Regulation of dye peroxidase gene expression in *Pleurotus ostreatus* grown on glycerol as the carbon source (#92673)

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Regulation of dye peroxidase gene expression in *Pleurotus ostreatus* grown on glycerol as the carbon source

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Dye peroxidases (DyePs) (E.C. 1.11.1.19) are heme peroxidases that catalyze oxygen transfer reactions similar to oxygenases. DyePs utilize hydrogen peroxide (H_2O_2) both as an electron acceptor co-substrate and as electron donor when oxidized to its respective radical. The production of DyePs as well as lignin-modifying enzymes (LME) are regulated by the carbon source, while less readily metabolizable carbon sources improve LME production. In this research, we analyzed the effect of glycerol on *Pleurotus ostreatus* growth, total DyePs activity and the expression of three *Pleos-DyeP* genes (*Pleos*-DyeP1, *Pleos*-DyeP2 and *Pleos*-DyeP4) through real time RT-qPCR, monitoring the time-course of *P. ostreatus* cultures supplemented either with glycerol or glucose and Acetyl Yellow G (AYG) dye. Our results indicate that glycerol negatively affects *P. ostreatus* growth resulting in a biomass production of 5.31 and 5.62 g/L with growth rates (µ) of 0.027 h^{-1} and 0.023 h^{-1} for fermentations in the absence and presence of AYG dye respectively. In contrast, 7.09 g/L and 7.20 g/L of biomass, with µ of 0.033 h^{-1} and 0.047 h^{-1} were observed in the equivalent control fermentations with glucose. Nevertheless, higher DyeP activity levels were obtained on glycerol, as 4043 IU/L and 4902 IU/L, equivalent to a 2.6-fold and 3.16-fold higher than the activity observed when glucose is used as carbon source. We explored the differential regulation of the DyeP genes in *P. ostreatus* by the carbon source, the growth phase, as well as the influence of the dye . Throughout the fermentation, we observed both up-and down- regulation of the three DyeP genes evaluated. In the control media, the highest induction was for the *Pleos*-DyeP1 gene, equivalent to a 11.1-fold increase in the relative expression ($\log_2$) at the stationary phase of the culture (360 h). In addition, glycerol preferentially induced *Pleos*-DyeP1 and *Pleos*-DyeP2 genes, leading to a 11.61 and 4.28-fold increase after 144 h respectively. On the other hand, an increase in the induction level of *Pleos*-DyeP1 and *Pleos*-DyeP2 of 12.86 and 4.02-fold after 360 h and 504 h of culture respectively were observed in the presence of AYG. To our knowledge, this is the first report describing the effect of a less metabolizable carbon source such as glycerol on the differential expression of DyeP encoding genes and the corresponding activity. These suggest that the observed glycerol effect on DyeP activity results from the absence of carbon catabolite repression, probably present in glucose cultures.

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Abstract

Dye peroxidases (DyePs) (E.C. 1.11.1.19) are heme peroxidases that catalyze oxygen transfer reactions similar to oxygenases. DyePs utilize hydrogen peroxide (H_2O_2) both as an electron acceptor co-substrate and as electron donor when oxidized to its respective radical. The production of DyePs as well as lignin-modifying enzymes (LME) are regulated by the carbon source, while less readily metabolizable carbon sources improve LME production.

In this research, we analyzed the effect of glycerol on *Pleurotus ostreatus* growth, total DyePs activity and the expression of three *Pleos-DyeP* genes (*Pleos*-DyeP1, *Pleos*-DyeP2 and *Pleos*-DyeP4) through real time RT-qPCR, monitoring the time-course of *P. ostreatus* cultures supplemented either with glycerol or glucose and Acetyl Yellow G (AYG) dye.

Our results indicate that glycerol negatively affects *P. ostreatus* growth resulting in a biomass production of 5.31 and 5.62 g/L with growth rates (μ) of 0.027 h^{-1} and 0.023 h^{-1} for fermentations in the absence and presence of AYG dye respectively. In contrast, 7.09 g/L and 7.20 g/L of biomass, with μ of 0.033 h^{-1} and 0.047 h^{-1} were observed in the equivalent control fermentations with glucose. Nevertheless, higher DyeP activity levels were obtained on glycerol, as 4043 IU/L and 4902 IU/L, equivalent to a 2.6-fold and 3.16-fold higher than the activity observed when glucose is used as carbon source.

We explored the differential regulation of the DyeP genes in *P. ostreatu*s by the carbon source, the growth phase, as well as the influence of the dye. Throughout the fermentation, we observed both up-and down- regulation of the three DyeP genes evaluated. In the control media, the highest induction was for the *Pleos*-DyeP1 gene, equivalent to a 11.1-fold increase in the relative expression ($\log_2$) at the stationary phase of the culture (360 h). In addition, glycerol preferentially induced *Pleos*-DyeP1 and *Pleos*-DyeP2 genes, leading to a 11.61 and 4.28-fold increase after 144 h respectively. On the other hand, an increase in the induction level of *Pleos*-DyeP1 and *Pleos*-DyeP2 of 12.86 and 4.02-fold after 360 h and 504 h of culture respectively were observed in the presence of AYG.

To our knowledge, this is the first report describing the effect of a less metabolizable carbon source such as glycerol on the differential expression of DyeP encoding genes and the corresponding activity. These suggest that the observed glycerol effect on DyeP activity results from the absence of carbon catabolite repression, probably present in glucose cultures.

