

EVALUATION OF ENZYMATIC ACTIVITY OF *Pleurotus ostreatus* REGARDING STAGES OF MYCELIUM DEVELOPMENT

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Abstract. The aim of the study was to evaluate cellulolytic and amylolytic enzymes activities in substrates, regarding the time of the *Pleurotus ostreatus* (Mori Hiratake no 39) B7 mycelium incubation, and the stage of physiological development. The experiment was divided into three parts which had different times of mycelium incubation before fructification: I – the time of incubation – 2 weeks; II – 4 weeks; III – 6 weeks. After the mycelium incubation, cold-shock and mechanical-shock was provided on each series. Researches proved that agents such as the time of mycelium incubation in substrate, agrotechnical treatment (cold-shock, mechanical-shock), and the type of mycelium layer had an impact on cellulolytic and amylolytic activity of mycelium in the cultivation's substrate. The biggest influence on change of enzymatic activity had agrotechnical treatment: cold-shock and mechanical-shock.

Key words: *Basidiomycetes*, *Pleurotus ostreatus*, amylases, cellulases, mushrooms

INTRODUCTION

Fungal bioconversion of wood is an important agent responsible for the environmental carbon circulation [Kirk and Farrell 1987, Eriksson et al. 1990]. Fungi, thanks to the enzymatic systems, are the most important organisms which have ability to decompose lignocellulosic substances [Kirk and Farrell 1987]. White rot fungi, including *P. ostreatus*, decompose effectively lignocellulosic materials because of the synthesis of hydrolytic enzymes (such as cellulase and hemicellulase) and thanks to unique composition of oxidative enzymes which decompose cellulose, hemicellulose and lignin to low-molecule components. The products of decomposition are easily absorbed by fungi [Carroll and Wicklow 1992, Hoff et al. 2004, Kues and Liu 2000]. The composition of the enzymes contains also lignin peroxidase (EC 1.11.1.14), Mn-dependant peroxidase (EC 1.11.1.13) and phenoloxidase containing copper-laccase (EC 1.10.3.2) [Elisashvili

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et al. 2001, Galhaup et al. 2002, Moldes et al. 2004, Mikiashvili et al. 2004, Vikineswary et al. 2006]. During cultivation on lignocellulosic substrates enzyme activities change when shift between substrate colonization and fructification stages of mushroom growth [Omoanghe and Mikiashvili 2009].

Many authors have carried out experiments to establish correlations between lignocellulosic substances degradation and specific enzymes synthesis [Eriksson et al. 1990, Valmaseda et al. 1991], biological efficiency [Wang et al. 2001] and lignocellulose decomposition [Hadar et al. 1992, Baldrian et al. 2005]. However, there is a limited knowledge on the changing in enzyme activities during different stages of the life of *Basidiomycetes*. A few authors observed that enzyme activities in *Agaricus bisporus* changed depending on stage of mushroom growth – fructifications, substrate colonization or periodic flushing [De Groot et al. 1998, Ohga et al. 1999].

Pleurotus spp., besides *Agaricus* spp. and *Lentinula* spp., are very commonly cultivated mushroom in the world. It can be naturally found in tropical and subtropical rainforests, and can be artificially cultivated [Dundar et al. 2009]. These days mushrooms are used extensively in many cuisines specially in Japanese, Chinese and European. They are popular for their taste and flavor. This mushrooms are known to have high nutritional values [Tshinyangu 2006]. Fruit bodies of *Pleurotus ostreatus* have high quantities of proteins, carbohydrates, minerals (calcium, phosphorus, iron) and vitamins (thiamin, riboflavin and niacin) as well as low fat [Manzi et al. 1999].

The aim of the study was to evaluate cellulolytic and amylolytic enzymes activity in cultivation's substrates regarding the time of the *Pleurotus ostreatus* B7 mycelium incubation, and the stage of physiological development.

MATERIALS AND METHODS

The experiment was set in the Department of Fruits, Vegetables and Mushrooms Technology on University of Life Sciences in Lublin.

Biological materials and substrate's components. For the research *Pleurotus ostreatus* Mori Hiratake no 39, designated as "B7", was used. Mycelium was received on Petri dishes with agaric substrate PDA (agar, glucose, potato residue), which were incubated for 14 days in 24°C. All was stored slantwise with the same substrate in 4°C.

