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The quest for fungal strains and their co-culture potential to improve enzymatic degradation of Chinese distillers' grain and other agricultural wastes

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ABSTRACT

Distillers' grain (DG) is one of the lignocellulosic agricultural waste streams having a high potential for biofuel production, however it is causing health and environmental problems in Southwest region of China because of improper treatment before being used for landfill. Here, we aimed to identify fungal strains that efficiently hydrolyze DG and explored the possibility to further improve the hydrolysis of DG and other agricultural wastes by co-culturing these fungi. The tested fungal strains included 11 strains that were isolated from DG (Maotai Town, Guizhou, China) and 19 strains that were selected from the CBS collection of Westerdijk Fungal Biodiversity Institute. *Aspergillus niger* CBS 110.42 and CBS 554.65 and *Phanerochaete chrysosporium* CBS 246.84 showed overall best cellulose and xylan degrading activity, whereas high laccase and peroxidase activities were detected for *Oidiodendron echinulatum* CBS 113.65 and *Bjerkandera adusta* CBS 143380, respectively. Furthermore, *A. niger* CBS 554.65 co-cultured with *Trichoderma reesei* CBS 383.78 and *A. niger* CBS 110.42 with *P. chrysosporium* CBS 246.84 showed the best improvement (up to 2- and 3-fold higher of β-glucosidase and β-xylosidase activities). These fungi and the combinations have strong potential to be further developed for local bioenergy production.

1. Introduction

Lignocellulosic biomass from agricultural waste is a promising and sustainable option as feedstock for second-generation biofuel production (Tan et al., 2014). Lignocellulosic agricultural waste is rich in cellulose (30–50% by weight) and hemicellulose (19–45% by weight) with certain amounts of lignin (15–35% by weight) depending on the plant source (Sorieul et al., 2016). Typically, four main steps are required for converting agricultural waste into bioethanol: pre-treatment, enzymatic hydrolysis (or saccharification), fermentation and product recovery (Harun et al., 2014; Norholm et al., 2009; Tan et al., 2014). Efficient hydrolysis of plant biomass is the bottleneck to achieve high yields in a cost-effective and sustainable manner (Carroll and Somerville, 2009; Lau and Dale, 2009). Direct inoculation of micro-organisms to pre-treat and hydrolyze biomass is one option (de Vries et al., 2004; Gupta et al., 2012; Li et al., 2014). Apart from requiring

Abbreviations: ABTS, 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); BGL, β-glucosidase; BXL, β-xylosidase; CMC-red, carboxymethyl cellulose Congo red agar medium; ITS, internal transcribed spacer; PDA-blue, potato dextrose agar containing 0.01% aniline blue; *p*NP-BGL, *para*-nitrophenyl β-glucoside; *p*NP-BXL, *para*-nitrophenyl β-xyloside; Xyl-red, xylan Congo red agar medium; YPD, yeast extract peptone dextrose medium; WB, wheat bran; WS, wheat straw; CS, corn stover; BW, birch; NS, Norway spruce; DG, distillers' grain; CM, complex medium; MM, minimal medium; CMC, carboxymethyl cellulose

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low energy and mild pH and temperature conditions (Chaturvedi and Verma, 2013; Rasmussen et al., 2010), direct use of microorganisms is inexpensive and less hazardous waste is produced compared to physical and (physico-)chemical methods, respectively (Maurya et al., 2015; Wan and Li, 2012). However, the main disadvantage of direct use of microorganisms as pre-treatment is the slow hydrolysis rate (Sun and Cheng, 2002). One way to improve this is to identify fungal strains with a higher ability to efficiently delignify agricultural waste (Maurya et al., 2015).

Distillers' grain is a lignocellulosic by-product of the distillation process for alcohol production for human consumption. Sorghum, rice and corn are commonly used in East Asia, particularly in China (Tan et al., 2014; Wang et al., 2013). Approximately two to four tons of distillers' grains are produced per ton of distilled alcohol (Ma et al., 2016). Distillers' grain contains mainly lignocellulose, but its degradation is complicated by its highly acidic nature (Liu et al., 2014; Tan et al., 2014). The cumulative impact of these factors causes environmental pollution, particularly in the Southwest region of China where there are many distilleries. Currently, this distillers' grain is used for landfill without prior treatment, which causes environmental problems within its vicinity (Wang et al., 2013). To resolve these issues, we aim to use fungi to degrade distillers' grain, after which the degraded materials can be used for the local bioenergy production.

In this study, we report 1) screening of fungal strains, which can efficiently degrade distillers' grain, using strains directly isolated from distillers' grain as well as from the CBS fungal collection (Westerdijk Fungal Biodiversity Institute), 2) establishing synergistic fungal combinations (co-cultivation) from the selected fungi, and 3) evaluating the hydrolysis ability of the selected (co-)cultures.

2. Materials and methods

2.1. Materials

Waste distillers' grain reused for fermentation several times was obtained from a distilling factory in Maotai Town, Guiyang, Guizhou China (personal communication). Fungal strains were directly isolated from this distiller's grain (see below). In addition, the distiller's grain was also used as the main C-source for growing the fungi. To prepare as a growth medium, the distillers' grains was dried at 30 °C for 24 h and ground in a blender (Conair Waring Pulverizer, Fisher Scientific). Wheat bran was purchased from Wageningen Mill, the Netherlands. Corn stover was provided by TNO, the Netherlands. Birch was obtained from a forest in the Netherlands. Wheat straw was purchased from pet shop. Norway spruce was a gift from Department of Microbiology, University of Helsinki. Carboxymethyl cellulose and xylan for solid media were purchased from Chaoyan and Yuancheng Biotechnology Company (Guiyang, China). *para*-Nitrophenyl β-glucoside (*p*NP-BGL), *p*NP β-xyloside (*p*NP-BXL), Avicel, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and other chemicals were purchased from Sigma-Aldrich.

2.2. Fungal isolation and identification

One gram of distillers' grain was added to liquid yeast extract peptone dextrose (YPD) medium and incubated at 25 °C for 5 days. The culture broth or visible mycelia were then transferred to YPD agar medium and incubated for 3–5 days. The fungi were isolated and purified several times until the pure cultures were obtained. gDNA of the fungi was isolated using Wizard® Genomic DNA Purification Kit (Promega) and used as template for internal transcribed spacer (ITS) regions amplification (Bellemain et al., 2010). ITS1–5.8S–ITS2 sequences were used for identification by nucleotide BLAST (Altschul et al., 1990). The fungi isolated from distillers' grains were deposited at the Westerdijk Fungal Biodiversity Institute or at the Chinese Academy of Science.

