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Mixed colonies of *Aspergillus niger* and *Aspergillus oryzae* cooperatively degrading wheat bran

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ABSTRACT

In both natural and man-made environments, microorganisms live in mixed populations, while in laboratory conditions monocultures are mainly used. Microbial interactions are often described as antagonistic, but can also be neutral or cooperative, and are generally associated with a metabolic change of each partner and cause a change in the pattern of produced bioactive molecules. *A. niger* and *A. oryzae* are two filamentous fungi widely used in industry to produce various enzymes (e.g. pectinases, amylases) and metabolites (e.g. citric acid). The co-cultivation of these two fungi in wheat bran showed an equal distribution of the two strains forming mixed colonies with a broad range of carbohydrate active enzymes produced. This stable mixed microbial system seems suitable for subsequent commercial processes such as enzyme production. XlnR knock-out strains for both aspergilli were used to study the influence of plant cell wall degrading enzyme production on the fitness of the mixed culture. Microscopic observation correlated with quantitative PCR and proteomic data suggest that the XlnR Knock-out strain benefit from the release of sugars by the wild type strain to support its growth.

1. Introduction

Among filamentous fungi, *Aspergillus* represents a large genus. Several species have a long history as cell factories for enzyme production with applications in bioethanol, pulp and paper, textile and food industries (Meyer, 2008). In particular, *A. niger* and *A. oryzae* are well-known species with substantial commercial values (Benoit et al., 2015). From laboratory to large scale production, fermentations are mostly carried on as pure cultures (Baker, 2006). However, in nature, complete lignocellulose degradation requires contributions from several microorganisms (Bayer and Lamed, 1992).

Aspergillus niger is found in highly diverse biotopes throughout all continents. It has a saprobic lifestyle, mainly decomposing plant materials but is also able to grow in man-made environments such as bathrooms and carpet dust (Buhari et al., 2012; Segers et al., 2016). It is occasionally an opportunistic pathogen of humans and animals. *A. niger* grows within a broad range of pH and temperature and tolerates low water activity (Krijgsheld et al., 2013). *Aspergillus oryzae*, also known as koji mold, has been used for oriental food fermentation for centuries, such as soy sauce, sake and miso, and produces numerous enzymes including amylases, proteases, lipases and cellulases (Machida et al., 2008). There are conflicting opinions on whether *A. oryzae* can be found in nature but most experts consider it to be a domesticated variant of *A. flavus* (Kurtzman et al., 1986) with the industrial strain RIB40 being a laboratory-adapted strain (Gibbons et al., 2012).

Mixed cultures of *A. niger* or *A. oryzae* together with other fungi have shown interesting features for enzyme production. Cellulase activity was increased by using mixed cultures of *A. niger* and *Trichoderma reesei* on sugar cane bagasse and cellulose enriched medium (Gutierrez-Correa et al., 1999), (Ahamed and Vermette, 2008). The interaction between these two fungi was recently described as competitive (Daly et al., 2017) and strongly dependent on the conditions of cultivation (Kolasa et al., 2014). The co-cultivation of *A. oryzae* with *T. reesei* was reported to enhance ellagic acid accumulation and to produce different levels of ellagitannin acyl hydrolase, cellulase and xylanase by these two fungi (Huang et al., 2008). Pectinase production was increased by solid state fermentation of *A. niger* and *A. terreus* co-cultures on banana peels (Rehman

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et al., 2014) and *A. niger* and *Saccharomyces cerevisiae* co-cultivated on orange peels (Zhou et al., 2011). The mixed culture of *A. niger* and *A. foetidus* showed synergistic effects on the treatment process of potato chip industry wastewater at all pH values, but the highest chemical oxygen demand (COD) reduction and fungal biomass was recorded at pH 6 (2004 Mishra).