Cultivation's substrate: – beech sawdust 80%, – wheat bran 20%, – distilled water – about 70% humidity of substrate.

Receiving inoculum and mycelium incubation. Inoculum, which was received on Petri dishes with the PDA substrate, served as a base for inoculation one-litre polypropylene bottles with the wheat grain substrate. To begin with the wheat grain substrate was cooked during one hour with the addition of 1% (w/w) of calcium sulfate. After that it was set in one-litre polypropylene bottles and sterilized in autoclave for two hours in 121°C. After cooling the wheat grain substrate was inoculated by transferring ¼ of PDA agar with mycelium. It was incubated for 30 days in 24°C. Next, the grain spawn was used to inoculation polypropylene bottles with the sawdust substrate (cultivation's substrate). 500 g of this substrate was set in one-litre polypropylene bottles, and then steril-

ized in autoclave for two hours in 121°C. And finally, the cultivation's substrate on the surface was inoculated by 2 g of grain spawn and then incubated in 22°C.

All bottles with inoculated cultivation's substrate were divided into three parts which had different times of mycelium incubation before fructification: I – the time of incubation – 2 weeks, II – 4 weeks, III – 6 weeks. After the mycelium incubation, cold-shock (the temperature was lowered to 8°C for 5 days) and mechanical-shock (about 0,5 cm upper layer was removed) was provided on each parts. After that, the cultivation set bottles were moved to cultivation's room and were incubated in 16–18°C where humidity reached 90% and the light was on for 12 hours. The activity of cellulase and amylase was determined before cold-shock and mechanical-shock, after cold-shock and mechanical-shock, during the primordial formation and after the first harvest. Experiments were performed in one series in three replicates (three bottles with inoculated cultivation's substrate) for each stages of mycelium development and for each time of incubation.

Dry matter assay. The content of dry matter of substrate was determined by drying the sample at 105°C for 24 h.

Preparation of samples for analysis. Cultivation's substrate with overgrown mycelium was divided into three parts: upper (Layer 1), medium (Layer 2) and lower layer (Layer 3). Each of the layers was next subjected to enzymatic evaluation. The samples (cultivation's substrate with overgrown mycelium) were exposed to homogenization process for 5 minutes in mortar.

Cellulolytic and amylolytic activity assay. The activity of cellulase and amylase was determined as follows: 5 g of homogenized substrate was treated with 10 cm³ of acetate buffer solution (pH 5.5). Next the mixture was centrifuged at 3000× g for 15 min and supernatant was filtered through Whatman No. 1 filter paper. After filtration to supernatant was added 10 cm³ of 1% CMC (carboxymethyl cellulose) solution (substrate for cellulase) or 10 cm³ of 1% starch solution (substrate for the amylase). The mixture was incubated at 50°C in thermostat for one hour. Control for determination of reductive sugars was consisted of 5 g of homogenized substrate and 20 cm³ of acetate buffer solution (pH 5.5).

Reductive sugars assay – DNS method [Miller 1959]. After incubation for enzymatic reactions the solutions were analyzed in the respect of reductive sugars quantity. To achieve the results 0,5 cm³ of incubated mixture was added to 1,5 cm³ 2% DNS (3,5-dinitrosalicylic acid) solution, then all was mixed and boiled in water bath for 5 minutes. After cooling process 6 cm³ of distilled water was added, mixed, cooling and the absorbance was read at 540 nm against a blank. Control material was a trial, where after-incubation solution was superseded by distilled water. To make calibration curve the glucose was used.

From quantity of reductive sugar in trials, where suitable substrate was taken (CMC or starch), the quantity of reductive sugars from the control materials was subtracted.

Enzymatic activity of amylase and cellulase was expressed in µg of released glucose in 1 g of dry matter of substrate per one hour under assay conditions.

The obtained research results were processed statistically by Standard Deviation using Statistica 9.0 software. All estimations were done in three replicates.

RESULTS AND DISCUSSION

Researches reported, that factors such as the time of mycelium incubation in substrate, agrotechnical treatment (cold-shock, mechanical-shock), and the type of substrate layer have an impact on cellulolytic and amylolytic activity of mycelium.

