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The optimization of propagation medium for the increase of laccase production by the white-rot fungus *Pleurotus ostreatus*

Miroslava Hazuchová, Daniela Chmelová and Miroslav Ondrejovič✉

Department of Biotechnology, Faculty of Natural Sciences, University of SS. Cyril and Methodius in Trnava, Nám. J. Herdu 2, Trnava, SK-917 01, Slovak Republic

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Abstract

The lignocellulolytic enzymes are routinely produced by submerged fermentation using lignocellulosic material, but for more effective production, it would be suitable to precede the production phase on the lignocellulose by propagation phase in the nutrition medium suitable for growth of the fungi. Therefore, the aim of this study was to increase the laccase production by the white-rot fungus *Pleurotus ostreatus* by two-step cultivation strategy. In the first step, propagation medium was optimized for the maximal biomass growth, the second step included the laccase production by produced fungal biomass in media with the selected lignocellulosic material (pine sawdust, alfalfa steam and corn straw). From our experiments, parameters such as glucose concentration, yeast extract concentration and pH of propagation medium were selected as key factors affecting growth of *P. ostreatus*. The optimal conditions of propagation medium for maximal fungal growth determined by response surface methodology were: glucose concentration 102.68 g/L, yeast extract concentration 43.65 g/L and pH of propagation medium 7.24. These values were experimentally verified and used statistical model of biomass production prediction was appropriate adjusted. Thus prepared fungal biomass produced in the media with lignocellulose approximately 9-16 times higher concentrations of the laccase in 3 times shorter time than the fungal biomass without propagation phase in optimized propagation medium.

Introduction

Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are multi-copper oxidases that require only molecular oxygen for oxidation of wide range of substrates. Therefore, these enzymes are potentially used for different industrial applications including agro-food, chemical or pharmaceutical industries (Rodríguez-Delgado *et al.* 2015; Legerská *et al.* 2016). The main problem of laccase applications for the commercial purposes is the high costs of their production. The key factors for laccase production are a selection of the suitable producer, medium composition and cultivation conditions.

Laccases are produced by various organisms such as bacteria, filamentous fungi, insects or higher plants. The most significant producers are white-rot fungi (Bhattacharya *et al.* 2011). From a group of white-rot fungi, *Pleurotus ostreatus* appears to be a usable microbial producer. Several studies have been reported on high laccase production by this fungal strain (Bhattacharya *et al.* 2011; El-Batal *et al.* 2015; Ergun and Urek 2017). Laccases produced by *P. ostreatus* exhibit significant differences in their properties. Molecular weights

✉ *Corresponding author:* miroslav.ondrejovic@ucm.sk

of produced isoenzymes varied from 46-86 kDa and pH and temperature optima varied from 2.0 to 8.0 and from 25 to 65 °C depending on used substrate, respectively (Baldrian 2006). The composition of cultivation medium (mainly carbon and nitrogen sources) and cultivation conditions (pH, temperature, aeration, agitation etc.) are important factors for successful production of laccases. Laccase production can be increased by the addition of various aromatic compounds. Arantes *et al.* (2011) found that the addition of lignocellulosic material to the medium enhanced lignolytic enzyme production in some white-rot fungi. Several authors have proved that higher laccase production was measured in the media contained lignocellulosic materials such as wheat straw, corn, coffee husk, cedar sawdust or wheat bran than this in the media without lignocellulose (Singh *et al.* 2013; Gonzáles *et al.* 2013; Kneževic *et al.* 2013). The advantages of the selection of lignocellulosic material as carbon source for laccase production are: low cost of raw material, the availability and content of potential laccase inductors in selected material.

Moreover, suitable cultivation conditions need to set up for optimal laccase production. Except of some physical factors (pH, temperature), the important factor for the laccase production is type of cultivation. Laccases can be produced by batch, fed-batch or continuous cultivation. For higher effectivity, it can be used a multi-step approach (Michelin *et al.* 2018). In first steps, the fungal biomass grows in propagation medium under favourable conditions resulting in the greatest fungal growth, followed by the second step in which are produced laccases in production medium (Chmelová and Ondrejovič 2013). This method is more appropriate used for the production of secondary metabolites, including laccases because the conditions for their production are often different than these for biomass growth.

The aim of this study was to increase laccase production by the white-rot fungus *P. ostreatus* by the two-step cultivation strategy, while in the first step propagation medium was optimized by response surface methodology (RSM) for the maximum fungal growth and the second step was adapted for laccase production in media with selected lignocellulosic biomass.

Experimental

Microorganism

Pleurotus ostreatus DSM 1833 was purchased from Leibniz-Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Germany). This strain was maintained on malt agar (Biolife, Italy) at 4 °C. For all experiments, the suspension of fungal mycelium was prepared by plaque scraping (1 cm^2) of culture from agar plate using microbiological loop and mixing in sterile deionized water (10 mL). The inoculum was used for the inoculation of media in ratio 1 : 10 (v/v).

Lignocellulosic materials

Lignocellulosic materials used as the carbon source for production media were pine sawdust (*Pinus nigra;* soft wood), alfalfa steam (*Medicago sativa;* forage) and corn straw (*Zea mays;* agricultural waste). These materials were extracted in methanol by Soxhlet extractor during 12 hours for removal of extractive compounds, then were dried at 100 °C to constant weight and were homogenized to the particle size ≤ 1.0 mm.

