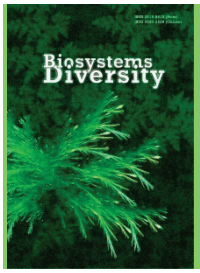

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Application of natural fungi in bioconversion of lignocellulosic waste to second-generation ethanol

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The second generation (2G) or cellulosic ethanol can help with diversification of the use of fossil energy sources. However, as bioconversion of plant waste into 2G bioethanol requires expensive additional steps of pre-treatment/hydrolysis of lignocellulosic materials, and this technology has not yet reached the technological readiness level which would allow it to be scaled-up, this process needs more interdisciplinary and comprehensive studies. This work was aimed at experimental study of a full cycle of successive processes of pre-treatment/saccharification using cellulolytic enzymes of filamentous fungi and fermentation of obtained syrups by xylose-fermenting yeast, using selected natural microorganisms for the fungal-based bioconversion of lignocellulosic agricultural waste to 2G ethanol. Using the Plackett-Burman and Box-Behnken methods of mathematical statistics, the optimal conditions for pre-treatment and enzymatic hydrolysis of wheat straw by a hemi- and cellulolytic multi-enzyme complex of the selected fungal strain *Talaromyces funiculosus* UCM F-16795 were established: microwave-assisted alkali pre-treatment with sodium hydroxide (NaOH) solutions (concentration range 4.6–4.8%), and saccharification conditions of medium pH 4, temperature 40 °C, hydrolysis duration 18 hours, and dilution of culture liquid with a buffer solution 1:1. The total energy of microwave irradiation 1.2 kJ and the ratio of substrate/enzyme solution 100 mg/1 mL were used. Under optimized conditions, wheat straw hydrolysates contained 5.0–7.5 g/L of reducing sugars, which, according to HPLC assessment, contained 0.7–1.0 g/L of glucose, 2.2–2.9 g/L of xylose and 0.7–0.8 g/L cellobiose. We used the selected strain of xylose-fermenting yeast in fermentation of mixtures of the most important monosaccharides in hydrolysates, xylose and glucose, in the concentration range relevant for syrups obtained by us during the optimized saccharification of lignocellulosic substrates with *T. funiculosus* enzymes. Based on sequencing and phylogenetic analysis, strain UCM Y-2810 was confirmed as *Scheffersomyces stipitis*; its nucleotide sequences of ITS region and 28S gene rDNA were deposited in GenBank under the accession numbers OP931914 and OP931915, respectively. The ethanologenic process for *S. stipitis* UCM Y-2810 was studied according to Box-Behnken design, assessing ethanol concentration by gas chromatography-mass spectrometry. Yeast fermentation under static microaerophilic conditions showed a 1.5 times higher rate of bioethanol production and 1.7 times greater efficiency of ethanologenesis per yeast biomass than for submerged cultivation. Optimization of the process of ethanologenesis resulted in the maximum rate of fermentation mixture of sugars, being 11.30 ± 0.36 g/L of ethanol, with optimal values of factors: 30 g/L of xylose, 5.5 g/L of glucose and cultivation for 5.5 days. It was revealed that the tested glucose concentrations did not significantly affect the process of xylose-fermentation by yeast, and non-competitive inhibition of xylose transport by glucose into yeast cells did not occur. This study demonstrated the potential of a full cycle bioconversion of lignocellulosic waste to 2G ethanol based on use of natural fungal strains and optimization of conditions for all steps.

Keywords: second-generation bioethanol; lignocellulosic waste; enzymatic hydrolysis; cellulolytic fungi; xylose-fermenting yeasts; *Scheffersomyces stipitis*.

Introduction

One of the ways to diversify away from the use of fossil energy sources can be the production of cellulosic ethanol (Soccol et al., 2010; Silveira et al., 2018; Esmaeili et al., 2020). Cellulosic ethanol or the second generation (2G) bioethanol is a biofuel in the form of ethyl alcohol obtained from the cellulose of plant cell walls (Sharma et al., 2020). Cellulosic ethanol can also solve the main problem of traditional grain-based bioethanol (first-generation bioethanol), which is the demand for grain crops used for food purposes, leading to competition between land use for food and energy production purposes (Demirbas, 2011; Anwar et al., 2014).

Cellulose is the most common carbohydrate polymer on the planet. Plants contain over 720 billion tons of cellulose, and meanwhile around 40 billion tons of plant biomass are produced annually. All this cellulose-rich biomass can be eventually naturally decomposed by microscopic fungi and other microorganisms. An important component of lignocellulosic biomass is agricultural waste. The kind of lignocellulosic agricultural waste varies depending of the region and the country (Esmaeili et al., 2020). In Ukraine, for example, the most important lignocellulosic waste includes wheat straw, and stalks and cobs of corn. According to research conducted by the US Department of Agriculture (USDA) and the US Department of Energy, up to 57% of corn post-harvest waste can be used to produce cellulosic ethanol without adversely affecting soil fertility.

The development and use of technologies for obtaining ethanol from cellulose-containing plant waste has significant advantages: increasing national energy security, reducing greenhouse gas emissions, involving the development of the latest biotechnologies (Guimarães et al., 2023). However, there are issues with the use of these materials including several technological and scale up constraints, which still need to be overcome (Dutta et al., 2023). As a result, the 2G bioethanol technology currently is not used on a large scale, representing only 3% of the bioethanol production worldwide (Sharma et al., 2020). Deep insights and more research on all steps of the process of 2G ethanol bioconversion, including thorough

study of microbial agents of this bioconversion, are needed to solve these issues. The most important microorganisms in 2G ethanol bioconversion are microscopic fungi. Biotransformation of the remains of agricultural plants with fungal enzymes makes it possible to obtain sugar mixtures (syrups), which can be used by yeasts simultaneously or subsequently with substrate conversion to produce bioethanol. The efficiency of syrups' or reducing sugars' (RS) formation during the substrate conversion largely depends on many process parameters: the type of lignocellulosic substrate, its humidity and grinding degree, temperature, medium pH, methods and conditions of pre-treatment of the substrate, duration of the process, activity of the individual components of the enzyme cellulolytic complex, etc.

The basics of biochemical conversion of cellulosic waste into 2G bioethanol include such steps as (1) pre-treatment of plant biomass, (2) saccharification of pre-treated lignocellulosic raw materials by enzymatic hydrolysis, and (3) fermentation of the obtained sugar mixtures to ethanol (Soccol et al., 2010).

Pre-treatment is a process used to free cellulose and hemicellulose from lignin and reduce the crystallinity index to ensure the availability of polysaccharides for the next stage of hydrolysis. Pre-treatment of lignocellulosic biomass is mainly carried out by acid hydrolysis. This method of pre-treatment leads to the formation of decomposition products with an inhibitory effect on the process of enzymatic hydrolysis and fermentation, mainly 5-hydroxymethylfurfural (HMF). Therefore, when using this method of pre-treatment, a detoxification step is necessary. Another common pre-treatment method is alkaline treatment of the substrate, which has been shown to be effective in removing lignin. The comprehensive method of pre-treatment of plant biomass that we have previously proposed avoids the process of detoxification of harmful substances (Syrchin et al., 2017).

Enzymatic hydrolysis of lignocellulosic biomass (saccharification) is carried out by cellulases and hemicellulases, which are a hydrolytic complex of enzymes produced mainly by fungi. These enzymes catalyze the stepwise hydrolysis of cellulose and hemicellulose to glucose and xylose, respectively, and act sequentially and synergistically (Kubicek & Kubicek, 2016; Liu et al., 2019).

Endoglucanase or endo-1,4-beta-D-glucanase (EC 3.2.1.4) randomly cleaves internal β (1→4) glycosidic bonds on the amorphous sections' regions of cellulose polysaccharide chains and forms new chain ends. Exoglucanases or cellobiohydrolases cleave cellulose at sites located two to four units from the ends of the open chains, resulting in mainly tetra- and disaccharides, in particular, cellobiose. Type I exoglucanases (EC 3.2.1.176) hydrolyze the reducing end of the cellulose chain, unlike exoglucanase type II (EC 3.2.1.91) hydrolyzing the non-reducing end. Cellobiases (EC 3.2.1.21) or β-glucosidases hydrolyze cellobiose, a product of exoglucanase, into individual monosaccharides (glucose). Hemicellulases are represented mainly by xylanases or endo-1,4-xylanases (EC 3.2.1.8), which hydrolyze β (1→4) glycosidic bonds in the linear sections of hemicellulose polymers (Payne et al., 2015).

