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Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation

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Summary

The effect of additional nitrogen sources on lignocellulolytic enzyme production by four species of white-rot fungi (*Funalia trogii* IBB 146, *Lentinus edodes* IBB 363, *Pleurotus dryinus* IBB 903, and *P. tuberregium* IBB 624) in solid-state fermentation (SSF) of wheat straw and beech tree leaves was strain- and substrate-dependent. In general, the yields of hydrolytic enzymes and laccase increased by supplementation of medium with an additional nitrogen source. This stimulating effect of additional nitrogen on enzyme accumulation was due to higher biomass production. Only xylanase specific activity of *P. dryinus* IBB 903 and laccase specific activity of *L. edodes* IBB 363 increased significantly (by 66% and 73%, respectively) in SSF of wheat straw by addition of nitrogen source to the control medium. Additional nitrogen (20 mM) repressed manganese peroxidase (MnP) production by all fungi tested. The study of the nitrogen concentration effect revealed that 10 mM peptone concentration was optimal for cellulase and xylanase accumulation by *P. dryinus* IBB 903. While variation of the peptone concentration did not cause the change in MnP yield, elevated concentrations of this nutrient (20–40 mM) led to a 2–3-fold increase of *P. dryinus* IBB 903 laccase activity. About 10–20 mM concentration of NH_4NO_3 was optimal for cellulase and xylanase production by *F. trogii* IBB 146. However, neither the laccase nor the MnP yield was significantly changed by the additional nitrogen source.

Introduction

White-rot fungi are the only group of microorganisms capable of the degradation of all the basic wood polymers, due to their capability to synthesize the relevant hydrolytic (cellulases and hemicellulases) and oxidative (ligninolytic) extracellular enzymes. These are responsible for the degradation of substrate major components, i.e. cellulose, hemicellulose and lignin into low molecular weight compounds that can be assimilated for fungal nutrition (Kirk & Farrell 1987; Eriksson *et al.* 1990). The major hydrolytic enzymes are endo-1,4-β-D-glucanase (EC 3.2.1.4), exo-1,4-β-D-glucanase (EC 3.2.1.91), and xylanase (EC 3.2.1.8). The ligninolytic enzyme complexes of white-rot fungi differ significantly in their composition. These fungi secrete one or more of three extracellular enzymes that are essential for lignin degradation: two glycosylated heme-containing peroxidases, lignin peroxidase (EC 1.11.1.14) and Mn-dependent peroxidase (EC 1.11.1.13), and a copper-containing phenoloxidase, laccase (EC 1.10.3.2). The potential application of ligninolytic enzymes in biotechnology has stimulated the investigation of their production with the purpose of selecting the most promising producers of enzymes (Elisashvili *et al.* 2001; Mikiashvili *et al.* 2004; Moldes *et al.* 2004). The physiology of ligninolytic enzymes has been extensively studied using submerged and solid-state fermentation of lignocellulosic substrates (Elisashvili *et al.* 2001; Baldrian & Gabriel 2003; Kapich *et al.* 2004; Mikiashvili *et al.* 2004; Moldes *et al.* 2004). However, not many comparative studies are available on ligninolytic enzyme production by white-rot basidiomycetes and especially little attention has been given to the evaluation of the

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hydrolytic system of these fungi. At the same time, it is important to evaluate the hydrolytic and oxidative enzyme activities of basidiomycetes under cultivation in the presence of lignocellulose, since in lignified plant substrates cellulose, hemicelluloses, and lignin are linked intra- and intermolecularly.

