

Extracellular Enzymatic Activity of *Tuber maculatum* and *Tuber aestivum* Mycelia

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Abstract

Truffle mycelia exhibit a complex interaction pattern with host plants and have been extensively studied over the last years as a source of new bioactive compounds. Fungal enzymes possess a wide use in food industry, confectionaries, textiles and leather industries in order to simplify the processing of raw materials. They are often more stable than enzymes derived from other sources. *Tuber maculatum* and *Tuber aestivum* mycelia were tested for enzymes production in Petri dishes solid medium conditions. The results showed that *Tuber maculatum* produced seven extracellular enzymes (amylase, xylanase, laccase, lipase, peroxidase, cellulase and catalase) while *Tuber aestivum* produced only three enzymes (amylase, peroxidase and catalase).

Keywords

Tuber maculatum, *Tuber aestivum*, Mycelia, Enzymes, Amylase, Xylanase, Laccase, Lipase, Peroxidase, Cellulase, Catalase

1. Introduction

Truffles are one of the most expensive edible fungi growing in symbiosis with several forest trees such as oak,

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hazel, beech and birch [1]. *Tuber melanosporum*, the Perigord black truffle, *Tuber magnatum* Pico, the Italian white truffle and *Tuber aestivum*, the summer truffles are among the renowned edible truffles having high ecological and commercial values [2]. *Tuber borchii vittad*, commonly called Bianchetto truffle, is the only truffle species with commercial value found naturally in Finland so far [3]. In Autumn 2006, the Truffle ascocarps collected from a natural forest located in Lahti, Finland (100 km north to Helsinki) by the help of a trained truffle dog, were identified as *Tuber maculatum* and *Tuber scruposu* [4]. Truffles are the most highly edible valued mycorrhizae fungi in gastronomic and economic terms. Some species of Truffle such as *Tuber maculatum* and *Tuber aestivum* are highly praised due to their organoleptaroma properties [5].

Truffles form filamentous mycelia in their initial phase of growth and further form symbiotic association with host plants [6]. Various biochemical changes occur during this phase and as a consequence, many enzymes are secreted in the extracellular form to degrade the organic matter surrounding the host plant into simple and soluble molecules. Extracellular enzymes help penetration and colonization of host root cells by the fungi and thus play a functional role in host symbiosis and nutrient acquisition strategy [7].

From the last century, enzymes have been used excessively in wide range of industrial applications such as food, dyes, textiles and agrochemical industries [8]. Thus there has been a wide interest to study the production of different enzymes from bacteria, fungi and plants [9]. For example, amylolytic enzymes are widely used in industries and have nearly 25% - 33% of the enzyme market [10]. Today amylase has almost totally replaced chemical hydrolysis of starch in starch processing industry. Starch-degrading amylytic enzymes are of great importance in biotechnological sector ranging from food, fermentation, textile to paper industries [11].

Generally, fungi are more preferred as producers enzymes than bacteria and plants due to their wide variety of catalytic activities, higher yields, ease of genetic manipulation and efficient production in inexpensive media. Other appealing factors include the fact that most enzymes are biodegradable and are active under mild conditions with respect to temperature and pH [12]. Many studies have been reported on enzyme production from edible mushrooms (Table 1).

The present study was carried out to evaluate the potential of *Tuber maculatum* and *Tuber aestivum* mycelia to secrete industrially important enzymes using specific indicative solid media.

2. Material and Methods

2.1. Isolation and Culture of the Mycelia

Truffle mycelia were produced from the ascocarps of *Tuber maculatum* and *Tuber aestivum/uncinatum*. Truffle fruiting bodies were topically sterilized with 70% ethanol and then small pieces of truffle tissue (1 - 2 mm) were aseptically excised from the inner part of the truffle ascocarps and cultivated on Malt Extract Agar Medium (MEA, Sigma Aldrich, Germany). Incubation was carried out in dark at room temperature. Mycelial growth and purity were investigated weekly microscopically. Mycelial discs of 5 mm diameter were transferred to fresh MEA plates and incubated at 4°C.

