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Screening of Novel Basidiomycetes for the Production of Lignocellulolytic Enzymes During Fermentation of Food Wastes

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Abstract

Nine species of white-rot basidiomycetes isolated from the forests of Georgia have been screened for the endoglucanase, xylanase, laccase, and manganese peroxidase production in both glycerol-containing synthetic medium and mandarin peels-based medium. *Irpex lacteus* and *Trametes ochracea* expressed the highest hydrolases activity in submerged and solid-state fermentation (SSF), respectively, of ethanol production residue (EPR). *Lenzites betulina* appeared to be very promising producer of laccase in both synthetic (52.5 U ml^{-1}) and complex (76.7 U ml^{-1}) media. The tested white-rot fungi exhibited quite different responses to growth substrates used. Mandarin peelings, ethanol production residue, and wheat bran appeared to be excellent growth substrates for the production of complex preparations of hydrolases and oxidases. The same substrates as well as walnut pericarp enhanced laccase and MnP secretion. In addition, the ratio of individual enzymes in final preparations significantly depended on growth substrate. Thus, the laccase/MnP ratio changed from 3 to 548 with substitution of EPR by walnut pericarp in submerged fermentation by *L. betulina*. Usually, the submerged fermentation of lignocellulosic materials was preferable for the target enzyme secretion. However, the SSF of wheat bran was essential for the CMCase production by *I. lacteus*, *L. betulina*, and *S. hirsutum*. The SSF of EPR was preferable for the laccase production by *G. resinaceum*, *L. betulina*, and *T. ochracea* while the SSF of wheat bran favored MnP accumulation as compared to submerged cultivation.

Keywords: Basidiomycetes, cellulase, xylanase, laccase, manganese peroxidase, lignocellulosic materials, fermentation.

Introduction

Basidiomycetes comprise very different ecological groups of white rot, brown rot, and leaf litter fungi that may insure their nutrition in different ways. White-rot basidiomycetes are the only group of organisms capable of degrading all basic wood polymers due to their capability to synthesize relevant hydrolytic (cellulases and hemicellulases) and oxidative (ligninolytic) extracellular enzymes, which are responsible for the degradation of substrate's major components, i.e., cellulose, hemicellulose, and lignin into low-molecular-weight compounds that can be assimilated for fungi nutrition (Kirk and Farrell, 1987; Eriksson et al., 1990). Their major hydrolytic enzymes are endo-1,4-ß-D-glucanase (EC 3.2.1.4), exo-1,4-ß-D-glucanase (EC 3.2.1.91), and xylanase (EC 3.2.1.8). In addition, 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), versatile peroxidase (EC 1.11.1.16), and laccase (EC 1.10.3.2). These lignocellulose-degrading enzymes of basidiomycetes are of fundamental importance for the efficient bioconversion of plant residues and they are promising for a great variety of biotechnological applications, including food, pulp and paper, textile, and dye industries, bioremediation, agriculture, and analytical biochemistry (Durán et al., 2002; Minussi et al., 2002; Wesenberg et al, 2003). The application of lignocellulolytic enzymes in industrial and environmental technologies requires significant amounts of these enzymes at low cost. Therefore, the increasing demand for lignocellulose-degrading enzymes has intensified the search for fungi with outstanding enzyme activity. Recently, several species of white rot fungi have been studied from both a basic and an applied viewpoint, some of them having shown high potential for the production of individual groups of hydrolytic and oxidative enzymes (Machuca and Ferraz, 2001; Galhaup et al., 2002; Elisashvili et al., 2006, 2008; Papinutti and Forchiassin, 2007; Osma et al., 2007; Levin et al., 2008). Further research

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using lignocellulosic wastes as the growth substrate holds promise since some of them may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis, ensuring efficient production of lignocellulolytic enzymes (Machuca and Ferraz, 2001; Rosales et al., 2005; Kachlishvili et al., 2006; Songulashvili et al., 2006).

