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CELLULOSE BIOSACCHARIFICATION BY *Irpex lacteus* WOOD DECAY FUNGUS

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ABSTRACT

Enzymatic hydrolysis is an environmentally friendly technology to produce sugars from pretreated biomass. Here, we show that the new Il-11 *Irpex lacteus* strain can synthesize cellulases in a high quantity. The peptone and filter paper contained in the medium significantly enhanced activity of endo-1,4-β-D-glucanases (app. 50 IU/mL) and total cellulases (app. 9 IU/mL), whereas the medium with peptone and sodium carboxymethyl cellulose stimulated activity of exo-1,4-β-D-glucanases (33 IU/mL). The expression of cellulases reached its maximum within 96–144 hours, and the optimum pH is 3,7. Thermal treatment at 30 °C for 60 minutes activated endo-1,4-β-D-glucanases and total cellulases, while exo-1,4-β-D-glucanases activity was enhanced following 40 °C treatment. In total, the cellulases complex (300 IU/g) saccharified untreated cellulose by 38 % in 48 hours. Concentrate with filter paper activity 100 IU/g is the more balanced enzyme-substrate ratio (2 %), which allows prolonging the saccharification process that will have a positive effect on the cost of the final product.

Keywords: Cellulose, endo-1,4-β-D-glucanases, exo-1,4-β-D-glucanases, *Irpex lacteus*, saccharification, thermostability.

INTRODUCTION

An environmental impact and finite reserves of fossil energy underlie a growing demand for the search for cheaper and environmentally-friendly fuel sources. A potential industrial process for bioethanol production is conversion of lignocellulosic biomass (Sharma *et al*. 2018). Cellulose, the major part of the plant cell wall, is the most common polysaccharide and an essential, renewable fuel source alternative. Cellulose has a simple chemical composition and includes D-glucose units joined by β-1,4-glycosidic bonds. The artificial conversion of lignocellulosic biomass, i.e., cellulose, hemicelluloses, and lignin, requires multiple successive stages, including pretreatment, enzymatic hydrolysis, and fermentation (Asgher 2013). Enzymatic hydrolysis is an efficient, cost-effective, and environmentally friendly technology to produce sugars from pretreated biomass (Wyman 1999).

After an experimental plant converted cellulosic feedstock to ethanol for the first time in 2014 (Menetrez 2014), POET-DSM and Abengoa Bioenergy (Hugoton, Kansas) founded a commercial-scale plant with

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cellulosic bioethanol producing capacity of up to 25 million gallons per year. However, the current cost of enzymes is a bottleneck for commercial-scale bioethanol production due to its significant contribution to the final product cost (Hahn-Hagerdal *et al*. 2006). Commercial cellulases are produced mainly from fungi *Aspergillus niger*, *Trichoderma reesei*, *T. viride* and *T. longibrachium* (Singhania *et al*. 2010, Reczey *et al*. 1996), although the Basidiomycota fungi are also able to synthesize enzymes which catalyze the breakdown of complex biopolymers, e.g., cellulose, hemicelluloses, lignin, pectin (Elisashvili *et al*. 2009, Floudas *et al*. 2012). The fungal ability to produce cellulase complexes is tied to the strains diversity, while available substrate and growing conditions induce the expression of the enzyme (Boiko 2018, Metreveli *et al*. 2017, Xiao *et al*. 2013). The cellulose breakdown requires the presence of the mix of glycoside hydrolases, e.g., GH GH1, GH3, GH5, GH6, GH7, GH9, GH12, and GH45, and additionally oxidative enzymes (AA): AA3_1, AA8, AA9, and AA10 (Cragg *et al*. 2015). Available information on the basidiomycetes enzyme systems composition and biochemical properties offers new advancements in the expansion of the possible sources of cellulases for bioethanol technology (Bentil *et al*. 2018).

