inoculum, and growth media, were investigated. Maximum DHD production (1.2 g/L) was achieved after 48 h incubation when 1% (w/v) of SHE in the 20-min-sterilized Gifu Anaeboic Medium media was inoculated with OD_{600} 0.3-0.4 of MRG-1. This is the first report that crude soy biomaterial, instead of pure compounds, such as daidzin and daidzein, is utilized for the production of the DHD enriched biomaterial.

Keywords Biomaterial · Biotransformation · Dihydrodaidzein · Gut bacterium · Soybean hypocotyl extract

Introduction

Beneficial effects of dietary isoflavones on human health have been extensively studied for the last decade, and the molecular-level understanding of their biotransformation, interaction with biological receptors, metabolism in body are now available [1-4].

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For example, a strong phytoestrogenic *S*-equol can be produced from daidzin and daidzein in the dietary soy foods [5], in which the contents of isoflavonones are varied depending on the processing [6]. The biotransformation of *S*-equol production is achieved only by a few intestinal bacteria and the biosynthetic pathway involves dihydrodaidzein (DHD) and tetrahydrodaidzein (THD) metabolites [7,8]. However, biological activity and industrial applications of DHD have not been fully investigated.

It was reported that DHD can improve metabolic disorders, such as obesity and high blood pressure, as well as enhance the biosynthesis of collagen to reduce skin wrinkle [9]. As the application of DHD in food and cosmetic industries has become more important, technology development for the industrial scale production of DHD is being sought. However, traditional fermentation of soy isoflavone does not produce DHD, but only anaerobic biotransformation of daidzein does.

Recently, we have reported a newly isolated DHD producer, human gut bacterium MRG-1, catalyzing the stereospecific production of *R*-DHD from daidzin and daidzein [10]. The new anaerobic bacterium has a short doubling time of 55 minutes under the anaerobic conditions, making MRG-1 a good candidate for the industrial DHD production. In this report, microbial DHD production from soybean hypocotyl extract (SHE) by MRG-1 under various culture conditions were investigated.

Materials and Methods

Chemicals

The main growth medium of MRG-1 was Gifu Anaeboic Medium (GAM) from Nissui Pharmaceutical Co. (Tokyo, Japan). Brain Heart Infusion (BHI), nutrient broth and tryptone were purchased from Becton, Dickinson and Company (38800 Le Pont de Claix, France), and Nutrient broth (NB) was purchased from Bacto Difco (Lawrence Kansas, KS, USA). The SHE, Isovone and Soluvone, were obtained from Bioland Nature, Science and Life (Gyeonggido, Korea). Daidzin, glycitin and glycitein were

purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Daidzein, genistin, genistein were from LC Laboratories (Woburn, MA, USA). DHD was synthesized according to the published method [11]. High performance liquid chromatography (HPLC) grade methanol was obtained from Fisher (Pittsburgh, PA, USA).

Microorganism and culture conditions

A Gram-negative anaerobic bacterium MRG-1, KCTC11894BP, was used for this study [10]. For the broth culture, GAM (5.9 g) in 100 mL of distilled water was used, and 1.5 g of agar was added for plate. MRG-1 culture was prepared from plate culture in anaerobic chamber (CO_2 5%, H_2 10%, N_2 85%) at 37 ºC. Single colony was selected from the plate by sterilized toothpick, and inoculated to GAM broth (800 µL). After 24 h, daidzin (10 mM) was added to the 100 µL of cell culture to yield 0.1 mM of daidzin solution. After incubation for 1 h, the culture broth was extracted with ethyl acetate (1.0 mL) for the activity check. The mixture was vortexed for 20 s at room temperature. After centrifugation at 10770× g (13000 rpm) for 5 min, 800 µL of supernatant was taken to the dryness under vacuum and re-dissolved in methanol (150 µL). Methanolic solution was filtered through a 0.2 µM filter and the filtrate was used for HPLC analysis. The broth culture (100 µL) exhibited highest DHD production activity was inoculated into GAM broth for the further experiments.

