Multi-omics analysis provides insights into lignocellulosic biomass degradation by *Laetiporus sulphureus* ATCC 52600

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Research

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Abstract

Background: Wood-decay basidiomycetes are effective for the degradation of highly lignified and recalcitrant substrates. Brown-rot strains produce carbohydrate-active enzymes involved in the degradation of lignocellulosic materials, along with a non-enzymatic mechanism via Fenton reaction. Differences in the lignocellulose metabolism among closely related brown rots are not completely understood. Here, a multi-omics approach provided a global understanding of the strategies employed by *L. sulphureus* ATCC 52600 in the degradation of lignocellulosic by-products derived from sugarcane and Eucalyptus.

Results: To evidence the oxidative-hydrolytic mechanism, the *Laetiporus sulphureus* ATCC 52600 genome was sequenced and the response to lignocellulosic substrates was analyzed by transcriptomics and proteomics. The transcriptomic profile in response to a short cultivation period on *in natura* sugarcane bagasse revealed 128 out of 12,802 upregulated transcripts. The high upregulated transcripts included a set of redox enzymes along with hemicellulases. The exoproteome produced in response to extended time cultivation on Avicel, and steam-exploded sugarcane bagasse, sugarcane straw, and Eucalyptus (*from Eucalyptus grandis*) revealed 121 proteins. Contrasting with the mainly oxidative profile observed in the transcriptome, the secretomes showed a diverse hydrolytic repertoire including constitutive cellulases and hemicellulases, in addition to 19 upregulated proteins relative to glucose. The secretome produced on sugarcane bagasse was evaluated in the saccharification of pretreated sugarcane straw by supplementing a commercial cocktail. Additionally, growth analysis revealed that *L. sulphureus* ATCC 52600 has higher efficiency to assimilate glucose than other mono- and disaccharides.

Conclusion: This study shows the singularity of *L. sulphureus* ATCC 52600 compared to other Polyporales brown rots, regarding the presence of cellobiohydrolase and peroxidase class II. The multi-omics analysis reinforces the oxidative-hydrolytic metabolism involved in lignocellulose deconstruction, providing insights into the overall mechanisms as well as specific proteins of each step.

Background

Wood-decay basidiomycetes are essential for the carbon cycle because of their highly specialized biomass degradation. Their metabolic systems include carbohydrate-active enzymes (CAZymes), but also non-CAZymes and other associated non-enzymatic compounds. This ability allows them to be potentially used for the production of value-added biocompounds derived from lignocellulosic biomass (1–3).

Traditionally, wood-decay basidiomycetes have been classified as brown rot or white rot based on the capacity to degrade plant cell wall components. Accordingly, brown rots degrade cellulose and hemicellulose while only modifying lignin. These two decay modes have been distinguished based on the reduction or absence of some enzymes, such as ligninolytic peroxidases (PODs) class II (manganese-, lignin- and versatile-peroxidases), as well as enzymes involved in cellulose degradation such as

cellobiohydrolase (CBH), lytic polysaccharide monooxygenase (LPMO) and cellobiose dehydrogenase (CDH) (3, 4). To compensate for the paucity of cellulolytic enzymes, some brown rots employ mechanisms for endoglucanases overproduction (5). In fact, the lignocellulose degradation performed by brown-rot fungi involves chemical, biological and spatial relationships between fungal hyphae and the plant cell wall to perform a two-step mechanism: earlier lignocellulose oxidative (LOX) degradation mediated by Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}\cdot$) followed by a late hydrolytic mechanism. Key requirements for Fenton systems include mechanisms for extracellular peroxide production and iron reduction, involving extracellular fungal enzymes and metabolites, to generate reactive oxygen species (ROS) (6–9).

Most brown-rot Agaricomycetes belong to the order Polyporales Gäum. Within this order, most brown-rot species belong to the “Antrodia clade”, which includes the families *Dacrybolaceae* Jülich, *Fomitopsidaceae* Jülich, *Laetiporaceae* Jülich, and *Sparassidaceae* Herter, as well as a few unsolved groups (10). *L. sulphureus* is considered a cosmopolitan species causing brown cubical heart rot in many deciduous and coniferous trees (11). It is known to produce metabolites with antioxidant and antimicrobial properties (12, 13) and natural dyes (14, 15), in addition to the potential for bioremediation of treated wood (16) and decolorization of textile effluents (11). During wood decay, *L. sulphureus* causes higher polysaccharide weight loss than lignin loss (17). This fungus has superior potential to produce cellulolytic and hemicellulolytic enzymes in comparison to other representative brown rots (18). The *L. sulphureus* enzymatic repertoire secreted in the presence of carboxymethyl-cellulose (CMC) was analyzed by mass spectrometry (19). However, the potential for lignocellulose degradation has not been explored at multi-omics level.

Omics approaches allow a deep understanding of the biology of an organism, including its behavior during growth on complex plant biomass (20). In this work, genetic sequencing followed by transcriptomic and proteomic analysis provided a global understanding of the strategies employed by *L. sulphureus* ATCC 52600 in the degradation of lignocellulosic by-products derived from sugarcane and *Eucalyptus*. In addition, a commercial enzymatic cocktail supplemented with the *L. sulphureus* secretome was evaluated for biomass saccharification.

Results

Sequencing, annotation, and phylogenetic analysis of L. sulphureus ATCC 52600

The *L. sulphureus* ATCC 52600 genome sequence was assembled by a combination of paired-end (45,000,408 sequences) and mate-pair libraries (13,294,823 and 13,280,039 sequences), corresponding to 43.4 Mb, as detailed in Table 1.

Table 1
Statistical information on the genome
assembly of *L. sulphureus* ATCC 52600.