The preliminary screening of wood-decay fungi sampled in the territory of Ukraine allowed us to reveal a perspective culture of *Irpex lacteus* that actively hydrolyzed cellulosic substrates. The fungus was isolated from a *Prunus armeniaca* deadwood. In comparison to other cultures, the strain Il-11 had the highest activity of extracellular cellulases and was able to maintain its expression under variable conditions (Boiko 2020). *I. lacteus* is one of the most common fungi and is an ordinary object in biotechnological research (Fujimoto *et al*. 2004, Svobodová *et al*. 2008).

The main goal of this work was to study the expression of cellulases by a new *I. lacteus* culture to evaluate its potential for saccharification of cellulosic biomass. In addition, we tested various conditions to obtain the highest yield of cellulases from the studied culture.

MATERIAL AND METHODS

Organism and inoculum preparation

The white-rot basidiomycetes strain Il-11 *Irpex lacteus* (Fr.) Fr. is deposited in the collection of the Institute for Evolutionary Ecology (Kyiv, Ukraine). Fungal inoculate was prepared by growing the mycelia on agar nutrient medium in Petri dishes of the following composition (g/L): agar - 9, glucose - 5, starch - 5, peptone - 3. After 7 days of cultivation, the mycelium was used to inoculate the liquid nutrient medium by transferring small mycelial-agar plugs into the culture vessels.

Cultivation conditions and optimization of the nutrient medium

The fungus was cultured under stationary conditions (surface culture) in a liquid glucose-peptone (GP) medium containing (g/L): glucose — 10; peptone — 3; KH_2PO_4 — 0,6; $MgSO_4 \times H_2O$ — 0,5; $CaCl_2$ — 0,05; K_2HPO_4 — 0,4; $ZnSO_4 \times H_2O$ — 0,001. The mineral composition of the GP medium (without peptone and glucose) was used as the base medium to include various concentrations of the tested components. The composition of the medium was optimized by carbon source (glucose, sodium carboxymethyl cellulose, Avicel® PH-101, Whatman® filter paper) and nitrogen (peptone, ammonium sulfate), at different concentrations. The cultivation period lasted 4, 6, 8 and 10 days from the time of liquid medium inoculation. Cultivation was carried out in 100 mL flasks containing 25 mL of the medium at the temperature of 27 °C. The medium pH was adjusted to 5.0 before sterilization. The medium containing peptone and filter paper with an initial pH of 4 to 7 was used to determine the influence of the medium acidity on the cellulases activity. The process was scaled (500 mL of medium in 2 L flasks) to obtain cellulase preparations. The enzyme concentrate was obtained using the system Vivaspin turbo 15, 30 kDa MWCO (Sartorius stedim biotech GmbH). Protein concentration was determined by a spectrophotometric method using Ulab-131UV (Stoscheck 1990).

Determination of cellulases thermostability

To assay the cellulases thermostability, we treated the samples in the temperature range of 30 ºC – 60 °C during 1 h – 2 h and measured enzymatic activity upon incubation.

Enzyme assays

The supernatants obtained by centrifugation (5000 g for 20 min) of medium after mycelium incubation were analyzed for enzymatic activity with corresponding substrates. The total cellulase activity (TCA) was measured with Whatman filter paper, for endo-1,4-β-D-glucanase activity (endo-) used 1 % carboxymethyl cellulose, for exo-1,4-β-D-glucanase activity (exo-) used cellulose microcrystalline (Avicel PH-101) (Ghose 1987, Eveleigh *et al*. 2009). One unit of cellulase activity (IU) was defined by the formation 1μM of glucose equivalents released per minute under the assay conditions. The quantity of reducing sugar was estimated using the Somogyi-Nelson method (Somogyi 1952).

Pretreatments of filter paper

The filter paper was sliced, immersed in a 4 % NaOH solution, and incubated at 30 °C for 24 hours. Treatment with sodium hydroxide increases the accessibility of cellulose chains to reagents, solvents or enzymes (Kapoor 2015). Pretreatment may separate fiber bundles and increase their porosity and surface area, as well as lead to a reduction in cellulose crystallinity and partial degradation of cellulosic chains. After the treatment, the cellulose fibers were washed thoroughly with distilled water until neutralization and then oven-dried at 70 °C.