2.3. Screening on solid media

The fungi were pre-grown on YPD agar medium at 25 °C for 3–5 days, then a plug of 0.8 mm mycelium was transferred to three different media 1) carboxymethyl cellulose Congo red agar medium (CMC-red: 1% sodium CMC, 0.05% KH_2PO_4, 0.05% $MgSO_4$•$7H_2O$, 1.5% agar, 0.02% Congo red, 0.2% gelatin) (Gupta et al., 2012; Li et al., 2014; Wu et al., 2016), 2) xylan Congo red agar medium (Xyl-red: 0.5% xylan, 0.01% Congo red, 0.1% $MgSO_4$•$7H_2O$, 0.5% yeast extract, 0.1% KH_2PO_4, 1.5% agar, 0.1% $(NH_4)_2SO_4$, 0.5% NaCl) (Liu et al., 2010), and 3) potato dextrose agar containing 0.01% aniline blue (PDA-blue) (Wang et al., 2009). The plates were incubated at 25 °C for 5–6 days. The growth of mycelium and hydrolytic diameter (a clear halo zone) were measured. Analysis of hydrolytic efficacy was performed by division of the diameter of mycelium growth by that of the clearance zone (see detail in Fig. A1).

2.4. Screening in liquid medium

The fungi showing high activity on solid media were grown in 50 ml liquid YPD medium at 25 °C, 120 rpm 3 days (ascomycetes) or 7 days (basidiomycetes). Inocula containing 380 mg mycelium (wet weight) were transferred to 50 ml of 6% (w/v) distillers' grains in demineralized water and the cultures were incubated at 25 °C without agitation. Culture supernatants were collected at day 3 and day 7 after inoculation for enzyme activity assays.

2.5. Co-cultivation and enzyme production optimization

For co-cultivation, two or three selected fungi were pre-cultured as mentioned above individually in YPD and the inocula containing 185 or 125 mg mycelium of each strain (total of ~380 mg), respectively, were transferred to 50 ml of 6% (w/v) distillers' grains.

For ligninolytic activity improvement (Mishra et al., 2017; Wang et al., 2016; Yang et al., 2013), 0.4 mM $CuSO_4$, gallic acid or syringic acid as well as a mix of all three (0.4 mM each) were supplemented to the 6% (w/v) distillers' grains prior to cultivation.

For improving the medium composition, 6% (w/v) distillers' grains was mixed with 50 ml minimal medium (MM) or complex medium (CM) for *Aspergillus* (de Vries et al., 2004) instead of demineralized water.

The cultures were incubated at 25 °C without agitation and the culture supernatants were collected up to 7 days after inoculation for enzyme activity assays.

2.6. Enzyme activity assay

β-glucosidase and β-xylosidase activities were measured with the substrates *para*-nitrophenyl β-glucoside (*p*NP-BGL) and *para*-nitrophenyl β-xyloside (*p*NP-BXL), respectively. The reaction contained 40 µl culture supernatant, 10 µl 0.1% *p*NP-BGL or *p*NP-BXL, and 50 µl 100 mM sodium acetate, pH 5.0. The reactions were performed overnight (~16 h) at 25 °C followed by addition of 100 µl 250 mM Na_2CO_3 prior to measurement of the absorbance at 405 nm using a microtiter plate reader (FLUOstar OPTIMA, BMG Lab Tech, Ortenberg, Germany). 0–100 µM *para*-nitrophenol was used as standard (Patyshakuliyeva et al., 2016). One unit was defined as the amount of enzyme releasing 1 µmol *p*NP from *p*NP-BGL or *p*NP-BXL under assay condition.

Cellulase and xylanase activities were measured towards avicel and birchwood xylan, respectively. The reaction contained 20 µl culture supernatant and 180 µl 1% avicel or birch xylan in 50 mM sodium acetate, pH 5.0, and was incubated overnight (~16 h) at 25 °C. 100 µl of reaction mixture was mixed with 150 µl 3,5-dinitrosalicylic acid solution (1% 3,5-dinitrosalicylic acid, 0.2% phenol, 0.05% Na_2SO_3 and 1% NaOH), incubated at 95 °C, 30 min and cooled on ice prior to measurement of the absorbance at 560 nm using a microtiter plate reader

(Miller, 1959). 2–20 mM D-glucose or D-xylose was used as standard (Patyshakuliyeva et al., 2016; Peciulyte et al., 2017).

Peroxidase and laccase activities were measured towards 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) with or without supplement of hydrogen peroxide, respectively. The peroxidase reaction contained 20 µl culture supernatant, 20 µl 140 µM ABTS, 10 µl 3% H_2O_2, 25 µl 400 mM Britton-Robinson buffer, pH 4.5, and 125 µl ddH_2O. Detection of initial rates of hydrolysis was performed at 440 nm with a 2 min interval up to 60 min, at 25 °C using a microtiter plate reader. The laccase reaction contained 20 µl culture supernatant, 20 µl 140 µM ABTS, 20 µl 500 mM glycine-HCl, pH 3.0 and 140 µl ddH_2O. The initial rates of hydrolysis were determined by measuring absorbance at 440 nm in a 2 min interval for 30 min, at 25 °C. Laccase and peroxidase activities were calculated based on Lambert-Beer law, where the extinction coefficient of $3.6{\cdot}10^4\,M^{-1}\,cm^{-1}$ was used (Childs and Bardsley, 1975; Srinivasan et al., 1995).

2.7. Hydrolytic activity towards polysaccharides

Wheat bran, corn stover, birch, wheat straw and Norway spruce as well as distillers' grains were used as substrates for hydrolytic activity measurement from selected culture supernatants. 1% of polysaccharide was prepared in 50 mM sodium acetate buffer, pH 4.5, with 0.02% sodium azide as described previously (Dilokpimol et al., 2017). A reaction containing 500 µl 1% substrate and 100 µl culture supernatant was incubated, at 30 °C, 24 h, 100 rpm. The enzyme was inactivated by heating at 95 °C, 10 min prior to monosaccharide analysis.

2.8. Monosaccharide analysis

The culture supernatant was diluted 10-fold in MilliQ water prior to analysis. The monosaccharides were analyzed from peak areas in HPAEC-PAD (Dionex ICS-5000 + system; Thermo Scientific) equipped with CarboPac PA1 column (2 × 250 mm with 2 × 50 mm guard column; Thermo Scientific). The column was pre-equilibrated with 18 mM NaOH followed by a multi-step gradient: 0–20 min: 18 mM NaOH, 20–30 min: 0–40 mM NaOH and 0–400 mM sodium acetate, 30–35 min: 40–100 mM NaOH and 400 mM to 1 M sodium acetate, 35–40 min: 100 mM NaOH and 1 M–0 M sodium acetate followed by re-equilibration of 18 mM NaOH for 10 min (20 °C; flow rate: 0.30 mL/min). 2.5–200 mM D-glucose and D-xylose were used as standards for quantification (Mäkelä et al., 2016).

3. Results

3.1. Screening for fungi that efficiently produce lignocellulolytic enzymes

Analyses from the Complex Carbohydrate Research Center at the University of Georgia (Athens, GA) indicated that distillers' grain (pH around 4) contained 89.8% carbohydrate (98.6% glucose, 0.8% xylose and 0.6% arabinose) and 8.4% lignin, indicating that nearly all the carbohydrates present were cellulose derivatives.