Since biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is the search for powerful producers of enzymes and the utilization of the potential of lignocellulosic wastes/by-products, some of which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis, ensuring efficient production of lignocellulolytic enzymes (Elisashvili *et al.* 2001; Rosales *et al.* 2002; Reddy *et al.* 2003; Kapich *et al.* 2004). Various lignocellulosic substrates and white-rot fungi have been used successfully in submerged and solid-state fermentation for lignocellulolytic enzyme production. The data obtained prove that the type and the composition of lignocellulosic substrate appear to determine the type and amount of enzyme produced by the wood-rotting basidiomycetes (Tsiklauri *et al.* 1999; Kapich *et al.* 2004; Moldes *et al.* 2004; Elisashvili *et al.* 2005). Various reports have indicated that lignocellulose fermentation method may considerably influence enzyme production (Elisashvili *et al.* 2001; Fujian *et al.* 2001). The activity of *Phanerochaete chrysosporium* ligninolytic enzymes in solid-state fermentation (SSF) of straw was far higher than in submerged fermentation (Fujian *et al.* 2001). Moreover, it has been shown that during SSF of lignocellulosic materials some fungi produce a different set of enzymes compared with synthetic liquid cultures (Vares *et al.* 1995). SSF is considered as the most appropriated method for filamentous fungi cultivation and lignocellulolytic enzyme production because they grow under conditions close to their natural habitats, due to which they may be more capable of producing certain enzymes and metabolites, which usually will not be produced or will be produced only at low yield in submerged cultures (Pandey *et al.* 1999).

Many previous studies have proved that both the nature and concentration of nitrogen sources are powerful nutrition factors regulating lignocellulolytic enzyme production by wood-rotting basidiomycetes (Zakariashvili & Elisashvili 1993; Sun *et al.* 2004). It is worth noting that the effect of these compounds depends not only on the fungi physiology but also on the cultivation medium. For example, it is well known that the model ligninolytic organism *P. chrysosporium* cultivated in synthetic medium produces lignin peroxidase and MnP only under nitrogen-limited conditions (Reddy & D'Souza 1994). However, Kapich *et al.* (2004) demonstrated that in the presence of lignocellulosic substrate a high concentration of organic nitrogen stimulates the production of these enzymes. Moreover, in these conditions they observed the highest enzyme productivity ever recorded for *P. chrysosporium*.

This paper describes the lignocellulolytic enzymes activities produced by four white-rot fungi under solid-state fermentation of beech tree leaves and wheat straw in dependence on nitrogen source in the medium.

Materials and methods

Organisms and inoculum preparation

Funalia trogii IBB 146, *Lentinus edodes* IBB 363, *Pleurotus dryinus* IBB 903, and *P. tuberregium* IBB 624 are maintained in Culture Collection of the Institute of Biochemistry and Biotechnology, Tbilisi on malt extract agar slants at 4 °C. The inoculum was prepared by growing mushrooms on a rotary shaker at 150 - rev min^{-1} and 27 °C in 250-ml flasks containing 100 ml of following synthetic medium (g l^{-1}): glucose 10; NH_4NO_3 1; KH_2PO_4 0.8; Na_2HPO_4 0.2; $MgSO_4 \cdot 7H_2O$ 0.5; yeast extract 2. The medium was adjusted to pH 6.0 with 2 M NaOH prior to sterilization. After 5 days of fungi cultivation mycelial pellets were harvested and homogenized with a Waring laboratory blender, three times 20 s with 1 min interval.

Culture conditions

The SSF of wheat straw and beech tree (*Fagus sylvatica*) beech leaves (peaces of 0.1–0.5 cm), both collected in the Brussels region, has been carried out at 27 °C in 125-ml flasks containing 4 g of lignocellulosic substrate moistened with 12 ml of the above-mentioned medium without glucose but supplemented by yeast extract at 4 g l_1. To study the effect of nitrogen sources, the same medium was used, but all nitrogen-containing inorganic and organic compounds were added to the medium in final concentrations equal to 20 mM of nitrogen. Controls without additional nitrogen source were run in parallel. The initial pH of the medium was adjusted to 6.0 prior to sterilization by adding 2 M NaOH. About 3 ml of homogenized mycelium was used to inoculate the flasks containing medium with lignocellulosic substrates. In general, all species needed at least 1 week to completely colonize the substrates. Therefore, after 7, 10 and 14 days of fungal growth the extracellular enzymes were twice extracted with 25 ml of distilled water (total volume 50 ml) on a mechanical extractor. The solids were separated by filtration through nylon cloth followed by centrifugation (7500×*g*; 15 min) at 4 °C. The supernatants obtained after biomass separations were assayed to determine the pH of extracts, and the activity of hydrolytic and oxidative enzymes.

Biomass protein estimation

The total nitrogen was determined according to the Kjeldahl method with Nessler reagent after pre-boiling of samples in 0.5% solutions of trichloroacetic acid for 15 min to remove non-protein content. True protein was calculated as the total nitrogen multiplied by 4.38.

