Journal of the Serbian Chemical Society
JSCS@tmf.bg.ac.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *87* (4) 437–450 (2021)
JSCS–5532
Original scientific paper
Published 12 November 2022

Examination and optimization of lignocellulolytic activity of *Stereum gausapatum* F28 on beechwood sawdust supplemented with molasses stillage

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(Received 1 June, revised 26 August, accepted 16 September 2021)

Abstract: This study provides a detailed analysis of the lignocellulolytic activity of a new isolate *Stereum gausapatum* F28, a Serbian autochthonous fungi, on beechwood sawdust supplemented with cheap waste, sugar beet molasses stillage. Advanced multiple response optimization techniques were applied to improve ligninolytic and reduce hydrolytic activity as a requirement for potential biorefinery use. The applied techniques were supposed to select cultivation conditions that would give manganese peroxidase and laccase activities above 0.84 and 0.12 U g^{-1} substrate, respectively, and cellulase and xylanase activities below 1.12 and 1.4 U g^{-1} substrate. The optimal cultivation conditions that met the set requirements included molasses stillage concentration of 10 %, substrate moisture content of 53 %, incubation temperature of 23.5 °C, and pH 5.2. The research showed that the addition of molasses stillage had a positive effect on enzyme production and that the optimal stillage concentration differed depending on the enzyme type (for laccase it was <5 %, manganese peroxidase ≈12 %, cellulase ≈21 % and xylanase ≈16 %), which should be taken into consideration when optimizing the desired process.

Keywords: response surface method; genetic algorithm; fungi; biomass; enzymes.

INTRODUCTION

The growth of enzyme usage in industry has increased the demand for novel microbial strains that could be used for the productions of various enzymes to meet the current requirements.[1] Wood-decay fungi are recognized as organisms suitable for the production of the industrially valuable enzymes laccase (EC

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https://doi.org/10.2298/JSC200601075J

1.10.3.2), manganese-dependent peroxidase (MnP; EC 1.11.1.13), versatile peroxidase (VP; EC 1.11.1.16), lignin peroxidase (LiP; EC 1.11.1.14), cellulase (EC 3.2.1.4), xylanase (EC 3.2.1.8), and other hemicellulases. These fungi have been extensively examined for application in wastewater treatment, in the pretreatment of biomass to facilitate its conversion to value-added chemicals, and production of lignocellulose-degrading enzymes, which can be used in various processes in the pulp and paper industry, food and beverage, personal care, healthcare, pharmaceutical and other industries.[2,3]

The application of fungi in the production of enzymes and other industrially and commercially valuable products from lignocellulosic waste and by-products from various industries has been emphasized in recent years. Lignocellulose has attracted attention as it is a renewable and inedible material that is generated in large amounts every year; the agro-industry alone produces about 5 billion metric tons of lignocellulosic waste per year.[1] By-products from various industries pose a danger to the environment; the demands for proper management of this waste have increased, thus enforcing the development of new technologies based on natural systems or processes to remove, purify, or convert this material into value-added products. However, for some agents, such as molasses stillage (MS) – a by-product of the alcohol industry that generates 8–15 L of MS per L of produced alcohol – adequate applications and disposal are still being sought.[4] The potential solutions include its use as a substrate or a supplement in a lingocellulosic substrate for enhancement of fungal enzyme production (laccase, amylase, xylanase).[5–7] Still, to recommend the use of this agent in the industry, new fungal isolates have to be examined, and particularly the influence of this agent on their enzyme production/activity.

