PRETREATMENT OF LIGNOCELLULOSIC BIOMASS WITH AUTOCHTHONOUS FUNGI FROM SERBIA

PREDTRETMAN LIGNOCELULOZNE BIOMASE POMOĆU AUTOHTONIH GLJIVA SRBIJE

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ABSTRACT

This research examined the potential use of isolated Serbian autochthonous fungi in lignocellulosic biomass pretreatment. Among 12 isolated fungi, the isolates identified as Trametes hirsuta F13 and Stereum gausapatum F28 stood out as ligninolytic enzyme producers and were selected for potential use in the pretreatment of a waste lignocellulosic biomass. An isolate identified as Myrmaecium fulvopruinatum *F14 showed high hydrolytic activity, but negligible ligninolytic activity, and it was selected as a potential producer of important industrial hydrolytic enzymes.*

Further, the breakdown of lignocellulosic waste, beechwood sawdust, by T. hirsuta F13 and S. gausapatum F28 was examined. Both isolates efficiently degraded biomass, but T. hirsuta F13 exhibited greater selectivity (selectivity coefficient of 1.7) than S. gausapatum F28 (1.1). The isolate F13 was considered a better candidate for the pretreatment, and it was selected for further analysis which involved the use of molasses stillage as a supplement to improve the pretreatment.

Keywords: *autochthonous fungi, lignocellulose, laccase, manganese peroxidase, cellulase, xylanase.*

REZIME

Predtretman gljivama se poslednjih godina intenzivno istražuje za biološku razgradnju lignoceluloze zbog prisustva enzima sposobnih da raskinu inter- i intrapolimerne veze u ovom supstratu i tako ga razgrade do fermentabilnih šećera. U ovom istraživanju su izolovane autohtone lignocelulolitičke gljive Srbije među kojima su odabrani i identifikovani izolati koji bi se mogli primenjivati u predtretmanu otpadne lignocelulozne biomase. Među 12 uspešno izolovanih gljiva, visokom ligninolitičkom aktivnošću su se istakli izolati identifikovani kao Trametes hirsuta F13 i Stereum gausapatum F28, dok je izolat identifikovan kao Myrmaecium fulvopruinatum F14 ispoljio visoku hidrolitičku aktivnost, ali zanemarljivu ligninolitičku aktivnost zbog čega nije razmatran kao potencijalni kandidat za primenu u predtretmanu, ali jeste razmatran kao proizvođač industrijski važnih hidrolitičkih enzima.

U daljem radu ispitan je potencijal razgradnje lignoceluloznog otpada – piljevine bukve – pomoću T. hirsuta F13 i S. gausapatum F28. Oba izolata su efikasno razgradila biomasu, ali je T. hirsuta F13 ispoljio veću selektivnost (koeficijent selektivnosti 1,7) nego izolat S. gausapatum F28 (1,1) – brže razgradio lignin nego celulozu – pa se smatra boljim kandidatom za predtretman. Kako je bitno da predtretman bude ekonomičan, za unapređenje je korišćena melasna džibra, otpad iz industrije alkohola. Dodatak melasne džibre je skratio vreme predtretmana i unapredio selektivnost razgranje lignocelulozne biomase izolatom T. hirsuta F13 – nakon 18 dana koeficijent selektivnosti je bio 1,9 uz dodatak melasne džibre i 1,7 bez dodatka melasne džibre.

Ključne reči: *autohtone gljive, lignoceluloza, lakaza, mangan peroksidaza, celulaza, ksilanaza.*

INTRODUCTION

Lignocellulosic waste as a renewable resource has attracted attention as a potential substrate for the production of value-added chemicals, but its complex structure prevents its direct use; it needs to be pretreated to remove lignin and hydrolyzed before it could be used, which can be done by chemical, physical, physicochemical, or biological methods (*Gauna et al., 2018; Nair and Sivakumar, 2020; Vučurović et al., 2019*). The use of fungi or their enzymes in the pretreatment and hydrolysis of the lignocellulosic substrate is an ecologically safe method, and there is no formation of toxic components that can be hostile to the production microorganism (*Nair and Sivakumar, 2020*). The biggest disadvantage of this method is the long pretreatment time, which can be 1-3 months, as well as the loss of a certain amount of sugar – an integral part of lignocellulosic biomass – required for fermentation processes (*Mielenz, 2020; Zhang et al., 2020*). These limitations can be overcome or mitigated by the use of organisms that efficiently degrade biomass, primarily by the use of selective decomposers, as well as by formulating the conditions that would improve the pretreatment process by shortening its duration and thus reducing the loss of necessary

sugars. So far, a sufficiently efficient fungal strain has not been found, nor the conditions that would enable the industrial application of this organism in the treatment of lignocellulosic biomass have been formulated. Fungi that belong to the genera *Trametes*, *Ganoderma*, *Pleurotus* have proved to be good candidates for lignocellulose pretreatment use (*Gauna et al., 2018; Isroi et al., 2011*).

