DYNAMICS OF PRETREATED WHEAT STRAW SACCHARIFICATION BY CELLULOSOME OF *TRICHODERMA VIRIDE*

MILICA GALIĆ, MIRJANA STAJIĆ AND JASMINA ĆILERDŽIĆ

University of Belgrade, Faculty of Biology, Takovska 43, 11000 Belgrade, Serbia
✉ *Corresponding author: J. Ćilerdžić, simonicj@bio.bg.ac.rs*

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The main goal of this study was to analyze the potential of *Trichoderma viride* BEOFB 1210m for cellulase production during solid-state fermentation of wheat straw pretreated with the ligninosome of a well-known white-rot delignificator – *Pleurotus pulmonarius* HAI 573. After only 7 days of *T. viridae* BEOFB 1210m cultivation on biologically pretreated wheat straw, this micromycete produced the most active xylanases, which were also the dominant enzymes, with a value of even 3730.10 U L^{-1}. Likewise, maximal but much lower values of exocellulases (155.83 U L^{-1}) and β-glucosidases (59.98 U L^{-1}) were detected after the same period of fermentation. However, much higher activity of endocellulase (2439.55 U L^{-1}) was obtained on the 10th day. The dynamics of enzyme activity was reflected on the level of substrate depolymerization. As much as 30.56% of the cellulose was degraded already on the 7th day, and that percentage did not change significantly until the end of the cultivation period. A significant loss of hemicelluloses was also measured at the beginning of the fermentation process, but it reached a maximum of approximately 50% by the 21st day. The results clearly showed that the selected *T. viride* strain has very good potential to synthesize highly active cellulases when grown on a cheap and available substrate, which is significant for further large-scale industrial applications.

Keywords: agricultural residues, cellulases, depolymerization, biological pretreatment, *Trichoderma viride*

INTRODUCTION

Constantly rising energy consumption and fuel prices at the world level, as well as the unstable and uncertain availability of petroleum sources, have triggered huge efforts in recent years to produce "greener" energy based on renewable resources. Biomass residues generated from agriculture, forestry, food production, or municipal waste represent an extremely significant potential renewable energy source. Lignocellulosic biomass is one of the most abundant renewable energy sources, as the growth of the world's population is accompanied by increasing agricultural production, which inevitably creates a huge amount of waste material.[1,2] The sustainability, accessibility, and great environmental benefits of lignocellulosic biomass herald the era of advanced biofuels, such as cellulosic ethanol, biomass-based diesel, bio-butanol and bio-oil, and biorefineries will have a significant share in the global energy market.[3,4] Thus, according to estimates from the International Energy Agency, biofuels could meet a quarter of the needs of all transportation fuels by 2050. However, to fully utilize the potential of plant biomass, it is necessary to overcome numerous challenges in the process of transforming lignocellulose into fermentable sugars.

Numerous studies have shown the importance of pretreatment or delignification of lignocellulose as an initial and key step, but the process of saccharification, whose efficiency affects the yield of fermentation, is not far behind.[5,6] The justification for using renewable energy sources could be undermined by harmful, energy-demanding, and ultimately expensive conventionally used physico-chemical methods of pretreatment and saccharification. Therefore, increasing importance has been attached to the optimization of alternative biological methods, which primarily include enzyme cocktails of different fungal species.[7,8] While a large number of studies have dealt with the ligninolytic potential of numerous macromycetes, much less scientific attention has been paid to species with cellulolytic potential, even though cellulases play a key role in the conversion of holocellulose into biofuels and biochemicals in sugar-based biorefineries.[3] The justification for finding efficient producers of these enzymes among fungi is supported by the fact that cellulases are currently the third most important industrial enzymes owing to their use in paper recycling, cotton processing, and production of detergents, juices, and feed additives. However, since biofuels are expected to be the dominant transportation fuels, cellulases will become the leading industrial-scale enzymes. Therefore,

it is necessary to remodel cellulase cocktails to improve their activity, hydrolysis rate and sugars yield, as well as adaptation to different lignocellulose sources and pretreatments.[9] Therefore, the technology for the conversion of biomass polysaccharides to fermentable sugars has to be optimized and cost-effective. A significant reduction in cellulase production costs is necessary in order to make the price of lignocellulose-based bioethanol competitive with the price of fossil fuels or bioethanol obtained from starchy biomass. Thus, the production of these enzymes on cheap substrates, as well as the increase of their efficiency and thermal stability, are necessary to make the most competitive hydrolysis process for obtaining bioethanol.[10,11]