Enzyme production and product quality can be improved using proper optimization techniques in the production process or system. Biological processes contain a large number of natural variations.[8] Different parts of a complex network of microbial reactions are affected by various environmental/cultivation factors and factor interactions, making the formulation of appropriate conditions difficult. Increased knowledge about the examined system or process and the reliability of experimental data are prerequisites for overcoming the mentioned difficulties, which can be accomplished by appropriate experimental design and statistical evaluation of the collected results.[8] The optimization methods can be divided into traditional (one factor at a time) and advanced (statistical/mathematical).[8] Traditional methods are still used in the initial research to determine the composition of the appropriate medium for the production of a new metabolite or when a new organism is used in the production of an already known metabolite. Advanced methods involve the application of experimental design, in which the experimental phase enables determination of potentially influential factors and development of a predictive model that can be used to optimize factor settings

that give the best value of the response variable.[9] The advanced methods can be used for the optimization of single or multiple responses. Optimization of a single response usually involves the application of response surface methods (RSM), such as central composite design (CCD) or Box–Behnken design (BBD), while for multiple response optimization, often used methods include desirability function and/or evolutionary algorithms (among which genetic algorithm (GA) is the most often used), or artificial neural networks.[10] This research used CCD of RSM, desirability function, and GA to evaluate the influence of factors and factor interactions on the laccase, manganese peroxidase (MnP), cellulase, and xylanase activity of a fungal isolate, create predictive models, and optimize the cultivation conditions.

The aim of the present research was a detailed analysis of the lignocellulolytic activity of *S. gausapatum* F28, a novel Serbian autochthonous fungal isolate, with the purpose of application in various biotechnological processes – from enzyme production to the pretreatment of lignocellulose. The study examined the effect of MS on the lignocellulolytic activity of this fungus to assess the possibility of using this agent as a supplement to improve the production of ligninolytic or hydrolytic enzymes. The research also examined the possibility of steering the lignocellulolytic activity of this isolate into the desired direction using advanced optimization methods.

EXPERIMENTAL

Organism and substrate

This research examined an isolate *Stereum gausapatum* F28 collected in southern Serbia (previously identified using molecular methods,[11] its DNA sequence was deposited in the NCBI GenBank database under the accession number KY264753[11]). The lignocellulosic substrate was beechwood sawdust obtained from a local sawmill. Sugar beet molasses stillage (MS) was a waste obtained from the local alcohol industry.

Inoculum preparation and cultivation conditions

The active culture was maintained at 30 °C on malt extract agar and every 2–3 weeks transferred to a fresh agar. The inoculum was prepared on an inoculum agar medium (the precise composition was given in a previous research[5]). Three mycelial discs, approximately 10 mm in diameter each, were taken from the edge of 7-day-old inoculum culture and transferred to 100-mL Erlenmeyer flasks containing 4.5 g dry substrate mass of the lignocellulosic substrate. The dry substrate mass was determined using the NREL/TP-510-42621 protocol.[12] The substrate moisture was adjusted using diluted MS (MS concentrations and moisture content are given in Table S-I of the Supplementary material to this paper) or distilled water. The substrate was autoclaved for 30 min before inoculation.

Enzyme extraction and assays

The enzymes were extracted with 50 mL of distilled water, which was added to Erlenmeyer flasks and subsequently shaken for 40 min at 220 rpm and 25 °C, and then filtered through a Whatman No. 1 filter paper and centrifuged at 4185*g* using a Z-206-A high capacity, compact centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany). The extracts

were stored at 4 °C until use. Enzyme activity assays were conducted within 24 h of the extraction on a Ultrospec 3300 pro spectrophotometer, Amersham Biosciences Ltd., Little Chalfont, UK. The laccase activity was determined using the guaiacol assay, the MnP and VP activities using the Phenol Red assay and the LiP activity using Azure B. These assays were performed according to the procedures provided in the research of Jović *at al.*[11] Cellulase (CMCase) and xylanase activity were measured using a modified dinitrosalicylic acid (DNS) reagent method proposed by Miller following the procedure described in detail in the research by Jović *at al.*[5]

Experimental design and optimization

Response surface method. A central composite design (CCD) of the response surface method (RSM) was used to examine the influence of four factors (MS concentration, substrate moisture, temperature and pH) and factor interactions on the ligninolytic and hydrolytic activity of *S. gausapatum* F28. The factors were selected using fractional factorial design before RSM. The CCD of RSM had two blocks, *i.e.*, a factorial block that contained 2^4 full factorial design (16 star points) and four replicates in the center point, and an axial block with eight axial points and three replicates in the center point. The response values were obtained in 31 experimental runs. The studied system was described using a second-order polynomial equation (Eq. (1)) for each examined response:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ij} x_{ii} x_{jj} + \Sigma\beta_{ii} x^2_{ii} \quad (1)$$

where $i = 1,2,\ldots,k$ and $j = 1,2,\ldots,k$.

