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Effect of Carbon, Nitrogen Sources, and Copper Concentration on the Ligninolytic Enzyme Production by *Coriolopsis gallica*

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Abstract

The effect of a wide range of culture conditions on production of laccase and manganese peroxidase by Coriolopsis gallica was studied. Copper, carbon and nitrogen sources, and their concentration had a strong impact on enzyme activities. Several industrially relevant features of the fungus were revealed. Very high enzyme activity was expressed in the cultivation in both synthetic (xylose, glycerol) and lignocellulose (wheat bran)-containing media, and it produced better laccase and MnP at high concentrations (2-3%) of carbohydrates in the absence of any specific inductor. Another feature of this fungus is its capability to secrete laccase during primary metabolism. The fungus proved to be a potent agent of Remazol Brilliant Blue R (RBBR) decolorization.

Keywords: Coriolopsis gallica, laccase, manganese peroxidase, synthesis, carbon and nitrogen sources, copper, RBBR

Introduction

Agro-industrial residues are produced worldwide in huge quantities as waste/by-products of crop cultivation and food processing. These materials can potentially be converted into variously different value-added products including biofuels, chemicals, enzymes, animal feeds, and human nutrients.

Higher basidiomycetes represent a taxonomically, ecologically, and physiologically diverse group of eukaryotic organisms. White-rot basidiomycetes are unique in their ability to degrade all components of lignocellulose due to their capability to synthesize relevant hydrolytic and oxidative extracellular enzymes (Eriksson et al., 1990; Aro et al., 2005). These fungi secrete one or more of three extracellular enzymes that are essential for lignin degradation: lignin peroxidase (EC 1.11.1.14), manganese-dependent peroxidase (EC 1.11.1.13), and laccase (EC 1.10.3.2). The ligninolytic enzymes of basidiomycetes are of fundamental importance for the efficient bioconversion of lignified plant residues and they are prospective for the various biotechnological applications in pulp and paper, food, textile and dye industries, bioremediation, cosmetics, analytic biochemistry, and many others (Cohen et al., 2002; Durán et al., 2002). It is evident that the potential applications of these enzymes in industrial and environmental technologies require huge amounts of these enzymes at low cost. The main issue delaying their implementation on an industrial scale is the low yield of ligninolytic enzymes in most white-rot fungi even when their genes

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are expressed in heterologous hosts. Therefore, numerous studies have been carried out recently to select new organisms with tremendous synthesis of these enzymes and to develop strategies for their overproduction (Galhaup et al., 2002; Kiiskinen et al., 2004; Revankar and Lele, 2006; Zhang et al., 2006; Elisashvili and Kachlishvili, 2009; Hailei et al., 2009; Bonugli-Santosa et al., 2010). Although some of the selected fungi have shown a high potential for the production of individual oxidative enzymes, the main issue delaying their application on an industrial scale is still the low yield of ligninolytic enzymes in most white-rot fungi.

Recently, we have isolated the novel white-rot fungus, Coriolopsis gallica (Fr.) Ryvarden, belonging to the family Polyporaceae. This fungus secreted extremely high levels of laccase along with appreciable MnP activity (Kenkebashvili et al., 2009). However, no information exists on the physiology of laccase and MnP synthesis by this promising enzyme producer. The aim of this study was to optimize the fungus cultivation conditions and to elucidate factors affecting and enhancing these enzymes production during cultivation in synthetic and lignocellulose-containing media.

Materials and Methods

Organism and Inoculum Preparation

Coriolopsis gallica (Fr.) Ryvarden-strain 1184 was maintained on malt extract agar slants at 4°C in the culture collection of the Laboratory of Cryptogamic Plants and Fungi, University of Haifa. The inoculum was prepared by growing mushrooms using a rotary shaker at 140 rpm and 26±1°C in 250-ml flasks containing 50 ml of the following nutrient medium : glucose 10 g/l; NH4NO3 2 g/l; KH2PO4 0.8 g/l; K2HPO4 0.4 g/l; MgSO4•7H2O 0.5 g/l; yeast extract 2 g/l. The pH of the medium was adjusted to 6.0 with 2M NaOH prior to sterilization at 121oC for 20 min. After 6 days of fungus cultivation, mycelial pellets were harvested and homogenized with a Waring laboratory blender, three times for 20 s with 1 min intervals.

