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ORIGINAL PAPER

Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes

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Abstract The exploration of seven physiologically different white rot fungi potential to produce cellulase, xylanase, laccase, and manganese peroxidase (MnP) showed that the enzyme yield and their ratio in enzyme preparations significantly depends on the fungus species, lignocellulosic growth substrate, and cultivation method. The fruit residues were appropriate growth substrates for the production of hydrolytic enzymes and laccase. The highest endoglucanase (111 U ml^{-1}) and xylanase (135 U ml^{-1}) activities were revealed in submerged fermentation (SF) of banana peels by *Pycnoporus coccineus*. In the same cultivation conditions *Cerrena maxima* accumulated the highest level of laccase activity (7,620 U l^{-1}). The lignified materials (wheat straw and tree leaves) appeared to be appropriate for the MnP secretion by majority basidiomycetes. With few exceptions, SF favored to hydrolases and laccase production by fungi tested whereas SSF was appropriate for the MnP accumulation. Thus, the *Coriolopsis polyzona* hydrolases activity increased more than threefold, while laccase yield increased 15-fold when tree leaves were undergone to SF instead SSF. The supplementation of nitrogen to the control medium seemed to have a negative effect on all enzyme production in SSF of wheat straw and tree leaves by *Pleurotus ostreatus*. In SF peptone and ammonium containing salts significantly increased *C. polyzona* and *Trametes versicolor* hydrolases and laccase yields. However, in most cases the supplementation of media with additional nitrogen lowered the fungi specific enzyme activities. Especially strong repression of *T. versicolor* MnP production was revealed.

Keywords White-rot basidiomycetes · Lignocellulose fermentation · Cellulase · Laccase · Manganese peroxidase

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Introduction

White-rot basidiomycetes are unique in their ability to degrade most components of wood due to their capability to synthesize the relevant hydrolytic and oxidative extracellular enzymes. 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 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 lignocellulolytic enzymes of basidiomycetes are of fundamental importance for the efficient bioconversion of 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. The potential applications of lignocellulolytic enzymes in industrial and environmental technologies require huge amounts of these enzymes at low cost. Therefore, there is need to select new organisms with tremendous synthesis of these enzymes and to

develop strategies for their overproduction. One of the appropriate approaches for this purpose is to utilize the potential of lignocellulosic wastes, some of which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis ensuring efficient production of ligninolytic enzymes [5, 22–24]. Thus, barley bran increased *Trametes versicolor* laccase activity almost 50-fold compared to the control culture with glucose [18]. Furthermore, isoenzymes proportion significantly depended on the type of lignocellulosic substrate in nutrient medium [14, 18]. In contrast to lignin-degrading enzymes, the information on basidiomycetes hydrolases is scarce. Moreover, little attention has been given to the study of simultaneous production of the hydrolytic and oxidative enzymes by these fungi [12, 15, 19, 25].

Among several approaches used to enhance lignocellulolytic enzyme synthesis in fermentation of plant raw materials were the supplementation of nutrient media with nitrogen sources and inducers [5, 9, 21, 25]. High nitrogen containing media gave the highest laccase activity in *Lentinus edodes*, *Rigidoporus lignonus*, *Trametes pubescens*, and *T. versicolor* while nitrogen-limited conditions enhanced the production of *Pycnoporus cinnabarinus*, *P. sanguineus*, and *Phlebia radiata* enzymes [9, 16]. Tekere et al. [26] 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. 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. It is clear that these data reflect the physiological diversity of fungi tested. As it turned out, the effect of nitrogen source on enzyme synthesis depends not only on the fungus physiology but also on the medium composition, especially on the presence of lignocellulosic substrate [2, 13]. Thus, lignin peroxidase and MnP production by *Phanerochaete chrysosporium* is completely suppressed by high nitrogen concentration in synthetic medium. However, in the lignocellulose-containing medium the presence of high concentration of peptone (3–4 g l^{-1}) was prerequisite for high production of ligninolytic peroxidases [13]. Sun et al. [25] showed that *T. gallica* needs a high nitrogen content to synthesize xylanase, laccase, and MnP under the SSF of wheat straw. Nevertheless, comprehensive data about the nitrogen source effect on the production of lignocellulolytic enzymes by many white-rot fungi are still lacking.