2.2. Detection of Enzymatic Activity

The qualitative investigation of the enzymes was carried out according to Hankin and Ananostakis (1975); and Sunitha *et al.*, (2013) methods [13] [14]. Typically about 5 mm of mycelial discs were cut from the edges of a culture plate and inoculated at the centre of agar plates containing separately substrates for specific enzymes dissolved in growth media. After 3 - 4 days of incubation at room temperature, the plates were flooded with the suitable indicator. Formation of clear zones or zones with different colours around the fungal colony indicated the presence of enzyme activity.

Table 1. List of enzymes produced by mycelia of edible mushrooms.

Enzymes by edible fungi	Enzymes	References
<i>P. ostreatus</i>	Xylanases, cellulase, laccase	Marquez <i>et al.</i> (2007) [21]
	Manganese peroxidase, lignin	Okamoto <i>et al.</i> (2000) [22]
	Peroxidase, tannanases	Shah and Nerud (2002) [23]

2.3. Test for Amylase

Amylase activity was assessed by growing the truffle mycelia on Malt Extract Peptone Agar (MPA, Sigma-Aldrich, Germany) medium supplemented with 0.2% soluble starch from potato (Sigma-Aldrich, Germany) at pH 6. After incubation for 4 days, the plates were flooded with 1% iodine in 2% potassium iodide. Formation of a clear zone surrounding the colony was considered as positive result for amylase production.

2.4. Test for Protease

For proteolytic activity, Malt Extract Agar medium amended with 0.4% gelatine was used for growing truffle mycelia. Degradation of gelatine is seen as clear zone around the colonies. Plates when flooded with aqueous ammonium sulphate result in the formation of a precipitate making the agar opaque and enhancing the clear zone.

2.5. Test for Lipase

For lipase activity measurement, the truffle mycelia were grown on Peptone Agar Medium (Peptone 10 g, NaCl 5 g, Agar 16 g, Distilled water added to make 1 L) supplemented with 1% w/v tween 20 (separately sterilized). Positive lipase activity was indicated by the formation of a visible precipitate of calcium salts of lauric acid.

2.6. Test for Peroxidase

For peroxidase activity, Malt Extract Agar Medium was used. After 4 days of incubation the presence of peroxidase was evaluated by flooding the plates with a freshly prepared mixture of 0.4% H₂O₂ and 1% pyrogallol dissolved in water. Plates were checked 3 and 24 hours after applying the substrate. A dark yellow brown color around the mycelium indicated peroxidase activity.

2.7. Test for Laccase

Laccase activity was determined by using Malt Extract Agar medium amended with 0.05 g/L 1-naphthol. As the mycelia grows the colorless medium changes to blue due to oxidation of 1-naphthol by laccase enzyme.

2.8. Test for Cellulase

For Cellulase test, Malt Extract Agar medium containing 1% of carboxymethyl cellulose sodium salt was used for growing truffle mycelia. After 4 days of incubation at room temperature, the plates were flooded with 0.2% of aqueous Congo red solution and destained with 1 M NaCl for 15 minutes. Appearance of yellow areas around the mycelia colony indicates the presence of cellulase activity.

2.9. Test for Xylanase

Xylanase activity was tested using Malt Extract Agar medium (25 g Malt, 10 g Yeast extract 16 g Agar contained 1% Natural Xylan (CAS: 58-86-6 HPLC 98% D-(+)-Xylose)). After 4 days of incubation the plates were flooded with Congo red solution (0.2%) and destained with 1 M NaCl solution. The plate was again incubated at room temperature for 15 minutes to obtain clear zone surrounding the colony, indicating xylanase activity.

2.10. Test for Catalase

For catalase activity, Malt Extract Agar medium was used. After 4 days of incubation the presence of catalase was evaluated by flooding the plates with a freshly prepared 0.4% H₂O₂, and the presence of bubbles indicates presence of catalase activity.

