Transcriptome Sequencing and Comparative Analysis of *Piptoporus betulinus* in Response to Birch Sawdust Induction

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Abstract: *Piptoporus betulinus*, a brown-rot parasitic fungus of birch trees (*Betula* species), has been used as a common anti-parasitic and antibacterial agent. The lack of genetic resource data for *P. betulinus* has limited the exploration of this species. In this present study, we used Illumina Hiseq 2500 technology to examine the transcriptome assembly of *P. betulinus* in response to birch sawdust induction. By de novo assembly, 21,882 non-redundant unigenes were yielded, and 21,255 (97.1%) were annotated with known gene sequences. A total of 340 responsive unigenes were highly homologous with putative lignocellulose-degrading enzyme candidates. Additionally, 86 unigenes might be involved in the chemical reaction in xenobiotics biodegradation and metabolism, which suggests that this fungus could convert xenobiotic materials and has the potential ability to clean up environmental pollutants. To our knowledge, this was the first study on transcriptome sequencing and comparative analysis of *P. betulinus*, which provided a better understanding of molecular mechanisms underlying birch sawdust induction and identified lignocelluloses degrading enzymes.

Keywords: *Piptoporus betulinus*; transcriptome; comparative analysis; lignocellulose degradation

1. Introduction

Piptoporus betulinus, a brown-rot parasitic fungus of birch trees (*Betula* species), has been used as a common anti-parasitic and antibacterial agent for the treatment of wounds and various diseases, such as cancer, inflammation and so on [1,2]. Its extract has been demonstrated to be effective in preventing fatigue, strengthen immunity and relieving pain. Previous findings have suggested that this fungus has the ability to degrade of wood components, including cellulose and hemicellulose [3–5].

Lignocellulose represents an abundant carbon-neutral renewable resource that can be used for the production of bioenergy and biomaterials. It could be efficiently converted into ethanol using fungi as biological pre-treatment [6]. Furthermore, published findings have shown that brown-rot fungi have the potential as a new biocatalyst with unprecedented fermentability and a microbial starter [7]. Moreover, brown-rot fungi primarily break down cellulose and hemicellulose with carbohydrate active enzymes (CAZy), which catalyze cellulose into 6-carbon sugars [8,9]. In addition, it could

greatly balance the conversion system of bioethanol production without genetic engineering support or external hydrolase. Therefore, it was considered as a promoter in lignocellulose biodegradation.

Although *P. betulinus* has various potential properties, the gene sequences in the publicly available databases for this fungus are rare. Only about 78 nucleotide sequences of *P. betulinus* to date have been deposited in National Center for Biotechnology Information's (NCBI) GenBank database, and most of them were isolated and cloned from ribosomal RNA genes [10,11]. In recent years, the lignocellulose-decaying transcriptomes of white-rot fungi have been thoroughly studied [12,13], while only few studies describing the transcriptomes of brown-rot fungi on wood have been reported [14,15]. This present study used Illumina Hiseq 2500 technology to examine the transcriptome of *P. betulinus* in response to birch sawdust induction. Transcriptome profiling of *P. betulinus* provides a better understanding of molecular mechanisms underlying birch sawdust induction and could identify lignocellulose-degrading enzymes in this species.

2. Materials and Methods

2.1. Fungal Strain

In August 2014, the fruiting body of the fungal strain was collected from *Betula platyphylla* at Liangshui Nature Reserve, Lesser Xing'an Mountains in Yichun city, Heilongjiang Province, China. The fruiting body was sterilized in sodium hypochlorite for 1 min, before being rinsed with sterile deionized water 3–5 times. This was cultured on potato dextrose agar plate and kept in the dark at 28 °C for 10 days. Following this, this strain was identified as *P. betulinus* according to Internal Transcribed Spacer (ITS) sequencing and alignment in NCBI (GenBank accession number MF967582).

