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Polysaccharide-hydrolysing secretome of Schizophyllum commune during growth on different carbon sources

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A B S T R A C T

In the present work, the polysaccharide-hydrolysing secretome of Schizophyllum commune BCC 632 was analysed in submerged fermentation conditions to elucidate the effect of chemically and structurally different carbon sources on the expression of cellulases and xylanase. Among polymeric substrates, crystalline cellulose appeared to be the best carbon source providing the highest endoglucanase (53.5 U/mL), total cellulase (9.2 U/mL), and xylanase (636.1 U/mL) activities. The use of mandarin pomace as a growth substrate also allowed to achieve high volumetric activities of all target enzymes whereas wheat straw and xylan turned out to be the weakest inducers of enzyme production. The supplementation of the Avicel or wheat straw-based medium with a low concentration of easily metabolizable carbon source (mandarin pomace or glycerol) favored enzyme secretion. The addition of 0.5% glucose to the Avicel-containing medium caused short-term catabolite repression of the synthesis of both cellulase and xylanase while the addition of α-deoxy-D-glucose prevented enzyme secretion. It was shown that the presence of compounds inducing the formation of cellulase and xylanase by S. commune BCC 632 depends on the age of the fungal culture.

Keywords: Schizophyllum commune, Submerged fermentation, Cellulase, Xylanase, Carbon sources, Regulation.

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Introduction

Agro-industrial plant residues accumulating worldwide in huge quantities as wastes/by-products of crop cultivation and food processing are renewable, abundant, and cheap resources for bioconversion to various value-added products including biofuels, chemicals, animal feeds, and human nutrients [1,2]. Hydrolysis of biomass polysaccharides into fermentable sugars by cellulases and hemicellulases is the key step for enzymatic conversion of lignocellulose. Cellulases comprise endoglucanases (EC 3.2.1.4) which cleave internal β-1,4-glucosidic bonds of cellulose chains, exoglucanases (EC 3.2.1.91) which processively act on the reducing and non-reducing ends of cellulose to release short-chain cello-oligosaccharides, and β-glucosidases (EC 3.2.1.21) which hydrolyze soluble cello-oligosaccharides to glucose. As far as hemicellulases are concerned, endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) and auxiliary enzymes are required for the complete hydrolysis of xylan.

The main challenges for wide and large-scale application of cellulases and xylanases are reducing their cost and developing more efficient enzyme cocktails with high specific activity and stability [3,4]. Therefore, considerable research efforts were devoted to the bioprospecting of enzyme producers from less studied environments and exploitation of alternative sources of cost-effective enzymes. Wood- and litter-degrading basidiomycetes produce a variety of extracellular enzymes including glycoside hydrolases. Moreover, some of them have shown exceptional potential for the production of individual groups of hydrolytic enzymes under appropriate cultivation conditions. Thus, Coprinellus disseminatus produced 469 U/mL of alkali-thermo-tolerant xylanase along with negligible cellulase activity [5] while Armillaria gemina secreted up to 146 U endoglucanase/mL, 15 U β-glucosidase/mL, and 1.72 U FPA/mL [6]. Moreover, Jagtap et al. [7] achieved very high β-glucosidase activity (45.2 U/mL) in the submerged cultivation of Pholiota adiposa in a medium containing rice straw and corn steep powder.

Recently, during the extensive screening of wood and litter-deconstructing basidiomycetes for lignocellulolytic enzyme production, Schizophyllum commune BCC 632 has been revealed as a potent enzyme producer [8]. Several studies already reported on the ability of S. commune to secrete cellulase and xylanase and optimization of these enzyme productions [9-12]. It is worth noting that the crude enzyme cocktail derived from S. commune demonstrated superior performance over a commercial enzyme preparation from Trichoderma longibrachiatum in the hydrolysis of pre-treated lignocellulosic biomass at low enzyme loadings [11]. Moreover, S. commune was exploited for ethanol production from wood chips by consolidated bioprocessing [13]. However, studies on physiological mechanisms of cellulase and xylanase production by S. commune have been limited and more in-depth studies are required to understand how specific environmental factors modulate the secretion of individual cellulases and xylanase isoenzyme and develop the fungus enzyme system for biotechnological application. Therefore, in the present work, the polysaccharide-hydrolysing secretome of S. commune BCC 632 was analysed in submerged fermentation conditions to elucidate the effect of chemically and structurally different carbon sources on the expression of cellulases and xylanase.

Materials and methods

Organisms and inoculum preparation

Schizophyllum commune BCC 632 isolated from a tree branch in Georgia and deposited in the Institute of Microbial Biotechnology basidiomycetes culture collection has been used in this study. The fungal inoculum was prepared by growing the mycelium on a rotary shaker at 150 rpm and 27 oC in 250 mL flasks containing 100 mL of standard medium (g/L): glucose – 15.0, KH2PO4 – 0.8, K2HPO4 – 0.6, MgSO4 x H2O – 0.5, peptone – 3.0, yeast extract – 3.0, pH 6.0. After 7 days of fungal cultivation mycelial pellets were harvested and homogenized using a Waring laboratory blender.

