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Decolorization and Degradation of Daunomycin by *Bjerkandera adusta* R59 Strain

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The ability of *Bjerkandera adusta* R59 strain to degrade anthraquinonic antibiotic (daunomycin) points on its possible aptitudes for decomposing of other anthraquinonic derivatives, *e.g.* lignocellulose subunits or metabolically related lipids, present in wood. This study was performed to investigate the possibility of *B. adusta* R59 to synthesize enzymes participating in decay of wood compounds (including lignin, celluloses, hemicelluloses and lipids). *Geotrichum*–like strain, anamorphic stadium of *B. adusta*, white–rot fungus, was isolated from soil. It was found to completely decolorize and degrade 10% daunomycin post–production effluent during 10 days of incubation at 26 °C. R59 strain produces only small activities of lignolytic enzymes when grown on wheat straw or beech sawdust–containing media but in the presence of humic acids derived from brown coal synthesizes significant activities of laccase and lipase. This phenomenon was coupled with entering the idiophase by this fungus and appearance of aerial mycelium. The ability of *B. adusta* R59 strain to degrade humic acids from brown coal could be useful in constructing of new generation of biologically active filters for purification of humic acids–contaminated comestible waters.

INTRODUCTION

Aromatic and colored compounds (*e.g.* textile, leather or wood dyes) are very resistant when faded upon exposure to many chemical agents or microbial attack. Therefore, dye–containing post–processing effluents are hardly decolorized by conventional biological wastewater treatments (Willmott *et al.*, 1998). Removal of these wastes is a challenging problem because about of 15% of used dyestuff, frequently revealing toxic, mutagenic or carcinogenic features, are released into the process waters (Vaidya and Datye, 1982; Chung *et al.*, 1992). Biotechnological approaches pointed on a fact that some microorganisms are highly potential in combating this recalcitrant pollution (Ollikka *et al.*, 1993; Cripps *et al.*, 1990; Robinson *et al.*, 2001). By far the only microorganisms capable of efficient degradation of dyes belong to white–rot fungi (*e.g. Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus* and *Bjerkandera adusta*), presenting also the abilities of lignin depolymerization. Lignolytic enzymes (laccase and peroxidase) produced by these species are non–specific ones and oxidize, aside from lignin, a lot of different xenobiotics, including chlorinated organic compouns, polycyclic aromatic hydrocarbons, nitrocompounds, azo–dyes, phtalocyanic dyes, anthraquinones and other aromatic compounds (Banat *et al.*, 1996; Bumpus *et al.*, 1985; Field *et al.*, 1993; Paszczynski *et al.*, 1995). However, there are no reports on degrading of anthraquinonic antibiotics by white–rot fungi. Daunomycin is a representative of this xenobiotic group and possesses not only germicidal and bacteriostatic properties but also chromophoric group, responsible for its red colour. This antibiotic is produces by actinomycete *Streptomyces peucetius* on industrial scale. Post–production effluent is neutralized by chemical method and then released as wastewater to natural water reservoirs. However it is still highly harmful, therefore the method of biological degradation of daunomycin could solve the problem of its toxicity.

Investigations in to white–rot fungus, *Bjerkandera adusta* R59, isolated from black earth soil, are very promising approach for its ability to decolorize and degrade post–production wastes containing daunomycin. These processes are accompanied by peroxidase biosynthesis and drop of free radicals and phenolics levels (Malarczyk *et al.*, 1998). This phenomenon is obvious in a light of a fact that white–rot fungi frequently synthesize lignolytic enzymes (laccase, LiP, MnP, versatile peroxidase and SOD). It is therefore possible that white–rot fungus *B. adusta* produces not only lignolytic but also cellulytic and hemi–cellulolytic enzymes.

Moreover it was reported that lignolytic activities of some fungi may be accompanied by lipolytic enzymes, as wood contains significant amounts of lipids–both triglycerides and sterol esters (Gutierez *et al.*, 2002). Lipid peroxidation reaction (*e.g.* MnP) could be a part of the lignin degradation process performed by white–rot basidiomycetes, as *Ceriporiopsis subvermispora* (Bao *et al.*, 1994; Kapich *et al.*, 1999) or *P. chrysosporium* (Kirk *et al.*, 1987). It was confirmed that laccase enzymes play a significant role in degradation of trilinolein (Zhang *et al.*, 2002) by oxidizing unsaturated fatty acids in this lipid. Moreover, the laccase possesses

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also an activity of lipase, showing the possibility of connection between lignolytic and lypolytic pathways.