In Eq. (1), Y is a response, x_i/x_j are coded value of the i^{th} factor, k is the number of examined factors, and β_0, β_i, β_{ii} and β_{ij} are the second order regression coefficients. Each factor was examined at five different levels (–2.098, –1, 0, 1, 2.098). The actual and coded values of the examined factors are given in Table S-I.

Linear regression analysis and diagnostics (R^2 value and diagnostic plots), ANOVA, and "adequate precision" (AP, calculated by Eq. (2)) were used to examine the model quality:

$$AP = (\max(\hat{Y}) - \min(\hat{Y}))/(\sqrt{(p\sigma^2/n)}) \quad (2)$$

where $\hat{Y}$ is prediction at the run settings, p is the number of terms in the model, σ^2 is the mean square of residuals, and n is the number of runs in the design.

Optimization using desirability function and genetic algorithm. A combination of desirability function and GA was used for the optimization of the lignocellulolytic activity of *S. gausapatum* F28. Two types of individual desirability functions were used, *i.e.*, the transformation of maximization for ligninolytic activities and transformation of minimization for hydrolytic activities.

Software and statistical analysis. The software used for the design analysis was R studio version 1.3.959, and R language version 3.6.3,[13] and R packages RSM,[14] desirability[15] and GA.[16] The values were expressed as mean ± standard deviation. ANOVA was used for comparison of the mean values. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

S. gausapatum is the primary colonizer of oak,[17] capable of producing various lignocellulose degrading enzymes. The enzymes of its lignocellulolytic system have been largely identified to date. Still, changes in its lignocellulolytic system have been insufficiently studied, both under different cultivation conditions and different incubation durations, and the information about the utilization of

waste material in lignocellulolytic enzyme production is limited. Understanding these changes could facilitate as well as improve the precision of the application of *S. gausapatum* in specific industrial processes, such as biomass conversion or enzyme production. This research examined the MnP, laccase, cellulase and xylanase activity on beechwood sawdust supplemented with MS. The LiP and VP activities were excluded as less relevant components of the lignocellulolytic system of *S. gausapatum* F28 for lignin degradation based on the screening analysis (fractional factor design), which showed that these activities were at the statistical error level, or under certain conditions completely undetectable.

Response surface method, model design, and model quality analysis

The influences of the examined factors and factor interactions on the activity of laccase, MnP, cellulase and xylanase were investigated using CCD of RSM. The CCD matrix and the predicted and measured response values of general laccase, MnP, cellulase, and xylanase activities and activities per dry substrate mass are given in Tables S-II, S-IIA, S-III and S-IIIA of the Supplementary material. To explain the correlation between the response variables and factors, second-order polynomial equations were created (Eqs. (S1)–(S4) in the Supplementary material) – laccase activity model (Eq. (S1)), MnP activity model (Eq. (S2)), cellulase activity model (Eq. (S3)), and xylanase activity model (Eq. (S4)).

The applied analysis of regression coefficients and model quality revealed that the generated models needed improvements, and that the effect of blocking was statistically significant in the case of the hydrolytic activities, but not in the case of ligninolytic activities. Statistically insignificant terms were removed gradually until the minimal adequate models with an improved quality and predictability were gained (Eqs. (S1)–(S4)). After each term removal, a model quality check was performed. A particular statistically-not-significant term would be retained in the equation only if the analysis revealed that its removal impaired the model quality and predictability (Tables S-IV–S-VII of the Supplementary material). The term removal reduced the R^2-value of the models to some extent (Table S-VIII) and lowered the difference between the R^2 and the adjusted R^2 of the obtained minimal models (Table S-IX). Diagnostic plots confirmed the assumptions of the regression (linearity, normal distribution, homoscedasticity and presence/absence of influential data points). The plots are presented in Figs. S-1–S-4 of the Supplementary material, while the calculated *AP*-values confirmed good signal (Table S-IX), which indicated a good quality of all four minimal adequate models.