In this study, *A. niger* and *A. oryzae* were chosen for co-cultures because of their industrial relevance and available molecular tools (Lubertozzi and Keasling, 2009). Moreover, knockout mutants for the (hemi-)cellulolytic regulator XlnR are available, allowing a detailed study of the mixed culture towards plant polysaccharides degradation. Both aspergilli are ascomycetes with a growth rate in the same range on various substrates. Using flow cytometry for large particles, microscopy and proteomics we assessed the effect of the interaction and its potential for enzyme production. We chose wheat bran, a byproduct of the flour milling industry because of its composition in polysaccharides (i.e. cellulose, hemicellulose) and aromatics (e.g. ferulic acids) (Laddomada et al., 2015) which makes it an excellent substrate to induce the production of plant biomass degrading enzymes.

2. Material and methods

2.1. Media and culture conditions

The strains used in this study are shown in Supplementary Table 1. *Aspergillus niger* CB-A119.1 and *Aspergillus oryzae* CBS 141650 harbour PgpdA-gfp and PgpdA-dtomato constructs respectively (de Bekker et al., 2011) and this study). Spores of *A. niger* strains CB-A119.1, N402 and ΔXlnR (UU-A062.10), and *A. oryzae* strains CBS 141,650, RIB40 and AoXlnR (JCM 16332) were isolated from complete medium plates containing 2% glucose. Liquid shaken cultures were inoculated with 5×10^8 spores ml^{-1} and incubated for 16 h at 30 °C and 250 rpm in 1L Erlenmeyer flasks with 250 ml transformation medium (TM) (Kusters-van Someren et al., 1991) or minimum medium with wheat bran 1%. The co-cultures and control experiments were done in triplicate.

2.2. Flow cytometry using COPAS PLUS

Microcolonies were harvested by filtration over a Büchner funnel with nylon gauze, washed with 50 ml PBS and fixed for 15 min at room temperature with 4% formaldehyde in PBS. The fixative was removed by washing 2 times in excess PBS using centrifugation at 3000*g* for 5 min to pellet the microcolonies. Microcolonies were simultaneously sorted based on size (Time of Flight, TOF), density (extinction, EXT) and fluorescence using a COPAS PLUS profiler equipped with a 1 mm nozzle (Union Biometrica). Sorting parameters were set in order to exclude clusters of colonies or debris from the analysis ([EXT] ≥ 25). GFP and dTomato fluorescence were detected with a 488 nm solid state laser combined with a Green PMT 514/23 nm optical emission filter.

2.3. Protein quantification

Samples from the liquid shaken cultures were centrifuged for 15 min at 12,000 rpm to remove the mycelia from the soluble fraction. Prior to preparing the samples for analysis, their protein content was precipitated with trichloroacetic acid/acetone, solubilized and measured using a BCA Protein Assay kit (Thermo, San Jose, CA, USA), to ensure that the same amount of protein was loaded onto each SDS-PAGE lane.

2.4. Sample preparation for proteome analysis

Mycelia grown in duplicate in wheat bran and pectin media (cf. media and culture conditions) for 16 h were washed in PBS and sorted using the COPAS Plus.

Proteomics samples were prepared as described in (Rupakula et al., 2014). In summary, samples (40 μg of protein) were boiled in SDS loading buffer (2% SDS, 10% glycerol, 50 mM Tris pH 6.8, 100 mM DTT, 0.05% bromophenol blue) and loaded on a SDS-polyacrylamide gel and separated on SDS-PAGE (Mini-PROTEAN gel electrophoresis, Bio-Rad). Proteins were stained with Coomassie PageBlue (ThermoFisher) revealing approximate equal amounts of proteins for each treatment. From each sample 5 slices containing the proteins were cut from the gel. Following in-gel digestion (Rupakula et al., 2014), the samples were dissolved into 50 μl 1 ml/l formic acid in water and the obtained peptides were analyzed by nanoLC-MS/MS as described before (Lu et al., 2011). Reversed hits were deleted from the MaxQuant protein groups result table. In the MaxQuant protein groups result file, only peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least 2 identified peptides of which at least one should be unique and at least one should be unmodified were accepted. Total non-normalised protein intensities corrected for the number of measurable tryptic peptides (intensity based absolute quantitation (iBAQ) intensity (Schwanhausser et al., 2011) were, after taking the normal logarithm, used for plotting on the y-axis in Fig. 3. The nLC-MSMS system quality was checked with PTXQC (Bielow et al., 2016) using the MaxQuant result files. *A. niger* ATCC 1015 and *A. oryzae* RIB40 protein databases from the Uniprot and Aspergillus Genome databases (AspGD) were used for peptides search, protein identification and CAZyme family prediction (Cerqueira et al., 2014). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD005532.

