

The Mechanism of Degradation of Alizarin Red by a White-Rot Fungus (*Lenzites Gibbosa*)

Jian Zhang

Northeast Forestry University

YuJie Chi (✉ chiyujienefu@126.com)

Northeast Forestry University

Lianrong Feng

Northeast Forestry University

Research Article

Keywords: Transcriptome, alizarin red degradation, white fungi, LC-MS, GC-MS

Posted Date: June 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-529050/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Alizarin red (AR) is a typical anthraquinone dye, and the resulting wastewater is toxic and difficult to remove. A study showed that the white rot fungus *Lenzites gibbosa* (*L. gibbosa*) can degrade dye wastewater by decolorization and has evolved its own enzyme-producing traits.

Methods

In this study, transcriptome sequencing was performed after alizarin red treatment for 0, 3, 7, 10, and 14 h. The key pathways and key enzymes involved in alizarin red degradation were found to be through the analysis of KEGG, GO and COG. The GST, MnP and Laccase enzyme activities of *L. gibbosa* treated with alizarin red for 0–14 h were detected. LC-MS and GC-MS analyses of alizarin red decomposition products after 7 h and 14 h were performed.

Results

The glutathione metabolic pathway ko00480, and the key enzymes GST, MnP, Laccase and CYP450 were selected. Most of the genes encoding these enzymes were upregulated under alizarin red conditions. The GST activity increased 1.8 times from 117.55 U/mg prot at 0 h to 217.03 U/mg prot at 14 h. The MnP activity increased 2.9 times from 6.45 U/L to 18.55U/L. The Laccase activity increased 3.7 times from 7.22 U/L to 27.28 U/L. Analysis of the alizarin red decolourization rate showed that the decolourization rate at 14 h reached 20.21%. The main degradation intermediates were found to be 1,4-butene diacid, phthalic acid, 1,1-diphenylethylene, 9,10-dihydroanthracene, 1,2-naphthalene dicarboxylic acid, bisphenol, benzophenol-5,2-butene, acrylaldehyde, and 1-butylene, and the degradation process of AR was inferred. Overall, 1,4-butene diacid is the most important intermediate product produced by AR degradation.

Conclusions

The glutathione metabolic pathway was the key pathway for AR degradation. GST, MnP, Laccase and CYP450 were the key enzymes for AR degradation. 1,4-butene diacid is the most important intermediate product. This study explored the process of AR biodegradation at the molecular and biochemical levels and provided a theoretical basis for its application in practical production.

Background

Alizarin Red (AR) is a typical anthraquinone dye with a high fixation rate and good dyeing fastness, and it has become an important dye because of its bright colour. Anthraquinone dyes are mostly aromatic compounds with stable conjugated structures, good light resistance and potential toxicity. Because of the

special properties of anthraquinone dyes, the wastewater produced is not only high in organic composition and not easily oxidized but also has high chromaticity, complex composition and toxicity, so it is difficult to oxidize and to degrade by chemical methods. It is easy to dissolve AR in water and difficult to remove it by conventional physical methods, which brings some difficulty to wastewater treatment. Anthraquinone dyes are difficult to remove as the main pollutants in wastewater, which affects the degradation of wastewater and the ecological environment [1–3]. The degradation methods of anthraquinone dye wastewater mainly include physical, chemical and biological methods. The chemical treatment of dyes easily causes secondary pollution, and physical bleaching has the disadvantages of high cost and low efficiency [4]; however, the biological treatment of dye wastewater is efficient and without secondary pollution [5]. According to previous research, white rot fungi have certain decolorization and degradation abilities in dye wastewater [6]. White rot fungi secrete a variety of intracellular and extracellular enzymes, such as extracellular manganese peroxidase (MnP), lignin peroxidase (LiP) and Laccase [7–10], and intracellular cytochrome P450 (CYP450) [11–12]. The extracellular oxidase produced in secondary metabolism can degrade all kinds of dyes in a broad spectrum, especially for many synthetic compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyl compounds. However, the mechanism of dye degradation by white rot fungi is not well studied. Using transcriptome technology to study various biological phenomena at the molecular level is a popular research method [13].

On the basis of previous studies, HiSeq high-throughput sequencing technology was used to study the changes in the transcription level of *Lenzites gibbosa* treated at 0, 3, 7, 10, and 14 h in an anthraquinone dye alizarin red environment. To explore the key pathways, genes and their regulated enzymes of *L. gibbosa* involved in alizarin red dye degradation were studied. Enzyme activity related to alizarin red degradation was detected. AR degradation intermediate products were analysed by LC-MS and GC-MS to explore the degradation pathway of AR. For white rot bacteria, the mechanism of *L. gibbosa* degradation of anthraquinone dye AR provides molecular-level data support and a theoretical basis.

Materials And Methods

2.1. Fungal culture and the hyphal collection

Source of materials: Our limited collection of the *Lenzites gibbosa* (*L. gibbosa*) CB1 subentities for research is allowed. The strain was collected by Professor Yujie Chi in Changbai Mountain, Jilin Province, China, and then underwent tissue separation in the laboratory. Store in the Forest Pathology Laboratory of School of Forestry, Northeast Forestry University. Samsampling site is located in Changbai Mountain Nature Reserve, Northeast China (127°42'55" E, 41°16'48" E, 41°49'42" N, 42°51'18" N) with an average altitude of 802 m. Professor Yujie Chi sequenced the collected subentities and the isolated mycelium extract DNA for ITS, and the PCR product sequencing of the rDNA-ITS region of the strain CB1 obtained a 601bp nucleotide sequence. Submit this sequence to NCBI's GenBank (NCBI Accession No: JF279440). BLAST homology comparison and identification results showed that 1-182bp was ITS1 sequence, 183–347 bp 5.8S rDNA sequence, 348–535 bp ITS2 sequence and 536-601bp 28S rDNA sequence. The

sequence has the highest similarity of ITS sequence coverage between 85–97%, all at 99%, indicating that the strain is *Trametes gibbosa* and can be represented as *Lenzites gibbosa* = = = *Trametes gibbosa*.

Lenzites gibbosa(*L. gibbosa*)CB1(NCBI Accession No: JF279440) was obtained by self-separation subphysical processes from the Changbai Mountains. Samples were preserved in the Northeast Forestry University Laboratory. The preserved strains were inoculated on PDA plate medium (9 cm) and cultured at 25°C for 7 days and to obtain hyphae from the edge of the plate with a 5 mm² punch. Hyphae were inoculated into a triangle bottle (250 mL) containing 5 ml filtered and sterilized 15% glucose (V/V) and 70 mL LNAS medium. After static culture at 25°C for 10 days, alizarin red (AR, C₁₄H₇NaO₇S·H₂O) dye was added to each bottle, and the final concentration of AR dye in the medium was 50 mg/L. Hyphae were extracted after 0 h, 3 h, 7 h, 10 h and 14 h of AR treatment. Among them, 0 h was set as the control group (no dye treatment) mark as CK, and the others were the experimental group, mark as QSH1, 2, 3 and 4. Every 5 bottles of hyphae of the same culture conditions were mixed as a repeat, with 3 replicates per group. After mixing the hyphae and then placed into the frozen storage tube, sealed, put into liquid nitrogen for freezing, and then put into the 80°C refrigerator and saved for future analysis.