This study for the first time evaluates the lignocellulolytic enzyme activity produced by seven white-rot fungi under both submerged and solid-state fermentation of various lignocellulosic residues. The effect of additional nitrogen source on the enzymes production by selected fungi was also assessed.

Materials and methods

Lignocellulosic substrates

Wheat straw and tree leaves (*Fagus sylvatica*) were collected in Brussels region. Mandarin, banana, and apple peals were available from the Brussels market. All residues were dried at 50 °C and milled for SF, while for SSF they were chopped in pieces of 0.2–1.0 cm.

Organisms and inoculum preparation

The following white-rot fungi were used in this study: *Cerrena maxima* IBB 681, *Funalia trogii* IBB 146, *Trametes pubescens* IBB 663, and *T. versicolor* IBB 897 from the Culture Collection of the Institute of Biochemistry and Biotechnology (Tbilisi), *Coriolopsis polyzona* 38443, *Pycnoporus coccineus* 38527 from the Culture Collection of the Free University of Brussels. *Pleurotus ostreatus* 2191 was purchased from the company "Mycelia" (Gent, Belgium). Fungal inocula were prepared by growing mushrooms on a rotary shaker at 150 rpm and 27 °C in 250-ml flasks containing 100 ml of following standard medium: glucose, 10.0 g l^{-1}; NH_4NO_3, 1.0 g l^{-1}; KH_2PO_4, 0.8 g l^{-1}; Na_2HPO_4, 0.2 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1}; yeast extract, 2.0 g l^{-1}. The medium was adjusted to pH 6.0–6.2 with 2 M NaOH. After 5–6 days of fungi cultivation mycelial pellets were harvested and homogenized with a Waring laboratory blender. The same identical inoculum was used to conduct the SF and SSF of selected lignocellulosic materials.

Culture conditions

Solid-state fermentation (SSF) of selected residues has been carried out at 27 °C in 125-ml flasks containing 4 g of lignocellulosic substrate moistened with 12 ml of above-mentioned medium without glucose but supplemented with 4 g l^{-1} yeast extract. To study the effect of nitrogen sources, all nitrogen containing inorganic and organic compounds were added to the medium in final concentration equal to 20 mM of nitrogen. Control without additional nitrogen source was run in parallel. The initial pH of the medium was adjusted to 6.0 prior to sterilization by adding 2 M NaOH. Three ml of homogenized mycelium was used to inoculate the flasks containing media with lignocellulosic substrates. After 7, 10, and 14 days of fungal growth the extracellular enzymes were extracted on a mechanical extractor two times with 25 ml of distilled water (total volume 50 ml). The solids were separated by filtration through nylon cloth followed by centrifugation at 6,000×*g* for 15 min at 4 °C.

Submerged fermentation (SF) of lignocellulosic substrates has been carried out on a rotary shaker at 150 rpm and 27 °C in 125-ml flasks containing 50 ml of above-mentioned medium with 40 g l^{-1} of lignocellulose instead glucose. To study the effect of nitrogen sources, all nitrogen containing compounds were added to the medium in final concentrations equal to 10 mM of nitrogen. Control without additional nitrogen source was run in parallel. The initial pH of the medium was adjusted to 6.0 prior to sterilization by adding 2 M NaOH. Three ml of mycelial homogenate was used to inoculate the flasks containing media with lignocellulosic substrates. After 3, 5, 7, and 10 days of mushrooms cultivation, when the cultures were at the beginning, middle, and end of logarithmic phase and at stationary phase of growth, respectively, biomasses were filtered and the solids were separated by centrifugation at 6,000×*g* for 15 min at 4 °C.

