Microbial Transformations of 6- and 7-Methoxyflavones in *Aspergillus niger* and *Penicillium chermesinum* Cultures

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A detailed study of the biotransformation of 6- and 7-methoxyflavones by four fungal strains (*Aspergillus niger* strains *MB*, *KB*, and *SBP*; *Penicillium chermesinum 113*) was carried out. Products of demethylation and also demethylation combined with hydroxylation at C-4' were identified. The biotransformation products were stronger antioxidants than the substrates.

Key words: Biotransformation, Flavones, *Aspergillus niger*

Introduction

Flavonoids, due to their high contents in plants and plant products, are inherent ingredients of the human diet. The main sources of flavonoids in food are vegetables and fruits, but they are also present in *e. g.* chocolate (cacao), tea, red wine, and beer (Aisling Aherne and O'Brien, 2002; Scalbert and Williamson, 2000). The therapeutic potential of flavonoids and their low toxicity, along with an insufficient knowledge of their metabolism, turned our attention to their biotransformation. Flavonoid-transforming microorganisms may be considered models imitating the metabolism of these compounds in mammals or in biodegradation processes (Abourashed *et al.*, 1999; Das and Rosazza, 2006).

Important information on O-demethylation of aromatic compounds was provided by Bache and Pfennig (1981), who used *Acetobacterium woodii*, isolated from the human intestinal flora. The same bacteria were used as a biocatalyst for demethylation of 2-methoxyphenol leading to catechol (Kalil and Stephens, 1997). Research by Hur and Rafii (2000) confirmed that the anaerobic bacterium *Eubacterium limosum* demethylates the isoflavonoids biochanin A, formononetin, and glycitein to estrogen derivatives with high antioxidant properties. Among filamentous fungi, regioselective demethylation was performed by *Aspergillus alliaceus* which transformed 2',3'-dimethoxyflavanone to 2'-methoxy-3'-hydroxyflavanone (Sanchez-Gonzalez and Rosazza, 2004). In another study, *A. niger* was found to catalyze the double demethylation of 7,4'-dimethoxyisoflavanone and the hydroxylation at C-6 with the methyl groups at C-7 and C-4' left intact (Miyazawa *et al.*, 2004).

The aim of this paper was to increase the antioxidant activity and to improve other biological properties of 6- and 7-methoxyflavones through structural modifications by microbial transformations. Additionally, detailed studies of the course of biotransformation were done in order to steps in the metabolism of methoxyflavones in the cultures of filamentous fungi.

Material and Methods

Analysis

The analytical procedures were as described previously (Kostrzewa-Susłow *et al.*, 2010).

Mass spectra were obtained using high-resolution electrospray ionization (ESI^+-MS) (Waters LCT Premier XE mass spectrometer; Milford, MS, USA). Melting points were determined with a Boetius apparatus (Kofler block) (Jena, Germany).

Materials

The substrates for biotransformation – 6-methoxyflavone (**1**) and 7-methoxyflavone (**4**) – were purchased from Sigma-Aldrich (Poznań, Poland). *6-Methoxyflavone* (**1**): M.p. 163–165 °C. – R_t 17.84 min (HPLC). *7-Methoxyflavone* (**4**): M.p. 110–112 °C. - R_t 16.98 min (HPLC).

Microorganisms

A wild strain of *Aspergillus niger* (*KB*) and two UV mutants (*MB*, *SBP*) were used. The *KB* strain came from the collection of the Department of Biotechnology and Food Microbiology of Wrocław University of Environmental and Life Sciences (Wrocław, Poland) and the strains *MB* and *SBP* came from Wrocław University of Economics (Wrocław, Poland). The microorganisms were maintained on potato slants at 5 °C.

The wild strain *Penicillium chermesinum 113* was obtained from the culture collection of the Department of Chemistry of Wrocław University of Environmental and Life Sciences (Wrocław, Poland). The microorganism was maintained on agar slants at 5 °C.

Screening procedure

Screening tests were performed according to the general procedure described earlier (Kostrzewa-Susłow *et al.*, 2010).

Biotransformations were carried out in two ways: the substrate was added to the cultivation medium, either at the time of inoculation with the microorganism or 24 h after the inoculation. The samples were taken 1, 2, 3, 4, 5, 6, 9, and 11 d after substrate addition. All experiments were performed in duplicate.

Preparative-scale biotransformation

Preparative-scale biotransformations and isolation of compounds were carried out according to the procedure described earlier (Kostrzewa-Susłow *et al.*, 2010). The spectral data of the products are consistent with the literature values (Ibrahim and Abul-Hajj, 1990):

6-Hydroxyflavone (**2**) ($C_{15}H_{10}O_3$): M.p. 231 – 232 °C. – R_t 14.02 min (HPLC). – Purity 99% (HPLC). – HRESI-MS: *m/z* = 239.0765 [M+H$^+$]; found 239.0760.

