Chemistry of Natural Compounds, Vol. 31, No. 5, 1995

STUDY OF THE CONDITIONS OF ENZYME FORMATION AND THE SECRETION OF EXTRACELLULAR LIGNINASES BY THE FUNGUS *Pleurotus ostreatus* UzBI-I108

Z. R. Akhmedova

UDC 577.152.1:663.152:582.284

The possibility has been shown of the rational use of lignocellulose wastes as components of a nutrient medium for the deep cultivation of some wood-destroying basidial fungi isolated from various sources and actively breaking down natural polysaccharides and lignin with the formation of ligninolytic enzymes and biologically valuable products. An active producing agent of ligninolytic enzymes (laccase, peroxidase, lignin peroxidase, polyphenoloxidase, Mn-dependent peroxidase) has been revealed as Pleurotus ostreatus, *strain UzBI-I108. The optimum conditions have been determined for the formation of enzymes and the secretion of total ligninase preparations with a fairly high specific activity of lignin peroxidase.*

In the breakdown of lignocellulose a special place is occupied by basidial fungi, which are responsible for the white rot of wood and possess a powerful enzyme system with hydrolytic, oxidative, transferase, and other activities, rapid growth, and a high penetrating capacity in relation to an insoluble substrate [1-3]. It is possible to activate the biosynthesis of ligninases by regulating the choice of active cultures of basidial fungi and optimizing the composition of the nutrient medium for cultivating the producing agents and also by selecting the conditions for isolating the enzymes from the producing agent's culture liquid.

The task of the present work was to optimize the conditions for the formation of enzymes and for the isolation of total ligninase preparations with a high specific activity of lignin peroxidase in the deep cultivation of some higher basidial fungi on media containing lignocellulose wastes.

The fungi tested were isolated from various sources and belonged to the orders Agaricales, Tricholomatales, and Aphyllophorales. The fungus oyster mushroom *Pleurotus ostreatus* UzBI-I108 (a xylosaprotroph) was isolated from the wood of a willow stump, *Panus tigrinus* UzBI-I3 (a xylosaprotroph) from the wood of a walnut tree trunk, *Fomes fomentarius* UzBI-Ya55 (a xyloparasite) from the wood of an apple-tree trunk, and *Inonotus hispidus* UzBI-T8 (xyloparasite) from the wood of a mulberry tree trunk. The fungi were grown on media containing various lignocellulose wastes: combined pulp from cottonseed meal (I), cottonplant stems (II), rice husks (III), and kenaf chaff (IV), and also an aqueous extract of the combined pulp of cottonseed meal.

The results of a study of the formation of the ligninolytic activities of the fungi selected (*P. tigrinus, P. ostreatus, F. Fomentarius* and *I. hispidus*) on media with different plant wastes showed that in the process of growth on lignocellulose wastes these fungi secreted lignin peroxidase into the culture medium as functions of the structure of the lignocellulose component of the medium (Fig. 1) and of the time of cultivation. Thus, it was found that the highest lignin peroxidase (LGP) activities were exhibited by the fungi *P. tigrinus* and *P. ostreatus*. As can be seen from Fig. 1, almost all the fungi had a relatively high LGP activity on media with the combined cottonseed pulp. The fungus *P. ostreatus* was particularly distinguished by its ligninolytic activity, forming LGP on almost all media. The results presented in Table 1 show that the formation of LGP by the fungus *P. ostreatus* was accompanied by an increase in the amount of protein and biomass in the culture liquid. The maximum LGP activity on medium I was observed on the 27th day of the growth of the fungus, i.e., at the end of the stationary phase, as shown by a slowing down of growth (2.54 g of biomass from 100 ml of culture medium);

Institute of Microbiology, Academy of Sciences, Republic of Uzbekistan, Tashkent, fax (3712) 41 71 29. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 739-747, September-October, 1995. Original article submitted November 11, 1994.