3. Results and Discussion

The results presented in **Table 2** showed *Tuber maculatum* mycelia to be able to produce seven different enzyme activities (amylase, xylanase, laccase, lipase, cellulase, peroxydase and catalase) while *Tuber aestivum* mycelia secreted only three of the enzyme activities studied (amylase, peroxydase and catalase). However, it is

important to note that *Tuber aestivum* showed an excessive production of these enzymes compared to *Tuber maculatum* (Table 2).

The Amylase activity was found to be higher with *Tuber aestivum* than with *Tuber maculatum* Figure 1. The absence of protease activity (Figure 2) may be due to the dependence of *Tuber maculatum* mycelium on another set of enzymes to break down nitrogen sequestered in organic molecules. Also, the absence in protease activity in *Tuber maculatum* may be related to repressing the enzyme product. e.g. protease since its formation may be repressed on the MEA medium, suggesting that the fungus did not get enough N from the extract to may sufficiently grow. Nevertheless, ME is a common N-source. But it seems to be difference between the two species, and Figure 2 showed that *Tuber maculatum* grow less than *Tuber aestivum* in ME medium.

Table 2. Enzymatic activity of *Tuber aestivum* and *Tuber maculatum*.

Enzymes	<i>Tuber aestivum</i>	<i>Tuber maculatum</i>
Amylase	++	+
Protease	-	-
Xylanase	-	+
Laccase	-	+
Lipase	-	++
Peroxidase	+	+
Cellulase	-	+
Catalase	++	+

+: Refers to the presence of the activity; -: Refers to absence of the activity; ++: Refers to an excessive presence of the activity).



Figure 1. Comparison of amylase activity. (A) Control; (B) *Tuber aestivum*; (C) *Tuber maculatum* the plates were flooded with 1% iodine in 2% potassium iodide. Formation of a clear zone surrounding the colony was considered as positive result for amylase production.

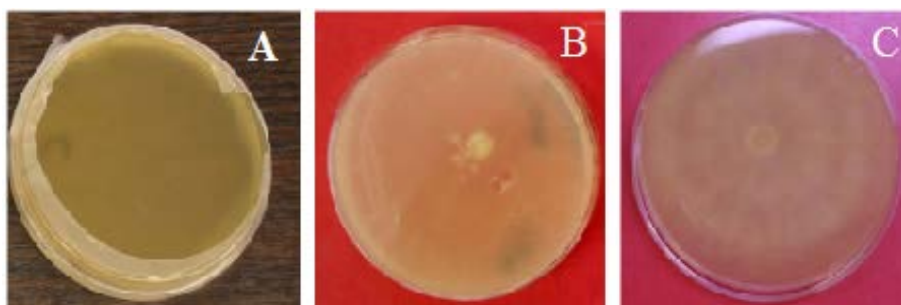


Figure 2. Comparison of protease activity. (A) Control; (B) *Tuber aestivum*; (C) *Tuber maculatum* Plates when flooded with aqueous ammonium sulphate result in the formation of a precipitate making the agar opaque and enhancing the clear zone.

Also, truffle fruiting bodies are rich in proteins, hence protease production might interfere and hamper the growth of the truffle fruit. The absence of protease production was also noted by Rajoriya *et al.* (2014) [15] on two of edible mushroom mycelia, *Pleurotus florida* and *Pleurotus sajor-caju*.

For *Tuber maculatum*, lipase activity was observed 4 days after incubation and it increased proportionally with mycelial growth (Figure 3). Lipase is one of the enzymes that has wide biotechnological applications. The ability of lipase secretion by *Tuber maculatum* mycelia was observed suggesting its capacity to use fats and lipids as energy sources. Similar results were obtained by Rajoriya *et al.*, (2014) [15], who studied the enzymatic activity of two edible mushrooms *P. florida* and *P. sajor-caju*, and *P. florida* showed higher amylase and lipase activity than *P. sajor-caju*, while both species showed negative results for the xylanase and protease activities.

