STARCH HYDROLYSES AND LACCASE ACTIVITY OF HEAT TOLERANT MUSHROOM ISOLATE

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Abstract: 34 isolates of oyster mushroom recovered from different regions of Egypt were screened on the bases of their growth rate at different temperatures. Only five isolates were able to growth intensively at 35 ºC and isolate P1 together with P2 showed the highest growth rate. According to morphological and cultural characteristics of two tested mushroom isolates both belonged to *Pleurotus ostreatus* species. Besides measuring their growth rates under wide range of temperatures (18ºC, 28ºC, 35ºC and 40ºC) the ability of starch hydrolyses was also tested. Isolate P1 showed almost same hydrolysis rates, however there was a steady increase in hydrolyses of starch by isolate P2 which began to decline over 35°C. Some mushrooms produce not only hydrolytic but also oxidative enzymes, such as laccase. After 15 days of growing isolate P2 showed high laccase activity at 35ºC suggesting that this heat tolerant isolate could be a good candidate for various industrial applications or mushroom producers in Egypt.

Introduction

Mushroom cultivation is one of the most important agribusiness, because some species are produced as human food and able to recycle for animal foodstuff. Oyster mushrooms have excellent taste and high-content of proteins, carbohydrates, vitamins and minerals moreover produce various secondary metabolites of medical interest. White-rot mushroom could prevent and reduce several diseases, including high blood pressure, cholesterols (AGRAWAL et al. 2010) breast and prostate cancer (JEDINAK and SLIVA 2008). There are more than 5000 mushroom varieties belonging to the class of the Basidiomycetes and *Pleurotus* genus is spreading all around the world gathering several species. *Pleurotus ostreatus* is the second most cultivated edible mushroom worldwide after *Agaricus bisporus*. However, often missing heat-tolerant mushroom isolates and many mushroom producers suffered from it in Egypt (GULER et al. 2006).

Nutritional and medicinal characteristics of *Pleurotus ostreatus* depend on the growth substrate. In nature, this fungus grows on dead wood, but it can be artificially cultivated on agricultural wastes. FASIDI and KADIRI (1993) reported successful growth of mushrooms on lignocellulose wastes and rapeseed meal may also find use as an inexpensive and efficient substrate for white-rot fungi (ZUCHOWSKI et al. 2013). The degradation of agricultural wastes involves some enzyme complexes made up of oxidative and hydrolytic enzymes. Moreover, applications of their oxidative enzyme, laccase in biotechnology include textile dye or stain bleaching, paper-pulp bleaching, synthetic dye decolorization (ANNUNZIATINI et al. 2005).

The mycoremediation of pollutants such as petroleum, polycyclic aromatic is also achieved by the lignolytic enzyme complex of *Pleurotus* species (PALMERI et al. 1997, TELLEZ-TELLEZ et al. 2013).

The objectives of this study were to isolate heat-tolerant oyster mushrooms, estimate their capacity of degrading starch and lignin which could be important factors in oyster mushroom spawn production in Egypt.

Materials and methods

Sources of materials

Samples of oyster mushroom varieties were collected from different areas of Egypt. Under aseptic conditions, samples of mushroom fruiting bodies were washed then wiped with alcohol (70%) and washed with sterile water several times. Small pieces of the core part of caps were separated, transferred on Potato dextrose agar plates (Duchefa Biochemie, Netherlands), and incubated at 28°C for 7 days. The culture transfer was conducted many times until obtaining the pure isolates. The clean fungal isolates were pre-cultured on potato dextrose agar (PDA) then new PDA plates were inoculated by 5 mm discs of 7 days old pure mycelia of isolates of *P. ostreatus*. Three replicates of each isolates were incubated separately at 18°C and 35°C temperatures for 10 days.
The *P. ostreatus* isolate P1 was originated from the collection of Agricultural Microbiology Department, at Agricultural Research Center, in Giza, Egypt.

Estimation the growth rates and the hydrolyses of starch by *P. ostreatus* isolates at different temperatures

Potato starch peptone agar (PPA) plates were prepared containing 0.5% soluble starch (Difco, USA) 0.1% of peptone and 1.5% of agar. PPA plates were inoculated separately by a 5mm disc of 7 days old pure mycelium of *P. ostreatus* and incubated at different temperatures (18, 28, 35 and 40°C) for 15 days.

