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Mycelial culture of *Xerocomus chrysenteron* and its metabolites

STANISŁAW KOHLMÜNZER, JANINA WĘGIEL, and JAN GRZYBEK

Department of Pharmaceutical Botany, Collegium Medicum Jagiellonian University
Medyczna 9, PL-30-688 Kraków, Poland

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Subject to analysis was mycelial culture of *Xerocomus chrysenteron* and its metabolites which night be of pharmaceutical on toxicological interest. Several groups of metabolites were analysed: steroids, fatty acids, indole desiratives, aminoacid, sugars – using chromatographic analytical methods. Amony others soma interesting idolic substances were detected.

Key words: mycelial culture, *Xerocomus chrysetneron*, metabolites.

INTRODUCTION

Mycelial cultures as well as carpophores of macromycetes may be a good source of bioactive substances. *Xerocomus chrysenteron* (Bull.: Fr.) Quél., a mycorrhizal fungus (*Boletaceae*) is known as an edible species, relatively common in Poland, occasionally being sold on the local markets.

Only few informations on chemical components of its carpophores were published. The pigments have been investigated by Steglich et al. (1968) and xerocomic acid lactate has been found as colouring phenolic substance in carpophores.

In our laboratory attempts have been undertaken to establish myceliel culture of this fungal species *in vitro*. The next aim was to examine the cultured mycelium on the occurrence of its metabolites possibly of pharmacological or toxicological interest. Special attention was given to indole deviratires, which night be essential bioactive compounds in the species under investigation.

MATERIAL AND METHODS

Carpophores of the fungal species under investigation were collected in leaved forest in southern region of Cracow in September 1997. Specimens are deposited in the Dept. of Pharmaceutical Botany Jagiellonian University in Cracow.

The mycelial culture was performed in two subsequent steps: 1) culture on solid medium, 2) culture in liquid medium.

To establish the first step of culture several nutrient media were tested. The best growth was achieved on nutrient medium with composition showed below:

Culture medium (pH 5.5)

Glucose	10 g	$MnSO_4 \times xH_2O$ (0.5%)	1.5 mL^3
Malt extract	5–10 g	$ZnSO_4 \times x7H_2O$ (0.3%)	1.5 mL^3
NH_4Cl	0.5 g	$CaCl_2 \times 6H_2O$ (2%)	5 mL^3
L-asparagine	1 g	Caseine hydrolysate	200 mg
KH_2PO_4	0.5 g	Yeast extract	30 mg
$MgSO_4 \times 7H_2O$	0.5 g	Adenine	12 mg
$FeCl_3$ (1%)	10 drops	Aq. dest.	ad 1000 mL^3

As inoculum explants of hymenial part of fresh fruiting bodies were taken (2–5 mm in size), sterilized by use of 70% ethanol, then aseptically placed on the at 120°C sterilized culture medium, solidified by addition of 3% of agar in Petri dishes. They were kept in dark at the temperature of 25° ± 1°C.

The mycelium growth was observed just after 3 days and lasted for about 21 days. After microscopical examination of the formed hyphae the mycelium was collected from the surface, freeze dried and weighed. Some parts of the fresh mycelium were subcultured into Erlenmayer flasks or fermentors to initiate liquid shake culture.

In Petri dishes mycelium of *X. chrysenteron* grows on the surface as cream-beige radially elongated clusters with good marked centre (Fig. 1). The liquid culture was performed in two ways: the shake aerated culture in 500 ml Erlemayer's flasks on the rotary shaker (Fig. 2) and in fermentor (6 l) aerated with sterile air.

Both experiments were performed at the pH 5.5 ± 0.2 liquid medium. The optimal time of culture was about 21 days, then the growth stopped and the mycelium was removed from the culture medium by filtration.

The yield of the obtained and freeze dried mycelium was 1.57–2.43 g/l respectively. To obtain a sufficient quantity of biomass for metabolite analysis the liquid culture was repeated 3 – fold and finally 19.3 g of dry mass have been obtained.

