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Antibacterial Activity of Macromycetes Mycelia and Culture Liquid

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The antibacterial activities of thirty mushroom species belonging to Basidiomycetes and Ascomycetes, cultivated on two liquid media, were evaluated against gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria by the disk diffusion method. All of the mushrooms, except *Auriporia aurea*, *Fomes fomentarius*, and *Lyophyllum shimeji*, showed different antibacterial activity levels—from 9.5 mm in diameter of the inhibition zone to full inhibition of growth of the test bacteria. The antibacterial activities of *Crinipellis schevczenkovi*, *Hohenbuehelia myxotricha*, *Oxyporus obducens*, and *Spongipellis litschaueri* were observed for the first time. The antibacterial potential of culture liquids of the investigated species was higher than that of their mycelia activity. Dependence of the intensity of antibacterial activity on the culture medium was shown. The antibacterial efficiency of the most active species (*Lentinus edodes*, *Piptoporus betulinus*, and *Phellinus igniarius*) was verified and compared with those of some commercial antibiotics and natural essential oils of *Salvia* and *Eucalyptus*. The culture liquid of *Piptoporus betulinus*, obtained after cultivation on glucose-peptone-yeast culture medium, is a potential substance for further creation of antibacterial products.

Keywords: **Macromycetes, mycelium, culture liquid, antibacterial activity**

Introduction

Creation of antibacterial drugs is a continuous process due to the appearance of multi resistant to existing antibiotics pathogens and new pathogen species. First of all, researches are interested in medications from natural sources having antibacterial activity and less side effects. In the past several decades there has been a growing interest in investigation of therapeutic potential of higher mushrooms (phylum Basidiomycota and some of phylum Ascomycota) including the study of antibacterial activity of their extracts. The results of numerous studies of mushrooms antibacterial activity were summarized in reviews [2, 28]. Antibacterial potential against 32 species of 18 genera of bacteria has been detected in 316 species of 150 genera of Basidiomycetes and Ascomycetes [28]. A significant number of investigations of mushrooms antibiotic activity indicate its varying intensity: mild, moderate, strong or its total absence [4, 6, 8, 9, 15, 21, 29, 30, 32, 35, 37, 43]. The majority of studies have been carried out on fruiting bodies [6, 9, 21, 29, 30, 32, 35], less – on mycelium [8, 15, 25, 26, 37, 39, 42] and/or culture liquid [3, 26, 34, 42] of Macromycetes. Of particular interest are the studies that allow not only to assess but also compare the antibacterial potential of different vegetative forms of mushrooms and products of their cultivation (fruiting bodies, mycelia, culture liquids) [8, 17, 20, 26, 41, 42]. Only few studies have been devoted to the effects of cultivation conditions of some

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mushroom species on their antibacterial activity [11, 22, 34, 39].

The objects for antibacterial activity determination are, first of all, mushroom extracts. The most used solvents for their preparation are: methanol, ethanol, water, and ethyl acetate, less often – chloroform, dichloromethane, heptane, diethylether, etc. Among the groups of biologically active substances with antibacterial effect identified by researchers: sesquiterpenes and other terpenoids, saponins, phenols, steroids, anthraquinones, quinolones, benzoic and oxalic acids derivatives, glycosides, peptides and proteins [2, 21].

The literature data on antibacterial effects of mushroom extracts are quite contradictory. This effect largely dependents on the mushroom species, their strains and vegetative forms, cultivation conditions, method of extract preparation, methods of evaluation and interpretation of the results. That's why it is quite difficult to compare available data.

The purpose of the present work was to evaluate the antibacterial potential of mycelia and culture liquids of some Macromycetes.

