ŻYWNOŚĆ. Nauka. Technologia. Jakość, 2015, 3 (100), 74 – 83

DOI: 10.15193/zntj/2015/100/041

ANNA GRYGIER, MAŁGORZATA MAJCHER, KAMILA MYSZKA

ANALYSIS OF THE ABILITY TO FORM 2-PHENYLETHYL ALCOHOL BY G*ALACTOMYCES GEOTRICHUM* MK017

S t r e s z c z e n i e

The aim of the study was to evaluate the ability of *Galactomyces geotrichum* MK017 for the biosynthesis of 2-phenylethyl alcohol and optimization of the culture medium composition in order to increase the yield of the product. The culture of *Galactomyces geotrichum* MK017 strain was carried out in the laboratory scale. For isolation of volatiles Solid Phase Microextraction (SPME) has been used. The identification and quantification of aroma compounds produced by examined microflora was determined by gas chromatography and mass spectrometry (GC/MS) system. The results showed that the tested mould *Galactomyces geotrichum* MK017 shows the potential for the production of 2-phenylethyl alcohol, but also for the synthesis of phenylacetaldehyde, phenylacetic and phenyllactic acids. For the optimization of the 2-phenylethyl alcohol production yield four different medium composition have been tested. The bioprocess of aroma compound production by tested microorganism was the most efficient on the medium composed of sucrose (80 g/l) and L-phenylalanine (21 g/l) and pH value of 5.0. Using this composition in a batch culture of 770 ml volume the highest concentration of 2-phenylethyl alcohol has been obtained – 6 mg/l. At the same time amount of phenylacetaldehyde, phenylacetic acid and phenyllactic acidhas reached 2.2 mg/l, 10.66 mg/l and 32.3 mg/l respectively.

Słowa kluczowe: *Galactomyces geotrichum*, 2-phenylethyl alcohol, GC/MS, SPME

Introduction

In recent years there has been an increase worldwide demand for aroma compounds. Their presence and diversity enhance the sensory attractiveness of a product, while simultaneously raising its price. For this reason natural aromas obtained from plant tissue extracts are frequently replaced with cheaper aroma compounds produced

Mgr inż. A. Grygier, dr inż. M. Majcher, Instytut Technologii Żywności Pochodzenia Roślinnego, Wydz. Nauk o Żywności i Żywieniu, Uniwersytet Przyrodniczy w Poznaniu, ul. Wojska Polskiego 31, 60-624 Poznań, dr inż. K. Myszka, Katedra Biotechnologii i Mikrobiologii Żywności, Wydz. Nauk o Żywności i Żywieniu, Uniwersytet Przyrodniczy w Poznaniu, ul. Wojska Polskiego 48, 60-627 Poznań.
Kontakt: agrygier@up.poznan.pl

by chemical synthesis. At the same time, the manufacturing process of flavors using chemical methods leads to the formation of many noxious waste products [3].

At present public attention has been increasingly focused on the composition of purchased products and consumer awareness of harmfulness of certain components has been growing. For this reason possibly the most natural products, sold at affordable prices are in greatest demand. In the case of production of aroma compounds an alternative is provided by the application of methods based on biotechnology [1]. Although these processes are connected with the need for adequate selection and optimization of strains capable of production aroma compounds at the genotype and phenotype levels, a process competitive in relation to conventional technologies may be developed thanks to the appropriate selection of medium composition and physical parameters of culture.

Eukaryotic microorganisms are valued producers of aroma compounds. The process of microbiological synthesis of these substances is relatively well described in yeasts. However, available literature provided limited information evaluating the above mentioned capacity in non-pathogenic moulds, common in the natural environment. Rapid growth of moulds and their relatively low growth requirements seem to guarantee high efficiency of synthesis in the case of aroma compounds.

2-phenylethyl alcoholis a fragrance compound with flowery/honey odor note, one of the most widely use din the food industry. Natural 2-phenylethyl alcohol obtained in the course of biotransformation may be a valuable alternative for consumers. The main goal of this studies was analysis of the ability to produce 2-phenylethyl alcohol by a mould*Galactomycesgeotrichum*MK017 and optimization of the culture medium composition in order to increase the yield of the product.