Cultivation conditions for hydrolases production

The submerged cultivation was carried out using rotary shakers Innova 44 (New Brunswick, USA) at 160 rpm and 27 °C in 250-mL flasks containing 100 mL of the medium of following composition (g/L): KH2PO4 – 0.8, K2HPO4 – 0.6, MgSO4 x H2O – 0.5, (NH4)2SO4 – 5.0, yeast extract – 5.0, pH 5.8. Crystalline cellulose (Avicel), soluble carboxymethyl cellulose (CMC, low viscosity, Sigma, USA), xylan from birchwood (Sigma, USA) at a concentration of 15 g/L and milled to powder wheat straw and mandarin peels at a concentration of 40 g/L were used as the fungus carbon sources. Moreover, the effect of adding glucose, α-deoxy-D-glucose, methyl-α-D-glucose, and cycloheximide to the 1.5% Avicel-containing medium on enzyme synthesis was assessed in short-term experiments. Also, in separate short-term experiments, the fungus was grown in a medium with cellulose for 5 and 10 days, then the solids (biomass and cellulose) were separated by filtration. The resulting filtrate was boiled 5 min to inactivate available enzyme activity, added with 1.5% Avicel, then inoculated with homogenized biomass of the fungus grown both in a medium with glucose and in a medium with cellulose.

During the fungus cultivation, at predetermined time intervals, 1-2-ml samples were taken from the flasks, the solids were separated by centrifugation at 10,000 g for 10 min at 4 °C and the supernatants were analysed for pH, reducing sugars, protein content, and enzyme activities.

Analytical methods

Protein concentration in culture liquids was determined using the Bradford Reagent (Serva, Heidelberg, Germany) according to the manufacturer's instructions. The total cellulase activity (filter paper activity, FPA) was measured with Whatman filter paper No. 1 according to IUPAC recommendations [14]. Endoglucanase (CMCase) activity was assayed using 1% low-viscosity carboxymethyl cellulose in 50 mM citrate buffer (pH 5.0) at 50 oC for five minutes [14]. Xylanase activity was determined using 1% birchwood xylan (Roth 7500) in 50 mM citrate buffer (pH 5.0) at 50 oC for 10 min [15]. Glucose and xylose standard curves were used to calculate the cellulase and xylanase activities. In all assays, the release of reducing sugars was measured using the dinitrosalicylic acid reagent method [16]. One unit of enzyme activity was defined as the amount of enzyme, releasing 1 μmol of reducing sugars per minute. To measure β-glucosidase and β-xylosidase activities, the reaction mixture containing 1.8 mL of 2 mM solutions of p-nitrophenyl-β-D-glucopyranoside or p-nitrophenyl-β-D-xylopyranoside in 0.05 M acetate buffer, pH 4.8, and 0.2 mL of the enzyme solution was incubated at 50 °C for 10 min [17]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per minute.

Zymogram analyses of CMCase activity

The native-PAGE was performed using 10% separating and 5% stacking gels. Separating gels were incorporated with 1% carboxymethyl cellulose. A sample containing 10 μg protein was loaded in a well. Electrophoresis was performed at a constant 100 V for 3 h using a Vertical Gel Electrophoresis System (MRC, Israel). After electrophoresis, the native gel was removed from the glass plates, rinsed twice with distilled water, and then incubated in sodium citrate buffer (0.1M, pH 5.0). CMCase bands were developed after staining with 0.1% Congo red (w/v) for 30 min followed by de-staining with 1 mM NaCl until the clear zone was observed. Staining and de-staining procedures were performed on Rocker Shaker MR-1 (Biosan). The reaction was fixed with 5% acetic acid solution for 5 min.

Statistical analysis

All experiments were performed twice using three replicates each time. The results are expressed as the mean ± SD. The mean values, as well as standard deviations, were calculated by the Excel program (Microsoft Office 2010 package) and only values of $p \leq 0.05$ were considered as statistically significant.