This research aimed to select the isolate with the good characteristics for application in the pretreatment of beechwood sawdust, to gain insight into the substrate breakdown by the selected isolate (the selectivity), and to examine the effects of sugar beet molasses stillage (MS) on the pretreatment for its potential use as a supplement.

MATERIALS AND METHODS

Isolates were collected in southern Serbia near the City of Leskovac. The strains were isolated from stumps, fallen trees and branches, fallen leaves, or living trees found in the oak forest and orchards around the forest. The three isolates with the best lignocellulolytic enzyme activities were identified using ITS sequences, and the sequences were deposited in the NCBI

GenBank database. Their accession numbers are KY264754.1 (*Trametes hirsuta* F13), KY264753.1 (*Stereum gausapatum* F28), and MF521930.1 (*Myrmaecium fulvopruinatum* F28) (*Jović et al., 2018*). Sugar beet MS was obtained from a local alcohol industry, and beechwood sawdust was obtained from a local sawmill. The activities of the produced enzymes were determined using spectrophotometric methods. Laccase activity was measured using guaiacol assay (*Abd El Monssef et al., 2016; Jović et al., 2018*), manganese peroxidase and versatile peroxidase activities were determined with phenol red (*Arora and Gill, 2005; Jović et al., 2018; Kuwahara et al., 1984*), lignin peroxidase activity with azure B (*Archibald, 1992; Jović et al., 2018*), and cellulase (carboxymethyl cellulase) and xylan activities were determined by the Miller's DNS method (*Jović et al., 2020; Miller, 2002*). The dry substrate mass was determined according to the NREL / TP-510-42621 protocol (*Sluiter et al., 2008*), and the share of acid-soluble and acid-insoluble lignin was determined according to the LAP-003 and LAP-004 protocols (*Ehrman, 1996; Templeton and Ehrman, 1995*). Experiments were performed in triplicates. Activity values are presented as mean±standard deviation. The differences between mean values were assessed using a one-way analysis of variance (ANOVA). The p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The purpose of the pretreatment is to remove lignin (*Gauna et al., 2018; Nair and Sivakumar, 2020*), thus when selecting organisms, attention was primarily focused on those isolates that were the best producers of ligninolytic enzymes. Among the 12 isolated fungi, the best ligninolytic activity (laccase, manganese peroxidase, and versatile peroxidase) showed two isolates, F13 and F28 (Figure 1 and 2), which were identified as *Trametes hirsuta* F13 and *Stereum gausapatum* F28, respectively. The best laccase producer was the isolate *T. hirsuta* F13 with activities above 40 U/l under submerged (SF) and solid-state fermentation (SSF). Research of pure activated culture of this isolate has shown that laccase activity can exceed 300 U/l in a liquid medium - after seven days of incubation, 315.9±0.7 U/l was recorded (*Jović et al., 2018*), which confirmed that for the successful use of this isolate the optimization of cultivation conditions is required. *S. gausapatum* F28 exhibited lower laccase activity but was the best producer of manganese peroxidase. This isolate yielded better ligninolytic enzyme activities under solid-state cultivation conditions than in submerged cultivation. MnP activity of 31.50 ± 1.58 U/l was recorded under SSF, while no MnP activity was detected in SF.

Fig. 2. Versatile peroxidase (VP) and lignin peroxidase (LiP) activities obtained by fungal isolates under solid-state (SSF) and submerged (SF) cultivation conditions.

The production of hydrolytic enzymes was also examined. Enzymatic hydrolysis of the pretreated substrate is considered a favorable method, primarily for environmental reasons, but also because it doesn't form toxic components that could harm the production microorganisms. However, the high price of industrial enzymes increases the cost of the lignocellulose conversion to value-added chemicals, so the industry still uses acid hydrolysis. The use of indigenous fungi in the pretreatment and for the production of enzyme cocktails that would be used in hydrolysis after lignin removal can be a good alternative in biorefinery or in other industries where the use of purified enzymes is not demanded (*Champreda et al., 2019; Choudhary et al.2016*).

