Agro-food waste employed to design and develop culture media for fungal growth

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Abstract

Aim: Waste from the agricultural and food industries could be used as a source of nutrients in the culture media for fungal growth and thus reduce environmental pollution and the cost of bioprocesses. In this work, it was determined if some agro-food waste can improve the mycelial growth of fungi and laccase production.

Methodology: Culture media with agar and vermicompost, vermiwash, calcined pork bones or fish waste for mycelial growth of fungi were elaborated. The laccase activity of each strain grown in all culture media was also estimated.

Results: *Pleurotus eryngii*, *Lentinula edodes*, *Pleurotus ostreatus*, *Flammulina velutipes*, *Pleurotus citrinopileatus* and *Ganoderma lucidum* grew in all culture media, except *Lentinula edodes*, obtaining in general higher biomass and radial growth rate than those observed on potato-dextrose agar. The maximum laccase activity was found in *Pleurotus citrinopileatus* cultivated in fish waste medium. The C: N ratio, ashes and pH were important factors for the growth of the strains.

Interpretation: There were significant differences in biomass concentration and laccase activity in the designed culture media. These results provide an interesting alternative for the use of wastes and metabolites production of industrial interest.

Key words: Agro-waste, Calcined bones, Fungi, Laccase activity, Vermicompost

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Introduction

Food waste includes inedible parts that in many cases represent a serious contamination problem due to their high production volumes and rich chemical composition. In most cases they have no application. Wastes from the agro-food industries are a potential ingredient for the development of fungal culture media since there is an increasing demand of economics culture media, obtained from renewable sources with similar efficiency to the commercial media. In this sense, the vermicompost, calcined pork bones and fish waste can be an alternative to obtain fungal biomass and enzymes. Vermicompost are effective for the growth of mushrooms such as *Agaricus blazei* (Matute et al., 2011) as they can improve the mycelium production capacity. The vermiwash has been used for the plant growth and for controlling some diseases caused by fungi (Pant et al., 2011). Calcined bones are rich in phosphorus (Saeid et al., 2014) and can be a source of micronutrients. Crustacean waste and fish waste have been used for microbial growth (Naraian et al., 2014; Choi et al., 2011; Basu et al., 2008). Oyster shells have been used as nutrient to obtain fruiting bodies of *Pleurotus eryngii* and *Pleurotus florida* (Naraian et al., 2014; Choi et al., 2011). Similarly, starfish powder was found effective for the growth of *Pleurotus eryngii* (Choi et al., 2009). Basu et al. (2008) reported that the scale powder is cheap and can be easily obtained, therefore, would be a promising source of complex nutrient instead of commercial peptone. Naraian et al. (2014) reported that low concentrations of oyster shell powder could be used as an alternative limiting supplement to induce enzymes production and increase the mushrooms production.

Among the enzymes produced by some basidiomycetes are laccases (Karp et al., 2012). Laccase (benzenediol: oxygen oxidoreductase) is an excellent multi-copper containing polyphenol oxidase which acts on various substrates from one laccase to other (Poornima and Velan, 2018). Fungal laccases have been extensively studied due to their relevance in diverse industrial applications including food, paper, textile, cosmetics, chemistry, synthetic nano-biotechnology and bio-remediation (Rodríguez-Couto and Toca-Herrera, 2006). This enzyme has been obtained by cultivating fungi in Petri dishes, solid and submerged fermentation and using synthetic or complex culture media such as agro-industrial wastes, however, it has been observed that their production depends on the fungus type, the physico-chemical conditions of the culture and substrate type used (Poornima and Velan, 2018; Karp et al., 2012; landolo et al., 2011; Sathishkumar et al., 2010). Keeping in view the above, the aim of this study, was to determine if some agro-food waste can improve the mycelial growth of fungi and laccase production.

Materials and Methods

Fungal strains and maintenance: Fungi of industrial interest were used to evaluate their growth in the media designed. The mycelium of fungi *Pleurotus eryngii* C11, *Lentinula edodes* H21, *Flammulina velutipes* A12, *Pleurotus ostreatus* B54, *Pleurotus*

citrinopileatus F43 and *Ganoderma lucidum* D12 were provided by the fungi culture collection of the Biotechnological Processes Laboratory, Universidad Politécnica de Tlaxcala. The fungi were reactivated in Petri dishes (100 mm) containing potato dextrose agar (PDA), incubated at 25 °C for 8 days and used as inoculum for the designed media in this work.