Zymogram analysis

Electrophoretic separation of enzymes was performed in 11,25 % polyacrylamide gel (PAGE) using Tris-glycine buffer (pH 8,3). Amount of added protein varied from 40 μg to 60 μg in each well. Detection of endo-1,4-β-D-glucanases in PAGE was performed in the presence of 0,1 % Na-carboxymethyl cellulose (Manchenko 2003). After electrophoresis, the gel was washed in 50 μM acetate buffer (pH 5,8) for 10 min and incubated at 50 °C for 60 min. Then the gel was rinsed in distilled water and placed in 0,1 % Congo Red solution for 10 min at room temperature. Finally, the gel was washed in 1 M NaCl solution for 10 min. PageRuler Plus Prestained Protein Ladder (Fermentas — Thermo Scientific) kit was used to establish the molecular weight of the proteins. The gel-documentation was performed on AlphaImager 2200 (Alpha Innotech). Electrophoretograms were assessed with the TotalLab TL 120 software.

Scanning electron (SEM) and polarized light microscopy (PLM)

SEM and PLM were carried out to observe the morphological changes of cellulose fiber ultrastructures after 24 hours of incubation with enzyme concentrate. Studied samples were thoroughly investigated under a transmitted light microscope (Ulab XY-B2T LED) with two “crossed” polarizing elements, and photographs were obtained with Canon EOS x50 camera. Micrographs were taken using a JEOL JCM-6000 (JEOL Ltd., Tokyo, Japan) scanning electron microscope under Semaphore software (JEOL, Sollentuna, Sweden).

Enzymatic saccharification of filter paper

The filter paper enzymatic hydrolysis experiments were conducted at a temperature of 40 °C with continuous shaking at 10,47 rpm for 24 h. The reaction mixture (10 mL) contained 200 mg substrate, cellulases 20 FPU/g, 100 FPU/g, and 300 FPU/g cellulose, and 100 mg tetracycline to prevent microbial contamination. After the saccharification, the reaction mixture was centrifuged at 5000 g for 15 min to remove the substrate, and then glucose content of the supernatant was determined. The amount of the released reducing sugars was calculated by using a standard curve of glucose. The percentage of saccharification was calculated using the equation (Mandels and Sternberg 1976) as follows Equation 1:

$$Saccharification = \frac{Reducing\ sugars\ x\ 0,9}{Initial\ substrate\ concentration} x\ 100 \quad (1)$$

Factor 0,9 was used to account for water uptake during hydrolysis in the conversion of a polysaccharide to a monosaccharide.

Statistical analysis

All experiments were performed with three replications each time. For statistical analysis we used environment R software (Verzani 2005). To determine the optimal nutrient medium, we normalized the activity data by dividing each value by the corresponding mean of each enzyme. The resulting matrix was sorted by hierarchical cluster analysis for environmental and time components. Distances in the dendrograms are Euclidean distances, the Vard method of construction. The mean values, as well as standard deviations, were calculated, and only values of $p \leq 0,05$ were considered statistically significant.

RESULTS AND DISCUSSION

Optimization of conditions and medium components

The selection of suitable cultivation conditions can intensify cellulases production by fungi. The cellobiose, a decomposition product of an insoluble substrate containing the β-1,4-glucosidic bond, is a possible trigger of fungi cellulases synthesis. A based activity of cellulases has been suggested to provide the primary cellulose hydrolysis and some amount of soluble products, e.g., cellobiose, sufficient to start the induced biosynthesis of enzymes.

The medium optimization matrix contained six primary component configurations (Table 1) and the corresponding cellulases activity was measured in 4, 6, 8, and 10 days of cultivation (Figure 1).

Table 1: Matrix of nutrient media with different components.

