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Lignin degradation in plant remnants under liquid-phase fermentation conditions

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Peroxidase, laccase and tyrosinase activities of micromycetes *Galactomyces geotrichum, Myrothecium verrucaria, Mortierella verticillata, Sporotrichum pruinosum* and *Papularia sphaerosperma* were investigated, and lignin degradation in rye straw by these fungi after 4, 8 12, 16, 20 days of cultivation under liquid-phase fermentation conditions was evaluated.

The maximum of peroxidase activity (136.25 a.u./ml) was detected in the case of *Myrothecium verrucaria* after 16 days of cultivation.

The highest tyrosinase activity was shown by *Galactomyces geotrichum* after 20 days (1.25 c.u./ml) and by *Myrothecium verrucaria* after 12 days (1.06 c.u./ml).

The laccase activity of all the micromycetes tested was weak. A higher enzymatic activity was found in *Galactomyces geotrichum* after 20 days (extinction coefficient 0.136).

A more significant lignin degradation (to 8.81%) was performed by *Myrothecium verrucaria* within 16 days. This corresponded with the highest peroxidase activity.

Key words: phenoloxidases: peroxidase, tyrosinase, laccase, lignin

INTRODUCTION

In natural environments, plant remnants are accumulated in rather high amounts. They are composed of heavily degrading complex organic polymeric matter that stores large amounts of photosynthetic energy. It has been stated that 60% of this energy lies in cellulose and hemicellulose. In nature, these polymers are tightly linked to lignin and are very resistant to external factors. Therefore, the energy accumulated in such compounds is hardly available to environmental biota. If these links were weakened, plant remnants would be easer available to other members of the ecological system, and soil biological activity would increase [1–3].

Presently, new measures are being searched for and new biotechnological methods developed to enhance destruction of plant remnants and to obtain physiologically active substances that could be used for enriching the feed with proteins and other valuable supplements and for obtaining ethanol and biogas, etc. Conversion of plant remnants is catalyzed by microbial enzymes released during their functional activity.

The main micromycete enzymes participating in the lignin degradation process are phenoloxidases and in cellulose degradation – endogluconase [4, 5]. Until now, the question about the lignin composition of herbaceous plants and microorganisms able to degrade them has been solved incompletely.

There are some works dealing with the lignin-cellulose complex degradation in plant substrates by micromycetes [6–9] and rye straw delignification using H_2O_2 [10]. Research has been done on lignin–cellulose complex degradation by micro- and macromycetes [6, 11, 12]. There are also data on lignin–cellulose complex degradation in plant remnants by complexes of micromycetes of various taxonomic groups [7, 13].

The ability of micromycetes to degrade the lignin–cellulose complex in plant remnants greatly depends on cultivation conditions.

The aim of the work was to investigate the enzymatic activity of micromycetes – phenoloxidase producers, and lignin degradation in plant remnants under liquid-phase fermentation conditions in order to use these remnants as a supplement in feed processing technologies.

MATERIALS AND METHODS

The object of the research was rye straw (*Secale*) remnants. For experiments we used micromycetes – the most promising destructors of the lignin–cellulose complex in plant remnants in the solid-phase fermentation phase. The following micromycetes were isolated, identified and investigated *Galactomyces geotrichum* (Bul. et Petersen) Redhead et Malloch, *Myrothecium verrucaria* (Alb. et Schweinitz) Ditmar ex Fries, *Mortierella verticillata* Linnem, *Sporotrichum pruinosum* (Gilman et Abbott), *Papularia sphaerosperma* (Pers) Höhn.

The identification was performed following Domsch et al. [14].

Galactomyces geotrichum was isolated from soil (Kaunas Botanical Garden), *Myrothecium verrucaria* from roots of cereal plants (Dotnuva), *Mortierella verticillata* from soil (timothy rhizosphere (Tr. Vokė)), *Sporotrichum pruinosum* from materials made from lignin, cellulose, aromatic caotchoucs and polyamids (Juodkrantė), *Papularia sphaerosperma* from polymeric materials made from copolymer (tetrafluorethylene–hexafluorpropylene (Juodkrantė).

For the investigation, micromycetes stored at the Laboratory of Biodeterioration Research, Institute of Botany, were used.

Micromycetes were cultivated in a standard Czapek medium (under liquid-state fermentation) for 20 days at 28 °C. The carbon source here was rye straw (1 g). The activity of phenoloxidases (peroxidases, laccases and tyrosinases) in the medium and the lignin degradation degree were fixed in rye straw after 4, 8, 12, 16 and 20 days.

