# MICROBIAL TRANSFORMATIONS OF 5-HYDROXY- AND 5-METHOXYFLAVONE IN *ASPERGILLUS NIGER* AND *PENICILLIUM CHERMESINUM* CULTURES

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**ABSTRACT**

Enzymatic systems of *Aspergillus niger MB, Aspergillus niger KB* and *Penicillium chermesinum 113* catalysed hydroxylation at C-4' in ring B of 5-hydroxyflavone and O-methylation at the same position in 5-methoxyflavone. Additionally, in the culture of *Aspergillus niger SBP* 5-methoxyflavone underwent demethylation. An important factor was the time of substrate addition to the cultivation medium. The substrates were added either at the time of inoculation of the medium with a microorganism, or 24 h after the inoculation. For 5-hydroxyflavone better yields of transformation were observed when the substrate was added to the cultivation medium at the time of the inoculation, whereas, for 5-methoxyflavone the situation was opposite. Antioxidant properties of the substrates and the products of biotransformations were evaluated spectrophotometrically by DPPH assay.

**Keywords:** *Aspergillus niger, Penicillium chermesinum*, biotransformation, 5-hydroxyflavone, 5-methoxyflavone

## INTRODUCTION

Flavonoids are plant components of the diet. Their daily intake is about 1g. In Poland the main source of flavonoids is apples, tea and onions. The great therapeutic potential of flavonoid compounds of natural origin is well known and widely described (**Bansal *et al*., 2009; Havsteen, 2002**). Although the presence of natural flavonoids in mammals has not been confirmed so far, their multidirectional influence on many physiological processes has been proved and described by many research institutes all over the world (**Cook and Samman, 1996; Szliszka *et al*., 2012**). However, from the pharmacological point of view, there is no the administered substrate itself, but the products of its metabolism that are most responsible for therapeutic properties (**Fura *et al*., 2004**). Therefore, it is important to determine possible ways of transformations of flavonoids in mammals. The first step may be biomimetic study using microorganisms, e.g. filamentous fungi (**Abourashed *et al*., 1999; Kostrzewa-Susłow *et al*., 2008; Kostrzewa-Susłow and Janeczko, 2012; Smith and Rosazza, 1974**). It has been proved that filamentous fungi of the genus *Aspergillus* and *Penicillium* are able to catalyze regio- and stereoselective hydroxylation and O-methylation of flavonoid compounds (**Kostrzewa-Susłow *et al*., 2014, Kostrzewa-Susłow *et al*., 2012, Kostrzewa-Susłow *et al*., 2007**). Enzymatic hydroxylations are catalyzed mostly by cytochrome P450 monooxygenases. In human body molecular forms of cytochrome P450 constitute a large group of hemoproteins capable of metabolizing many exogenous compounds (drugs, toxic substances, stimulants), and taking part in synthesis and biotransformation of many endogenous compounds of great physiological importance (fatty acids, steroid hormones, prostaglandins, leukotrienes). O-methylation is catalyzed by S-adenosylmethionine-dependent O-methyltransferases. Therefore, flavonoid compounds which possess several hydroxyl groups are good substrates for an O-methyltransferase (**Kostrzewa-Susłow *et al*., 2007**). By O-methylation they are transformed into a more soluble, convenient transportable form. In mammals O-methylation is a well known reaction in detoxification processes.

In the paper by Ibrahim and Abul-Hajj concerning biotransformation of 5-hydroxyflavone, except for mono- and dihydroxylation the authors described also sulfation at C-4', which was catalysed by *Streptomyces fulvissimus* (**Ibrahim and Abul-Hajj, 1989**). The study on metabolism and pharmacokinetics of selected flavones, conducted on male Sprague-Dawley rats after oral administrations of 40mg/kg of bw of 5-hydroxyflavone, proved the presence of its glucuronide form (**Shia *et al*., 2009**). 5-Methoxyflavone in the culture of *Beauveria bassiana* was transformed into 5-methoxyflavanone,

whereas the strain of *Aspergillus alliaceus* performed hydroxylation at C-4' with the methoxy group at C-5 untouched (**Herath *et al*., 2009**).

Biotransformations described in this paper, using natural enzymatic systems of microorganisms of the genera *Aspergillus* and *Penicillium,* were carried out in order to determine the capability of selected filamentous fungi to perform biotransformation of 5-hydroxyflavone and 5-methoxyflavone, to determine the effect of substrate addition time on the biotransformation course, and also to establish antioxidant properties of substrates and products and their UV absorption capacity.

