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RESEARCH ARTICLE

Biosynthesis of novel daidzein derivatives using *Bacillus amyloliquefaciens* whole cells

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ABSTRACT

Biotransformation of daidzein was performed by using *Bacillus amyloliquefaciens* KCTC 13588, *Lactococcus lactis* subsp. *lactis* KCTC 3769, *Leuconostoc citreum* KCTC 13186, *Kluyveromyces lactis* var. *lactis* KCTC 17704, *Pediococcus pentosaceus* KCTC 3116, and *Lactobacillus sakei* KCTC 13416 cells as a biocatalyst. Four derivatives of daidzein such as daidzein-7-*O*-phosphate, daidzein-7-*O*-*β*-D-glucoside, daidzein-7-*O*-*β*-(6′′-*O*-succinyl)-D-glucoside, and 4′-Ethoxy-daidzein-7-*O*-*β*-(6′′-*O*-succinyl)-D-glucoside were isolated from the biotransformation reaction mixture. The structures of the molecules were elucidated by HPLC, HR-QTOF-ESI/MS and ^{1}H-NMR analyses. Among them 4′-Ethoxy-daidzein-7-*O*-*β*-(6′′-*O*-succinyl)-D-glucoside derivative is novel compound and not reported elsewhere till now.

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Biotransformation; Resting cells; Daidzein; Novel compound; *Bacillus amyloliquefaciens*

Introduction

Flavonoids and isoflavonoids, also known as phenolic compounds, are extracted from plants and have various structures and biological functions. Several studies have shown that biological activities such as vasodilation, anti-inflammatory, anti-viral and anti-allergic properties are associated with flavonoids (Pietta 2000). Isoflavones exhibit potent anti-cancer effects and are used in the prevention and treatment of chronic diseases. Recently, isoflavones were found to reduce the risk of breast cancer (Kucuk 2006; Laurenz et al. 2017). They are also known to be effective in mitigating menopausal disorders and preventing osteoporosis (Miyazawa et al. 2006).

Daidzein, one of the major isoflavones of soybeans, has several beneficial properties related to human health (Kulling et al. 2001). It inhibits malignant angiogenesis and tyrosine kinase (Foti et al. 2005). However, its pharmacological use is limited because of low water solubility (Shimoda et al. 2008). In an effort to enhance the water solubility of daidzein, researchers have used microbial biotransformation (Chen and Reese 2002), enzymatic transformation (Pandey et al. 2014), and chemical modification (Hsu et al. 2013). Among these, the chemical method is associated with safety and waste disposal issues that adversely affect the environment. In addition, some chemical reactions are not cost-effective with low final yields (Wang et al. 2010). Therefore, many researchers are investigating biotransformation as a practical alternative to the chemical synthesis process (Muñoz Solano et al. 2012).

Biotransformation has been successful in the large industrial production of novel drugs and cosmetic products, with advantages over chemical synthesis of higher conversion rates, higher specificity, better reaction conditions and lower chemical contamination (Venisety and Ciddi 2003; Tong and Dong 2009). The biotransformation of daidzein has been accomplished by modifying a simple technique using resting bacterial cells in buffer supplemented with glycerin and glucose. In this study, we employed six different non-pathogenic Gram-positive bacteria for whole cell biocatalysis. Among them, *Bacillus amyloliquefaciens* produced different derivatives of daidzein.

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Supplemental data for this article can be accessed here.

Materials and methods

Chemicals and bacterial strains for daidzein biotransformation

Daidzein was purchased from Sigma-Aldrich (St. Louis, MO). Peptone, beef extract, glucose and yeast extract as components of nutrient medium were all purchased from Difco (Baltimore, MD, USA). *Bacillus amyloliquefaciens* KCTC 13588, *Lactococcus lactis* subsp. *lactis* KCTC 3769, *Leuconostoc citreum* KCTC 13186, *Kluyveromyces lactis* var. *lactis* KCTC 17704, *Pediococcus pentosaceus* KCTC 3116, and *Lactobacillus sakei* KCTC 13416, was purchased from KCTC (Korean Collection for Type Cultures, Republic of Korea). All other reagents used were purchased from Sigma-Aldrich.

