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АНАЛИЗ АКТИВНОСТИ ОКИСЛИТЕЛЬНЫХ ФЕРМЕНТОВ МЕТОДОМ МНОГОМЕРНОЙ РЕГРЕССИИ В ПРИСУТСТВИИ MG^{2+} МИЦЕЛИЯ ГРИБА ВЕШЕНКА

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Проведены исследования по оптимизации процесса глубинного культивирования мицелия гриба вешенка. Усовершенствован процесс получения мицелия как посевного материала для выращивания плодовых тел грибов. Изучено влияние различных концентраций карбоната магния (Magnesium carbonates) на ростовые характеристики мицелия гриба *Pleurotus ostreatus* при глубинном культивировании. Выявлена зависимость активности ферментов от концентрации металла в питательной среде прорастания мицелия вешенки. Адаптирована методика определения активности каталазы спектрофотометрическим методом для изучаемых объектов. Впервые получены данные активности ферментов каталазы и супероксиддисмутазы мицелия в присутствии добавки карбоната магния. Установлено, что применение карбоната магния в малых концентрациях положительно влияет на рост биомассы мицелия гриба *Pleurotus ostreatus,* поскольку с увеличением концентрации Mg^{2+} отмечено уменьшение скорости роста биомассы и активности каталазы, предположительно, за счет участия магния в создании определенной ионной концентрации, при которой начинается инактивация каталазы. Изучена возможность применения метода многомерной регрессии в виде метода главных компонент (МГК). Проведен анализ редокс-состояния культуры *Pleurotus ostreatus* на уровне ферментных компонентов системы антиоксидантной защиты при погруженном культивировании базидомицетов, который показал, как взаимосвязаны между собой полученные переменные с разными единицами измерений. Графики счетов также наглядно указывают на зависимость роста мицелия от концентрации применяемой добавки. Введение предложенных в работе условий культивирования в практику грибоводства потенциально способствует более успешному противостоянию макромицетов биотическому и абиотическому стрессу. Результаты исследований актуальны для развития фундаментальных основ науки о грибах.

Ключевые слова: мицелий, каталаза, супероксиддисмутаза, активность ферментов, ионы, магний, гриб вешенка

ANALYSIS OF THE ACTIVITY OF OXIDATIVE ENZYMES BY MULTIVARIATE REGRESSION IN THE PRESENCE OF MG^{2+} OYSTER MUSHROOM MYCELIUM

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Studies have been conducted to optimize the process of deep cultivation of the Oyster mushroom mycelium. The process of obtaining mycelium as a seed for cultivation of fruiting bodies of mushrooms has been improved. The effect of different concentrations of magnesium carbonate (Magnesium carbonates) on the growth characteristics of mycelium of the fungus *Pleurotus ostreatus* during deep cultivation has been studied. The dependence of enzyme activity on the concentration of metal in the nutrient medium of germinating mycelium of oyster mushrooms has been revealed. The method for determining the activity of catalase by spectrophotometric method has been adapted for the studied objects. For the first time the data on the activity of mycelium catalase and superoxide dismutase

enzymes in the presence of magnesium carbonate additive have been obtained. It has been found that the application of magnesium carbonate in low concentrations has a positive effect on the growth of mycelial biomass of the fungus *Pleurotus ostreatus*, since with increasing concentration (Mg2+) a decrease in biomass growth rate and catalase activity has been observed, presumably due to the participation of magnesium in creating a certain ionic concentration at which catalase inactivation begins. The possibility of applying the method of multivariate regression in the form of the principal components analysis (PCA) has been studied. The redox state of *Pleurotus ostreatus* culture at the level of enzyme components of the antioxidant defense system during submerged cultivation of basidomycetes has been analyzed, which showed how the obtained variables with different measurement units are interconnected. The account graphs also clearly indicate the dependence of mycelial growth on the concentration of the additive used. The introduction of the cultivation conditions proposed in this work in the practice of mushroom production potentially contributes to a more successful resistance of macromycetes to biotic and abiotic stress. The results of the research are relevant to the development of the fundamentals of the science of fungi.

