

Junko Sugano*, Riikka Linnakoski, Seppo Huhtinen, Ari Pappinen, Pekka Niemelä and Fred O. Asiegbu

Cellulolytic activity of brown-rot *Antrodia sinuosa* at the initial stage of cellulose degradation

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Abstract: The initial stage of cellulose degradation has been studied via *in vitro* assays of fungi isolated from rotten wood in a boreal forest. Among the 37 isolates, *Antrodia sinuosa* appeared to be an effective cellulose degrader and was selected for studying the initial degradation process. In the liquid cultivation with carboxymethylcellulose (CMC), the increase of the mycelial dry weight coincided with the pH decrease of the culture medium from pH 5.7 to 3.9, between the 3rd and 6th cultivation day. At the same time, the cellulolytic activity increased; the CMCase activity increased sharply and the reducing sugars reached their maximum concentration in the culture medium. It seems that the decreasing pH enables the cellulose degradation by *A. sinuosa* at an early stage of the process. The results of this study may be useful for a more efficient industrial application of biomass by means of brown-rot fungi.

Keywords: *Antrodia sinuosa*, biomass saccharification, brown-rot, cellulose degradation, CMCase, enzymatic activity, fungal pretreatment, initial stage

Introduction

Renewable biomasses are promising alternative resources for the production of biofuels and bio-materials, such as

***Corresponding author: Junko Sugano**, Department of Microbiology, University of Helsinki, P.O. Box 56, FI-00014 Helsinki, Finland; and Department of Biology, University of Turku, FI-20014 Turku, Finland, e-mail: junko.sugano@helsinki.fi. <https://orcid.org/0000-0002-7678-867X>

Riikka Linnakoski: Natural Resources Institute Finland (Luke), Latokartanonkaari 9, FI-00790 Helsinki, Finland; and Department of Forest Sciences, University of Helsinki, P.O. Box 27, FI-00014 Helsinki, Finland. <https://orcid.org/0000-0002-3294-8088>

Seppo Huhtinen and Pekka Niemelä: Herbarium, Biodiversity Unit, University of Turku, FI-20014 Turku, Finland

Ari Pappinen: Faculty of Science and Forestry, School of Forest Sciences, University of Eastern Finland, P.O. Box 111, FI-80101 Joensuu, Finland

Fred O. Asiegbu: Department of Forest Sciences, University of Helsinki, P.O. Box 27, FI-00014 Helsinki, Finland

nanocellulose and chemical feedstocks (Deepa et al. 2015; Guerriero et al. 2016), but the related processes are not easy routines. Biomasses from forestry and agricultural residues are complex and contain an interpenetration network of polysaccharides and lignin (shortly: lignocellulosics) (Kumar et al. 2016). Cellulose with its long chains of anhydroglucose is the major component (roughly around 43%) of plant biomass. Cellulose can be degraded into the fermentable glucose by wood-rot fungi, but the degradation is slow because of the complex structure of lignocellulosics (Himmel et al. 2007). The same is true for industrial processes via several chemical and physical pretreatment steps (Mosier et al. 2005), which are expensive and polluting (Shirkavand et al. 2016). The direct biological degradation of cellulose with living fungi is an alternative and environmentally friendly saccharification method (Wan and Li 2012; van Kuijk et al. 2015).