The selection of appropriate plant residues suitable for fungal growth and target enzyme synthesis is a key step in the development of an efficient technology of enzyme production. Various raw plant materials have been used successfully for the lignocellulolytic enzyme production in submerged and solid-state fermentations (Reddy et al., 2003; Kachlishvili et al., 2006; Elisashvili et al., 2006, 2008; Rosales et al., 2007; Osma et al., 2007; Levin et al., 2008). Thus, barley bran increased Trametes versicolor laccase activity almost 50-fold compared to the control culture with glucose (Moldes et al., 2004). Recently, we have proven that the presence of lignocellulosic substrate is mandatory for MnP production by several fungi since there was no enzyme production when they were cultivated in synthetic media with different carbon sources (Elisashvili et al., 2006; Elisashvili and Kachlishvili, 2009). Furthermore, the ratio of individual hydrolytic and oxidative enzymes in the culture liquid and even the proportion of isoenzymes significantly depended on the type of lignocellulosic substrate in the medium (Moldes et al., 2004; Songulashvili et al., 2006; Elisashvili et al., 2008). However, in contrast to lignin-degrading enzymes, the information on hydrolases produced by basidiomycetes is still scarce. In this respect, only few reports are concerned with the simultaneous production of hydrolytic and oxidative enzymes by white-rot fungi (Machuca and Ferraz, 2001; Baldrian and Gabriel, 2003; Nazareth and Sampy, 2003; Sun et al., 2004; Kachlishvili et al., 2006; Elisashvili et al., 2006, 2008; Levin et al., 2008). Taking into account that cellulose and hemicelluloses are the main carbon and energy sources for the wood-rotting basidiomycetes, it is clear that the hydrolytic enzymes play a decisive role in steadily supplying nutrients to the growing fungi.

The mycobiota of Georgia comprise more than 7000 species widespread in various ecosystems, including alpine and subtropics habitats, mountains, forests, and steppes (Nakhutsrishvili, 1975). They represent an untapped source of new productive genotypes, interesting from both a basic and an applied viewpoint. The aims of this study were (a) to characterize the lignocellulolytic systems of new basidiomycetes strains isolated from the forests of Georgia, (b) to evaluate their cellulase, xylanase, laccase, and manganese peroxidase activities in submerged and solid-state (SSF) fermentations of lignocellulosic materials, and (c) to assess the potential of selected local food residues to serve as growth substrates for enhanced enzyme production.

Materials and Methods

Lignocellulosic substrates

Solid residue after ethanol production from wheat grains (REP) was available from the enterprise in Kaspi (Georgia). Mechanically de-stoned olive pomace (OMW) obtained from Escobedo y Garcia Asesores (Jaén, Spain) was used exactly as it was produced in two-phase system. Wheat bran, mandarin peelings, banana peelings, and walnut pericarp were available from the local market. All residues were dried at 50°C and milled to dust extent (<1 mm).

Organisms and Inoculums Preparation

The following white-rot fungi isolated from dead and living trees in the forests of Georgia have been used in this study: *Fomes fomentarius* IBB 37, *Fomitopsis rosea* IBB 67, *Ganoderma australe* IBB 69, *Ganoderma resinaceum* IBB 80, *Irpex lacteus* IBB 104, *Lenzites betulina* IBB 159, *Stereum hirsutum* IBB 13, *Trametes ochracea* IBB 76, *Trametes versicolor* IBB 78. The above isolates have been deposited in the culture collection of the Durmishidze Institute of Biochemistry & Biotechnology under the numbers given above. Fungal inocula were prepared by growing mycelium on a rotary shaker at 150 rpm and 27°C in 250-ml flasks containing 100 ml of standard medium with the following composition (g l^{-1}): glucose, 10.0; ammonium nitrate, 2.0; KH_2PO_4, 0.8; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 3.0. The medium was adjusted to pH 6.0 with 2M NaOH. After five to six days of fungal cultivation mycelial pellets were harvested and homogenized with a Waring laboratory blender. The same identical inoculum was used to conduct both the SF and SSF of selected lignocellulosic materials.