2.2. RNA Extraction

The strain was inoculated into different liquid media (Potato dextrose broth (PDB) and PDB + birch sawdust (*Betula platyphlla*) (5 g/L)), which were cultured with continuous shaking (150 rpm) at 28 °C for 10 days. For each treatment (PDB and PDB + birch sawdust, respectively), the mycelia of *P. betulinus* were harvested by filtration, before being frozen in liquid nitrogen and prepared for RNA extraction. Total RNA was extracted using TRNzol reagent according to the manufacturer's protocol (TIANGEN, Beijing, China). Total RNA was extracted from each sample in triplicate, before synthesized cDNA were pooled together for sequencing. A quantitative real-time PCR (qRT-PCR) using RNA samples as templates was performed to detect genomic DNA. Samples with no amplified qRT-PCR products were used as a template for cDNA synthesis. The extracted RNA yield and purity were checked by NanoDrop 2000 (Thermo Scientific, Hudson, NH, USA). The qualified RNA samples were used for cDNA synthesis using PrimeScript™ RT reagent Kit (TakaRa). Subsequently, cDNA fragments were selected for PCR amplification and cDNA library were used for sequence analysis via Illumina HiSeq™ 2500(Illumina, San Diego, CA, USA).

2.3. Transcriptome Analysis

Raw reads were cleaned by removing adaptor sequences, short sequences and low-quality reads (reads containing Ns >5). The remaining clean reads were assembled into unigenes using short reads assembling program, which is also known as SOAPdenovo [16]. TIGR Gene Indices clustering tool (TGICL) was used to acquire a set of non-redundant unigenes [17]. After this, all the non-redundant unigenes were used for blast search (E-value < 10^{-5} or 10^{-3}) and annotation in various databases, including NCBI Nr database, SwissProt database, Kyoto Encyclopedia of Genes and Genomes (KEGG) database and Cluster of Orthologous Groups (COG) database. For the functional annotation, gene ontology (GO) terms were analyzed using the Blast2GO program [18]. Finally, Web Gene Ontology Annotation Plot (WEGO) was used to classify GO function for all unigenes [19].

A differential expression analysis between two treatments was performed using the DESeq2 R package (http://www.bioconductor.org/package/DESeq2/). The false discovery rate (FDR) control

method was applied in the Benjamini and Hochberg method to correct the results for *p*-values. An FDR <0.01 and FC (fold change) $\geq$2 was set as the threshold to determine the significance of gene expression differences. The fragments per kilo bases per million reads (FPKM) were used to evaluate expressed values and quantify transcript levels, which normalizes and eliminates the influence of gene length and sequencing depth in calculating gene expression, allowing for direct comparison of gene expression between different treatments [20]. Genes with an adjusted *p*-value < 0.05 was assigned as differentially expressed according to the reports of Deng et al. [21]. For pathway enrichment analysis, all differentially expressed unigenes were submitted in the online KEGG Automatic Annotation Server (http://www.genome.jp/kegg/) and we searched for significantly enriched KEGG terms to obtain the pathway annotation (adjusted *p*-value < 0.05).

2.4. Quantitative Real-Time PCR Validation

A total of 12 representative birch sawdust induced-relevant unigenes (carbohydrate-active enzyme genes and lignocellulose-degrading enzyme genes) with significantly differential expression (adjusted *p*-value < 0.05) identified by RNA-seq were chosen for experimental validation using qRT-PCR with gene specific primers (Table S1). The qRT-PCR was performed using SYBR® Premix Ex Taq™ II Kit (Tli RNaseH Plus) (TakaRa, Tokyo, Japan) in a volume of 20 µL, which contained 10 µL of SYBR Premix Ex Taq (2×), 0.4 µL of ROX Reference Dye II (10×), 2 µL of cDNA template and 0.5 µM of each primer. The amplification was performed as follows: 95 °C for 30 s, 45 cycles of 95 °C for 5 s and 60 °C for 40 s. The qRT-PCR amplifications were conducted in an ABI 7500 detection system (Applied Biosystems, Carlsbad, CA, USA). All samples and reactions were performed in triplicate and the results were expressed relative to the expression levels of actin in each gene by using the $2^{-\triangle\triangle Ct}$ method [22].

2.5. Availability of Data

All transcriptome data files were submitted to the Sequence Read Archive database with the accession number SRP117136.