Another important research direction is bioprospecting for potential producers of superior cellulases, and previous studies have shown that species of the genera *Trichoderma*, *Aspergillus* and *Penicillium* have the greatest potential for the development of biofactories of these enzymes.[12,13] However, compared to other cellulase-producing micromycetes, species of the genus *Trichoderma* particularly stand out for their enormous cellulolytic potential during growth on a cheap and available substrate, such as wheat straw.[14] According to the UN Food and Agricultural Organization, wheat production has been in constant growth for more than a century, it is grown in more than 115 countries under different environmental conditions, which makes it the world's most widely cultivated crop. Based on these data, the importance of wheat straw as the most abundant renewable energy source is completely clear. All of the above helped us in defining the goal of this study – the determination of the dynamics of *T. viride* cellulolytic enzymes activity during the fermentation of wheat straw pretreated with a *P. pulmonarius* ligninolytic cocktail.

EXPERIMENTAL

Organism and growth conditions

The culture of *Trichoderma viride* BEOFB 1210m is maintained on Malt agar medium at 4 °C in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB). The inoculum preparation was performed by inoculating 100.0 mL of the synthetic medium (glucose, 10.0 g L^{-1}; NH_4NO_3, 2.0 g L^{-1}; K_2HPO_4, 1.0 g L^{-1}; NaH_2PO_4 x H_2O, 0.4 g L^{-1}; $MgSO_4$ x $7H_2O$, 0.5 g L^{-1}; yeast extract, 2.0 g L^{-1}; pH 6.5) with 25 mycelial disks (Ø 0.5 cm) of 7-day-old culture. The incubation was performed on a rotary shaker (22 ± 2 °C, 160 rpm) for 7 days. Using sterile distilled water (dH_2O), the obtained biomass was washed three times and then homogenized with 100.0 mL of dH_2O in a laboratory blender (Waring, USA). Solid-state fermentation by the selected species was carried out in 100 mL flasks, containing 2.0 g of wheat straw (previously pretreated with *Pleurotus pulmonarius* HAI 509) and 10.0 mL of the modified synthetic medium (without glucose) inoculated with 3.0 mL of homogenized inoculum. Incubation was performed at 25 °C in the dark and the samples were harvested after 7, 10, 14, 17, and 21 days of cultivation.[15]

Assays of enzyme activity and total protein production

The cellulolytic enzymes were extracted by sample stirring with 50.0 mL of dH_2O at 4 °C for 10 min. The obtained supernatant after extract centrifugation (4 °C, 3000 rpm, 15 min) was used for measurement of the activity of exo- and endocellulases, β-glucosidases and xylanases, spectrophotometrically (BioQuest CECIL CE2501, UK). The activities of exo- and endocellulases were determined according to the methods of Bernfeld[16] using microcrystalline cellulose (1%) and medium viscosity carboxymethyl cellulose (1%), respectively, as the substrates, with glucose as a standard. The activity of β-glucosidases was estimated using 4-nitrophenyl β-D-glucopyranoside and p-nitrophenol as a substrate and standard, respectively, while the activity of xylanases was determined using birchwood xylan (1%) as a substrate and xylose as a standard.[17] One unit of exo-, endocellulase, and xylanase activity was defined as the amount of enzyme required to produce 1.0 μmol of glucose or xylose, respectively, per min at 39 °C, while one unit of β-glucosidase activity was defined as the amount of enzyme required to produce 1.0 μmol of p-nitrophenol per min at 37 °C.