Introduction

The ligninolytic enzymes of white rot fungi are mainly produced during secondary metabolism. Activation of this metabolism occurs under limiting conditions, such as concentration and origin of bioavailable carbon and/or nitrogen sources (*Alfaro et al., 2020; Aro et al., 2005; Elisashvili et al., 2002; Faison & Kirk, 1985; Mikiashvili et al., 2006; Stajić et al., 2006*). It has been reported that the ligninolytic isoenzymes encoded by members of a gene family, often exhibit variations in their differential expression, catalytic properties, regulation mechanisms, and cellular location (*Fernández-Fueyo et al., 2014; Garrido-Bazán et al., 2016; Janusz et al., 2013; Knop et al., 2015*). Furthermore, analyses of the promoters of ligninolytic enzymes encoding genes in the *P. ostreatus* genome have revealed the presence of different putative responsive elements (*Janusz et al., 2013; Knop et al., 2015; Piscitelli et al., 2011*). These elements include carbon catabolite repressor binding elements (CRE), nitrogen response (Nit2), xenobiotic-response elements (XRE), metal-response elements (MRE), and heat-shock elements (HSE), among other elements, which may be involved in the regulation of gene expression in response to environmental conditions (*Jiao et al., 2018; Todd et al., 2014*). Carbon catabolite repression (CCR) in combination with different signaling pathways play a crucial role in the utilization of different carbon sources in *P. ostreatus* and other Basidiomycota fungi (*Daly et al., 2019; Suzuki et al., 2008; Toyokawa et al., 2016; Yoav et al., 2018*). The existence of an ortholog of Cre1, the main transcriptional regulator in the CCR pathway, has also been demonstrated and may participate in this regulatory process (*Alfaro et al., 2020; Pareek et al., 2022; Yoav et al., 2018*). Furthermore, it has been shown that Cre1 is regulated by cAMP-dependent protein kinase A(PKA) (*Boominathan & Reddy, 1992; de Assis et al., 2020; Pareek et al., 2022*), and both Cre1 and PKA may be involved in the induction of genes encoding lignin-modifying enzymes in *P, ostreatus* (*Toyokawa et al., 2016*).

As part of their wood degradation system *P. ostreatus* produces dye-decolorizing peroxidases (DyePs; EC 1.11.1.19). These enzymes are heme peroxidases, and their name reflects their ability to degrade several anthraquinone dyes. They utilize the heme group as redox cofactor to catalyze the hydrogen peroxide-mediated oxidation of a wide range of molecules, including dyes, aromatic and lignin models compounds, some of which are poorly metabolized by other heme peroxidases (*Catucci et al., 2020; Singh & Eltis, 2015; Xu et al., 2021*). Within the *P. ostreatus* genome, four DyeP genes coding for dye-decolorizing peroxidase activity have been identified: *Pleos*-DyeP1, *Pleos*-Dyep2, *Pleos*-PyeP3 and *Pleos*-DyeP4 (*Ruiz-Dueñas et al., 2011*). Up to date, limited reports on factors regulating DyeP production exist. In a previous study we explored the effect of dyes on the differential expression of *P. ostreatus* DyeP encoding genes and DyeP activity, showing that dyes had an induction effect on DyeP activity (*Cuamatzi-Flores et al., 2019*). Additionally, an extracellular proteome analysis during *P. ostreatus* growth on lignocellulosic material revealed the exclusive synthesis of *Pleos*-DyeP4 together with several versatile peroxidases (VPs) and manganese peroxidases (MnPs) enzymes (*Fernández-Fueyo et al., 2015*). Glycerol can be used as a carbon and energy source for several basidiomycetes including *P. ostreatus*. Furthermore, the activity of some LMEs increases when glycerol or other less metabolizable carbon sources are used instead of glucose which could imply that the glycerol mediates carbon catabolite de-repression of LMEs. Giving the physiological relevance of DyePs enzymes in several group of organisms and their potential biotechnological applications, this research aims to investigate the impact of glycerol as a carbon source in the production and differential regulation of DyePs in *P. ostreatus*.

Materials & Methods

Microorganism

P ostreatus from the American Type Culture Collection (ATCC 32783) (Manassas, Virginia, U.S.A.) was used in this research. The white rot fungus strain was grown and maintained in potato dextrose agar (PDA).

Dye Decolorization on Agar Plate

Petri dishes containing glucose or glycerol as carbon source, 500 ppm of Acetyl yellow G (AYG) (dye content 95%) (SIGMA-ALDRICH 250309) and agar 15g/L were inoculated with 0.4 cm^2 mycelia plugs taken from the periphery of a *P. ostreatus* colony growing on PDA at 25 °C and incubated during seven days. The inoculum was placed mycelium facing down, on the center of the plate. Then, the plates were incubated at 25 °C for eight days. The fungal colony growth and the effect on the dye were documented by daily photographs throughout the period of incubation.

Submerged culture conditions and growth kinetics characterization

The composition of the medium, selection of Acetyl yellow G dye and the conditions for the submerged cultures were adapted from *Cuamatzi-Flores et al.,(2019)*. In this study we conducted four type of *P. ostreatus* cultures: with either glucose (GM) or glycerol (GlyM), as carbon source, as well as with the complementary addition of 500 ppm of Acetyl yellow G (GAYG and GlyAYG) (dye content 95%) (SIGMA-ALDRICH 250309). Each flask out of three per fermentation type was inoculated with three mycelial plugs (4 mm diameter) taken a steel punch from the periphery of *P. ostreatus* colonies grown for 7 d at 25°C in Petri dishes containing potato dextrose agar. The cultures were incubated at 25°C for 23 days on a rotary shaker (SEV-PRENDO 650M) with constant shaking of 120 rpm. Three flasks were taken as samples every 24 h from 120 h (5 d) to 552 h (23 d) of fermentation. The supernatant was obtained by filtering the cultures using Whatman No. 4 filter paper and stored at -20°C. Glucose was determined by the DNS Method (*Miller, 1959*), while glycerol consumption was assessed as described by *Kuhn et al., (2015)*. Dye peroxidase activity was measured by following the degradation of ABTS (*Salvachúa et al., 2013*), while the percentage of dye decolorization was determined at fixed time intervals as proposed by *Upadhyay & Przystas, (2023)*. The mycelium was rinsed with 0.9% NaCl and stored at -70°C until subjected to the total RNA extraction or dry weight measurement (X, g/L). The specific growth rate (μ) was obtained for each replicate from the logistic equation ($X = X_{max}/(1 + (X_{max} - X_0/X_0)\cdot e^{-m\cdot t})$) using 100 permutations in R software, version 4.3.0 (*R Development Core Team, 2023*).

The decolorization of AYG dye was monitored spectrophotometrically at λ_{max} (390 nm). All experiments were performed in triplicate. The growth curves were established with dry biomass measurements from each fermentation.

RNA extraction and RT-qPCR

The total RNA was isolated from frozen mycelia harvested at different time-points of the fermentation, using the NTES extraction protocol. The concentration was quantified spectrophotometrically, and the purity was determined by the absorbance ratio at OD 260/280. The RNA was treated with RNAse-free DNase I (Invitrogen). The final RNA concentration was set to 300 ng/μl, after which 3 μg of total RNA was reverse-transcribed into cDNA in a volume of 20 μl using M-MuLV Reverse Transcriptase (Fermentas), following the manufacturer's protocol.

