Ligninolytic enzymes activities of Oyster mushrooms cultivated on OMW (olive mill waste) supplemented media, spawn and substrates

Alejandro Ruiz-Rodríguez[2#], Isabel Polonia[1#], Cristina Soler-Rivas[2*] and Harry J. Wichers[1]

[1] Food & Biobased Research. Wageningen University and Research Centre, Bornse Weilanden 9, 6708 WG Wageningen. The Netherlands.

[2] Research Institute in Food Science (CIAL). c/Nicolás Cabrera 9. Universidad Autónoma de Madrid. 28049 Madrid, Spain.

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[#]Equal contribution

*Corresponding author: Research Institute in Food Science (CIAL). c/Nicolás Cabrera 9. Universidad Autónoma de Madrid. 28049 Madrid, Spain. Tel: +34914973776. Fax: +34914978255. E-mail address: cristina.soler@uam.es

Abbreviations: AAO: aryl-alcohol oxidase, iMMP: gamma-irradiated olive mill waste, MMP: malt mycological peptone, OMW: olive mill waste.

Abstract

Ligninolytic enzymes activities (laccases, peroxidases (total, MnP and MiP) and aryl-alcohol oxidase (AAO)) were measured during the cultivation of six commercial *Pleurotus* sp. strains on MMP media, on cereal grains (spawn) and on straw substrates (the three commonly utilized cultivation steps to obtain fruiting bodies) supplemented with several concentrations of autoclaved (OMW) or gamma-irradiated (iOMW) olive mill waste. Results indicated that all the strains were able to grow on MMP media and spawn containing up to 30% OMW and iOMW and on straw substrates mixed with 50% OMW. None of the strains showed AAO activity and there was not a single strain which showed the highest laccases and peroxidases activities, independently of the utilized substrate. *Pleurotus* mycelia adjusted their enzymatic mechanisms depending on their variety, type of substrate, concentration of OMW or iOMW added. OMW was a better supplement to use than iOMW because OMW induced higher exo-enzymes activities.

1. Introduction

The modern ecological manufacture of olive oil involves technological processes, which generate, besides the oil, a residue with high moisture content (between 50 and 70%) named olive mill waste (OMW). Valorisation of OMW is not an easy task. The expensive chemical extraction of the reminiscent oil due to the high level of moisture content and the heterogeneous composition, in particular the fibrous material, makes it a difficult material to handle.

White rot fungi, and particularly Oyster mushrooms (*Pleurotus* sp.) mycelia, can grow on and detoxify olive mill waste waters or vegetation waters (OMWW) (Fountoulakis et al. 2002; Kalmis and Sargin 2004), the major residue obtained when the traditional olive oil production system is followed (using a three-phase centrifugation process). Nowadays, the modern olive oil factories produce more OMW than OMWW, thus, many of the latest investigations have been aimed to study the ability of *Pleurotus* mycelia to colonise and degrade/detoxify substrates supplemented with different OMW concentrations (Saavedra et al. 2006; Sampedro et al. 2007). In some cases, a double objective (ecological and economical) was achieved such as to obtain good quality *Pleurotus* fruiting bodies by means of the OMW degradation (Ruiz-Rodriguez et al. 2010).

However, the production process for Oyster mushroom fruiting bodies involves many steps. Firstly, the mycelia mother cultures are sub-cultivated on specific liquid or semi-solid media. Then, mycelia are inoculated on solid carriers (which usually are pre-treated cereals grains such as wheat, rye, millet, etc.) and incubated until the grains are fully colonized. This process is called spawn preparation or spawning. Finally, specific substrate mixtures are prepared (wheat straw and many other lignocellulosic wastes (Yildiz et al. 2002)), inoculated with the spawn, homogeneously distributed, packed in bags or blocks and incubated under controlled conditions until the mycelia fully colonize the substrate and is able to initiate the fruiting bodies production. Each step involves a different substrate to be colonized indicating that the mycelium has to adapt and re-adapt their metabolic pathways in order to grow on them. Oyster mushrooms have the interesting ability of producing and secreting specific lignolytic enzymes to the surrounding environment enabling them to use a wide range of substrates (including OMW supplemented substrates) as source of nutrients and energy necessary for the fruiting bodies production. Thus in principle, the mushrooms strains which are able to produce larger quantities of these enzymes might be better suited to colonize the substrates and to produce higher fruiting bodies yields. Moreover, perhaps, their need or/and time to adapt their enzymatic mechanisms from lab medium to spawn and from spawn to substrate could be reduced if OMW would have been added from the first cultivation steps accelerating the substrate colonization, increasing the enzyme production and the mushroom yields. Thus, in this work, cultivation medium, spawn and substrates were supplemented with OMW to investigate the effect of this residue on the mycelial growth and on the lignolytic enzyme production using six different *Pleurotus* strains. Particularly, laccases, peroxidases and aryl-alcohol oxidases (Ander and Marzullo 1997) that have been usually pointed as the enzymes mainly responsible for the degradation of lignocellulosic residues such as OMW.

The use of γ-irradiation of lignin-containing substrates was an approach previously followed to facilitate microbial lignin degradation. This pre-treatment can also facilitate disruption of lignocellulose polymers, allowing an easier and faster fungal enzymes attack (Awafo et al. 1995; Gbedemah et al. 1998; Al-Masri and Zarkawi 1999; Lam et al. 2000). Thus, in this work, two different treatments were applied to raw OMW, the standard high temperature sterilisation (autoclaving) and the application of γ-rays as a cold sterilisation process.

2. Material and methods

2.1 Biological material

Mushroom strains used in this study were the commercial strains 2191 and 2171 of *Pleurotus ostreatus* and *Pleurotus pulmonarius* 2204 obtained from the mother culture collection of Mycelia (Gent, Belgium). *Pleurotus ostreatus* K15 and *Pleurotus pulmonarius* P17 were supplied by Micelios Fungisem S.A. (Autol, Spain). *Pleurotus ostreatus,* strain 1111 was kindly donated by INETI (Intituto Nacional de Engenharia Tecnologia e Inovaçao) (Lisboa, Portugal).

Olive mill waste (OMW) was obtained from a continuous olive oil production process with a two-phase centrifugation system (Cooperativa de Olivicultores de Fatima, Fatima, Portugal), frozen at –25°C as it was acquired and lyophilised. A part of the freeze dried OMW was irradiated with γ-rays in a cobalt-60 source, until reached an average dose of 5 kGy, and stored at –25º C. The irradiation was performed in Isotron Netherland BV (Ede, The Netherlands). Irradiated OMW in these conditions will be mentioned as iOMW.

2.2 Fungal growth on semi-solid media

Mycelia obtained from the mother cultures were inoculated on Petri plates containing MMP medium (10 g L^{-1} of malt extract (Difco), 5 g L^{-1} of mycological peptone (Difco) and 15 g L^{-1} of agar (nº 3, Oxoid)) and sterilised cellophane circles at the top of the semi-solid medium. To test the effect of olive mill waste addition, MMP medium was supplemented with 5, 15 and 30 % (w/v) OMW (20 min autoclaved olive mill waste) or iOMW (irradiated OMW). Plates were inoculated in triplicate from non-adapted inocula and incubated at 25 ^{o}C.

Mycelial growth (minor and major diameters) was measured twice a day until mycelia reached the plates edge (after 9 days). Growth was later expressed as mm day^{-1} using the slope at the linear growth phase of the fungi (2 to 7 days). Mycelial biomass was also quantified by weighting the produced mycelia scratched from the cellophane after 9 incubation days. Afterwards, fresh mycelia were frozen, freeze-dried and weighted again to calculate their dry weight. They were later ground in a mortar with liquid nitrogen and stored at -20ºC for ligninolytic enzymes determinations.

2.3 Fungal growth on liquid media

Liquid MMP medium (25 mL) was supplemented with 0, 5, 15 and 30 % (w/v) OMW or iOMW on 100 mL Erlenmeyer flasks and inoculated with non-adapted inocula from mother cultures of the *Pleurotus* strains. Erlenmeyer flasks were incubated in triplicate for each strain and media and placed at 25 ^{o}C, in darkness without shaking, during 9 days.

2.4 Lab- and commercial scale spawn preparation

Lab-scale spawns were prepared by mixing rye grains with tap water in a ratio (2 : 1) (w/v), supplemented with 0, 5, 15 and 30% OMW (w/w) and sterilized in an autoclave 30 min. Irradiated-OMW was added after sterilisation of the cereal grains. Sterilized and γ-irradiated spawns (20 g) were added to the top of colonised Petri plates (after 7 incubation days) including OMW or iOMW to allow further mycelial colonisation of the grains. Afterwards, plates were incubated at 25 ^{o}C during 7 days. Colonized grains were extracted from the plate and used to inoculate commercial scale spawn bags.