For determination of the composition of each selected lignocellulosic material, 1 g of this material was mixed with 2.5 mol/L of NaOH in ratio of 1 : 20 (w/v). This mixture was incubated at the laboratory temperature for 16 hours on rotary shaker (150 RPM) and after this time, the mixture was centrifuged (10 min, 4 000 RPM). Hemicelluloses were precipitated from the supernatant by addition of ethanol in ratio 1 : 4 (w/v). The mixture was incubated for 24 hours at 4 °C, centrifuged (10 min, 4 000 RPM) and decanted. Precipitates were dried at 100 °C to the constant weight (hemicellulose content). The residue of lignocellulosic material was washed to neutral reaction by deionized water and dried. Dry precipitate (0.5 g) was mixed with 72 % (v/v) sulphuric acid in ratio of 1 : 10 (w/v) and the mixture was incubated for 2.5 hours at the laboratory temperature. Then, deionized water was added to the mixture to final volume of 100 mL and after this, it was incubated for 1 hour at 100 °C. The mixture was filtered through filtrate paper from glass fibres. In the filtrate neutralized by solid

Table 1. Levels of the factors tested in response surface methodology.

Factor	Coded levels				
	-1.682	-1	0	1	1.682
Glucose concentration (g/L)	74.75	100	137.5	175	200.25
Yeast extract concentration (g/L)	24.9	35	50	65	75.1
pH	5.3	6.0	7.0	8.0	8.7

$NaHCO_3$, reducing saccharide content was measured by DNS method. Solid residues were washed by warm deionized water and dried to the constant mass (Klason lignin content).

Propagation

The influence of different carbon sources (glucose, xylose, lactose, fructose, saccharose, cellulose, lignin or xylan) in the concentration of 10 g/L with ammonium sulphate as nitrogen source (2 g/L) in phosphate buffer (pH 7.0) and the influence of different nitrogen sources (ammonium sulphate, yeast extract, peptone, tryptone, albumin, casein or potassium nitrate) in the concentration of 2 g/L with glucose as carbon source (10 g/L) in phosphate buffer (pH 7.0) on fungal growth were tested. Cultures were incubated at 30 °C on a rotary shaker (150 RPM) for 14 days. The same cultivation conditions were used for the determination of effect of suitable concentration of carbon and nitrogen sources on growth of the fungal biomass. Glucose and yeast extract concentrations were tested in concentration range from 2 – 200 g/L and 1 – 100 g/L, respectively.

Effects of initial pH and temperature of the cultivation on fungal growth were evaluated in the medium contained glucose and yeast extract as carbon and nitrogen sources in concentration 10 g/L and 2 g/L, respectively, for 14 days. The pH values (4.0 – 9.0) were modified by 1 mol/L HCl or 1 mol/L NaOH. Tested temperatures varied in range of 15 – 37 °C.

RSM was used for the optimization of three selected factors: glucose concentration, yeast extract concentration and pH values for enhancing of biomass growth of *P. ostreatus*. The three independent variables were investigated at five different levels (-1.682, -1, 0, 1, 1.682) (Table 1).

Dry biomass [g/L] of *P. ostreatus* was fitted using a second-order polynomial equation (Equation 1) and a multiple regression of the data was carried out for obtained an empiric model related to the factors.

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i<j}^{k-1} \sum_{j=2} b_{ij} X_i X_j \quad \text{(Eq. 1)}$$

where X_i are independent variables causing the Y response and b_i are regression coefficients describing dependences between measured properties and coded values of observed factors.

Production

Biomass of *P. ostreatus* produced in the optimized propagation medium after 5× re-washed by sterile deionized water (two step cultivation strategy) as well as biomass from slant agar used for the storage of fungi (one step cultivation) were used for laccase production in the medium with lignocellulosic biomass (pine sawdust, alfalfa steam or corn straw). The composition of production medium is shown in Table 2. The laccase production by the white-rot fungus *P. ostreatus* was evaluated in liquid production medium containing lignocellulosic material for 14 days at 30 °C.

Table 2. The composition of production medium (Sánchez and Viniegra-Gonzáles 1996).

Component	Concentration (g/L)
Lignocellulosic material	10
Yeast extract	2
K_2HPO_4	0.5
$MgSO_4.7\ H_2O$	0.5
$FeSO_4$	0.02
NaCl	0.01
$ZnSO_4$	0.02
$MnSO_4$	0.02
$CuSO_4$	0.025

Determination of glucose concentration by DNS method

The sample (0.1 mL) was pipetted to 0.8 mL DNS reagent (3,5-dinitrosalicylic acid) (Miller 1959).