## MATERIAL AND METHODS

### Analysis

The course of microbial transformation was monitored by TLC (SiO$_2$, DC Alufolien Kieselgel 60 F$_{254}$, Merck, Darmstadt, Germany). Chromatograms were developed using the following developing systems: hexane : ethyl acetate (7:3), dichloromethane : ethyl acetate (1:1), toluene : diethyl ether (4:1). Column chromatography (SiO$_2$, Kieselgel 60, 230 - 400 mesh, 40 – 63 μm, Merck) was performed using the same eluents. $^1$H NMR and $^{13}$C NMR spectra were recorded with a Bruker Avance DRX 300 spectrometer. Mass spectra were obtained using high-resolution electrospray ionization (ESI$^+$-MS) (Waters LCT Premier XE mass spectrometer, Milford, MS, USA). HPLC analyses were performed with a Waters 2690 instrument equipped with Waters 996 photodiode array detector, using ODS 2 column (4.6 × 250 mm, Waters) and a Guard-Pak Inserts μBondapak C18 pre-column. Separation conditions were as follows: gradient elution, using 80% of acetonitrile in 4.5% formic acid solution (eluent A) and 4.5% formic acid (eluent B); flow, 1 mL/min; detection wavelength 280 nm; program: 0 – 7 min, 10% A 90% B; 7 – 10 min, 50% A 50% B; 10 – 13 min, 60% A 40% B; 13 – 15 min, 70% A 30% B; 15 – 20 min 80% A 20% B; 20 – 30 min 90% A 10% B; 30 – 40 min, 100% A. Melting points were determined with a Boetius apparatus (Kofler block) (Jena, Germany). Antioxidant properties were measured with a Cintra 20 spectrometer (Melbourne, Australia).

### Materials

The substrates for biotransformation – 5-hydroxyflavone (**1**) and 5-methoxyflavone (**3**) – were purchased from Sigma-Aldrich (Poznań, Poland).

5-Hydroxyflavone (**1**). C$_{15}$H$_{10}$O$_3$; melting point 158-160°C; R$_t$ 20.29 min (HPLC). $^1$H NMR (DMSO-d$_6$) δ: 6.83 (1H, d, *J$_{6,7}$*=8.2 Hz, H-6), 7.14 (1H, s, H-

3), 7.22 (1H, d, $J_{8,7}$=8.5 Hz, H-8), 7.65 (4H, m, H-3', H-4', H-5', H-7), 8.12 (2H, d, $J$=7.8 Hz, H-2', H-6'), 12.8 (1H, s, 5-OH); $^{13}$C NMR (DMSO-d$_6$) δ: 105.6 (C-3), 107.5 (C-8), 110.1 (C-10), 110.9 (C-6), 126.6 (C-2', C-6'), 129.1 (C-3', C-5'), 130.5 (C-1'), 132.3 (C-4'), 135.9 (C-7), 155.9 (C-9), 159.8 (C-5), 164.0 (C-2), and 183.2 (C-4).

5-Methoxyflavone (**3**). $C_{16}H_{12}O_3$; melting point 128-130°C; $R_t$ 14.99 min (HPLC). $^1$H NMR (CD$_3$OD) δ: 3.87 (3H, s, 5-OCH$_3$), 6.69 (1H, s, H-3), 6.92 (1H, d, $J_{6,7}$=8.3 Hz, H-6), 7.16 (1H, d, $J_{8,7}$=8.5 Hz, H-8), 7.47 (3H, m, H-3', H-4', H-5'), 7.62 (1H, t, $J$=8.4 Hz, H-7), 7.91 (2H, m, H-2', H-6'); $^{13}$C NMR (CD$_3$OD) δ: 108.1 (C-3), 109.1 (C-8), 111.2 (C-6), 114.9 (C-10), 127.4 (C-2', C-6'), 130.2 (C-3', C-5'), 132.4 (C-1'), 132.9 (C-4'), 136.0 (C-7), 159.7 (C-9), 161.0 (C-5), 163.7 (C-2), and 180.6 (C-4).

### Microorganisms

A wild strain of *Aspergillus niger KB* and two UV mutants (*MB* and *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 (Poland) and the strains *MB* and *SBP* came from Wrocław University of Economics (Poland). The microorganisms were maintained on potato slants at 5 °C. A wild strain of *Penicillium chermesinum 113* was obtained from the culture collection of the Department of Chemistry of Wrocław University of Environmental and Life Sciences (Poland). The microorganism was maintained on agar slants at 5 °C.

### Biotransformations

#### Screening procedure (initial biotransformations)

Cultivation media consisted of 3% glucose (Gliwice, Poland) and 1% peptobac (Warszawa, Poland) in water. The microorganisms were transferred from the slants to 500 mL Erlenmayer flasks, each containing 200 mL of the medium. Preincubation was performed at 25 °C for 24 – 48 h. Then portions of 1 mL of the culture solution were transferred to inoculate 500 mL flasks, each containing 200 mL of the medium and the mixtures were cultivated at 25 °C on a rotary shaker. 10 mg of a substrate, dissolved in 0.5 mL of THF, was added to the cultures. 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 hours after the inoculation. Control cultivation with no substrate was also performed. After 3, 4, 5, 6, 7, 8, 9 and 11 days of incubation under the above conditions, portions of 5 mL of the transformation mixture were withdrawn and extracted with ethyl acetate (3 × 3 mL). The extracts were dried over MgSO$_4$ (5 min), concentrated *in vacuo* and analyzed by TLC. Quantitative analyses of the mixtures were performed by means of HPLC. Calibration curves for quantitative analyses were prepared using isolated and purified biotransformation products as standards. All experiments were performed in duplicate.