0009-3130/95/3105-0619$12.50

TABLE 1. Formation of LGP by the Fungus *P. ostreatus* During Cultivation on Media Containing Various Lignocellulose Wastes

Substrate	pH of the medium	Protein, mg/ml of cult. medium	Biomass, g/100 ml	LGP, units/ml	Time of cultivation, days
Combined cottonseed pulp (I)	3.24	13.3	2.54	24.0	27
Cottonplant stems (II)	3.47	12.4	4.8	13.0	6
Rice husks (III)	4.52	9.8	2.82	16.0	24
Kenaf chaff (IV)	4.85	11.3	4.02	13.4	12

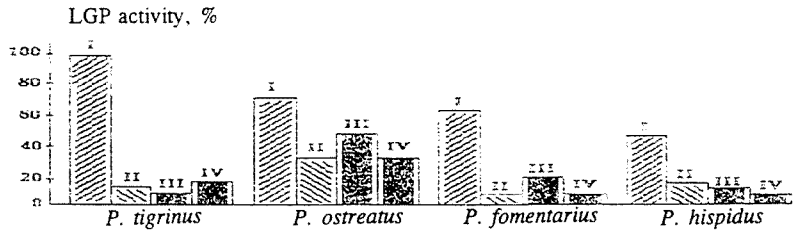

Fig. 1. Formation of lignin peroxidase by some basidial fungi on media containing plant wastes: I) combined cottonseed wastes; II) cottonplant stems; III) rice husks; IV) kenaf chaff.

on medium II on the 6th day, i.e., in the exponential growth phase (4.8 g of biomass); on medium III on the 24th day, just at the end of the stationary growth phase; and, finally, on the 12th day of the cultivation of the fungus on medium IV, i.e., at the end of the exponential phase of the growth of the producing agent (see Table 1).

It must be mentioned that during the cultivation of the fungus on all the media, a fall in the pH of the culture medium from 5.6-5.4 to 2.3 and even to 1.5 was observed. The acid nature of the culture medium was apparently due to the formation of oxidizing enzymes, organic acids, and new carboxyl groups in the lignin decomposition products, and also to the phenolcarboxylic and phenoxyacetic acids formed under the action of the ligninases (unpublished results). In addition, the culture liquid (c.l.) of the fungus was found to contain glucose oxidase — an enzyme participating in the formation of hydrogen peroxide and fulfilling the function of a key enzyme in the breakdown of lignocellulose.

The appearance of LGP activity mainly at the end of the stationary phase of the growth of the fungus shows a stepwise degradation first of the cellulose and hemicellulose and then of the difficulty metabolizable lignin substrate by the corresponding enzymes, i.e., cellulases, hemicellulases, pectinases, ligninases, etc. It has been shown for *Ph. chrysoporium*, as an example, that the ligninolytic activity of a culture is not exhibited in the period of primary growth but appears in response to a deficiency of nitrogen, carbon, or sulfur in the medium [4]. It must be considered as proved that, although lignin is also a potentially energy-rich material, it cannot serve as the sole source of carbon and energy for the growth and development of lignin-destroying fungi. For the fungi of white rot to decompose lignin, it is necessary to add cellulose, hemicellulose, glucose, or other sugars to the medium [5].

According to an observation of Kirk et al. [6], the decomposition of lignin ceases when the cellulose in the medium is exhausted. A similar pattern has been observed by other researchers [7, 8], as well, in the study of the decomposition of aspen sawdust and wheat straw. Analogous results have been obtained in a study of the decomposition of lignin isolated birchwood and wheat straw by the fungi *Ph. chrysosporium* 1764 and *P. tigrinus* 144 [9]. While the actual fact of the necessary presence of a co-substrate in the degradation of lignin must be regarded as established, the optimum ratio of growth substrate and co-metabolizable substrate is not yet known for all lignin-decomposing fungi. The co-substrate apparently provides the energy for the synthesis of the enzymes promoting degradation, the production of hydrogen peroxide connected with the functioning of these enzymes, and the synthesis of possible effectors of the liginolytic system of the type of veratryl alcohol (an inductor of the biosynthesis of lignin peroxidases) and other low-molecular-mass compounds [10].