The specific cellulase activities were determined using Bradford reagent (Coomassie brilliant blue (CBB) G-250) and bovine serum albumin by the method of Silva *et al.*[18]

Defining the amount of lignocellulosic polymers

The loss of substrate dry matter (%) was determined by the formula (Mi – Mf)/Mi x 100, where Mi represents the initial lignocellulosic mass and Mf is the mass after fermentation by the studied species. The determination of hemicellulose, cellulose and lignin contents was performed using the modified methods of Kirk and Obst[19] and Van Soest *et al.*[20] The dried ground sample (1.0 g) was treated with a neutral detergent/Na_2SO_3 mixture under refluxing conditions to remove soluble sugars, proteins, lipids and vitamins, and the obtained biomass presented neutral detergent fibers (NDF). Acidic detergent fibers (ADF) were obtained by treatment of these samples with acidic detergent. The difference between the obtained fibers represented the hemicellulose

content. The lignin content (LC) was defined after ADF incubation with 72% H_2SO_4 at 30 °C and its hydrolysis at 120 °C, and was expressed as the percentage of quantity present in the initial sample. The difference between ADF and LC represented the cellulose content.

Statistical analysis

All the experiments were done in three replicates and the results were expressed as mean ± standard error. One-way analysis of variance (ANOVA) and Tukey's test were performed using STATISTICA, version 6.0 (StatSoft, Inc., Tulsa, USA) to test any significant differences ($p<0.05$) among means.

RESULTS AND DISCUSSION

The selected *T. viride* strain produced all tested cellulolytic enzymes, but they differed significantly in both production dynamics and level of activity during the period of fermentation of biologically pretreated wheat straw. However, if we consider the activity of each enzyme individually, there were no significant variations in activity at different measurement points (Fig. 1A-D). Thus, the maximum level of exocellulase activity was detected on the 7th day (155.83 ± 0.48 U L^{-1}) and thereafter declined moderately (~130 U L^{-1}) by the end of the cultivation period (Fig. 1A). On the other hand, endocellulases were much more active, but also with no great variations between maximal and minimal values (2439.55 ± 68.45 U L^{-1} on day 10 to 1894.19 ± 18.93 U L^{-1} on day 21), while the activities were about 2000 U L^{-1} at other measurement points (Fig. 1B). The activity of synthesized β-glucosidase was many-fold lower than that of exo- and especially endocellulases, with the maximum of only 59.98 U L^{-1} obtained at the beginning of the cultivation process, which sharply declined in two weeks, increased again on the 17th day and then fell to approximately 30 U L^{-1} to the end of fermentation (Fig. 1C).

On the other hand, the level of xylanase activity was significantly higher than the levels of endocellulases and especially exocellulases and β-glucosidases. *T. viride* synthesized xylanases with an activity peak of even 3730.10 U L^{-1} detected on day 7, after which a slight decrease in activity was recorded until the 21st day, when the measured value was insignificantly below 3000 U L^{-1} (Fig. 1D). Generally, pretreated wheat straw was proven as a good stimulator of cellulolytic enzymes synthesis by *T. viride* BEOFB 1210m, uniformly inducing the most active isoforms at the beginning of the fermentation period. The substrate and cultivation period also affected proteins production and, consequently, the specific enzyme activities. Total protein concentration ranged from 1.24 mg mL^{-1} on day 10 to 2.08 mg mL^{-1} on day 17, which reflected on specific enzyme activities. Thus, significant values of xylanase and endocellulase specific activities were noted on the 7th and 10th days (2.72 U mg^{-1} and 1.97 U mg^{-1}, respectively), while the specific activities of the other two tested enzymes were insignificant (<1.0 U mg^{-1}).