Materials and Methods

Fungal species

Thirty mushroom species: *Agrocybe aegerita* (V. Brig.) Singer 1853, *Auriporia aurea* (Peck) Ryvarden 5048, *Cordyceps sinensis* (Berk.) Sacc. 1928, *C. militaris* (L.) Fr. 207, *Coprinus comatus* (O.F. Müll.) Pers. 137, *Crinipellis schevczenkovi* Bukhalo 31, *Flammulina velutipes* (Curt.) Sing. 1878, *Fomes fomentarius* (L.) Fr. 355, *Fomitopsis pinicola* (Sw.) P. Karst. 1523, *Ganoderma applanatum* (Pers.) Pat. 1701, *G. lucidum* (Curtis) P. Karst. 1900, *Grifola frondosa* (Dicks.) Gray 976, *Hericium erinaceus* (Bull.) Pers. 970, *Hohenbuehelia myxotricha* (Lév.) Singer 1599, *Hypsizygus marmoreus* (Peck) H.E. Bigelow 2006, *Inonotus obliquus* (Ach. ex Pers.) Pilát. 1877, *Laetiporus sulphureus* (Bull.) Murrill 352, *Lentinus edodes* (Berk.) Sing. 355, *Lepista luscina* (Fr.) Singer 64, *Lyophyllum shimeji* (Kawam.) Hongo 1662, *Morchella esculenta* (L.) Pers. 1853, *Oxyporus obducens* (Pers.) Donk 5085, *Phellinus igniarius* (Fr.) Quél. 29, *Piptoporus betulinus* (Bull.) P. Kast. 327, *Pleurotus djamor* (Rumph. ex Fr.) Boedijn 455, *P. eryngii* (DC.) Quél. 2015, *P. ostreatus* (Jacq.) P. Kumm. 551, *Schizophyllum commune* Fr. 1768, *Spongipellis litschaueri* Lohwag 5312, *Trametes versicolor* (L.) Lloyd 353 were kindly supplied by the Culture Collection of Mushrooms (IBK) of the M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine [5]. Stock cultures were maintained on beer-wort-agar slants at 4℃.

Growth conditions

Mycelial cultures were initially grown in Petri dishes (90 mm diameter) on glucose-peptone-yeast agar culture medium (GPY) with pH 6.0, composed of (g/l): 20.0 glucose, 3.0 yeast extract, 2.0 peptone, 1.0 K_2HPO_4, 1.0 KH_2PO_4, 0.25 $MgSO_4 \cdot 7H_2O$, and 20.0 agar. The liquid culture medium GPY without agar and natural liquid medium with CO_2-extraction waste – amaranth flour (60 g amaranth flour in 1 L distilled water) were sterilized by autoclaving for 20 min at 121℃. Flasks (250 ml) with 50 ml liquid medium were inoculated with three mycelial plugs of 8 mm diameter cut from the Petri dishes using a sterile borer at the stage of actively growing mycelia. Mycelia were grown at static cultures (without agitation and in the dark) in flasks for 14 days at 26 ± 2℃.

Separation

Mycelium was separated from the medium by filtration through Whatman filter paper № 4 and washed with distilled water. Then, it was ground into native homogenized mycelium by using mortar and pestle. Cultural liquid (GPY medium) and supernatant after centrifugation (at 3000 rpm for 5 min) of the natural medium with amaranth flour were evaporated on sand bath until the volume of the solution was reduced by a factor of 5.

Bacterial strains

The bacterial cultures *Bacillus subtilis* ATCC 6633, *Escherichia coli* 06, and *Staphylococcus aureus* 209 were kindly supplied by the Culture Collection of Microorganisms of the Department of Industrial Biotechnology of the National Technical University of Ukraine (Kyiv Polytechnic Institute). Tested microorganisms were activated in Mueller Hinton agar (MHA) (37°C, 24 h). Each microorganism was suspended in sterile saline and diluted to 10^6 Colony Forming Units (CFU) per ml. Afterwards, Petri dishes containing MHA were inocu-

lated with the bacterial suspensions.

Screening of antibacterial activity

The antibacterial activity was determined by the agar disk diffusion method. Sterile paper disks (8 mm) impregnated with tested mushroom preparations (mycelium suspension or evaporated cultural liquid) were placed into the Petri dishes with MHA previously inoculated with the bacterial suspensions. The inoculated Petri dishes were incubated overnight at 37°C. Antibacterial activity was assessed by measuring of the inhibition zone diameter (in mm) – clear zones formed around each disc. Antibacterial activity was recorded in cases when the zone of inhibition was greater than 8 mm. Filter discs impregnated with some commercial antibiotics and natural essential oils were used for antibacterial potential comparison. The distilled water was used as negative control. The bioassay was performed in triplicate in order to calculate the mean value.