Visualization of starch hydrolyses was done by flooding 1% iodine solution on PPA plates, 7 days after inoculation to produce deep blue colored starch-iodine complex. Hydrolysis rate of starch was calculated at different temperatures (18, 28, 35 and 40°C) measuring the clean hydrolyses zone (mm). Each treatment had five replicates.

Culture conditions and assay for laccase activity

Laccase activity was estimated by cultivating isolate P2 in a complex broth [30 g of glucose, 15 g of tryptone, 7.5 g of yeast extract, 30 mg of $CuSO_4 \cdot 5\ H_2O$, and 40 mg of lignin-sulfonic acid (pH: 5.6) in one liter (HUBLIK and SCHINNERA 2000)] then incubated at 35°C in a rotary shaker (150 rpm). After 20 days of growth the culture was separated from the mycelium by filtration using Whatman No.1 filter paper. The culture filtrate was used directly for enzyme activity determination.

Laccase activity was measured by the oxidation of 2,2'-azino-bis (3-ethylbenzthiazoline-6- sulphonate) (ABTS) (Sigma, St. Louis, MO, USA) at 35°C according to BUSHWELL et al. (1996). The reaction mixture (1 ml) contained 600 µl fungal extract, 300 µl 0.1 M sodium acetate buffer (pH 5.0) and 100 µl 1 mM ABTS solution. The oxidation was followed via the increase of the absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme able to oxidize 1 µmol of ABTS per minute.

Statistical analysis

Data were subjected to statistical analysis of variance by ANOVA test in SPSS, 11 software statistical packages. The effects of experimental factors were evaluated by the analysis of variance (ANOVA), and comparisons between means were carried out using Tukey HSD test at the significance level of $P < 0.05$.

Results and discussion

Of the 34 isolates recovered from different places of Egypt, only five were able to growth intensively at 35 ºC (data not show). On the basis of its highest growth rate, isolate P2 was selected for the study of starch hydrolyses, laccase production and together with isolate P1for their morphological characterization. According to the morphological classification system of oyster mushrooms (SHARDA 1989, DUNG 2003), both tested isolates were found to be belonged to *Pleurotaceae ostreatus*.

To evaluate their efficacy, growth rates of both *P. ostreatus* isolates were compared under wide range of temperatures (18, 28, 35 and 40ºC) on potato starch peptone agar plates. However mycelia developments of both isolates started one day after inoculation on PGA, applying starch as nutrient, the mycelium growth of P1 and P2 isolates could be recognized only after 24 h of inoculation (Figure 1). The mycelium developments of both isolates were the same at 18ºC. However, increasing the incubation temperature there was only a small decrease in the growth rate of P1 isolates. The relationship between developments of mycelia at different incubation-temperatures showed that 28ºC was the most suitable temperature for mycelium growth of P_1 and 35ºC was the best for P2 isolate. As well as isolate P2 had a great ability to grow even at the highest tested temperature (40ºC) where the growth of isolate P1 was stopped. These results are in agreement with the results of FRITSCHE (1981) and ISIK (1996) who reported that 28-30ºC were suitable temperatures for mushroom mycelium development. On the other hand SONG (1975) reported that 39ºC was the optimum temperature for *A. bitorquis* and showed that the growth of mycelia declined under 15ºC and over 40ºC.

There are various reports on starch degrading microorganisms from different sources and respective amylase activity (DOODNATH et al. 2000, KATHIRESAN et al. 2006, NWAGU and OKOLO 2011, ALARIYA et al. 2013). Temperature is an important factor of amylase production and these two pure isolates performed significantly different ability of starch degradation at different incubation temperatures (Figure 2). There was a steady increase in hydrolyses of potato starch caused by isolate P2 which began to decline over 35°C. Figure 2. illustrates slow and almost same hydrolysis rates of isolate P1. Consequently, potato starch was slowly degraded during the incubation period, indicating lower hydrolysis activities. The highest activity was found at 35°C by P2 strain, double more than the activity of isolate P1. However starch degradation of both isolates was decreased at 40ºC, the difference was increased from two to four times, indicating high-temperature resistant isolate, P_2.