<i>L. sulphureus</i> ATCC 52600	
Estimated coverage	125x
# contigs (≥ 5000 pb)	428
# contigs (≥ 10000 pb)	375
# contigs (≥ 25000 pb)	275
# contigs (≥ 50000 pb)	213
# scaffolds	785
Total length (pb)	43,372,605
Largest contig (pb)	1,372,164
GC (%)	51.22
N50	211,056
N75	102,005
L50	53
L75	1,129
Number of predicted genes	12,802

Genomic features were similar to *L. sulphureus* var. *sulphureus* v1.0 (21). Comparative analysis showed the strains sharing 8,419 clusters of orthologous genes, with 7,724 single-copy genes, which accounted for 60% and 56% of all coding sequences for the strains ATCC 52600 and var. *sulphureus* v1.0, respectively. The phylogenomic analysis considering whole-genome information strongly supported monophyletic clades for all families within the order Polyporales (Fig. 1). The strain ATCC 52600 clustered with var. *sulphureus* v1.0 and *Wolfiporia cocos* in the family *Laetiporaceae*, which, in turn, appears as a sister clade of *Fibroporiaceae* (*Fibroporia radiculosa*) and closely related to *Dacryobolaceae* (*Postia placenta*).

sulphureus ATCC 52600 genome: non-enzymatic mechanism for biomass deconstruction and non-canonical brown-rot CAZymes

The CAZymes content was constituted of 271 modules, including 133 glycoside hydrolases (GH), 51 auxiliary activities (AA), 67 glycosyltransferases (GT), 13 carbohydrate esterases (CE), and 4 polysaccharide lyases (PL). Regarding carbohydrate-binding modules, CBM20 was found associated with GH, in addition to the non-appended CBM13 and CBM21. GHs comprise 49% of CAZymes, and the most abundant families were GH16 (19 members) and GH5 (16 members). Further analysis revealing the GHs

repertoire for cellulose degradation included endoglucanases (GH5), beta-glucosidases (GH1, GH3), and, notably, one predicted cellobiohydrolase (GH7). A wide range of GHs associated with the degradation of hemicelluloses such as xylan (GH10, GH43, GH115), glucans (GH16, GH55), and mannans (GH53) was identified, in addition to enzymes active on starch (GH13, GH15), pectin (GH28), chitin (GH18), and trehalose (GH37) (Fig. 2 and **Additional file 1: Table S1**).

The analysis showed 19% of the predicted CAZymes assigned to AAs. Prominent among them was the large number of AA3 (28 members) grouped into the subfamilies AA3_2 (25 aryl/glucose oxidases) and AA3_3 (3 alcohol oxidases). Also, 6 members of AA1 were identified and categorized into the subfamilies AA1_1 (3 laccases), AA1_2 (1 ferroxidase), and AA1_3 (2 laccase-like multicopper oxidases). Three AA5_1 glyoxal oxidases and one AA6 benzoquinone reductase were also identified. Moreover, two genes coding for AA2 PODs were predicted, as well as AA members acting on cellulose/hemicellulose, including seven AA7 glucooligosaccharide oxidases, two AA9 lytic polysaccharide monooxygenases (LPMO), and two AA14 LPMOs (Fig. 2 and **Additional file 1: Table S1**).

The most prevalent CE family was CE16 (6 acetyl esterases), in addition to CE4 (2 chitin deacetylase and 1 acetyl xylan esterase), CE1 (1 acetyl xylan esterase and 1 carboxylesterase), CE8 (1 pectin methylesterase), and CE9 (N-acetylglucosamine deacetylase). Three PL14 and one PL35 were also identified. The most abundant GT family was GT2 encompassing 15 members, followed by GT8 (5 members), GT4 (4 members), and GT15, GT20, GT21, GT39, and GT69 (3 members each) (Fig. 2 and **Additional file 1: Table S1**).

A wide diversity of genes involved in Fenton reaction and oxidative mechanisms was identified in the *L. sulphureus* ATCC 52600 genome, including alcohol dehydrogenases, aldo-keto reductases, catalases, ferroxidase, cytochrome P450, peroxidases not assigned to the CAZy domain, oxidoreductases, oxalate decarboxylase, and hydroquinone dehalogenases, the last two involved in the production of low-molecular-weight compounds (LMW) (**Additional file 1: Table S1**).

L. sulphureus ATCC 52600 displays reduced growth in non-glucose saccharides and slow glucose consumption

Further analysis of the *L. sulphureus* ATCC 52600 primary metabolism was motivated by the presence of transporters for different carbohydrates such as glucose, mannose, and trehalose in the genome (**Additional file 1: Table S1**). The strain was able to grow on a wide range of mono- and disaccharides (**Additional file 2: Figure S1A**) and the growth rate was reduced by approximately 50% on arabinose, xylose, and galacturonic acid, and by 60% on galactose, cellobiose, and lactose in comparison with glucose (**Additional file 2: Figure S1B**). Growth in liquid medium with glucose showed an extended lag period, with glucose consumption starting only after 48 h of cultivation, and then decreasing at a slow rate, to around 40% at 168 h of cultivation (**Additional file 2: Figure S1C**). These data raised questions about the biological behavior of this basidiomycete on complex carbon sources in terms of protein expression and secretion, especially because of the presence of some non-canonical brown-rot CAZymes such as CBH and AA2 peroxidase in the genome.

Transcriptomic analysis reveals the early response of L. sulphureus ATCC 52600 for the deconstruction of in natura sugarcane bagasse

A total of 10,015 transcripts were identified with at least one transcript per million (TPM), with 6,920 sequences presenting statistical significance. Differentially expressed genes (DEGs) comprised 1,120 up- and 1,455 downregulated genes, out of which 64 up- and 32 downregulated transcripts corresponded to predicted CAZymes. Among the CAZyme upregulated transcripts, five and seventeen genes with predicted cellulolytic and hemicellulolytic functions, respectively, were identified, including glucanases (GH5, GH16, GH55, and GH71), β -glucosidases (GH1 and GH3), α -xylosidase (GH31), α/β -mannosidases (GH2 and GH47), and α/β -galactosidases (GH27, GH35, and GH71). In addition, several genes with predicted activity on starch (GH13 and GH31), chitin (GH18 and CE4), and pectin (GH28, GH78, and GH105) were also upregulated, whereas transcripts predicted for xylan-active xylanase (GH30) and β -xylosidase (GH43) were downregulated.

Among the AAs, two AA1_1 laccases and one AA1_2 ferroxidase were upregulated; AA3 members such as the 11 members of the subfamily AA3_2 were downregulated, while one AA3_3 alcohol oxidase was upregulated. In addition, two AA7 glucooligosaccharide oxidases, one AA6 benzoquinone reductase, one AA9 LPMO, and one AA14 LPMO were upregulated (Fig. 3A).