Commercial-scale spawns were prepared by boiling rye grains at 100ºC during 30 minutes. Afterwards, grains were collected with a sieve and placed on filter paper to drain excess of water during 10 minutes. Cooked grains (130 g) were mixed with 0 (control) or 15% OMW (w/w) and 6% calcium carbonate / calcium sulphate (1:3) (w/w). The mixture (150 g) was placed in special thermo-resistant bags (13 x 7 cm^2) and sterilised in an autoclave during 30 minutes. Spawn bags were inoculated with 10% (w/w) of fully colonized grains produced as above described (lab-scale spawn including 0 or 15% OMW) and incubated at 25 ^oC during four weeks in darkness. After 7, 14, 21 and 28 days, two bags per strain and substrate were separated and a fraction lyophilized to determine the ligninolytic enzyme activities.

2.5 Pleurotus cultivation on wheat straw substrates

Substrates for *Pleurotus* cultivation were prepared as follows: wheat straw was chopped (2 – 5 cm) and left overnight soaking up tap hot water. The excess of water was drained on a sieve during 20 min. Afterwards, the soaked straw was mixed and homogenized with 0% (control) or 50% OMW (expressed in dry weights of both straw and OMW). Homogenized substrates (60 g) were placed in plastic bags (15 x 25 x 3 cm) and sterilized in autoclave during 30 minutes. Sterilized substrates were inoculated with 10% (w/w) of fully colonized commercial-scale spawn from the selected strains and left incubated in dark at 25^oC during 60 days. Every five days, two bags per strain and substrate were separated and a fraction lyophilized to determine the ligninolytic enzyme activities.

2.6 Determination of ligninolytic enzymes activities

Dried mycelia powders (10 mg) obtained from the semi-solid media, were mixed with 1 mL buffer (0.1 M citric-phosphate buffer (pH 5) for laccase, 0.1 M succinic-lactic acid buffer (pH 4.5) for peroxidases, or 0.1 M phosphate buffer (pH 6) for aryl-alcohol oxidase determination) to measure the intracellular ligninolytic enzymes activities. The mixture was stirred in a Vortex for 10 minutes and centrifuged at 14 000 rpm during 5 minutes (Hermle Z200 M/H). Obtained supernatants were used as source of enzymes for determination of laccase, peroxidase and aryl-alcohol oxidase activities.

Extracellular ligninolytic activities were measured on the liquid media after 9 incubation days. Media (1 mL) were centrifuged at 14000 rpm, 2 min (Hermle Z200 M/H) and the supernatants used as source of extracellular enzymes.

Freeze-dried grains or straws from the different spawn or substrate types were ground with liquid nitrogen on a miller (Moulinex Masterchef 20, France) during 1 min at maximum speed and sieved until the particle size was smaller than 0.3 mm. The obtained powder (2 g) was vigorously mixed with 8 mL (for spawn samples) or (10 mL for straw samples) of the above described buffers and similarly stirred. Suspensions were centrifuged at 5000 rpm for 10 min at 4ºC (Sigma Laborzentrifuge 3-10, Germany) and supernatants were used as ligninolytic enzyme source.

Ligninolytic activities were monitored using a Perkin Elmer UV/vis Spectrometer Lambda 2S, by measuring absorbance of the oxidation products. Activities were measured in duplicate and calculated as the slopes between absorbance and time of the first linear stage of reaction. Enzymatic activities were defined as the amount of enzyme that transforms 1 mol of substrate second^{-1} (katal) per gram of dry weight of mycelia, or per mL of liquid media (in case of extracellular enzymes).

Laccase activity was measured using ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid, Sigma) as substrate, following the method of Niku-Paavola et al. (1988).

Absorbance was measured at 436 nm and 25 ^{o}C, using a mixture of enzyme extract and 5 mM ABTS in 0.1 M citric-phosphate buffer (pH 5). The molar extinction coefficient of the oxidation product from ABTS was ε_{436} = 29 300 M^{-1} cm^{-1}.

Peroxidases activities (POD) were measured as total peroxidase (total POD), manganese dependent peroxidases (MnP) and manganese independent peroxidases (MiP) according to the method described in Martínez et al. (1996) using as substrate 3-Methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH, Fluka) which interact with 3-dimethylaminobenzoic acid (DMAB, Aldricht) producing a purple coloured reaction in the presence of the enzyme, H_2O_2 and manganese (Mn). The reaction was followed at 590 nm and 30 ^{o}C. The molar extinction coefficient of the oxidation product from MBTH/DMAB was ε_{590} = 32 900 M^{-1} cm^{-1}. Aryl-alcohol oxidasa (AAO) was measured using veratryl alcohol (3,4-Dimethoxy benzyl alcohol) as substrate following the method of Gutierrez et al. (1994).

Statistical analysis

One way analysis of variance (anova) was performed using a Statgraphics® Plus 3.1 for Windows software (Statistical Graphics Corporation, MD, USA). The mean comparison test used was Fisher's least significant differences procedure (LSD).

3. Results

3.1 Pleurotus sp. on OMW containing media

The growth of six *Pleurotus* strains was evaluated on Petri dishes containing MMP media and MMP supplemented with different OMW and iOMW concentrations up to 30% (Table 1). The *Pleurotus pulmonarius* strains showed a faster growth than the selected *P. ostreatus* strains independently of the cultivation media. OMW supplementation up to 5% enhanced a significantly higher growth rate in all the analyzed strains compared with MMP control media. Higher OMW supplementations (15%) did not increase the mycelial growth. On the contrary, some strains grew even slower than on MMP control medium. This decrease was significant when 30% OMW was added. However, additions of iOMW did not significantly stimulate or reduce the mycelial growth because rates were similar to those on MMP media.

When the fungal growth was measured as the produced biomass, results confirmed that *P. pulmonarius* produced more mycelial mass than *P. ostreatus* strains in MMP control medium (Table 2). In the medium where the mycelium seemed to spread their hyphae faster (5% OMW supplementation) their fresh biomass was similar or even higher (if expressed in dry weight) than the control for *P. ostreatus* strains and similar or lighter for *P. pulmonarius* strains. Additions of 15 or 30% OMW induced a higher fresh weight than in control medium for four of the strains and similar percentage of dry weight as on the 5% OMW containing medium. On average, additions of iOMW to MMP media showed similar or a slight biomass reduction compared to control.

The levels of the ligninolytic enzymes (laccases and peroxidases) were measured inside and outside the fungal hyphae of *Pleutorus* strains cultivated on MMP or OMW / iOMW supplemented media. Results differed depending on the considered strain and enzyme location (intra- or extracellular activities). Strains such *as P. ostreatus* 2171, 2191 and k15 showed significantly higher intracellular laccase activities in control media than the rest of the analyzed strains (Fig. 1a). All the strains showed higher laccase levels when cultivated on control medium than on 5 and 15% OMW supplemented media. Only when they were grown on medium including 30% OMW, intracellular laccase levels increased up to similar levels to when they were cultivated on MMP for some strains (PO1111, PP-P17 and PP2204) and in all cases, higher than when they were cultivated on media including 5 or 15% OMW. When the *Pleurotus* strains were cultivated on media containing iOMW, their laccase activities were in all the cases lower than the control but independently of the iOMW added.

The levels of extracellular laccases seemed to increase with the OMW concentration added for the *P. pulmonarius* varieties (Fig. 1b). A remarkable increase was observed for the P-17 strain, the increase was also observed with increasing concentrations of iOMW. *P. ostreatus* varieties showed extracellular laccase activities too but their activity was strain dependent and did not correlate with the presence or absence of OMW or iOMW in their cultivation media.

The three *Pleurotus* strains that showed high intracellular laccase activities on MMP medium showed also higher endo-peroxidases (POD) levels than the rest of the strains except for *P. ostreatus* 1111 that showed low laccase levels and very high peroxidases activities (Fig. 2a). For the latter strain and for *P. ostreatus* K-15 and 2171, the peroxidases levels decreased with increasing OMW concentrations. This was not observed with media containing iOMW. The *P. pulmonarius* varieties showed very low POD activities compare with *P. ostreatus* samples in all the utilized media.

The above described values for the intracellular peroxidases activities were the sum of manganese-dependent (MnP) and independent (MiP) peroxidase activities. The activity of one or other type depended more on the strain than of the cultivation media (data not shown). For instance, the total POD activity observed in *P. ostreatus* K-15, 1111, 2191 and 2171 were mostly due to their MiP (respectively 88, 80, 71 and 69% on average) however, the *P. pulmonarius* strains showed 40-42% MnP.