Previous studies have already shown different potentials of *Trichoderma* species/strains for the synthesis of cellulolytic enzymes, depending on the type of plant residues used as a substrate, as well as on the length of the cultivation period, but, in general, similarly to our study, a significantly lower activity of β-glucosidases was detected compared to other cellulolytic enzymes.[21,22] Thus, Adsul *et al.*[23] fermented chemically pretreated sugarcane with *T. viride* strain for 10 days and obtained very active xylanases, whose values reached even ~80000 U L^{-1}, and also very active endocellulases, which could be explained by the substrate composition, genetic basis of the species/strain, the pretreatment type and the hydrolysis period. On the other hand, Asgher *et al.*[24] reported that *T. harzianum* produced cellulases of significantly lower activities than in our study during the hydrolysis of wheat straw pretreated with *G. lucidum* ligninolytic enzymes cocktail, namely the obtained activities ranged from 39.0 U mL^{-1} to 53.5 U mL^{-1}. Likewise, in the study of Grujić *et al.*,[17] *T. harzianum* strain cultivated on spent compost of *Agaricus bisporus* synthesized highly active xylanase isoforms (>2.0 U mL^{-1}), as well as *T. atroviride* whose endocellulase production reached ~0.8 U mL^{-1}, while the best β-glucosidase producer was *T. koningi* (~6.0 U mL^{-1}). However, Vázquez *et al.*[25] detected significantly lower values of endo- and especially exocellulase activities during the whole period of wheat straw fermentation by *T. viride*, compared to our results. Furthermore, the same species synthesized very low active xylanase isoforms during submerged cultivation on various agricultural residues, such as wheat, sorghum, and corn.[26] According to Garcia-Kirchner *et al.*[21] and Singhania *et al.*,[22] species of the genus *Trichoderma* were generally weak producers of β-glucosidases that are necessary for the completion of cellulose depolymerization, primarily owing to the final products of the substrate hydrolysis, *i.e.* glucose and cellobiose, which inhibit the synthesis of this enzyme.