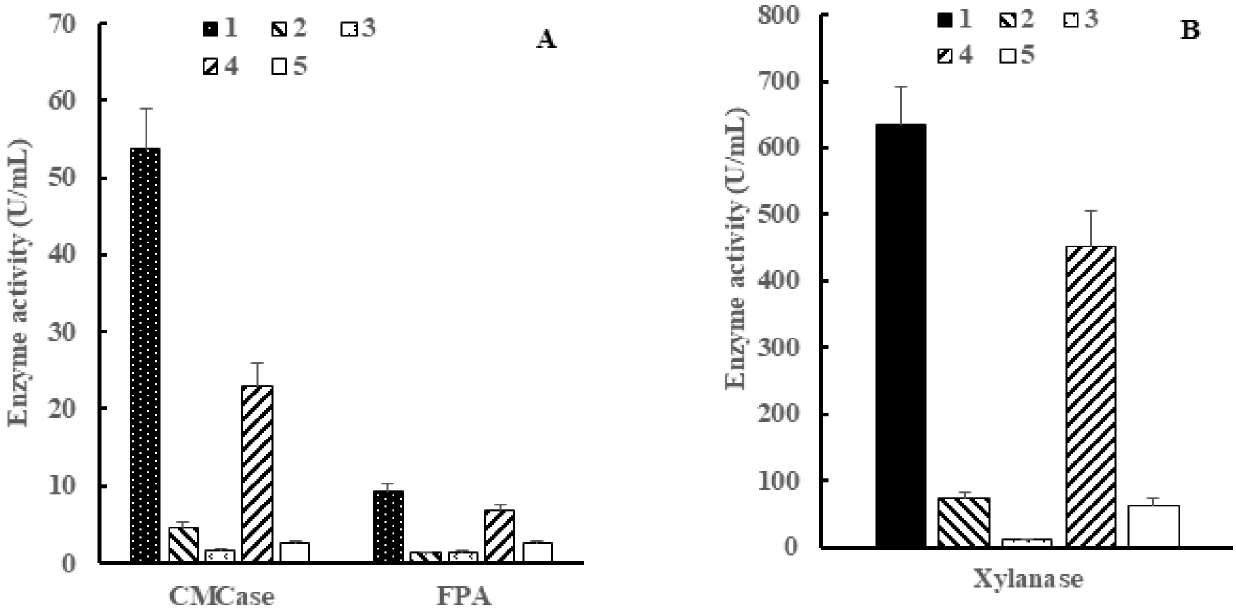


Fig. 1. *Effect of the polymeric carbon sources on the S. commune BCC632 cellulases (A) and xylanase (B) activities. Legend: 1 – 1.5% Avicel, 2 – 1.5% CMC, 3 – 1.5% xylan, 4 – 4% mandarin pomace, 5 – 4% wheat straw.*

Results

Effect of the polymeric carbon sources on the S. commune BCC632 enzyme activity

Initially, *S. commune* BCC 632 was cultivated in media containing crystalline cellulose, carboxymethyl cellulose, xylan, wheat straw, and mandarin pomace as carbon sources and the potential stimulators of cellulase and xylanase activities production. Growth on mandarin pomace followed by Avicel occurred at a faster rate while that on wheat straw being lowest. S. commune BCC 632 secreted cellulases and xylanase activities regardless of the material tested; however, the enzyme yield differed significantly. Crystalline cellulose appeared to be the best carbon source providing the highest endoglucanase (53.5 U/mL), total cellulase (9.2 U/

mL), and xylanase (636.1 U/mL) activities (Fig. 1). The use of mandarin pomace as a growth substrate also allowed to achieve high volumetric activities of all target enzymes. These results were far superior to those achieved with other materials used as substrates for fungal growth and enzyme production. Among them, xylan turned out to be the weakest inducer of enzyme production, including xylanase activity - 59 times lower than that in the microcrystalline cellulose-containing medium. It is worth noting that CMC and wheat straw induced comparatively significant xylanase activity secretion by S. commune BCC632, although the fungus cellulase activity was low.

Table 1. *Effect of easily metabolizable additional carbon sources on the S. commune BCC632 enzyme activity*

Substrate (carbon source)	CMCase (U/mL)	Xylanase (U/mL)	FPA (U/mL)	β-glucosidase (U/mL)	β-xylosidase (U/mL)
1.5% Avicel	52.5 ± 6.1	673.1 ± 70.2	9.0 ± 1.3	7.7 ± 0.6	0.14 ± 0.02
4% Mandarin pomace (MP)	22.4 ± 2.8	460.7 ± 57.3	7.1 ± 0.8	10.1 ± 0.9	0.18 ± 0.03
4% Wheat straw (WS)	3.8 ± 0.5	63.8 ± 8.6	2.2 ± 0.3	3.4 ± 0.4	0.14 ± 0.03
1.5% Avicel+4% MP	32.5 ± 3.8	780.1 ± 52.7	13.5 ± 1.5	18.0 ± 1.5	0.37 ± 0.07
1.5% Avicel+0.6% glycerol	50.8 ± 4.7	869.4 ± 86.4	14.0 ± 1.6	11.5 ± 0.9	0.41 ± 0.06
4% WS+4% MP	11.6 ± 1.4	364.8 ± 41.0	5.2 ± 0.5	5.9 ± 0.6	0.07 ± 0.01
4% WS+0.7% glycerol	16.4 ± 1.9	617.0 ± 51.8	6.8 ± 0.8	2.5 ± 0.4	0.17 ± 0.03