Enzyme assays

The total cellulase activity ('filter paperase' activity, FPA) was assayed according to IUPAC recommendations by using filter paper as the substrate (Ghose 1987). A reaction mixture containing a string of filter paper (Whatman No. 1), 0.8 ml of a 50 mM citrate buffer (pH 5.0) and 0.2 ml appropriately diluted supernatant was incubated at the 40 °C for 30–120 min.

Carboxymethylcellulase (CMCase) activity was determined by mixing 70 μl appropriately diluted sample with 630 μl of 1% carboxymethylcellulose (Sigma, low viscosity) in 50 mM citrate buffer (pH 5.0) at the 40 °C for 10 min (Ghose 1987). The xylanase activity was determined by mixing 70 μl appropriately diluted sample with 630 μl of 1% birchwood xylan (Roth 7500, Karlsruhe, Germany) in 50 mM citrate buffer (pH 5.0) at 40 °C for 10 min (Bailey *et al.* 1992). Glucose and xylose standard curves were used to calculate the cellulase and xylanase activities. In all assays, the release of reducing sugars was measured using the dinitrosalicylic acid reagent method (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars per minute.

Laccase activity was determined by monitoring the A_{420} change related to the rate of oxidation of 1 mM 2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS) in 50 mM Na-acetate buffer (pH 4.2). Assays were performed in 1-ml cuvettes at 20 ± 1 °C with 50 μl of adequately diluted culture liquid. One unit of activity was defined as the amount of enzyme, which leads to the oxidation of 1 μmol of ABTS per minute.

Manganese peroxidase (MnP) activity was measured by oxidation of phenol red (Glenn & Gold 1985). The 1-ml reaction mixtures were incubated for 1–5 min at 20 ± 1 °C in the presence of 0.1 mM H_2O_2, then the reaction was terminated with 50 μl 4 M NaOH and 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 H_2O_2 were subtracted from the values obtained in the presence of hydrogen peroxide to establish the true peroxidase activity.

The extracellular enzyme activities were measured after 7, 10 and 14 days of fungal growth. The experiments were performed at least twice using three replicates. The data presented in the tables correspond to mean values of maximal enzyme activities expressed per g of extracted fermented substrate with a standard error less than 12%.

Results

Effect of nitrogen source on the fungal enzyme activity in fermentation of beech tree leaves

When beech tree leaves were used as the lignocellulosic substrate, growth of fungi was accompanied by acidification of the nutrition medium from pH 6.0–6.2 to 4.4–4.8 in the case of *L. edodes* IBB 363 and to 5.2–5.8 in the cultures with other fungi. All fungi grew well and produced significant enzyme activities when no nitrogen source was present in the medium because of the existence of nitrogen in the lignocellulosic substrate and yeast extract (Table 1). All tested nitrogen sources stimulated the growth of the mushrooms, increasing the protein content in final biomasses (after 14 days of SSF) by 89–137% as compared with the control medium. In general, maximal laccase activity was revealed after 7 days of fungal cultivation, whilst the highest activity of other enzymes accumulated after 10–14 days of beech tree leaves SSF. The effects of nitrogen sources on mushroom enzymatic activity was variable, depending on the fungus and the compound tested. A very weak positive effect of additional nitrogen source on *P. tuberregium* IBB 624 enzyme yield was observed, although the fungal biomass increased 2-fold. Peptone and $(NH_4)_2SO_4$ were the best nitrogen sources for endoglucanase and xylanase production by *L. edodes* IBB 363 and *P. dryinus* IBB 903, while KNO_3 appeared to be the most appropriate compound for the accumulation of these enzymes by *F. trogii* IBB 146. FPA of three fungi only weakly depended on the nitrogen source while in the culture of *F. trogii* IBB 146 supplemented with NH_4NO_3, the maximum value of this enzyme was 3-fold higher than in the control medium. Of all the nitrogen sources investigated, peptone, NH_4NO_3, and $(NH_4)_2SO_4$ exhibited significant influence on the laccase activity of all fungi, with a maximal value of 20 U g^{-1} in the culture of *L. edodes* IBB 363. In contrast to laccase, secretion of MnP was not stimulated by supplementation of an additional nitrogen source in the medium.