Biotransformation of daidzein

Bacillus amyloliquefaciens KCTC 13588 colony was picked from the nutrient agar medium and inoculated in a culture tube containing 4 mL of nutrient broth. Two hundred microlitre of the seed culture was inoculated into fresh 100 mL of nutrient broth and incubated at 37 °C and 200 rpm for 17 h. After incubation, the culture broth was centrifuged at 6000 rpm for 15 min, and the submerged cells were washed twice using 5 mL of 50 mM phosphate glycerin (PG) buffer. The PG buffer is a phosphate buffer containing 2% glycerin, adjusted to pH 7.2. Finally, the cells were resuspended in 5 mL of PG buffer. To the cell, daidzein was added to a final concentration of 4 μg/mL. Thereafter, the reaction was carried out at 30 °C and 200 rpm for 48 hours. After completion of the reaction, the culture broth was centrifuged at 6000 rpm for 10 min. The supernatant was concentrated using an evaporator and dissolved in methanol. The exactly similar protocol was followed while performing reactions using the PG buffer supplemented with glucose. However, 2% glycerin was replaced by 2% glucose in the PG buffer. In the conventional biotransformation method, daidzein was directly added to the growing culture of *B. amyloliquefaciens* KCTC 13588 and then further cultivation was carried out.

HPLC analysis of daidzein biotransformation products

High performance liquid chromatography (HPLC) analysis was performed using a Shimadzu Prominence-i (LC-2030C) equipped with a photo-diode array (PDA) detector with a Phenomenex Synergy® C18 analytical column (250 × 4.6 mm, 4.0 μm). The mobile phases were water containing 0.1% TFA (solvent A) and acetonitrile (solvent B). Analytical conditions were based on solvent B and in gradient condition as follows: 70% (0–12) min, 100% (12–16) min, 100% (16–20) min, 10% (20–25) min and 10% (25–30) min. The flow rate was 0.8 mL/min. 10 μL of sample was injected using an auto-sampler.

Purification of daidzein products

All the new peaks were separated by preparative-HPLC to determine the structure of the compounds. A Shimadzu SPD-20A prominence UV/Vis detector equipped with a Phenomenex Luna® C18 column (250 × 10 mm, 5.0 μm) was used. Purification was performed under the following conditions. The mobile phases were water containing 0.1% TFA (solvent A) and acetonitrile (solvent B). The flow rate was 5 mL/min and it was gradient condition based on solvent B as follows-70% (0–24) min, 100% (24–30) min, 100% (30–33) min, 10% (33–35) min. The amount of sample injected was 450 μL.

LC/MS and NMR analysis of daidzein derivatives

High-resolution quantitative time-of-flight electrospray ionization mass spectrometry (HRQTOF-ESI-MS) was performed in positive ion mode using an ACQUITY mass spectrometer (Waters, Billerica, MA, USA) coupled with SYNAPT G2-Si column (Waters).

The NMR spectra data were measured using a VNMRS 400 NMR spectrometer (Agilent Technology, Santa Clara, CA), and the residual solvent peaks (DMSO-$d_6 = \delta_H 2.50$) of deuterated NMR solvents (Sigma-Aldrich, St. Louis, MA) were used as reference peaks.