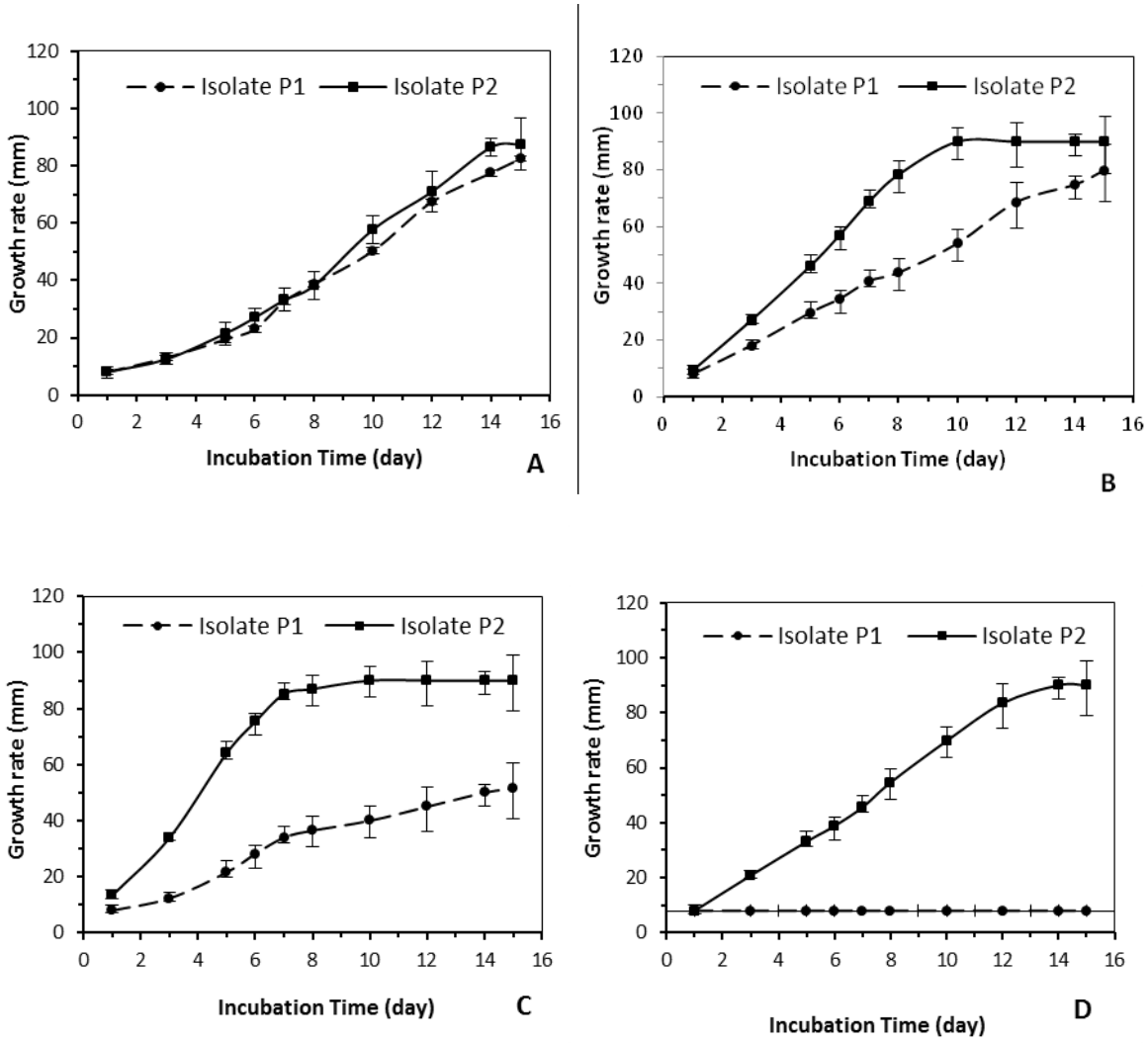

Figure 1. Growth of two isolates of *Pleurotus ostreatus* (P1 and P2) at 18ºC (A), 28ºC (B), 35ºC (C) and 40ºC (D)

1. ábra A két *Pleurotus ostreatus* (P1 és P2) növekedése különböző hőmérsékleten (18ºC (A), 28ºC (B), 35ºC (C), 40ºC (D)

Mushrooms as saprophytic fungi are producing not only hydrolytic but also oxidative enzyme complexes. Moreover ELSAYED et al. (2012) showed that soluble starch was the best inducer for their laccase formation. Interestingly, the specific activity of laccase was increased with increasing soluble starch concentration up to 15 g/l, which was the best concentration of soluble starch for laccase formation.

