

# EVALUATION OF *Pleurotus ostreatus* BASIDIOMES PRODUCTION ON *Pinus* SAWDUST AND OTHER AGRICULTURAL AND FORESTRY WASTES FROM PATAGONIA, ARGENTINA

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## ABSTRACT

The basidiomes production of two strains of *Pleurotus ostreatus* (one native from Patagonia, Argentina, isolated from the conifer *Araucaria araucana* and the other one a commercial strain) grown in different agricultural and forestry wastes was studied. The ability of the native strain generates interest to know its degradative capabilities for its use with conifer waste from the regional forest industry, which is dominated by the use of exotic *Pinus* species. In addition, white poplar (*Populus alba*), southern beech (*Nothofagus pumilio*), wheatgrass (*Thinopyrum ponticum*) and residues from beer brewing were also explored as substrates. The native strain showed better productivity of basidiomes with a biological efficiency of 17,86 % ± 3,74 % on pine wood shavings in comparison with the commercial strain used as control. The best substrates were poplar and wheatgrass with biological efficiency between 35,28 % to 88,5 % and 27,84 % to 84,01 %, respectively. Supplemented substrates showed better productivity than those without supplement. The low biological efficiency on pine could be attributed to the aromatic compounds contents because, in growth test, the diameter of the fungal colony was affected negatively by the addition of pine resin volatiles, mainly with  $\alpha$ -limonene and  $\delta$ -3-carene. In spite of the low biological efficiency, promising inter-breeding or hybrids generation could improve yields in future assays.

**Keywords:** Edible mushroom, pine, sawdust, spent beer grain, wood wastes.

## INTRODUCTION

Leftover agricultural and forest material can be used for edible mushroom production. The accumulation of lignocellulosic residues from cultivated areas is usually a problem because they generate large amounts of biomass that are disposed of by burning in fields (El-Haggag 2007) can be toxic. This modifies the phosphorous content in the soil, generating water quality issues such as excess nutrient and sodium concentrations from leachates. Moreover, it changes pH in soil for some chemical compounds like phenols (Bonanomi *et al.* 2006a), where it may be harmful for beneficial microbes and pollinators affecting growth of some crops (Ramírez-García *et al.* 2019) or can be a source of pathogenic organisms (Bonanomi *et al.* 2006b). However, all these waste products constitute a huge source of energy that could be exploited by xylophagous fungi, which use peroxidases and Cu-dependent laccases in the case of white-rot fungi and iron (Fenton reaction) in the case of brown-rot fungi, for degradation of aromatic chemical compounds (Kubecik 2013). White-rot fungi such as *Pleurotus ostreatus* are capable of degrading lignin until its complete mineralization due to the extracellular enzymatic groups they secrete (Chang and Miles 2004), such as ligninperoxidases, manganeseperoxidases and laccases. Ligninolytic enzyme production with emphasis in the induction of laccase, has been studied in other genera of white rot fungi as *Trametes* (Hess *et al.* 2002) and *Ganoderma* (Kuhar and Papinutti 2014) due to its efficiency as lignin degraders, using metals such as copper.

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*Pleurotus ostreatus* is worldwide cultivated since many years. In Argentina, it has been cultivated for more than 30 years, using principally wheat straw with annual production (Lechner and Albertó 2011). In the Río Negro valley and Neuquén (Patagonia), it is cultivated on poplar logs in autumn and spring (Albertó *et al.* 2010). The productive yields of *P. ostreatus* depend on the substrate's degradation and the nutrient content (carbon, nitrogen and phosphorus). Generally, commercial cultivation is made with wheat straw or *Salix* sp. and *Populus* sp. sawdust because they are abundant, show shorter growing period, high productivity and also, in the case of straw, allows a process of pasteurization at 60 °C (Yildiz *et al.* 2002, Lechner and Albertó 2011).

There are only few records of *Pleurotus ostreatus* cultivation on resinous substrates, in spite of the interest to use pine sawmills wastes around the world. This is due to usually contain a high concentration of wood extractives or pitch deposits (Croan 2003). Schmidt (1986) obtained basidiomes of *P. ostreatus* in outdoors cultures on softwoods chips of Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) but he did not report yields or productivity data.