The results of a study of the chemical composition of a lignocellulose substrate before and after fermentation by the fungus *P. ostreatus* showed a substantial difference in the amounts of biopolymers: cellulose, hemicellulose, and lignin. The

TABLE 2. Destruction of Lignocellulose by the Fungus *P. ostreatus* in Deep Cultivation on Media Containing Lignocellulose Wastes

Substrate	Time of cultivation, days	Destruction, %		
		lignin	cellulose	hemicellulose
Combined pulp cottonseed	Control	28.0 (100)	41.5(100)	26.1(100)
	10	9.6	22.4	19.6
	20	11.4	25.6	27.4
	30	23.2	29.6	29.6
	40	29.5	31.8	33.6
	50	36.8	37.4	40.7
	60	41.4	44.5	51.4
Cottonplant stems	Control	25.5(100)	38.3(100)	28.9(100)
	10	17.0	17.8	24.8
	20	19.0	30.0	26.7
	30	21.0	33.3	27.4
	40	26.5	38.5	30.6
	50	27.1	44.7	36.4
	60	29.4	54.0	29.8
Kenaf chaff	Control	27.0(100)	37.4(100)	33.4(100)
	10	20.7	20.4	25.0
	20	22.5	22.5	33.4
	30	24.6	24.7	36.3
	40	27.9	25.2	37.8
	50	29.7	31.4	39.5
	60	31.9	37.6	42.7
Rice husks	Control	19.3(100)	29.0(100)	18.3(100)
	10	7.5	15.4	20.0
	20	9.7	16.9	32.7
	30	11.4	18.5	28.6
	40	16.7	21.4	32.7
	50	20.9	26.9	36.5
	60	23.5	32.7	24.3
Birch sawdust	Control	39.2(100)	37.1(100)	26.9(100)
	10	12.3	17.8	22.4
	20	17.8	24.7	26.8
	30	24.3	27.4	28.5
	40	25.3	29.5	31.5
	50	26.7	31.3	33.7
	60	28.3	33.1	30.5

TABLE 3. Change in the Chemical Composition of Kenaf Chaff During the Cultivation of the Fungus *P. ostreatus* (% on the dry weight)

Substrate	Cell-ulose	Hemicell-ulose	Lignin	RHPSs	DHPSs	Proteins	Lipids	Carbo-hydrates
0 days	38.2	27.8	28.8	12.7	34.4	1.9	1.7	4.3
10 days	21.0	19.2	22.7	14.4	30.7	7.6	2.5	14.7
20 days	12.5	15.4	20.4	16.9	5.3	12.6	5.4	23.0
30 days	10.8	8.4	15.7	23.7	4.8	9.0	7.3	17.3

greatest concentration of lignin was found in birch sawdust (39.2%), of cellulose in cottonplant stems (38.3%), and of hemicellulose in kenaf chaff (33.4%). After the deep cultivation of the fungus *P. ostreatus* UzBI-I108 for 60 days on media with lignocellulose substrates, their utilization had taken place to different degrees, depending on the nature of the substrate and the time of cultivation of the fungus. The greatest degradation was suffered by the lignin and hemicellulose of the combined cottonseed pulp (41.4 and 51.4%, respectively) and also by the cellulose of cottonplant stems (54%) (see Table 2).

The change in the composition of the polysaccharides of kenaf chaff lignin after the growth of *P. ostreatus* is shown in Table 3. In 3-30 days the cellulose content fell by 45-72%, that of hemicellulose by 17-54%, that of lignin by 21-45%, and that of difficultly hydrolyzable polysaccharides (DHPSs) by 11-85%. The decrease in the amount of natural biopolymers was accompanied by an almost 2-fold increase in the amount of readily hydrolyzable polysaccharides (RHPSs), an almost 3.1-fold increase in the amount of lipids, and an almost 4.2-fold increase in the amount of proteins.

In the culture liquid of the fungus we detected free sugars (glucose, mannose, xylose, rhamnose, arabinose, fructose, and galactose) and also free amino acids, including almost all the essential amino acids.