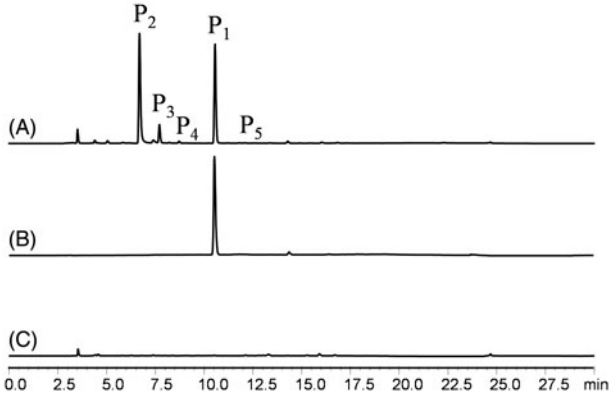

Figure 1. Biotrasnformation of daidzein using (A) *Bacillus amyloliquefaciens* KCTC 13588; (B) Daidzein standard; and (C) Negative control.

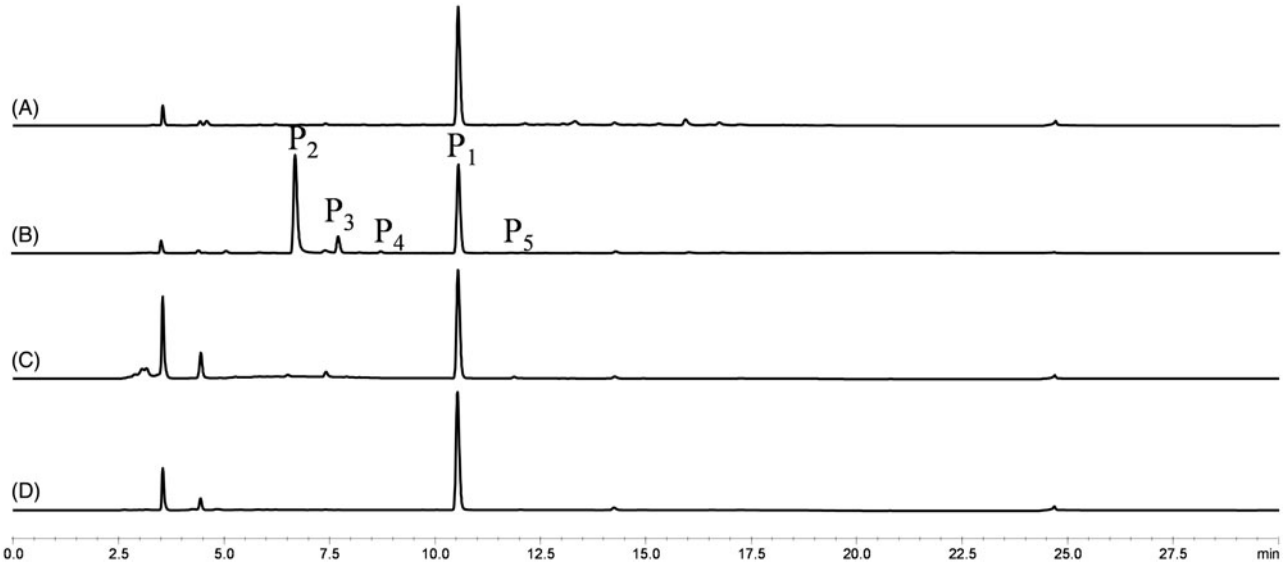

Figure 2. Biotransformation by growing cells and biotransformation using resting cells in buffer. Daidzein conversion at 48 h using *Bacillus amyloliquefaciens* KCTC 13588 cells. (A) Biotransformation (no carbon source supplement); (B) Resting cells in buffer supplementing 2% glycerol in PG buffer; (C) Biotransformation supplementing 2% glucose while adding daidzein; and (D) Biotransformation in PG buffer supplementing 2% glucose.

Results and discussion

HPLC analysis of the biotransformation reaction mixtures of daidzein revealed multiple peaks in the *B. amyloliquefaciens* KCTC 13588 reaction mixture (Figure 1(A)). The reaction chromatograms were compared with standard daidzein (Figure 1(B)) and the control reaction (Figure 1(C)). However, no novel peaks were observed with other strains such as *Lactococcus lactis* subsp. *lactis* KCTC 3769, *Leuconostoc citreum* KCTC 13186, *Kluyveromyces lactis* var. *lactis* KCTC 17704, *Pediococcus pentosaceus* KCTC 3116, and *Lactobacillus sakei* KCTC 13416 (data not shown). Therefore, the *B. amyloliquefaciens* KCTC 13588 strain was chosen for further production of daidzein biotransformed derivatives.