Agricultural production in the Patagonian Andean region generates large amounts of wastes. With the increase of beer brewing and wine production, spent beer grains and grape vines and stalks are very abundant and have low cost. These residues have already been tested as substrates or supplements (Wang *et al.* 2001, Lara *et al.* 2002, Lechner and Albertó 2002, Gregori *et al.* 2008). The exploitation of southern beech (*Nothofagus pumilio*) wood to manufacture products (Bava and López Bernal 2006) also generates sawdust or wood shavings. However, the main waste from the northwest Patagonian Andean region are chips, sawdust and shavings from pine plantations, and these also could be used for the cultivation of this mushroom.

*Pleurotus ostreatus* has been found in Argentina fruiting naturally on deciduous trees, mainly from the genera *Populus* and *Salix*, especially on fallen trunks or very old standing trees (Lechner *et al.* 2004). It has also been registered and isolated on the native conifer *Araucaria araucana* (Lechner *et al.* 2002), producing basidiomes and rots. This tree species presents a strong antimicrobial activity, for which reason few species of Basidiomycota and Ascomycota have been reported so far (Céspedes *et al.* 2005). The oleoresin complex (mixture of resins and essential oils) is the primary defense system in conifers and is the main mechanism of resistance against the invasion of insects and pathogens (Paine and Hanlon 1994). *Pleurotus ostreatus* strains growing on *Araucaria araucana* generate interest to know its potential degradative abilities for their eventual use with conifer wastes from the regional *Pinus* forest industry, mainly dominated by *Pinus ponderosa*.

The aim of this work is to explore the ability of a native strain of *P. ostreatus* to grow, degrade and develop basidiomes on substrates generated from conifer debris of the Patagonian forest industry. In addition, we explore poplar (*Populus*), southern beech (*N. pumilio*), wheatgrass (*Thinopyrum*) and residues from beer brewing as potential substrates.

## MATERIALS AND METHODS

### Fungal strains and culture conditions

Two strains of *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm. (Agaricales, Basidiomycota) were used: 1) *P. ostreatus* CIEFAPcc 619 (Fungal Collection of the *Centro de Investigación y Extensión Forestal Andino Patagónico - CIEFAP*): Argentina, Neuquén, Pehuenia, 1 km from Pulmarí gate to Lanín National Park, on *Araucaria araucana* tree, leg. M. Rajchenberg 12517 and M. Rugolo, 2 May 2013. 2) *P. ostreatus* strain CIEFAPcc 129; commercial strain from Italy.

Strains were incubated at 23 °C and kept at 4 °C on malt-extract (ME) agar (1,2 % malt extract, 1 % glucose, and 2 % agar) until use.

### Spawn production

Spawn was produced in 350 ml glass jars filled with boiled oat grains (*Avena sativa*) and 1 % (w/w) CaCO<sub>3</sub>. Jars were sterilized for 1,5 h at 121 °C, cooled and inoculated with a 1 cm diameter plug of mycelium and then they were incubated at 23 °C in the dark for 20 d, with periodical shaking (once every three days).

## Substrate preparation

Polypropylene bags of 25 cm × 15 cm were filled with 100 g (dry weight) of substrate: wood shavings (1 cm × 1 cm) of the regional residues as base substrate: *Pinus ponderosa* (pine), *Populus alba* (white poplar), *Nothofagus pumilio* (southern beech), and *Thinopyrum ponticum* (wheatgrass) supplemented with spent beer grain (malted barley) at 40 % w/w. Humidity was adjusted (w/w) to 75 %. Bags were closed with cotton plugs held by polyvinyl chloride (PVC) cylinders and sterilized in autoclave at 121 °C for 1,5 h (Zadrazil 1978). The control was made with pine wood shavings (*Pinus ponderosa*) residues without supplement.

## Inoculation and basidiomes induction

After cooling, bags were inoculated with 5 % (wet weight) of spawn, and were incubated at 23 °C in the dark until total substrate colonization (25-35 days).