Cultivation Conditions

Submerged cultivation of the fungus was carried out on a rotary shaker at 140 rpm and 27oC in 250-ml flasks containing 50 ml of standard medium: carbon source 15 g/l; peptone 3 g/l; KH2PO4 0.8 g/l; K2HPO4 0.4 g/l; MgSO4•7H2O 0.5 g/l; CuSO4•5H2O 0.125 g/l; yeast extract 4 g/l. When the effect of carbon sources on fungus growth and enzyme production was studied, crystalline cellulose (Avicel), glucose, xylose, gluconic acid, mannitol, and glycerol were used at a concentration of 15 g l−1. A complex growth substrate, wheat bran, promoting the enzyme synthesis, had also been used for comparison in a concentration of 40 g l-1. A control without a carbon source was run in parallel. To study the effect of nitrogen sources, the same standard medium containing 4% wheat bran was used, but all nitrogen containing inorganic and organic compounds were added to the medium at a final concentration of 10 mM in nitrogen equivalent in addition to yeast extract and lignocellulosic substrates. A control without a nitrogen source was run in parallel. To study the effect of copper on enzyme production, the same nutrient medium containing favorable carbon and nitrogen sources at optimal concentrations was supplemented with 0.5-2 mM CuSO4•5H2O at the time of inoculation. In several other experiments conducted in parallel, the effect of 0.2% Remazol Brilliant Blue R (RBBR) had been evaluated because in preliminary tests this synthetic dye favored the enzyme production. The initial pH of all media was adjusted to 6.0 prior to sterilization by adding 2M NaOH. Three milliliters of mycelial homogenate were used to inoculate the flasks containing media. After 5, 7, 10, and 14 days of fungus cultivation, when the cultures reached the middle and end of logarithmic, early and late stationary phases of growth, respectively, the samples (1 ml) were taken aseptically from the flasks, and the solids were separated by centrifugation (14,000 x g; 5 min) at 4°C.

Biomass Estimation

After 10 and 14 days of cultivation, the mycelial biomasses were separated by centrifugation at 6,000 x g (15 min, 4°C). The collected biomasses were dried at 60°C to a constant weight.

Decolourization Assay

In submerged cultivation of fungus, the residual dye concentration in supernatants was measured spectrophotometrically at the maximum absorbance of Remazol Brilliant Blue R (595 nm). The time of dye addition (after four days of cultivation) was considered to be day zero, and the absorbance of the dye in the medium on day zero was considered to be 100%.

Enzyme Assay

The supernatants obtained after separation of the biomasses were analyzed for pH and enzyme activity. Laccase activity was determined by monitoring the A420 change related to the rate of oxidation of 1mM 2,2-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS) in 25 mM Na-acetate buffer (pH 3.8) (Bourbonnais and Paice, 1990). Assays were performed in 1-ml cuvette at 20±1°C with adequately diluted culture liquid. One unit of laccase activity was defined as the amount of enzyme that leads to the oxidation of 1μM of ABTS per minute.

Manganese peroxidase (MnP) activity was measured by oxidation of Phenol Red (Glenn and Gold, 1985). The 1-ml reaction mixtures contained a 0.89 ml lactate-succinate buffer (50 mM, pH 4.5) with manganese sulphate (0.1 mM), phenol red (0.1 mM), egg albumin (0.1%), and 0.1 ml of appropriately diluted enzyme preparation. The reaction was initiated with 0.1 mM H2O2, mixtures were incubated for 1-5 min at 20±1°C, and then the reaction was terminated with 50μl 4M NaOH. Absorbance was read at 610 nm. One unit of enzyme activity was expressed as the amount of enzyme required to oxidize 1 μmol of Phenol Red in 1 min. Activities in the absence of H2O2 were subtracted from the values obtained in the presence of hydrogen peroxide to establish true peroxidase activity.

The experiments were performed at least two times using three replicates. The data presented in the tables correspond to mean values with standard deviations less than 17%.