#### Preparative-scale biotransformation

Portions of 1 mL of the preincubation culture solution were used to inoculate three 2000 mL flasks, each containing 500 mL of the cultivation medium. The cultures were incubated at 25 °C for 48 hours on a rotary shaker. Then 50 mg of the substrate dissolved in 2.5 mL of THF was added to each flask (50 mg of the substrate per 1 L of the cultivation mixture). After 9 days of incubation the mixtures were extracted with ethyl acetate (3 × 200 mL), dried (MgSO$_4$) and concentrated *in vacuo*. The transformation products were separated by column chromatography. Pure products were identified by means of spectral analyses (TLC, $^1$H NMR, $^{13}$C NMR). Physical and spectral data of the products obtained are presented next.

5,4'-Dihydroxyflavone (**2**). $C_{15}H_{10}O_4$; melting point 240-243°C; $R_t$ 15.63 min (HPLC); purity 99% (HPLC). $^1$H NMR ((CD$_3$)$_2$CO) δ: 6.78 (1H, d, $J_{6,7}$=8.3 Hz, H-6), 6.99 (1H, s, H-3), 7.04 (2H, dd, $J_{3',2'(5',6')}$=8.9 Hz, $J_{3',5'}$=2.0 Hz, H-3', H-5'), 7.12 (1H, d, $J_{8,7}$=8.5 Hz, H-8), 7.63 (1H, dd, $J_{7,8}$=8.4 Hz, $J_{7,6}$=8.3 Hz, H-7), 7.99 (2H, dd, $J_{2',3'(6',5')}$=8.9 Hz, $J_{2',6'}$=2.0 Hz, H-2', H-6'), 8.11 (1H, s, 4'-OH), 12.83 (1H, s, 5-OH); $^{13}$C NMR (DMSO-d$_6$) δ: 103.4 (C-3), 107.5 (C-8), 110.0 (C-10), 110.8 (C-6), 116.1 (C-3', C-5'), 121.0 (C-1'), 128.8 (C-2', C-6'), 135.6 (C-7), 155.8 (C-9), 159.4 (C-5), 161.5 (C-4'), 164.7 (C-2), and 182.9 (C-4); HRESI-MS [M+H$^+$] (calculated/found) (*m/z* = 255.0853/255.0849).

5,4'-Dimethoxyflavone (**4**). $C_{17}H_{14}O_4$; oily liquid; $R_t$ 12.0 min (HPLC); purity 99% (HPLC). $^1$H NMR ((CD$_3$)$_2$CO) δ: 3.30 (3H, s, 4'-OCH$_3$), 3.90 (3H, s, 5-OCH$_3$), 6.56 (1H, s, H-3), 6.99 (1H, d, $J_{6,7}$=8.1 Hz H-6), 7.02 (2H, dd, $J_{3',2'(5',6')}$=8.9 Hz, $J_{3',5'}$=2.1 Hz, H-3', H-5'), 7.21 (1H, d, $J_{8,7}$=8.4 Hz, H-8), 7.65 (1H, t, $J$=8.3 Hz, H-7), 7.92 (2H, dd, $J_{2',3'(6',5')}$=8.9 Hz, $J_{2',6'}$=2.1 Hz, H-2', H-6'); $^{13}$C NMR (THF-d$_8$) δ: 55.4 (4'-OCH$_3$), 56.9 (5-OCH$_3$), 108.1 (C-3), 109.3 (C-8), 111.3 (C-6), 114.7 (C-10), 115.2 (C-3', C-5'), 122.7 (C-1'), 126.6 (C-2', C-6'),

136.1 (C-7), 159.6 (C-9), 160.7 (C-5), 162.1 (C-4'), 163.8 (C-2), and 180.8 (C-4); HRESI-MS [M+H$^+$] (calculated/found) (*m/z* = 283.1127/283.1123).

### Measurement of antioxidant properties of the substrate and the products

A methanolic solution of DPPH (1,1-diphenyl-2-picryl-hydrazyl) with an absorbance of about 1.00, was mixed with a proper amount of a tested flavonoid (**1 – 4**). After 20 min, disappearance of absorbance at 520 nm was measured. The initial concentration of DPPH was determined by means of 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 (**Kostrzewa-Susłow et al., 2010; Kostrzewa-Susłow et al., 2012**).

## RESULTS AND DISCUSSION

### Biotransformations

This study on microbial transformation of 5-hydroxy- (**1**) and 5-methoxyflavone (**3**) is the continuation of the research on transformation of monosubstituted flavones (**Kostrzewa-Susłow et al., 2012**). In our previous paper we described biotransformation of 6- and 7-methoxyflavones. The initial, small-scale biotransformations allowed to check whether the strains of *A. niger* (*MB, KB, SBP*) and *P. chermesinum 113* were capable of transformation of flavones with hydroxyl (**1**) and methoxy (**3**) groups at C-5. Additionally, yields of products (HPLC) were assessed during reaction course, between the 3$^{rd}$ and the 11$^{th}$ day, for both substrate addition times (substrate added to the cultivation medium at the time of inoculation with a microorganism or after 24 h from the inoculation). When the time of biotransformation in the culture of *A. niger* MB exceeded 11 days, we observed that the amounts of both the product and the unreacted substrate slightly decreased, which indicated loss of activity of the catalyzing enzyme. Therefore, to standardize our experiments, we chose 11 days as the maximum reaction time for all our biotransformations.