The isolate F14 (Figure 3) identified as *Myrmaecium fulvopruinatum* F14 showed the best hydrolytic activity. It was determined that solid-state cultivation was more suitable for enzyme production than submerged cultivation. Carboxymethyl cellulase activity of 5253.42±262.67 U/l was recorded on solid, while 3159.98±158.00 U/l was recorded in the liquid medium. The SSF cultivation gave xylanase activity of 6831.15±304.15 U/l, and SF yielded the activity of 3792.97±189.65 U/l. Isolates *T. hirsuta* F13 and *S. gausapatum* F28 showed a moderate activity of hydrolytic enzymes of about 1000 U/l (Figure 3). *Myrmaecium fulvopruinatum* F14 exhibited negligible ligninolytic activity, which makes it unsuitable for lignin removal, but it showed high potential for use in the production of cocktails of hydrolytic enzymes that could be used in the next biomass conversion step – hydrolysis.
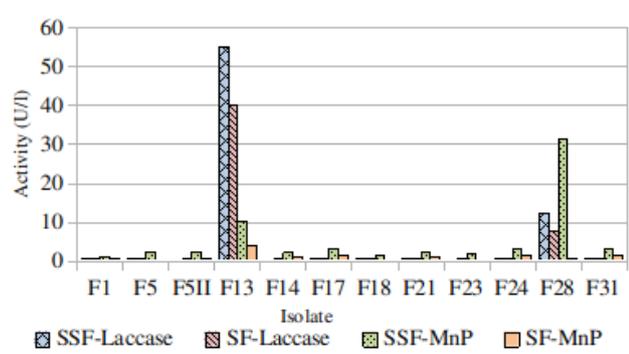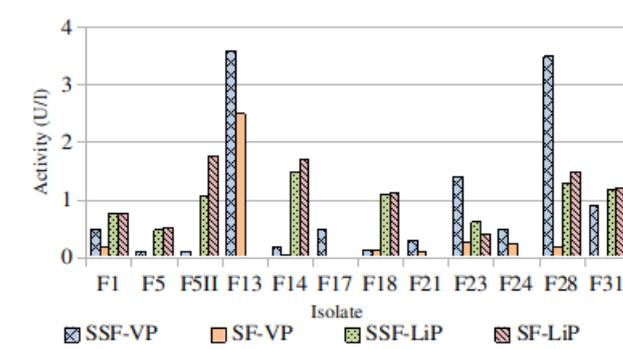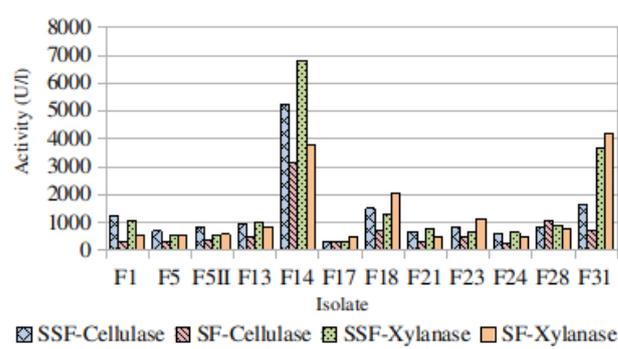
Fig. 1. Laccase and manganese-dependent peroxidases (MnP) activities obtained by fungal isolates under solid-state (SSF) and submerged (SF) cultivation conditions.

Fig. 3: Cellulase and xylanase enzyme activities obtained under solid-state (SSF) or submerged (SF) cultivation conditions.

The current research has generally shown that solid-substrate cultivation is more convenient for the production of ligninolytic and hydrolytic enzymes of studied isolates, except in the case of laccase production by *T. hirsuta* 13, for which the cultivation in

liquid medium is more favorable (Figures 1, 2 and 3). Applied cultivation conditions affect the type of enzyme activity that will dominate. It was found that high substrate moisture content (>75%) promotes laccase production, while moisture below 70% promotes MnP production. It was also found that the addition of minerals can improve enzyme production (*Jović et al., 2018*).

The successful pretreatment of biomass requires the activity of all ligninolytic enzymes; MnP is particularly important (*Reina et al., 2019*). Therefore, in further research, which included testing the selectivity of biomass degradation, SSF cultivation was applied. In the first step, when the best candidate for application in the pretreatment was selected, no minerals were added to the substrate, only distilled water was used. Substrate moisture content was 70%. The difference in biomass reduction achieved by these isolates after 35 days of the pretreatment was not statistically significant (p> 0.05), while lignin reduction was statistically significant (p <0.05). More lignin was degraded by *T. hirsut* F13 than *S. gausapatum* F28: its selectivity coefficient, relative to the total lignin, was 1.7, while the selectivity coefficient obtained for *S. gausapatum* was 1.1 (*Jović et al., 2018*). Values of the selectivity coefficient, relative to Klason's lignin, were 1.47 for the isolate F13 and 0.8 for the isolate F28, which confirmed that *T. hirsuta* F13 was the best candidate among the isolated fungi for use in biomass pretreatment. This isolate was further used in the pretreatment research in which sugar beet MS was used instead of a basic medium for ligninolytic enzyme production (LBM). The initial results showed that the addition of MS could improve ligninolytic activity, but also that the type of a dominant enzyme activity differed depending on the MS concentration. The concentrations of 10 and 20% were tested and compared with a substrate that contained LBM and with a substrate without any supplements other than distilled water (Figure 4).
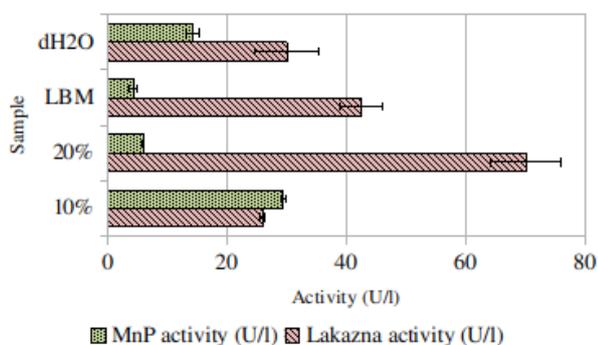

