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SCREENING OF EXTRACELLULAR ENZYMATIC ACTIVITY OF MACROFUNGI

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ABSTRACT

Six types of extracellular enzymes activities in thirty cultures of Macrofungi (Macromycetes) of different ecophysiological (wood decaying, saprotrophic, entomophilous, and leaf-litter decaying) and taxonomic fungi groups were studied. Amylase activity was detected in all investigated mushroom cultures, lipase – in 26, laccase – 21, urease – 20. Protease activity was revealed in 6 species and nitrate reductase activity only in *Lepista luscina* and *Morchella esculenta*. As a whole, *L. luscina* with its amount of detected enzymes and their good visualization seemed to be a promising species. Some of investigated mushrooms are reported as species producing extracellular enzymes investigated in this study for the first time: *Hohenbuehelia myxotricha*, *L. luscina*, *Lyophyllum schimeji*, *Phellinus igniarius*, *Piptoporus betulinus*, and *Spongipellis litschaueri* (amylase); *L. luscina*, *Crinipellus schevczenkovi*, *Auriporia aurea*, *Hypsizygus marmoreus*, *L. schimeji*, *Oxyporus obducens*, and *S. litschaueri* (laccase); *A. aureus*, *C. schevczenkovi*, *H. myxotricha*, *L. luscina*, *L. schimeji*, *O. obducens*, and *S. litschaueri* (lipase); *A. aureus*, *C. schevczenkovi*, *H. myxotricha*, *H. marmoreus*, *L. luscina*, *L. schimeji*, *Pleurotus djamor*, and *S. litschaueri* (urease) as well as *L. luscina* (nitrate reductase).
Characterization of enzymatic activity of individual fungal cultures increases the value of the respective culture collection, and simultaneously makes an easier choice to select fungal strains possessing desired enzymatic potential.

Keywords: Macrofungi, amylase, laccase, lipase, protease, urease, nitrate reductase