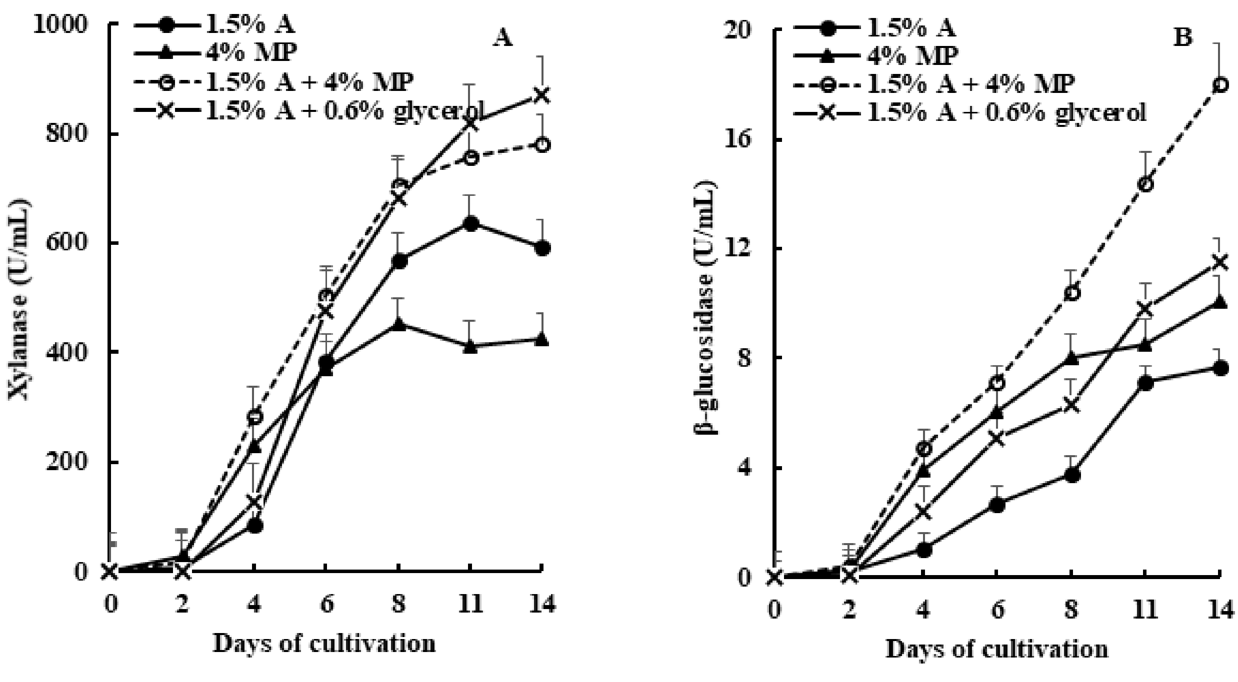
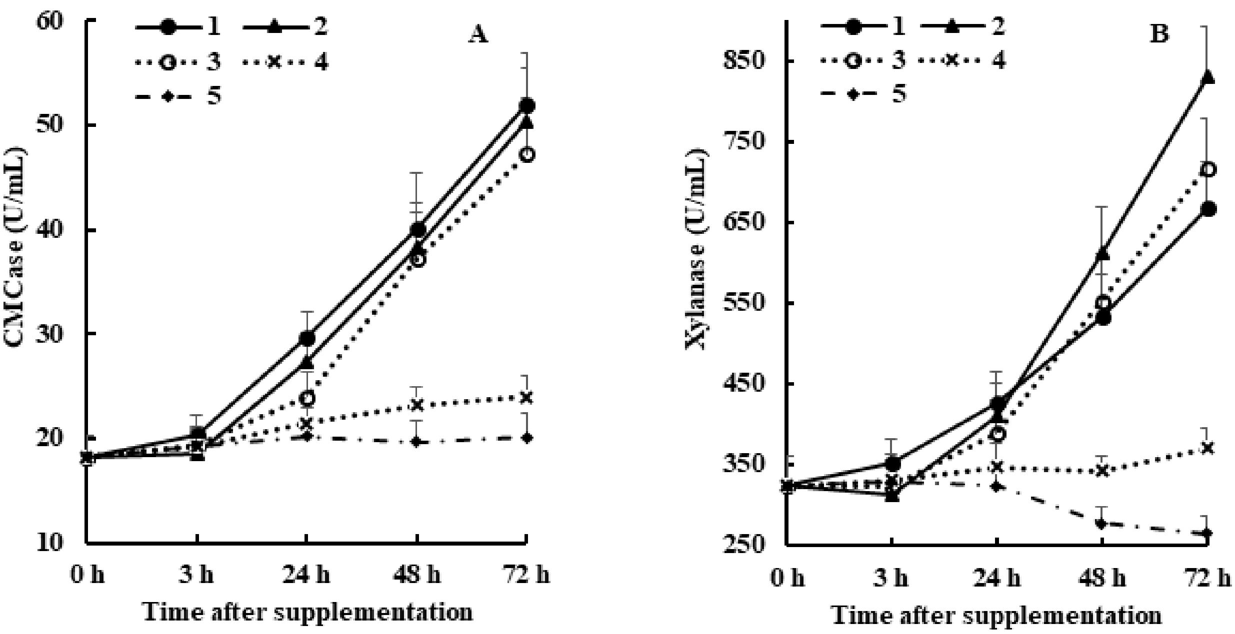