Results and Discussion

The objects of our study (mycelia and culture liquids of thirty Basidiomycetes and Ascomycetes species) were selected according to the following principle: the antibacterial activity of some of them has been confirmed by researchers, others – by our preliminary studies, part of them has not been explored for this activity. All mushrooms except *A. aurea*, *F. fomentarius*, and *L. shimeji* showed the different antibacterial activity levels – from 9.5 mm in diameter of inhibition zone to full inhibition of test bacteria growth. The majority of studied species showed generally the weak antibacterial properties. The most active species (full inhibition of bacterial growth) were: *L. edodes*, *P. betulinus* and *P. igniarius* (Table 1).

The highest inhibitory activity against *B. subtilis* (Fig. 1A, B) was observed in *P. betulinus* culture liquid produced by cultivation on GPY and *L. edodes* culture liquid obtained by cultivation on both media. *P. igniarius*

Table 1. Antibacterial activity of mushroom species.

Mushroom species		Zone of inhibition (mm in diameter)					
		B. subtilis		*E. coli*		*S. aureus*	
		GPY medium	Amaranth flour medium	GPY medium	Amaranth flour medium	GPY medium	Amaranth flour medium
A. aegerita	M	11.5 ± 0.5	-	-	-	12.5 ± 0.5	-
	CL	14.0 ± 0.0	14.5 ± 0.5	12.0 ± 0.0	-	15.4 ± 1.0	-
A. aurea	M	-	-	-	-	-	-
	CL	-	-	-	-	-	-
C. sinensis	M	-	-	-	-	-	-
	CL	11.0 ± 1.0	-	10.5 ± 0.5	-	11.5 ± 0.5	-
C. militaris	M	-	-	-	-	-	-
	CL	15.0 ± 0.1	-	14.1 ± 0.9	-	13.0 ± 1.0	-
C. comatus	M	-	-	15.0 ± 0.0	-	-	-
	CL	-	-	-	-	-	-
C. schevczenkovi	M	-	-	-	-	-	-
	CL	12.0 ± 0.1	11.5 ± 0.3	14.6 ± 0.5	11.5 ± 0.4	12.0 ± 0.0	-
I. obliquus	M	-	-	-	11.5 ± 0.6	-	11.4 ± 0.6
	CL	-	-	-	12.0 ± 0.0	-	10.8 ± 0.4
F. velutipes	M	-	-	-	-	-	-
	CL	-	-	9.7 ± 0.3	-	-	-
F. fomentarius	M	-	-	-	-	-	-
	CL	-	-	-	-	-	-
F. pinicola	M	-	15.5 ± 0.9	-	13.6 ± 0.4	-	-
	CL	20.0 ± 0.0	12.7 ± 1.0	19.8 ± 0.2	12.8 ± 0.7	21.8 ± 0.8	13.8 ± 1.2

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Table 1. Continued.