Vermicompost and vermiwash production: Vermicompost was produced to use its nutrients in a culture medium. The vermicompost was made from wheat straw and dried sheep manure (5:1), these materials were piled and composted for 25 days with a mechanical aeration every 5 days. After that time, the organic material was set in wooden boxes of 50 cm³ and inoculated with 1000 red Californians earthworms (*Eisenia fetida*). The boxes were kept at room temperature and the organic matter was kept hydrated with 40% humidity by sprinkling water. The vermicompost was collected after 40 days and dried at 25 °C. The vermiwash was obtained by leaching the vermicompost with deionized water at 25 °C (1:2 ratio), the water was re-circulated twice a day for 1 week to obtain the concentrate.

Calcined pork bones and fish waste: Cortical pig bones were used to develop this experiment. The bones were collected from a slaughter house located in Puebla City, Mexico. The bones were sterilized at 130 °C per 20 min and then boiled in deionized water for 30 min, the remains of meat were removed by hand. The bones were cut into small pieces and dried at 120 °C per 12 hr (Thermo Fisher Scientific, Heratherm OGM) and calcined at 750 °C per 6 hr (Thermo Fisher Scientific, F48015-60). The calcined bones were ground until a fine powder was obtained. Fish waste was also used to design the culture medium. Viscera, heads and skin of tilapia and trout were obtained from a fish market in Puebla City, Mexico. The fish wastes were washed and dried at 80 °C per 24 hr (Thermo Fisher Scientific, Heratherm OGM), and their size was reduced with a mill (Hamilton Beach, 80335R).

Preparation of culture media: Five different media were designed for fungal growth: M1 (20 g l⁻¹ vermicompost, and 15 g l⁻¹ bacteriological agar), M2 (39 g l⁻¹ PDA in vermiwash), M3 (39 g l⁻¹ PDA and 5 g l⁻¹ calcined bones), M4 (20 g l⁻¹ fish waste and 15 g l⁻¹ bacteriological agar) and PDA alone. The media were sterilized and dispensed in Petri dishes (100 mm). The plates were inoculated in the center with 5 mm diameter discs of mycelium and these were incubated at 25 °C until the fungi completely invaded the Petri dish. The radial growth rate was obtained as the slope of graphing the mycelial growth in cm vs. time (hr) of growth. The mycelium was separated from the culture medium according to the previously reported methodology (Suárez-Segundo et al., 2013) and dry biomass (X) was determined by dry weight (AOAC, 1990).

Laccases activity: The enzymatic extract was obtained from the Petri dishes with biomass, 5 ml of deionized water were added to each Petri dish and stirred for 1 hr/100 rpm. In each extract, laccases activity was estimated following the method of Téllez-Téllez et al., (2005), using 2,6-dimethoxyphenol in phosphate

buffer at pH 6.5 as substrate, one enzymatic unit (U) of laccase was defined as the amount of enzyme which increases by 1 unit of absorbance per min in the reaction mixture.

Chemical analysis: The proximal chemical composition of the culture media was determined. Total nitrogen was determined by the Kjeldahl method (Pomeranz and Meloan, 1994) in a digester and distiller (Novatech, KJ6C). Total organic carbon was estimated by the rapid titration method of Walkley and Black (1934). Fat concentration was determined through extraction with petroleum ether in an equipment Soxhlet (Novatech, VH-6) and the concentration of ashes was determined by calcination of the samples at 550 °C per 5 hr (Thermo Fisher Scientific, F48015-60) (Pomeranz and Meloan, 1994). The pH was determined with a digital pH metro (Hanna, HI 2211).

Statistical analyses: The results were analyzed by ANOVA with the software Statistical Analysis Software (SAS) to determine the significant differences between the treatments. The radial growth rate with pH, C:N ratio, ashes and fats values were analyzed by a principal component analysis using the software Statistical Analysis Software; the number of components was determined by the Eigenvalue-one criterion. The matrix contained 5 columns (radial growth rate, pH, C:N, ashes and fats) and 33 lines.

Results and Discussion

Table 1 shows the radial growth rate for all strains. In the M1 medium all strain grew with radial growth rate higher than those observed in PDA, except for *Flammulina velutipes*, obtaining an increase of 9.5, 23.5, 25.0, 89.0 and 200 % in *Lentinula edodes*, *Pleurotus eryngii*, *Ganoderma lucidum*, *Pleurotus ostreatus* and *Pleurotus citrinopileatus*, respectively. The radial growth rate in *Pleurotus ostreatus* was similar to the values reported by Sainos et al. (2006), who cultivated this fungus in straw and wheat grains (0.036-0.049 cm hr⁻¹). The biomass of all strains is shown in Fig. 1. The biomass obtained with M1 showed an increase ($p < 0.05$) in *Pleurotus citrinopileatus* (103%) and *Lentinula edodes* (132%), *Flammulina velutipes*, *Pleurotus ostreatus* and *Pleurotus eryngii* a reduction of 92, 245 and 341 % (respectively) was found and in *Ganoderma lucidum* no significant difference was found as compared to the PDA medium.