The levels of extracellular peroxidases were increasing with increasing OMW concentrations and the effect was observed in all the analyzed strains (Fig. 2b). Moreover, the peroxidases activities showed similar values in all the strains ranging from (on average) 101 nkat mL^{-1} on control medium up to more than 10 fold the control values on media including 30% OMW (1383 nkat mL^{-1}). Similar values were also observed within the *Pleutorus* strains cultivated on increasing iOMW supplementations but slightly lower POD levels were achieved than for OMW (except for *P. ostreatus* 1111). The peroxidases secreted to the extracellular media were mainly MnP since their activities were, on average, on MMP medium 52% the total POD activity while on MMP supplemented with 30% OMW increased up to 67.3%. The percentage of MnP was even higher when iOMW was utilized as MMP additive (71 – 78%).

3.2 Pleurotus sp. on OMW containing spawn

Rye grains supplemented with OMW or iOMW were inoculated with adapted mycelia grown on media including the same olive mill waste concentration and type. When the spawn were fully colonized (after 5 days in the lab-scale experiment) ligninolytic enzymes were measured. The strains which showed an increase of exo-laccase activity with increasing OMW concentration during their cultivation in medium such as *i.e. P. pulmonarius* P-17 and 2204 showed a similar increasing laccase profile when they were grown on spawn supplemented with OMW and iOMW (Fig. 3a). Similarly *P. ostreatus* 1111, one of the strains which showed high peroxidase levels on OMW and iOMW containing media presented the highest levels of all the analyzed strains when they were cultivated on wheat grains supplemented with the same supplements (Fig. 3b). However, in this case, the strain showed higher peroxidase activity in control spawn than in the supplemented samples. The rest of the analyzed strains also showed POD activities but lower values and independent of the OMW or iOMW concentration added.

The effect of 15% OMW supplementation was also tested using a large scale spawning procedure. Similarly, ligninolytic enzymes activities were recorded during the

28 incubation days necessary for the complete grain colonization. On average, the laccase activities, detected on control spawns, were lower than on spawn including 15% OMW during the complete cultivation time but, the levels were strain dependent (Fig. 4). *Pleurotus pulmonarius* 2204 showed the highest laccase activity of all the analyzed strains on control spawn showing a peak of activity after 21 days (Fig. 4a). However, *P. pulmonarius* P-17 was the strain which showed the highest activity on OMW supplemented spawn (Fig. 4b) peaking also after 21 days. In fact, except for *P. pulmonarius* 2204, all the strains grown on supplemented spawn showed a maximum of laccase activity at approx 21 days.

The values of the total peroxidase activities during spawning on control grains showed higher differences than laccase activities from strain to strain (Fig. 5a). The strain with the highest laccase activity (*P.pulmonarius* 2204) showed very low peroxidases levels compared with other strains. *P.ostreatus* 1111 showed the highest activity of all the analyzed strains after 14 days of incubation mostly due to MiP (3 fold higher values than MnP) although a second activity peak was noticed after 28 days because of a significant increase of MnP compensating the MiP activity decrease. *P.ostreatus* K15 showed a high peak after 14 days but it was due to the high MnP levels since the levels of MiP activity were always very low. However, the total peroxidase activity *of P. ostreatus* 2191 was very high after 28 incubation days and it was mostly due to the presence of MiP.

When the grains were supplemented with OMW the total peroxidase activity profiles became more similar within the studied strains. Some strains showed a low peroxidase level through the complete incubation time (*P. pulmonarius* 2204 and *P ostreatus* 2191) and the rest showed a pronounced increase almost at the end of the incubation time (Fig. 5b). However, the type of peroxidases involved in the observed activities was strain dependent. The activity peak observed after 14 incubation days observed for *P. ostreatus* 2171 was produced mostly by MiP while the peak after 28 days of *P. pulmonarius* P-17 was due to MnP. *P. ostreatus* K-15 showed a small activity peak after 14 days due to its MnP while after 28 days the higher activity peak was due to MiP.

3.3 Pleurotus sp. on OMW containing straw substrates

Wheat straw substrates or substrates supplemented with 50% OMW were inoculated with adapted fully colonized spawn without or with 15% olive mill waste. The ligninolytic enzymes activities were measured during 60 days. The *Pleurotus* strains cultivated on wheat straw showed lower levels of laccase activity (Fig. 6a) than when they were cultivated on the substrate supplemented with OMW (Fig. 6b). In both type of substrates, most of strains showed a maximum of laccase activity after 10 days except *P. ostreatus* 1111 and *P. pulmonarius* 2204. The latter strains showed a laccase maximum after 15 cultivation days. The maxima of total peroxidase activities appeared in all the analyzed strains and in both substrate types after the laccase peak. MnP accounted for almost 100% of the total POD activity in all the studied strains since the levels of MiP were insignificant during the complete cultivation time and independent of the substrate type. When the strains were cultivated on control substrate, except for *P. ostreatus* 2171 (POD peaked after 15 incubation days), most of the strains showed a POD maximum after 20 days and *P. ostreatus* 1111 and K-15 after 25 days (Fig. 7a). On wheat straw supplemented with OMW, all cultivated strains showed more POD activities than on control substrates (Fig. 7b). *P. ostreatus* 2171 and *P. pulmonarius* 2204 showed a maximum of activity at the same incubation day than when they were cultivated on control substrate (respectively 15 and 20 days) but higher POD levels were measured in *P. ostreatus* 2171 at the days following the peaking and *P.*

pulmonarius 2204 doubled its POD activity at the maximum level. The rest of strains showed a time shift in the activity peaking, POD activity of *P. ostreatus* K-15 and 2191 was maximal in this substrate after 30 incubation days but P. *pulmonarius* P-17 anticipated the POD secretion 5 days before its production on control substrate.

4. Discussion

The mechanisms for degradation of lignin-containing substrates followed by *Pleurotus* and other white rot fungi is still not completely understood but apparently mushrooms need the combination of several enzymes to effectively degrade such a complex material. All *Pleurotus* strains were able to colonize and grow on media containing up to 30% OMW suggesting that they were able to synthesize and secrete ligninolytic enzymes. The faster or slower growth could be caused by the higher or lower activities depending on the isoforms synthesized by the different strains. It is now well known that some compounds present in a culture medium might induce their synthesis and secretion facilitating media colonization (*i.e.* ferulic acids, veratryl alcohol etc. (Chen et al. 2003; Jaouani et al. 2006)).

Aryl alcohol oxidases were measured but not detected in any of the strains and any of the three cultivation steps, probably because this enzyme only appears when the nitrogen source is nearly exhausted (Gutiérrez et al. 1995). Results suggested that both laccases and peroxidases were synthesized and secreted as they were needed for nutrient mobilization, since their intracellular levels depended only on mushroom strain but extracellular levels correlate with increasing OMW concentrations. This effect was observed in all the studied strains for peroxidase activities although it was less pronounced for the exo-laccases activities of some *P. ostreatus* strains. Some authors mentioned that endo-enzymes were different isoforms than the exoenzymes secreted in the medium (Dittmer et al. 1997). If this was the case, the endo-laccases and –peroxidases were not influenced by the evolving media although in some strains their endo-peroxidases levels seemed to decrease with increasing concentrations of OMW added to the medium. Other results that might be partially in concordance with Dittmer et al. (1997) was the fact that inside the *P. ostreatus* strains more MiP than MnP were detected while outside the hyphae MnP was mostly observed.

The induction of lignolytic exo-enzymes by OMW supplementation was also observed when the *Pleurotus* mycelia were grown on rye grains to produce the spawn, but it was only remarkable on the laccase activities measured on *P. pulmonarius* strains.

Irradiated-OMW supplementation of MMP media or rye grains enhanced similar effects as OMW, but results were more variable between strains suggesting that either the sterilization process yield a more homogeneous material when it is autoclaved than irradiated or that the heat treatment might have modified some OMW compounds transforming them in more powerful ligninolytic enzyme enhancers or inducers. Thus, the use of iOMW was discarded and in the following experiments were performed using only OMW.

The low laccase and peroxidase activities observed for some of the strains cultivated on control or OMW supplemented spawns could be due to the fact that these enzymes are mainly produced in a specific growth stage and usually laccases are produced before peroxidases (Fu et al. 1997; Fenice et al. 2003). When the enzyme production profile was studied during 28 cultivation days on spawns or 60 days on substrates, results confirmed this hypothesis since a maximum of laccase activity was found during only a few days (depending on the strain and depending on whether it was cultivated using a standard formulation as control or supplemented with OMW) followed by an increase in peroxidase activities a few days later.