2.5. Microscopy

Green fluorescent protein (GFP) and dTomato fluorescence was monitored with an Axioskop 2 plus microscope (Zeiss, Germany) equipped with a HBO 100 W mercury lamp and a photometrics Cool SNAP camera (1392 × 1024 pixels) using standard FITC and tetramethylrhodamine filters respectively. The GFP protein was excited with the 488 nm laser line and fluorescence was detected at 500–550 nm bandpass. The dTomato protein was excited with the 543 nm laser line and fluorescence was detected with a long pass LP560 emission filter. Bright field images were made using the transmission channel. Laser intensity was kept to a minimum to reduce photo bleaching and phototoxic effects. Images were captured as z-series of optical sections. The data sets were displayed as maximum intensity projections (1024 × 1024 pixels) using ZEISS software. The DAPI (Sigma-Aldrich) concentration in the working solution was 10 μg/ml in distilled water.

2.6. Quantitative PCR experiment for determination of the A. niger and A. oryzae ratio

Mycelia was pooled on Miracloth and immediately frozen in liquid nitrogen. Chromosomal DNA was isolated from frozen-ground mycelia by Trizol extraction according to the manufacturer's instructions. DNA quantity and quality were determined on a Nanodrop spectrophotometer. Wheat bran monocultures and mixed cultures were done in triplicate resulting in nine samples (three *A. niger*, three *A. oryzae* and three mixed cultures). Glyceraldehyde-3-phosphate dehydrogenase primer pairs (Supplementary Table 2) for each species were designed using the software Primer express

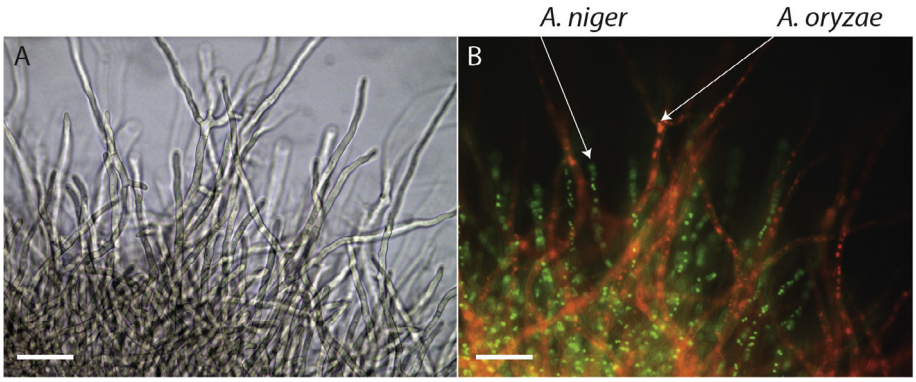

Fig. 1. *A. niger* and *A. oryzae* mixed microcolony. Panel (A) shows a bright field image, Panel (B) is the corresponding confocal image of *A. niger* expressing green fluorescent protein and *A. oryzae* expressing red fluorescent protein. Scale bars indicate 10 μm.