The main commercial producer of cellulolytic enzyme preparations is currently the ascomycetous filamentous fungus *Trichoderma reesei*, which has a high level of synthesis and secretion of cellulases in quantities exceeding 100 g/L (Raj et al., 2022).

According to the secretome analysis data, the fungi of the *Trichoderma* genus, as a rule, are characterized by high productivity, mainly in relation to cellulases, but to a much lesser extent in relation to other carbohydrases, in particular, β-glucosidases. Under optimal conditions, the induction of β-glucosidases is only 1% of the total number of secreted *T. reesei* cellulases. Such low level of β-glucosidase synthesis reduces the efficiency of transformation of lignocellulosic biomass and the feasibility of the use of *T. reseei*-based enzyme preparations (Gong et al., 2015). There are several approaches to prevent this *T. reesei* disadvantage. For example, the addition of β-glucosidase preparations synthesized by other fungi significantly increases the efficiency of hydrolysis of cellulosic substrates. On the other hand, the activity *T. reesei* β-glucosidase was increased using of genetic engineering by expressing heterologous β-glucosidase genes from other fungi, such as *Penicillium decumbens*, *Aspergillus aculeatus* and *Periconia* sp. Despite many years of research, to date a strain of *T. reesei* able to synthesize a complete cellulolytic enzymes complex effective on natural lignocellulosic biomass has not been created. Therefore, the search for new natural strains of micromycetes with a high level of cellulose-degrading ability remains relevant today (Cai et al., 2021). The final step of bioconversion of cellulosic waste into 2G bioethanol is fermentation when the sugars that have been obtained by hydrolysis (or saccharifcation) of lignocellulosic materials are transformed into ethanol and carbon dioxide by yeasts (Jansen et al., 2017). The bioconversion can use the classic scheme of successive processes of saccharification and fermentation (SHF) or less commonly used simultaneous saccharification and fermentation (SFF).

Regarding fermentation process, the yeast *Saccharomyces cerevisiae* is traditionally used by the alcohol industry to produce ethanol from hexoses. However, the process of hydrolysis of cellulosic materials results not only in the hexoses easily fermented by a wide variety of yeasts (glucose and galactose), but also in pentoses (D-xylose and L-arabinose), which cannot be assimilated and fermented by *S. cerevisiae* dominant in the alcohol industry (Kręgiel et al., 2017).

Particular interest is being drawn to xylose, which is the main sugar composing hemicellulose and the second most common sugar of lignocellulose, and certainly xylose-fermenting yeasts (Jeffries & Kurtzman, 1994; Kurtzman et al., 2011; Morais et al., 2013). Yeasts capable of fermenting pentoses such as xylose can be isolated from various natural sources, including plant sap, dead wood, insect guts, rotting fruits, etc; and they are less common in nature than hexose-fermenting ones (Cadete et al., 2017; Moremi et al., 2020; Ianieva, 2022). To enhance the natural performance of xylose-fermenting yeasts,projects have been conducted on the creation of recombinant yeast strains capable of D-xylose fermentation by cloning xylose isomerase genes from mycelial fungi. However, the efficiency of fermentation of glucose-xylose mixtures by recombinant yeast under conditions of high sugar concentrations still remains insufficient. As engineering microbial cell factories for efficient biofuel production continues to be only a prospect, the search for new natural strains as a basis for further research is required (Liu et al., 2022). Therefore, one of the promising directions of xylose fermentation is the use of yeasts, such as *Scheffersomyces stipitis*, which demonstrate the natural ability to assimilate and ferment xylose into ethyl alcohol.

The aim of this work was an experimental study of the full cycle of sequential processes for the production of second-generation bioethanol from lignocellulosic agricultural waste using selected natural microorganisms and the methods of statistical optimization. The steps of pre-treatment of lignocellulose substrates, processes of saccharification using cellulolytic enzymes of filamentous fungi and fermentation of obtained syrups by xylose-fermenting yeast were studied.

Materials and methods

Microorganism used in the optimization of pre-treatment and enzymatic hydrolysis of lignocellulosic agricultural waste. Previously, the selected strain of cellulolytic filamentous fungus *Talaromyces funiculosus* (Thom) Samson, Yilmaz, Frisvad & Seifert 2011 (anamorph *Penicillium funiculosum* Thom 1910), strain UCM F-16795 (IMV F-100111) from the Ukrainian Collection of Microorganisms was used as a producer of a complex of cellulolytic and xylanolytic enzymes with high β-glucosidase activity (Yurieva et al., 2017). Cellulase and xylanase production by this strain has been previously optimized by us (Syrchin et al., 2023).

To obtain fungal inoculum, the strain was cultivated on potato dextrose agar (PDA) at 26 ± 2 °C for 14 days. Then a standard suspension of conidia (1 x 10^6 spores/mL) was prepared to be added (5% v/v) to the potato dextrose liquid medium in 750 mL Erlenmeyer flasks and cultivated under submerged conditions at the shaker at 210–230 rpm and 26 ± 2 °C for 48 hours (Methods of experimental mycology, 1982).

To obtain the enzymatic preparation, cultivation of the fungus was carried out under submerged conditions at 210–230 rpm and 26 ± 2 °C for 48 hours, on the nutrient medium of the following composition (g/L): corn stalk 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 obtained *T. funiculosus* culture liquid was decanted, centrifuged for 10 min at 3000 rpm (926 g), and the supernatant was used for the hydrolysis process.

Wheat straw was chosen to simulate the process of hydrolysis of the lignocellulosic substrate. The dry substrate was crushed using a laboratory mill and subsequently sifted through sieves with different pore diameters.

A fraction with a size of 0.5 to 5.0 mm was used for hydrolysis. The stage of preliminary processing of crushed wheat straw was carried out by a complex physico-chemical method. First, the substrate was treated with a 4–6% NaOH solution in the ratio of 400 mL per 100 g of straw. Then the straw, moistened this way, was irradiated for 2–10 min with microwave irradiation at frequency of 2.45 GHz and power of 600 W. Taking into account the mode of magnetron operation at this power, this corresponds to 58 and 288 kJ of radiation energy. After that, the substrate was washed from alkali with distilled water to neutral pH and dried to constant weight.

Hydrolysis of the pre-treated wheat straw (WS) was carried out with the culture liquid of *T. funiculosus* in a dilution of 1:1 and 1:4, at a temperature of 40–60 °C and pH values 4.0–6.0 for 18–30 hours.

The total amount of reducing sugars (RS) in the syrups obtained from hydrolysates filtered from straw was determined by Bertrand's method (Chandraju et al., 2016).

The qualitative and quantitative composition of the main mono- and disaccharides in the mixture of the sugars in the syrups was determined in some cases using the method of high-performance liquid chromatography (HPLC, liquid chromatograph Shimadzu LC-20, Kyoto, Japan) with Rezex RPM-Monosaccharide column and water as a mobile phase, according to the manufacturer recommendations.

Important factors affecting the hydrolysis of cellulosic substrates by enzyme preparations are (i) the temperature and acidity of the process environment, which, as a rule, coincide with the optimum temperature and pH of the reaction of enzymes that destroy β-glucosidase bonds; as well as (ii) the ratio between the substrate and the liquid phase, (iii) the activity of the enzyme preparation, (iv) the duration of hydrolysis and (v) the method of pre-treatment of the substrate. Pre-treatment of the plant residues by physico-chemical methods helps remove lignin and partially hemicellulose, and increase the number of reactive sites available for cellulolytic enzymes.

Previously, when using an enzyme preparation from *Fennellia* sp. UCM F-2806, we have shown that combined pre-treatment of wheat straw with alkali and microwave irradiation can be successfully applied for increasing the amount of reducing sugars at the enzymatic hydrolysis process (Syrchin et al., 2017). In addition, such combined pre-treatment of the substrate allowed us to reduce the duration and intensity of conditions of substrate chemical treatment, compared to other methods. As a result, for example, during the hydrolysis of wheat straw with an enzyme preparation from *Fennellia* sp. UCM F-2806 the main process of hydrolysis was completed during the first 18 hours.

Along with substrate pre-treatment processing, the impact of mixing of the components of the reaction (substrate and liquid phase) is regarded as an important factor of the hydrolysis process. Also, instead of mixing, the ratio of the substrate to the liquid phase can be maximized. Besides, the comprehensive studies of various stages of plant substrate hydrolysis as a whole process of obtaining syrups with sugar mixtures are also of interest. Therefore, in order to establish optimal conditions for the process of hydrolysis of refined wheat straw using enzymes of *T. funiculosus* we used parameters shown in Table 1.