6,4'-Dihydroxyflavone (**3**) ($C_{15}H_{10}O_4$): M.p. 339 – 340 °C. – R_t 11.33 min (HPLC). – Purity 98% (HPLC). – HRESI-MS: *m/z* = 255.0855 [M+H$^+$]; found 255.0850.

7-Hydroxyflavone (**5**) ($C_{15}H_{10}O_3$): M.p. 245 – 247 °C. – R_t 13.87 min (HPLC). – Purity 99% (HPLC). – HRESI-MS: *m/z* = 239.0761 [M+H$^+$]; found 239.0755.

7,4'-Dihydroxyflavone (**6**) ($C_{15}H_{10}O_4$): M.p. 321 – 323 °C. – R_t 11.52 min (HPLC). – Purity 99% (HPLC). – HRESI-MS: *m/z* = 255.0867 [M+H$^+$]; found 255.0862.

Measurement of antioxidant properties of the substrate and the products

A methanolic solution of DPPH (2,2-diphenyl-1-picryl-hydrazyl), with an absorbance of about 1.00, was mixed with a proper amount of a tested flavonoid. After 20 min, disappearance of absorbance at 520 nm was measured. The initial concentration of DPPH was determined by means of a calibration curve. The IC_{50} value (antiradical activity) was determined graphically – DPPH radical reduction (expressed in %) as a function of concentration of the tested compound. IC_{50} means concentration of the antioxidant that reduces the initial concentration of DPPH by half.

Results and Discussion

At the beginning of the study, screening tests were performed with 27 filamentous fungal strains of the genera *Aspergillus*, *Penicillium*, *Piptoporus*, *Spicaria*, *Absidia*, *Coryneum*, *Nectria*, *Cryptosporiopsis*, and *Chaetomium*. The strains of *Aspergillus niger* and *Penicillium chermesinum* were effective catalysts of the transformations of flavones, flavanones (Kostrzewa-Susłow *et al.*, 2006, 2008), and their hydroxy and methoxy derivatives. The only strains capable of transforming 6-methoxyflavone (**1**) and 7-methoxyflavone (**4**) were a wild strain of *A. niger KB*, two UV mutants of this strain (*A. niger MB* and *A. niger SBP*), and the strain *P. chermesinum 113*.

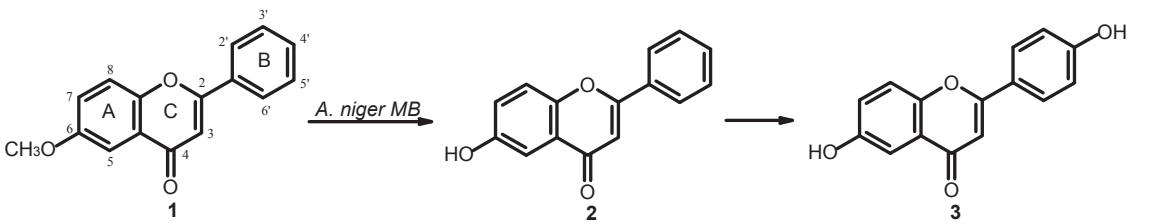
Biotransformations of substrates **1** and **4** on a preparative scale were performed in a 9-days reaction using *A. niger MB*. The substrates were added to the cultivation medium at the time of inoculation with the microorganism. Transformation of 6-methoxyflavone (**1**) gave two products: 6-hydroxyflavone (**2**) and 6,4'-dihydroxyflavone (**3**) in yields of 33.0% (16.5 mg) and 15.2% (7.6 mg), respectively (Scheme 1). Biotransforma-

tion of 7-methoxyflavone (**4**) in the culture of *A. niger MB* led to 7-hydroxyflavone (**5**) and 7,4'-dihydroxyflavone (**6**) in yields of 13.8% (6.9 mg) and 48.1% (24 mg) (Scheme 2).

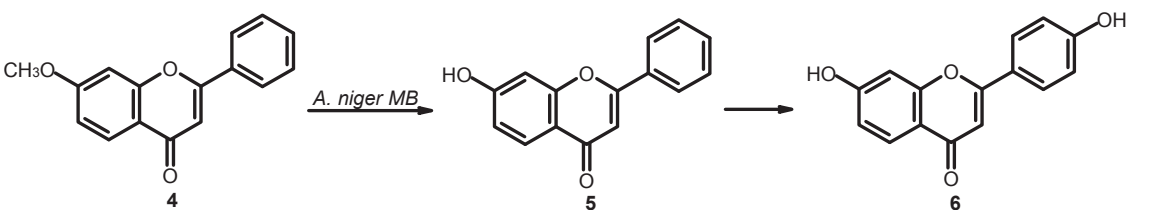
The products isolated after preparative-scale biotransformations were used as standards for quantitative analysis by means of high-performance liquid chromatography (HPLC).