3. Results

3.1. Raw Reads Processing and de novo Assembly

For a better understanding of molecular mechanisms underlying birch sawdust induction and comparative transcriptome analysis in *P. betulinus*, two cDNA treatments prepared from PDB media and PDB + sawdust were sequenced with Illumina HiSeq™ 2500. An overview of the sequencing and assembly is given in Table 1 and Table S2. After filtering adapters and short sequences or low-quality bases, a total of 94,942,136 clean reads with 9,510,783,105 nucleotides were obtained in the two treatments (Table S2). Finally, de novo assembly yielded 21,882 non-redundant unigenes with an average length of 1949 bp and an N50 of 3007 bp (Table 1). Of these unigenes, 17,081 (88.1%) were >500 bp and 10,670 (48.8%) were >1500 bp. These obtained sequences provided abundant information on transcriptomes for further analysis of the birch sawdust-induced genes in *P. betulinus*.

Table 1. Overview of transcriptome sequencing and assembly for *P. betulinus.*

| Length (bp)	Total Number	Percentage (%)
<200	0	0.0
200–500	4800	21.9
500–1000	3362	15.4
1000–1500	3049	23.9
1500–2000	2626	12.0
$\geq$2000	8044	36.8
Total	21,882	
Total length of all unigenes		42,652,276
Median length of all unigenes (N50)		3007
Average length of all unigenes		1949.17

3.2. Functional Annotation

To identify the putative functions of unigenes in *P. betulinus*, we annotated all the assembled unigenes against the Nr, Swiss-port, KEGG and COG databases. With comparison against those four databases, a total of 21,255 (97.1%) unigenes were successfully annotated with known gene sequences. The number of unigenes with significant similarity to sequences in Nr, Swissport, KEGG, and COG databases were 21,255 (97.1%), 3351 (15.3%), 6256 (28.6%) and 7955 (36.4%), respectively (Table 2).

Table 2. Annotation of non-redundant unigenes.

Database	Number of Annotated Unigenes	Percentage of Annotated Unigenes
Nr	21,255	97.1%
Swissport	3351	15.3%
KEGG	6256	28.6%
COG	7955	36.4%

The GO analysis classified the functions of predicted unigenes into three main categories: biological processes, molecular function and cellular components (Figure 1). A total of 3351 sequences were assigned with 9111 GO terms, among which 2000 unigenes (22.0%) were assigned at least one GO term in the biological processes, 4253 (46.7%) in the cellular components and 4658 (51.1%) in molecular functions. Additionally, these unigenes were further classified into functional subcategories. In cellular components, the largest subcategory was cells (29.8%) and the second largest was cell parts (13.6%). Regarding molecular function, the largest number were found in catalytic activity (63.5%) and binding (46.8%). According to biological processes, genes involved in metabolic process and cellular process were highly represented, which accounted for 54.3% and 42.9% of the matched unigenes in the subcategory, respectively.
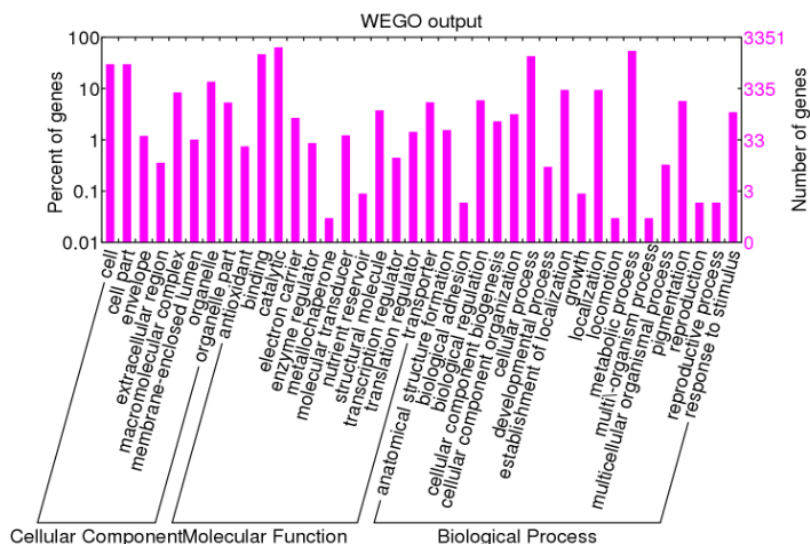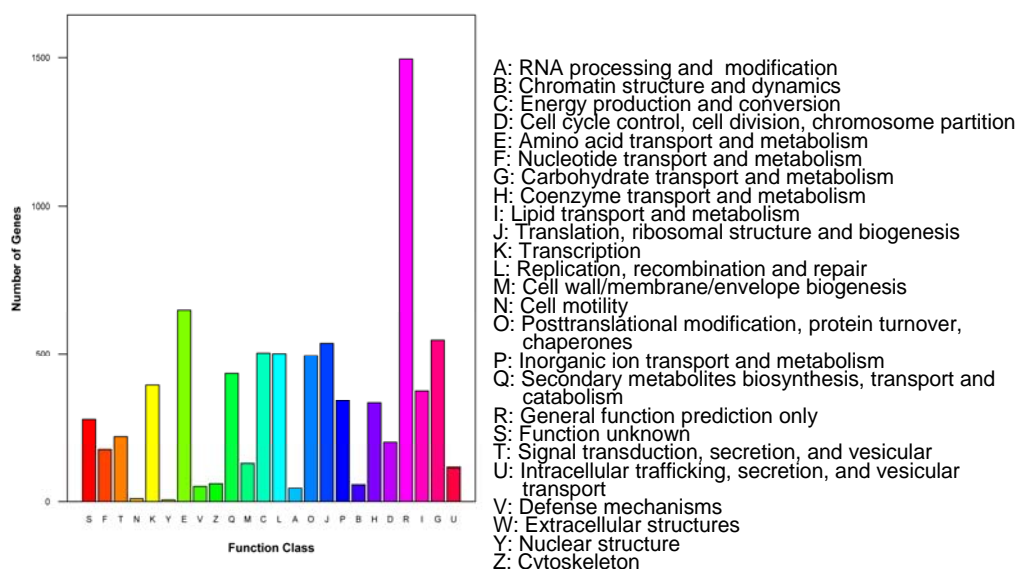