TABLE 4. Enzymes of the Ligninolytic Complex Formed by the Fungus *P. ostreatus* in Media Containing Lignocellulose

Lignocellulose	Activities of the enzymes, units/ml of culture liquid			
	laccase	peroxidase	polyphenoloxidase	Mn-dependent peroxidase
Combined cottonseed pulp	108.2	37.1	46.3	17.6
Cottonplant stems	40.2	21.9	22.0	14.7
Rice husks	21.2	3.9	28.3	15.9
Kenaf chaff	36.2	7.8	9.6	25.4

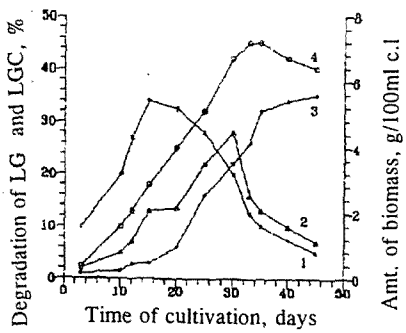

Fig. 2. Degradation of LCS (*1*) and LG (*2*) in the dynamics of the growth of the fungus and accumulation of biomass in a medium with LG (*3*) and with LCS (*4*).

The results obtained showed that enzymatic attack of natural polysaccharides takes place with the formation of biologically valuable products and enzymes. As reported by L. A. Golevleva et al. [11], the initial stages of the decomposition of lignin must be effected by diffusing attacking agents, since the polymeric lignin cannot penetrate within the cells. Such diffusing enzymes may be extracellular enzymes and (or) activated forms of oxygen produced by the fungi themselves.

An important role in the decomposition of lignin is also played by extracellular phenoloxidases (laccases, peroxidases, and other oxygenases), a particular role being assigned to laccases. In spite of the fact that under the action of a laccase no such deep changes take place in the structure of lignin, it is necessary for the oxidation of the aromatic compounds formed in the depolymerization of the lignin. The results of an investigation of the activities of the phenol-oxidizing enzymes in the culture liquid of the fungus *P. ostreatus* have shown that the fungus likewise forms a laccase, a peroxidase, an Mn-dependent peroxidase, and a polyphenoloxidase (Table 4).

The lignin-decomposing capacity of the fungus was confirmed by experiments with the addition to the nutrient medium (Czapek) of dioxane lignins (from kenaf chaff, cottonplant stems, and birch sawdust) as the sole source of carbon in concentrations of 10-20 mg/ml. Figure 2 shows comparative results for the growth of the fungs *P. ostreatus* on media with the dioxane lignin of kenaf chaff (LG) and with kenaf chaff as such (lignocellulose — LCS). The best growth of the fungus was observed on the medium containing LCS, and it was also accompanied by greater degradation of the lignin (2-34%). The loss of lignin in the LCS amounted to about 20-54%, and in the LG to about 10-32%.

It can be seen from Fig. 2 that destruction of the chaff lignin took place more intensively. Thus during 15 days of cultivation an abundant growth of the fungus took place (5.1 g/100 ml of c.l.) on the medium with kenaf chaff and a moderate one (0.7 g/100 ml of c.l.) on the medium with LG. After 20 days of cultivation, growth on the medium with LCS began to decrease, while on the medium with LG it increased, reaching a maximum (4.2 g) on the 30th day of cultivation. The active degradation of both substrates continued even in the later period of the growth of the fungus, reaching a maximum (47%) on the 35th day of growth. After 45 days of cultivation the growth of the fungus had almost ceased.

It is likely that nitrogen starvation causes the "switching on" of a secondary metabolism, which is expressed by the transition to the stationary phase (or the idiophase) of the growth of the culture, by the appearance of secondary growth of the fungal hyphae, and by the formation of extracellular polysaccharides [12]. The effective action of a deficiency of nitrogen is

TABLE 5. Isolation of Total Preparations of Extracellular Ligninases from a Filtrate of the Culture Liquid (c.l.) of the Fungus *P. ostreatus* by Various Methods