In the quantitative study we assessed the effect of the time of substrate addition on the biotransformation. The substrate was either added at the time of inoculation of the cultivation medium with the microorganism or 24 h after inoculation. The reactions were monitored by thin-layer chromatography (TLC) and HPLC. The results of these experiments are presented in Figs. 1 to 8, in which the mean values of two replicates are given. The ratios of products formed and the rates of substrate consumption are different in each case.

Microbial transformations of 6-methoxyflavone (**1**) in the cultures of *A. niger MB*, *A. niger KB*, and *P. chermesinum 113* led to the formation of 6-hydroxyflavone (**2**) and 6,4'-dihydroxyflavone (**3**). *A. niger SBP* catalyzed only demethylation of **1** at C-6. The best biocatalyst for transformation of **1** proved to be the strain *KB* of *A. niger*. Products **2** and **3** were produced by this strain faster and in higher yields than by any other strain (Figs. 1 and 2). Addition of the substrate to *A. niger KB* 24 h after inoculation resulted in an increase of both the rate and yield of the biotransformation process (Fig. 1). In the transformation of **1** by *A. niger MB* demethylation at C-6 preceded hydroxylation at C-4' of the B-ring between days 5 and 11 of culture. Starting from day 6 of the biotransformation, a gradual decrease in the amount of **2** was observed, along with a proportional increase in the amount of 6,4'-dihydroxyflavone (**3**) (Figs. 3 and 4). After 6 days of biotransformation, **1** no longer appeared



Scheme 1. Microbial transformations of 6-methoxyflavone (**1**) in *A. niger MB* culture.



Scheme 2. Microbial transformations of 7-methoxyflavone (**4**) in *A. niger MB* culture.

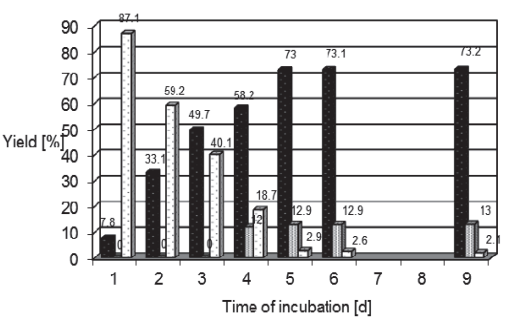


Fig. 1. Biotransformation of 6-methoxyflavone (**1**) in *A. niger KB* culture – yield (%) of products determined by HPLC (substrate added 24 h after inoculation).

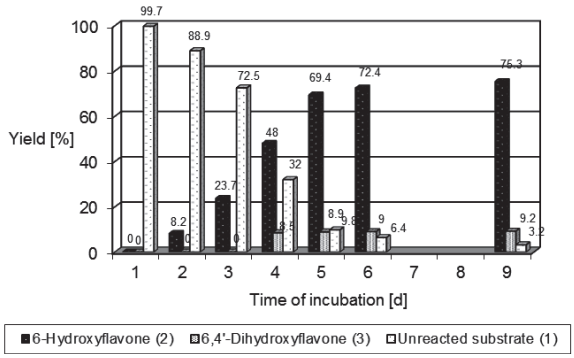


Fig. 2. Biotransformation of 6-methoxyflavone (**1**) in *A. niger KB* culture – yield (%) of products determined by HPLC (substrate added at the time of inoculation).

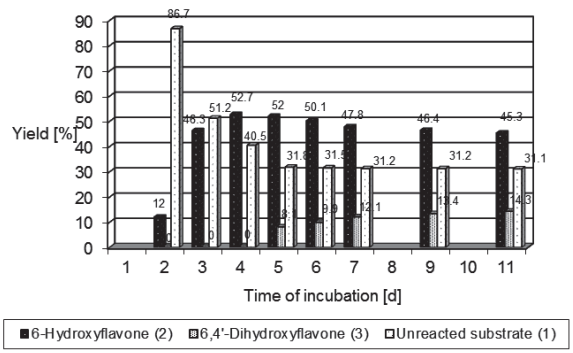


Fig. 3. Biotransformation of 6-methoxyflavone (**1**) in *A. niger MB* culture – yield (%) of products determined by HPLC (substrate added 24 h after inoculation).

to be metabolized, while the previously formed **2** was converted to **3** (Scheme 1). The weakest biocatalyst for transformation of **1** was *P. chermesinum 113*. The substrate was consumed to only about 20%. Biotransformation of **1** by the strain of *A. niger SBP* led to a single product, *i. e.* 6-hydroxyflavone (**2**) which was formed in high yield (84.2% yield, when **1** was added at the time of inoculation).