Biomass protein estimation

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

Enzyme assays

The supernatants received after biomass separation were analyzed for pH, reducing sugars content, and enzyme activity. The total cellulase activity (filter paper activity, FPA) was assayed according to IUPAC recommendations by using filter paper as the substrate [10]. 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 40 °C for 30–120 min. Carboxymethyl cellulase (CMCase) activity was determined by mixing 70 µl appropriately diluted sample with 630 µl of carboxymethylcellulose low viscosity (1% w/v) in 50 mM citrate buffer (pH 5.0) at 40 °C for 10 min [10]. Xylanase activity was determined by mixing 70 µl appropriately diluted sample with 630 µl of birch wood xylan (Roth 7500) (1% w/v) in 50 mM citrate buffer (pH 5.0) at 40 °C for 10 min [1]. 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 [17]. 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-ethyltiazoline-6-sulfonate] (ABTS) in 50 mM Na-acetate buffer (pH 4.0). Assays were performed in 1 ml cuvette at 20 ± 1 °C with 50 µl of adequately diluted culture liquid. One unit activity was defined as the amount of enzyme, which leads to the oxidation of 1 µmol of ABTS per minute.

MnP activity was measured by oxidation of phenol red [11]. 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, 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.

To compare the enzyme activity of fungi grown in submerged (SF) and solid-state conditions (SSF) all enzyme activities were expressed in international units per ml or per liter of culture liquid. The experiments were performed at least two times using three replicates. The data presented in the tables correspond to mean values with a standard error less than 15%.

Results and discussion

Species-dependent enzyme production

The white-rot basidiomycetes have a capability to produce simultaneously the hydrolytic and ligninolytic enzymes in fermentation of lignocellulose [7, 12, 25]. In this study, the activity of main lignocellulose-degrading enzymes of seven white-rot fungi was evaluated for the first time in both submerged and solid-state fermentation of five readily available plant residues. The data represented in Tables 1 and 2 show that the enzyme yield significantly depended on the fungus species although all fungi exhibited quite different responses to lignocellulosic substrates used. In the SSF of lignocellulosic materials the highest CMCase (62 U ml^{-1}) and xylanase (64 U ml^{-1}) activities were revealed in *T. versicolor* followed by *F. trogii* and *T. pubescens* (Table 1). It is interesting that all fungi expressed appreciable FPA with the highest in culture of *C. maxima.* The maximum laccase and MnP activities varied from 273 U l^{-1} (*C. maxima*) to 988 U l^{-1} (*F. trogii*) and from 152 U l^{-1} (*C. maxima*) to 685–690 U l^{-1} (*F. trogii*, *T. pubescens*, *T. versicolor*), respectively.

In SF of lignocelluloses CMCase and xylanase activities of studied basidiomycetes varied from 9 U ml^{-1} (*T. versicolor*) to 111 U l^{-1} (*P. coccineus*) and from 14 U l^{-1} (*C. polyzona*) to 135 U l^{-1} (*P. coccineus*), respectively, while FPA activity ranged from 1.5 to 7.1 U ml^{-1} (Table 2). These data indicate that in appropriate cultivation conditions some basidiomycetes species are excellent

Table 1 White-rot fungi enzyme activity in lignocellulosic residues solid-state fermentation