Keywords: mycelium, catalase, superoxide dismutase, enzyme activity, ions, magnesium, oyster mushroom

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INTRODUCTION

The question of the possibility of artificial cultivation of edible and medicinal mushrooms has occupied mankind for over two millennia. More than 100,000 species are subjects of scientific research worldwide. The functional importance of fungi in various biogeocenoses is well-known, where, due to their wide range of enzymes, they actively participate in the processes of organic matter destruction and mineralization [1]. Although fungi are a very intriguing group of living organisms, both theoretically and practically, they remain relatively under-researched to date. Macromycetes produce biologically active substances, such as polysaccharides, glycoproteins, terpenes, sterols, and carotenoid pigments, which can demonstrate antibacterial, antiviral, anticancer, antiparasitic, and immunomodulatory properties.

Significant research into the physiology of higher fungi began between the 1980s and 2020s. The complex physiological and biochemical processes occurring during the growth and development of a fungal organism, their intensity, determined by the hereditary and potential qualities of the organism itself and external environment factors, require further study [2]. The study of basidiomycete growth in liquid environments focuses on understanding the nutritional needs and physiology of the species in pure culture and developing submerged mycelium cultivation techniques to obtain feed and food biomass. Deep cultivation is also proposed as a quick and effective method for producing seeding material for mushroom cultivation. Significantly fewer works focus on the biochemistry of deep cultivation, its relationship with the physiology of growth and development of the fungal organism in submerged culture. In particular, the antimicrobial activity of higher fungi is clearly understudied, and only a few studies address this activity in relation to physiological aspects, mushroom morphogenesis issues [3-

15]. Research aimed at creating growing conditions for basidiomycetes, to achieve maximum biomass yield, reduce cultivation time, reduce seeding material, etc., is actively developing. Analyzing the change in the enzymatic activity of basidiomycetes during varying cultivation conditions is one of the main effective indicators of the competitiveness of mycelial biomass growth [16].

In the world of higher plants, enzymes act as antioxidants, protecting cellular components from oxidation by reactive oxygen species (ROS). Primarily, these include the enzyme superoxide dismutase (SOD), catalase, and glutathione peroxidase. Superoxide is one of the most common ROS produced by mitochondria, while SOD converts superoxide anions into hydrogen peroxide, thus serving as a central regulator of ROS levels. The enzyme catalase is a hemin enzyme. The biological role of catalase is to catalyze the decomposition of hydrogen peroxide. *In vivo* (in the cell), most enzymes are spatially organized into so-called "multienzyme systems." They are either associated with cellular structures or are free in various cell organelles.

The practice of adding nutrient supplements for mushroom cultivation during spawning or casing to maximize yield emerged in the 1960s [17] and has been widely recognized and disseminated, but its use may be limited in some sectors due to technical and economic factors. Additives, as a nutrient substance, are defatted plant flour obtained from soybean meal and also bran, which is an organic source of protein enriched with minerals or vitamins, often used for growing *Agaricus* and *Pleurotus* species.

Higher fungi's growth and development are favorably influenced by trace elements in small quantities. Absence of trace elements causes various developmental disturbances in the organism. At specific concentrations of zinc, iron, manganese, copper, calcium, and some other trace elements, there is a stimulation of mycelium formation and growth. For nutrition, i.e., for the main metabolism of fungi, about 17-18 elements are needed, including nitrogen, carbon, oxygen, hydrogen, sulfur, phosphorus, potassium, magnesium, iron, copper, zinc, manganese, molybdenum, calcium. Fungi require the following main elements in large amounts: nitrogen, carbon, oxygen, hydrogen, phosphorus, potassium, sulfur, and magnesium. Therefore, nutrient media with sufficient trace elements, in addition to sources of nitrogen and carbon, add potassium and magnesium. Magnesium plays a significant role in carbohydrate metabolism and all syntheses based on the use of phosphorus bond energy. About 50 enzymatic reactions in fungi involve magnesium.

The purpose of the research is to analyze the redox state of the mycelium of the *Pleurotus ostreatus* culture at the level of enzymatic components of the antioxidant defense system during cultivation with different concentrations of magnesium carbonate $MgCO_3$ using multivariate regression and to determine the activity of catalase and superoxide dismutase in extracts from mycelial biomass.

MATERIAL AND METHODS

The chosen strain for the research was *Pleurotus ostreatus* strain NK-35 from the collection of the agrochemical laboratory of TRPC "Agrocenter" of the Vavilov University. This strain has the following characteristics: during maturation, fruiting waves are roughly uniform - 10-12% for the first wave, 7-10% for the second, and 5-7% for the third. The optimal growing parameters are temperature of 16-17°C; humidity at the time of primordia formation is 90%, after massive formation it decreases to 88%, and during fruiting it is maintained at 85-87%; the level of carbon dioxide should not exceed 850 ppm.

