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STATISTICS-BASED OPTIMIZATION OF CELLULASE AND XYLANASE PRODUCTION BY THE ENDOPHYTIC FUNGUS *TALAROMYCES FUNICULOSUS* USING AGRICULTURAL WASTE MATERIALS

Lignocellulosic biomass can be utilized as a low-cost, renewable, and sustainable feedstock for obtaining non-fossil energy sources with low CO_2 emission. One of the most promising technologies for producing 2G biofuels is the saccharification of agricultural waste materials with the help of cellulolytic enzymes, followed by yeast fermentation of sugars into cellulosic ethanol. Cellulases are multi-component enzymes involved in the degradation of cellulose, which can synergistically degrade cellulose and includes three major categories: endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). The core enzyme used for the degradation of the xylan skeleton of hemicellulose is endo-β-1,4-xylanase (EC 3.2.1.8). The high cost of enzymes synthesized by fungi is a bottleneck for the production of cellulosic ethanol. Optimization of the nutrient medium composition is an important factor in increasing the production of enzymes and the efficiency of lignocellulosic biomass hydrolysis. ***The aim*** *of the current study was to optimize the production of cellulolytic and xylanolytic enzymes through cultivation of filamentous fungus Talaromyces funiculosus on low-cost nutrient media with non-pretreated agricultural waste materials.* ***Methods.*** *Filamentous fungus Talaromyces funiculosus was grown on potato-dextrose agar for 10—14 days at 26±2 °C. To obtain the culture filtrate, the fungus was cultivated under submerged conditions in an Erlenmeyer flask for 4 days. The nutrient medium composition was varied according to the factor experiment design. A two-step optimization of the nutrient medium composition was used. A screening experiment with the Plackett-Burman fractional factorial design and response surface methodology with the Box-Behnken design were used to optimize cellulase production. The enzymatic activity was determined by measuring the reduced sugar production after the enzymes hydrolysis with specific substrates: exoglucanase with filter paper, endoglucanase with carboxymethylcellulose, and xylanase with beech wood xylan, using the colorimetric DNS method with glucose or xylose as a standard. The activity of β-glucosidase was*

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determined by the hydrolysis reaction of p-nitrophenyl-β-D-glucopyranoside, which results in the formation of p-nitrophenol, quantified at 410 nm. ***Results.*** *As a result of experiments with using agricultural waste, including wheat straw, corn stalk, and corn cob as carbon sources of the culture medium, it was shown that T. funiculosus is able to grow and produce cellulase and xylanase on all non-pretreated substrates studied. The two-step sequential optimization of the nutrient medium composition for T. funiculosus cultivation according to the Plackett-Berman and Box-Behnken designs made it possible to increasethe activity of cellulolytic and xylanolytic enzymes by 2.4—2.6 times. The optimized cultivation medium does not contain such expensive components as Avicel, peptone, and yeast extract and has the following composition, g/L: corn stalks — 50.0; urea — 0.86;* $NaNO_3$ *— 1.0;* KH_2PO_4 *— 6.0; KCl — 0.25;* $MgSO_4$ *— 0.25;* $FeSO_4$ *— 0.01.* ***Conclusions.*** *The studied strain of T. funiculosus produces a lignocellulosic enzyme complex with a high level of β-glucosidase activity when cultivated on an optimized nutrient medium with untreated agricultural waste and is promising for the conversion of lignocellulosic biomass into fermentable sugars.*

Keywords: *Talaromyces funiculosus, Plackett-Burman design, Box-Behnken design, exoglucanase, endoglucanase, β-glucosidase, xylanase.*

According to the European Biofuels Technology Platform, Ukraine is one of the largest grain exporters in the world. It has many sustainable lignocellulosic raw materials, which creates opportunities to reach the target of 10% renewable energy, mainly relying on the domestic production of biofuels. In recent years, the growing instability of the region has been exacerbated, which threatens Ukraine's energy security. Thus, today particular importance is given to the promotion of energy efficiency and renewable energy sources. The efficient conversion and utilization of renewable lignocellulosic biomass are of a global research interest, primarily due to the possibility of obtaining non-fossil energy sources with low CO_2 emission. Second-generation (2G) or cellulosic ethanol can be obtained from cheap agricultural waste and has advantages over corn-derived bioethanol as no food sources are used in its production [1].

Plant biomass contains lignocellulose, which is resistant to biodegradation. Lignocellulosic plant cell walls are composed predominantly of cellulose, hemicellulose, and lignin [2]. Cellulose and hemicellulose are promising substrates for fermentation processes. Cellulose is the most abundant and affordable renewable energy source on earth; it can be converted to glucose and then fermented by yeast into cellulosic ethanol. The transformation of cellulose into valuable products is performed by various microbes, mainly fungi, which secrete enzymes that break down cellulose fibers [3].

The complete transformation of cellulose to glucose is carried out by the cellulase enzyme complex. Cellulases play an important role in industrial processes, accounting for about 20% of the world market for enzymes and offering a wide range of applications — from the bakery, animal feed, laundry and detergent, textile and pulp industries to biofuel production. The use of cellulases to convert biomass into cellulosic ethanol production has been on the rise in recent years. Cellulases act on insoluble cellulose, catalyzing the degradation of β-1,4-glycosidic bonds and includes three types that act synergistically: endoglucanases (EC 3.2.1.4), which randomly hydrolyze β-1.4 bonds in the cellulose molecule; cellobiohydrolases or exoglucanases (EC 3.2.1.91), which release a cellobiose unit and act at the end of the chain, and β-glycosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21), the key components of the cellulase complex, which complete the last step in cellulose hydrolysis by converting cellobiose to glucose [4—6].