In the biotransformation of 7-methoxyflavone (**4**) by *A. niger KB* and *A. niger MB* (Scheme 2) demethylation in ring A also occurred prior to hydroxylation at C-4' of ring B (Figs. 5 – 8).

A. niger SBP demethylated 4 to 5 in 41.1% yield within the first day of incubation, and then the yield of 5 increased gradually until day 9. The time of substrate addition was very important for the reaction course, reaction rates and yields being much higher when the substrate was added 24 h after inoculation. Biotransformation of **4** by *P. chermesinum 113* led to the formation of 7,4'-dihydroxyflavone (**6**). There was no 7-hydroxyflavone (**5**) observed as an intermediate product. Also in this case, the addition of the substrate 24 h after inoculation resulted in a higher yield of **6**.

The xenobiotic substrates 6- and 7-methoxyflavones underwent two types of reactions: O-demethylation and hydroxylation. In most cases demethylation of ring A took place prior to hydroxylation at C-4' of ring B, or the process stopped at the demethylation stage. In no case was the hydroxylation at C-4' observed in the presence of the methoxy group at C-6 or C-7.

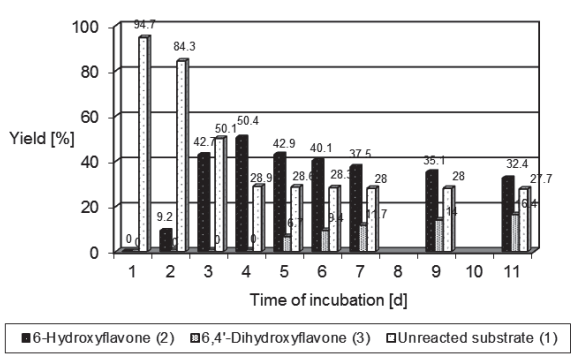


Fig. 4. Biotransformation of 6-methoxyflavone (**1**) in *A. niger MB* culture – yield (%) of products determined by HPLC (substrate added at the time of inoculation).

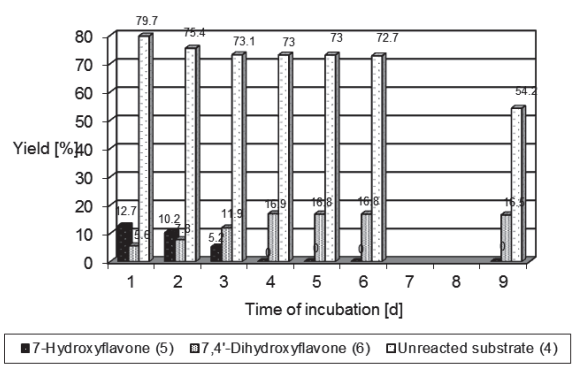


Fig. 5. Biotransformation of 7-methoxyflavone (**4**) in *A. niger KB* culture – yield (%) of products determined by HPLC (substrate added 24 h after inoculation).

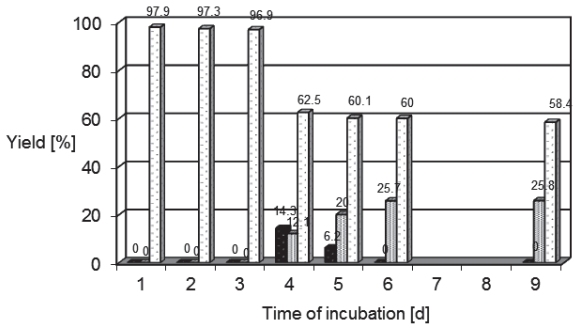


Fig. 6. Biotransformation of 7-methoxyflavone (**4**) in *A. niger KB* culture – yield (%) of products determined by HPLC (substrate added at the time of inoculation).