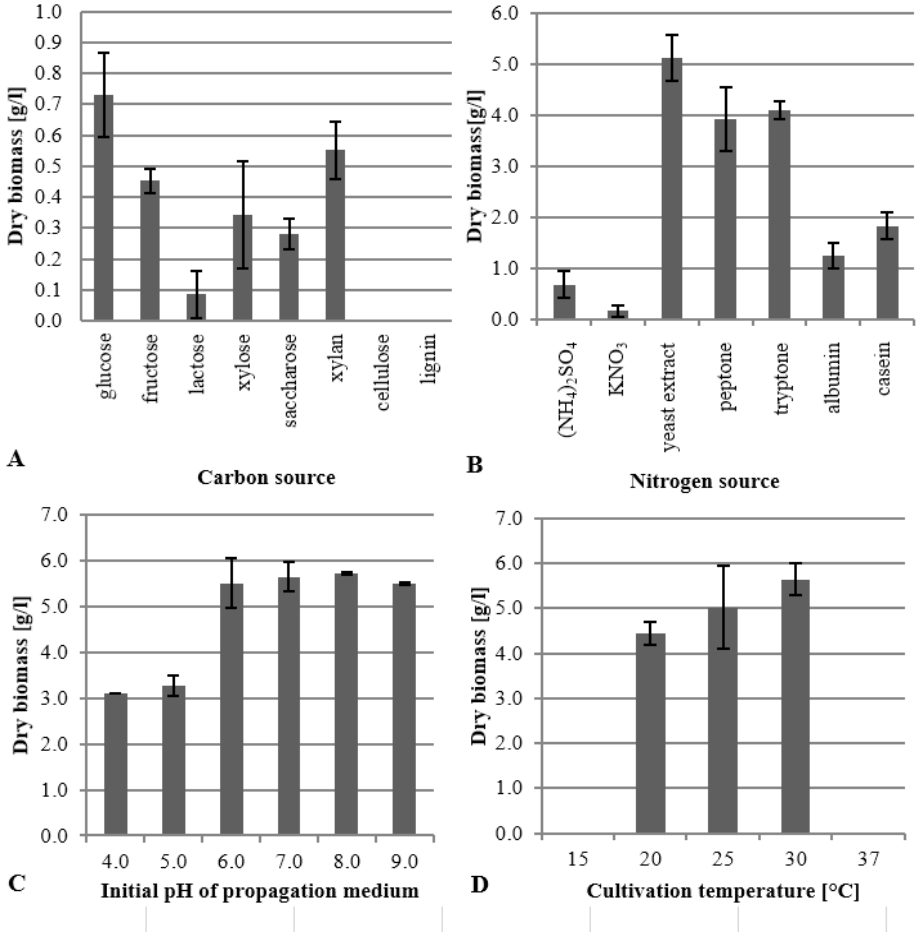

Fig. 1. The effect of carbon source (A), nitrogen source (B), initial pH of the propagation medium (C) and cultivation temperature (D) on the production of dry biomass of the white-rot fungus *Pleurotus ostreatus*.

After thorough mixing, the mixture was boiled in water bath for 5 min and cooled to the laboratory temperature. After 10 min 8.0 mL of deionized water was added to the reaction mixture and mixed. Absorbance was measured at 540 nm (Microplate Reader, Biotek EL 800) and glucose concentration was evaluated from calibration curve of glucose.

Determination of enzymatic activities

Laccase activity was determined by oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Shin *et al.* 1987). The assay mixture contained 150 µL of 50 mmol/L phosphate buffer (pH 5.0) with 1 mmol/L ABTS and 50 µL of enzyme extracts. The oxidation of ABTS was monitored by measuring of absorbance at 450 nm. Activity of laccase was expressed in unit [U] as the amount of enzymes able to oxidation of 1µmol of ABTS per minute. Cellulase activity from the production medium (0.5 mL) was determined with α-cellulose (50 mg) as substrate in 0.1 mol/L McIlvain buffer (pH 4.8) with 0.1 % (w/v) sodium azide (1 mL). The mixture was cultivated for 24 hours at 30 °C and 150 RPM. After this time, the mixture was centrifuged (4 000 RPM, 10 min) and cellulase activity was determined at 540 nm as the amount of reducing saccharides releasing during reaction with α-cellulose.

Native-PAGE

The laccase isolation procedure was performed according to the modified method by Chefetz *et al.* (1998). The supernatant from the production medium was concentrated (Vivaspin1® 30 kDa) and applied to native-PAGE. Native-PAGE was performed with 5 % (w/v) stacking gel and 12 % (w/v) separation gel using a vertical gel electrophoretic system (5 W, 180 V, 20 mA, 30 min) (Bio-Rad) (Laemmli 1970). Gel from

native-PAGE was stained with phosphate buffer (0.1 mol/L; pH 5.0) with 1 mmol/L of ABTS (for laccase detection); 1 mmol/L ABTS and 10 µmol/L H_2O_2 (for laccase and lignin peroxidase detection) or 1 mmol/L ABTS, 10 µmol/L H_2O_2 and 1 mmol/L Mn^{2+} ions (for laccase, lignin peroxidase and manganese peroxidase detection).

Statistical analysis

All experiments were carried out in triplicate. Data are presented at the mean with the standard deviation. Statgraphics Plus 5.1 (Statpoint Technologies, Inc. USA) was used to evaluate regression equations in the optimization by RSM.

Results and Discussion

The most common method for the production of laccase and other lignocellulolytic enzymes is based on the cultivation of appropriate microbial producer, such as white-rot fungus, on the lignocellulosic material such as wood, straw or hay (Gonzáles *et al.* 2013; Kneževic *et al.* 2013; Chmelová and Ondrejovič 2016). The results of this method are not suitable for the commercial production of target enzymes. The fermentation production of lignocellulolytic enzymes can be improved by the simple propagation of microbial producer within two-step cultivation process. During the first step of cultivation, conditions favouring the microbial growth are generated, while during the second step conditions beneficial for laccase production are established.

Propagation

Selection of optimization range

The propagation medium must comply for the growth of biomass and therefore, it is necessary its optimization. In order to optimize fungal growth, we first assessed the effect of different carbon and nitrogen sources on *P. ostreatus* growth. Similarly, cultivation conditions, such as pH and temperature of the cultivation, are able to affect the biomass growth. The results are showed in Fig. 1.

From the results (Fig. 1), the most suitable carbon source was glucose (0.73 ± 0.14 g/L) and yeast extract as nitrogen source (5.12 ± 0.45 g/L) for the biomass growth. Lihua *et al.* (2009) observed that glucose was the best substrate for the fungal biomass growth. *P. ostreatus* was able to utilize pentoses (fructose, xylose) and hexose (glucose) (Fig. 1A). The polysaccharide xylan was also suitable substrate for biomass production (0.55 ± 0.09 g/L). The lowest production of biomass was noted in the media with lactose (0.08 ± 0.08 g/L). From organic nitrogen sources, namely yeast extract, peptone, tryptone, albumin and casein, the most suitable substrate was yeast extract (Fig. 1B) for biomass growth. Inorganic nitrogen sources were not appropriate for the effective biomass growth. Organic sources can provide proteins and amino acids resulting to more abundant growth of the fungus (Das *et al.* 2016). El-Batal *et al.* (2015) similarly found that organic nitrogen sources were better alternative for the biomass growth than inorganic nitrogen sources. For the selection of cultivation conditions, the biomass production in the propagation medium was comparable within pH range 6.0-9.0 (Fig. 1C). Bettin *et al.* (2011) observed that suitable pH value for biomass growth was 7.4-7.5. Kalmis *et al.* (2008) suggested that optimum pH for biomass growth is at between 6.5 and 7.0. Temperatures of 15 °C and 37 °C were not suitable for the biomass production. The most suitable temperature for cultivation of *P. ostreatus* appears 30 °C (Fig. 1D). Similar to our results, Bellettini *et al.* (2016) described the optimal temperatures for *Pleurotus spp.* growth in the range of 25-30 °C.