Reagents	Ratio of c.l. to reagent by vol.	Enzyme activities, units/ml					Yield of protein, mg
		per-oxidase	laccase	poly-phen-oloxidase	Mn-per-oxidase	ligninase	
Ethanol	1:2	750	1009	967	847	519	550
	1:3	1572	1327	1761	2031	863	1520
	1:4	2500	2250	2419	3341	1027	1947
Acetone	1:2	697	903	703	1831	461	469
	1:3	865	1403	918	1969	714	1114
	1:4	1607	2117	2450	4417	897	1503
Isopropyl alcohol	1:2	643	814	669	717	314	869
	1:3	851	1340	1241	1904	664	1097
	1:4	1504	1716	1019	2200	712	1469
Ammonium sulfate, %	20	820	927	742	4201	819	987
	40	3200	1510	230	1749	1264	1535
	60	1600	2240	1110	2343	493	747
	80	2700	1100	3050	1147	198	501
lyophilized c.l.	1000	2970	2460	3250	5320	898	5200

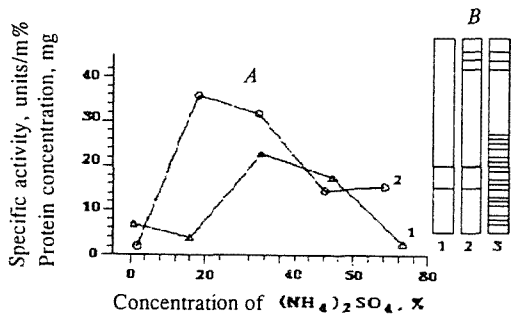

Fig. 3. *A*. Distribution of ligninase activity and protein in the fractionation of the culture liquid of the fungus *P. ostreatus* with ammonium sulfate: 1) LPG activity; 2) protein concentration. *B*. Isoelectric focusing of the total LGP preparation with ampholines in 7.5% PAAG in the pH range of 3-10: 1) LGP; 2) oxidase; 3) protein.

understandable, since the nitrogen content of the wood that is the sole substrate in the growth of the fungus is very low (for example, in kenaf chaff there is 1.9% of protein in the initial substrate) [13].

The next stage of our work was a determination of the optimum conditions for obtaining total preparations of lignin-degrading enzymes. Starting from the fact that the highest activity of lignin-degrading enzymes, especially LGP, was observed in a medium with the combined cottonseed pulp, to obtain the enzyme preparations the fungus was grown on a medium containing 1% of cottonseed pulp (substrate 1).

To isolate the LGP preparation we used the 15-day culture liquid of the fungus *P. ostreatus*. As the first step in obtaining the enzyme we used traditional approaches to the isolation of enzymes, i.e., precipitation of the proteins from filtrates of the culture liquid with organic solvents and with ammonium sulfate.

Table 5 gives information on the production of a total preparation of ligninases. The best method for their isolation from the c.l. of the fungus *P. ostreatus* is precipitation of the enzymes with ethanol in a ratio of 1:4, and fractional precipitation of the c.l. with 20 and 40% ammonium sulfate (Fig. 3, *A*). By these methods, highly active preparations of LGP were obtained, with activities of 1027 units/g (ethanol precipitation) and 1264 units/g (40% saturation with ammonium sulfate), respectively. As can be seen from Table 5, in the precipitation of the LGP with organic precipitants it is desirable to use c.l.:precipitant ratios of 1:4 and 1:5, and, in fractionation with ammonium sulfate, 40% saturation (Fig. 3, *A*).

Thus, by fractionating with ammonium sulfate the 15-day c.l. of the fungus *P.ostreatus* grown on medium No. 1 we obtained 5.2 g/liter of a total preparation of lignin peroxidase with a specific activity of 5.94 units/mg of protein.

Isoelectric focusing of the desalted total preparation in PAAG with ampholines (pH 3-10) showed the presence of about 20 proteins, of which five gave a specific coloration for an oxidase and two for a lignin peroxidase (Fig. 3, *B*).

Thus, the possibility has been shown of a rational utilization of lignin-containing wastes as components of a nutrient medium for the cultivation of higher basidial fungi in the production of LGP. It has been established that the fungus *P. ostreatus* possesses the highest activity of ligninolytic enzymes. Natural lignin undergoes greater destruction than lignin isolated from its natural sources thanks to the presence of co-substrates in LCS. The biosynthesis of LGP depends both on the components of the nutrient medium and on the time of cultivation of the producing agent.