Eleven fungal strains isolated from distiller's grain and 19 selected fungi from the CBS collection, including ascomycetes and basidiomycetes, which were previously reported to efficiently or potentially hydrolyze plant biomass (Borin et al., 2015; Kolasa et al., 2014; Tian et al., 2012; Yao et al., 2016), were grown on three different solid media containing carboxymethyl cellulose (CMC), xylan or aniline blue to screen for their cellulolytic, xylanolytic or ligninolytic activity, respectively (Fig. 1, Fig. A1.). *Bjerkandera adusta* CGMCC 5.2195, CBS 143335, CBS 143380 and *Moniliella* sp. CBS 143337 were the active lignocellulolytic enzyme producers on solid media isolated from distillers' grain. The most promising strains from the CBS collection were *Oidiodendron echinulatum* CBS 113.65, *Phanerochaete chrysosporium* CBS 246.84, *Aspergillus niger* CBS 110.42, *Doratomyces nanus* CBS 882.68, *Aspergillus niger* CBS 554.65, *Stachybotrys cylindrospora* 878.68 and *Trichoderma reesei* CBS 383.78.

The fungi that showed high lignocellulolytic activity on solid media (growth and activity ratio higher than 2 in Fig. 1) were selected to test for enzyme production on distillers' grain. The selected fungi were grown in water containing 6% distillers' grain as the sole nutrient and their culture supernatants were collected on the third and seventh day after inoculation to determine the cellulolytic, xylanolytic and ligninolytic activities (Fig. 2). Mycelium inoculation was used instead of spores because some fungi did not produce spores and this therefore provided a better comparison of the strains. *A. niger* CBS 110.42 produced very high and broad cellulolytic and xylanolytic activities. *A. niger* CBS 554.65 and *P. chrysosporium* CBS 246.84 also produced a broad set of enzymes, but lower than those of *A. niger* CBS 110.42. In addition, *B. adusta* CBS 143335 produced high β-glucosidase activity, while *Penicillium simplicissimum* CBS 138.65 produced high β-xylosidase activity. Only a few fungi produced high ligninolytic activity, i.e. *O. echinulatum* CBS 113.65 and *B. adusta* CBS 143380 produced high laccase and peroxidase activities, respectively (Fig. 2).

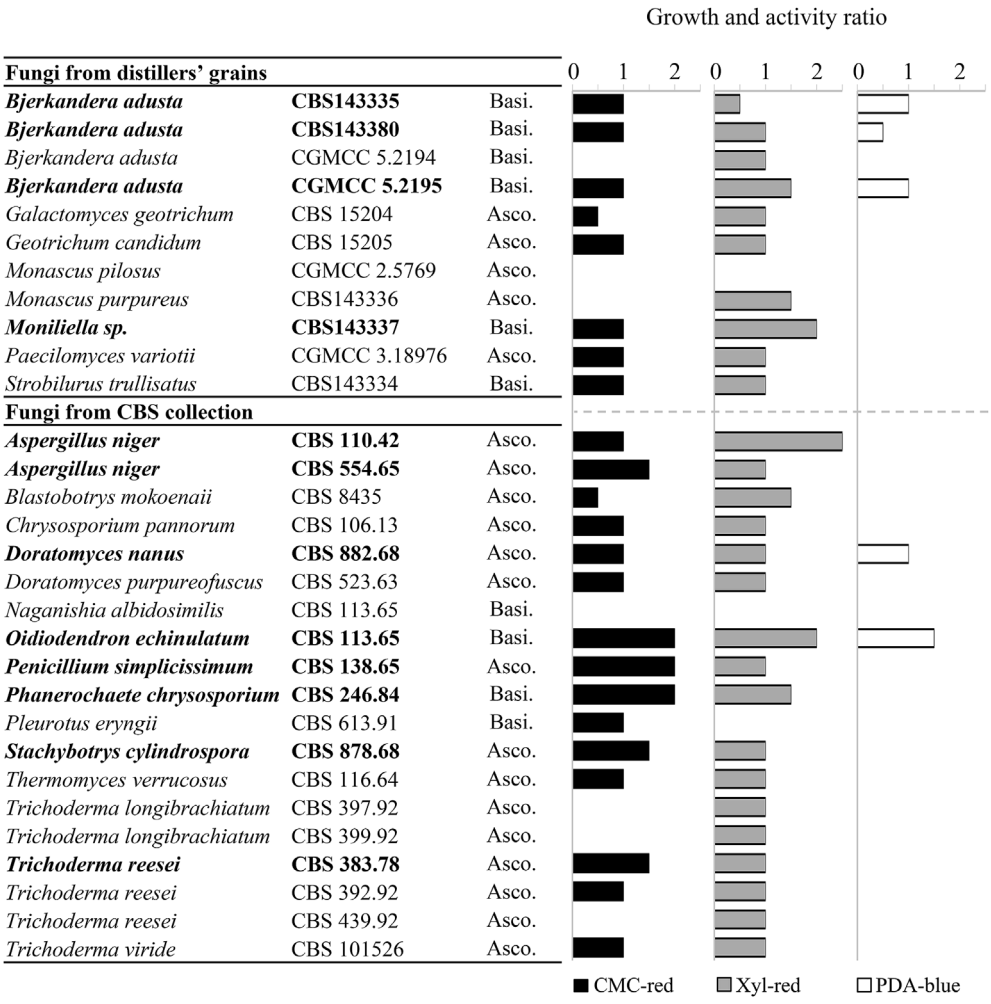
3.2. Improving enzyme activity by co-cultivation

To improve enzyme activity by co-cultivation, six selected strains (*A. niger* CBS 110.42, *A. niger* CBS 554.65, *P. chrysosporium* CBS 246.84, *T. reesei* CBS 383.78, *O. echinulatum* CBS 113.65 and *B. adusta* CBS 143380) were combined in pairs or triplets with the aim to improve cellulolytic and xylanolytic activities (Fig. 3 a-e, Table A.1) or overall lignocellulolytic activity (Fig. 3 f-p, Table A.1). The co-cultures (using 50% or 33.3% of each inoculum for two or three strains combinations, respectively) were grown on 6% distillers' grain in demineralized water. To assess the improved enzyme activity, we compared the activity of the co-cultivation with the highest activity from the individual strains present in the co-cultivation, e.g. in case of *A. niger* CBS 554.65 + *T. reesei* CBS 383.78, the co-culture showed twice the cellulolytic and xylanolytic activity in comparison to *A. niger* CBS 554.65, which showed higher enzyme activity than *T. reesei* CBS 383.78. In most cases, the co-cultivation with *A. niger* CBS 554.65 improved the β-glucosidase activity and to a certain extent xylanase activity (Fig. 3 c, e, m, n). The co-cultivation with *A. niger* CBS 110.42 mainly improved the β-xylosidase activity (Fig. 3 a, b, f, i, o), which was three-fold higher when combining this strain with *P. chrysosporium* CBS 246.84 or *T. reesei* CBS 383.78 (Fig. 3 a, b). To improve ligninolytic activity, *O. echinulatum* CBS 113.65 and/or *B. adusta* CBS 143380 were co-cultivated with other selected strains, but none of the combinations showed improvement compared to the individual strains. One explanation could be that *O. echinulatum* CBS 113.65 and *B. adusta* CBS 143380 might be overgrown by the other fungi.