Xylans, the major components of hemicellulose, are composed of a backbone of β-1,4-linked D-xylopyranosyl residues and side chains containing different substituents. The complete breakdown of xylans requires a variety of hydrolytic enzymes that form the xylanolytic complex. Among the xylanolytic enzymes, xylanase

(endo-1,4-xylan 4-xylanohydrolase, EC 3.2.1.8) is an important enzyme for the cleavage of internal β-(1,4)-linked D-xylosyl glycosidic bonds in heteroxylan to generate short xylo-oligosaccharides [7]. The potential use of xylanases in numerous biotechnological processes has been proposed, such as pulp bleaching, animal feed quality improvement, the textile and fuel industries, and production of prebiotic xylooligosaccharides [8].

Many industrial applications require the synergistic action of cellulase and xylanase complexes, predominantly in the bioconversion of lignocellulosic materials and agro-wastes into fermentative sugars for the 2G bioethanol production [9, 10].

Filamentous fungi, in particular *Trichoderma* sp., *Aspergillus* sp., and *Talaromyces* (*Penicillium*) sp., have efficient systalks for the synthesis and secretion of plant cell wall-degrading enzymes which can be used to convert lignocellulosic biomass to fermentable sugars [10, 11].

The filamentous fungus *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is an efficient industrial cell factory for the production of cellulolytic enzymes for biofuel and other applications (Accelerase, Celluclast 1.5L, CellicCTec2, Novozyme 188). Despite the fact that the use of commercial *T. reesei*- and *A. niger*-based enzymes to produce fermentable sugars from lignocellulose has shown good results, high cost of media components is a limiting factor for their large-scale application. In addition, such enzyme mixtures do not show the best results for each type of biomass due to the diverse compositional variability. Therefore, it is very important to develop an enzyme complex with low cost and good synergistic characteristics for the hydrolysis of a specific substrate [12].

Another filamentous fungus that has been widely studied for cellulolytic and xylanolytic enzyme production is *Talaromyces funiculosus* (or *Penicillium funiculosum*), which is capable to convert various pretreated lignocellulosic feedstocks into fermentable sugars [13, 14]. Genus *Talaromyces*, early regarded as a teleomorph state of *Penicillium*, forms a monophyletic clade distinct from the other *Penicillium* subgenera [15], and many *Talaromyces* strains have also been studied as cellulase and xylanase producers [6,16]. Filamentous fungi *T. funiculosus* strains X33 and NCIM1228 has been widely studied for enzyme production at the secretome level. Interestingly, the genome of *T. funiculosus* is the smallest of the known cellulase producers of other fungal genera. Analysis of the genome sequence of *T. funiculosus* has established that the genome size of the strain X33 is 28.49Mb and ASM429976v1 — 28.5282 Mb, unlike *A. nidulans*, *A. oryzae,* and *T. reesei* genomes are 30.5, 36.5, and 34.3 Mb respectively. *T. funiculosus* genome contains around genes 92 Glycoside hydrolases (GHs) and 113 Carbohydrate-Active Enzymes (CAZymes) [17], which is much lower than the number of *GH other producers*: 247 genes in *A. nidulans*, 285 genes in *A. oryzae*, and 200 genes in *T. reesei*. The proteomic analysis of *T. funiculosus* secretome revealed that more than half the total proteins secreted under the cellulase-inducing conditions belongs to the CAZymes families. It was found that cellobiohydrolase1 of *T. funiculosus* can hydrolyze crystalline biomass with about five-fold higher efficiency than its counterpart from *T. reesei* [18]. Both *T. funiculosus* and *T. reesei* demonstrate high cellulase production capacity even though the number of CAZyme coding genes is substantially different, which indicates the importance of studying mechanisms of regulation of cellulase and hemicellulase synthesis in filamentous fungi [19, 20].

Previously, we have shown that a number of studied *T. funiculosus* strains produce an active complex of cellulolytic enzymes [21]. The ascomycete *T. funiculosus* IMV F-100111 is the most promising strain with high catalytic activity of the enzyme complex and was chosen for optimization experiments. Optimization strategies

include the selection of a sustainable lignocellulosic raw material as a cheap carbon source for fungal growth, which simultaneously acts as an inducer of the enzyme synthesis [22].

It is known that the synthesis of cellulases and xylanases can be induced by the same transcription regulators (Xyr1, Ace2), so the use of statistical approaches has a theoretical basis in terms of studying different enzyme complexes in one optimization experiment [23].

The aim of this study is to optimize the composition of the nutrient medium using lignocellulosic biomass waste from the main agricultural crops of Ukraine in order to increase the production of inexpensive enzyme complexes of cellulase and xylanase.