Magnesium carbonate is used as a food additive E 504. In Russia, additive E 504 is permitted for the production of cocoa and chocolate products, cheeses, and other products according to the technical requirements of production. Magnesium is an element required for oxidation processes, which does not form stable organic compounds in mushroom mycelium.

In Russia, the largest share in the range of magnesium fertilizers is attributed to lime-magnesium and potassium-magnesium fertilizers.

The use of organic fertilizers, the chemical composition of which contains magnesium within 0.01-0.09% (according to GOST R 58658-2019 Agricultural products, raw materials, and food with improved environmental characteristics), is notable (see Table 1).

Cultivation was performed periodically using a deep method at a temperature of (28 ± 1)°C from 6 to 14 days with continuous stirring (rotation frequency of 200 rpm) in the dark on a shaker-incubator in flasks with a capacity of 750-1000 ml. Cultivation was carried out in liquid nutrient media containing different concentrations of magnesium carbonate, in a 2% solution of first-grade flour. The nutrient media were autoclaved at 1.2 atm for 0.5 hours.

Total activity of superoxide dismutase was determined by the enzyme's ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to Giannopolitis and Ries[1], with some modifications as described by O.G. Poleskaya et al.[2] The amount of soluble protein in the supernatant was determined by the Bradford method. Superoxide dismutase activity was expressed in arbitrary units per milligram of protein. The activity of catalase in liquid samples was measured by the decrease in the concentration of H_2O_2 upon contact with protein extracts from the mycelium. Catalase activity was determined by the spectrophotometric method at a wavelength of 240 nm. The amount of soluble protein in the supernatant was determined by the Bradford method[3]. Catalase activity was expressed in units of $mM \cdot min^{-1} \cdot mg^{-1}$ of protein. The analysis of the kinetic curves of catalase activity was carried out using the UVWin5 program. Growth characteristics (growth rate) during deep cultivation were determined in accordance with the recommendations[4] based on the accumulation of dry biomass per unit of time depending on the duration of cultivation.

Biomass measurement experiments were conducted in 5-10 repetitions, all others in 3 repetitions. For quantitative data processing, the principal component analysis (PCA) method was used, with particular attention paid to score plots. On the score plot, each sample is depicted in coordinates (t_1, t_2), denoted by GC1 and GC2. An essential property of PCA is the orthogonality (independence) of the principal components. The proximity of two points indicates their similarity, i.e., a positive correlation. The dependence of the average value of the quantity on some other quantity or several other quantities is considered. Unlike the purely functional dependence $y = f(x)$, where each value of the independent variable x corresponds to one specific value of the dependent variable y, with regression linkage, the same value of the independent variable (factor) x can correspond to different values of the dependent variable (response) y depending on the specific case. If at each value $x = x_i$ there are ni values of y_{ij}; $j = 1, n_i$, then the dependence of arithmetic mean values: $y_i = \frac{1}{n_i} \sum_{j=1}^{n_i} y_{ij}$ on x_i

Табл. 1. Дизайн эксперимента
Table 1. Experiment design

No	Group	Initial concentration of $MgCO_3$ in nutrient medium, mol/l	Designation color
1	Control	–	Green©
2	1st	$1 \cdot 10^{-6}$	Lilac©
3	2nd	$1 \cdot 10^{-5}$	Red©
4	3rd	$1 \cdot 10^{-4}$	Yellow©
5	4th	$1 \cdot 10^{-3}$	Blue©

[1]*Giannopolitis C. N., Ries S.K.* Superoxide Dismutases: I. Occurrence in Higher Plants // Plant Physiology. 1977, Vol. 59, pp. 309–314.

[2]*Polesskaya, O.G., Kashirina, E.I., Alekhina, N.D.* Changes in the activity of antioxidant enzymes in wheat leaves and roots depending on the form and dose of nitrogen in the medium // Plant Physiology. 2004, No. 5, P. 686–691.

[3]*Bradford* GPM.1.2.3.0012.15. Determination of protein, GENERAL PHARMACOPEIAN MORNING, Instead of Art. GF XII, part 1.