From the previous results, it is evident that the most suitable carbon and nitrogen sources for the growth of *P. ostreatus* glucose and yeast extract, respectively. In the next step, it was necessary to find the effects of glucose and yeast extract concentrations of fungal biomass growth. Results are showed in Fig. 2.

It was observed that 100 g/L of glucose concentration and 50 g/L of yeast extract concentration stimulated fungal growth. Gem *et al.* (2008) found that the increase of yeast extract concentration had the positive effect on the biomass production. Wang *et al.* (2005) found the best conditions for the biomass production were: glucose concentration of 40 g/L and corn steep liquor concentration of 20 g/L. It seems that high concentrations of glucose (200 g/L) and yeast

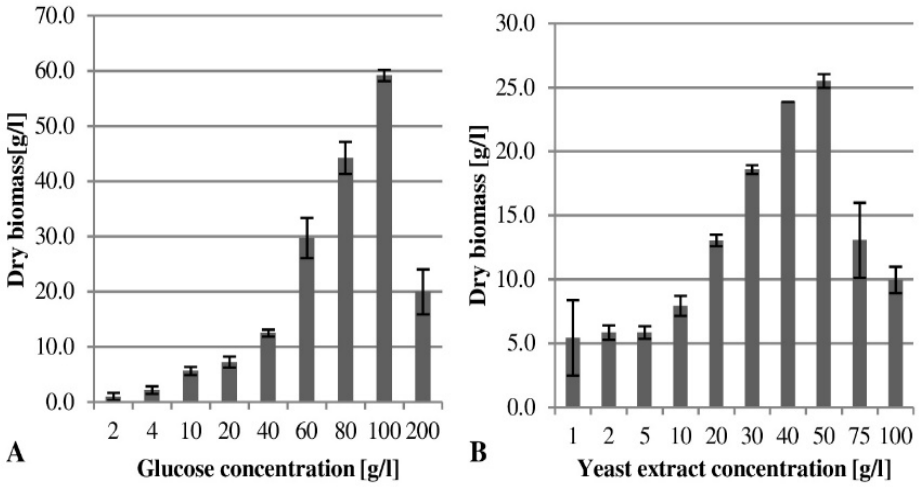

Fig. 2. The effect of glucose (A) and yeast extract concentration (B) on production of dry biomass of the white-rot fungus *Pleurotus ostreatus* at 30 °C and pH 5.0 for 14 days.

extract (75 – 100 g/L) had the inhibition effect on the biomass growth (Fig. 2).

Optimization of propagation medium

In this work, 17 experiments were carried out according to the RSM (Table 3). Based on the results from Fig. 1 and Fig. 2, independent variables were glucose concentration, yeast extract concentration and initial pH of propagation medium. Dependent variable was dry biomass. The RSM was used for the evaluation of relationships between variables (Myer and Montgomery 2001). Table 3 shows the design and the results of experiments carried out by RSM. The second-order polynomial model (Equation 1) was used to evaluate the results of optimization. The high value of the coefficient of determination (R^2 = 0.899) indicates that only 10.1 % of total variation was not explained by the model. The fitted response for the above regression model is shown in Fig. 3.

The fungal biomass growth increased with higher glucose and yeast extract concentration. Thereafter, the fungal growth decreased as the pH decreased (pH < 7.2).

Table 3. The experimental matrix for the optimization of dry biomass production (g/L) with coded levels of independent variables.

Exp.	Glucose concentration (g/L)	Yeast extract concentration (g/L)	pH	Dry biomass (g/L)
1	100 (-1)	35 (-1)	6.0 (-1)	73.5
2	100 (-1)	65 (1)	8.0 (1)	82.9
3	137.5 (0)	50 (0)	7.0 (0)	113
4	175 (1)	65 (1)	6.0 (-1)	44.4
5	175 (1)	35 (-1)	8.0 (1)	46.0
6	100 (-1)	65 (1)	6.0 (-1)	20.1
7	175 (1)	65 (1)	8.0 (1)	33.9
8	137.5 (0)	50 (0)	7.0 (0)	85.6
9	100 (-1)	35 (-1)	8.0 (1)	89.5
10	175 (1)	35 (-1)	6.0 (-1)	62.9
11	137.5 (0)	50 (0)	7.0 (0)	118.5
12	137.5 (0)	24.9 (-1.682)	7.0 (0)	82.4
13	200.25 (1.682)	50 (0)	7.0 (0)	116.3
14	74.7505 (-1.682)	50 (0)	7.0 (0)	80.1
15	137.5 (0)	75.1 (1.682)	7.0 (0)	49.1
16	137.5 (0)	50 (0)	5.3 (-1.682)	18.6
17	137.5 (0)	50 (0)	8.7 (1.682)	27.6