A set of genes encoding non-CAZy enzymes and proteins with a predicted function in the oxidative mechanism and Fenton reaction were also regulated, corresponding to 159 transcripts, out of which 62 were up- and 97 downregulated (Fig. 3A). The importance of this mechanism became clearer when the regulation level of individual genes was analyzed, i.e., among the highly upregulated transcripts ($\log_2$ -fold change ≥ 2 , $n = 43$), 67% were associated with this metabolism, including both AAs and non-CAZymes. The remaining CAZymes (33%) were mostly hydrolases including miscellaneous hemicellulases (acting on glucan, mannan, galactan), amylases, pectinase, and chitinases. Remarkably, the top upregulated transcripts included a series of AA oxidoreductases belonging to the families AA3_3, AA1_2, AA6, and AA7, as well as non-CAZy oxidoreductases, dehydrogenases, cytochrome, and enzymes involved in LMW metabolism. In turn, transcripts of cellulose- or xylan-active enzymes were absent (Fig. 3B).

Proteins secreted by L. sulphureus ATCC 52600 during cultivation on pretreated plant biomass

The exoproteomes of *L. sulphureus* ATCC 52600 cultivated on lignocellulosic biomass were analyzed by MS. A total of 3,328 spectra were identified, accounting for 121 proteins. This set of proteins was composed of 42 CAZymes (34%), 9 peptidases/proteases (7%), 10 non-CAZy oxidoreductases (8%), 7 esterases (6%), 5 dehydrogenases (4%), 36 miscellaneous proteins/domains (30%), and 9 hypothetical proteins (8%) with unknown function (Fig. 4A and **Additional file 1: Table S3**). Most of the proteins identified were predicted with SP (**Additional file 1: Table S3**).

GHs were predominant among the secreted CAZymes, accounting for 78% (33 members), followed by AA members with 6 representatives (14%) (Fig. 4A and **Additional file 1: Table S3**). Almost 50% of the GHs corresponded to enzymes with predicted activity on hemicelluloses, while 5 were cellulose-active GHs. In

addition, GHs with predicted activity on pectin, starch, chitin, and trehalose, as well as one PL35 alginate lyase, were also secreted (**Additional file 1: Table S3**). Overrepresented GH families corresponded to GH18 with predicted chitinolytic activity (g5150, g834, and g10854), and GH3 represented by β -glucosidases (g2032 and g11777) and β -xylosidase (g7390). The secreted AAs array consisted of AA3 aryl alcohol oxidases (g5677, g5675, g5206, and g10342), AA5 glyoxal oxidase (g4370), and AA7 glucooligosaccharide oxidase (g9758) (Fig. 4B).

The highest number of CAZymes was identified in the secretome produced on SCB (41 proteins), followed by 40, 39, 30, and 20 proteins identified on *Eucalyptus*, Avicel, SCS, and glucose, respectively. Differences in the CAZyme arsenal produced by *L. sulphureus* ATCC 52600 for lignocellulose degradation became evident when comparing the secretomes (Fig. 4C and **Additional file 1: Table S3**). A total of 19 enzymes were secreted in all conditions (43% of the CAZymes), indicating constitutive secretion. The secretome produced on SCB showed the highest number of upregulated CAZymes, comprising 16 hits, out of which 10 were exclusively secreted in this condition. These hits corresponded to exo-type enzymes mainly related to hemicellulose degradation such as GH3 β -xylosidase (g7390), GH35 β -galactosidase (g11423), and GH47 α -mannosidase (g10983), along with GH3 β -glucosidase (g11777) and AA7 oxidoreductase (g9758). Moreover, the secretomes produced on SCB and SCS showed common upregulation of GH3 (g2032), AA3_2 (g5675), and GH20 (g8819). Interestingly, some enzymes with basal constitutive secretion on glucose such as GH7 CBH (g8442), GH92 α -1,2-mannosidase (g9634), and GH18 chitinase (g834) were upregulated on Avicel. Additionally, one xylanase (g4476) was upregulated on all polymeric substrates, and one AA3_2 aryl alcohol oxidase (g10342) was exclusively upregulated on SCS. Despite the high number of proteins secreted on *Eucalyptus*, only one β -L-arabinofuranosidase (g6508) was upregulated in this condition as well as on SCB. Another remarkable characteristic is the complete absence of AAs in the secretome produced on Avicel. Finally, 16 non-CAZymes belonging to the “others” classification were upregulated on at least one condition, mostly on SCB. Prominent among them were galactose oxidase (g7548) and another oxidoreductase (g9473), both showing upregulation with Avicel, SCB, and *Eucalyptus*.

Performance of the *L. sulphureus* ATCC 52600 secretome on biomass conversion

Enzymatic activities were validated on the different *L. sulphureus* ATCC 52600 secretomes. Activities were detected on arabinoxylan, beta-glucan, starch, and xylan from beechwood, and the secretome produced on SCB presented higher activity in all substrates comparing to the other secretomes (**Additional file 2: Figure S2**). This secretome was applied for supplementing commercial enzymatic cocktails in the saccharification of pretreated sugarcane straw. Glucan conversion was increased by 9% when 15% of the commercial cocktail was replaced by the secretome produced on SCB, while no differences were observed in xylan conversion. The commercial cocktail resulted in 40–55% glucan conversion and 30% xylan conversion (Fig. 5).

Discussion

sulphureus ATCC 52600 genome does not resemble typical brown-rot fungi

Genomic sequencing of filamentous fungi followed by transcriptomic and proteomic approaches has been widely employed to understand the strategies of microorganisms to degrade plant biomass (22–28). Overall, the *L. sulphureus* ATCC 52600 genome revealed only subtle differences compared to the previously sequenced *L. sulphureus* var. *sulphureus* v1.0 (21), indicating that the strains might have undergone some changes in their ecological niches to shape their genomes to the environmental conditions. Our phylogenetic analysis, providing high resolution on the evolutionary history of organisms by considering whole-genome information (28), complements the previous phylogeny of the order Polyporales (10). The phylogenetic tree (**Figure 1**) strongly supports monophyletic clades for the families within the order Polyporales. *L. sulphureus* ATCC 52600 clusters with *L. sulphureus* var. *sulphureus* v1.0 and *W. cocos* giving further support to the existence of the family *Laetiporaceae* Jülich, as proposed previously by Justo and colleagues and currently present in Mycoguide, but retrieved as an invalid name in MycoBank and Index Fungorum.