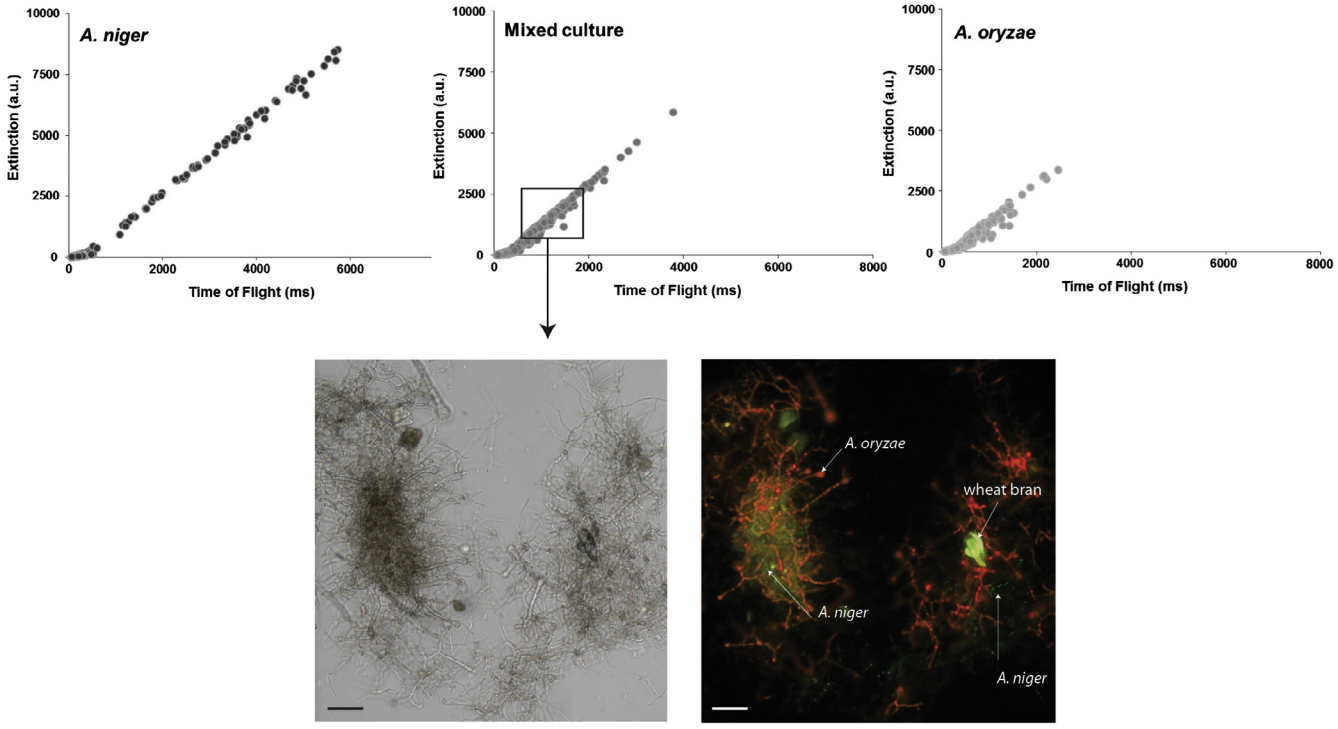

Fig. 2. (A) Distribution of diameter (time of flight in milliseconds) and optical density (extinction in arbitrary units) of microcolonies from *A. niger*, *A. oryzae* and the mixed culture from wheat bran liquid cultures. (B) Microcolonies from the mixed culture, sorted by COPAS. Scale bars indicate 10 μm.

3.0 (Applied Biosystems, Warrington, UK). The primers were tested to determine the optimal primers concentrations and efficiency. Primers had between 98% and 107% efficiency. 20 ng was assayed in triplicate in a final volume of 20 μl of qPCR reaction containing the optimized concentration of each primer and 10 μl of ABI Fast SYBR Master Mix (Applied Biosystems, Foster City, USA). Reactions were carried out in a ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, USA), with the following cycling conditions: 95 °C 20 s, followed by 40 cycles of 95 °C 3 s and 60 °C 30 s. Standard curves of both primer pairs of *A. oryzae* and *A. niger* were made from TM cultures (Supplementary Fig. 1).