Influence of the examined factors on the ligninolytic and hydrolytic activity

The influence of factors and factor interactions on the ligninolytic and hydrolytic activities of the fungal isolate was examined and visualized using contour plots. The contour plots were created by plotting the response values against any

two independent variables while keeping the other variables at their central (0) level. It was revealed that the MS concentration and substrate moisture had the greatest influence on the ligninolytic and hydrolytic activity.

The laccase model is a min–max function with stationary points at an MS concentration of 16.6 %, a substrate moisture of 67.9 %, a temperature of 24.5 °C, and a pH of 6.4. The contour plots show that high laccase activity could be obtained at higher temperature (above 25 °C), low MS concentration (below 5 %), pH around 5.5, and substrate moisture between 70 and 80 % (Figs. S-6a and c and S-7 of the Supplementary material). Stationary points obtained for the MnP activity – an MS concentration of 12.62 %, a substrate moisture of 70 %, a temperature of 27.5 °C, and a pH of 5.6 – are points of maximum; hence, they represent the optimal cultivation conditions for MnP activity (Figs. S-6b and d and S-8). In general, lower values of temperature and substrate moisture are expected for MnP production.[5] However, the optimal temperature depends on the fungal species or strain, and it can vary from 25 (recorded for some species such as *Trametes hirsuta*[5,18]) to 35 °C (for species such as *Trametella trogii*[18]). According to the studies conducted by Humphrey and Siggers, and Herrick,[19,20] *S. gausapatum* is a species that grows at lower temperatures. Its optimal growth temperature is around 24–25 °C, but it can grow at temperatures between 5 and 33 °C, although at maximum or minimum values of this range, it grows very little, while at 38 °C, its growth is completely inhibited. In this research, *S. gausapatum* F28 grew poorly at 33 °C, while at 37 °C, its growth was completely inhibited. Therefore, the optimal temperature for laccase production could be expected at temperatures up to 30 °C.

The optimal substrate moisture for the production of ligninolytic enzymes also depends on the fungal species. Different studies reported good ligninolytic enzyme production at a substrate moisture content between 65 to 90 %,[21,22] while the optimal moisture content for fungal growth and biomass utilization can range from 30[22] to 90 %.[21] The level of the initial substrate moisture influences the production of ligninolytic enzyme, more specifically, the type of ligninolytic enzyme. A higher moisture level (80–90 %) is often reported as more suitable for laccase production,[5,11,21] while a lower moisture level (50–65 %) for MnP production.[5,21] A good production of ligninolytic enzymes is often reported at pH 5.[22] MS is not extensively examined as a supplement for the production of ligninolytic enzymes. A previous study with *Trametes hirsuta* F13 showed that a high concentration of this agent (above 20 %)[5] was required for laccase production. In the case of *S. gausapatum* F28, examined in the current research, the laccase production requires a low concentration of this agent (below 5 %), while the optimal MS concentration for MnP production obtained for *S. gausapatum* F28 (12.62 %) was close to that obtained for *T. hirsuta* F13 (14.36 %).[5] The

optimal concentration of MS required for the production of laccase may depend on the fungal species.