Many investigators reported different incubation periods for optimum production of laccase. SIVAKUMAR et al. (2010) estimated that maximum laccase production at the 7th and 10th day of incubation in case of *Lentinus edodes* and *Ganoderma sp*, respectively. Oppositely, CAVALLAZZI et al. (2005) found maximum laccase activity with *Lentinula edodes* after 30 days of incubation. According to CAVALAZZI et al. (2005) our result showed high laccase activity only after 15 days of growing (Figure 3). The lignolytic enzyme system of white rot fungi, although may be present in the primary phase of growth, usually is triggered in response to N or C depletion, attaining its maximum in the idiophase when the mycelial dry weight is decreasing (KAAL et al. 1995, ELSAYED et al. 2012).

Our results show that *P. ostreatus* isolate P2 has great potential to transform easy degradable agro-industrial wastes at higher temperature.

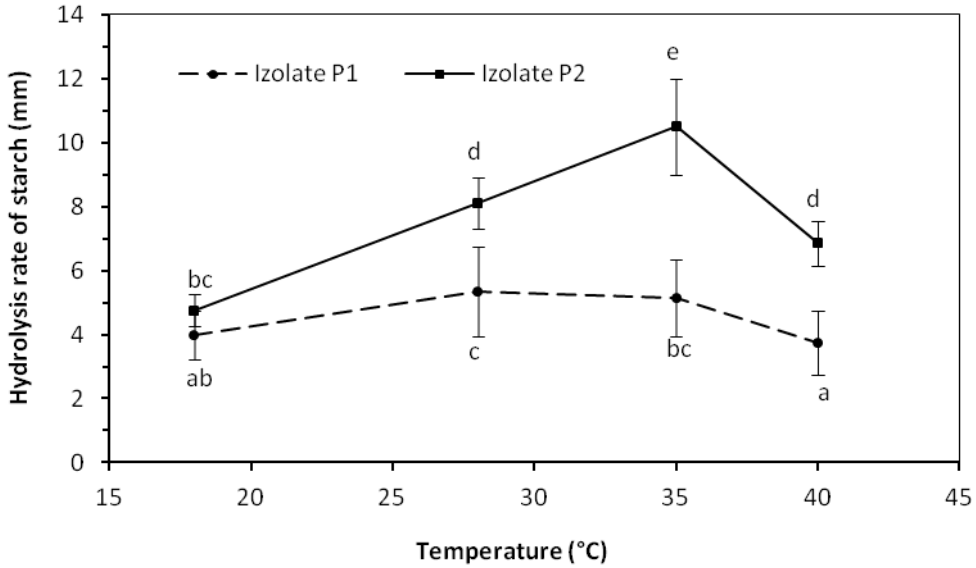

Figure 2 Hydrolysis of potato starch by both isolates (P1 and P2) of *Pleurotus ostreatus* at different temperatures

1. ábra A két *Pleurotus ostreatus* (P1 és P2) keményítő bontó képessége különböző hőmérsékleten