Preparative biotransformations (scaling up the process) allowed to isolate the products and to establish their structures by means of $^1$H NMR and $^{13}$C NMR. The products isolated from the preparative-scale biotransformations were used as standards for quantitative analysis by means of high-performance liquid chromatography (HPLC).

The strains of *A. niger (MB* and *KB)* and *P. chermesinum 113* transformed 5-hydroxyflavone (**1**) to 5,4'-dihydroxyflavone (**2**) (Fig 1, 2).
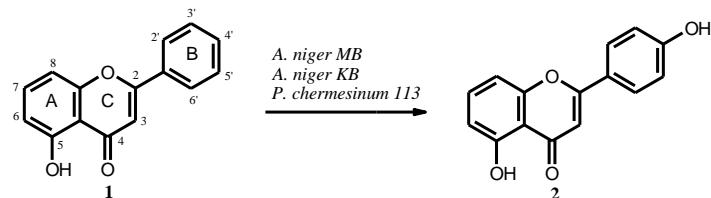


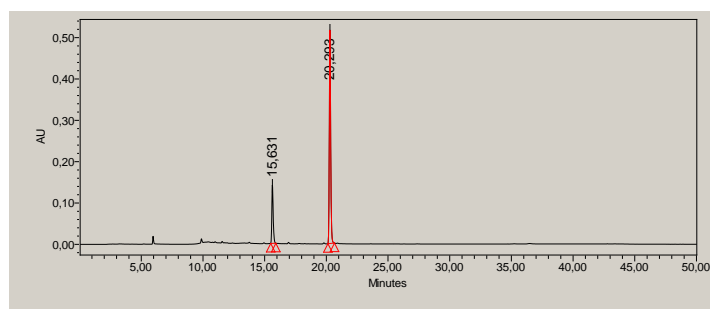Figure 1 **Microbial transformations of 5-hydroxyflavone** (1)



**Figure 2** HPLC chromatogram – biotransformation of 5-hydroxyflavone in the culture of *A. niger MB* (Rt=20,293 5-hydroxyflavone, Rt=15,631 5,4'-dihydroxyflavone)

The biotransformation product was formed at a higher rate and in a higher yield in the case when the substrate was added to the cultivation medium at the time of the inoculation (Figs 4-6). In the culture of *A. niger MB* when the substrate was added at the time of the inoculation, the product appeared on the 8$^{th}$ day (18,7%). Its amount was increasing with time to reach 39,4% on the 11$^{th}$ day (Fig 3, 4).
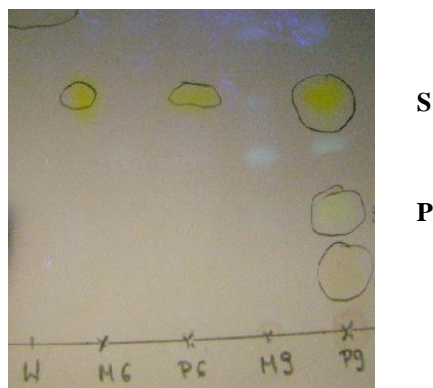
**Figure 3** Biotransformation of 5-hydroxyflavone (**1**) in the culture of *A. niger MB*. TLC chromatogram (the photograph of the plate placed under a UV lamp) (S-substrate (**1**), P-product; 5,4'-dihydroxyflavone (**2**)).

In the case when the substrate was added 24 h after the inoculation the yield was much lower. The biotransformation product appeared no sooner than on the 11th day (7.9%).
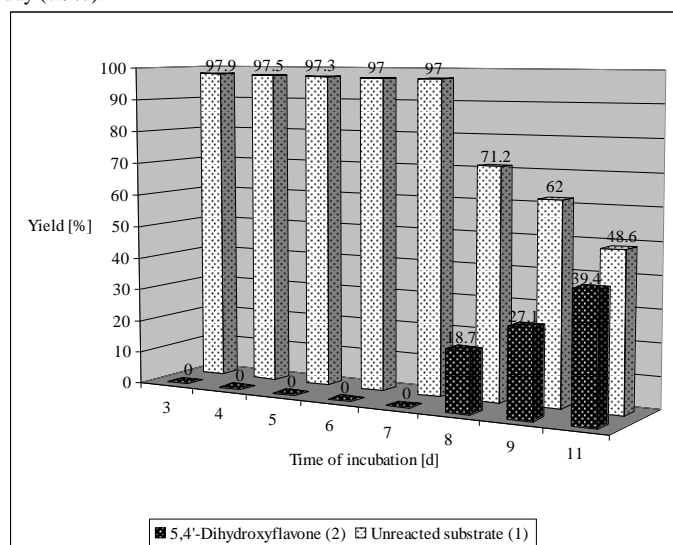


**Figure 4** Biotransformation of 5-hydroxyflavone (**1**) in *A. niger MB* culture – yield (%) of products determined by HPLC (substrate added at the time of the inoculation)