According to the performed RSM analysis, only two factors had a statistically significant influence on the cellulase activity, *i.e.*, the MS concentration and the substrate moisture. The stationary points obtained for the cellulase activity model – MS concentration of 21.58 % and substrate moisture of 82.76 % – were the points of maximum and represent the optimal conditions for cellulase production (Fig. S-5d). Three factors had a statistically significant influence on the xylanase activity, *i.e.*, the MS concentration, the substrate moisture, and pH (Fig. S-5a–c). The stationary points obtained for the xylanase model were also the points of maximum, which indicates optimal cultivation conditions. These conditions included an MS concentration of 16.26 %, a substrate moisture of 70 %, and a pH of 5.6. The moisture content is a critical parameter for the production of cellulases and xylanases, and depending on the fungal species and origin of the lignocellulosic substrate, the optimal moisture content could vary from 60 to 80 %.[23] The optimal pH value also depends on the fungal species; pH values between 4 and 6.5 are often reported as optimal for the production of hydrolytic enzymes.[23,24] Studies that examined the use of stillage in the production of fungal cellulase or xylanase are still rare. In a study conducted by Acharya *et al.*,[7] an increase in the concentration of anaerobically treated distillery spent wash improved cellulase production by *Aspergillus ellipticus*. In the study conducted by Shahryari,[6] the addition of tin stillage from a residual stream of the whole-wheat ethanol process positively affected the production of xylanase by *Neurospora intermedia*. The influence of MS on the fungal production of xylanase has not hitherto been reported. According to the results obtained in the current research with *S. gausapatum* F28, the addition of the appropriate MS concentration can improve the production of xylanase and cellulase. However, a higher MS concentration is required for the production of cellulase (≈21 %) than for xylanase production (≈16 %).

Optimization of the lignocellulolytic activity

The optimization process took into consideration changes in the enzyme content under different cultivation conditions and the role and importance of certain lignocellulolytic enzymes in biomass degradation. The aim was to determine cultivation conditions that would promote ligninolytic and reduce hydrolytic activities, bearing in mind the potential application in the pretreatment of lingocellulosic biomass. Variations that exist among lignocellulose of different origins and the complex structure of this material make selection and optimization of a proper pretreatment method difficult. However, some requirements, such as efficient lignin removal and recovery of a high percentage of holocellulose, apply to all pretreatment methods.[25] In the case of the biological process, the production

organism would need a certain amount of consumable carbohydrates to survive during the pretreatment, which further complicates process optimization.

The dominant ligninolytic activity of *S. gausapatum* F28 on beechwood sawdust supplemented with MS was MnP activity. This enzyme is crucial for the initial lignin degradation.[26] The optimization goal was to improve ligninolytic activity; yet, the research showed that different cultivation conditions promoted laccase or MnP activity, but not both simultaneously. However, the differences observed with *S. gausapatum* F28 were less extreme than in a previous study with *T. hirsuta* F13.[5]

The optimization took the next best approach by prioritizing MnP production and adjusting laccase production accordingly. The laccase activity values varied between 0.04 and 0.24 U g^{-1} of dry substrate, but the most common activity values ranged between 0.11 and 0.17 U g^{-1}. Under the conditions that yielded moderate laccase activity (0.12–0.17 U g^{-1}), a fairly high MnP activity was observed (≥0.84 U g^{-1}). The cultivation conditions that yielded laccase activity below 0.06 U g^{-1} were hostile for ligninolytic and hydrolytic enzymes. It was also noticed that, under the conditions that yielded laccase activity above 0.17 U g^{-1}, the recorded MnP activity was extremely low (the values were below 0.34 U g^{-1}, see Table S-II). Laccase and MnP activity values of 0.12 and 0.84 U g^{-1}, respectively, were chosen as the desired minimum activities and set as lower limits in the function of maximization. The values were selected based on the obtained results, observations, and the enzyme importance for lignin degradation.[26] The chosen upper limits of MnP and laccase activities were the values that, when transformed by the function of maximization, gave the highest possible value below 1 to enable the selection of a single and objective solution. The scale factor for the laccase model was set at 0.1 to slow growth toward 0.17 U g^{-1}. The scale factor for the MnP model was set at 3 to enable rapid achievement of high MnP activities.