Materials and Methods. *Talaromyces funiculosus* IMV F-100111 deposited in the Depositary of Microorganisms of the Zabolotny Institute of Microbiology and Virology of NAS of Ukraine and stored in the collection of cultures of filamentous fungi of the Department of Physiology and Taxonomy of Micromycetes. The fungus was isolated from the leave of *Oxycoccus palustris* Pres. of sphagnum bogs of the Zhytomyr Polissya region of Ukraine and identified by cultural and morphological characteristics and rDNA ITS region sequence. The nucleotide sequence of the ITS region for the *T. funiculosus* IMV F-100111 was deposited in the GenBank database under an accession number of KY620212. The strain was grown on potato-dextrose agar (PDA) for 10—14 days at 26±2 °C. A spore suspension (1×10^6 spore/mL) was cultivated in a 750 mL Erlenmeyer flask with a potato-dextrose medium under submerged conditions at 26±2 °C for 48 h [24]. To obtain the culture filtrate (CF), filamentous fungus was cultivated under submerged conditions at 26±2°C for 4 days, the amount of inoculum introduced into the medium was 5%. The nutrient medium composition varied according to the factor experiment. After cultivation, the fungi mycelium was separated by filtration through ashless paper filters (blue tape).

Enzyme activities. The endoglucanase and exoglucanase activities of the CFs were assayed according to Meng [27].

Endoglucanase activity was determined in a reaction mixture containing 0.5 mL of CF with 0.5 mL of 2.0% solution of Na-CMC (Sigma-Aldrich, medium viscosity) in 0.05 M acetate buffer (pH 4.5) and incubated for 30 min at 50 °C.

Exoglucanase activity was determined by hydrolysis of filter paper (1×6 cm) in 1 ml of 0.05 M acetate buffer (pH 4.5) and 1 mL of CF, incubation time 1 hour at 50°C.

Xylanase activity was determined by hydrolysis of 0.18 mL of 1% solution of beech wood xylan (Sigma-Aldrich) in 0.05 M acetate buffer (pH 4.5) and 0.02 mL CF at 50°C for 5 min [28].

The reducing sugar in the reaction mixture after the enzymatic hydrolysis of Na-CMC, filter paper, or xylan was determined by the colorimetric method with a 3,5 dinitrosalicylic acid (DNS, Sigma-Aldrich) reagent [29]. One unit of endo-, exoglucanase, or xylanase activities was defined as the amount of enzyme catalyzing the release of 1 μmol of glucose or xylose equivalent per 1 min, respectively, under the assay conditions.

β-Glucosidase activity was determined by measuring the hydrolysis of p-nitrophenyl-β-D-glucopyranoside (pNPG) according to the Parry method [30]. The reaction mixture contained 0.5 mL of 10 mM pNPG (Sigma-Aldrich) in 50 mM acetate buffer (pH 5.0) and 0.5 ml CF was incubated at 50 °C for 30 min. The reaction was terminated by adding 1 mL of 0.5 M Na_2CO_3, and the absorbance was measured at 425 nm. One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1 μmol of p-nitrophenol per 1 min under the assay conditions.

Optimization of the nutrient medium composition consisted of two steps. In the first step, a 2-level Plackett-Burman fractional factorial Design (PBD) was used for 8 variables to determine

medium components that significantly affectcellulase and xylanase production (Table 1) [25].

The following first-order model was used for the Plackett-Burman design:

$$Y = \beta_o + \Sigma \beta_i X_i$$

where Y is the response which is expressed in units of enzyme activities, β_o is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable.

The factors identified as having a significant effect on xylanase production from the Plackett-Burman experiments were further optimized with a response surface methodology (RSM) using a 3-level Box-Behnken design (BBD) (Table 2) [26].

A second-order polynomial equation corresponding to the Box-Behnken design is given below:

$$Y = \beta_o + \Sigma \beta_i X_i + \Sigma \beta_{ij} X_i X_j + \Sigma \beta_{ii} X_i^2$$

where Y is the dependent variable (response in terms of enzyme activities); β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the intercept, linear, quadratic, and interaction terms, respectively; and X_i and X_j are independent variables.

Table 1. **The experimental factors and their levels in Plackett-Burman design**

No.	Code	Factor	Low level, g/L	High level, g/L
1	A	Wheat straw	5.0	10.0
2	B	Empty corn cobs	5.0	10.0
3	C	Corn stalks	5.0	10.0
4	D	Urea	0.5	2.0
5	E	KH_2PO_4	2.0	6.0
6	F	KCl	1.0	2.0
7	G	$MgSO_4$	1.0	2.0
8	H	$NaNO_3$	1.0	4.0

Table 2. **The influencing factors and their levels in Box-Behnken design**

No.	Code	Factor	Level, g/L		
			Low	Center	High
1	A	Corn stalks	20.0	35.0	50.0
2	B	Urea	0.5	1.25	2.0
3	C	KH_2PO_4	2.0	4.0	6.0

Statistical Analysis. All experiments were carried out with three independent replicates, and the results are presented as mean ± standard error of the mean. The creation of experimental designs and statistical analysis of data were performed using the MiniTab 17 software (Minitab Ltd. UK). Data were statistically analyzed using Student's *t*-test to compare the means applying a significance level of $p < 0.05$.