EXPERIMENTAL

Cultures. We tested cultures of the basidial fungi *Panus tigrinus* UzBI-013, *Pleurotus ostreatus* UzBI-I108, *Fomes fomentarius* UzBI-Ya55, and *Inonotus hispidus* UzVI-IT8, isolated from rotting plant wastes and died-off parts of certain trees. A local strain of the fungus *P. ostreatus* UzBI-I108 was isolated from the trunk of a poplar. The fungus was seeded from slope wort-agar media into a previously sterilized nutrient medium continuing 0.3% of wort and 1% of LCS.

Cultivation was carried out by the deep method at 280-290°C [sic] in 500-ml conical Erlenmeyer flasks containing 200 ml of nutrient medium with pH 6.5 for 3-60 days on circular shaking machines at a speed of 250 rpm.

To isolate the enzyme preparation LGP we used a filtrate of the culture liquid of the fungus *P. ostreatus* containing 1% of an aqueous extract of combined cottonseed pulp obtained by pressing the substrate (with a particle size of 0.01-0.1 nm [sic] ground in a ball mill) at high pressures in an IBFM press. Samples were taken every 24 h.

The amount of protein was determined gravimetrically. For this purpose, the filtered mycelium was carefully washed with distilled water and dried, and the yield of biomass from 100 ml of culture medium was determined.

The protein contents of the samples were determined by Lowry's method, and also spectrophotometrically [14].

The activity of the lignin peroxidase (LGP) in a filtrate of the culture liquid was determined spectrophotometrically from the rate of oxidation of 3,4-dimethoxybenzyl (veratryl) alcohol to veratraldehyde [15]. The method was based on the increase in absorption at 310 nm (absorption maximum of veratraldehyde, ε_{310} = 9300 $mM^{-1}cm^{-1}$). Composition of the reaction mixture: 0.1 mM Na tartrate buffer, pH 2.65, 0.4 mM H_2O_2, 2 mM veratryl alcohol, and 100 ml of c.l. The total volume of the reaction mixture was 1 ml. The absorption kinetics were measured on a Shimdzu (Japan) scanning double-beam spectrophotometer for 3 min. As the unit of activity we took the amount of enzyme required to form 1 μmole of veratraldehyde in 1 min per 1 ml (mg) of enzyme [sic] at 20°C.

The total ligninolytic activity in EP gels and on IEF in PAAG was determined from the decoloration of the polymeric dye Poly B-411, oxidase activity from the staining of the gel with 4-chloronaphthol, and the loss of lignin spectrophotometrically [16].

Laccase activity was determined from the oxidation of syringaldehyde [17], **peroxidase activity** from the oxidation of *o*-dianisidine [18], **Mn-dependent peroxidase activity** from the oxidation of NADH [19], and **polyphenoloxidase activity** from the oxidation of guaiacol or pyrocatechol [20].

The lignins (LGs) of kenaf, the cotton plant, and birchwood (LGs) were added to the culture medium on the sowing of the fungus in a concentration of 2-20 mg/ml of medium, dissolved in dioxane or dimethylformamide.

Production of Ligninase Preparations. The enzymes were isolated from 15-day culture liquid. As the first stage we used traditional methods of isolating enzymes. Total LGP preparations were obtained by precipitating cooled (4°C) c.l. with organic solvents: acetone, isopropyl alcohol, and ethanol, and also by fractionation with ammonium sulfate. After 24 hours' standing in a cold room at 0-4°C the precipitate that had deposited was separated by centrifugation, washed several times with distilled water, and dried in the air.

The enzyme preparation obtained by fractional precipitation with ammonium sulfate was dissolved in the minimum volume of distilled water and desalted on a column (5 × 50 cm) of Bio-Gel P-6 (Bio-Rad, USA) equilibrated with 0.005 M Na acetate buffer, pH 4.65. The protein fractions containing enzyme activity were combined and lyophilized.

Electrophoresis of the technical enzyme preparations was conducted under denaturing conditions according to Laemmli [21].

ISF points were determined by analytical isoelectric focusing in PAAG with ampholines or farmalits at pH 3-10.