All selected strains showed higher laccase activity peaks on spawn supplemented with OMW than on control. On the contrary, the peroxidase activities were higher on control than on OMW supplemented spawn and depending on the strains some produced more MnP than MiP or vice versa or first they showed a peak of MiP and later another of MnP. The laccase and peroxidase activities profile when mushrooms were cultivated on substrates were also different than on spawn since higher laccase levels were found on OMW supplemented substrates than in control. POD levels on substrates were similar between control and OMW supplemented substrates for some strains: some produced more POD in control substrates and others more in supplemented substrates. These results might indicate that mushrooms can easily adjust their enzymatic pathways to generate those enzymes better suited to degrade the surrounding environment depending on its precise composition and/or degree of degradation during all the steps of their cultivation and their growth. Thus, there was no need to include OMW in all the cultivation steps for a better adaptation of the mushroom mycelium. Moreover, there was not a specific *Pleurotus* strain which showed the highest laccase or peroxidase activity in the three studied cultivation steps neither a specific strain which produced the highest levels of both enzymes. However, if only the last cultivation step was observed *Pleurotus ostreatus* K-15 could be considered as better suited to grow on OMW supplemented substrates than the others since its showed high levels of both laccase and peroxidase activities. This strain was also identified in previous studies (Ruiz-Rodriguez et al. 2010) as one of the best *Pleurotus* strain able to grow on OMW and to produce good quality fruiting bodies, probably because of the large amount of lignolytic enzymes produced.

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Table 1 – Slopes (mm day^{-1}) of six *Pleurotus* strains obtained by linear regression of mycelium growth between 2 and 7 days (linear growth curve) on petri plates containing MMP media suplemented with 0, 5, 15 and 30% of olive mill waste (OMW) or irradiated OMW (iOMW).

	Pleurotus ostreatus					***Pleurotus pulmonarius***			
	PO 2191	*PO-K15*	*PO 1111*	*PO 2171*	**Average *P. ostreatus***	*PP 2204*	*PP-P17*	***Average P. pulmonarius***	***Average all strains***
MMP	13[a]	12.5[a]	15.0[a]	13.2[a]	**13.4**	16.1[a]	16.6[a]	**16.4**	**14.9**
MMP+5% OMW	17.4[b]	17.9[b]	17.3[b]	16.3[b]	**17.2**	18.4[b]	18.1[b]	**18.3**	**17.7**
MMP+15% OMW	13.6[a]	14.4[a]	13.8[c]	15.8[b]	**14.4**	16.0[a]	14.5[c]	**15.3**	**14.8**
MMP+30% OMW	11.8[c]	13.2[a]	12.0[c]	12.7[a]	**12.4**	11.6[c]	12.5[c]	**12.1**	**12.2**
MMP+5% iOMW	11.7[c]	14.4[a]	16.3[a]	13.4[a]	**14.0**	16.4[a]	16.6[a]	**16.5**	**15.2**
MMP+15% iOMW	14.7[a]	15.4[a]	16.0[a]	14.5[a]	**15.2**	17.9[b]	16.3[a]	**17.1**	**16.1**
MMP+30% iOMW	13.9[a]	14.1[a]	15.7[a]	13.6[a]	**14.3**	16.0[a]	14.9[c]	**15.5**	**14.9**

Values are the mean of three separate experiments. [a,b,c] Different superscript denotes statistically significant differences ($p<0.05$) among data in the same column.

Table 2 – Fresh weight and percentage of dry weight of six *Pleurotus* strains after 9 days of incubation on petri plates containing MMP media suplemented with 0, 5, 15 and 30% of olive mill waste (OMW) or irradiated OMW (iOMW).

	Pleurotus ostreatus					***Pleurotus pulmonarius***			
Growth Media	*PO 2191*	*PO-K15*	*PO 1111*	*PO 2171*	***Average***	*PP 2204*	*PP-P17*	***Average***	***Average all strains***
MMP	0.48^{a} (12.5%)	0.63^{a} (9.5%)	0.31^{a} (12.9%)	0.30^{a} (6.7%)	**0.43 (10.4%)**	0.79^{a} (11.4%)	0.58^{a} (13.8%)	**0.69 (12.6%)**	**0.56 (11.5%)**
MMP+5% OMW	0.37^{b} (16.2%)	0.56^{a} (12.5%)	0.35^{a} (17.1%)	0.48^{c} (14.6%)	**0.44 (15.1%)**	0.50^{b} (12.0%)	0.55^{a} (12.7%)	**0.53 (12.4%)**	**0.48 (13.7%)**
MMP+15% OMW	0.57^{c} (12.3%)	0.54^{a} (14.8%)	0.48^{c} (14.6%)	0.67^{c} (14.9%)	**0.57 (14.2%)**	0.83^{a} (9.6%)	0.72^{c} (9.7%)	**0.78 (9.7%)**	**0.67 (11.9%)**
MMP+30% OMW	0.57^{c} (12.3%)	0.45^{b} (13.3%)	0.47^{c} (12.8%)	0.50^{c} (14.0%)	**0.50 (13.1%)**	0.46^{b} (15.2%)	0.70^{c} (11.4%)	**0.58 (13.3%)**	**0.54 (13.2%)**
MMP+5% iOMW	0.36^{b} (13.9%)	0.37^{b} (13.5%)	0.29^{a} (17.2%)	0.35^{a} (17.1%)	**0.34 (15.4%)**	0.52^{b} (11.5%)	0.43^{a} (16.3%)	**0.48 (13.9%)**	**0.41 (14.7%)**
MMP+15% iOMW	0.51^{a} (13.7%)	0.52^{a} (11.5%)	0.50^{c} (14.0%)	0.54^{c} (11.1%)	**0.52 (12.6%)**	0.54^{b} (9.3%)	0.63^{a} (11.1%)	**0.59 (10.2%)**	**0.55 (11.4%)**
MMP+30% iOMW	0.44^{a} (13.6%)	0.63^{a} (12.7%)	0.40^{a} (12.5%)	0.50^{c} (12.0%)	**0.49 (12.7%)**	0.42^{b} (11.9%)	0.44^{a} (13.6%)	**0.43 (12.8%)**	**0.46 (12.7%)**

Values are the mean of three separate experiments. ^{a,b,c} Different superscript denotes statistically significant differences ($p<0.05$) among data in the same column.

Fig. 1: A) Intra- and B) extracellular laccase activities in six *Pleurotus* sp. strains cultivated on MMP control medium or MMP media supplemented with OMW or iOMW

Fig. 2: A) Intra- and B) extracellular total peroxidase activities in six *Pleurotus* sp. strains cultivated on MMP control medium or MMP media supplemented with OMW or iOMW.

Fig. 3: A) Laccase and B) total peroxidase activities in six *Pleurotus* sp. strains cultivated on rye grains (spawn) supplemented with 0, 5, 15 or 30% OMW or iOMW.

Fig. 4: Laccase activities in six *Pleurotus* sp. strains cultivated on rye grains (spawn) supplemented with a) 0 or b) 15% OMW during 28 days.

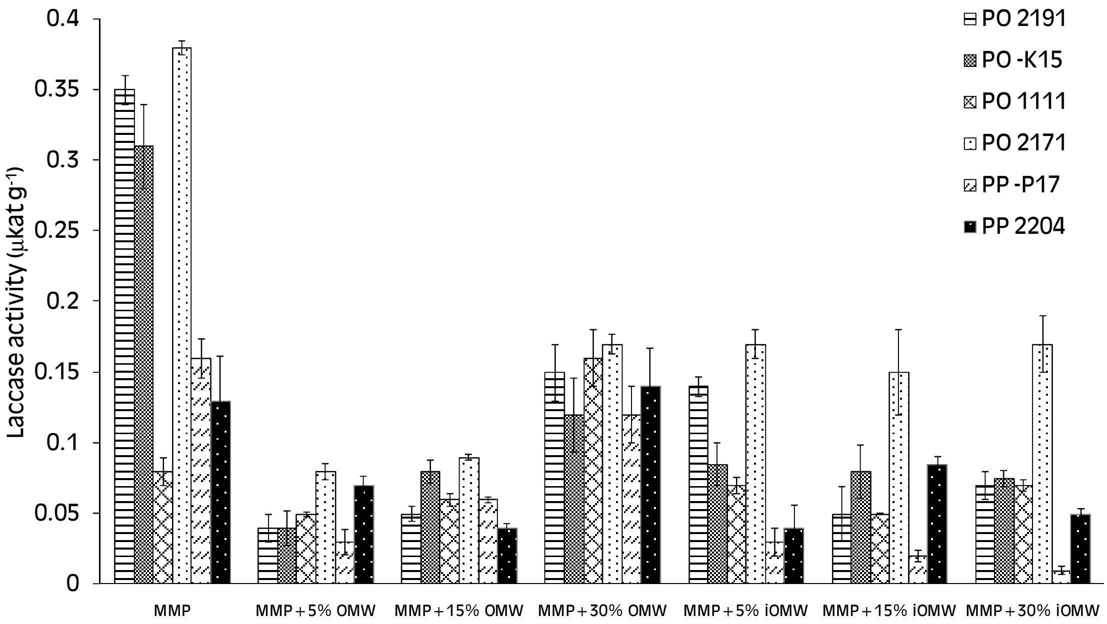
Fig. 5: Total peroxidase activities in six *Pleurotus* sp. strains cultivated on rye grains (spawn) supplemented with a) 0 or b) 15% OMW during 28 days.