Fig. 4: Laccase and MnP production by T. hirsut F13 on the substrate supplemented with 10% or 20% of MS lignin basal medium (LBM), and the substrate without supplements other than distilled water (dH2O).

Optimization of cultivation conditions could additionally improve enzyme production, and thus the pretreatment, but because the substrate moisture content and MS concentration influence the type of dominant enzyme activity, the enzyme production should be directed toward the activity that mostly contributes to the pretreatment. The cultivation conditions were optimized in our previous research. The research also confirmed that the addition of MS improved ligninolytic activity and the improvement was statistically significant (*Jović et al., 2020*). However, changes in the substrate related to lignin and biomass reduction have not been analyzed. The current research examined the reduction of Klasson lignin and biomass, and the selectivity achieved by *T. hirsut* F13 under optimal pretreatment

conditions (substrate moisture of 63%, temperature of 25 °C, MS concentration of 13% (*Jović et al., 2020*)) after 18 and 35 days. The results showed that after 18 days the reduction of lignin was higher under the optimal cultivation conditions compared to the pretreatment before optimization and that the supplementation with MS further improved the pretreatment (Table 1), the difference between treatment in the presence and absence of MS was statistically significant (p <0.05). After 35 days, the selectivity coefficient obtained under optimal conditions was similar to that achieved with the pretreatment before optimization. However, more lignin was degraded when optimal cultivation conditions were applied, especially when MS was used as a supplement. Between the 18th and 35th day of pretreatment in the presence of MS, lignin was reduced only slightly, regardless of the long duration of this phase of the pretreatment. After 18 days of pretreatment, 29.2% of Klason's lignin was removed, and for the remaining 17 days, only 3.5% was removed. At the same time, the total biomass was reduced by an additional 8.1%, which is probably a consequence of the predominant degradation of holocellulose. The biomass and lignin degradation under optimal conditions in the absence of MS was evenly distributed during the entire pretreatment duration, but the degree of lignin reduction achieved after 18 days in the presence of MS, was achieved after 35 days in the absence of MS. The previous research has shown that the use of MS can improve the enzymatic activity of the fungus (*Jović et al., 2020*), while the analysis performed in this study showed that the application of MS shortened the pretreatment duration from 35 to 18 days.

Table 1: Biomass and lignin reduction and selectivity coefficient achieved after 18 and 35 days of pretreatment in the presence (MLS) and absence (dH₂O) of MS under optimal conditions and after 35 days without the addition of supplements (dH₂O) under conditions that were not optimal.

Monitored parameters	Optimal cultivation conditions				Before optimization
	18 days		35 days		35 days
	MLS	dH₂O	MLS	dH₂O	dH₂O
Biomass reduction (%)	15	13,9	22	19,6	19
Klason's lignin reduction (%)	29,2	23,5	32,7	29,1	28
Selectivity coefficient	1,9	1,7	1,49	1.48	1,47

(Selectivity coefficient) = (Klason's lignin reduction)/ (Biomass reduction)

CONCLUSION

Serbian autochthonous fungi are great unexplored potential for application in various industries (from the pulp and paper industry, to the textile industry and biofuel production). This study selected and examined three isolates, *T. hirsuta* F13, *M. fulvopruinatum* F14, and *S. gausapatum* F28, which can be used in the production of industrially important lignocellulolytic enzymes and/or in biomass pretreatment. The candidate with the best potential for application in the pretreatment was *T. hirsuta* F13, which showed a high selectivity of lignocellulosic biomass degradation. It was also shown that the use of sugar beet MS as a supplement can improve pretreatment. With its use as a supplement, the pretreatment time was shortened from 35 to 18 days, and the selectivity of biomass degradation was improved.

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