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# Investigations on the Degradation of Lignin by *Pholiota mutabilis*

## Badania nad rozkładem ligniny przez *Pholiota mutabilis*

## Исследования над разложением лигнина грибом *Pholiota mutabilis*

### 1. INTRODUCTION

The degradation of lignin in plant material remains in soil, wood litter, and peats has considerable economic importance, as it is closely connected with the biogenesis of humic substances (11, 21). The decomposition of lignin in wood by saprophytic fungi is also important from the practical point of view. The chemical composition of lignin is not yet fully known. It is probably a polymer composed of basic building stones of cumaric, coniferyl and sinapic alcohol, for which the relation and linking depend on the type of biological material available. A model diagram of the structure of coniferyl lignin can be seen in Fig. 1, according to (1). The content of the methoxyl groups in lignin may range from 10% to 25% (12).

Wood lignin is decomposed by „white rot" fungi of the *Hymenomycetes* type (9, 10). Lindeberg (18) reports that a strain of the fungus *Collybia butyracea* grown on dead leaves and pine needles causes a loss of lignin from this material of up to 77% after seven months' culture. Fähraeus (8) reported a loss of lignin amounting to 80% in beech sawdust, on which he grew the species *Polyporus abietinus, Stereum rugosum* and *Marasmius scorodonius*. Intensive decomposition of wood lignin in aspen is caused by *Polyporus paragamesus* (17). Harris (16) observed similar effects in experiments with *Marasmius peronatus* strain on beech wood.

Mikola (20) names the following species of *Basidiomycetes* as decomposing lignin in wood litter: *Clavaria ligula, Clitocybe cerussata, Clitocybe odora, Collybia*

*butyracea, Collybia dryophila, Hypholoma fasciculare, Marasmius perforans, Mycena galopas, Mycena lactea, Mycena sanguinoleta, Pholiota mutabilis, Polyporus annosus, Polyporus betulinus, Stropharia depilata.*

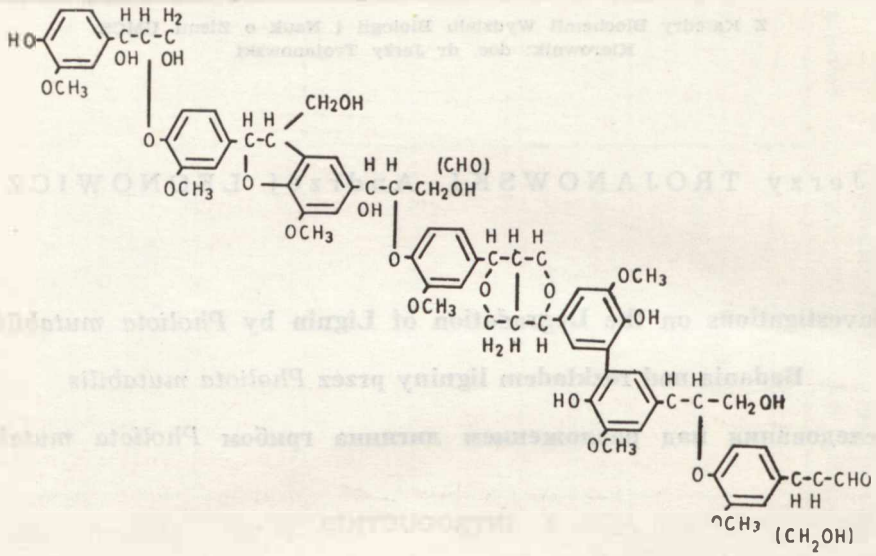

Fig. 1. Chemical structure of lignin composed of basic building stones of coniferyl alcohol according to Adler (1)

The decomposition of lignin by fungi may be considered proved. It remains an open question, however, whether lignin can be the sole source of carbon for the fungi, and the problem of the enzymatic mechanism of this decomposition remains also to be solved.