After total colonization by the mycelium, polypropylene bags were opened with small cuts and transferred to the basidiome production rooms. Cropping conditions to induce basidiome formation were 18 °C ± 2 °C, 12 h light/12 h dark photoperiod (2000 lux of fluorescent light), 75 % to 85 % humidity levels and watering by automatic spray provided for 2 min, 3 times every day (modified from Albertó 2008).

Up to three flushes were harvested during the cropping period (45 days between the induction day and the last harvest) in the different substrates. Mature basidiomes (80 % of expanded pileus) were collected manually. Biological efficiency (BE), calculated as the percentage ratio of fresh mushrooms weight over the dry weight of the substrate, was used to test the suitability of the substrates (Rugolo *et al.* 2016).

## Fiber analysis

A representative portion of raw materials and residual substrates were dried, ground (1 mm grid size) and pooled. Samples were studied for their fiber fractions (hemicellulose, cellulose and lignin), extractives (gums, tannins, resins and latexes among others) and mineral composition (carbon, nitrogen and phosphorous). Fibers analyses were done by the Van Soest acid detergent fiber method (Van Soest 1963) employing an automated Fiber Analyzer (Ankom A2000) with filter bags F57 and mineral composition was obtained using inductively coupled plasma mass spectrometry.

## Volatile compounds effects on growth

The effects of individual volatile compounds on the growth of the native *P. ostreatus* strain were determined using a modification of López *et al.* (2013) methods. Five volatile compounds commonly found in *Pinus* sp. were assayed, including  $\alpha$ -limonene, 4-carvomenthenol,  $\delta$ -3-carene,  $\beta$ -citronellol, and *p*-cymen-8-ol purchased from Pherotech International Inc. (Delta, BC, Canada). Malt Extract Agar (MEA) medium was used as control and MEA medium with 0,1 mM of copper (CuSO<sub>4</sub> Sigma-Aldrich) was used as promotor (modified from Kuhar and Papinutti 2014).

## Statistical treatments

For basidiome production, each treatment consisted of ten replicates. Analysis of variance (ANOVA) was used with BEs measurements and significant differences between treatments were compared by Tukey's test at 5 % level probability. Kruskal Wallis non parametric method was used when normality (Shapiro Wilks) or homogeneity (Levene) did not comply. Statistical treatments were tested with InfoStat software (InfoStat 2017).

# RESULTS

## Waste nutritional composition and basidiome production

The values for carbon, nitrogen and phosphorus of different local agricultural and forest waste by-products are shown in Table 1. The C, N and P values were similar between the wastes of beer production (spent beer grain) and the industrial wheat bran (traditionally used as supplement), indicating that this residue is an economic alternative for the formulation of substrates, contributing N and P principally (Table 1). In the case of

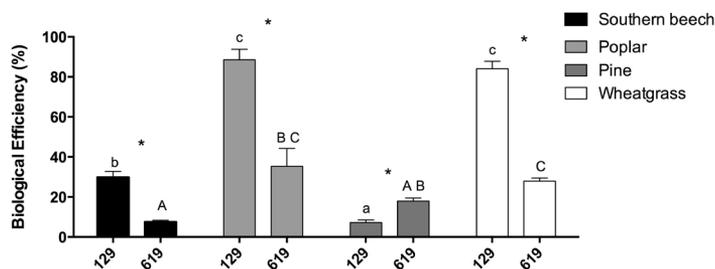
base substrate, C content was similar in all materials, the N content was higher in pine sawdust (0,264 %) and wheatgrass (0,208 %) in comparison with poplar (0,138 %) and southern beech sawdust (0,131 %); whereas P was higher in wheatgrass and poplar content.