2.2. RNA extraction, cDNA library sequencing of the transcriptome, and the assembly and splicing of sequences

Frozen hyphal samples were sent to Beijing BMK Biotechnology Co., Ltd. for transcriptome sequencing by Illumina HiSeq X Ten high-throughput sequencing platform. Data were analysed after quality control of raw data obtained by sequencing (clean reads). Sequence alignment of clean reads with JGI *Trametes gibbosa* reference genomes using software. The genome was published in December 2016. (The ITS1 and ITS2 fragments of *L. gibbosa* strain CB-1 were 601 bp long (GenBank accession number: JF279440). A BLAST search of the 601 bp sequence indicated that the sequence showed 99% homology with 11 strains of *Trametes gibbosa*. Thus, *Lenzites gibbosa*===*Trametes gibbosa*.) The resulting mapped data and Cufflinks were compared with mapped data reads. The transcripts were assembled according to the GFF documents of the reference genome, and the Cuffmerg transcripts were merged to obtain a complete set of transcript information and the expression value of each gene. Using BLAST [14] software, the genes obtained from the transcriptome were functionally annotated in the COG, eggNOG, GO, KEGG, NR, and SwissProt gene functional annotation databases.

2.3. Analysis of gene differential expression in AR

BMKCloud (www.biocloud.net) and edgeR software (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>) were used, and FDR = 0.01 and FC = 2 were selected as the DEG screening criteria[15]. Analysis of differential expression was performed in the following groups: CK vs QSH1; CK vs QSH2;CK vs QSH3;CK vs QSH4;QSH1 vs QSH2; QSH1vs QSH3;QSH1 vs QSH4;QSH2 vs QSH3; QSH2vs QSH3;QSH3 vs QSH4. The differentially expressed gene (DEG) sets between the groups were obtained. Then, the total number of DEGs was obtained, and GO, COG and KEGG analyses of DEGs were performed.

2.4. Detection of AR decolorization rate

The maximum absorption peak and its wavelength were determined by scanning 50 mg/L AR between 300–800 nm with a spectrophotometer. The absorbance value of AR was measured at wavelength at the maximum absorption peak with the average of 3 sets of data, according to the absorption value of the test group minus the absorption value of the control group, as A_i and the absorptivity of the 50 mg/L AR minus the absorptivity of the control group as A_0 . The decolorization rate was calculated by the following formula.

$$\text{Decolourization rate (\%)} = \frac{A_0 - A_i}{A_0} \times 100\%$$

2.5. Detection of extracellular enzyme MnP and Laccase activity

The extraction of 3 mL of culture medium was performed in 5 stages (three replicates per group), followed by 13000 r/min centrifugation for 10 minutes, and extraction of the supernatant. Extracellular enzyme Laccase and MnP activity related to AR degradation were detected. Laccase and MnP activity detection methods were refer to Y. P. Shi [16] and Y. L.Zhang [17].

2.6. Detection of intracellular GSH content and intracellular GST activity

L. gibbosa hyphal treatment was extracted by AR at 5 stages and rinsed and dried in phosphate-buffered saline (PBS). Hyphae were ground into powder with liquid nitrogen, and then an equal proportion of PBS homogenate was used. Then, the samples were centrifuged to extract the supernatant and set aside. Glutathione S-transferase (GST), Glutathione (GSH) and the Total protein (TP) assay kit (Cargo Number: A004, A006-1-1 and A045-2, Nanjing Jiancheng Bioengineering Institute, China) methods and formulas were used to detect the GST and GSH activity of *L. gibbosa* mycelium at each stage.

2.7. LC-MS and GC-MS were used to detect decomposition products AR at different time points

Degradation of dye molecules during the *Lenzites gibbosa* decolourization process passes through some intermediate products that were analysed by LC-MS and GC-MS and identified by interpretation of their mass spectral data presenting their molecular ion peaks with respect to m/z (where m is the molecular weight of the intermediates in the mass spectra). Thus, the molecular cleavage pathway of the AR dye is deduced. The *L. gibbosa* culture medium (100 mL) was extracted after AR treatment for 7 and 14 h, and the culture medium was pre-treated.

The sample pre-treatment of culture medium was as follows: Dichloromethane were taken and activated in the C18 column stationary phase. Methanol was taken through the column to remove impurities.

Finally, the column was washed with deionized water. The culture medium over the C18 column and mixture solution were prepared using 2 mL methanol and 8 mL dichloromethane, and the intermediate product was eluted from the C18 column and coexisted in the test tube.

Descriptions of various test parameters and data processing procedure are found below.

(1) LC-MS test parameters: Chromatographic column: Agilent SB-C18RRHD, 1.8 μ m, 2.1*100 mm, column temperature: 35°C. Mobile phase: A 5 mM ammonium acetate solution, B methanol. Flow rate: 0.3 mL/min. Injection: 5 μ L. Automatic sampler temperature/TEM: 25°C, DAD detection wave: 519 nm. Mass Spectrometry Acquisition Parameters: Ion source type: ESI, Pattern: MS2 Scan, Polarity: neg, m/z range: 100–370, Ion source temperature: 350°C.

(2) GC-MS test parameters: Sampler: 7683B, The mass spectrometry used an EI ion source, electron energy of 70 eV, scanning range of 50 ~ 500, four-stage rod temperature of 150°C, ion source temperature of 230°C, and transmission line temperature of 280°C. For related parameters see Table 1.

Table 1
The relevant parameters of LC-MS operation

| Controlling factor | Parameter conditions | Controlling factor | Parameter conditions |
|------------------------|----------------------|--------------------|----------------------|
| Maximum scanning speed | 10.0 mAu/sec | Carrier gas | He |
| Detector | MSD | Sample size | 10 μ L |

Results

3.1. Results of transcriptome data and quality control evaluation

A total of 109.29 Gb clean data were obtained from transcriptome analysis of 15 samples, the percentage of Q30 bases was 91.32% and above, and the quality of transcriptome data was up to standard. The clean reads of each sample were compared with the designated reference genome, and the alignment efficiency was 87.22%-91.32%, and reference genome selection was correct and efficient (Table S1). A total of 12921 genes were obtained of which 10560 genes were annotated, and the annotation efficiency was 81.72%. There were 883 new genes, 162 of which were annotated. (Table S2). Three of the 15 samples (QSH1-0, QSH2-0, and QSH3-0) were significant outliers compared with the other 12 samples in the same group. Except for these 3 outlier samples, the other 12 samples showed good correlation (Fig. 1-a).