Two different patterns of brown color crystal formation were observed when the selected truffle species were assayed for peroxidase production (Figure 4). On one hand, *Tuber aestivum* showed the formation of a circle of brown crystals inside the colony. On the other hand, *Tuber maculatum* exhibited a pattern characterized by the formation of crystals in a circle outside the colony. However, the present study showed the presence of peroxidase activity. Such different peroxidase enzyme secretion patterns have been observed in white rot wood fungi and white rot humus fungi [16]. *Tuber maculatum* showed the presence of cellulase and laccase activities belonging to the lignocellulolytic group of enzymes as well as xylanase activity. Laccase activity has been demonstrated in many fungal species and the enzyme has already been purified from tens of species. This might lead to the conclusion that laccases are extracellular enzymes generally present in most fungal species. However, this conclusion is misleading as there are many taxonomic or physiological groups of fungi that typically do not produce significant amount of laccase or where laccase is only produced by a few species. For *Tuber maculatum*, laccase activity was detected at day 4 without any sign of mycelial growth from the mycelial plug (Figure 5).

An increase of laccase activity was observed after 7 days incubation. A study of Miranda *et al.*, (1992) [17], showed that truffle species such as *Tuber melanosporum*, *T. aestivum*, *T. brumale*, *T. magnatum* and *T. excavatum* possess tyrosinase (EC 1.14.18.1) and laccase (EC 1.10.3.2) activities.

Secretion of cellulase (Figure 6) and xylanase activities (Figure 7) observed in the case of *Tuber maculatum* suggests that they are mutualistic and may have a major saprophytic approach for nutrition [18]. Xylanase production has been observed in several edible fungi such as *Pleurotus eryngii* (oyster mushrooms) [19].

Figure 8 shows the presence of catalase enzyme in the form of bubbles as a result of the decomposition of hydrogen peroxide to water and oxygen. In general, Catalase works as antioxidant defense in organisms living in

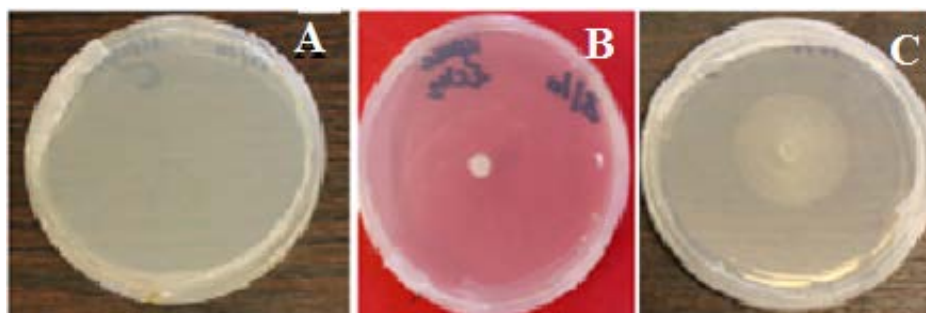


Figure 3. Lipase activity observed only in *Tuber maculatum*. (A) Control; (B) *Tuber aestivum*; (C) *Tuber maculatum* production of lipase activity was indicated by the formation of a visible precipitate of calcium salts of lauric acid.



Figure 4. Comparison of peroxidase activity and the presence of brown crystals. (A) Control; (B) *Tuber aestivum*; (C) *Tuber maculatum*.

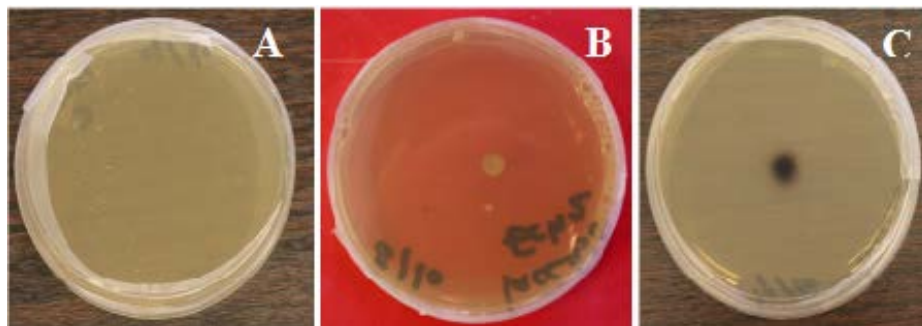


Figure 5. Laccase activity production by *Tuber maculatum*. (A) Control; (B) *Tuber aestivum*; (C) *Tuber maculatum*.