Materials and methods

Analyses were conducted on a strain *Galactomycesgeotrichum*MK017, coming from the collection of the Fats and Flavors Group, the Faculty of Food Science and Nutrition, the Poznań University of Life Sciences. The tested strain was stored on solid medium with chloramphenicol (BTL, Łódź, Poland) at 4 ºC. Solvents, such as ethyl acetate and hexane; the reference aroma compounds: 2-phenylethyl alcohol, phenylacetaldehyde, phenylacetic acid, phenyllactic acid and [$^2H_6$]-phenol were purchased from Sigma-Aldrich (Poznań, Poland). The purity of solvents and reference standards was no lower than 99 and 97 %, respectively.

For the medium optimization, every vial was inoculated with *Galactomyces geotrichum* mould. The inoculum was retrieved from a culture of microorganisms usinga sterile smear loop. For each vial containing medium one tube with *Galactomyces geotrichum* MK017 moulds was used. Tested microorganisms were inoculated in the 40 ml vials containing 20 ml of different media and cultured at 30 ºC for 18 days with

aeration. Four variants of culture media, pre-selected in terms of environment optimization for the synthesis of 2-phenylethyl alcohol, were tested. The composition of the media was presented in Tab. 1.

Table 1. The composition of the media 1 - 4 used for the culture of *Galactomyces geotrichum* MK017 to produce 2-phenylethyl alcohol

Tabela 1. Skład pożywek 1 - 4 wykorzystanych do produkcji 2-fenyloetanolu w hodowli *Galactomyces geotrichum* MK017

Composition of the media [g/l] Skład pożywek [g/l]	Medium 1 Pożywka 1	Medium 2 Pożywka 2	Medium 3 Pożywka 3	Medium 4 Pożywka 4
Glucose / Glukoza	10	-	10	-
Sucrose / Sacharoza	-	60	-	120
L-phenylalanine / L-fenyloalanina	-	7	10	10
Yeast extract / Ekstrakt drożdżowy	3	0,17	-	5
Soy peptone / Peptone sojowy	5	-	-	-
Maltose extract / Ekstrakt maltozowy	3	-	-	-
Na_2HPO_4 x $2H_2O$	-	22,8	-	-
Citric acid / Kwas cytrynowy	-	10,3	-	-
$MgCl_2$	-	0,5	-	-
KH_2PO_4	-	-	1	7,5
Urea / mocznik	-	-	1	-
$MgSO_4$	-	-	0,3	-
$CaCl_2$	-	-	0,2	-
K_2HPO_4	-	-	-	9,6
$MgSO_4x7H_2O$	-	-	-	0,5
Distilled water / Woda desytlowana	1	1	1	1

The concentration of 2-phenylethyl alcohol was estimated using Solid Phase Microextraction (SPME) with gas chromatography and mass spectrometry analysis. For the compound isolation Carboxene/PDMS fiber (Supelco, Bellefonte, USA) was exposed in the vial for 30 min at 30 ºC. Before SPME extraction 100 µl of internal standard (IS) of [$^2H_6$]-phenol has been added reaching the concentration of 2.5 mg/l. Next the efficiency of synthesis was assessed using gas chromatography (GC) and mass spectrometry (MS) performed on an Agilent Technologies 7890A GC coupled to an Agilent Technologies 5975 VL MSD (Agilent, Santa Clara, USA). The chromatograph was equipped with DB-5 MS columns (30 m × 250 µm × 0.25 µm, Agilent, Santa Clara USA). The initial oven temperature was 40 ºC, followed by an increase in temperature by 8 ºC/min to 180 ºC and next by 35 ºC/min and it was maintained for 3 min.

The temperature at the injection port was 260 ºC. Separation was performed using helium at a flow rate of 1.5 ml/min. Compounds extracted using SPME fiber were analyzed in the splitless mode (1 min). In the course of separation the temperature of the transfer line was 300 ºC. The mass spectrometer worked in the scan mode. Ions were collected between 30 and 330 m/z. The ion source temperature was 220 ºC. Volatiles were identified by a comparison of mass spectra with the NIST library and respective standards.