The RT-qPCR reactions were performed in a StepOne Plus thermal cycler (Applied Biosystems), using Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher) to detect the amplification of product. Specific primers were designed to amplify the transcripts of the thre *Pleos*-DyeP genes identified in the genome (Table 1). The reaction mixture, the amplification program, the melting curve, and the selection of the reference genes were adapted from *Garrido-Bazán et al., (2016)*. According to their expression stability under the studied culture conditions and the reference index consisting of the geometric mean of the best-performing housekeeping genes, peptidase (pep) gene was used for RT-qPCR data normalization.

The RT-qPCR reactions were carried out in triplicates with a template-free negative control performed in parallel.

Results

Effect of carbon source on *P. ostreatus* growth and AYG Dye decolorization in Plate Assays

Figure 1 shows the growth and decolorization capacity of *P. ostreatus* on agar plates of glucose and glycerol supplemented with 500 ppm of AYG dye. Although *P. ostreatus* was able to grow in both glucose and glycerol media, a higher growth rate is clearly observed on glucose, either alone or supplemented with AYG dye. This differential growth pattern led to the development of larger fungal colony over the 192 h of incubation. Furthermore, different changes in the color of the AYG dye were observed during the experiment. In media containing glucose, the AYG dye transitioned from yellow to reddish hues over time, whereas such color alterations were not evident in the glycerol-supplemented medium.

These findings suggest a significant influence of the carbon source on both, growth kinetics of *P. ostreatus* and AYG dye oxidation, highlighting the role of carbon substrate in shaping fungal metabolism.

Effect of glycerol on *Pleurotus ostreatus* growth, dye peroxidase activity and Acetyl Yellow G dye decolorization in submerged fermentation

Growth kinetics characterization and AYG decolorization

To quantitatively analyze the changes observed on plate assays, submerged cultures were conducted. The comparison of *P. ostreatus* growth in submerged fermentation with glucose or glycerol as carbon source alone or supplemented with AYG dye is shown in Figure 2.

Variation on the maximal biomass (Xmax) reached were both higher in glucose and glucose/AYG cultures (7.09 g/L and 7.20 g/L, respectively) compared to glycerol or glycerol/AYG (5.31g/L and 5.62 g/L, respectively). The same differences were observed in growth rate (μ), with values for μ of 0.033 h^{-1} and 0.047 h^{-1} were obtained for glucose and glucose/AYG -media higher than 0.027 h^{-1} and 0.023 h^{-1} observed for glycerol and glycerol/AYG media, respectively. Interestingly, the addition of the AYG dye does not significantly affect biomass production (Xmax), as no substantial differences were observed when compared both cultures with and without the dye.

Consequently, carbon source depletion is faster in the presence of AYG dye when glucose is used as the carbon source (Figure 3). In effect, glucose depletion was observed after 336 h of culture, compared to 240 h in the fermentation with the AYG dye, as expected considering the already reported higher growth rate. However, although the specific growth rates were rather similar with glycerol as carbon source, depletion occurred at 400 h, as opposed to 312 h in the fermentation supplemented with the AYG dye. These findings suggest that *P. ostreatus* can metabolize the carbon source more efficiently in the presence of the dye, leading to accelerated carbon source depletion.

Furthermore, the decolorization percentage during glucose fermentations increased gradually over the fermentation-course, reaching percentages of 100 % after 552 h, contrary to the glycerol fermentation where 10.8 % decolorization was observed just after 48 h and a maximum of 22 % discoloration after 552 h (Figure 4). Markedly, the carbon source had a discernible impact on the rate of dye decolorization.

Effect of glycerol and AYG dye on DyeP activity production

The effect of glycerol and AYG dye on dye peroxidases production by *P. ostreatus* is shown in Figure 5. The highest titers of dye peroxidase activity (4043 and 4903 UI/L) were observed when glycerol and glycerol with AYG were employed as carbon source, reaching maximum levels at 408 h and 360 h, respectively. On the other hand, lower activity levels were obtained in glucose and glucose with AYG cultures (1551 and 2882 UI/L) at 312 and 288 h, respectively. It is noteworthy that independently of the carbon source, the addition of AYG dye consistently induced the production of DyeP, as concluded from the higher activity levels observed early in the fermentation.

De-repression of *Pleos*-DyeP genes expression and differential regulation in response to glycerol and AYG dye

The transcriptional response of *Pleos*-DyeP genes to glycerol as carbon source and to the addition of a synthetic dye (AYG) was also evaluated. Figure 6 and supplementary table 2, show the influence of the carbon source and the AYG dye on the expression patters of DyeP genes, revealing a dynamic up-/down-regulation pattern over the course of fermentation for the three evaluated DyeP genes (*Pleos*-DyeP1, *Pleos*-DyeP2 and *Pleos*-DyeP4).

The highest induction levels in the control media were for the *Pleos*-DyeP1 and *Pleos*-DyeP4 genes, with 11.12 and 8.28-fold increase in the relative expression level ($\log_2$) after 360 h and 168 h respectively. Additionally, gene expression profiles indicated that glycerol induced *Pleos*-DyeP1 and *Pleos*-DyeP2 genes, with a 11.61- and 4.28-fold increase observed after 144 h, respectively. On the other hand, AYG addition results in a 12.86 and 4.02-fold increase induction levels after 360 h and 504 h of culture for *Pleos*-DyeP1 and *Pleos*-DyeP2, respectively. Interestingly, under these experimental conditions, expression of *Pleos*-DyeP4 was not detected. These findings underscore the intricate dynamics of gene expression in response to different carbon sources and the presence of AYG dye, shedding light on the regulatory mechanisms governing dye peroxidase production in *P. ostreatus*.

Discussion

To test the influence of glycerol on growth, dye peroxidase expression/production, and the effectiveness on AYG dye decolorization compared to glucose, we cultured *P. ostreatus* both on plate and in liquid cultures.

Effect of carbon source on growth

In this research, we first evaluated the effect of glycerol as a carbon source on *P. ostreatus* growth, both on plate assays and submerged fermentation. Glycerol exhibited lower efficiency as a carbon source compared to glucose, significantly affecting both growth rate and biomass production. Glycerol can be used as a carbon and energy source for several groups of fungi; however, its utilization efficiency varies among fungi and, compared to glucose, often glycerol metabolism results less efficient (*Klein et al., 2017; Liu et al., 2012; Mikiashvili et al., 2006; Urek & Pazarlioglu, 2007*). Our findings confirm previous research demonstrating that *P. ostreatus* growth is affected by a complex carbon source such as glycerol resulting in reduced biomass production and altered growth rates (*Mikiashvili et al., 2006; Tinoco et al., 2011*).

