

INFLUENCE OF THE CULTURE MEDIUM pH ON THE ACTIVITY OF SOME OXIDOREDUCTASES IN *MONILINIA LAXA* (ADERH.&RUHL) HONEY PARASITE ON PLUM

ALEXANDRU MANOLIU^{1*}, ELENA TUTU², LĂCRĂMIOARA OPRICĂ²,
ELENA CIORNEA², PETRONELA GRĂDINARIU²

Keywords: *Monilinia laxa*, plum, catalase, peroxidase activity, pH culture medium

Abstract: The paper presents the influence of culture medium pH on the peroxidase and catalase activities in *Monilinia laxa* (Aderh.&Ruhl.) Honey parasite on plum. The fungus was cultivated “*in vitro*” on Leonian medium, different concentrations of hydrogen ions being achieved by means of appropriate buffers. The peroxidase and catalase activity was determined from mycelium and culture liquid culture at 7 days and 14 days after inoculation. Experiment consisted of three consecutive measurements and showed that oxidoreductase activity was influenced by the culture medium pH and culture age.

INTRODUCTION

Enzymatic activity can be directly associated with growing microorganism. These enzymes may be contained within the cell and are often located within the cell membrane or microbody tip of peroxisomes. Enzymes also be released from the cell, in which case they are called extracellular enzymes.

As long as environmental factors is strictly conditioned by the species of pathogen, their rigorous study is necessary. Hydrogen- ion concentration of the reaction medium can be irreversibly affect the enzymes activity (when the distortion occurs at extreme values of the physico-chemical parameter, be reversible when influence the degree of ionization of the enzyme substrate, complex ES or all these molecular species (Cojocaru, 2007).

In this paper we presented the dynamic of the activity of catalase and peroxidase in fungus *Monilinia laxa* (Aderh.&Ruhl.) Honey in conditions of cultivation on media with different pH values, that displays on a scale from 2.0 to 9.0. Most research ranks catalase pH optimum between 4 and 11, depending on the species (Brenda, 2009) and they were described likened versatile peroxidases (*Pleurotus pulmonarius*) whose instability coincides with the increase concentration of H₂O₂ and decreases with decreasing pH of culture medium (Bockle *et al.* 1999). Other recent studies signals in *Aspergillus nidulans* a protein PalF, involved in signaling pathway of ambient pH and contains arrestin N-terminal and C-terminal domains and binds strongly to two different regions within the C-terminal cytoplasmic tail of the 7TM, putative pH sensor PalH. Upon exposure to alkaline ambient pH, PalF is phosphorylated and, like mammalian β -arrestins, ubiquitinated in a signal-dependent and 7TM protein-dependent manner. Substitution in PalF of a highly conserved arrestin N-terminal domain Ser residue prevents PalF-PalH interaction and pH signaling *in vivo* (Herranz *et al.*, 2005). Also, on *Candida albicans* homologous PalF genes have been described Palfi, PHR1 and PHR2. Their expression is dependent on pH culture medium.

MATERIALS AND METHODS

Research was effected at the isolates of *Monilinia laxa* (Aderh.&Ruhl.) Honey harvested from the Experimental Orchard of Pomiculture Research Station Miroslava, county Iasi. For fungi cultivation was used basal synthetic medium Leonian (Leonian, 1924), the modified formula Bonnar (Both, 1971), a standard medium for cultural studies of Ascomycetes, but lacking the agar: KH₂PO₄, 1,25g; MgSO₄·7H₂O; 0.625g; peptone 0.625g; dextrose, 6.25g; malt extract, 6.25g; distilled water, 1000 ml.

