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CAMOMILE AUTOFERMENTATION IN POLYETHYLENE GLYCOL/DEXTRAN TWO-PHASE SYSTEM

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The objective of this study was the investigation of the extractive bioconversion of apigenin-7-O-β-glucoside in camomile ligulate flowers into apigenin by autofermentation in polyethylene glycol 6000 / dextran 200000 two-phase system. In 22.5% polyethylene glycol / 14% dextran aqueous two-phase system the obtained yield of apigenin in the top phase was 96.5%. In the presence of plant material that partiotioned to the interphase, the yield of apigenin in the top phase was 3.5 times higher in comparison to the model system.

KEYWORDS: Camomile; autofermentation; apigenin, apigenin-7-O-β-glucoside; aqueous two-phase system

INTRODUCTION

Aqueous two-phase systems (ATPSs) are ideal media in which clarification, concentration, and partial purification of different materials can be integrated in one step. Moreover, this method can be made highly selective and can be easily scaled up, thus allowing wider biotechnological applications. An aqueous two-phase system is an aqueous, liquid–liquid, biphasic system which can be obtained by mixing aqueous solutions of two polymers. Since these phase components are inert towards biological materials, these can therefore be employed for partitioning of biomolecules, and cell organelles and whole cells as well. The basis of partitioning depends upon surface properties of the particles and molecules, which include size, charge, and hydrophobicity. Moreover, the most characteristic feature of a two-phase system is that the water content in it is as high as 85–99%, which when complemented with suitable buffers and salts results in providing a suitable milieu for biological materials (1). Besides partitioning and purification,

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two-phase systems have also been used for extractive bioconversions, in which integration of bioconversion and product removal is achieved, with the possibility of repeated use of its components. Such systems have been proven to be a convenient medium for bioconversation of pharmacologically active compounds (2,3).

Apigenin, 4',5,7-trihydroxyflavone, is a flavonoid found in parsley, artichoke, basil and other plants. This flavonoid is a major constituent of chamomile, which is recognized for its antiphlogistic, antispasmodic, and antibacterial effects. Also, apigenin exhibits a distinct anxiolytic activity without evidencing sedation or muscle relaxant effects at doses similar to those used for classical benzodiazepines without the anticonvulsant action (4). Apigenin can be metabolically activated to produce toxic prooxidant and phenoxyl radicals. Pure apigenin is used primarily in research as a protein kinase inhibitor that may suppress tumor promotion and that has anti-proliferating effects on human breast cancer cells and inhibitory actions on MAP kinase (5) . Moreover, studies showed the topical use of apigenin reduced UV-induced skin tumoregenesis (6). Like the most flavones, apigenin found in plants is usually bound to sugars as glycosides or in its ester form with tannin acids. Pharmacological activity of apigenin-glucoside is much lower than that of aglycone apigenin. The conversation of apigenin-*7-O-β*-glucoside is possible by autofermentation (7).

The aim of this study was to investigate the possibility of extractive bioconversion of apigenin-glucoside to apigenin by autofermentation of plant material in the aqueous two-phase system. Conditions concerning concentrations of the phase-forming components - polyethylene glycol and dextran, which enable highest possible separation of apigenin from plant material by their partitioning into opposite phases, were previously established in model systems.

EXPERIMENTAL

Plant Material

Camomile ligulate flower: tetraploid camomile produced by the Institute for Hops, Broomcorn and Medicinal Herbs, Bački Petrovac (2001).

Chemicals

Standard samples of apigenin used in the present work were purchased from Sigma-Aldrich Chemie GmbH. Dextran 20000 ($\overline{M}_w$15000–20000) (DEX) from *Leuconostoc* spp, (Sigma-Aldrich Production GmbH, Switzerland) and polyethylene glycol 6000 ($\overline{M}_w$ 6000) (PEG) from Merck (Germany) were used. All other chemicals were of analytical reagent grade.

Sample Preparation

Autofermentation of camomile ligulate flowers: Camomile ligulate flowers (10 g) were moistened with 0.1 mol/dm^3 Na-acetate buffer pH 5.5 (50 cm^3) and autofermented at 37°C for 72 hours. After that, the sample was dried at 40°C.

Autofermentated camomile ligulate flowers were extracted with 70% ethanol (5 g of sample and 250 cm^3 of solvent) using ultrasound (40 min). After filtration, extract (100 cm^3) was dried by solvent evaporation under vacuum. The dry extract was dissolved in 8 cm^3 70% ethanol (Extract A).