Results. At the first stage of this study, the PBD was used to determine the most important factors influencing *T. funiculosus* cellulase and xylanase production. This design allowed us to evaluate the influence of a large number of nutrient medium components with a minimum number of experiments on the production of cellulolytic and xylanolytic activity of this strain. Czapek medium was selected as basal, which provides as fungal growth as cellulosolytic and xylanolytic enzymes complex production by *T. funiculosus* when the carbon source of glucose replacing on a cellulose-containing plant substrate [13, 32]. Previously, we have shown that various lignocellulosic raw materials, widely distributed in Ukraine in the form of different types of agro-industrial wastes, including wheat straw, corn cobs, and stalks, are the best inducers of the cellulase and xylanase complexes production compared to synthetic ones [21]. Urea was added to the nutrient medium as an additional source of nitrogen. According to the literature data, this compound can improve the production of cellulosolytic enzymes complex in *T. funiculosus*. Protein hydrolyzates are usually used as a nitrogen source in the culture medium such as peptone, yeast extract, products of soy protein

processing, but we excluded such sources as peptone and yeast extract from the nutrient medium because of their high cost.

The first step in multidimensional optimization, matrices for 8 variables with 13 experimental runs were generated using the two-level PBD. The results of optimization experiments are presented in Table 3. The relevance order of the variables influencing enzyme production is depicted in the Pareto chart (Fig. 1). Analysis of the data obtained in the fractional factorial PBD showed that the main factors in the nutrient medium that influenced the enzymatic activity of the *P. funiculosum* strain were urea (CH_4N_2O) and corn stalks among carbon sources and potassium dihydrogen phosphate (KH_2PO_4). The other components of the nutrient medium did not significantly affect any of the studied activities. Urea had a significant negative effect on the studied activities; the addition of it to the nutrient medium at the previous stage was carried out through the literature on the possible positive effect on the exoglucanase, endoglucanase, and β-glucosidase activities, which has been found for another strain of *P. funiculosum*. Our preliminary results indicate that for Czapek medium, this component is not significant for improving cellulase or xylanase activities (Table 3).

The use of ground corn stalks as a carbon source had a significantly positive effect on all studied activities except exoglucanase one, while an increase in potassium dihydrogen phosphate significantly improved the production of β-glucosidase activity only. Based on the first stage of optimization by the BBD, three factors of the nutrient medium were se-

Table 3. **Enzymatic activities of *T. funiculosus* on nutrient media generated for 8 variables according to Plackett-Burman design**

No.	Factor of nutrient medium, g/L								Enzymatic activity, U/mL			
	Wheat straw	Corn cobs	Corn stalks	$NaNO_3$	KH_2PO_4	KCl	$MgSO_4$	Urea	Endoglucanase	Exoglucanase	Xylanase	β-glucosidase
1	10.0	5.0	10.0	4.0	2.0	2.0	1.0	0.5	0	0.05 ± 0.030	4.4 ± 0.99	2.79 ± 0.128
2	5.0	10.0	5.0	1.0	2.0	2.0	2.0	2.0	0	0.06 ± 0.007	0	4.10 ± 0.709
3	5.0	10.0	10.0	1.0	6.0	1.0	1.0	0.5	0.30 ± 0.049	0.20 ± 0.007	10.3 ± 3.30	9.15 ± 1.251
4	5.0	5.0	10.0	4.0	6.0	1.0	2.0	2.0	0.83 ± 0.072	0.27 ± 0.028	24.3 ± 0.66	8.54 ± 0.975
5	5.0	5.0	5.0	4.0	6.0	2.0	1.0	2.0	0.21 ± 0.021	0	11.8 ± 2.5	5.07 ± 0.393
6	5.0	5.0	5.0	1.0	2.0	1.0	1.0	0.5	0	0	2.3 ± 1.24	4.21 ± 0.254
7	5.0	10.0	10.0	4.0	2.0	2.0	2.0	0.5	0.62 ± 0.175	0.24 ± 0.078	25.7 ± 3.70	12.04 ± 1.389
8	10.0	10.0	10.0	1.0	6.0	2.0	1.0	2.0	0.49 ± 0.047	0.12 ± 0.021	17.7 ± 2.91	4.40 ± 0.176
9	10.0	5.0	5.0	1.0	6.0	2.0	2.0	0.5	0.57 ± 0.120	0.13 ± 0.042	15.1 ± 2.57	9.87 ± 1.131
10	10.0	10.0	5.0	4.0	6.0	1.0	2.0	0.5	0.52 ± 0.0021	0.21 ± 0.0092	13.5 ± 3.30	12.27 ± 0.474
11	10.0	5.0	10.0	1.0	2.0	1.0	2.0	2.0	0.48 ± 0.071	0.18 ± 0.028	19.1 ± 1.98	8.93 ± 0.170
12	10.0	10.0	5.0	4.0	2.0	1.0	1.0	2.0	0.85 ± 0.085	0.10 ± 0.035	20.5 ± 9.90	11.15 ± 1.737
13	7.5	7.5	7.5	2.5	4.0	1.5	1.5	1.25	0.35 ± 0.085	0.18 ± 0.044	16.8 ± 2.64	5.49 ± 1.466