Biotransformation by growing cells and biotransformation using resting cells in buffer were compared using daidzein as a substrate while supplementing with glucose and glycerin as carbon sources. Interestingly, the reaction mixture of resting cells suspended in buffer showed new peaks within 48 h (Figure 2). No additional peaks were observed for up to one week when daidzein was directly added into the growth medium (Figure 2(A)). These results showed that the use of resting cells in buffer is a suitable method to generate new daidzein compounds effectively, even during short reaction times.

Additional experiments were performed to investigate the effect of carbon supplements in the buffer and biotransformation medium. In the case of resting cells in PG buffer, cells were supplemented with glucose instead of the previously added glycerin. In the biotransformation reactions by growing cells, glucose was added together with daidzein. Unlike biotransformation using glycerin PG buffer, PG buffer supplemented with glucose did not produce new peaks (Figure 2(C,D)). Similarly, no new peaks were found in the biotransformation reaction with glucose supplementation. These preliminary experiments revealed the prime importance of glycerin to generate different derivatives of daidzein by the biotransformation approach using resting cells in buffer.

Biotransformation in buffer supplemented with glycerin was replicated several times and a large amount of reaction mixture was collected for purification of each product for structural elucidation. All products were purified using preparative HPLC (Figure 3(A)). The purity of each product was over 98%. The purified compounds were subjected to HRQTOF-ESI/MS and NMR analyses.

In HPLC analysis, compound P_2 was eluted at 6.8 min with a UV maximum absorbance (λ_{max}) at 250 nm. The peak showed a mass that matched phosphorylated daidzein in positive mode (i.e. $[C15H11O7P + H^+]$ *m/z* of 335.0325) and was suspected to be a phosphate-conjugated derivative of daidzein for which the calculated mass was 335.0321 Da (Figure 3) (Kanakubo et al. 2001). The mass data were further supported by ^{1}H-NMR analysis that confirmed the conjugation of a phosphate unit at the 7-OH of daidzein (Table 1; Figure S1), indicating the molecule was daidzein-7-*O*-phosphate. Similarly, compound P_3 appeared at t_R of 7.7 min (λ_{max}: 251 nm)