HPLC analysis of soy isoflavones and DHD

For the HPLC analysis, samples in 10 µL of methanol were used for injection. Finnigan Surveyor Plus HPLC system (Thermo Scientific, Waltham, MA, USA) equipped with a photodiode array detector (PDA Plus) and a C18 reversed-phase column (Hypersil GOLD 5 µm, 4.6 by 100 mm; Thermo Scientific) was employed and monitored at 291 nm. The mobile phase was composed of 0.1% acetic acid (A) and methanol (B). The elution profile started with an A/B ratio at 80:20 (v/v), and linearly changed to 50:50 (v/v) for 30 min. The flow rate was 1.0 mL/min. Standard solutions of daidzin, genistin, daidzein, genistein and DHD in methanol at four concentrations were used for the calibration curves. Regression equation of the calibration curves were $Y = 8120X + 50$ ($R^2 = 0.9993$) for daidzin, $Y = 8528X + 96$ ($R^2 = 0.9974$) for genistin, $Y = 10250X + 94$ ($R^2 = 0.9987$) for daidzein, $Y = 13304X + 57$ ($R^2 = 0.9993$) for genistein and $Y = 11620X + 177$ ($R^2 = 0.9979$) for DHD, where Y was the integrated peak area and X was concentration (mM) of standard compound (Supplementary materials Fig. 1).

Validation of isoflavone extraction procedure

To establish and test the extraction method, ethyl acetate and chloroform were used as extraction solvents and isoflavone contents by single and triple extraction were analyzed by HPLC. Distilled water was used as a control of GAM broth. Soybean meal (50 mg) was dispersed in 5.0 mL of distilled water and GAM broth, respectively, and sterilized at 121 ºC for 20 min using autoclave (MAC-601, EYELA, Tokyo, Japan). For the single extraction, ethyl acetate (1.0 mL) was added to the sterilized solution (100 µL) and the mixture was vortexed for 20 s at room temperature. After centrifugation at 10770× g (13000 rpm) for 5 min, 800 µL of supernatant was taken to the dryness under vacuum and re-dissolved in methanol (150 µL). Metanolic solution was filtered through a 0.2 µM filter and the filtrate was used for HPLC analysis. For the triple extraction, the precipitate from the first extraction was subjected to the additional extractions twice. Total 2.4 mL of the combined supernatant was taken to the dryness under vacuum and re-dissolved in methanol (150 µL) for the following HPLC analysis.

Soluble isoflavone content of soy products

To analyze the soluble isoflavone contents of soy products, soybean meal, isoflavone-rich dietary supplement (Genistein, Source Naturals, USA), Isovone and Soluvone (Bioland, Cheongju, Korea) were selected. Each sample (50 mg) was dispersed in 5.0 mL of distilled water and GAM broth, and sterilized at 121 ºC for 20 min. Each solution (100 µL) was extracted with 1.0 mL of ethyl acetate by triple extraction as described above.

Effect of autoclaving time on the soluble flavonoid content in media

To test whether autoclaving time changes the concentration of soluble isoflavones, Isovone (50 mg) was dispersed in 5.0 mL of distilled water and GAM broth, and autoclaved at 121 ºC for 20, 30, 40 and 50 min, respectively. For the control, one sample was not autoclaved and kept at room temperature.

DHD production by different growth stage inoculums

MRG-1 was cultured until the optical density at 600 nm (OD_{600}) reached at 0.2, 0.3, 0.4 and 0.5. Two independent bacterial cultures (25 µL) with the same OD were inoculated to the sterilized GAM broth (5.0 mL) containing Isovone (50 mg), and incubated under anoxic conditions (5% CO_2, 10% H_2, and 85% N_2) at 37 ºC. Media (100 µL) were taken for the analysis after 0, 3, 6, 9, 12, 24 and 48 h incubations, and analyzed by HPLC.

DHD production in different media

MRG-1 was cultured until OD_{600} reached at between 0.3-0.4. Bacterial cultures (25 µL) were inoculated to five different media (5.0 mL) of GAM (59 g/L), LB (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L), BHI (37 g/L), NB (8 g/L), and distilled water containing Isovone (50 mg), and incubated under anoxic conditions (5% CO_2, 10% H_2, and 85% N_2) at 37 ºC. Media (100 µL) were taken after 0, 3, 6, 9, 12, 24 and 48 h and the isoflavone content was analyzed by HPLC.