3.3. Optimizing enzyme activity from co-cultivations by adjusting the medium composition

Because none of the combinations resulted in improved peroxidase and laccase activities, we attempted to improve the ligninolytic activity by adjusting the medium composition. Previously, it was reported that addition of $CuSO_4$, gallic acid, and syringic acid helped boosting ligninolytic activity in certain basidiomycetes (Mishra et al., 2017; Wang et al., 2016; Yang et al., 2013), hence we first added those compounds in the individual cultures (Table A.2). Addition of $CuSO_4$ improved ligninolytic activity for both *O. echinulatum* CBS 113.65 and *B. adusta* CBS 143380, while addition of gallic acid and syringic acid only increased the activity of a single strain (*O. echinulatum* CBS 113.65 and *B. adusta* CBS 143380, respectively).

In addition, because most co-cultures that contained *A. niger* CBS 110.42 or CBS 554.65 showed improvement of cellulolytic and xylanolytic activities, and the lack of prior knowledge on suitable medium composition for basidiomycetes (*O. echinulatum* CBS 113.65, *B. adusta* CBS 143380 and *P. chrysosporium* CBS 246.84), we used complete or



Fungi from distillers' grains		
Bjerkandera adusta	**CBS143335**	Basi.
Bjerkandera adusta	**CBS143380**	Basi.
Bjerkandera adusta	CGMCC 5.2194	Basi.
Bjerkandera adusta	**CGMCC 5.2195**	Basi.
Galactomyces geotrichum	CBS 15204	Asco.
Geotrichum candidum	CBS 15205	Asco.
Monascus pilosus	CGMCC 2.5769	Asco.
Monascus purpureus	CBS143336	Asco.
Moniliella sp.	**CBS143337**	Basi.
Paecilomyces variotii	CGMCC 3.18976	Asco.
Strobilurus trullisatus	CBS143334	Basi.
Fungi from CBS collection		
Aspergillus niger	**CBS 110.42**	Asco.
Aspergillus niger	**CBS 554.65**	Asco.
Blastobotrys mokoenaii	CBS 8435	Asco.
Chrysosporium pannorum	CBS 106.13	Asco.
Doratomyces nanus	**CBS 882.68**	Asco.
Doratomyces purpureofuscus	CBS 523.63	Asco.
Naganishia albidosimilis	CBS 113.65	Basi.
Oidiodendron echinulatum	**CBS 113.65**	Basi.
Penicillium simplicissimum	**CBS 138.65**	Asco.
Phanerochaete chrysosporium	**CBS 246.84**	Basi.
Pleurotus eryngii	CBS 613.91	Basi.
Stachybotrys cylindrospora	**CBS 878.68**	Asco.
Thermomyces verrucosus	CBS 116.64	Asco.
Trichoderma longibrachiatum	CBS 397.92	Asco.
Trichoderma longibrachiatum	CBS 399.92	Asco.
Trichoderma reesei	**CBS 383.78**	Asco.
Trichoderma reesei	CBS 392.92	Asco.
Trichoderma reesei	CBS 439.92	Asco.
Trichoderma viride	CBS 101526	Asco.

Fig. 1. Growth and activity ratio of the selected fungi grown on solid media. The media contains 1% cellulose (CMC-red), 0.5% xylan (Xyl-red) or 0.01% aniline blue (PDA-blue). Fungi in bold showed > 2.0 growth ratio and were chosen for cultivation in liquid media. Growth and activity ratio was estimated by hydrolytic diameter/colony diameter after 5 days of culture. The experiment was done in duplicate. 3, big clear zone; 2, medium clear zone; 1, small clear zone. Asco., Ascomycota; Basi., Basidiomycota.

minimal medium for *Aspergillus* (CM or MM, respectively (de Vries et al., 2004),) to prepare 6% distillers' grains culture, instead of demineralized water, to test whether these further increased the enzyme activities (Table A.3). Both *A. niger* strains produced several enzyme activities at higher levels in CM and MM than in the water medium, in particular β-glucosidase and β-xylosidase activities on day seven. *P. chrysosporium* CBS 246.84 and *T. reesei* CBS 383.78 showed a similar trend as *A. niger*. CM and MM also helped improving laccase activity of *B. adusta* CBS 143380, but not ligninolytic activities of *O. echinulatum* CBS 113.65.

We selected four combinations based on the increasing enzyme activity for further optimization by cultivation in MM containing 6% distillers' grains and $CuSO_4$ (Fig. 4, Table A.4). In most cases, MM with or without $CuSO_4$ improved β-glucosidase and β-xylosidase activities in particular at day seven (Fig. 4 d, f, h), but almost no improved cellulase and xylanase activities and these activities were even reduced in one combination (Fig. 4 a). Using MM with $CuSO_4$ also improved the peroxidase activity in all combinations at day three, which decreased on day seven, whereas the laccase activity was increased on day seven for *A. niger* CBS 110.42 + *O. echinulatum* CBS 113.65 and *A. niger* CBS 554.65 + *T. reesei* CBS 383.78 + *O. echinulatum* CBS 113.65 (Fig. 4 b, f). Addition of $CuSO_4$ alone showed the opposite effect. Surprisingly, cellulase activity was almost absent at day seven in all combinations.

3.4. Release of monosaccharides by the co-cultures

The culture broths from the co-cultures grown in MM containing 6% distillers' grains and $CuSO_4$ were further tested for their suitability for saccharification of distillers' grain (Fig. 5 a, b). Since *A. niger* CBS 554.65 + *T. reesei* CBS 383.78 + *B. adusta* CBS 143380 or *A. niger* CBS 554.65 + *T. reesei* CBS 383.78 + *O. echinulatum* CBS 113.65 showed similar overall enzyme activity, except that the first combination showed higher peroxidase activity (Fig. 4), we only tested the combination with *B. adusta* CBS 143380. Among the combinations, the 3rd day filtrates of *A. niger* CBS 554.65 + *T. reesei* CBS 383.78 with and without *B. adusta* CBS 143380 released around 20% glucose from the distillers' grain. However, the monoculture of *A. niger* CBS 554.65 and *A. niger* CBS 110.42 released glucose at similar or slightly higher levels (Fig. 4 a). In comparison among the three days, all culture filtrates showed quite similar level of glucose release, but those from day 3 were slightly more active (around 5%) than the others. Even though distillers' grain contains only a low amount of xylose, we detected the highest xylose release by the monoculture of *A. niger* CBS 554.65 followed by *A. niger* CBS 110.42 (Fig. 4 b).