Fig. 6: Laccase activities in six *Pleurotus* sp. strains cultivated on wheat straw substrates supplemented with a) 0 or b) 50% OMW during 60 days.

Fig. 7: Total peroxidase activities in six *Pleurotus* sp. strains cultivated on wheat straw substrates supplemented with a) 0 or b) 50% OMW during 60 days

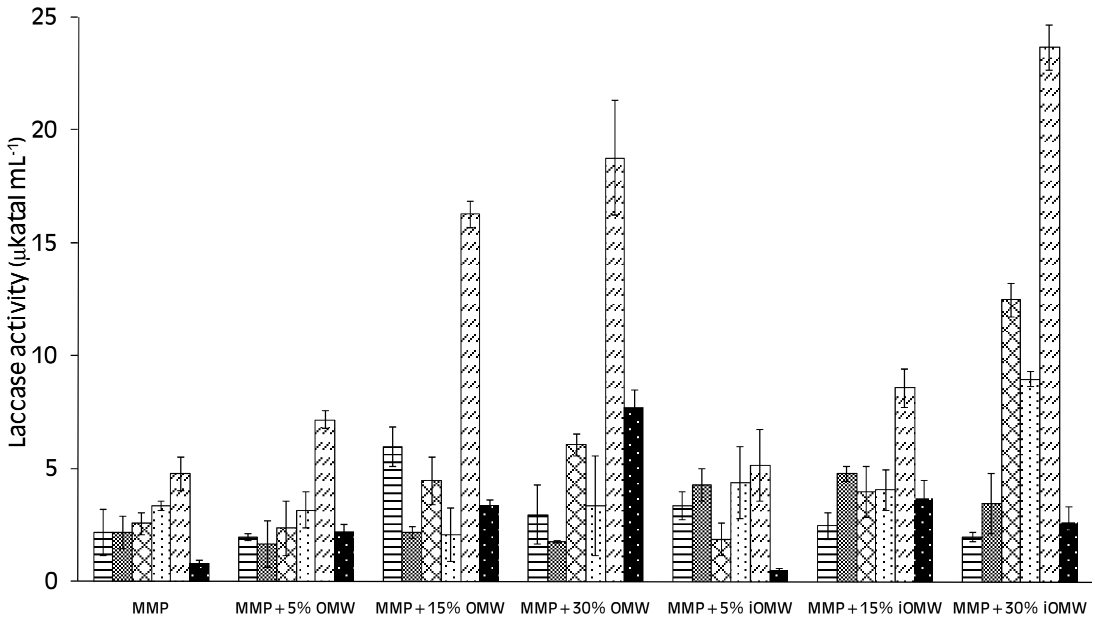
Figure 1:

A)



Laccase activity (μkat g⁻¹)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
PO 2191
PO -K15
PO 1111
PO 2171
PP -P17
PP 2204
MMP
MMP+5% OMW
MMP+15% OMW
MMP+30% OMW
MMP+5% iOMW
MMP+15% iOMW
MMP+30% iOMW

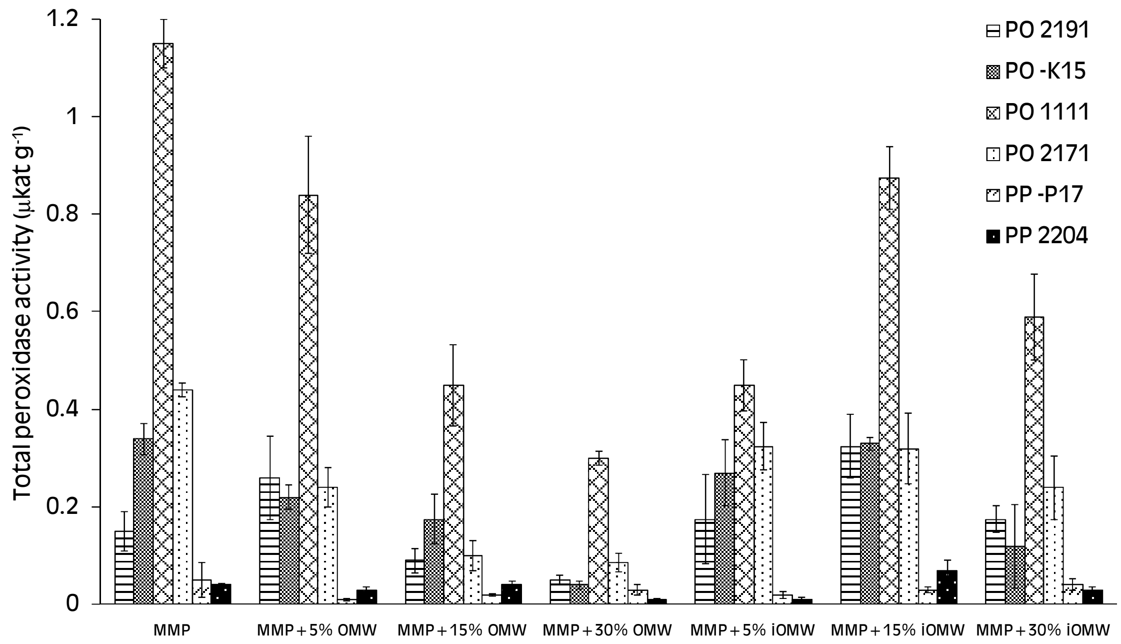
B)



Laccase activity (μkatal mL⁻¹)
25
20
15
10
5
0
MMP
MMP+5% OMW
MMP+15% OMW
MMP+30% OMW
MMP+5% iOMW
MMP+15% iOMW
MMP+30% iOMW

Figure 2:

A)


Total peroxidase activity (µkat g-1)
1.2
1
0.8
0.6
0.4
0.2
0
PO 2191
PO -K15
PO 1111
PO 2171
PP -P17
PP 2204
MMP
MMP+5% OMW
MMP+15% OMW
MMP+30% OMW
MMP+5% iOMW
MMP+15% iOMW
MMP+30% iOMW

B)

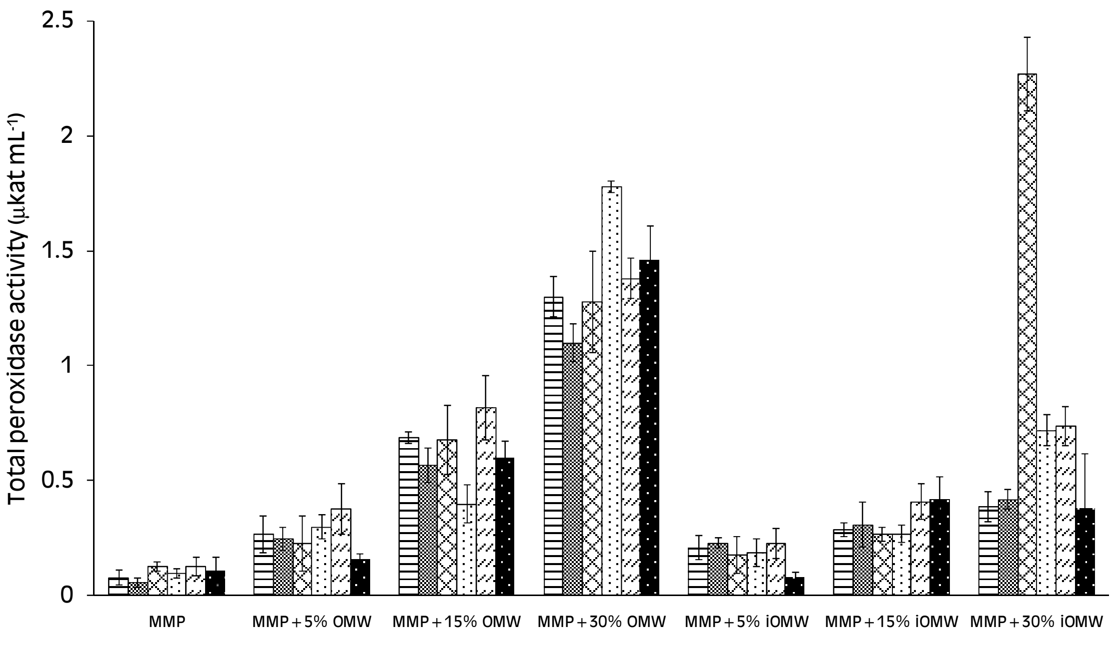
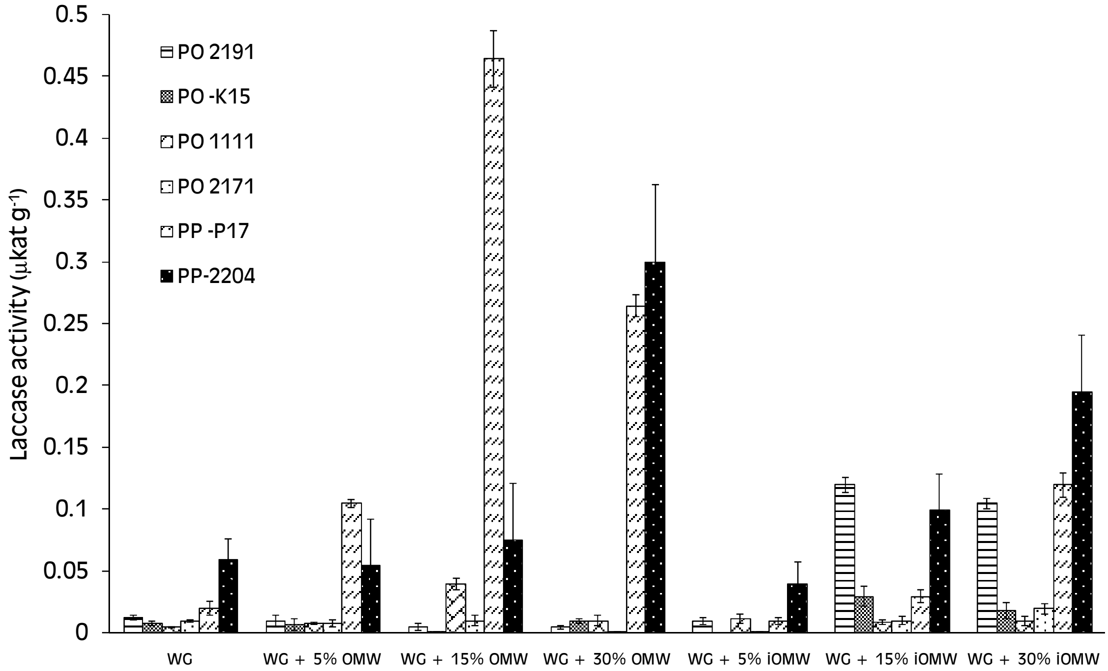