INTRODUCTION

The attention of researchers in recent decades has been focused on the comprehensive study of different mushroom species, which belong particularly to Macrofungi (Macromycetes). Ascomycetes and Basidiomycetes are two the most diverse and exceedingly numerous group of Macrofungi that have been intensive investigated in various aspects. Exploration of the enzymatic activity of these mushrooms is one of the important trend for understanding their physiological, biochemical features and in order to clear up considerable promising potential for industrial and biotechnological applications. Practical value and perspective directions of the use of enzymes from Macromycetes was shown in the reviews of the last years (**Madhavi and Lele, 2009; Maciel *et al*., 2010; Shraddha *et al*., 2011; Ray, 2012; Thakur, 2012**). Numerous reports devoted to the investigations of lignolytic enzyme production by Basidiomycetes (**Jarosz-Wilkołazka *et al*., 2002; Elisashvili *et al*., 2002, 2008, 2009; Kiiskinen *et al*., 2004; Kalmiş *et al*., 2008; Fernandes *et al*., 2008; Erden *et al*., 2009; Jang *et al*., 2009; Rana and Rana, 2011; Kumari *et al*., 2012; Seshikava and Singara**). A number of works devoted to the exploration of proteolytic (**Sabotič *et al*., 2007; Goud *et al*., 2009; Denisova, 2010), amylase (Goud *et al*., 2009; Jonathan and Adeoyo, 2011; Chen *et al*., 2012**), cellulose (**Elisashvili *et al*., 1999; Jonathan and Adeoyo, 2011; Kobakhidze *et al*., 2012**), and xylanase (**Kubacková *et al*., 1975; Elisashvili *et al*., 1999, 2008, 2009; Kobakhidze *et al*., 2012**) activities. Few articles showed results of investigation of other extracellular hydrolytic enzymes − phytase (**Collopy, 2004; Goud *et al*., 2009**). Despite the importance of lipase practical use there has been only a few investigations of Macrofungi lipase activity (**Goud *et al*., 2009; Verma *et al*., 2012; Thakur, 2012**). Only lipase from basidiomycete *Bjerkandera adusta* has been elucidated in detail (**Bancerz and Ginalska, 2007**). Besides, production of lipase by *Antrodia cinnamonea* was reported by Shu et al. (2006). Such enzymes as nitrate reductase (**Ali and Hipkin 1989; Mukchaylova and Buchalo, 2012**), urease (**Mukchaylova and Buchalo, 2012**) etc. have been studied seldom. The most of the works are devoted to the survey of the enzymatic activity of one ecophysiological group of mushroom, particularly white-rot fungi (**Kachlishili *et al*., 2006; Elisashvili *et al*., 2009; Isroi *et al*., 2011; Nagadesi and Arya, 2013**). Numerous papers devoted to the investigation of: one class of enzymes in different mushroom species (**Kubacková *et al*., 1975; Saparrat *et al*., 2000; Elisashvili *et al*., 2002, 2008, 2009; Jarosz-Wilkołazka *et al*., 2002; Kiiskinen *et al*., 2004; Nakamura *et al*., 2011; Chen *et al*., 2012**), various classes of enzymes in one (**Buchalo *et al*., 2011a, 2012; Chmelová and Ondrejovič, 2012**) or two mushroom species (**Rana and Rana, 2011; Majolagbe *et al*., 2012/13**), or particular enzyme in one fungi (**Shu *et al*., 2006; Bancerz and Ginalska, 2007**). Paradoxically, there have been few studies of extracellular enzymes of fungi belonging to different ecophysiological and taxonomical groups (**Saparrat *et al*., 2000; Denisova, 2010; Floudas *et al*., 2012**). Admittedly, the qualitative determination of enzyme activity is effective simple method, which is particularly useful for exploration of large number of research objects. Besides, the color reactions with different indicators were successfully used to determine enzyme activity in some screening (**Jarosz-Wilkołazka *et al*., 2002; Kiiskinen *et al*., 2004; Kalmiş *et al*., 2008; Saparrat *et al*., 2000**). The literature data on the enzymatic activity not always coincide because the enzymes were analyzed using different conditions (pH values, temperature, solid or liquid culture media, incubation period, sets of substrates, indicators, inhibitors) and mushroom samples (fruit bodies, spores, vegetative mycelium, cultural liquid). Considering that the amount of enzyme depends on the species and strain, the selection of mushrooms with substantial synthesis of enzymes is always actual and is a first necessary step of the screening. The results of our previous work (**Krupodorova and Barshteyn, 2011**) on the study of the growth of mushrooms on different substrates have led us to the next stage of research – the study of mushroom enzymatic activity.
The aim of this work was to get information on the enzymatic properties of Macrofungi from different ecophysiological and taxonomical groups.

MATERIAL AND METHODS

Fungal species

Thirty macrofungi from different ecophysiological (wood decaying, saprotrophic, entomophilous, and leaf litter decaying) and taxonomic groups belonging to 6 Orders and 18 Families were screened (Tab 1). All the mushroom species used in this study were kindly donated by the Culture Collection of Mushrooms (IBK) of the National Academy of Sciences (NAS) of Ukraine of M.G. Kholodny Institute of Botany NAS of Ukraine, Kyiv, Ukraine (**Buchalo *et al*., 2011b**). Stock cultures were maintained on beer-wort-agar slants at 4 ºC.

