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INDUCTION OF LIGNINOLYTIC ENZYMES OF THE FUNGUS *SCHIZOPHYLLUM COMMUNE*

The study focuses on the induction of ligninolytic enzymes that can replace alkaline solutions used to separate lignin from cellulose. *Schizophyllum commune* pure rot fungus culture was identified as a producer and wheat was used as a substrate in the solid phase fermentation process. The experiment was carried out in three parallels: a control sample without a biological object, a sample with the *S. commune* fungus, a sample with the *S. commune* fungus and an inductor in the form of $CuSO_4$ at 30 ± 2°C for 30 days, the moisture content of the nutrient substrate was 70%. Every 10 days, a sample was taken from each sample for quantitative analysis of lignin, which was carried out according to the Klarson method modified by Komarovsky. The initial amount of lignin in the control sample was 20.16% on the 30th day, the content of lignin with the *S.commune* fungus without the inductor was 10.15%, while in the sample with the glaucoma and the introduction of the $CuSO_4$ inductor, a decrease to 8.6% was observed, which much more efficient. The high activity of ligninolytic enzymes produced by the fungus during induction proves the possibility of partial or complete replacement of chemical reagents with natural enzymes, which will significantly reduce the amount of toxic emissions into the environment pulp and paper manufacturing.

Key words: *S.commune*, ligninases, manganese peroxidase, lignin peroxidase, inductor, copper, ecology, toxic emissions.

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Schizophyllum Commune саңырауқұлағының лигнинолитикалық ферменттерін индукциялау

Бұл зерттеу лигнинді целлюлозадан бөлу үшін қолданылатын сілтілі ерітінділерді алмастыра алатын лигнинолитикалық ферменттердің синтезін индукциялауға арналған. Ақ шірік қоздырғышы *Schizophyllum commune* таза саңырауқұлақ дақылы продюсер ретінде бөлініп, қатты фазалық ашыту процесінде бидай субстрат ретінде пайдаланылды. Эксперимент үш параллельде жүргізілді: биологиялық объектісіз бақылау үлгісі, *S.commune* саңырауқұлағы бар үлгі, *S.commune* саңырауқұлағы бар және $CuSO_4$ түріндегі индуктор үлгі. Үлгілер 30 ± 2°C температурада 30 күн сақталды, қоректік субстраттың ылғалдылығы 70% құрады. Комаровский өзгерткен Кларсон әдісі бойынша лигниннің сандық талдауы үшін әрбір 10 күн сайын әр үлгіден сынама алынды. Бақылау үлгісінде лигниннің бастапқы мөлшері 20,16% құрады, 30-шы күні индукторсыз *S.commune* саңырауқұлақтары бар лигнин мөлшері 10,15% болды, ал *S.commune* және $CuSO_4$ индукторын енгізу үлгісінде 8,6%-ға дейін төмендеу байқалды, бұл әлдеқайда тиімді. Индукторды қолдану кезінде саңырауқұлақтар шығаратын лигнинолитикалық ферменттердің жоғары белсенділігі химиялық реагенттерді табиғи ферменттерге ішінара немесе толық ауыстыру мүмкіндігін дәлелдейді, бұл қоршаған ортаға (экожүйеге) улы шығарындыларды айтарлықтай азайтады) целлюлоза-қағаз өндірісі.

Түйін сөздер: *S.commune*, лигниназалар, марганец пероксидаза, лигнинпероксидаза, индуктор, мыс, экология, улы шығарындылар.