To see the effect of variation of pH on catalase activity, the pathogen was grown in synthetic medium with pH range from 2.0 to 9 and a version control. The pH of the medium was adjusted with 1N NaOH and 0,1N HCl. The pathogen was grown in 500 ml conical flasks containing 100 ml of various culture media. These flasks were sterilized to 0.5 atmospheres for 20 minutes. The inoculum in each case consisted of a single disc of 8 mm diameter cut-out from the margin of a freshly grown colony at the age of 7 days of *Monilinia laxa* on PDA medium. The flasks were incubated at 28°C. Experimental measurements of the activity of these enzymes have been made with three replicates at intervals of 7 and 14 days from seeding the culture medium, so the fungus mycelium and the culture liquid. After incubation the culture filtrate and the mycelium were harvested. After 30 minutes of extraction with 0.1 M disodium phosphate solution homogenates were centrifuged at 4000 rpm for 15 minutes to obtain supernatant which served as source of enzyme.

The catalase activity was determined by the method of Sinha which is based on the determination of hydrogen peroxide remaining after interrupting action of catalase with a mixture of potassium dichromate and acetic acid, chromic acetate product can be determined spectrophotometrically at a wavelength of 570 nm (Artenie & al. 2008). Peroxidase activity was determined by Moller's method (1966), using o- dianisidine as action substrate for the enzyme and measuring the optical density of the product of oxidation at 540 nm (green filter) (Cojocaru, 2009). Protein concentration was determined according to Bradford method with bovine serum albumin as a standard (Bradford, 1976; Artenie, 2008).

RESULTS AND DISCUSSION

The data regarding the influence of the culture medium on catalase activity at 7 days and 14 days after inoculation, from mycelium and culture liquid are presented in figures 1-2.

Results of investigations the catalase activity under the influence of variations of pH culture medium in fungus mycelium of *Monilinia laxa* (Aderh.& Ruhl.) Honey exhibited in figure 1.

At 7 days after inoculation, the highest value of the catalase activity in mycelium was found in variant V2(pH 3) - 4382.318 CU/μmoli/min. and the lower value of catalase activity in the mycelium was detected in variant V1 (pH 2), which was zero. Between these two limits, these values were found in descending order: the V3 (pH 4) - 1717.57 CU/μmoli/min., V7 (pH 8)- 122.6054 CU/μmoli/min, V8 (pH 9) - 1107.442 CU/μmoli /min., the V6 (pH 7) - 745.2482 CU/μmoli/min., the V4 (pH 5) - 435.108 CU/μmoli / min. and the V5 (pH 6) - 277.4911 CU/μmoli/min. At 14 days, the activity of catalase in the mycelium in all variants was relatively uniform, except V9 (control) that had the lowest value-39.2421 CU/μmoli/min, after V1 variant (pH = 2) whose catalase activity in the mycelium had a zero value. Compared with the control the highest point reached by the endocellular oxidoreductiv enzyme was found at variant V4 (pH =5) - 1788, 497 CU/μmoli/min, followed in decreasing order by the variants V6 (pH =7) - 1184.957 CU/μmoli/min, V3 (pH 4) - 1154.528 CU/μmoli/min, V8 (pH= 9) - 913.0205 CU/μmoli/min., V7 (pH 8) - 590.9558 CU/μmoli/min., V5 (pH 6) - 550.8963 CU/μmoli/min. and V2 (pH 3) - 462.7991 CU/μmoli/min.

