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Enzymatic activity of *Phlebiopsis gigantea* isolates

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Initial experiment was made in order to test wood-decomposing fungus *Phlebiopsis gigantea* for enzymatic activity. Laccase, peroxidase, cellulase, phosphatase, dehydrogenase were marked using different methods for *P. gigantea* isolates growing on Scots pine wood.

Key words: *Phlebiopsis gigantea*, laccase, peroxidase, cellulase, phosphatase, dehydrogenase

INTRODUCTION

Studies on biology and application possibilities of *Phlebiopsis gigantea* (Fr.: Fr.) Jülich [=*Phanerochaete gigantea* (Fr.: Fr.) Rattan et al.] with the aim of control of parasite infections caused in Scots pine and Norway spruce stands by *Heterobasidion annosum* sensu lato were initiated back in the fifties and the sixties (Rishbeth 1951, 1959, 1963). Due to remaining threat of *H. annosum* on roots, these studies are being continued in the U.K. and have been commenced and conducted in other countries too, such as Finland, Poland, Sweden and Italy (Korhonen et al. 1994; Sierota 1995; Nicolotti et al. 1999; Pratt et al. 2000; Westlund, Nohrstedt 2000; Łakomy 2001; Pettersson, Rönnberg 2003; Berglund, Rönnberg 2004; Sierota, Małecka 2004; Thor 2005; Sierota et al. 2007). One of the studied issues is selection of the most efficient at propagation and decomposition of stump wood isolates of *P. gigantea*. The causes for the study are clear at least in the realization of the EU DIR 91/414 and the notification of this species into Annex 1 of the Commission Regulation (EC) 2229/2004.

These are appropriate enzymes that enable fungi to decompose wood (Zeller 1916, after Cartwrigh, Findlay 1951) as one of those first among scientists took up studies of fungi enzymes that decompose wood. In comparison with studies of enzymes in other organisms those of fungi enzymes are not yet sufficiently substantiated.

The aim of the present study is to define the enzymatic activity of dehydrogenase and phosphatase in the chosen *P. gigantea* fungi isolates grown on pine wood, as well as assertion and detecting of presence of enzymatic activity that would decompose lignincellulitic bonds in wood; cellulase, laccase and peroxidase. For comparison similar enzymatic activity test was done for pathogen of *H. annosum* sensu lato.

MATERIALS AND METHODS

The materials of the study were six isolates of *P. gigantea* (marked below as *P.g.* with an index) and one isolate of *H. annosum* sensu lato as control (Tab. 1).

Sterilized at 120°C pine wood pieces of 1 x 2 x 3 cm were placed in dishes containing pure cultures of appropriate isolates and incubated at 24°C afterwards. After 30 days the pieces of wood with the mycelium grown on their surface were mechanically crashed (wood mixer, 6 000 rpm) into particles not exceeding 1 mm. Next, the mass was ground in buffer of the appropriate type. Dry mass of each piece of wood was determined upon drying at 105°C. In samples of appropriate measurement variants dehydrogenase, phosphatase and cellulase were marked.

To mark laccase and peroxidase activities the samples were homogenized in buffer of Tris-HCl with pH=7.5 in proportion of 1:10 and spun at 8 000 rpm, enzymatic activity and protein were marked in supernatant. The laccase and peroxidase were also marked in liquid cultures of isolates based on mineral nutrient with microelements enriched by 0.5% glucose with addition of so called ligninsulphanians (paper and cellulose industry waste). This waste in solid state was solved in 0.1 M NaOH at concentration of 10% and 1 cm^3 of it was added to 100 cm^3 of nutrient. The samples were incubated at 26°C.