Lignin obtained by drastic industrial methods, e. g. by action of sulphuric acid on wood is used by *Polyporus* sp. with difficulty (14, 15, 17). Lignin isolated by the gentler method of Brauns (5) may play the role of the sole source of carbon for adapted mycelia of *Poria subacida* and *Polyporus abietinus* (7) and *Corriolus versicolor* (22). Brauns's lignin cannot, however, be considered the material identical to the native lignin, as it is a mixture of lignans (13). For biochemical experiments lignin isolated by Björkman's method (3, 4) is more suitable because of the gentler conditions of extraction and a great yield amounting to 30%.

In the present investigations the authors examined the decomposition of Björkman's lignin by the fungus *Pholiota mutabilis*, which is known as a decomposer of lignin (20). The aim of the research was to estimate quantitatively the loss of lignin mass and the changes in the content of the methoxyl groups in the purified preparation of lignin, being in contact with the mycelium or with the enzymes secreted by it.

## 2. EXPERIMENTS

### A. MATERIAL

The lignin was isolated from rye straw (variety „Ludowa") by Björkman's method (3, 4). Cut straw was dried at a room temperature and ground in a disc-mill to granules of average diameter 0.1 mm. The milled product was washed three times with methanol and twice with ethyl ether and dried in an exsiccator over $P_2O_5$. 6 g portions were then placed in a ball mill, and 50 ml. of toluene was added to obtain relatively thick suspension; this was then milled again for 96 hours. The product was filtered off and dried in a vacuum exsiccator. The dried product was extracted at a room temperature with dioxane containing 10% of water. The extraction was repeated 5 times, each time using 30 ml. of solvent for each 6 g of milled straw. The solvent was filtered off at G-3 and the extracts mixed. The mixed extracts were evaporated in a vacuum to smaller volume and slowly poured into a 10 times greater quantity of ethyl ether, while stirring vigorously. The precipitated yellow-brown sediment of lignin was centrifuged out, and dried in air. In order to purify the lignin powder, it was dissolved in 90% acetic acid, forming a saturated solution. Next the solution was slowly poured in small portions into a 10 times greater volume of saturated $Na_2SO_4$ solution.

We used this solution, instead of water used by Björkman, to facilitate coagulation. The precipitate of lignin thus obtained was centrifuged, washed with water several times, and dried by air. Next the lignin preparation was dissolved in dichloroethane + alcohol (I : I) and again precipitated in a 10 times greater volume of ethyl ether. The purified preparation of Björkman's lignin obtained in this way was dried in a vacuum over $P_2O_5$.

A pure culture of the fungus *Pholiota mutabilis* (Schff. ex Fr.) Quel. was received through the courtesy of doc. H. Orłoś, Department of Plant Physiopathology, I.B.L., Warsaw.

### B. FUNGUS CULTURE ON LIQUID MEDIUM

The nutrient solution was inoculated from plum agar stock cultures. The following nutrient solution was used: glucose — 5 g, asparagine — 1 g, $MgSO_4 \cdot 7H_2O$ — 0.5 g, $KH_2PO_4$ — 0.45 g, $Na_2HPO_4 \cdot 12H_20$ — 0.47 g, Ca — 20 ppm., Mn — 2.7 ppm., Fe, Zn, Cu — 1 ppm., thiamine — 50 micrograms, yeast extract — 100 mg, distilled water up to 1 000 ml. The composition of this medium is chiefly according

to Lindeberg (19), only the glucose content has been halved. The method of surface stationary cultures was applied according to Trojanowski and Dernałowicz*.

### C. THE ACTION OF THE FUNGI UPON LIGNIN

To incorporate the lignin into Lindeberg's liquid medium in sterile conditions the technique proposed by Day was used (7). 0.25 g of the preparation of Björkman's pure lignin was powdered in a mortar to fine powder and next mixed with 50 ml. of the medium as described above (in B.). The suspension was thoroughly shaken and transferred to sterilized flasks, the tops of which were then plugged with gauze. The flasks with the lignin suspension were sterilized three times at intervals of 24 hrs. in Koch's apparatus at 100°C.

Analyses carried out by Brauns's method (6) showed that the content of methoxyl groups in the lignin after sterilization in these conditions did not undergo any observable change.