The optimization of the conditions for obtaining sugar mixtures was carried out by a two-stage method with the consistent use of Plackett-Burman and Box-Behnken factorial designs (Infanzón-Rodríguez et al., 2020; Ozbek et al., 2021; Devesa-Rey et al., 2023). To establish the significant parameters of WS hydrolysis, an experimental matrix was compiled according to the Plackett-Burman method, where seven influencing factors and two levels (minimum and maximum) of their values were used (Table 1). This design is especially practical in the case of a large number of factors and when it is unclear which settings are likely to be nearer to the optimum responses (Devesa-Rey et al., 2023). All variants of the experiment were carried out in three replicas. The first stage of optimization established factors that had a significant effect on the formation of reducing sugars during the WS hydrolysis with the cultural liquid of *T. funiculosus*. The first stage was followed by the second stage, where the Box-Behnken method was applied to get deeper insights into WS hydrolysis, assessing the possible mutual influence of the studied factors (Box & Behnken, 1960; Saulawa et al., 2023; Thuy et al., 2023). The second stage is presented in the Results section.

Statistical processing of data in the optimization of pre-treatment/ enzymatic hydrolysis, including construction of experimental plans and their mathematical processing, was carried out using the computer program MiniTab 16 (Minitab Ltd. UK).

Endoglucanase activity was determined at 50 °C and pH 5 (i) by hydrolysis of a 2.0% solution of sodium carboxymethyl cellulose (Na-CMC) after 30 min of incubation of 0.5 mL of culture filtrate with 0.5 mL of substrate; (ii) exoglucanase – by hydrolysis of filter paper (1 x 6 cm) for 60 min; and (iii) xylanase – by hydrolysis of 1% xylan solution for 5 min (Bailey at al., 1992; Meng et al., 2021). The amount of reducing sugars formed in the reaction mixture after the enzymatic hydrolysis of Na-CMC, filter paper or xylan was determined by the method using 3,5-dinitrosalicylic acid (DNS) (Miller, 1959). A unit of endo-, exoglucanase or xylanase activity was taken to be the amount of enzyme that, under the given conditions, formed 1 μmol of glucose or xylose per 1 mL of culture liquid in 1 min.

Table 1
Factors of the model of wheat straw hydrolysis according to Plackett-Burman method

No	Code of factor	Factors	Minimum value "–"	Maximum value "+"
1	A	Temperature, °C	40	60
2	B	Medium pH	4.0	6.0
3	C	Total energy of microwave irradiation, kJ	58	288
4	D	NaOH concentration, %	1	5
5	E	Enzyme dilution in a buffer solution, v/v	1:1	1:4
6	F	Ratio of substrate/enzyme preparation, mg/mL	50	100
7	G	Hydrolysis duration, h	18	30

β-glucanase activity was determined by the formation of p-nitrophenol as a result of hydrolysis of a 10 mM p-nitrophenyl-glucopyranoside (pNPG) solution. For this, 100 μL of pNPG solution was incubated with 100 μL of the correspondingly diluted enzyme preparation and 200 μL of acetate buffer (pH 5) for 10 min at a temperature of 50 °C. The amount of enzyme capable of forming 1 μmol of p-nitrophenol in 1 min under the given conditions was taken as a unit of β-glucanase activity (Parry et al., 2001).

Xylose-fermenting yeasts were used in the research on optimization of the ethanologenesis on the mixtures of sugars resulting from enzymatic hydrolysis of lignocellulosic agricultural waste. Previously, of all strains of non-conventional yeasts of the genera *Kluyveromyces*, *Pichia*, *Debaryomyces*, *Scheffersomyces*, *Candida*, etc., both newly isolated and taken from the Ukrainian Collection of Microorganisms (UCM), only two strains were capable of fermenting xylose to alcohol. According to preliminary biochemical identification, these strains belonged to the species *Scheffersomyces stipitis* (Pignal) Kurtzman & Suzuki 2010. These strains were isolated in 2021 from decaying wood with working codes w18 and w20.1, and later they were assigned codes of the Ukrainian Collection of Microorganisms UCM Y-2810 and UCM Y-2811, respectively (Ianieva et al., 2022). Of these two strains, the average values of ethanol production on medium with 40 g/L xylose were slightly higher for *S. stipitis* UCM Y-2810 (6.1 g/L) than for *S. stipitis* UCM Y-2811 (5.5 g/L). Therefore, for a more in-depth study at this stage, we selected the strain *S. stipitis* UCM Y-2810 as a model.

Two genetic markers of the ribosomal operon were used for accurate molecular genetic identification of yeast isolate w18 (collection code UCM Y-2810) from rotten wood, which was previously identified by biochemical methods as *Scheffersomyces stipitis* (formerly known as *Pichia stipitis* species) (Yamada et al., 1994; Ianieva et al., 2022). There were (1) ITS-fragment containing the 5'-end of the 18S rRNA gene, ITS1, the 5.8S rRNA gene, ITS2, and the 3'-end of the 28S rRNA gene, and (2) D1/D2 locus of the 28S rRNA large subunit gene. Nucleotide sequences of the amplicons: 1) the ITS-fragment, obtained using the universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for amplification, and 2) 28S rRNA, obtained using primers NL-1 (5 ′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′), were sequenced (Macrogen, Seoul, South Korea) and analyzed using BLASTN (Kwiatkowski et al., 2012; Schoch et al., 2012; Raja et al., 2017).

In the choice of cultivation methods for *S. stipitis* UCM Y-2810, we relied on the experience of our previous studies of this strain, including

data on the duration of its cultivation necessary to obtain ethanol (Ianieva et al., 2022). At this stage of the work, yeast cultivation was carried out on liquid media at 25 °C under following conditions: 1) under static microaerophilic conditions in Dunbar tubes, which are traditionally used for qualitative evaluation of yeast ability for fermentation, with a maximum duration of up to 7 days, as well as 2) under submerged conditions in conical flasks (with total volume 250 mL) on a shaker with rotation rate 120 rpm, with a maximum duration of up to 3 days. The inoculum was a suspension of yeast grown for two days on malt extract agar (MEA) at 25 °C. The yeast suspension was added to the experimental liquid medium to make up the concentration $1 \cdot 10^6$ cells/mL. Modified yeast extract-peptone-dextrose (YPD) media were used in the experiments, with volume of 3.5 mL for Dunbar tubes and 80 mL for flasks. In these media, the mixtures of pentose and hexose monosaccharides: xylose and glucose were added to yeast extract (10 g/L) and peptone (5 g/L) at the concentrations characteristic of syrups obtained in this project during saccharification of lignocellulosic substrates with fungal enzymes.

The application of mathematical statistics methods with the determination of significant factors and optimal values of significant factors was used for a more in-depth study of the ethanologenesis process for yeast *S. stipitis* UCM Y-2810 cultivated on the mixtures of xylose and glucose. A three-factor three-level experimental design according to Box-Behnken Plan (BBP) was used in combination with the desirability function (Statistica 14, TIBCO Software Inc. 2020). The BBP matrix field was chosen in accordance with the actual concentrations of these monosugars in syrups, which were obtained in this project as a result of the use of fungal enzymes. According to the Box-Behnken plan, 15 variants of the experiment were performed, each of which meant a certain combination of optimization factors, where the concentration of ethanol in the medium served as an optimization parameter. The independent variables were the following factors: X_1 – xylose concentration in the medium (g/L); X_2 – glucose concentration in the medium (g/L); X_3 – duration of yeast cultivation (days, Table 2). Depending on the type of cultivation, either under static conditions in Dunbar tubes or submerged growth in conical flasks with stirring, two different experimental Box-Behnken designs were developed, where the duration of cultivation differed (both shown in Tables 2 and 3).

We also employed the desirability function in the analysis of the overall effects of the combinations of studied factors on the ethanologenesis. The desirability function is based on the transformation of all responses into a dimensionless value (Rahman & Nasir, 2018). In the optimization process, each response is transformed into an individual desirability function (d_i) that varies between 0 and 1, indicating an undesirable response ($d_i = 0$) and a fully desirable response ($d_i = 1$).

Table 2
Conditions of *S. stipitis* cultivation in Dunbar tubes (D) and conical flasks (C) according to Box-Behnken plans

Factor designation	Factor		Minimum value "–"	Average value "0"	Maximum value "+"
X_1	Xylose, g/L		3	16.5	30
X_2	Glucose, g/L		1	5.5	10
X_3	Cultivation duration, day	D	1	4.0	7
		F	1	2.0	3

Yeast concentration was estimated by the number of cells (colony-forming units – CFU) per mL of suspension.