Nutrient medium #	Peptone	$(NH_4)_2SO_4$	Glucose	Na-CMC	Avicel® PH-101	Filter paper
1	+	-	-	+	-	-
2	+	-	+	+	-	-
3	+	-	-	-	+	-
4	-	+	+	+	-	-
5	-	+	+	-	+	-
6	+	-	-	-	-	+

Medium #6 significantly enhanced the activity of the endo-1,4-β-D-glucanase (app. 50 IU/mL) and total cellulase (app. 9 IU/mL). Medium #2 heightened expression of the exo-1,4-β-D-glucanase (app. 33 IU/mL). We found that the optimal cultivation time to provide highest cellulases activity is 144 hours (six days), although during the whole period of observation time (240 hours, ten days) their expression was still detected (Figure 2), which makes the studied strain of *I. lacteus* more valuable than other commercial strains (Balat *et al*. 2008, Balsan *et al*. 2012).

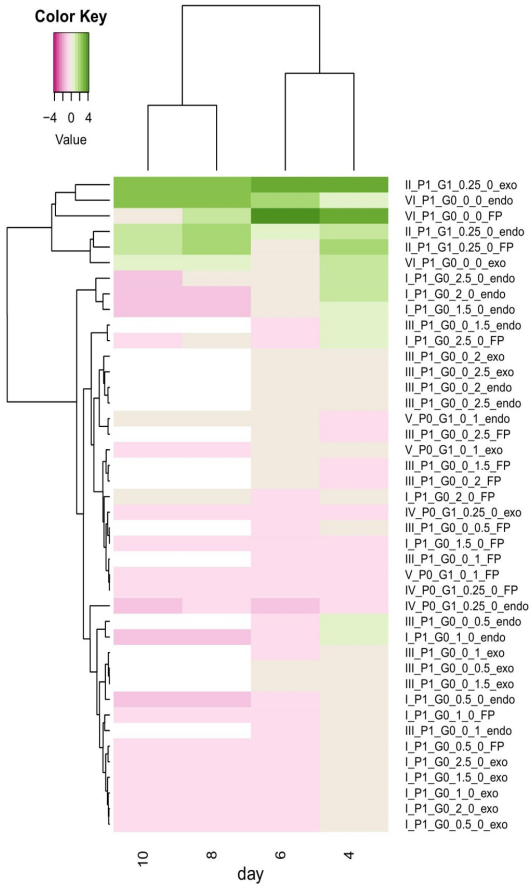

Figure 1: Heatmap of values of cellulase activity at the different compositions of nutrient medium and cultivation time. The order of rows and columns corresponds to the results of cluster hierarchical analysis - dendrograms at the top (clustering by time) and at the left (clustering by nutrient medium). Activity values are normalized.

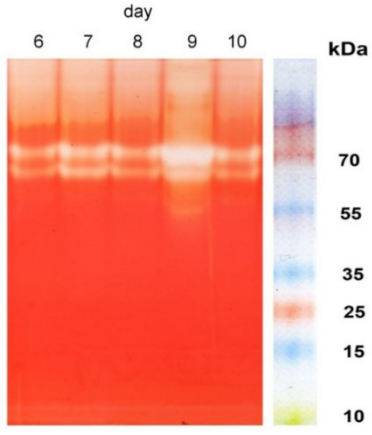

Figure 2: Endo-1,4-β-D-glucanases in cultural filtrate of Il-11 *Irpex lacteus* at 6-10 days of cultivation.

The obtained results agreed with earlier research showing that cellulose and peptone were the critical nutrients for another endoglucanases producer, *Trichoderma harzianum* (Lee *et al*. 2015). However, the enzyme's activity of the *T. harzianum* was substantially lower compared to the studied strain of *I. lacteus.*

As known, the temperature is a key factor influencing the rate of biochemical reactions (Sohail *et al*. 2009, Bansal *et al*. 2014). Typically, the use of multi-stage processes makes biotechnology less time-consuming and improves product yield. In most cases, each stage requires a specific temperature determined by the technological protocol. The gross cellulase activity of the studied strain Il-11 of *I. lacteus* was highest at 28 °C (Figure 3). The activity of endo-1,4-β-D-glucanases, exo-1,4-β-D-glucanases, and total cellulases of *I. lacteus* reached 52-60 IU/ml, 30-32 IU, and 17-19 IU/ml, respectively. These values were higher than the reported 0,35 IU/ml for endo-1,4-β-D-glucanases and 1,5 IU/ml for total cellulases in commercial strains of *Aspergillus niger* (Sohail *et al*. 2009) and *Trichoderma reesei* (Juhasz *et al*. 2004), respectively.