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Индуцирование лигнинолитических ферментов гриба *Schizophyllum Commune*

Данное исследование посвящено индуцированию синтеза лигнинолитических ферментов, способных заменить щелочные, вредные для окружающей среды растворы, используемые для отделения лигнина от целлюлозы. В качестве продуцента была выделена чистая культура гриба возбудителя белой гнили *Schizophyllum commune* а в качестве субстрата использовали пшеничную солому в процессе твердофазной ферментации. Эксперимент проводился в трех параллелях: контрольный образец без биообъекта, образец с грибом *S. commune*, образец с грибом *S. commune* и индуктором в виде $CuSO_4$ при 30 ± 2°С на протяжении 30 суток, уровень влажности питательного субстрата составляла 70%. Каждые 10 дней с каждого образца отбиралась проба на количественный анализ лигнина, который проводился по методу Кларсона в модификации Комаровского. Изначальное количество лигнина в контрольном образце составляла 20,16% на 30-е сутки содержание лигнина с грибом *S.commune* без индуктора составило 10,15%, тогда как в образце с грибом и внесением индуктора $CuSO_4$ наблюдалось снижение до 8,6%. Высокая активность лигнинолитических ферментов, продуцируемых грибом при внесении индуктора, доказывает возможность частичной или полной замены химических реагентов на природные ферменты, что значительно сократит количество токсичных выбросов в окружающую среду в целлюлозно-бумажном производстве.

Ключевые слова: *S.commune*, лигниназы, марганецпероксидаза, лигнинпероксидаза, индуктор, медь, экология, токсичные выбросы.

Introduction

Biotechnology is currently at the peak of its popularity in various fields of human activity: health, agriculture, processing, food processing, etc. One of the industries in need of biotechnology is pulppapermaking. Currently, more than 390 million tons of paper and paperboard [1] are produced worldwide, which entails a huge amount of toxic emissions that adversely affect the environment.

The key stage of pulp and paper production is the separation of the target product – cellulose from lignin. Today, two methods are most often used to achieve this goal: less mechanical, more chemical. However, both methods have the same enormous negative environmental impact. For example, mechanical method implies separation of lignin from cellulose using high temperature and pressure [2-3], with thermal emissions to the atmosphere, and chemical method requires use of chemical reagents, alkaline solutions [4-5] which then drain into wastewater, polluting the hydrosphere and the soil layers of the lithosphere. branches.

The walls of plant cells consist of structural components which are represented as cellulose, lignin and hemicellulose. The dry mass of the cell wall is 40-60% cellulose, 20-40% hemicellulose and 20-35% lignin, depending on the plant, so soft wood is 25-35% lignin and deciduous wood 20-25% [6].

The chemical structure of lignin is represented by a highly branched polymer molecule consisting of many monomers, the base of which is phenylpropane. Three types of alcohol are involved in the synthesis of lignin: p-coumaryl alcohol (I), coniferyl alcohol (II), and sinapyl alcohol [7]. By determining the content of alcohols, it is possible to determine the nature of the origin of lignin. For example, three types of lignin are distinguished: the lignin of coniferous plants consists practically only of coniferyl alcohol; hardwood lignin includes coniferyl and sinapyl alcohols; Lignin of herbaceous plants consists of absolutely all alcohols [8]. Lignin is a macromolecule that includes many functional groups, such as hydroxyl (-OH), methoxyl (CH3-O), carboxylic (-C=O-), carboxylic (-COOH) and gasoline (C_6H_6), which increases its uniqueness [9]. In particular, their ratios depend on the source and extraction methods. The presence of a covalent bond between lignin and cellulose and hemicellulose makes it practically impossible to completely purify lignin from impurities [10]. The inability of hydrolytic enzymes to break down lignin is due to its bulky three-dimensional structure and the heterogeneity of C-C and C-O ether bonds. Also, enzymes with low

potential, for example, plant oxidases, which initiate the process of lignin polymerization, are not able to oxidize non-phenolic aromatic subunits of lignin [11].

Lignin acts as a link in the cellulose-hemicellulose matrix through the network structures of lignin-carbohydrates, providing rigidity to cell walls and gluing cells together, thereby protecting polysaccharides from microbial degradation. Due to its hydrophobic property, lignin acts as a dam, preventing water from penetrating into internal structures. The above biological properties of lignin make it unique and one of the most resistant to enzymatic degradation of natural compounds [12].