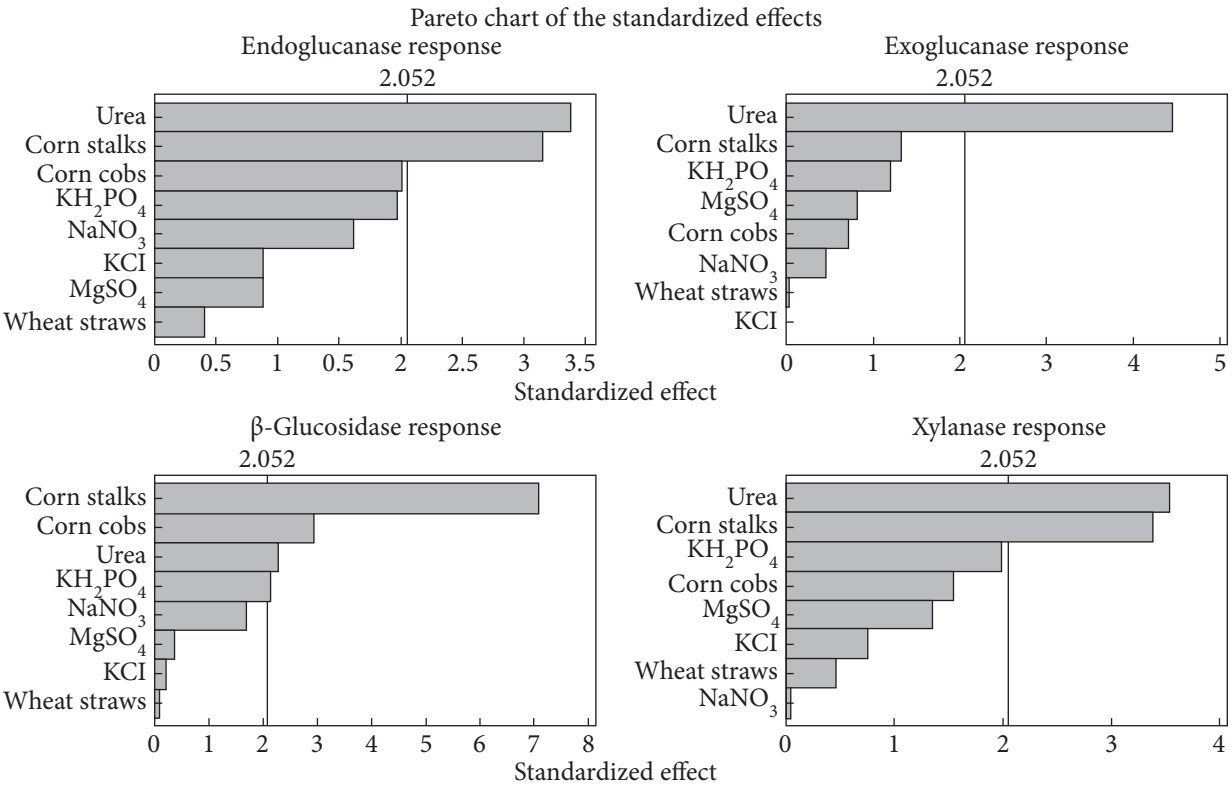

Fig. 1. Pareto charts of the effect of media components on the endoglucanase, exoglucanase, xylanase, and β-glucosidase enzymatic activities of T. *funiculosus* (alpha = 0.05)

lected for the second stage, namely corn stalks, urea, and potassium dihydrogen phosphate. The compositions of the experimental nutrient media are shown in Table 4. Corn stalks were used as the only source of carbon in the media, because they had the strongest positive effect on all studied enzymatic activities. Analysis of the four studied enzymatic activities production *by T. funiculosus* on nutrient media of various compositions (Table 4) indicates that the main components influencing the production of cellulolytic enzymes of this microscopic fungus are: corn stalks as a source of carbon and urea as a source of nitrogen.

Experiments planned by PBD revealed the following regularity: the effect of the corn stalks was mostly positive, while that of urea was mostly negative in the studied concentration range. Since an increase in concentration of carbon source (corn stalks) leads to an increase in all enzymatic activities studied, so the optimal concentration of carbon source for the synthesis of enzymes of the xylan and cellulosolytic complex was set at 50 g/L. The optimal concentration of urea in the nutrient medium for maximum production of xylanase and endoglucanase activities was found to be of 0.65 and 1.1 g/L, respectively, while for exoglucanase and β-glucosidase activities, the optimal concentration was the minimal concentration of urea in the medium, i.e. 0.5 g/L. Potassium dihydrogen phosphate had no significant effect on the exoglucanase and xylanase activities of *T. funiculosus*. Its significant effect was found by BBD in the interaction with the other significant components of the nutrient medium.

The use of the optimization module of the statistical program MiniTab 17 (Fig. 2) to obtain a maximum activity of all studied enzymes of *T. funiculosus* under the submerged cultivation conditions allowed us to obtain the optimal medium of the following composition, g/L: corn stalks — 50.0; urea — 0.86; $NaNO_3$ — 1.0; KH_2PO_4 — 6.0; KCl — 0.25; $MgSO_4$ — 0.25; $FeSO_4$ — 0.01. The results of theoretically calculated by BBD and experimentally obtained enzymatic activities with the optimized cultivation medium are given in Table 5. For comparison, Table 5 includes the enzyme activities on the initial cultivation medium.