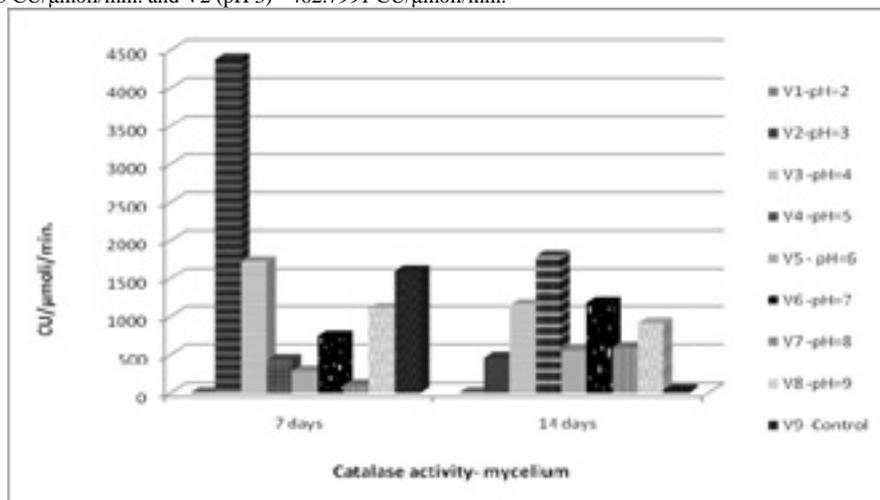


Fig.1. The influence of variations of pH culture medium on the activity of catalase in mycelium of *Monilinia laxa* (Aderh.& Ruhl.)Honey

Following the dynamic activity of catalase in the mycelium fungus between the two intervals, it was observed that the most intense decrease in this enzyme activity was recorded in case of variant V2 (pH 3) - from 4382.312 CU/μmoli/min.to 462.7991 CU/μmoli/min., followed by V9 (martor) - 1599.026 from CU/μmoli/min. to 39.24212 CU/μmoli/min., V3 (pH 4) at 1717.57 CU/μmoli/min. to 1154, 528 CU/μmoli/min. and V8 (pH 9) - from 1107.442 CU/μmoli/min to 913.0205 CU/μmoli / min. Increasing of catalase activity observed at V4 (pH 5) from 435.108 CU/μmoli/min.to 1788.497 CU/μmoli/min., V6 (pH 7) from 745.2482 CU/μmoli/min. to1184.957 CU/μmoli/min., V5 (pH 6) from 277.4911 CU/μmoli/min. to550.8963 CU/μmoli/min. and V7 (pH 8) from 99.72476 CU/μmoli/min.to590.9558 CU/μmoli/min. ; the variant V2 (pH 2) was zero in both intervals, because this variant with extreme acid pH, mycelium fungus could not developed.

Experimental data on catalase activity in liquid culture are presented graphically in figure 2, which showed that extracellular catalase values recorded in cultures of *Monilinia laxa* 7 days old, grown in different pH conditions, were relatively uniform, meeting the minimum activity at pH extremely acid V1 (pH 2) where, because culture medium that has allowed the development mycelium fungus, the catalase was noted as zero. The peak value of extracellular enzyme activity was detected in variant V3 (pH 4) - 371.2575 CU / $\mu\text{moli} / \text{min}$, followed by variants V4 (pH 5) - 351.7915 CU / $\mu\text{moli} / \text{min}$., V6 (pH 7) - 179.1045 CU/ $\mu\text{moli} / \text{min}$, V5 (pH 6) - 175.4386 CU / $\mu\text{moli} / \text{min}$,V7 (pH 8) - 122.6054 CU / $\mu\text{moli} / \text{min}$. and variant V9 (marmor) - 63.89776 UC / $\mu\text{moli} / \text{min}$.

In cultures aged 14 days the maximum catalase activity was at variant V4 (pH5) -1498.049 CU/ $\mu\text{moli}/\text{min}$ and the minimum was at V1(pH 2),with zero. Between these limits, catalase in liquid culture recorded the following values in descending order as follows: variant V9 (marmor) - 1179.768 CU/ $\mu\text{moli}/\text{min}$., followed by V2 (pH 3) - 885.4533 CU/ $\mu\text{moli}/\text{min}$, V5 (pH 6) - 435.5412 CU/ $\mu\text{moli}/\text{min}$, V7 (pH 8) - 176.4519 CU/ $\mu\text{moli}/\text{min}$. Other variants have suffered reductions in relatively modest extracellular catalase activity as follows: V3 (pH 4) - 217, 4479 CU/ $\mu\text{moli}/\text{min}$., V7 (pH 8) - 176.4519 CU/ $\mu\text{moli}/\text{min}$. and V8 (pH 9) - 67.96117 CU/ $\mu\text{moli}/\text{min}$.