The genomic CAZyme content in both *L. sulphureus* strains and the closely related brown-rot Polyporales shows a fairly typical number of GHs, CEs, PLs, and GTs compared to *W. cocos*, *P. placenta* and *F. radiculosa*, whereas a lower number of CAZymes, particularly GHs, were observed in comparison with *Fomitopsis pinicola* (Fomitopsidaceae). In turn, *L. sulphureus* ATCC 52600 shows a higher AA content than in the other genomes (9). Additionally, the genome reveals several similarities with other brown-rot genomes associated with the evolutionary reductions and losses in key enzymes involved in biomass breakdown, especially cellulases and lignin-modifying enzymes (3). Accordingly, it presents a reduced number of genes coding CAZymes from the families GH1, GH3, GH5, GH7, GH10, AA9, and CE1 along with the absence of GH6, GH11, AA3_1, CBM1, and CE15 (**Additional file 1: Table S1**).

Considering these reductions/absences, other enzymes may also be necessary to achieve an effective breakdown of cellulose/hemicellulose, such as the identified AA9 and AA14 LPMOs. AA9s perform oxidative cleavage on cellulose and other glucans with great importance in lignocellulose degradation (29), presenting an average number of 3 genes in Polyporales genomes (3,25). The recently established family AA14 also groups LPMOs that are widespread in fungi. Within the order Polyporales, white-rot strains present 4.5 genes on average, whereas brown-rot strains present 2.5 genes. This reduction pattern can also be added to the other gene reductions associated with the evolution of the brown-rot lifestyle (30). One AA14 member characterized from the white rot *Pycnoporus coccineus* presents oxidative activity on xylans of xylan-coated cellulose fibers (30), and its sequence aligns with the *L. sulphureus* ATCC 52600 AA14 LPMO. One of the sequences, however, lacks the amino terminus that can be attributed to a gap in the genome sequencing analysis (**Additional file 2: Figure S3**).

Regarding the enzymes involved in the oxidative mechanism, AA3_1 CDHs are absent in *L. sulphureus*, as verified in *P. placenta*, *W. cocos*, and *F. pinicola* (31). In turn, a large number of genes coding for AA3_2 (aryl alcohol oxidase and glucose 1-oxidase) and AA3_3 (alcohol oxidase) were identified, and the products H₂O₂ (reduction of oxygen by the oxidases) and hydroquinones (reduction of quinones) can

support other enzymes or reactions that are important for the deconstruction of lignocellulose (32). Similarly, AA5_1 glyoxal oxidases and AA6 benzoquinone reductase, which are also involved in the generation of Fenton reagents (33–35), were identified (**Additional file 1: Table S1**). Notably, the absence of CDH may also suggest the presence of other redox partners for AA9 and AA14 LPMOs, such as AA3_2 flavoenzymes (36) and GMC oxidoreductases, among others (37), or the peroxide produced might be driving LPMOs reaction (38).

The *L. sulphureus* genome also revealed some distinctions in the enzymatic lignocellulolytic repertoire. For example, the well-known lack of cellulases in brown-rot fungi is generally attributed to a reduced number of GH6/GH7 CBHs (31,39,40), which are recognizably absent in the genomes of brown-rot Polyporales (41). Our sequencing, however, identified one putative GH7 CBH (g8442) in the *L. sulphureus* ATCC 52600 genome, in accordance with a GH7 CBH previously identified in the secretome of *L. sulphureus* growing on CMC (19). Sequence analysis shows these enzymes sharing more than 90% identity, and the phylogeny using predicted and characterized fungal CBHs reveals 65% similarity with other fungal CBHs. The sequence identity is higher compared to basidiomycete CBHs (44%) than to the characterized counterparts (33%) that are mainly from ascomycetes (**Additional file 2: Figure S4**). Additionally, analysis of 42 fungal genomes indicates that brown rots generally have a reduced number of GH45, in a 3:1 ratio in comparison with white rots (9). Our initial search parameters identified one putative GH45 (g10751), coinciding with a GH45 (ID 174393) previously identified in the *L. sulphureus* secretome (19). These sequences share 92.5% identity, having an expansin domain predicted by InterPro v.78.1 (42), despite the previous classification as GH45 class C (19). Expansins are indeed closely related to GH45 endoglucanases and have been widely found in brown-rot strains (9), performing an important function in reducing biomass recalcitrance, thus increasing the deconstruction of lignocellulose in synergism with cellulases (43).

Lignin degradation and the presence and importance of different lignin-active enzymes in brown rots is a matter of debate, but it is widely recognized that brown rots present a reduced number of laccases and absence of PODs class II in comparison with white-rot strains (8). *L. sulphureus* ATCC 52600 has AA1_1 and AA1_3 laccases, similarly to *F. pinicola*, *P. placenta*, and *W. cocos* (44). Additionally, 13 predicted PODs were identified in the *L. sulphureus* ATCC 52600 genome, and two of them presented AA2 domain predicted by dbCAN. InterPro annotation classified one of them as an intracellular POD class I, while the other (g11846) was classified as a fungal ligninase/POD class II. The enzyme presents a predicted SP, and a BLAST search retrieved 87% and 66% identity with PODs class II from *L. sulphureus* var. *sulphureus* v1.0 and *W. cocos* MD-104SS10 v1.0, respectively. POD class II has been reported as a single copy in *P. placenta*, *W. cocos* and *F. pinicola* genomes (31), and the *P. placenta* peroxidase (Ppl44056) was classified as a basal peroxidase, not closely related to LiP and MnP (45). Laccases in Polyporales are multigenic (46) and have been characterized as functional enzymes in *P. placenta* and *F. pinicola* (47–49), playing a role in wood decay performed by *P. placenta* (47). Significant lignolysis has been observed in *Gloeophyllum trabeum* (Gloeophyllales) and *P. placenta* without considering the involvement of PODs class II (6,50). Nevertheless, the biological importance or the precise role of these PODs II found

specifically in *L. sulphureus* and other closely related brown rots are uncertain since these enzymes have not been characterized to date.