The ability of *B. adusta* R59 strain to degrade anthraquinonic antibiotic (daunomycin) points on its possible aptitudes for decomposing of other anthraquinonic derivatives, *e.g.* lignocellulose subunits or metabolically related lipids, present in wood. This study reports the results of the effect of *Bjerkandera adusta* R59 to synthesize enzymes participating in decay of wood compounds (including lignin, cellulose, hemicellulose and lipid).

MATERIALS AND METHODS

Fungus isolation

Bjerkandera adusta R59 strain was isolated from black earth soil (Phaeozems, FAO) of medium loam texture from a field near Lublin (South–Eastern Poland). Isolation procedure was described by Ginalska *et al.*'s method (2004).

Daunomycin decolorization

Post–production effluent (culture liquid) after industrial daunomycin (Fig. 1) extraction was kindly provided by the Biotechnology and Antibiotics Institute in Warsaw (Poland). Estimation of daunomycin decolorization rate by *B. adusta* R59 was performed on petri dishes containing agarized Park and Robinson medium (1969) with 5 and 10% of daunomycin post–production effluent. Round pieces (ø=1 cm) of *B. adusta* R59 mycelium after 7 days of growth on PDA medium were used as inocula. Incubation was performed at 26 °C. Extents of decolorization spheres were measured after 4, 7 and 10 days. Daunomycin biodegradation was tested also in liquid Park and Robinson (1969) medium (50 ml) with 10% daunomycin post–production effluent. Cultures were inoculated with 1 ml homogenized *B. adusta* R59 (10^5 c.f.u./ml) from 7 day–culture of fungus on potato–glucose (20% potato, 2% glucose) medium. Stationary cultures and controls (media without inoculation) were incubated at 26 °C. Periodical analyses aimed at determining:

- concentration of daunomycin, measured spectrophotometrically at 480 nm (maximum absorption) against the standard curve on pure daunomycin
- the level of phenolic compounds ($A_{500\,nm}$) according to Malarczyk (1984). The standard curve was performed using vanillic acid
- peroxidase activity measured using o–dianisidine as a substrate according to Maehly and Chance (1954)
- morphology of mycelium based on macroscopic and microscopic observations.

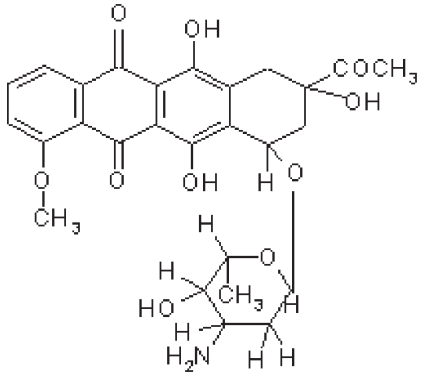

Fig. 1. Daunomycin structure.

Growth conditions

Fungus was cultured in Fahreus medium (Fahreus and Reinhmmar, 1976) with 1% (control cultures and inoculum) or 0.25% glucose (in appropriate cultures). Except of control, medium was enriched with 0.25% glucose and other compounds, as follows:

Version 1–12.5% (w/v) wheat straw (from fields near Lublin)
Version 2–12.5% (w/v) beech sawdust (from local forests)
Version 3–0.01% (w/v) humic acids from brown coal (Fluka, USA)
Version 4–0.01% (w/v) humic acids from chernozem (acc. FAO)
Version 5–0.01% (w/v) humic acids from lessive soil (Haplic Luvisols acc. FAO)
Version 6–0.5% Norlig (Lignotech, USA) and 1mM ferulic acid (sterilized separately under UV–rays for 12 h and added separately after autoclaving).