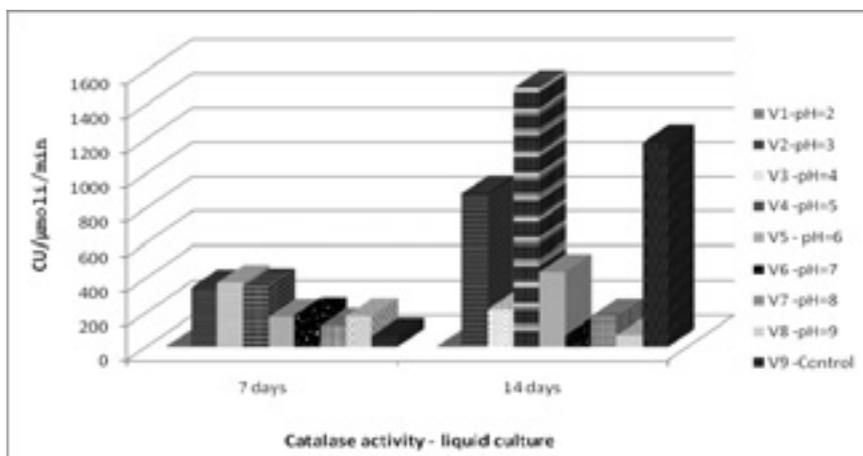


Fig.2. Influence of pH on catalase activity in liquid culture of *Monilinia laxa* (Aderh.&Ruhl.) Honey

It reveals that the culture in the age of 14 days, extracellular catalase activity in control variant V9 is close to the values of variant V5 (pH 5), which can be explained by the fact that the pH of the culture medium of version control has a value close to 5, namely, 4.8.

The analysis of catalase activity in dynamics of culture, in liquid culture, reveals that, in the second half of the time, unlike endocellular catalase, the extracellular may had developed momentum. Thus, the highest increase of activity of this enzyme was found while the variant V4 (pH 5) - from CU 351.7915/ $\mu\text{moli}/\text{min}$ to 1498.049 CU/ $\mu\text{moli}/\text{min}$., followed by the V9 variant (control) from 63.89776 CU/ $\mu\text{moli}/\text{min}$. to 1179.768 CU $\mu\text{moli}/\text{min}$, V2 (pH 3) from 344.6154 CU/ $\mu\text{moli}/\text{min}$. to 885.4533 CU/ $\mu\text{moli}/\text{min}$. and V5 (pH 6) from 175.4387 CU/ $\mu\text{moli}/\text{min}$. to 435, 5412CU/ $\mu\text{moli}/\text{min}$. The other variants have suffered reductions in the time variations of catalase activity and extracellular variables V3 (pH 4) at 371.2575 CU/ $\mu\text{moli}/\text{min}$. to 217.4479 CU/ $\mu\text{moli}/\text{min}$., variant V6 (pH 7) at 184,0491CU/ $\mu\text{moli}/\text{min}$. to 65.57377 CU/ $\mu\text{moli}/\text{min}$., V8 (pH 9) at 179.1045 CU/ $\mu\text{moli}/\text{min}$. to 67.96117 CU / $\mu\text{moli}/\text{min}$. It can be concluded that both extracellular and endocellular catalase have to *Monilinia laxa* optimal pH range 3-5 at 28^oC, which demonstrates the speciality findings.

Data on the influence of different variations of pH on peroxidase activity in the mycelium and culture liquid in *Monilinia laxa* (Aderh.&Ruhl.) Hone are presented in figures 3 and 4.

Figure 3 shows that at 7 days after sowing, in mycelium, the peroxidase activity has a maximum value in V8 (pH 9) - 0.84727 PU/ g^*10^{-3} and minimal activity in variant V6 (pH 7) - 0.07628 PU/ g^*10^{-3} ; in variant V1 (pH 2) has not developed and the value was null. Between these two limits of peroxidase activity were included the following: the variant V5 (pH 6) - 0.6395 PU/ g^*10^{-3} , V4 (pH 5) - 0. 3994 PU/ g^*10^{-3} , V2 (pH 3) - 0.31654 PU/ g^*10^{-3} , V3 (pH 4) - 0.13672 PU/ g^*10^{-3} , and V7 (pH 8) - 0.11256 PU/ g^*10^{-3} .