The similar course of biotransformation of 5-hydroxyflavone (**1**) was observed for *A. niger KB*. When the substrate was added at the time of the inoculation, the biotransformation product (**2**) was observed as soon as on the 4th day (3,2%) and its amount gradually increased with time (Fig 5). Whereas, in the case when **1** was added 24 h after the inoculation product **2** was noticed on the 9th day (9,3%) and the increase in its amount during the next few days was small (14,7% on the 11th day).
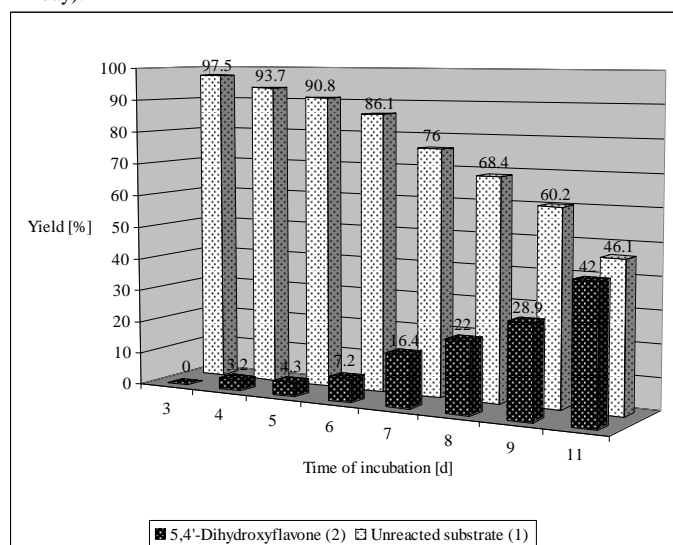


**Figure 5** Biotransformation of 5-hydroxyflavone (**1**) in *A. niger KB* culture – yield (%) of products determined by HPLC (substrate added at the time of the inoculation)

In biotransformation with the help of *P. chermesinum 113* 5,4'-dihydroxyflavone (**2**) was observed on the 6th day (32,4%) (Fig 6). Since then its amount was gradually increasing up to 64,7% on the 11th day. In the case when the substrate was added 24 h after the inoculation, it was late as on the 11th day of biotransformation when the small amount of the product was observed (5,5%). The strain of *A. niger SBP* did not transform 5-hydroxyflavone (**1**) at all.
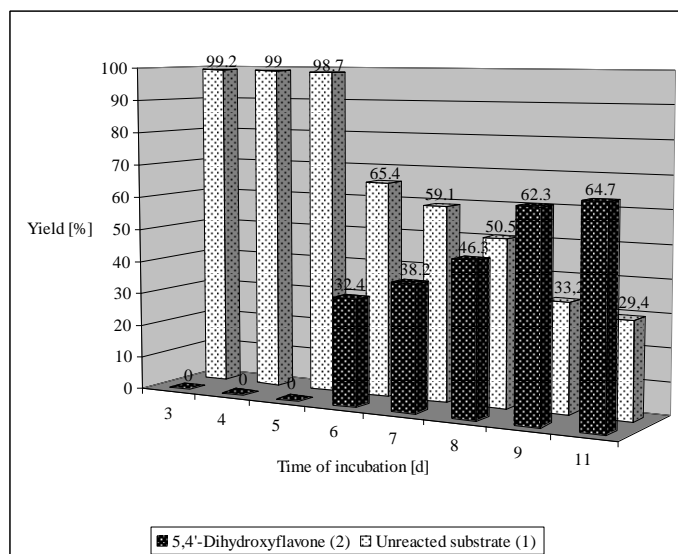


**Figure 6** Biotransformation of 5-hydroxyflavone (**1**) in *P. chermesinum 113* culture – yield (%) of products determined by HPLC (substrate added at the time of the inoculation)

In order to confirm the structure of 5,4'-dihydroxyflavone (**2**), the compound was isolated after a 9-day biotransformation of 5-hydroxyflavone (**1**) in the culture of *A. niger MB* in 15% yield (22,5 mg). In the $^1$H NMR spectrum of **2** there is a singlet of the C-5 hydroxyl group observed at δ=12,83 ppm, and also an additional signal of the hydroxyl group at C-4' is also visible at δ=8,11 ppm, compared to **1**. The multiplet of protons at C-3', C-4', and C-5' in ring B of the substrate (**1**) disappeared, whereas a new doublet of doublets at δ=7,04 ppm was observed, integrating for two protons and attributed to C-3' and C-5'protons ($J_{3',2'}$= 8,9 Hz and $J_{3',5'}$=2,0 Hz). The presence of a hydroxyl group at C-4' was additionally confirmed by $^{13}$C NMR, where the signal of C-4' moved from δ=132,3 ppm for the substrate (**1**) to δ=161,5 ppm for the product (**2**).