Despite the fulfillment of important functional properties, lignin is a by-product of such industries as the pulp and paper industry and the production of bioethanol. One aspect of biotechnology is the replacement of the traditional separation of cellulose from lignin by a method using biological facilities to reduce toxic emissions. For example, on the example of decaying trees, it can be concluded that tree-destroying fungi have the ability to biodegradate components of the plant cell wall. Scientists have carried out research on the pathogens of white and brown rot and the enzymes synthesized by them [7-8,13]. In the course of research, it was revealed that white rot fungi differ from brown rot fungi in their ability to produce phenylloxydases that accelerate the oxidative processes of phenols contained in lignin [14]. Wood affected by the white rot fungus acquires a white color due to the biodegradation of lignin and bleaching of wood during oxidative reactions occurring under the action of fungal enzymes [15]. To date, such enzymes as lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Lac) are known to be capable of biodegradating lignin [16]. The mentioned enzymes are synthesized by both bacteria and fungi, including fungi that cause white rot. Some of the most common white rot fungi are: *Trametes versicolor*, *Phanerochaete chrysosporium* and *Schizophyllum commune*. *T. versicolor* affects many angiosperms, but sometimes it also affects gymnosperms. This type of fungus is especially widespread in countries with a temperate climate. *P. chrysosporium* affects the wood of broad-leaved and coniferous trees and is common in countries with a temperate climate [17]. Of the above-mentioned fungi, *Schizophyllum commune* is common in the South of Kazakhstan [18-19]. Its ability to synthesize lignin-destroying enzymes has been repeatedly proven: manganese peroxidases, lignin peroxidases and laccases [20-22].

The last mentioned enzyme, laccase, is widespread in higher plants, is also present in insects, bacteria [23] and cyanobacteria [24], but their abundance is observed mainly in white rot fungi [25], especially *Schizophyllum commune* produces laccases with wide industrial and biotechnological applications. The widespread use of laccase can be evidenced by its use from the food industry [26] up to bioremediation of the soil, and the production of paper [27]. Due to the multidisciplinary use of laccase, there is an urgent need to stimulate its expression and productivity.

Thanks to genetic engineering methods, a large number of transgenic microorganisms with altered expression of genes responsible for lignin synthesis have been obtained [28-29], however, such methods are somewhat more expensive than the use of chemical inductors. Another advantage of inductor metals is the absence of the need for special equipment and easy accessibility, which significantly reduces economic costs. Among the numerous laccase inducers, copper is the most common. The prerequisites for this are: the need for copper by microorganisms is met by very low concentrations, within 1-10 microns [30] and their easy availability. Binding and absorption of copper in fungi usually consists of two phases: first, surface binding, independent of metabolism, and then an energy-dependent influx of metal [31].

In this paper, we investigated the process of biodegradation of straw lignin by the white rot fungus *Schizophyllum commune*, and the importance of copper in the synthesis of ligninolytic enzymes.

Materials and methods

1. Isolation of a pure culture of the fungus S. commune

A sample of the fungus was taken from the surface of the tree in a sterile bag, the next day under sterile conditions it was transferred to Sabouraud's solid nutrient medium and thermostated for five days at 25°C. The resulting mycelium characteristic of the fungus *Schizophyllum commune* was reseeded five times until a homogeneous growth was obtained, indicating the purity of the culture. The inoculum was obtained by washing off the *Schizophyllum commune* culture from the surface of the Sabouraud nutrient medium with 0.9% sodium chloride solution.

2. Straw sampling for microbial treatment

Straw samples were collected in wheat fields near the city of Shymkent, cut into pieces 3-5 cm long and then crushed in an electronic laboratory

mill LK-200 to a size of 100 microns. The particle size was determined by the grid method.

3. Fermentation

Solid phase fermentation was carried out in 250 ml Erleinmer flasks in three parallels: a control sample without inoculum, a sample with inoculum, and a final sample with inoculum and 1.5 mM $CuSO_4$ inducer.

The nutrient substrate containing the inductor contained 20 g of the obtained straw powder moistened with distilled water to provide 70% humidity, 0.5 g of glucose and 0.05 g of $CuSO_4$. The composition of the substrate without the inductor was characterized by the absence of $CuSO_4$, the control sample contained only 20 g of straw powder with 70% humidity. All three substrates were autoclaved at 121°C for 15 min.