Cultivation Conditions

Submerged fermentation of lignocellulosic substrates was carried out on a rotary shaker at 150 rpm and 27°C in 250-ml flasks containing 50 ml of the following medium (g l^{-1}): lignocellulosic substrate, 40.0; peptone, 3; KH_2PO_4, 0.8; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.25; yeast extract, 3.0. For comparison, the selected fungi have been evaluated in submerged cultivation in synthetic medium containing one percent glycerol as a carbon source. The initial pH of the media was adjusted to 6.0 prior to sterilization by adding 2 M NaOH. The flasks were inoculated with 3 ml of mycelial homogenate. After five, eight, and twelve days of growth, when the cultures existed in the middle-end of logarithmic and stationary phases of growth, respectively, samples (1 ml) were taken from flasks and the solids were separated by centrifugation at 14,000 g for five minutes at 4°C. The separated biomasses were dried at 60°C to constant weight.

Solid-state fermentation (SSF) of selected residues was carried out at 27°C in 100-ml flasks containing 4 g of lignocellulosic substrates moistened with 12 ml of the above-mentioned medium. The flasks were inoculated with 3 ml of mycelial homogenate. After seven and fourteen days of cultivation, when the cultures existed in the logarithmic and stationary phases of growth, respectively, the extracellular enzymes were extracted from whole biomass twice with 25 ml of distilled water (total volume 50 ml). The solids were separated by filtration through nylon cloth followed by centrifugation at 14,000 g for five minutes at 4°C.

Enzyme Assays

The supernatants obtained after biomass separation were analyzed for the enzyme activity. Endoglucanase (CMCase) activity was assayed according to IUPAC recommendations using low-viscosity carboxymethyl cellulose (one percent w/v) in 50 mM citrate buffer (pH 5.0) at 50°C for ten minutes (Ghose, 1987). Xylanase activity was determined by mixing 70 µl appropriately diluted samples with 630 µl of birch wood xylan (Roth 7500) (one percent w/v) in 50 mM citrate buffer (pH 5.0) at 50°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 spectrophotometrically the change in absorbance at 420 nm (A420) related to the rate of oxidation of 1 mM 2,20-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS) in 50 mM Na-acetate buffer (pH 3.8) (Bourbonnais and Paice, 1990). Assays were performed in 1-ml cuvettes at room temperature with 50 µl of adequately diluted culture liquid. Manganese peroxidase (MnP) activity was measured at 270 nm by following the formation of a Mn3+-malonate-complex (Wariishi et al., 1992). One unit of laccase or MnP activity was defined as the amount of enzyme, which led to the oxidation of 1 µmol of substrate per minute.

To compare the enzyme activity of fungi grown in submerged and SSF, all enzyme activities were expressed in international units per ml of culture liquid. The experiments were performed twice using three replicates. The data presented correspond to mean values, the standard deviation being lower than 17%.

Results

Screening of Basidiomycetes in Submerged Fermentation of Mandarin Peelings

Nine strains have been screened for the lignocellulose-degrading enzyme activities in both glycerol-containing synthetic medium and mandarin peels-based medium. All fungi grew well in form of pellets producing in the presence of glycerol 3.6-6.9 g l^{-1} mycelial biomass (Table 1). The cellulase and xylanase activities were tested only in the cultures grown in the presence of mandarin peelings.

The data presented in Table 1 show that the hydrolases production varied widely among individual fungal strains. CMCase and xylanase activities of studied basidiomycetes varied from 0.7 to 2.9 U ml^{-1} and from 2.6 to 15.1 U ml^{-1}, respectively. *T. ochracea* and *L. betulina* appeared to be the best producers of hydrolases. Very high xylanase activity (13.2-15.1 U ml^{-1}) was revealed in cultures of these fungi, although other fungi also secreted significant levels of this enzyme in submerged fermentation of mandarin peelings.

Table 1. Screening of basidiomycetes for the lignocellulolytic enzyme production in submerged fermentation of glycerol and mandarin peels (MP).