Species	Substrate	CMCase (U ml^{-1})	Xylanase (U ml^{-1})	FPA (U ml^{-1})	Laccase (U l^{-1})	MnP (U l^{-1})
C. maxima	Tree leaves	19 ± 0.5	35 ± 3.1	1.8 ± 0.2	253 ± 24	152 ± 18
	Wheat straw	4 ± 0.4	3 ± 0.3	1.3 ± 0.1	33 ± 4	21 ± 3
	Apple peels	24 ± 3.1	53 ± 4.3	4.3 ± 0.4	183 ± 12	55 ± 5
	Banana peels	21 ± 2.2	41 ± 3.3	5.3 ± 0.4	273 ± 18	81 ± 9
C. polyzona	Tree leaves	9 ± 0.8	7 ± 0.6	1.0 ± 0.1	119 ± 15	454 ± 37
	Wheat straw	5 ± 0.4	3 ± 0.3	1.2 ± 0.1	27 ± 2	31 ± 4
	Apple peels	16 ± 2.1	17 ± 2.0	1.8 ± 0.2	173 ± 19	308 ± 22
	Banana peels	21 ± 2.1	23 ± 2.9	3.0 ± 0.3	290 ± 31	375 ± 44
F. trogii	Tree leaves	10 ± 0.8	16 ± 3.5	1.7 ± 0.2	458 ± 54	303 ± 41
	Wheat straw	24 ± 1.2	23 ± 1.7	1.3 ± 0.2	760 ± 70	639 ± 58
	Apple peels	35 ± 3.0	47 ± 6.2	3.7 ± 0.5	211 ± 24	685 ± 77
	Banana peels	55 ± 6.4	51 ± 6.0	3.6 ± 0.3	988 ± 74	165 ± 12
P. coccineus	Tree leaves	17 ± 1.9	18 ± 1.7	1.0 ± 0.1	167 ± 14	0
	Wheat straw	18 ± 1.9	19 ± 1.8	2.2 ± 0.2	252 ± 21	0
	Apple peels	18 ± 2.0	17 ± 2.0	2.0 ± 0.2	404 ± 47	0
	Banana peels	32 ± 4.1	37 ± 3.8	2.5 ± 0.3	573 ± 47	0
P. ostreatus	Tree leaves	26 ± 2.2	29 ± 1.8	2.6 ± 0.2	289 ± 36	234 ± 38
	Wheat straw	13 ± 1.6	15 ± 1.8	1.6 ± 0.2	339 ± 40	203 ± 17
	Apple peels	12 ± 0.8	17 ± 2.3	3.1 ± 0.3	231 ± 19	164 ± 14
	Banana peels	17 ± 2.1	26 ± 3.1	3.4 ± 0.4	183 ± 24	198 ± 35
T. pubescens	Tree leaves	29 ± 3.1	26 ± 2.8	4.3 ± 0.4	205 ± 25	464 ± 42
	Wheat straw	13 ± 1.3	15 ± 1.4	2.7 ± 0.3	162 ± 20	149 ± 16
	Apple peels	24 ± 3.0	28 ± 2.9	3.6 ± 0.4	280 ± 23	227 ± 29
	Banana peels	45 ± 6.1	64 ± 7.4	3.0 ± 0.3	188 ± 20	690 ± 73
T. versicolor	Tree leaves	22 ± 2.5	38 ± 4.1	4.1 ± 0.4	662 ± 71	160 ± 19
	Wheat straw	5 ± 0.6	3 ± 0.3	1.2 ± 0.1	137 ± 14	179 ± 20
	Apple peels	45 ± 5.8	64 ± 7.0	3.0 ± 0.3	188 ± 20	690 ± 77
	Banana peels	62 ± 5.3	58 ± 4.1	4.1 ± 0.4	203 ± 24	120 ± 13

producers of CMCase and xylanase. Moreover, in contrast to *Ganoderma australe* [7] the tested fungi accumulated many-fold higher yields of total cellulase activity. The capacity of these basidiomycetes to produce high levels of cellulases and hemicellulases is important to supply the growing cultures with materials essential for their biosynthetic activity. *C. maxima* appeared to be the best producer of laccase (7,620 U l^{-1}) in SF of lignocelluloses accumulating 12-fold higher enzyme activity as compared with *P. ostreatus* (Table 2). This appreciable laccase activity was obtained in absence of specific aromatic compound or microelement. Hence, *C. maxima* has high potential as an efficient producer of cheap laccase. In the same cultivation conditions, MnP activity of tested fungi varied from 1,263 U l^{-1} (*F. trogii*) to 55 U l^{-1} (*P. ostreatus*).