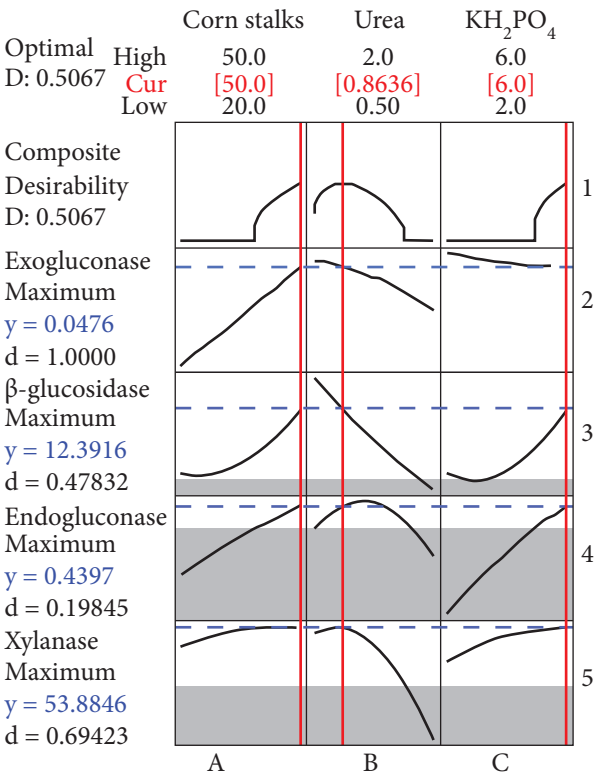

Fig. 2. Optimization plot of the influence of nutrient medium components on the *T. funiculosus* enzymatic activities production according to mathematical models generated by the Box-Behnken design. Symbols: A — corn stalks; B — urea; C — KH_2PO_4; 1 — Composite desirability; enzyme activities: 2 — exoglucanase, 3 — β-glucosidase, 4 — endoglucanase, 5 — xylanase

Table 4. **Enzyme activities of *T. funiculosus* on nutrient media according to Box-Behnken design**

No	Factors			Enzyme activities, U/mL			
	Corn stalks	Urea	KH_2PO_4	Endoglucanase	Exoglucanase	Xylanase	β-glucosidase
1	20.0	2.00	4.0	1.1±0.14	0	9.1±1.06	4.10±0.12
2	20.0	1.25	6.0	3.9±0.14	0.02±0.01	44.5±2.40	9.31±0.41
3	20.0	0.50	4.0	2.7±0.42	0.09±0.01	45.5±2.68	8.76±0.71
4	50.0	1.25	2.0	1.7±0.21	0.54±0.05	46.0±6.22	8.85±0.59
5	50.0	2.00	4.0	1.9±0.20	0.37±0.03	31.3±6.93	9.18±0.11
6	35.0	2.00	6.0	2.8±0.35	0.32±0.03	20.6±1.98	6.43±0.59
7	35.0	1.25	4.0	3.2±0.38	0.30±0.01	44.9±8.06	7.70±0.14
8	35.0	1.25	4.0	3.0±0.14	0.29±0.05	50.0±2.83	7.36±0.57
9	35.0	2.00	2.0	2.1±0.34	0.32±0.04	36.7±4.95	6.56±0.18
10	50.0	1.25	6.0	5.4±0.78	0.49±0.04	54.2±4.53	11.17±0.72
11	35.0	1.25	4.0	3.2±0.07	0.27±0.01	44.6±1.41	7.22±0.40
12	35.0	0.50	2.0	2.1±0.16	0.31±0.04	32.9±5.52	9.77±0.94
13	50.0	0.50	4.0	3.7±0.24	0.48±0.04	51.1±4.61	11.57±0.52
14	35.0	0.50	6.0	2.2±0.35	0.37±0.04	46.4±7.64	11.93±1.41
15	20.0	1.25	2.0	0.5±0.09	0.03±0.02	24.3±1.34	4.18±0.11

Discussion. A two-step statistical optimization with PBD and BBD approaches was used to improve cellulase and xylanase production. PBD is essentially a screening design that allows one to determine the significant main effect of factors from a list of many potential ones, but it does not take into account the mutual influence of factors, in our case – the components of the cultivation medium on studied enzyme activities. The response surface methodology (RSM) is designed to allow estimation of interactions of selected factors. Central composite designs (CCDs) were first described by Box and Wilson and practically are the most common response surface designs. The BBD base on the Doehlert matrix is slightly more efficient than the central composite design and other response surface designs [26]. Like RSD, BBD requires three levels (−1, 0, 1) and can be applied to a number of factors within the range of physiological values. For three factors, BBD offers some advantage in requiring a fewer number of runs, but not for 4 or more factors. BBD was specially designed to fit a second-order regression model (quadratic model) for RSM. It uses the 2^2 full factorial design to generate for the higher number of factors by systematically adding a mid-level between the low and the high levels of the factors. BBD allows not only to evaluate the interaction of factors but also to avoid extreme values for combinations of factor levels that cannot be tested due to the physiological limitations of the process.

Table 5. **Enzyme activities (U/mL) of *T. funiculosus* on basal and optimized media**

Enzyme activity	Basal medium	Optimized medium	
		Theoretically calculated value	Practically obtained value
Exoglucanase	0.16±0.09	0.48	0.49±0.09
Endoglucanase	2.1±0.24	4.4	5.4±0.94
β-glucosidase	6.3±0.65	12.4	15.2±2.99
Xylanase	25.0±3.56	53.9	59.5±6.1

As a result of statistical optimization, data were obtained (Table 4) that indicate that cellulase production by *T. funiculosus* IMV F-100111 using the optimized media composition results in the highest production of cellulase and xylanase complex (Table 5).