These results indicate that the vermicompost can improve the mycelial growth of some fungi, this effect was also observed by Matute et al. (2011) who used vermicompost to supplement non-composted substrates for the growth of same *Agaricus blazei*. Table 2 shows the chemical and physical composition of culture media. The C: N ratio found in M1 medium was in the range reported by Khatua et al. (2018) who produced vermicompost. Chikae et al. (2006) indicated that use of immature compost can inhibit the plant growth due to production of phytotoxic substances. The vermicompost concentration (20 g l⁻¹) used in the medium M1 did not inhibit the fungal growth. The mycelial growth of some edible mushrooms has been reported at C: N ratios of 5: 1 to 25: 1 (Carlile et al., 2001). The M1 medium presented a C:N

ratio in this range. The laccases activity of all strains obtained in each medium is shown in Fig. 2. This activity was higher in M1 medium for *Pleurotus eryngii*, *Flammulina velutipes* and *Pleurotus ostreatus* as compared to PDA medium ($p < 0.05$), *Lentinula edodes* and *Pleurotus citrinopileatus* showed reduction of 61 and 245 %. *Pleurotus eryngii* cultivated in this medium presented the lowest biomass concentration (43.9 ± 0.04 mg per plate) and higher laccases activity (897.54 ± 11.7 U gX⁻¹) than that observed in PDA. pH is an important factor for the fungal growth and production of laccases. Diaz et al. (2013) indicated that the initial pH of the growing medium is an important factor for regulating the expression of laccase genes. These authors reported the highest laccase activity in cultures grown at initial pH of 4.5 and the lowest in cultures grown at initial pH of 8.5. The initial pH of the M1 medium was 6.1 (Table 2) and the enzymatic activity was determined at pH 6.5. The M1 medium was suitable for the growth of all fungi and some case promoted high enzyme production. *Pleurotus ostreatus* grown in M1 medium showed a laccases activity similar to that reported by Sainos et al. (2006) who reported laccase activity of 703 U gX⁻¹.

The mycelial growth on M2 was observed in 5 of the 6 strains evaluated, except for *Lentinula edodes*. The radial growth rate values were higher ($p < 0.05$) in *Pleurotus eryngii* and *Pleurotus citrinopileatus* with an increase of 47 and 209 % in comparison with that observed in PDA medium. *Flammulina velutipes* and *Ganoderma lucidum* showed reduction in the radial growth rate values of 29 and 200 %, however, increase in biomass contents of 16 and 44 % were observed in these strains. In *Pleurotus eryngii* and *Pleurotus ostreatus*, biomass production was less in comparison to PDA. *Flammulina velutipes* strain cultivated in the M2 medium, showed a higher radial growth rate in comparison to the results reported by Furlan et al. (1997). Gea et al. (2012) reported mycelial growth of 5.1 cm after 7 days of incubation of *Agaricus bisporus* in compost extract made from spent mushroom substrate, the growth rate was overcome with the vermiwash (*Pleurotus eryngii*: 7.3 cm/7 d, *Pleurotus ostreatus*: 6.5 cm/7 d and *Pleurotus citrinopileatus*: 7.3 cm/7 d). Hanc et al. (2017) reported that the vermicompost leachate has the capacity to solubilize nutrients and other chemical components from the solid phase and this is why they are optimal for some plant growth. In this work, the vermiwash helped mycelial fungi growth.