Fungi	Biomass (g l^{-1})	CMCase (U ml^{-1})	Xylanase (U ml^{-1})	Laccase (U ml^{-1})		MnP (U ml^{-1})	
	Glycerol	MP	MP	Glycerol	MP	Glycerol	MP
F. fomentarius	3.7±0.2	1.8±0.22	4.5±0.38	0.4±0.1	5.7±0.6	0	0.14±0.02
F. rosea	3.6±0.1	2.3±0.25	5.0±0.53	3.8±0.5	5.8±0.8	0	0
G. australe	6.9±0.3	1.7±0.13	3.5±0.45	0.9±0.1	1.3±0.1	0	0.38±0.05
G. resinaceum	4.3±0.2	0.7±0.09	2.6±0.37	0.2±0.1	19.0±2.3	0	0.06±0.01
I. lacteus	4.6±0.2	0.9±0.14	4.4±0.40	0	0	0	0
L. betulina	5.0±0.3	2.9±0.24	13.2±0.92	52.5±7.3	37.8±5.1	0	0.29±0.04
S. hirsutum	3.8±0.2	2.1±0.27	3.9±0.52	3.8±0.5	9.8±0.7	0	0
T. ochracea	6.4±0.3	2.8±0.36	15.1±1.67	14.5±1.9	22.0±2.0	0	0.16±0.03
T. versicolor	6.9±0.3	0.9±0.07	3.0±0.40	2.8±0.4	10.1±1.4	0	0.06±0.01

All white-rot basidiomycetes tested, with the exclusion of *I. lacteus*, produced laccase in both media (Table 1). When the fungi were grown in synthetic medium, very low enzyme activity (0.2-0.4 U ml-1) was detected in cultures of *G. resinaceum* and *F. fomentarius*. In the same cultivation conditions, *L. betulina* followed by *T. ochracea* accumulated especially high enzyme activity (52.5 and 14.5 U ml^{-1}, respectively). In fermentation of mandarin peels the laccase activity of tested fungi varied from 1.3 to 37.8 U ml^{-1}. In this case, all strains, with the exclusion of *L. betulina*, secreted much higher laccase activity as compared to synthetic medium. Unexpectedly, none of white-rot basidiomycetes screened secreted detectable levels of MnP in submerged cultivation in glycerol-containing medium. However, in submerged fermentation of mandarin peels several fungi appeared to be capable to secrete MnP. Among them, *G. australe* and *L. betulina* demonstrated the highest activity of this enzyme (0.29-0.38 U ml^{-1}).

Effect of lignocellulosic substrates in submerged fermentation

New, unexplored fungi which expressed significant lignocellulolytic enzyme activity in screening experiment were selected for the further evaluation of their enzymatic potential in fermentation of various food wastes (Table 2). All lignocellulosic substrates tested in this study, with the exception of OMW promoted excellent growth of fungi tested. In general, the enzyme

Table 2. Effect of lignocellulosic materials on the enzyme production in submerged fermentation by selected fungi.

