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Valorization of bioethanol by-products to produce unspecific peroxygenase with *Agrocybe aegerita:* Technological and proteomic perspectives

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ABSTRACT

Unspecific peroxygenase (UPO) presents a wide range of biotechnological applications. This study targets the use of by-products from bioethanol synthesis to produce UPO by *Agrocybe aegerita*. Solid-state and submerged fermentations (SSF and SmF) were evaluated, achieving the highest titers of UPO and laccase in SmF using vinasse as nutrients source. Optimized UPO production of 331 U/L was achieved in 50% (v:v) vinasse with an inoculum grown for 14 days. These conditions were scaled-up to a 4 L reactor, achieving a UPO activity of 265 U/L. Fungal proteome expression was analyzed before and after UPO activity appeared by shotgun mass spectrometry proteomics. Laccase, dye-decolorizing peroxidases (DyP), lectins and proteins involved in reactive oxygen species (ROS) production and control were detected (in addition to UPO). Interestingly, the metabolism of complex sugars and nitrogen sources had a different activity at the beginning and end of the submerged fermentation.

Introduction

The discovery of the unspecific peroxygenases family is relatively recent, being first described in the basidiomycete fungus *Agrocybe aegerita* in 2004 [1]. Although initially cataloged as haloperoxidase or aromatic peroxygenase (APO), due to the partial similarity of its amino acid sequence to heme chloroperoxidase (CPO) from the ascomycete *Caldariomyces fumago,* and due to the hydroxylation activity found on halides and aromatic compounds, a few years later this class of enzymes was renamed unspecific peroxygenases (UPOs, E.C. 1.11.2.1) [1–3]. These heme-thiolate enzymes are of particular interest because of their combination of the catalytic cycle of heme-peroxidase (one electron oxidation) with the ability to perform selective C-H oxyfunctionalization reactions commonly performed by cytochromes P450 monooxygenases [4]. However, unlike cytochrome P450 monooxygenases, which are usually integrated into the endoplasmic reticulum, UPOs are extracellular enzymes that exhibit greater stability and a simpler catalytic cycle, requiring only H_2O_2 as co-substrate, which acts as both electron acceptor and oxygen donor [5,6].

UPOs are capable of oxidizing a wide range of compounds, including alkenes, chlorinated benzenes, halogenated biphenyl ethers, nitroaromatics, polycyclic aromatic hydrocarbons (PAHs), phthalates, steroids, etc [7–9]. Their versatility to catalyse a wide variety of reactions has increased interest in studying new methods to produce these enzymes. So far, they has been secreted by several fungi, such as *Agrocybe* (*Cyclocybe*) *aegerita*, *Coprinellus* (*Coprinus*) *radians*, *Marasmius rotula*, *Chaetomiun globosum*, *Coprinus verticillatus*, *Leptoxyphium fumago*, *Marasmius wettsteinii* and *Psathyrella aberdarensis* [4]. Nevertheless, fungal fermentations usually require long incubation times to obtain the highest enzyme titers, so some studies have focused on the use of recombinant microorganisms. Heterologous expression allows reducing the period needed to obtain the enzyme, hence lowering the cost of the process, and importantly, engineering of UPOs can result in new functionalities, activities and selectivities [10]. On the other hand, heterologous expression may involve some negative aspects such as the need for more complex and expensive further processing, the formation of aggregates of the recombinant protein that can inactivate the enzyme or the lack of a post-translational glycosylation necessary for the

Abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); ANOVA, one-way analysis of variance; APO, aromatic peroxygenase; CPO, chloroperoxidase; DyP, dye-decolorizing peroxidase; DDGS, distillery dry grain soluble; PASEF-MSMS, parallel accumulation-serial fragmentation mass spectrometry; PAH, polycyclic aromatic hydrocarbons; SmF, submerged fermentation; SSF, solid-state fermentation; TIMS, trapped ion mobility spectrometry; UPO, unspecific peroxygenase.

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functionality of the enzyme when using bacteria as host [11,12]. Nevertheless, several studies have achieved high UPO concentrations by heterologous expression in yeast such as *Saccharomyces cerevisiae* or *Pichia pastoris* (using as origin microorganisms *Aspergillus niger* and *Candolleomyces aberdarensis)*, with values of 0.3 g/L, but lower than the maximum values achieved by homologous expression (0.6 g/L) [1,13,14].

Since this peroxygenase is produced during secondary metabolism, its production requires a long time, from 2 to 4 weeks of cultivation [4]. In addition, *A. aegerita* is also capable of producing other interesting enzymes such as laccase and carboxylmethyl cellulase [15]. The composition of the culture medium has a direct impact on the accomplishment of UPO production. Previous reports suggest that a high nitrogen content, which can be provided by plant-derived compounds such as soybean meal or soybean peptone, may trigger UPO expression [1,16], although this relationship has not yet been elucidated. Therefore, it seems essential to investigate the effects of new substrates with high nitrogen content. Considering that most reports on UPO production propose fermentation methods that require the addition of high-cost compounds [1,16], it is particularly relevant to consider alternative methods based on the valorisation of waste streams. In this regard, the liquid side-stream (vinasse) from the bioethanol production process is usually treated and dried to obtain distillery dry grain soluble (DDGS). This stream, despite its deficit in certain assimilable nutrients (e.g. lysine), is used as a high-protein feed supplement for livestock [17,18]. In particular, this high nitrogen concentration makes it a promising substrate for use in UPO production.

In recent years, bioethanol synthesis has gained considerable importance as a green alternative to fossil fuels. Among the advantages compared to the production of conventional fuels are the reduction of CO_2 emissions, the use of renewable raw materials and the greater stability of the product obtained [19]. However, first-generation bioethanol, which is obtained by fermentation of soluble sugars or starch, entails a series of negative impacts on the environment, such as competition with food and feed production, impacts on land use, water resources and potential contamination of soils due to the use of distillation residues as fertilizers [20]. In this context, the study of the revalorization of the byproducts derived from this process assumes more relevance to balance these negative aspects. Specifically, one-third of the total dry matter obtained in the bioethanol synthesis process is recovered as co-products, which are mainly wet cake (a.k.a. vinasse) or DDGS [21]. For this reason, the revaluation of DDGS may become more important in the coming years with the aim of increasing the economic value of this product and trying to integrate the first-generation process into the framework of the circular economy.

In addition, it is important to analyze the factors involved in UPO expression in order to identify the most suitable culture medium conditions to induce the expression of this enzyme. The physiological role of UPO in nature is still uncertain, different activities having been proposed, including metabolite synthesis, detoxification processes of lignin degradation and interaction with antimicrobial peptides released by host plants [3]. It has been suggested [14] that, in addition to external culture conditions, a kind of fungal autoregulation may influence UPO synthesis and secretion. In this direction, proteomic analysis allows exploration of the changes of proteome expression at different times of the fungal culture with the aim of relating other cellular activities to the induction of these enzymes.

In this work an alternative use of these by-products from the bioethanol industry is proposed in the formulation of the fermentation medium suitable for producing UPO by *A. aegerita* (AaeUPO), demonstrating that both solid and liquid fermentations are optimal for the fungal growth and UPO expression. In addition, the proteome of *A. aegerita* was analyzed by shotgun mass spectrometry at two different timepoints of the submerged fermentation to explore the fungal enzymatic set expressed in these experimental conditions and potential changes in the metabolic activities during this biotechnological process.