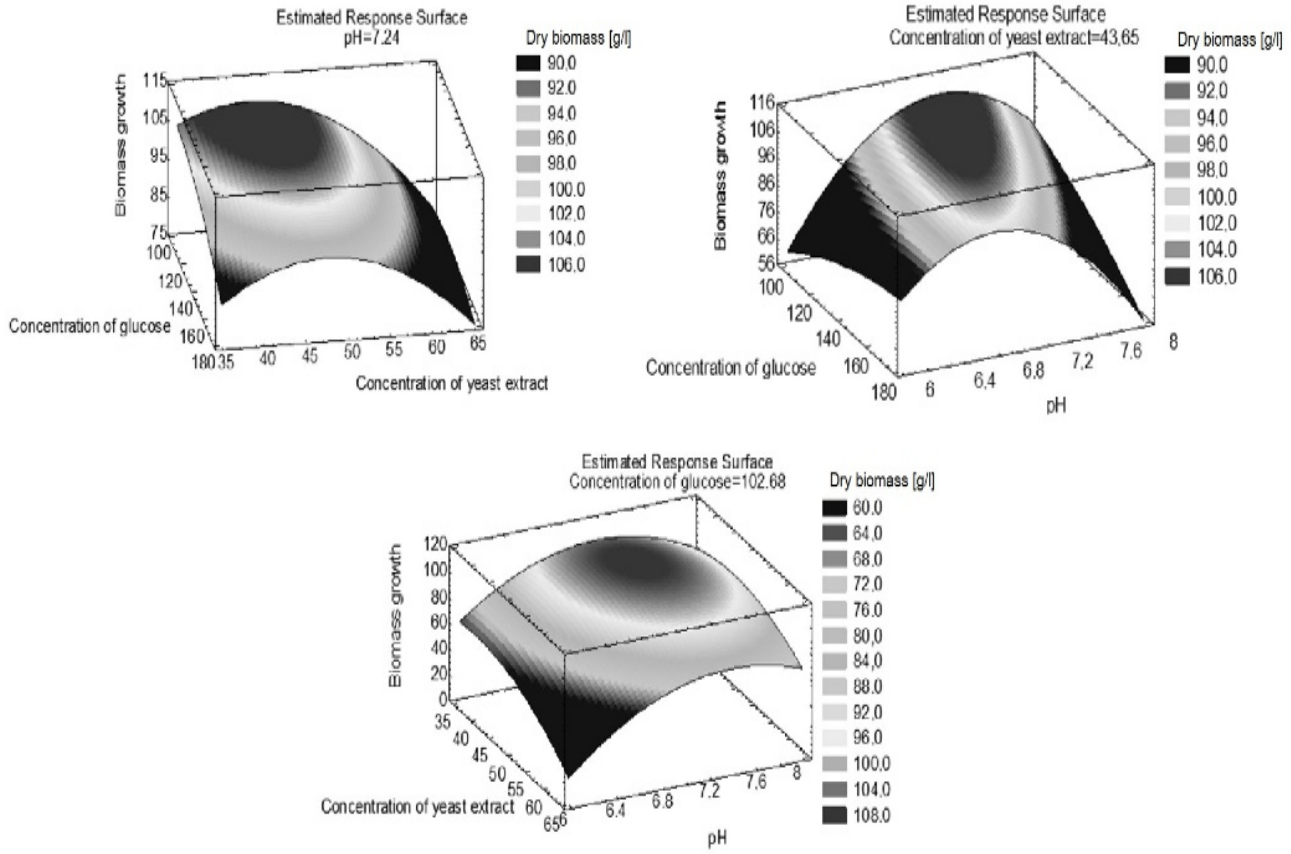

Fig. 3. 3D surface graphs showing the effect of yeast extract concentration, glucose concentration and initial pH value on the growth of *P. ostreatus* expressed as dry biomass (g/L) for 14 days at 30 °C.

The optimal values of selected variables were chosen to the evaluation of variable interactions and to determine the optimal level of each variable for the maximal response. According to the studies of RSM, the maximum biomass growth produced by *P. ostreatus* can be reached at optimal conditions, namely glucose concentration 102.68 g/L, yeast extract concentration 43.65 g/L and pH 7.24 for predicted dry biomass recovery of 109 g/L. These conditions were experimentally verified and predicted value of dry biomass (109 g/L) coincided with experimentally measured values (99.5 g/L).

Production of lignocellulolytic enzymes

Chemical composition of lignocelluloses

Lignocellulose belongs to natural substrates for growth of white-rot fungi in the environment. It proved to be a suitable substrate for the fungal growth and attractive feedstock for the laccase production. The composition of lignocellulosic material directly affect the efficiency of laccase production (Levin *et al.* 2008; Elisashivili *et al.* 2009) and therefore, selected lignocellulosic materials were analysed for cellulose,

Table 4. The content of cellulose, hemicelluloses and lignin in selected lignocellulosic materials.

Lignocellulosic material	Cellulose content (%)	Hemicellulose content (%)	Lignin content (%)
Pine sawdust (softwood)	30.1 ± 4.6	22.4 ± 3.8	23.7 ± 1.4
Alfalfa steam (forage)	27.0 ± 2.0	48.4 ± 1.6	16.7 ± 0.7
Corn straw (agricultural waste)	41.7 ± 2.0	52.3 ± 0.3	5.0 ± 0.0