Table 1 Enzymatic activity of Macrofungi

Fungal species	Strain	Ecophysiological groups	Amylase	Laccase	Protease	Lipase	Urease	Nitrate reductase
Basidiomycetes								
Agrocybe aegerita (V. Brig.) Singer	1853	Wood-decay fungi (brown rot)	1	1	0	0	3	0
Auriporia aurea (Peck) Ryvarden	5048	Wood-decay fungi (brown rot)	2	1	0	1	1	0
Coprinus comatus (Mull.) S.F. Gray	137	Soil saprotrophic	2	3	0	2	0	0
Crinipellus schevczenkovi Bukhalo	31	Leaf litter decay	2	2	0	3	2	0
Inonotus obliquus (Pers.) Pilat.	1877	Wood-decay fungi (white rot)	2	1	0	0	2	0
Flammulina velutipes (Curt.) Sing.	1878	Wood-decay fungi (white rot)	3	1	0	1	1	0
Fomes fomentarius (Fr.) Gill.	355	Wood-decay fungi (white rot)	2	2	0	0	2	0
Fomitopsis pinicola (Sw.) P. Karst.	1523	Brown-rot fungi	2	1	0	3	0	0
Ganoderma applanatum (Pers.) Pat.	1701	Wood-decay fungi (white rot)	1	2	0	2	1	0
Ganoderma lucidum (Curtis) P. Karst.	1900	Wood-decay fungi (white rot)	2	2	0	3	0	0
Grifola frondosa (Dicks.) S.F. Gray	976	Wood-decay fungi (white rot)	2	1	3	3	0	0
Hericium erinaceus (Bull.: Fr.) Pers.	970	Wood-decay fungi (white rot)	3	1	0	2	1	0
Hohenbuehelia myxotricha (Lev.) Singer	1599	Wood-decay fungi (white rot)	3	0	0	2	1	0
Hypsizygus marmoreus (Peck) H.E. Bigelow	2006	Wood-decay fungi (white rot)	1	1	0	1	3	0
Laetiporus sulphureus (Bull.) Murr.	352	Wood-decay fungi (brown rot)	1	0	2	3	0	0
Lentinus edodes (Berk.) Sing.	502	Wood-decay fungi (white rot)	2	3	1	3	0	0
Lepista luscina (Fr.) Singer	64	Soil saprotrophic	3	3	0	1	3	3
Lyophyllum schimeji (Kawam.) Hongo	1662	Soil saprotrophic	2	1	0	3	3	0
Oxyporus obducens (Pers.) Donk	5085	Wood-decay fungi (white rot)	2	1	0	2	0	0
Phellinus igniarius (Fr.) Quel.	1589	Wood-decay fungi (white rot)	2	1	0	3	0	0
Piptoporus betulinus (Bull.) P. Kast.	327	Wood-decay fungi (brown rot)	2	0	3	1	0	0
Pleurotus djamor (Rumph. ex Fr.)	1526	Wood-decay fungi (white rot)	2	0	0	1	3	0
Pleurotus eryngii (DC.) Quel.	2015	Wood-decay fungi (white rot)	2	1	0	0	0	0
Pleurotus ostreatus (Jacq.) Kumm.	551	Wood-decay fungi (white rot)	3	1	0	3	3	0
Schizophyllum commune Fr.: Fr.	1768	Wood-decay fungi (white rot)	2	0	0	2	3	0
Spongipellis litschaueri Lohwag	5312	Wood-decay fungi (white rot)	2	1	0	3	2	0
Trametes versicolor (L.: Fr.) Quel.	353	Wood-decay fungi (white rot)	2	0	0	2	1	0
Ascomycetes								
Cordyceps militaris (L.) Link.	1862	Entomophilous	2	0	2	2	3	0
Cordyceps sinensis (Berk.) Sacc.	1928	Entomophilous	3	0	2	2	2	0
Morchella esculenta (L.) Pers.	1843	Soil saprotrophic	2	0	0	2	2	2

Legend: 0 – negative reaction, 1 – weak positive reaction, 2 – positive reaction, 3 – strong positive reaction

Screening Methods

During this screening experiment, the presence of six extracellular enzymes was determined qualitatively according to the method (with modification) described by **Molitoris and Schaumann (1986)**.

The screening of amylase, laccase, protease, and urease activity was performed using Petri-dishes (90 mm in diameter) with 25 ml of the basal medium GPDA, g/L: glucose – 5, peptone – 2.5, yeast extract – 0.5. Each Petri-dish was inoculated with one agar disk (8 mm diameter) obtained from the edge of actively growing mycelium of mushrooms (6 or 7-days old) and incubated for 14 days at 26±2 ºC. Petri-dishes were sealed by film Parafilm to save the humidity of the nutrient medium. Exploration of lipase and nitrate reductase was conducted in test tubes.

