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Substrate-based differential expression analysis reveals control of biomass degrading enzymes in *Pycnoporus cinnabarinus*

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ABSTRACT

White rot fungi possess a powerful ability to degrade recalcitrant lignin within plant biomass. *Pycnoporus cinnabarinus* PB 94 accomplishes lignin degradation through the combined activity of laccases, peroxidases, and their supporting enzymes. Assembly of the *de novo* transcriptome for PB 94 resulted in identification of 45,286 transcripts, including isoforms, and a predicted 27,990 genes. Differential expression analysis revealed how the expression of these ligninolytic enzymes was dictated by the nature of the substrate. Growth on lignin containing poplar and switchgrass resulted in as much as a 285-fold increase in expression of peroxidases (auxiliary activity family AA2) and a 36-fold increase in expression of alcohol oxidases (auxiliary activity family AA3) compared to growth on soluble sugars. These findings suggest that expression of these enzyme classes was controlled by the presence of lignin, or triggered by an aromatic break out product of lignin degradation. Laccases showed little significant regulation under these growth conditions, but one laccase transcript (TR10024|c0_g1_i1), similar to laccase gene *lcc2* in *Pycnoporus sanguineus*, was greatly upregulated during growth on cellobiose, suggesting that this sugar may be a mechanism for enhanced expression. The findings presented here reveal regulation patterns of enzymes critical for the lignin degrading activity of *P. cinnabarinus* PB 94 providing insight into how the white rot fungus controls lignocellulolytic activity.

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1. Introduction

Production of chemicals and fuels from biomass is critical for a renewable economy. While cellulose and hemicellulose have been valuable sugar feedstocks for microbial production [1,2], lignin has proven difficult to convert into profitable products. Lignin valorization aims to convert lignin into renewable chemical feedstocks or biofuels [3–5], but lignin's heterogeneous nature often results in complex product distributions upon depolymerization.

Incorporating biological lignin depolymerization processes into valorization efforts is a promising opportunity in making lignin a commercially viable renewable chemical feedstock [6]. An in depth understanding of biological lignin breakdown could help streamline bio-reactors and provide inspiration for biomimetic approaches to lignin depolymerization.

White rot fungi degrade woody plant materials, breaking down lignin to leave soft, white, rotted wood behind. The common mechanisms by which white rot fungi accomplish this is through the use of lignin peroxidase, manganese peroxidase, versatile peroxidase, and laccase enzymes to oxidatively degrade lignin [7,8]. The fungus *Pycnoporus cinnabarinus* was once thought to only generate laccase enzymes to facilitate lignin breakdown [9,10]. However, the presence of lignin peroxidases has recently been detected in extracellular assays and in the genome of *P. cinnabarinus* BRFM137, which contained the genes necessary to express lignin peroxidases as well as their support enzymes [11,12]. Based on these new insights, further exploration of *P. cinnabarinus* PB 94's role in lignin disassembly was undertaken through RNA sequencing and differential expression analysis [13,14].

Next generation sequencing (NGS) is a powerful tool for understanding biological processes, including the breakdown of lignin, through transcriptomic analysis [15–17]. In particular, global analysis of gene expression under different physiological conditions can give detailed insights into the enzymatic profile of lignin-degrading fungi in response to lignin-rich substrates [18]. Transcriptome analysis has previously been employed to study the closely related species of white rot fungi, *Pycnoporus coccineus,* detailing the metabolic changes that occur between hard and soft wood lignin degradation [19]. We have used RNAseq to explore the changes in lignin-degrading capabilities of a previously uncharacterized strain of *P. cinnabarinus,* PB 94, comparing expression during growth on sugar and biomass of varying lignin content. Differential expression analysis reveals genes that are significantly regulated in response to growth on lignin-rich substrates and presents a picture of the metabolic changes that occur in response to growth on lignin for this white rot fungus. The regulation of carbohydrate active enzymes (CAZymes) [20], particularly members of the auxiliary activity (AA) family containing enzymes responsible for lignin degradation, was of particular interest in these investigations [21].