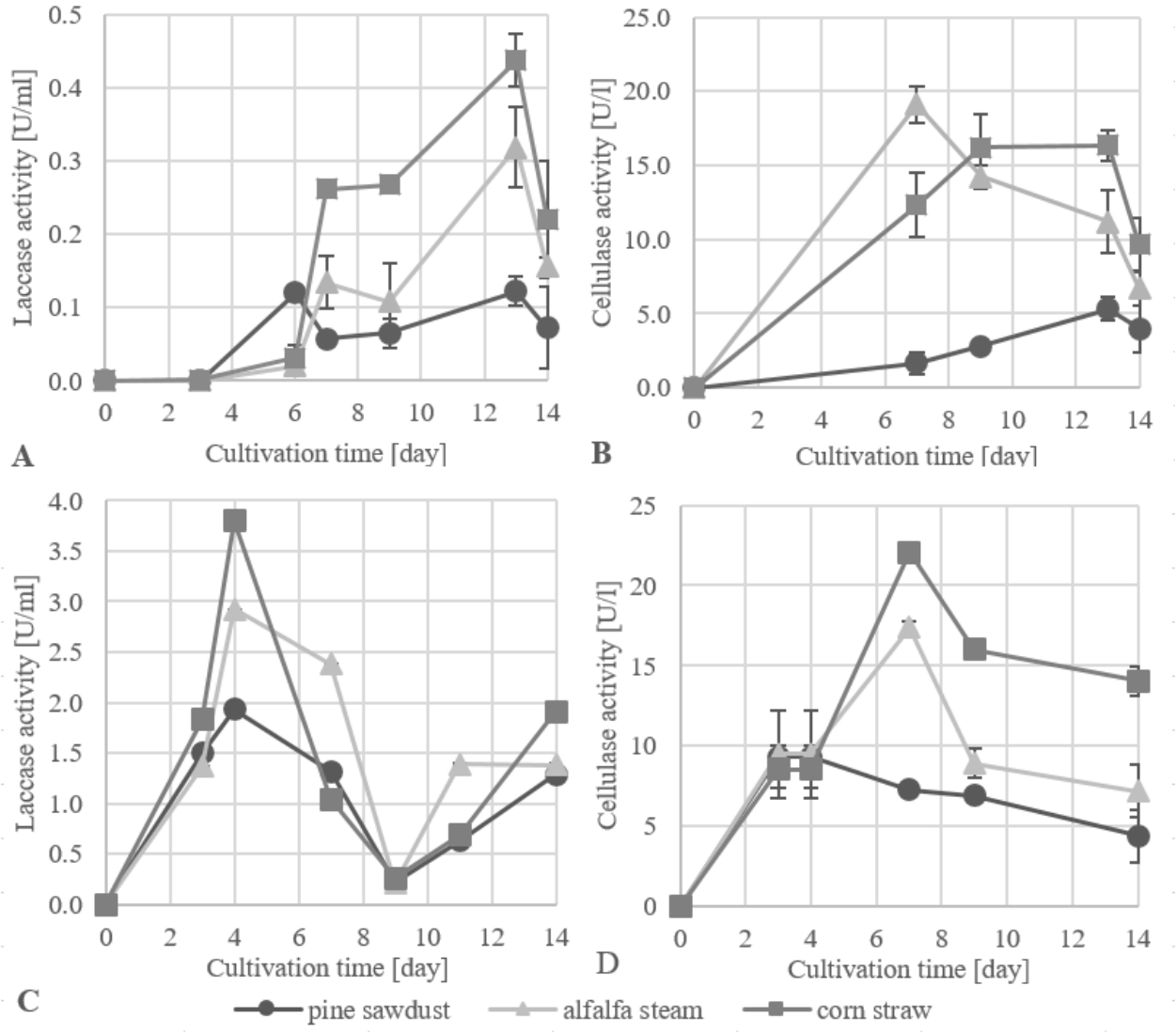

Fig. 4. The production of laccase (A, C) and cellulases (B, D) by the white-rot fungus *Pleurotus ostreatus* in the production medium with selected lignocellulosic material at 30 °C, pH 7.0 during 14 days (A, B – the production of lignocellulolytic enzymes by the one-step cultivation; C, D – the production of lignocellulolytic enzymes by the two-step cultivation strategy).

hemicelluloses and lignin content (Table 4).
The highest content of cellulose and hemicelluloses were measured at corn straw (41.7 and 52.3 %, respectively). The lowest amount of lignin was observed in corn straw (5.0 %). Pointer *et al.* (2014) determined the comparable amount of selected components of lignocellulose in straw corn, specifically 38.8 % of cellulose, 44.4 % of hemicelluloses and 11.9 % of lignin. Alfalfa steam is composed from 27.0 % of cellulose, 48.4 % of hemicelluloses and 16.7 % of lignin. Bidlack and Buxton (1992) determined similar content of lignin in alfalfa steam (17.4 %). From selected material, the highest content of lignin was determined in pine sawdust (23.7 %). In general, similar to our results, Sjöström (1993) determined that cellulose, hemicelluloses and lignin contents varied among selected softwood among 33-42 %, 22-40 % and 27-32 %, respectively.

Production of laccase

The effect of propagation step on increasing of the laccase production by the white-rot fungus *P. ostreatus* was tested in the media contained selected lignocellulosic material, namely pine sawdust, alfalfa steam or corn straw (two-step cultivation strategy). For the comparison, media with lignocellulosic biomass was directly

inoculated from slant agar with *P. ostreatus* culture (one-step cultivation) (Fig. 4).

The results from laccase production in the media with lignocellulose without pre-cultivation of biomass in the propagation medium are shown in Fig. 4A. The highest laccase activity was measured in the production medium with corn straw (0.44 U/mL) and the lowest was observed in the medium with pine sawdust (0.12 U/mL). The laccase production was noted at 6th day of cultivation in the media with all tested lignocellulosic materials. The greatest cellulase activity (Fig. 4B) was measured at 7th day of cultivation in the medium with alfalfa stems (19.1 U/L). Higher cellulase activities were observed in the production medium with corn straw (16.3 U/L) and the lowest cellulase activity was measured in the media with pine sawdust (5.3 U/L). The results in Fig. 4 also demonstrate that the composition of lignocellulosic material leads to changes of the enzyme production. The laccase production seems to be stimulated by lower lignin content in lignocellulosic material (Fig. 4A). Specifically, the lowest lignin content was determined in corn straw, following by alfalfa steam and pine sawdust (Table 4). Similarly, cellulase production was higher in the cultivation medium containing lignocellulosic material with lower lignin content (Fig. 4B) than that in the media with higher lignin content. These findings were observed in works of other authors (Sun *et al.* 2004; Elisashvili and Kachlishvili 2009).