In this study, M2 medium presented a suitable C: N ratio for the growth of 5 strains, however, this effect was not same for *Lentinula edodes*. Reeve et al. (2010) reported that the compost extracts can affect growth rate of some plants. Furlan et al. (1997) reported the *Lentinula edodes* growth at pH 4-6.4. The basic medium (pH 8.5) of M2 was not favorable for *Lentinula edodes*. In the M2 medium, 4 of the 5 strains showed higher laccase activity compared to that obtained in PDA medium ($p < 0.05$), with values of 25, 61, 356 and 673 % for *Pleurotus ostreatus*, *Flammulina velutipes*, *Pleurotus eryngii* and *Ganoderma lucidum*, respectively. As mentioned above, the optimum pH reported for the laccase production is 4.5, however, at pH 8.5 (M2 medium) a

high enzymatic activity was also found in *Ganoderma lucidum* (1187.26 U/gX) and *Pleurotus eryngii* (1165.13 U/gX). Since fungal growth is multifactorial, it is necessary to evaluate and optimize the culture medium because different results can be obtained with the modification of nutrients or physico-chemical parameters. The six strains evaluated grew in the medium M3. The radial growth rates observed in M3 medium showed an increase of 16, 17, 27, 59 and 117 % in *Lentinula edodes*, *Ganoderma lucidum*, *Pleurotus eryngii*, *Pleurotus ostreatus* and *Pleurotus citrinopileatus*, respectively. The radial growth rate found in M3 medium overcame the reported values by Zervakis et al. (2001) for *Lentinula edodes* (0.011-0.017 cm hr⁻¹) and *Pleurotus eryngii* (0.013-0.020 cm hr⁻¹) cultivated in wheat, poplar straw, cotton gin-trash, peanut shells, sawdust, oak sawdust, corn cobs and olive press-cake. Yang et al. (2003) reported that *Ganoderma lucidum* grew 7.8 cm after 16 days of incubation in stillage grain and calcium carbonate. In this study, *Ganoderma lucidum* presented similar growth (7.6 cm but after 7 days of incubation). The calcined bones increased the biomass concentration in 5 strains, however, in *Ganoderma lucidum* a reduction of 24 % was found in comparison with PDA medium.

The highest ashe content was observed in M3 media comparison with M1, M2 and M4. Bacteria and fungi have the capacity to break phosphate bonds using specific enzymes. (Saeid et al., 2014). The calcined bones at 5% concentration (M3) increased the pH of PDA from 5.6 to 6.7, however, mycelial growth was not affected by pH change. Nair et al. (2015) used phosphoric acid to degrade lignin for the subsequent fungal

growth, further phosphoric acid can also be a source of nutrient for microorganisms. Laccases activity in *Pleurotus eryngii* grown in M3 medium was highest (1383.14 U g⁻¹x; $p < 0.05$). Laccases can be induced by adding ferulic acid to the medium (Karp et al., 2012). In this work, an inductor was not intentionally added to the culture media, however, the substrates used for its elaboration contained molecules that could induce laccases expression.

The radial growth rate of *Pleurotus eryngii*, *Pleurotus ostreatus* and *Pleurotus citrinopileatus* cultivated in M4 medium presented a significant increase of 31, 59 and 100% ($p < 0.05$) as compared with PDA (Table 1). Similarly, the biomass production increased in *Flammulina velutipes* (15%), *Pleurotus ostreatus* (50 %), *Pleurotus citrinopileatus* (110 %) and *Lentinula edodes* (224 %) in comparison with PDA. *Pleurotus ostreatus* in the M4 medium presented the highest biomass concentration in this study (439.2 mg per plate). The M4 medium showed the highest concentration of nitrogen and fats compared to other media evaluated (Table 2). High fat content can be attributed to the fact that fish waste was not pressed to extract the oil.

Fish waste can be an alternative to obtain an economical source of nitrogen for cultivating microorganisms. Shirahigue et al. (2018) reported that products from fish silage were effective for bacterial growth by providing the main source of nitrogen and carbon. The viscera, heads and skin of tilapia and trout can promote fungal growth and are a rich source of nitrogen. Yang et al. (2003) reported that carbon, nitrogen and mineral sources are essential for fungal growth. In this study,

Table 1: Radial growth rate of fungi grown on M1- M4 media

Media	Radial growth rate (cm hr ⁻¹)					
	<i>P. eryngii</i>	<i>L. edodes</i>	<i>F. velutipes</i>	<i>P. ostreatus</i>	<i>P. citrinopileatus</i>	<i>G. lucidum</i>
PDA	0.026 (2.0) ^a	0.019 (0.1) ^b	0.031 (1.0) ^a	0.017 (0.1) ^c	0.012 (0.1) ^c	0.036 (2.0) ^c
M1	0.032 (0.1) ^b	0.021 (2.0) ^a	0.027 (0.2) ^b	0.032 (0.1) ^a	0.036 (1.0) ^a	0.045 (0.1) ^a
M2	0.038 (1.0) ^a	NG	0.022 (1.0) ^c	0.028 (1.0) ^b	0.037 (0.2) ^a	0.012 (2.0) ^e
M3	0.033 (1.0) ^b	0.022 (1.0) ^a	0.027 (0.1) ^b	0.027 (0.1) ^b	0.026 (1.0) ^b	0.042 (1.0) ^b
M4	0.034 (1.0) ^b	0.019 (0.1) ^b	0.026 (1.0) ^b	0.027 (4.0) ^b	0.024 (3.0) ^b	0.023 (1.0) ^d