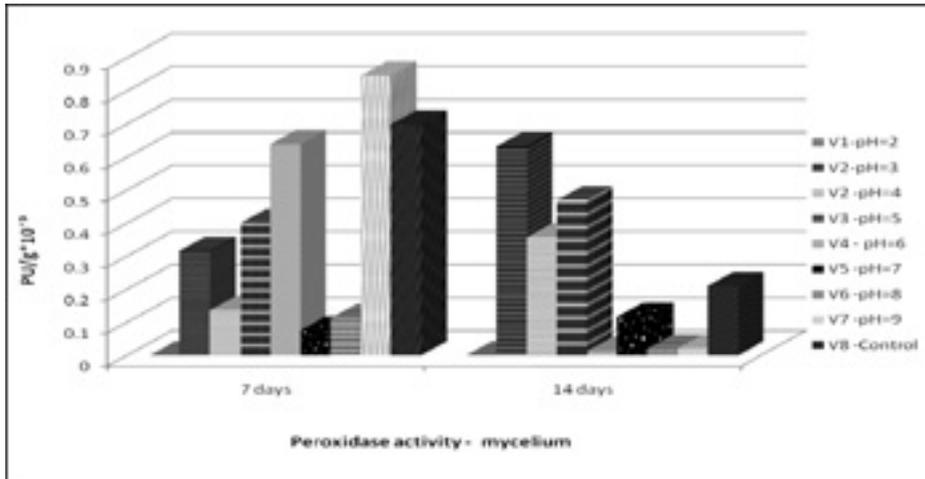


Fig.3. Influence of pH on peroxidase activity in mycelium of *Monilinia laxa* (Aderh.&Ruhl.) Honey ($PU/g \cdot 10^{-3}$)

At 14 days after sowing, at V9 (control) endocelulare peroxidase activity was $0.21061 PU/g \cdot 10^{-3}$. Compared to these, the highest values were found in acidic extreme values, the highest point being reached at the variant V2 activity (pH 3) - $0.63320 PU/g \cdot 10^{-3}$, closely followed by V4 (pH 5) - $0.47003 PU/g \cdot 10^{-3}$, V3 (pH 4) - $0.35669 PU/g \cdot 10^{-3}$. At the variant V6 (pH 7) was recorded as the peroxidase activities in mycelium have suffered reductions with increasing alkaline culture medium. Thus, variant v5 (pH 7) is one notable exception, the peroxidase activity to it being $.11684 PU/g \cdot 10^{-3}$ and then at V8 (pH 9) were found $0.02181 PU/g \cdot 10^{-3}$ and V6 (pH 8) - $0.02073 PU/g \cdot 10^{-3}$.

Examining the peroxidase activity in the fungus mycelium between the two time intervals (dynamics), it was found that the highest increase of enzyme activity was found in V2 (pH 3) - from $0.31654 PU/g \cdot 10^{-3}$ to $0.63320 PU/g \cdot 10^{-3}$, followed by V4 (pH 5) with an increase from $0.39947 PU/g \cdot 10^{-3}$ to $0.47003 PU/g \cdot 10^{-3}$ and V3 (pH4) from $0.13672 PU/g \cdot 10^{-3}$ to $0.35669 PU/g \cdot 10^{-3}$. Variant V6 (pH 7) had a slight increasing in dynamic peroxidase activity in the mycelium, from $0.07628 PU/g \cdot 10^{-3}$ to $0.11684 PU/g \cdot 10^{-3}$. At others variants were recorded decreases of enzyme activity under study. Thus, the variant V5 (pH 6) noted a decrease from $0.63952 PU/g \cdot 10^{-3}$ to $0.01403 PU/g \cdot 10^{-3}$ at V7 (pH 8) from $0.11256 PU/g \cdot 10^{-3}$ to $0.02073 PU/g \cdot 10^{-3}$ and the V8 variant (pH 9) from $0.84727 PU/g \cdot 10^{-3}$ to $0.02181 PU/g \cdot 10^{-3}$.