Total peroxidase activity (µkat mL-1)
2.5
2
1.5
1
0.5
0
MMP
MMP+5% OMW
MMP+15% OMW
MMP+30% OMW
MMP+5% iOMW
MMP+15% iOMW
MMP+30% iOMW

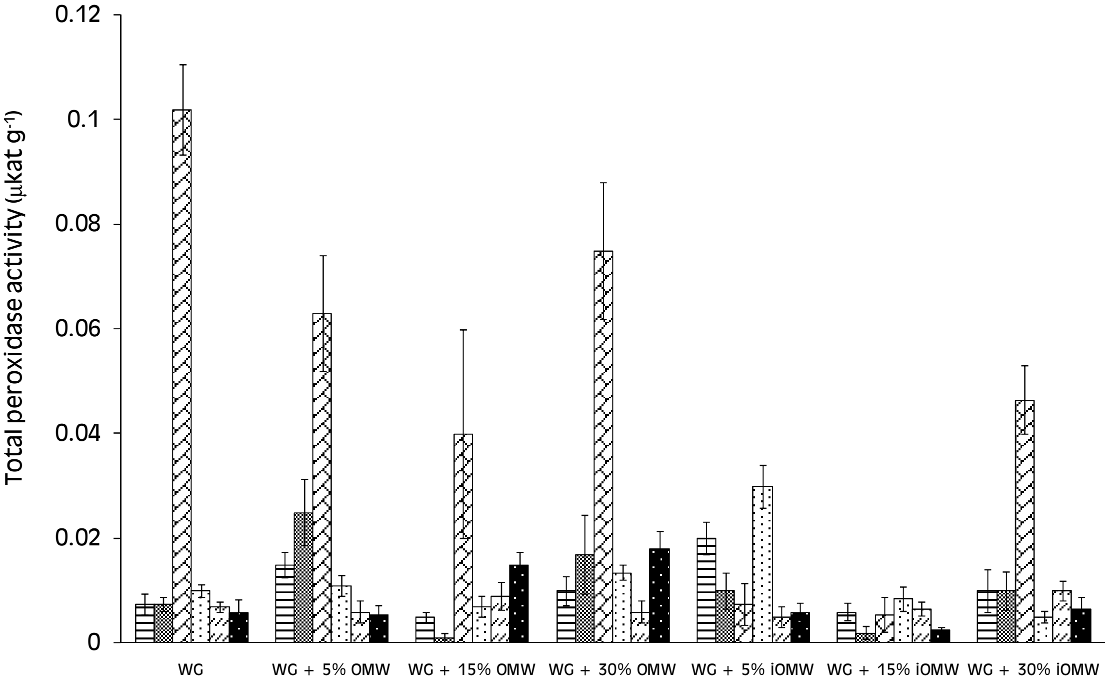
Figure 3:

A)



Laccase activity (μkat g⁻¹)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
PO 2191
PO -K15
PO 1111
PO 2171
PP -P17
PP-2204
WG
WG + 5% OMW
WG + 15% OMW
WG + 30% OMW
WG + 5% iOMW
WG + 15% iOMW
WG + 30% iOMW

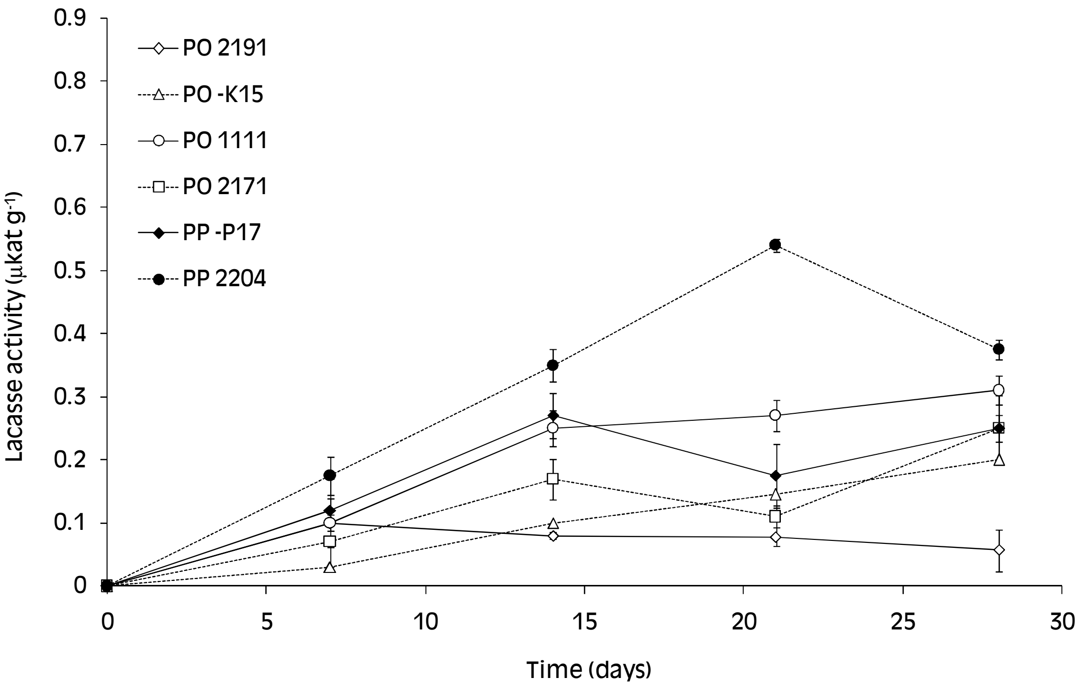
B)



Total peroxidase activity (μkat g⁻¹)
0.12
0.1
0.08
0.06
0.04
0.02
0
WG
WG + 5% OMW
WG + 15% OMW
WG + 30% OMW
WG + 5% iOMW
WG + 15% iOMW
WG + 30% iOMW

Figure 4:

A)


PO 2191
PO -K15
PO 1111
PO 2171
PP -P17
PP 2204
Lacasse activity (μkat g⁻¹)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
25
30
Time (days)

B)