Media in volumes of 100 ml were sterilized in Erlenmayer flasks (300 ml) and inoculated with 2 ml of inoculum prepared as follows: 200 ml Erlenmayer flask with 100 ml of Fahreus medium (1% glucose) was inoculated with a piece of *B. adusta* R59 mycelium and left at 26 °C until the surface of medium was totally covered with fungal mycelium (7 days). Then it was homogenized in a homogeniser (MPW–309, Precise Mechanics, Warsaw, Poland) in two cycles (1000 rpm, 30 seconds) and passed into culture flasks (2 ml/100 ml of medium). Stationary cultures were grown at 26 °C for: 12 days (control), 14 days (Versions 1, 4, 5, 6) or 18 days (Version 2, 3). Criterion of culturing time was the moment of a total overgrowth of liquid medium surface by mycelium. Shaken cultures were grown for 7 days (time sufficient for intensive fungal growth) at 26 °C an a rotary shaker at 130 rpm. Post–culture liquids were separated from fungal mycelium by filtration, desalted on Sephadex G–25 column and lyophilized. Fungal intracellular extracts were prepared by disintegration of mycelim with alumina (type 305, Sigma) and 5 ml of distilled water followed by centrifugation at 7,000 g (5 min) and 12,000 g (10 min). Enzymatic activities were determined in intracellular extracts and extracellular lyophilizates after dissolving in distilled water.

Enzymatic assays

Endopolygalacturonase activity was estimated according to Lobarzewski *et al.* (1985). 1 U of enzymatic activity was the amount of enzyme that releases 1 μmol of reducing groups from polygalacturonic acid per

minute under reaction conditions. Glucose oxidase activity was estimated according to Fiedurek (1986). 1 U of enzymatic activity was an amount of enzyme releasing 1 μmol H_2O_2 per 1 minute under assay conditions. Laccase activity was estimated according to Leonowicz and Grzywnowicz (1981). 1 U of enzymatic activity was an amount of enzyme that oxidizes 1 μmol of syringaldazine per minute under assay conditions. HRP–like peroxidase activity was measured after Lobarzewski (1981). 1 U of enzymatic activity was an amount of enzyme that oxidizes 1 μmol of guaiacol per minute under assay conditions. Manganese peroxidase was estimated according to Wariishi *et al.* (1991). 1 U of enzymatic activity was an amount of enzyme that oxidizes 1 μmol of guaiacol per minute under assay conditions. Cellulase activity was was estimated according to Mandels and Weber (1969) with cellulose (Whatman 1). 1 U was the amount of enzyme that releases 1 μmol of reducing groups from cellulose per minute under reaction conditions. Lipase activity was estimated according to Sokolovska (1998). Superoxide dismutase activity was estimated according to Marklund (1974). Enzymatic activity was expressed as a percent of inhibition of pyrogallol dimerization under assay conditions. Endo–β–1,4–xylanase activity was estimated according to Bailey *et al.* (1992). 1 U of enzyme activity was expressed as an amount of enzyme that releases 1 μmol of reducing groups from birchwood xylan (Sigma) per minute under assay conditions. Endo–β–1,4–mannanase activity was estimated according to Zakaria *et al.* (1998). 1 U of enzyme activity was expressed as an amount of enzyme that releases 1 μmol of reducing groups from locust bean gum (Fluka) per minute under assay conditions. Protein concentration assay was performd according to Schacterle and Pollack (1973).

RESULTS AND DISCUSSION

The capability of *B. adusta* R59 strain to decolorize daunomycin is a very interesting and possible to applicate feature of this fungus. In this experiment, the time of decolorization of liquid daunomycin wastes depended on antibiotic concentration and development stage of the fungus (Tables 1 and 2). Complete daunomycin decolorization was observed just after 10 days of *B. adusta* growth in media containing 5 and 10% of post–production antibiotic effluent (containing 8.9 μg of daunomycin cm^{-3}) (Fig. 2).

Simultaneously with the increase of antibiotic effluent concentration in medium, lag phase of the fungal growth extended. Decolorization of the medium was found to happen paralelly with the appearance of aerial mycelium and thus paralelly with transformation of the vegetative growth phase (trophophase) to the fructification phase (Table 2) which corresponds with induction of secondary metabolic phase (idiophase). Maximum decolorization of daunomycin in medium was simultaneous with a total covering the culture surface by aerial mycelium. Decolorization of daunomycin has been found to be coupled with peroxidase biosynthesis. A drop of peroxidase activity was coupled with a complete overgrowth of medium surface by fungal hyphae (Table 2). This proves the existence of connections between peroxidase biosynthesis or daunomycin degradation with secondary metabolism of *B. adusta* R59. These observations confirm the results of other authors' (Glenn and Gold, 1983; Cripps *et al.* 1990) investigations into decolorization of synthetic dyes including anthraquinons by white–rot fungus *P. chrysosporium*. They proved that decolorization of those dyes takes place