Materials and methods

Chemicals and raw material

Vinasse and DDGS were provided by Bioetanol Galicia S.A. (Teixeiro-Curtis, Spain). The vinasse was stored at − 18 °C, whereas DDGS was stored at room temperature until use. The characterization of DDGS kindly provided by Bioetanol Galicia S.A. is shown in Supplementary Table S1. H_2O_2, veratryl alcohol, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and $MgCl_2$ were purchased from Sigma-Aldrich (Barcelona, Spain), malt extract from Biolife italiana (Milano, Italy), KH_2PO_4 and EDTA from Panreac (Barcelona, Spain), yeast extract from iNtRON Biotechnology (Seongnam, South Korea), $MgSO_4{\cdot}7\ H_2O$ from Fluka (Steinheim, Germany) and sodium dodecyl sulfate (SDS) from Invitrogen (Massachusetts, EEUU).

Fungal strain and inoculum preparation

The fungus *A. aegerita*, strain DSM 22459, was obtained from the German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig. For mycelium production in static cultures, three plugs of active mycelia were transferred from Petri dishes to Fernbach flasks with 100 mL of malt extract-based medium (malt extract 7.5 g/L, yeast extract 2 g/L, KH_2PO_4 1 g/L, $MgSO_4{\cdot}7\ H_2O$ 0.5 mg/L) and maintained at 28 °C for 14–28 d. After this step, the content of the Fernbach flasks was crushed and used as inoculum for the Erlenmeyer flasks.

Fermentations

Solid-state fermentation

DDGS was used as support and substrate for the solid-state fermentation (SSF) of *A. aegerita*. The DDGS pellets were crushed using a pestle to increase the surface area of the substrate. Each 100 mL Erlenmeyer flask containing 3 g of DDGS, and 7.5 mL of distilled water was sterilized for 20 min at 121 °C (RAYPA AES-75, Barcelona, Spain) and inoculated with 750 μL of inoculum. The Erlenmeyer flasks were incubated for 42 d at 28 °C in a humidity-saturated environment under static conditions. The moisture content was replenished weekly to ensure optimal humidity conditions for fungal growth. All experiments were conducted in duplicate.

For enzyme extraction, 30 mL of distilled water (dH_2O)was added to each Erlenmeyer flask and incubated for 1 h at 175 rpm on an orbital shaker (C24 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) to promote separation of the adsorbed enzyme on the DDGS. The extracted liquid was then filtered through a cellulose filter and centrifuged for 10 min at 10,000 rpm before measuring enzyme activity and pH in the supernatant.

Submerged fermentation

Different dilutions of vinasse were evaluated as fermentation media: 25%, 50% and 75% (v:v) of vinasse. Next, the inoculum age was evaluated using fungal biomass grown in Fernbach flasks for 14, 21 and 28 d, respectively. Erlenmeyer flasks containing 112.5 mL of the final medium were autoclaved at 110 °C for 40 min and inoculated with 12.5 mL of inoculum. The flasks were incubated on an orbital shaker (Innova 4000 Incubation Shaker, New Brunswick Scientific, New Jersey, USA) at 28 °C and 120 rpm for 40 days. Fermentations were carried out in triplicate at an initial pH of 5.5 that was adjusted by addition of 1 M NaOH. Periodically, 750 μL samples were withdrawn and centrifuged for 10 min at 13,500 rpm to remove fungal biomass. The supernatant was used to monitor culture pH, H_2O_2 concentration and to analyze UPO and laccase activities.

Bioreactor operation

Enzyme production was scaled-up to a 4 L stirred tank bioreactor (Biostat MD, B. Braun Biotech, Melsungen, Germany). The fungus grown in Erlenmeyer flasks for 3 weeks was used as inoculum at 20% (v:v). The medium was prepared with a 50% dilution of vinasse in dH_2O (v:v) and adjusted to a pH of 5.5 with 1 M NaOH. The conditions of medium sterilization and inoculum percentage were selected after testing different sterilization times (40–70 min) and temperatures (110–121 °C), selecting as optimal conditions 70 min of sterilization at 121 °C. Shorter times and lower temperatures led to the contamination of the culture by yeasts, which probably came from the vinasse used for the formulation of the culture medium. In addition, a percentage of 20% of inoculum was used in order to promote faster fungal growth and avoid contamination. The bioreactor was operated for 19 d at controlled temperature (25 ± 3 °C), under mechanical agitation (100–120 rpm), air sparging (2 L/min) and pH monitoring.

Protein identification and shotgun proteomic analysis

Biomass sampled from Erlenmeyer flasks of submerged fermentation (SmF) before and after UPO activity was detected (7 and 16 d of fermentation, respectively) was lyophilized. Each extraction was performed in triplicate using aliquots of 100 mg dry biomass, that were finally pooled for mass spectrometry (MS) analysis.

To obtain the total proteome fractions, 1.5 mL of extraction buffer containing 1.5% SDS, 10 mM EDTA, 20 mM $MgCl_2$ in 50 mM Tris buffer was added to the dry biomass that was further digested at 90 °C for 20 min, cooling the samples each 5 min. Next, zirconium glass beads were added to the sample and cell disruption was performed in a bead beater for a total of 15 min (alternating 3 min beating and 1 min in ice). Samples were then centrifuged at 5000 g, 4 °C for 20 min. Supernatants were collected, proteins were precipitated with two consecutive steps of cold acetone (approximately 4 vol per sample vol) at − 20 °C and resuspended in 1 mL resuspension buffer (molecular grade water with 1 M urea and 10 mM EDTA).

Aliquots of 0.3 μg of protein were processed in solution by trypsin digestion, reduction-alkylation and finally desalted using ZipTip-μC18 material (Merck Millipore). The peptide samples were injected onto a timsTOF Pro (Bruker, Berlin, Germany) equipped with a nano-electrospray source (CaptiveSpray) and a tims-QTOF analyzer. The chromatographic analysis were performed using a nanoELUTE chromatograph (Bruker) with a ReproSil C18 column (50 × 0.075 mm, 1.9 μm, 120 Å, Bruker). The nHPLC was configured with binary mobile phases that included solvent A (0.1% formic acid in ddH_2O), and solvent B (0.1% formic acid in acetonitrile). The analysis time was 20 min, in which B/A solvent ratio was gradually increased.

For MS acquisition, a collision induced dissociation (CID) fragmentation and a nanoESI positive ionization mode was used. Parallel accumulation serial fragmentation (PASEF)-MSMS scan mode was applied for an acquisition range of 100–1700 *m/z*. MS/MS spectra were processed by PEAKS Studio (Bioinformatics Solutions) software for protein identifications using a homemade database with all protein sequences available in NCBI for *A. aegerita* and *Cyclocybe aegerita* taxonomies. Amino acid sequences from those accessions corresponding to 'Unnamed protein' were manually blasted in NCBI BLASTP against all fungal kingdom and the protein description with the highest identity was included in the column Description. The label-free module from PEAKS Studio was used for protein semiquantification. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [22] partner repository with the dataset identifier PXD041675.

Analytical protocols

UPO activity was measured by monitoring the oxidation of 20 mM veratryl alcohol into veratraldehyde at 310 nm (ε = 9.3/M cm) in presence of 10 mM H_2O_2. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of product per min. Laccase activity was determined by measuring the oxidation of 0.267 mM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to its cation form ($ABTS^{+\bullet}$) at 420 nm (ε = 36000/M cm) in McIlvaine buffer (pH 3) at room temperature, with the addition of 980 μL of ABTS and 20 μL of sample. A possible oxidation of ABTS by UPO was discarded, considering the maximum H_2O_2 concentration detected in the fermentation medium and the dilution of the sample, reaching a final H_2O_2 concentration lower than 0.01 mM, insufficient to initiate UPO catalytic cycle. Changes in absorbance over time were monitored using a UV–visible spectrophotometer (Shimadzu UV-1800, Shimadzu, Europa GmbH, Duisburg, Germany)·H_2O_2 was measured with semi-quantitative peroxide test strips (colorimetric, 0.5–25 mg/L (H_2O_2), MQuant®, Merck).