Figure 1. Functional annotation of non-redundant unigenes based on Gene Ontology (GO) classification. The results are summarized in three functional categories: cellular components, molecular function and biological process.

The genes belonging to the same biological pathway synergistically participated to accomplish the biological functions. The KEGG database was used to identify and predict the metabolism pathways in *P. betulinus*. Details of sequences involved in KEGG annotation are listed in Table S3. In KEGG metabolic pathways, a total of 6256 unigenes were matched in 309 different KEGG pathways (Table S3). Among these pathways, the most common was amino acid metabolic pathways with 240 members,

followed by ribosome biogenesis (202) and energy metabolism (197). These might be involved in maintaining the basic metabolic process of *P. betulinus*.

In addition, in COG classification analysis, out of 21,882 Nr hits, 7955 unigenes were grouped into 25 COG categories. Among them, the prediction of the general function was the most populated group (18.8%), followed by amino acid transport and metabolism (8.16%), and carbohydrate transport and metabolism (6.9%). Two hundred and seventy-six unigenes were mapped into unknown functions, which might be involved in a specific gene in *P. betulinus*. The smallest groups were found in cell wall/membrane/envelope biogenesis and nuclear structures (Figure 2).

A: RNA processing and modification
B: Chromatin structure and dynamics
C: Energy production and conversion
D: Cell cycle control, cell division, chromosome partition
E: Amino acid transport and metabolism
F: Nucleotide transport and metabolism
G: Carbohydrate transport and metabolism
H: Coenzyme transport and metabolism
I: Lipid transport and metabolism
J: Translation, ribosomal structure and biogenesis
K: Transcription
L: Replication, recombination and repair
M: Cell wall/membrane/envelope biogenesis
N: Cell motility
O: Posttranslational modification, protein turnover, chaperones
P: Inorganic ion transport and metabolism
Q: Secondary metabolites biosynthesis, transport and catabolism
R: General function prediction only
S: Function unknown
T: Signal transduction, secretion, and vesicular
U: Intracellular trafficking, secretion, and vesicular transport
V: Defense mechanisms
W: Extracellular structures
Y: Nuclear structure
Z: Cytoskeleton

Figure 2. Histogram presentation of clusters of orthologous groups (COG) classification (7955 sequences have a COG classification among the 25 categories).

Based on GO classification, KEGG pathway and COG annotation, the catalytic function accounted for the highest percentage in the molecular functions, while amino acid transport and metabolism, carbohydrate transport and metabolism were the predominant categories. Therefore, further analysis was needed with specific focus on those pathways.