Sterilized substrates were inoculated with a 20% (wt) suspension of fungal mycelium and incubated at 30 ± 2°C for 30 days.

4. Lignin content

Samples were taken for analysis at intervals of 10 days for 30 days. 2 grams of the sample were taken for quantitative analysis of lignin according to the Clarson method modified by Komarovsky using 72% sulfuric acid. Pre-dried 1 g of sample (a) was poured into an Erleinmer flask, poured into 150 ml of deionized water and brought to 90–100°C, kept for 1 hour, then the solution was filtered, washed with 300 ml of hot distilled water, and dried to constant weight. The dried precipitate was added to a flask with 150 ml of 1N H_2SO_4 and brought to 90-100°C after 1 hour, the solution was filtered and washed with 300 ml of hot distilled water, dried to constant weight (s). The resulting dry precipitate was moistened with 10 ml of 72% H_2SO_4 and left for 4 hours at room temperature, then 150 ml of 1N H_2SO_4 was added to the mixture and kept at 90–100°C for 1 hour, then the solution was filtered, washed with 400 ml of distilled water, and dried in oven at 105°C to constant weight (d). Then the same precipitate was dried to ash and weighed (e).

The calculation was performed according to the following formula:

$$\% \text{ cellulose} = \frac{c-d}{a} \times 100\%,$$

$$\% \text{ lignin} = \frac{d-e}{a} \times 100\%,$$

where: a is the weight of the sample,

c – weight of the mass at the second weighing,

d – weight of the mass at the third weighing,

e is the weight of the ash.

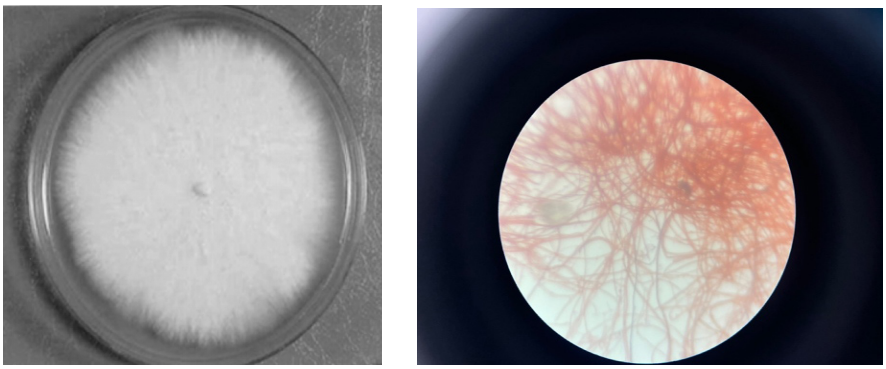
Scanning electron microscopic analysis

Examination of samples and images were obtained using an electron microscope at an accelerating potential of 20 kV with a magnification of 100 times.

Results and discussion

Identification of S. commune

S. commune isolate was identified based on morphological features obtained by microscopic method. The growth of the isolate was rapid at 28° C. on Sabouraud's solid growth medium and the resulting colonies were white. Microscopically, mycelium was found in aggregated hyphal bundles. The hyphae system was monolithic and non-agglutinated (Fig. 1). The morphological characteristics of the obtained were similar to the morphological characteristics of the *S. commune* culture.



a b

Figure 1 – a) Growth on a Saburo nutrient medium;
b) S. commune under a microscope (× 100)

Lignin content

During solid-phase biodegradation of straw lignin by *S. commune.* within 10, 20, and 30 days, degradation of 21, 42, and 48% of lignin, respectively, was recorded. Regarding the samples with the addition of the inductor, lignin degradation was 21, 48, 58% on days 10, 20, and 30, respectively. Comparative data with the control sample are presented below (Table 1) (Fig. 2).