Preparation of model aqueous two-phase system

The phase systems for model system experiments were prepared by weighing the required PEG and DEX quantities and their dissolving in acetate buffer (0.1 mol/dm^3 Na-acetate buffer pH 5.5), and then extract A (0.1cm^3) was added and mixed thoroughly. The total mass of the two-phase system was 5 g. Model ATPS was left to equilibrated at room temperature for 12 hours, then the upper phase was carefully collected with a syringe.

Extraction of autofermented camomile

To previously established ATPS, autofermented camomile ligulate flowers (250 mg) were added. Total mass of ATPS was 50 g. Extraction was performed at ambient temperature for 24 hours with constant mixing. The two phases were allowed to separate before sampling and then the upper phase was collected and analyzed.

Extractive autofermentation of camomile

To previously established ATPS, camomile ligulate flowers (250 mg), previously moistened with acetate buffer, were added. Total mass of ATPS was 50 g. Extractive bio-conversion was performed at 37^{0}C for 72 hours with constant stirring. The two phases were allowed to separate before sampling and then the upper phase was collected and analyzed.

Phase sample preparation for TLC and HPLC:

Samples of the top phases (2 cm^3) were added to the solvent (70% ethanol, 6 cm^3). Ethanol was used to stop further bioconversion and as a preservative. Solution was dried by solvent evaporation at 35^{0}C. Dried sample was then dissolved with 70% ethanol (12 cm^3) and mixed thoroughly, using Vortex, for 15 min. Apigenin was dissolved in ethanol while PEG, insoluble in alcohol, precipitated. Sample was centrifuged (3000 rpm) for 10 minutes and the supernatant was collected and analyzed by HPLC method.

Qualitative and quantitative analyses

HPLC: Agilent Technologies 1100 HPLC system, equipped with a binary pump, UV-Diode Array detector, autosampler, Chem station software was used. Chromatographic conditions were: temperature 25°C thermostated, column Zorbax, SB-C18 (4.6 x 150 mm, 5 μm), guard column Zorbax, SB-C18 (4.6 x 12.5 mm, 5 μm). Elution profile was isocratic: acetonitrile/H_2O (3/7, v/v) with addition of acetic acid (1% of total volume). Injection volume was 0.01 cm^3, flow rate 1.0 cm^3/min, and stop time 40 min. Detection and quantification were conducted at UV 340 nm. Peak purity and identity were checked by comparison of the UV spectra (DAD-detector) and co-chromatography (TLC, HPLC) with reference substance (apigenin and apigenin-7-O-β-glucoside) and the literature data (8). Quantification of apigenin and apigenin-glycoside was performed by HPLC method using a single point calibration with apigenin as an external standard (8).

Partition parameters

The yield of apigenin in the top phase was defined as:

$$Y_T(\%) = \frac{C_T V_T}{C_o V_o} 100 \qquad [1]$$

where: C_T and C_o are the concentrations of apigenin in the top phase and in the extract loaded to ATPS, respectively; V_T and V_o are volumes of the top and the extract loaded to ATPS, respectively.

RESULTS AND DISCUSSION

Previous study showed that PEG 6000/ DEX 20000 two-phase system is a convenient medium for the extractive enzyme conversion of apigenin-glucoside to apigenin by β-galactosidase (9). Apigenin and apigenin-7-O-β-glucoside had affinity to the top and the bottom phase, respectively, while the enzyme partitioned to the bottom phase, which opened the possibility of its recycling. Even though the possible enzyme reuse decreases the cost of bioconversion, still the application of enzyme might be considered as an expensive technology. So, the idea was to take advantage of the presence of β-glucosidase in the plant material and to run autofermentation of camomile in an aqueous two-phase system.

In the first set of experiments, selection of two-phase system having different concentrations of its constituents was performed (Table 1). The concentration of apigenin in the top phase was determined by HPLC and yield of apigenin in the top phase was calculated from its quantity in the extract from autofermented camomile that was loaded in ATPS.