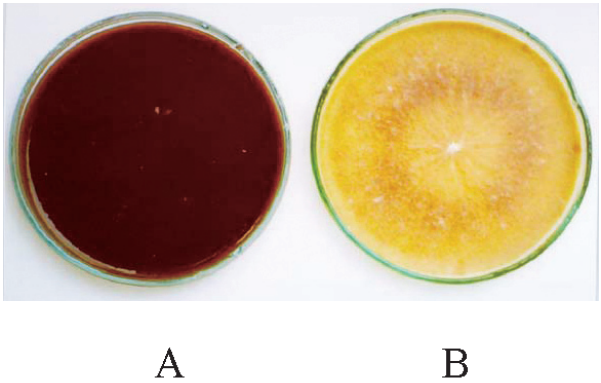

Fig. 2. Decolorization of agarized Park and Robinson medium with 10% post–production daunomycin effluent.
A–Control (without *Bjerkandera adusta* R59 mycelium);
B–after 10 days of *Bjerkandera adusta* growth.

Table 1. The extent of decolorization spheres (diameters in cm) on plates (ø=9 cm) with agarized Park and Robinson medium with post–production daunomycin effluent (5 and 10%) by mycelia of *Bjerkandera adusta* R59 strain

Effluent concentration in agarized medium, %	Culture, day		
	4	7	10
5	2.0	8.5	9.0
10	0.0	6.0	9.0

Table 2. Changes in the features of liquid cultures of *Bjerkandera adusta* R59 in medium with 10% post–production daunomycin effluent

Culture day	Growth characteristics	Daunomycin $\mu g\ ml^{-1}$	Phenolics level μg of vanillic acid ml^{-1}	Peroxidase activity U mg of protein
0	–	8.9	11.0	0
7	g.g.	3.6	7.6	0.294
10	i.g.	1.53	7.37	0.228
14	i.g.	0.5	6.28	0.207
18	i.g.	0.5	4.99	0.089
21	i.g.	0.5	486	0.074

– lack of growth
g.g. intensive growth of submerged mycelium; slight appearance of aerial mycelium
i.g. intensive growth of aerial mycelium (medium totally covered with mycelium)

during secondary metabolism phase (idiophase) and depends on peroxidase biosynthesis.

Daunomycin contains in its structure methoxyl, amino, hydroxyl and quinonic groups (Fig. 2). It is therefore structurally related to lignin subunits and also humic acids polymers. It seems possible that *B. adusta*–as white–rot fungi–will show a tendency for degradation of compounds being wood derivatives–ligninocellulose and hemicellulose. Culture conditions (stationary growth and temperature of 26 °C) imitated natural growth conditions in upper soil layer within a cultivation period. Therefore it was probable that enzymatic activities measured in culture filtrates could resemble those existing in soil extracts after *B. adusta* growth in its natural environment. Lignolytic enzymes reached in stationary cultures small activities or did not appear at all. HRP–like peroxidase showed small activities in cultures containing Norlig A (calcium lignosulfonates originating from sulfite pulping of hardwoods)–0.009 U mg^{-1} of protein and humic acids isolated from soils (chernozem and lessive soil)–0.0054–0.0078 U mg^{-1} of protein. MnP appeared only in cultures grown in medium enriched with beech sawdust (0.06 U mg^{-1} of protein). The only culture versions revealing extracellular laccase activities were humic acids from brown coal (6.7 U mg^{-1} of protein) and from chernozem (0.0053 U mg^{-1} of protein) (Table 3).

Activity of superoxide dismutase (SOD) was not detected in these extracellular fractions, but was present abundantly in intracellular fractions from *B. adusta* R59 mycelia. It reached the activity from 15.9% of inhibition for cultures with beech sawdust to 97% of inhibition for media containing wheat straw, when in control culture reached the value of 52.5%. Fact of solely intracellular presence of SOD is confirmed by publications in world scientific literature (Keele *et al*., 1970). In eucaryotic cells SOD is synthesized in cytosol and then transported into mitochondrial matrix (Bannister *et al*., 1987; Belinky *et al*., 2002) where serves as a scavenger of superoxide radicals, resulting from the final combustion of organic substances and reduction of oxygen to water. Oxidase presenting its activity against glucose shows the highest activity in a culture containing 0.01% humic acids from brown coal. Other versions of culture, including a control, revealed only traces of activity of this enzyme, playing a role of a "helper" during lignin degradation by fungi (Table 3).