Mushroom species		Zone of inhibition (mm in diameter)					
		B. subtilis		*E. coli*		*S. aureus*	
		GPY medium	Amaranth flour medium	GPY medium	Amaranth flour medium	GPY medium	Amaranth flour medium
G. applanatum	M	-	-	-	-	-	-
	CL	10.0 ± 0.1	10.5 ± 0.5	-	-	12.0 ± 0.0	10.5 ± 0.5
G. lucidum	M	19.1 ± 0.9	10.0 ± 0.0	-	13.0 ± 0.9	-	10.0 ± 0.0
	CL	10.0 ± 0.1	-	-	11.5 ± 0.5	-	-
G. frondosa	M	-	-	-	-	-	-
	CL	-	14.5 ± 0.5	-	14.0 ± 2.0	-	11.5 ± 0.5
H. erinaceus	M	-	-	-	12.0 ± 0.3	-	-
	CL	-	14.0 ± 1.1	-	-	-	10.5 ± 0.5
H. myxotricha	M	-	-	-	-	-	-
	CL	13.3 ± 0.7	19.0 ± 1.0	-	10.0 ± 0.0	-	15.0 ± 1.0
H. marmoreus	M	-	-	-	-	-	-
	CL	12.0 ± 0.4	12.0 ± 0.6	-	-	14.0 ± 1.0	14.2 ± 0.8
L. sulphureus	M	-	-	-	-	-	-
	CL	-	14.3 ± 0.4	-	14.6 ± 0.7	-	11.2 ± 0.9
L. edodes	M	-	14.0 ± 1.0	-	11.5 ± 0.5	-	-
	CL	FI	FI	13.0 ± 1.0	12.5 ± 0.5	-	19.5 ± 0.5
L. luscina	M	-	-	-	-	11.0 ± 0.0	10.5 ± 0.4
	CL	10.0 ± 0.0	11.5 ± 0.5	15.0 ± 0.0	11.0 ± 0.0	10.0 ± 0.0	15.0 ± 1.0
L. shimeji	M	-	-	-	-	-	-
	CL	-	-	-	-	-	-
M. esculenta	M	11.0 ± 0.0	10.6 ± 0.4	-	10.3 ± 0.7	23.8 ± 1.2	-
	CL	11.5 ± 0.5	-	-	-	-	14.1 ± 0.9
O. obducens	M	10.0 ± 0.0	-	-	11.5 ± 0.5	-	10.5 ± 0.5
	CL	9.5 ± 0.5	-	-	-	-	10.5 ± 0.5
P. igniarius	M	-	-	FI	-	-	-
	CL	-	-	FI	-	-	-
P. betulinus	M	-	10.0 ± 0.0	-	10.0 ± 0.0	-	-
	CL	FI	19.0 ± 0.9	-	20.0 ± 0.8	FI	20.0 ± 0.2
P. djamor	M	-	-	-	-	-	-
	CL	-	-	-	19.0 ± 1.0	-	11.5 ± 0.5
P. eryngii	M	-	10.0 ± 0.0	-	-	-	-
	CL	-	-	-	-	-	-
P. ostreatus	M	-	-	-	-	-	-
	CL	12.0 ± 0.0	-	12.0 ± 0.2	-	13.0 ± 0.4	-
S. commune	M	11.0 ± 1.0	-	-	12.0 ± 0.8	-	-
	CL	11.0 ± 1.0	-	-	12.0 ± 1.1	-	-
S. litschaueri	M	-	-	-	-	-	11.0 ± 0.5
	CL	-	-	-	-	-	-
T. versicolor	M	16.8 ± 1.2	11.5 ± 0.5	-	-	18.6 ± 1.4	22.0 ± 2.0
	CL	-	-	11.4 ± 0.4	-	-	11.4 ± 0.7

Note: FI – full inhibition of bacterial growth; «-» – the lack of antibacterial activity.

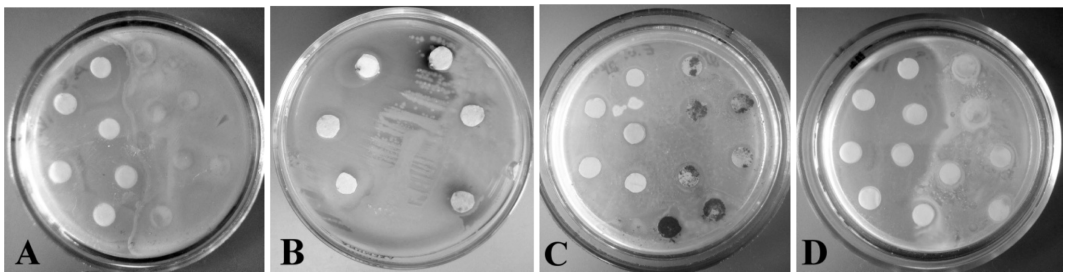

Fig. 1. Inhibition of bacteria growth by mushroom mycelia and culture liquids (cultivation on GPY media): *B. subtilis* ((A) and (B) - effect of *P. betulinus* and *L. edodes*, respectively), *E. coli* ((C) - action of *P. igniarius*) and *S. aureus* ((D) - effect of *P. betulinus* using). Note: left – disks impregnated with the culture liquid of mushrooms, right – native homogenized mycelium of the same mushroom.