Determination of laccase activity. Laccase activity was marked according to Leonowicz and Grzywnowicz (1981) method, 0.5 mM of ethanol solution of syringaldasine was used as substrate. The reaction mixture contained 0.2 cm^3 of enzyme (supernatant) + 1.6 cm^3 H_2O + 2 cm^3 of citrinian-phosphoran buffer with pH=5.0. The reaction was initiated by adding 0.2 cm^3 of syringaldasine solution. Next, absorbency gain was measured at wavelength of λ=525 nm for 1 minute. The results were converted into activity units, i.e. $U \cdot min^{-1}$ according to following pattern:

$(\Delta/min)/(0.0065 \cdot n \cdot c)$ with: 0.0065 – absorbency coefficient for syringaldasine; n – sample thinning; c – protein concentration in $mg \cdot cm^{-3}$.

Table 1
The isolates of *P. gigantea* and *H. annosum* sensu lato used in the experiment

Isolate	Localization (Forest District)	Date of sample collection	Place of collection	Collection
P. gigantea				
P.g.$_1$	Jabłonna	08.06.2001	wood of pine stump	Żółciak
P.g.$_2$, *P.g.*$_3$	Chojnów	23.10.2002	wood of 2 pine stumps	
P.g.$_4$, *P.g.*$_5$	Chojnów	04.11.2003	wood of 2 pine stumps	
P.g.$_6$	Nidzica	19.11.2004	fruit body	
H. annosum sensu lato				
H.a. (control)	Pisz	07.11.2002	fruit body	Żółciak

Determination of peroxidase activity. Peroxidase activity was marked according to Maehly and Chance (1954) method, modified by Malarczyk (1984). The substrate was prepared by mixing 1 cm^3 of 1% methanol solution of orthodianisidine and 99 cm^3 of 0.003% H_2O_2 in 0.1 M octane buffer with pH=5.5. Most often for marking 3.8 cm^3 substrate and 0.2 cm^3 of enzyme were used. The gain of absorbency was measured at wavelength of λ=460 nm for 1 minute. The resulting absorbency values were converted into proper activity units: $U \cdot min^{-1}$ according pattern:

$(\Delta E/min)/(11.3 \cdot n \cdot c)$ with: 11.3 mole absorbency coefficient for o-dianisidine; n – sample thinning; c – protein concentration in $mg \cdot cm^{-3}$.

Protein was marked by Lowry et al. (1951) method modified by Schacterle and Pollack (1973).

Determination of cellulase activity. Cellulase activity was analyzed according to Pancholy and Rice (1973) method using carboximethylcellulose (CMC) as a substrate. To 1g of crashed mass of wood with mycelium, 0.25 cm^3 of toluen was added and 15 minutes later - 5 cm^3 of 1% CMC solution in octane buffer with pH=5.3. The control ones were those samples with the buffer additive and no substrate. The incubation was run at 30°C for 24 hrs. On cooling down the samples were filtered and reduction sugars were marked in the filtrate by Samogyi and Nelson (Nelson 1944) method using 1 cm^3 of the filtrate and 1 cm^3 of Samogyi reagent in the reactive mixture. After 15 min. of warming up in water bath 1 cm^3 of Nelson reagent and 2 cm^3 H_2O were added. 20 min. later absorbency was measured at wavelength of λ=520 nm. The results were read from the model graph drawn for glucose and were expressed in μg of glycoside for 1 g of dry wood mass.

Determination of phosphatase activity. Phosphatase activity of studied isolates was marked according to Tabatabai and Bremner (1969) method with p-nitrophenol phosphorane as a substrate. 4 cm^3 of mallein buffer with pH=6.5 and 1 cm^3 of substrate (1 tablet of PNP in 20 cm^3 buffer) were added to 1 g wood sample. The substrate was not added to the test sample. The samples were incubated at 37°C for 60 min. The enzymatic reaction was stopped in 15 minutes by cooling. After incubation, 4 cm^3 of 0.5 M NaOH buffer was added as well as 1 cm^3 of M $CaCl_2$. Afterwards, the mixture was filtered and filtrate absorbency was marked at wavelength of λ=400 nm. The results were calculated according to pattern: $(E \cdot 66.43)$/dry mass with: 66.43 – absorbency coefficient.