The medium thus prepared with the addition of lignin was inoculated in sterile conditions from the fungus culture previously grown for 2 weeks on a liquid medium of the above mentioned composition and then incubated in a thermostat at 20°C for 0, 1, 2, 3, 4, 5, and 6 weeks, each variant in 3 replications. Simultaneously control variants without lignin were incubated and kept in identical conditions.

After the allotted time had elapsed the mycelial mat and the remaining lignin were filtered on G-4, and the sediment and filtrate were dried in air and then in the exsiccator over $P_2O_5$; next the whole was weighed. The lignin was then washed out of the filter with a 90% water solution of dioxane. The mycelium remaining on the filter after several rinsings with dioxane was again dried in a vacuum exsiccator and weighed. From the difference between the first and second weight we calculated the loss of lignin decomposed by the fungus. The solution of lignin was evaporated to small volume and poured in portions into a 10 times greater volume of ether to precipitate the lignin. The precipitate thus obtained was again dissolved in dioxane and precipitated into ether to purify it, dried over $P_2O_5$ and the content of methoxyl groups was determined according to (6). The results are set out in Tables 1 and 2, and in Fig. 2.

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* J. Trojanowski, E. Dernałowicz: Badania nad lakazą z *Pholiota mutabilis*. Typed copy. Department of Biochemistry UMCS, Lublin.

Table 1. Loss of mass of Björkman's lignin depending on the time of incubation with the mycelium of *Pholiota mutabilis*

| Time of incubation in weeks | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Dry remains of lignin mass in grams: 1st replication | 0.25 | 0.23 | 0.24 | 0.20 | 0.18 | 0.16 | 0.16 |
| Dry remains of lignin mass in grams: 2nd replication | 0.25 | 0.24 | 0.22 | 0.22 | 0.15 | 0.14 | 0.14 |
| Dry remains of lignin mass in grams: 3rd replication | 0.25 | 0.24 | 0.23 | 0.21 | 0.17 | 0.15 | 0.11 |
| Dry remains of lignin mass in grams. average | 0.25 | 0.24 | 0.23 | 0.21 | 0.17 | 0.15 | 0.14 |
| Dry remains of lignin mass, average in % | 100 | 96 | 92 | 84 | 68 | 60 | 58 |
| Mass of lignin degraded in % | 0 | 4 | 8 | 16 | 32 | 40 | 42 |

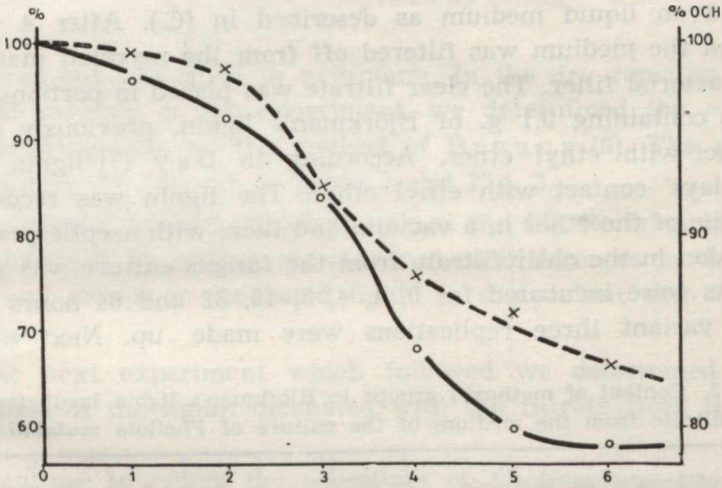


Fig. 2. Degradation of Björkman's lignin by growing mycelium of *Pholiota mutabilis* depending on the time of culture (in weeks); 1 — mass of lignin after incubation (in %), 2 — changes in the content of $OCH_3$ in the lignin left after incubation (in % in relation to the control)

In order to ensure that, besides lignin, other components of the dried mycelium do not dissolve in dioxane, we also extracted the mycelium from the control variant without lignin, using dioxane. We did not, after evaporation, observe the presence of any non-volatile substances.