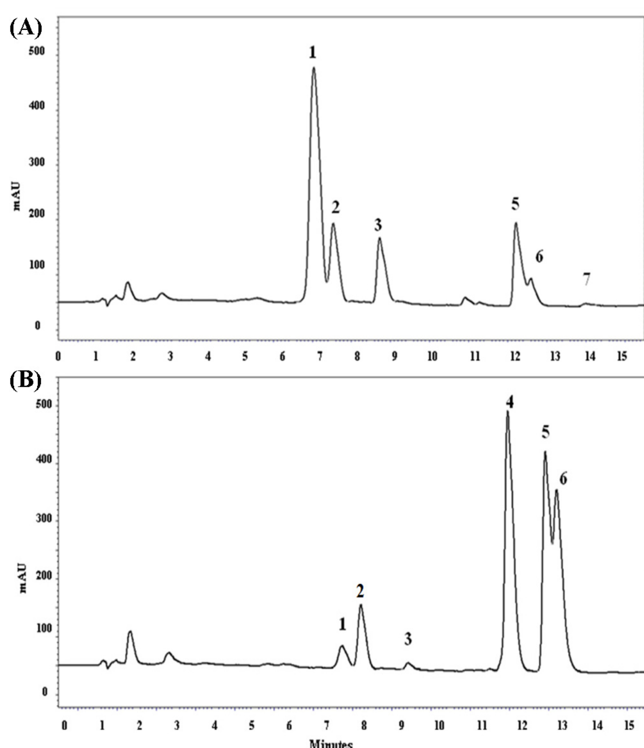

Fig. 1 HPLC chromatograms of isoflavonone extracted from SHE at 0 h (A) and at 48 h (B). 1, daidzin; 2, glycitin; 3, genistin; 4, dihydrodaidzein; 5, daidzein; 6, glycitein; 7, genistein

Results and Discussion

Soluble isoflavone content of soy products

Major isoflavones in soy products are isoflavone glucosides (daidzin, genistin and glycitin) and the aglycones (daidzein, genistein and glycitein). Defatted soybean meal (crude protein 44%), two different soybean hypocotyl extracts (Isovone and Soluvone), and dietary soy isoflavone supplement (Genistein) were selected for the analysis of isoflavone content. Because these soy isoflavones are the substrates of MRG-1 in the medium, soy products were dissolved in GAM broth medium and autoclaved at 121 °C for 20 min. Soy products in distilled water were also prepared and analyzed by HPLC in the same way as controls (Fig. 1). As shown in Table 1, daidzin was most abundant and daidzein was least abundant in all soy products. Genistein was detected from HPLC chromatogram, but quantization was not significant due to the low concentration. Under the analytical conditions, glycitein and glycitin were not detected by HPLC. Although defatted soybean meal is being widely used as feed and food supplements, isoflavone content was lowest. Daidzin concentration of soybean hypocotyl extracts, Isovone and Soluvone (3.32±0.37 g/L and 1.00±0.09 g/L) in GAM broth medium were much higher than dietary soy isoflavone supplement and soybean meal. Soluble isoflavone contents of soy products in water were practically same. Because Isovone showed highest daidzin and daidzein contents, Isovone was selected for further study with autoclave time, sequential extractions and biotransformation in the different culture media.

Effect of solvent and sequential extractions on the isoflavonoids analysis

Soybean hypocotyl extract, Isovone, in distilled water and GAM broth were autoclave at 121 °C for 20 min. The amount of isoflavones analyzed after single extraction (E1) and triple extraction (E3) with ethyl acetate and chloroform is shown at Supplementary materials Fig. 2. Repeating the extraction of media three times increased extraction efficiency of isoflavones in GAM broth medium and water. Each isoflavone concentration after triple extraction was found as 3.78±0.39, 0.77±0.09, 0.40±0.04, and 0.01±0.01 g/L for daidzin, genistin, daidzein and genistein, respectively, in dH_2O. In the GAM broth, genistein was not detected, and daidzin, genistin, and daidzein were found at the concentration of 2.97±0.28, 0.67±0.07, and 0.31±0.03 g/L, respectively. In general, less soluble isoflavones were determined in GAM broth, probably because isoflavones might have interacted with the medium during the sterilization.