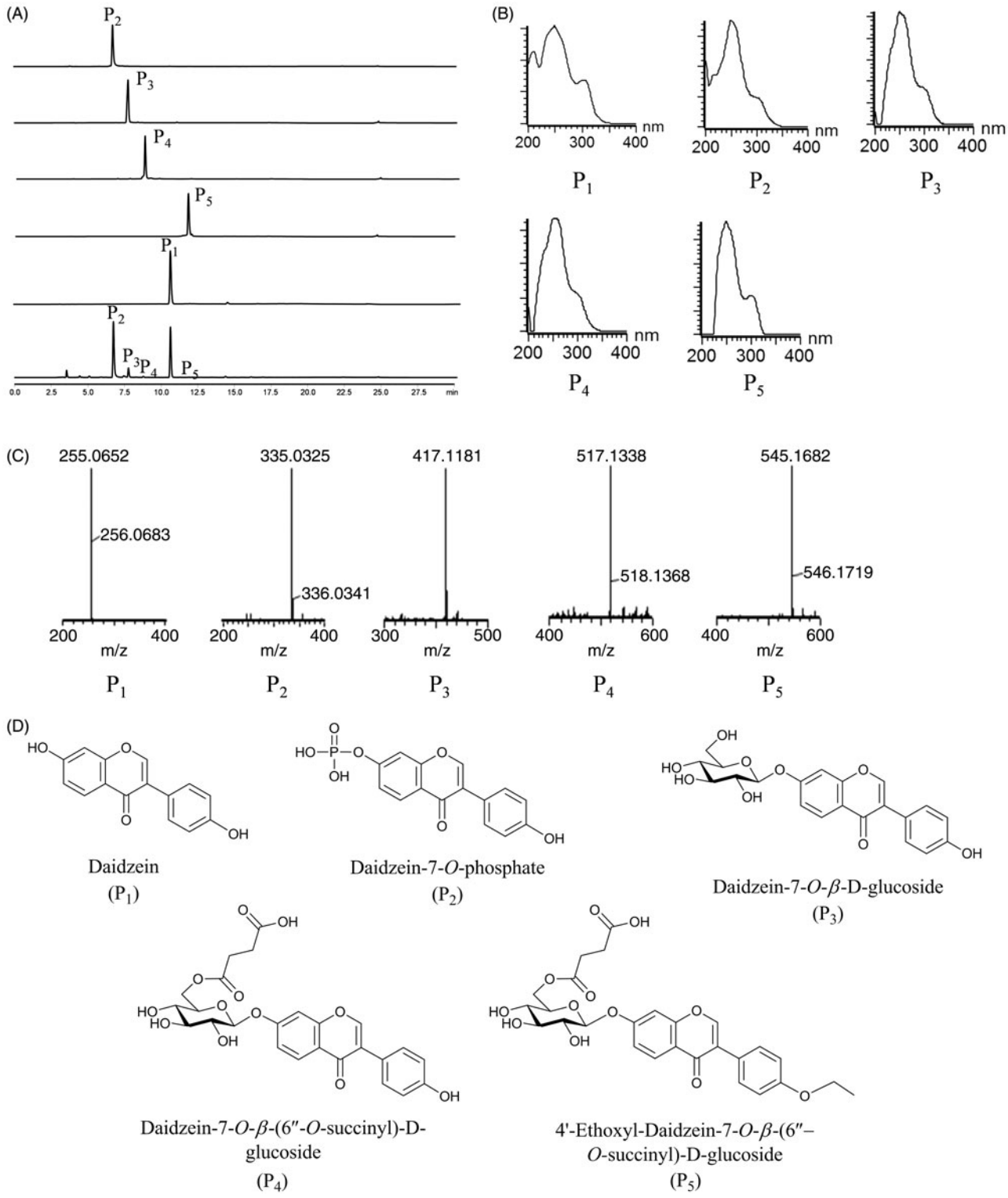

Figure 3. (A) HPLC chromatogram of biotransformation reaction mixture in buffer and purified products. (B) UV spectra of the purified compounds (P_1-P_5). (C) HRQTOF-ESI/MS spectra of each peak (P_1-P_5). (D) Structures of the identified daidzein derivatives from biotransformation reaction mixture.

in the HPLC chromatogram and its mass suggested that this compound was a glycosylated daidzein derivative in positive mode (i.e. [C21H20O9 + H^+] *m/z* of 417.1181) for which the calculated exact mass was 417.1186 Da.

^{1}H-NMR showed the presence of a doublet spectrum at a chemical shift of δ 5.10 ppm, with a coupling constant value of 7.2 Hz. This spectrum was confirmed to be the anomeric proton of a sugar attached to

Table 1. 1H-NMR of daidzein biotransformed products.