The cellulases expression rate significantly depends on the initial acidity of the medium (Figure 4). The gross activity of cellulases indicates that their synthesis has the highest level between the 4th and 6th days of cultivation at the initial pH value of 4,0. The pH changes towards alkaline values reduce the synthesis of the enzymes. A similar pattern was found for *Aspergillus niger* (Ascomycota), which has the highest endoglucanase and β -glucosidase expression at pH 4.0 (Sohail *et al*. 2009).

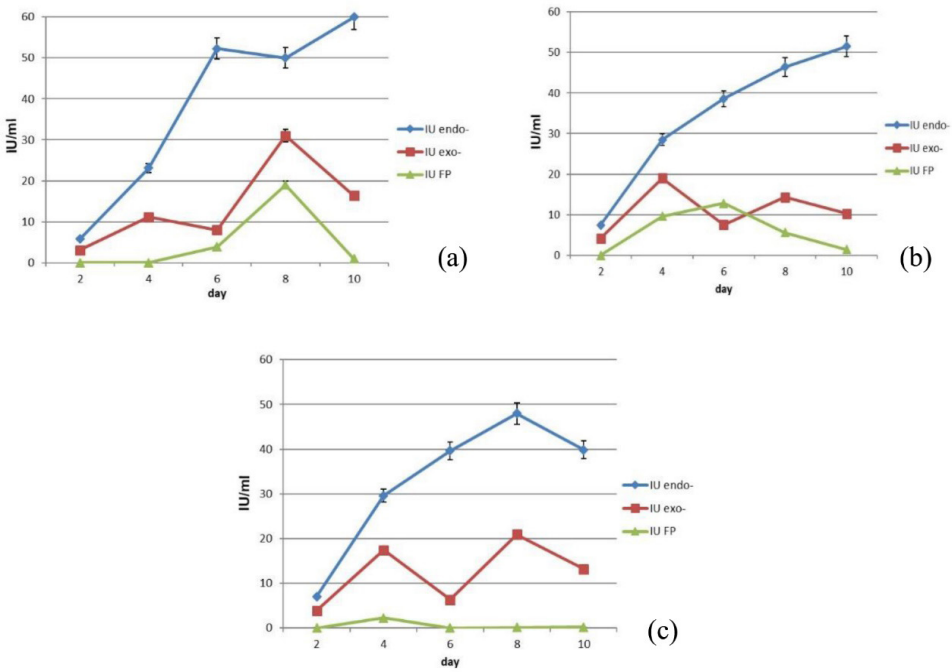

Figure 3: The effect of cultivation temperature on the cellulases activity Il-11 *Irpex lacteus.*
(a) – 28 °C; (b) – 32 °C; (c) – 36 °C.

Changes in the production of reducing sugars in the culture filtrate confirmed that the expression rate reached a peak on the 4-6th day of cultivation, and the dynamics of the filtrate acidity indicate that the optimal pH value was approx. 3.7. These results are consistent with published data on co-cultivated *I. lacteus* and *S. commune*, where enzymatic activity was highest on the 6-7 days, although the medium was less acidic (Metreveli *et al*. 2017).