Cellulase and hemicellulase are enzymes decomposing cellulose and hemicellulose, constituting 69–91% of total organic substances in wood (46–56% cellulose; 23–35% hemicellulose). In soils, these polymers make about 8–14% of total soil carbohydrates. Cellulase activity in all tested versions of experiment were close to the value estimated for control medium (0.018 U mg^{-1} of protein; Table 3). Among hemicellulolytic enzymes, endo–β–1,4–xylanase and endo–β–1,4–mannanase activities were estimated in this experiment. These enzymes hydrolyse crystalline, hardly hydrolyzing fractions of hemicellulose–xylan (pentosan) and mannan (hexosan). Xylanase was at relatively similar activities in all versions of culture media including control (about 0.02–0.03 U mg^{-1} of protein) (Table 3). This confirms the observation that this enzyme is a constitutive one (Sachslehner, 1998) which did not undergo an induction in presence of hemicellulose–containing media. Mannanase activities, however, were induced about 10 times in versions with wheat straw, beech sawdust and humic acids isolated from soil (Table 3). Presence of humid acids extracted form brown coal increased mannanase activity 100 times in comparison with control. Soluble lignosulfonates (Norlig A) in culture medium did not influence the activity of this enzyme (Table 3).

Extracellular lipase activity was found to increase even 8 times in comparison to the control when B. adusta R59 strain grew in media containing humid acids form brown coal (16.7 U mg^{-1} of protein), chernozem (14.97 U mg^{-1} of protein) or lessive soil (8.27 U mg^{-1} of protein) (Table 3). Lipolytic activity did not change in other versions of culture media, when compared with control. Extracellular activities of endo–polygalacturonase–decomposing easily hydrolysed substances–were very small and present only in control and versions of media containing wheat straw and beech sawdust (Table 3). Wheat straw and beech sawdust were the sole substrates insoluble in medium, therefore the contact of *B. adusta* mycelium with these organic substances was hindered. To avoid this deficiency, shaken cultures of media containing these substrates (together with a con-

Table 3. Extracellular activities of enzymes from *Bjerkandera adusta* R59 after stationary growth on culture media in versions: 1 – with 12.5% (w/v) wheat straw, 2 – with 12.5% (w/v) beech sawdust (from forests), 3 – with 0.01% (w/v) humic acids from brown coal (Fluka, USA), 4 – with 0.01% (w/v) humic acids from chernozem, 5 – 0.01% (w/v) humic acids from lessive soil, 0.5% Norlig (w/v) and 1 mM ferulic acid

Enzyme	Extracellular specific activities in culture versions Umg^{-1} of protein						
	Control	Version 1	Version 2	Version 3	Version 4	Version 5	Version 6
Endopolygalacturonase	0.0034	0.014	0.0049	0	0	0	0
Glucose oxidase	0	0.012	0.005	1.024	0.0071	0.009	0.006
Laccase	0	0	0	6.7	0.0053	0	0
HRP–like peroxidase	0.011	0	0	0	0.0078	0.0054	0.009
MnP	0	0	0.060	0	0	0	0
Cellulase	0.018	0.007	0.0164	0.016	0.0115	0.0093	0
Lipase	2.37	2.84	2.9	16.7	14.97	8.27	2.37
Endo–β–1,4–xylanase	0.003	0.032	0.0195	0.0195	0.023	0.019	0.018
Endo–β–1,4–mannanase	0.102	1.05	0.949	9.936	1.56	0.745	0.111

trol) were performed. The results were presented in Table 4. It is obvious that intensified contact of fungal mycelium with straw and sawdust did not particularly influence the biosynthesis of lignolytic enzymes (laccase, MnP, HRP–like peroxidase) Only activity of glucose oxidase increased to 0.107 U mg^{-1} of protein for sawdust–containing culture, in comparison with 0.006 U mg^{-1} of protein for control. The activities of lipase and mannanase has increased in comparison with control–up to 8 times (for lipolytic activity) and 20 times (for mannan–hydrolyzing enzyme) (Table 4).