Table 1. Apigenin concentration and yield in the top phase of ATPSs having different polymer concentrations

ATPS	PEG (%, w/w) / DEX (%, w/w)	C_T (mg/ml)	Y_T (%)
A	7.5 / 15.5	0.024	10.7
B	10.0 / 5.0	0.013	15.0
C	22.5 /14.0	0.019	27.5

After partitioning in the two-phase systems the results of analysis revealed that the highest concentration of apigenin in the top phase of system was achieved in system A. Although the composition of ATPS in this system was the most appropriate for the apigenin to be partitioned to the top phase, the highest yield was achieved in the system C: 22.5% PEG / 14% DEX, mainly because of the higher top phase volume (Fig. 1).

Hence, in the next experiments the system 22.5% PEG / 14% DEX was chosen to perform the extractive biotransformation of apigenin-7-O-β-glucoside, from camomile ligulate flowers into apigenin by autofermentation. At the same time, the extraction of apigenin from camomile ligulate flowers, previously autofermented, was investigated in order to compare these two processes. Yield of apigenin in the top phase was calculated considering the amounts of both apigenin and apigenin-glucoside present in the plant material, determined by HPLC.

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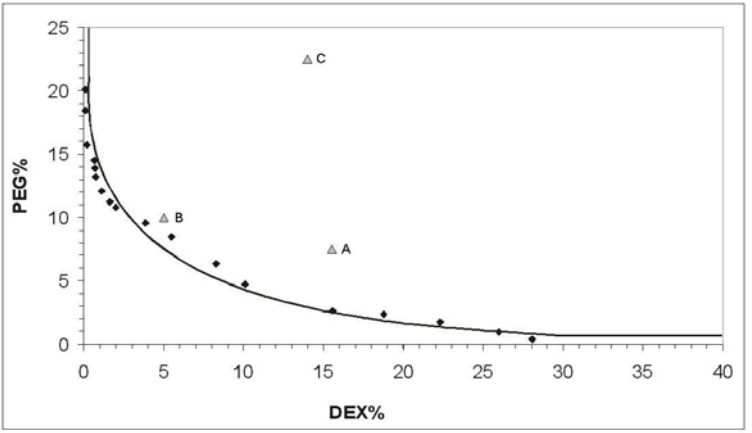

Figure1. Phase diagram of polyethylene glycol 6000 / dextran 20000 aqueous two-phase system

The bioconversion of camomile ligulate flowers by autofermentation under these conditions was successful and it resulted in apigenin yield in the top phase of 96.5% (Table 2). Extraction of previously autofermented camomile ligulate flowers resulted in a yield of 97.3%. In both systems, plant material was partitioned between two phases which opens the possibility for its removal from the system, and adding fresh material. Moreover, the yield in the top phase of apigenin was higher than in the model ATPS, proving that the presence of plant material favoured partitioning of apigenin towards the top phase. The obtained results showed that bioconversion of apigenin-glucoside into apigenin by autofermentation and *in situ* apigenin extraction in ATPS from plant material can be integrated in one unit operation.

Table 2. Apigenin concentration and yield in the top phase of 22.5% PEG / 14.0% DEX two-phase system after (1) extraction of previously autofermented camomile and (2) extractive autofermenation of camomile

Process		C_T (mg/ml)	Y_T (%)
1	Extraction of autofermented camomile	0.165	97.3
2	Extractive autofermentation of camomile	0.146	96.5

CONCLUSION

Polyethylene glycol 6000 / dextran 20000 two-phase system appeared to be a convenient medium for the bioconversion of apigenin-7-O-β-glucoside contained in camomile flower into apigenin by autofermentation and subsequent apigenin extraction into the top phase. At the same time, plant material is partitioned between the phases, allowing its easy removal. Moreover, successful extractive biocenversion would be a more cost effec-

tive procedure because of the process integration. This opens a lot of possibilities such as production of apigenin from camomile flower by autofermentation using ATPS and obtaining the product - the top phase that contains apigenin and PEG, which could be used in pharmaceutical industry for production of pills and creams.

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АУТОФЕРМЕНТАЦИЈА КАМИЛИЦЕ У ПОЛИЕТИЛЕН ГЛИКОЛ / ДЕКСТРАН ДВОФАЗНОМ СИСТЕМУ

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У раду је испитана екстрактивна биоконверзија апигенин-7-О-β-глукозида из латица камилице у апигенин, аутоферментацијом у воденом двофазном систему полиетилен гликол 6000 / декстран 20000. У воденом двофазном систему 22,5% PEG / 14% DEX постигнути принос апигенина у горњој фази је био 96,5%. С биљним материјалом у систему, који се расподелио између фаза, принос апигенина у горњој фази је био 3,5 пута већи него у модел систему.

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