Effect of glycerol and AYG dye on DyeP activity production and dye decolorization

We demonstrate here that the use of glycerol instead of glucose resulted in a 3-fold increase in dye peroxidase activity. Similar observations were reported by *Roch et al., (1989)* with *Phanerochaete chrysosporium* growing under carbon limitation with glycerol as carbon source, affecting both *P. chrysospoorium* growth and increased lignin peroxidase activity. Many studies on medium composition effects concerning lignin-modifying enzymes (LME) production have primarily focused on optimizing laccase activity or global LME induction, rather than specifically examining dye decolorizing peroxidase (DyeP). *Tinoco et al., (2011)* optimized a culture medium for laccase production by *P. ostreatus*, using copper and lignin as inducers. In contrast to our findings, they did not observe significant influence of glucose or glycerol on laccase production. However, they noted a positive effect of xylose on laccase activity. On the other hand, they did observe a positive effect of peptone and yeast extract as nitrogen sources. Several reports indicate that the production of lignin modified enzymes (LME) in basidiomycetes is dependent on the carbon and nitrogen sources, as well as the presence of aromatic compounds in the culture medium (*Elisashvili et al., 2018; Faison & Kirk, 1985; Mansur et al., 1998; Mikiashvili et al., 2006; Stajić et al., 2006; Thiribhuvanamala et al., 2017*). For instance, *Elisashvili et al., (2002)*, demonstrated the effect of different carbon sources and aromatic compounds on the lignocellulolytic enzyme activity of different edible and medicinal basidiomycetes, concluding that it is possible not only to substantially increase the lignocellulolytic activity, but also to lead their preferential synthesis by supplementing with nutritional compounds in the culture medium. In many basidiomycetous fungi laccases are expressed constitutively and this constitutive low expression is often enhanced by inducers such as aromatic compounds. In effect, the addition of cellobiose, mannitol and xylan as carbon sources and the aromatic compound 2,5-xylidine, increased up to 20-fold the induction of laccase activity depending on the fungal strain (*Armas et al., 2019; Castanera et al., 2012; Scheel et al., 2000; Téllez-Téllez et al., 2005*). According with *Jiao et al., (2018)*, the addition of small aromatic molecules in the culture medium could increase the yield of laccase activating the laccase gene transcription by binding on the xenobiotic response element (XRE) of the target gene.

In our study, the production of DyeP was primarily observed during the stationary phase (268-552 h) of the *P. ostreatus* culture. In general, ligninolytic enzymes are produced as secondary metabolites (*Elisashvili et al., 2020; Hammel, 1997; Mester & Field, 1998; Thiribhuvanamala et al., 2017*). It is assumed that the metabolized substrate is essential for fungi not only for the synthesis of lignin degrading enzymes, but also to produce peroxide as well as effectors of the ligninolytic system (*Faison & Kirk, 1985*). According with *Buswell et al., (1984)*, *Phanerochaete chrysosporium* growth on glycerol leads to carbon limitation which can affect the onset of secondary metabolism. That condition has been reported as favoring the carbon catabolite de-repression of both CAZ and ligninolytic enzymes *(Kern, 1990; Peng et al., 2021; Suzuki et al., 2008*). *Aro et al., (2005)* reported that the expression of gene encoding ligninolytic enzymes, is generally triggered by the depletion of nutrient nitrogen, carbon or sulfur. However, this does not apply to laccase-encoding genes, whose production can be detected at early fermentation stages.

On the other hand, the efficiency of AYG decolorization was considerably affected by the carbon source. We observed decolorization percentages of 45 % and 10 % decolorization after 48 h in glucose and glycerol, respectively (Figure 4). Furthermore, in glucose, complete decolorization was observed after 552 h. The efficiency of dye decolorization can be favored by co-metabolism with different carbon sources. Glucose, sucrose, fructose and glycerol are among the most extensively studied carbon sources (*Casas et al., 2013; Civzele et al., 2023; Haider et al., 2019; Leung & Pointing, 2002; Merino et al., 2019; Rao et al., 2019*). Furthermore, analysis of *Pleos*-DyeP1 expression on both carbon sources evaluated suggests the decolorization associated with DyeP1 activity.

The ability of *P. ostreatus* to metabolize a wide variety of toxic compounds is primarily attributed to their non-specific multi-enzyme oxidative system (*Eichlerova et al., 2002; Garrido-Bazán et al., 2016; George et al., 2023; Grandes-Blanco et al., 2013; Kunjadia et al., 2016; Šlosarčiková et al., 2020*). *Cuamatzi-Flores et al., (2019)* reported that when glucose was the sole carbon source, addition of Acetyl Yellow G (AYG), Remazol Brilliant Blue R (RBBR) or Acid Blue 129 (AB129) dyes increased DyeP activity, ultimately achieving complete decolorization. When grown in liquid media, transformation of RBBR dye by *P. ostreatus* seems to be mainly via laccase oxidation. However, dye decolorization peroxidase and veratryl alcohol oxidase were also produced (*Palmieri et al., 2005*). *Eichlerová et al., (2006)*, evaluated the decolorization capacity of Orange G and Remazol Brilliant Blue R (RBBR) dye and ligninolytic enzyme production of eight different *Pleurotus* species. The main enzymes detected were Lac and MnP whose production was strongly influenced by the type of cultivation media and the presence of a dye. *Ottoni et al., (2014)*, reported that in *Trametes versicolor,* glycerol is an important substrate for oxidative metabolism, promoting higher laccase production and consequently, increasing the decolorization process of Reactive Black 5.

De-repression of *Pleos*-DyeP genes expression and differential regulation in response to glycerol and AYG dye

In this study, glycerol was used as an alternative substrate to examine the transcriptional responses of *P. ostreatus* DyeP genes, and to investigate the potential participation of carbon catabolite de-repression in their regulation. Our analysis of DyeP gene expression profiles revealed significant variations influenced by the carbon source, growth phase, and the presence of AYG dye. These variations led to up- and down-regulation patterns over the fermentation period in the three evaluated *Pleos*-DyeP genes. Interestingly, the addition of glycerol and AYG dye induced early-stage expression of *Pleos*-DyeP1 (144 hours). In contrast, the expression of *Pleos*-DyeP4 was not detected in glycerol cultures.