This topic has been frequently investigated (Mathews et al. 2016; Agematu et al. 2017) and the feasibility of a fungal pretreatment as an alternative to physical and chemical cellulose conversion has been described (Keller et al. 2003; Bak et al. 2009; Shi et al. 2009; Xu et al. 2009; Dias et al. 2010; Sindhu et al. 2016). The challenges of an industrial application are the long pretreatment time and the substantial loss of cellulose and hemicelluloses (Balan 2014), and the optimization of the cultivation conditions and sugar yields (Kumar et al. 2008; Wan and Li 2012). The process development begins by the selection of suitable fungal species followed by more detailed analyses of their enzymatic capacity. The hypothesis for the present work was that a better knowledge of the initial degradation processes of cellulose is useful for the optimization of lignocellulose pretreatment for wood saccharification, for example, in terms of cellobiose and glucose formation, which could be further utilized by yeast in ethanol fermentation. This is the reason why, in the present work, a screening experiment was performed. Among the 37 fungal strains, *Antrodia sinuosa* (a brown-rot fungus) was found to be the most effective cellulose degrader and thus this fungus will be analyzed with more detail. This fungus was already studied concerning its growth rate, wood decay ability and culturing parameters (Schmidt and Moreth 1996; Bigelow et al.

1998; Matheron et al. 2006). Renvall (1995) observed the early stages of conifer trunk decay by *A. sinuosa* but its cellulose degradation performance was not yet studied. The present study will fill this gap, with carboxymethyl-cellulose (CMC; amorphous cellulose) and a crystalline cellulose (CrC) as model substrates. CMC is well suited to the observation of microbial cellulose degradation, while the CMCase activity can be assayed by measuring reducing sugars as a result of endoglucanase activity (Tomšovský et al. 2009; Dashtban et al. 2010; Longoni et al. 2012). CrC is a substrate similar to natural cellulose in the cell wall (Highley 1980; Tanaka et al. 2009; Kogo et al. 2017). During the degradation process, mycelial growth, pH, cellulose degrading products (reducing sugars and glucose), cellulose related enzyme activities (CMCase and β -glucosidase) and total extracellular proteins will be measured.

Materials and methods

Fungal isolates: Samples of rotten woods and fungal fruiting bodies were collected from five different locations (Tervola, Rovaniemi, Joensuu, Viitasaari and Turku) in boreal forests in Finland. Samples were plated onto 2% malt extract agar (MEA) and incubated at 25°C until fungal growth was observed. Pure cultures were obtained by transferring the mycelium from the edges of single colonies. The pure fungal cultures were stored at 4°C.

Fungal isolates were identified by molecular methods. The isolates were cultured on 2% MEA prior to DNA extraction. Fungal DNA was extracted with the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following the kit user's manual. The DNA was amplified via polymerase chain reaction (PCR) with the internal transcribed spacer region (ITS1 and ITS2, including the 5.8S gene) primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The DNA was amplified in a 25 μ l reaction mixture that contained 0.25 μ l of Phusion® High-Fidelity DNA polymerase (2 U μ l⁻¹) (Finnzymes, Espoo, Finland), 5 μ l of Phusion® HF reaction buffer (5 \times), 0.75 μ l of 100% DMSO (supplied with the enzyme), 0.5 μ l of dNTP's (10 mM) (Finnzymes, Espoo, Finland) and 0.50 μ l of each primer (10 mM) (Invitrogen, Carlsbad, CA, USA). PCR reactions were performed on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR conditions: an initial denaturation step at 98°C for 30 s, followed by 35 cycles per 10 s at 98°C, 30 s at 57°C and 30 s at 72°C, and a final chain elongation at 72°C for 8 min. Amplified products were visualized by the Lonza FlashGel System (Lonza Rockland Inc., Rockland, ME, USA).

PCR products were purified and sequenced at Macrogen Europe (Amsterdam, The Netherlands). Sequences were edited, and consensus sequences were determined by Geneious R8 8.0.3. All sequences obtained in this study were deposited in the GenBank (Table 1, Clark et al. 2015). The fungal isolates were identified by Megablast, searching the nucleotide database in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Identification was conducted with caution due to existence of misidentified sequences in the GenBank.