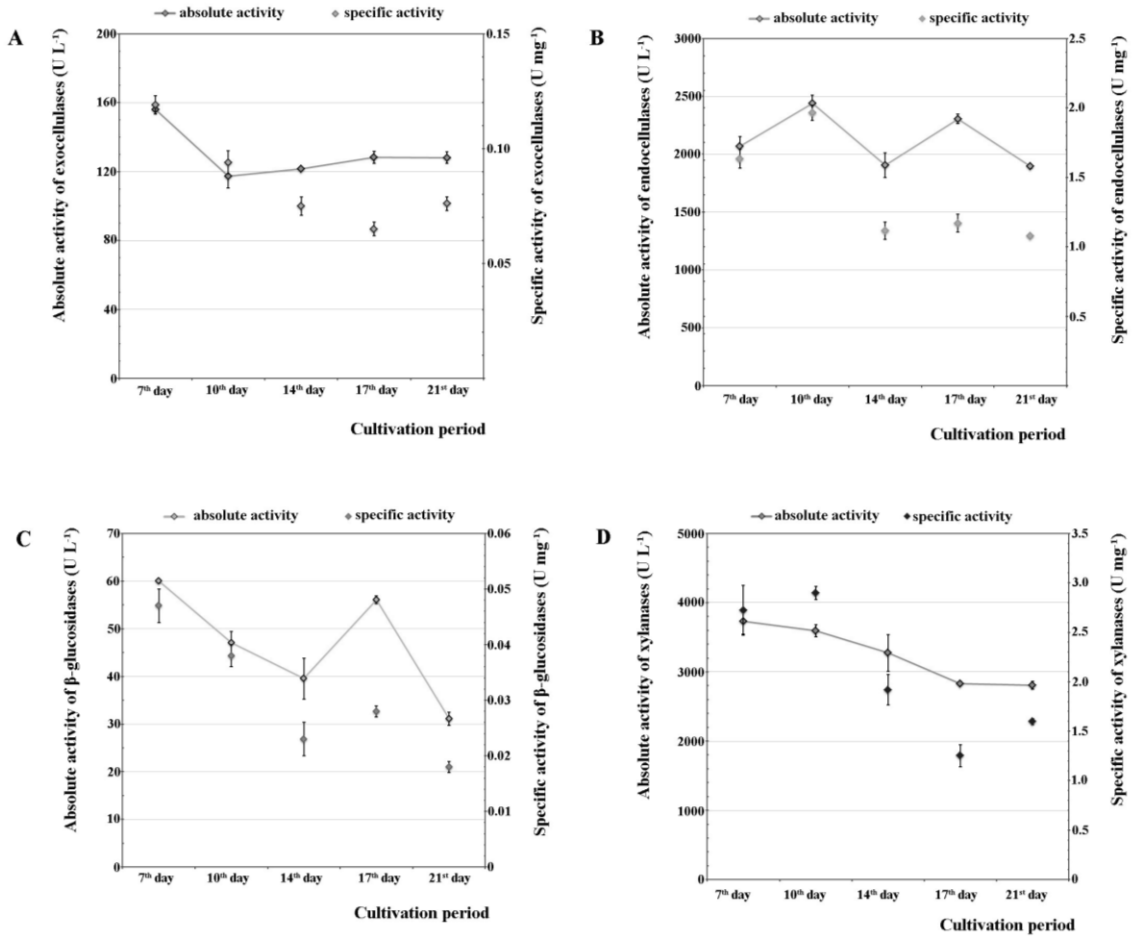

Figure 1: Activity of exocellulases (A), endocellulases (B), β-glucosidases (C) and xylanases (D) produced by *Trichoderma viride* during 21 days of biologically pretreated wheat straw fermentation

Considering that hemicellulose hydrolysis products (xylose, xylooligosaccharides, galactose, and mannose) are strong inhibitors of β-glucosidases and cellulases,[27] further research should be focused on optimizing the relative proportions of these enzymes in the entire cellulosome. The importance of such efforts is clear if we take into account that, contrary to cellulose, whose depolymerization produces exclusively fermentable glucose, the degradation of hemicelluloses yields a mixture of different sugars, among which difficultly fermentable pentoses are present in different proportions.[28] On the other hand, highly active xylanases are important enzymes found in numerous industrial enzyme cocktails used for lignocellulose processing.[29] Future studies should also be focused on the identification and profiling of some accessory enzymes that would ensure the complete utilization of plant biomass in energy- and chemically less-intensive processes. Namely, it was shown that the activity of cellulolytic enzymes of *T. reesei* could be considerably enhanced with crude enzyme preparations of other fungal species.[9]

Thanks to its high ability to synthesize cellulolytic enzymes, *T. viride* BEOFB 1210m was also an exceptional degrader of *P. pulmonarius* pretreated wheat straw, with different mineralization efficiency of its polymers. Thus, almost a quarter of the total mass of wheat straw was degraded already on the 7th day of fermentation, while by the end of the process that percentage had increased to as much as 36.25% (Table 1). The degree of holocellulose removal was also extremely high already at the beginning of the cultivation. Namely, a very high level of cellulose depolymerization (30.56%) was measured already at the beginning of the fermentation process, but it did not increase significantly during the cultivation period, considering that only a few more percent of this polymer were degraded until the day 21 (Fig. 2). On the other hand, the percentage of decomposed hemicelluloses almost doubled from the 7th (24.59%) to the 21st day (49.87%) (Fig. 2). Although the efficiency of delignification was significantly lower, comparing to holocellulose removal, the percentage of

degraded lignin at the end of fermentation is not inconsiderable (10.19%), indicating a certain ligninolytic potential of this micromycete, especially since the substrate was largely delignified during its pretreatment.

Table 1
Extent of degradation of *Pleurotus pulmonarius* pretreated wheat straw by *Trichoderma viride*

Cultivation period	Studied treatment	Sample weight (g)	Fiber content of samples (mg)			Dry matter loss (%)
			Lignin	Cellulose	Hemicelluloses	
7th day	Control*	0.80	70.79	329.60	182.40	/
	BEOFB 1210m	0.61	69.97	228.89	137.54	23.75[a]
10th day	Control*	0.80	70.79	329.60	182.40	/
	BEOFB 1210m	0.60	66.00	228.84	136.41	25.00[a]
14th day	Control*	0.80	70.79	329.60	182.40	/
	BEOFB 1210m	0.58	65.28	224.67	127.66	27.50[b]
17th day	Control *	0.80	70.79	329.60	182.40	/
	BEOFB 1210m	0.54	64.80	220.66	106.08	32.50[c]
21st day	Control *	0.80	70.79	329.60	182.40	/
	BEOFB 1210m	0.51	63.58	218.41	91.43	36.25[c]