Determination of dehydrogenase activity. Dehydrogenase activity was marked according to Thalmann (1968) method with 2,3,5-triphenyltrasol chloride (TTC) as a substrate. 5 cm^3 of 1% solution of TTC in Tris-HCL buffer with pH=7.4 was added to 0.5 g of wood mass. Only the buffer was added to the test sample. The samples were incubated at 37°C for 24 hours. Phormasane ensuing the reduction (TPF) was extracted in 20 cm^3 of methyl. The samples were filtered and filtrate absorbency was marked at wavelength of λ=485 nm. The results were calculated according to the model graph drawn for phormasane and were converted into mg of phormasane for 1 g dry mass of wood for 24 hours.

The markings were made in three parallel repetitions.

RESULTS

The analysis performed showed that laccase had been found exclusively for isolate of *H. annosum* sensu lato (Tab. 2). The enzyme was present in case of isolates growing on pine wood pieces as well as of isolates growing in liquid medium containing lignin waste. The highest activity of laccase occurred between the 14th and 21st day of growing. *P. gigantea* isolates did not show ability to produce laccase except for $P.g._1$, $P.g._3$, in case of which trace quantities of laccase were obtained.

All studied isolates showed ability to produce peroxidase (Tab. 3). Cellulase activity differed compared with *H. annosum*, so did phosphatase and dehydrogenase activities (Tabs 4-6). Among enzymes of the isolates in question *P. gigantea* that directly participated in decomposition of lignin laccase enzyme had not been found. The absence of laccase was stated in case of both isolates set on wood and the so called "fresh" ones (trace quantities in case of $P.g._1$, $P.g._3$ isolates, 100 times lower than in case of isolate of *H. annosum*).

The highest peroxidase activity was shown by: isolate of $P.g._6$ obtained in November 2004 from the fruiting body developing on a pine stump and a set isolate $P.g._2$ obtained in October 2002 from pine stump wood. Isolate $P.g._2$ shows the same share of peroxidase as does isolate *H. annosum* obtained from the fruiting body in November 2002.

As far as cellulase activity was concerned isolates of $P.g._1$ and $P.g._2$ obtained from pine tree stump showed the highest values, whereas $P.g._5$ isolate indicated the lowest values of this parameter.

Table 2
Laccase activity of *P. gigantea* and *H. annosum* isolates

Isolate	Laccase activity of *P. g.* and *H. a.* isolates growing on pine wood		Laccase activity of *P. g.* and *H. a.* isolates growing in liquid medium containing lignin waste (in $U \cdot cm^{-3}$)
	$U \cdot g^{-1}$ fresh mass	$U \cdot g^{-1}$ dry mass	
$P.g._1$	6.976	14.310	0
$P.g._2$	0	0	0
$P.g._3$	5.245	11.174	0
$P.g._4$	0	0	0
$P.g._5$	0	0	0
$P.g._6$	0	0	0
H.a.	590.00	1482.785	46.882

Table 3
Peroxidase activity of *P. gigantea* and *H. annosum* isolates

Isolate	Peroxidase activity of *P. gigantea* and *H. annosum* isolates growing on pine wood		Peroxidase activity of *P. gigantea* and *H. annosum* isolates growing in liquid medium containing lignin waste (in $U \cdot cm^{-3}$)
	$U \cdot g^{-1}$ fresh mass	$U \cdot g^{-1}$ dry mass	
$P.g._1$	1.337	2.734	$7.850 \cdot 10^{-1}$
$P.g._2$	2.910	7.029	$9.833 \cdot 10^{-1}$
$P.g._3$	1.246	2.654	$8.989 \cdot 10^{-1}$
$P.g._4$	$1.950 \cdot 10^{-1.}$	$4.060 \cdot 10^{-1}$	$1.279 \cdot 10^{-1}$
$P.g._5$	$2.828 \cdot 10^{-1}$	$6.075 \cdot 10^{-1}$	$2.548 \cdot 10^{-1}$
$P.g._6$	3.011	8.802	$16.761 \cdot 10^{-1}$
H.a.	2.903	7.296	$41.919 \cdot 10^{-1}$