Table 1 – Lignin content in samples on days 10, 20, 30

Sample	Substrate with S. commune (%)	Substrate with S. commune and $CuSO_4$ inducer (%)
Control	20,16	20,16
10th day	16,9	15,9
20th day	11,5	10,4
30th day	10,5	8,6

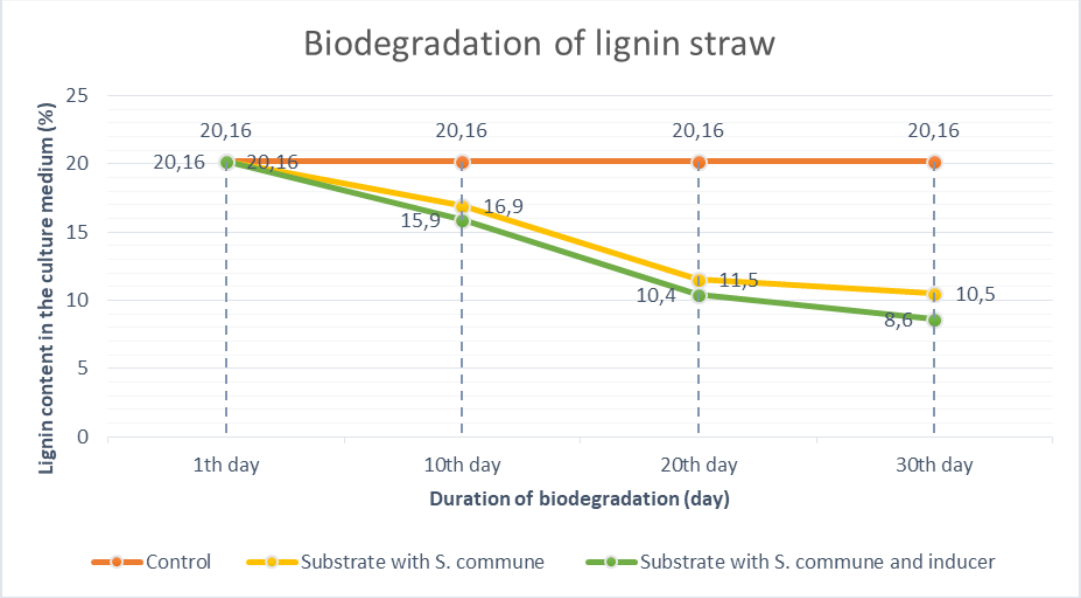


Figure 2 – Lignin content in samples on days 1th, 10th, 20th, 30th

Examination of samples under a scanning electron microscope

The process of biodegradation of straw samples exposed to *S. commune* mushroom culture for 30 days was studied using a scanning electron microscope JSM-6490LM with an energy dispersive X-ray microanalysis system Energy INCA 350 and the HKL Basic system.

On scanned electron micrographs of straw samples degraded for 30 days by the culture of the fungus *S. commune*, a coating of fungal spores was observed that occupied cell cavities and small cell breaks. Some fungal hyphae were also scattered over the surface (Fig. 3a). Samples degraded for 30 days by the fungus culture *S. commune* with the addition of an inductor in the form of $CuSO_4$ showed the most loose structure of cells and cell walls on the surface (Fig. 3b). In some places, you can see the glow of crystals of the $CuSO_4$ inductor. Scanned electron micrographs of control straw samples (Fig. 3c) showed the correct cell structure without any damage to cells and cell wall components.