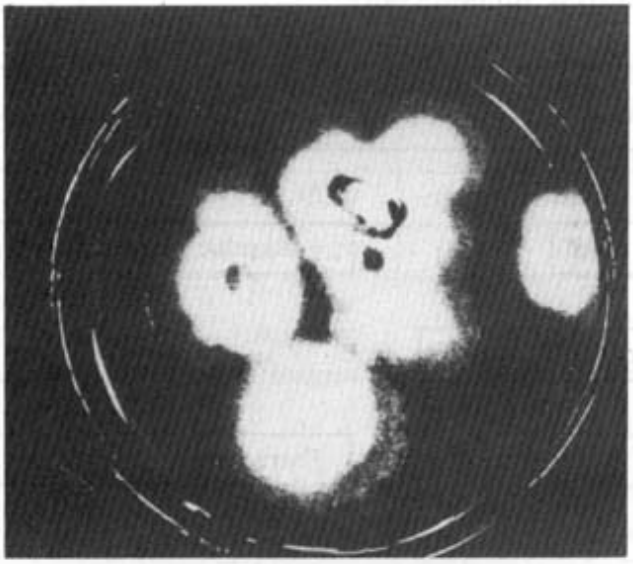

Fig. 1. Static mycelial culture of *Xerocomus chrysenteron* on agar medium

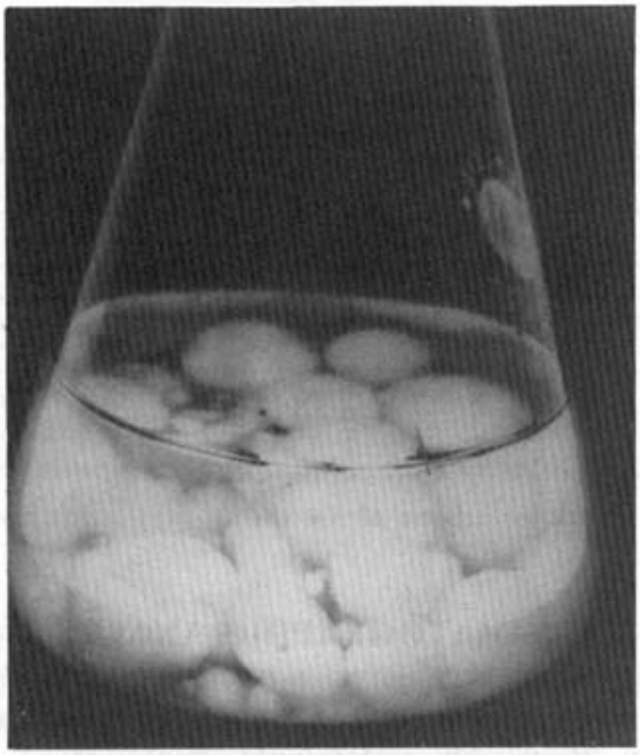

Fig. 2. Shake mycelial culture of *Xerocomus chrysenteron* on liquid medium

ANALYSIS OF MYCELIUM METABOLITES

Three kinds of extraction procedures were used in order to detect lipophilic, less lipophilic and hydrophilic metabolites: petrol ether extraction, methanol extraction, water extraction (Fig. 3).