Insights of the L. sulphureus ATCC 52600 biomass deconstruction mechanism

Several omics studies analyzing brown-rot fungi with significant taxonomic and niche distances such as *W. cocos*, *F. radiculosa*, *P. placenta*, *G. trabeum*, and *Serpula lacrymans* (Boletales) cultivated in different conditions show the common presence of a two-step mechanism employed in biomass deconstruction (8,24,45,51–57). The initial oxidoreductive step persists for 48 h (8), which can be correlated with both the observed slow growth of *P. placenta* in cellulose and spruce (53) and the *L. sulphureus* growth and glucose consumption in liquid medium (**Additional file 2: Figure S1C**).

Based on these studies, our transcriptomics analysis performed in a short cultivation period reveals a series of upregulated genes related to the oxidative mechanism induced by recalcitrant *in natura* sugarcane bagasse (**Figure 3** and **Additional file 1: Table S2**). The most highly upregulated transcripts include alcohol dehydrogenase, cytochrome P450, aldo/keto reductases, and redox genes involved in the generation of hydrogen peroxide, while hydroquinone dehalogenase is involved in hydroquinone production which, in turn, initiates Fenton reaction by the carrying of Fe^{3+} (58). Moreover, the presence of AA6 quinone reductases points to this enzyme as the main component in the quinone redox cycle supporting Fenton chemistry, as previously observed in *P. placenta* (45), while also playing a role in the detoxification process (5). Such observations are consistent with a biodegradative role of Fenton chemistry occurring during early cultivation, as verified in other brown-rot transcriptomes (9,59,60).

Regarding CAZymes (**Figure 3A** and **Additional file 1: Table S2**), previous brown rot transcriptomic studies also revealed a small set of cellulases and a very similar and general set of hemicellulases with predicted activity on glucans and mannans (8,45). The upregulation of some cellulases and hemicellulases supports the existence of inducing mechanisms, which rely mostly on substrate exposure and availability, occurring even at the beginning of the degradation process. Additionally, two AA1 laccases-encoding genes were upregulated suggesting the microorganism's ability to partially oxidize lignin substructures. On the other hand, two other AA1 laccases as well as two non-CAZy peroxidases were downregulated, so the importance of ligninases for this fungus remains unclear (**Figure 3A** and **Additional file 1: Table S2**). Transcripts of AA9 and AA14 LPMOs were upregulated in the transcriptome, while not being detected in the proteomes, corroborating the concept of LPMOs being naturally produced by fungi during early biomass degradation (59,61). The biological importance of LPMOs for brown-rot fungi remains unclear since their secretion has only been identified in *G. trabeum* growing on lignocellulose (45). Furthermore, the early upregulation of different pectinases observed in *L. sulphureus* is similarly verified in *P. placenta* and *G. trabeum*. Pectinases act by facilitating the access of other enzymes to the plant cell wall components after pectin removal (8,53).

In contrast to the transcriptome, the secretome data (7-day cultivation) reflects the late hydrolytic decay profile (53), which is supported by the absence of AAs in the secretome produced on Avicel. Comparative

analysis of the secretomes allows the identification of a core set of constitutive CAZymes, comprising some GHs with predicted activity on cellulose and a wide diversity of GHs acting on glucans, xylan, mannans, trehalose, starch, and chitin (**Additional file 1: Table S3**). Apart from the xylan-active enzymes, the hemicellulases set is very similar to the profile observed in the transcriptome. This wide-range enzymatic core allows the fungus to obtain energy sources from substrates with diversified composition, providing an increase in survival capability under different environmental conditions.

Regulatory mechanisms take place after the sensing and transport of inducers, resulting in the secretion of a series of regulated CAZymes directed to substrate degradation. Here, comparing grass and wood substrates, which typically present different compositions (62,63), differences in the secretion of specific enzymes were mainly observed in the cultivation of *L. sulphureus* on SCB, which indeed resulted in the highest number and diversity of secreted and upregulated proteins. This result may be related to substrate recalcitrance and pretreatment (64,65).

Endoglucanases were poorly secreted by *L. sulphureus*, apparently playing a minor role in cellulose degradation, despite the importance of processive endoglucanases in brown rots (66). Two GH3 β -glucosidases were upregulated on SCB while GH7 CBH was upregulated on Avicel. These data, in addition to the basal secretion of some CAZymes found in all evaluated substrates, show that CBH is inducible and it is not under catabolite repression, as verified for the endoglucanase from *G. trabeum* (67) or cellulases from *P. placenta* (53). However, the gene encoding GH7 CBH was not differentially expressed (transcriptome - early stage), and the secretion of endoglucanases and β -glucosidases, as well as oxidative agents, can compensate for this absence, characterizing less effective biomass decay in early stages (4,6).

In addition to the constitutive hemicellulases, a diversity of mannanases and glucanases were upregulated at both early and late response to biomass degradation and can be explained by a natural preference of brown rots for softwoods (1,3,7,56). There is evidence that hemicellulose loss progresses faster than cellulose loss in coniferous wood decay caused by *G. trabeum* and *F. pinicola* (68). Additionally, our secretome data clearly show that *L. sulphureus* targets hemicellulose as part of the hydrolytic late response. Several enzymes active on xylan, the main hemicellulose found in grasses, were secreted, *i.e.*, one GH10 xylanase is upregulated on Avicel and SCB, while the production of another GH10 xylanase is constitutive. Also, one beta-xylosidase is upregulated on SCB, while two α -L-arabinofuranosidases are widely secreted on the polymeric substrates. Corroborating this orientated inducible mechanism, transcripts of most of these enzymes do not show early upregulation; rather, one GH30 xylanase and one GH51 arabinofuranosidase are downregulated. This result shows that *L. sulphureus* shifts its metabolism to the degradation of grass, however other brown rots from the *Antrodia* clade have been reported to be inefficient in the degradation of corn stalk (69).