Effect of the lignocellulosic substrate

All lignocellulosic substrates tested in this study promoted an excellent growth of fungi. The first well visible signs of growth in SSF were seen 2 days after inoculation and total colonization of substrates was completed within 7–9 days. The SF of these substrates by tested fungi occurred in form of pellets. In general, the enzyme activity appeared after 2–3 days of cultivation and gradually increased achieving a maximal values on days 7–10 during SF and on days 10–14 of SSF. As it was indicated, the levels of extracellular enzyme activities produced during fermentation of different plant raw materials varied among the fungi studied, but several general features may be noted (Tables 1, 2). Firstly, the fruit residues are appropriate growth substrates for the production of hydrolytic enzymes in both SF and SSF by five basidiomycetes species. On the contrary, tree leaves ensured highest CMCase and xylanase activities of *P. ostreatus* (in SSF). Secondly, fungi distinguished with their response to growth substrate supplementation. Thus, the CMCase activity of *T. versicolor* varied from 5 to 62 U ml^{-1} whereas that of *P. coccineus* fluctuated only from 17 to 32 U ml^{-1} depending on lignocellulosic material (Table 1). The maximum activity of hydrolytic enzymes

Table 2 Basidiomycetes enzyme activity in lignocellulosic residues submerged fermentation

Species	Substrate	CMCase (U ml^{-1})	Xylanase (U ml^{-1})	FPA (U ml^{-1})	Laccase (U l^{-1})	MnP (U l^{-1})
C. maxima	Tree leaves	27 ± 3	21 ± 2	2.7 ± 0.2	1,153 ± 134	171 ± 14
	Mandarin peels	87 ± 10	29 ± 3	4.9 ± 0.4	2,671 ± 38	113 ± 10
	Apple peels	25 ± 3	18 ± 2	3.5 ± 0.3	4,701 ± 502	262 ± 29
	Banana peels	26 ± 3	15 ± 2	3.4 ± 0.3	7,620 ± 883	367 ± 30
C. polyzona	Tree leaves	32 ± 3	33 ± 4	2.8 ± 0.3	1,780 ± 191	896 ± 97
	Mandarin peels	94 ± 9	98 ± 8	5.6 ± 0.6	769 ± 72	516 ± 61
	Apple peels	14 ± 2	18 ± 2	1.5 ± 0.2	67 ± 7	46 ± 5
	Banana peels	39 ± 4	14 ± 1	1.9 ± 0.2	119 ± 13	124 ± 11
F. trogii	Tree leaves	42 ± 3	48 ± 3	3.6 ± 0.3	1,417 ± 119	1,263 ± 147
	Mandarin peels	65 ± 5	109 ± 8	7.1 ± 0.5	2,564 ± 190	817 ± 77
	Apple peels	39 ± 4	71 ± 5	3.6 ± 0.4	2,832 ± 339	116 ± 9
	Banana peels	70 ± 8	118 ± 14	6.4 ± 0.8	901 ± 67	Traces
P. coccineus	Tree leaves	35 ± 4	48 ± 5	4.3 ± 0.3	46 ± 5	0
	Mandarin peels	38 ± 4	70 ± 9	3.8 ± 0.4	428 ± 51	0
	Apple peels	37 ± 4	83 ± 6	2.3 ± 0.3	1,418 ± 50	0
	Banana peels	111 ± 13	135 ± 12	4.8 ± 0.6	2,619 ± 57	0
P. ostreatus	Tree leaves	15 ± 2	29 ± 3	3.0 ± 0.4	73 ± 7	0
	Mandarin peels	13 ± 1	32 ± 4	3.3 ± 0.4	340 ± 36	Traces
	Apple peels	18 ± 2	36 ± 3	3.5 ± 0.4	507 ± 37	Traces
	Banana peels	27 ± 5	83 ± 5	5.9 ± 0.6	631 ± 78	55 ± 6
T. pubescens	Tree leaves	64 ± 5	56 ± 6	2.7 ± 0.3	630 ± 54	81 ± 9
	Mandarin peels	49 ± 6	81 ± 9	3.7 ± 0.4	1,084 ± 92	Traces
	Apple peels	30 ± 3	39 ± 5	3.8 ± 0.3	834 ± 61	96 ± 7
	Banana peels	29 ± 3	38 ± 3	4.0 ± 0.3	1,680 ± 190	172 ± 13
T. versicolor	Tree leaves	29 ± 3	45 ± 5	5.1 ± 0.5	769 ± 84	202 ± 15
	Mandarin peels	42 ± 4	67 ± 6	5.6 ± 0.5	3,008 ± 325	127 ± 13
	Apple peels	9 ± 1	21 ± 3	2.1 ± 0.2	540 ± 59	81 ± 9
	Banana peels	38 ± 3	45 ± 5	4.8 ± 0.4	1,294 ± 149	109 ± 12

expressed by each of tested fungi in SF varied two- to six-fold with substitution of growth substrate.