3.2. Gene Expression Pattern Analysis and Clustering of DEGs.

The transcriptome data were divided into 10 groups, among which 6 groups had more downregulated genes than upregulated genes (Table S3). Ten differential groups received a total of 1480 DEGs of which 1370 DEGs were annotated. A total of 4 expression trends were obtained by analysing 1480 DEG expression trends (Fig. 1-b). Profile 1 and Profile 2 genes were downregulated, for a total of 761 genes. Profile 3 and Profile 4 genes belonged to the upregulation type, for a total of 719 genes. There were more downregulated genes than upregulated genes. The expression of Profile 4 genes increased at 0–3 h and decreased slightly at 3–14 h. Profile 1 and Profile 2 genes were inhibited after exposure to AR stimulation and stress. The expression of the Profile 3 and Profile 4 genes increased after AR induction.

According to the differential trend analysis of DEGs, it was found that for a total of 66.3% of DEGs (Profile 2 and Profile 4) at 0–3 h were the most variable stages of DEG expression, 3–14 h expression was stable, and 17.1% of DEGs (Profile 1) continued to change at 0–14 h. The expression of 16.6% of DEGs (Profile 3) at 0–3 h was stable, and the expression changed greatly at 3–14 h, and 0 h, 3 h and 14 h are important nodes for DEG expression. The expression of all DEGs can be divided into two stages: 0–3 h and 3–14 h. Therefore, the analysis of CK vs QSH1 (0 h vs 3 h) and QSH1 vs QSH4 (3 h vs 14 h) were more representative of the role of *L. gibbosa* DEGs in AR degradation.

3.3. Differential grouping of DEGs in the GO database analysis

The DEGs of the different groups CK vs QSH1 and QSH1 vs QSH4 were GO enriched. The enrichment categories of $\log_{10}(KS) \geq 2$ are intercepted (Table S4). KS: the significant statistics of the enrichment of the GO category, the smaller the KS value is the larger the $\log_{10}(KS)$ is, indicating the more significant the enrichment. At 0–3 h, upregulated DEGs were significantly enriched to GO:0020037 (heme binding), GO:0000041 (transition metal ion transport), GO:0005741 (mitochondrial outer membrane), GO:0046274 (lignin catabolic process) and GO:0004601 (peroxidase activity) categories, and downregulated DEGs were significantly enriched to GO:0020037 (heme binding), GO:0004521 (endoribonuclease activity) and GO:0006536 (glutamate metabolic process) categories (Fig. 2-a). At 3–14 h, upregulated DEGs were significantly enriched in the GO:0016620 (oxidoreductase activity, acting on the aldehyde or oxo group of donors, and NAD or NADP as the acceptor) and GO:0020037 (heme binding) categories, and downregulated DEGs were significantly enriched in the haem binding, lignin catabolic process and reactive oxygen species metabolic process categories (Fig. 2-b). Four important categories were enriched in both phases: GO: 0020037 (heme binding), GO: 0046274 (lignin catabolical process), GO: 0016620 (oxidoreduction activity, acting on the aldehyde or oxo group of donors, and NAD or NADP as the acceptor) and GO: 0004601 (period activity). Haem is the cofactor of haemoglobin and myoglobin, cytochrome, peroxidase, and catalase, which controls the synthesis and expression of redox enzymes such as the MnP haem-binding category, which can be determined to be associated with redox reactions. Lignin is a polymer in which phenylpropanoid structural units are irregularly coupled by ether and carbon

bonds[18]. However, most dyes are similar to lignin structures and are composed of heterocycles and aromatic rings, all of which are aromatic compounds. Therefore, the significant enrichment of the lignin catabolic process class can be determined to be related to AR degradation.

Based on two stages of GO enrichment analysis, at 0–3 h, *L. gibbosa* secondary metabolic activity and redox reactions become active, and basic life activities such as carbohydrate utilization and protein synthesis are inhibited. At approximately 14 h, the redox reaction activity decreased relative to that at 3 h and carbohydrate transport and metabolism as well as energy production and transformation functions at 14 h.

All 1480 DEGs were compared to the four important categories enriched above (Table 2). The 20 DEGs enriched in haem-binding classes encoded 8 enzymes: 4-hydroxysphinganine ceramide fatty acyl 2-hydroxylase, Versatile peroxidase, MnP, Acyl-CoA dehydrogenase, Acyl-CoA desaturase, Cytochrome P450 (CYP450), Fumarate reductase, L-lactate dehydrogenase (cytochrome) and nitric oxide dioxygenase. Laccase of all 3 DEGs was enriched in the lignin catabolic process category. Two DEGs were enriched in oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor categories, encoded 1-pyrroline-5-carboxylate dehydrogenase and aldehyde dehydrogenase (NAD). Peroxidase activity of four DEGs encoded Versatile peroxidase (VP) and MnP genes, and all of them were redox enzymes. Among them, MnP and VP appear in two categories. The CYP450 enrichment genes were the most. Laccase-enriched functional categories were most correlated with AR degradation. According to the GO enrichment analysis, the redox reaction plays a role in AR degradation. MnP, VP, CYP450 and Laccase are key enzymes for the redox decomposition of AR.

Table 2
DEGs from four important GO terms.

| GO term | Gene function | Gene No. |
|---|--|--|
| Heme binding | 4-hydroxysphinganine ceramide fatty acyl 2-hydroxylase | gene_5570 |
| | Versatile peroxidase | gene_11851, _11537, _713 |
| | MnP | gene_8611 |
| | Acyl-CoA dehydrogenase | gene_22, _4 |
| | Cytochrome P450 | gene_7628, _9113, _4488, _5216, _11472, _4487, gene_5139, _7522, _10935, _6568 |
| | Fumarate reductase | gene_8119 |
| | L-lactate dehydrogenase (cytochrome) | gene_4787 |
| | nitric oxide dioxygenase | gene_10651 |
| Lignin catabolic | Laccase | gene_3889, _3902, _1741 |
| oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as the acceptor | 1-pyrroline-5-carboxylate dehydrogenase | gene_1202 |
| | aldehyde dehydrogenase (NAD ⁺) | gene_3674 |
| Peroxidase activity | Versatile peroxidase | gene_11851, _11537, _713 |
| | MnP | gene_8611 |

3.4. Differential grouping DEG KEGG metabolic pathway analysis

The DEGs of CK vs QSH1 (0 h vs 3 h) and QSH1 vs QSH4 (3 h vs 14 h) were KEGG enriched, rich factor ≥ 4 , and q value ≥ 1 . Upregulated DEGs at 0–3 h were significantly enriched in the proteasome, terpenoid backbone biosynthesis, sphingolipid metabolism and glutathione metabolism pathways. Downregulated DEGs at 0–3 h were significantly enriched in alanine, aspartate and glutamate metabolism, regulation of mitophagy - yeast, arginine biosynthesis, starch and sucrose metabolism, glyoxylate and dicarboxylate metabolism, galactose metabolism and the nitrogen metabolism pathways (Fig. 2-c).