Peroxidase activity was determined with the o-dianisidine reagent [15]. The assay method for peroxidase (E.C. 1.11.1.7) activity is based on the colorimetric evaluation of the oxidation product of o-dianisidine in the presence of H_2O_2. The reaction mixture contained 0.1–0.5 ml of the enzyme extract, 3 ml of o-dianisidine reagent comprising 50 ml phosphate buffer (0.4 M, pH 5.9), 2 ml of o-dianisidine and 200 ml of distilled water, and 0.2 ml of 0.05% H_2O_2. Tests and controls were incubated at 20 °C for 5 min in a water bath. The reaction was stopped by adding till 10 ml 50% H_2SO_4 and the absorbance read colorimetrically using a green filter. Activity was calculated according to the coefficient of micromolar extinction, which is 0.0128. Peroxidase activity is expressed as activity units (AU) ml.

Laccase (E.C. 1.10.3.2) activity [16] was measured according to Ravin and Harward (1956). The reaction mixture contained 0.1 ml of the enzyme extract, 1 ml of 0.5% p-phenilenediaminechloride and 2 ml of 0.1N acetate buffer (pH 6).The reaction was stopped by adding 1 ml of 0.1% sodium azide solution. The absorbance was read at 530 nm. Laccase activity is expressed as the extinction coefficient.

Tyrosinase (E.C. 1.14.18.1) activity [17] was measured spectrophotometrically using a method based on the estimation of the optical density of reaction products formed during oxidation of pyrokatechin (or other substratum) over a given period. The reaction mixture contained 0.1 ml of the enzyme extract, 2 ml of phosphate buffer (pH 7.4) and 0.5 ml of pyrokatechin (0.05 M), and the absorbance was read at 420 nm every 20 s for 2 min. Enzyme activity is expressed as conditional units (CU) ml.

Lignin content was estimated in rye straw according to Khudiakova's method [18].

The data were computed using the Excel 98 program.

RESULTS AND DISCUSSION

Peroxidase activity. After 4 days of cultivation peroxidase activity was detected only in *Myrothecium verrucaria* (6.87 a.u./ml) (Fig. 1). The other micromycetes showed no activity.

While cultivating further for 8, 12 and 16 days, peroxidase activity was found only in *Galactomyces geotrichum* and *Myrothecium verrucaria*. The maximum peroxidase activity was achieved in 16 days of fungal growth (46.87 and 136.25 a.u./ml).

In the case of *Myrothecium verrucaria*, two maximums of peroxidase activity after 8 and 16 cultivation days were detected. This could be explained by the fact that while growing micromycetes in the medium where the C sourse is complex natural polymers, mycelium grows and dies irregularly, manitesting an uneven enzymatic activity. This phenomenon is also confirmed by other authors' [11] data on *Tyromyces lacteus* on rye straw (solid-phase fermentation).

Tyrosinase activity. Tyrosinase activity of micromycetes altered variably (Fig. 2). Two maximums of *Galactomyces geotrichum* tyrosinase activity were observed – after 12 and 20 days of cultivation (0.80 and 1.5 c.u./ml, respectively).

A higher tyrosinase activity of *Myrothecium verrucaria* was detected after 8 and 12 cultivation days (1.0 and 1.06 c.u./ml). A lower tyrosinase activity of this micromycete was found after 20 days.

The micromycete *Sporotrichum pruinosum* showed no tyrosinase activity after 4, 12, 16 days, whereas the highest activity (0.87 c.u./ml) of this micromycete was observed after 8 days, and it was low after 20 days.

The most evident tyrosinase activity of *Papularia sphaerosperma* was detected after 4 days of growth when enzymatic activity decreased, and slightly grew up (to 0.098 c.u./ml) after 16 days.

Activity of tyrosinase and other enzymes is highly influenced by destruction metabolites formed during the degradation of plant remnants. When these metabolites enter the environment at particular periods, some of them enhance enzymatic activity, while others supress it. Therefore, enzymatic activity in the course of cultivation alters.