Lignocellulosic substrates	*G. resinaceum*	*L. betulina*	*S. hirsutum*	*T. ochracea*
			CMCase (U ml^{-1})	
EPR	4.3±0.47	2.8±0.25	2.5±0.36	2.3±0.25
Banana peels	3.7±0.55	2.6±0.19	1.0±0.14	3.6±0.43
Mandarin peels	1.4±0.19	3.1±0.28	2.6±0.38	3.3±0.42
Wheat bran	0.7±0.09	2.1±0.24	0.7±0.06	2.4±0.26
Walnut pericarp	1.9±0.20	2.2±0.17	2.7±0.33	1.5±0.23
OMW	3.3±0.28	2.4±0.21	0.8±0.10	2.8±0.34
		Xylanase (U ml^{-1})		
EPR	26.9±3.7	17.5±2.1	7.9±0.82	11.9±1.7
Banana peels	13.2±1.8	8.2±1.1	21.8±2.5	11.5±1.3
Mandarin peels	4.9±0.71	12.5±1.3	5.2±0.68	14.3±1.7
Wheat bran	8.1±0.84	7.0±0.57	4.2±0.37	16.2±2.1
Walnut pericarp	10.6±0.91	5.9±0.63	7.6±0.62	4.0±0.53
OMW	15.2±1.7	9.4±1.2	3.9±0.53	10.2±1.4
			Laccase (U ml^{-1})	
EPR	3.7±0.5	6.6±0.6	0.9±0.1	7.2±1.0
Banana peels	1.7±0.2	7.6±0.8	1.0±0.1	6.3±0.6
Mandarin peels	16.2±2.3	43.6±5.9	13.0±1.7	25.8±3.0
Wheat bran	5.2±0.7	6.1±0.5	2.4±0.3	5.4±0.6
Walnut pericarp	11.8±1.6	76.7±8.7	13.6±1.2	18.1±2.2
OMW	2.4±0.3	1.0±0.2	1.2±0.2	0.6±0.1
		MnP (U ml^{-1})		
EPR	0.34±0.04	2.12±0.27	0.07±0.01	1.16±0.17
Banana peels	0	0.19±0.02	0.03±0.01	0.16±0.02
Mandarin peels	0.10±0.02	0.42±0.06	0	0.29±0.04
Wheat bran	0	1.55±0.22	0.10±0.01	0.46±0.05
Walnut pericarp	0.04±0.01	0.14±0.02	0.23±0.02	0.14±0.02
OMW	0	0.60±0.09	0.14 ±0.02	0.18±0.03

activity appeared after 2–3 days of cultivation and gradually increased to reach maximum values on days seven through ten, depending on the substrate. The levels of extracellular enzyme activities produced during fermentation of different plant materials varied among the fungi studied, but several general features may be noted. First, the food residues are appropriate growth substrates for the production of hydrolytic enzymes under submerged fermentation (Tables 2). Second, the tested fungi distinguish with their response to growth substrate added to the medium. Thus, the CMCase and xylanase activities of selected fungi varied from 0.7 to 4.3 U ml^{-1} and from 3.9 to 26.9 U ml^{-1}, respectively. The ethanol production residue (EPR) appeared to be the best growth substrate for the production of cellulase and xylanase by I. lacteus. This residue, along with mandarin peels, favored these enzymes production by *L. betulina*. It is interesting that *S. hirsutum* secreted equally high levels of CMCase in presence of EPR, mandarin peels, and walnut pericarp, whereas banana peels promoted extremely high secretion of xylanase. All tested lignocellulosic materials, with the exclusion of walnut pericarp, provided significant production of endoglucanase and xylanase by *T. ochracea*.

Evaluation of the fungi ligninolytic enzymes activity showed that the maximum activity of laccase and MnP expressed by each of tested fungi varied depending on the nature of growth substrate. The highest laccase activity (76.7 U ml^{-1}) was revealed in submerged fermentation of walnut pericarp by *L. betulina*. The same substrate and mandarin peels favored the laccase secretion by *S. hirsutum*. On the contrary, mandarin peelings followed by walnut pericarp ensured the highest laccase activity of *G. resinaceum* and *T. ochracea*. Substitution of these substrates in nutrient medium with other materials caused a manifold decrease of these fungi laccase activity. By contrast, among the six lignocellulosic residues tested, EPR favored MnP secretion, especially by *G. resinaceum*, *L. betulina*, and *T. ochracea*. Among the four fungi tested, *L. betulina* appeared to be an excellent producer of this enzyme in submerged fermentation of both EPR (2.12 U ml^{-1}) and wheat bran (1.55 U ml^{-1}). The data received indicate that *G. resinaceum* and *S. hirsutum* are poor producers of MnP, and these fungi could not secrete this enzyme in submerged fermentation of several tested substrates.