As in the case of 5-hydroxyflavone (**1**), also for the second substrate: 5-methoxyflavone (**3**) a detailed quantitative and qualitative study was carried out, so as to find the optimal biotransformation time. It was observed that the strains of *A. niger (MB* and *KB)* and *P. chermesinum 113* transformed 5-methoxyflavone (**3**) to 5,4'-dimethoxyflavone (**4**), whereas *A. niger SBP* performed only demethylation of **3** at C-5, leading to **1** (Fig 7).
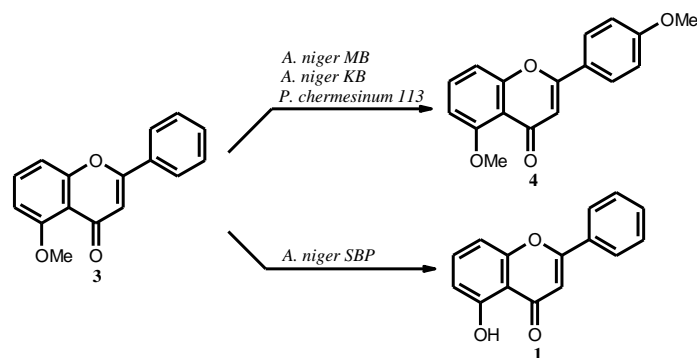


Figure 7 **Microbial transformations of 5-methoxyflavone** (3)

In biotransformation using the strain *A. niger MB*, when the substrate was added to the cultivation medium at the time of inoculation with the microorganism, no transformation product was observed. Whereas, in the case when the substrate (**3**) was added after 24 hours from the inoculation, product **4** was formed on the 5th day (12,4%) and its amount gradually increased up to the 8th day (44,2%) (Fig 8).
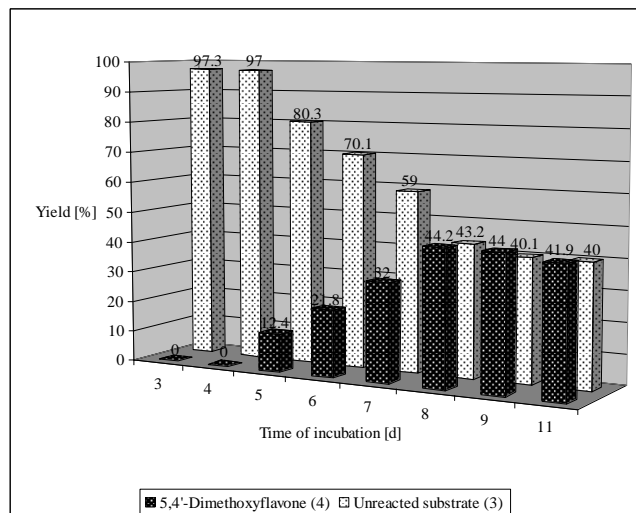
**Figure 8** Biotransformation of 5-methoxyflavone (**3**) in *A. niger MB* culture – yield (%) of products determined by HPLC (substrate added 24 h after the inoculation)

In the culture of *A. niger KB*, in the case when the substrate (**3**) was added after 24 hours from the inoculation, 5,4'-dimethoxyflavone (**4**) was observed in the reaction mixture earlier (on the 4th day) and with higher yield (40,7% on the 11th day) (Fig 9). Under the second reaction conditions (the substrate added at the time of the inoculation) the biotransformation product (**4**) appeared on the 7th day (7,1%), to reach the yield of 22,4% on the 11th day.
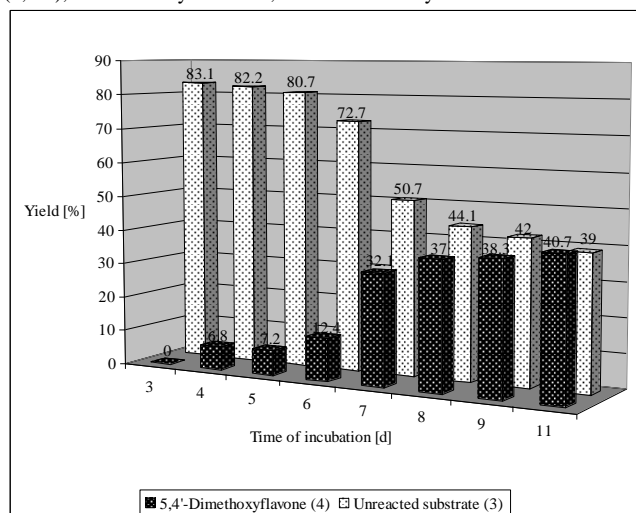


**Figure 9** Biotransformation of 5-methoxyflavone (**3**) in *A. niger KB* culture – yield (%) of products determined by HPLC (substrate added 24 h after the inoculation)

In the culture of *P. chermesinum 113* the product of biotransformation (**4**) in both experiments: with the substrate addition at the time of the inoculation and after 24 h, appeared as late as after 11 days and the yields did not exceed 13% (Fig 10, 11).
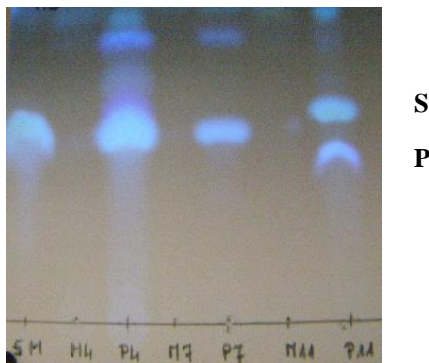