Results

Effect of Carbon Source

To study the effect of carbon sources on enzyme production, the basal medium was supplemented with 1% simple carbohydrates or a cellulose and 4% wheat bran. All carbon sources supported the fungus growth in the form of pellets increasing the biomass yield 4-8-fold as compared to the control medium (Table 1). The fungal growth in media supplemented with gluconic acid and glycerol was accompanied by a significant increase in medium pH when cultures reached a stationary growth phase. Laccase activity was detected in culture liquids from the second day of fungus cultivation, then gradually increased and peaked at days 7-10. Laccase activity in xylose and glycerol-containing media exceeded that in control medium more than 6-fold and attained the enzyme activity level in wheat bran-based medium. Crystalline cellulose, glucose, and mannitol were poorer carbon sources for laccase production, because only a three-fold increase of the enzyme yield was attained. The calculation of laccase-specific activity showed that the fungus cultivation in the presence of glucose and mannitol decreased enzyme production by C. gallica from 10980 U g−1 in the control medium to 5160 and 7900 U g−1, respectively (Table 1). At the same time, the supplementation of the control medium with gluconic acid resulted in the highest specific laccase activity (14190 U g−1).

No MnP or very low enzyme activity was detected in submerged cultivation of C. gallica in synthetic medium with vari-

Table 1. Effect of carbon source on Coriolopsis gallica growth and enzyme activity.

Carbon Source	Final pH	DW (g l^{-1})	Laccase		MnP (U l^{-1})
			(U ml^{-1})	(U g^{-1})	
Control	5.7±0.2	0.9±0.1 (7)	9.8±1.3 (10)	10.8	0
Avicel	6.1±0.1	ND	32.1±5.7 (7)	ND	0
Avicel+RBBR	5.7±0.1	ND	28.4±3.1 (7)	ND	0
Glucose	6.1±0.2	6.6±0.2 (7)	34.1±4.5 (10)	5.1	0
Glucose+RBBR	5.4±0.2	6.8±0.2 (7)	43.5±6.8 (10)	6.4	0
Xylose	6.3±0.2	6.0±0.1 (7)	60.2±8.3 (10)	10.0	110±16 (4)
Xylose+RBBR	5.9±0.2	6.2±0.2 (7)	76.2±8.6 (10)	12.2	100±17 (14)
Gluconic acid	7.8±0.1	3.6±0.1 (10)	51.1±5.3 (14)	14.1	0
Gluconic acid+RBBR	7.0±0.1	3.2±0.1 (10)	45.8±6.2 (10)	14.3	0
Mannitol	5.0±0.2	4.4±0.1 (10)	34.8±4.0 (7)	7.9	0
Mannitol+RBBR	5.3±0.2	4.0±0.2 (10)	40.2±5.7 (10)	10.0	50±8 (10)
Glycerol	6.9±0.1	6.0±0.1 (7)	63.4±8.2 (10)	10.5	90±11 (10)
Glycerol+RBBR	6.0±0.2	6.2±0.1 (7)	73.1±9.7 (10)	11.7	100±14 (10)
Wheat bran	6.4±0.1	ND	57.2±7.1 (10)	ND	620±80 (10)
Wheat bran+RBBR	6.4±0.1	ND	64.0±7.8 (10)	ND	850±142 (10)

The numbers in parentheses indicate the day of peak activity; DW – dry weight; ND – not determined

Table 2. Effect of carbon source concentration on Coriolopsis gallica growth and enzyme activity.

Carbon Source	%	DW (g l^{-1})	Laccase		MnP (U l^{-1})
			(U ml^{-1})	(U g^{-1})	
Control	0	0.8±0.1	9.2±1.3	11.5	0
Xylose	1	3.7±0.3	40.8±6.9	11.0	170±20
	2	6.0±0.3	59.4±6.7	9.9	200±28
	3	8.4±0.4	52.6±7.2	6.3	120±9
Glycerol	1	4.2±0.3	43.8±5.2	10.4	120±15
	2	6.4±0.4	65.3±7.0	10.2	180±26
	3	7.9±0.4	77.1±11.9	9.8	290±47
Wheat bran	2	ND	45.7±6.0	ND	590±67
	4	ND	66.2±7.3	ND	760±88
	6	ND	51.6±8.3	ND	280±49

ous carbon sources. However, the submerged fermentation of wheat bran favored the secretion of MnP. It is worth noting that the supplementation of actively growing cultures with RBBR on day 4 caused a negative effect on enzyme synthesis. This dye promoted laccase production by C. *gallica* by 15-28% when the fungus was grown in media containing glucose, xylose, mannitol, and glycerol. Moreover, the addition of RBBR to lignocellulose-containing medium stimulated the MnP accumulation by 35%. The addition of RBBR to the cultures grown in the presence of Avicel or gluconic acid decreased the laccase yield but did not affect the enzyme-specific activity.