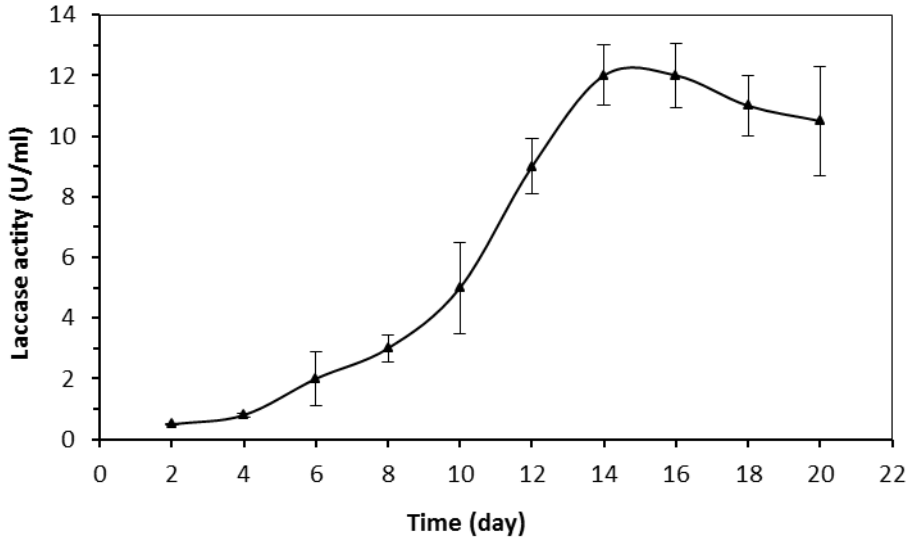

Figure 3 Effect of incubation time on laccase activity of *Pleurotus ostreatus* isolate P2 at 35 °C .

3. ábra Az induklációs idő hatása a *Pleurotus ostreatus* (P2) laktáz aktivitására 35 °C -on

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References

AGRAWAL R. P., CHORPA A., LAVEKAR G. S., PADHI M. M., SRIKANTH N., OTA S., JAIN S. 2010: Effect of oyster mushroom on glycemia, lipid profile and quality of life in type 2 diabetic patients. Australian Journal of Medic Herbalism 22(2): 50-54.

ANNUNZIATINI C., BAIOCCO P., GERINI, M. F., LANZALUNGA O., SJÖGREN B**.** 2005: Aryl substituted N-hydroxyphthalimides as mediators in the laccase-catalyzed oxidation of lignin model compounds and delignification of wood pulp. Journal of Molecular Catalysis B: Enzym 32: 89-96.

ALARIYA S. S., SETHI S., GUPTA S., GUPTA B. L. 2013: Amylase activity of a starch degrading bacteria isolated from soil. Archives of Applied Science Research 5 (1): 15-24.

BUSWELL J. A., CAI Y. J., CHANG S. T., PEBERDY J. F., FU S. Y., YU H. S. 1996: Lignocellulotytic enzyme profiles of edible mushroom fungi. World Journal of Microbiology and Biotechnology 12: 537-542.

CAVALLAZZI J. R. P., KASUYA C. M., SOARES M. A. 2005: Screening of inducers for laccase production by Lentinula edodes in liquid medium. Brazilian Journal of Microbiology 36: 383-387.

DOODNATH V., BACCOUS-TAYLOR G. S. H., BADRIE N., MELLOWES W. A. 2000: Production of alpha amylase from *Bacillus subtilis*: Effects of enzyme hydrolysis on starches. West Indian Journal of Engeneering 23(1): 36-41.

DUNG L. B. 2003: Mushrooms in Tay Nguyen (in Vietnamese). Ha Noi: Science and Technique.

ELSHAFEI A. M., HASSAN M. M., HAROUN, B. M., ELSAYED M. A., OTHMAN A. M. 2012. Optimization of laccase production from *Penicillium martensii* NRC345. Advances in Life Sciences 2(1): 31-37.

FASIDI I. O., KADIRI M. 1993: Use of agricultural wastes for the cultivation of *Lentinus subnudus* in Nigeria. Revista de Biologia Tropical 41: 411-415.

FRITSCHE G. 1981: Some remarks on the breeding maintenance of strains and spawn of *Agaricus bisporus* and *Agaricus bitorquis* Mushroom Science XI, 367-385. Proceedings of the Eleventh International Scientific Congress on the Cultivation of Edible Fungi, Australia.

GULER P., ERGENE A., TAN S. 2006: Production of high temperature-resistant strains of *Agaricus bitorquis*. African Journal of Biotechnology 5(8): 615-619.

HUBLIK G., SCHINNERA F. 2000: Characterization and immobilization of the laccase from *Pleurotus ostreatus* and its use for the continuous elimination of phenolic pollutants. Enzyme and Microbial Technology 27: 330-336.