Amino acid (AA) profile was determined for a lyophilized dilution 50% (v:v) of raw vinasse, a lyophilized sample at the beginning of the SmF and a lyophilized sample obtained at the end of the SmF. For this analysis, an acid hydrolysis of the proteins was carried out for 24 h at 110 °C using HCl 6 N. After protein hydrolysis was completed, the hydrolysate was diluted with distilled water and filtered through a 0.45 μm filter (Filter Lab, Barcelona, Spain). The derivatisation of standards and samples was carried out according to [23]. The identification of AA was done through HPLC (Alliance 2695 model, Waters, Milford, MA, USA), using a scanning fluorescence detector (model 2475, Waters) according to [24]. The quantification was done using the external standard technique with AA standard (Amino Acid Standard H, Thermo, Rockford, IL, USA). The results were expressed as g per 100 g protein.

Statistical analysis

In order to determine any significant differences between the maximum UPO production values in SmF with inoculum at different inoculum ages, a one-way analysis of variance (ANOVA) was performed using the R software (version 4.0.5, R Core Team, 2021). A significance level (α) of 0.05 was considered for all the statistical analysis. Results from the statistical analysis are shown in Suppl. Fig. S1.

Results and Discussion

Solid-state fermentations

DDGS was used as the sole substrate and support for the growth of *A. aegerita*, achieving a complete colonization of the substrate in the Erlenmeyer flask after 14 d (Suppl. Fig. S2), indicating that DDGS is a suitable substrate for *A. aegerita* growth without requiring external nutrient supplementation. Other substrates used for *A. aegerita* culture under solid-state conditions included agricultural lignocellulosic wastes such as wheat straw and cotton waste [25], kiwi fruit peels and peanut shells [26] or orange peels and distillery grape stalks [27].

However, it is important to highlight that fungal growth is not always indicative of peroxygenase production by *A. aegerita*, as previously reported [28]. In DDGS medium, laccase and UPO, were produced during SSF (Fig. 1). Laccase was expressed before UPO, reaching its maximum activity on day 21 (10.2 ± 1.6 U/g DDGS). It has been previously reported that nitrogen rich materials, with high protein content, can stimulate laccase production [29]. These values agree with those reported in [30] where a maximum laccase activity of 11.31 U/g was reached in the third week of *A. aegerita* cultures. In contrast, in the present study UPO activity was first detected 14 d after the onset of laccase production once laccase activity began to decline. Maximum UPO activity titers (2.4 ± 0.2 U/g DDGS) were reached at 33 d fermentation. The pH showed a gradual increase to reach a value of 7.6 at 20 d fermentation, being maintained thereafter and finally decreasing to pH 7.2 at the end of the culture. As far as we are aware, only two previous studies evaluated UPO production under SSF [31,32]. In these

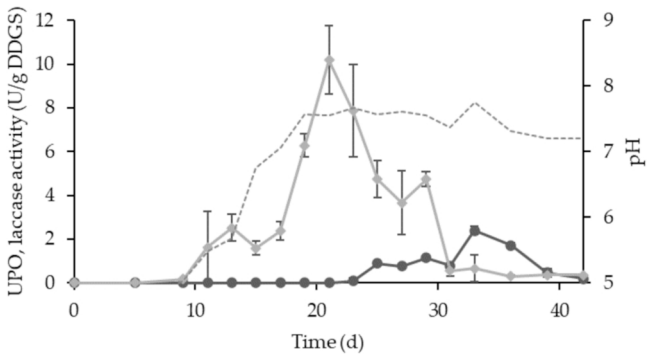

Fig. 1. Parameters measured during solid state fermentation of *A. aegerita* with distillery dry grain soluble as substrate: UPO activity (●), laccase activity (◆) and pH (- -).

reports, a maximum value of 1.2 U/g was obtained by *A. aegerita* growing on barley medium supplemented with dried olive-mill residue [31], while UPO activities below 0.4 U/g were reached using different media and/or with different strains. Thus, DDGS proved to be an efficient substrate for laccase and UPO production, with UPO production being the highest reported to date in SSF.

Submerged fermentations

Different dilutions of vinasse were evaluated in order to establish an optimal concentration to maximize UPO production under submerged conditions. In addition, the influence of inoculum age was evaluated, as it may affect the maximum enzyme activity and the time of peak production [33].

Effect of vinasse concentration

Concentrations between 25% and 75% v:v (vinasse:H_2O) were evaluated as fermentation medium. The objective was to study whether any of the compounds present in the substrate could have an inhibitory effect on fungal growth and/or enzyme production. Fig. 2 shows the evolution of enzyme production over 38 d of fermentation. UPO activity (Fig. 2a) seems to be affected by the vinasse concentration of the medium, with the highest production titers in 50% (v:v) assay (331 ± 229 U/L) on day 23. The medium with 25% (v:v) of vinasse showed the lowest UPO production (47 ± 4 U/L), which can be related to a protein deficit, since previous studies reported that high concentrations of organic nitrogen were required for UPO production by *A. aegerita* [1, 16]. In this sense, it has been observed [1] that a 2-fold increase in N source concentration (soybean meal) led to an increase of about 40% in AaeUPO production. Nevertheless, the authors also showed that UPO production was highly dependent on the strain of *A. aegerita* employed, since by evaluating 8 different strains, activities were reached ranging between 5 and 1336 U/L, employing the same fermentation medium [1].

It is noteworthy that the time required to obtain maximum production (19 d) in 25% v:v medium was shorter than in the other dilutions tested. In the case of the 75% (v:v) experiment, the maximum UPO production of 167 ± 191 U/L was reached on day 32. The large difference between the replicate values could be anticipated, given the long fermentation periods. Previous works where fermentation times exceeded 30 d have also shown significant differences between replicates [31].

With reference to laccase production (Fig. 2b), a similar trend was observed. The time required to reach maximum activity increased with vinasse concentration. The highest production was obtained in 50% (v:v) medium, reaching 11,252 ± 4501 U/L on day 21. This activity was much higher than those previously achieved in our laboratory also using

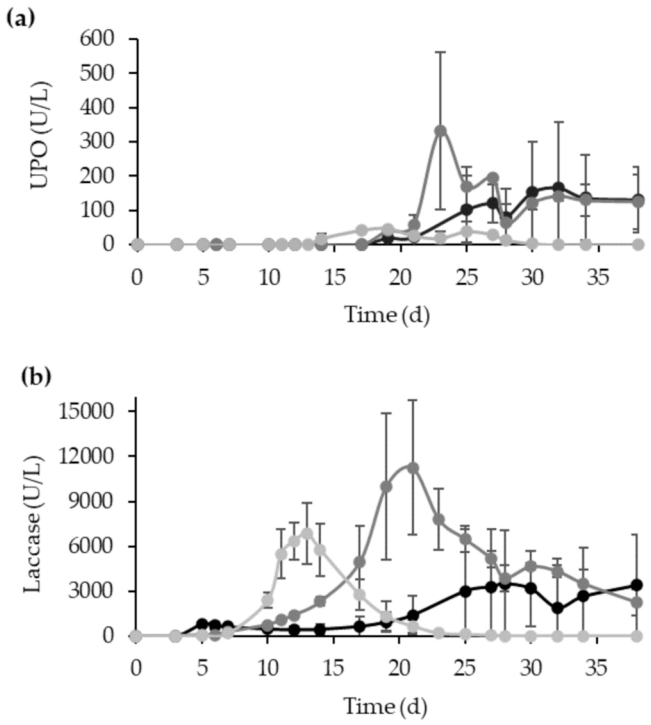

Fig. 2. Evolution of **(a)** UPO and **(b)** laccase activity in the submerged fermentation of *A. aegerita* with 25% (●), 50% (●) and 75% (●) v:v vinasse medium.

