ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN—POLONIA
VOL. XIX, 16 SECTIO C 1964

Z Katedry Biochemii Wydziału Biologii i Nauk o Ziemi UMCS
Kierownik: doc. dr Jerzy Trojanowski

Elżbieta DERNAŁOWICZ i Jerzy TROJANOWSKI

The Exoenzyme Laccase in *Pholiota mutabilis* (Schff. ex Fr.) Quel.

Egzoenzym lakaza u *Pholiota mutabilis* (Schff. ex Fr.) Quel.

Экзоэнзим лаказа у *Pholiota mutabilis* (Schff. ex Fr.) Quel.

INTRODUCTION

The fungi of the *Hymenomycetes* type are able to decompose lignin in dead plant tissues in natural conditions, thus participating in the decomposition of wood litter, straw, and dead leaves in the soil (humification). The mycelium of *Hymenomycetes* excretes exoenzyme laccase (C. E. — 1. 10. 3. 2. — oxydase p-diphenol: 0_2) into the medium on which it grows (3, 9).

According to Fähraeus (2) the substrates of laccase produced by *Polyporus versicolor* may be:

1 — Diphenols (orto and para),
2 — monophenols,
3 — m-diphenols,
4 — monoamines.

The role of laccase in the decomposition process is not yet fully known. According to some authors (5, 6, 7, 11) laccase oxidizes products of lignin decomposition (basic building guaiacyl-propanol stones) thus making it responsible for the metabolism of the fungus. Various species and strains of *Hymenomycetes* differ considerably with regard to their ability to produce laccase and their sensitivity to inducers, responsible for the formation of the enzyme.

The present paper deals with *Pholiota mutabilis*, the mycelium of which decomposes lignin in wood litter in natural conditions (10). In liquid mediums of this fungus the authors observed the formation of laccase depending on the time of culture, experimental conditions, and the inducers added.

The results were compared with the data obtained by other workers with other species of *Hymenomycetes*.

MATERIAL AND METHODS

A. CULTURES OF THE FUNGUS

Pure culture of the fungus *Pholiota mutabilis* was obtained from the Department of Plant Phytopathology IBL in Warsaw. The mycelium was kept on plum agar stock and inoculated to a fresh agar every month. The continuous capacity of the mycelium to produce laccase (oxidase p-diphenol: O_2) was assessed by the Bevendamm test. The following liquid medium, similar to that described by L i n d e b e r g (8), but free from yeast extract, was used in the formation of exoenzyme laccase by the mycelium:

> glucose 10 g, asparagine 1 g, $MgSO_4 \cdot 7H_20$ — 0.5 g, KH_2PO_4 — 0.45 g, $Na_2HPO_4 \cdot 12H_2O$ — 0.47 g, Ca — 20 ppm, Mn — 2.7 ppm, Fe, Zn, Cu — 1 ppm, thiamine — 50 µg, distilled water up to 1000 ml.

The mycelium from plum agar stock was inoculated into medial nutrient the composition of which was as described above. After five days the culture of the mycelium was crushed by shaking with glass beads, and used to start preparatory cultures. A part of the mycelial suspension was taken from the medial culture under sterile conditions, and inoculated into a 500 ml nutrient solution as above, in Fernbach flasks. Preparatory cultures were grown mainly as stationary. Apart from them a variant of preparatory culture was grown which was constantly shaken by a rotary shaker.

The activity of laccase (p-diphenoloxidase) secreted by the mycelium into the nutrient was determined by the standard Warburg technique. The main vessel contained 2.3 ml of the grown nutrient and the side arm 0.5 ml of the substrate solution at a concentration of 16 mg/ml. The control measurement was made with a medium in which the enzyme had been inactivated by boiling. The optimal pH value determined experimentally was 5.5.

B. THE EFFECTS OF CONDITIONS UNDER WHICH THE CULTURE WAS GROWN ON THE FORMATION OF LACCASE

When growing *Pholiota mutabilis* the addition of yeast extract in an amount ranging from 70 to 1400 mg of dry weight per 100 ml of nutrient, has no effect on the formation of laccase either in the stationary or in the shaking culture (submersed mycelium). Therefore in all variants of the culture this growth factor was disregarded. This made it easier to observe the changes of protein concentrations in growing cultures.