Upregulated DEGs at 3–14 h were significantly enriched in the pentose and glucuronate interconversion pathways. Downregulated DEGs at 3–14 h were enriched in biosynthesis of antibiotics, synthesis and degradation of ketone bodies, terpenoid backbone biosynthesis, tyrosine metabolism and valine, leucine and isoleucine degradation (Fig. 2-d).

According to DEG enrichment of KEGG pathways at 0–3 h, genes involved in glutathione metabolism expression were upregulated by AR at this stage. Downregulation of the gene enrichment pathway found that the addition of AR affected the metabolism of substances needed for basic life activities such as glucose metabolism and energy metabolism in *L. gibbosa*. At the 3–14 h stage, glucose metabolism genes began to be upregulated, and the metabolism of amino acids and lipids was downregulated. Terpenoid synthesis gene transcription began to be downregulated.

Glutathione metabolism and redox reactions are the main processes involved in AR degradation by COG and GO database analysis.

3.5. Redox reactions and glutathione metabolism are involved in AR degradation

Some important reactive redox reactions and glutathione metabolism pathway related to AR degradation were identified by transcriptome analysis (Fig. 3). Exogenous chemicals are generally eliminated in two ways: one is discharged directly from the body without metabolism, and the other is excreted in the form of metabolites after metabolism. The metabolism of exogenous chemicals in vivo mainly undergoes a two-step reaction. The first step is the phase I reaction in which exogenous chemicals are oxidized, reduced or hydrolysed to more polar metabolites. The key enzyme to catalyse the phase I reaction is the CYP450 enzyme system. The second step is the phase II reaction in which exogenous chemicals and their metabolites are combined with endogenous substances and discharged in vitro. There are many enzymes that catalyse the phase II reaction. The important enzymes are glucuronic acid transferase, GST and N-phthalyl transferase. When AR enters *L. gibbosa*, it is first oxidized by CYP450 and other phase I enzymes. Then, bound to reduced glutathione (GSH) under GST catalysis it is excreted (this process is an important detoxification process in organisms). AR metabolites were treated in vitro with CYP450 and GST and then oxidized and decomposed by the extracellular enzyme Laccase and MnP and other oxidases.

During this study, 1 MnP gene (gene_8611) was upregulated; three Laccase genes (gene_3902,_3889, and _1741) 2 upregulated and 1 downregulated.; three VP genes (gene_11851,_11573, and _713), 2 upregulated and 1 downregulated; 4 GST genes (gene_9280,_6690,_11173 and _393), 3 upregulated and 1 downregulated and 30 CYP450 genes (gene_7522, _5557, _11278, _9592, _6093, _11472, _6568, _3430, _4487, _5809, _6800, _7521, _5139, _7593, _7, _6521, _5321, _6115, _6076, _9114, _9113, _7628, _4486, _5216, _10935, _9118, _4482, _7594, _4488, and _6573), 23 had upregulated expression and 7 had downregulated expression. These inferences may be involved in most of the upregulation of genes encoding AR-degrading enzymes under AR conditions. The glutathione peroxidase gene and isocitrate dehydrogenase gene, of genes involved in GSH production, also showed differential expression and

upregulated expression. The molecular level shows that MnP, Laccase, CYP450, VP, GST and GSH are involved in AR degradation.

3.5.1. GST and GSH activity determination results

To verify whether the GST and GSH responds to AR, the GST activity and GSH content of hyphae in the 5 treatment stages were detected. The GSH content decreased slightly from 0 h 51.71 gGSH/L to 14 h 44.04 gGSH/L (Fig. 4-a). The GST activity of 0–3 h decreased, 3–7 h increased sharply, 7–10 h decreased, and 10–14 h increased violently. GST activity showed an overall upward trend, from 0 h 117.55U/mg prot to 14 h 217.03U/mg prot, and enzyme activity increased 1.8 times (Fig. 4-b). GST activity and GSH content showed that the GSH content slightly decreased GST, and a large amount of secretion promoted the binding of GSH and AR. Thus, glutathione metabolism responds to AR and participates in intracellular AR degradation.

3.5.2. Detection of redox enzyme activity participates in AR degradation

Extracellular enzyme Laccase and MnP activity were determined at the 5 AR treatment stages. The results showed that Laccase and MnP enzyme activities decreased slightly at 0–3 h, increased sharply at 3–10 h and then the upward trend at 10–14 h slowed down (Fig. 4-c). Laccase activity increased 3.7 times from 0 h 7.22 U/L to 14 h 27.28 U/L. MnP activity from 0 h 6.45 U/L to 14 h 18.55 U/L, and enzyme activity increased 2.9 times. The results showed that with the increasing *L. gibbosa* time in the AR dye environment, the activities of Laccase and MnP increased. These two enzymes respond to AR and act extracellularly.

3.6. *L. gibbosa* Decolourization of AR

AR decolourization was determined at 0, 3, 7, 10, and 14 h (Fig. 4-d). The decolourization rate increased at 0–10 h. By 10 h, the decolourization rate reached 20.06%. At 10–14 h, the decolourization rate was stable and unchanged. At 14 h, the final decolourization rate was 20.21%. The explanation for these findings is that *L. gibbosa* has a decolourization effect on AR.

3.7. Inference of AR Decomposition

3.7.1. Analysis of AR degradation products by LC-MS and GC-MS techniques

To explore the possible degradation pathways of AR, LC-MS was carried out by sampling the intermediates as well as the final and stable degraded products. The mass-to-load ratio (m/z) of particle fragments can be obtained by LC-MS. The results of LC-MS analysis showed that the types of intermediate products obtained at 7 h and 14 h were the same, but the contents of each substance at 14 h were obviously less than those at 7 h. Seven substances (Table 3 and Fig. 4-e, f, g and h) were obtained with mass-to-load ratios of 318.9, 117.1, 165.0, 179.1, 215.0, 225.0, and 304.9. On the basis of m/z, 318.9 is AR (alizarin red removal Na), and 304.9, 179.1, 225 and 165 are benzophenone-5, 1,1-diphenylethylene,

bisphenol and phthalic acid, respectively. These four substances may be formed by AR from the benzene ring opening in the middle. The m/z 215.0 is 1,2-naphthalene dicarboxylic acid, and the carbonyl addition reaction may occur from AR and form ring openings from sulfite roots. The m/z 117.1 is a 1,4-butene diacid, which is a small molecular substance formed by cracking of the benzene ring. Figure 4-e,f,g and h shows that 1,4-butene diacid is the most abundant AR decomposition intermediate and is the most important intermediate product of *L. gibbosa* degradation of AR. The m/z 179.1 can also be inferred to be 9,10-dihydroanthracene, which is derived from AR de-sodium ions, hydroxyl groups and sulfite ions. The anthraquinone structure of AR is destroyed, and the anthraquinone structure is the hair colour group of AR. The anthraquinone structure was destroyed, and the dye colour disappeared.