Quantification of odorants has been done by stable isotope dilution assay with an IS [$^2H_6$]-phenol using characteristic ions presented in Tab. 2. The obtained results were adjusted by the detector response factor between labelled and unlabelled compound presented in Tab. 2.

Table 2. Characteristic ions and response factors (Rf) used for the quantitation of analyzed volatiles
Tabela 2. Charakterystyczne jony oraz współczynnik odpowiedzi wykorzystane do ilościowej analizy związków lotnych

No. / Nr	Compound Związek	Ions Jony	Rf
1.	phenylacetaldehyde aldehyd fenylooctowy	120	3,1
2.	2-phenylethyl alcohol 2-fenyloetanol	122	3,2
3.	phenylacetic acid kwasfenylooctowy	136	2,4
4.	phenyllactic acid kwasfenylomlekowy	148	8,7
5.	IS – phenol [$^2H_6$] IS – fenol [$^2H_6$]	99	-

In the second stage of the study the culture was scaled-up to bioreactors with max. capacity of 1 l (Duran, Wertheim, Germany). Total culture volume was 770 ml and included 700 ml of optimized medium and 70 ml of the inoculate. The culture was run on the media composed of: 80 g/l of sucrose (Chempur, Piekary Śląskie, Poland) and 21g/l of L-phenylalanine (Sigma-Aldrich, Poznań, Poland) and pH value of 5.0, which has been optimized and selected in the previous stage of the study and described as medium 2. The experiments were run for 120 h. It was a batch culture. Samples for analyses were collected at fixed intervals throughout the culture period and identification and quantitation of volatiles was determined. Aeration rate in the bioreactor was 0.52 l/dm^3/min at 30 ºC. Extraction was performed using 5 ml of ethyl acetate added to 20 ml of the culture medium. Additionally, for the purpose of quantitative analyses of

determined compounds an internal standard was introduced, i.e. [$^2H_6$] phenol at 100 µl. Next the solution was mixed for 30 min and then centrifuged for 7 min at 1000 rpm. The amount of 1 µl was collected from the supernatant and it was injected into the injection port of the gas chromatograph. The results were statistically analyzed using the standard deviation.

Results and discussion

In the first stage of the study the optimal growth environment was selected for the synthesis of 2-phenylethyl alcohol by *Galactomyces geotrichum* MK017. For the optimization purposes four different mediums previously used for the production of 2-phenylethyl alcohol by yeast were evaluated. Media compositions were selected on the basis of published papers by Białecka-Florjańczyk [1], Etschmann [4], Mei [7] and Wang [9] with small modifications. Preparing medium 1 bacto-peptone was changed into soy peptone and 3 g/l maltose extract was added. In medium 3 dose of L-phenylalanine was decreased into 10 g/l. The biggest difference in between selected mediums was in the course of the carbon and nitrogen source, the quantity of L-phenylalanine and a mineral composition. For many microorganisms saccharides are the basic energy compounds and the source of a carbon. In turn, L-phenylalanine is the most important component in the production of 2-phenylethyl alcohol, since in the Ehrlich pathway [2] it is the precursor of the process. Minerals are required for growth and proliferation. Phosphorus is used to build cell membranes and certain enzymes. Magnesium, another component of the medium, activates numerous enzymes and stabilizes nucleic acids [8].

Comparing chosen media. The results have shown that the most efficient synthesis of 2-phenylethyl alcohol, based on the peak area, was obtained when using medium 2 containing 60 g/l of sucrose and 7 g/l of L-phenylalanine (Fig. 1). Based on those results medium 2 was selected for further optimization. The experiments were conducted changing the composition of medium constituents, i.e. sucrose at 60 g/l, 80 g/l and 100 g/l, L-phenylalanine at 7 g/l, 14 g/l and 21 g/l, and pH at the value of 4, 5, 6 and 7.