Screening of fungal isolates: All fungal isolates were tested for their ability to metabolize CMC as a carbon source by cultivating them on agar plates with and without CMC with three biological replicates. The agar plates were prepared with CMC (0.1% CMC, Alfa Aesar, Ward Hill, MA, USA) (1 g of CMC, 1 g of K₂HPO₄, 0.5 g of MgSO₄ · 7 H₂O, 0.5 g of KCl, 0.01 g of FeSO₄ · 7 H₂O and 15 g of agar in 1 l of pure water at pH 6.0) and without CMC. Isolates were cultivated at 22°C, which is the median optimum for fungal growth, in the dark for 15 days. The radial growth was followed for 3 days. After the 15-days' cultivation, the agar plates were melted in hot water and filtered through Whatman qualitative filter paper Grade 1 (GE Healthcare, Chicago, IL, USA). The mycelium was separated from the filter paper with a pair of tweezers, dried on a weighing paper at 40°C overnight and weighed. To determine the optimal cultivation period, a preliminary 18-days' cultivation had been performed for four fungal isolates, one from each phyla or subphyla: *Trichoderma polysporum* (Ascomycota), *A. sinuosa* (Basidiomycota), *Mortierella zychae* (Mortierellomycotina) and *Mucor* sp. (Mucoromycotina).

The significance of the difference between fungi grown (dry weight) with and without CMC was analyzed by Student's t-test (P-value < 0.05) performed with R Version 3.3.1 (R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria). The species (*A. sinuosa*) that had the greatest difference between the agars with and without CMC was chosen for further experiments.

In vitro cultivation of *A. sinuosa*: The fungus was cultivated in 100 ml Erlenmeyer flasks with 25 ml of a liquid modified basal Douglas fir cotyledon revised medium [modified DCR: 400 mg of NH₄NO₃, 340 mg of KNO₃, 85 mg of CaCl₂ · 2 H₂O, 556 mg of Ca(NO₃)₂ · 4 H₂O, 170 mg of KH₂PO₄, 370 mg of MgSO₄ · 7 H₂O, 39 mg of Na-Fe-EDTA, 22.3 mg of MnSO₄ · H₂O, 0.25 mg of CuSO₄ · 5 H₂O, 8.6 mg of ZnSO₄ · 7 H₂O, 6.2 mg of H₃BO₃, 0.25 mg of Na₂MoO₄ · 2 H₂O, 0.025 mg of CoCl₂ · 6 H₂O, 0.83 mg of KI, 0.025 mg of NiCl₂, 1 mg of thiamine-HCl, 0.5 mg of pyridoxin-HCl, 0.5 mg of nicotinic acid and 2 mg of glycine in 1 l of pure water at pH 6.0] (Gupta and Durzan 1985) containing 1 g of CMC as a sole carbon source. The pH value was adjusted to 5.7. The liquid cultivations were inoculated from freshly cultured MEA plates with a 7 mm diameter agar plug. A total of 18 flasks of *A. sinuosa* in the medium was prepared; three biological replicates for each six destructive sampling. Fungi were incubated under constant normal conditions at 25°C with shaking (100 rpm) (MaxQ 4000, Thermo Fisher Scientific, Waltham, MA, USA) for 15 days and sampled every 3 days.

After 15 days cultivation, the culture pH was measured, and visible fungal mycelium was weighed after filtering as described. The remaining culture medium was sterile filtered through a 0.2 μ m syringe filter (VWR international, Radnor, PA, USA) to remove spores and all mycelium and stored at -20°C for further analyses.

The increase of mycelial dry weight reflects the amount of CMC consumption, which was determined via measuring reducing sugars by means of the dinitrosalicylic acid (DNS) method (Wood and Bhat 1988; Fu et al. 2010). The concentration of glucose was measured via the Glucose (GO) Assay Kit (Sigma-Aldrich, St Louis, MO, USA) according to manufacturer's instructions. The absorbance at 540 nm was measured with a spectrophotometer (UV-1800 UV Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) with glucose as standard.

To assess the enzyme production of the fungal isolate, the concentration of total extracellular proteins was determined by the Coomassie (Bradford) Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions, with bovine serum albumin (BSA) as standard. The extracellular enzyme

Table 1: Fungal species isolated from rotten wood and fruit bodies in Finland.