Subsequently, the effect of carbon source concentration on the C. *gallica* growth and enzyme yield was evaluated. Two carbon sources (xylose and glycerol) providing the highest yield of laccase were selected. The data presented in Table 2 show that gradually increasing the amount of carbon source in nutrient medium from 0 to 3% resulted in an 8-fold increase in fungal biomass yield compared to the control medium. Laccase activity correlated with the biomass accumulation when C. *gallica* was grown in medium containing glycerol. In this medium, the fungus-specific laccase activity varied insignificantly (from 9760 to 10440 U g−1), and the highest enzyme activity was detected in medium containing 3% glycerol. When the fungus was cultivated in xylose-based medium the productivity of mycelia decreased from 11030 to 6260 U g−1 with the elevation of sugar concentration in the nutrient medium, and highest laccase activity was attained in the presence of 2% xylose. Supplementing the control medium with carbon source promoted MnP production by C. *gallica* with the highest enzyme yield in media containing 2% xylose or 3% glycerol.

Effect of Nitrogen Source

To study the effect of different nitrogen sources on enzyme production by C. *gallica* in submerged fermentation of 4% wheat bran, physiologically basic (potassium nitrate) and acid (ammonium sulphate) salts, as well as ammonium nitrate were selected in addition to organic compounds, casein hydrolysate and peptone at a concentration of 10 mM in nitrogen equivalent. The data presented in Table 3 show that in submerged fermentation of wheat bran the supplementation of the control medium with additional nitrogen source did not significantly affect the enzyme yield. Nevertheless, the fungus cultivation in the presence of physiologically acid salt and ammonium nitrate caused the decrease of laccase yield compared to the control medium by 13-18%. Supplementation of the control medium with peptone resulted in a 20% increase of C. *gallica* laccase activity. Moreover, Fig. 1 indicates that during the first four days, the enzyme accumulation in media containing KNO_3, casein hydrolysate, and peptone (slightly higher medium pH) was faster compared to the control medium, whereas in media supplemented with $(NH_4)_2SO_4$ and NH_4NO_3 (slightly lower pH) it was slower during the entire period of fungus cultivation compared with the medium without an additional nitrogen source. Obviously, the medium pH affected the fungus growth favoring or delaying laccase accumulation. None of the nitrogen sources favored MnP secretion by C. *gallica* in submerged fermentation of wheat bran. On the contrary, casein hydrolysate caused a 19% decrease of this enzyme yield. It is worth noting that the supplementation of 0.2% RBBR to the nutrient medium on day 4 enhanced both enzyme secretions. Furthermore, the presence of this synthetic dye in media containing ammonium sulphate or ammonium nitrate resulted in a 22-25% increase in laccase yield compared to the corresponding control media.

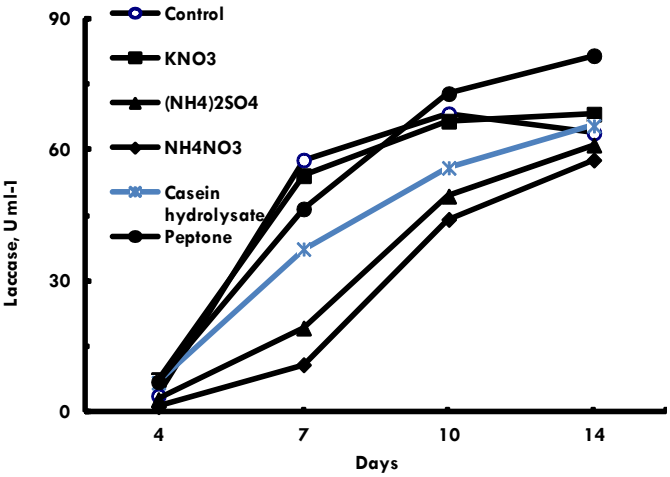

Figure 1. Effect of N source on laccase production during submerged fermentation of wheat bran by C. *gallica*.