The effect of chemical dyes on *P. ostreatus* on DyeP activity and gene expression profile has been previously reported. The addition of dyes results in an induction effect on the enzyme activity and the expression profiles of DyeP genes, with maximum induction level detected for DyP4 gene at the end of the fermentation (*Cuamatzi-Flores et al., 2019*). The potential XRE, Cre1 and Nit2 binding sites motifs identified in the promoter of the three DyeP genes analyzed (Supplementary Table 1), suggest that DyeP genes transcription among others can be regulated by xenobiotics, carbon and nitrogen sources respectively. The frequency of these cis-acting elements varies between genes from 0 for XRE in DyeP2 gene, 1 for Cre1 in the three genes and 3 for Nit2 in DyeP4.

Carbon catabolite repression (CCR) has been a focal point of research in the Ascomycota (*Adnan et al., 2018; De Assis et al., 2021; Strauss et al., 1999*). However, several studies on the Basidiomycota have demonstrated that CCR, in combination with different signaling pathways, plays a key role in the utilization of different carbon sources in these group of fungi (*Hu et al., 2020; Janusz et al., 2013; Nakazawa et al., 2019; Toyokawa et al., 2016; Zhang et al., 2022*). The presence of an ortholog of Cre1 and its participation as main regulator in CCR has been also demonstrated (*Daly et al., 2019; Jiao et al., 2018; Pareek et al., 2022; Yoav et al., 2018*). *Yoav et al., (2018)*, conducted genetic modifications on the transcriptional regulator Cre1 in *Pleurotus ostreatus* PC9 strain, observing that the secretion level of CAZymes, including lignin modifying enzymes, were not exclusively dependent on Cre1 activity. According with *Pareek et al., (2022)*, Cre1 in the basidiomycete *Coprinopsis cinerea*, similarly as in ascomycetes mediates regulation of CAZymes (carbohydrate-active enzymes) however, LMEs were downregulated in the *C. cinerea cre1* mutants, indicating that these enzymes fall under different regulation from that of CAZymes. *Daly et al., (2019)* shed light on the widespread glucose-mediated CCR of plant biomass utilization in the white-rot basidiomycete *Dichomitus squalens.* Glucose-mediated repression of secreted CAZyme genes but not of genes encoding proteases suggested that CCR has been maintained by the fungus to strongly conserve nitrogen use in its nitrogen-scarce woody biotope. In *Aspergillus nidulans* cross-talk between signaling pathways, such as the one between the protein kinase A (PKA), high osmolarity glycerol (HOG) and mitogen-activated protein kinases (MAPK) pathways, are required for the utilization of different carbon sources. Furthermore, MAPKs regulate the carbon catabolite repressor CreA activating HOG pathway, PKA activity and CCR (*de Assis et al., 2020; de Assis et al., 2021*).

Furthermore, regulation of Cre1 by cAMP-dependent protein kinase A(PKA) has been well-established (*Boominathan & Reddy, 1992; de Assis et al., 2020; Pareek et al., 2022*). This regulatory mechanism also extends to the induction of genes encoding lignin-modifying enzymes in *Pleurotus ostreatus* (*Toyokawa et al., 2016*).

Conclusions

The fungus *Pleurotus ostreatus* can grow on glycerol as carbon source inducing the production of dye peroxidase activity. The analysis of the promoters of DyeP encoding genes has revealed the presence of several putative cis-regulatory elements, including the CCR Cre1-binding site. Notably, the induction of DyeP genes at earlier stages in the presence of glycerol suggests a potential regulation by CCR. However, as has previously been reported, alternative regulation mechanisms may be involved, requiring further studies to establish the overall mechanisms by which DyeP and other oxidases produced by *P. ostreatus* are transcriptionally regulated.

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Table 1(on next page)

Primers used in this study

qPCR primers (type – forward or reverse – and sequences) and amplification length and efficiency for the *P. ostreatus* dye peroxidase genes and the selected reference gene.

Table 1. Primers used in this study**.** qPCR primers (type – forward or reverse – and sequences) and amplification length and efficiency for the *P. ostreatus* dye peroxidase genes and the selected reference gene.

Gene	Transcript ID[a]	Direction[b]	Sequence (5' to 3')	Product size (bp)	Efficiency[c]
Pleos_DyeP1	62271	Fw	CGCTTGAGTTGATCCAGAAA	104	2.21
		Rv	TATTTCCTTCGGCTTCCTCA		
Pleos_DyeP2	1092668	Fw	TACATTCTTGCCGCTGGAT	117	1.87
		Rv	GCGAGAACCTGCTTGAACTT		
Pleos_DyeP4	1069077	Fw	ATGAACACTTCGGCTTCCTC	64	2.03
		Rv	GGCAAGTACCGCAGATAAG		
pep	1092697	Fw	CGGAGGACATTCTTGTTCAC	142	1.89
		Rv	AGATCGGTAACCCACACGAG		

Note: [a]Transcript ID and gene nomenclature refer to the annotation of *P. ostreatus* PC15 genome version 2.0 (http://genome.jgi-psf.org/ PleosPC15_2/PleosPC15_2. home.html), [b]Fw, Forward; Rv, reverse, [c]Efficiency for primers used in qPCR

Figure 1

Plate decolorization of AYG dye by *P. ostreatus*

The fungus was incubated on agar plates supplemented with glucose or glycerol as carbon source as well as complemented with the AYG dye. Reverse (**R**) and front side (**F**)