Effect of nitrogen source on fungal enzyme activity in fermentation of wheat straw

The accumulation of *P. tuberregium* IBB 624 lignocellulolytic enzymes activity in SSF of wheat straw proceeded the same manner as in fermentation of beech tree leaves. In spite of a 2-fold increase of fungal protein in the presence of the additional nitrogen sources, no significant stimulation of enzyme production was observed (Table 2). In contrast, the culture medium supplemented with 20 mM nitrogen almost 2–4-fold improved cellulase and xylanase activity of *P. dryinus* IBB 903, but did not enhanced laccase activity and apparently inhibited MnP accumulation. No increase of cellulases and xylanase yield was detected in cultures of *L. edodes* IBB 363 supplemented by an additional nitrogen source. In contrast, extracellular laccase activity of this fungus increased more than 2–3-fold as compared to the control medium. The highest MnP titre resulted when the mushroom was grown on peptone-containing medium. *F. trogii* IBB 146 growth on media with different nitrogen source showed variable levels of enzyme activity. In this culture, NH_4NO_3 supported maximum CMCase, xylanase, and laccase production. By contrast, KNO_3

Table 1. Effect of nitrogen source on fungal lignocellulolytic enzyme activities in beech tree leaves in solid-state fermentation.

Nitrogen sources	Protein (mg)	CMCase (U g^{-1})	Xylanase (U g^{-1})	FPA (U g^{-1})	Laccase (U g^{-1})	MnP (U g^{-1})
P. dryinus IBB 903						
Control	19 ± 2.0	181 ± 16	308 ± 26	24 ± 2.0	5.6 ± 0.4	0.6 ± 0.04
KNO_3	38 ± 3.9	211 ± 22	247 ± 27	25 ± 2.3	6.2 ± 0.6	0.7 ± 0.05
$(NH_4)_2SO_4$	41 ± 3.7	256 ± 20	369 ± 31	26 ± 1.9	8.4 ± 0.6	1.0 ± 0.07
NH_4NO_3	38 ± 3.0	233 ± 22	304 ± 23	18 ± 1.8	10.0 ± 0.9	0.5 ± 0.05
Peptone	44 ± 3.2	284 ± 27	341 ± 35	32 ± 3.0	12.4 ± 1.3	0.6 ± 0.07
P. tuberregium IBB 624						
Control	19 ± 1.7	41 ± 2.9	60 ± 4.7	14 ± 1.2	3.4 ± 0.3	1.4 ± 0.11
KNO_3	37 ± 2.8	36 ± 3.1	46 ± 4.0	25 ± 2.6	5.1 ± 0.4	1.4 ± 0.13
$(NH_4)_2SO_4$	36 ± 3.4	60 ± 6.2	86 ± 9.1	17 ± 1.6	3.5 ± 0.4	0.9 ± 0.08
NH_4NO_3	41 ± 3.5	53 ± 4.4	54 ± 4.1	14 ± 1.2	4.6 ± 0.4	1.5 ± 0.13
Peptone	45 ± 3.8	51 ± 5.0	88 ± 7.8	17 ± 1.8	3.8 ± 0.4	1.4 ± 0.12
L. edodes IBB 363						
Control	17 ± 1.5	60 ± 4.6	109 ± 8.7	14 ± 1.0	8.4 ± 0.6	0.2 ± 0.02
KNO_3	33 ± 2.9	87 ± 7.0	150 ± 11	16 ± 0.9	10.7 ± 0.7	0.3 ± 0.03
$(NH_4)_2SO_4$	35 ± 3.1	58 ± 6.7	114 ± 12	15 ± 1.2	18.9 ± 1.1	0.4 ± 0.03
NH_4NO_3	34 ± 2.6	76 ± 6.3	134 ± 10	17 ± 1.2	20.1 ± 1.5	0.3 ± 0.03
Peptone	37 ± 3.0	158 ± 12	183 ± 14	19 ± 1.6	14.8 ± 1.3	0.3 ± 0.03
F. trogii IBB 146						
Control	19 ± 1.7	56 ± 6.4	68 ± 7.1	6 ± 0.4	3.6 ± 0.4	0.2 ± 0.02
KNO_3	37 ± 3.9	135 ± 10	167 ± 12	6 ± 0.6	4.8 ± 0.5	0.2 ± 0.02
$(NH_4)_2SO_4$	41 ± 2.8	100 ± 9.7	107 ± 8.3	8 ± 0.9	5.4 ± 0.5	0.1 ± 0.01
NH_4NO_3	40 ± 3.8	113 ± 9.0	116 ± 10	17 ± 1.1	6.9 ± 0.5	0.1 ± 0.01
Peptone	42 ± 3.5	87 ± 6.7	106 ± 9.3	11 ± 0.9	4.1 ± 0.4	0.2 ± 0.02