Table 1

Type of inducer	Concentration in Mol/l	Oxygen uptake / hour	
		Shaking culture estimation after 12 days	Stationary culture estimation after 42 days
medium without inducer		46	60
p-cresol	0.5×10^{-4}	150	—
p-cresol	5×10^{-4}	180	30
p-cresol	10^{-3}	0	—
α-naphtol	10^{-4}	0	—
gallic acid	10^{-4}	175	50
saturated solution of resin in ethanol	0.2 ml	190	120
saturated solution of resin in ethanol	0.05 ml	150	—
p-xylidine	5×10^{-4}	40	220
p-xylidine	10^{-4}	45	220

To increase the formation of enzyme the inducers suggested by Fähraeus (1) were added to the nutrient. Their concentration is presented in Table 1. In addition the inducing activity of pine resin in ethyl alcohol was tested. After the mycelium was 4 days old the inducers were introduced as ethanol solution in the amount of 0.2 ml/500 ml of the nutrient under sterile conditions. The inducers were found to have different effects on stationary and shaking cultures. The most effective for the stationary and shaking cultures proved to be p-xylidine at a concentration of 5×10^{-4} M and resin in the amount of 0.2 ml of the saturated ethanol solution per 500 ml of the nutrient, respectively. No effect of xylidine was found in the shaking cultures. The quantitative data of this experiment are presented in Table 1.

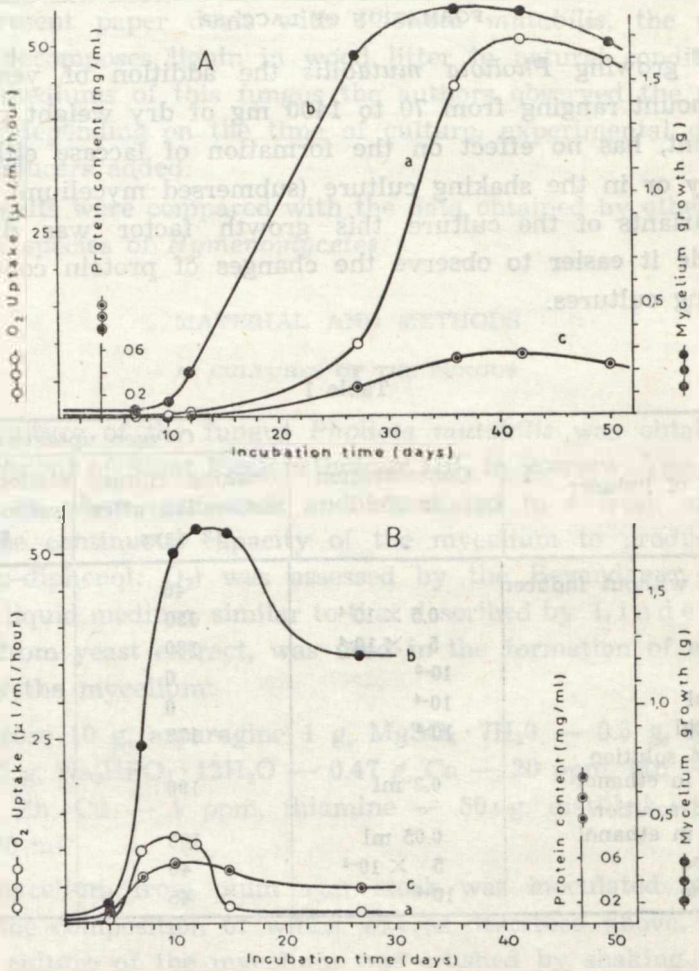

Fig. 1. The effect of conditions under which a culture is grown on the oxygen uptake — a, yield of dry weight of mycelium — b, and protein content — c; A — stationary culture, B — shaking culture

C. THE EFFECT OF THE CONDITIONS UNDER WHICH CULTURE WAS GROWN ON THE CONTENT OF PROTEIN AND FORMATION OF MYCELIUM

Determinations were made of the protein content, dry weight of the mycelium, and the activity of laccase in stationary and shaking cultures. In all these experiments p-xylidine was used as an inducer at a concentration of 5×10^{-4}, after the culture was 4 days old. The cultures were incubated at a temperature of 23°C in 6 repetitions. Dry weights of the mycelium from the various flasks were determined by drying the separated mycelium, at a temperature of 105° up to its steady