Evaluation of the fungi ligninolytic enzymes activity showed that fruit residues are also appropriate growth substrates for the laccase production. This observation agrees with the recently reported findings [22–24]. A substitution of banana peels with wheat straw in SSF caused eight- and tenfold decrease of *C. maxima* and *C. polyzona* laccase activity, respectively. At the same time, tree leaves and wheat straw provided the highest laccase activity of *T. versicolor* and *P. ostreatus*, respectively. Especially distinct data on the role of complex lignocellulosic substrates in laccase secretion were obtained when measuring enzyme activity in SF. For example, *P. coccineus* laccase activity varied from 46 U l^{-1} in medium with tree leaves to 2,619 U l^{-1} in banana peels containing medium (Table 2). The lignified materials, wheat straw and tree leaves, appeared to be appropriate for the MnP secretion by majority basidiomycetes. Thus, *F. trogii* MnP activity varied from traces in medium with banana peels to 1,263 U l^{-1} in tree leaves containing medium (Table 2).

The fact that the fruit residues yielded highest cellulase and xylanase activities, as compared with lignified wheat straw and tree leaves, may be related to their composition, namely to the presence of high concentrations of soluble sugars and to the easy availability of their polysaccharides which promoted an abundant growth of fungi. On the other hand, it is not inconceivable that these lignocellulosic materials contain specific aromatic compounds or microelements liberating during fermentation or some specific compounds appeared during substrates fermentation and stimulated ligninolytic enzyme synthesis. Thus, the presence of extractive substances, derived from straw, was essential for the production of MnP by *Phanerochaete chrysosporium* [13].

D'Souza et al. [4] showed that *Ganoderma lucidum* produced laccase as the major enzyme during pine wood decay, whereas MnP was the major enzyme in poplar

containing culture. Like these and other data [13, 18, 24], our results indicate that the ratio of individual enzymes in final preparation depends on the type of growth substrate. Thus, the laccase/CMCase ratio changed from 283 to 31 with substitution of banana peels with mandarin peelings in SF by *C. maxima*. Moreover, the laccase/MnP ratio changed from 4:1 to 1:3 with substitution of tree leaves by banana peels in SSF by *T. versicolor*. Only traces of MnP were produced during SF of banana peals by *F. trogii* whereas the highest enzyme level (1,263 U l^{-1}) accumulated in fermentation of tree leaves. Hence, these data prove that the fungus-specific growth substrate should be selected to maximally express the target enzyme activity.

Effect of the cultivation method

Few reports indicate that the lignocellulose fermentation method may considerably influence the enzyme production by white-rot fungi [6, 8]. Sun et al. [25] showed that no MnP activity could be detected under agitated cultures of *Trametes gallica*. Low enzyme activity could be obtained while the fungus was grown in the stationary liquid culture. The substitution of stationary cultivation with the SSF of wheat straw provided more than tenfold increase of MnP activity. On the contrary, laccase level observed in agitated culture was much greater than that seen in stationary conditions. In shake culture of *Panus tigrinus* no measurable xylanase and laccase activities were observed in contrast to the marked enzyme activity under static cultural conditions [19]. Our study also underlines that the expression of basidiomycetes biosynthetic potential highly depends on the method of fungi cultivation. The SSF is considered as the most appropriated method for basidiomycetous fungi cultivation because they grow under conditions close to their natural habitats [20]. Indeed, the SFF promoted abundant growth of fungi and enzyme production. The comparison of volumetric enzyme activities indicates that the SSF of tree leaves was appropriate for the laccase production by *P. coccineus* and *P. ostreatus*, while SSF of apple and banana peels favored to this enzyme accumulation by *C. polyzona* as compared with SF (Tables 1, 2). The SSF of substrates tested was essential for MnP production by most fungi. Moreover, *T. pubescens* and *T. versicolor* gave the highest MnP yields in SSF of banana and apple peelings, respectively. In this case, the maximum MnP activity of *Trametes* species was more than threefold higher than that achieved in SF of lignocellulosic residues. Furthermore, no MnP was detected in SF of tree leaves by *P. ostreatus* and only traces or very low enzyme activity was revealed in SF of other substrates. Regarding hydrolytic enzymes, the SSF was preferable only for the *C. maxima* xylanase and *T. versicolor* CMCase and xylanase secretion in fermentation of apple and banana peels, respectively. As compared with SSF, the SF of lignocellulosic residues favored to the maximum hydrolases and laccase accumulation by all other fungi tested.