Varying the amount of sucrose added into the medium no significant effect on the efficiency of 2-phenylethyl alcohol synthesis by a mould *Galactomyces geotrichum* MK017 was recorded – Tab. 3. Therefore the average sucrose content in the culture medium of 80 g/l was chosen for further experiments. In the experiment with a variable content of L-phenylalanine in the medium the highest level of 2-phenylethyl alcohol was recorded at the substrate concentration of 21 g/l. The next experiment assessed the effect of culture medium pH on the capacity of a mould *Galactomyces geotrichum* MK017 to synthesize 2-phenylethyl alcohol. Results have shown that the highest

amount of obtained 2-phenylethyl alcohol by the tested microorganisms was observed at pH 5.0 – Tab. 3.

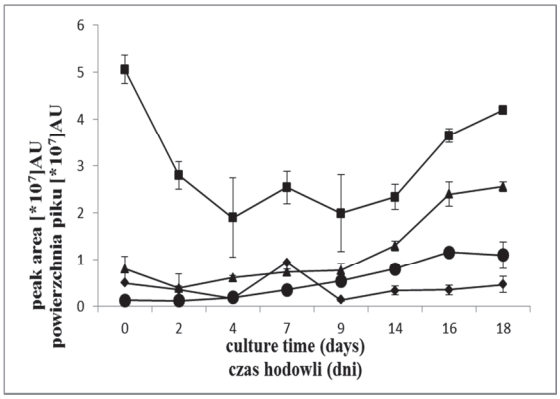

Fig. 1. Changes in 2-phenylethyl alcohol content, based on peak areas, during *Galactomycesgeotrichum*MK017 culture on different media 1 – 4. The composition of media can be found in Table 1. Medium 1 – diamonds; medium 2 – squares; medium 3 – triangles; medium 4 – circles

Rys. 1. Zmiany zawartości 2-fenyloetanolu w hodowli *Galactomyces geotrichum* MK017 na pożywkach 1 - 4, na podstawie powierzchni pików. Skład podłoży hodowlanych przedstawiono w tab. 1. Pożywka 1 – romby; pożywka 2 – kwadraty; pożywka 3 – trójkąty; pożywka 4 – koła

Table 3. Changes in 2-phenylethyl alcohol content, based on peak areas, during culture of *Galactomyces geotrichum* MK017 on media with a different sucrose and L-phenylalanine content and with a different pH (n = 2)

Tabela 3. Zmiany zawartości 2-fenyloetanolu w hodowli *Galactomyces geotrichum* MK017 na pożywkach z różną zawartością sacharozy i L-fenyloalaniny oraz różnym pH pożywki, na podstawie powierzchni pików (n = 2)

Item Wyszczególnienie	Sucrose [g/l] Sacharoza [g/l]			L-phenylalanine [g/l] L-fenyloalanina [g/l]			pH			
	60	80	100	7	14	21	4	5	6	7
2-phenylethyl alcohol peakarea [10^6] AU Powierzchnia piku 2-fenyloetanolu [10^6] AU	3.51 ± 0.24	3.84 ± 0.57	3.87 ± 0.03	4.43 ± 1.56	5.62 ± 0.71	8.45 ± 0.51	2.11 ± 0.32	2.36 ± 0.64	1.46 ± 0.01	1.11 ± 0.01

In further experiment culture of a mould*Galactomycesgeotrichum*MK017, containing 700 ml of previously optimized medium with 80 g/l sucrose, 21 g/l L-phenylalanine, 3 g/l yeast extract, 22,8 g/l Na_2HPO_4 x $2H_2O$, 10,3 g/l citric acid, 0,5 g/l $MgCl_2$; 1 g/l distilled water was prepared. The experiment have been conducted through four days and it confirmed the capacity of selected moulds to synthesize the following compounds: phenylacetaldehyde, phenylethyl alcohol, phenylacetic acid and phenyllactic acid, which corresponds to the Ehrlich pathway [2], (Fig 2). According to described pathway, the first transformation product is phenylpyruvate, followed by phenylacetaldehyde, 2-phenylethyl alcohol, which with the participation of dehydrogenase may again convert 2-phenylethyl alcohol to phenylacetaldehyde and further to phenylacetic acid. However it does not describe further transformation into phenyllactic acid. Generally in the Ehrlich pathway amino acids are slowly assimilated by microorganisms during fermentation. Prior to the secretion of the product outside the cell the compound is converted to alcohol or acid [5].