Using GC-MS methods, the degradation products can be more comprehensively analysed to supplement LC-MS results. It can be observed in the above total ion chromatography (Fig. 4-i,j) that the abundance of material absorption ranges from 10500 at 7 h to 9000 at 14 h, indicating that the pollutants that exist in the solution are decreasing. The detected substances in the GC-MS will be referenced to the NISETO mass spectrometry database for material matching. Intermediate products related to AR decomposition were detected as some small molecular substances (Table 4) such as 2-butene, acrylaldehyde and 1-butylene.

The pathway of AR degradation was inferred from the analysis of *L. gibbosa* intermediate products of AR degradation by LC-MS and GC-MS (Fig. 4-k). The AR hydroxyl groups and sulfite roots were removed first, then the anthraquinone structure was broken, and finally, the benzene ring cracked to form small molecular inorganic salts. At this point, AR is completely degraded.

Table 3 Relevant information of the intermediates by LC-MS

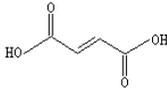
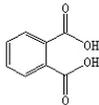
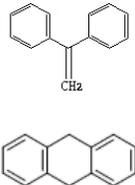
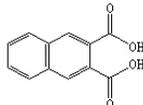
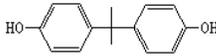
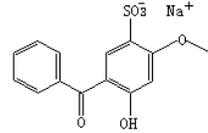
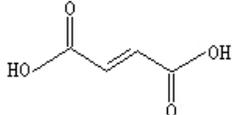
| Number | Molecular formula | Molecular mass | m/z | Constitutional formula | Name |
|--------|--|----------------|-------|--|--|
| a | C ₄ H ₄ O ₄ | 116 | 117.1 |  | 1,4-Butene diacid |
| b | C ₈ H ₆ O ₄ | 166 | 165.0 |  | Phthalic acid |
| c | C ₁₄ H ₁₂ | 180 | 179.1 |  | 1,1-Diphenylethylene And 9,10-Dihydroanthracene |
| d | C ₁₂ H ₈ O ₄ | 216 | 215.0 |  | 1,2- Naphthalene dicarboxylic acid |
| e | C ₁₅ H ₁₆ O ₂ | 228 | 225.0 |  | Bisphenol |
| f | C ₁₄ H ₁₁ O ₆ SNa | 330.29 | 304.9 |  | Benzophenone-5 |
| g | C ₁₄ H ₇ O ₇ SNa | 342.26 | 318.9 |  | Alizarin red |

Table 4 Relevant information of the intermediates by GC-MS

| | CAS NO. | Name | Molecular formula | Molecular weight | Molecular structure |
|------|----------|---------------|---------------------------------|------------------|---|
| 7 h | 590-18-1 | 2-butene | C ₄ H ₈ | 56.1063 |  |
| | 107-02-8 | Acrylaldehyde | C ₃ H ₄ O | 56 |  |
| | 106-98-9 | 1-Butylene | C ₄ H ₈ | 56.11 |  |
| 14 h | 590-18-1 | Butene | C ₄ H ₈ | 56.1063 |  |
| | 106-98-9 | 1-Butylene | C ₄ H ₈ | 56.11 |  |

Discussion

For the present study, we aimed to examine the gene transcriptional changes of *L. gibbosa* and AR metabolites at different processing times. To determine the degradation of key AR genes and enzymes and the AR degradation process, a transcriptomic analysis of *L. gibbosa* with different AR processing times was performed for the first time of genes that encode enzymes from GST, MnP, Laccase, lignin peroxidases, and VP. Of the oxidoreductase types, AR treatment resulted in higher expression levels in *L. gibbosa*. White rot fungi are by far the most efficient ligninolytic organisms described to date. This capability to degrade lignin is due to their extracellular nonspecific and nonstereoselective enzyme system is composed of Laccase, lignin peroxidases and manganese peroxidases, which function together with H₂O₂-producing oxidases and secondary metabolites [19]. The same unique nonspecific mechanisms that give these fungi the ability to degrade lignin also allow them to degrade a wide range of pollutants. They are able to degrade polycyclic aromatic hydrocarbons (PAHs), chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides, explosives and dyes[20–21]. Purified Laccases, lignin (LiP) and MnP are able to decolourize dyes of different chemical structures. It has been shown that these enzymes can degrade different dyes [22–23]. In our study, the activity of MnP and Laccase increased significantly after AR treatment. MnP, Laccase, LiP and other genes were differentially expressed, and most of them were upregulated.

Since one of the basic functions of GST is detoxification, GST in metabolic aromatic compounds may be expressed in conjunction with degradation enzyme genes, so they may be used as reporter genes to monitor the expression of degradation pathway genes[24]. L Loyd-Jones et al. believe that genes encoding GST may be widely present in PAH-degrading bacteria and can be used as molecular tools for the detection of such bacteria. The authors obtained amplified bands from PAH-degrading bacteria isolated from sources in the United States, New Zealand and Antarctica. Successful amplification in contaminated soils also makes GST coding genes a useful tool for evaluating PAH-contaminated soils[25]. This study used transcriptome differential analysis to enrich the glutathione metabolic pathway (ko00480) in which GST genes are the most differentially enriched genes in this pathway, and most of them are upregulated. GST activity is also rising, which is consistent with previous studies.

Conclusion

A transcriptome analysis of AR *L. gibbosa* showed the following: (1) The differentially expressed genes encoding oxidoreductase in the AR environment combined with the MnP and Laccase activity results indicate that oxidoreductase is the key enzyme for AR degradation. (2) The glutathione metabolic pathway (ko00480) was screened, and binding GST activity and GSH content indicated that the glutathione metabolic pathway is involved in AR degradation. The AR degradation process was inferred by LC-MS and GC-MS, and 1,4- Butene diacid was the most intermediate. This study explored the process of AR biodegradation at the molecular and biochemical levels and provided a theoretical basis for its application in practical production.

Abbreviations

L. gibbosa: Lenzites gibbosa; AR:alizarin red; MnP:manganese peroxidase; LiP:lignin peroxidase; Cytochrome P450:CYP450; GSH:glutathione; GST:Glutathione S-transferase; TP:Total protein; VP:Versatile peroxidase

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals that were performed by any of the authors.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request. This study data is being uploaded to the NCBI. NO. : SUB9461105.

Competing Interest

The authors declare that they have no conflicts of interest.

Funding

This work was financially supported by a grant from Natural science foundation of Heilongjiang province (2016006). Financing entity had a role in the study design, data collection, or analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

Conceived and designed experiments: YC, JZ. Acquired Funding: YC. Collected the data and performed the analysis: JZ, LF. Administrated resources for the project: YC, JZ. Drafted the manuscript: JZ and YC. Performed critical review of the manuscript with editions: YC. All authors read and approved the final manuscript

Acknowledgements

Authors thank to Elsevier Language Editing Services for English editing. Transcriptome sequence and some analysis were performed using BMKCloud (www.biocloud.net), which are gratefully acknowledged.