Analysis of the Plant Substrates. The plant substrates — pulp, cottonplant stems, rice husks, and kenaf chaff — were separated from the fungal culture liquid, carefully washed with distilled water, and dried, and then their contents of carbohydrates, lipids, proteins, difficultly- and readily-hydrolyzable polysaccharides, lignin, cellulose, and hemicellulose were determined.

Carbohydrates were determined by the method of [22], **lipids** as in [23], **lignin** as in [24], and **cellulose** and **hemicellulose** as in [25]. Amounts of readily- and difficultly-hydrolyzable polysaccharides were determined as described in [24].

REFERENCES

1. A. Raszczynski, V. Huyhn, and R. Crawford, FEMS Microbiol. Lett., **29**, 37 (1985).
2. P. J. Dodson, C. S. Evans, P. J. Harvey, and J. M. Palmer, FEMS Microbiol. Lett., **42**, 17 (1987).
3. T. Johanson and P. O. Nyman, Acta Chem. Scand., **41**, 766 (1990).
4. T. Jeffries, S. Choi, and T. Kirk, Appl. Environ. Microbiol., **42**, No. 2, 290 (1981).
5. G. F. Leatham, Appl. Microbiol. Biotechnol, **24**, No. 1, 51 (1986).
6. T. K. Kirk, W. J. Connors, and J. G. Zeikus, Appl. Environ. Microbiol., **32**, No. 1, 192 (1976).
7. I. D. Reid, Can. J. Bot., **57**, 2050 (1979).
8. D. C. Ulmer, M. S. A. Leisola, B. H. Schmidt, and A. Fiechter, Appl. Environ. Microbiol., **45**, No. 6, 1795 (1983).
9. L. A. Golovleva, Kh. G. Ganbarov, and G. K. Skryabin, Mikrobiologiya, **51**, No. 4, 543 (1982).
10. B. D. Faison and T. K. Kirk, Appl. Environ. Microbiol., **49**, No. 2, 299 (1985).
11. L. A. Golovleva, V. N. Pertsova, I. E. Fedechkina, Kh. G. Ganbarov, and V. P. Baskunov, Prikl. Biokhim. Mikrobiol., **19**, No. 6, 709 (1983).
12. R. Liwicki, A. Peterson, M. J. MacDonald, and P. Broda, J. Bacteriol., **162**, No. 2, 641 (1985).
13. W. Merril and E. B. Cowling, Can. J. Bot., **44**, 1555 (1966).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and F. J. Randall, J. Biol. Chem., **193**, No. 1, 265 (1951).
15. M. Tien and T. K. Kirk, Science, **221**, No. 46, 661 (1983).
16. J. K. Glenn and M. N. Gold, Appl. Environ. Microbiol., **45**, 1741 (1983).
17. A. Leonowicz and K. Grzywnowicz, Enzyme Microbiol. Technol., **3**, No. 1, 55 (1981).
18. L. V. Gudkova and R. G. Degtyar', Enzymes in Medicine, the Food Industry, and Agriculture [in Russian], Naukova Dumka, Kiev (1968), p. 172.
19. Y. Asada, M. Miyabe, M. Kirkava, and M. Kuwachara, Agric. Biol. Chem., **50**, 525 (1986).
20. E. G. Bekker, Isolation, Properties, and Main Features of the Action of Ligninolytic Enzymes [in Russian], Dissertation ... Candidate of Biological Sciences, Moscow State University, Moscow (1993).
21. U. K. Laemmli, Nature (London), **227**, 680 (1970).
22. M. Dubois, R. A. Gilles, and G. K. Hamilton, Anal. Chem., **28**, No. 3, 350 [sic].
23. É. F. Solomko, L. P. Panchenko, and R. K. Sil'chenkova, Prikl. Biokhim. Mikrobiol., **20**, No. 20, 273 (1984).
24. A. V. Obolenskaya, V. P. Shchegolev, G. L. Akim, et al., Practical Work in the Chemistry of Wood and Cellulose [in Russian], Lesnaya Prom-st', Moscow (1965), p. 411.
25. M. Kyurshner and N. Ganek, Methods of Biochemical Analyses [in Russian], Nauka, Moscow (1974), p. 15.