The function of minimization was used to lower the cellulase and xylanase activity. A cellulase activity of 1.12 U g^{-1} and a xylanase activity of 1.4 U g^{-1} were set as the upper limits. Each of them makes about 40 % of the highest achieved corresponding activity value (Table S-II). These values were selected based on the RSM analysis as the values that would not jeopardize achieving an MnP activity above 0.84 U g^{-1}. The lower limits were chosen as values that, when transformed by the function of minimization, give the highest possible value below 1. As the production organism requires some sugar from the substrate to survive, the fast achievement of too low activity values could be counterproductive, therefore, the scale factors were set at 0.1.

The cultivation conditions selected using a GA were an MS concentration of 10 %, a substrate moisture of 53 %, a temperature of 23.5 °C, and a pH of 5.2. These conditions were expected to yield a laccase activity of 0.13 U g^{-1}, an MnP

of 1.28 U g^{-1}, a cellulase value of 0.93 U g^{-1}, and an xylanase value of 1.27 U g^{-1}. The overall desirability value was 0.568 (Fig. S-9 of the Supplementary material).

Validation of the predicted optimum and importance of molasses stillage

The optimal cultivation conditions gave the following activity values: laccase 0.17±0.01, MnP 1.05±0.05, cellulase 1.11±0.05 and xylanase 1.40±0.12 U g^{-1}. The differences between experimental and predicted values were not statistically significant ($p > 0.05$; Fig. 1).

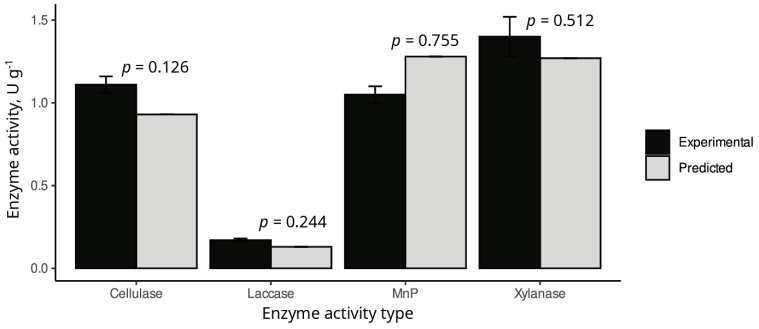

Fig. 1. Data validation, comparison of the experimental and predicted laccase, MnP, cellulase, and xylanase activity values after seven days of incubation under the optimal cultivation conditions.

The same optimal conditions, but without the addition of MS, were applied to examine the contribution of MS to the enzyme production. The results showed that the addition of MS improved laccase activity by about 18 % and MnP activity by about 55 %, which confirmed that MS could be used as a supplement for improved production of ligninolytic enzymes of *S. gausapatum* F28.

The improvement in the laccase activity was not statistically significant ($p = 0.811 > 0.05$), while the improvement in the MnP activity, which was prioritized in the optimization, was significant ($p = 0.022 < 0.05$). The achieved hydrolytic activities were low and in accordance with the set optimization conditions; although, the activity values produced in the presence of MS were similar to those obtained in its absence (Fig. 2).

Cultivation conditions change over time, which directly affects the production of enzymes of the fungal lignocellulolytic system and indirectly affects the processes in which these enzymes participate, such as biomass decomposition. In previous research, *S. gausapatum* F28 showed high efficiency in biomass decomposition but low selectivity in comparison to *T. hirsuta* F13.[11] That research did not investigate fluctuations and profile of lignocellulolytic activities involved in a more than 30-day long biomass treatment. However, current research examined changes in the lignocellulolytic system of *S. gausapatum* F28 to provide more

