J Korean Soc Appl Biol Chem (2014) 57(4), 523–527
DOI 10.1007/s13765-014-4145-6

Online ISSN 2234-344X
Print ISSN 1738-2203

ARTICLE

Biotransformation of Isoflavones by *Aspergillus niger* and *Cunninghamella elegans*

Ji-Ho Lee · Eun-Tae Oh · Se-Chul Chun · Young-Soo Keum

Received: 19 May 2014 / Accepted: 25 June 2014 / Published Online: 31 August 2014

Abstract Isoflavones are well-known flavonoids in many Legumes. Numerous biological activities are reported, including antioxidant, anti-inflamatory, anti-cancer, and antifungal activities. Their metabolic fates of natural isoflavones were studied in mammalians and several microorganisms. However, no detailed analyses have been reported on the isoflavone and its synthetic analogues. Recently, application of microorganism on natural products bioconversion has gained strong attentions due to their advantages over plants and animals. The metabolism of isoflavone and 4'-fluoroisoflavone were tested with *Aspergillus niger* and *Cunninghamella elegans*. The structures of selected metabolites were confirmed by synthetic standards. Both fungi rapidly transformed isoflavone into several metabolites. The half-lives of isoflavone (40 mg/L) were 1.6 and 4.2 days for *A. niger* and *C. elegans*, respectively. Overall, *A. niger* gave much more complex metabolite profiles. Approximately 23 metabolites were tentatively identified. The major metabolites were mono- and di-hydroxylated isoflavones at initial period, whereas those of 10 days were di- and tri-hydroxy-isoflavones. Hydroxylation usually occurred in B-ring of isoflavone, confirmed by authentic standards. Among dihydroxyisoflavones, 3',4'-dihydroxy analogue was the most abundant metabolite, followed by daidzein (4',7'-dihydroxyisoflavone). Methoxylated metabolites slowly accumulated during culturing. In addition, several glycosides were found, including hexose conjugates of mono-/di-hydroxyisoflavone and minor amount of pentose conjugates during culturing. However, 4'-fluoroisoflavone was not transformed during the culturing period, indicating the region-selective hydroxylation on initial metabolism of isoflavones.

Keywords 4'-chloroisoflavone · *Aspergillus* · *Cunninghamella* · daidzein · fungal metabolism · Isoflavone

J.-H. Lee · E.-T. Oh · S.-C. Chun · Y.-S. Keum (✉)
Department of Bioresource and Food Technology, Konkuk University, 1 Hwayang-dong, Gwanjin-Gu, Seoul, 143-701, Republic of Korea
E-mail: rational@konkuk.ac.kr

Introduction

Isoflavones are commonly found in numerous plants, especially from *Leguminosae*. However, recent studies revealed that some microorganisms can also produce flavones and isoflavones through the *de novo* biosynthesis (Maskey et al., 2003). In general, natural isoflavones usually contain several substituents, namely hydroxyl, methoxy, isoprenyl, and benzopyranyl groups. However, isoflavones with other functional groups has not yet been reported. Their biological activities include antioxidant, anti-inflamatory, anti-cancer, and antifungal activities (Rimbach et al., 2003; Rice-Evans, 2004). Some isoflavones have protective roles against pathogenic fungi and bacteria (e.g., glyceollins from soybean). Although the anti-microbial roles are documented, several fungi and bacteria can metabolize these compounds. For example, some anaerobic bacteria can reductively transform isoflavones to isoflavans (Kim et al., 2008). Several fungi can oxidatively transform natural isoflavones to multi-hydroxy metabolites (Das and Rosazza, 2006). For example, biochanin A and formononetin were rapidly transformed, whereas daidzein was relatively stable to *Fusarium oxysporum* (Mackenbrock and Barz, 1983). In comparison with natural isoflavones, there were limited numbers of studies about the metabolism of isoflavone and its non-natural analogues (Ibrahim and Abul-Hajj, 1990). Microbial conversion usually results in poly-hydroxy or methoxylated metabolites with interesting biological activities (Atkinson et al., 2005). Transformation with biological systems now became a promising method to increase the diversities of natural products in drug discovery or related areas (Michels et al., 1998). In addition, numerous fungi are already in use for many industrial processes (e.g., food industry, enzyme preparation). In the present study, metabolism of isoflavone and 4'-fluoroisoflavone was tested with *Cunninghamella elegans* and *Aspergillus niger*. *C. elegans* is a representative fungus with strong metabolic potential over diverse natural and synthetic chemicals, whereas *A. niger* was selected, because the fungus is well-known to transform many flavonoids (Asha and Vidyavathi, 2009; Das and Rosazza, 2006). Detailed metabolisms were