vinasse but with another basidiomycetous fungus [34] and with different substrates such as a residual stream from the organosolv process [35]. Production reached in 25% and 75% (v:v) media was also significant (6848 ± 2062 and 3499 ± 3574 U/L on days 13 and 28, respectively), but notably lower than that reached in the 50% (v:v) medium. The high laccase activity might also be a consequence of the residual ethanol remaining in the vinasse, which has been suggested to act as inducer of laccase production [36]. In this regard, it was reported [37] that ethanol only functions as an inducer of laccase when simple nitrogen sources are unavailable in the culture medium, but in the presence of complex nitrogen sources, such as those present in vinasse, ethanol can have a positive synergistic effect on laccase production. Other studies focusing on UPO production with different strains of *A. aegerita* in conventional medium have reported higher UPO expression (2021 U/L) but lower laccase production, supporting the hypothesis of laccase induction by ethanol from vinasse [1].

As for the pH trend (Suppl. Fig. S3), UPO production began at pH values close to 8, with a maximum activity around pH 8.5. This pH profile is similar to that reported in fermentations with *A. aegerita* in soybean meal-based medium [1].

Effect of inoculum age

Inoculum age can play a crucial role in fungal metabolic activity and growth [38]. In order to evaluate its potential effect on UPO expression, inocula from 2 to 4 weeks were used to cultivate *A. aegerita* under submerged conditions. Additionally, H_2O_2 concentration was monitored since its production may be related to UPO secretion. As depicted in Fig. 3 and Fig. 4, inoculum age did not appear to have a strong effect on the onset of UPO and laccase production in the vinasse based medium. As observed in SSF, laccase production in SmF (Fig. 4) started earlier in the culture (day 3), whereas UPO production (Fig. 3) started between days 11 and 16.

Although production values using inocula of different ages did not show significant differences (statistical analysis in Suppl. Fig. S1, p-

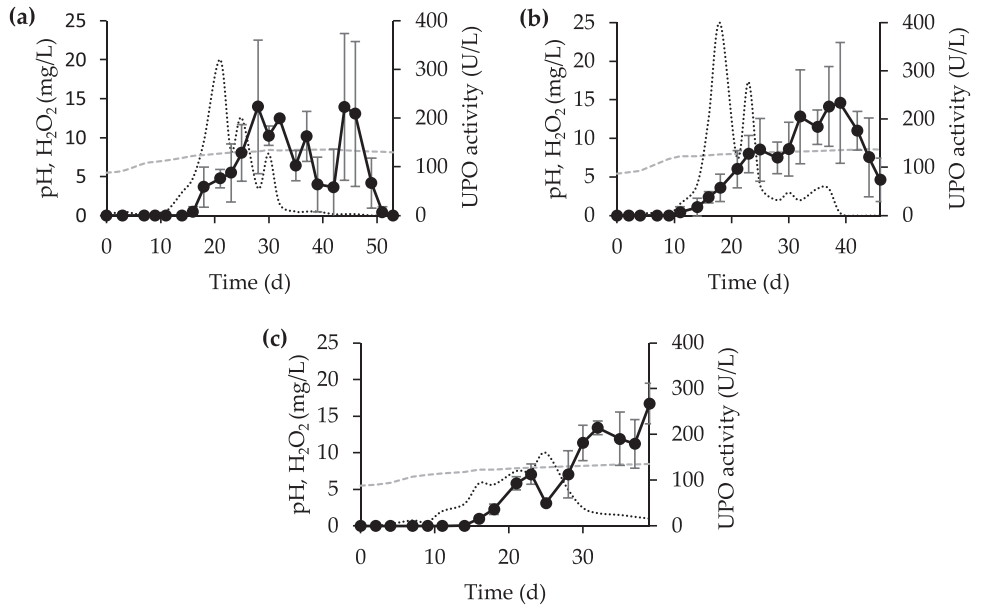

Fig. 3. Evolution of UPO activity (•), pH (–) and H_2O_2 (◦) in submerged fermentations of *A. aegerita* in vinasse-based medium 50% v:v employing inocula of different age: **(a)** 14-days, **(b)** 21-days and **(c)** 28-days.

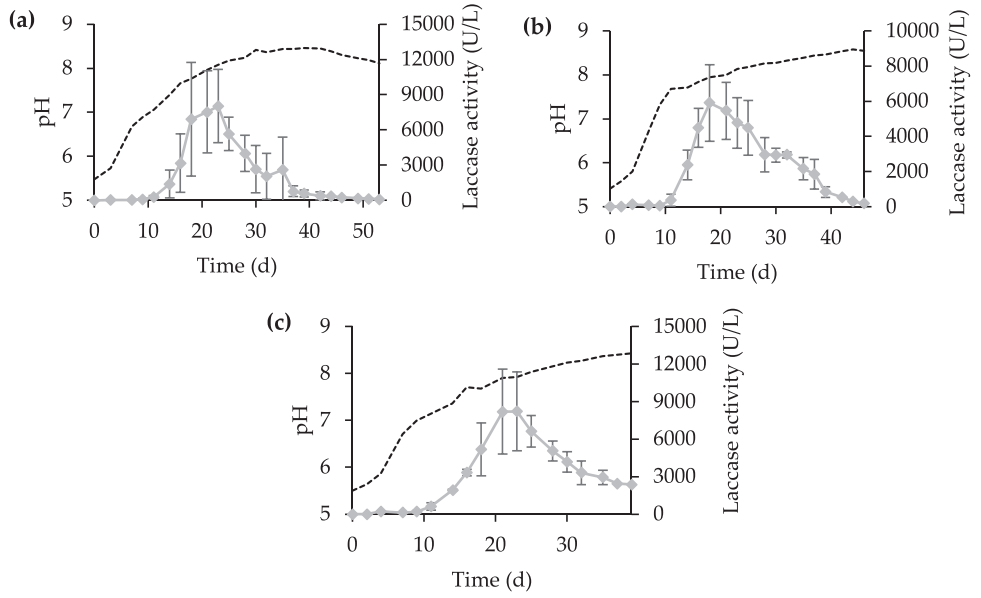

Fig. 4. Evolution of laccase activity (◆) and pH (-) in submerged fermentation of *A. aegerita* in vinasse-based medium 50% (v:v) employing inocula of different age: **(a)** 14-days, **(b)** 21-days and **(c)** 28-days.

value 0.88) the time required to reach maximum UPO production was shorter in the fermentation with 14-day-old inoculum, where a production of 223 ± 137 U/L was obtained on day 28. In terms of laccase activity, younger and older inocula led to similar titers (8030 ± 3124 and 8219 ± 3148 U/L on day 23, respectively). On the other hand, the intermediate-aged inoculum required a shorter period to obtain the highest laccase activity (18 days), but only an activity of 5899 ± 2176 U/L was achieved.