Phylum/Subphylum Species name	Pers.ID no.	CBS ^a no.	Host	Origin	GenBank acc. no.
Ascomycota					
<i>Trichoderma polysporum</i>	1	142256	<i>Picea abies</i> (L.) H. Karst.	Tervola	KP174729
<i>T. polysporum</i>	27		Coniferous wood ^b	Tervola	KP174753
<i>T. polysporum</i>	14		Mycorrhizal	Tervola	KP174741
<i>Trichoderma pulvinatum</i>	40		Polypore	Tervola	KP174763
<i>T. pulvinatum</i>	13	142265	<i>Fomitopsis pinicola</i>	Joensuu	KP174740
<i>Trichoderma paraviridescens</i>	36	142275	Coniferous wood ^b	Joensuu	KP174761
<i>Trichoderma viride</i>	18	142269	<i>Phellinus</i> sp.	Tervola	KP174745
<i>T. viride</i>	19		<i>Pholiota</i> sp.	Tervola	KP174746
<i>T. viride</i>	20		Coniferous wood ^b	Tervola	KP174747
<i>T. viride</i>	22		<i>Phellinus</i> sp.	Tervola	KP174749
<i>Trichoderma hamatum</i>	26	142271	<i>Prunus padus</i> L.	Turku	KP174752
<i>Trichoderma atroviride</i>	4	142259	Unknown	Tervola	KP174732
<i>Monographella lycopodina</i>	11	142263	<i>Phellinus</i> sp.	Tervola	KP174738
<i>Scytalidium lignicola</i>	32	142274	Stump of <i>Betula</i> sp. L.	Viitasaari	KP174757
<i>Phoma</i> sp.	37		<i>Pinus sylvestris</i> L. (d.w. ^c)	Rovaniemi	KP174762
<i>Phoma</i> sp.	15	142266	Mycorrhizal	Tervola	KP174742
Basidiomycota					
<i>Antrodia sinuosa</i>	43	142277	Coniferous wood ^b	Tervola	KP174765
<i>Antrodia xantha</i>	24	142270	Coniferous wood ^b	Tervola	KP174751
<i>Fomitopsis pinicola</i>	42	142276	Coniferous wood ^b	Tervola	KP174764
<i>Gloeophyllum sepiarium</i>	31	142272	<i>Picea abies</i> (L.) H. Karst.	Tervola	KP174756
<i>G. sepiarium</i>	34		Coniferous wood ^b	Tervola	KP174759
<i>Irpex lacteus</i>	2	142257	<i>Prunus padus</i> L.	Turku	KP174730
<i>Pleurotus</i> sp.	29	142273	Unknown	Joensuu	KP174755
<i>Trametes pubescens</i>	44	142278	Deciduous wood	Joensuu	KP174766
<i>Bjerkandera adusta</i>	45	142279	Unknown	Joensuu	KP174767
Mortierellomycotina					
<i>Mortierella hyalina</i>	9	142262	<i>Salix caprea</i> L.	Tervola	KP174737
<i>M. hyalina</i>	21		Deciduous wood	Tervola	KP174748
<i>M. hyalina</i>	23		Deciduous wood	Tervola	KP174750
<i>M. hyalina</i>	35		Coniferous wood ^b	Tervola	KP174760
<i>Mortierella alpina</i>	3	142258	Unknown	Tervola	KP174731
<i>Mortierella zychnae</i>	6		<i>Phellinus</i> sp.	Joensuu	KP174734
<i>Mortierella verticillata</i>	5	142260	Coniferous wood ^b	Tervola	KP174733
Mucoromycotina					
<i>Mucor</i> sp.	7	142261	<i>Crustoderma dryinum</i>	Tervola	KP174735
<i>Umbelopsis ramanniana</i>	16	142267	<i>Picea abies</i> (L.) H. Karst.	Tervola	KP174743
<i>Umbelopsis isabellina</i>	12	142264	<i>Prunus padus</i> L.	Tervola	KP174739
<i>U. isabellina</i>	28		Unknown	Viitasaari	KP174754
<i>Absidia psychrophilia</i>	17	142268	<i>Picea abies</i> (L.) H. Karst.	Tervola	KP174744

^aCBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; ^b*Picea abies* or *Pinus sylvestris*;

^cdry wood.

activity measurements were based on the release rate of reducing sugars [indicating CMCase and filter paper cellulase (FPase) as endo-glucanases for amorphous and CrC degradation] and glucose (indicating β -glucosidase). The methods of literature have been applied with slight modifications as indicated in the following: For the CMCase activity measurement (Ghose 1987; Fu et al. 2010), 0.5 ml of each sample medium was mixed with 0.5 ml of 2% (w/v) CMC solution in 0.05 M citrate buffer (pH 4.8) and incubated at 50°C for 30 min. Then, 0.5 ml of the incubated solution was reacted with 1.5 ml of DNS reagent at 100°C for 5 min. After cooling down, water (8 ml) was added.

For FPase activity (Wood and Bhat 1988), a rolled strip of Whatman No. 1 filter paper (1×6 cm), 1 ml of 0.05 M citrate buffer (pH 4.8) and 0.5 ml of sample medium were introduced into 50 ml capacity test tube. After incubating the test tube at 50°C for 60 min, 3 ml of DNS was added and boiled for 5 min. After cooling, 20 ml of distilled water was added and kept in the test tubes for 20 min. For β -glucosidase activity (Sternberg 1976; Lowe et al. 1987; Yoon et al. 2008), 0.5 ml of each sample medium and 0.5 ml of 10 mM cellobiose solution in 100 mM sodium acetate buffer (pH 5.0) were mixed and incubated at 50°C for 30 min. Then the incubated samples were boiled for 5 min.

The spectrophotometric analyses were performed as described. The enzymatic activities were calculated as $1 \mu\text{mol of Glc min}^{-1} \text{ml}^{-1}$ of medium and expressed as 1 unit ml^{-1} . The same experiments were conducted with CrC instead of CMC in the culture medium.

Results and discussion

Screening of the species

In total, 37 fungal strains (from 25 species) were isolated from rotten wood or fruiting bodies in a boreal forest in Finland (Table 1). Many of them were wood-decay polypores (Polyporales, Basidiomycota) that were already described as lignocellulose degrading fungi (Mansfield et al. 1998; Elisashvili et al. 2008; Tomšovský et al. 2009; Casieri et al. 2010; Quiroz-Castañeda et al. 2011). The species represent the members of the fungal phyla and subphyla Ascomycota, Basidiomycota, Mortierellomycotina and Mucoromycotina.

In the preliminary growth test concerning the cultivation period of fungi, mycelial dry weights of *T. polysporum* and *A. sinuosa* were higher in the case when grown on agar with CMC (Figure S1a,b). The difference became larger with longer cultivation time. The mycelial dry weights of *M. zychae* and *Mucor* sp. were about the same with or without CMC (Figure S1c, d). The fungal growth, except for *T. polysporum*, reached a steady state within 15 days. Therefore, the species were cultured for 15 days in the screening test, in which all species were able to grow on the plate in the absence of CMC and some species even preferred agar

as a carbon source (Figure 1). However, several fungal species had higher mycelial dry weight after growing on agar plates with CMC than without CMC. Five out of the 37 isolates, namely two isolates of *T. polysporum* (personal isolate No. 1 and 14), *A. sinuosa* (No. 43, CBS 142277), *Mucor* sp. (No. 7) and *Absidia psychrophilia* (No. 17), showed significantly higher mycelial dry weight on the plates with CMC than without (t-test, $P < 0.05$). Obviously, these five species preferred CMC instead of agar as a carbon source.