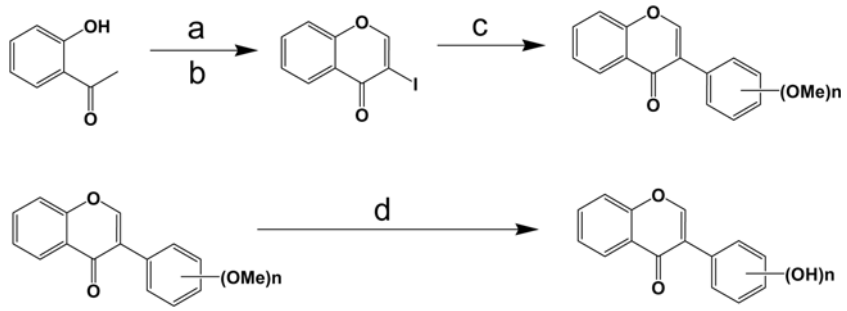

Fig. 1 Synthesis of isoflavone and mono-/di-hydroxy isoflavone metabolites. a: *N*,*N*-dimethylformamide dimethyl acetal, 140°C, 3 h; b: I_2, $CHCl_3$, 12 h, room temperature (rt); c: substituted benzeneboronic acid, $Pd(PPh_3)_4$, K_2CO_3, ethanol/water, 12 h, reflux; d: BBr_3, CH_2Cl_2, rt, 16 h; substituents, n =0, 1, or 2.

investigated with several synthetic standards Regioseletive aspects of kinetic parameters were also given. The results will help for better understand fungal metabolism of isoflavones.

Materials and Methods

Chemicals and reagents. Daidzein and other reagents were obtained from Alfa Aesar (Korea). Potato dextrose broth was from BD Korea (Korea). Solvent were of High-performance liquid chromatography grade or higher.

Synthesis of isoflavone. To a solution of 3-iodo-4H-chromen-4-one (2.71 g) in toluene (30 mL), sodium carbonate (3.18 g) were added in distilled water (15 mL) , followed by $Pd(PPh_3)_4$ (52 mg, 5 mol %), and benzeneboronic acid (1.45 g) in EtOH (10 mL). The resulting mixture was refluxed overnight at 100°C. The mixture was extracted twice with ethyl acetate, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by column chromatography (10% ethyl acetate (EtOAc)/hexane) to give isoflavone as off-white solid. Similar procedures were applied for the preparation of isoflavone metabolites to give methoxy(or dimethoxy)-isoflavones. The methoxylated isoflavones were de-protected with BBr_3 to give hydroxyl (or dihydroxy-isoflavones) (Fig. 1). 4'-Fluoroisoflavone was prepared from 4'-fluorobenzeneboronic acid with the above mentioned procedures.

Culture of fungi. *A. niger* and *C. elegans* (KACC 42713) were obtained from Department of Bioresource and Food Technology, Konkuk University (Korea) and RDA-Genebank Information Center (Korea), respectively. The seed culture was grown on potato dextrose broth, containing 0.4% (w/v) potato starch, 2% (w/v) dextrose (Difco Laboratories, Korea). Metabolic studies of isoflavone were performed as follows. Aliquots (0.5 mL) of isoflavone solution (40 mg/L in dimethyl sulfoxide) were treated to the 3-day culture medium (100 mL). The cultures were further incubated at 30°C, 200 rpm.