Table 2. Changes in the content of methoxyl groups in Björkman's lignin depending on time of incubation with *Pholiota mutabilis*

| Time of incubation in weeks | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Content of —$OCH_3$ in % 1st repl. | 14.9 | 14.6 | 14.8 | 13.8 | 12.6 | 12.9 | 12.6 |
| Content of —$OCH_3$ in % 2nd repl. | 14.9 | 14.9 | 14.5 | 13.7 | 13.2 | 12.8 | 12.5 |
| Content of —$OCH_3$ in % 3rd repl. | 14.9 | 14.7 | 14.6 | 13.9 | 13.4 | 12.6 | 12.3 |
| Content of —$OCH_3$ in % average | 14.9 | 14.8 | 14.7 | 13.8 | 13.1 | 12.8 | 12.4 |
| Content of —$OCH_3$ in % if control value = 100% | 100 | 99.3 | 98.6 | 92.6 | 87.9 | 85.9 | 83.2 |
| Loss of —$OCH_3$ in % if control value = 100% | 0 | 0.7 | 1.3 | 7.4 | 12.1 | 14.1 | 16.8 |

### D. THE ACTION OF THE CULTURE FILTRATE ON LIGNIN

In the experiments set out below we observed enzymatic activity in the liquid medium from fungus culture. *Pholiota mutabilis* was cultivated on liquid medium as described in (C.). After 4 weeks of incubation the medium was filtered off from the mycelial mat through a Seitz bacterial filter. The clear filtrate was placed in portions of 5 ml in flasks containing 0.1 g. of Björkman's lignin, previously sterilized by contact with ethyl ether. According to Day (7) lignin is sterile after 3 days' contact with ethyl ether. The lignin was recovered by evaporation of the ether in a vacuum and then, with aseptic precautions, a suspension in the clear filtrate from the fungus culture was prepared. The flasks were incubated for 0, 1, 4, 8, 16, 32 and 64 hours at 20°C. In each variant three replications were made up. Next the lignin

Table 3. Content of methoxyl groups in Björkman's lignin incubated with filtrate from the medium of the culture of *Pholiota mutabilis*

| Time of incubation in hours | 0 | 1 | 4 | 8 | 16 | 32 | 64 |
|---|---|---|---|---|---|---|---|
| Content of —$OCH_3$ in % 1st repl. | 14.9 | 14.9 | 14.5 | 14.1 | 13.6 | 12.8 | 12 |
| Content of —$OCH_3$ in % 2nd repl. | 14.9 | 14.7 | 14.1 | 13.5 | 13.4 | 12.7 | 12.2 |
| Content of —$OCH_3$ in % 3rd repl. | 4 9 | 14.8 | 14.5 | 14.9 | 13.2 | 12.8 | 12.1 |
| average | 14.9 | 14.8 | 14.4 | 13.8 | 13.4 | 12.8 | 12.1 |
| Content of —$OCH_3$ in % if control value = 100% | 100 | 99.3 | 96.6 | 92.6 | 89.9 | 85.9 | 81.2 |
| Loss of —$OCH_3$ in % if control value = 100% | 0 | 0.7 | 3.4 | 7.4 | 10.1 | 14.1 | 18.8 |

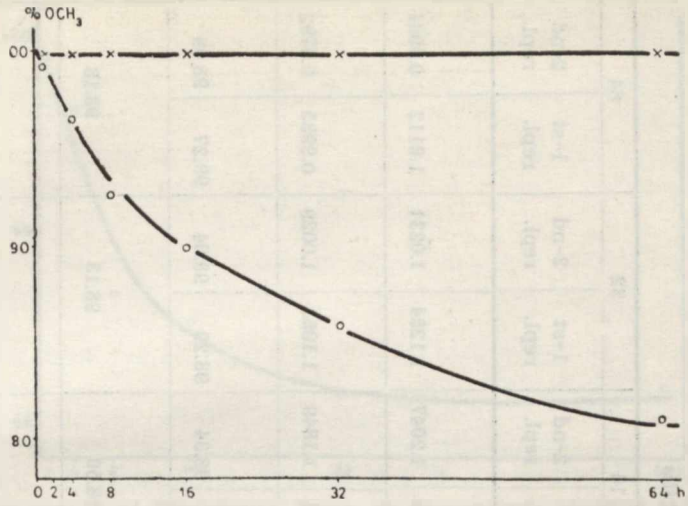


Fig. 3. Demethylation of Björkman's lignin (in % in relation to the control), by exoenzymes secreted into the liquid medium by *Pholiota mutabilis*, depending on time of incubation (h) with the filtrate of the medium; 1 — active filtrate, 2 — filtrate inactivated by boiling