The presence of H_2O_2 was detected before the onset of UPO production (Fig. 3), increasing to a maximum before the peak of UPO activity. This trend in the H_2O_2 concentration is probably related to its consumption by UPO and/or other enzymes (e.g., catalases) in different fungal metabolic pathways, leading to a decrease in the concentration of available H_2O_2 as enzymatic production increases. In fact, the maximum levels of UPO coincided with the minimum concentration of H_2O_2, suggesting that enzyme production could be affected by the lack of H_2O_2 required for its catalytic cycle.

Inactivation of UPO due to a high H_2O_2 concentration could be ruled out because a maximum concentration of 0.74 mM (25 mg/L) was detected, which is lower than the concentration reported to completely inactivate UPO from *A. aegerita* at pH 7 [39]. On the other hand, H_2O_2 concentration could affect laccase activity, as 1 mM H_2O_2 was reported to inactivate 76% of laccase from *Pleurotus pulmonarius* [40].

Unspecific peroxygenase production in a 4L bioreactor

The SmF with the medium based on a dilution of 50% of vinasse was successfully scaled up to a total volume of 4 L. After several episodes of yeast contamination, probably from the stillage, medium sterilization was intensified, and inoculum concentration doubled to reduce the

possibility of contamination. Fermentation was monitored for 17 d. Both enzymes were expressed, obtaining maximum values of 265 U/L for UPO on day 16 of fermentation, and 9639 U/L for laccase on day 11 (Fig. 5). As observed in the Erlenmeyer flask cultures, pH increased throughout fermentation, with the greatest increase coinciding with the maximum UPO activities. The maximum UPO activity was obtained at pH 8.6, which was similar to the pH reached in the Erlenmeyer scale SmF (8.4), so that pH could be used as an optimal indicator of the enzymatic production stage.

The UPO and laccase activity values achieved in the reactor were similar to those obtained with Erlenmeyer flasks. Therefore, mechanical stress due to agitation, which affects fungal morphology causing pellet breakage did not seem to affect enzyme production [41,42]. Interestingly, these maximum titers were achieved in shorter time than at Erlenmeyer flask scale, which could be related to the higher inoculum concentration or to the more efficient aeration system enhancing mass transfer in the bioreactors [43]. UPO production from *A. aegerita* achieved in [1] was higher and required shorter fermentation periods (maximum UPO activity of 1550 U/L, about 10 mg/L, at day 10 in a 5 L bioreactor), but the laccase production was significantly lower, reaching only 290 U/L compared to 9639 U/L achieved with a vinasse-based medium.

Heterologous expression has allowed higher AaeUPO production values. For example, in [13] a maximum AaeUPO production of 300 mg/L was reached after 7 d (activity of 30,000 U/L by ABTS assay) using *Pichia pastoris* as host microorganism. This suggests that heterologous expression seems to be the most suitable option to produce AaeUPO. However, it should be noted that obtaining enzyme cocktails with a predominance of several enzyme activities, such as laccase and UPO here, can result in a synergistic effect when applying these cocktails as biocatalysts in different reactions. Furthermore, homologous expression may be useful in the study of the natural functions of UPO, which remain unclear. The elucidation of its role in nature is not merely a scientific question but is also important for biotechnological development and ecophysiological considerations of the enzyme [14].

Protein identification

To explore the enzymatic set expressed by the fungus and its cellular activities in different stages of the biotechnological process, shotgun proteomic analysis was performed at two different periods of *A. aegerita* growth in Erlenmeyer flasks: on day 7 of the culture (no UPO activity had yet been detected) and on day 16, when UPO activity was measured in the supernatant. A total of 328 proteins were identified in this study (after considering only those with a minimum of 2 peptides detected). The complete list of individual proteins identified at both timepoints is shown in Suppl. Table S2, whereas Table 1 summarises the selected

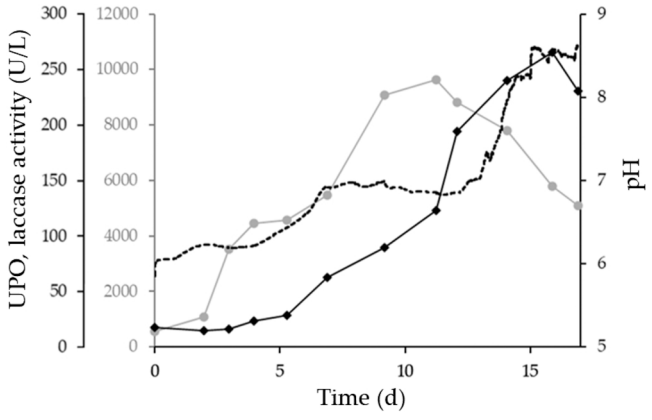

Fig. 5. Evolution of pH (-), UPO activity (◆) and laccase activity (●) during the submerged fermentation of *A. aegerita* in a 4 L bioreactor.

identified proteins. The amino acid sequences from the detected peptides were analysed by Unipept and grouped into Interpro protein categories. Those categories activated, supressed, or upregulated at day 16 compared to day 7 are shown in Suppl. Table S3, Suppl. Table S4 and Suppl. Fig. S4, respectively.

First, shotgun proteomics served to corroborate the production of unspecific peroxygenases (accession numbers: pdb|2YP1|B and CAA7262403.1) and a laccase (CAA7265189.1) by *A. aegerita*. According to the specific activities measured on site, the expression of unspecific peroxygenases was detected at day 16 whereas at day 7 no UPO activity was detected. Laccase activity was already detected on day 7 of fermentation, although the values were lower than those obtained on day 16. Interestingly, a DyP-type peroxidase (CAA7264490.1) was also found on day 6, which is known to reduce H_2O_2 to water and oxidize phenolic compounds as well as non-phenolic lignin model dimers and veratryl alcohol [44].

A catalase (CAA7262553.1), responsible for the degradation of H_2O_2 to H_2O and O_2, was detected and its expression was negatively correlated with both UPO expression and the H_2O_2 concentration in the medium. It was found to be more abundant before the UPO activity was detected (when the H_2O_2 concentration had not yet been detected) than in the second period (H_2O_2 concentration detected). This observation might suggest that UPO and catalase follow different induction patterns. Nevertheless, at the stage in which catalase was detected, the production of laccase has already started. According to others [45], catalase can be expressed in response to the presence of recalcitrant colouring compounds derived from vinasse degradation and also to the degraded products produced by ligninolytic enzymes, such as laccase.

NADP-dependent oxidoreductases were overexpressed when UPO was active. These enzymes are involved in providing reducing power for the decomposition of ROS. Furthermore, two superoxide dismutases (CAA7266156.1 and CAA7263457.1) involved in the intracellular ROS control [44], catalysing the reduction of superoxide (O^{-2}) or molecular oxygen (O_2) into intracellular H_2O_2, were also detected. However, in this case no significant change in their expression was found among the two periods studied.

Regarding Interpro protein categories, thioredoxin-like family (which contains UPO) was overexpressed at day 16, according to results above. Interestingly, other proteins involved in ROS defence were only found at day 16, when UPO activity was detected, among them alkyl hydroperoxide reductases, cupredoxins, peroxiredoxins and redoxins. Peroxiredoxins are thiol-dependent redox enzymes responsible for quenching of intracellular ROS and building up the effective cellular defence system in living organisms against oxidative stress. The expression of these enzymes is induced by stressful conditions resulting from the presence of ROS from vinasse degradation, coinciding with the observations described previously [45].

Another interesting finding was the presence of lectins at the time in which UPO production started (sp|Q6WY08.1|ATLE_AGRAE and CAA7264511.1). This type of protein is proposed as protein reserve in mushrooms and also as a defence mechanism [46]. Moreover, these proteins have been increasingly studied due to their antitumour and antimicrobial activity [47,48].