Extraction of metabolites. The whole culture medium and mycelia were treated with dilute HCl (1 N, 15 mL) and saturated sodium chloride solution (100 mL). After maceration with blender for 2 min, the medium was extracted with EtOAc (400 mL×2). The combined organic layer was dried over anhydrous Na_2SO_4, and concentrated. The residue was re-dissolved in methanol (10 mL).

Derivatization of metabolites. Concentrated extracts (1 mL) were evaporated under reduced pressure. The residue was dissolved in dry pyridine (0.9 mL) and mixed with *N*,*N*-bis-trimethylsilyl-trifluoroaacetamide/trimethylsilyl chloride (BSTFA-TMCS, 0.1 mL). The mixture was heated to 75°C for 1.5 h.

Instrumental analysis. Metabolites were analyzed with gas chromatograph-mass spectrometer (GC-MS, Shimadzu, Japan, GC-2010 with GCMS-2010 SE) equipped with Rtx-5MS column (30 m, 0.25 μm film thickness, 0.25 nm i.d.; Resteck, USA). Helium was the carrier gas at a flow rate of 1 mL/min. The column temperature were programmed as follows; 160°C (10 min), raised to 295°C at 2.5°C/min, and held for 30 min. The mass spectra of metabolites were obtained in full scan mode.

Results and Discussion

Synthesis of metabolites. 4'-Fluoro-, 4'-hydroxy, 7-hydroxy and 3',4'-dihydroxyisoflavones were prepared by Suzuki coupling, followed by BBr_3-mediated deprotection. Their physicochemical properties well coincided with those from literature (Jung et al., 2003). The purities of synthetic standards were over 95% by GC-MS analyses.

Metabolite profiles. Both fungi could rapidly transform isoflavone (Fig. 2, Table 1). Approximately 23 isoflavone metabolites were tentatively identified from cultures (Table 1). These metabolites can be classified into three groups, a) with hydroxylated isoflavones with up to 4 hydroxyl groups, b) hydroxylated isoflavones with methoxy substituents, and c) pentose and/or hexose conjugates of the above metabolites (Table 1, Supplementary Figs. 1 and 2). There were notable differences of mass spectra of trimethylsilyl (TMS) derivatives of hydroxylated isoflavones with hydroxyl groups in A- or B-ring. For example, 4'-hydroxyisoflavone TMS derivative (**2**, Table 1) gave several fragment ions, indicating 4'-trimethylsilyloxyphenyl fragments (*m/z* 190 and 175) and the ion

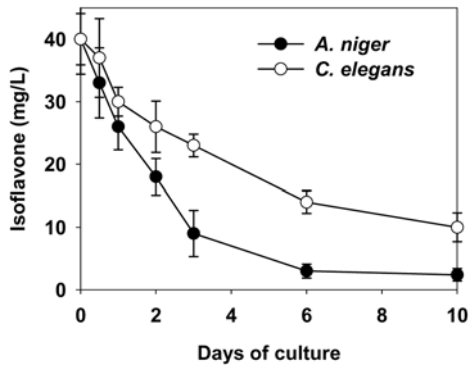

Fig. 2 Transformation of isoflavone by *A. niger* and *C. elegans*. Data were the averages of triplicate experiments.