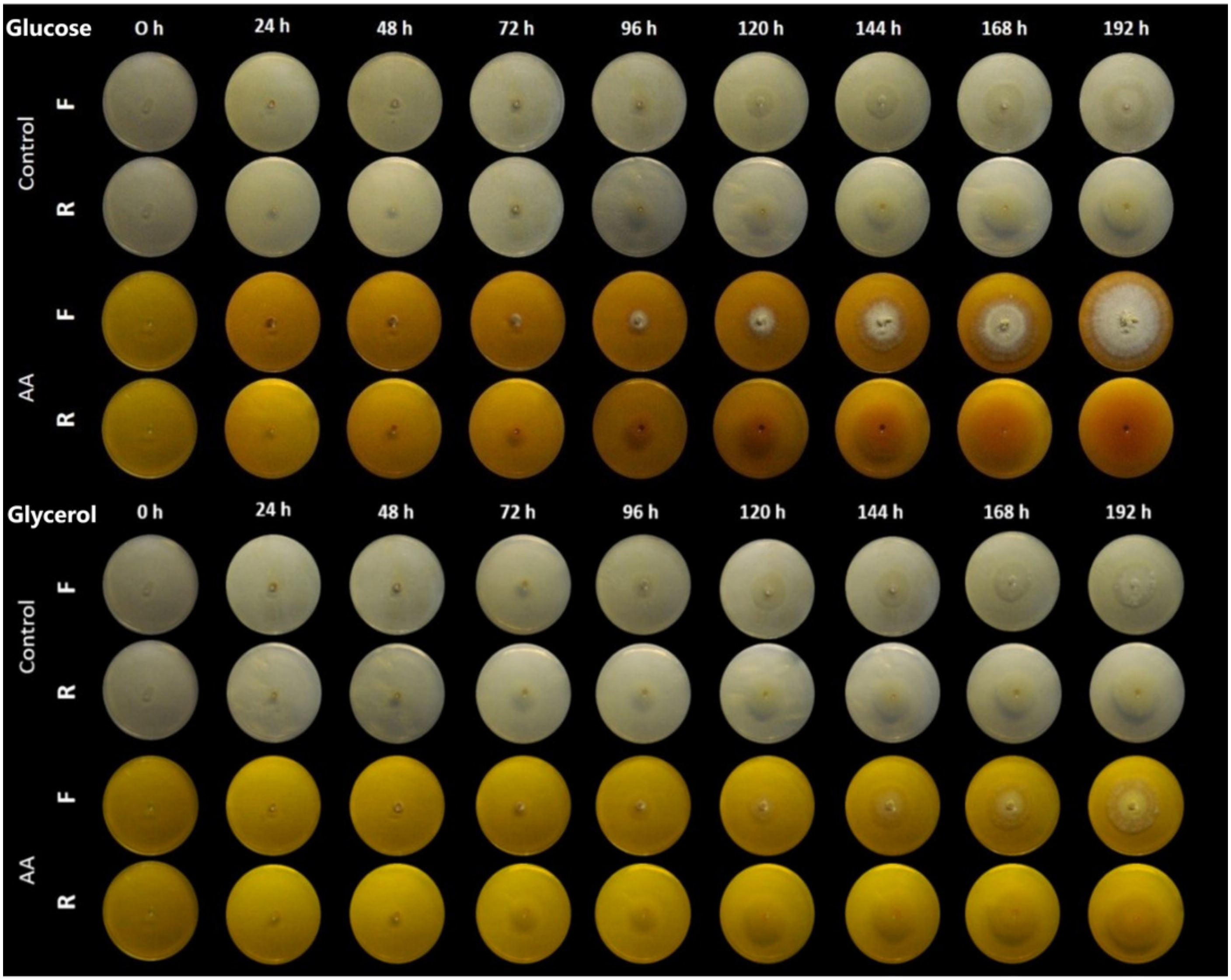

Figure 2

Growth kinetics and specific growth rate values (µ) for *P. ostreatus* fermentations

(**A**) Growth curve for glucose (blue circles), glucose supplemented with 500 ppm of AYG (yellow squares), glycerol (darkcyan diamonds), and glycerol supplemented with 500 ppm of AYG (orange triangles). Each point represents the mean of three replicates. The continuous line represents the best fit of the measured data to the logistic model. (**B**) Specific growth rate (h^{-1}) in each fermentation. Each bar represents the mean of three replicates. Statistical significance was calculated with *t-test* (* = p < 0.05, ** p < 0.01). The error bars in both panels represent the standard error.