Table 3. Effect of nitrogen source on Coriolopsis gallica enzyme activity in submerged fermentation of wheat bran.

Nitrogen Source	Laccase (U ml^{-1})	MnP (U l^{-1})
Control	68.4±6.1	760±93
KNO_3	68.3±8.7	770±129
KNO_3+RBBR	69.2±7.4	860±126
$(NH_4)_2SO_4$	60.2±6.8	780±133
$(NH_4)_2SO_4$+RBBR	74.8±9.8	770±128
NH_4NO_3	57.6±6.5	760±83
NH_4NO_3+RBBR	71.8±7.3	780±107
Casein hydrolysate	65.6±6.9	640±94
Casein hydrolysate+RBBR	70.2±7.8	660±107
Peptone	81.9±10.2	810±116
Peptone+RBBR	93.6±11.7	960±145

The effect of peptone concentration on the enzyme production by C. *gallica* in submerged fermentation of wheat bran was also studied. The concentration of this nitrogen source in the medium varied from 0 to 40 mM in nitrogen equivalent. The data presented in Table 4 show that only a low peptone concentration (10 mM) increased laccase and MnP yields by 21% and 31%, respectively, compared with the control medium without additional nitrogen sources. The further elevation of peptone concentration hampered enzyme secretion. Namely, the highest concentration of peptone (40 mM) resulted in a decrease of laccase and MnP activity by 60% and 48%, respectively. The supplementation of nutrient media with 0.2% RBBR permitted an additional increase in the yields of both enzymes. The cultivation of C. *gallica* in the presence of synthetic dye raised lac-

Table 4. Effect of nitrogen source concentration on *Coriolopsis gallica* enzyme activity.

Peptone Concentration	Laccase (U ml^{-1})	MnP (U l^{-1})
Control	71.5±7.2	725±68
10 mM peptone	86.2±9.3	950±153
10 mM peptone+RBBR	102.4±12.7	1080±140
20 mM peptone	63.2±6.8	650±74
20 mM peptone+RBBR	72.6±8.4	920±138
40 mM peptone	44.7±5.2	490±72
40 mM peptone+RBBR	54.4±6.1	580±88

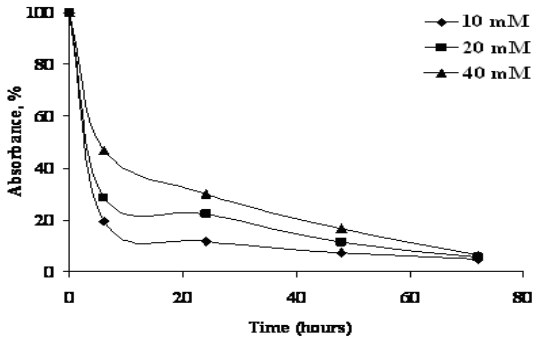

Figure 2. Decolorization of RBBR in submerged fermentation of wheat bran by C. *gallica*. 0.2% RBBR was added on day 4 in media containing 10 mM, 20 mM, and 40 mM peptone.

Table 5. Effect of copper concentration on Coriolopsis gallica growth, laccase and manganese peroxidase activities in wheat bran or glycerol-based media.

Copper concentration	Wheat Bran-based Medium		Glycerol-based Medium			
	Laccase (U ml^{-1})	MnP (U l^{-1})	DW (g l^{-1})	Laccase (U ml^{-1})	(U g^{-1})	MnP (U l^{-1})
Control	52.3±5.9	530±81	7.7±0.2	47.3±5.2	6140	210±17
0.5 mM	81.9±7.8	760±138	7.5±0.2	72.1±6.9	9610	240±32
1 mM	109.6±11.9	810±135	7.9±0.2	79.9±8.9	10110	110±16
2 mM	105.2±11.2	770±118	7.8±0.2	82.5±9.3	10570	100±14

case yields by 15-22% while those of MnP were increased by 14-42% compared with the corresponding control media. It is interesting that the rate of RBBR decolorization correlated with the enzyme activity. Accordingly, the dye decolorization rate decreased with increasing peptone concentrations (Fig. 2).