This hemicellulase-enriched profile was also reported for *L. sulphureus* ATCC 52600 secretome produced on CMC (19). Despite that, the saccharification analysis showed higher glucan conversion than xylan conversion (**Figure 5**). A similar result was previously reported using *L. sulphureus* ATCC 52600

secretome for sugarcane bagasse hydrolysis, in which the xylan conversion was around 50% of the glucan conversion (70). The lower biomass conversion efficiency, when compared to commercial cocktail, could be related to slow growth rate and glucose consumption results (**Additional file 2: Figure S1C**). These results allow us to hypothesize that slow growth and low sugar consumption is an adaptive mechanism reducing the advantages of competitors that demand sugar and grow at high rates.

In **Figure 6**, an overview of the *L. sulphureus* strategies for biomass deconstruction is proposed based on the multi-omics data. Our results are consistent with a temporal two-step oxidative-hydrolytic mechanism for the degradation of lignocellulose, while also demonstrating that this fungus does not resemble typical brown-rot fungi in many aspects, thus contributing to the weak dichotomy between white- and brown-rot strains, as previously proposed (25). Additional data applying biological approaches such as gene deletion and analysis of wood decay, as well as biochemical characterization of the enzymes would contribute to addressing this question.

Conclusions

Genome sequencing and analysis of expression and secretion patterns elucidated a repertoire of genes/proteins involved in lignocellulose degradation by *L. sulphureus* ATCC 52600. In many aspects, this brown-rot fungus presents similarities with other model brown rots, while not resembling typical brown rots regarding the notable presence of cellobiohydrolase and POD class II. The transcriptomic analysis using highly recalcitrant biomass and a short cultivation period demonstrated the presence of an early oxidative response, as well as other hallmarks of brown-rot early cultivation such as the presence of LPMO and pectinases. The proteomic analysis, in turn, provided the late response profiles of proteins secreted in response to cultivations with different substrates such as cellulose, lignocellulose from grass subproducts (sugarcane bagasse and straw), and woody residue from *Eucalyptus*. Overall, the secretome profile showed a set of CAZymes commonly secreted in different conditions, with subtle differences in the secretion of specific enzymes. Constitutive secretion and induction occur for some cellulases and a more complex regulatory mechanism of induction and repression occurs for enzymes acting on xylan degradation. Another remarkable characteristic is the absence of AAs in the degradation of pure cellulose, but not for the degradation of complex substrates.

Methods

Strain maintenance

sulphureus ATCC 52600 was purchased from Fundação André Tosello (CCT 4694). The strain was routinely maintained on solid media composed of 20 g/L malt extract and 2 g/L yeast extract and incubated for 7-10 days at 30 °C.

DNA extraction and sequencing

DNA extraction from mycelia was performed using phenol-chloroform (71), followed by RNase treatment. High-quality DNA was obtained using DNeasy Kit (Qiagen). Three Illumina libraries were constructed, a paired-end library with a 300 bp insert and two mate-pair libraries with 5 to 7 kb and 8 to 11 kb, according to the manufacturer's instructions. The libraries were sequenced on an Illumina HiSeq 2500 platform.

Genome assembly and annotation

Paired-end and mate-pair reads (2x100pb) were filtered by quality and presence of adaptors using Trimmomatic v0.32 (72) and NextClip (73) default parameters, respectively. The genome was *de novo* assembled using Velvet v.1.2.10 (74) with $kmer=55$. The resulting assembly was scaffolded by SSPACE v3.0 (75) and the mate-pair reads, and further refined by Pilon version 1.16 (76). The completeness of the genome was assessed using Benchmarking Universal Single-Copy Orthologs (Busco) (77) and prediction was performed using BRAKER1 (78), which applies GeneMark-ES and AUGUSTUS along with RNA-seq alignments for gene prediction. Predicted protein sequences were functionally annotated by searching for homologous sequences in the SwissProt (79), EggNOG (80), and Pfam (81) databases. Signal peptides (SP), transmembrane regions, and ribosomal genes were predicted using SignalP v.4.0 (82), TMHMM (83), and ITSx using fungal models, respectively. Carbohydrate transporters were identified and classified according to the PFAM 00083.21 (28) and enzymes associated with LMW metabolism were classified using Gene Ontology (<http://geneontology.org>). Comprehensive analysis of CAZymes was carried out using HMM-based dbCAN v.8 (84), HMMER (E-Value < $1e-15$, coverage > 0.35), DIAMOND (E-Value < $1e-102$), and Hotpep (Frequency > 2.6, Hits > 6).

Phylogenetic Analysis

The phylogenomic relationship of *L. sulphureus* ATCC 52600 and its closest described relatives of the family *Laetiporaceae* was determined based on orthologs single-copy genes using FastOrtho tool (<https://github.com/olsonanl/FastOrtho>). The protein sequences of each 601 single-copy orthologous genes present in 31 basidiomycete genomes closely related to the family *Laetiporaceae* and *L. sulphureus* ATCC 52600 were aligned by Mafft v.7.299 (85) and the resulting individual alignments were concatenated to create a supermatrix using FASconCAT-G v.1.02 (86). Evolutionary distance was inferred using maximum likelihood with RAxML v.8.2.0 (87), implementing PROTGAMMAWAG model and performing 1,000 bootstrap replicates to evaluate the reliability of the reconstructed phylogenetic tree.

Cultivation conditions for transcriptome analysis

Pre-inoculum, consisting of 15 discs (8 mm diameter) of *L. sulphureus* ATCC 52600 pre-cultivated on agar plates, was inoculated into 100 mL of liquid medium and incubated under 180 rpm for 7 days at 30 °C. Mycelia were then filtered and washed with water and transferred to liquid medium containing 1.0 g of *in natura* sugarcane bagasse and 100 mL of medium pH 7.0 composed of 6 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4 , 1 g/L KCl, and 1 g/L $MgSO_4$. Cultivation was performed under 180 rpm for 24 h at 30 °C. Mycelia and substrate mixtures were collected by filtration, washed with sterile water, manually dried in

filter paper, and stored at -80 °C before RNA extraction. Mycelium from pre-inoculum was used as a standard before induction (T₀).