Fig. 2. *Effect of easily metabolizable additional carbon sources on the S. commune BCC632 xylanase (A) and β-glucosidase (B) activities.*

Effect of easily metabolizable carbon sources on the S. commune BCC632 enzyme activity

Subsequently, the effect of easily metabolizable carbon sources on hydrolases production by S. commune during submerged fermentation of Avicel and wheat straw was studied. We hypothesized that microcrystalline cellulose and lignified straw are recalcitrant growth substrates that retard the initial development of mushroom culture. Therefore, the use of mandarin pomace or glycerol as additional sources of easily metabolized carbon can accelerate fungal growth and rapid biomass accumulation, thus favoring enzyme accumulation. Indeed, supplementation of the Avicel-based medium with mandarin pomace caused a significant increase in both xylanase and FPA as well as a more than 2-fold increase of β-glucosidase and β-xylosidase activities of S. commune BCC632, although a 65% decrease of CMCase activity was observed (Table 1). Moreover, when mandarin pomace was added to the wheat straw-based medium, the fungus CMCase activity increased 3-fold along with the raised activity of other enzymes with the exclusion of β-xylosidase activity. The supplementation of the wheat straw-based medium with glycerol was even more beneficial for cellulase, xylanase, and β-xylosidase production, although the β-glucosidase activity was decreased. The addition of glycerol to the Avicel-containing medium did not change the fungus endoglucanase activity but gave the highest xylanase, FPA, and β-xylosidase yields. It is worth noting that the presence of glycerol in the Avicel-based medium caused a short-term delay in cellulases and xylanase accumulation, obviously due to catabolite repression of enzyme synthesis (Fig. 2). When this easily metabolizable carbon source was depleted, the fungal culture switched to cellulose metabolism and cellulase and xylanase induction took place with a gradual increase of the enzyme activity until the end of the experiment. In general, the addition of glycerol to the Avicel medium resulted in a 29% and 56% increase of xylanase and FP activities of S. commune BCC632, respectively. Interestingly, the supplementation of a cellulose-based medium with mandarin pomace especially promoted an increase in β-glucosidase secretion during the entire cultivation period of the fungus, ensuring the accumulation of as high as 18 U/mL of enzyme activity (Fig. 2B).