[4]*Dudka I.A., Vasser S.P., Ellanskaya I.A. and others.* Methods of experimental mycology: a Handbook // Pod. ed. IN AND. Bilay. Kyiv: Naukova Dumka, 1982. 549 p.

and is regression in the statistical sense of the term[5, 6].

RESULTS AND DISCUSSION

To determine the possibility of using the principal component method for processing data on the activity of the enzyme catalase and superoxide dismutase, experimental studies were conducted on the cultivation of mycelium in the media containing different concentrations of magnesium carbonate, as indicated in Table 1, in a 2% solution of first-grade flour. The nutrient media were autoclaved at 1.2 atm for 0.5 hours.

Figure 1 shows score plots based on catalase activity data with several variables, including mass (m) of protein in mg, Σ (total protein mass in the sample); catalase activity per milligram of protein (cat/mg protein); catalase activity per microgram of protein (cat/μg protein); error for activity per 1 mg of protein; error for activity per 1 μg protein; ΔD/min – measurement weight errors). The data obtained by the spectrophotometric method at a wavelength of 240 nm for catalase activity, for *V* sample – 2 ml; ε – molar extinction coefficient of H_2O_2 – 0.039 mol^{-1}; *l* – optical path length – 1 cm; *t* – 1 min.

The obtained data are well modeled by the PCA method with two main components. In this analysis, the indicator of the sample and variable dependencies, representing catalase activity and its spectral range, is essential. Analysis of the score plots in Figure 1 shows a clear dependence of catalase activity on the concentration of magnesium carbonate in the nutrient medium. The proximity of the points highlighted in red (with $MgCO_3$ in the nutrient medium of $1 \cdot 10^{-5}$ mol/l) and purple ($1 \cdot 10^{-6}$ mol/l) demonstrates rapid mycelial biomass growth and a high catalase activity indicator compared to the green points (control). When constructing regression dependence by the least squares method (LSM), it is required that the sum of the squares of the experimental values deviations from those calculated by the approximating dependence be minimal.

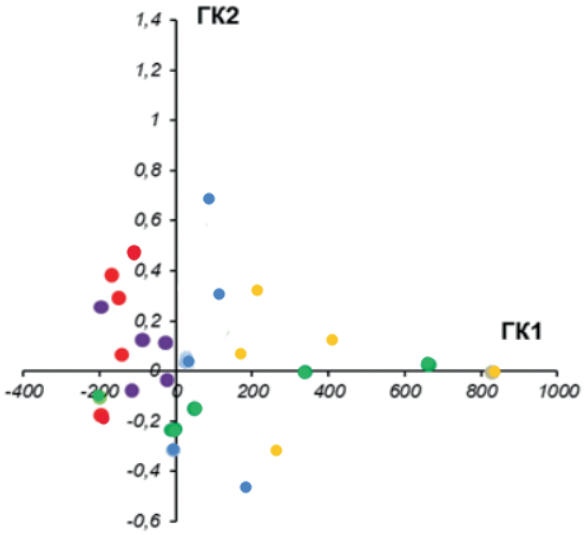

Рис. 1. Графики счетов (ГК1 и ГК2) по активности каталазы mM· мин$^{-1}$ · мг$^{-1}$ белка (контроль – зеленый©; $1 \cdot 10^{-6}$ – сиреневый©; $1 \cdot 10^{-5}$ – красный©; $1 \cdot 10^{-4}$ – желтый©;$1 \cdot 10^{-3}$ – синий©)

Fig. 1. Score plots (GS1 and GS2) for catalase activity mM·min–1·mg–1 of the protein (control-green©; $1 \cdot 10^{-6}$ – lilac©; $1 \cdot 10^{-5}$ – red©; $1 \cdot 10^{-4}$ – yellow©; $1 \cdot 10^{-3}$ – blue©)

Hence, it is clear how the points highlighted in yellow and blue are scattered, with higher magnesium concentrations in the nutrient medium (from $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ mol/l). This indicates a decrease in catalase activity and mycelial mass growth, which may be caused by damage to the enzyme structure or disruption of the catalase biosynthesis pathway in the presence of a high magnesium concentration. Toxins entering plant cells can bind to -SH, -NH_2, -COOH groups of amino acids that are part of the enzyme, which can lead to the suppression of enzyme activity. The concept of the coordinated action of active forms of oxygen (AFO) and metabolites essential for regulating growth, development, and stress tolerance of plant organisms is well known [18-20]. AFOs are multifunctional signaling molecules that contribute to adaptability, and the effect of any compound - a pronounced antioxidant - leads to weak oxidative stress development. Likely, counteracting AFO, agents

[5] *Pomerantsev A.L.* Chemometrics in Excel: textbook. Tomsk, TPU Press. 2014, 435 p.