weight. The content of protein in the nutrient, after separating the mycelium, was determined by the Folin method. The activity of laccase was determined by the Warburg apparatus. The results are presented in Figs. 1A and 1B. The maximal activity of laccase in the stationary culture was found to occur after 42 days ($Qo_2 = 200$). At that time the maximal dry weight of the mycelium was 1.8 g (500 ml of the nutrient). The activity of laccase in the shaking culture proved to be maximal after 12 days ($Qq_2 = 40$). At the same time the maximal dry weight of mycelium and the maximal dry protein content were found; they were: 1.75 g/500 ml of the nutrient and 30 mg/500 ml, respectively. The results showed that the specific activity of laccase in the stationary culture was 5 times greater than that in the shaking culture, while the formation of dry weight of mycelium was almost identical. Calculated per 1 mg of dry weight of mycelium, the activity of laccase in stationary culture was 4 times greater than in the shaking culture.

D. PARTIAL PURIFICATION OF ENZYME

A 42-day-old culture was used as the starting material for the purification of the enzyme. After collecting the mycelium by filtering on a Büchner funnel the medium was chilled to 0°, and precipitated with $(NH_4)_2SO_4$ or acetone.

a. Precipitation with $(NH_4)_2SO_4$

The chilled medium was saturated with ammonium sulphate *in substantia* to 100% saturation. After adding the necessary quantity of $(NH_4)_2SO_4$ in small portions during a period of 6 hours the liquid was put in the cold for 6 to 8 hours. After that time the protein of the enzyme was precipitated. The solution was centrifuged; the sediment was dissolved in redistilled water and dialysed against distilled water in the cold. After dialysis the solution was centrifuged in order to free it of undissoluble protein and the supernatant was removed from the sediment and subjected to liophilisation.

b. The procedure of precipitation with acetone

Chilled acetone (−20°) was gradually added to the medium, the mixture being kept under 4°. For the procedure of total precipitation of the enzyme 3 volumes of acetone to 1 volume of the solution were used. The sediment precipitated with acetone was allowed to deposit for 1/2 hour. Next, the precipitate was collected by filtering on a Büchner funnel No G 4. The precipitate was immediately dissolved

Table 2

1. Qo_2 of medium	2. Qo_2 of enzyme Precipitation with acetone	2./1.	3. Qo_2 of enzyme Precipitation with $(NH_4)SO_4$	3./1.
50[1]	255	5.1	110	2.5
120[2]	600	4.7	—	—
200[3]	810	4.1	450	2.25

The oxide uptake assessed by the Warburg method in the presence of 8 mg of catechol, 2.3 ml of medium or 2.3 mg of purified preparation at 25° during 10 min. $_{1, 2, 3}$ — examples of activity values obtained in the separate cultures

Table 3. Formation of laccase by mycelium of *Pholiota mutabilis* compared with that of other species of *Hymenomycetes*

Species	Activity of laccase in liquid culture (uptake of O_2)	Maximal activity attained after n days	Most effective inducer	Effect of inducer on the increase in the laccase activity	Author
Pholiota mutabilis	50 µl/ml/hour	42	p-xylidine 5×10^{-4} M	400 %	—
Polyporus versicolor strain No. 11a	1880 µl/ml/hour	7	p-xylidine 2×10^{-4} M	2000 %	(4)
Polyporus versicolor strain No. 1	161 µl/ml/hour	10	p-xylidine 10^{-4} M	8000 %	(4)
Polyporus zonatus strain No. 1	425 µl/ml/hour	21	without inducer	—	(4)
Polyporus zonarius strain No. 1	300 µl/ml/hour	14	—	—	—
Polyporus hirsutus strain No. 1	100 µl/ml/hour	10	—	—	—
Polyporus hirsutus strain No. 1	300 µl/ml/hour	14	—	—	—

with chilled redistilled water, its volume being 25 times smaller than the volume of the medium used as starting material. Insoluble impurities were removed from the liquid. The solution obtained was precipitated once more with chilled acetone using 3 volumes of acetone against 1 volume of the solution. The sediment was centrifuged and suspended in distilled water and then subjected to dialysis in 0.005 M acetate buffer, at pH 5.5. The liquid obtained after dialysis was centrifuged in order to remove impurities, and the supernatant was liophilised.