3. Results and discussion

3.1. Solid media cultures show mycelium segregation

The two aspergilli were inoculated from a mixed spore solution on complete medium, TM and wheat bran agar plates. Both fungi were growing radially, forming a single mixed colony. However, based on sporulation, a segregation of the mycelia was observed as reported previously (Hu et al., 2011). Although some parts of the mixed colony seemed exclusively *A. niger* dominated (black sporulation) and other parts seemed exclusively *A. oryzae* dominated (green sporulation), microscopic observation of cross sections showed both mycelia embedded into one another (Supplementary Fig. 2).

3.2. Culture profiling by flow cytometry

All *Aspergillus* strains were grown in TM and in wheat bran liquid shaken cultures for 16 h. The resulting microcolonies (also known as pellets) were analyzed on basis of their diameter as expressed as the time of flight (TOF) in milliseconds and on the basis of their density as expressed as extinction (EXT) in arbitrary units (de Bekker et al., 2011). A mixture of hyphae and microcolonies was observed in the extinction range 150–200. Events

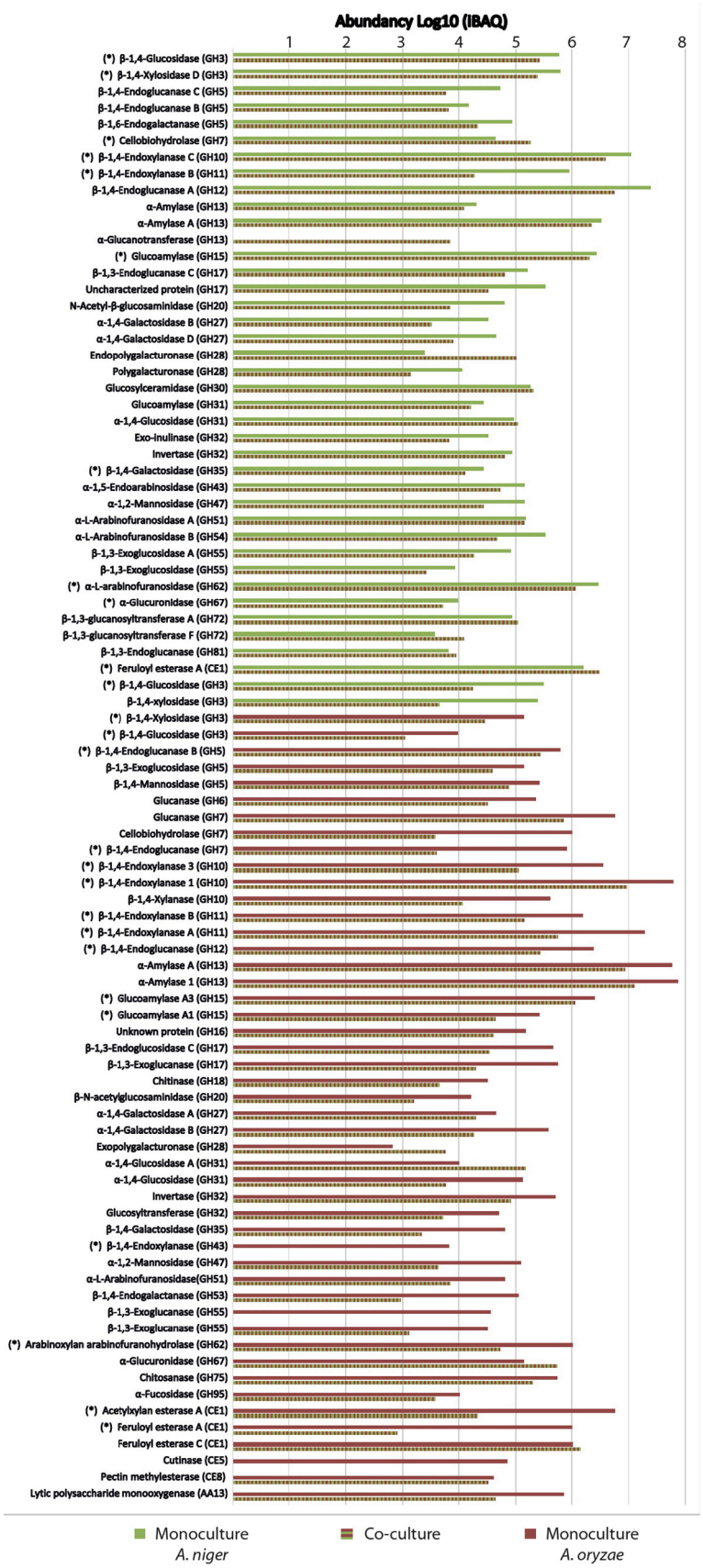

Fig. 3. Proteins secreted by *A. niger* (green), *A. oryzae* (red) monocultures and mixed culture (green-red dashes) in wheat bran as determined by mass spectrometry. Samples were taken after 16 h and are the same samples used for COPAS measurements. Log (Intensity Based Absolute Quantification) information is shown as the bar heights. (*) Proteins regulated by XlnR are marked with an asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with EXT ≥ 200 were considered microcolonies (van Veluw et al., 2013). Solid particles of wheat bran were measured with an EXT ≤ 110 (Supplementary Table 2). Additionally, microcolonies were sorted by fluorescence, CB-A119.1 and CBS 141650 harboring respectively GFP and dTomato. From microscopic observations, microcolonies from the mixed cultures appeared to be a combination of both aspergilli tightly embedded into one another (Fig. 1).

Single species microcolonies from *A. niger* were smaller and more compact than the microcolonies from *A. oryzae* which were more dispersed. The difference in microcolonies morphology is shown by the dot plot graph obtained from the flow cytometry analysis (Fig. 2). The shape of the mixed cultures was intermediate between the two pure cultures (Fig. 2). The ratio of *A. niger* and *A. oryzae* in the mixed culture was measured with GFP and dTomato fluorescence and showed an equal distribution between the two aspergilli (Fig. 3). This equal distribution was also reflected by the pH of the mixed culture which was in between the significantly different pH of the two monocultures (Table 1).