Effect of autoclaving time on the soluble isoflavonoids content in media

To examine whether sterilization time of isoflavone-containing media affects the concentration of soluble isoflavones, isoflavone concentration in the autoclaved media was analyzed, after different sterilized times (20, 30, 40 and 50 min). All the isoflavones were analyzed slightly higher in water than GAM broth as shown at Fig. 2. For example, the concentrations of the most abundant soy isoflavone daidzin in dH_2O were found as 3.78±0.39, 2.86±0.17, 3.00±0.41, and 3.13±0.38 g/L after 20, 30, 40 and 50 min sterilizations, respectively, and which were higher than those in GAM broth as 2.97±0.28, 1.56±0.10, 2.85±0.33, and 2.77±0.29 g/L, respectively. Even though it is not clear why the

Table 1 Soluble isoflavone content of soy products

Sample	Isoflavone (g/L) in GAM			Isoflavone (g/L) in water		
	Daidzin	Genistin	Daidzein	Daidzin	Genistin	Daidzein
Soybean meal	0.01±0.00	0.01±0.00	nd	0.01±0.00	nd	nd
Dietary supplement	0.12±0.02	0.01±0.00	nd	0.07±0.01	0.01±0.00	nd
Isovone	3.32±0.37	0.48±0.09	0.05±0.02	3.39±0.55	0.40±0.06	0.06±0.03
Soluvone	1.00±0.09	0.21±0.02	nd	1.11±0.04	0.21±0.01	nd

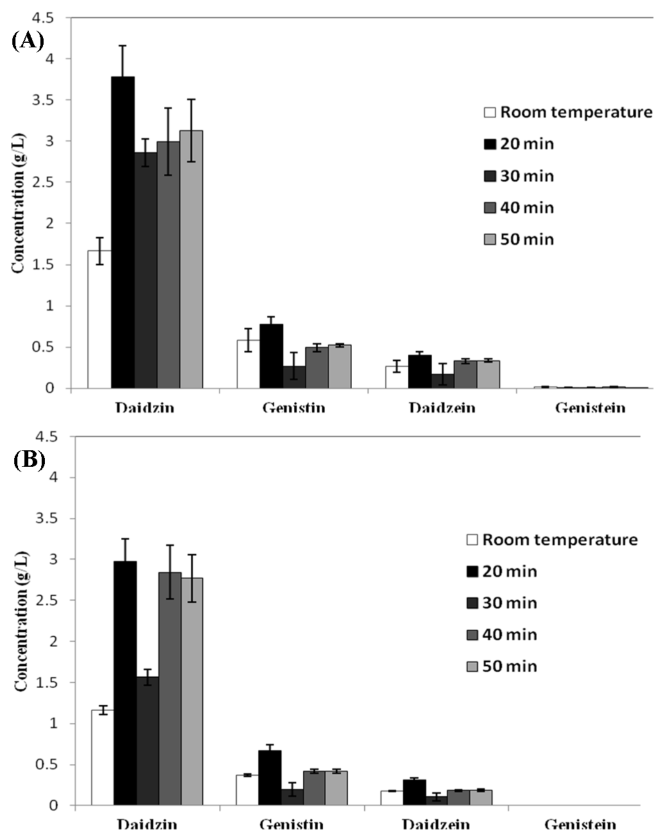

Fig. 2 Concentration (g/L) of soluble soy isoflavone in dH_2O (A) and GAM broth (B) after sterilization at 121 °C for 20, 30, 40, and 50 min. Controls were kept at room temperature