Previously, it was shown that the main factor affecting the induction and synthesis of cellulases are structural features of the cellulose substrate, in particular agricultural waste. The effect of the culture medium composition on cellulase production has been studied using various feedstocks as a carbon source, including sugarcane bagasse, wheat bran, corn stover, and rice straw [31, 32].

T. funiculosus, a filamentous fungus, secretes a complete set of enzymes (exo- and endoglucanase, β-glucosidase, and xylanase) for the effective saccharification of lignocellulosic biomass. The focus of research on this fungus was the optimization of media components and the optimization of process parameters.

Castro and co-authors [13] studied the synthesis of cellulases by the microscopic fungus *P. funiculosum* ATCC 11797 and found that the activity of each component of the enzymatic complex depends mainly on the type of substrate. The cellulolytic complex produced by this strain showed well-balanced amounts of β-glucosidase, endo- and exoglucanase, resulting in a high efficiency of enzyme production in the presence of lignocellulosic material. The highest activity of all components of the cellulose complex was observed during growth on Avicel (microcrystalline cellulose) and delignified substrates, while the activities were in the range: exoglucanase 0.25–0.35; endoglucanase 1.8–3.5, and β-glucosidase 0.8–1.8 U/ mL.

Carvalho and co-authors [33] carried out optimization of the culture media composition for strain *P. funiculosum* ATCC11797 in accordance with the full factorial (2^4 FFD) design. The se-

quential optimization strategy allowed the attainment of an enzyme pool with exoglucanase 0.508 U/mL; endoglucanase 9.2 U/mL, and 2.4 U/mL for β-glucosidase activities with peptone and Avicel in the medium.

Maeda and co-authors under submerged cultivation conditions of *P. funiculosum* on medium with peptone and Avicel obtained concentrated 25 times enzyme mixtures with β-glucosidase activity of 26.6 U/mL, which corresponds to the activity of 1.1 U/mL of CF. In other experiments, β-glucosidase activity in CF was increased to 2.26 U/mL [14, 31].

The ability of *P. funiculosum* to grow and synthesize cellulases on various substrates, including lignocellulosic ones, has been shown by Vasquez-Montoya et al. [34]. Moringa straw was established to be an inducer and source of carbon for *P. funiculosum* cellulase production. Cultivation of the fungus in submerged conditions led to the production of cellulases with an endoglucanase activity of 1.68 U/mL and β-glucosidase one of 0.93 U/mL [34].

It should be emphasized that β-glucosidase activity of studied *T. funiculosus* IMV F-100111 was 15.2 U/mL CF. Thus, a great advantage of this strain is the ability to produce high levels of β-glucosidase activity when cultivated on media without valuable synthetic components such as microcrystalline cellulose Avicel, peptone, or yeast extract. Comparison of the cellulolytic activity of the studied *T. funiculosus* strain with the other strains showed that it has average values of the exo- and endoglucanase activities but an order of magnitude higher than the activity of β-glucosidase, the key enzyme that determines the degree of cellulose conversion into glucose (but not into di- and oligosaccharides).

It should be especially noted that studies on the optimization of the production of enzymes of the cellulase-xylanase complex of this species have hardly been carried out yet. Burugu et al. [35] investigated the optimization of xylanase production on nutrient media with corn cobs, peptone, and yeast extract. The xylanase activity of the studied *P. funiculosum* strain was 668 U/g of corn cobs, which, in terms of the activity of our strain, was about 1200 U/g.

Chavan et al. employed the RSM approach (PBD followed by BBD) to optimize media components, which successfully increased cellulase and xylanase production from *P. funiculosum* NCIM 1228. Xylanase activity was 36.5 U/mL on nutrient media with Avicel, pepton, and yeast extract. According to the graphical data of specific enzyme activities, *T. funiculosus* IMV F-100111 strain was twice as high xylanase-active as NCIM 1228 [36].

It should also be noted that when comparing the activity values in the theoretical model and under experimental conditions, no statistically significant values are observed (Table 5). Among the possible reasons for this phenomenon, it should be taken into account that experiments on optimizing the production of cellulases were carried out on natural lignocellulosic waste without a pretreatment stage, which significantly reduces the biomass recalcitrance and non-productive adsorption of enzymes on lignin [37]. Previously, we studied the complex pretreatment of wheat straw and showed that this process makes it possible to obtain an adequate model for optimizing the production of cellulases with agricultural waste [38].

Proteome and secretome analyses of *T. funiculosus* have revealed a significant genetic potential of this species of fungi and have shown the prospects for its use in the technology of saccharification of natural substrates [19, 32]. *T. funiculosus* has been shown to have great potential for using in bioengineering technologies to create effective recombinant strains. However, so far these studies only demonstrate the capabilities of bioengineering methods, and the resulting recombinant strains have not revealed full biotechnological properties [39].

When studying fungi that have the ability to biotransform natural lignocellulosic raw mate-

rials, it should be taken into account that the enzyme activity is not the most critical factor influencing sugar yield, since synergistic effects between cellulases, hemicelluloses, and the accessory enzymes complex may be more important, in particular for *T. funiculosus* [20, 40]. Therefore, in the future, it is of interest to study the optimization of the activity of the enzyme complex in terms of the maximum yield of sugars from pretreated lignocellulosic biomass [12].