Positive reaction on amylase activity was observed when light (cream) zones were emerged after addition of 3 % Lugol's solution; the negative reaction was set on a violet color. Growth medium consisted of 900 ml GPDA and 100 ml of 2 % starch solution, pH 6.0.

Visualization of laccase activity was registered in case of occurring reddish-brown color in the medium owing to guaiacol oxidation by generated laccase. Prepared medium consisted of 1 L of GPDA and 0.05 g of guaiacol, pH 6.0.

Protease activity was confirmed by transparent zones around mushroom colony. The first solution for activity evaluation was 900 ml of GPDA, the second solution consisted of 6g of gelatin in 100 ml of water. Two solutions sterilized separately, cool down to 45 ºC and mixed together.

Positive reaction on lipase activity manifested if we observed residue of saponificated compounds under the mushroom colony. Growth medium consisted of GPDA – 1L, $CaCl_2$ – 0.5 g, Tween 80 – 10 ml, pH 6.0. In this experiment we used test tubes with uncut medium.

Urease converts urea to ammonium, which changes pH of the medium and color of phenol red indicator from yellow to pink. Growth medium were: GPDA – 1 L, NaCl – 5 g, KH_2PO_4 – 2 g, phenol red – 0.012 g. Aseptic solution of 20 % urea was added to growth medium after sterilization, pH 5.2.

Nitrate reductase converted nitrate to nitrite and changed the color of medium, after addition of sulfanilic acid and α-naphtylamine, to bright pink. Growth medium consisted of 1 L of GPDA and 15 g of $NaNO_3$, pH 7.4, and control medium was GPDA. The first solution composed of 0.5 g sulfanilic acid and 150 ml of 5N acetic acid. For preparation of the second solution we diluted 0.1 g of α-naphtylamine in 20 ml of distilled water and appended to 150 ml with 5N acetic acid. We added 0.2 ml of each solution in the test tubes with cultures and inspected color alterations in 10 and 60 min.

All the experiments were carried out in triplicates.

Evaluation of enzymatic activity

Extracellular enzymatic activity was conditionally divided by us in the following scale: 0 – negative reaction (no activity was observed, the color of medium without changes); 1 – weak positive reaction (zone of activity for amylase and lipase ≤ 2 mm; for protease – hardly appreciable clear zone around the colony; in case of nitrate reductase and urease the color of medium changed to pale pink); 2 – positive reaction (zone activity for amylase and lipase 2.1−6.9 mm, for protease – 1–2 mm; in case of nitrate reductase and urease the color of medium changed to pink); 3 – strong positive reaction (zone activity for amylase and lipase ≥7 mm; for protease – 4–5 mm; in case of nitrate reductase the color of medium changed to scarlet, and urease – to raspberry-red). Areas of activities photographed with a digital camera Panasonic DMC-FZ7 (Japan).

RESULTS AND DISCUSSION

The studied mushrooms species showed the presence of different enzyme activities. In keeping with our previous results **(Krupodorova and Barshteyn, 2011)** about the ability of mushrooms to the active assimilation of macaroni production waste, we are primarily interested in the presence of amylase in the studied species. In confirmation to this fact, in all investigated mushrooms were detected instantaneous amylase activity, an enzyme that is one of the widely used