Due to the high enzyme activity from the selected co-cultures, we also tested their saccharification efficiency on different plant biomass, i.e. birch, corn stover, Norway spruce, wheat straw and wheat bran (Fig. 5 c-l). These biomasses are agricultural or industrial by-products,

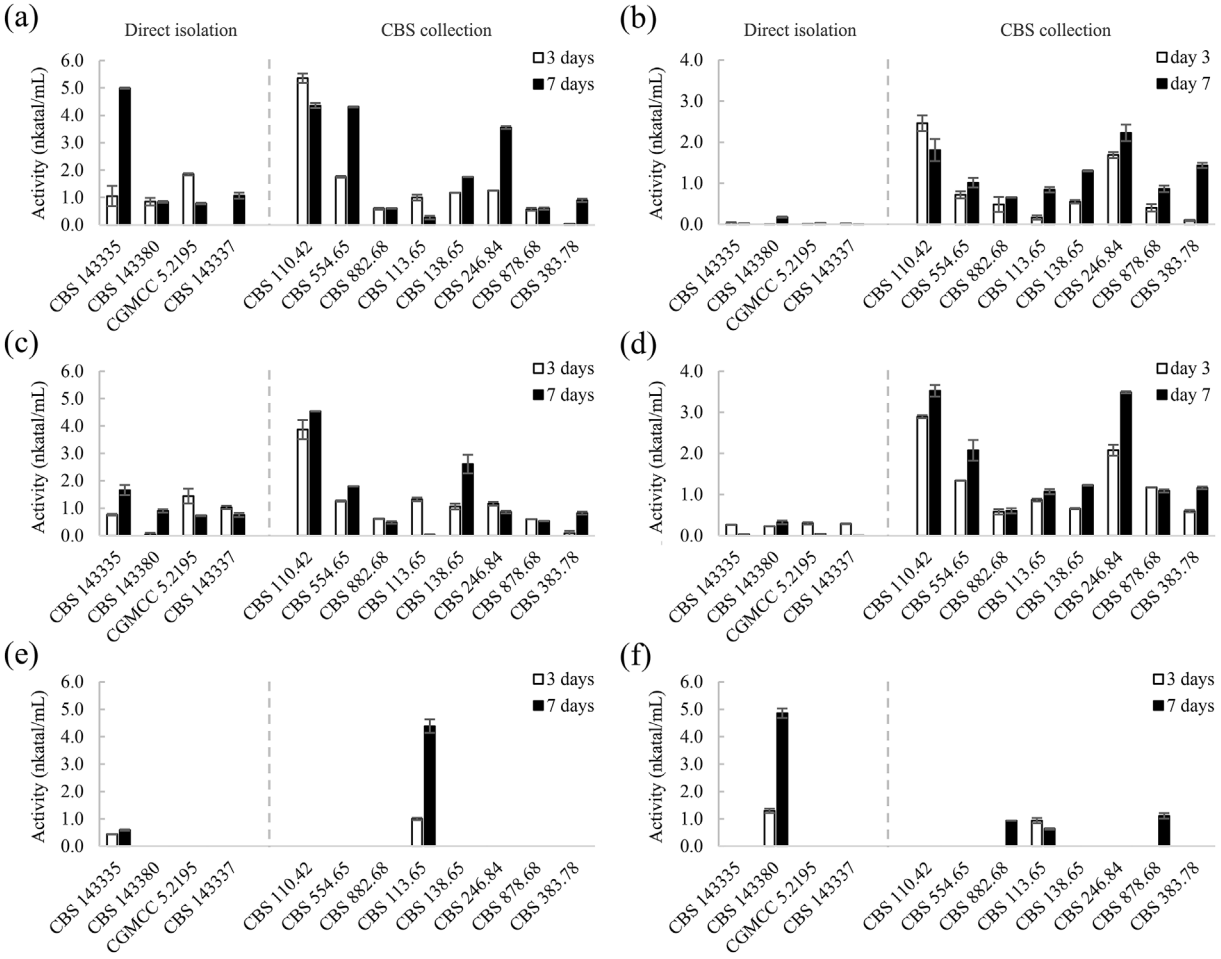


Fig. 2. Extracellular enzyme activities of the culture supernatant of selected fungi grown on 6% distillers' grain (in water) as the sole nutrient. (a) β-Glucosidase, (b) cellulase, (c) β-xylosidase, (d) xylanase, (e) laccase, and (f) peroxidase activities. The culture supernatants were collected at three (white bar) and seven (black bar) days after inoculation and used for measurement of enzyme activities. Each experiment was made with two biological duplicates. Standard deviations are shown as error bars.

and their main polysaccharide components are cellulose and xylan (Wyman et al., 2004; Yang et al., 2011). Glucose release from birch, corn stover, Norway spruce and wheat straw showed similar patterns, i.e. the 3rd day filtrate of the monoculture of *A. niger* CBS 554.65 and *A. niger* CBS 110.42 released around 40% of the total glucose content, followed by the co-cultures *A. niger* CBS 554.65 + *T. reesei* CBS 383.78 and *A. niger* CBS 554.65 + *T. reesei* CBS 383.78 + *B. adusta* CBS 143380 (Fig. 5 c, e, g, i). Surprisingly, the release of glucose by these mono- and co-cultures reduced severely on day seven. In addition, apart from *A. niger* CBS 110.42 + *O. echinulatum* CBS 113.65 for which the glucose release was also severely decreased on day seven, the other conditions showed similar glucose release among the three days. In case of wheat bran, the glucose release pattern was similar to that of the distillers' grain with almost double efficiency. The highest release of xylose was obtained from wheat bran treated with the 7th day culture filtrate from *A. niger* CBS 554.65. In addition to wheat bran, birch and corn stover showed similar patterns, i.e. the monoculture of *A. niger* CBS 554.65 released the highest amount of xylose, followed by *A. niger* CBS 110.42, *A. niger* CBS 554.65 + *T. reesei* CBS 383.78 and *A. niger* CBS 554.65 + *T. reesei* CBS 383.78 + *B. adusta* CBS 143380, respectively, by the 7th (or 5th) day filtrate (Fig. 5 d, f, l). For Norway spruce, the monoculture of *A. niger* CBS 110.42 release the highest amount of xylose from 5th day filtrate, and xylose release was reduced on day seven. The culture filtrates released only a small amount of xylose from wheat straw.

4. Discussion

4.1. Rational selection strains are more preferred than the direct isolation

To screen for fungal strains that can degrade the distillers' grain efficiently, we used two approaches, i.e. direct isolation and rational (or literature-based) selection. The isolation of fungi directly from the distillers' grain was used because of the expectation that this waste material might be difficult to degrade and require specific fungi as it has low pH and nutrient content (Liu et al., 2014; Tan et al., 2014). For the literature-based selection, we selected the strains from the CBS collection that were previously reported to efficiently degrade agricultural by-products (Borin et al., 2015; Kolasa et al., 2014; Tian et al., 2012; Yao et al., 2016). Even though most fungi from both approaches showed to produce lignocellulolytic enzymes on solid media (Fig. 1), the literature-based selection from the culture collection was more superior for the degradation of distillers' grain (Fig. 2). The main reason to use solid media containing defined substrates was to screen the distillers' grain-derived isolates for efficient lignocellulolytic enzyme producers, even though this was not specific for degradation of distillers' grain. Unsurprisingly, the fungi that could efficiently degrade distillers' grain included *A. niger* and *T. reesei*, because these fungi and their enzyme cocktails are widely used for plant biomass valorization (Gupta et al., 2016; Pel et al., 2007). In addition, the advantage for using the literature-based strains is the known growth conditions. To handle a newly isolated strain requires greater effort to establish proper growth