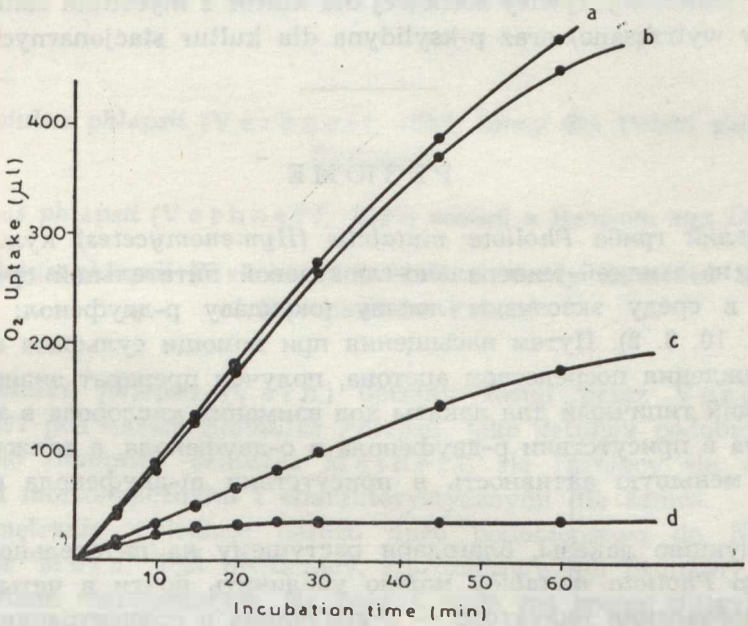

Fig. 2. The substrate specificity of partly purified laccase of a stationary culture of *Pholiota mutabilis;* the determination by the method of Warburg; 1 mg of the enzyme in 2.3 ml of buffer solution, pH 5.5 (main vessel), 8 mg of substrate in 0.5 ml of water (side arm); a — hydrochinon, b — catechol, c — resorcine, d — p-cresol

Table 2 presents the changes in the enzyme activity during the process of purification. Precipitation with ammonium sulphate yields a twofold purification, while that with acetone yields four- or fivefold purification. Figure 2 presents the process of oxidation of various substrates of phenol type with a preparation obtained through precipitation with acetone. In Table 3 the capability to form laccase of the mycelium of *Pholiota mutabilis* and that of other species of *Hymenomycetes* are compared.

STRESZCZENIE

Badano wytwarzanie enzymu lakazy przez grzyb *Pholiota mutabilis* do pożywki płynnej w zależności od czasu hodowli, zanurzenia mycelium oraz dodatku induktorów.

Zauważono, że wytwarzanie lakazy było intensywniejsze w kulturach z mycelium nie zanurzonym (kultury stacjonarne), a optymalną aktywność enzymu w tych kulturach stwierdzono po 6 tygodniach. Najskuteczniej działającym induktorem spośród pięciu badanych okazał się roztwór etanolowy żywicy sosnowej dla kultur z mycelium zanurzonym (kultury wytrząsane) oraz p-ksylidyna dla kultur stacjonarnych.

РЕЗЮМЕ

Мицелий гриба *Pholiota mutabilis (Hymenomycetes)* культивированный на жидкой минерально-глюкозовой питательной среде, выделяет в среду экзоэнзим лаказу (оксидазу р-двуфенол: O_2 — С. Е. — 1. 10. 3. 2). Путем насыщения при помощи сульфата аммония или осаждения посредством ацетона, получен препарат энзима, проявляющий типичный для лаказы ход взимания кислорода в аппарате Варбурга в присутствии р-двуфенола и о-двуфенола, а также значительно меньшую активность в присутствии m-двуфенола и монофенола.

Продукцию лаказы, благодаря растущему на питательной среде мицелию *Pholiota mutabilis* можно увеличить почти в четыре раза путем добавления индуктора — р-ксилидина в концентрации $5 \cdot 10^{-4}$ М (в стационарных культурах), либо путем добавления р-крезола в концентрации $5 \cdot 10^{-4}$ М или сосновой смолы (в встряхиваемых культурах). Максимум активности лаказы в стационарных культурах гриба установлен спустя 42 дня (Qo_2 питательной среды равняется 200); в встряхиваемых культурах максимальная активность достигнута спустя 12 дней ($Qo_2 = 40$). В такие же сроки достигала максимума сухая масса мицелия.

Papier druk. sat. III kl. 80 g | Format 70 × 100 | Druku str. 8
Annales UMCS Lublin 1964 | Lub. Zakł. Graf. Lublin, Unicka 4 | Zam. 2468. 31.VII.64
1000 + 125 egz. H-3 | Manuskrypt otrzymano 31.VII.64 | Data ukończenia 14.VIII.65