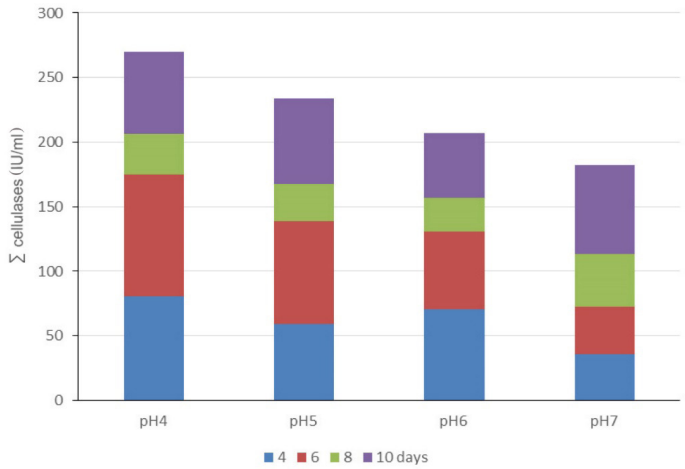

Figure 4: Cellulases activity of *Irpex lacteus* Il-11 in media with different pH.

Thermostability of cellulases

Cellulases of different types and origins vary in the temperature optimum that allows fungi to transform plant substrates more efficiently under constantly changing natural conditions. This property of enzymes makes it possible to control biopolymer degradation reactions in technological processes. To evaluate cellulases thermostability, we used concentrates obtained by ultrafiltration (30 kDa MWCO) which simplifies reaching the required value of the activity of the enzymes and pre-incubated at 30 ºC, 40 ºC, 50 ºC, or 60 °C for 60 min and 120 min. On average, the protein concentration in the culture liquid and in the concentrate reached the value of 1000 µg/ml -1100 µg/ml and 1500 µg/ml - 1600 µg/ml, respectively. Cellulases of different types varied in the pattern of their thermostability. Thermal treatment for 60 minutes activated endoglucanases and total cellulases at 30 °C and exoglucanases at 40 °C. Increasing the temperature to 50 °C inactivated all cellulases of Il-11 *I. lacteus*. A temperature of 30 °C during 120 minutes of pre-incubation did not change the endo- and exoglucanases activity, while 40 ºC – 50 °C reduced it by 7 % – 9%. A 60 °C temperature decreased the endoglucanases and exoglucanases activity by 70 % – 78 % and 23 % – 30%, respectively.

Polarized light (PLM) and Scanning electron microscopy (SEM)

Polarized and scanning electron microscopy have been used to study the degradation of cellulose fibers. Figure 5 shows the cellulose fibers before (Figure 5a) and after (Figure 5b) the treatment with enzymes concentrate. Cellulosic fibers treated for 24 hours at 40 °C became highly fragmented (Figure 5b). Sites of fibers destruction were visible in polarized light (Figure 5c).