Table 2. Effect of nitrogen source on fungal lignocellulolytic enzymes activity in wheat straw in solid-state fermentation.

Nitrogen sources	Protein (mg)	CMCase (U g^{-1})	Xylanase (U g^{-1})	FPA (U g^{-1})	Laccase (U g^{-1})	MnP (U g^{-1})
P. dryinus IBB 903						
Control	18 ± 1.2	135 ± 11	154 ± 16	18 ± 1.4	3.5 ± 0.4	2.0 ± 0.19
KNO_3	39 ± 4.0	306 ± 21	440 ± 32	38 ± 3.7	3.9 ± 0.4	1.1 ± 0.09
$(NH_4)_2SO_4$	42 ± 2.8	401 ± 28	602 ± 46	41 ± 3.6	4.4 ± 0.4	1.2 ± 0.09
NH_4NO_3	44 ± 3.3	341 ± 27	527 ± 43	46 ± 4.0	4.1 ± 0.3	0.9 ± 0.07
Peptone	45 ± 4.1	369 ± 23	543 ± 40	43 ± 4.2	4.2 ± 0.3	0.8 ± 0.07
P. tuberregium IBB 624						
Control	18 ± 1.7	41 ± 3.8	65 ± 6.9	24 ± 2.7	1.9 ± 0.2	1.4 ± 0.13
KNO_3	39 ± 4.1	39 ± 3.2	67 ± 5.6	27 ± 2.1	2.7 ± 0.3	2.5 ± 0.23
$(NH_4)_2SO_4$	40 ± 3.2	42 ± 2.7	59 ± 4.8	22 ± 1.9	2.6 ± 0.3	1.5 ± 0.17
NH_4NO_3	43 ± 3.8	60 ± 4.0	68 ± 4.3	30 ± 2.8	3.0 ± 0.3	1.4 ± 0.12
Peptone	42 ± 2.8	49 ± 3.2	79 ± 6.2	24 ± 2.0	2.4 ± 0.2	1.5 ± 0.16
L. edodes IBB 363						
Control	18 ± 2.0	97 ± 9.8	134 ± 15	26 ± 2.5	3.9 ± 0.3	1.4 ± 0.15
KNO_3	35 ± 3.7	80 ± 8.7	117 ± 14	22 ± 2.0	10.2 ± 0.8	1.2 ± 0.14
$(NH_4)_2SO_4$	38 ± 3.1	76 ± 6.4	98 ± 10	16 ± 1.3	12.2 ± 0.8	1.1 ± 0.09
NH_4NO_3	37 ± 2.7	84 ± 6.6	103 ± 10	25 ± 2.4	14.0 ± 1.0	0.8 ± 0.09
Peptone	39 ± 4.0	93 ± 9.7	104 ± 12	23 ± 2.5	8.6 ± 1.0	3.1 ± 0.33
F. trogii IBB 146						
Control	20 ± 1.5	203 ± 14	228 ± 15	18 ± 1.7	12.4 ± 0.8	1.0 ± 0.09
KNO_3	46 ± 5.0	208 ± 23	219 ± 23	15 ± 1.7	9.0 ± 0.7	0.9 ± 0.09
$(NH_4)_2SO_4$	50 ± 3.8	269 ± 24	274 ± 25	16 ± 1.4	10.1 ± 0.9	1.1 ± 0.09
NH_4NO_3	51 ± 4.8	330 ± 35	341 ± 38	13 ± 1.5	15.8 ± 1.7	1.2 ± 0.13
Peptone	48 ± 4.5	322 ± 32	285 ± 25	18 ± 1.4	11.9 ± 0.8	1.1 ± 0.10

was rather a poor nitrogen source for synthesis of all enzyme activities.

