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Transcriptomic responses of mixed cultures of ascomycete fungi to lignocellulose using dual RNA-seq reveal inter-species antagonism and limited beneficial effects on CAZyme expression

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ABSTRACT

Gaining new knowledge through fungal monoculture responses to lignocellulose is a widely used approach that can lead to better cocktails for lignocellulose saccharification (the enzymatic release of sugars which are subsequently used to make biofuels). However, responses in lignocellulose mixed cultures are rarely studied in the same detail even though in nature fungi often degrade lignocellulose as mixed communities.

Using a dual RNA-seq approach, we describe the first study of the transcriptional responses of wild-type strains of *Aspergillus niger*, *Trichoderma reesei* and *Penicillium chrysogenum* in two and three mixed species shake-flask cultures with wheat straw.

Based on quantification of species-specific rRNA, a set of conditions was identified where mixed cultures could be sampled so as to obtain sufficient RNA-seq reads for analysis from each species. The number of differentially-expressed genes varied from a couple of thousand to fewer than one hundred. The proportion of carbohydrate active enzyme (CAZy) encoding transcripts was lower in the majority of the mixed cultures compared to the respective straw monocultures. A small subset of *P. chrysogenum* CAZy genes showed five to ten-fold significantly increased transcript abundance in a two-species mixed culture with *T. reesei*. However, a substantial number of *T. reesei* CAZy transcripts showed reduced abundance in mixed cultures. The highly induced genes in mixed cultures indicated that fungal antagonism was a major part of the mixed cultures. In line with this, secondary metabolite producing gene clusters showed increased transcript abundance in mixed cultures and also mixed cultures with *T. reesei* led to a decrease in the mycelial biomass of *A. niger*. Significantly higher monomeric sugar release from straw was only measured using a minority of the mixed culture filtrates and there was no overall improvement.

This study demonstrates fungal interaction with changes in transcripts, enzyme activities and biomass in the mixed cultures and whilst there were minor beneficial effects for CAZy transcripts and activities, the competitive interaction between *T. reesei* and the other fungi was the most prominent feature of this study.

Abbreviations: An, *Aspergillus niger*; CAZy, carbohydrate active enzyme or protein, as found in the CAZy database; CAZyme, carbohydrate active enzyme; FPKM, uniquely mapped **F**ragments **P**er **K**ilobase of gene model per **M**illion uniquely mapped fragments; MWCO, molecular weight cut-off; p_{adj}, p value from DESeq analysis with adjustment for multiple hypothesis testing; Pc, *Penicillium chrysogenum*; pNP-Ara, 4-Nitrophenyl-α-L-arabinofuranoside; pNP-α-Glc, 4-Nitrophenyl-α-D-glucopyranoside; pNP-β-Glc, 4-Nitrophenyl-β-D-glucopyranoside; pNP-Cel, 4-Nitrophenyl-β-D-cellobioside; pNP-Xyl, 4-Nitrophenyl-β-D-xylopyranoside; Tr, *Trichoderma reesei*.

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1. Introduction

Lignocellulose is an abundant raw material that has the potential to be a cost-effective source of liquid biofuels. A key limitation of the process is the saccharification stage which requires costly enzymes (Klein-Marcuschamer et al., 2012). The enzymes required are lignocellulose degrading carbohydrate active enzymes (CAZymes) which include cellulases, hemicellulases and accessory activities (van den Brink and de Vries, 2011). One approach to reduce the costs is to exploit a better understanding of the fungal responses to lignocellulose and understanding the responses of fungi in mixed cultures forms part of this approach. Fungal mixed cultures are mixtures of two or more individual fungal species or strains. Part of the rationale for using mixed cultures to degrade lignocellulose is that different fungal species can be found in the same lignocellulose-containing ecological niche such as a hollow tree stump (Tian et al., 2010) or leaves (Das et al., 2007). The inter-species fungal interaction are most likely to be based on antagonism and competition (Boddy, 2000) although co-operative interactions are also possible that could benefit the saccharification of lignocellulose. Enzymes from lignocellulose-containing mixed cultures could be advantageous over combining enzymes from monocultures because of enzymatic and gene regulatory reasons. Unique activities from one fungus could expose sugar inducers from the lignocellulose that the enzyme activities of the other fungus might have been incapable of exposing such as in a complex pectin structure thus resulting in a more complete induction of CAZymes in that other fungus. We explored the interactions of wild-type strains of *Aspergillus niger*, *Trichoderma reesei* and *Penicillium chrysogenum* in two and three mixed species shake-flask cultures with wheat straw using a range of approaches including dual RNA-seq.

Genome-wide transcriptomic studies could elucidate how transcript abundance changes in a mixed culture compared to monocultures. Arfi et al. (2013) studied, using a standard complex laboratory medium, mixed cultures with basidiomycetes that competed. RNA-seq analysis was performed only on the out-competing fungus from the mixed culture, *Pycnoporus coccineus*, showing that genes involved in detoxification of secondary metabolites had higher transcript abundance compared to the monoculture (Arfi et al., 2013). Jonkers et al. (2012) analysed mixed cultures of two plant-infecting fungi but their use of microarrays did not facilitate an accurate measure of total RNA from each species in their mixed cultures. To our knowledge, there is no literature on the genome-wide transcriptional responses of mixed cultures to lignocellulose degradation or of the antagonistic responses of different species when exposed to lignocellulose as mixed cultures.