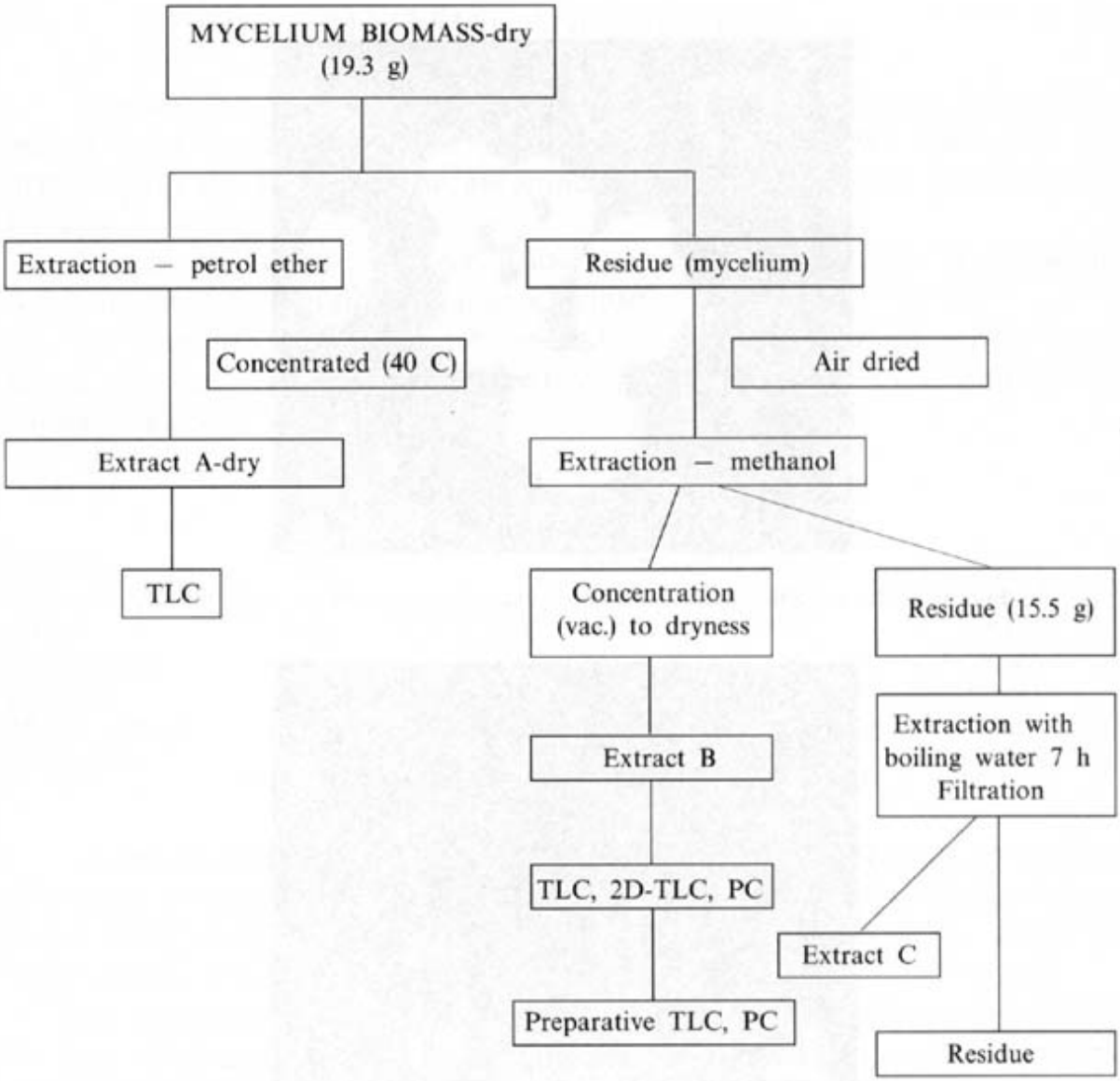

Fig. 3. Extraction scheme of *Xerocomus chrysenteron* mycelium

CHROMATOGRAPHIC ANALYSIS

E x t r a c t A – was analyzed by use of thin layer chromatography (TLC) for the occurrence of steroids and fatty acids.

S t e r o i d s

TLC was performed on DC Alufolien 60 Merck Platten in following developing systems: I. Petrol ether – ethyl acetate – benzene 7:2:1 (v/v/v), II. Acetone – benzene 1:1 (v/v), III. Chloroform – acetone 9:1 (v/v).

The steroid spots (brown) were visualized by spraying with 50% solution of O-phosphoric acid with following heating at 105°C by 5′ (Tab. 1).

Table 1
TLC of steroids (Rf values)

Substance	I		II		III	
	S	Sx	S	Sx	S	Sx
Ergosterol	0.59	0.59	0.73	0.73	0.59	0.59
Ergosterol superoxide	0.24	9.24	0.83	0.83	0.49	0.49

S – Standard substance
Sx – Extract under investigation
I, II, III – Developing systems (see text), reagent: 50% phosphoric acid (105°C)

Table 2
TLC of fatty acids (Rf values)

Substance	I		IV		V	
	S	Sx	S	Sx	S	Sx
Oleic acid	0.41	0.41	0.36	0.36	0.92	0.92
Myristic acid	0.47	0.47	0.49	0.49	–	–
Linolic acid	0.17	0.17	0.23	0.23	0.82	0.82
Stearic acid	0.39	–	–	–	–	–
Lauric acid	0.92	0.92	0.67	0.67	0.76	0.76
Arachidonic acid	0.33	0.33	0.3	0.3	–	–
Palmitic acid	0.09	0.09	0.94	0.94	0.36	0.36

S – Standard substance
Sx – Extract under investigation
I, IV, V – Developing systems (see text), reagent: 55% sol. of $K_2Cr_2O_7$ in H_2SO_4 (110°C)

Fatty acids

TLC with developing systems was used: IV. Cyclohexan – chloroform 25:75 (v/v), V. Petrol ether – diethylether – acetic acid glac. 7:2:1 (v/v/v).
System I (as above)
The fatty acids spots (dark blue) were visualized by spraying with $K_2Cr_2O_7$ 55% solution in H_2SO_4 followed by heating at 110°C by 25′ (Tab. 2).