from A-ring (*m/z* 121), whereas the mass spectra of 7-hydroxy-isoflavone mono-TMS and daidzein di-TMS derivatives were shown to have ions (*m/z* 208) as diagnostic fragments for A-ring mono-hydroxylation (Fig. 3). Due to low abundance of diagnostic ions, it was difficult to identify the structures of polyhydroxy metabolites with more than 2 hydroxyl groups. However, the retention properties were used to determine whether the multiple hydroxylation is either on both rings (A and B rings of isoflavone) or on a single ring. The retention time (*Rt*) of daidzein (**6**, 4',7-dihydroxy) was 48.77 min, whereas metabolite **5** (dihydroxy on the same ring system) was eluted at 42.81 min. It can be suggested that metabolites with multiple hydroxyl groups on the same ring usually show lower retention under GC condition than those with hydroxyl groups on both rings. Detailed analyses indicated that 4'-hydroxyisoflavone (**2**) was the sole mono-hydroxy metabolite in *A. niger*, while **2** and 7-hydroxy derivatives (**3**) were found in *C. elegans*. Further hydroxylation and other metabolism were observed during longer culture periods. However, 4'-hydroxyisoflavone was the major metabolite in *C. elegans*, followed by 3',4'-dihydroxy-isoflavone (**5**) after 10 days. *A. niger* gave more complex metabolite profiles. For example, only trace amount of **2** was observed in 10 days, whereas di- and tri-hydroxy metabolites (**5**, **7**, and **8**) accumulated in the same culture. It can be suggested that **2** was rapidly metabolized into **5**, **7**, and **8** by *A. niger*. In consideration of the general retention properties, metabolites **7** (*Rt*, 46.1 min) and other trihydroxy derivatives (**9**, **11-14**, *Rts* 51.3-54.7 min) were tentatively assigned to trihydroxy groups on B-ring (**7**) and both A- and B-rings (**11-14**). Due to the low abundance, detailed structural identifications of **15-17** were not possible. However, the mass spectra of these metabolites suggested that they were isoflavones with 4 hydroxyl groups. Methoxylated metabolites (**4**, **8**, and **10**) gradually accumulated during culture. They were not observed at the initial culture period

Table 1 Retention times (Rts) and fragmentation of isoflavone metabolites from the cultures of *A. niger* and *C. elegans*

ID	Retention time (Rt, min)	Fragment ions	Structure[a]
1	34.970	310 (M^+,12), 295 (18), 73(100)	1 OH
2	37.325	310 (M^+, 100), 295 (88), 267(42)	4'-Hydroxy
3	37.742	310 (M^+, 96), 309(100), 295(88), 208(7)	7-Hydroxy
4	41.620	340(M^+, 70), 325(17), 310(100), 73(23)	1 OH, 1 MeO
5	42.815	398(M^+, 65), 383 (67), 208(10), 73(100)	3',4'-Dihydroxy
6	48.770	398(M^+, 100), 383 (87), 208(10), 73(70)	Daidzein (2 OH)
7	46.090	486(M^+, 43), 471 (50), 73(100)	3 OH
8	49.530	428(M^+, 57), 413(68), 73(100)	2 OH, 1 MeO
9	50.250	486(M^+, 43), 471 (50), 73(100)	3 OH
10	51.005	428(M^+, 82), 413(35), 398(89), 383(30)	2 OH, 1 MeO
11	51.265	486(M^+, 49), 471 (50), 73(100)	3 OH
12	53.115	486(M^+, 50), 471 (52), 73(100)	3 OH
13	53.555	486(M^+, 68), 471 (60), 73(100)	3 OH
14	54.665	486(M^+, 42), 471 (53), 73(100)	3 OH
15	55.590	574(M^+, 45), 559(51), 73(100)	4 OH
16	56.860	574(M^+, 50), 559(32), 73(100)	4 OH
17	57.735	574(M^+, 57), 559(40), 73(100)	4 OH
18	63.335	577(15), 398(25), 383(20), 259(30), 73(100)	Pentose, 2 OH[b]
19	67.765	577(15), 398(25), 383(20), 259(30), 73(100)	Pentose, 2 OH
20	68.105	486(23), 471 (17), 361(40), 73(100)	Hexose, 3 OH
21	69.295	398(30), 383(14), 361(31), 73(100)	Hexose, 2 OH
22	69.715	398(10), 383(24), 361(25), 73(100)	Hexose, 2 OH
23	71.295	361(30), 340(29), 325(11), 310(5), 73(100)	Hexose, 1 OH, 1 MeO

[a]Structure: Tentatively identified structure of metabolites, 2, 3, 5, and 6 were isoflavone with hydroxyl groups in specified position. Others denote the numbers of hydroxy or methoxy groups on isoflavone skeleton.
[b]Pentose and hexose in metabolite **18-23** were the carbohydrate moiety in each metabolite.