Effect of nitrogen source concentration on fungal enzyme activity

Subsequently, the effect of varying nitrogen concentrations on enzyme production by *P. dryinus* IBB 903 and *F. trogii* IBB 146 was studied. These fungi were selected because they appeared to be the best producers of hydrolases and oxidases in SSF of lignocellulosic substrates. By gradually increasing the nitrogen concentration from 0 to 10–40 mM, fungal protein content in the final biomass was about doubled (Table 3). In SSF of beech tree leaves CMCase and xylanase activity of *P. dryinus* IBB 903 gradually increased from 138 and 205 U g^{-1} to 308 and 344 U g^{-1}, respectively, i.e. by 2.2 and 1.7 times by increasing the peptone concentration in

the medium from 0 to 10 mM (expressed as nitrogen equivalent). Further increase in the concentration of nitrogen source did not increase these enzyme activities. Concerning FPA, the secretion of this enzyme appeared to be only slightly dependent on nitrogen content in the medium. While the variation of peptone concentration did not cause the change of MnP yield, an elevated concentration of this nutrient (20–40 mM) led to 2–3-fold increase in the laccase activity of *P. dryinus* IBB 903.

Variation of NH_4NO_3 concentration from 0 to 40 mM showed that 10–20 mM concentration of this compound was optimal for cellulase and xylanase production by *F. trogii* IBB 146 (Table 3). Neither laccase activity nor MnP activity was significantly changed by an additional nitrogen source, indicating that this nutrient does not positively affect the enzyme yield.

Discussion

In this study, we have shown that four different white-rot fungi produce cellulases, xylanase, laccase, and MnP when grown under solid-state fermentation of wheat straw and beech tree leaves. Among them, *L. edodes* secreted the highest laccase activity, while *F. trogii* IBB 146 and *P. dryinus* IBB 903 accumulated the highest amounts of hydrolases for bioconversion of lignocellulosic substrates. In fungal cultivation on lignocellulosic substrates, hydrolases play a crucial role supplying the culture with a carbon and energy source and providing microorganisms with the materials for biosynthetic activity. The low production of these enzymes may limit the rate of polysaccharide degradation into soluble sugars. In this study, the CMCase, FPA, and xylanase activities of the tested fungi appeared to be much higher than that in cultures of other basidiomycetes (Tsiklauri *et al*. 1999; Baldrian & Gabriel 2003; Reddy *et al*. 2003; Silva *et al*. 2005). As a result, *F. trogii* IBB 146 and *P. dryinus* IBB 903 showed abundant accumulation of biomass during SSF of both substrates.

In addition, we have shown that lignocellulosic substrates in the medium significantly affect the mushroom enzymatic activities. For example, when *P. dryinus* IBB 903 and *F. trogii* IBB 146 were grown in the presence of nitrogen source the substitution of beech tree leaves by wheat straw caused a significant increase (in some cases 2–3-fold) of CMCase and xylanase activity. It is worth noting that the SSF of wheat straw without additional nitrogen source decreased the maximal values of *P. dryinus* IBB 903 CMCase and xylanase activities compared with these enzyme yields obtained in fermentation of beech tree leaves. The maximal accumulation of laccase by *P. dryinus* IBB 903 in wheat straw SSF appeared to be three times lower than that in fermentation of beech leaves. Analogically, the laccase activity in SSF of barley bran by *Coriolopsis rigida* was around 25-fold higher than the value attained in the chestnut shell cultures (Gómez *et al*. 2005).