**Table 1:** Nutritional composition (C, N and P) of different local agricultural waste by-products.

Substrate	Carbon (%)	Nitrogen (%)	Phosphorus (mg/kg)
White poplar sawdust	45,60	0,138	176
Southern beech sawdust	47,53	0,131	88
Pine sawdust	47,40	0,264	68
Wheatgrass	43,98	0,208	170
Spent beer grain	46,46	2,898	5190
Wheat bran (extra regional)	42,90	2,702	5892

The commercial strain of *Pleurotus ostreatus* CIEFAPcc 129 was more productive than CIEFAPcc 619 native strain in white poplar, wheatgrass, and southern beech substrates ( $p < 0,05$ ). Biological efficiency was over 80 % with poplar ( $88,5 \% \pm 12,9 \%$ ) and with wheatgrass ( $84,01 \% \pm 9,32 \%$ ), and close to 30 % with southern beech ( $29,92 \% \pm 6,96 \%$ ), while the native strain produced BE close to 40 % in white poplar ( $35,28 \% \pm 21,9 \%$ ) and in wheatgrass ( $27,84 \% \pm 3,98 \%$ ) and close to 10 % in southern beech ( $7,56 \% \pm 2,05 \%$ ) (Figure 1).

However, in the treatment with pine as a base substrate, the strain with the highest production was the native CIEFAPcc 619, with a BE of  $17,86 \% \pm 3,74 \%$  in comparison with CIEFAPcc 129 which achieved a BE of  $7,15 \% \pm 3,14 \%$  ( $p < 0,05$ ) (Figure 1). On the other hand, in the substrate without supplement, the mycelium grew fully in 30-40 days, but there was not development of basidiomes.

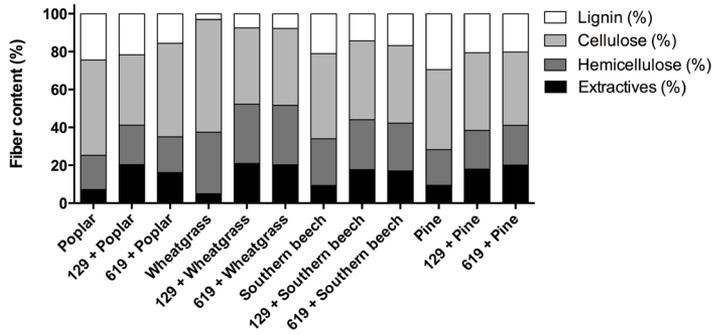


**Figure 1:** Biological efficiency of *Pleurotus ostreatus* cultivated on different local substrates

Different capital letters indicate significant differences between the substrates used with CIEFAPcc 619 strain ( $p < 0,05$ ). Different lowercase letters indicate significant differences between the substrates used with CIEFAPcc 129 strain ( $p < 0,05$ ). Asterisks indicate significant differences between strains CIEFAPcc 129 and CIEFAPcc 619 for each substrate ( $p < 0,05$ ).

### Fiber analysis of the substrates after crop period

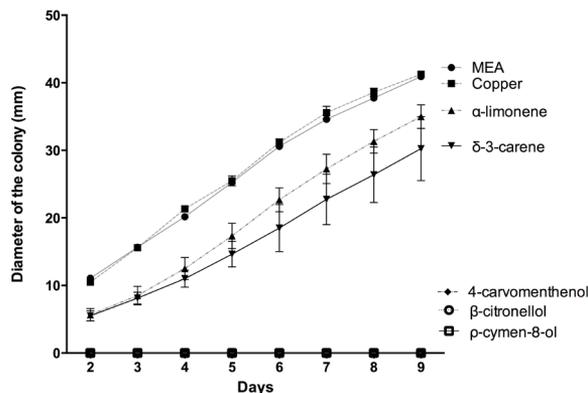
The proportional of lignin, cellulose and hemicellulose was lower after degradation by both strains in white poplar, while in southern beech and pine the degradation was greater on lignin. In wheatgrass, the degradation was mainly on cellulose. In all substrates, the percentage of extractives (gums, tannins, resins and latexes among others) increased after fungal degradation (Figure 2).



**Figure 2:** Fiber analysis of lignin, cellulose, hemicellulose and extractives (gums, tannins, resins and latexes) of different substrates after *P. ostreatus* degradation. The control was the substrate without fungal colonization.

### Volatile compounds effects

The diameter of the fungal colony was negatively affected by the addition of resin volatiles. The treatment with copper ( $\text{CuSO}_4$ , 41,25 mm  $\pm$  0,5 mm) in the evaluated concentration did not show differences with the MEA medium control (40,91 mm  $\pm$  0,14 mm) after 9 days. However, it was lower in treatments with  $\alpha$ -limonene (35,00 mm  $\pm$  3,04 mm) and  $\delta$ -3-carene (30,25 mm  $\pm$  6,72 mm); and null with 4-carvomenthenol,  $\beta$ -citronellol and  $p$ -cymen-8-ol (Figure 3).