was filtered out and dried in a vacuum. In the dry remains of lignin in several variants of the experiment, we determined the content of the methoxyl groups by the method of B r a u n s (6). The results of the estimation are set out in Table 3 and Fig. 3.

In the control variant, which contained the filtrate from the fungus culture inactived by boiling, we observed no differences in the content of methoxyl groups in incubated lignin in comparison with the initial preparation.

In the next experiment which followed we determined the loss of dry mass of the lignin incubated with the filtrate from the fungus culture.

The culture in which the mycelium of *Pholiota* was grown for 4 weeks, was filtered through a Seitz bacterial filter into sterilized flasks. 1 g. of Björkman's lignin was placed in each flask, after previous sterilization with ethyl ether. In each flask there were 10 ml. of filtered liquid medium from the fungus culture. The flasks were kept at 20° for 0, 1, 4, 8, 16, 32 and 64 hours. Each variant had 2 replications. After the allotted time of incubation, the remaining lignin was filtered out on G-4, next dried and weighed. The loss of lignin mass depending on the time of contact with the filtrate of the liquid medium from fungus culture is given in Table 4 and Fig. 4.

Table 4. Loss of mass of the preparation of Björkman's lignin depending on the time of incubation with filtrate from the medium of the culture of *Pholiota mutabilis*

| Time of incubation in hours | 1 | | 4 | | 8 | | 16 | | 32 | | 64 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1-st repl. | 2-and repl. | 1-st repl. | 2-nd repl. | 1-st repl. | 2-nd repl. | 1-st repl. | 2-nd repl. | 1-st repl. | 2-nd repl. | 1-st repl. | 2-nd repl. |
| Mass of lignin in grams before incubation | 1.0261 | 0.9941 | 1.0431 | 1.0521 | 1.0253 | 0.9834 | 0.9926 | 1.2047 | 1.1254 | 1.0231 | 1.0112 | 0,9984 |
| Mass of lignin in grams after incubation | 1.0232 | 0.9938 | 1.0385 | 1.0482 | 1.0107 | 0.9701 | 0.9793 | 1.1849 | 1.1053 | 1.0029 | 0.9935 | 0.9792 |
| Dry remains of non-degraded lignin in % | 89.81 | 99.99 | 99.62 | 99.62 | 98.64 | 98.65 | 98.66 | 98.34 | 98.22 | 98.04 | 98.27 | 98.08 |
| Dry remains of non-degraded lignin, average in % | 99.90 | | 99.62 | | 98.65 | | 98.50 | | 98.13 | | 98.18 | |
| Mass of degraded lignin, average in % | 0.10 | | 0.38 | | 1.35 | | 1.50 | | 1.87 | | 1.82 | |

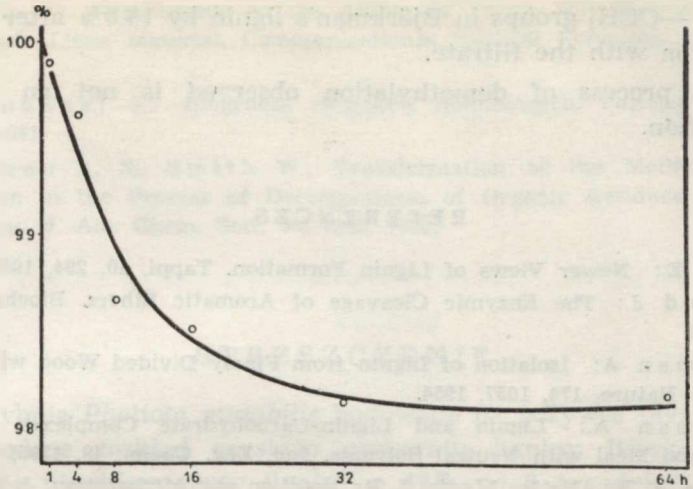


Fig. 4. Degradation of Björkman's lignin by exoenzymes secreted into the liquid medium by *Pholiota mutabilis*. The remains of the lignin mass after incubation (in %) depending on time of incubation (h) with the filtrate from the medium