Antrodia sinuosa was selected for further investigation because the difference of the dry weight between the plates with and without CMC was twice as great among the isolated species. Cultivation on agar plates confirmed the ability of *A. sinuosa* for cellulose degradation. The mycelial dry weight on CMC plates was 2.4 mg and without CMC 1.2 mg (Figure 1). Moreover, the growth rate of the mycelia on CMC plates was 2.3 mm day^{-1} and without CMC 2.1 mm day^{-1} . Accordingly, *A. sinuosa* needed CMC to build cell biomass although it was able to also grow without CMC. *Antrodia sinuosa* was studied before for its wood decay efficiency and agricultural influence (Bigelow et al. 1998; Matheron et al. 2006; Brischke et al. 2008) but neither its cellulose degradation *in vitro*, nor its biochemical changes in the initial stage of cellulose degradation were hitherto observed.

Cellulolytic activity of *A. sinuosa* *in vitro* cultivation

The mycelial dry weight of *A. sinuosa* showed an increasing trend until the 12th day during the 15-days'

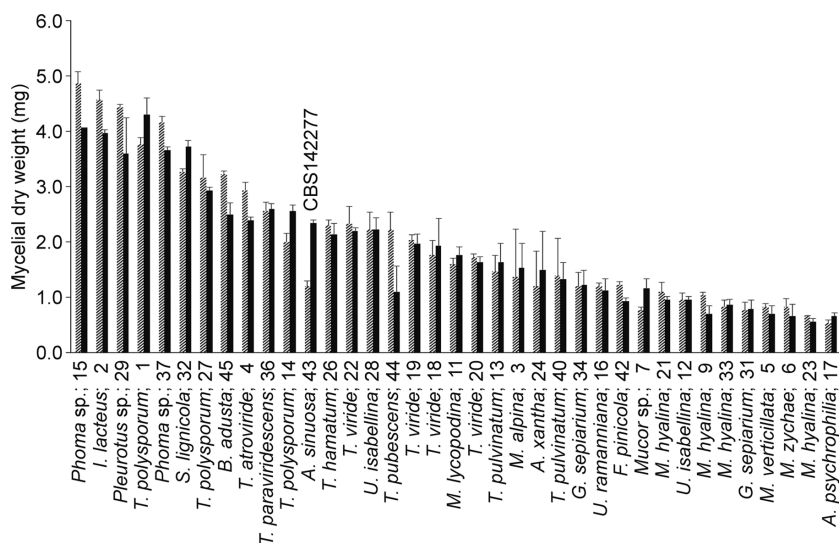


Figure 1: Mycelial dry weight of fungal isolates grown on agar without (shaded bars) and with (black bars) CMC for 15 days. The numbers after species refer to the personal isolation number. Error bars refer to standard deviation (SD) ($n = 3$).

cultivation in liquid medium with CMC as a sole carbon source (Figure 2a). The maximum growth rate was 0.6 mg day^{-1} from the 3rd to the 6th day, which was slowed down after 6 day. The maximum dry weight (3.9 mg) was observed on the 12th growing day. The pH value of the culture medium decreased after the inoculation day (pH 5.7) (Figure 2a). A considerable drop in the pH was observed from the 3rd to the 6th day, from pH 5.4