In media with produced fungal biomass from the optimized propagation medium, the laccase activity was the highest at 4th day of cultivation (Fig. 4C). The maximal laccase activity was observed in the medium with corn straw (3.80 U/mL). The laccase activity in the production medium with alfalfa steam and pine sawdust was approximately two times lower (1.93 and 1.76 U/mL, respectively) than those in the media with corn straw. Widiatuti *et al.* (2008) reached the maximal laccase activity in first week of the cultivation. In the comparison with the one-step of cultivation (Fig. 4A), laccase activities were greater 9-16 times by the two-step cultivation strategy. Moreover, the slightly increase of laccase production was also noted in 14th day of the cultivation. The use of different substrates for the cultivation of white-rot fungi can affect the secretion of different lignolytic enzymes. It is obvious, that the most suitable substrate for laccase production is corn straw. This material shows the lowest content of lignin (Table 4) although, lignin is considered as laccase inductor. Cellulase activities (Fig. 4D) detected in all tested media under the two-step cultivation strategy were similar to the one-step of cultivation (Fig. 4B). Cellulases are probably constitutively produced during the cultivation.

P. ostreatus is a white-rot fungus which can produce lignolytic enzymes, namely manganese peroxidases (MnP) and laccases (Das *et al.* 2016) which can oxidize ABTS used as the substrate. Therefore, the presence of these enzymes in the media was determined by native-PAGE. Results are shown in Fig. 5.

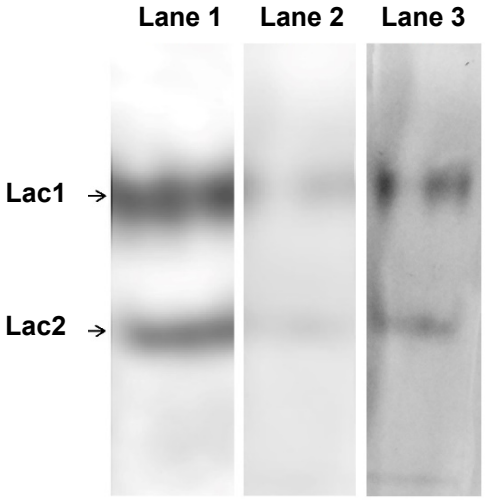

Fig. 5. The presence of lignolytic enzymes in the production medium. Lane 1 – ABTS (presence of laccases), Lane 2 – ABTS and H_2O_2 (presence of laccases and lignin peroxidases) and Lane 3 – ABTS, H_2O_2 and Mn^{2+} (presence of laccases, lignin peroxidases and manganese peroxidases).

From native-PAGE (Fig. 5), only two laccase isoforms were detected (Fig. 5, Lanes 1-3). Lane 1 in Fig. 5 shows two bands of laccases stained by ABTS solution. Lane 2 of PAGE gel stained with ABTS and hydrogen peroxide for lignin peroxidase (LiP) presence shows the same bands corensponding only the presence of laccase isoforms. Similarly, Lane 3 stained with the solution of ABTS, hydrogen peroxide and Mn^{2+} ions also shows two laccase isoforms without the presence of other bands. Therefore, it can be written that *P. ostreatus* cultivated at the described conditions produces only two isoforms of laccase.

Similar to our results, Muñoz *et al*. (1997) did not detect LiP and MnP activities during the cultivation of *P. eryngii*.

Conclusions

The special attention has been devoted the laccase production from cheap sources, such as agricultural wastes or agro-industrial residues. The suitable technique of laccase production by the white-rot fungus *P. ostreatus* seems to be the two-step cultivation strategy. In the first step, it is advisable to cultivate of fungal producer at the optimal propagation conditions. The RSM prediction shows that the optimal propagation conditions were: glucose concentration 102.68 g/L, yeast extract concentration 43.65 g/L and pH 7.24. In the second step, *P. ostreatus* produced laccases with 9-16 times higher activities than laccases produced by the one-step cultivation directly on the lignocellulose material without the previous propagation step. Moreover, the cultivation time of laccase production by the two-step cultivation strategy was three times shorter than this by one-step cultivation of *P. ostreatus*. *P. ostreatus* under the selected cultivation conditions produces two isoforms of laccases without the presence of other lignolytic enzymes.

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References

Arantes V, Silva EM, Milagres AMF (2011) Optimal recovery process conditions for manganese-peroxidase obtained by solid-state fermentation of eucalyptus residue using *Lentinula edodes*. Biomass Bioenergy 35: 4040-4044.

Baldrian P (2006) Fungal laccases-occurrence and properties. FEMS Microbiol. 30: 215-242.

Bellettini MB, Fiorda FA, Maieves HA, Teixeira GL, Ávila S, Hornung PS, Júnior AM, Ribani RH (2016) Factors affecting mushroom *Pleurotus* spp. Saudi J. Biol. Sci., in press.

Bettin F, Osório da Rosa L, Montanari Q, Calloni R, Gaio TA, Malvessi E, Moura da Silveira M, Dillon AJP (2011) Growth kinetics, production, and characterization of extracellular laccases from *Pleurotus sajor-caju* PS-2001. Process Biochem. 46: 758-764.

Bhattacharya SS, Garlapati VK, Banerjee R (2011) Optimization of laccase production using response surface methodology coupled with differential evolution. New Biotechnol. 28: 31-39.

Bidlack JE, Buxton DR (1992) Content and deposition rates of cellulose, hemicelluloses and lignin during regrowth of forage grasses and legumes. Can. J. Plant Sci. 72: 809-818.

Chefetz B, Kerem Z, Chen Y, Hadar Y (1998) Isolation and partial characterization of laccase from a thermophilic composted municipal solid waste. Soil Biol. Biochem. 30: 1091-1098.