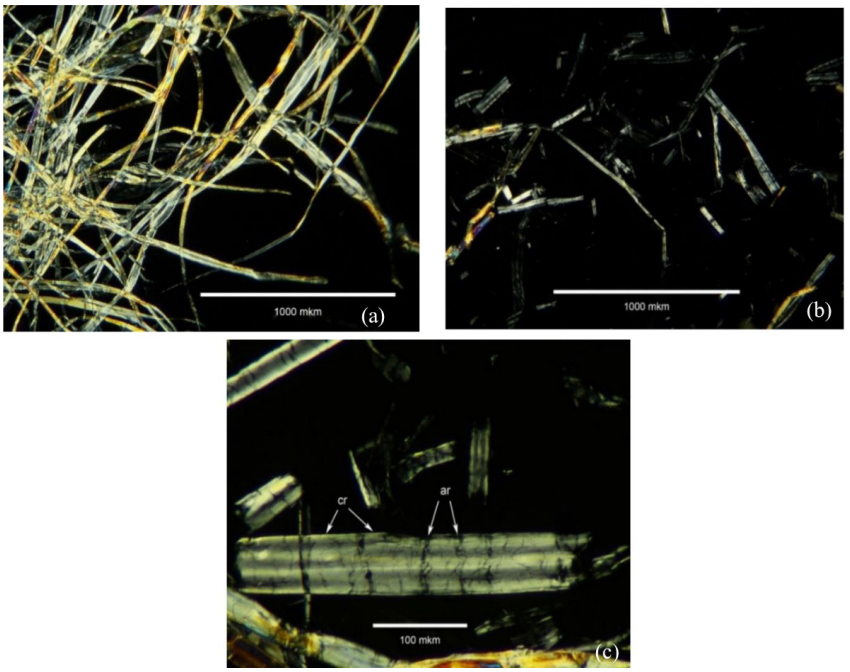

Figure 5: Polarization microscopy of the cellulose fibers before (a) and after (b, c) 24 hours in the enzyme concentrate. The arrows point to crystalline regions (cr) and amorphous regions (ar).

Ruptures occurred primarily in low-density amorphous regions (ar) of fibers due to hydrogen bonds breaking (Orłowski *et al*. 2015, Lynd *et al*. 2002), while crystalline regions (cr) with dense packaging required a longer time for fragmentation. Scanning electron microscopy revealed (Figure 6) that the destruction of cellulose fibers by enzymes produced by the Il-11 *I. lacteus* occurred not only in the amorphous region due to endo-1,4-β-D-glucanases effect, but also over the entire surface of the fiber due to other enzyme systems, i.e., exo-1,4-β-D-glucanases and β-glucosidases. After treatment with enzymes, the rough fibers became thinner, and the surface acquired a smooth structure.

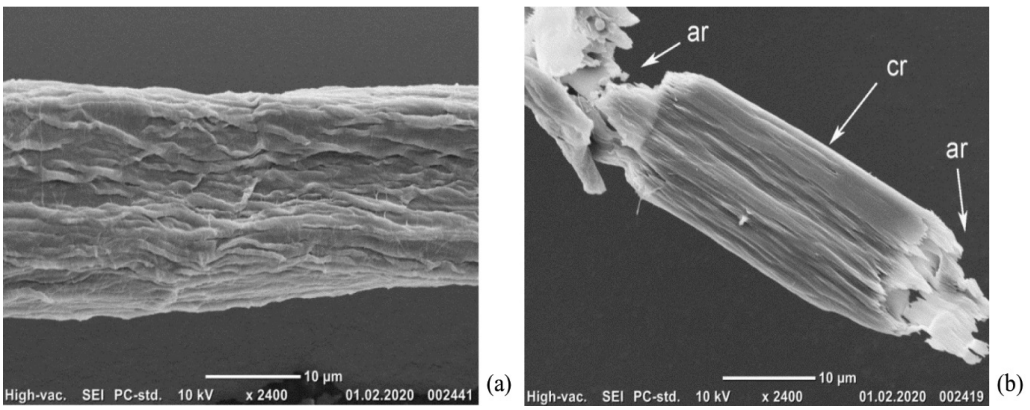

Figure 6: (a) Scanning electron micrographs of cellulose fiber (b) and its degradation after 24 hours. The arrows point to crystalline region (cr) and amorphous regions (ar).

Features of cellulase concentrate

The best means to investigate the features of the enzymes complex is to use its concentrate. Fungi express the complex of cellulases to perform the cellulose saccharification and the exclusion of any enzymatic component could inhibit or terminate this process. The final volume of concentrate obtained from culture liquid was five times less than the initial one, leading to an increase in the protein concentration by 23 % - 28 % and a decrease by 33 % - 38 % in the filtrate.

As a result, the culture liquid had 62 % - 67 % of proteins with a molecular weight of <30 kDa. Both the culture liquid and the concentrated preparation were tested for cellulase activity. Exo-1,4-β-D-glucanases and endo-1,4-β-D-glucanases activities were respectively five times and 1.7 times higher than that in the culture liquid. In both liquids, the activity of endo-1,4-β-D-glucanases gradually decreased after the first hour, whereas exo-1,4-β-D-glucanases and total cellulases activity grew slowly and then remained at a high level for 24 hours. Thus, the saccharification began under the initial activity of endo-1,4-β-D-glucanases, which transformed cellulose fibers for further hydrolysis by subsequent enzyme systems.