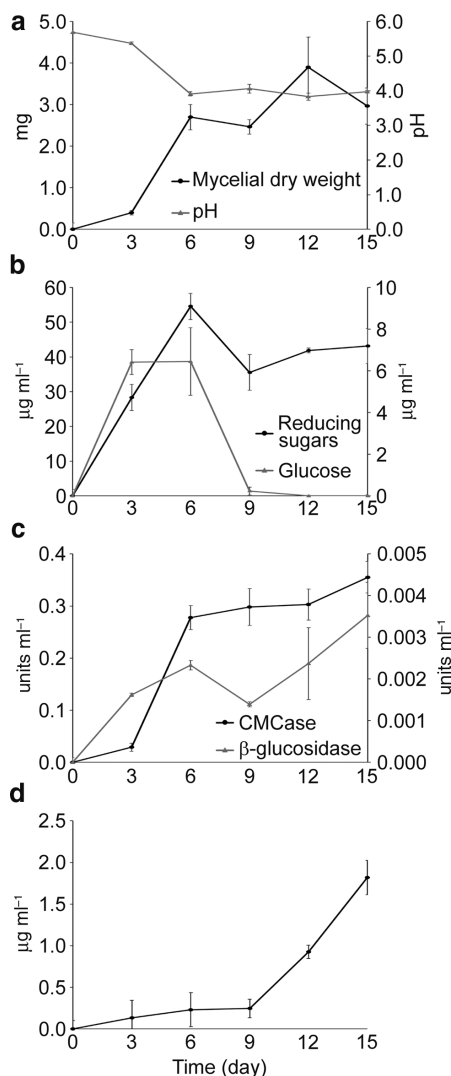


Figure 2: Correlations of cell growth of *A. sinuosa* and CMC degradation activities. (a) Mycelial dry weight (left y-axis) of *A. sinuosa* grown in liquid culture medium with CMC and the pH (right y-axis) of the culture medium during the incubation. (b) Concentrations of reducing sugars and glucose (the left and right y-axes, respectively) in *A. sinuosa* culture medium with CMC during the incubation. (c) Extracellular CMCCase and β -glucosidase (the left and right y-axes, respectively) activity during the incubation. (d) Total extracellular protein concentrations in *A. sinuosa* culture medium with CMC during the incubation. Error bars refer to SD ($n=3$).

to pH 3.9 and the pH was equilibrated to a nearly constant pH 4.

In the incubation with CMC, the increase of *A. sinuosa* mycelial weight coincided with the decrease of culture medium pH, from pH 5.7 to 3.9. Thus, \approx pH 4 was the optimum for the *A. sinuosa* growth. The same optimum was previously observed for *A. camphorata* (Shu and Lung 2004) and the optimal pH of cellulolytic hydrolase in general seems to be between 2.5 and 4.5 (Baldrian 2008). Several acidic compounds were identified in the culture medium of another *Antrodia* species, e.g. *A. cinnamomea* (Wu et al. 2011). Our results can be interpreted that *A. sinuosa* produces acid metabolites at the early stage of cellulose degradation, which are leading to an optimized pH. The oxalic acid production of brown-rot fungi is known in the context of Fenton reaction (Arantes and Goodell 2014). Nevertheless, the causality of the pH and cellulase activities in the case of *Antrodia* species needs to be studied in more detail.

The concentration of reducing sugars increased after inoculation but decreased from the 6th to the 9th day and slightly increased again until the 15th day (Figure 2b). The maximum concentration of the reducing sugars was $54.6 \mu\text{g ml}^{-1}$ on the 6th day. The glucose concentration increased from the inoculation day to the 3rd day and was relatively high until the 6th day (Figure 2b), with a maximum of $6.5 \mu\text{g ml}^{-1}$ and then decreased to a relatively low level. The CMCCase activity increased slightly until the 3rd day (Figure 2c) and increased rapidly between the 3rd and 6th day and later the increment was moderate and ended up with a maximum of 0.36 U ml^{-1} . The β -glucosidase activity increased relatively sharply until the 6th day (Figure 2c) and decreased between the 6th and 9th day and increased again beyond this time to reach a maximum at the 15th day (0.0035 U ml^{-1}). The FPase activity, which indicates CrC degradation, hardly changed during the cultivation in the CMC medium, only a slight increment was observed (Figure S2). It can be assumed that the fungus does not have enzymatic activity against CrC as the cultivating substrate was CMC. Therefore, FPase activity measurement was not especially useful in this context.