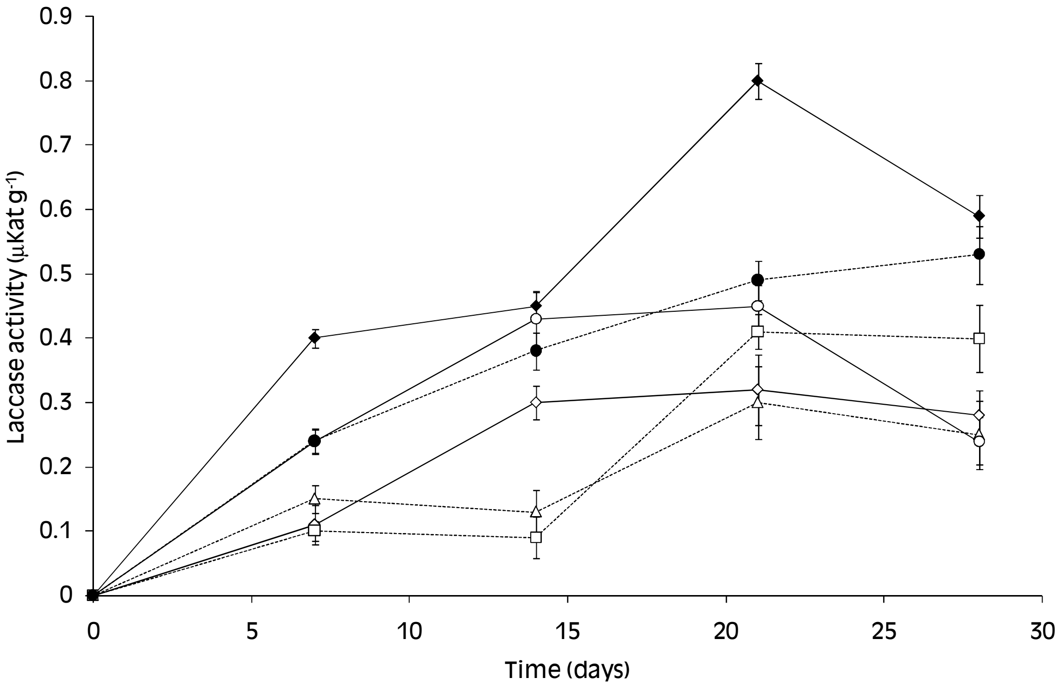
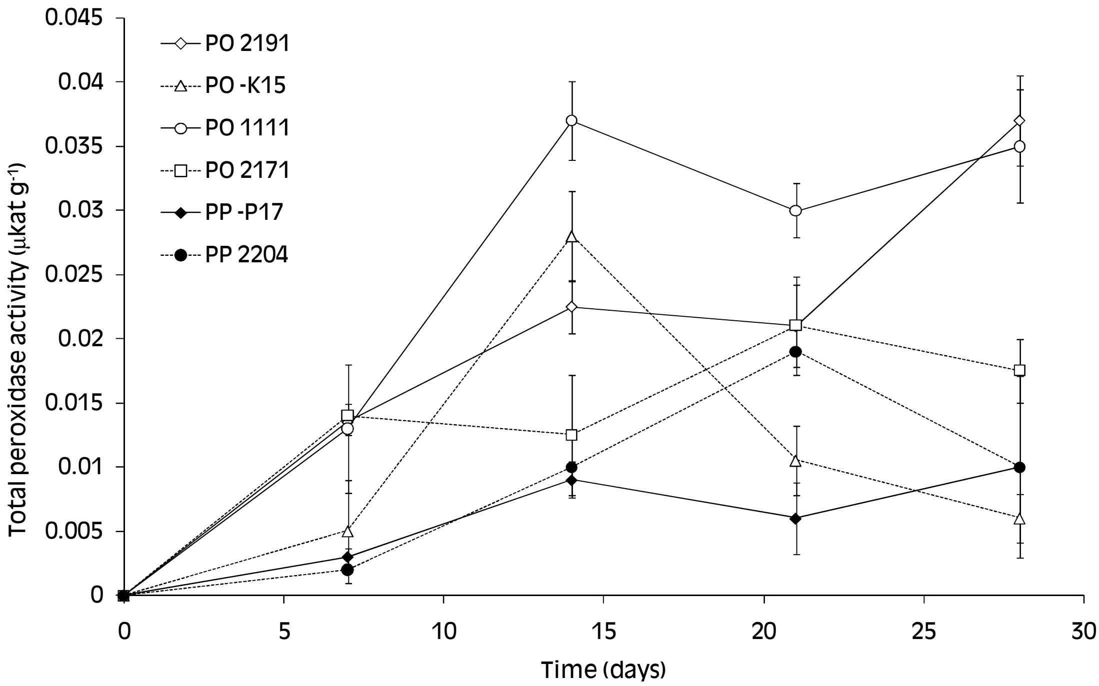

Laccase activity (μkat g⁻¹)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
25
30
Time (days)

Figure 5:

A)


PO 2191
PO -K15
PO 1111
PO 2171
PP -P17
PP 2204
Total peroxidase activity (µkat g-1)
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
5
10
15
20
25
30
Time (days)

B)

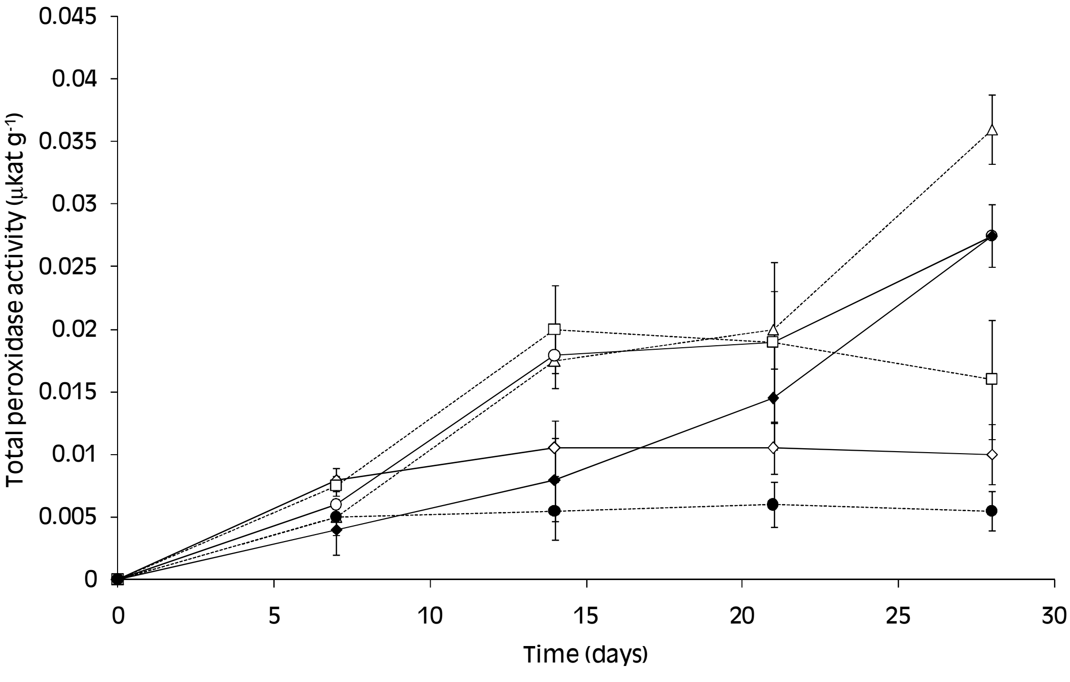
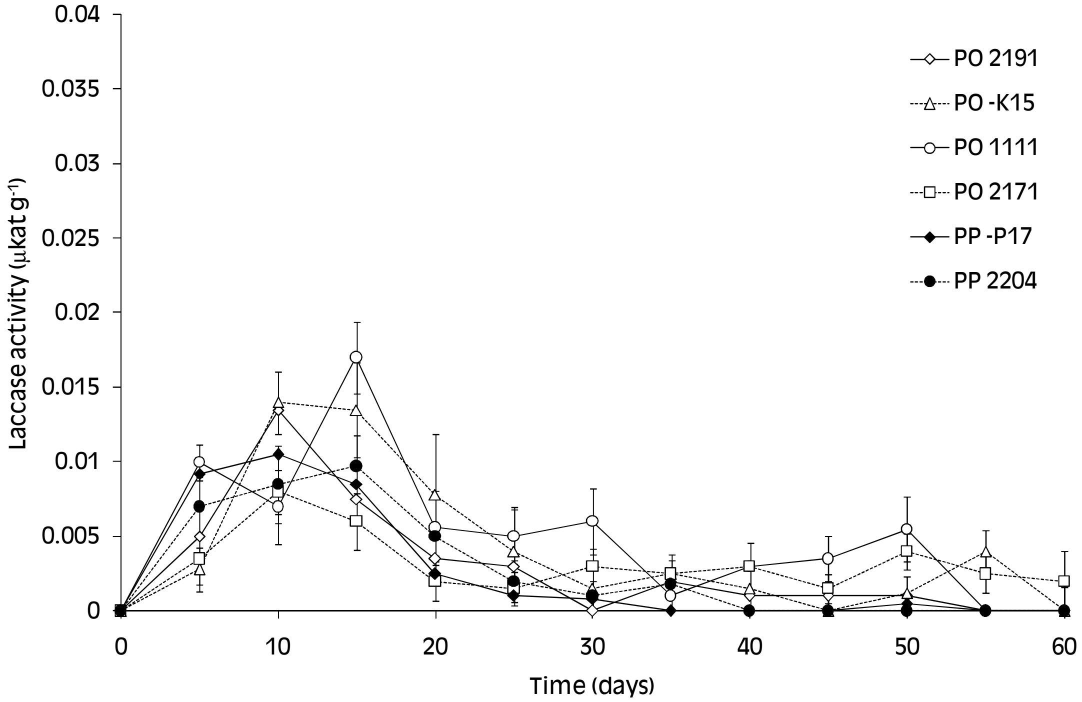