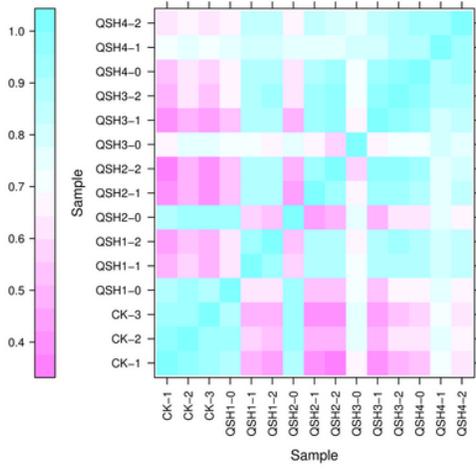
References

1. Mahmoodi NM, Arami M. Degradation and toxicity reduction of textile wastewater using immobilized titania nanophotocatalysis. *J Photochem Photobiol B*. 2009;94(1):20–4.
2. Devi LG, Rajashekhar KE, Anantha Raju KS, et al. Kinetic modeling based on the non-linear regression analysis for the degradation of Alizarin Red S by advanced photo Fenton process using zero valent metallic iron as the catalyst. *J Mol Catal A: Chem*. 2009;314(1–2):88–94.
3. Namasivayam C, Kavitha D. Removal of Congo Red from water by adsorption onto activated carbon prepared from coir pith, an agricultural solid waste. *Dyes Pigm*. 2002;54:47–58.
4. Kwok WY, Xin JH, Sin KM. Quantitative prediction of the degree of pollution of effluent from reactive dye mixtures. *Color Technol*. 2010;118(4):174–80.
5. Modi HA, Rajput G, Ambasana C. Decolorization of water soluble azo dyes by bacterial cultures, isolated from dye house effluent. *Biores Technol*. 2010;101(16):6580–3.
6. Assadi MM, Azin M, Shahvali M. Decolorization of textile wastewater by *Phanerochaete chrysosporium* Desalination. 2001;141(3):331–6.
7. Robinson T, Chandran B, Nigam P. Studies on the production of enzymes by white-rot fungi for the decolourisation of textile dyes. *Enzyme Microbial Technology*. 2001;29:575–9.
8. Campos R, Kanelbauer A, Robra KH, et al. Indigo degradation with purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii*. *J Biotechnol*. 2001;89:131–9.
9. Wong Y, Yu J. Laccase-catalyzed decolorization synthetic dyes. *Water Res*. 1999;33:3512–20.
10. Xu H, Guo MY, Gao YH, Bai XH, Zhou XW. Expression and characteristics of manganese peroxidase from *Ganoderma lucidum* in *Pichia pastoris* and its application in the degradation of four dyes and phenol. *BMC Biotechnol*. 2017;17:19.
11. Doddapaneni H, Yadav JS. Differential regulation and xenobiotic induction of tandem P450 monooxygenase genes pc-1(CYP63A1) and pc-2(CYP63A2) in the white-rot fungus *Phanerochaete chrysosporium*. *Appl Microbiol Biotechnol*. 2004;65(5):559–65.
12. Teramoto H, Tanaka H, Wariishi H. Fungal cytochrome P450s catalyzing hydroxylation of substituted toluenes to form their hydroxymethyl derivatives. *FEMS Microbiol Lett*. 2004;234(2):255–60.
13. Mills JD, Janitz M. Alternative splicing of mRNA in the molecular pathology of neurodegenerative diseases. *Neurobiol Aging*. 2012;33(5):1012 e1011–24.
14. Altschul SF, Madden TL, Schäffer A, Zhang J, et al. Gapped BLAST and PSI BLAST: A New Generation of Protein Database Search Programs. *Nucleic Acids Res*. 1997;25(17):3389–402.
15. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139–40.
16. Shi YP, Chi YJ, Yu C. Induction on Laccase Activity Produced by *Lenzites gibbosa*. *Scientia Silvae sinicae*. 2016;52(12):150–5.
17. Zhang YL, Chi YJ, Feng LR. Enzymology Characteristics and Dye Decolorization of Manganese Peroxidase from *Lenzites gibbosa*. *Scientia Silvae sinicae*. 2017;53(9):73–80.

18. Kamo T, Matsue M, Kashiwabara M, et al. 1, 2-Dehydrolactarolide A, a new plant growth regulatory lactarane sesquiterpene from *Lactarius vellereus*. *Journal of the Agricultural Chemical Society of Japan*. 2006;70(9):2307–9.
19. Kirk TK, Farrell RL. Enzymatic “combustion”: the microbial degradation of lignin. *Ann Rev Microbiol*. 1987;41:465–505.
20. Pointing SB, Vrijmoed LLP. Decolorization of azo and triphenylmethane dyes by *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase. *World J Microbiol Biotechnol*. 2000;16:317–8.
21. Pasti-Grigsby MB, Paszczynski A, Goszczynski S, Crawford DL, Crawford RL. Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp. And *Phanerochaete chrysosporium*. *Appl Environ Microbiol*. 1992;58(11):3605–13.
22. Cripps C, Bumpus JA, Aust SD. Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol*. 1990; 56:1114–1148.
23. Rodriguez E, Pickard MA, Vazquez-Duhalt R. Industrial dye decolorization by laccases from ligninolytic fungi. *Curr. Microbiol*. 1999; 38: 27–32.
24. Vuilleumier S, et al. The glutathione S-transfease od the dioxin degrading bacterium *Sphingomonas* sp RW1 displays maley1pyruvate isomerase activity. *Chem Biol Int*. 2001; 333: 265–267.
25. Gareth LJ, Andrew DL, David WFH. F Rhonda, Analysis catabolic genes for Phenanthrene and Naphthalene degradation in contaminated New Zealand soils. *FEMS Microbiol Ecol*. 1999; 29: 69–79.