for breaking down starch. High amylase activity has been marked for 6 species: *Auriporia aurea* (Fig 1A), *Cordyceps sinensis*, *Hericium erinaceus*, *Hohenbuehelia myxotricha*, *Lepista luscina*, and *Pleurotus ostreatus* (Tab 1). Amylase activity has been detected in **Goud *et al.* (2009)** investigations in 28 out of 60 studied Basidiomycetes. These researchers haven't detected amylase in *Ganoderma applanatum*, *Laetiporus sulfureus*, *Pleurotus eryngii*, *Schizophyllum commune*, and *Trametes versicolor*. As opposite to the results of these scientists we have noted positive reaction for amylase in *P. eryngii*, a weak activity in *L. sulphureus* and *G. applanatum*. We also noted the presence of positive reaction in *G. lucidum*. The results published previously have coincided with those obtained by us for amylase activity of *Morschella esculenta* **(Mukchaylova and Buchalo, 2012)**, *G. frondosa* **(Buchalo *et al.*, 2011a)** and *Sch. commune* **(Buchalo *et al.*, 2012)**. The data, that 6 species (*H. myxotricha*, *L. luscina*, *Lyophyllum schimeji*, *Phellinus igniarius*, *Piptoporus betulinus*, and *Spongipellis litschaueri*) have extracellular amylase activity, were received by us for the first time.

Taking into account that about 60% of studied by us fungi cause white rot, we have seen the necessity to conduct the screening for the presence of laccase − one of the most studied and key enzyme, which accountable for lignin degradation. According to the observation results (Tab 1), positive reaction was detected in 21 species (not only marked as lignolytic) mostly in 2-4 days after inoculation and formed reddish-brown zone around the mycelium. The most active producers of laccase were *Lentinus edodes* (Fig 1B), *L. luscina* and *Coprinus comatus* (the latter two mentioned species are referred to the soil saprotrophic). We should note the fact of laccase presence in two (*Agrocybe aegerita* and *Fomitopsis pinicola*) out of four brown-rot species. **Souza *et al.* (2008)** found in the genome of brown-rot silencing genes of laccase. Our investigations showed laccase activity of species *L. luscina*, *Crinipellus schevczenkovi*, *A. aurea*, *Hypsizygus marmoreus*, *L. schimeji*, *Oxyporus obducens*, and *S. litschaueri* for the first time. The existence of laccase has been detected in earlier studies in similar species: *A. agerita* **(Saparrat *et al.*, 2000; Erden *et al.*, 2009; Jang *et al.*, 2009)**, *C. comatus* **(Kalmiş *et al.*, 2008)**, *Fomes fomentarius* **(Elisashvili *et al.*, 2009)**, *Fomitopsis pinicola*, *Flamulina velutipes*, *Ganoderma lucidum* **(Jarosz-Wilkołazka *et al.*, 2002)**, *G. applanatum* **(Jarosz-Wilkołazka *et al.*, 2002; Elisashvili *et al.*, 2009)**, *Grifola frondosa* **(Jang *et al.*, 2009; Buchalo *et al.*, 2011a)**, *L. edodes* **(Jarosz-Wilkołazka *et al.*, 2002; Kalmiş *et al.*, 2008; Buchalo *et al.*, 2011a; Seshikava and Singara, 2012)**, *P. eryngii* **(Kalmiş *et al.*, 2008; Jang *et al.*, 2009; Seshikava and Singara, 2012)** and *P. ostreatus* **(Erden *et al.*, 2009; Jang *et al.*, 2009; Elisashvili *et al.*, 2002, 2008, 2009; Shraddha *et al.*, 2011; Seshikava and Singara, 2012)**. This enzyme has been found unlike our data in *P. djamor* **(Kalmiş *et al.*, 2008)**, *H. myxotricha* **(Jang *et al.*, 2009)**, *L. sulphureus* **(Shraddha *et al.*, 2011; Seshikava and Singara, 2012)**, and *T. versicolor* **(Jarosz-Wilkołazka *et al.*, 2002; Elisashvili *et al.*, 2008, 2009; Erden *et al.*, 2009; Jang *et al.*, 2009; Shraddha *et al.*, 2011; Seshikava and Singara, 2012)**.