3.3. The mixed culture produced A broad range of secreted enzymes

The supernatant of the two mono-cultures and the mixed culture grown in wheat bran were analyzed for secreted proteins by mass spectrometry. Thirty-seven and forty-nine Carbohydrate Active enZymes (CAZymes) (Lombard et al., 2014) were identified in *A. niger* and *A. oryzae* mono-cultures respectively (Supplementary Table 3). Several studies have reported the secretomes of *A. niger* and *A. oryzae*. For instance, *A. oryzae* grown in wheat bran under submerged and solid-state culture conditions and on soy sauce and rice wine koji (Oda et al., 2006; Liang et al., 2009; Zhang et al., 2012) and *A. niger* grown in rapeseed meal (Shi et al., 2016) However, the variation in growth conditions and substrates as well as the method used to analyze the secreted proteins only give an idea of which protein are produced in a particular condition. The two secretomes of these study were consistent with a previous study on wheat bran (Benoit et al., 2015). Eighty-three CAZymes were identified from the mixed culture (Fig. 3). From *A. niger*, ten enzymes were involved in cellulose degradation, eight were acting on xylan and xyloglycan, five on pectin, one on galactomannan, five on starch, two on inulin and five were involved in fungal cell wall remodeling. From *A. oryzae*, fourteen enzymes were acting on cellulose, eleven on xylan and xyloglycan, four on pectin, four on galactomannan, six on starch, two on inulin and seven were involved in fungal cell wall remodeling. One protein was solely found in the mixed culture, a GH13 from *A. niger*. Three proteins from *A. oryzae*, a GH43, a GH55 and a CE5 were not found in the mixed culture but in the monoculture. Five out of the eighty-three CAZymes had a significantly higher abundancy in the mixed culture than in the monoculture, they consisted of a GH7, GH13 and GH28 enzyme from *A. niger* and a GH28 and GH31 enzyme from *A. oryzae*. The other seventy-eight CAZymes were found equally or more abundant in the monocultures (Fig. 3). During vegetative growth, *A. niger* is known to naturally acidify the medium from pH 6 to pH 2.5–3 by producing organic acids. The pH value of the culture medium during germination and early development of *A. oryzae* has been shown to be around 3 (Zhu et al., 2004) which is, for instance, the pH required for the acidic activation of the *A. oryzae* protyrosinase to tyrosinase (Ichishima, 2016), then during vegetative growth, the optimum pH of *A. oryzae* rises to 7. Protein production is influenced by the medium composition and the pH. For each protein of interest, the optimum pH of production may vary and conditions of production should be adapted as such.