Laccase activity. Laccase activity of the micromycetes was not high. A slightly higher activity was noted after cultivation

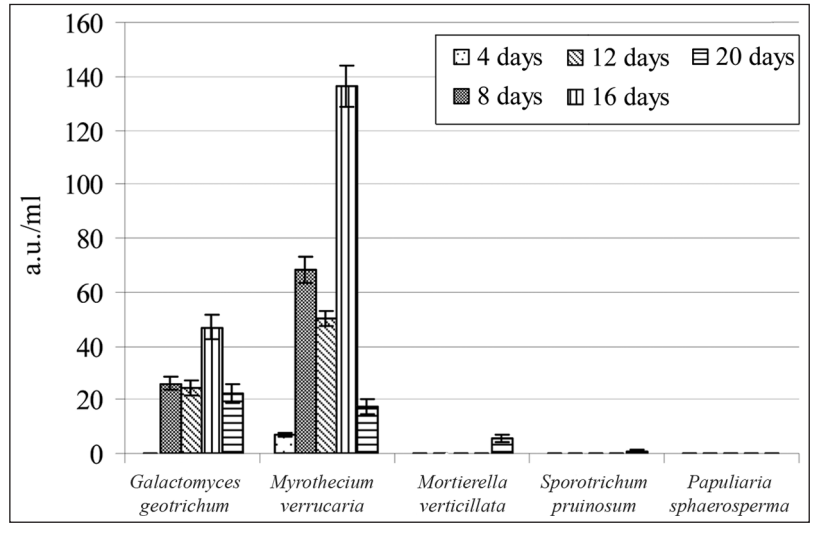

Fig. 1. Peroxidase activity of micromycetes after 4, 8, 12, 16 and 20 days of cultivation under liquid-phase fermentation conditions

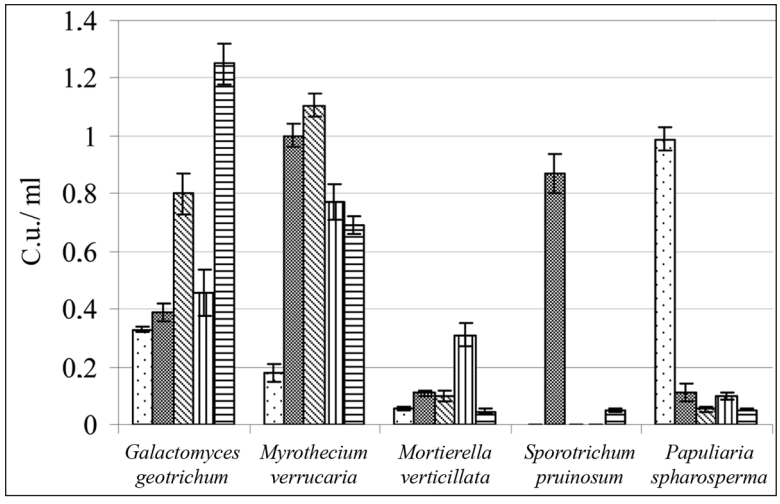

Fig. 2. Tyrosinase activity of micromycetes after 4, 8, 12, 16 and 20 days of cultivation under liquid-phase fermentation conditions (legend as in Fig. 1)

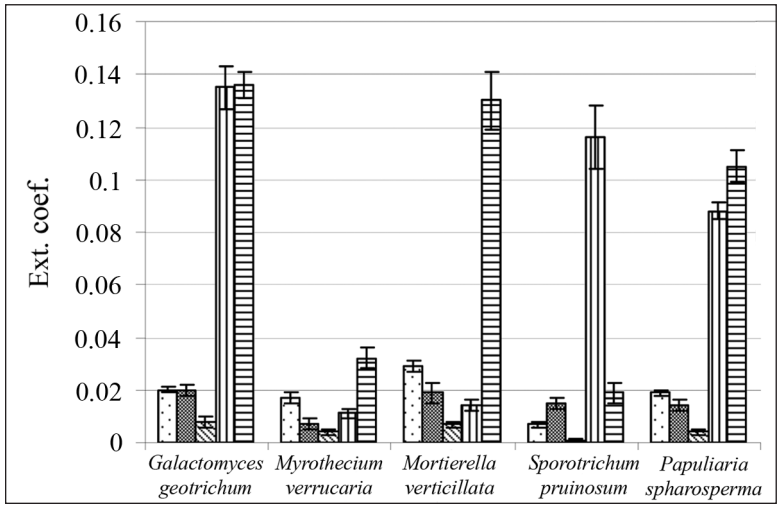

Fig. 3. Laccase activity of micromycetes after 4, 8, 12, 16 and 20 days of cultivation under liquid-phase fermentation conditions (legend as in Fig. 1)