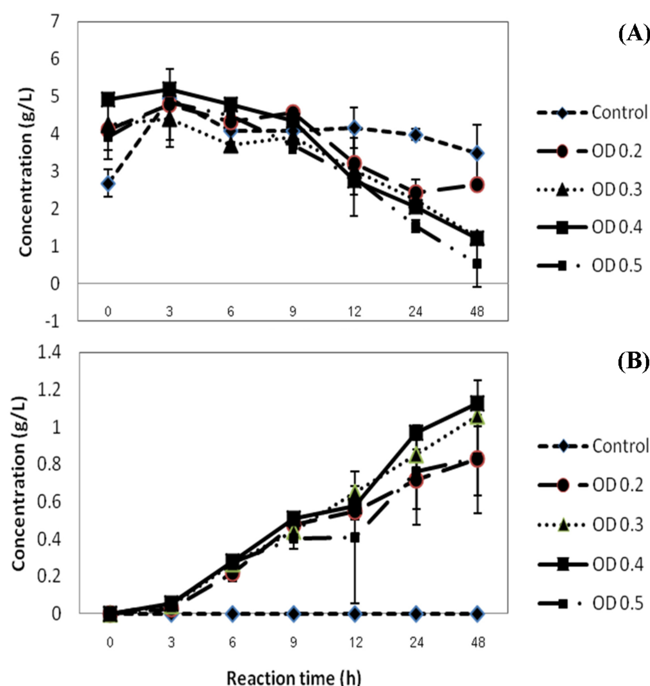

Fig. 3 Concentration (g/L) changes of isoflavones in GAM broth were monitored by daidzin consumption (A) and DHD production (B) when the different growth phases of MRG-1 were inoculated.

isoflavone concentration in the 30 min sterilization was lowest, it was found that 20 min sterilization time resulted in the highest soluble isoflavone concentration. The controls at room temperature were found to contain much low concentration of isoflavone in both solutions. Therefore, sterilizations of media for the further experiments were carried out at 121 °C for 20 min.

DHD production by different growth stage inoculums

MRG-1 was cultured in GAM broth until the OD_{600} reached at 0.2, 0.3, 0.4 and 0.5, and inoculated into GAM broth containing isovone (10 mg/mL). As shown in Fig. 3, daidzin consumption and DHD production increased proportional to the incubation time. In 24 hours, 0.72±0.24, 0.85±0.03, 0.97±0.04, and 0.76±0.20 g/L of DHD productions were observed for OD_{600}=0.2, 0.3, 0.4 and 0.5, respectively. After 48 hours' incubation, DHD productions increased to 0.83±0.29, 1.06±0.00, 1.13±0.12, and 0.83±0.20 g/L, respectively. From these results, it was concluded that MRG-1 inoculum at the range between OD_{600} 0.3 and 0.4 is most suitable for the maximum DHD production.

DHD production in different media

DHD productions by MRG-1 in five different anaerobic media, including water, were monitored by HPLC for 48 hours (Fig. 4). Although GAM is the most popular anaerobic medium, it is expensive than the other growth media and the biotransformation of isoflavone by MRG-1 in other media has never been tried. Daidzin concentration in GAM, LB and BHI media decreased over the incubation time, from 2.78±1.07, 4.51±0.03, and 3.66±0.25 g/L at the beginning to 1.40±0.28, 2.96±0.56, and 0.98±0.30 g/L, respectively, for GAM, LB and BHI media. Daidzin is the substrate of MRG-1 for the production of DHD and 1.16±0.11, 0.29±0.21, and 1.13±0.01 g/L of DHD was produced from GAM, LB, and BHI media, respectively, after 48 h reaction. Biotransformation of SHE with MRG-1 in NB medium and dH_2O did not change the concentration of daidzin significantly, and accordingly no DHD production was observed. From the results, it was found that BHI medium is as efficient as GAM for the DHD production.

Since the biosynthetic pathway of *S*-equol was elucidated [7], large scale production of DHD and THD are required for the industrial application. Because traditional fermentation of soy food cannot provide these metabolites, we have investigated the optimum biotransformation conditions for the production of DHD enriched biomaterial with the selected SHE. The human gut bacterium MRG-1 (NCBI accession no. HQ687764) showed high 16S rDNA sequence homology to *Massilimicrobiota*, *Longibaculum*, and *Clostridium* genus from Blastn search [12]. In conclusion, maximum DHD production (1.2 g/L) was achieved after 48 h