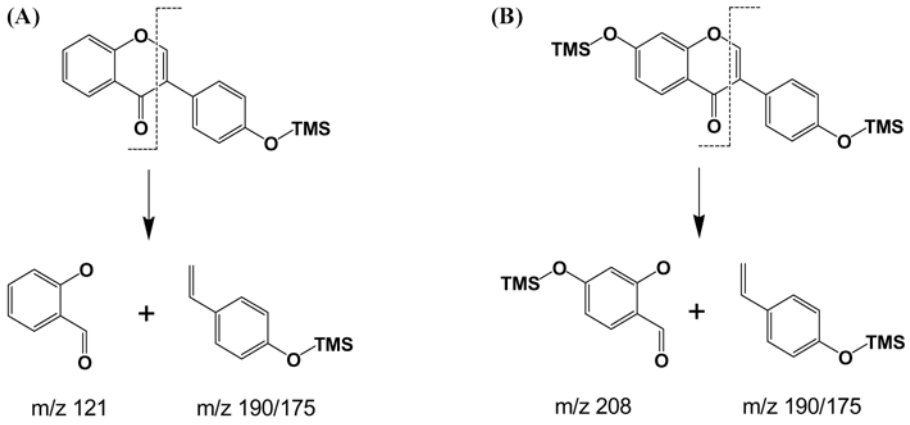

Fig. 3 MS-fragmentation of TMS-derivatized 4'-hydroxyhydroxyisoflavone (A) and daidzein (B).

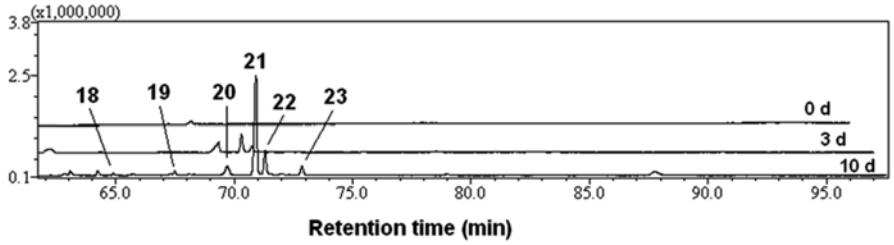

Fig. 4 Overlayed GC-MS total ion chromatograms of culture extracts of A. niger, indicating the accumulation of glycoside. From top to bottom, 0, 3, and 10 days.

(0-3 days), but clearly observed within 7–10 days. Compound **4** was tentatively identified as isoflavone with 1 hydroxy and 1-methoxy groups. The ion (*m/z* 340, 315, and 309) were assigned for parent, des-methyl, and des-methoxy structures.

Many fungal species are known to give several types of phase II metabolites (conjugates), including glycoside, sulfates, and glucuronides (Sutherland et al., 1992; Sun et al., 2004; Keum et al., 2009). Several glycosides (**18-23**) were found after 3 days and the amounts gradually increased (Fig. 4). Due to the characteristic fragmentation in mass spectra, TMS-derivatives of aromatic glycosides can easily be identified. There were two indicative ions, namely those from carbohydrates (e.g., *m/z* 217, 361) and from aglycones (Keum et al., 2010). The mass spectra of **18-23** also gave such ions (*m/z* 217, 259, and 361 for carbohydrates; *m/z* 310, 398, and 340 for aglycone form of isoflavones). In general, glucosides were the most commonly found phase II metabolites in fungal xenobiotic metabolism. However, some fungi can produce pentose-conjugated metabolites (Sutherland et al., 1992). There were large differences of mass spectra and retention properties between metabolite **18-19** and **20-23**. The latter showed characteristic fragment ions (*m/z* 361), indicating hexose conjugates, whereas ions (*m/z* 259) in **18-19** suggested the metabolites as pentose conjugates. Interestingly, similar hexose conjugates (**20-23**) were found in the culture of *C. elegans*, whereas **18-19** were found only in *A. niger*. The accumulation of glycosides in *A. niger* was quite unexpected due to numerous reports about the deconjugation of natural product glycosides, using *Aspergillus* species (e.g., Nam et al., 2011; Lee et al., 2013). It is well known that *A. niger* and others in the same genus have strong glucosidase and related hydrolase activities.