Determination of the amount of ethanol synthesized by yeast was carried out by gas chromatography-mass spectrometry (Laboratory of Biological Polymer Compounds, D. K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine) on an Agilent 6890N/5973 inert GC/MSD system (Agilent Technologies, USA) with capillary column HP- INNOWax (30 m × 0.25 mm × 0.25 μm) (J&W Scientific, USA). The separation was carried out with a temperature gradient of 20 °C/min from 40 to 120 °C, carrier gas helium, and flow rate through the column 1 mL/min.

General statistical processing of the obtained data was carried out using Minitab 16 (Minitab, LLC., 2010) and trial version Statistica 14 (TIBCO Software Inc., 2020, http://tibco.com) programs. While employing methods of mathematical statistics, including optimization, the triplicated measurements (n = 3) were used to estimate the standard deviation. Using one-way ANOVA comparative analysis of the yeast biomass growth and the efficiency of ethanol production by this biomass we used n = 15. The obtained results were considered statistically significant at $P \leq 0.05$.

Table 3
Planning matrix for *S. stipitis* cultivation conditions in Dunbar tubes (D) and conical flasks (F) according to Box-Behnken plan

No of varian	(X_1) Xylose concentration, g/L	(X_2) Glucose concentration, g/L	(X_3) Cultivation duration, day	
			D	F
1	3	1	4	2
2	30	1	4	2
3	3	10	4	2
4	30	10	4	2
5	3	5.5	1	1
6	30	5.5	1	1
7	3	5.5	7	3
8	30	5.5	7	3
9	16.5	1	1	1
10	16.5	10	1	1
11	16.5	1	7	3
12	16.5	10	7	3
13	16.5	5.5	4	2
14	16.5	5.5	4	2
15	16.5	5.5	4	2

Results

Establishing the main optimal parameters of T. funiculosus UCM F-16795 enzymatic hydrolysis of wheat straw. In order to establish the optimal conditions for the process of WS enzymatic hydrolysis using the culture liquid of *T. funiculosus*, as mentioned earlier, the following parameters were studied: (A) temperature; (B) medium pH; (C) total energy of microwave irradiation; (D) NaOH concentration; (E) enzyme dilution in a buffer solution; (F) ratio of substrate/enzyme preparation; (G) hydrolysis duration (Tables 1, 4). The results of application of the Plackett-Burman method with comparison of theoretical and experimentally obtained data on amount of reducing sugars resulting from hydrolysis are presented in Table 4.

The regression model of reducing sugars (RS) formation was theoretically calculated using coded experimental data according to the Plackett-Burman plan:

$$RS_{theor} = 1.667 - 0.987 \cdot A - 1.273 \cdot B + 1.008 \cdot D + 0.932 \cdot E - 0.388 \cdot G.$$

The maximum concentration of reducing sugars formed as a result of hydrolysis of wheat straw according to the model was 6.265 g/L.

According to the obtained model, the main significant factors were temperature, medium pH, NaOH concentration, enzyme dilution with probability $P < 0.001$, and duration of hydrolysis with $P = 0.013$. These five factors were used in the following second stage of this study employing by 3-level 5-factor Box-Behnken experimental plan. Application of the Box-Behnken model clarified further obtained data of mathematical modelling of the process of WS hydrolysis by using the culture liquid of *T. funiculosus*, as well as possible relationships between individual significant factors (A) NaOH concentration, (B) medium pH, (C) enzyme dilution in a buffer solution, (D) temperature, and (E) duration of hydrolysis (Table 5).

Based on coded data resulting from the Box-Behnken experiment, the mathematical model of the formation of reducing sugars (RS) was calculated:

$$RS_{theor} = 1.14 + 0.58 \cdot X_1 - 1.54 \cdot X_2 + 0.56 \cdot X_3 - 0.67 \cdot X_4 - 0.69 \cdot X_5 - 0.93 \cdot X_3 \cdot X_4 - 0.93 \cdot X_4 \cdot X_5 + 0.5 \cdot X_2^2$$

The maximum value of reducing sugars according to this model was 7.4298 g/L, and the experimentally obtained actual value was 7.64 ± 0.42 g/L. The latter experiments were performed under conditions with chosen constant parameters: (i) total energy of microwave irradiation 1.2 kJ and (ii) ratio of substrate/enzyme solution 100/1 mg/mL.

That is, the Box-Behnken-based mathematical model more accurately described the process of wheat straw hydrolysis by culture filtrate of *T. funiculosus*. According to this model, all studied factors individually had a significant effect on the wheat straw hydrolysis process with $P = 0.004$ for alkali concentration, $P < 0.001$ for medium pH, $P = 0.006$ for enzyme concentration, $P = 0.001$ for temperature, and $P = 0.001$ for dura-

tion of hydrolysis. In addition, considerable effect was also shown for the joint action of medium pH with enzyme concentration (X_2*X_3) and enzyme concentration with temperature (X_3*X_4) with P = 0.019 for each interaction. As a result of the consistent use of the Plackett-Burman and Box-Behnken statistical plans, there were established optimized conditions of WS enzymatic hydrolysis using the enzyme complex of *T. funiculosus* UCM F-16795, which included alkali (NaOH) concentration 4.6–4.8%; medium pH 4; enzyme dilution in a buffer solution 1:1; temperature 40 °C; and duration of hydrolysis 18 hours. Under such conditions of pretreatment, the hydrolysates of wheat straw contained from 5.0 to 7.5 g/L of reducing sugars, of which 0.7–1.0 g/L glucose, 2.2–2.9 g/L xylose and 0.7–0.8 g/L cellobiose.

Table 4

Formation of reducing sugars according to experimental matrix by 2-level 7-factor Plackett-Burman plan with coded data for factors affecting wheat straw hydrolysis

No	Factors and their codes (presented in backets)							Amount of reducing sugars, g/L	Theoretical amount of reducing sugars, g/L
	(A) Temperature, °C	(B) pH	(C) Total energy of microwave irradiation, kJ	(D) NaOH concentration, %	(E) Enzyme dilution, v/v	(F) Substrate/enzyme preparation ratio, mg/mL	(G) Hydrolysis duration, h		
1	–	+	–	–	–	+	+	0.18 ± 0.32	0.0
2	–	–	–	–	–	–	–	1.11 ± 0.35	2.4
3	+	+	+	–	+	+	–	0.00 ± 0.00	0.0
4	–	+	+	–	+	–	–	1.25 ± 0.47	1.7
5	+	–	+	+	–	+	–	2.04 ± 0.22	2.4
6	+	–	+	–	–	–	+	0.44 ± 0.00	0.0
7	+	+	–	+	+	–	+	0.29 ± 0.37	1.7
8	–	–	–	+	+	+	–	7.64 ± 0.42	6.3
9	–	–	+	+	+	–	+	5.45 ± 0.16	5.5
10	+	–	–	–	+	+	+	1.02 ± 0.88	1.5
11	–	+	+	+	–	+	+	0.35 ± 0.16	1.1
12	+	+	–	+	–	–	–	0.35 ± 0.31	0.0
13	0	0	0	0	0	0	0	5.36 ± 0.35	1.7

Table 5

Formation of reducing sugars according to experimental matrix by 3-level 5-factor Box-Behnken plan with uncoded data for factors affecting wheat straw hydrolysis