Effect of Copper Concentration

The effect of copper concentration was studied in submerged cultivation of C. *gallica* in optimized media, containing 4% wheat bran or 3% glycerol as a carbon source and 0.2% peptone as a nitrogen source. When the fungus was grown in glycerol-based medium, no effect of copper concentration on the C. *gallica* biomass yield was observed (Table 5). However, the enzyme yield significantly depended on the microelement concentration. Stepwise increase of copper concentration in nutrient medium from 0 to 2 mM resulted in a gradual increase in laccase yield from 47.3 U ml^{-1} to 82.5 U ml^{-1}. The calculation of laccase-specific activity indicates that this microelement stimulated enzyme expression by C. *gallica* increasing the laccase-specific activity from 6140 U g-1 to 10570 U g^{-1}. The supplementation of synthetic medium with 1-2 mM copper resulted in a two-fold decrease of MnP activity of C. gallica. The supplementation of wheat bran medium with copper promoted not only laccase production, but also intensified the MnP accumulation. Laccase activity doubled while the MnP activity increased by 50% with the elevation of microelement concentration from 0 to 1-2 mM.

Discussion

Optimization of the cultivation conditions for ligninolytic enzyme production is extensively explored with selected organisms. Few reports have described the effects of carbon sources on ligninolytic enzyme production clearly indicating that basidiomycetes display a wide diversity in their response to carbon sources and their concentration in nutrient medium. (Elisashvili et al., 2002, 2006; Galhaup et al., 2002; Wang et al., 2008). Thus, in Phanerochaete chrysosporium, the ligninolytic gene expression is triggered only by the depletion of nutrient carbon (Wang et al., 2008). Significant laccase secretion by Trametes pubescens started when the glucose concentration in the growth medium reached a certain low, critical concentration (Galhaup et al., 2002). Glucose and cellobiose that were efficiently and rapidly utilized by this fungus resulted in high levels of laccase activity. Both lactose and cellulose, which were only poorly utilized for growth, resulted in low laccase levels. Analogically, the laccase activity obtained in cultivation of Pleurotus sajor-caju in media containing fructose or glucose (37 U ml−1 and 36 U ml−1, respectively) was significantly higher than that obtained with lactose (3 U ml−1) (Bettin et al., 2008). On the contrary, lactose appeared to be the best carbon source for laccase secretion by Pseudotrametes gibbosa and was quite appropriate for the enzyme production by Cerrena unicolor and Fomes fomentarius (Elisashvili, Kachlishvili, 2009). This study showed that C. *gallica* is an interesting producer of laccase because, in contrast to

many other WRB, it expresses very high enzyme activity in both synthetic and lignocellulose-containing media (Table 1). Of the carbon sources tested, xylose and glycerol provided the highest laccase activity of the fungus achieving the enzyme activity level in wheat bran-based medium. Significant MnP activity was found in wheat bran-containing medium suggesting that some compounds in this material or products of its degradation likely play an important role in the biosynthesis of this enzyme. One salient feature of C. gallica is that this fungus, in contrast to T. pubescens (Galhaup et al., 2002) and P. chrysosporium (Wang et al., 2008), produces better laccase and MnP at high concentrations of carbohydrates in absence of any specific inducer (Table 2). Naturally, the enzyme purification would be easier with culture grown on such medium, containing no lignocellulose and pigments, making the process more economical.

Among several approaches used to enhance the ligninolytic enzyme synthesis were the supplementation of nutrient media with nitrogen sources and inducers. Peptone turned out to be the best nitrogen source for laccase and MnP accumulation by C. gallica (Table 3). This is in accordance with other reports on the stimulating effect of peptone on the ligninolytic enzyme production by Pleurotus ostreatus (Mikiashvili et al., 2006) and Polyporus sanguineus (Bajwa and Arora, 2009). It is worth noting that only the high concentration of peptone (0.8%) inhibited both laccase and MnP production by C. gallica (Table 4). The fungus most actively secreted both enzymes at a moderate concentration of peptone (0.2%) providing sufficient growth of biomass. Moreover, although C. gallica secretes high levels of laccase in absence of a carbon source or copper, the comparison of this enzyme-specific activity indicates that the fungus productivity might be regulated by varying the carbon source or copper concentration in the nutrient medium.

Further studies are needed to investigate on the physiological mechanisms enhancing ligninolytic enzymes synthesis by this fungus and to develop efficient technology of laccase production.

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