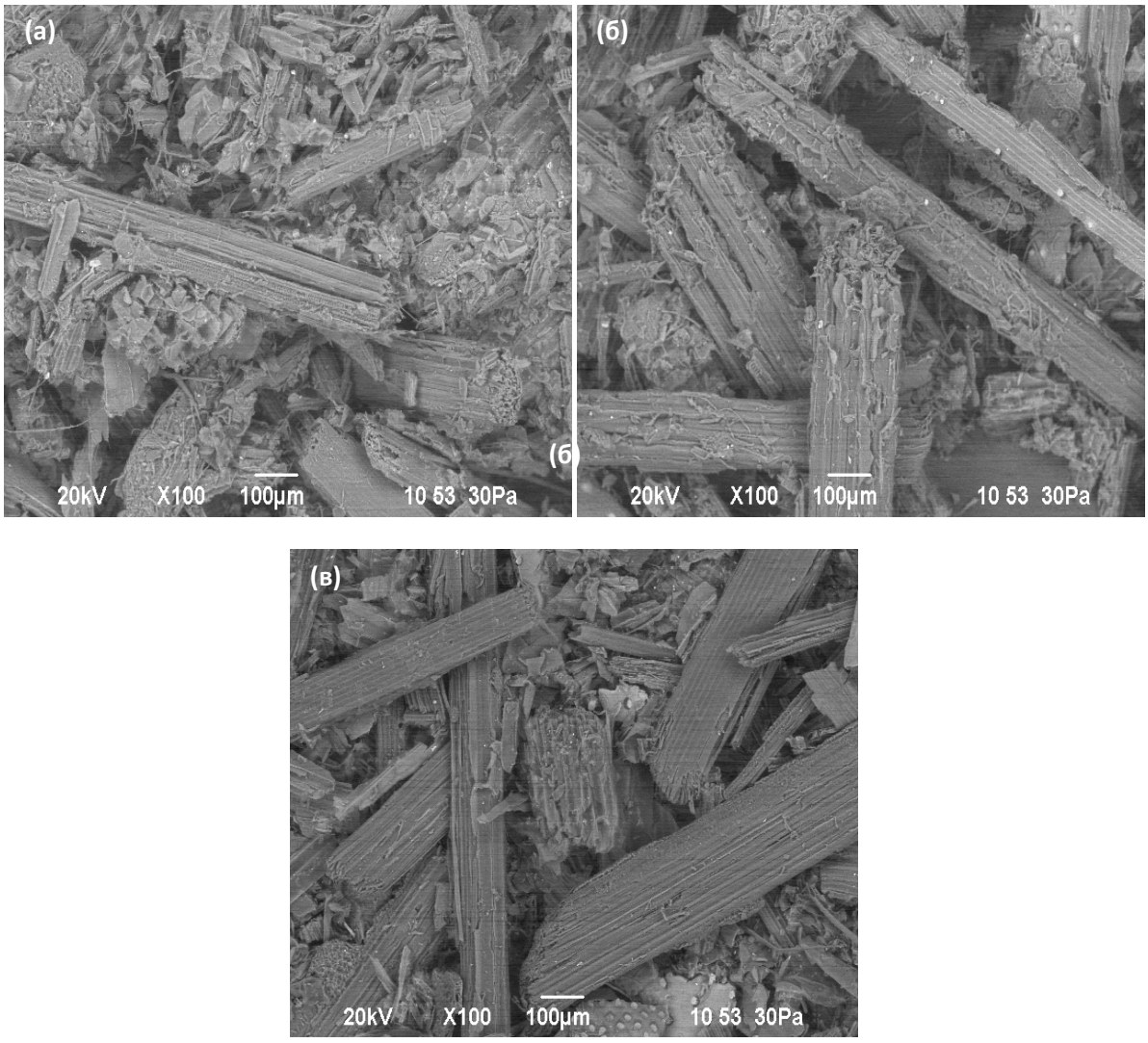


Figure 3 – Samples of straw under magnification of electron raster microscope

Conclusion

The presented results of this work indicate the possibility of considering $CuSO_4$ as an inductor for the synthesis of lignin-degrading enzymes by the fungus S. commune. According to the studies, on the 30th day of cultivation of the fungus at 30 ± 2°C, in the sample without the addition of the inductor, the lignin content decreased from 20.16% to 10.5%, while in the sample with the addition of the $CuSO_4$ inductor, a decrease was observed from 20.16% up to 8.6%. The addition of inductors to microorganism nutrient media will increase the enzyme exposure, which proves the probability of replacing alkaline solutions during the process of separation of lignin from cellulose on natural ligninolytic enzymes, that have no adverse impact on the ecosystem of our planet.

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