No	Factors and their codes (presented in brackets)					Amount of reducing sugars, g/L	Theoretical amount of reducing sugars, g/L
	(X_1) NaOH concentration, %	(X_2) Medium pH	(X_3) Enzyme dilution, v/v	(X_4) Temperature, °C	(X_5) Hydrolysis duration, h		
1	3	6.0	1:2	60	24	0.00	0.00
2	5	5.0	1:2	60	24	1.31	1.05
3	3	5.0	1:4	50	18	2.37	2.39
4	3	4.0	1:1	50	24	1.97	1.69
5	3	4.0	1:2	60	24	1.44	2.51
6	3	4.0	1:4	50	24	5.42	4.67
7	5	5.0	1:2	40	24	2.90	2.39
8	3	5.0	1:1	50	18	0.64	1.27
9	3	5.0	1:4	50	30	0.00	1.01
10	3	6.0	1:4	50	24	0.00	0.00
11	1	5.0	1:2	50	18	0.51	1.25
12	1	5.0	1:2	50	30	0.00	0.00
13	5	5.0	1:2	50	30	0.64	1.03
14	3	5.0	1:1	50	30	0.00	0.00
15	1	5.0	1:2	40	24	1.31	1.23
16	5	5.0	1:2	50	18	1.57	2.41
17	3	6.0	1:2	40	24	0.64	0.77
18	3	6.0	1:1	50	24	0.25	0.47
19	3	4.0	1:2	40	24	3.43	3.85
20	1	5.0	1:2	60	24	0.00	0.00
21	3	5.0	1:1	60	24	0.78	0.84
22	1	4.0	1:2	50	24	1.44	2.60
23	5	4.0	1:2	50	24	3.56	3.76
24	3	5.0	1:1	40	24	0.64	0.32
25	3	5.0	1:4	40	24	3.56	3.30
26	5	5.0	1:1	50	24	0.38	1.16
27	5	5.0	1:4	50	24	2.24	2.28
28	1	6.0	1:2	50	24	0.00	0.00
29	5	6.0	1:2	50	24	0.00	0.68
30	3	5.0	1:2	60	18	2.9	1.16
31	3	6.0	1:2	50	30	0.00	0.00
32	3	6.0	1:2	50	18	0.00	0.79
33	1	5.0	1:4	50	24	0.00	1.12
34	3	5.0	1:2	40	30	0.91	1.12
35	3	4.0	1:2	50	30	3.69	2.49
36	1	5.0	1:1	50	24	0.00	0.00
37	3	5.0	1:4	60	24	0.00	0.10
38	3	4.0	1:2	50	18	4.62	3.87
39	3	5.0	1:2	40	18	4.62	2.50
40	3	5.0	1:2	60	30	0.91	0.00
41	3	5.0	1:2	50	24	1.14	1.14

Assessment of the ability of xylose-fermenting yeast for ethanologenesis on media with a mixture of glucose and xylose and initial optimization of the process. Recently isolated xylose-fermenting yeast strain UCM Y-2810 (working code w18 (Ianieva et al., 2022), which belonged to the species *Scheffersomyces stipitis* according to preliminary phenotypic evaluations of its physiological and biochemical characteristics, served in this assessment as a model yeast in such experiments. We performed precise molecular genetic identification of this strain to confirm whether it belongs to species *S. stipitis*. Two genetic markers of the ribosomal operon, ITS fragments and the 28S rDNA gene (or LSU) were used in the analysis of

the NCBI GenBank database to search the sequences similar to the reference sequences of the yeast strain UCM Y-2810. As a result of this search, there were found 28 *S. stipitis* strains with ITS-fragment sequences with 100% identity, and 71 *S. stipitis* strains with LSU sequences with ≥ 99.5% identity to the reference strain UCM Y-2810. Some *S. stipitis* strains with ITS and LSU sequences the most similar to those of studied strain UCM Y-2810 are presented in Table 6. There were both ITS and LSU sequences available in GenBank for nine strains (CBS:6054 ch 3, D141_1, D6, CECT 1922, CBS:7124, CBS:7125, CBS:7126, DCBM3, DCBM5) with identity 99.7–100.0% and 99.5–100.0%, respectively. The type *S. stipitis* strains for each genetic marker with 100% similarity to the studied xylose-fermenting strain were also presented. Thus, we confirmed that yeast strain UCM Y-2810 belonged to the species *S. stipitis*, and its nucleotide sequences of the ITS-fragment and LSU were deposited in the GenBank (NCBI) under accession numbers OP931914 and OP931915, respectively.

The effects established in the classic one-dimensional studies may differ from the results of multidimensional optimization using mathematical statistics methods, which take into account the statistical significance of the influence of the studied factors and the significant effects of interaction between experimental factors, as well as considerably reduce the duration and cost of experiments. Therefore, at this stage of a more in-depth study of the influence of the cultivation conditions of unconventional pentose-fermenting yeast on the process of ethanologenesis on model media with mixtures of glucose and xylose, we preferred the methods of mathematical statistics for the planning and analysis, which included the Box–Behnken design and response surface methodology. The data of yeast *S. stipitis* growth and ethanol production, cultivated under either static microaerophilic conditions in Dunbar tubes or submerged cultivation in conical flasks, which were obtained using the Box-Behnken experimental plan are presented in Table 7.

Table 6

S. stipitis strains from GenBank database with the highest ITS and LSU sequences identity with those of the studied xylose-fermenting yeast strain UCM Y-2810

Codes of *S. stipitis* strains	GenBank accession numbers	Identity (I), %	Query coverage (Qc), %	Additional information
		ITS		
ATCC 62970 *	GU256745.1	100	100	Type material
ATCC 58376 *	NR_165942.1	100	98	Type material
CBS:6054 ch 3	CP000497.1	100	100	Xylose-fermenting yeast
D141_1	JQ026363.2	100	100	Bulgaria
D6	HM627151.2	99.0	92	Bulgaria
CECT 1922	DQ409167.1	100	100	N/A
CBS:7124	KY105360.1	99.3	98	Soil
CBS:7125	KY105359.1	99.8	98	Soil
CBS:7126	KY105358.1	99.8	98	Soil
DCBM3	AB862498.1	99.7	98	Japan, insect
DCBM5	AB862501.1	99.7	98	Japan, insect
EH001	HQ652053.1	100	98	USA, insect
CBS 5776	KX525669.1	100	98	France, insect larva
KTGY31.5	ON954672.1	100	98	Guatemala, insect gut
		LSU		
NRRL Y-7124 *	JN943257.1	100	96	Type material
CBS:6054 ch 3	CP000497.1	100	100	Xylose-fermenting yeast
D141_1	JQ026364.1	100	99	Bulgaria
D6	HM627089.1	99.5	100	Bulgaria
CECT 1922	DQ409152.1	100	100	N/A
CBS:7124	KY109581.1	99.5	99	Soil
CBS:7125	KY109583.1	99.5	100	Soil
CBS:7126	KY109584.1	100	100	Soil
DCBM3	AB862499.1	99.5	98	Japan, insect
DCBM5	AB862502.1	99.5	98	Japan, insect
BG10-06-16-04-9	JQ025391.1	100	98	Thailand

Notes: * – type strains; N/A – not available.

The level of significance of the effects (individual linear and quadratic effects as well as interaction effects) of experimental factors: concentrations in the medium of xylose and glucose, and the duration of yeast cultivation, was determined using analysis variance (ANOVA) (Table 8). A considerable difference between the cultivation conditions was revealed: static microaerophilic conditions in Dunbar tubes and submerged cultivation in conical flasks on a shaker. The obtained data indicated that in the case of Dunbar tubes a significant ($P \leq 0.05$) influence on ethanol yield was characteristic for the concentration of xylose (linear effect, $F = 18.932$, $P = 0.005$), the duration of yeast cultivation (quadratic effect, $F = 7.288$, $P = 0.033$) and the linear interaction of these two factors ($F = 13.119$, $P = 0.015$). At the same time, such an optimization factor as the concentration of glucose did not statistically reliably affect the yield of ethanol in contrast to concentration of xylose, which had the maximum reliable effect under these conditions. In contrast to yeast cultivation in Dunbar tubes, it was found that only one experimental factor, the duration of cultivation, had a statistically reliable linear effect on ethanol production ($F = 4.927$, $P = 0.040$) for submerged conditions of yeast growth in conical flasks with stirring. The influence of the rest of the investigated factors was not significant.

Table 7

Ethanol production and yeast growth of *S. stipitis* in Dunbar tubes (D) and conical flasks (F) according to Box-Behnken plans

No of variant	Ethanol concentration, g/L		Yeast biomass, LgCFU/mL	
	D	F	D	F
1	0	0	8.079	9.279
2	9.796	2.799	7.845	9.580
3	4.692	0	8.176	9.279
4	11.278	3.375	7.602	9.279
5	4.034	2.716	7.279	9.491
6	3.210	2.305	7.279	8.491
7	0	2.963	8.000	9.699
8	9.878	3.787	8.114	9.398
9	3.951	0	9.204	8.447
10	5.433	0	8.959	8.643
11	3.046	5.350	8.633	9.398
12	4.363	7.738	8.362	9.643
13	7.902	4.198	7.954	9.643
14	7.655	3.128	7.778	9.908
15	8.149	3.210	7.845	9.519

Table 8

Analysis of significance of factors affecting ethanol production by yeast, cultivated under either static microaerophilic conditions in Dunbar tubes or submerged conditions in conical flasks, according to 3-level 3-factor Box-Behnken experimental plan

Cultivation conditions	Factor	Fisher's criterion	Probability
Dunbar tubes	(X_1) Xylose concentration [L+Q]	18.932	0.005
	(X_2) Glucose concentration [L+Q]	2.809	0.152
	(X_3) Duration of cultivation [L+Q]	7.288	0.033
	X_1*X_2	1.180	0.327
	X_1*X_3	13.119	0.015
	X_2*X_3	0.003	0.958
Flasks	(X_1) Xylose concentration	1.768	0.231
	(X_2) Glucose concentration	0.615	0.565
	(X_3) Duration of cultivation	4.927	0.040

Notes: L – linear effects of factor; Q – nonlinear (quadratic) effects of factor; * – joint action of several factors; other statistically insignificant effects are not shown in the table.