Table 4
Cellulase activity of *P. gigantea* and *H. annosum* isolates

Isolate	μg glucose·g^{-1} fresh mass		μg glucose·g^{-1} dry mass	
	Repetition	Average	Repetition	Average
Pg.$_1$	2015.0 2054.0 1950.0	2006.33	4133.33 4213.33 4000.00	4115.55
Pg.$_2$	1976.0 2059.2 2080.0	2038.00	4772.95 4973.91 5024.15	4923.67
Pg.$_3$	1911.0 1586.0 1716.0	1737.67	4071.15 3378.78 3655.73	3701.89
Pg.$_4$	1092.0 1313.0 1066.0	1157.0	2273.58 2733.71 2219.45	2408.91
Pg.$_5$	455.0 390.0 117.0	320.0	977.44 837.81 251.34	688.86
Pg.$_6$	1183.0 1079.0 1105.0	1122.33	3458.05 3154.05 3230.05	3280.72
H.a.	1833.0 1508.0 1755.0	1698.0	4606.69 3789.90 4410.66	4269.08

Table 5
Phosphatase activity of *P. gigantea* and *H. annosum* isolates

Isolate	μg p-PNP·g^{-1} fresh mass·h^{-1}		μg p-PNP·g^{-1} dry mass·h^{-1}	
	Repetition	Average	Repetition	Average
Pg.$_1$	136.12 134.72 136.38	135.74	279.22 276.75 279.75	278.44
Pg.$_2$	25.04 26.71 27.17	26.31	60.49 64.50 65.63	63.54
Pg.$_3$	127.63 128.14 127.08	127.62	272.99 271.90 270.73	27187
Pg.$_4$	132.00 138.77 134.45	135.07	274.83 288.92 279.93	281,23
Pg.$_5$	132.73 133.39 146.01	137.38	285.13 286.55 313.66	295.11
Pg.$_6$	104.76 101.51 101.97	102.75	306.23 296.73 298.07	300.34
H.a.	142.23 135.92 141.89	140.01	357.45 341.59 356.59	351.88

The highest activity as regards phosphatase formation showed the isolate of *H. annosum;* the lowest – *P.g.*$_2$ isolate. Similar values as regards phosphatase formation were shown by *P.g.*$_1$, *P.g.*$_4$ and *P.g.*$_5$ isolates. The highest dehydrogenase activity along with *H. annosum* was shown by the isolate of *P.g.*$_5$ and the lowest by *P.g.*$_6$ isolate obtained from the fruiting body.

Table 6
Dehydrogenase activity of *P. gigantea* isolates and *H. annosum* isolates

Isolate	mg TPF·g^{-1} fresh mass·24 h^{-1}		mg TPF·g^{-1} dry mass·24 h^{-1}	
	Repetition	Average	Repetition	Average
P.g.$_1$	25.65 17.99 21.86	21.84	52.63 36.90 44.85	44.80
P.g.$_2$	15.41 15.08 14.68	15.06	37.22 36.44 35.46	36.37
P.g.$_3$	19.44 15.25 22.67	19.12	41.42 32.48 48.29	40.74
P.g.$_4$	19.36 20.17 16.14	18.56	40.31 41.99 33.60	38.63
P.g.$_5$	25.82 24.20 21.78	23.93	55.47 51.99 46.79	51.42
P.g.$_6$	7.26 8.07 10.48	8.60	21.22 23.59 30.63	25.15
H.a.	18.56 24.20 26.62	23.13	46.64 60.82 66.90	58.12