In the literature, contradictory evidence exists for the effects of the nature and concentration of the nitrogen source on ligninolytic enzyme production. While high nitrogen media gave the highest laccase activity in *L. edodes*, *Rigidoporus lignonus*, and *Trametes pubescens*, nitrogen-limited conditions enhanced the production of the enzyme in *Pycnoporus cinnabarinus*, *P. sanguineus*, and *Phlebia radiata* (Mester & Field 1997; Gianfreda *et al*. 1999; Galhaup *et al*. 2002). The role of these compounds in the regulation of enzyme synthesis depends not only on the physiology of the tested fungi but also on the medium composition, especially on presence of lignocellulosic substrate (Couto *et al*. 2004; Kapich *et al*. 2004). Tekere *et al*. (2001) showed that some *Trametes* species, *T. cingulata*, *T. elegans* and *T. pocas* produced the highest MnP activities in a medium containing high carbon and low nitrogen conditions. At the same time, high MnP activity was notable for *T. versicolor* when both carbon and nitrogen in the medium were present at high levels. Laccase production by these species was highest under conditions of high nitrogen. Sun *et al*. (2004) demonstrated that stationary cultivation conditions and low nitrogen concentration favoured MnP production by *T. gallica*, while during

Table 3. Effect of nitrogen source concentration on lignocellulolytic enzyme activities of *P. dryinus* IBB 903 and *F. trogii* IBB 146.

Nitrogen source (mM)	Protein (mg)	CMCase (U g^{-1})	Xylanase (U g^{-1})	FPA (U g^{-1})	Laccase (U g^{-1})	MnP (U g^{-1})
P. dryinus IBB 903[a]						
0	20 ± 1.6	138 ± 14	205 ± 17	18 ± 1.6	5.2 ± 0.4	0.6 ± 0.07
5	31 ± 2.2	220 ± 20	272 ± 23	20 ± 1.9	5.9 ± 0.7	0.7 ± 0.08
10	41 ± 2.9	308 ± 24	344 ± 29	21 ± 2.2	7.6 ± 0.7	0.8 ± 0.06
20	43 ± 4.1	281 ± 31	290 ± 28	23 ± 2.2	10.1 ± 1.2	0.6 ± 0.04
40	46 ± 5.0	307 ± 33	275 ± 30	26 ± 2.1	15.6 ± 1.3	0.6 ± 0.07
F. trogii IBB 146[b]						
0	21 ± 1.8	218 ± 15	281 ± 19	20 ± 1.5	13.4 ± 0.9	1.1 ± 0.12
5	30 ± 2.3	308 ± 23	298 ± 27	22 ± 1.9	15.3 ± 1.4	1.3 ± 0.11
10	43 ± 2.9	356 ± 39	371 ± 39	26 ± 2.7	16.5 ± 1.3	1.0 ± 0.07
20	47 ± 3.5	347 ± 34	426 ± 40	27 ± 2.4	12.8 ± 1.5	1.0 ± 0.10
40	47 ± 3.9	336 ± 38	437 ± 49	23 ± 2.5	12.1 ± 1.5	0.9 ± 0.11

[a] Beech tree leaves and peptone were used for SSF by *P. dryinus* IBB 903.
[b] Wheat straw and NH_4NO_3 were used for SSF by *F. trogii* IBB 146.

solid-state fermentation of wheat straw, lignocellulolytic enzyme production needed a high nitrogen content. Our study has shown that both lignocellulosic substrates tested and yeast extract, without additional nitrogen source, ensure high-level formation of all lignocellulolytic enzymes by the four different fungi tested. The additional nitrogen sources stimulated the growth of the fungi, and peptone stimulated the growth most extensively. The yield of lignocellulolytic enzymes largely varied in cultures of basidiomycetes when they were grown under different nitrogen sources. In general, the yields of hydrolytic enzymes and laccase were increased by supplementation of the medium with an additional nitrogen source. However, the comparison of the specific activities of the tested cultures evidenced that the stimulating effect of additional nitrogen on enzyme accumulation may simply be due to a higher biomass production. Moreover, our calculations showed that the addition of nitrogen source repressed production of all enzymes by *P. tuberregium* IBB 624 as well as the production of MnP by all tested mushrooms. Only xylanase specific activity of *P. dryinus* IBB 903 and laccase specific activity of *L. edodes* IBB 363 was increased significantly (by 66% and 73%, respectively) in SSF of wheat straw by addition of nitrogen to the control medium.

In general, this and other studies (Tsiklauri *et al.* 1999; Kapich *et al.* 2004; Moldes *et al.* 2004; Gómez *et al.* 2005; Silva *et al.* 2005) underline the need to explore not only more organisms but also lignocellulosic substrates with different composition to express and evaluate the real potential of fungi producing hydrolases and oxidases. In addition, the medium composition must be optimized for each enzyme producer.

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