2. Materials and methods

2.1. Growth and Maintenance of P. cinnabarinus *PB 94*

P. cinnabarinus PB 94 was obtained from the ATCC and maintained on Remel malt extract agar plates (33.6 g/L). Experimental cultures were grown on five different carbon sources: glucose, maltose, cellobiose, switchgrass, and Poplar wood chips, as well as a control culture consisting solely of the semi-minimal media (sMM) and the agar plug from the *P. cinnabarinus* PB 94 maintenance plate. The sMM consisted of diammonium tartrate (1.84 g/L), disodium tartrate (2.3 g/L), KH_2PO_4 (1.33 g/L), $CaCl_2 \cdot 2H_2O$ (0.1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $FeSO_4 \cdot 7H_2O$ (0.07 g/L), $ZnSO_4 \cdot 7H_2O$ (0.046 g/L), $MnSO_4 \cdot H_2O$ (0.035 g/L), $CuSO_4 \cdot 5H_2O$ (0.007 g/L), and yeast extract (1 g/L). Liquid cultures consisted of 45 mL sMM (autoclaved) combined with 5 mL of maltose or cellobiose dissolved in sMM (final concentration 20 g/L). Maltose and cellobiose solutions added to the sMM were filtered through a 0.22 μM sterile filter. Biomass cultures contained 2 g biomass (Switchgrass or Poplar) and 20 mL of sMM. Each culture was inoculated with a 5-mm cube of agar overgrown with *P. cinnabarinus* PB 94. The maltose and control cultures were grown for five days and the cellobiose culture was grown for nine days. Liquid cultures were centrifuged at 3220 RPM for ten minutes at 4 °C to isolate the fungal cells from the sMM, 1 mL of RNAlater[TM] was added to the cells and the samples were stored at −80 °C. Biomass cultures were grown for twelve days and the RNA was immediately isolated at the time of harvest.

2.2. RNA isolation

Total RNA was isolated using a Qiagen[TM] RNeasy mini kit with Qiagen[TM] Qiashedder spin columns. Fungal cells were homogenized via grinding with a mortar and pestle under liquid nitrogen. RNA isolation then proceeded following the plant and fungi protocol with on column DNA digest. The RNA concentration was determined using a Qubit fluorometric assay and the RNA integrity number equivalent (RINe) was determined using an Agilent 2200 tape station. RNA was isolated from three biological replicates for each growth condition.

2.3. cDNA library preparation and sequencing

cDNA libraries for cultures grown on soluble substrates were prepared using the TruSeq[TM] Stranded mRNA Library Prep Kit (Illumina, San Diego, CA) following the standard protocol. mRNA from cultures grown on solid biomass was selected using the Ribo Zero Gold[TM] rRNA removal kit for yeast due to low RINe scores (∼5). The yeast kit was selected after comparing ribosomal RNA sequences for *P. cinnabarinus* to those used in the Ribo-zero kit using the RNA MatchMaker tool from Epicentre (www.epibio.com/rnamatchmaker). After cDNA library preparation, each sample was normalized and pooled together for a final concentration of 4 pM and sequenced on an Illumina NextSeq 500 the v2 mid-output 150 cycle kit, with paired end, 75 base reads.

The transcriptome of *P. cinnabrainus* was assembled *de novo* using the Trinity algorithm[22] and reads from one sample under each growth condition. Transcript IDs are formatted as 'TR##|c#_g#_i#' where 'TR##|c#_g#' indicates a gene and 'i#' after the gene indicates isoforms, if any are identified. Blast2GO was used to perform BLASTx and InterPro scan annotation of the *de novo* transcriptome to provide insight into the predicted function of transcripts.

2.4. Differential expression analysis

Transcripts were quantified for differential expression using the Trinity utility function, align_and_estimate_abundance.pl, to perform RSEM analysis [23]. Differential expression analysis was then performed using the DESeq2 package for the R programming platform [24]. The threshold for a gene to be considered regulated was a Log2 fold change value of at least 1 and a p-value of 0.01. Gene set enrichment analysis (GSEA) [25] was performed using gene sets for CAZy predicted function determined based on the protein domain annotations assigned by the InterPro scan. To compare expression between transcripts, counts measured in transcripts per million (TPM) were used as determined by the RSEM analysis.

3. Results and discussion

3.1. De novo *assembly of* Pycnoporus cinnabarinus *PB 94 transcriptome yields unexpectedly large exome*

The transcriptome of PB 94 was assembled *de novo* using RNA isolated from cultures grown on five substrates: glucose (G), maltose (M), cellobiose (CB), Poplar (Po), and switchgrass (SG), as well as a control (C) culture without substrate (Table S1). Initially, the exome of *Pycnoporus cinnabarinus* BRFM137 was used as a reference for alignment and abundance estimation using RSEM analysis [11]. However, alignment to BRFM137's genome was poor and resulted in a significant number of genes with no aligned reads, therefore the *de novo* transcriptome was used for all analyses. Cultures grown on soluble substrates yielded high quality RNA as determined by Agilent TapeStation RINe scores of 7.5 or greater while cultures grown on biomass, Po and SG, demonstrated isolation of lower quality RNA, yielding partially degraded RNA with RINe scores between 5.5 and 6.6. The RNA degradation observed on biomass substrates, which was not observed on soluble substrates, may be due to the generation of radical species by the lignin degrading-enzymes expressed under these conditions, resulting in lower stability of RNA. To accommodate the partially degraded RNA samples, ribosomal depletion, rather than the poly-A enrichment used on high quality RNA, was used to remove ribosomal RNA from Po and SG cultures [26]. Poly-A enrichment was used to isolate mRNA from samples with high RINe scores.

The *de novo* assembly used approximately 30 million reads from each of the five different growth conditions for equal representation of each substrate. This resulted in a transcriptome comprised of 45,286 transcripts, including isoforms, of a predicted 27,990 genes (Supplementary Table S2), much larger than the previous