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Quantitative multiplexed profiling of *Penicillium funiculosum* secretome grown on polymeric cellulase inducers and glucose

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ABSTRACT

Filamentous fungi respond to the need to secure utilisable carbon from their growth milieu by secreting unique extracellular proteins depending upon the types of polymeric substrates. We have here profiled the variations in the secretome pattern of a non-model hypercellulolytic fungus – *Penicillium funiculosum*, grown in minimal media containing four different polymeric cellulase inducers, i.e., Avicel, wheat bran, ammonium-pretreated wheat straw and Avicel & wheat bran, and glucose over its early and late log phases of growth. Of the 137 secreted proteins validated at 1% FDR, we identified the quantified proteins in three clusters as early, persistently or lately expressed. The type of carbon substrate present in the culture media significantly affected the levels of cellulolytic enzymes expression by the fungus. The top abundant proteins quantified in the secretome for Avicel and wheat bran were cellobiohydrolaseI [GH7-CBM1], cellobiohydrolaseII [GH6-CBM1], β-glucosidase [GH3], arabinofuranosidase [GH51] and β-xylosidase [GH3], with bicupin being highest in case of wheat straw. Our results further suggested that the fungus secreted the extracellular proteins in waves, such that the initial responders act to hydrolyse the composite substrates in the culture environment before the second wave of proteins which tend to be more tailored to the specific substrate in the cultivating media.

Biological significance: In this article, we have comprehensively examined the dynamics of the secretome of a non-model hypercellulolytic fungus produced in response to model and composite cellulase inducers. Our study has provided additional insights into how the fungus enzyme machinery responds to the presence of different polymeric cellulase inducers over the two different growth phases (early growth and late growth phase). The comprehensive typing and quantification of the different proteins present in the secretomes of the cellulolytic fungal strains in response to diverse nutrient sources hold many prospects in understanding the fungus unique enzyme machinery and dynamics for the downstream biotechnological applications.

1. Introduction

Lignocellulosic biomass represents a vast, renewable and sustainable resource to produce biofuels and bio-based chemicals [1–3]. One way to harness it is by exploiting the natural toolbox of microorganisms that have evolutionarily been developed to use lignocelluloses as carbon and energy sources [1,4–6]. The most common efficient degraders of lignocelluloses are saprophytic ascomycetes and basidiomycetes [1–4]. They secrete diverse enzymes and associated proteins to break down structural polymers of biomass to sugars, followed by the conversion of these sugars to products using dedicated sugar metabolizers [1,2,5].

The nature of the enzymes secreted by plant biomass-degrading fungi, however, are usually reflective of the growth environment [5–10]. The hydrolysis of biomass by filamentous fungi occurs in stages - starting from nutrient sensing, to transmission and modulation of signals, to activation of transcription factors and gene induction, to enzyme complement and function [6,7]. With the advancements in high-throughput technologies bordering genomics, proteomics and systems-level studies; our current understanding of the mechanisms involved in fungal lignocellulosic biomass deconstruction been broadened. As such, we now have the frameworks for unravelling new enzymatic strategies employed by such microbes in deconstructing the plant cell wall [2,5–7,11].

While the importance of secreted proteins as the microbial “strike force” – network of carbohydrate-active enzymes or proteins - for

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lignocellulosic biomass deconstruction is not new, surprisingly, not many studies incorporate qualitative and quantitative information about cellulose inducers related changes with time. Most have focused on mass spectrometry-based identification and cataloguing of secretomes over one-time point. Notwithstanding, the comprehensive typing and quantification of the different proteins of secretomes by cellulolytic fungal strains relative diverse nutrient sources hold many prospects in understanding each fungus unique enzyme machinery and dynamics [12,13].

In our earlier report on the non-model ascomycete - *Penicillium funiculosum* NCIM 1228 - we showcased the fungus as being a hypercellulolytic in nature, with the biomass saccharification potential excelling industrial workhorse – *Trichoderma reesei* RUT C-30, and whose crude secretome competes favourably with commercial cellulase cocktail during biomass hydrolysis [14,15]. We identified the arrays of active proteins, their relative abundance and preferential associations within the crude secretome from the fungus when grown on a composite mixture of wheat bran and microcrystalline cellulose. However, in this study, we have proceeded with evaluating the secretome dynamics during cultivation on different polymeric cellulase inducers and glucose, to complement our understanding of the fungus secretome regulation on different polymeric cellulase inducers. The studies of the secretomes of microbes grown on insoluble substrates are essential for the discovery of novel proteins involved in biomass conversion [2,16,17].

Based on the preliminary experiments in our lab, we evaluated microcrystalline cellulose (Avicel), ammonium pre-treated wheat straw, wheat bran and a composite mixture of Avicel with wheat bran as polymeric cellulase inducers. Avicel is a pure crystalline cellulose, mainly homogeneous, and a known inducer of core cellulases [18,19]. Ammonium pre-treated wheat straw is heterogeneous, but majorly crystalline cellulose, and it is the primary biomass available to us for scale up. Wheat bran is heterogeneous, and it is an inducer of hemicellulases [20,21]. A composite mixture of Avicel with wheat bran in equal proportion served as model substrate for induction based on the previous study [14]. Glucose is a soluble sugar and the most preferred substrate for growth of fungi and is considered as a repressor for cellulase expression [22–25].