3.3. Changes in Gene Expression under Sawdust Induction

To characterize the differences of molecular response to the PDB (control) and PDB + birch sawdust medias, unigene expression levels were calculated by the FPKM method. Based on FPKM values, 776 unigenes were identified as differentially expressed genes (Table S4). Among them, 444 genes were up-regulated and 332 were down-regulated, which were identified by DESeq2 (http://www.bioconductor.org/package/DESeq2/) with adjusted p-value $\leq$ 0.05 and fold change value >2. Following this, all differentially expressed genes were mapped into 26 different metabolic pathway categories (Table S5). Of these annotated pathways, phenylpropanoid biosynthesis, starch and sucrose metabolism and lysosome were the main pathways that accounted for 7.8%, 7.3% and 6.4%, respectively. Notably, the pathway enrichment analysis revealed that phenylpropanoid biosynthesis as well as starch and sucrose metabolism were the important pathways in the degradation of wood components by *P. betulinus*.

3.4. Detection of Lignocellulose-Degrading Enzyme-Relevant Gene Sequences and qRT-PCR Analysis

For further insight into birch sawdust induction in *P. betulinus*, we detected and analyzed induction-related genes in this study. These matched enzymes were mainly involved in the degradation of cellulose, hemicellulose and starch. There were 340 highly homologous unigenes

in putative wood-degrading enzymes (Table 3 and Table S6). Among them, 109 unigenes were identified as glycoside hydrolases (GHs), 37 belonged to glycosyl transferases (GTs), while 12 and 13 genes were assigned to carbohydrate esterases (CEs) and polysaccharide lyases (PLs), respectively. Fifty-three unigenes shared a similar identity to glucosidase, among which 52 homologs belonged to beta-glucosidase. Twenty-three unigenes were similar to 3-(3-hydroxy-phenyl) propionate hydroxylase, 20 unigenes to chitinase, 16 unigenes to mannosidase, 11 unigenes to polygalacturonase, 9 unigenes to mannosidase, 9 unigenes to pectinesterase.

Table 3. Putative wood-degrading genes in the *P. betulinus* transcriptome.

Enzymes	Number of Unigenes
glycoside hydrolases	109
glycosyl transferases	37
carbohydrate esterases	12
polysaccharide lyases	13
beta-glucosidase	52
alpha-glucosidase	1
mannosidase	16
glycosyltransferase	4
polygalacturonase	11
alpha-galactosidase	7
D-xylose 1-dehydrogenase (NADP)	4
N-glycosylase	2
Pectinesterase	9
3-(3-hydroxy-phenyl)propionate hydroxylase	22
alpha-amylase	5
xylose isomerase	7
beta-xylosidase	2
xyloglucan:xyloglucosyl transferase	2
glucosylceramidase	5
chitinase	20
Total	**340**

Several putative unigenes were involved in xenobiotic biodegradation and metabolism (Table S6). Enzymes involved in chloroalkane and chloroalkene degradation were *S*-(hydroxymethyl) glutathione dehydrogenase/alcohol dehydrogenase and aldehyde dehydrogenase with 7 and 41 unigenes, respectively [23]. One unigene was identified as carboxymethylenebutenolidase, which catalyzed the chemical reaction in toluene and chlorobenzene degradation [24]. Two unigenes, 1 and 23 unigenes, were found to exhibit a striking homology to amidase, nitrilase and 3-(3-hydroxy-phenyl) propionate hydroxylase, respectively. These above enzymes were related to styrene degradation. Alcohol dehydrogenase, which mainly degrade naphthalene compounds, were detected in *P. betulinus* transcriptome with 7 unigenes. Four unigenes belonged to aflatoxin B1 aldehyde reductase, which regulated the metabolism of xenobiotics by cytochrome P450.