**Figure 10** Biotransformation of 5-methoxyflavone (**3**) in *P. chermesinum 113* culture. TLC chromatography (the photo of the plate taken under a UV lamp) (S-substrate (**3**), P-product; 5,4'-dimethoxyflavone (**4**))
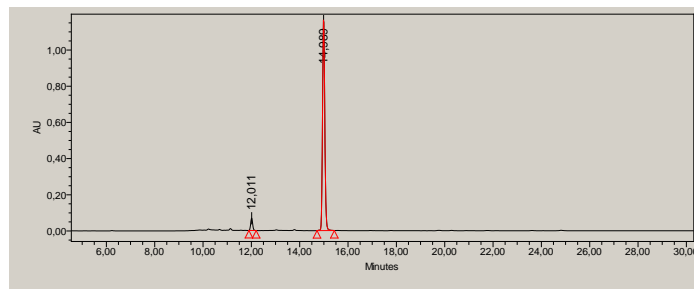


**Figure 11** HPLC chromatogram – biotransformation of 5-methoxyflavone in the culture of *P. chermesinum 113* (R$_t$=14,989 5-methoxyflavone, R$_t$=12,011 5,4'-dimethoxyflavone)

The only strain that performed demethylation of 5-methoxyflavone (**3**) was *A. niger SBP* (Fig 7, 12). In biotransformation with this species in the experiment with the substrate added 24 h the after the inoculation, biotransformation product **1** was observed after 4 days (5,4%), whereas for substrate addition at the time of the inoculation the product appeared after 8 days (7,5%). In both cases the yield of demethylation was low and the content of products in post-reaction medium was about 8,5% on the eleventh day of biotransformation.
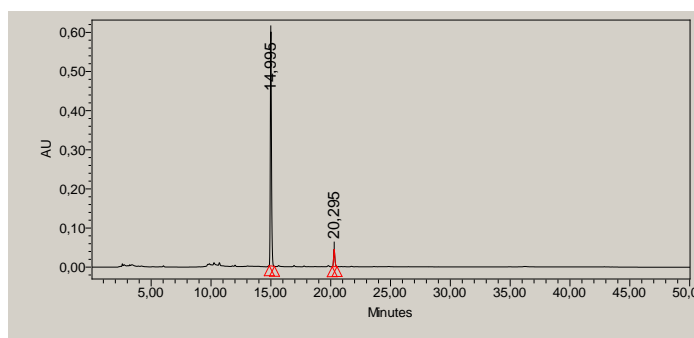


**Figure 12** HPLC chromatogram – biotransformation of 5-methoxyflavone in the culture of *A. niger SBP* (R$_t$=14,995 5-methoxyflavone, R$_t$=20,295 5-hydroxyflavone)

5,4'-Dimethoxyflavone (**4**) for spectral analysis was isolated in 68% yield (102 mg) after a 9-day biotransformation of **3** catalysed by *A. niger MB*. In the ^{1}H NMR of **4** the singlet of C-5 methoxy protons is shifted to δ=3,90 ppm, compared to δ=3,87 ppm for the substrate. An additional signal of the C-4' methoxy group protons appears at δ=3,30 ppm. The multiplet of C-3', C-4'and C-5' protons of ring B disappears, whereas a new doublet of doublets is observed at δ =7,02 ppm, integrating for two protons and attributed to C-3' and C-5'protons (J$_{3',2'}$= 8,9 Hz and J$_{3',5'}$=2,1 Hz). The presence of a methoxy group at C-4' in rig B is additionally confirmed by the ^{13}C NMR spectrum. The C-4' signal is moved from δ=132,9 ppm for substrate **3** to δ=162,1 ppm for product **4**.

The detailed qualitative study provided important information about the course of biotransformation of 5-hydroxy- and 5-methoxyflavone. They revealed that the addition of 5-hydroxyflavone to the cultivation mixtures of the strains *A. niger MB*, *A. niger KB* and *P. chermesinum* at the time of inoculation of the cultivation medium with the microorganisms resulted in activation of their enzymatic systems – the biotransformation was more efficient and the product was formed at higher rate. For 5-methoxyflavone the situation was opposite – the biotransformation was faster and more efficient when the substrate was added 24 h after the inoculation. In the culture of *A. niger SBP*, despite the fact that it is not 5,4'-dimethoxyflavone, but 5-hydroxyflavone that is the product of this biotransformation, it is better for the reaction rate and efficiency to add the substrate 24 h after the inoculation.

An important factor in the transformations of methoxyflavones is the position of the methoxy group in ring A. In biotransfomations with the help of *A. niger (MB, KB, SBP)* and *P. chermesinum 113* the course of the reaction was completely different for flavones with methoxyl groups at C-6 or C-7 in ring A (**Kostrzewa-Susłow et al., 2012**). Unlike in the case of 5-methoxyflavone (**3**), there were hydroxy- and dihydroxyflavones that were the products of these biotransformations. The fastest and the most efficient transformation was observed for 6-methoxyflavone (**Kostrzewa-Susłow et al., 2012**). In the first step of biotransformation of 6-methoxy- and 7-methoxyflavone demethylation in ring A occurred, followed by hydroxylation at C-4'. The strains of *A. niger (MB, KB)* and *P. chermesinum 113* did not perform demethylation of 5-methoxyflavone (**3**). Introduction of the additional methoxyl group at C-4' proceeded probably via O-methylation of the hydroxyl group, which had been earlier introduced at C-4'. O-Methylation of flavonoids modifies chemical reactivity of phenolic hydroxyl groups and increases their lipophilicity. This may be very important with respect to achieving optimal hydrophilic-lipophilic properties of newly formed flavones. Biological activity of compounds to a great degree depends on their hydrophilic-