Visually, the effects of combinations of the studied factors on the ethanol concentration were investigated using the method of response surfaces with integration of all obtained data using desirability function. The resulting visualization of the general effects of the combinations of the studied factors on ethanol concentration using the method of response surfaces and desirability function is presented in Figure 1. The response surfaces illustrated well the great difference between the method of cultivation of yeast, on the one hand, in Dunbar tubes, where the significant factors were the xylose concentration and the duration of cultivation in the absence of influence of the glucose concentration in the medium, and, on the other hand, under submerged conditions with stirring in conical flasks, where the only significant factor affecting ethanologenesis was the duration of cultivation.

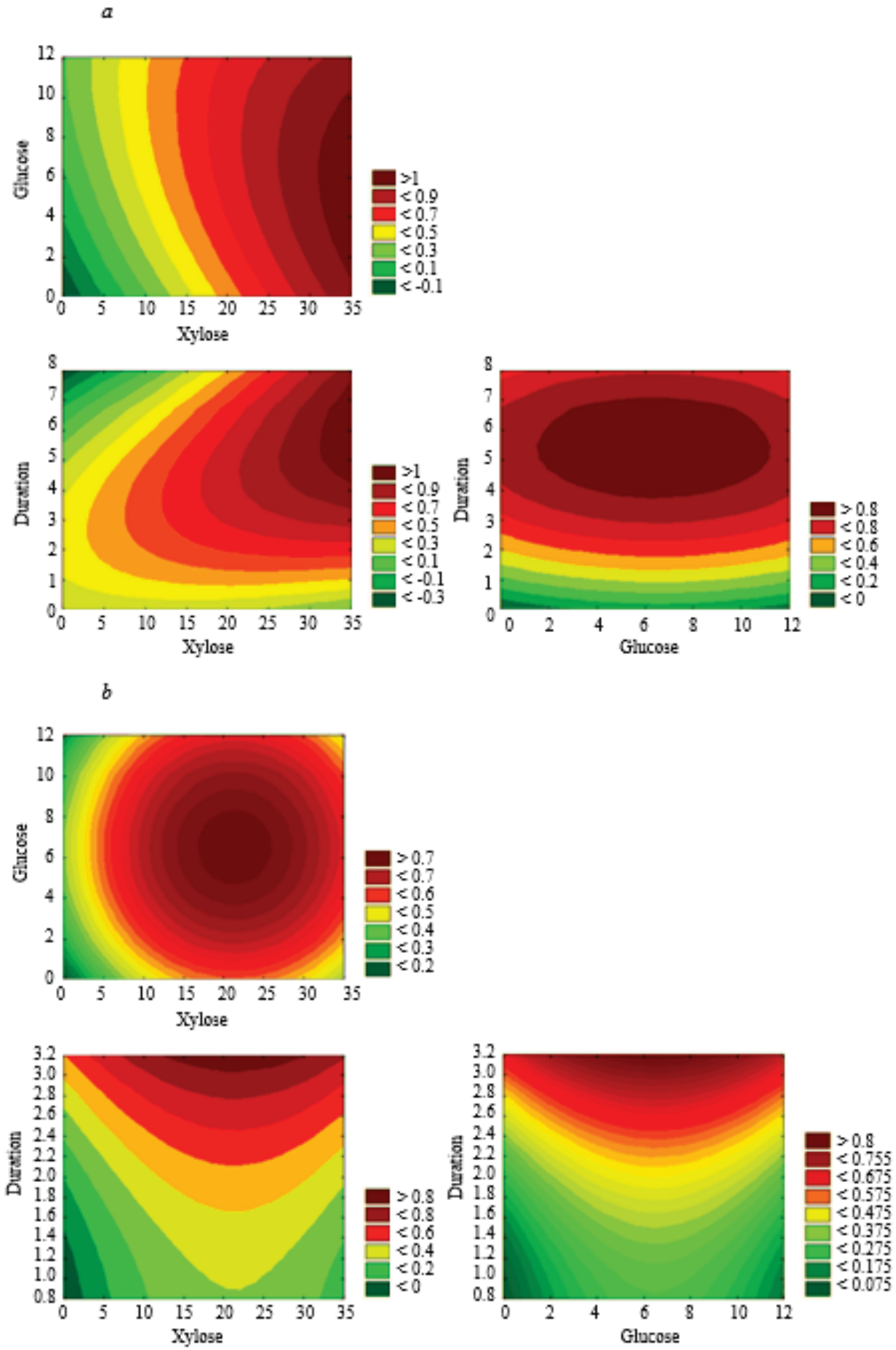

Fig. 1. Visualization of the general effects by method of response surfaces: the dependence of the desirability indicators of ethanol production on the concentration of xylose (g/L), glucose (g/L), and the duration of yeast cultivation (days) using Dunbar tubes (D) and conical flasks (F)

Application of the optimization module of the statistical program (Statistica) to obtain the maximum yield of ethanol during the fermentation of mixtures of xylose and glucose by yeast *S. stipitis* UCM Y-2810 also demonstrated a significant difference between the cultivation of yeast in Dunbar tubes and in conical flasks with stirring. In the mathematical models we obtained, the optimal calculated values for the investigated factors under the conditions of cultivation in Dunbar tubes were 30 g/L of xylose and 5.5 g/L of glucose in the medium, and duration of 5.5 days. Whereas, during submerged cultivation in flasks with stirring, the optimal values were 23.25 g/L xylose, 5.50 g/L glucose and 3 days. Resulting values of the maximum ethanol yield were 11.28 ± 0.20 g/L (x ± SE) in the experiments with Dunbar tubes and 7.74 ± 0.76 g/L for submerged cultivation in conical flasks.

The obtained data indicated that as a result of optimization of the ethanologenesis process and the use of static cultivation of yeast under microaerophilic conditions, it is possible to increase the yield of ethanol production by 1.5 times compared to the submerged cultivation with stirring, as well as approximately twice compared to the maximum rates of ethanol production (5.5–6.0 g/L) according to the results of the previous stage of our research on *S. stipitis* yeasts, in which process optimization has not yet been applied.

Yeast growth did not statistically significantly differ for all investigated variants of specific cultivation method (Table 9). However, the comparative one-way ANOVA analysis (Minitab 16) showed that, on average, the biomass of yeast grown in Dunbar tubes was significantly lower ($P < 0.001$) than in conical flasks on a shaker (Fig. 2a). A comparative analysis of the efficiency of ethanol production by biomass (or specific ethanol production), which was calculated as the maximum ethanol values per the number of yeast cells (LgX) for each variant of the corresponding experiment, also demonstrated a significant difference ($P < 0.001$) between cultivation methods, static and submerged conditions (Fig. 2b). For this we used the above-mentioned values of maximum ethanol production determined as a result of optimization: 11.28 g/L for Dunbar tubes and 7.74 g/L for flasks. Consequently, it was demonstrated that, under static conditions in Dunbar tubes, a smaller amount of biomass produced a larger amount of ethanol than under submerged conditions in conical flasks with stirring (Fig. 2a, 2b).

Since static microaerophilic conditions for cultivating yeast in Dunbar tubes turned out to be more effective, we chose these conditions for a more detailed analysis of the effect of the different ratios of the two studied monosaccharides, glucose and xylose, on the ethanologenesis of *S. stipitis* using the response surfaces approach, which could give us more information on physiological peculiarities of yeast consumption of glucose and xylose while producing ethanol (Fig. 3).

It was observed that, at all studied glucose concentrations, the significance of xylose concentration in the process of ethanologenesis increased with the duration of yeast cultivation under static conditions (Fig. 3a–c). The patterns were observed for xylose: first, a sharp increase in ethanol production at increased xylose concentration and duration of yeast cultivation, and second, the ethanol concentration sharply decreased at the lowest xylose concentration in combination with the longest duration of cultivation, which testified to its assimilation by yeast under these conditions.