**Figure 3:** Mycelium growth in MEA medium with copper ( $\text{CuSO}_4$ ) and volatile compounds addition.

### Discussion

The studied residues of agricultural and forest wastes from Patagonia were found to be useful for the production of *Pleurotus ostreatus* basidiomes. The highest yields were obtained with a commercial strain, except in pine substrate, where the native strain showed better mycelium growth and productive yields. When pine was used, the BEs reached by the native strain was 30 % higher than the commercial strain used, as expected. Although these BEs were less than 50 %, minimum value suggested for a profitable production (Patra and Pani 1995), the use of sawdust and shavings of the most commonly used wood in Patagonia, would allow the generation of high value food from a high available waste. This result allows to propose new assays with some additional additive to fine-tune the substrate and obtain higher yields, or look for promising crosses to take advantage of the growth capacity in resinous substrates of the local strains. Previously, different Mexican strains obtained for monosporic inter-breeding of *P. ostreatus* were cultivated in pine wood shaving, with biological efficiencies between 27,98 % to 53,53 % (Pérez Merlo and Mata 2005). In the present work, the best substrate to both strains was white poplar sawdust. However, the disposition of poplar wastes were lower than

the others. Regarding the substrate, in general, willow shavings or wheat straw were the best substrates for the cultivation of *Pleurotus* (Lechner and Albertó 2011) in comparison with hardwoods or resinous woods. Wheat straw requires a shorter period of fermentation and fewer food supplements because it contains high cellulose content, 39 % to 51% (Yildiz *et al.* 2002).

The residues of the brewing production were suitable supplements for edible mushrooms production and could easily be replaced by the traditional wheat bran. In addition to the yields obtained with this supplement, the analysis of the nutrient content shows that the spent beer grain presented 2,89 % of nitrogen against 2,70 % of wheat bran and 5190 mg/kg against 5892 mg/kg of phosphorus. White poplar sawdust and wheatgrass seems to be the best option for the production of *P. ostreatus* in Patagonia. For both mycelium growth and basidiomes, development on lignin cellulose material within C/N value of 50/1 are sufficient (Block *et al.* 1958). Straw substrate has proved in practice to be the simplest nutrient base (Zadražil 1978) with low percentage of lignin content and nitrogen level (Philippoussis *et al.* 2001). Addition of the supplements with basal substrate has been a common practice to shorten the crop period and enhance the yield, nutritional and medicinal values (Naraian *et al.* 2016). In our work, when supplemented substrates were rich in N content, colonization was greater and more intense, and this positive relation is consistent with previous reports indicating the positive effect of nitrogen (lower C:N ratio) during *Pleurotus ostreatus* cultivation (Belletini *et al.* 2016). Moreover, the final constitution of spend substrate is altered by the removal of nutrients by *Pleurotus* mycelium. In this process N, K and P macroelements decreased because they are used for basidiomes development (aminoacids, vitamins and other biologically active products) (Zadražil 1978).

In the future, we should evaluate the willow chips made from rods, in replacement of poplar, because *Salix* is more invasive and has a high occurrence in riversides (Datri *et al.* 2016); moreover, *Pleurotus* has been found in willow trunks fruiting naturally (Lechner *et al.* 2004). We could use pruning remains of the poplar trees planted as windbreaker or shavings and sawdust generated in the sawmill processing of this wood because it is important in fruits packaging and furniture manufacturing (García *et al.* 2017, Jovanovski *et al.* 2011).

The EBs on wheatgrass, which averaged 80 %, showed the excellent aptitude of this material as substrate, being similar to reports on wheat straw (Salmones *et al.* 2005, Kumari and Achal 2008, Lechner and Albertó 2011, Jaramillo and Albertó 2013, Garuba *et al.* 2017). This grass could replace the wheat straw traditionally used as cattle forage in the region and with limited availability in comparison with Pampas region (Albertó *et al.* 2010). Southern beech was not a good substrate to *Pleurotus* production (BEs < than 30 %), probably for the recalcitrant compounds present in lignin and extractives fractions (Gallo *et al.* 2019).