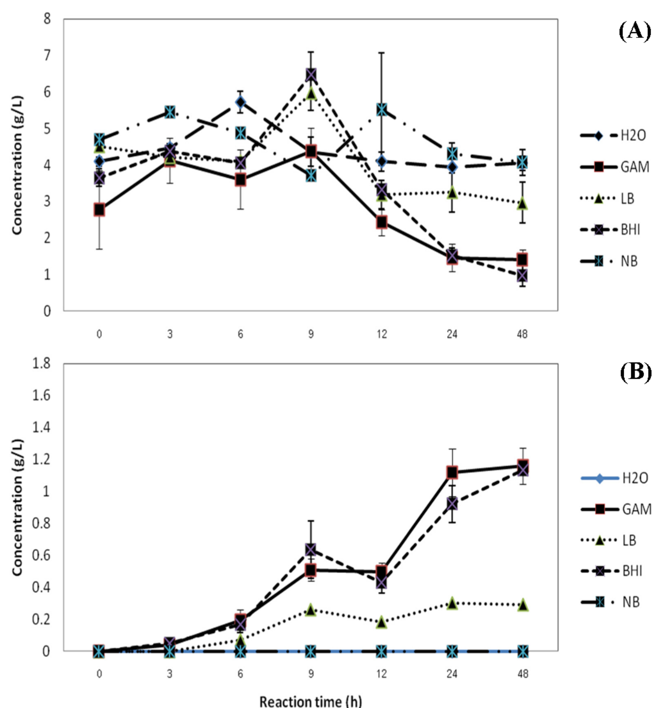

Fig. 4 Concentration (g/L) changes of isoflavonones in SHE, daidzin (A) and dihydrodaidzein (DHD) (B) in the five different media

incubation when 1% (w/v) of SHE in the 20-min-sterilized GAM media was inoculated with OD_{600} 0.3-0.4 of MRG-1.

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Supplementary material Calibration curves for the standard compounds and isoflavone concentration depend on the extraction times.

References

1. Han J (2019) Chemical aspects of gut metabolism of flavonoids. Metabolites 9: 136. doi: 10.3390/metabo9070136
2. Pabich M, Materska M (2019) Biological effect of soy isoflavones in the prevention of civilization diseases. Nutrients 11: 1660. doi: 10.3390/nu11071660
3. Kim M, Han J (2016) Isoflavone metabolism by human intestinal bacteria. Planta Med 82: S784. doi: 10.1055/s-0036-1596810
4. Kim M, Lee J, Han J (2015) Deglycosylation of isoflavone C-glycosides by newly isolated human intestinal bacteria. J Sci Food Agric 95: 1925–1931. doi: 10.1002/jsfa.6900
5. Sekikawa A, Wharton W, Butts B, Veliky CV, Garfein J, Li J, Goon S, Fort A, Li M, Hughes TM (2022) Potential protective mechanisms of S-equol, a metabolite of soy isoflavone by the gut microbiome, on cognitive decline and dementia. Int J Mol Sci 23:11921. doi: 10.3390/ijms231911921.
6. Rostagno MA, Villares A, Guillamón E, García-Lafuente A, Martínez JA (2009) Sample preparation for the analysis of isoflavones from soybeans and soy foods. J Chromatogr A 1216: 2–9. doi: 10.1016/j.chroma.2008.11.035
7. Kim M, Marsh ENG, Kim SU, Han J (2010) Conversion of (3S,4R)-tetrahydrodaidzein to (3S)-equol by THD reductase: Proposed mechanism involving a radical intermediate. Biochemistry 49: 5582–5587. doi: 10.1021/bi100465y
8. Wang XL, Hur HG, Lee JH, Kim KT, Kim SI (2005) Enantioselective synthesis of S-equol from dihydrodaidzein by a newly isolated anaerobic human intestinal bacterium. Appl Environ Microbiol 71:214–219. doi: 10.1128/AEM.71.1.214-219.2005
9. Kim JS, Lee H, Nirmala FS, Jung CH, Kim MJ, Jang YJ, Ha TY, Ahn J (2019) Dihydrodaidzein and 6-hydroxydaidzein mediate the fermentation-induced increase of antiosteoporotic effect of soybeans in ovariectomized mice. FASEB J 33:3252–3263. doi: 10.1096/fj.201800953R
10. Park HY, Kim M, Han J (2011) Stereospecific microbial production of isoflavanones from isoflavones and isoflavone glucosides. Appl Microbiol Biotechnol 91:1173–1181. doi: 10.1007/s00253-011-3310-7
11. Won DH, Shin BK, Han J (2008) Synthesis and the absolute configurations of isoflavanone enantiomers. J Appl Biol Chem 51: 17–19. doi: 10.3839/jabc.2008.004
12. Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7: 203–214. doi: 10.1089/10665270050081478