Screening of Basidiomycetes in Solid-State Fermentation of Mandarin Peelings

Simultaneously with submerged fermentation, eight strains have been screened for the lignocellulose-degrading enzymes production in SSF of mandarin peels. The data presented in Table 3 show that usually the enzyme yield in SSF was lower compared to submerged fermentation of mandarin peels. Thus, the CMCase activity of studied basidiomycetes varied from 0.4 to 1.3 U ml^{-1} while the xylanase activity reached 0.2-1.0 U ml^{-1} with highest activity in cultures of *I. lacteus* and *F. rosea*. Like submerged fermentation, all white-rot basidiomycetes tested, with the exclusion of *I. lacteus*, produced laccase in SSF of mandarin peels. All strains, with the exclusion of *T. ochracea*, accumulated much lower laccase yields as compared to submerged fermenta-

Table 3. Screening of basidiomycetes for the lignocellulolytic enzyme production in solid-state fermentation of mandarin peels.

Fungi	CMCase (U ml^{-1})	Xylanase (U ml^{-1})	Laccase (U ml^{-1})	MnP (U ml^{-1})
F. fomentarius	0.8±0.11	0.6±0.09	3.5±4.4	0
F. rosea	1.1±0.12	0.7±0.10	0	0
G. resinaceum	0.8±0.09	0.7±0.08	9.4±1.3	0
I. lacteus	1.3±0.18	1.0±0.14	0	0.12±0.01
L. betulina	0.6±0.09	0.2±0.03	6.9±1.0	0.34±0.05
S. hirsutum	0.5±0.07	0.4±0.05	3.0±0.4	0.36±0.05
T. versicolor	0.4±0.05	0.6±0.11	3.6±0.5	0.26±0.03
T. ochracea	1.0±0.12	0.1±0.02	11.6±1.5	0.21±0.02

tion. The levels of enzyme activity among the fungi tested varied from 0 to 11.6 U ml^{-1}. *T. ochracea* followed by *G. resinaceum* were the best laccase producers. Only five white-rot fungi appeared to be capable to secrete MnP in SSF of mandarin peels. Among them, *S. hirsutum* and *L. betulina* secreted the highest activity of this enzyme (0.34-0.36 U ml^{-1}).

Effect of lignocellulosic substrates in solid-state fermentation

The first well visible signs of fungal growth in SSF were seen two days (three to four days in the presence of walnut pericarp and OMW) after inoculation and the total colonization of substrates was completed within 9-12 days. The data in Table 4 indicate that the basidiomycetes CMCase activity widely varied in media with different growth substrates, although the *I. lacteus* enzyme activity little depended on plant materials. The CMCase activity of L. betulina varied from 0.4 U ml^{-1} in walnut pericarp-containing medium to 3.0 U ml^{-1} in EPR-based medium. It is worth noting that EPR appeared to be the most appropriate growth substrate for the endoglucanase production by tested fungi. Among the white-rot basidiomycetes screened, *T. ochracea* followed by *L. betulina* expressed the highest enzyme activity (4.1U ml^{-1} and 3.0 U ml^{-1}, respectively). Moreover, the EPR favored the xylanase accumulation by all fungi (8.0-13.0 U ml^{-1}). Again, *T. ochracea* followed by *S. hirsutum* showed the highest xylanase activity.

All fungi were capable of expressing laccase and MnP activities in SSF of selected substrates (Table 4). Evidently, the lignino-lytic enzyme production by tested basidiomycetes was affected by the nature of the lignocellulosic material added to the me-

Table 4. Effect of lignocellulosic substrates on the hydrolases production in their solid-state fermentation.