Saccharification of treated and untreated cellulose filter paper

To investigate the effect of cellulose pretreatment on its saccharification, we used mercerized paper in comparison with untreated filter paper as substrates, and added the enzymes concentrate up to FPA levels of 20 IU/g, 100 IU/g, and 300 IU/g. The experiments lasted 24 h and 48 h. The saccharification of both substrates increased with FPA (Figure 7), while 48 h incubation doubled the total reducing sugars yield.

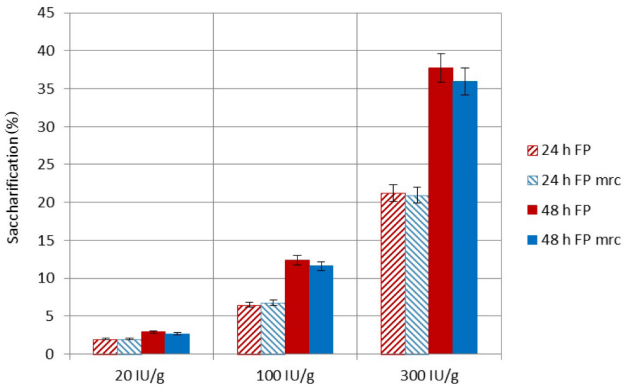

Figure 7: The effect of *Irpex lacteus* cellulase concentration on the saccharification process.

After 48 h, the product content was slightly higher in the reaction mixture with the untreated substrate than those in the pretreated one. The observed braking of the base-treated substrate hydrolysis could be associated with a Na^+ residue that may inhibit the cellulases activity (Bansal *et al*. 2014, Prajapati *et al*. 2017). Even though the total product yield reached a maximum of 38 % at an FPA load of 300 IU/g, cellulases activity was not constant during the experiment. Cellulases lost 26 % (untreated paper) and 34 % (treated paper) of their activity after 48 h at an FPA load of 20 IU/g, 5 % and 14 % at an FPA load of 100 IU/g, 12 % and 15 % at an FPA load of 300 IU/g, respectively, compared to its value after 24 h. These results indicated a more balanced ratio of the enzyme-substrate complex (100 FPA/g) and revealed perspectives to prolong the saccharification process, which reduces the cost of the final product.

Comparison of existing data on cellulase activity of known commercial strains (Alrumman 2016, Balsan *et al*. 2012, Zhang *et al*. 2019) with the new strain *I. lacteus* Il-11 points to the biotechnological value and high potential of this culture. It should also be noted that cellulases synthesis by this culture and the yield of reducing sugars can potentially be increased by further research and development.

CONCLUSIONS

A high synthetic potential of the cellulase complex of the fungus *I. lacteus* has been described. The content of peptone and filter paper in the nutrient medium significantly enhanced the activity of endo-1,4-β-D-glucanases and total cellulases, while the peptone and sodium carboxymethyl cellulose induced the activity of exo-1,4-β-D-glucanases. The cellulase activity of the new strain supersedes that of commercial cultures. The optimal cultivation time for obtaining cellulase complex was 144 hours. Destruction of cellulose fibers by *I. lacteus* Il-11 cellulases occurred not only in the amorphous region, which was caused by endo-1,4-β-D-glucanases effect but also over the entire surface of the fiber due to other enzyme systems. There is no significant difference between treated and untreated fiber at 48 hours of saccharification by enzymes concentrate. The established data indicates that the new strain of *I. lacteus* Il-11 is a potential source of highly demanded enzymatic systems in ecological technologies of bioconversion.

AUTHORSHIP CONTRIBUTIONS

S. B.: Conceptualization, data curation, investigation, validation, methodology, visualization, writing – original draft, writing – review & editing; M. N.: Formal analysis, software, visualization, writing – review & editing; V. R.: Conceptualization, funding acquisition, investigation, project administration, resources, visualization, writing – review & editing**.**

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