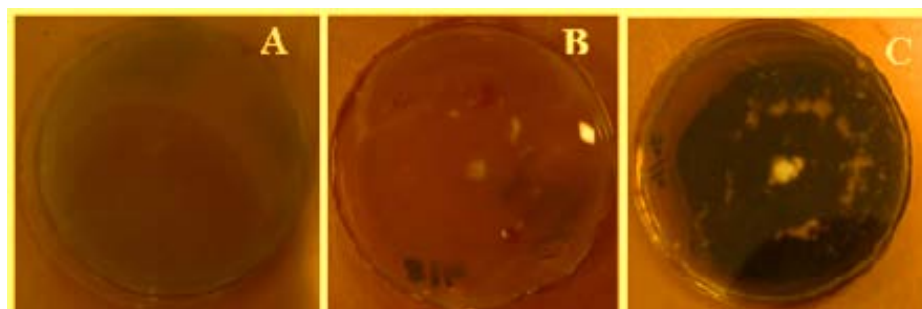


Figure 6. Cellulase activity positive only for *Tuber maculatum*. (A) Control; (B) *Tuber aestivum*; (C) *Tuber maculatum* the plates were flooded with 0.2% of aqueous Congo red solution and destained with 1 M NaCl for 15 minutes. Appearance of yellow areas around the mycelia colony indicates the presence of cellulase activity.

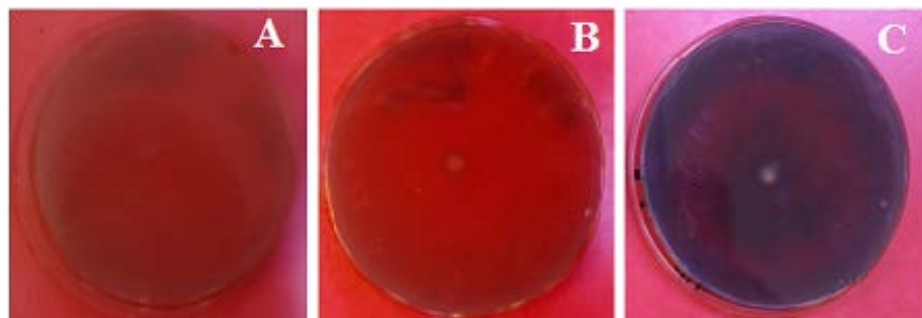


Figure 7. Xylanase enzyme production by *Tuber maculatum*. (A) Control; (B) *Tuber aestivum*; (C) *Tuber maculatum* the plates were flooded with Congo red solution (0.2%) and destained with 1 M NaCl solution. The plate was again incubated at room temperature for 15 minutes to obtain clear zone surrounding the colony, indicating xylanase activity.

aerobic environment although truffles live in poor oxygen environment. A study has been performed on *T. melanosporum* (immature and mature), *T. brumale* (immature and mature), *T. aestivum* (mature) and *T. magnatum* (mature) showing the presence of the catalase activity with different values with all these species [20].

4. Conclusion

Tuber maculatum has higher extracellular enzyme activities compared with *Tuber aestivum* that secreted only three enzymes (amylase, peroxidase and catalase) under the conditions applied in this study. Further studies should include patterns of substrate to understand the interrelations between the fungi and host plant, physiology of *Tuber* species and the formation of fruiting bodies. Also, the potential for these enzymes may be useful in bioremediation and in food biotechnology. The preliminary study on the enzyme production by mycelium of