Total peroxidase activity (µkat g-1)
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
5
10
15
20
25
30
Time (days)

Figure 6:
A)


Laccase activity (µkat g-1)
Time (days)
PO 2191
PO -K15
PO 1111
PO 2171
PP -P17
PP 2204
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
10
20
30
40
50
60

B)

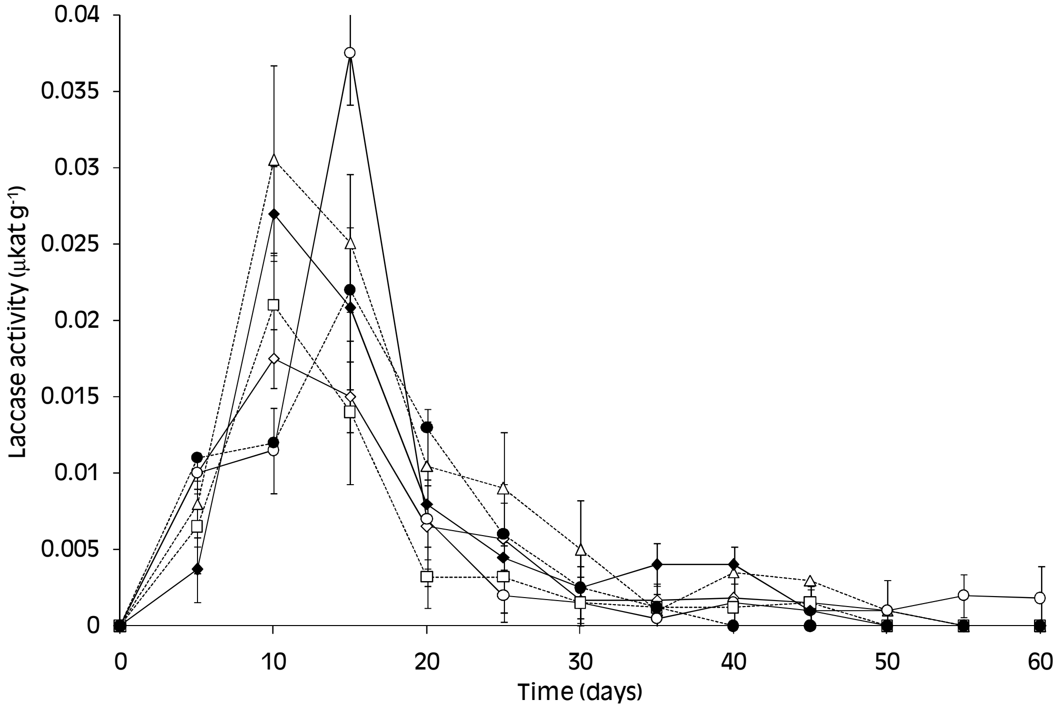
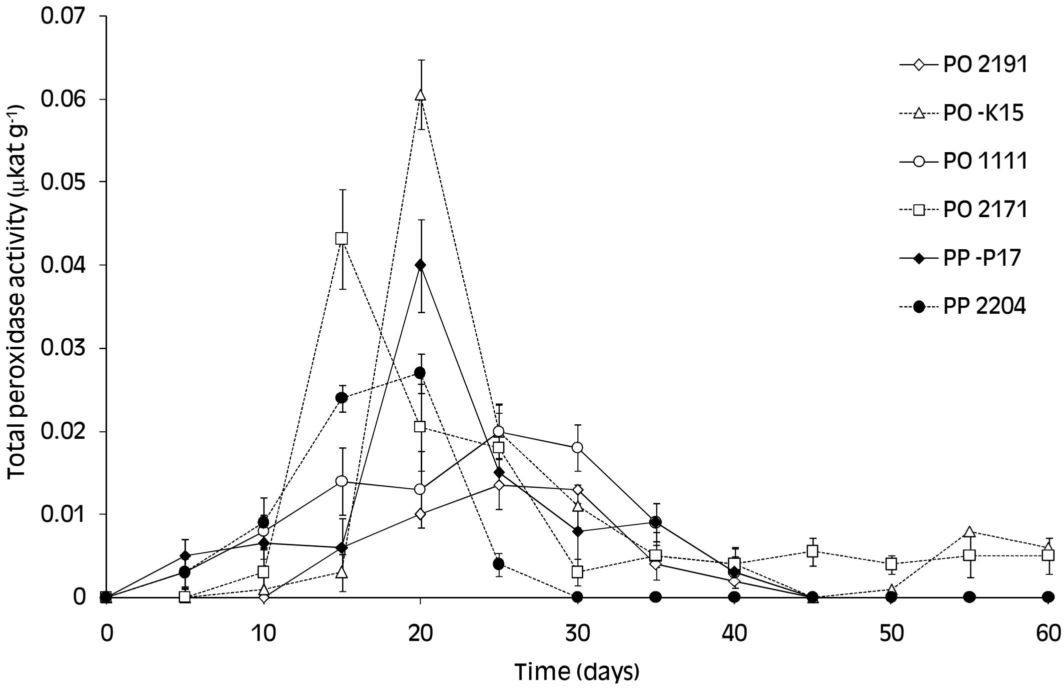
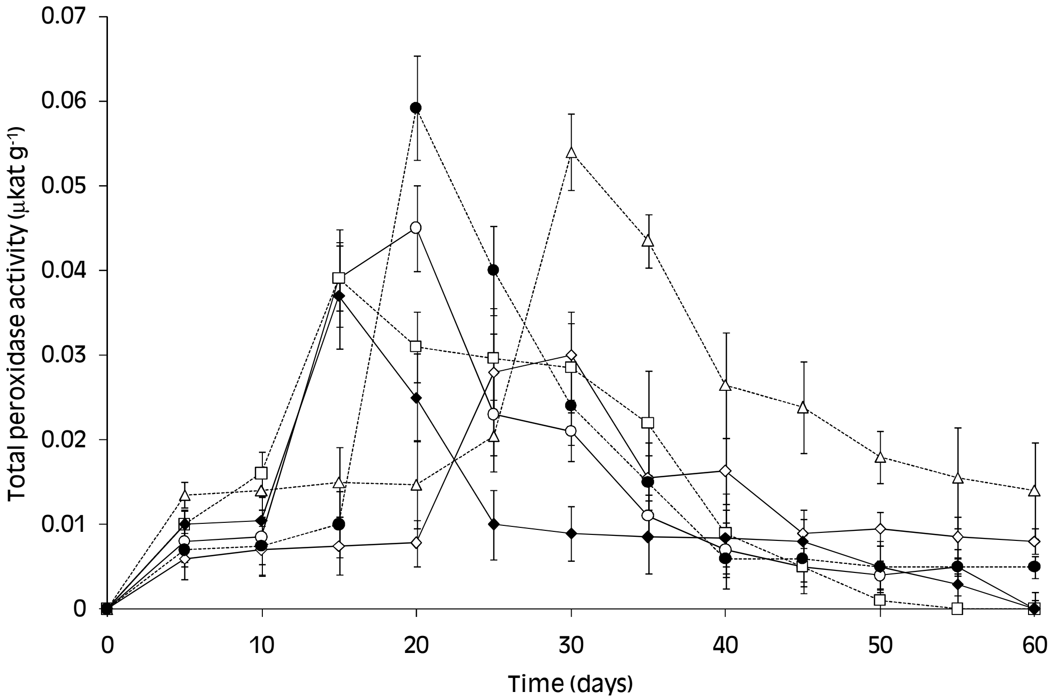

Laccase activity (µkat g-1)
Time (days)
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
10
20
30
40
50
60

Figure 7:

A)


0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Total peroxidase activity (μkat g-1)
0
10
20
30
40
50
60
Time (days)
PO 2191
PO -K15
PO 1111
PO 2171
PP -P17
PP 2204

B)


0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Total peroxidase activity (μkat g-1)
0
10
20
30
40
50
60
Time (days)