There is another unique hydrolase – protease, which deserves much attention due to broad potential of application in different areas **(Ray, 2012)**. Protease activity was revealed in 6 species of the tested fungi (Tab 1). The most prominent protease activity of *P. betulinus* (Fig 1C) became apparent on the 2-d day, while other species (*L. edodes, Grifola frondosa, Cordyceps militaris,* and *C. sinensis*) exhibited transparent zone around their colonies on the 4-th day. Protease activity of *L. edodes* and *G. frondosa* disappeared on the 7-th day, but appeared around the colony of *L. sulphureus*. The results (presence or absence of protease) in *G. lucidum*, *L. sulphureus*, *T. versicolor* (unlike species *G. applanatum*, *P. eryngii*, and *Sch. commune*) are in agreement with report of **Goud *et al.* (2009)**. The manifestations of protease activity of *L. edodes* and *G. frondosa* were found by **Nakamura *et al.* (2011)**. This enzyme in contrast to our data has been detected in *P. ostreatus*, *A. agerita*, *F. velutipes* **(Denisova, 2010)** and *Morchella esculenta* **(Mukchaylova and Buchalo, 2012)**.

The wide versatility of lipase applications in different branches of industry **(Verma *et al.*, 2012; Thakur, 2012)** motivated us to investigate selected fungi species on lipase activity. We found lipase in 26 (about 87 % of investigated) species, though it was not detected in *A. agerita*, *F. fomentarius*, *Inonotus obliquus*, and *P. eryngii* (Tab 1). The presence of this enzyme in fungi demonstrates their ability to use lipids as a source of carbon. We observed lipase activity in a while after the test tubes were fully colonized. The earliest and the most intense in color and quantity residue revealed in *C. shevchenkovi* and *G. lucidum* (Fig 1D) trials on the 4-th day, *G. applanatum* and *F. pinicola* showed less activity on this day. *G. applanatum* colony consumed all medium with residue under the colony on the 14-th day, but the residue emerged again on the 25-th day of cultivation. The latest appearance of activity recorded on the 25-th day of incubation for *P. betulinus* and *H. myxotricha*. Some species, namely, *A. aureus*, *C. schevczenkovi*, *H. myxotricha*, *L. luscina*, *L. schimeji*, *O. obducens*, and *S. litschaueri* haven't yet been examined as lipase producers before our research. **Goud *et al.* (2009)** also evaluated lipase in *G. lucidum, Sch. commune*, but not detected it in *P. eryngii*. On the contrary to their results we found this enzyme in *G. applanatum*, *L. sulphureus*, and *T. versicolor*. In studies of the same strains of *G. frondosa* and *Sch. commune* and by **Buchalo *et al.* (2011a, 2012)** have been detected the identical existence of lipase.

Despite the immense importance of the availability of nitrogen-containing components for the growth of fungi, the information about nitrate assimilation by basidiomycetes is still limited. In this aspect we studied urease and nitrate reductase enzymes in investigated fungi. In mushrooms urease and nitrate reductase contribute to the transformation of nitrogen sources of media for the formation of nitrogen-containing cell components.

The presence of urease noted in about 67 % of studied species of fungi (Tab 1). Urease activity of *C. militaris* (Fig 1E), *P. ostreatus*, *L. luscina*, *S. litschaueri*, *A. aegerita*, *C. comatus*, and *M. esculenta* revealed on the 2-d day of cultivation in Petri dishes. The presence of urease activity in our studies was sufficiently close to results obtained for *M. esculenta* **(Mukchaylova and Buchalo, 2012)** higher for *Sch. commune* **(Buchalo *et al.*, 2012)** and lower for *Gr. frondosa* **(Buchalo *et al.*, 2011a)**. The latest appearance of activity was observed for *A. aureus* and *G. applanatum* on the 20-th day of cultivation. The secretion of urease by *A. aureus*, *C. schevczenkovi*, *H. myxotricha*, *H. marmoreus*, *L. luscina*, *L. schimeji*, *P. djamor*, and *S. litschaueri* observed in our investigations for the first time.

As a rule, nitrate reductase is the basic enzyme in regulation of nitrate assimilation in most organisms. Only two soil saprotrophic species: *L. luscina* (Fig 1F) and *M. esculenta* showed direct nitrate reductase activity (Tab 1). Quite similar results for *M. esculenta* have been received by **Mukchaylova and Buchalo (2012)**. *L. luscina* have never been presented in the literature as producer of this enzyme.