The fiber analysis revealed that strains differed in their degradative action depending on the growth medium. This differential action according to the substrate used was also reported by Postemsky *et al.* (2017) with *Ganoderma lucidum* cultivated in wheat straw and sunflower seed husks. Compared with the control substrate in poplar, the cellulose fraction decreases when strain CIEFAPcc 129 acts and the lignin fraction decreases in the case of strain CIEFAPcc 619. The behavior of both strains in wheatgrass showed a marked decrease in cellulose content, and a proportional increase in lignin. When the substrate used was southern beech or pine, both strains degraded lignin principally, decreasing its fraction in relation to cellulose or hemicellulose. This is typical process of a white rot fungus belonging to the type of simultaneous white-rot fungi (Martínez *et al.* 2005, Kubecik 2013). The lignin percentages in these substrates are a very important fraction that displays an inhibitor role for microbial action, protecting hemicellulose and cellulose from fungal attack (Ruiz-Dueñas and Martínez 2009). The extractives fraction, mainly in pine and southern beech also plays a recalcitrant and toxic role, principally due for the resins (Himejima *et al.* 1992) and tannins contents (Serrano *et al.* 2009).

In spite of the low BEs obtained in the pine substrates, we were able to demonstrate that the native strain of *Pleurotus ostreatus* growing on *Araucaria araucana* presents an adaptation to the resinous contents in *Pinus ponderosa* because it presented a better behavior in comparison to the commercial strain used as control. The aromatic fraction compounds could be responsible for the mycelial growth decrease and the low BE obtained when strains were grown in formulations with conifer residues. Precisely, cultures in agar medium showed a growth decrease when they were exposed to different conifer aromatic compounds. This coincided with López *et al.* (2013), who showed that the growth of the lignivorous fungi *Amylostereum areolatum* and *A. chailletii* was adversely affected in the presence of *Pinus ponderosa* and *Pseudotsuga menziesii* essential oils. For *A. areolatum* this had a delay in growth of 86,52 % and 85,28 %, respectively, and for *A. chailletii* it had a delay of 97,1 % and 92,36 %, respectively. However, in the control culture with copper growth was induced, probably due to laccase enzyme induction. This induction by metallic ions has already been demonstrated in numerous works. Studies on *Pleurotus* showed the induction effect of laccase activity with  $\text{CuSO}_4$  150  $\mu\text{M}$  (Palmieri *et al.* 2000) and similar results were obtained with *Coriolus hirsuta* (Koroljova-Skorobogat'ko *et al.* 1998) and

*Neurospora crassa* (Schilling *et al.* 1992). A study with a white-rot *Trametes versicolor* strain, the optimal copper concentration ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) for the highest laccase production was 0,5 mM (Birhanli and Yesidala 2017). In addition, Lorenzo *et al.* (2006) showed that a slight stimulatory effect of heavy metals on laccase production from *T. versicolor* was observed in the presence of  $\text{Ag}^+$ ,  $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  whereas the cultures supplemented with copper sulphate (2 mM) showed the highest values, which is about 9-fold higher than that attained in the control. For other white-rot fungi, as *Ganoderma lucidum*, Kuhar and Papinutti (2014) showed laccase induction with phenolic and metallic inducers (copper, manganese, cadmium, chrome). In this case,  $\text{CuSO}_4$  1mM appeared to be the best inducer, and the effect becomes more marked in combination with ferulic acid. These results clearly showed the positive effect of copper sulphate as an inducer of laccase activity.

## CONCLUSIONS

A native strain of *Pleurotus ostreatus* growing on conifer *Araucaria araucana* had the ability to grow, degrade and develop basidiomes on substrates generated from conifer debris of the regional forest industry. In spite that the aromatic compounds showed growth inhibition, these advances in the cultivation of this edible mushroom on resinous substrates could be optimized to enable the development of large-scale production with higher yields.

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