lipophilic properties, which are responsible for transport across cell membranes and for their affinity to the active site. Therefore, optimal hydrophilic-lipophilic properties are important for designing new compounds of the desired activity, which might serve as potential drugs. The products obtained from biotransformation of 5-hydroxyflavone (**1**) and 5-methoxyflavone (**3**) – 5,4'-dihydroxyflavone (**2**) and 5,4'-dimethoxyflavone (**4**), respectively, confirm the model of flavonoid-enzyme interaction proposed by Ibrahim and Abul-Hajj, who suggest that the oxygen atom  belonging to ether, carbonyl or hydroxyl groups binds to hydrophilic site on the enzyme surface, which enables hydroxylation of the respective carbon atom in ring B to occur (**Ibrahim and Abul-Hajj, 1990**). The biotransformation products with hydroxyl and methoxyl groups at C-4' confirm the regiospecificity of the reactions catalyzed by enzymatic systems of the strains *A. niger (MB, KB)* and *P. chermesinum 113*.

**Antioxidant properties**

In order to determine the impact of different functional groups and their positions in a molecule on antioxidant activity of flavones, the substrates and the obtained biotransformation products were tested for antioxidant properties, using the spectrophotometric 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging method (**Kostrzewa-Susłow *et al.*, 2010**). Comparison of $IC_{50}$ values of the substrates and the products revealed that hydroxylation at C-4' of 5-hydroxyflavone (**1**) resulted in a small increase in antioxidant properties (lower $IC_{50}$ value), compared to the substrate. 5,4'-Dimethoxyflavone (**4**), the product of biotransformation of 5-methoxyflavone (**3**), has lower antioxidant activity (higher $IC_{50}$ value) (Table 1).

**Table 1** $IC_{50}$ values of the flavonoid substrates and the biotransformation products

| Substrate | Product | $IC_{50}^{a}$ (± SD) [µM] |
|---|---|---|
| 5-Hydroxyflavone (**1**) | | 9.78 (± 0.02) |
| | 5,4'-Dihydroxyflavone (**2**) | 9.30 (± 0.03) |
| 5-Methoxyflavone (**3**) | | 9.99 (± 0.02) |
| | 5-Hydroxyflavone (**1**) | 9.78 (± 0.02) |
| | 5,4'-Dimethoxyflavone (**4**) | 10.97 (± 0.03) |

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

**UV absorption**

For the substrates and the biotransformation products UV absorption spectra were measured and the influence of newly introduced by microbial transformations functional groups on shifts of absorption maxima was checked, as well as the changes in molar extinction coefficients of substrates and products were determined. The additional hydroxyl group at C-4' introduced to 5-hydroxyflavone (**1**) resulted in changes of absorption maxima in **2** towards shorter wavelength (hypsochromic shift). Molar extinction coefficient of 5,4'-dihydroxyflavone (**2**) for the first band slightly increased compared to the substrate (**1**) (Table 2). In the case of product **4** the additional methoxy group at C-4' resulted in shifts of absorption maxima towards longer wavelength compared to **3** (bathochromic effect). A small increase of molar extinction coefficient for the second band was observed (Table 2).

**Table 2** UV absorption of the flavonoid substrates and the biotransformation products

| Compound | 1st band | | 2nd band | | 3rd band | |
|---|---|---|---|---|---|---|
| | $\lambda_{max}$ [nm] | log ε | $\lambda_{max}$ [nm] | log ε | $\lambda_{max}$ [nm] | log ε |
| 5-Hydroxyflavone (**1**) | 226 | 4,59 | 270 | 4,66 | 336 | 4,17 |
| 5,4'-Dihydroxyflavone (**2**) | 219 | 4,65 | 268 | 4,65 | 327 | 3,76 |
| 5-Methoxyflavone (**3**) | 228 | 4,51 | 264 | 4,52 | 325 | 4,19 |
| 5,4'-Dimethoxyflavone (**4**) | 230 | 4,34 | 267 | 4,93 | 331 | 3,72 |

**CONCLUSION**

1. The strains: *A. niger MB*, *A. niger KB* and *P. chermesinum 113* transform 5-hydroxyflavone to 5,4'-dihydroxyflavone. The process is more efficient and faster when the substrate is added to the cultivation medium at the time of the inoculation.
2. The above-mentioned species transform 5-methoxyflavone to 5,4'-dimethoxyflavone. The strain of *A. niger SBP* performs transformation of 5-methoxyflavone to 5-hydroxyflavone. The most profitable with respect to yield and the reaction rate is addition of the substrate to the cultivation medium 24 h after the inoculation.
3. 5,4'-Dihydroxyflavone has higher antioxidant properties than the starting substrate. 5-Hydroxyflavone and its dihydroxy derivative have stronger antioxidant activity than 5-methoxyflavone and the product of its biotransformation – 5,4'-dimethoxyflavone.

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