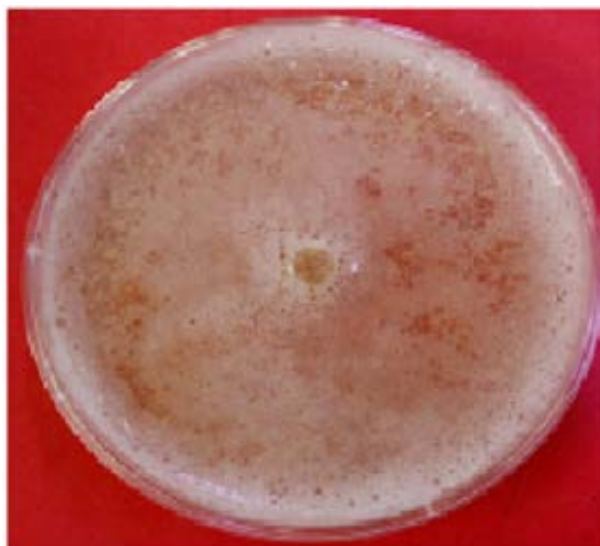


Figure 8. Catalase enzyme production by *Tuber aestivum*. The presence of catalase was evaluated by flooding the plates with a freshly prepared 0.4% H₂O₂, and the presence of bubbles indicates presence of catalase activity.

truffle species can be a future prospective for the exploration of these truffle mycelia as producers of various bioactive compounds.

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References

- [1] Shamekh, S., Grebenc, T., Leisola, M. and Turunen, O. (2014) The Cultivation of Oak Seedlings Inoculated with *Tuber aestivum* Vittad. in the Boreal Region of Finland. *Mycol Progress*, **13**, 373-380. <http://dx.doi.org/10.1007/s11557-013-0923-5>
- [2] Streiblová, E., Gryndlerová, H., Valda, S. and Gryndler, M. (2010) *Tuber aestivum*—Hypogeous Fungus Neglected in the Czech Republic. *Czech Mycology*, **61**, 163-173.
- [3] Kosonen, L. (2002) Mitä kasvaako Suomessakin tryffeleitä. *Sienilehti*, **54**, 105-110.
- [4] Shamekh, S., Donnini, D., Zambonelli, A. and Leisola, M. (2009) Wild Finnish Truffles. *Acta Botanica Yunnanica*, **31**, 69-71.
- [5] Culleré, L., Ferreira, V., Chevret, B., Venturini, M.E. and Sánchez-Gimeno, A.C. (2010) Characterization of Aroma Active Compounds in Black Truffle (*Tuber melanosporum*) and Summer Truffle (*Tuber aestivum*) by Gas Chromatographyolfatometry. *Food Chemistry*, **122**, 300-306. <http://dx.doi.org/10.1016/j.foodchem.2010.02.024>
- [6] Frank, B. (2005) On the Nutritional Dependence of Certain Trees on Root Symbiosis with Belowground Fungi. *Mycorrhiza*, **15**, 267-275. <http://dx.doi.org/10.1007/s00572-004-0329-y>
- [7] Rejón-Palomares, A., García-Garrido, J.M., Ocampo, J.A. and García-Romera, I. (1996) Presence of Xyloglucan-Hydrolyzing Glucanases (Xyloglucanases) in Arbuscular Mycorrhizal Symbiosis. *Symbiosis*, **21**, 249-261.
- [8] Karigar, C.S. and Rao, S.S. (2011) Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. *Enzyme Research*, **2011**, Article ID: 805187. <http://dx.doi.org/10.4061/2011/805187>
- [9] Neelam, G., Sumanta, R., Sutapa, B. and Vivek, R. (2013) A Broader View: Microbial Enzymes and Their Relevance in Industries, Medicine, and beyond. *BioMed Research International*, **2013**, Article ID: 329121.
- [10] Swargiari, B.N. and Baruah, P.K. (2013) Isolation and Screening of Amyolytic Penicillium Species from Soil. *International Journal of Pharma and Bio Sciences*, **4**, 575-581.