PDA: Control medium; M1: Vermicompost; M2: Vermiwash and PDA; M3: Calcined bone and PDA; M4: Fish waste. *Media ($\pm$ SD 10-3). Different lowercase letters indicate least significant differences ($p < 0.05$). Three experiments were carried out with four replicates. NG= No growth

Table 2: Proximal chemical analysis and pH of M1-M4 culture media

Culture media	pH	C:N ratio	Organic Carbon (%)	Total nitrogen (%)	Fat (%)	Ash (%)
M1	6.1 (0.1) [*]	20.97 (0.4)	23.16 (2.3)	1.16 (0.3)	0.63 (0.0)	12.02 (0.0)
M2	8.5 (0.1)	18.21 (1.4)	47.64 (4.5)	2.66 (0.2)	2.54 (0.1)	11.96 (0.3)
M3	6.7 (0.0)	15.64 (0.5)	43.80 (7.8)	2.80 (0.0)	5.09 (1.4)	15.66 (1.4)
M4	6.5 (0.1)	6.24 (3.6)	45.11 (6.5)	7.23 (0.2)	25.84 (3.1)	1.37 (0.1)

M1: Vermicompost; M2: Vermiwash and PDA; M3: Calcined bone and PDA; M4: Fish waste. *Media ($\pm$ SD). Three experiments were carried out with three replicates

Fig. 1: Production of mycelial biomass by fungal species in different agro-food wastes: (A) *P. eyngii*, (B) *L. edodes*, (C) *F. velutipes*, (D) *P. ostreatus*, (E) *P. citrinopileatus* and (F) *G. lucidum*. PDA: control medium, M1: vermicompost, M2: vermiwash and PDA, M3: calcined bones and PDA, M4: fish waste. Different lowercase letters indicate least significant differences ($p < 0.05$). Three experiments were carried out with four replicates.

the culture media M2, M3 and M4 presented similar organic carbon concentrations, however, these media did not always produce high biomass as compared to PDA. The maximum laccase activity of $2178.01 \text{ U gX}^{-1}$ was found in *Pleurotus citrinopileatus* grown in M4 medium. *Pleurotus ostreatus* in this medium showed a lower enzyme activity. *Pleurotus ostreatus* cultivated in M4 medium, produced the highest biomass, however, showed lowest laccase activity. The laccases

production by basidiomycete fungi has been evaluated on agro-industrial waste. Karp *et al.* (2012) reported laccase enzyme activity of $1.06\text{--}167 \text{ U g}^{-1}$ for *Pleurotus ostreatus* grown on a sugarcane bagasse. landolo *et al.* (2011) cultivated *Pleurotus ostreatus* on tomato residues and reported enzyme activity of $15.5 \text{ U g}^{-1} \text{ dm}$. Sathishkumar *et al.* (2010) cultivated *Pleurotus florida* of banana peels, mandarin and cantaloupe and reported laccases activity of 5.4 U g^{-1} after 10 days of incubation.

Fig. 2: Laccase activity by fungi species in different agro-food wastes: (A) *P. eyngii*, (B) *L. edodes*, (C) *F. velutipes*, (D) *P. ostreatus*, (E) *P. citrinopileatus* and (F) *G. lucidum*. PDA: control medium; M1: vermicompost; M2: vermiwash and PDA; M3: calcined bones and PDA; M4: fish waste. Different lowercase letters indicate least significant differences ($p < 0.05$). Three experiments were carried out with four replicates.

The principal component analysis for radial growth rate revealed that factor 1 (46.90 %) can be related to C: N ratio and ash content in culture media. Factor 2 (26.04 %) can be related to the pH of culture media; Factor 3 (20.72 %) to ash and fat content. Three factors represented the analysis and explained 93.66% variance. In this study, it was observed that vermicompost and vermiwash, calcined bones and fish waste promote the growth and laccase activity of some fungi. The best culture media were selected based on the radial growth rate,

biomass and laccases activity. Thus, it can be concluded that the results of this study provide alternatives for the use of agro-food waste for fungal growth and its subsequent use in bioprocesses.

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