insights for a better understanding of previous results, which could help to develop methods to improve a process during an extended course of cultivation. The laccase and MnP activities decreased drastically after 18 and 33 days of incubation (Fig. 2). The laccase activities measured in the absence of MS were similar to those obtained in the presence of MS (the values were below 0.03 U g^{-1} in both cases). By the 18th day of incubation, in the presence of MS, the MnP activity dropped to 0.39±0.02 U g^{-1}, and then, by the 33rd day, it had increased to 0.68±0.01 U g^{-1}. These activities were higher than the corresponding activities obtained without MS supplementation by ≈44 and ≈66 %, respectively. The cellulase activity increased after 18 days of incubation to 1.58±0.11 U g^{-1} on a substrate supplemented with MS, and by the 33rd day, it had decreased by 12 % to the value of 1.41±0.22 U g^{-1}. Without MS supplementation, the cellulase activity changed only slightly, it first rose to 1.15 U g^{-1} (18th day) and then decreased to 1.11 U g^{-1} (33rd day). After 18 days of cultivation on the substrate supplemented with MS, the xylanase activity decreased only slightly (≈6 %), and by the 33rd day, it had dropped by an additional 19 % to 1.05 U g^{-1}. After 18 days of incubation, without MS supplementation, the xylanase activity decreased by ≈42 %, and by the 33rd day by an additional 3 % to the final value of 0.71 U g^{-1}.

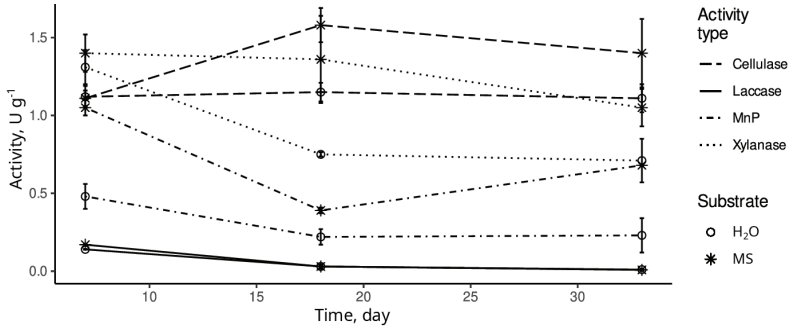

Fig. 2. Influence of MS and changes in lignocellulolytic activity during 33 days of incubation.

Potential for industrial use

Fungi that are capable to produce highly active enzymes and to withstand harsh conditions are targeted for industrial use. Most of the fungi already used in industry have been genetically modified to meet these requirements, but new organisms able to produce enzymes in higher amounts or with new characteristics are still being sought. Fungi often examined for industrial use belong to genera *Pleurotus*, *Daedaleopsis*, *Ganoderma*, *Irpex*, *Polyporus*, *Pycnoporus*, *Bjerkandera*, *Lentinus*, *etc*. They are examined for the production of industrial enzymes or other industrial applications such as biomass conversion to valuable chemicals, dye removal and for degradation of organic phenolic and non-phenolic compounds, or wastewater treatment. For example, Tripathi *et al.*[27] examined the

production of ligninolytic enzymes by *Bjerkandera adusta* and *Lentinus squarrosulus* and reported laccase activities of ≈5.5 and 64 U L^{-1}, and MnP activities of ≈215 and 370 U L^{-1}, respectively. A study conducted by Eichlerová and Baldrian.[28] examined potential candidates for industrial use that belong to the genera *Pleurotus*, *Daedaleopsis*, *Ganoderma*, *Irpex, Polyporus*, *Pycnoporus*, *etc*. This study was focused on laccase and MnP enzyme production and fungal application in dye degradation. The enzyme activities differed depending on the fungal species and ranged from 0.04–106 U L^{-1} for laccase, and 0.09–20.36 U L^{-1} for MnP enzymes with the best laccase activity values obtained with *P. ostreatus* (≈106 U L^{-1}), *Cyclocybe erebia* (≈114 U L^{-1}), and *Abortiporus biennis* (≈103 U L^{-1}), and the best MnP values were recorded for *Omphalina mutila* (≈20 U L^{-1}), *Hericium erinaceus* (≈17 U L^{-1}), and *Mycetinis alliaceus*, *Phellinus robustus*, *Inonotus obliquus* and *Fomitiporia mediterranea* each with an activity of about 16 U L^{-1}. *S. gausapatum* used in the current research showed the maximum laccase activity of 0.23 U g^{-1} (20.1 U L^{-1}) and maximum MnP activity of 1.38 U g^{-1} (123.3 U L^{-1}; see Table S-IIA) which puts this isolate in the group of good MnP and laccase producers, and as a candidate for industrial application.