Kinetics of isoflavone transformation. The half-lives of isoflavone under the specified culture conditions were 1.6 and 4.2 days for *A. niger* and *C. elegans* (Fig. 3). During the culturing, the biomass increased approximately 193 and 175% for *A. niger* and *C. elegans*, respectively (Data not shown). The mass balance with isoflavone and selected metabolites are presented in Fig. 5. The mass balance of *C. elegans* showed that most isoflavone metabolites were limited to mono- and di-hydroxy analogues, whereas those in *A. niger* indicates several additional metabolism occurred in prolonged cultures. Because no authentic standards were available for tri- and tetra-hydroxy metabolites, exact quantitation were not performed. However, rapid accumulation of these metabolites was observed in *A. niger*, of which the amount was estimated at approximately the same as dihydroxy metabolites (Supplementary Fig. 1). Another interesting aspect of isoflavone metabolisms was the slow onset of glycoside accumulation. Hydroxylated metabolites were detected immediately after culture. However, glycosides were observed after 3 days. Although *C. elegans* is well known to exert its extensive metabolic activities over diverse xenobiotics (Asha and Vidyavathi, 2009), lower amount of glycosides were obtained than *A. niger*.

In comparison with the isoflavone, 4'-fluoroisoflavone was resistant to metabolic transformation-Almost 100% of starting 4'-fluoroisoflavone was found in 10 and 14 day culture media.

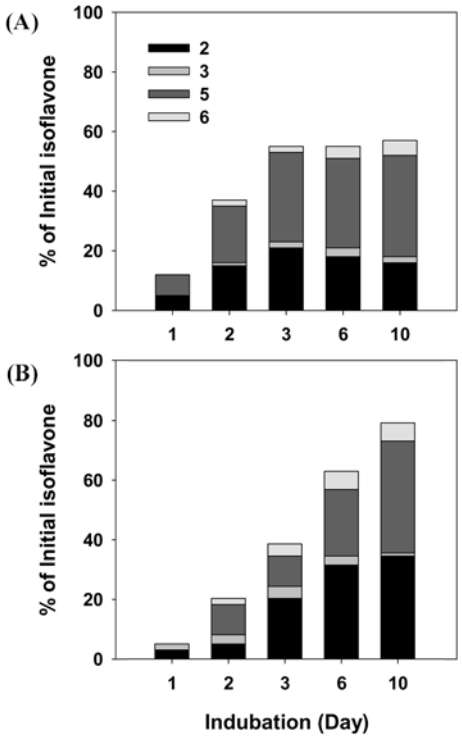

Fig. 5 Mass balances of selected metabolites (**2**, **3**, **5**, and **6**) in *A. niger* (A) and *C. elegans* (B) at 1, 2, 3, 6, and 10 days of incubation. Vertical axis is for the amount of metabolites in percent of initial amount (40 mg/L) of isoflavone.

According to the observation, it can be suggested that enzymes for isoflavone metabolism may have strong regioselectivity (e.g., 4'-hydroxylation on isoflavone skeleton). Poor absorption can also be another cause of such phenomenon. However, estimated logPs with ClogP software was almost same for isoflavone and 4'-isoflavone (3.0 and 3.1, respectively).