In summary, our research has expanded the knowledge of enzymatic activity of studied mushrooms. The investigation of enzyme profiles of fungi showed their qualitative variations and level of its visualization. Enzymatic activity of mushroom species can be represented in our work as follows (from often to rarely encountered in studied fungi): amylase > lipase > laccase > urease > protease > nitrate reductase. The information of different enzyme presence in mushrooms may be useful for further experiments with bioconversion of various substrates.

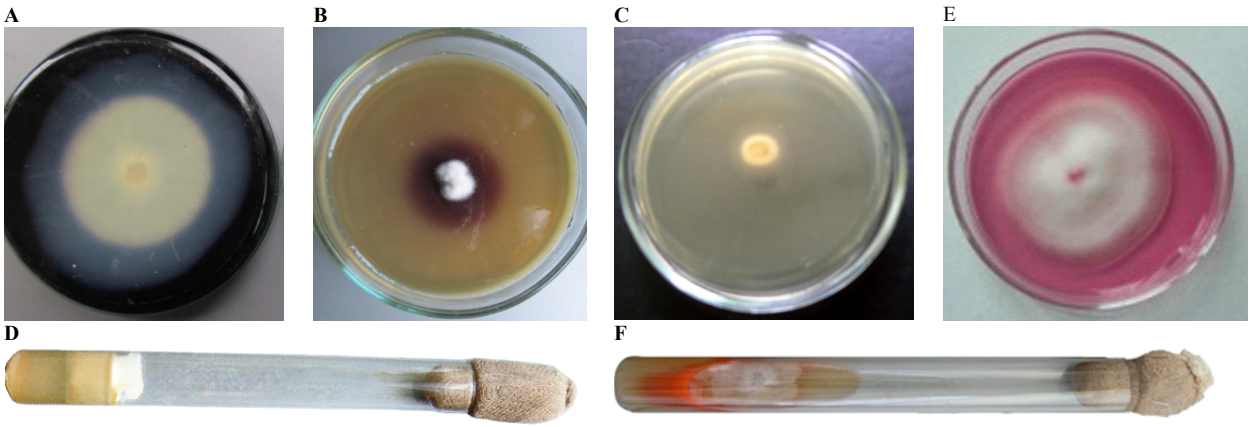

Figure 1 (A-F) The illustration of different enzymatic activity of selected mushrooms: amylase – *Auriporia aurea* (A); laccase – *Lentinus edodes* (B); protease – *Piptoporus betulinus* (C); lipase – *Ganoderma lucidum* (D); urease – *Cordyceps militaris* (E), nitrate reductase – *Lepista luscina* (F).

CONCLUSIONS

The study of mushroom enzymatic activity is one of the important stages of understanding their physiological and biochemical features, and the revealing of perspective ways of their further use. The possibility of various enzyme syntheses is encoded in the genome of mushrooms, and it can be realized under certain conditions of cultivation. The results of the present study showed different potential for producing the range of extracellular enzymes by thirty investigated

Macrofungi from different ecophysiological and taxonomical group. The investigated species of fungi contain from 2 to 5 enzymes (out of 6 studied). Basidiomycete *L. luscina* can be considered the most interesting because of its enzyme amount and their good visualization. One of the main results of the study was obtaining for the first time data about the ability of some fungi to produce one or the other extracellular enzymes. Our screening has helped us to select for future quantitative enzymatic determination of some perspective mushrooms: *H. erinaceus*, *A. aureus*, and *C. sinensis* (amylase activity); *L. edodes*, *L. luscina*, and *C. comatus* (laccase activity); *G. frondosa* and *P. betulinus* (protease activity); *C. militatis*, *L. luscina*, and *P. ostreatus* (urease activity); *L. luscina* and *M. esculenta* (nitrate reductase activity). Our research has shown the feasibility of investigation of other important enzyme presence in studied fungi.

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