ISIK S. E. 1996: Kültür mantarnnin ekolojik istekleri, Türkiye 5. Yemeklik Mantar Kongresi. Yalova. pp. 14-24.

JEDINAK A., SLIVA D. 2008: *Pleurotus ostreatus* inhibits proliferation of human breast and colon cancer cells through p53-dependent as well as p53-independent pathway. International Journal of Oncology 33: 1307-1313.

KAAL E. E. J., FIELD J. A. 1995: Joyce TW. Increasing ligninolytic enzyme activities in several white-rot basidiomycetes by nitrogen-sufficient media. Bioresource Technology 53: 133-139.

KATHIRESAN K., MANIVANNAN S. 2006: α-Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. African Journal of Biotechnology 5: 829-832.

NWAGU T. N., OKOLO B. N. 2011: Extracellular amylase production of a the termotolerant *Fusarium* sp. isolated from Eastern Nigerian soil. *Brazilian* Archives of Biology and Technology 54(4): 649-658.

PALMIERI G., GIARDINA P., BIANCO C., SCALONIA A., CAPASSO A., SANNIA G. *1997:* A Novel White Laccase from Pleurotus ostreatus. Jornal of Biological Chemistry 272*:* 31301-31307.

SHARDA O. P. 1989: Textbook of Fungi. 5th edn. New Delhi: Tata McGraw-Hill.

SIVAKUMAR R., RAJENDRAN R., BALAKUMAR C., TAMILVENDAN M. 2010: Isolation, screening and optimization of production medium for thermosta ble laccase production from Ganoderma sp. International Journal of Engineering Science and Technology 2(12): 7133-7141.

SONG S. F. 1975: Studies on Agaricus bitorquis, The preparation and amount of spawn needed for spawning, Taiwan Agriculture Quarterly 11(2):130- 137.

TÉLLEZ-TÉLLEZ M., DÍAZ-GODÍNEZ G., AGUILAR M. B., SÁNCHEZ C., FERNÁNDEZ F. 2012: Description of a laccase gene from *Pleurotus ostreatus* expressed under submerged fermentation conditions. Bioresources 7(2): 2038-2050.

ZUCHOWSKI J., PECIO L., JASZEK M., STOCHMAL A. 2013: Solid-State Fermentation of Rapeseed Meal with the White-Rot Fungi Trametes versicolor and Pleurotus ostreatus. Applied Biochemistry and Biotechnology Sep 11.

HŐTOLERÁNS GOMBA IZOLÁTUMOK KEMÉNYÍTŐ HIDROLÍZISÉNEK ÉS LAKKÁZ AKTIVITÁSÁNAK VIZSGÁLATA

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Kulcsszavak: *Pleurotus ostreatus*, keményítő hidrolízis, lakkáz, hő toleráns

Összefoglaló: Egyiptom különböző régióiból izolált 34 db gomba növekedési rátáját vizsgáltuk különböző hőmérsékleten. Az izolátumok közül öt mutatott intenzív növekedést 35 ºC-on, ezek közül is kiemelkedett a P1 és P2 izolátumok növekedési intenzitása. A telepek morfológiai és mikroszkópikus sajátosságai alapján mind a két izolátumot *Pleurotus ostreatus*-ként azonosítottuk.
A gombák növekedése mellett a keményítő bontó képességük mértékének meghatározására is sor került, igen széles hőmérsékleti tartományban (18 ºC, 28 ºC, 35 ºC és 40 ºC). Az eltérő hőmérsékleteken a P1-es izolátum keményítő bontó képessége nem mutatott jelentős eltérést, ezzel szemben a P2-es jelű laskagomba keményítő hidrolízisének intenzitása a hőmérséklet növekedésével nőtt, és csak 35 °C fölött csökkent drasztikusan. A hőtoleráns P2 izolátum lakkáz aktivitásának mérését is elvégeztük a növekedés különböző fázisaiban. A 15 napos tenyészet mutatott legmagasabb lakkáz aktivitást 35 °C-on, mely eredmény alapján igen kedvező lehet termesztésük az egyiptomi klímaviszonyok között, illetve ipari célú felhasználásuk is nagy perspektívát mutat.