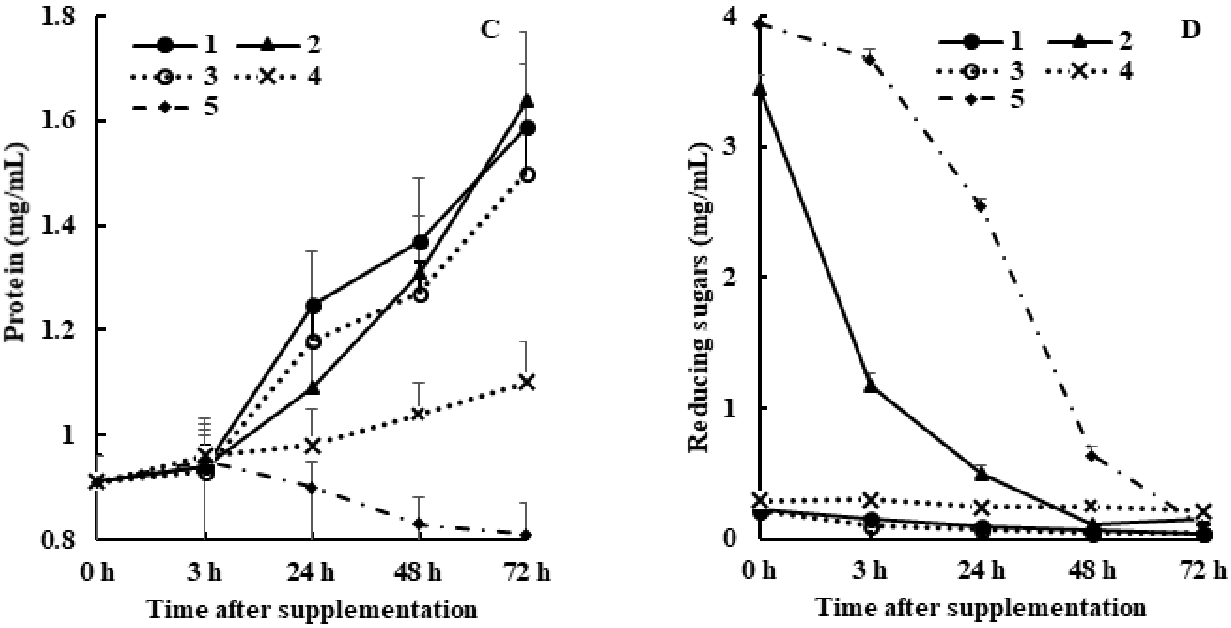


Fig. 3. *Effect of glucose analogues and cycloheximide on the S. commune BCC632 xylanase activity (A) and reducing sugars content. Legend: 1 – 1.5% Avicel, 2 – 1.5% Avicel + 0.5% glucose, 3 – 1.5% + 0.25% methyl-α-D-glucose, 4 – 1.5% Avicel + 0.25% α-Deoxy D-glucose, 5 – 1.5% Avicel + 1 μg/mL cycloheximide.*

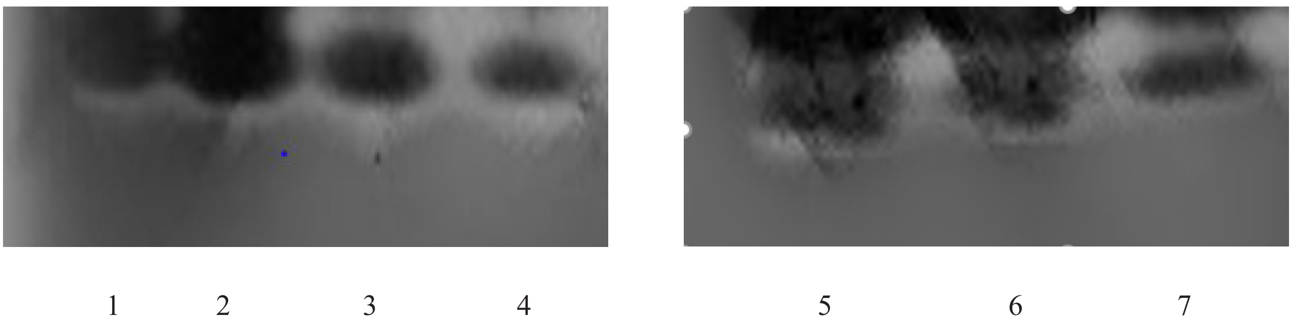


Fig. 4. *CMCase activity intensity in native electrophoresis of culture liquids after short-term cultivation of S. commune BCC632 with different carbon sources. Legend: 1 – 1.5% Avicel, 0 h; 2 – 1.5% Avicel, 24 h; 3 – 1.%5 Avicel + 0.5% glucose, 24 h; 4 – 1.5% Avicel + 0.5% glucose + 1 μg/mL cycloheximide, 24 h; 5 – 1.5% Avicel, 72 h; 6 – 1.5% Avicel + 0.5% glucose, 72 h; 7 – 1.5% Avicel + 0.5% glucose + 1 μg/mL cycloheximide, 72 h. The arrow shows bands of CMCase activity.*

Effect of glucose, glucose analogues, and cycloheximide on the S. commune BCC632 enzyme activity

In the next step, cellulolytic secretome was assessed in short-term experiments after the addition of glucose, α-deoxy-D-glucose, methyl-α-D-glucose, and cycloheximide to the *S. commune* BCC632 culture grew during 6 days in the 1.5% Avicel-containing medium. Monitoring of the pH values of the media did not reveal significant differences between cultures containing Avicel and cultures with added sugars.