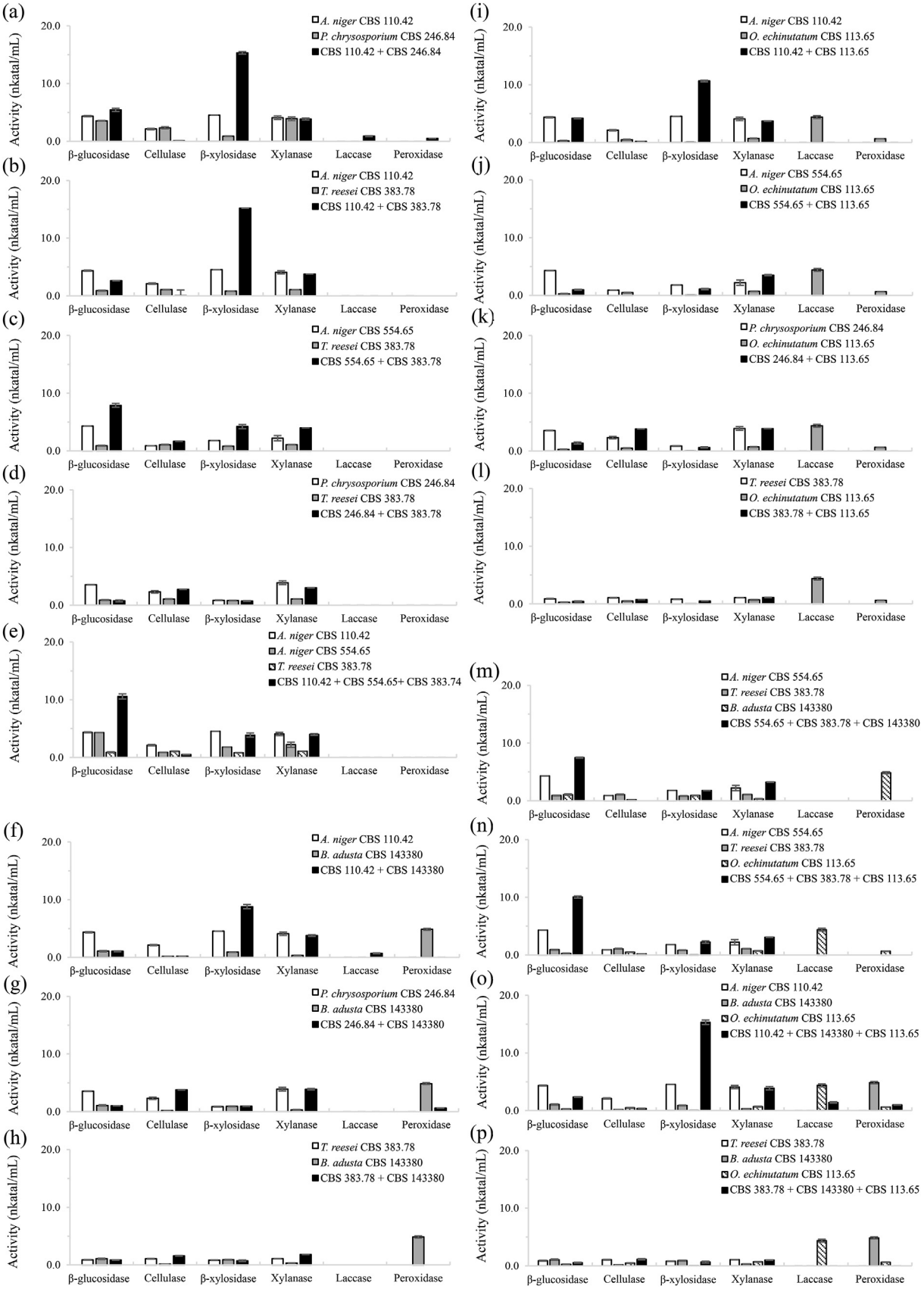


Fig. 3. Extracellular enzyme activity of the selected co-cultivated fungi grown on 6% distillers' grains for seven days. The combination of co-cultivated fungi is indicated in each graph. White, gray and striped bars: activities in individual cultures. Black bar: activity in the combined culture (see Table A.1 for full data). Each experiment was made with at least one duplicate. Standard deviations are shown as error bars.