To verify that the unigenes from sequencing were indeed differentially expressed genes and to analyze the difference of gene expression profile between control samples and induced samples, twelve unigenes related to lignocellulose-degrading enzyme-encoding genes were selected for qRT-PCR analysis (Figure 3). The results showed that 8 unigenes were up-regulated and 4 unigenes were down-regulated. Interestingly, the expression of c10553_g1_i5 and c10553_g1_i6 (mannosidase, EC: 3.2.1.21) under sawdust-induced samples increased by 6.3-fold and 1.5-fold compared to controls, suggesting that most of mannosidases were induced by sawdust in the lignocellulose-degrading process. Similarly, the expression of pectinesterase (c8512_g1_i14 and c8512_g1_i8) was also increased after being cultivated in birch sawdust. Several lignocellulose-degrading enzymes were detected to be down-regulated, including c7080_g1_i5 and c8929_g1_i1 (polygalacturonase); as well as c9335_g2_i1

and c5710_g1_i1 [3-(3-hydroxy-phenyl) propionate hydroxylase]. Therefore, those enzymes were jointly involved in the degradation of lignocellulose.
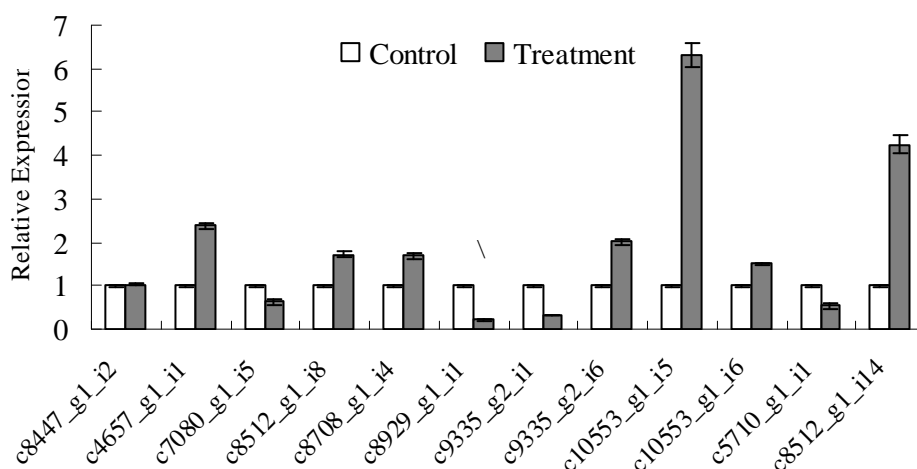
Figure 3. Quantitative real-time PCR validation of differentially expressed unigenes in the *P. betulinus* transcriptome. All reactions were performed in biological triplicate, and the error bars represent the standard deviations. This figure included c8447_g1_i2, c9355_g2_i6 β-D-Glucosidase; c10553_g1_i6, c10553_g1_i5,β-mannosidase; c8512_g1_i14, c8512_g1_i8, pectinesterase c4657_g1_i1, c8708_g1_i4, α-glycosidase; c7080_g1_i5, c8929_g1_i1,polygalacturonase; c9335_g2_i1 and c5710_g1_i1 [3-(3-hydroxy-phenyl) propionate hydroxylase].

4. Discussion

Transcriptomics, an important part of functional genomics, clarify the genetic transcription of cells at the overall level [25]. Data obtained by Illumina high-throughput sequencing has many advantages due to its large storage size, high efficiency and low cost, which is suitable for a species that has not had its whole genome sequenced. In this study, we firstly sequenced the *P. betulinus* transcriptome using Illumina Hiseq 2500 technology. After de novo assembly, 21,882 non-redundant unigenes were obtained, among which 21,255 were annotated into known functions involved in the lignocellulose-degrading process. These findings showed that a considerable number of genes were successfully identified through high-throughput sequencing and would be useful for the further functional analysis.

Based on KEGG pathway database, 6256 unigenes were mapped into 309 types of enzyme-encoding genes. Among them, many unigenes participated in degradation of wood components, which would provide a better platform for cloning of cellulose-degrading genes and related functional validation. The KEGG pathway results showed that amino acid transport and metabolism, carbohydrate transport and metabolism as the predominant pathways were closely associated with cellulose and hemicellulose degradation.

Annotation analysis of unigenes in the *P. betulinus* transcriptome showed that 340 transcripts were identified as putative wood-degrading genes, which is less than other brown-rot fungi [26,27]. Additionally, 53 unigenes were found to be similar to glucosidase, demonstrating that *P. betulinus* could degrade wood components. Interestingly, 2 unigenes were identified as pectinesterase in our transcriptome, which is comparable with other brown-rot fungi [28] and suggests that this enzyme was relatively conservative. Although we found that 20 unigenes had homology with chitinase, further confirmation of its ability to degrade chitin is required.