Figures

a



b

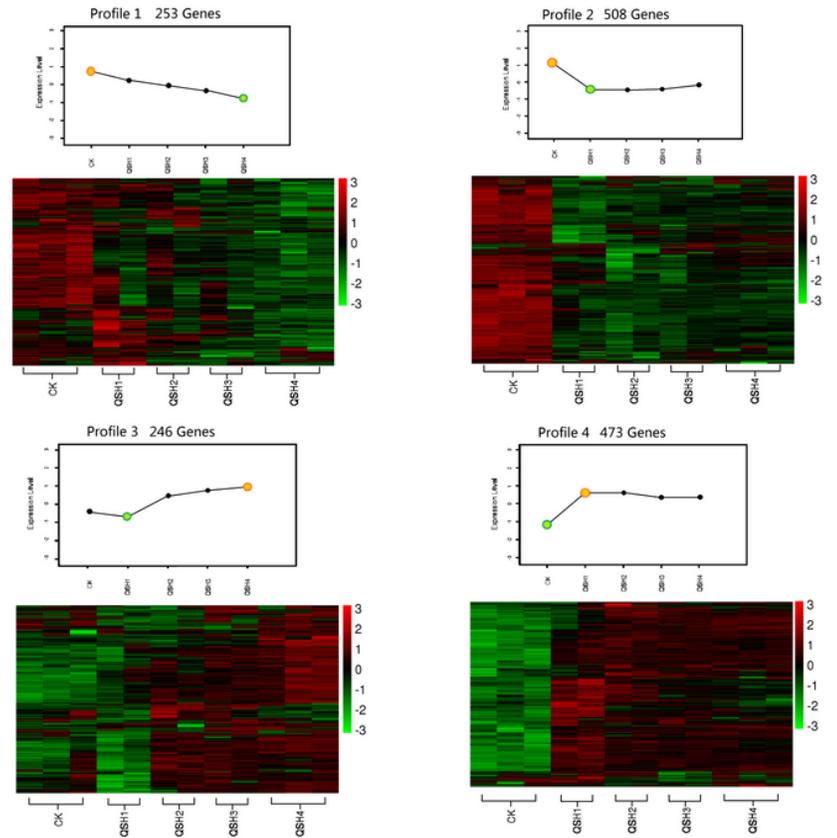


Figure 1

(a): RNA-seq correlation detection. Rows and columns are all 15 sample names, and the intersection of rows and columns is the square of the correlation coefficient between samples (Pearson correlation coefficient square, R^2). The larger the value is, the stronger the correlation is. (b): Gene expression patterns at five time points in the AR environment. Yellow indicates the highest expression of this pattern, and green indicates the lowest level.

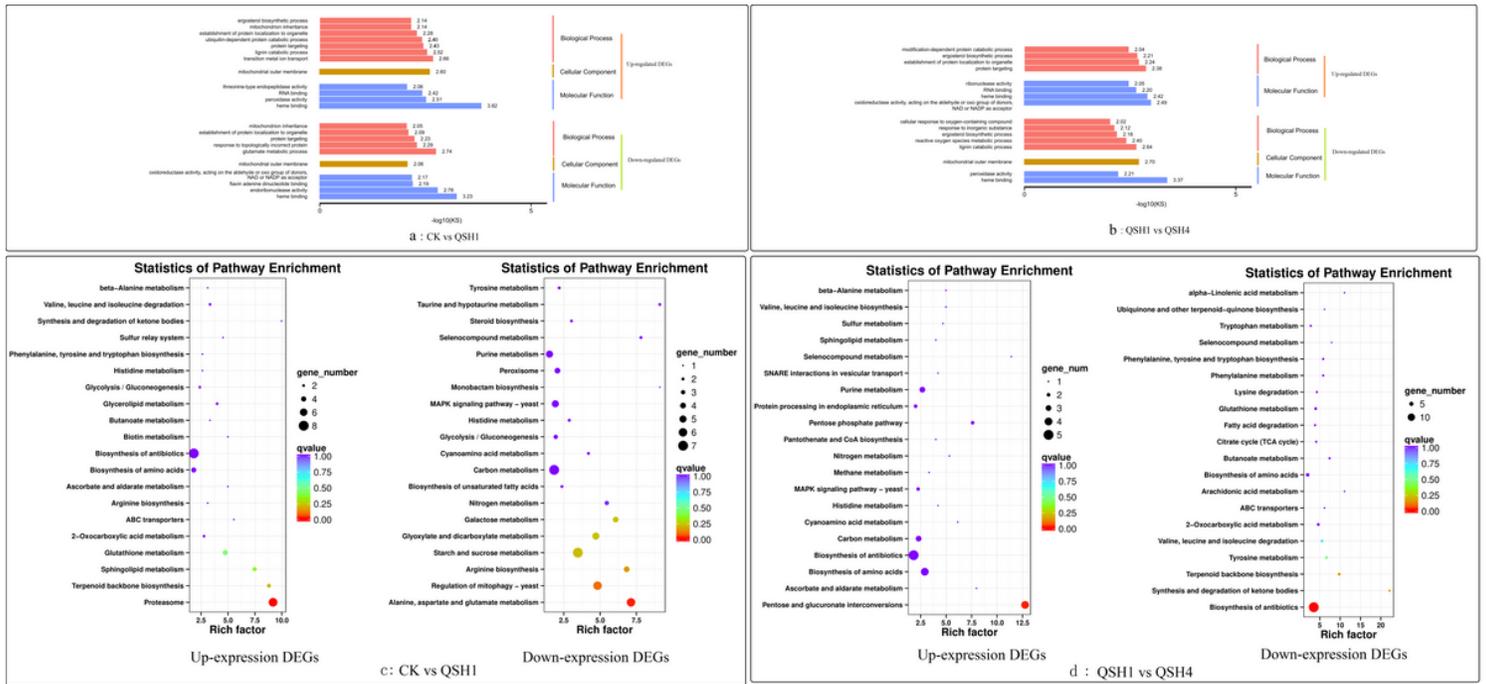


Figure 2

Enrichment analysis GO and KEGG to DEGs. (a) GO enrichment of CK vs QSH1 DEGs. (b) GO enrichment of QSH1 vs QSH4 DEGs. (c) KEGG enrichment of CK vs QSH1 DEGs. (d) KEGG enrichment of QSH1 vs QSH4 DEGs.