^{1}H Position	Daidzein-7-*O*-phosphate (P_2)	Daidzein-7-*O*-β-D-glucoside (P_3)	6″-*O*-Succinyldaidzin (P_4)	4′-Ethoxyl-daidzein-7-*O*-β-(6″-*O*-succinyl)-D-glucoside (P_5)
2-H	8.40, S	8.39, s	8.35, s	8.42, s
5-H	8.10, d, $J = 8.8$ Hz	8.05, d, $J = 8.8$ Hz	8.05, d, $J = 8.9$ Hz	8.03, d, $J = 8.9$ Hz
6-H	7.30, dd, $J = 8.8$ Hz, 2.2 Hz	7.14, d, $J = 8.9$ Hz	7.13, dd, $J = 8.9$ Hz, 2.4 Hz	7.05-7.10, m
8-H	7.38-7.43, m	7.23, d, $J = 2.4$ Hz	7.23, d, $J = 2.3$ Hz	7.15, d, $J = 2.3$ Hz
2′-H	7.38-7.43, m	7.41, d, $J = 8.6$ Hz	7.40, d, $J = 8.7$ Hz	7.52, d, $J = 8.7$ Hz
3′-H	6.82, d, $J = 8.6$ Hz	6.81, d, $J = 8.4$ Hz	6.81, d, $J = 8.6$ Hz	7.05-7.10, m
5′-H	6.82, d, $J = 8.6$ Hz	6.81, d, $J = 8.4$ Hz	6.81, d, $J = 8.6$ Hz	7.05-7.10, m
6′-H	7.38-7.43, m	7.41, d, $J = 8.6$ Hz	7.40, d, $J = 8.7$ Hz	7.52, d, $J = 8.7$ Hz
1″-H		5.10, d, $J = 7.2$ Hz	5.14, d, $J = 7.1$ Hz	4.94, d, $J = 7.2$ Hz
2″-H		3.18-3.70, overlapped	3.80-3.20, m	3.80-3.20, m
6″-H		3.18-3.70, overlapped		
6″-H_A			4.40, d, $J = 11.4$ Hz	
6″-H_B			4.02, dd, $J = 11.8$ Hz, 7.5 Hz	
5″-H			3.80-3.20, m	3.80-3.20, m
2‴-H			2.50-2.57, overlapped, m	2.50-2.57, m
3‴-H			2.50-2.57, overlapped, m	2.50-2.57, m
1⁗-H				4.19, q, $J = 7.0$ Hz
2⁗-H				1.38, t, $J = 7.0$ Hz
4′-OH	9.57, s	9.57, s	9.55, s	

daidzein in the β configuration. Other spectra for the glucose moiety appeared between 3.0-5.0 ppm. Broad singlet spectra for 4′-OH and 7-OH in daidzein appear at δ 9.57 ppm and δ 10.82 ppm, respectively. Because the spectrum for the 7-OH proton at δ 10.82 ppm was missing, we confirmed the conjugation of glucose at the 7-OH position of daidzein (Table 1; Figure S2). Moreover, the spectra were compared with previously published results (Hur et al. 2000) and the molecule was identified as a daidzein-7-*O*-β-D-glucoside derivative.

Peak P_4, which was at t_R of 7.7 min (λ_{max}: 250 nm), corresponded with the succinylated daidzein glucoside in positive mode (i.e. $[C25H24O7 + H^+]$ *m/z* of 517.1338) and had mass of 517.1346 Da (Toda et al. 1999). ^{1}H-NMR analysis further supported the ESI/MS analysis. Interestingly, ^{1}H-NMR analysis revealed conjugation of the succinyl group at the 6″-OH position of the glucose moiety attached at the 7-OH of daidzein in β-configuration. This identified the molecule as daidzein-7-*O*-β-(6″-*O*-succinyl)-D-glucoside (6″-*O*-succinyl daidzein) (Table 1; Figure 3 and S3).

The remaining peak, P_5, appeared at 11.9 min (λ_{max}: 249 nm) and was also found to be a derivative of daidzein. The mass in positive mode (i.e. $[C27H28O12 + H^+]$ *m/z* of 545.1682) matched an ethoxy succinyl daidzein derivative which has the exact mass of 545.1659 Da (Figure 3). ^{1}H-NMR analysis revealed conjugation of a 6″-succinylated glucose moiety at the 7-OH of daidzein in β-configuration, and the presence of an ethyl group at the 4′-OH of daidzein (Table 1; Figures. 3, S4). The molecule was identified as 4′-ethoxy-daidzein-7-*O*-β-(6″-*O*-succinyl)-D-glucoside (Figure 3(D)). This was a novel molecule not reported previously from any natural sources, or by chemical synthesis or biotransformation approaches.