RNA extraction and sequencing

The mycelium was ground with liquid nitrogen and total RNA extraction was performed with mirVana™ Total Isolation Kit (Thermo Fisher), according to the manufacturer's instructions. The resulting solution was treated with DNase (DNA-Free RNA Kit, Zymo Research) and purified with RNeasy Kit (Qiagen), and quality was verified using RNA Nano Bioanalyzer 2100 chip (Agilent). cDNA libraries were prepared according to the manufacturer's instructions and sequenced on the Illumina HiSeq 2500 platform.

Bioinformatics analysis of RNA-seq data

Reads were processed as described previously for the genome libraries and evaluation and filtration of the rRNAs were performed using SortmeRNA. The filtered data were mapped into the *L. sulphureus* ATCC 52600 reference genome sequenced in this work using Tophat2 algorithm (88). Differential gene expression analysis was based on counting data and performed with the Bioconductor DESeq2 package (89) using the R platform, by paired comparisons against the control condition. Transcripts showing differential expression ($\log_2$ -fold change ≥ 1 and ≤ -1) relative to the non-induced condition (T₀) were determined by applying $p \leq 0.05$ as the threshold.

Carbohydrate metabolism and glucose consumption

Mycelia discs were excised from the border of the colony growing on potato dextrose agar plates and transferred to the center of minimal medium agar plates (90) supplemented with 1% (w/v) of the following substrates: glucose, arabinose, galacturonic acid, xylose, lactose, cellobiose and galactose. Cultivation was performed in triplicate for 7 days at 30 °C, and growth rates were estimated from the daily measurement of the colony diameters. For cultivation in liquid medium, 15 mycelial discs were transferred into 250 mL Erlenmeyer flasks containing 100 mL of liquid minimal medium pH 5.5 supplemented with 1% (w/v) glucose for 7 days under static conditions at 30 °C. Samples were taken at 6, 12, 24, 48, 72, 96, and 120 h of cultivation, and residual glucose was measured by high-performance liquid chromatography (HPLC). Cultivation was performed in triplicate.

Cultivation conditions for proteomic analysis

Pre-inoculum: *L. sulphureus* ATCC 52600 was grown on potato dextrose agar plates pH 5.5 at 25 °C. After 7 days of cultivation, 15 mycelium discs (8 mm diameter) were excised from the colony border and transferred to 250 mL Erlenmeyer flasks containing 50 mL of liquid medium composed of 0.5 g/L NH₄CH₃CO₂, 0.5 g/L NaNO₃, 0.5 g/L MgSO₄, 0.2 g/L Na₂HPO₄, 0.8 g/L KH₂PO₄, 4.0 g/L yeast extract, and 10.0 g/L glucose. The pre-inoculum was incubated for 21 days under static conditions at 30 °C.

Cultivation: pre-grown mycelia were removed by filtration, washed with distilled water, transferred to a 50 mL conical tube, and manually macerated with 2 g of glass beads. The macerated mycelia were then

transferred to 250 mL Erlenmeyer flasks containing 50 mL minimal medium pH 5.5 supplemented with 1% (w/v) steam-exploded sugarcane bagasse (SCB), steam-exploded sugarcane straw (SCS), steam-exploded *Eucalyptus* residue (*Eucalyptus grandis*), Avicel® PH-101 (Sigma), and glucose. Cultivation was performed in triplicate under static conditions for 7 days at 30 °C.

Mass spectrometry and data analysis

Cultivation supernatants (secretomes) were filtered with Miracloth (Millipore), centrifuged (13,000*g*, 20 min, 4 °C), and concentrated using 10 kDa cut-off Amicon Centrifugal Filter Units (Millipore). Protein concentration was measured with the Pierce BCA Protein Assay kit (Thermo Scientific) using BSA as standard. Secretomes (20 µg) partially resolved on 12% SDS-PAGE (91) were excised, reduced, and digested with 20 mg/ml trypsin (Promega) (92). After extraction, samples were dried under vacuum and peptide mixtures were analyzed in LTQ Velos Orbitrap-activated, as described elsewhere (93).

Spectra data were annotated based on the *L. sulphureus* ATCC 52600 genome. The adjusted conditions to validate protein identification were protein probability thresholds higher than 99% and at least 2 different peptides identifying a protein, each with 95% certainty. Once the parameters were defined, a 0.0% false discovery rate (FDR) was generated and spectrum counting data were analyzed in a semi-quantitative method. Spectra counts are equivalent to the total number of standard spectra assigned to each protein and are commonly used to determine relative abundance (94). As the spectra counting methodology was used for analysis, FDR was designated as one of the parameters to determine the reliability of the experimental data. FDR was defined as the expected correspondent percentage of each peptide spectrum (95). Initially, a score was assigned to each peptide (primary analysis) performed with Mascot Distiller software. Subsequently, Mascot data were analyzed by Scaffold 4 Proteomic software attributing the number of spectra to the abundance and FDR to the reliability of the results. By using average spectra outputs from Scaffold 4, differentially secreted proteins were identified according to their spectra counting and quantitative values were applied to normalize the counts. The statistical analysis of the spectra was performed by the t-test ($p \leq 0.05$) and fold change by category, using data from cultivation with glucose as standard.

GO terms were analyzed and identified in the topGO platform

(<https://bioconductor.org/packages/release/bioc/html/topGO.html>) using the following tools: basic local alignment search (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), PFAM (<https://pfam.xfam.org/>), and MEROPS (<https://merops.sanger.ac.uk/>). Parameters used to run BLASTp were: E-value ≤ 40 , identity $\geq 40\%$, and consultation coverage $\geq 80\%$. Classification of CAZymes and carbohydrate-binding modules (CBM) was performed on dbCAN v.8 (csbl.bmb.uga.edu/dbCAN). The presence of at least three representative members was established to define a classification group. Prediction of signal peptide (SP) and non-classical protein secretion were verified using SignalP 4.1 (www.cbs.dtu.dk/services/SignalP) (96) and SecretomeP 2.0 (www.cbs.dtu.dk/services/SecretomeP/), respectively.