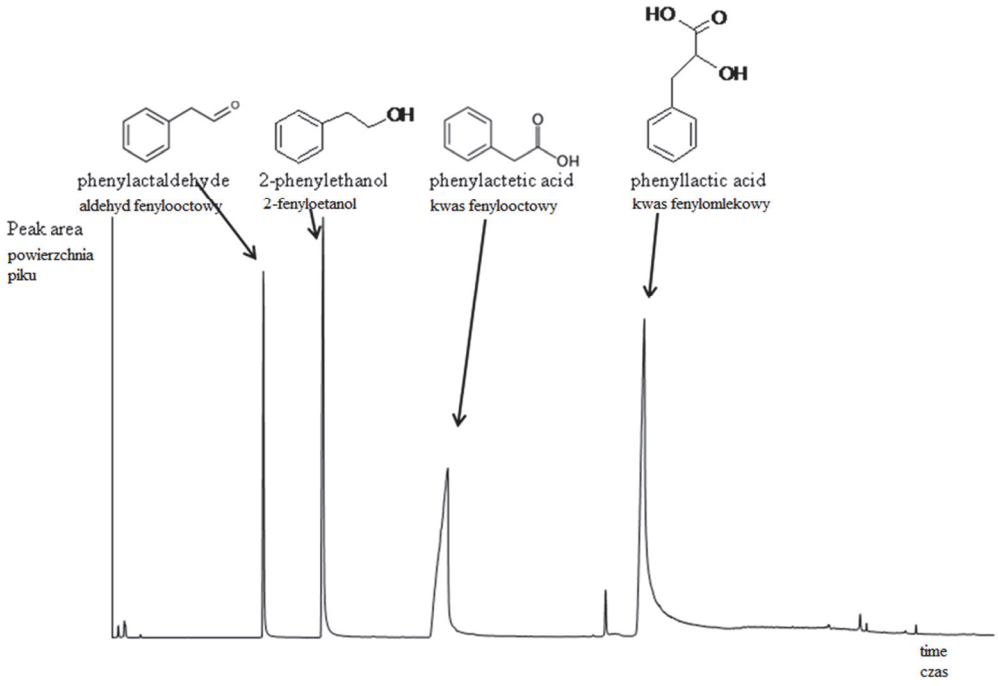

Fig. 2. Exemplary chromatogram (extracted ion 91) obtained from the extract of 4 days biomass production by *Galactomycesgeotrichum*MK017 in a 1000 ml bioreactor

Rys. 2. Przykładowy chromatogram (wybranych 91 jonów) uzyskany z ekstraktu z biomasy z 4. dnia hodowli *Galactomyces geotrichum* MK017 w 1000 ml bioreaktorze

In the presented experiment, during the four days of the bioreactor culture low concentrations of phenylacetaldehyde and 2-phenylethyl alcohol were already produced in the first hours of the trial (1.6 and 2.0 mg/l respectively). The amount of phenylacetaldehyde did not change significantly later on. On the other hand the concentration of 2-phenylethyl alcohol almost tripled after the first day up to 5.9 mg/l and remained at the same level in the course of successive days (Fig. 3).