By understanding the differential expression pattern of the various essential biomass hydrolysing proteins from this non-model fungus, one can prioritise their inclusion while designing active enzyme mixture for more efficient lignocellulose biomass bioconversion as well as utilise them as possible targets for modulation during rational strain improvement regimens for biotechnological applications.

2. Experimental procedures

2.1. Experimental design and statistical rationale

The experimental setup consisted of two flasks for each carbon substrates (glucose, Avicel, wheat bran, ammonium hydroxide pretreated wheat straw, and a composite combination of Avicel with wheat bran) and for each time points (early and late log phase of the fungus growth). Thus, there were 20 flasks in all evaluated (Fig. 1). After cultivation, the flasks were assessed for pH and growth of the fungus via intracellular ATP measurement. Following a satisfactory evaluation, secretomes from duplicate flasks were pooled together as a single set partly to minimise biological variations [9] as well as to provide a sufficient pool of concentrated protein for downstream analysis and validation experiments. Endoglucanase and cellobiohydrolase activities for secretomes from each carbon substrate and time points (early phase and late log phases) were evaluated while the mass spectrometry data acquisition of appropriately labelled samples was determined in duplicates. For proteomic statistical analysis, the log-normalised corrected reporter ion intensities from MaxQuant output files were processed in Perseus version 1.5.3.2 [26,27]. The statistical methods used at each step of data processing are described in detail in the corresponding paragraph of experimental or analysis procedure. Average values and standard errors of the mean are reported for all studies.

2.2. Microorganism, cultivation conditions, and secretome extraction

Penicillium funiculosum NCIM 1228 previously identified as a hypercellulolytic strain [14] was maintained on potato dextrose agar plates. Conidial suspensions were prepared by growing *P. funiculosum* NCIM 1228 potato dextrose agar for seven days at 30 °C. Spores were collected from lawns of fungi culture in sterile distilled water, filtered through a glass wool plug to remove hyphal fragments and counted on a hemocytometer. For enzyme production, conidia at 10^6 spores·mL^{-1} were inoculated in a base medium - KH_2PO_4 2.0 g·L^{-1}; $(NH4)_2SO_4$ 1.4 g·L^{-1}; Urea 0.3 g·L^{-1}; $MgSO_4{\cdot}7H_2O$ 0.3 g·L^{-1}; $FeSO_4{\cdot}7H_2O$ 5.0 mg·L^{-1}; $MnSO_4{\cdot}H_2O$ 1.6 mg·L^{-1} and $ZnSO_4{\cdot}7H_2O$ 1.4 mg·L^{-1} [28], containing 2% carbon. Submerged culture experiments were carried out in 100 mL shake flasks containing 20 mL cultures in duplicates. The flasks were kept at 30 °C for 3 and 6 days (corresponding to the early phase and late log phases of the fungus) respectively with orbital shaking at 150 rpm (Innova 44, Eppendorf AG – Germany). Induced cultures were centrifuged at 7000 rpm for 10 min at 4 °C, and the supernatants were filtered using syringe filters with a 0.45-µm PVDF membrane (Millipore, Germany). The obtained secretome was concentrated using Vivaspin columns with a 5 kDa MWCO (GE Healthcare, USA).

2.3. Biochemical assays and validations

Unless otherwise indicated, all enzymatic activities were measured in sodium acetate buffer (50 mM, pH 5.0) at 50 °C. The activities of individual secretome towards amorphous cellulose (CMC) and microcrystalline cellulose (Avicel PH-101) were measured by using the dinitrosalicylic acid (DNSA) method, as described before [29]. Briefly, 30 µL of crude secretome were mixed with 100 µL of substrates at 1% concentration and incubated for 30 min. The reaction was terminated by the addition of DNSA reagent [30] and boiled for 10 min. The absorbance at 540 nm was measured relative to a glucose standard curve. One unit of enzyme activity was defined as the amount of protein that released one µmol of reducing sugar per min. The protein concentrations of the obtained secretome were determined by the Bicinchoninic acid (BCA) method using bovine serum albumin as a standard. Sodium dodecyl sulphate (SDS)-polyacrylamide gels (10%) were prepared, and proteins were separated via SDS - polyacrylamide gel electrophoresis (PAGE) as described by Laemmli [31]. Proteins of the gel were stained with Coomassie blue R-250 (Sigma-Aldrich, USA). The molecular mass under denaturing conditions was determined with reference standard proteins (Thermo Scientific, USA). For growth estimation on the different carbon sources, the intracellular adenosine triphosphate (ATP) concentration of fungal cells from the respective cultures was used [32]. Briefly, an aliquot of the fermentation broth containing mycelium was extracted from the flasks, and the amount of ATP released following mycelium lysis was determined using a kit (CheckLite 250 Plus; Kikkoman, Japan). The luminescence based on the luciferin-luciferase reaction measured using luminescence mode of the multimode plate reader (Spectra Max M3, USA).

2.4. Protein preparation for digestion and TMT labelling

For relative protein quantitation, the protein samples were separately derivatised with 6-plex TMT reagents (ThermoFisher Scientific), according to the instructions provided by the manufacturer. In brief, secretome from each test samples was obtained via centrifugation and clarified by filtering them through 0.2 µm PVDF filters. The clarified supernatants were further subjected to ultrafiltration using a 5 kDa MWCO Vivaspin (GE Healthcare, USA) to obtain concentrated proteins.