Lignocellulosic Substrates	*G. resinaceum*	*L. betulina*	*S. hirsutum*	*T. ochracea*
			CMCase (U ml^{-1})	
EPR	2.6±0.23	3.0±0.29	1.2±0.13	4.1±0.53
Banana peels	1.0±0.13	1.1±0.15	0.6±0.10	0.2±0.02
Mandarin peels	1.8±0.23	1.0±0.15	0.7±0.09	1.4±0.15
Wheat bran	1.6±0.24	2.5±0.34	1.3±0.12	1.8±0.16
Walnut pericarp	2.2±0.21	0.4±0.05	0.2±0.03	0.1±0.01
OMW	1.5±0.20	0.6±0.08	0.4±0.06	0.7±0.10
		Xylanase (U ml^{-1})		
EPR	8.0±1.1	9.1±1.3	10.7±1.1	13.0±1.6
Banana peels	0.4±0.06	1.9±0.25	0.7±0.10	0.6±0.08
Mandarin peels	1.6±0.27	0.6±0.09	0.7±0.11	0.2±0.03
Wheat bran	1.6±0.20	3.5±0.51	2.0±0.21	4.4±0.57
Walnut pericarp	2.2±0.21	1.0±0.14	0.6±0.07	0.4±0.05
OMW	1.5±0.24	0.4±0.06	0.2±0.03	0.3±0.05
			Laccase (U ml^{-1})	
EPR	7.4±1.0	15.3±1.9	0.6±0.1	12.1±1.3
Banana peels	1.1±0.1	1.9±0.3	0.4±0.1	3.1±0.3
Mandarin peels	8.6±1.2	9.7±1.4	4.2±0.6	14.9±1.6
Wheat bran	1.8±0.2	18.5±2.1	2.8±0.4	29.8±2.8
Walnut pericarp	6.2±0.9	10.6±1.5	8.2±0.9	8.2±1.1
OMW	1.9±0.3	1.1±0.2	4.2±0.6	1.3±0.2
		MnP (U ml^{-1})		
EPR	0.09±0.01	0.16±0.02	0.10±0.01	0.10±0.01
Banana peels	0	0.31±0.04	0.17±0.02	0.24±0.02
Mandarin peels	0	0.34±0.04	0.36±0.05	0.21±0.03
Wheat bran	0.04±0.01	0.74±0.06	0.05±0.01	0.21±0.02
Walnut pericarp	0	0.50±0.08	0.28±0.04	0.04±0.01
OMW	0	0.31±0.05	0.43±0.07	0.36±0.07

dium. The highest laccase activity (29.8 U .l^{-1}) was revealed in SSF of wheat bran by *T. ochracea*. The same substrate followed by EPR promoted the laccase accumulation by *L. betulina* while mandarin peels followed by EPR and walnut pericarp favored the laccase secretion by *G. resinaceum*. Finally, walnut pericarp, like in submerged fermentation conditions, ensured the highest laccase activity of *S. hirsutum*, whereas EPR and banana peels appeared to be very poor growth substrates for the laccase production in SSF. Three fungi secreted appreciable levels of MnP. Among them, *L. betulina* accumulated the highest enzyme activity (0.74 U ml^{-1}) in SSF of wheat bran. It is interesting that nutrient medium with OMW resulted in highest MnP activity of *S. hirsutum* and *T. ochracea* although several other substrates also supported production of this enzyme. Of four fungi tested, obviously, *G. resinaceum* is the poorest producer of MnP, since only very low or no enzyme activity was detected in cultivation of this fungus.

Discussion

Thus, the screening results obtained in this study and the literature data (Sun et al., 2004; Kachlishvili et al., 2006; Elissetche et al., 2007) show that the simultaneous synthesis of hydrolytic and ligninolytic enzymes in fermentation of lignocellulose is a common feature of white-rot basidiomycetes. This study evaluated for the first time the lignocellulose-degrading enzyme activity of not previously described species and notable intergeneric differences were revealed with regard to the extent of hydrolases and oxidases activity. Among the white-rot basidiomycetes screened, *L. betulina* appeared to be very promising producer of laccase in both synthetic and complex media. Moreover, this fungus in appropriate media secretes high level of MnP. It is worth noting that these significant laccase and MnP activities were obtained in the absence of a specific exogenous inducer. Furthermore, *L. betulina* expressed high endoglucanase and xylanase activities in both submerged and SSF of lignocellulosic materials. The capacity of this fungus to produce high levels of cellulases and xylanase is of importance in supplying the growing culture with a carbon source and nutrients essential for their biosynthetic activity.