mycelium and culture liquid (cultivation on GPY) showed the maximal suppression of *E. coli* (Fig. 1C). The highest activity against *S. aureus* (Fig. 1D) has been established for *P. betulinus* culture liquid (cultivation on GPY). Previous experiments showed that *P. betulinus* (strain Lu9-1) synthesized by submerged cultivation antibiotic piptamine effective against different bacteria, including *B. subtilis* and *S. aureus* [31]. Ethyl acetate extract of the *P. betulinus* fruit bodies have also been reported to contain some metabolites with antibacterial effect: 3β-acetoxy-16α hydroxyl-24-oxo-5α-lanosta-8-ene-21-oic acid – against *B. subtilis*, *S. aureus* and *E. coli* and polyporenic acid C – against *B. subtilis* and *S. aureus* [1]. Isolates from *L. edodes* mycelium [13] and particularly filtrates of this fungus (strain Le-1) [11, 13] suppressed the growth of *B. subtilis*. Filtrates of *L. edodes* inhibited growth of *S. aureus* but were not active against *B. subtilis* and *E. coli* in another study [7]. Generally it was established that different antibacterial compound such as cortineline [27], lentinamicin, β-ethyl phenyl alcohol [19], and lentionin [12, 19] were isolated from *L. edodes*. *Phellinus* spp. cultivated under submerged conditions showed antibacterial activity against *B. subtilis* [10]. The aqueous extract of *P. igniarius* fruit bodies exerted inhibitory action on growth of *S. aureus* including methicillin sensitive strains [36].

The sensitivity of the bacteria to some of commercial antibiotics and natural essential oils (*Salvia* and *Eucalyptus*) has been investigated for comparison (positive control) and assess of the prospects of the selected mush-

Table 2. The assays of antibiotic drugs and essential oils against studied bacteria.

Drugs and essential oils	*B. subtilis*	*E. coli*	*S. aureus*
Sulfadimethoxinum (50 mg/ml), Ukraine	20.3 ± 0.6	-	-
Laevomycetinum (500 mg/ml), Ukraine	FI	FI	FI
Erythromycinum (100 mg/ml), Ukraine	FI	16.0 ± 1.0	FI
Groseptol (480 mg/ml), Poland	FI	-	-
Tetracyclinum (100 mg/ml), Ukraine	FI	18.0 ± 1.4	23.8 ± 2.0
Lincomycin (300 mg/ml), Ukraine	FI	FI	FI
Gramox A (25 mg/ml),Ukraine	FI	25.0 ± 0.2	17.6 ± 2.0
Zitrotsin (40 mg/ml), India	25.0 ± 0.0	-	19.0 ± 0.1
Ceftriaxone (100 mg/ml), Ukraine	FI	FI	FI
Benzylpenicillin (100 mg/ml), Ukraine	FI	FI	FI
Gentamycin sulphate (40 mg/ml), Ukraine	FI	FI	FI
Essential oil of *Salvia*, Ukraine	FI	9.5 ± 0.5	FI
Essential oil of *Eucalyptus*, Ukraine	FI	11.5 ± 0.9	14.5 ± 0.5

Note: FI – full inhibition of bacterial growth; «-» – the lack of antibacterial activity.

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room species (Table 2). So, the activity of *P. betulinus* and *L. edodes* culture liquids was comparable with the action of all tested antibiotics and essential oils. Antibacterial effect of *P. igniarius* mycelium and culture liquid was not only at the level of some antibiotics, but even exceeded the result of other antibiotics and was much higher than that of essential oils (Table 1, 2). The ability of *P. betulinus* culture liquid to inhibit the growth of *S. aureus* was similar to the effect of the most used antibiotics and essential *Salvia* oil (Table 1, 2). Gram-negative bacteria *E. coli* was more resistant to tested antibiotics than Gram-positive bacteria *B. subtilis* and *S. aureus*. This tendency is in line with literature data [40]. The distilled water as negative control showed the absence of antibacterial activity.

Our results confirm existing studies data about the antibacterial activity of extracts from mycelium and/or culture liquid of some mushroom species against similar bacteria: *F. velutipes* [3, 14], *G. lucidum* [16, 17, 20, 23], *Ganoderma* spp. [10, 42], *H. erinaceus* [24, 41], *M. esculenta* [15], *P. eryngii* [15], *P. ostreatus* [15, 38], *T. versicolor* [42]. Some of our results correspond to data of other researchers who did not observe antibacterial activity of the following fungi mycelium: *C. comatus*, *L. sulphureus* [8], *P. ostreatus* [26].

Antibacterial activity of *C. schevczenkovi*, *H. myxotricha*, *O. obducens*, *S. litschaueri* has been observed in our study for the first time. However, antibacterial compounds have been found in some species of genus *Hohenbuehelia*: *H. mastrucata* [37], *H. atrocaerulea* [33], and *Hohenbuehelia* spp. [4].