Table 1
pH values of the mono- and mixed cultures, 16 h after inoculation.

	pH		
	A. niger	*A. oryzae*	Mixed culture
Transformation medium	3	7	5.5
Wheat bran	3	7	5.5

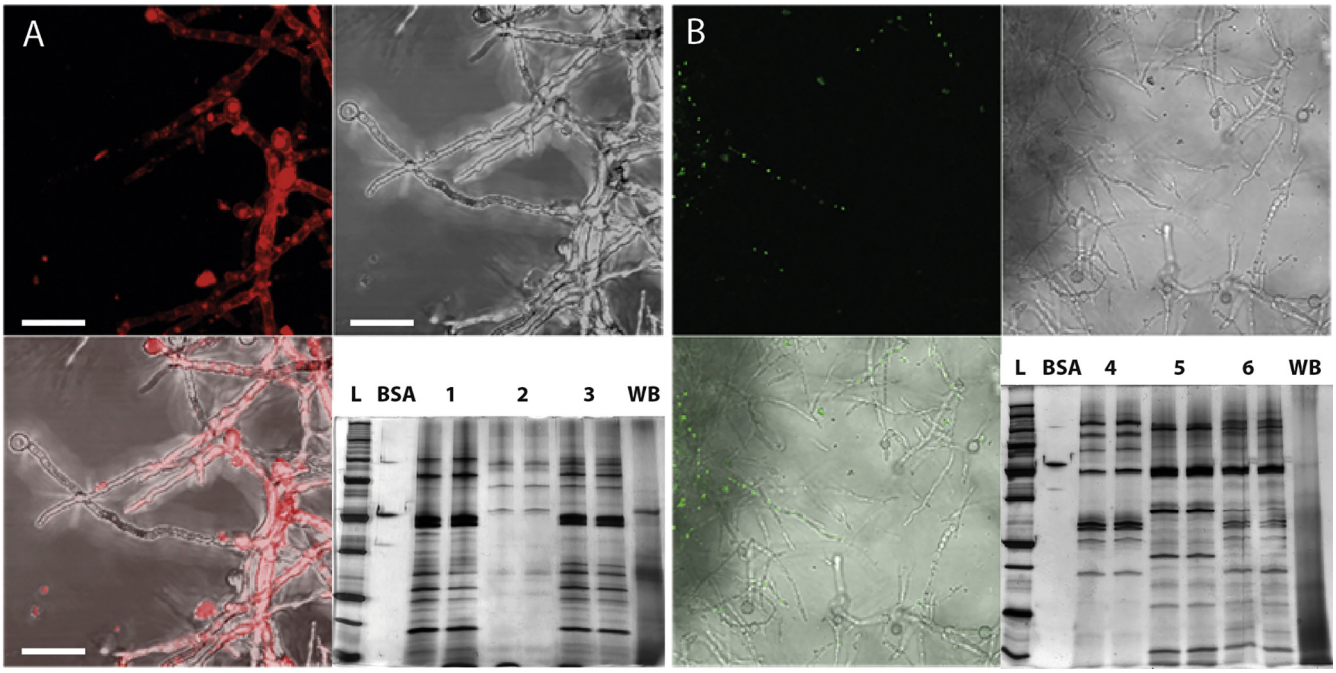

Fig. 4. XlnR Knockout and wild type mixed cultures. (A) ΔXlnR *A. niger* and wild type *A. oryzae* mixed culture; (B) ΔXlnR *A. oryzae* and wild type *A. niger* mixed culture. Upper left panels show the confocal images of dTomato-labelled *A. oryzae* (CBS 141650) or GFP-labelled *A. niger* (CB-A119.1), while the upper right panels show the corresponding bright field images. Scale bars indicate 12 μm. Lower right panels are exoproteome profiles on SDS-PAGEs of mono and mixed cultures; (L) ladder, (BSA) Protein standard Bovin Serum Albumin, 100 ng loaded, (1) *A. oryzae* WT CBS 141650; (2) *A. niger* XlnR knockout mutant; (3); mixed culture of *A. oryzae* WT and *A. niger* ΔXlnR. (4) *A. niger* CB-A119.1; (5) *A. oryzae* XlnR knockout mutant; (6); mixed culture of *A. niger* WT and *A. oryzae* ΔXlnR. Supernatants from biological duplicates, (WB) wheat bran medium prior inoculation.

Nonetheless, fermentations at pH 6 compared to pH 3 greatly reduces protease activity (O'Donnell et al., 2001). Therefore, the resulting pH 5.5 of the mixed culture medium is suitable for the production of a broad range of enzymes and can be further optimized.

3.4. XlnR knockout mutants and wild type strains grown in mixed cultures

The transcriptional activators AnXlnR and AoXlnR control the expression of several cellulases and xylanolytic enzymes (Van Peij et al., 1998; Marui et al., 2002). The deletion of these regulators results in impaired growth on hemicellulose rich media and also an increased sensitivity to oxidative stress (Raulo et al., 2016). Mixed cultures of *A. niger* ΔXlnR with *A. oryzae* CBS 141650 and *A. niger* CB-A119.1 with *A. oryzae* ΔXlnR were grown in wheat bran. Wheat bran is rich in D-xylose which has been shown to trigger reversible phosphorylation of XlnR (Noguchi et al., 2011). The expression of ten and fourteen CAZymes respectively identified in the *A. niger* and *A. oryzae* monocultures, are known to be regulated by the transcriptional activator XlnR (Fig. 3). In the monocultures of the XlnR knockout strains and in the mixed cultures, the expression of these enzymes was lower (or not detected) than in the monocultures of the wild type strains. In the mixed cultures, the knockout strains were growing well in both cases as depicted in Fig. 4 and an even distribution between the two aspergilli was confirmed by quantitative PCR (Supplementary Fig. 1). The exoproteome profiles from the XlnR knockout strains showed a clear reduction in protein production compared to the wild-type strains (Fig. 4). This suggests that the ΔXlnR *Aspergillus* strains benefit from the release of sugars by the wild type strain to support their growth. The pH of the monocultures and the mixed cultures was the same as the wild type cultures. Similarly, no sign of competition could be detected, even though the knockout strain is benefitting from the enzyme production of the wild type strain.

4. Conclusion

A. niger and *A. oryzae* mixed cultures in wheat bran secreted a broader range of plant cell wall degrading enzymes than respective monocultures. The interaction between the two aspergilli was stable and there was no significant sign of competition which makes this two industrial fungi compatible for co-cultivation in a single fermenter. Engineering these two strains combined with a mixed culture method would be an interesting option to optimize enzyme cocktails.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2017.02.006.

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