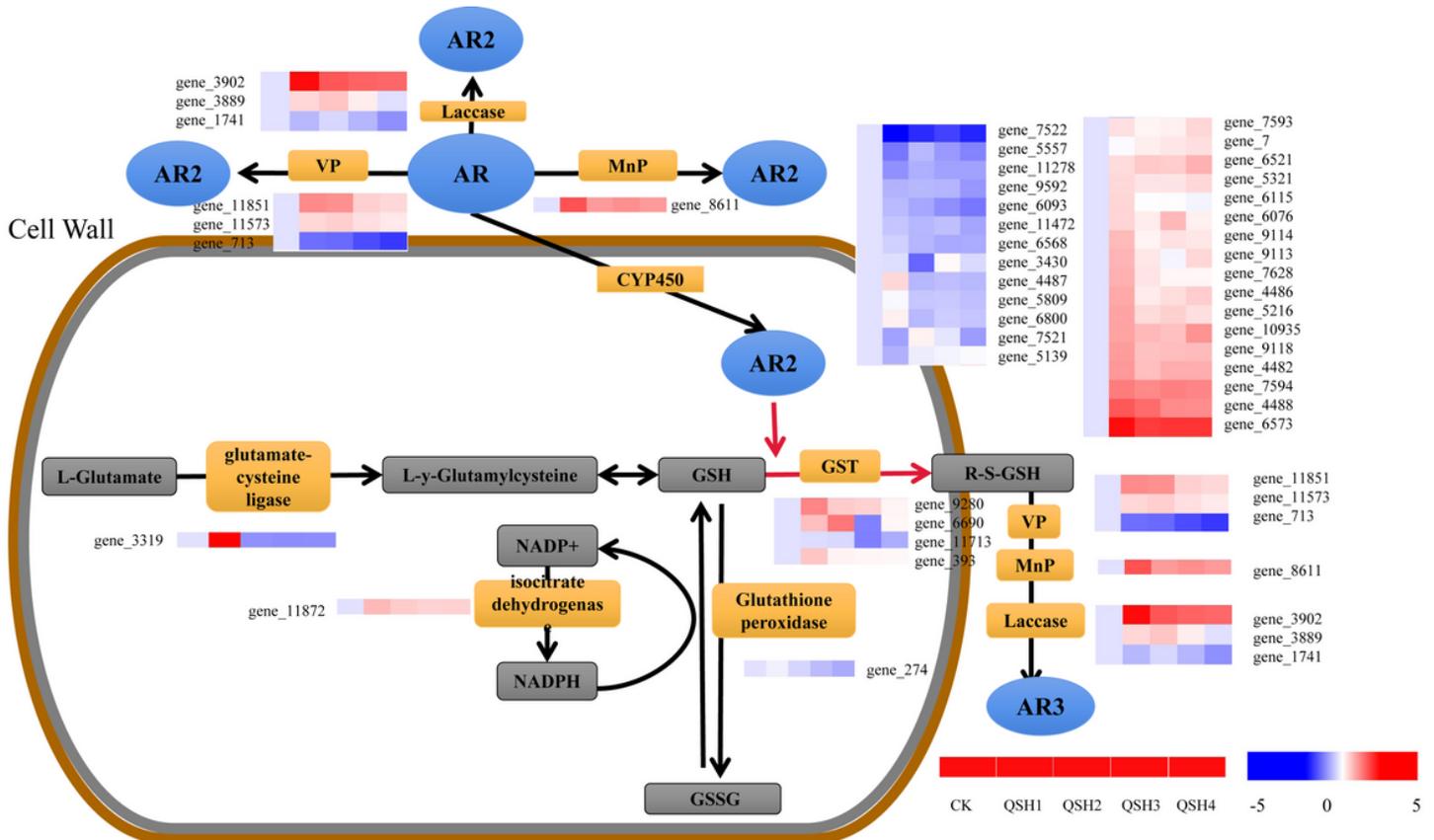


Figure 3

L.gibbosa DEG transcriptional changes in the AR metabolic pathway. The enzymes in response to AR are represented in orange boxes, whereas the other metabolites are in grey boxes. For enzyme reactions, the arrows between two metabolites represent the directions of catalytic reactions. The expression patterns over the five time points of the genes encoding corresponding enzyme(s) are given above or under the arrows (based on the CK expression of each gene, set to 0). AR2 and AR3 represent metabolite production of AR under enzymatic action.

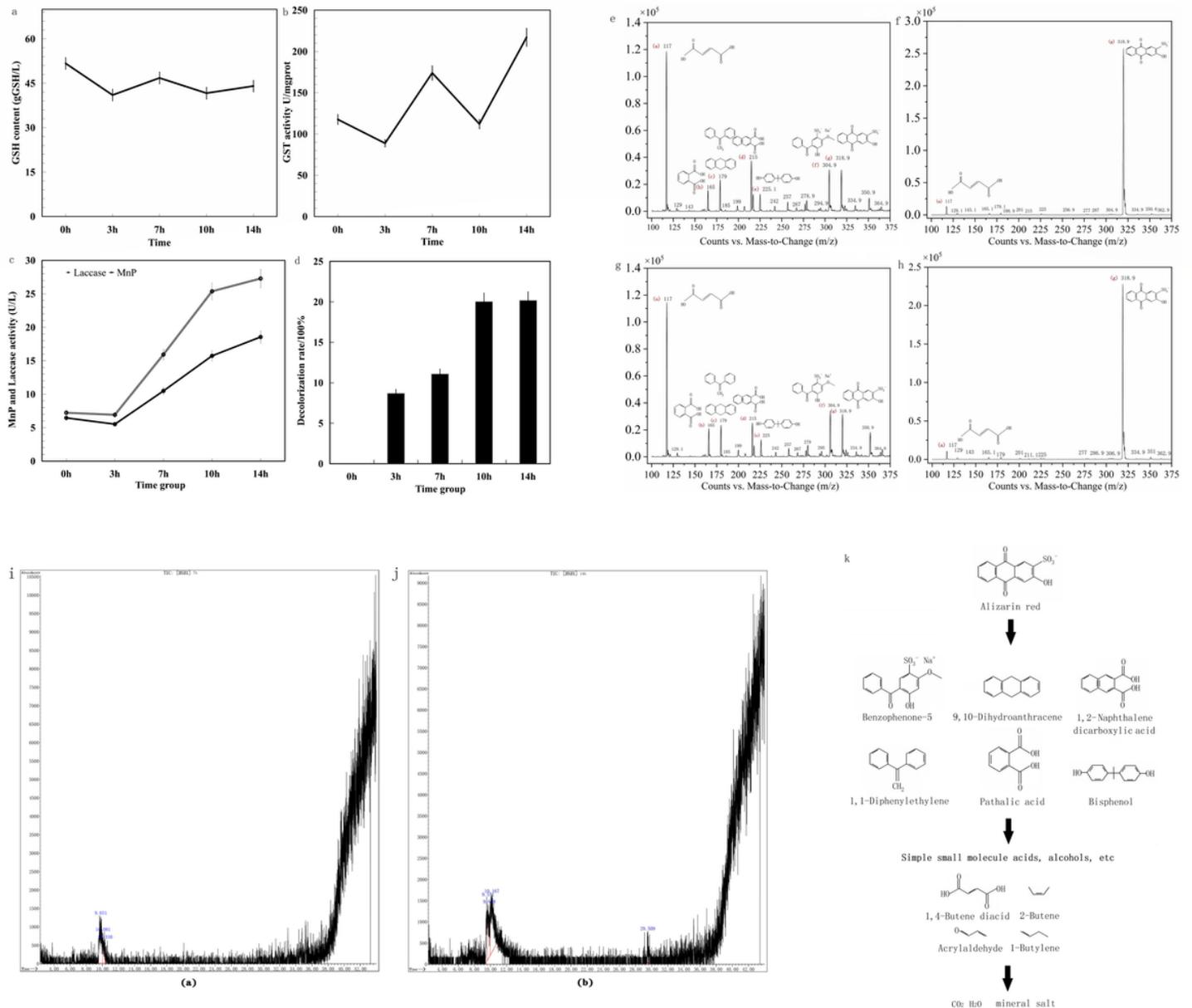


Figure 4

Detection of the activity of various substances of *L. gibbosa* and AR degradation production by *L. gibbosa* at various stages. (a) GSH content. (b) GST activity (c) MnP and Laccase activity. (d) AR

decolourization rate. (e) and (f): The decomposition products of AR at 7 h were analysed by LC-MS, (g) and (h) 14 h production spectrum of AR. (i) 7 h GC-MS Total ion chart. (j) 14 h GC-MS Total ion chart. (k): Extrapolation of AR Degradation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementarytable2.docx](#)