[6] *Pomerantsev A.L.* Chemometrics in Excel, John Wiley and Sons, 2014, 336 p.

with antioxidant properties – metal-containing additives – influenced the biochemical processes of macromycetes, could be one of the reasons for the decrease in growth indicators due to the absence of stress-dependent activation of some antioxidant enzymes in fungi. Moreover, catalase is a chromoprotein and has an oxidized heme as a non-protein group. One catalase molecule can cause the decomposition of 6 × 106 molecules of H_2O_2 per second. However, catalase has low affinity for hydrogen peroxide, so it starts to function only at its high content in the cell. Increasing the concentration of catalase in cells enhances the decomposition of hydrogen peroxide, which, in turn, positively affects the speed of redox reactions. Possibly, the increase in enzyme activity accelerates cell metabolism processes, affecting growth rate. Therefore, an increase in magnesium concentration leads to a decrease in the growth rate of biomass and catalase activity due to the cells' response at the level of enzyme components of the antioxidant defense system during mushroom cultivation.

Key antioxidant enzymes in cells are SOD, catalase, and peroxidase. SOD provides the "first line" of cell protection against AFOs, catalyzing the dismutation reaction of the superoxide radical in various cell compartments. The hydrogen peroxide formed as a result of superoxide reduction, whose molecule also belongs to AFOs, is neutralized in turn by catalase and PO. It is known from the literature that plants resistant to various adverse environmental factors are characterized by higher antioxidant enzyme activity compared to susceptible ones [20, 21]. In our studies, the determination of superoxide dismutase activity was carried out by the spectrophotometric method at a wavelength of 560 nm; to plot scores based on SOD activity (c.u./mg protein), the above variables were also involved.

The significant increase in SOD activity observed at magnesium concentrations in the nutrient medium (from $1 \cdot 10^{-6}$ to $1 \cdot 10^{-5}$ mol/l), marked on the graph in purple and red points, obviously provides protection of cells from the increasing amount of superoxide radicals. The analysis results in Figure 2 indicate that different concentrations of magnesium carbonate differently affect the protein concentration during the cultivation of the *Pleurotus ostreatus* fungus.

From the score plots, it's evident that concentrations of $1 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$ had a negative impact on the superoxide dismutase (SOD) activity of the mycelium of the fungus *Pleurotus ostreatus*. A low level of SOD when increasing the magnesium concentration in the nutrient solution indicates that the intensification of oxidative processes in their cells doesn't occur. Presumably, the reduction in the intensity of the SOD enzyme and biomass growth in this case may be associated with a decrease in the number of active forms of oxygen (AFO). This, on the one hand, is related to the interaction of metal ions with SH-groups of membrane proteins, causing a change in their properties and, on the other hand, with the ability of the metal to indirectly influence the generation of excessive amounts of AFO.

Analysis of the biomass growth of basidiomycetes, cultivated by immersion in the presence of magnesium carbonate at different concentrations 14 days after sowing nutrient media, revealed a noticeable increase in growth at concentrations of $1 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ of magnesium carbonate, amounting to 120.5% and 112.9%, respectively, compared to control indicators (see Table 2).

However, higher concentrations, on the contrary, showed a decrease in biomass in percentage terms compared to the control. It's known that increasing the content of metals in nutrient media leads to inhibition of physiological processes, and the degree of inhibition largely depends on the metal resistance of the species [20, 21].