The strongest reaction caused by cold-shock and mechanical-shock was observed in mycelium after 2 weeks and 4 weeks of incubation. After such treatment the activity of cellulase decreased considerably in all layers, and in third (the deepest layer) the lowest activity were reported: $9.4 \pm 1.30 \mu\text{g}$ of glucose·g⁻¹ dry matter of substrate per 1 h in mycelium after 2 weeks of incubation and $4.8 \pm 0.61 \mu\text{g}$ glucose·g⁻¹ d.m. per 1 h in mycelium after 4 weeks of incubation (tab. 1 and 2). Also in mycelium after 6 weeks of incubation the lowest activity of cellulase was observed in Layer 3 of substrate during cold-shock and mechanical-shock (30.4 ± 2.17 $0.61 \mu\text{g}$ glucose·g⁻¹ d.m. per 1 h).

Table 1. Amylolytic (A) and cellulolytic (C) activity of *Pleurotus ostreatus* B7 mycelium after 2 weeks of incubation in various layers of cultivation's substrate

Tabela 1. Aktywność amylolytyczna (A) i celulozytyczna (C) grzybni *Pleurotus ostreatus* B7 po 2 tygodniach inkubacji w różnych warstwach podłoża uprawowego

	Enzymatic activity (μg glucose·g ⁻¹ d.m. of substrate per 1 h) \pm SD Aktywność enzymatyczna (μg glukozy·g ⁻¹ s.m. w ciągu 1 h) \pm SD					
	layer 1 of substrate – upper warstwa 1 podłoża – zewnątrzna		layer 2 of substrate – medium warstwa 2 podłoża – środkowa		layer 3 of substrate – lower warstwa 3 podłoża – wewnętrzna	
	C	A	C	A	C	A
a	89.8 \pm 2.92	64.1 \pm 0.95	66.5 \pm 6.33	40 \pm 2.48	41.2 \pm 2.77	37.9 \pm 2.42
b	35.5 \pm 1.36	20.5 \pm 2.30	21.9 \pm 2.62	21.6 \pm 2.01	9.4 \pm 1.30	4.4 \pm 1.21
c	107.4 \pm 3.0	30.2 \pm 1.13	59.8 \pm 2.65	55.5 \pm 2.69	55.3 \pm 3.99	39.6 \pm 4.98
d	57.2 \pm 2.10	63.8 \pm 2.23	163.3 \pm 5.34	30.8 \pm 2.26	74.4 \pm 3.05	57.9 \pm 3.58

SD – standard deviation (n = 3); A – amylolytic activity; C – cellulolytic activity; a – before cold-shock and mechanical-shock; b – after cold-shock and mechanical-shock; c – during the primordia formation; d – after the first harvest; d.m. of substrate. – dry matter of substrate

SD – odchylenie standardowe (n = 3); A – aktywność amylolytyczna; C – aktywność celulozytyczna; a – przed szokiem termicznym i mechanicznym; b – po szoku termicznym i mechanicznym; c – podczas formowania zawiązków owocników; d – po pierwszym zbiorze; s.m. – sucha masa podłoża

After increasing the temperature up to 16–18°C during primordial and fructification stages, the activity of cellulase increased in mycelium after 2 and 4 weeks incubation more than before the shock, aside from the second layer in mycelium after 2 weeks of incubation. In this case, cellulolytic activity before the shock gained $66.5 \pm 6.33 \mu\text{g}$ of glucose·g⁻¹ d.m. per 1 h and decreased to $59.8 \pm 2.65 \mu\text{g}$ of glucose·g⁻¹ d.m. per 1 h.

Weaker reaction for both shocks was observed in mycelium after 6 weeks of incubation, which was a possible cause of mycelium obsolescence and smaller vulnerability for extreme environmental changes. Our result from cellulase activity support the find-

ing of Omoanghe and Mikiashvili [2009], who observed that cellulase activities increased during primordia formation and fruiting. Ohga et al. [1999, 2000] reported that high CMCase (CMCase carboxymethylcellulase) activity was strongly associated with the fruiting period. These authors observed that cellulase messenger RNA transcripts in *Lentinula edodes* or *Agaricus bisporus* were the biggest during the fruiting body formation.