Extract B – was analyzed on the occurrence of more hydrophilic metabolites.
Special attention was given to detect indole derivatives which might be of pharmacological or toxicological interest.

In this aim TLC were used in the same way as above with following developing systems: VI. n-butanol-acetic acid-H_2O 12:3:5 (v/v/v), VII. Izopropanol--ammonia – H_2O 8:1:1 (v/v/v).

In addition two-dimensional (2D-TLC) technique was used.

As selective reagent for visualization of indole derivatives a 10% solution of p.-dimethylamino-benzaldehyd (DAB) in conc. HCl (1.19) diluted 1:4 with aceton was used. It gave very characteristic coloured spots (rosa--blue-violet) (W ę g i e l and K o h l m ü n z e r 1998). Standard reference substances were used for comparison.

For preparative purposes the UV active zones were removed from the plates and extracted exhaustively with pure methanol, evaporated (red. press.) to establish their chemical properties by spectroscopic methods (UV, H^1NMR, EIMS spectra).

The results are discussed on the basis of obtained data in the next chapter.

In addition routine TLC, 2D-TLC, PC methods were used to detect amino-acids and free sugars in this extract (Tab. 3 and 4).

E x t r a c t C – was elaborated by special procedure to isolate poly-saccharide fractions, as shown (Fig. 4).

T w o d i f f e r e n t p o l y s a c c h a r i d e fractions A and B have been obtained with the yield: A 202 mg (1.05%) and B 181 mg (0.94%) respectively.

After acid hydrolysis with 2N H_2SO_4 $2^h/120^\circ$ both fractions showed to be heteroglucans composed of glucose, galactose and mannose with a small peptidic part. This was supported by TLC, 2D-TLC and PC analysis.

T a b l e 3

2D – TLC of aminoacids (Rf values)

Substance	VIII		IX	
	S	Sx	S	Sx
Lysine	0.11	0.11	0.23	0.23
Alanine	0.17	0.17	0.2	0.2
Leucine	0.45	0.45	0.45	0.45
Isoleucine	0.4	0.4	0.34	0.34
Serine	0.12	0.12	0.18	0.18

S – Standard substance

Sx – Extract under investigation

VIII – Developing system (first direction) n-butanol – acetic acid – H_2O 4:1:5 v/v/v

IX – Developing system (second direction) n-propanol – H_2O 7:3 v/v, reagent: 1% ninhydryne sol. Ethanol

Table 4
PC of sugars (Rf values; Whatman Nr 3 paper)

Substance	IX		X	
	S	Sx	S	Sx
Glucose	0.15	0.15	0.3	0.3
Mannose	0.31	0.31	0.17	0.17
Galactose	0.25	0.25	0.28	0.28
Fucose	0.41	0.4	0.47	0.48

S – Standard substance; Sx – Extract under investigation
IX – Developing system; n-propanol – H_2O 7:3 v/v
X – Developing system; n-propanol – ethyl acetate – H_2O 7:2:1 v/v/v, reagent: aniline oxalate 1% sol. (105°C)