- [11] Pandey, A., Soccol, C.R. and Mitchell, D. (2000) New Developments in Solid State Fermentation: I-Bioprocesses and Products. *Process Biochemistry*, **35**, 1153-1169. [http://dx.doi.org/10.1016/S0032-9592\(00\)00152-7](http://dx.doi.org/10.1016/S0032-9592(00)00152-7)
- [12] Mckelvey, M.S. and Murphy, A.R. (2011) Biotechnological Use of Fungal Enzymes. In: Kavanagh, K., Ed., *Fungi: Biology and Applications*, John Wiley & Sons, Ltd., Chichester, 179-202.
- [13] Hankin, L. and Anagnostakis, S.L. (1975) The Use of Solid Media for Detection of Enzyme Production by Fungi. *Mycology*, **67**, 597-607. <http://dx.doi.org/10.2307/3758395>
- [14] Sunitha, V.H., Nirmala Devi, D. and Srinivas, C. (2013) Extracellular Enzymatic Activity of Endophytic Fungal Strains Isolated from Medicinal Plants. *World Journal of Agricultural Sciences*, **9**, 1-9.
- [15] Ashutosh, R., Panda, A. and Gupta, N. (2014) Comparative Evaluation of Nutritional, Biochemical and Enzymatic Properties of the Mycelium of Two *Pleurotus* Species. *Tropical Plant Research*, **1**, 22-26.
- [16] Barrasa, J.M., Blanco, M.N., Esteve-Raventós, F., Altés, A., Checa, J., Martínez, A.T. and Ruiz-Dueñas, F.J. (2014) Wood and Humus Decay Strategies by White-Rot Basidiomycetes Correlate with Two Different Dye Decolorization and Enzyme Secretion Patterns on Agar Plates. *Fungal Genetics and Biology*, **72**, 106-114. <http://dx.doi.org/10.1016/j.fgb.2014.03.007>
- [17] Miranda, M., Bonfigli, A., Zarivi, O., Ragnelli, A.M., Pacioni, G. and Botti, D. (1992) Truffle Tyrosinase: Properties and Activity. *Plant Science*, **81**, 175-182. [http://dx.doi.org/10.1016/0168-9452\(92\)90040-S](http://dx.doi.org/10.1016/0168-9452(92)90040-S)
- [18] Pointing, S.B. (1999) Qualitative Methods for the Determination of Lignocellulolytic Enzyme Production by Tropical Fungi. *Fungal Divers*, **2**, 17-33.
- [19] Altaf, S.A., Umar, D.M. and Muhammad, M.S. (2010) Production of Xylanase Enzyme by *Pleurotus eryngii* and *Flammulina velutipes* Grown on Different Carbon Sources under Submerged Fermentation. *World Applied Sciences Journal*, **8**, 47-49.
- [20] Amicarelli, F., Bonfigli, S., Colafarina, A., Cimini, A.M., Pruiti, B., Cesare, P., et al. (1999) Glutathione Dependent Enzymes and Antioxidant Defenses in Truffles: Organisms Living in Microaerobic Environments. *Mycological Research*, **103**, 1643-1648. <http://dx.doi.org/10.1017/S0953756299008928>
- [21] Márquez, A.A.T., Mendoza, M.G.D., González, M.S.S., Buntins, D.S.E. and Loera, C.O. (2007) Actividad fibrolítica de enzimas producidas por *Trametes* sp. EUM1, *Pleurotus ostreatus* IE8 y *Aspergillus niger* AD96.4 en fermentación sólida. *Interciencia*, **32**, 780-785.
- [22] Okamoto, K., Yanagi, S.O. and Sakai, T. (2000) Purification and Characterization of Extracellular Laccase from *Pleurotus ostreatus*. *Mycoscience*, **41**, 7-13. <http://dx.doi.org/10.1007/BF02464380>
- [23] Shah, V. and Nerud, F. (2002) Lignin Degrading System of White-Rot Fungi and Its Exploitation for Dye Decolorization. *Canadian Journal of Microbiology*, **48**, 857-870. <http://dx.doi.org/10.1139/w02-090>