There is substantial literature on the effects at the enzymatic level of mixed cultures and in particular with *T. reesei*, which is one of the most studied fungal species in mixed cultures (Ahamed and Vermette, 2008; Duff et al., 1987; Kolasa et al., 2014) as well as two studies that combined ascomycetes and basidiomycetes (Hu et al., 2011; Ma and Ruan, 2015). Culture filtrates from a mixed culture of *T. reesei* and a basidiomycete *Coprinus comatus* had a clear synergistic effect on saccharification compared to that of the monoculture filtrates, likely in part due to the de-lignifying activities of the basidiomycete (Ma and Ruan, 2015). Several studies show beneficial effects of mixed cultures for enzymatic activities but those studies did not culture the fungi with lignocellulose or did not perform saccharification assays with lignocellulose. One of the earlier studies showed there were beneficial effects when *T. reesei* was cultured with *Aspergillus* spp. where *T. reesei*, which is deficient in secreting β-glucosidases (or transcribing genes that encode β-glucosidases), was complemented by the secreted activities of *A. niger* (Duff et al., 1987). In the study of Hu et al. (2011) beneficial effects on enzymatic activities relevant to lignocellulose degradation were highest in mixed cultures of *Aspergillus oryzae* and *Phanerochaete chrysosporium*. Ahamed and Vermette (2008) reported a beneficial effect with higher volumetric filter paper activity in a mixed culture of *A. niger* and *T. reesei*, but not a higher filter paper activity per amount of fungal biomass compared to an *A. niger* monoculture.

There are several genome-wide transcriptomic studies of the response of monocultures of fungi to lignocellulose. These studies are in *A. niger* (de Souza et al., 2011; Delmas et al., 2012; Pullan et al., 2014; van Munster et al., 2014), *Aspergillus nidulans* (Brown et al., 2013; Coradetti et al., 2013), *T. reesei* (Bischof et al., 2013; Foreman et al., 2003; Hakkinen et al., 2012; Ries et al., 2013), *Neurospora crassa* (Benz et al., 2014; Tian et al., 2009) and *Myceliophthora thermophila* (Kolbusz et al., 2014). The regulatory basis for how fungi respond to lignocellulose consists of activating and repressing transcription factors that themselves respond to sugar molecules derived from the lignocellulose (for reviews see Daly et al. (2016), Glass et al. (2013), Kowalczyk et al. (2014)). In *A. niger*, the main activating transcription factor is XlnR which is activated by xylose (van Peij et al., 1998). In *T. reesei* the orthologous transcription factor XYR1 is also the main activating transcription factor but it is responsive to other sugars as well as xylose, such as cellobiose and sophorose (Stricker et al., 2008, 2006).

The three species chosen for this study were ascomycetes from three different genera; *A. niger*, *T. reesei* and *P. chrysogenum*. *T. reesei* is the dominant CAZyme producer used by industry (Martinez et al., 2008). *A. niger*, also a CAZyme producer, is an extensively studied species with regard to regulation of biomass-degrading CAZy genes and has a large repertoire of CAZymes (Andersen et al., 2011; Kowalczyk et al., 2014; Pel et al., 2007). *P. chrysogenum* is best known as a producer of β-lactam antibiotics, but *Penicillium* species are also enzyme producers and degrade lignocellulose in nature (Chávez et al., 2006; Gusakov and Sinitsyn, 2012) and *P. chrysogenum* is one of the *Penicillium* species that has a sequenced and annotated genome (van den Berg et al., 2008). Wild-type strains rather than industrial production strains were used so to be more similar to the sensing of the lignocellulose in nature. Industrial strains such as *T. reesei* RUT-C30 are modified in their regulatory circuits involved in carbon sensing as well as having an increased ability to secrete protein (Peterson and Nevalainen, 2012). Differences in enzymatic activities provide part of the rationale for combining fungal species in mixed cultures with lignocellulose and *T. reesei* lacks important activities. The absence of genes encoding pectin methylesterase activity and feruloyl esterase activity in *T. reesei* was noted by Martinez et al. (2008). Akel et al. (2009) also noted the absence of endo-arabinases in *T. reesei*.

At the genome-wide level, dual RNA-seq or simultaneous RNA-seq is a technique that allows the quantification of transcripts from multiple organisms simultaneously and the technique is primarily applied to host pathogen interactions (Westermann et al., 2012). Dual RNA-seq has the potential to be applied to mixed species fungal cultures, provided that there are sufficient nucleotide differences of the RNA-seq reads sequenced from each fungus and that sufficient reads can be obtained from each fungus in a mixed culture.

We will describe differences in gene content and enzymatic activities between the three fungal species that informed our experimental conditions for subsequent dual RNA-seq analysis. We used a method, which could be widely applicable, to quantify species-specific rRNA in mixed cultures to predict the number of RNA-seq reads that could be obtained. Subsequently, we will describe the limited beneficial effects and more widespread antagonistic effects on mycelial biomass, transcript abundance and enzyme activities of combining the three fungi in two and three species mixed cultures.