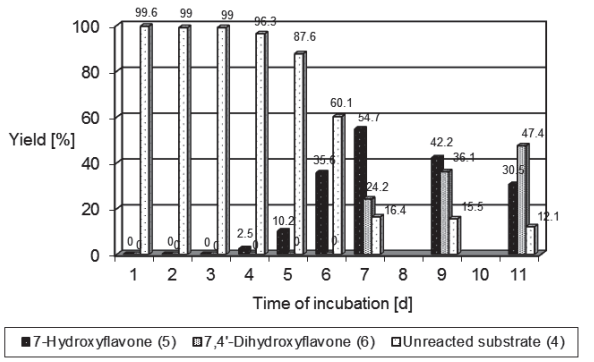


Fig. 7. Biotransformation of 7-methoxyflavone (**4**) in *A. niger MB* culture – yield (%) of products determined by HPLC (substrate added 24 h after inoculation).

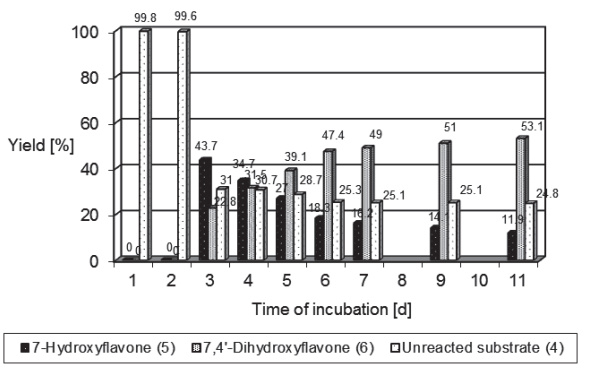


Fig. 8. Biotransformation of 7-methoxyflavone (**4**) in *A. niger MB* culture – yield (%) of products determined by HPLC (substrate added at the time of inoculation).

Cytochrome P-450 monooxygenases are responsible for demethylation and/or hydroxylation of many hydrophobic xenobiotics by microorganisms (Das and Rosazza, 2006). These oxygenases are also widely present in plants and animals (Hodek *et al.*, 2002) and form a large family of membrane-bound enzymes which are difficult to isolate and separate from each other in a functional state which makes it difficult to determine their substrate specificity.

In the case of flavones monosubstituted at the A-ring hydroxylation occurs regioselectively at C-4' of the B-ring.

In order to assess and compare antioxidant properties of the tested flavonoids, their IC_{50} values in the DPPH radical scavenging assay were determined. The results confirm the observation that the presence of aromatic hydroxy groups is associated with a considerable increase in antioxidant activity (Jeong *et al.*, 2007) (Table I). Demethylation of the A-ring of a flavonoid uncovers a hydroxy group which confers stronger antioxidant properties to the respective products. The strongest enhancement of antioxidant activity was observed in the case of the hydroxylation of the B-ring. Thus, 7,4'-dihydroxyflavone (**6**) was the most potent antioxidant of the compounds tested (Table I).

Natural products are a very important source of promising leads for the development of novel cancer therapeutics. Recently, some flavonoids have been implicated in the modulation of P-gp-type multidrug resistance (MDR) in cancers and shown to inhibit a variety of ATP-binding proteins

Table I. IC_{50} values of the flavonoid substrates and the biotransformation products in the DPPH radical scavenging assay.

Substrate	Product	IC_{50}[a] (± SD) [μM]
6-Methoxyflavone (**1**)		9.74 (± 0.02)
	6-Hydroxyflavone (**2**)	9.46 (± 0.04)
	6,4'-Dihydroxyflavone (**3**)	8.80 (± 0.06)
7-Methoxyflavone (**4**)		9.64 (± 0.04)
	7-Hydroxyflavone (**5**)	8.78 (± 0.07)
	7,4'-Dihydroxyflavone (**6**)	7.66 (± 0.07)

[a] Mean values of IC_{50} calculated as an average of at least three measurements.

such as plasma membrane ATPase, cyclic AMP-dependent protein kinase, and protein kinase C (Boumendjel *et al.*, 2002; Havsteen, 2002). It was shown that these compounds interact with transporter proteins (Bansal *et al.*, 2009; Nissler *et al.*, 2004) as well as with membrane lipids (Michalak *et al.*, 2007; Środa *et al.*, 2008). It is well known that biological activities of flavonoids strongly depend on their chemical structure. Especially important are methoxy and hydroxy substituents in the B-ring of the flavonoid molecule (Jeong *et al.*, 2007).

Conclusions

1. The results of the biotransformations show that flavones with a methoxy group at the A-ring undergo first demethylation and then hydroxylation at C-4' of the B-ring.

2. The strains of *A. niger MB* and *A. niger KB* transform both of the tested substrates, *i. e.* 6-methoxy- and 7-methoxyflavones, respectively, into two products: the demethylation product and its C-4'-hydroxylated derivative.

3. For both substrates the course of the microbial transformation depends on the time of substrate addition, *i. e.* in which growth stage of the microorganism it was added.

Acknowledgements

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