Conclusions. Thus, as a result of studies using the two-step optimization of the composition of the nutrient medium with the Plackett-Burman and Box-Behnken designs, the cellulosolytic and xylanolytic activities of *T. funiculosus* have been increased by 2.4—2.6 times. The optimized medium has the following composition, g/L: corn stalks — 50.0; urea — 0.86; $NaNO_3$ — 1.0; KH_2PO_4 — 6.0; KCl — 0.25; $MgSO_4$ — 0.25; $FeSO_4$ — 0.01.

The studied strain of *T. funiculosus*, when cultivated on non-pretreated lignocellulosic agricultural wastes, synthesizes cellulolytic and hemicellulolytic enzyme complexes with a high level of β-glucosidase activity without adding such expensive components to the nutrient medium as Avicel, peptone, or yeast extract and is promising for the transformation of agricultural waste into fermentable sugars. The strain can be further improved by optimizing the production of lignocellulosic enzymes using the proposed strategy to develop a cost- efficient and sustainable agricultural waste biotransformation.

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ОПТИМІЗАЦІЯ ПРОДУКЦІЇ ЦЕЛЮЛАЗ ТА КСИЛАНАЗИ ЕНДОФІТНИМ ГРИБОМ *TALAROMYCES FUNICULOSUS* НА ОСНОВІ СТАТИСТИЧНИХ ПРОЄКТІВ ПРИ КУЛЬТИВУВАННІ НА СЕРЕДОВИЩАХ З ВІДХОДАМИ СІЛЬСЬКОГОСПОДАРСЬКИХ РОСЛИН

Лігноцелюлозна біомаса може бути використана як дешева відновлювана сировина для отримання невикопних джерел енергії з низькими викидами CO_2. Однією з найперспективніших технологій виробництва біопалива є оцукрювання сільськогосподарських відходів за допомогою целюлозолітичних ферментів з наступним дріжджовим зброджуванням цукрів у целюлозний етанол. Целюлази — це багатокомпонентні ферменти, залучені до розкладання целюлози, які діють синергетично та включають три основні категорії: ендоглюканази (ЕС 3.2.1.4), екзоглюканази або целлобіогідролази (ЕС 3.2.1.91) і β-глюкозидази (ЕС 3.2. 1.21). Основним ферментом, який гідролізує ксилан геміцелюлози, є переважно ендо-β-1,4-ксиланаза (ЕС 3.2.1.8). Висока вартість ферментів, синтезованих грибами, є головною проблемою виробництва целюлозного етанолу. Оптимізація складу середовища культивування з поживними залишками є важливим фактором підвищення ефективності гідролізу лігноцелюлозної біомаси. **Мета** цього дослідження полягала в підвищенні синтезу целюлолітичних та ксиланолітичних ферментів шляхом оптимізації середовища культивування гриба *Talaromyces funiculosus* з необробленими відходами сільськогосподарських рослин. **Методи.** Мікроскопічний гриб *Talaromyces funiculosus* вирощували на картопляно-декстрозному агарі протягом 10—14 діб при 26±2 °С. Для отримання культуральних фільтратів гриб культивували у зануреному стані в колбах Ерленмейера протягом 4 днів. Склад поживного середовища змінювався залежно від плану факторного досліду. Викорис-

товували двоетапну оптимізацію складу живильного середовища. Для оптимізації виробництва целюлази було використано скринінговий експеримент із факторним планом Плакетта-Бермана та методологію поверхні відгуку за планом Бокса-Бенкена. Ферментативну активність визначали за гідролізом специфічних субстратів: екзоглюканаза з фільтрувальним папером, ендоглюканаза з карбоксиметилцелюлозою, ксиланаза з ксиланом деревини бука. Кількість відновлювальних цукрів визначали методом з 3,5-динітросаліциловою кислотою (DNS) з використанням глюкози або ксилози як стандартів. Активність β-глюкозидази визначали в реакції гідролізу п-нітрофеніл-β-D-глюкопіранозиду за утвореним п-нітрофенолом. **Результати.** В результаті експериментів з використанням сільськогосподарських відходів, зокрема пшеничної соломи, кукурудзяних стебел та качанів в якості джерела вуглецю культурального середовища, було показано, що *T. funiculosus* здатний рости та продукувати целюлази та ксиланазу на всіх досліджених субстратах, що не зазнали попередньої обробки. Двоетапна послідовна оптимізація складу живильного середовища для культивування *T. funiculosus* за планами Плакетта-Бермана та Бокса-Бенкена дозволила підвищити активність целюлолітичних та ксиланолітичних ферментів у 2,4—2,6 рази. Оптимізоване середовище не містить таких дорогих компонентів, як Авіцел, пептон, дріжджовий екстракт, і має наступний такий склад у г/л: стебло кукурудзи — 50,0; сечовина — 0,86; NaNO3 — 1,0; KH2PO4 — 6,0; KCl — 0,25; MgSO4 — 0,25; FeSO4 — 0,01. **Висновки.** Досліджений штам *T. funiculosus* продукує лігноцелюлозолітичний ферментний комплекс із високим рівнем β-глюкозидазної активності за умови культивування на оптимізованому живильному середовищі з відходами сільського господарства, що не були попередньо оброблені, та є перспективним для біоконверсії лігноцелюлозної біомаси на збродживані цукри.

Ключові слова: *Talaromyces funiculosus, Плакетта-Бермана і Бокса-Бенкена плани, екзоглюканаза, ендоглюканаза, β-глюкозидаза, ксиланаза.*