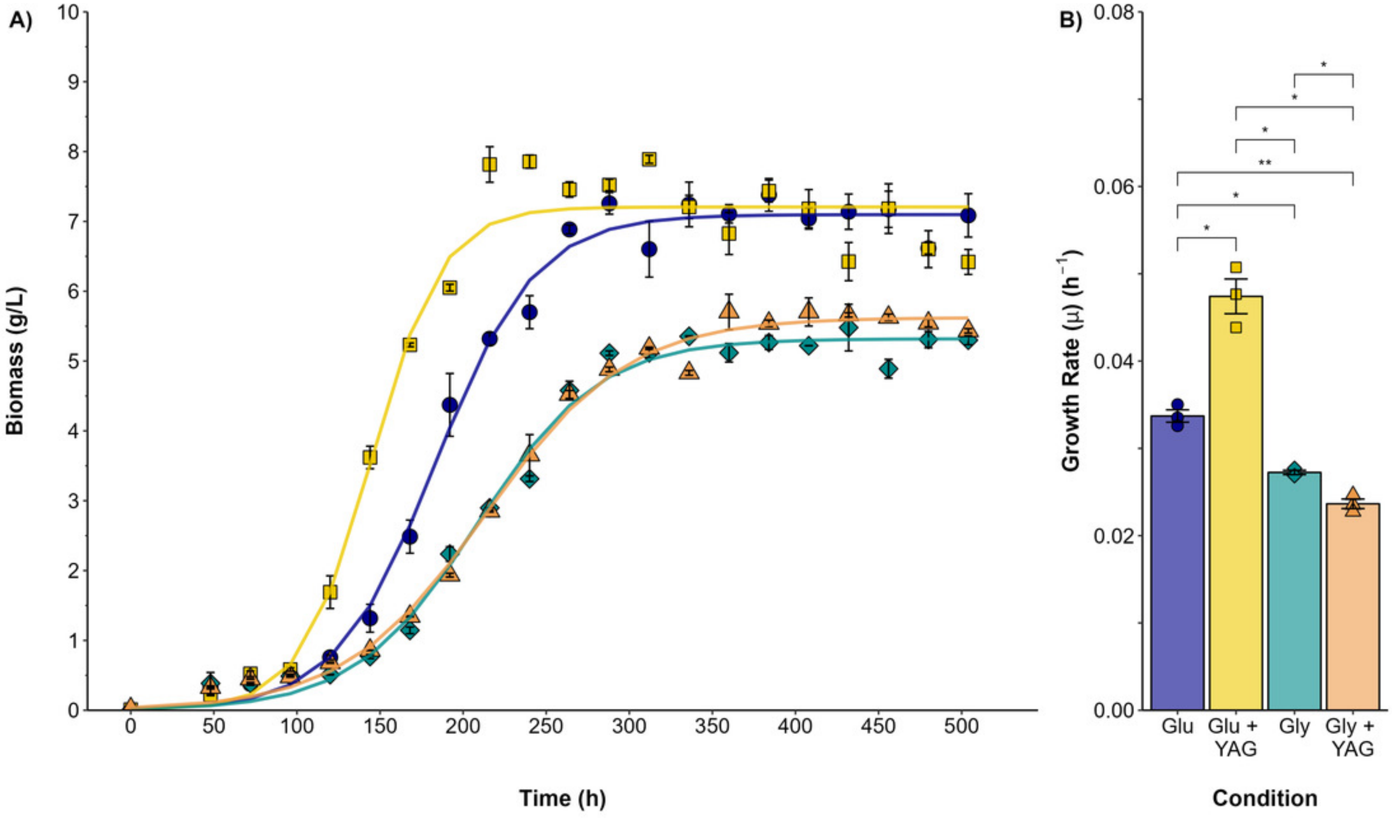

Figure 3

Carbon source consumption

During the fermentation with glucose (blue circle), glucose supplemented with 500 ppm of YAG (yellow squares), glycerol (green diamonds), and glycerol supplemented with 500 ppm of YAG (orange triangles). Each point represents the mean of three replicates. Error bars indicate the standard error.

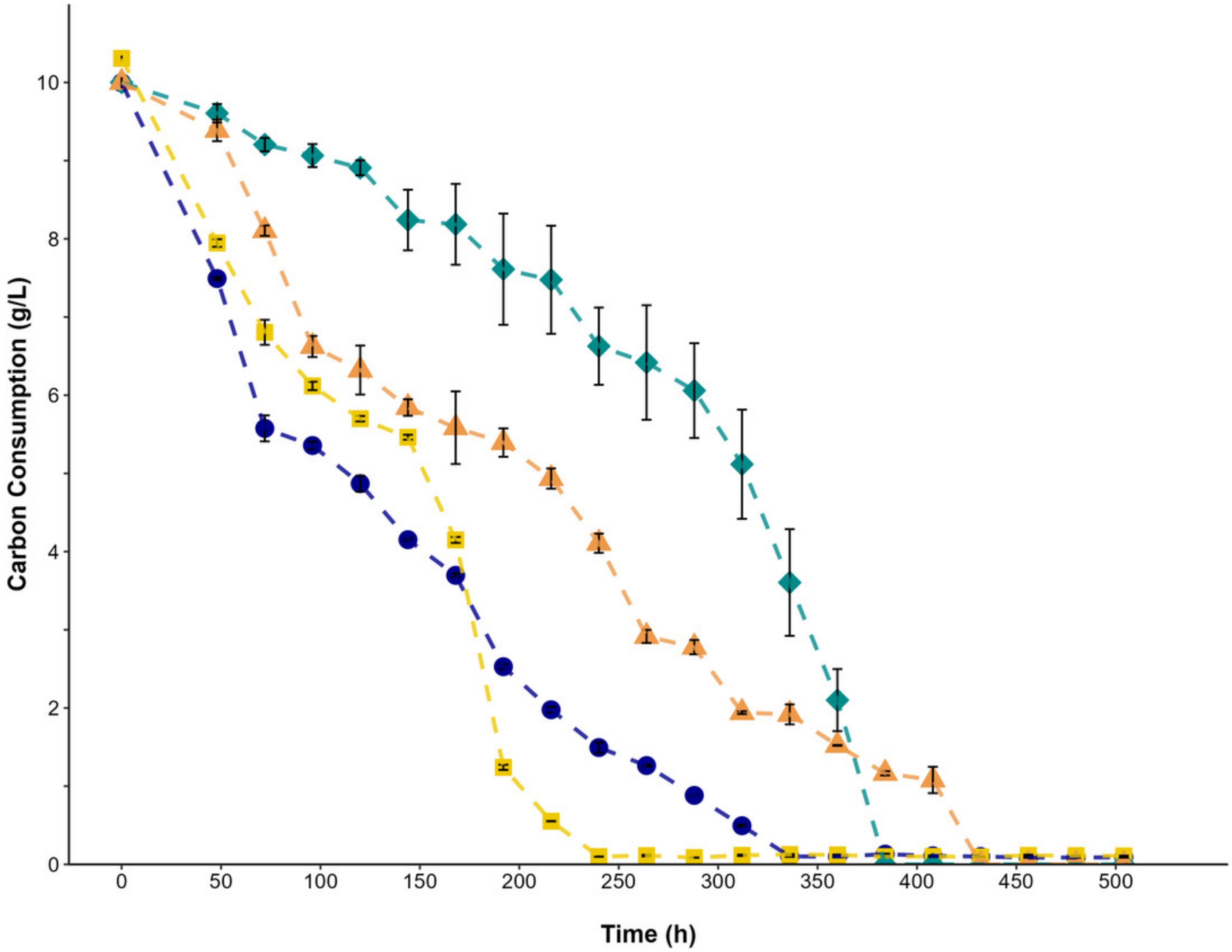

Figure 4

Decolorization percentage

Effect of carbon source on AYG dye decolorization during the time course of fermentation