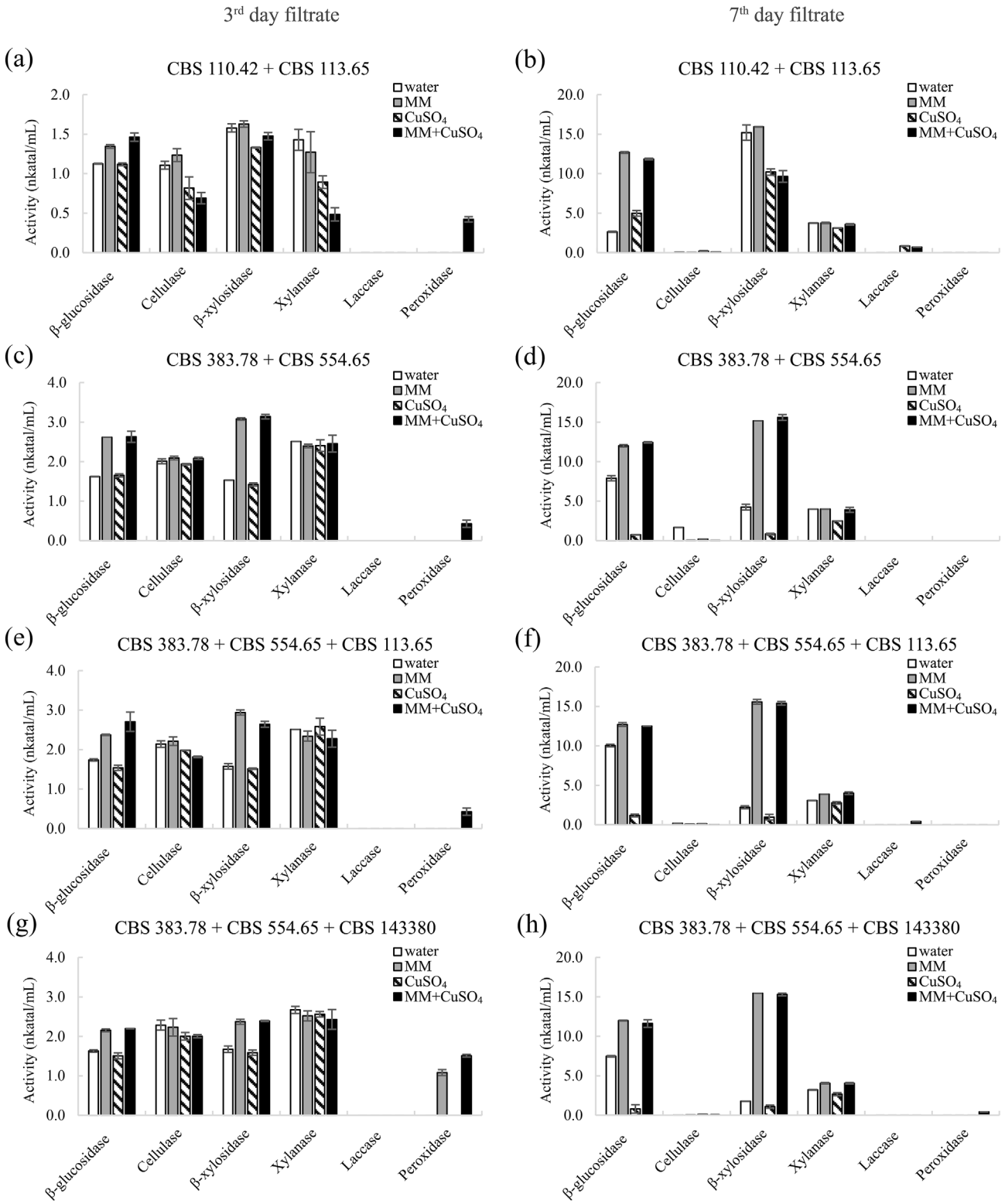


Fig. 4. Effect of minimal medium (MM) and $CuSO_4$ on enzyme activity of four selected fungal combinations. The fungi were grown in 6% distillers' grain in water, MM, $CuSO_4$ or MM + $CuSO_4$ for three (a–d) and seven (e–h) days after inoculation. Each experiment was made with two biological duplicates. Standard deviations are shown as error bars.

conditions. Hence in this study, rational selection was more effective than the direct isolation.

4.2. Strategy for improving co-cultivation

The main aim for the co-cultivation is to improve the overall lignocellulolytic activity, since lignocellulose is the main component in the agricultural waste. However, as it is not possible to predict which

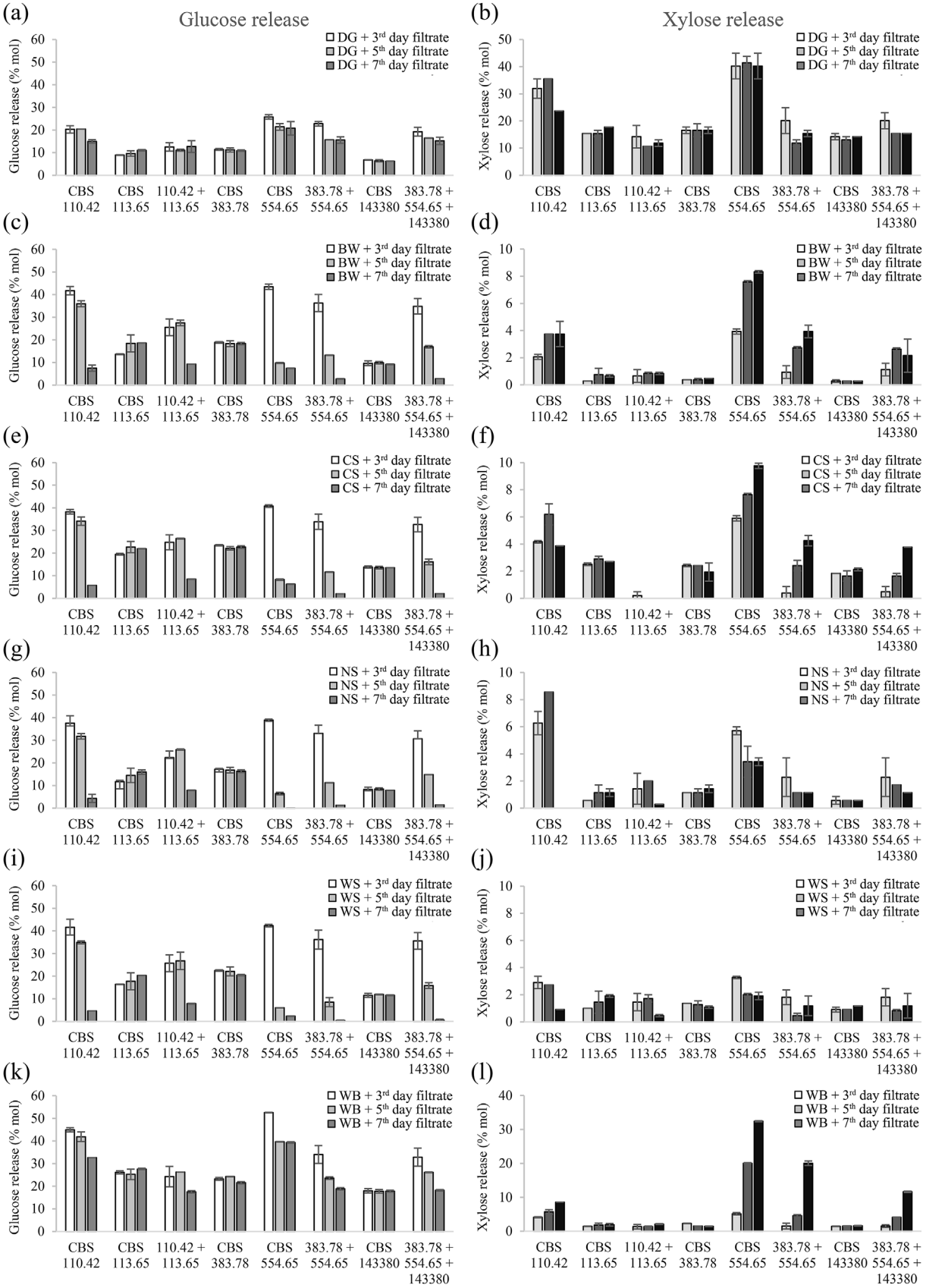


Fig. 5. Release of glucose and xylose from biomass treated with culture filtrates of selected co- and mono-cultures. Glucose (a–c) and xylose (d–f) released from treated biomass: distillers' grain (DG), birch (BW), corn stover (CS), Norway spruce (NS), wheat straw (WS) and wheat bran (WB). The fungi were grown in minimal medium containing 6% DG and 0.4 mM $CuSO_4$ for three (a, d), five (b, e) and seven (c, f) days after inoculation and the culture filtrates were used for treatment of the biomass. Each experiment was made in with least one technical duplicate. Standard deviations are shown as error bars.