In order to determine whether, during the incubation of the lignin with the mycelium mat, there is a process of oxidative demethylation, we analysed the medium after 4 week's incubation with lignin; in the second case we added fresh lignin to filtrate of liquid medium and similarly analysed it for the presence of formic aldehyde according to (2). In both cases the reaction with chromotropic acid to detect the presence of formic aldehyde (2) was negative. The sensitivity of reaction is $6 \cdot 10^{-4}$ %.

## CONCLUSIONS

1. The mycelium of *Pholiota mutabilis* grown on liquid medium with glucose causes the degradation of Björkman's lignin to be seen in the decrease of the lignin mass by 42 % after 6 weeks (Table 1).

2. In Björkman's lignin, which was incubated for 6 weeks with the growing mycelium mat of *Pholiota mutabilis* we observed a decrease in the content of methoxyl groups by 16.8 % (Table 2.).

3. The liquid medium filtered off from the 4-week culture of *Pholiota mutabilis*, contained exoenzymes causing a decrease in mass of Björkman's lignin of 1.82 % after 64 hours' incubation with the filtrate (Table 4).

4. The liquid medium filtered off from the 4-week culture of *Pholiota mutabilis* contains exoenzymes causing a decrease in the

content of —$OCH_3$ groups in Björkman's lignin by 18.8% after 64 hours of incubation with the filtrate.

5. The process of demethylation observed is not an oxidative demethylation.

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## STRESZCZENIE

1. Grzybnia *Pholiota mutabilis* hodowana na pożywce płynnej z glikozą powoduje rozkład czystego preparatu ligniny Björkmana wyrażający się ubytkiem masy ligniny o 42% po 6 tygodniach (tab. 1).

2. W ligninie Björkmana, która pozostawała przez 6 tygodni w kontakcie z rosnącą grzybnią *Pholiota mutabilis* stwierdzono zmniejszenie zawartości grup metoksylowych o 16,8% (tab. 2).

3. Odsączona od 4-tygodniowej grzybni *Pholiota mutabilis* pożywka płynna zawierała egzoenzymy powodujące ubytek masy ligniny Björkmana o 1,82% po 64 godzinach inkubacji z przesączem (tab. 4).

4. Odsączona od grzybni pożywka po 4-tygodniowej hodowli *Pholiota mutabilis* zawiera egzoenzymy wywołujące zmniejszenie zawartości grup —$OCH_3$ w ligninie Björkmana o 18,8% po 64 godzinach inkubacji z przesączem.

5. Obserwowany proces demetylacji nie ma charakteru demetylacji oksydatywnej.

## РЕЗЮМЕ

1. Мицелий *Pholiota mutabilis* выращиваемый на жидком субстрате с глюкозом вызывает разложение чистого препарата лигнина Бёркмана, что выражается уменьшением веса лигнина на 42% после шести недель (табл. 1).

2. В лигнине Бёркмана, который находился в контакте с растущим мицелием *Pholiota mutabilis*, в течение 6 недель, установлено снижение содержания метоксильных групп на 16,8% (табл. 2).

3. Отфильтрованный от четырехнедельного мицелия *Pholiota mutabilis* жидкий субстрат содержал эгзоферменты, вызывающие потерью веса лигнина на 1,82% после 64 часов инкубации.

4. Отфильтрованный от четырехнедельного мицелия *Pholiota mutabilis* питательный субстрат содержит экзоферменты приводящие к уменьшению содержания групп $OCH_3$ в лигнине на 18,8%, после инкубации с фильтратом в течение 64 часов.

5. Протекающий процесс деметиляции не является окислительной деметиляцией.



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