Activity on different substrates

Enzymatic assays were performed using 50 µl of the following substrates: 5 mM 4-nitrophenyl β-D-cellobioside, 4-nitrophenyl β-D-xylopyranoside and 4-nitrophenyl β-D-glucopyranoside, and 0.5% (w/v) polygalacturonic acid, starch, CMC, xylan from beechwood (Sigma), wheat arabinoxylan, β-glucan, and galactomannan (Megazyme). Assays were performed using 1 µg protein of the concentrated secretomes in 50 mM ammonium acetate buffer pH 5.5 for 4 h at 50 °C. Assays with 4-nitrophenol (pNP) substrates were stopped with 100 µl of 1 M sodium bicarbonate and the released 4-nitrophenolate was measured at 405 nm. Assays with polymeric substrates were stopped with 100 µl of 3,5-dinitrosalicylic acid (DNS) and the released reducing sugars were measured at 540 nm (97). All assays were performed in triplicate.

Enzymatic saccharification

Sugarcane straw, composed of leaves and green tops, was submitted to a mild alkaline treatment (10% w/w solids load, 0.8% NaOH w/w, 30 min, 60 °C) followed by hydrothermal treatment (10% w/w solids load, 20 min, 190 °C). Compositional analysis of the pretreated biomass (98) corresponded to 53% glucan, 13% arabinoxylan, 22% lignin, and 10% ashes. Enzymatic saccharification was performed in 1 mL working volume with 2% (w/w) solids substrate load in 50 mM sodium acetate buffer pH 5.0 at 50 °C up to 48 h in a Thermomixer under 1000 rpm agitation. The secretome was evaluated by replacing 15% of protein load from a commercial enzymatic cocktail by the *L. sulphureus* secretome produced on SCB. The total protein load (equivalent to 15 FPU) was the combination 5:1 (p/p) Celluclast[®]:glucosidase from *Aspergillus niger* (Merck) per gram of dry substrate (Celluclast[®] 115 FPU/mL = 150 mg protein/mL). FPAse activity was assayed (99, with modification of 100) and biomass conversion was calculated using glucan/xylan content in the biomass (pretreated/native) and anhydrous correction factors of 1.13 for xylose and 1.1 for glucose (98,101,102). Assays were performed in triplicate and the released sugars were measured by HPLC.

HPLC analysis

Glucose, xylose, and cellobiose were quantified in a liquid chromatography system (Waters 515 Pump, Water 717 plus Injector/Sampler) coupled to an Aminex HPX87H (300x7.8 mm) column and equipped with a refractive index (RI) detector (Waters 410). Detector and column temperatures were set, respectively, to 40 and 45 °C; 50 mM H₂SO₄ was used as a mobile phase at 0.6 ml/min flow rate; and 20 µl injection volume.

Declarations

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

ACPO, TAG, and FMS designed and performed the experiments related to genomics and transcriptomics, analyzed data, and wrote the manuscript. FLF, CRFT, and AD designed and performed the experiments related to proteomics and biomass degradation experiments. FLF, JAG, MVR, and CRFT designed and performed experiments, analyzed data, and wrote the manuscript. GT performed and analyzed the phylogenetic data and wrote the manuscript. GFP performed the bioinformatics analysis. MACS and RG performed HPLC analysis and collected data. TTF provided resources. AD and FS supervised the project. All authors read and approved the final manuscript.

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Data Availability Statement

The datasets generated for this study can be found in the Gene Expression Omnibus with the GEO accession number GSE151004.

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Figures

Figure 1

Phylogenomic analysis of *L. sulphureus* ATCC 52600 and related genera. The tree was built using the maximum likelihood (ML) method implemented in FastTree and WAG evolutionary model. A total of 601 single copy ortholog genes from 31 genomes of basidiomycetes belonging to the order Polyporales were analyzed. Bootstrap values (1000 resamples) above 0.8.

Figure 2

L. sulphureus ATCC 52600 CAZyme-coding genes. Genome profile represented the number of predicted genes encoding CAZymes. CAZy classes: GH, glycoside hydrolases; CBM, carbohydrate-binding module; CE, carbohydrate esterase; PL, polysaccharide lyase and AA, auxiliary activities.

Figure 3

Enzymes differentially expressed by *L. sulphureus* ATCC 52600 cultivated on in natura sugarcane bagasse. (A) Total up- and downregulated CAZymes and selected non-CAZymes, grouped according to their predicted function. (B) Highly expressed transcripts ($\log_2$ -fold change ≥ 2) of CAZymes and proteins related to the *L. sulphureus* oxidative mechanism. CAZy classes: GH, glycoside hydrolases; CBM, carbohydrate-binding module; CE, carbohydrate esterase; PL, polysaccharide lyase; and AA, auxiliary activities.

Figure 4

Overview of the *L. sulphureus* ATCC 52600 secretomes. (A) Total proteins identified in the secretomes (CAZy and non-Cazy). (B) CAZy classes: GH, glycoside hydrolases; CBM, carbohydrate-binding module; CE, carbohydrate esterase; PL, polysaccharide lyase; and AA, auxiliary activities. (C) Venn diagrams grouping upregulated CAZymes relative to glucose. SCB: sugarcane bagasse; Eucalyptus: *Eucalyptus grandis* residue; SCS: sugarcane straw.

Figure 5

Saccharification of pretreated sugarcane straw. Glucan and xylan conversion of total biomass. The reaction was performed with a Celluclast®: glucosidase from *Aspergillus niger* (5:1 w/w) supplemented with *L. sulphureus* ATCC 52600 secretome produced by cultivation with SCB. The 100% protein load corresponds to 15 FPU/g of substrate. Reactions contained 2% (w/v) solids load in 50 mM sodium acetate pH 5.0 and were incubated for 48 h at 50 °C. Data correspond to mean values and standard deviations of triplicates. Significance was analyzed using two-way ANOVA with Tukey's test against 85+15% (95% confidence interval) and is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6

Biomass degradation mechanism from *L. sulphureus* ATCC 52600. Multi-omics analysis showing the range of CAZymes induced in response to sugarcane lignocellulose. The scheme represents the main CAZymes found in the transcriptome (in the basidium stem) and secretome analysis responsible for lignocellulose deconstruction and lignin modification by the oxidative mechanism, involving CAZymes, low molecular weight (LMW) compounds, and Fenton reaction. In parallel, monomers released from holocellulose are metabolized, unlike lignin, which remains partially degraded.

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