It is worth noting that the enzyme productivity/g substrate in SF was much higher taking into account that in this case the substrate quantity was two times less as compared with that in SFF. Moreover, the maximum of enzyme activity in SF was reached 3–5 days earlier as compared with SFF.

Effect of nitrogen source

The data submitted in Table 3 show that in SSF of tree leaves or wheat straw the supplementation of media with peptone as the additional nitrogen source provided twofold increase of protein content compared to control medium. The supplementation of media with an additional nitrogen source in some cases significantly affected the enzyme yield. Thus, in SSF of tree leaves peptone increased *P. ostreatus* CMCase and xylanase activities from 20 U ml^{-1} to 28–35 U ml^{-1}, whereas $(NH_4)_2SO_4$ diminished those to 13–17 U ml^{-1} although the fungus biomass protein gain was almost the same. All nitrogen sources, especially peptone, considerably decreased MnP yield. In SSF of wheat straw no significant effect of additional nitrogen on CMCase and xylanase yield was detected. At the same time, $(NH_4)_2SO_4$ and peptone twofold increased FPA and augmented laccase and MnP yields by 64 and 51%, respectively. However, the enzymes specific activity comparison evidences that this positive effect of additional nitrogen on enzyme accumulation is due to the higher biomass yield. It is obviously that in SSF of both substrates by *P. ostreatus* the supplementation of nitrogen to the control medium seemed to have a negative effect on all enzyme production. Even the specific CMCase and xylanase activities (41 U mg^{-1} and 52 U mg^{-1} protein, respectively) reached in SSF of tree leaves in presence of peptone appeared to be lower than those (56 U mg^{-1} protein) in control medium. With the supplementation of nitrogen to control medium the specific laccase and MnP activities decreased from 0.78 U mg^{-1} to 0.37–0.58 U mg^{-1} protein and from 0.84 U mg^{-1} to 0.26–0.42 U mg^{-1} protein, respectively. These data disagree with finding that the addition of organic nitrogen (such as tryptone, peptone, and yeast extract) into culture media manifold improved the *Trametes gallica* laccase specific activity [3].

In SF of tree leaves by *C. polyzona* and mandarin peels by *T. versicolor* the yields of true protein in final biomasses obtained in the presence of additional nitrogen sources increased by 48–65% as compared with control medium (Table 4). The addition of peptone to the control medium twofold increased CMCase and xylanase activity in tree leaves SF by *C. polyzona*. Cultures containing $(NH_4)_2SO_4$

Table 3 Effect of nitrogen source on *P. ostreatus* 2191 lignocellulolytic enzymes activity in solid-state fermentation of tree leaves and wheat straw