Chmelová D, Ondrejovič M (2016) Purification and characterization of extracellular laccase produced by *Ceriporiopsis subvermispora* and decolorization of triphenylmethane dyes. J. Basic Microbiol. 56: 1173-1182.

Chmelová D, Ondrejovič M (2013) Repeated-batch production of laccase by *Ceriporiopsis subvermispora*. Nova Biotechnol. Chim. 12: 120-128.

Das A, Bhattacharya S, Panchanan G, Navya BS, Nambiar P (2016) Production, characterization and Congo red dye decolourizing efficiency of a laccase from *Pleurotus ostreatus* MTCC 142 cultivated on co-substrates od paddy straw and corn husk. J. Genet. Eng. Biotechnol. 14: 281-288.

El-Batal AI, Elkenawy NM, Yassin AS, Amin MA (2015) Laccase production by *Pleurotus ostreatus* and its application in synthesis of gold nanoparticles. Biotechnol. Rep. 5: 31-39.

Elisashvili V, Kachlishvili E, Tsiklauri N, Metreveli E, Khardziani T, Agathos SN (2009) Lignocellulose-degrading enzyme production by white-rot Basidiomycetes isolated from the forests of Georgia. World J. Microbiol. Biotechnol. 25: 331-339.

Elisashvili V, Kachlishvili E (2009) Physiological regulation of laccase and manganese peroxidase production by white-rot *Basidiomycetes*. J. Biotechnol. 144: 37-42.

Ergun SO, Urek RO (2017) Production of ligninolytic enzymes by solid state fermentation using *Pleurotus ostreatus*. Ann. Agrar. Sci. 15: 273-277.

Gern RMM, Wisbeck E, Rampinelli JR, Ninow JL, Furlan SA (2008) Alternative medium for production of *Pleurotus ostreatus* biomass and potential antitumor polysaccharides. Bioresour. Technol. 99: 76-82.

Gonzalez JC, Medina SC, Rodriguez A, Osma JF, Alméciga-Díaz CJ (2013) Production of *Trametes pubescens* laccase under submerged and semi-solid culture conditions on agro-industrial wastes. PLOS One 8: 1-9.

Kalmis E, Azbar N, Yildiz H, Kalyoncu F (2008) Feasibility of using olive mill effluent (OME) as a wetting agent during the cultivation of oyster mushroom, *Pleurotus ostreatus*, on wheat straw. Bioresour. Technol. 99: 164-169.

Kneževic A, Milovanovic I, Stajic M, Vukojevic J (2013) Potential of *Trametes species* to degrade lignin. Int. Biodeterior. Biodegrad. 85: 52-56.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 15: 680-685.

Legerská B, Chmelová D, Ondrejovič M (2016) Degradation of synthetic dyes by laccases – a mini-review. Nova Biotechnol. Chim. 15: 90-106.

Levin L, Herrmann C, Papinutti VL (2008) Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. Biochem. Eng. J. 39: 207-214.

Lihua L, Lin Z, Zheng T, Lin L, Zheng C, Lin Z, Wang S, Wang Z (2009) Fermentation optimization and characterization of the laccase from *Pleurotus ostreatus* strain 10969. Enzyme Microb. Technol. 44: 426-433.

Michelin M, Ruiz HA, Polizeli MLTM, Teixeira JA (2018) Multi-step approach to add value to corncob: production of biomass-degrading enzymes, lignin and fermentable sugars. Bioresour. Technol. 247: 582-590.

Miller GD (1959) Use of dinitrosalicyl acid regent for determination of reducing sugar. Anal. Chem. 31: 426-428.

Myer RH, Montgomery DC (2001) Response surface methodology: process and product optimization using designed experiments: 2nd edition, Wiley-Interscience, New York, 856 pp.

Pointer M, Kuttner P, Obrlik T, Kahr H (2017) Composition of corncobs as a substrate for fermentation of biofuels. Agron. Res. 12: 391-396.

Rodríguez-Delgado MM, Alemán-Nava GS, Rodríguez-Delgado JM, Dieck-Assad G, Martínez-Chapa SO, Barceló D, Parra R (2015) Laccase-based biosensors for detection of phenolic compounds. Trends Analyt. Chem. 74: 21-45.

Sánchéz C, Viniegra-Gonzáles G (1996) Detection of highly productive strains of *Pleurotus ostreatus* by their tolerance to 2-deoxy-D-glucose in starch-based media. Mycol. Res. 100: 455-461.

Shin T, Murao, S., Matsumura, E (1987) A chromogenic oxidative coupling reaction of laccase: application for laccase and angiotensin I converting enzyme assay. Anal. Biochem. 166: 380-388.

Singh MP, Vishwakarma SK, Srivastava AK (2013) Bioremediation of direct blue 14 and extracellular ligninolytic enzyme production by white rot fungi: *Pleurotus* spp. BioMed Res. Int. 1-4.

Sjöström E (1993) Wood chemistry, second edition: Fundamentals and Applications. Academic Press, San Diego, 293 pp.

Sun X, Zhang R, Zhang Y (2004) Production of lignocellulolytic enzymes by *Trametes gallica* and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels. J. Basic Microbiol. 44: 220-231.

Wang JC, Hu SH, Liang ZC, Yeh CJ (2005) Optimization for the production of water-soluble polysaccharide from *Pleurotus citrinopileatus* in submerged culture and its antitumor effect. Appl. Microbiol. Biotechnol. 67: 759-766.

Widiastuti H, Suharyanto S, Agustina W, Sutamihardja S (2008) Activity of ligninolytic enzymes during growth and fruiting body development of white rot fungi *Omphalina sp*. and *Pleurotus ostreatus*. HAYATI J. Biosci. 15: 140-144.