The fungus produced CMCCase that degraded the polymeric structure of CMC into oligomers, i.e. reducing sugars. The increase of glucose concentration indicate that these oligomers were further degraded into monomers, i.e. glucose, by β -glucosidase, as described by Lynd et al. (2002) and Rytioja et al. (2014). Concerning the initial stage of CMC degradation by *A. sinuosa*, the cellulose conversion into oligomers is more efficient than that of oligomers into glucose. This result is meaningful considering of industrial applications, where the requirement

to use lignocellulosic biomass is that after the fungal saccharification, sugars should be converted into ethanol by yeasts. It was reported that an engineered *Saccharomyces cerevisiae* can consume cellobiose effectively and ferment it to ethanol (Ha et al. 2011). Therefore, *A. sinuosa* could be a promising fungus as cellobiose provider for ethanol production.

The concentration of total extracellular proteins remained on a relatively low level until the 9th day (Figure 2d) and then increased rapidly until the maximum ($1.8 \mu\text{g ml}^{-1}$) was reached on the 15th day. *Antrodia* species produce endoglucanase and β -glucosidase (Tomšovský et al. 2009) and the gene models for these enzymes were detected in the *A. sinuosa* genome [Joint Genome Institute (JGI) website (<https://jgi.doe.gov/>)]. Therefore, the CMCase and β -glucosidase activities detected in the present paper are in line with the literature data.

No significant changes were observed in the presence of CrC in the culture medium but all values were lower than the respective values with CMC in the culture medium. The mycelial dry weights could not be measured accurately because the aggregates with the CrC powder and the mycelia could not be separated. The pH value slightly decreased after the inoculation day and was the lowest (pH 5.05) on the 12th day (Figure S3). The concentration of reducing sugars increased after inoculation and decreased from the 3rd to the 6th day and slightly increased again from the 12th day on (Figure S4a). The glucose concentration increased from the inoculation day to the 3rd day and then decreased to a low level (Figure S4a). The CMCase activity increased gradually from the inoculation day and reached a maximum on the 15th day (0.081 U ml^{-1}) (Figure S4b). The β -glucosidase activity increased from the inoculation day to the 3rd day to its maximum (0.0008 U ml^{-1}) and then decreased (Figure S4b). The total extracellular protein concentration increased until the 3rd day and then decreased until the 12th day and increased rapidly after the 12th day again (Figure S5). The end point of total protein concentration reached similar amount as seen in the CMC culture medium. Filter paper is composed of CrC and thus, it can be assumed that the FPase activity should have been higher for CrC than CMC. However, the FPase activity was almost steady and slightly lower for the CrC culture medium compared to that with CMC (Figure S2). In contrast to previous reports which showed efficient CrC degradation by cellulases of some brown-rot species (Cohen et al. 2005; Lee et al. 2008), CrC did not induce production of cellulases in *A. sinuosa*. Therefore, the conclusions can be drawn based on CMC degradation alone.

Conclusions

Antrodia sinuosa, a brown-rot fungus, was found to be a promising species for industrial cellulose degradation. *Antrodia sinuosa* metabolized CMC to support its growth and produced cellulolytic enzymes in the initial cultivation period. Cellulase activities were seen at the initial stage of CMC degradation, but the optimization of reducing sugars was not achieved. This illustrated the challenge for industrial scale application of fungi. The species specific interactions must be better understood, and the culture condition and the degradation process must be further optimized. *Antrodia sinuosa* needs an optimal pH for its effective growth. By self-regulating its pH environment, the fungus can survive and grow under poor conditions even when it lacks the perfect nutrient. It can colonize and metabolize the biomass faster than other species. After the first steps of the present research, more investigation is needed to evaluate the full potential of *A. sinuosa* in biotechnological applications. Co-cultivation studies of several species and finding a suitable species combination could be helpful in this context.

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