Comparing cellulolytic activity of mycelium which was growing 2, 4 and 6 weeks without any other treatment (the first analysis from each series and each layer) small cellulolytic activity fluctuation in time and in one layer may be reported. Possibly it means that during the time of mycelium growth, in the same temperature, during six weeks, the balance between synthesis and decomposition of enzyme establishes. It covers all three layers, but in deeper layers – second and third – cellulolytic activity is lower than in the first one. In lower layers of substrate it may be caused by limited oxygen and humidity access and influenced on slow synthesis of enzymes. Omoanghe and Mikiashvili [2009] reported that during long-term monitoring CMCase activity of tested *P. ostreatus* strains in ligninocellulosic substrates were not a significant difference were observed in a level of enzymatic activity. In the study of Singh et al. [2003] the highest cellulase production was observed also during the fruiting phase of the *Pleurotus* spp.

Table 2. Amylolytic (A) and cellulolytic (C) activity of *Pleurotus ostreatus* B7 mycelium after 4 weeks of incubation in various layers of cultivation's substrate

Tabela 2. Aktywność amylolityczna (A) i celulozyczna (C) grzybni *Pleurotus ostreatus* B7 po 4 tygodniach inkubacji w różnych warstwach podłoża uprawowego

	Enzymatic activity ($\mu\text{g glucose}\cdot\text{g}^{-1}$ d.m. of substrate per 1 h) \pm SD Aktywność enzymatyczna ($\mu\text{g glukozy}\cdot\text{g}^{-1}$ s.m. w ciągu 1 h) \pm SD					
	layer 1 of substrate – upper warstwa 1 podłoża – zewnątrzna		layer 2 of substrate – medium warstwa 2 podłoża – środkowa		layer 3 of substrate – lower warstwa 3 podłoża – wewnętrzna	
	C	A	C	A	C	A
a	101.3 \pm 2.88	38.1 \pm 2.43	33.9 \pm 3.00	20.1 \pm 1.74	37.3 \pm 2.49	22.2 \pm 2.26
b	30 \pm 2.07	12.9 \pm 2.19	19 \pm 1.66	10 \pm 1.21	4.8 \pm 0.61	2.5 \pm 0.56
c	123.1 \pm 4.51	60 \pm 2.41	110.2 \pm 5.24	48.1 \pm 2.29	69.7 \pm 1.90	50.1 \pm 2.11
d	139.1 \pm 3.10	71.7 \pm 3.12	89.5 \pm 2.51	43.8 \pm 3.03	110.3 \pm 2.31	42.9 \pm 1.91

Explanation – see table 1 – Objaśnienia – patrz tabela 1

The biggest influence on amylolytic activity had agrotechnical treatments, also changes related to the process of mycelium growth. In the literature a little is known about amylolytic activity of the higher fungi species what is probably connected with the growth in specific lignocellulosic substrates. Ligninocellulosic waste are rich in cellulose, lignin and polyoses and amylases are not specific enzymes involving in ligninocellulosic substrates utilization.

The strongest reaction on amylolytic activity caused by the shocks was observed in mycelium after 4 weeks (tab. 2) and 6 weeks of incubation (tab. 3) (likewise cellulolytic activity). After the treatment, activity of amylase decreased in all layers, and in the last layer lowered to $4.4 \pm 1.21 \mu\text{g}$ of glucose·g⁻¹ d.m. per 1 h in mycelium after 2 weeks of incubation (tab. 1) and $2.5 \pm 0.56 \mu\text{g}$ of glucose·g⁻¹ d.m. per 1 h in mycelium after 4 weeks of incubation (tab. 2). Smaller differences were reported in amylase activity changes in mycelium after 6 weeks of incubation (tab. 3) in different physiological stages of mycelium. It was previously mentioned, that this may be the reason for the mycelium obsolescence.

Discussing the results of amylase activity it may be stated, that the highest activity was observed after 6 weeks of overgrowing of mycelium in the first layer (tab. 3), after the first harvest ($87.2 \pm 2.96 \mu\text{g}$ of glucose·g⁻¹ d.m. per 1 h).