*untreated plant residue; [a-d] Values superscripted with the same letter are not significantly different ($p < 0.05$)

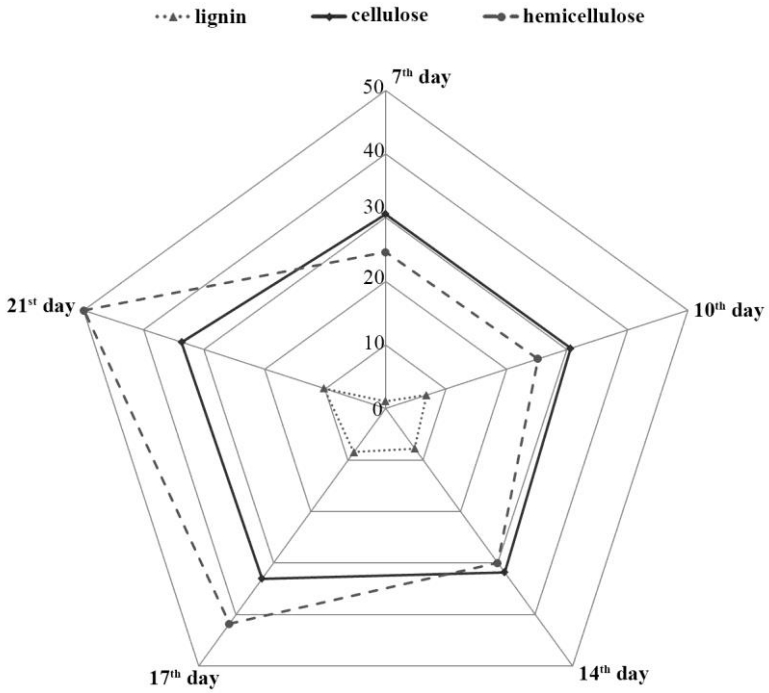

Figure 2: Extent of polymer degradation (%) in wheat straw after treatment with *Pleurotus pulmonarius* and *Trichoderma viride*

Compared to some previously published results on different *Trichoderma* species/strains, the strain used in our study was not an effective mineralizer. Thus, *T. harzianum* studied by Asgher *et al*.[24] mineralized even 88.3% of cellulose during only 5 days of hydrolysis of wheat straw pretreated with *Ganoderma lucidum* ligninolytic enzyme cocktail, while Nawaz *et al*.[30] recorded 83% of cellulose loss from wheat straw hydrolyzed with *T. viride* strain. The other micromycetes were also studied for their wheat straw depolymerization capacity, so Wang and Fan[31] showed that *Penicillium expansum* W4 degraded even 59.06% of cellulose and 78.75% of hemicelluloses after 10 days of wheat straw fermentation, while Bowen and Harper[32] analyzed mycobiota of wheat straw used as a natural organic fertilizer and found numerous populations of *Fusarium culmorum*, *Trichoderma* spp. and *Chaetomium globosum*, showing to be very effective degraders of this residue. Moreover, *Myrothecium verrucaria*, *Galactomyces geotrichum* and *Mortierella verticillata* depolymerized even higher percentage (~92%) of rye straw hemicelluloses, but after 60 days of cultivation.[33]

CONCLUSION

The obtained results clearly show the exceptional cellulolytic potential of *T. viride* BEOFB1210m during the fermentation of biologically pretreated wheat straw. The special significance of the results lies in the fact that the highest activities were measured at the beginning of the cultivation process, which is an important parameter for the production of enzymes on an industrial scale. Considering the availability and low price of wheat straw as a substrate, it is clear that the obtained results inspire further research to optimize its saccharification to fermentable sugars.

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