FINAL REMARKS

Laccase is responsible for toxic phenols oxidation. The phenols are exuded by plants as a defensive reaction to fungi infection. It participates in lignin oxidation as well as in degradation and detoxification of antifungal phenols in plant tissues (Mayer, Harel 1979; Mayer 1987; Mayer, Staples 2002). It can oxidize many substrates including mono-, di-(o-di- and p-di-), and tri-phenols. The absence of that enzyme in the wood being decomposed by *P. gigantea* isolates confirms the idea that *P. gigantea* is not a pathogenic organism, like fungus *H. annosum* sensu lato that show high activity of phenoloxidase and no peroxidase activity (Sierota, Miseikyte 2000). In joint pure cultures of *H. annosum* sensu lato and *P. gigantea,* on malt extract agar medium with guaiacol *H. annosum* mycelium causes discoloration of substrate into intense cherry and dark brown color as a result of guaiacol oxidation by phenoloxidase (Boidin 1951; Havličková, Rypáček 1957). Laccase activity has been reported in mycelial and rhizomorphs extracts of *Armillaria* that is - from pathogenic organisms as well (Kaarik 1965; Marsh, Wargo 1989).

Peroxidase catalyzes the oxidation of phenols by hydrogen peroxide (H_2O_2) and is non-specific for phenols. It was detected in exudates from *Armillaria* rhizomorphs (Mallett, Colotelo 1984). It makes one of the agents that determine pathogenic feature of *Armillaria* (Robene-Soustrade et al. 1992). In roots and bark of the butt of spruce roots ridden by *Armillaria* significantly increased peroxidase activity was affirmed in comparison with healthy trees (Feiler, Tesche 1991).

No similarities were found between diversified cellulase isolate activity and place or time of sample taking. Fungi can lose their abilities for enzyme production when cultured for long time (Szklarz et al. 1989).

Sierota and Miseikyte (2000) indicated that with overgrowth of pine tree roots by mycelium *P. gigantea* and secretion of enzymes (cellulase, phosphatase,

dehydrogenase, peroxidase) the tree became attractive for pine weevil imago for its females to lay eggs (second generation of pine weevil).

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Aktywność enzymatyczna wybranych izolatów *Phlebiopsis gigantea*

Streszczenie

Celem badań było określenie aktywności enzymatycznej dehydrogenaz i fosfataz u wybranych izolatów grzyba *P. gigantea* hodowanych na drewnie sosnowym, jak również stwierdzenie obecności oraz oznaczenie aktywności enzymów rozkładających połączenia ligninocelulozowe w drewnie – celulazy oraz lakazy i peroksydazy. Dla porównania, wykonano analogiczne testy aktywności enzymatycznej grzybni patogena – *H. annosum* sensu lato.

Przeprowadzone analizy wykazały, że lakaza była wykrywana w hodowlach izolatu grzyba *H. annosum.* Największą aktywność peroksydazy wykazywały: izolat $P.g._6$ pozyskany w listopadzie 2004 r. z owocnika rozwijającego się na pniaku sosnowym oraz pasażowany izolat $P.g._2$ pozyskany w październiku 2002 r. z drewna pniaka sosnowego. U izolatu $P.g._2$ stwierdzono podobny udział peroksydazy, jak u izolatu *H. annosum*, pozyskanego z owocnika w listopadzie 2002 r. Pod względem aktywności celulolitycznej wyróżniały się izolaty $P.g._1$ i $P.g._2$ pozyskane z drewna pniaka sosnowego, najmniejsze zaś wartości tego wskaźnika wykazywał izolat $P.g._5$. Nie stwierdzono przy tym zbieżności między zróżnicowaną aktywnością celulolityczną izolatów, a miejscem czy terminem pobrania próbek.

Największą aktywnością pod względem tworzenia fosfatazy wykazywał izolat *H. annosum,* najmniejszą zaś izolat $P.g._2$. U izolatów: $P.g._1$, $P.g._4$ oraz $P.g._5$ odnotowano podobne wartości fosfatazy. Najwyższą aktywnością dehydrogenazy, obok izolatu *H. annosum*, odznaczał się izolat $P.g._5$, a najmniejszą zaś izolat $P.g._6$ pozyskany z owocnika.