The addition of 0.5% glucose to the Avicel medium caused catabolite repression of the synthesis of both cellulase (Fig. 3A) and xylanase (Fig. 3B) during at least 3 hours of cultivation. Subsequently, the production of cellulase resumed at the same rate as in the control variant, while the accumulation of extracellular xylanase was even more intense. This phenomenon is confirmed by the data on the concentration of reducing sugars in the fungal culture (Fig. 3C), which decreased after 3 hours to less than 0.1%, apparently sufficient for the derepression of enzyme synthesis. Supplementation of growing culture with methyl-α-D-glucose, a non-metabolizable *glucose* analogue, slightly reduced the secretion of both enzymes only within 24 h, especially in the first 3 hours of cultivation. On the contrary, the addition of α-deoxy-D-glucose to the *S. commune* BCC632 culture actively synthesizing target enzymes prevented the active secretion of cellulase and especially that of xylanase. As was expected, no CMCase production occurred in the presence of cycloheximide whereas xylanase activity gradually decreased obviously due to partial inactivation of the available enzyme. It is important to note that the protein content in the culture liquids obtained after cultivation of the fungus in media with Avicel and additives largely correlated with the dynamics of enzymatic activity (Fig. 3D). Finally, zymogram analyses showed that proteins with hydrolytic activity on CMC were detected in all gels. Native electrophoresis confirmed that crystalline cellulose acted as an inducing substrate for endoglucanase production by *S. commune* BCC632. In parallel with volumetric CMCase activity, the size and intensity of bands with CMCase activity after 24 h and 72 h cultivation increased (Fig. 4, lanes 2 and 5) compared with that inoculation time (Fig. 4, lane 1). The intensity of the band in lane 3 correlated with the CMCase activity measured after 24 h after the addition of 0.5% glucose but it was increased after 72 h of the fungus cultivation (Fig. 4, lane 6). When cycloheximide was used as an inhibitor of protein synthesis, significantly decreased size and intensity of bands with CMCase activity were observed after 24 h and 72 h cultivation (Fig. 4, lanes 4 and 7).

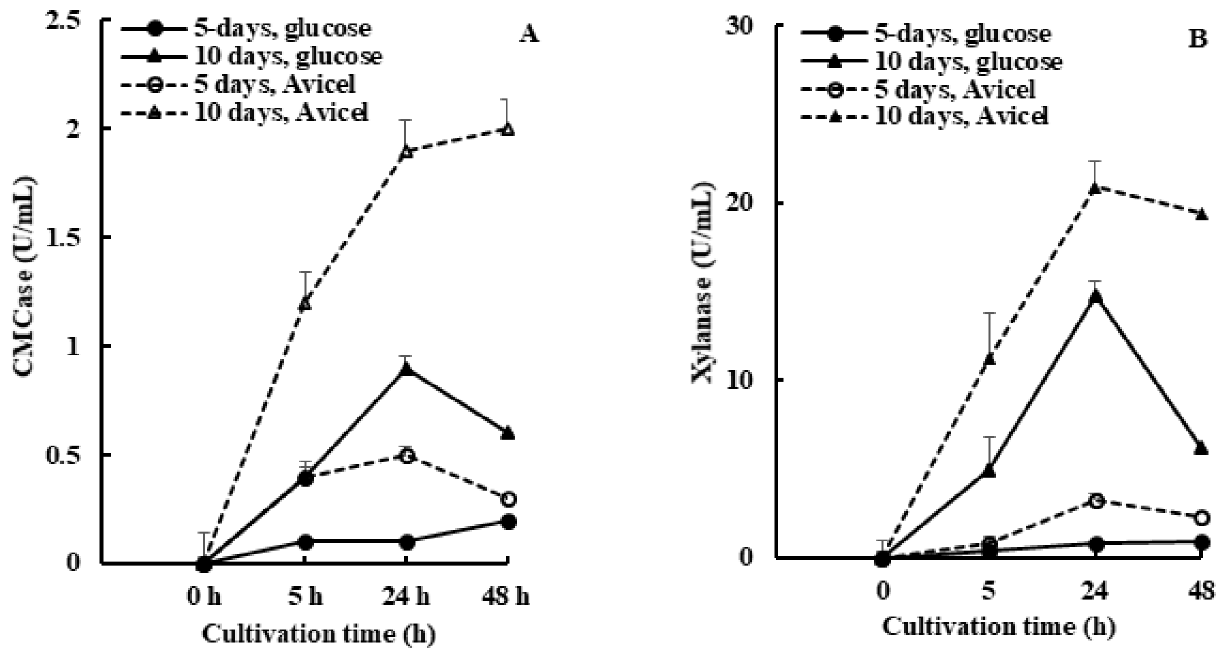


Fig. 5. *Secretion of cellulase (A) and xylanase (B) activity of S. commune BCC632 depending on the "age" of the filtrate and the carbon source in the inoculum culture.*

Testing the culture filtrate as a source of enzyme synthesis inducers

The results obtained in this study show that the secretion of cellulases and xylanases in the Avicel-containing medium takes place throughout the entire cultivation period, even when the growth of the fungus has practically ended. It can be assumed that the culture constantly contains substances that initiate the expression of genes responsible for the synthesis of these enzymes. To test this hypothesis, the filtrate of *S. commune* BCC632 grown in a medium with cellulose for 5 (logarithmic phase of growth) and 10 (stationary phase of growth) days was inoculated with homogenized biomass of the fungus grown both in a medium with glucose and in a medium with cellulose. The results shown in Fig. 5 indicate that the activity of both enzymes was significantly higher when the filtrate from the stationary growth phase was used for the cultivation of the fungus. Moreover, the kinetics of endoglucanase and xylanase secretion and the magnitude of the enzyme activity were preferable if the inoculated biomass was grown in a cellulose-containing medium.