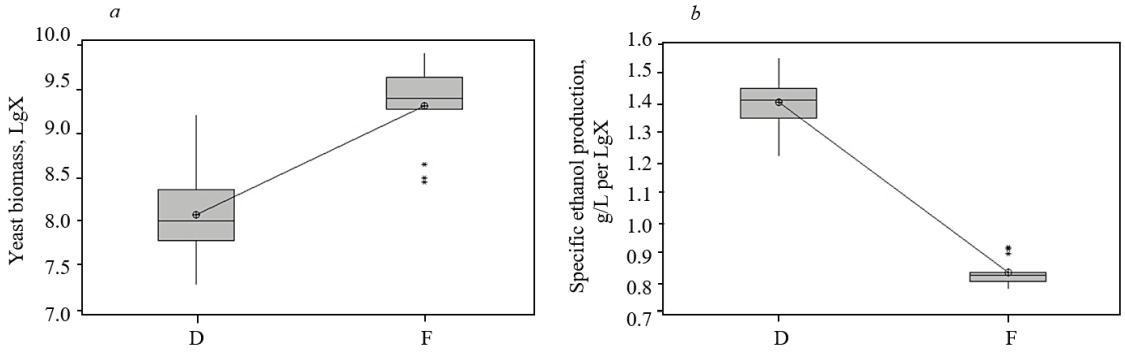

Fig. 2. Boxplots with whiskers showing one-way ANOVA comparative analysis of the yeast biomass growth (*a*) and the efficiency of ethanol production by this biomass (*b*) under static cultivation conditions in Dunbar tubes (D) and submerged cutivation in flasks on a shaker (F): grey box – interquartile range between 1st (25%) and 3rd (75%) quartiles, horizontal line within a box – median, crossed circle – mean value, upper and lower whiskers (vertical lines) – maximum and minimum values, separate asterisks – outliers data which are more extreme than the expected variation; n = 15

At all studied xylose concentrations, while analyzing the effect of interaction of glucose concentration/cultivation duration on ethanologenesis, the duration of cultivation had a strong effect on the ethanol production (Fig. 3d–f). At the lowest xylose concentration, a small effect of glucose concentration on ethanol production was observed over time, but with increasing xylose concentration this effect was eliminated and at the highest xylose concentration it was completely absent.

At the studied terms of cultivation duration, the greatest influence of glucose concentration on ethanologenesis was observed on the first day of cultivation, when there was a clear positive correlation between glucose concentration and ethanol production (Fig. 3g–i). However, this effect was not statistically considerable, and, over time, the importance of glucose concentration in the process of ethanologenesis was further decreasing. Unlike glucose, the concentration of xylose did not lose its considerable influence on ethanologenesis during the entire time of cultivation. All this indicated the rapid assimilation of glucose by yeast first, and then xylose, because the rate of consumption of glucose is higher than that of xylose.

Discussion

The present study explored the scientific principles of the bioconversion of lignocellulosic agricultural waste to second-generation ethanol using cellulolytic filamentous fungi and xylose-fermenting yeast, where the full biotechnological cycle from the hydrolysis of wheat straw by fungal enzyme complex to bioethanol production by yeast-based fermentation was studied using natural, non-genetically modified microorganisms. Besides, the conditions of the ethanologenesis study were based on our own data on the content of such monosaccharides as glucose and xylose, including their concentration and ratio.

We applied the methods of mathematical statistics to refine and optimize conditions at both hydrolysis and fermentation processes. Additionally, these methods helped us to elucidate some physiological patterns intrinsic, for example, for fermentation of mixtures of glucose and xylose by yeast.

The significant factors found in the present study affecting the wheat straw hydrolysis using *T. funiculosus* UCM F-16795 enzymes were somewhat different from our previous results on the treatment of wheat straw with an enzyme preparation from *Fennellia* sp. UCM F-2806 (Syrchin et al., 2017). In both cases the temperature and pH of the medium were, as expected, the main significant factors influencing the process of WS hydrolysis. However, unlike the data on *Fennellia* sp.-based enzymatic hydrolysis, in the present study, surprisingly, there was no significant effect of the total dose of microwave irradiation. Also, a feature of this process was the significant negative effect of increasing the duration of hydrolysis, despite our expectation of some positive input from this factor.

In addition, it is important to note that for the enzyme complex of *T. funiculosus* UCM F-16795 the cellulase activity is most likely to have the lower values of temperature and pH optimums compared to *Fennellia* sp. UCM F-2806, being 40 °C and 4.0 versus 50 °C and 5.0, respectively. This can be regarded as an advantage for *T. funiculosus* UCM F-16795

use because its enzyme complex would require less energy in the process of hydrolysis of plant residues.

The feasibility of simultaneous application of two enzyme preparations produced by strains *T. funiculosus* UCM F-16795 and *Fennellia* sp. UCM F-2806 to enhance the process of hydrolysis is under discussion and will require further investigation.

It should be highlighted that the choice of xylose concentration in the medium and the ratio of glucose and xylose in the mixtures of hexoses and pentoses is of great importance in the studies of the ethanologenic potential of xylose-fermenting yeasts (Agbogbo et al., 2006; Unrean & Khajeeram, 2015). When in the mid-1980s some yeasts were discovered with the natural genetic ability to ferment sugars of wood hydrolysates, in particular both hexoses and pentoses, the question arose of the sensitivity of such yeasts to varying degrees of inhibition of xylose utilization by glucose (Panchal et al., 1988). At that time, among such yeasts, the highest level of xylose-fermenting activity was found for representatives of the genus *Pichia*, especially the species *Pichia stipitis*, which was later assigned to the species *Scheffersomyces stipitis* (Kurtzman, 2011). It should be mentioned that xylose-fermenting yeasts like *S. stipitis* occupy a very specific econiche in nature and information on their sequences in GenBank database is still rather scanty compared to the ubiquitous species of yeast, especially conventional yeasts such as of genus *Saccharomyces* (Jeffries & Kurtzman, 1994; Morais et al., 2013; Ren et al., 2014; Ianieva et al., 2022). Obviously, in GenBank, there were provided many sequences of *S. stipitis* strains that are contained in well-known collections of microorganisms: ATCC (American Type Culture Collection), CBS (Westerdijk Fungal Biodiversity Institute), UCDEST (Phaff Yeast Culture Collection). In our case, to reduce species identification error we used two genetic markers: first, the ITS that was proposed and accepted as a universal DNA barcode for Fungi (Schoch et al., 2012), and, second, the LSU (D1/D2 domain of the large ribosomal subunit) that was also proposed to be used as a secondary barcode (Vu et al., 2016). In the species identification, we took into consideration the recommendations on taxonomic thresholds for discrimination of yeast species to be 98.5% similarity for ITS-fragment, and 99.5% similarity for LSU (Irinyi et al., 2015; Vu et al., 2016; Hoang et al., 2019; Nilsson et al., 2019; Salem-Bango et al., 2023). Thus, we confidently identified the studied yeast strain as *S. stipitis* UCM Y-2810.