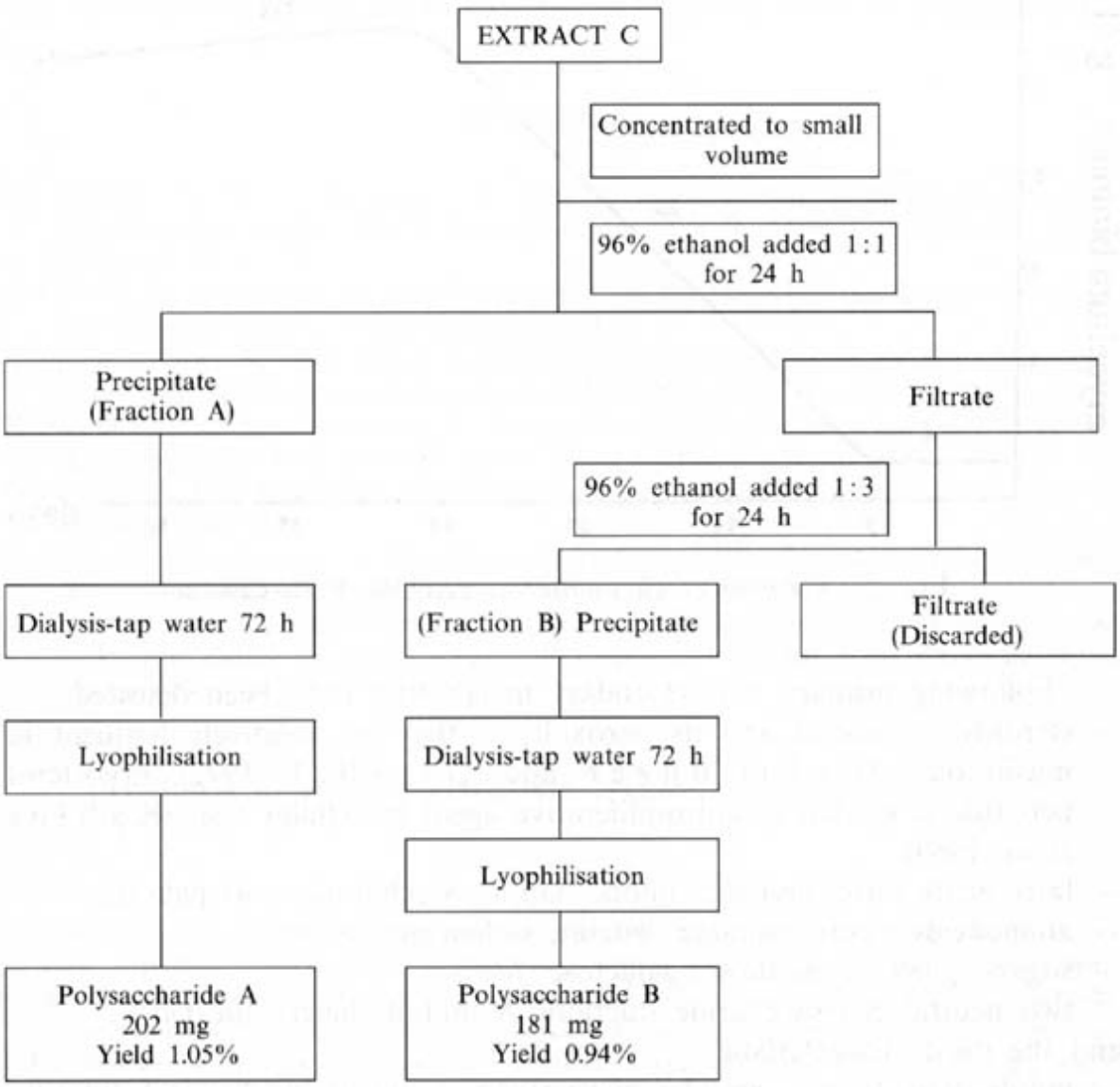

Fig. 4. Extraction scheme of polysaccharide fractions from *Xerocomus chrysenteron* mycelium

RESULTS AND DISSCUSION

The difficulties of growing mycelial cultures of mycorrhizal higher fungi are well known. In this investigation these difficulties have been overcome. The best results so far as mycelial growth is concerned were obtained with nutrient medium acc. to O d d o u x (1960) with some modifications. The yield of dry biomass obtained in liquid culture was 1.5–2.4 g/l respectively.

Three subsequent growth phases have been observed: 1) initial – lasting about 5 days; 2) logarythmic growth – lasting 5–16 days; 3) stationary – after about 20 days of cultivation (Fig. 5).