Resting *B. amyloliquefaciens* KCTC 13588 cells biotransformed daidzein to generate four products (P_2-P_5). All products were secreted into the medium. This strain biotransformed more than 50% of the substrate into products that were conjugated with different bulky hydrophilic groups such as phosphate, glucose, succinylated glucose and hydrophobic ethoxy groups. Several studies have been carried out to biotransform flavonoids and other plant-derived secondary metabolites using diverse microbes, fungi, actinomycetes and plant cell cultures (Roh et al. 2009; Pandey and Sohng 2013; Cao et al. 2015; Parshikov and Sutherland 2015). Most of the modified products are conjugated with sugars, methyl groups, hydroxyl groups and prenyl groups (Pandey et al 2016). Moreover, recently, microbial cells such as *Escherichia coli* were rationally engineered to produce target products by biotransformation of flavonoids. For example, the production of flavonoids conjugated to specific sugars (Kim et al. 2015), methyl group (Parajuli et al. 2018; Wen et al. 2017), and hydroxyl group (Pandey et al. 2011; Choi et al. 2012; Chu et al. 2016) has yielded post-modified compounds by the simple fermentation approach.

Bacillus amyloliquefaciens is one of the best candidate strains to use as a biocatalyst to produce different derivatives of daidzein and related molecules. The biosynthetic process for the new derivatives is very simple and easy to scale-up. In addition, the approach is sustainable and utilizes inexpensive carbon sources. Because endogenous microbial cofactors are utilized, there is no need to add expensive cofactors as

required in enzymatic biosynthesis. Moreover, the system is much superior to chemical synthesis because of the absence of hazardous chemicals.

Four derivatives of daidzein were obtained in this study. All of the molecules were structurally characterized. The mass and NMR spectra were validated by comparing with previously published data. One of the molecules, 4′-ethoxy-daidzein-7-*O*-*β*-(6′′-*O*-succinyl)-D-glucoside, was a new derivative isolated from the biotransformation reaction mixture of resting cells of *B. amyloliquefaciens* and is a novel candidate for the chemical library. The physiochemical and biological activities of these molecules will be studied in further research.

The gene expression of microorganisms differs greatly depending on their growth stage and composition of the medium. Recently, *E. coli* K12 in the stationary phase was shown to express different numbers of genes depending on the composition of the medium (Arunasri et al. 2014). In the present study, *B. amyloliquefaciens* was grown in buffer containing either glycerol or glucose. Both supplements are precursors of the primary metabolic glycolysis pathway in cells. The results demonstrated that only the glycerol-containing buffer medium was suitable and selective for biotransforming daidzein. Selective bioconversion in glycerol-containing buffer could be because of an unknown role of the type of the carbon source or catabolite repression in expressing *B. amyloliquefaciens* genes that play a crucial role in daidzein bioconversion.

Though glycerin is utilized by cells as a carbon source, recently, very high concentrations of glycerin (above 90 g/L; 9%) were found to be inhibitory for the growth of *Clostridium butyricum* (Szymanowska-Powałowska 2015). However, no evidence on switching biotransformation capabilities of microbial cells was reported previously. Thus, this subject could be of interest among microbiologists to identify potential targets of glycerol in *Bacillus* growth, metabolism and gene expression.

In conclusion, the method using resting cells in buffer to achieve biotransformation is suitable for the generation of biologically beneficial derivatives from a single precursor in a short period of time. Further transcriptome analysis will identify the genes responsible for newly generated daidzein derivatives.

Disclosure statement

No potential conflict of interest was reported by the authors.

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