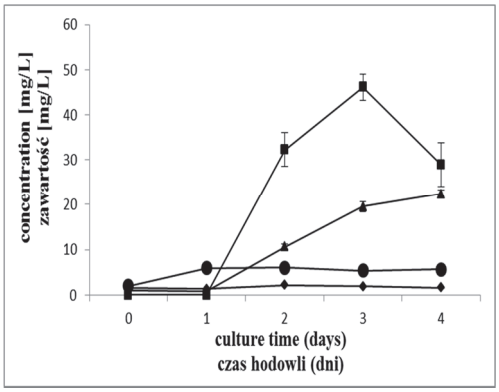

Fig. 3. Changes in concentration of 2-phenylethyl alcohol, phenylacetaldehyde, phenylacetic acid and phenyllactic acid content, during culture of *Galactomyces geotrichum* MK017 in bioreactor. Phenylacetaldehyde – diamonds; 2-phenylethyl alcohol – circles; phenylacetic acid – triangles; phenyllactic acid – squares

Rys. 3. Zmiany zawartości 2-fenyloetanolu, aldehydyfenylooctowego, kwasufenylooctowego i kwasu fenylomlekowego w hodowli *Galactomyces geotrichum* MK017 w bioreaktorze. Aldehyd fenylooctowy – romby; 2-fenyloetanol – koła; kwas fenylooctowy – trójkąty; kwas fenylomlekowy – kwadraty

At the same time observation of the phenylacetic and phenyllactic acids has revealed very small amounts of phenylacetic acid during the first day of the trial. However, starting from the second day of the culture its quantity considerably increased up to 22.4 mg/l at the fourth day of biosynthesis. In compare, during the first hours of the culture phenyllactic acid was not detected, while at day 2 it was found at a concentration of 7.6 mg/l which stayed at similar level till the completion of the trial. The presence of the following compounds: 2-phenylethyl alcohol, phenylacetaldehyde, phenylacetic and phenyllactic acids, confirms the transformation of L-phenylalanine by the *Galactomyces geotrichum* MK017 in the Ehrlich pathway (Fig. 3).

In the course of the experiment the highest concentration of 2-phenylethyl alcohol was 6 mg/l culture medium. At the same time this sample contained 2.2 mg/l phenylacetaldehyde, 10.66 mg/l phenylacetic acid and 32.3 mg/l phenyllactic acid, which gives mainly acids (84 %). In contrast, Hazelwood et al. [5] showed that in the case of *Sac-*

charomyces cerevisiae grown in a batch culture with L-phenylalanine a mixture of 90 % of alcohol (2-phenylethyl alcohol) and only 10 % of acids has been obtained. Higher results of the biosynthesis of 2-phenylethyl alcohol were acknowledged by Lomascolo et al. [6]. In this case the moulds *Aspergillusniger* grown in the medium consisting of 60 g/l glucose and 6 g/l L-phenylalanine, production of 2-phenylethyl alcohol was obtained at the 1375 mg/l. In this study the optimal substrate for the synthesis of 2-phenylethyl alcohol by a mould *Galactomyces geotrichum* MK017 was provided by the medium composed of sucrose, L-phenylalanine, yeast extract, citric acid, $MgCl_2$ and Na_2HPO_4 x $2H_2O$. A very similar composition of the medium was used by Etschmann et al. [4]. In that study the researchers assessed the capacity to synthesize 2-phenylethyl alcohol by 14 microbial strains. The best among the tested strains was *Kluyveromyces marxianus* CBS 600, which produced 1.8 g/l 2-phenylethyl alcohol and the culture medium contained sucrose from molasses, L-phenylalanine, Na_2HPO_4 x $2H_2O$, citric acid, $MgSO_4$ $7H_2O$ and Bacto Yeast Nitrogen Base without amino acids and ammonium sulfate [4]. The amount of 2-phenylethyl alcohol obtained by Etschmann was much higher than the one achieved in this research, however the authors did not mentioned any other compounds such as phenylacetic acid or phenyllactic acid, which were synthesized by *Galactomyces geotrichum* MK017.

Summary

The bioprocess of aroma compound production by tested microorganism was most efficient on the medium composed of sucrose (80 g/l) and L-phenylalanine (21 g/l) and pH value of 5.0. Additionally the tested mould*Galactomycesgeotrichum*MK017 shows the potential for the production of 2-phenylethyl alcohol, but also for the synthesis of phenylacetic and phenyllactic acids.