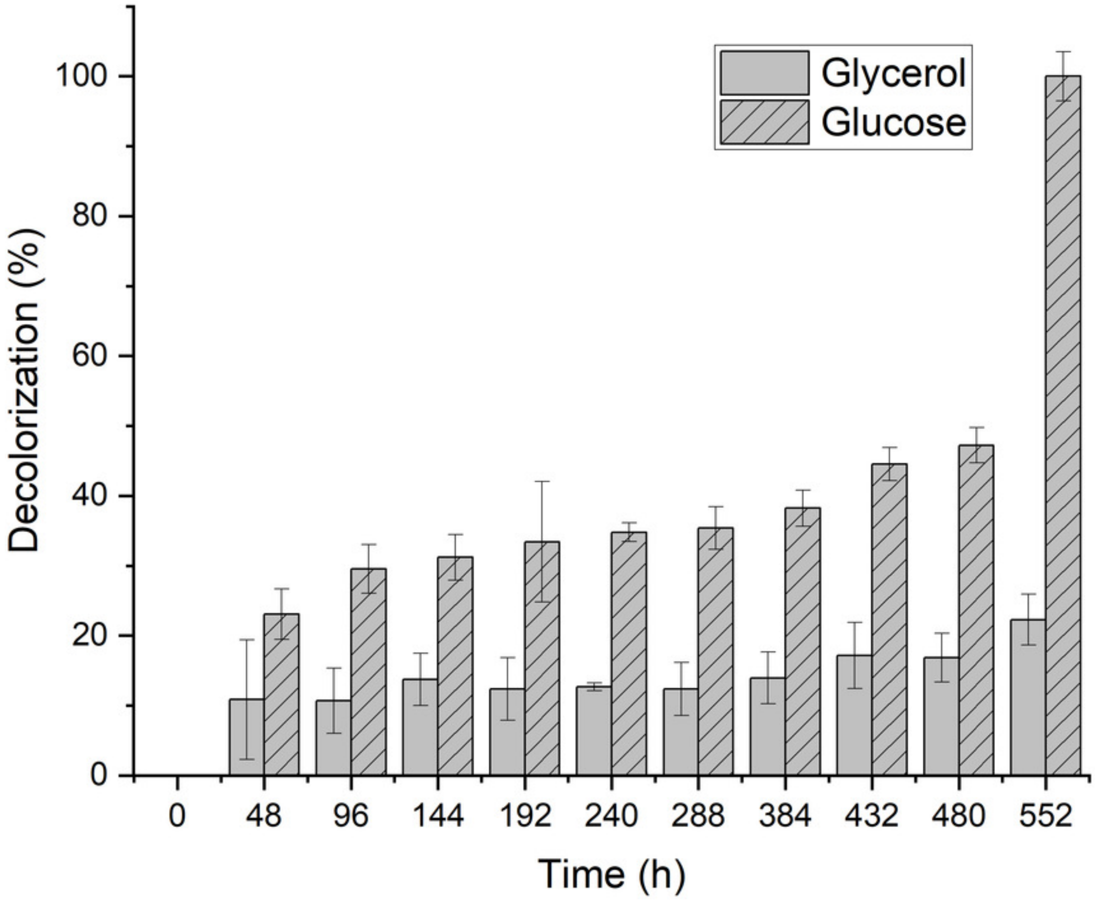

Figure 5

DyeP activity in the cultures of *P. ostreatus*

Enzymatic activity of DyeP in fermentation with glucose (blue circle), glucose supplemented with 500 ppm of AYG (yellow squares), glycerol (green diamonds), and glycerol supplemented with 500 ppm of AYG (orange triangles). Each point represents the mean of three replicates. Error bars indicate the standard error.

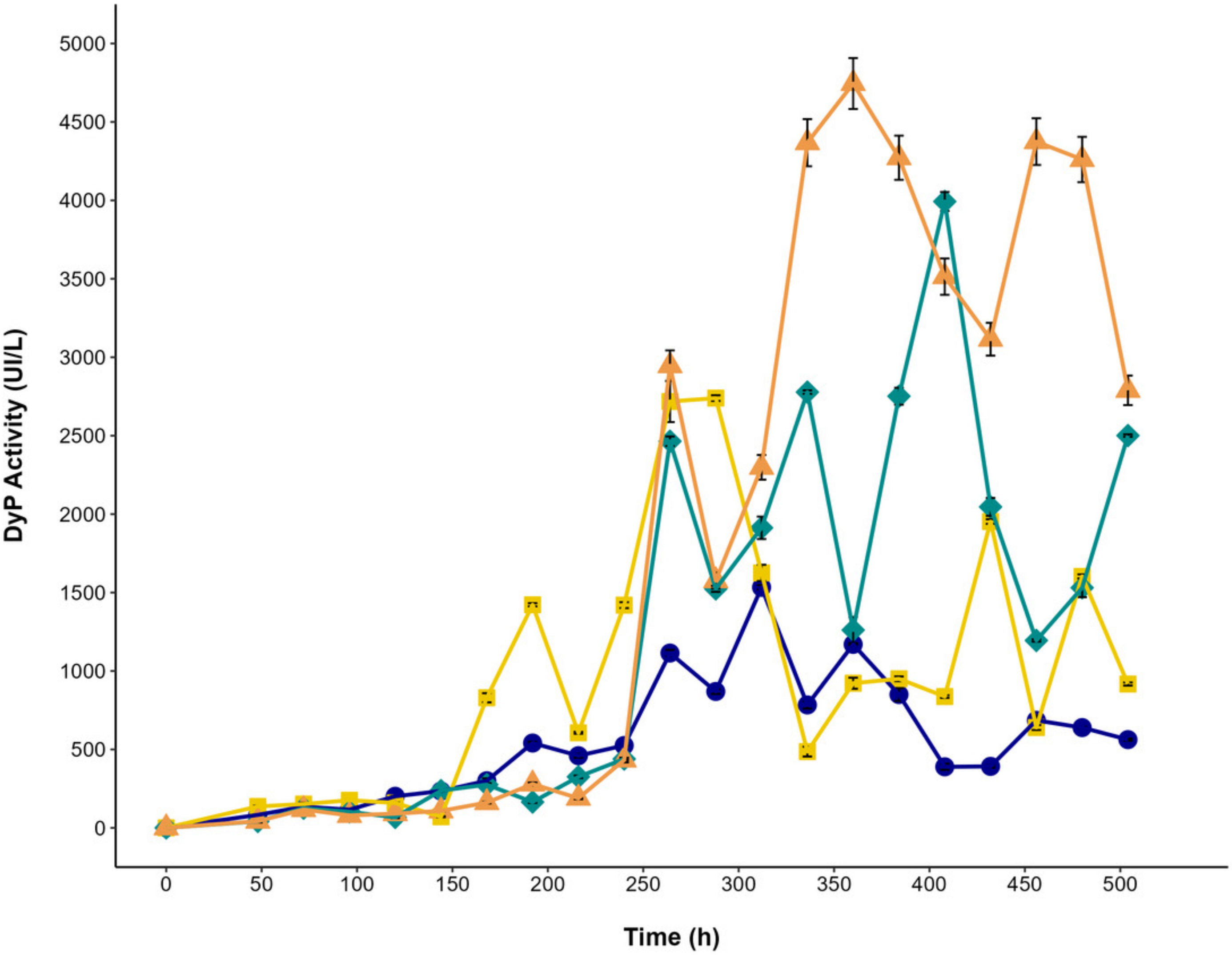

Figure 6

Analysis of gene expression of the three *P. ostreatus* dye peroxidases

Heatmap representing gene expression profiles of *Pleos*-DyeP genes during fermentation with Glucose, Glycerol, and Glycerol Supplemented with 500 ppm of AYG.