Production of enzymes depends on the substrate type and fungal isolate. Substrates with a lower amount of lignin and a higher amount of polysaccharides are more suitable for the production of hydrolytic enzymes than substrates with a higher share of lignin, such as beechwood sawdust. A study conducted by Namnuch *et al.*[29] examined CMCase and xylanase production by *A. flavus* KUB2 on various substrates. The best activity was obtained on sugarcane bagasse (CMCase 1.04 U mL^{-1} and xylanase 258.38 U mL^{-1}) while the lowest values were reported for production on sawdust (CMCase 0.06 U mL^{-1} and xylanase 14.07 U mL^{-1}). The maximum CMC and xylanase activities obtained with *S. gausapatum* F28 used in the current research were 1.87 U g^{-1} (166.54 L^{-1}) and 1.66 U g^{-1} (148.09 U L^{-1}), respectively, showing potential for CMC production; however, more investigation of various substrates to find the most suitable substrate for hydrolytic enzyme production is needed.

CONCLUSIONS

This research examined and optimized the lignocellulolytic system of Serbian autochthonous isolate *S. gausapatum* F28 on beechwood sawdust supplemented with MS. Advanced multiple response optimization methods were used to enhance ligninolytic and reduce hydrolytic activity. Based on the results, *S. gausapatum* F28 can grow and produce enzymes laccase, MnP, cellulase and xylanase on waste biomass (beechwood sawdust), while cheap waste, MS, can be used to improve the production of these enzymes. The optimal MS concentration is different for the production of each enzyme type. Another important cultivation factor, substrate moisture content, also differed depending on the enzyme

type. Variations in the optimal pH and temperature were less distinct and therefore less influential. *S. gausapatum* is an insufficiently researched species, but based on the results obtained for the isolate F28, its characteristics are promising for use in biorefinery or enzyme production.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/10808, or from the corresponding author on request.

Acknowledgements. The Research presented in this paper was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project #I-1). It is a part of Scientific and Technological Collaboration of Republic of Serbia and the People's Republic of China

ИЗВОД

ИСПИТИВАЊЕ И ОПТИМИЗАЦИЈА ЛИГНОЦЕЛУЛОЛИТИЧКЕ АКТИВНОСТИ *S. GAUSAPATUM* F28 НА ПИЉЕВИНИ БУКВЕ СА ДОДАТКОМ МЕЛАСНЕ ЦИБРЕ

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Ово истраживање детаљно анализира лигноцелулолитичку активност изолата *S. gausapatum* F28, аутохтоне гљиве Србије, на пиљевини букве суплементисане јефтиним отпадом – меласном цибром пореклом од шећерне репе. Примењене су напредне технике оптимизације вишеструких одговора ради унапређења лигнинолитичке и смањења хидролитичке активности фунгалног изолата као услова за потенцијалну примену у биорафинеријским поступцима. Примењеном техникама требало је да се одаберу услови култивације који би дали активности ензима манган пероксидаза и лаказа изнад 0,84 U g^{-1}, односно 0,12 U g^{-1} супстрата, и активности целулаза и ксиланаза испод 1,12 U g^{-1}, односно 1,4 U g^{-1} супстрата. Оптимални услови којима су испуњени постављени захтеви укључују концентрацију цибре од 10 %, влажност супстрата од 53 %, температуру инкубације од 23,5 °C и pH 5,2. Истраживање је показало да је додатак меласне цибре позитивно утицао на производњу ензима, али и да се оптимална концентрација цибре разликује за различиту врсту ензима (лаказа <5 %, манган пероксидаза ~12 %, целулаза ~21 %, ксиланаза ~16 %), на шта треба обратити пажњу приликом оптимизације жељеног процеса.

(Примљено 1. јуна, ревидирано 26. августа, прихваћено 16. септембра 2021)

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