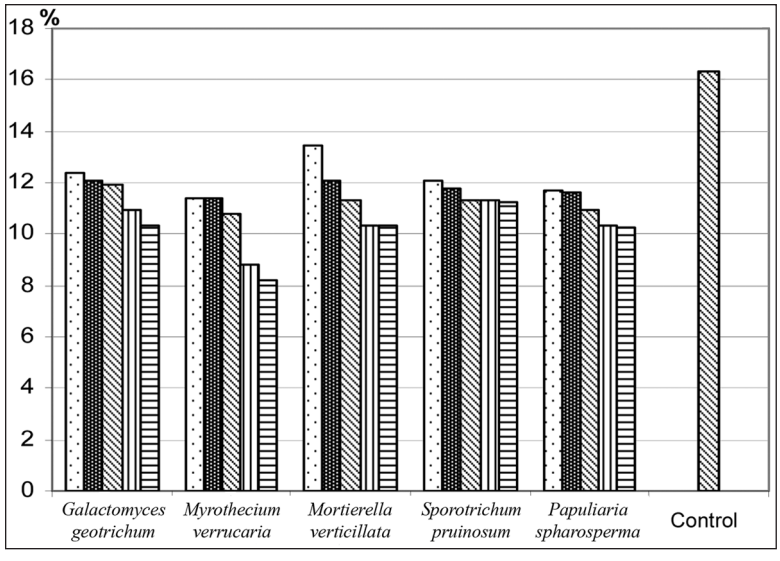

Fig. 4. Lignin degradation in rye straw by micromycetes under liquid-phase fermentation conditions after 4, 8, 12, 16 and 20 days of cultivation (legend as in Fig. 1)

of *Galactomyces geotrichum* for 16 and 20 days (extinction coefficients 0.135 and 0.136, respectively). Laccase activity of the other micromycetes varied from the extinction coefficient values of 0.001 to 0.130 (Fig. 3).

Lignin. Lignin in the control rye straw made up 16.2%. Under these conditions, lignin degradation in rye straw was observed after cultivation of all the micromycetes studied (Fig. 4). A more significant degradadation of lignin (to 8.81%) was found after *Myrothecium verrucaria* cultivation for 16 days, and this corresponded with the highest peak of its peroxidase activity. A deeper lignin degradation at this cultivation time was noted in cases of *Papularia sphaerosperma* (10.33%), *Mortierella verticillata* (10.36%) and *Galactomyces geotrichum* (10.9%).

The regularities of alterations of enzymatic activity and lignin degradation during the cultivation of *Galactomyces geotrichum, Myrothecium verrucaria* and *Papularia sphaerosperma*

under solid-state fermentation differed from those determined under liquid-state fermentation [19]. This can be explained by the fact that, while growing on substrata containing a hardly available carbon source, fungi produce peroxidase as soon as after 4 days. Peroxidase activity of these micromycetes was 31.1; 52.7; 5.2 a.u/ml, respectively, and lignin degradation at this peroxidase activity was 12.88, 13.98 and 14.19%.

Our investigation [9] and works of other authors [20] have shown that a rapid lignin degradation in plant remnants under solid-state fermentation conditions occurs in a later stage.

The obtained results have shown that under liquid-state fermentation conditions particular fungi are also able to degrade lignin in earlier stages of their cultivation.

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LIGNINO SUMAŽĖJIMAS AUGALŲ ATLIEKOSE SKYSTAFAZĖS FERMENTACIJOS SĄLYGOMIS

Santrauka

Ištirta mikromicetų *Galactomyces geotrichum, Myrothecium verrucaria, Mortierella verticillata*, *Sporotrichum pruinosum* ir *Papularia sphaerosperma* peroksidazės, lakazės ir tirozinazės aktyvumas, taip pat lignino sumažėjimas rugių šiauduose po 4, 8, 12, 16, 20 parų mikromicetų kultivavimo skystafazės fermentacijos sąlygomis. Peroksidazinio aktyvumo maksimumas (136,25 a.v./ml) buvo nustatytas po 16 parų mikromiceto *Myrothecium verrucaria* kultivavimo. Didesniu tirozinaziniu aktyvumu išsiskyrė *Galactomyces geotrichum* po 20 (1,25 sąl.v./ml) ir *Myrothecium verrucaria* – po 12 parų kultivavimo (1,106 sąl.v./ml). Lakazinis visų tirtų mikromicetų aktyvumas buvo nedidelis. Didesnis fermentinis aktyvumas buvo nustatytas po *Galactomyces geotrichum* 20 parų kultivavimo (ekstinkcijos koeficientas 0,136). Didesnė lignino degradacija (iki 8,81%) buvo nustatyta po *Myrothecium verrucaria* 16 parų kultivavimo. Tai atitiko ir didžiausią peroksidazinio aktyvumo maksimumą.