Interestingly, those enzymes involved in the transformation of complex sugars, such as β-galactosidase and glycoside hydrolase, were activated at day 16 (when UPO was active). Pyruvate kinase and ATP synthase activities were, however, supressed, indicating that glycolysis and growth may be compromised at this stage, which could be attributed to total reducing sugars depletion in the fermentation medium.

In addition, different proteins involved in the metabolism of nitrogen compounds, such as those belonging to the Interpro protein categories arginase (IPR014033) and ureohydrolase (IPR006035; IPR023696; IPR020855) were active only in the second condition (when UPO was active). This suggests that complex nitrogen compounds present in vinasse are decomposed into simpler molecules such as urea or ammonia, that can be responsible for the higher pH value of the medium

Table 1
List of selected proteins identified in the shotgun proteomic analyses of Erlenmeyer SmF of *A. aegerita* grown with vinasse and their relative abundances on the proteome before and after UPO activity was spectrophotometrically confirmed. The Spec value is based on peptide spectrum matches (PSM) and was used as indicator for the relative abundance of the proteins in each sample. NPT= number of total peptides identified in the sample and corresponding to that protein. NPU= number of unique peptides (i.e. high-confidence supporting peptides) that are mapped to only one protein group identified in the sample and corresponding to this protein.

NCBI Accession	Description	No UPO activity in the medium			UPO activity in the medium		
		Spec	NPT	NPU	Spec	NPT	NPU
CAA7262553.1	Catalase	43	21	21	25	14	14
CAA7263457.1	Superoxide dismutase	10	7	6	10	8	0
pdb|2YP1|B	Unspecific peroxygenase	0	0	0	13	8	0
CAA7265189.1	Laccase	0	0	0	11	5	0
CAA7270351.1	Thioredoxin-dependent peroxidase	0	0	0	7	5	0
CAA7269993.1	Lectin	2	2	2	5	3	0
CAA7266156.1	Superoxide dismutase	3	3	2	2	2	0
CAA7268902.1	Lectin	2	2	2	2	2	0
CAA7262403.1	Unspecific peroxygenase	0	0	0	2	2	0
CAA7264511.1	Lectin	2	2	2	0	0	0
CAA7264490.1	Dyp-type peroxidase	2	2	2	0	0	0
CAA7260134.1	Peptidase	5	4	4	7	4	0
CAA7264546.1	Peptidase	3	1	1	8	2	0
CAA7265101.1	Peptidase	2	2	2	3	2	0
CAA7266636.1	Peptidase	1	1	1	4	2	0
CAA7265216.1	Peptidase	0	0	0	2	2	0
CAA7264859.1	Oxidase	0	0	0	2	0	2
CAA7260317.1	Phosphate synthase	0	0	0	9	5	0

in the stage where UPO is produced. It is remarkable that arginine, the substrate for arginase, is the amino acid with the highest nitrogen content (32%). Results from amino acid profiling of the solid fermentation residue from SmF showed a decrease of 71.8% in arginine content at the end of the experiment (Suppl. Fig. S5).

Other enzymes related to nitrogen metabolism and overexpressed after UPO production were peptidases (CAA7260134.1, CAA7264546.1, CAA7265101.1, CAA7266636.1 and CAA7265216.1) which interact with amino acid residues for proteolysis [49]. This could be related to the reduction of amino acids other than arginine. Specifically, peptidases CAA7264546.1 and CAA7265101.1 use leucine-containing proteins as substrate, so are probably involved in the decline of leucine content (49.1%) at the end of the fermentation in submerged conditions (Suppl. Fig. S5). Compounds resulting from the proteolysis carried out by peptidases include short-chain peptides, which can act as inhibitors of the fungal growth [50]. Short-chain peptides from the metabolism of complex nitrogen compounds could induce UPO expression. In fact, it has been observed that short-chain peptides from storage proteins of soybean induced UPO production [51]. The author hypothesized that UPO production in ligninolytic fungi might play a role in the 'chemical warfare' between fungi and plants in real environmental conditions, acting as a response to antimicrobial peptides released by plants preventing a fungal attack.

Finally, the presence of peroxisomal copper amine oxidase (CAA7264859.1) indicates that the metabolism of primary or secondary amines was also activated at day 16. These enzymes can convert amino acids to keto-acids, with the subsequent release of ammonium and H_2O_2. Therefore, they could produce H_2O_2 required for UPO and other enzymes.

The overexpression of myo-inositol-1-phosphate synthase (CAA7260317.1) also supports the activation of metabolic pathways focused on the degradation of nitrogen compounds. Apart from their role in fungus growth, this enzyme is also secreted to confer resistance to abiotic stress at high concentration of H_2O_2 or NO [52], the latter being produced in the metabolism of complex nitrogen compounds.

Based on the results obtained, the metabolic pathways related to the glycolytic process that employs simpler sugars were suppressed during the second period, when UPO was active, whereas different enzymes involved in the degradation of more complex sugars were overexpressed at this stage. In addition, the metabolism of complex nitrogen sources was activated at this stage, where a variety of enzymes responsible for the metabolism of these compounds (ureohydrolases and peptidases) were detected. Previous literature reports have shown by operational approaches the relevance of nutrient concentrations in the environment to activate UPO production [1,16]. Data obtained in this proteomic analysis is in line with previous hypotheses pointing that the metabolism of complex sugars and complex nitrogen compounds could be related to the expression of UPO. Moreover, it is worth noting the presence of different enzymes related to ROS production and defence. The production of ROS in Basidiomyceta is related to different stress agents, such as mechanical damage and starvation [53], whereas the presence of enzymes related to ROS defence is part of the fungal strategy to maintain homeostasis in response to the oxidative stress during vinasse degradation [45]. However, this first biochemical evidence deserves further research efforts to reach conclusive theories. Specific time courses, allowing the collection of more proteome samples at different timepoints, may help to explain the mechanism of UPO expression.

Conclusions

Bioethanol synthesis by-products are adequate substrates for the growth of *A. aegerita* and the production of an enzymatic cocktail with UPO and laccase in solid and submerged fermentations due to their high protein content. The highest titer of UPO production was achieved in SmF, where the percentage of vinasse influenced enzyme production. The scale-up of this fermentation to a 4 L bioreactor allowed reducing the production time, reaching similar concentrations to those obtained in flasks. Proteomic analysis corroborated the presence of UPO, laccase, DyP, lectins, enzymes related to ROS production and control, as well as changes in the metabolism of complex sugars and nitrogen sources during the submerged fermentation.

Ethical approval

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

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CRediT authorship contribution statement

S.G., TA.L. and G.E. designed the experiments; S.G. performed the experiments; S.G., A.T. and T.AL. analyzed the data; S.G. and G.E. prepared the original draft; A.T., TA.L., MT.M and G.E. performed the revision and editing of the original draft. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.05.001.

References

[1] Ullrich R, Nueske J, Scheibner K, Spantzel J, Hofrichter M. Novel haloperoxidase from the agaric Basidiomycete *Agrocybe aegerita* oxidizes aryl alcohols and aldehydes. Appl Environ Microbiol 2004;70:4575–81. https://doi.org/10.1128/AEM.70.8.4575.

[2] Hofrichter M, Ullrich R. Heme-thiolate haloperoxidases: versatile biocatalysts with biotechnological and environmental significance. Appl Microbiol Biotechnol 2006;71:276–88. https://doi.org/10.1007/s00253-006-0417-3.