Discussion

The secretome of *S. commune* BCC632 grown on crystalline cellulose showed a combination of all tested enzymes: endoglucanase, xylanase, β-glucosidase, and β-xylosidase. This fungus is a promising producer of cellulases and xylanase that play a crucial role in biomass polysaccharide hydrolysis and supplying the mushroom culture with a carbon and energy source. It is worth noting that this strain is an excellent producer of β-glucosidase. Another *S. commune* KUC9397 secreted as high as 43.51 U/mL β-glucosidase in the medium containing 2.96% cellulose, 2.3% soy peptone, and 0.11% thiamine [18]. Besides, Villavicencio et al. [19] showed remarkably high levels of xylanase and β-glucosidase activities of S. commune in solid-state fermentation of birch wood.

Like in Fomes fomentarius BCC 38, Panus lecometei BCC 903, Pseudotrametes gibbosa BCC 17, and Trametes versicolor BCC 13 [20], production of cellulase and xylanase by *S. commune* BCC 632 requires the presence of inducing substrate in the culture medium. However, similar to the listed mushrooms, xylan and carboxymethyl cellulose appeared to be poor substrates for *S. commune* BCC 632 growth and enzyme secretion whereas cellulose was the most appropriate growth substrate. Similarly, wild and mutant strains of S. commune produced cellulolytic and hemicellulolytic enzyme activities and as high as 1145 U xylanase/mL in the fermentation of microcrystalline cellulose Avicel-PH101 [12]. Likewise, cellulose caused the highest inductive effect on Ganoderma applanatum LPB MR-56 cellulase and xylanase production [21]. However, unlike *S. commune* BCC632, xylan and CMC appeared to be potent inducers of G. applanatum hydrolases synthesis.

Literature data indicate that cellulolytic and xylanolytic activities of basidiomycetes depend on lignocellulosic substrate chemical composition. Rice straw was the best growth substrate for endoglucanase production by Armillaria gemina [6]. This enzyme activity reached 146 U/mL when the fungus was cultivated in the presence of yeast extract at 10 g/l as a nitrogen source. Among lignocellulosic materials tested, rice straw provided the highest CMCase (44.4 U/mL) and β-glucosidase (6.5 U/mL) activities of S. commune, while alkaline pre-treated sugarcane bagasse promoted accumulation of 1326 U/mL xylanase [12]. Unlike these studies, in our work, wheat straw appeared to be a poor substrate for both cellulases and xylanase secretion by *S. commune* BCC632 while mandarin pomace provided high enzyme activity and contained some compounds favoring enhanced xylanase production. We assumed that one of the reasons for the low enzymatic activity of the fungus in the presence of wheat straw may be the lack of easily metabolizable sugars necessary to accelerate the initial fungal growth. In fact, the supplementation of wheat straw-based medium with mandarin pomace or glycerol resulted in a significant secretion of cellulase and xylanase by the fungus.

As seen, the production of xylanase by *S. commune* BCC632 is strictly linked to the presence of cellulose. Hence, this fungus released low molecular weight soluble catabolites from the cellulose polymer, which served as signaling molecules promoting the simultaneous formation of both cellulase and xylanase. Based on the results, it can be concluded that the formation of cellulase and xylanase is inducible in S. commune BCC632 and it is under common control. At the same time, one should pay attention to significant differences in the regulatory control of these enzyme activities. The most obvious distinction concerns the predominant production of xylanase by the fungus regardless of the growth substrate composition.

The results obtained in this study show that in the microcrystalline cellulose-based medium the fungal culture contains compounds initiating/maintaining the secretion of cellulase and xylanase. Obviously, a metabolically less active 10-days culture of*S. commune* BCC632 that has reached the stationary growth phase contains a higher concentration of inducing compounds (cellooligosaccharides and xylooligosaccharides) than an intensively developing 5-days culture. Upregulation of cellobiohydrolase and endoglucanase gene expression in culture medium containing cellotriose and cellotetraose derived from cellulose was shown for Polyporus arcularius [22]. Likewise, the addition of cellotriose and cellotetraose increased transcript levels of several cellulolytic genes in Phanerochaete chrysosporium [23]. It is worth noting that in our short-term experiments, intensive secretion of CMCase and xylanase was observed during 24 h of cultivation. Subsequently, the production of enzymes sharply decreased, as we assume, due to the hydrolysis of oligosaccharides by β-glucosidase and β-xylosidase. Further research is needed to understand the mechanisms of regulation of cellulases and hemicelluloses by *S. commune* BCC632, which may have potential in large-scale applications of lignocellulose degradation.

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