The results obtained during the investigation show that both stationary and shaken cultures of *B. adusta* R59 with natural or post–industrial aromatic wood derivatives did not contain significantly increased enzymatic activities in comparison to the control. It is easy to understand in a light of a fact that R59 strain is an anamorphic stadium (*Geotrichum*–like) of haploidal hyphae, living in soil. The teleomorphic stadium of this fungus (*B. adusta*), of dicaryotic hyphae, as a white–rot fungus settles the wood leftovers. Similar organisms were already reported to live in natural conditions (Müller and Loeffler, 1982). Their nutritional demands are subordinated to their natural habitats and accessibility of organic compounds. R59 (anamorphic stadium of *B. adusta*) colonizes soil–an environment containing less recalcitrant and more easily accessible organic substances than wood. Probably this anamorphic fungal stadium possesses less developed and less efficient enzymatic system than its teleomorphic stadium.

Humic acids (HAs) were found to stimulate the synthesis of glucose oxidase, laccase, endo–β–1,4–mannanase and lipase, but only when these polymeric compounds were isolated from brown coal (Fluka). Humic acids extracted from soil did not induce any particular activity of these enzymes. Most probably these interesting observations come from the differences in source and isolation method for particular preparations of humic acids. Table 5 presents some data concerning chemical composition of three humic acids used in this experiment. They show that humic acids isolated from coal and soil differ, specially in their carbon, nitrogen and ashes content. HAs from coal (Fluka) were extracted using HCl (pH 1), while HAs from soils–by alkaline digestion (with 0.5 M NaCl; Schnitzer, 1989). It is possible that HA preparation obtained from brown coal contains some mediators–low–molecular weight compounds inducing laccase synthesis. These hypothesis could be supported by results of Fakoussa and Frost (1990). They found that *Trametes versicolor* grown in presence of brown coal decolorizes humic acids and synthesizes significant amounts of laccase, but not peroxidase. Redox potential of laccase is lower than that of peroxidase because the latter requires H_2O_2 as cofactor, in the contrary to laccase (Kersten *et al.*, 1990; Feng, 1996). Moreover, Facoussa (1990) suggests that decolorization of brown coal–derived humic acids depends on laccase–catalyzed oxidation of low–molecular mediators, participating afterwards in degradation of high molecular complexes of humic acids. *B. adusta* R59 possesses similar enzymatic properties as *T. versicilor* (Table 3); it is therefore possible that humic acids isolated from soils (grey–brown podzolic and chernozem) did not contain low–molecular mediators indispensable for laccase synthesis by *B. adusta*. Biosynthesis of lipase by R59 strain induced by presence of HAs from brown coal appears probably due to the presence of lipids complexed with natural humic substances (Schnitzer and Khan, 1972).

Humic acids, for their high adsorbing abilities, are capable of creating of toxic complexes with other substances present in soils or waters. This phenomenon creates a considerable problem, specially in a case of groundwaters collected in public water supplies. Drinking waters are frequently contaminated with humic acids eluted from brown coals present in soils originating from miocenic sediments. Chlorination of these waters (for elimination of bacteria or microorganisms) may result in creation the halogenic derivatives of mutagenic or carcinogenic properties (Grabinska–Loniewska *et al.*, 2002). These potentially harmful humic acids are usually eliminated from waters by absorption using so called biologically active EX–extra coal filters (Perchuc *et al.*, 2000). The possible application of *B. adusta* R59 in constructing of new generation of biologically active filter is highly promising and worth further research.

Table 4. Extracellular activities of enzymes form *Bjerkandera adusta* R59 after shaking culture on culture media in versions: 1 – with 12.5% (w/v) wheat straw, 2 – with 12.5% (w/v) beech sawdust

Enzyme	Extracellular specific activities in culture versions U mg^{-1} of protein		
	Control	Version 1	Version 2
Glucose oxidase	0.006	0.0125	0.107
Laccase	0	0	0
HRP–like peroxidase	0.013	0	0
MnP	0	0	0
Cellulase	0.037	0.166	0.013
Lipase	1.5	4.16	13.27
Endo–β–1,4–xylanase	0.036	0.017	0.11
Endo–β–1,4–mannanase	0.142	2.1	1.92

Table 5. Partial characterization of chemical composition of humic acids

Humic acid's source	C (%)	H (%)	N (%)	Ashes (%)
Brown coal	44.03	4.32	0.75	20
Lessive soil	51.54	4.41	2.9	4.14
Chernozem	51.4	3.96	3.58	2.87

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