[3] Hofrichter M, Kellner H, Pecyna MJ, Ullrich R. Fungal unspecific peroxygenases: Heme-thiolate proteins that combine peroxidase and cytochrome P450 properties. Adv Exp Med Biol 2015;851:341–68. https://doi.org/10.1007/978-3-319-16009-2_13.

[4] Kinner A, Rosenthal K, Lütz S. Identification and expression of new unspecific peroxygenases – Recent advances, challenges and opportunities. Front Bioeng Biotechnol 2021:9. https://doi.org/10.3389/fbioe.2021.705630.

[5] Črešnar B, Petrič Š. Cytochrome P450 enzymes in the fungal kingdom. Biochim Biophys Acta - Proteins Proteom 2011;1814:29–35. https://doi.org/10.1016/j.bbapap.2010.06.020.

[6] Ullrich R, Hofrichter M. The haloperoxidase of the agaric fungus *Agrocybe aegerita* hydroxylates toluene and naphthalene. FEBS Lett 2005;579:6247–50. https://doi.org/10.1016/j.febslet.2005.10.014.

[7] Peter S, Kinne M, Ullrich R, Kayser G, Hofrichter M. Epoxidation of linear, branched and cyclic alkenes catalyzed by unspecific peroxygenase. Enzyme Microb Technol 2013;52:370–6. https://doi.org/10.1016/j.enzmictec.2013.02.013.

[8] Karich A, Ullrich R, Scheibner K, Hofrichter M. Fungal unspecific peroxygenases oxidize the majority of organic EPA priority pollutants. Front Microbiol 2017:8. https://doi.org/10.3389/fmicb.2017.01463.

[9] Ullrich R, Poraj-Kobielska M, Scholze S, Halbout C, Sandvoss M, Pecyna MJ, et al. Side chain removal from corticosteroids by unspecific peroxygenase. J Inorg Biochem 2018;183:84–93. https://doi.org/10.1016/j.jinorgbio.2018.03.011.

[10] Beltrán-Nogal A, Sánchez-Moreno I, Méndez-Sánchez D, Gómez de Santos P, Hollmann F, Alcalde M. Surfing the wave of oxyfunctionalization chemistry by engineering fungal unspecific peroxygenases. Curr Opin Struct Biol 2022;73:102342. https://doi.org/10.1016/j.sbi.2022.102342.

[11] Gifre L, Arís A, Bach À, Garcia-Fruitós E. Trends in recombinant protein use in animal production. Micro Cell Fact 2017;16:1–17. https://doi.org/10.1186/s12934-017-0654-4.

[12] Ferreira RDG, Azzoni AR, Freitas S. Techno-economic analysis of the industrial production of a low-cost enzyme using *E. coli*: The case of recombinant β-glucosidase. Biotechnol Biofuels 2018;11:1–13. https://doi.org/10.1186/s13068-018-1077-0.

[13] Tonin F, Tieves F, Willot S, Van Troost A, Van Oosten R, Breestraat S, et al. Pilot-Scale Production of Peroxygenase from *Agrocybe aegerita*. Org Process Res Dev 2021;25:1414–8. https://doi.org/10.1021/acs.oprd.1c00116.

[14] Bormann S, Kellner H, Hermes J, Herzog R, Ullrich R, Liers C, et al. Broadening the biocatalytic toolbox—screening and expression of new unspecific peroxygenases. Antioxidants 2022:11. https://doi.org/10.3390/antiox11020223.

[15] Isikhuemhen OS, Mikiashvili NA, Kelkar V. Application of solid waste from anaerobic digestion of poultry litter in *Agrocybe aegerita* cultivation: mushroom production, lignocellulolytic enzymes activity and substrate utilization. Biodegradation 2009;20:351–61. https://doi.org/10.1007/s10532-008-9226-y.

[16] Gröbe G, Ullrich R, Pecyna MJ, Kapturska D, Friedrich S, Hofrichter M, et al. High-yield production of aromatic peroxygenase by the agaric fungus *Marasmius rotula*. AMB Express 2011;1:1–11. https://doi.org/10.1186/2191-0855-1-31.

[17] Ho DP, Ngo HH, Guo W. A mini review on renewable sources for biofuel. Bioresour Technol 2014;169:742–9. https://doi.org/10.1016/j.biortech.2014.07.022.

[18] Youssef AW, El-Magid SSA, El-Gawad AHA, El-Daly EF, Ali HM. Effect of inclusion of distillers dried grains with solubles (DDGs) on the productive performance of growing rabbits. Am J Agric Environ Sci 2012;12:321–6.

[19] Pulyaeva VN, Kharitonova NA, Kharitonova EN. Advantages and disadvantages of the production and using of liquid biofuels. IOP Conf Ser Mater Sci Eng 2020:976. https://doi.org/10.1088/1757-899X/976/1/012031.

[20] Bertrand E, Vandenberghe LPS, Soccol CR, Sigoillot JC, Faulds C. First generation bioethanol. In: Soccol C, Brar S, Faulds C, Ramos L, editors. Green Fuels Technology. Green Energy and Technology. Cham: Springer; 2016. https://doi.org/10.1007/978-3-319-30205-8_8.

[21] Kononoff PJ. Nutritional science and practical challenges of feeding DDGS. Proc Penn State Dairy Cattle Nutr Penn State Dairy Cattle Nutr 2010:85–91.

[22] Perez-Riverol Y, Bai J, Bandla C, Hewapathirana S, García-Seisdedos D, Kamatchinathan S, Kundu D, Prakash A, Frericks-Zipper A, Eisenacher M, Walzer M, Wang S, Brazma A, Vizcaíno JA. The PRIDE database resources in 2022: A Hub for mass spectrometry-based proteomics evidences. Nucleic Acids Res 2022;50(D1):D543–52. PubMed ID: 34723319.

[23] Gálvez F, Maggiolino A, Domínguez R, Pateiro M, Gil S, De Palo P, et al. Nutritional and meat quality characteristics of seven primal cuts from 9-month-old female veal calves: a preliminary study. J Sci Food Agric 2019;99:2947–56. https://doi.org/10.1002/jsfa.9508.

[24] Munekata PES, Pateiro M, Domínguez R, Zhou J, Barba FJ, Lorenzo JM. Nutritional characterization of sea bass processing by-products. Biomolecules 2020;10:1–18. https://doi.org/10.3390/biom10020232.

[25] Philippoussis A, Zervakis G, Diamantopoulou P. Bioconversion of agricultural lignocellulosic wastes through the cultivation of the edible mushrooms *Agrocybe aegerita*, *Volvariella volvacea* and *Pleurotus* spp. World J Microbiol Biotechnol 2001;17:191–200. https://doi.org/10.1023/A:1016685530312.

[26] Vipotnik Z, Michelin M, Tavares T. Ligninolytic enzymes production during polycyclic aromatic hydrocarbons degradation: effect of soil pH, soil amendments and fungal co-cultivation. Biodegradation 2021;32:193–215. https://doi.org/10.1007/s10532-021-09933-2.

[27] Nicolini L, Volpe C, Pezzotti A, Carilli A. Changes in in-vitro digestibility of orange peels and distillery grape stalks after solid-state fermentation by higher fungi. Bioresour Technol 1993;45:17–20. https://doi.org/10.1016/0960-8524(93)90137-Z.

[28] Pecyna MJ, Ullrich R, Bittner B, Clemens A, Scheibner K, Schubert R, et al. Molecular characterization of aromatic peroxygenase from *Agrocybe aegerita*. Appl Microbiol Biotechnol 2009;84:885–97. https://doi.org/10.1007/s00253-009-2000-1.