In summary, fungal metabolism of non-substituted isoflavone was initiated with B-ring hydroxylation, followed by further reaction on both A- and B-rings. Additional metabolisms, other than hydroxylation include methylation and conjugate formation. *A. niger* were found to be a superior fungus for biotransformation of isoflavones. The information obtained from the present study will help better understand the application of fungi to increase the chemical diversity of natural and man-made analogues of isoflavones.

Supplementary material. GC-MS chromatograms and mass spectra of isoflavone metabolites are presented in supplementary material.

References

Asha S and Vidyavathi M (2009) *Cunninghamella*-a microbial model for drug metabolism studies-a review. *Biotechnol Adv* **27**, 16–29.

Atkinson C, Frankenfeld CL, and Lampe JW (2005) Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol Med (Maywood)* **230**, 155–70.

Das S and Rosazza JPN (2006) Microbial and enzymatic transformations of flavonoids. *J Nat Prod* **69**, 499–508.

Ibrahim AR and Abul-Hajj YJ (1990) Microbiological transformation of flavone and isoflavone. *Xenobiotica* **20**, 363–73.

Jung SH, Cho SH, Dang TH, Lee JH, Ju JH, Kim MK et al. (2003) Structural requirement of isoflavonones for the inhibitory activity of interleukin-5. *Eur J Med Chem* **38**, 537–45

Keum YS, Lee HR, Park HW, and Kim JH (2010) Biodegradation of bisphenol A and its halogenated analogues by *Cunninghamella elegans* ATCC36112. *Biodegradation* **21**, 989–97.

Keum YS, Lee YH, and Kim JH (2009) Metabolism of methoxychlor by *Cunninghamella elegans* ATCC36112. *J Agric Food Chem* **57**, 7931–7.

Kim MH, Han JH, and Kim SU (2008) Isoflavone daidzein: chemistry and bacterial metabolism. *J Appl Biol Chem* **51**, 253–61.

Lee SH, Seo MH, and Oh DK (2013) Deglycosylation of isoflavones in isoflavone-rich soy germ flour by *Aspergillus oryzae* KACC 40247. *J Agric Food Chem* **61**, 12101–10.

Mackenbrock K and Barz W (1983) 3'-Hydroxylation of 4'-methoxyisoflavones by *Fusarium oxysporum* f. lycopersici. *Z Naturforsch* **38c**, 708–10.

Maskey RP, Asolkar RN, Speitling M, Hoffmann V, Grun-Wollny I, Fleck WF et al. (2003) Flavones and new isoflavone derivatives from microorganisms: isolation and structure elucidation. *Z Naturforsch* **58b**, 686–91.

Michels PC, Khmelnitsky YL, Dordick JS, and Clark DS (1998) Combinatorial biocatalysis: a natural approach to drug discovery. *Tr Biotechnol* **16**, 210–5.

Nam DH, Kim HJ, Lim JS, Kim KH, Park CS, Kim JH et al. (2011) Simultaneous enhancement of free isoflavone content and antioxidant potential of soybean by fermentation with *Aspergillus oryzae*. *J Food Sci* **76**, 194–200.

Rice-Evans C (2004) Flavonoids and isoflavones: absorption, metabolism, and bioactivity. *Free Radic Biol Med* **36**, 827–8.

Rimbach G, De Pascual-Teresa S, Ewins BA, Matsugo S, Uchida Y, Minihane AM et al. (2003) Antioxidant and free radical scavenging activity of isoflavone metabolites. *Xenobiotica* **33**, 913–25.

Sun L, Huang HH, Liu L, and Zhong DF (2004) Transformation of verapamil by *Cunninghamella blakesleeana*. *Appl Environ Microbiol* **70**, 2722–7.

Sutherland JB, Selby AL, Freeman JP, Fu FF, Miller DW, and Cerniglia CE (1992) Identification of xyloside conjugates formed from anthracene by *Rhizoctonia solani*. *Mycol Res* **96**, 509–17.