Nitrogen sources	Protein gain (mg/flask)	CMCase (U ml^{-1})	Xylanase (U ml^{-1})	FPA (U ml^{-1})	Laccase (U l^{-1})	MnP (U l^{-1})
Tree leaves						
Control	18 ± 2	20 ± 2	20 ± 2	1.4 ± 0.1	281 ± 35	304 ± 29
KNO_3	27 ± 3	17 ± 2	26 ± 3	1.4 ± 0.1	234 ± 21	207 ± 24
$(NH_4)_2SO_4$	30 ± 3	13 ± 1	17 ± 2	1.6 ± 0.2	329 ± 31	221 ± 16
NH_4NO_3	29 ± 2	23 ± 2	25 ± 2	1.7 ± 0.1	336 ± 29	274 ± 27
Peptone	34 ± 3	28 ± 3	35 ± 3	1.5 ± 0.1	252 ± 28	179 ± 20
Wheat straw						
Control	16 ± 2	10 ± 1	15 ± 2	1.3 ± 0.1	311 ± 36	290 ± 32
KNO_3	25 ± 3	13 ± 1	13 ± 1	1.4 ± 0.1	275 ± 34	317 ± 23
$(NH_4)_2SO_4$	27 ± 3	12 ± 1	18 ± 1	2.6 ± 0.3	551 ± 42	225 ± 25
NH_4NO_3	28 ± 3	15 ± 1	17 ± 1	2.0 ± 0.2	293 ± 25	268 ± 33
Peptone	33 ± 3	13 ± 1	20 ± 2	2.6 ± 0.2	357 ± 29	437 ± 40

Table 4 Effect of nitrogen source on fungi lignocellulolytic enzymes activity in submerged fermentation of lignocellulose

Nitrogen sources	Protein gain (mg $flask^{-1}$)	CMCase (U ml^{-1})	Xylanase (U ml^{-1})	FPA (U ml^{-1})	Laccase (U l^{-1})	MnP (U l^{-1})
C. polyzona 38,443 (tree leaves)						
Control	37 ± 4	15 ± 2	18 ± 2	1.6 ± 0.2	1,096 ± 132	482 ± 43
KNO_3	55 ± 4	20 ± 2	23 ± 2	2.5 ± 0.2	1,313 ± 138	591 ± 48
$(NH_4)_2SO_4$	57 ± 6	21 ± 2	23 ± 3	1.9 ± 0.2	1,675 ± 158	602 ± 67
NH_4NO_3	59 ± 7	26 ± 3	29 ± 2	2.1 ± 0.2	1,924 ± 220	762 ± 71
Peptone	60 ± 5	30 ± 3	39 ± 4	2.6 ± 0.3	1,884 ± 160	718 ± 78
T. versicolor IBB 897 (mandarin peels)						
Control	46 ± 5	30 ± 3	56 ± 7	5.3 ± 0.4	894 ± 95	201 ± 22
KNO_3	68 ± 8	34 ± 4	41 ± 7	6.0 ± 0.5	1,479 ± 117	87 ± 10
$(NH_4)_2SO_4$	73 ± 7	47 ± 3	82 ± 6	6.8 ± 0.8	3,801 ± 253	234 ± 18
NH_4NO_3	69 ± 5	46 ± 3	72 ± 4	5.9 ± 0.7	2,736 ± 301	148 ± 17
Peptone	76 ± 7	39 ± 4	65 ± 5	7.2 ± 0.7	4,108 ± 494	153 ± 14

and peptone gave the highest laccase activity (3,800–4,100 U l^{-1}) in mandarin peels SF by *T. versicolor*. These values were more than fourfold higher than that achieved in the control culture. It is interesting that the specific MnP activity of *T. versicolor* decreased from 0.22 U mg^{-1} protein in control medium to 0.06–0.16 U mg^{-1} protein in the presence of nitrogen sources while the supplementation of control medium with nitrogen source increased the specific laccase activity of *T. versicolor* from 0.97 U mg^{-1} to 1.09–2.70 U mg^{-1} protein. Thus, the response of *T. versicolor* on addition of comparatively high nitrogen concentration to the medium was similar to that of *Phellinus robustus* [24].

In conclusion, this study indicates the need to explore more lignocellulosic materials with different composition to select growth substrate adequate for target enzyme synthesis with the aim to fully express and correctly evaluate the lignocellulolytic potential of fungi. Utilization of some residues provides an opportunity to produce simultaneously high yields of lignocellulolytic enzymes in simple medium without supplementation of the culture medium with specific inducers. However, further studies are required to elucidate the reason by which some complex substrates stimulate enzyme production.

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