[29] Mishra A, Kumar S. Cyanobacterial biomass as N-supplement to agro-waste for hyper-production of laccase from *Pleurotus ostreatus* in solid state fermentation. Process Biochem 2007;42:681–5. https://doi.org/10.1016/j.procbio.2006.09.022.

[30] Vipotnik Z, Michelin M, Tavares T. Rehabilitation of a historically contaminated soil by different laccases and laccase-mediator system. J Soils Sediment 2022;22:1546–54. https://doi.org/10.1007/s11368-021-03125-4.

[31] Reina R, Liers C, García-Romera I, Aranda E. Enzymatic mechanisms and detoxification of dry olive-mill residue by *Cyclocybe aegerita*, *Mycetinis alliaceus* and *Chondrostereum purpureum*. Int Biodeterior Biodegrad 2017;117:89. https://doi.org/10.1016/j.ibiod.2016.11.029.

[32] Liers C, Arnstadt T, Ullrich R, Hofrichter M. Patterns of lignin degradation and oxidative enzyme secretion by different wood- and litter-colonizing basidiomycetes and ascomycetes grown on beech-wood. FEMS Microbiol Ecol 2011;78:91–102. https://doi.org/10.1111/j.1574-6941.2011.01144.x.

[33] Rola B, Karaśkiewicz M, Majdecka D, Mazur I, Bilewicz R, Rogalski J, et al. Scale up of *Cerrena unicolor* laccase production. J Fac Agric Kyushu Univ 2013;58:231–8. https://doi.org/10.5109/27352.

[34] Pena R, Lú-Chau TA, Lema JM. Use of white-rot fungi for valorization of stillage from bioethanol production. Waste Biomass Valoriz 2012;3:295–303. https://doi.org/10.1007/s12649-012-9130-x.

[35] García-Torreiro M, Martínez-Patiño JC, Gullón B, Lú-Chau TA, Moreira MT, Lema JM, et al. Simultaneous valorization and detoxification of the hemicellulose rich liquor from the organosolv fractionation. Int Biodeterior Biodegrad 2018;126:112–8. https://doi.org/10.1016/j.ibiod.2017.10.010.

[36] Manavalan T, Manavalan A, Thangavelu KP, Heese K. Characterization of optimized production, purification and application of laccase from *Ganoderma lucidum*. Biochem Eng J 2013;70:106–14. https://doi.org/10.1016/j.bej.2012.10.007.

[37] Hernández CA, Sandoval N, Mallerman J, García-Pérez JA, Farnet AM, Perraud-Gaime I, et al. Ethanol induction of laccase depends on nitrogen conditions of *Pycnoporus sanguineus*. Electron J Biotechnol 2015;18:327–32. https://doi.org/10.1016/j.ejbt.2015.05.008.

[38] Shah IJ, Gami PN, Shukla RM, Acharya DK. Optimization for α -amylase production by *Aspergillus oryzae* using submerged fermentation technology. Basic Res J Microbiol 2014;1:1–10.

[39] Karich A, Scheibner K, Ullrich R, Hofrichter M. Exploring the catalase activity of unspecific peroxygenases and the mechanism of peroxide-dependent heme destruction. J Mol Catal B Enzym 2016;134:238–46. https://doi.org/10.1016/j.molcatb.2016.10.014.

[40] Marques De Souza CG, Peralta RM. Purification and characterization of the main laccase produced by the white-rot fungus *Pleurotus pulmonarius* on wheat bran solid state medium. J Basic Microbiol 2003;43:278–86. https://doi.org/10.1002/jobm.200390031.

[41] Jafari AR, Sarrafzadeh MH, Alemzadeh I, Vosoughi M. Effect of stitter speed and aeration rate on the production of glucose oxidase by *Aspergillus niger*. J Biol Sci 2007;7:270–5. https://doi.org/10.3923/jbs.2007.270.275.

[42] Lopez-Ramirez N, Volke-Sepulveda T, Gaime-Perraud I, Saucedo-Castañeda G, Favela-Torres E. Effect of stirring on growth and cellulolytic enzymes production by *Trichoderma harzianum* in a novel bench-scale solid-state fermentation bioreactor. Bioresour Technol 2018;265:291–8. https://doi.org/10.1016/j.biortech.2018.06.015.

[43] Lú-Chau TA, Martínez-Patiño JC, Gullón B, García-Torreiro M, Moreira MT, Lema JM, et al. Scale-up and economic analysis of the production of ligninolytic enzymes from a side-stream of the organosolv process. J Chem Technol Biotechnol 2018;93:3125–34. https://doi.org/10.1002/jctb.5664.

[44] Mattila H, Österman-Udd J, Mali T, Lundell T. Basidiomycota fungi and ROS: Genomic perspective on key enzymes involved in generation and mitigation of reactive oxygen species. Front Fungal Biol 2022:3. https://doi.org/10.3389/ffunb.2022.837605.

[45] Romanholo Ferreira LF, Torres NH, de Armas RD, Fernandes CD, Vilar D, da S, et al. Fungal lignin-modifying enzymes induced by vinasse mycodegradation and its relationship with oxidative stress. Biocatal Agric Biotechnol 2020:27. https://doi.org/10.1016/j.bcab.2020.101691.

[46] Sun H, Chen GZ, Tong X, Yi PQ. A lectin with mycelia differentiation and antiphytovirus activities from the edible mushroom *Agrocybe aegerita*. J Biochem Mol Biol 2003;36:214–22. https://doi.org/10.5483/bmbrep.2003.36.2.214.

[47] Yang N, Li DF, Feng L, Xiang Y, Liu W, Sun H, et al. Structural basis for the tumor cell apoptosis-inducing activity of an antitumor lectin from the edible mushroom *Agrocybe aegerita*. J Mol Biol 2009;387:694–705. https://doi.org/10.1016/j.jmb.2009.02.002.

[48] Heim C, Hertzberg H, Butschi A, Bleuler-Martinez S, Aebi M, Deplazes P, et al. Inhibition of *Haemonchus contortus* larval development by fungal lectins. Parasites Vectors 2015;8:1–10. https://doi.org/10.1186/s13071-015-1032-x.

[49] Neto YAAH, Garzon NG da R, Pedezzi R, Cabral H. Specificity of peptidases secreted by filamentous fungi. Bioengineered 2018;9:30–7. https://doi.org/10.1080/21655979.2017.1373531.

[50] Mahindra A, Bagra N, Wangoo N, Khan SI, Jacob MR, Jain R. Discovery of short peptides exhibiting high potency against *Cryptococcus neoformans*. ACS Med Chem Lett 2014;5:315–20. https://doi.org/10.1021/ml500011v.

[51] Pecyna M.J. Molekularbiologische Charakterisierung von Häm-Thiolat- und DyP-type-Peroxidasen ausgewählter Basidiomyceten. Technische Universität Dresden, 2015. ⟨https://nbn-resolving.org/urn:nbn:de:bsz:14-qucosa-209428⟩.

[52] Tan J, Wang C, Xiang B, Han R, Guo Z. Hydrogen peroxide and nitric oxide mediated cold- and dehydration-induced myo-inositol phosphate synthase that confers multiple resistances to abiotic stresses. Plant, Cell Environ 2013;36:288–99. https://doi.org/10.1111/j.1365-3040.2012.02573.x.

[53] Gessler NN, Aver'yanov AA, Belozerskaya TA. Reactive oxygen species in regulation of fungal development. Biochemistry 2007;72:1091–109. https://doi.org/10.1134/S0006297907100070.