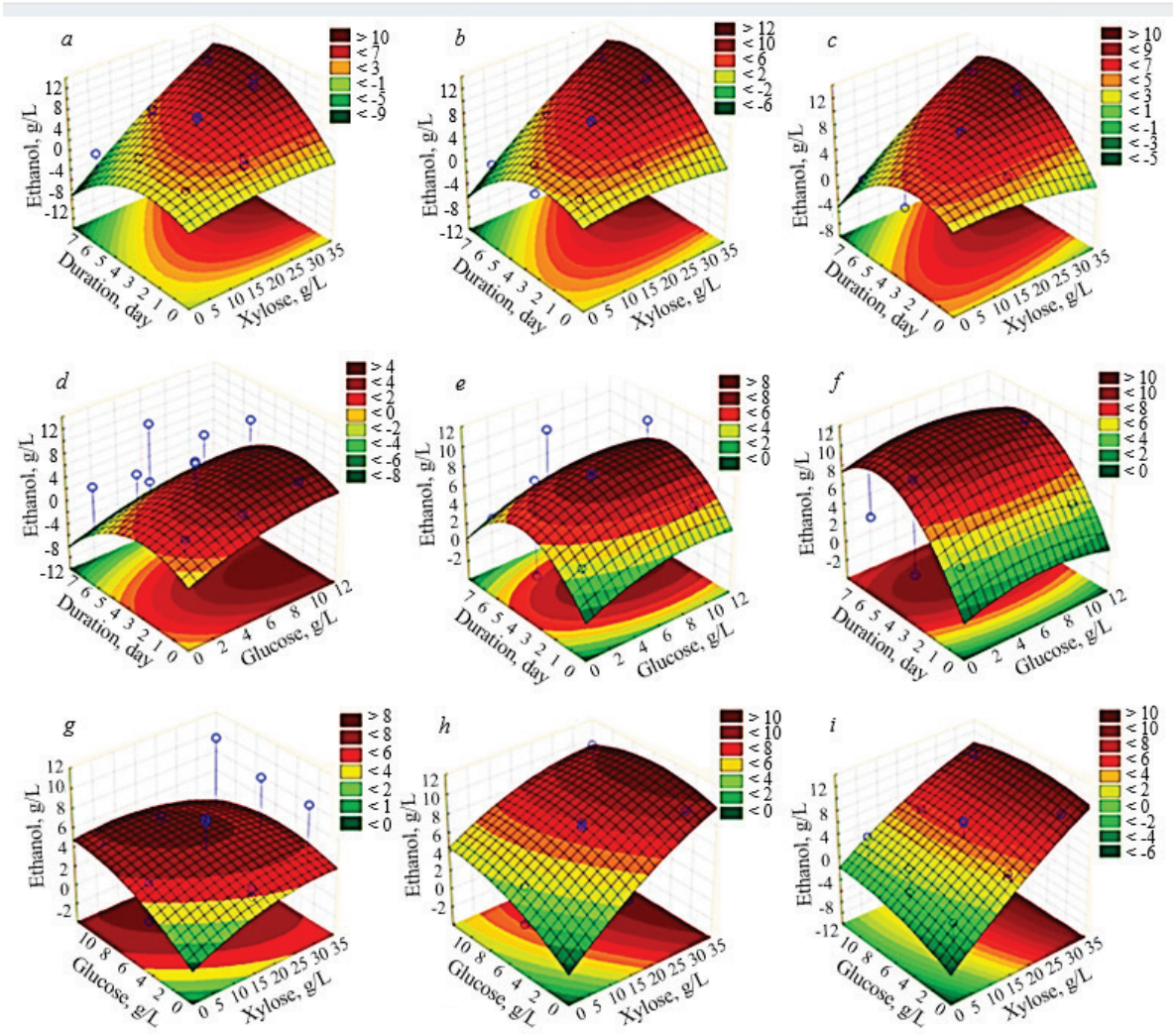

Fig. 3. Visualization by response surfaces of the detailed effects on ethanol production of different concentrations of glucose (*a–c*) and xylose (*d–f*) in the medium at different duration of yeast cultivation (*g–i*) under static microaerophilic conditions using Dunbar tubes: glucose concentration: *a* – 1 g/L, *b* – 5.5 g/L, *c* – 10 g/L; xylose concentration: *d* – 3 g/L, *e* – 16.5 g/L, *f* – 30 g/L; cultivation duration: *g* – 1 day, *h* – 4 days, *i* – 7 days

For representatives of the species *S. stipitis*, some researchers discovered the phenomenon of repression of xylose utilization by glucose under certain conditions (Panchal et al., 1988). The main prerequisites for the effective fermentation of cellulose hydrolysate to ethanol by a suitable microorganism were then formulated: 1) the ability to ferment both glucose and xylose, 2) the ability to overcome inhibition of xylose utilization by glucose, and 3) resistance to cellulose hydrolysis products such as acetic acid and furfural (Panchal et al., 1988).

Our data demonstrated that static microaerophilic conditions of yeast cultivation in Dunbar tubes were more effective than the submerged conditions in the flasks on a shaker, presumably because the stirring created the more aerobic and less favorable conditions for ethanologenesis of *S. stipitis*. That is why elucidation of physiological peculiarities of yeast consumption of glucose and xylose while producing ethanol was focused on the data obtained while cultivating yeast under static microaerophilic conditions.

As was mentioned before, the concentration of glucose and the ratio of xylose to glucose in pentose/hexose mixture are key factors that can affect ethanol production by yeast of *S. stipitis* because of the possibility of inhibition of xylose utilization by glucose (Panchal et al., 1988; Agbogbo et al., 2006; Unrean & Khajeeram, 2015). However, in most studies in the past, researchers used 3–4 times higher concentrations of glucose than xylose, and sometimes rather unrealistically high concentrations for lignocellulose waste hydrolysate glucose, for example 120 g/L glucose and 30 g/L xylose (Panchal et al., 1988; Unrean & Khajeeram, 2015). At the same time Panchal et al. (1988) demonstrated that in case of the pentose/hexose mixtures with 50 g/L xylose where glucose concentration varied from 0 to 10 g/L no negative effect on xylose utilization was observed; and only when glucose concentration reached 20 g/L did inhibition of xylose utilization start to manifest and gradually increase with increasing glucose concentration. It should be emphasized that in our experiments, we used monosaccharide concentrations and xylose to glucose ratio (3:1) in the mixtures, based on our data, obtained as a result of saccharification of wheat straw with fungal enzymes. It was revealed that, under such pentose/hexose ratio and concentration range relevant for the real syrups, glucose did not significantly affect the fermentation process of the studied strain *S. stipitis*, and did not inhibit the utilization and fermentation of xylose.

Even some weak effect of glucose concentration on ethanologenesis in the very beginning of cultivation significantly decreased with increasing cultivation duration, which may indicate a rapid initial glucose assimilateon by *S. stipitis* UCM Y-2810 compared to xylose (Agbogbo et al., 2006).

The influence of xylose on ethanologenesis was characterized by common patterns at all tested glucose concentrations: ethanol production positively correlated with xylose concentration and cultivation time, but the lowest concentration of xylose in the end of cultivation caused a decrease of ethanol concentration. The latter could indicate that in the end the yeast cells started to assimilate ethanol that has been previously produced by them.

At the lowest xylose concentrations, some weak influence of glucose concentrations on ethanologenesis was also observed. However, at xylose concentrations in the middle and highest range, the glucose concentration had almost no effect on the fermentation process. This may indicate that at the used glucose concentrations (≤ 10 g/L) there was not a non-competitive inhibition of xylose transport by glucose into yeast cells through the low-affinity proton-symport system (Panchal et al., 1988). Thus, under these conditions, *S. stipitis* UCM Y-2810 demonstrated the ability to overcome glucose repression of xylose utilization, which is one of the necessary conditions for effective fermentation of cellulose hydrolysates by yeasts of this species (Agbogbo et al., 2006).

Conclusion

At the steps of wheat straw pre-treatment and saccharification by enzymatic hydrolysis using the enzyme complex of *Talaromyces funiculosus* UCM F-16795, the alkali (NaOH) concentration of 4.6–4.8%; medium of pH 4; enzyme dilution in a buffer solution of 1:1; temperature of 40 °C; and duration of hydrolysis of 18 hours were established as optimized parameters, resulting in an increase of reducing sugars concentration in the wheat straw hydrolysates up to 5.0–7.5 g/L, of which 0.7–1.0 g/L were glucose, 2.2–2.9 g/L xylose and 0.7–0.8 g/L cellobiose.

For fermentation of the obtained sugar mixtures to ethanol at the final step of bioconversion, the taxonomy of the selected strain UCM Y-2810 of xylose-fermenting yeast was confirmed to belong to the species *Scheffersomyces stipitis* by means of molecular genetics: its nucleotide sequences of ITS-fragment and 28S rDNA gene were submitted to the GenBank under accession numbers OP931914 and OP931915, respectively.

The enhanced ethanol production by *S. stipitis* UCM Y-2810 on the sugar mixtures was obtained under static microaerophilic conditions, where xylose concentration and cultivation duration positively correlated with ethanologenesis. At the studied range of glucose concentrations, which are typical for the enzymatic saccharification of lignocellulosic raw materials, glucose did not significantly affect either growth or ethanologenesis, particularly xylose-fermentation by yeast. This indicated that, at glucose concentrations ≤ 10 g/L, non-competitive inhibition by glucose of xylose transport into yeast cells through the low-affinity proton-symport system did not occur. Thus, we experimentally showed the fundamental possibility of a full SHF cycle of the fungal-based bioconversion of lignocellulosic agricultural waste to second-generation ethanol, where the natural potential of non-genetically modified microorganisms was explored, and conditions of, on the one hand, plant waste pre-treatment followed by enzymatic hydrolysis by cellulolytic filamentous fungi and, on the other hand, fermentation of resulting glucose/xylose-contaning syrups by xylose-fermenting yeast were refined.

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