From the biotechnology point of view, namely, the non-waste using of mushrooms mycelia and their culture liquids, following species are also of interest: *F. pinicola* (efficient against *B. subtilis*) and *T. versicolor* (expedient against *S. aureus*), which have been cultivated on amaranth flour media.

It should be noted that the spectrum of antibacterial activity was varied. The studied mushrooms can be divided into three groups based on the number of bacteria strains, whose growth their mycelia and/or culture liquids inhibited: all three bacteria, two and only one bacteria strain. The results depended on the culture media (Table 1, Fig. 2). It should be noted which species inhibited growth of all three bacteria strains at the same relatively high level (zone of inhibition ca 20.0 mm): culture liquid of *F. pinicola* obtained on GPY medium and culture liquid of *P. betulinus* (amaranth flour medium) (Table 1). The use of the substrate on the basis of amaranth flour for the cultivation of mushrooms promotes the synthesis of antimicrobial metabolites (Fig. 2). The influence of substrate for mushrooms cultivation on antibacterial compounds formation has been established in some studies [11, 22]. Stationary cultivation of *L. edodes* in the liquid media on the basis of agricultural wastes was suitable for producing of antibacterial substances against *B. subtilis* [11]. Addition of free-range chicken droppings manure to the sisal waste substrate for *Coprinus cinereus* (Schaeff) Gray cultivation led to the production of active metabolites against *S. aureus* and *Pseudomonas aeruginosa* [22].

Comparison of the antibacterial activity of mycelium and culture liquid showed that the culture liquid has a higher activity (Fig. 3). This tendency is preserved regardless of the nutrient culture medium of cultivation. The culture liquid obtained on GPY medium exhibited

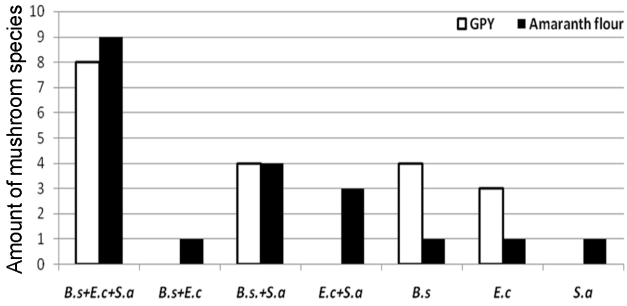

Fig. 2. Antibacterial spectrum of mushroom species. Note: *B.s.* + *E.c.* + *S.a.* – inhibition of growth of all bacteria: *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*; *B.s.* + *S.a.*, *E.c.* + *S.a.*, *B.s.* + *E.c.* – inhibition of growth of two respective bacteria; *B.s.*, *E.c.*, *S.a.* – inhibition of growth of one respective bacteria.

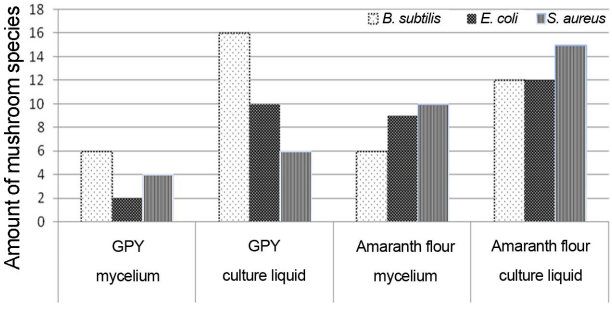

Fig. 3. Antibacterial activity of mushrooms mycelia and their culture liquid on different medium of cultivation.

the highest activity against *B. subtilis*. The use of amaranth flour as the basis of culture medium increased the expression of antibacterial activity of mycelium and culture liquid against *E. coli* and *S. aureus*. The highest antibacterial activity of the mushrooms culture liquid compared with their mycelium was expected due to the ability of mushrooms to produce antibacterial components needed to compete and survive in natural habitat.

Obtained data expanded the knowledge of biopharmaceutical properties of studied mushrooms. Culture liquid of *Piptoporus betulinus* obtained after cultivation on GPY medium is a perspective substance for further development of antibacterial products.

More research is needed not only to identify of the antibacterial compounds but also to better understand of the mechanism of mushrooms antibacterial action in culture condition.

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