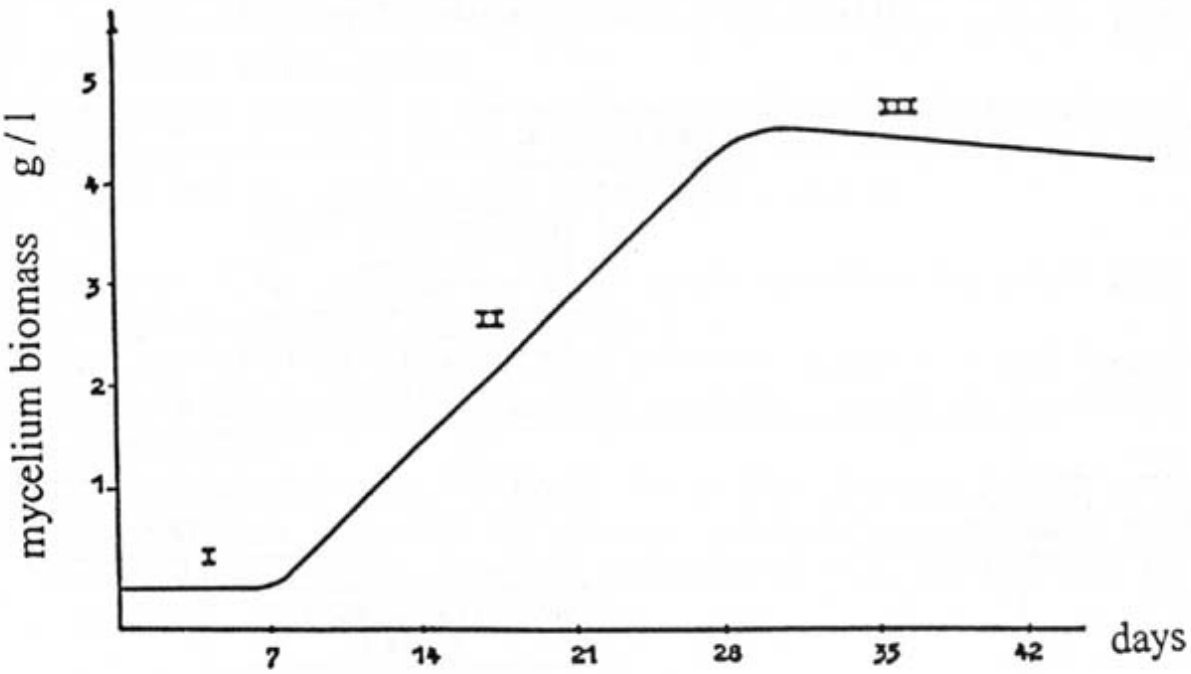

Fig. 5. *Xerocomus chrysenteron* growth of mycelium

Following primary and secondary metabolites have been detected:

- steroids: ergosterol and its peroxide – they are relatively frequent in mushrooms (K o h l m ü n z e r and G r z y b e k 1972). Ergosterol peroxide is known as antiproliferative agent in cellular test (K a h l o s et al. 1989);
- fatty acids oleic, myristic, linolic, lauric, arachidonic and palmitic;
- aminoacids: lysine, alanine, leucine, isoleucine, serine;
- sugars: glucose, mannose, galactose, fucose;
- two neutral polysaccharide fractions A and B -heteroglucans;

and the most characteristic:

- indole derivatives – basides of tryptophan and its biochemical degragation product kynurenine, two other substances with characteristic UV

absorption in the region of 220–290 nm and fragmentation patterns in EIMS spectra have been detected. They were not identical with any known fungal metabolite. Their exact chemical structure is now under investigation.

The polysaccharide fraction A isolated with good yield (1.05%) is interesting for testing of its biological activity, which is attributed to some fungal glucans as e.g. tylopilan isolated in this laboratory (K o h l m ü n z e r et al. 1980; G r z y b e k and K o h l m ü n z e r 1983).

The characteristic pigment of *Xerocomus chrysenteron* – xerocomic acid has not been detected in mycelial culture under investigation.

This is a first report on some mycelial culture metabolites of *Xerocomus chrysenteron.*

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Hodowla mycelialna *Xerocomus chrysenteron* i jej metabolity

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

Zaprowadzono hodowlę mycelialną grzyba mikoryzowego Xerocomus chrysenteron na pożywce stałej i płynnej, stosując podłoże hodowlane według Oddoux z własnymi modyfikacjami. Osiągnięto dobry wzrost mycelium w ciągu 3 tygodni 1.57–2.43 g suchej biomasy na 1 litr pożywki.

Zbadano metabolity produkowne przez mycelium metodami chromatograficznymi i spektralnymi. Wśród metabolitów podstawowych znaleziono aminokwasy i cukry, natomiast wśród metabolitów wtórnych ergosterol i jego nadtlenek, kwasy tłuszczowe, związki indolowe pochodne tryptofanu. Nie wykryto występującego w owocnikach tego gatunku kwasu kserokomowego.