Lignin biodegradation was mainly detected in white-rot fungi, which produces multiple isoenzymes of lignin peroxidase, manganese peroxidase and laccase [29]. Although *P. betulinus* is unable to degrade lignin, it could secrete enzymes which may modify lignin or break lignin seal by the methylation pathway and sufficiently expose cellulose and hemicellulose for enzymatic

action. Recently, most of the studies were focused on *Postia placenta*, such as genome, transcriptome, secretome analysis and nuclear magnetic resonance analysis [27,30]. Martinez et al. first reported transcriptional profile of brown-rot fungus *P. placenta*, and their results revealed that this fungus possessed unique extracellular enzyme systems, including an unusual repertoire of glycoside hydrolases, while exocellobiohydrolases and cellulose-binding domains were absent in this fungus [27]. When *P. placenta* was grown in medium containing cellulose as the sole carbon source, transcripts corresponding to many hemicellulases and endoglucanases were expressed at high levels compared to the glucose-grown culture [31]. It is well known that although brown-rot and white-rot fungi are both basidiomycetes, white-rot fungi can produce complex ligninolytic systems to degrade lignin [32]. The different degradation pathway mechanisms in these two fungi have attracted the attention of scientists. However, lignin peroxidase, manganese peroxidase, versatile peroxidase and laccase were not detected in the *P. placenta* genome [31], suggesting that this fungus could not induce lignin depolymerization, which was consistent with our finding.

In addition, several metabolic pathways might be involved in polycyclic aromatic hydrocarbon degradation, including phenylpropanoid biosynthesis, polycyclic aromatic hydrocarbon degradation, limonene and pinene degradation. However, in the past published papers, *P. betulinus* was thought to modify the structure of lignin instead of degrading lignin, which involve complex phenolic polymers composed of cinnamyl alcohol subunits, phenylpropanol and its derivatives [33]. Therefore, our paper speculated that those metabolic pathways in *P. betulinus* transcriptome were likely to participate in lignin modification. Importantly, we also found 8 potential wood-degrading related genes consisting of 3 laccases, 1 cellobiose dehydrogenase and 4 glucose oxidases. This indicated that *P. betulinus* could efficiently degrade wood components (Table S6), which is consistent with a previous report [34]. Although we detected wood-degrading enzymes encoding genes in transcriptome, enzymatic activity should be measured in a future experiment to confirm its enzyme activity level.

Eighty-six unigenes were matched with xenobiotics biodegradation and metabolism, including chloroalkane and chloroalkene degradation, toluene and chlorobenzene degradation, styrene degradation, naphthalene degradation and metabolism of xenobiotics by cytochrome P450. Prince and Roger [35] reported bioremediation using microbiological processes to clean-up organic and inorganic environmental pollutants by chloroalkane and chloroalkene degradation. Published findings have evaluated the effect of white-rot fungi on removal of gaseous chlorobenzene and naphthalene, while no reports have been published regarding this degradation using brown-rot fungi. Therefore, our next experiment would determine the ability of gaseous chlorobenzene or naphthalene in *P. betulinus* [36–38]. The findings of Krueger et al. [38] indicated that brown-rot fungus *Gloeophyllum trabeum* might be suitable for the biodegradation of styrene. Although white-rot fungi degrading xenobiotic compounds was originally attributed to its lignin-degrading enzyme system, Ide et al. [39] suggested that cytochrome P450 showed a novel catalytic property in xenobiotic metabolism. All annotated enzymes or degradation processes could be found in *P. betulinus* transcriptome, demonstrating that *P. betulinus* could not only harbor metabolic xenobiotic materials but also potentially degradation of environmental pollutants.

5. Conclusions

To our knowledge, this was the first study to describe the changes in transcriptome of brown-rot fungus *P. betulinus* when growing on birch saw dust. After an in-depth RNA-seq analysis, we obtained and annotated 3351 assembled unigenes to at least one database. A large number of potential lignicellulose-degrading enzyme-relevant genes were identified, suggesting that this species is suitable for wood degradation. In summary, our analysis results obtained from the transcriptome data provide an insight into the genetic background of brown-rot fungus *P. betulinus*.

Author Contributions: Qiuyu Wang conceived and designed the experiments; Mu Peng and Syed Sadaqat Shah performed the experiments and analyzed the data; Lixia Yang wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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