The conducted research showed that different concentrations of magnesium differently affect the protein concentration of this type of fungus. Analysis of the results indicates that antioxidant enzymes play an important role in accelerating the growth of *Pleurotus ostreatus* mycelium biomass when using magnesium carbonate in the nutrient solution at concentrations of $1 \cdot 10^{-6}$

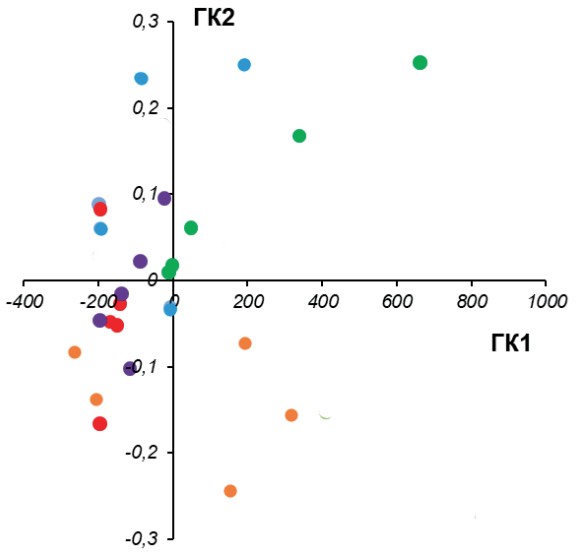

Рис. 2. Графики счетов (ГК1 и ГК2) по активности СОД, усл. ед. /мг белка (контроль – зеленый©; $1{\cdot}10^{-6}$ – сиреневый©; $1{\cdot}10^{-5}$ – красный©; $1{\cdot}10^{-4}$ – желтый©; $1{\cdot}10^{-3}$ – синий©)

Fig. 2. Score plots (GS1 and GS2) by SOD activity, c. u. per mg of protein (control-green©; $1{\cdot}10^{-6}$ – lilac©; $1{\cdot}10^{-5}$ – red©; $1{\cdot}10^{-4}$ – yellow©; $1{\cdot}10^{-3}$ – blue©)

and $1{\cdot}10^{-5}$. Their activity is well modeled by the multivariate regression method.

The data obtained show that the increase in enzyme activity under the influence of metal ions, in particular, in our study of magnesium carbonate can occur in the following ways:

– metal ions directly constitute the active center of the enzyme (catalase, peroxidase);

– metal ions participate in the formation of the enzyme-substrate complex (alcohol dehydrogenase);

– metal ions contribute to maintaining the specific catalytically active conformation of the enzyme molecule, primarily its active center, etc. Specificity is explained by the correspondence of the structure of the enzyme's active center and the substrate.

Thus, for the first time, we processed absorption spectrum data for catalase and superoxide dismutase activity using multivariate regression, depending on the growth factor of mycelial biomass. As the results of this study show, a different metabolic status of the studied magnesium concentrations is formed at the earliest stages of ontogenesis, and this is expressed in different values of enzyme activity indicators and different directions of metabolic strategies. Changes in the activity of catalase, as an important enzyme involved in forming donor-acceptor relationships, also reflect the different metabolic growth status of the fungus mycelium. The results provide a basis for considering the multivariate regression method as a promising approach for the rapid assessment of spectrophotometric data.

Табл. 2. Биомасса мицелия *Pleurotus ostreatus*, культивируемого в присутствии $MgCO_3$

Table 2. Biomass of mycelium *Pleurotus ostreatus* cultivated in the presence of $MgCO_3$

Concentration in the nutrient medium (*C*), mol/l	Biomass, mg	Biomass, % to the control*
Control*	836,6	100
$1{\cdot}10^{-6}$	1008,4	120,5
$1{\cdot}10^{-5}$	944,7	112,9
$1{\cdot}10^{-4}$	825,1	98,6
$1{\cdot}10^{-3}$	785,9	93,9

*Absence of $MgCO_3$ in the nutrient medium.

CONCLUSIONS

1. Based on the conducted research, it can be concluded that the use of magnesium carbonate in the nutrient medium at concentrations of $1{\cdot}10^{-5}$ and $1{\cdot}10^{-6}$ not only influences the enzymatic activity of the basidiomycete *Pleurotus ostreatus*, but also the growth characteristics, as well as the protein concentration in mycelial cells.

2. The use of multivariate regression shows the interrelationships of the obtained variables. Score plots clearly indicate the dependence of mycelium growth on the concentration of the applied additive.

3. Introducing the cultivation conditions proposed in this work into mushroom cultivation practice could potentially lead to more successful resistance of basidiomycetes to biotic and abiotic stress and make a significant contribution to the development of the fundamental basics of mycology. Cultivated higher fungi are

known as a natural biofactory of biologically active compounds, including antioxidant compositions. The research shows the possibility of increasing the yield of basidiomycete biomass by changing their cultivation conditions.

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