One of the appropriate approaches in fermentation technology development is the utilization of lignocellulosic wastes, some of which may contain significant concentrations of soluble carbohydrates and inducers ensuring an abundant growth of fungi and efficient production of lignocellulolytic enzymes (Sun et al., 2004; Rosales et al., 2005; Kachlishvili et al., 2006; Elisashvili et al., 2008; Levin et al., 2008; Winquist et al., 2008). Therefore, to gather more knowledge about the possible role of complex carbon sources in hydrolytic and oxidative enzyme production, several structurally and chemically different raw materials were assessed as growth substrates under both submerged and SSF conditions. In this study, evaluation of the fungi enzymes activity showed that several residues of food industry might be appropriate growth substrates for the production of lignocellulolytic enzymes. This observation agrees with the recently reported findings (Rosales et al., 2005; Songulashvili et al., 2006; Elisashvili, Kachlishvili, 2009). However, the tested white-rot fungi exhibited quite different responses to growth substrates used. Mandarin peelings, EPR, and wheat bran appeared to be excellent growth substrates for the production of hydrolases and oxidases in submerged and SSF. The same substrates as well as walnut pericarp enhanced the laccase and MnP secretion by tested fungi. Moreover, like several other studies (Moldes et al., 2004; Songulashvili et al., 2006; Elisashvili et al., 2008) we showed that the ratio of individual enzymes in final preparations significantly depended on growth substrate. Thus, the laccase/MnP ratio changed from three to 548 with substitution of EPR by walnut pericarp in submerged fermentation by *L. betulina*. These results suggest that the type of lignocellulosic substrate appears to determine the types and amounts of ligninolytic enzymes produced by the white-rot fungi. The most likely explanation for such different laccase and MnP production may be related to the presence in some plant raw materials of high concentration of specific aromatic compounds or microelements liberating during fermentation. Thus, the mandarin peels served as an appropriate growth substrate favoring efficient production of lignocellulolytic enzymes by wood-rotting fungi from various taxonomic and ecological groups (Elisashvili et al., 2002, 2008; Osma et al., 2007). In addition to cellulose (21%), hemicellulose (13%), lignin (2%), and nitrogen (1.2%), mandarin peels have a significant content of free sugars and organic acids ensuring abundant growth of different fungi as well as water-soluble aromatic compounds (flavones and flavonols) capable of inducing or stimulating biosynthesis of ligninolytic enzymes. On the other hand, it is not inconceivable that some specific compounds stimulating ligninolytic enzyme secretion appear during growth substrate degradation/transformation.

The analysis of data received also underlines that the expression of basidiomycetes biosynthetic potential highly depends on the method of fungi cultivation. The SSF is considered as the most appropriate method for the basidiomycetes cultivation because they grow under conditions close to their natural habitats. Indeed, during SFF some substrates promoted both abundant growth of fungi and enzyme production. However, the comparison of volumetric enzyme activities indicates that only the SSF of wheat bran was preferable for the CMCase production by *I. lacteus, L. betulina*, and *S. hirsutum*. The SSF of EPR was essential for the laccase production by *G. resinaceum*, *L. betulina*, and *T. ochracea* while the SSF of wheat bran favored this enzyme accumulation compared to submerged cultivation. It is interesting that the laccase and MnP yields in SSF of OMW by *S. hirsutum* and *T. ochracea* appeared to be two-three-fold higher than those in submerged fermentation of this highly toxic waste. In other media and cultures, the submerged fermentation of lignocellulosic materials was most preferable for the target enzyme secretion. Moreover, the enzyme productivity/g substrate in submerged fermentation was much higher taking into account that in this case the substrate quantity was two times less as compared to that in SSF.

The data presented in this study demonstrate that the search for the appropriate strain to achieve high enzyme yield remains feasible and can become less empirical and more rational if one follows the trends suggested by the variation of substrate and fermentation mode. In this study, high yields of cellulase, xyla-

nase, laccase, and MnP were obtained in submerged and SSF of cheap plant wastes by several white-rot basidiomycetes isolated from the forests of Georgia.

We showed that the selection of an appropriate plant residue adequate for fungus growth and target enzymes synthesis plays an important role in the development of an efficient technology of enzyme production. However, further studies are required to elucidate the reason by which some complex substrates stimulate the individual enzyme production.

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