strains will be able to grow together, we first grew different combinations on solid complex media (data not shown) (Hu et al., 2011). Strain combinations showing a separation zone or barrage line indicated incompatibility between the strains. These strain combinations were excluded from liquid cultivation. For the co-cultivation, the first set of combinations was aimed to improve cellulolytic and xylanolytic activities, chosen from the strains having high β-glucosidase, cellulase, β-xylosidase and/or xylanase activities (Fig. 3 a-e). The combinations containing *A. niger* CBS 110.42 showed 3-fold higher β-xylosidase (Fig. 3 a, b), whereas those containing *A. niger* CBS 554.65 showed 2-fold higher β-glucosidase activity (Fig. c, e). *Trichoderma reesei* CBS 383.78 showed rather low lignocellulolytic enzyme activity compared to other selected strains, but it grew well with *A. niger*, which enhanced the enzyme activities (Fig. 3 b, c, e, m, n). None of the combinations increased the main-chain degrading enzyme activities, i.e. cellulase or xylanase, which are crucial for the hydrolysis of agricultural waste. The second set of combinations was aimed to improve overall lignocellulolytic activity with focus on ligninolytic activity, because removal of lignin can reduce the biomass recalcitrance (Li et al., 2016). We added *O. echinulatum* CBS 113.65 and/or *B. adusta* CBS 143380 to the co-cultivations as their monocultures showed high laccase and peroxidase activities, respectively (Fig. 2 e, f). However, none of the combinations improved ligninolytic activity, which could be due to the low lignin content in the distillers' grain that was used as a sole substrate for growing the fungi. Even though we supplemented the cultures with chemicals, e.g. $CuSO_4$ that could increase peroxidase activity up to 2-fold (Fig. 4 g), using high lignin content biomass as a carbon source will be a more efficient strategy to improve the ligninolytic activity. In addition, we also observed that the ligninolytic strains were overgrown by the other strains, hence these selected basidiomycetes may not be a good choice for co-cultivation because of their slow growth or being dominated by other strains.

Overall, the co-cultivations highly improved β-xylosidase or β-glucosidase activity when they contain either *A. niger* CBS 110.42 or CBS 554.65, respectively (Fig. 3 f, i, m, n, o), although combination of the two *A. niger* strains did not result in improving those activities (Fig. 3 e). In addition, using only half or one-third of the amount of mycelium from each strain, the enzyme activity of the co-cultivations was higher than the sum of the activity from the individual strains, indicating an effective approach to achieve high level of selective enzyme production. Moreover, since increased activity was not observed for all enzymes, this indicated that the co-cultivation did not trigger a general protein production mechanism, but rather induced specific enzymes, as was also demonstrated in a previous study (Hu et al., 2011).

4.3. A. niger is the suitable organism for industrial saccharification

The co-cultures showed higher enzyme activities in comparison with their individual cultures (Fig. 3, Table A.4), however the highest glucose (day 3) and xylose (day 5–7) release was detected from the monocultures of *A. niger* CBS 554.65 and *A. niger* CBS 110.42. A possible explanation could be that the conditions used for the saccharification were not suitable for the enzymes from the co-cultures or the improved activity was not complemented. This also confirmed that *A. niger* was already the appropriate choice for current industrial saccharification. The reduction of the glucose release at later time points using the co-cultivation samples can be attributed to the drastically reduced cellulase activity at day 5–7 (Fig. 4). Hence, the optimal cultivation time is also crucial for production of suitable enzyme cocktails.

5. Conclusion

In this study, we aimed to identify fungal strains that can efficiently hydrolyze the Chinese distillers' grain and explore the possibility to further improve their efficiency by using co-cultivations of these fungi. The co-cultivation is an effective approach to achieve high production of certain enzymes. Several fungi were isolated from distillers' grain from China, some of which showed promising lignocellulolytic enzyme activity, even though *A. niger* proved to be the most effective in saccharification of Chinese distillers' grain and other agricultural wastes. The optimization for overall lignocellulolytic enzyme activity was challenging, because different fungal strains and enzymes prefer different conditions and the cultivation time was quite critical to make a suitable enzyme cocktail. Prolonging the cultivation time could result in severe reduction of certain enzymes (e.g. cellulase). By optimizing the growth conditions, high-enzyme activity co-cultures could be obtained, but this requires careful selection of time points, strains and enzyme activities.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2019.104765.

Appendices

Fungi	Accession No.	Phylum	CMC -red	Xyl -red	PDA -blue
Bjerkandera adusta	**CBS 143335**	Basidiomycota			
Bjerkandera adusta	**CBS 143380**	Basidiomycota			
Bjerkandera adusta	CGMCC 5.2194	Basidiomycota			
Bjerkandera adusta	**CGMCC 5.2195**	Basidiomycota			
Galactomyces geotrichum	CBS 15204	Ascomycota			
Geotrichum candidum	CBS 15205	Ascomycota			
Monascus pilosus	CGMCC 2.5769	Ascomycota			
Monascus purpureus	CBS 143336	Ascomycota			
Moniliella sp.	**CBS 143337**	Basidiomycota			
Paecilomyces variotii	CGMCC 3.18976	Ascomycota			
Strobilurus trullisatus	CBS 143334	Basidiomycota			
Medium plate without fungal inoculation					

Examples of measurement of mycelium growth (dash line) and hydrolytic zone (solid line).

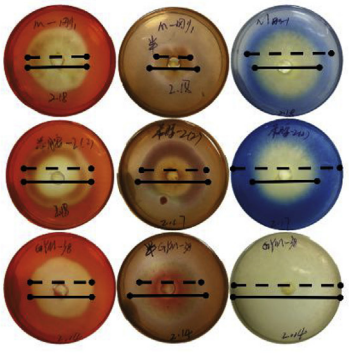
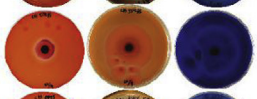
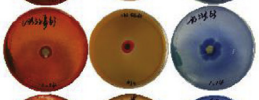


Figure A.1 Growth and activities of the selected fungi grown on solid media. The media contains 1% cellulose (CMC-red), 0.5% xylan (Xyl-red) or 0.01% aniline blue (PDA-blue).

Fungi	Accession No.	Phylum	CMC -red	Xyl -red	PDA -blue
Aspergillus niger	**CBS 110.42**	Ascomycota			
Aspergillus niger	**CBS 554.65**	Ascomycota			
Blastobotrys mokoenaii	CBS 8435	Ascomycota			
Chrysosporium pannorum	CBS 106.13	Ascomycota			
Doratomyces nanus	**CBS 882.68**	Ascomycota			
Doratomyces purpureofuscus	CBS 523.63	Ascomycota			
Naganishia albidosimilis	CBS 8626	Basidiomycota			
Oidiodendron echinulatum	**CBS 113.65**	Basidiomycota			
Penicillium simplicissimum	**CBS 138.65**	Ascomycota			
Phanerochaete chrysosporium	**CBS 246.84**	Basidiomycota			
Pleurotus eryngii	CBS 613.91	Basidiomycota			
Stachybotrys cylindrospora	**CBS 878.68**	Ascomycota			
Thermomyces verrucosus	CBS 116.64	Ascomycota			
Trichoderma longibrachiatum	CBS 397.92	Ascomycota			
Trichoderma longibrachiatum	CBS 399.92	Ascomycota			
Trichoderma reesei	**CBS 383.78**	Ascomycota			
Trichoderma reesei	CBS 392.92	Ascomycota			
Trichoderma reesei	CBS 439.92	Ascomycota			
Trichoderma viride	CBS 101526	Ascomycota			

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