This research has been financed by the Polish National Science Center project: N N312 157 134

Literatura

[1] Białecka-Florjańczyk E., Krzyczkowska J., Stolarzewicz L., Kapturowska A.: Synthesis of 2-phenylethyl acetate in the presence of *Yarrowia lipolytica* KKP 379 biomass. J Mol. Catal B-Enzym, 2012, **74**, 241-245.

[2] Ehrlich F.**:** Über die Bedingungen der Fuselölbildung und überihren Zusammenhangmitdem Eiweißaufbau der Hefe. Ber Dtsch. Chem. Ges, 1907, **40**, 1027-1047.

[3] Etschmann M.M.W., Bluemke W., Sell D., Schrader J.: Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biot, 2002, **59**, 1-8.

[4] Etschmann M.M.W, Sell D., Schrader J.: Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. Biotechnol. Lett, 2003, **25,** 531-536.

[5] Hazelwood L.A., Daran J.M, Maris A., Pronk J.T., Dickinson J.R.: The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on *Saccharomyces cerevisiae.* Metabolism. Appl Environ Microb, 2008,**74,** 2259-2266.
[6] Lomascolo A., Lesage-Meessen L., Haon M., Navarro D., Antona C., Faulds C., Marcel A.: Evaluation of the potential of *Aspergillus niger* species for the bioconversion of L-phenylalanine into 2-phenylethanol. World J. Microb. Biot, 2001, **17**, 99-102.
[7] Mei J., Min H., Lu Z.: Enhanced biotransformation of L-phenylalanine to 2-phenylethanol using an in situ product adsorption technique. Process Biochemistry, 2009, **44,** 886-890.
[8] Raczyńska-Cabaj A., Lipińska E., Sobczak E., Stosio A.: Wpływ jonów magnezu na rozwój bakteryjnej mikroflory występującej w prasowanych drożdżach piekarskich. Acta Sci. Pol. Technol. Aliment., 2004, **3,** 111-118.
[9] Wang H., Dong Q., Meng C., Shi X., Guo Y.: A continuous and adsorptive bioprocess for efficient production of the natural aroma chemical 2-phenylethanol with yeast. Enzyme Microbiol. Technol, 2011, **48**, 404-407.

ANALIZA ZDOLNOŚCI PRODUKCJI 2-FENYLOETANOLU PRZEZ *GALACTOMYCES GEOTRICHUM* MK017

S u m m a r y

Celem pracy było określenie zdolności pleśni *Galactomyces geotrichum* MK017 do biosyntezy 2-fenyloetanolu oraz optymalizacja składników podłoża produkcyjnego w celu zwiększenia ilości uzyskanego produktu. Hodowlę pleśni *Galactomyces geotrichum* MK017 przeprowadzono w skali laboratoryjnej. Do identyfikacji związków lotnych zastosowano metodę mikroekstrakcji do fazy stałej (SPME). Identyfikacji oraz oceny ilościowej uzyskanego związku zapachowego dokonano z wykorzystaniem chromatografii gazowej sprzężonej ze spektrometrią mas (GC/MS). Uzyskane wyniki potwierdziły zdolność badanej pleśni *Galactomyces geotrichum* MK017 do produkcji 2-fenyloetanolu oraz do syntezy aldehydu fenylooctowego, kwasu fenylooctowego oraz kwasu fenylomlekowego. W celu optymalizacji produkcji 2-fenyloetanolu przetestowano cztery różne podłoża hodowlane. Największą produkcję 2-fenyloetanolu otrzymano na pożywce zawierającej sacharozę (80 g/l) i L-fenyloalaninę (21 g/l) oraz przy pH pożywki na poziomie 5.0. Uwzględniając powyższe zawartości poszczególnych składników pożywki, uzyskano w hodowli okresowej w 770 ml pożywki, najwyższą koncentrację 2-fenyloetanolu – 6 mg/l. Równocześnie w próbie otrzymano aldehyd fenylooctowy, kwas fenylooctowy oraz kwas fenylomlekowy odpowiednio w ilościach: 2.2 mg/l, 10.66 mg/l i 32.3 mg/l.

Key words: *Galactomyces geotrichum*, 2-fenyloetanol, GC/MS, SPME