Table 3. Amylolytic (A) and cellulolytic (C) activity of *Pleurotus ostreatus* B7 mycelium after 6 weeks of incubation in various layers of cultivation's substrate

Tabela 3. Aktywność amylolityczna (A) i celulozyczna (C) grzybni *Pleurotus ostreatus* B7 po 6 tygodniach inkubacji w różnych warstwach podłoża uprawowego

	Enzymatic activity (μg glucose·g ⁻¹ d.m. of substrate per 1 h) \pm SD Aktywność enzymatyczna (μg glukozy·g ⁻¹ s.m. w ciągu 1 h) \pm SD					
	layer 1 of substrate – upper warstwa 1 podłoża – zewnątrzna		layer 2 of substrate – medium warstwa 2 podłoża – środkowa		layer 3 of substrate – lower warstwa 3 podłoża – wewnętrzna	
	C	A	C	A	C	A
a	87.4 ± 2.26	53.5 ± 1.47	44.8 ± 1.65	58.5 ± 1.71	30 ± 1.90	35.6 ± 2.77
b	50.1 ± 2.25	44.8 ± 1.99	34.6 ± 2.10	30.4 ± 2.17	30 ± 2.42	28.9 ± 1.47
c	96.6 ± 2.88	38.6 ± 1.64	60.8 ± 2.34	38.2 ± 1.51	41.5 ± 2.18	31.5 ± 4.22
d	138.3 ± 2.46	87.2 ± 2.96	102.9 ± 3.30	67.2 ± 2.45	136.7 ± 3.68	55.6 ± 3.96

Explanation – see table 1 – Objaśnienia – patrz tabela 1

There were also respective differences in the activity between the layers. In the upper layer amylase activity was slightly higher, which may be the effect of weaker overgrowth of mycelium in deeper layers.

P. ostreatus B7 mycelium tended to show higher cellulase activity than amylase one. Maybe it is related with decomposition of substrate which was mainly consisted from sawdust.

Researches proved that earliness and pace of overgrowth of mycelium in cultivation's substrate depends on enzymes which decompose lignocellulotic substrates. During the process it was observed, that mycelium reaction on extreme physical changes was tightly related to enzymatic activity. After 6 weeks incubation, the reaction on physical agents was noticeably weaker.

CONCLUSIONS

After the research on the amylase and cellulase activity of *P. ostreatus* mycelium it may be stated that:

1. The biggest influence on change of enzymatic activity had external conditions: cold-shock and mechanical-shock.
2. The highest cellulolytic and amylolytic activity was observed during primordia formation and after the first harvest.
3. Cellulase activity of *P. ostreatus* mycelium was higher than amylase activity.
4. Stronger reaction to external physical agents as change of enzymatic activity was observed in mycelium after 2 and 4 weeks of incubation than in mycelium after 6 weeks of incubation.

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OCENA AKTYWNOŚCI ENZYMATYCZNEJ *Pleurotus ostreatus* W ZALEŻNOŚCI OD STADIUM ROZWOJOWEGO GRZYBNI

Streszczenie. Celem przeprowadzonych badań była ocena aktywności enzymów celulozowych i amylolitycznych w podłożu w zależności od czasu inkubacji grzybni szczepu *Pleurotus ostreatus* (Mori Hiratake no 39) B7 i określonego etapu rozwoju rozwojowego grzybni. Doświadczenie podzielono na trzy części różniące się od siebie długością czasu inkubacji grzybni w podłożu uprawowym przed wywołaniem owocowania: I – czas inkubacji 2 tygodnie, II – czas inkubacji 4 tygodnie, III – czas inkubacji 6 tygodni. Po zakończeniu inkubacji grzybni wykonywano szok termiczny i mechaniczny. Badania wykazały, że czynniki takie jak czas inkubacji grzybni w podłożu, zabiegi agrotechniczne (szok termiczny i mechaniczny), rodzaj warstwy grzybni mają wpływ na aktywność celulozową i amylolityczną grzybni w podłożu uprawowym. Największy wpływ na zmianę aktywności enzymatycznej grzybni miały zabiegi agrotechniczne – szok termiczny i mechaniczny.

Słowa kluczowe: *Basidiomycetes*, *Pleurotus ostreatus*, amylazy, celulazy, grzyby wyższe

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