Results on the influence of pH on peroxidase activity in liquid cultures *Monilinia laxa* species are shown graphically in the figure below. It thus appears that the first interval of time measurements of extracellular peroxidase activity from *Monilinia laxa* species most variants have a higher activity of this enzyme in liquid culture, compared with values obtained in the second set of enzyme determinations.

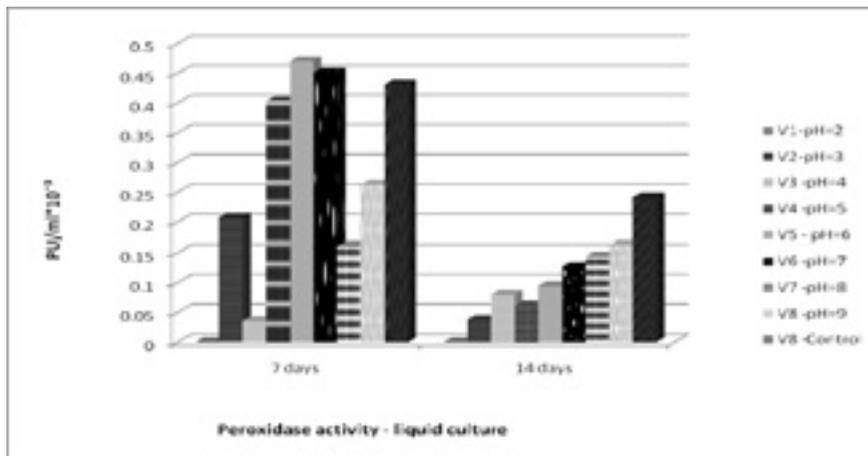


Fig.4. Influence of pH on peroxidase activity in liquid culture of *Monilinia laxa* (Aderh.&Ruhl.) Honey (PU/ml*10⁻³)

Thus, at 7 days, V9 (control) had a value of peroxidase activity in liquid culture among the highest, the 0.43125 PU/ml*10⁻³, being surpassed only by the V5 (pH 6) with 0.470 313 PU/ml*10⁻³, the V6 (pH 7) to 0.451563 PU/ml*10⁻³ and V4 (pH 5) -0.403125 PU/ml*10⁻³, followed in descending order by V8 (pH 9) 0.2625 PU/ml*10⁻³ and V2 (pH 3) with PU/ml*10⁻³. Variant V7 (pH 8) 0.160938 PU/ml*10⁻³ preceding variant, lowest enzymatic activity V3 (pH 4) 0.035585 PU / ml * 10-3.

At 14 days, extracellular peroxidase activity lower than that set by the V9 (control) – 0.241 484 PU/ml*10⁻³ following in ascending order, from variant V2 (pH 3) - 0.037813 PU/ml*10⁻³, V3 (pH 4)- 0.061875 PU/ml*10⁻³, V2 (pH 4) - 0.079922 PU/ml*10⁻³, V4 (pH 5) 0.094531 PU/ml*10⁻³, V5 (pH 6) - 0.126328 PU/ml*10⁻³, the V6 - 0.143516 PU/ml*10⁻³, and V7 - 0.162422 PU/ml*10⁻³.

Studying the evolution of the peroxidase activity in liquid culture of *Monilinia laxa* species has been observed that, except variant V3 (pH 4) where increased extracellular peroxidase activity of the enzyme - monitored from 0.035585 PU/ml*10⁻³ at 0.079922 PU/ml*10⁻³, the rest of the variants showed that enzyme activity decreases. Thus, it has been observed that the witness went V8 (pH 9) from 0.43125 PU/ml*10⁻³ to 0.241484 PU/ml*10⁻³ V5 (pH 6) from 0.470313 PU/ml*10⁻³ to 0.0945 PU/ml*10⁻³, V6 (pH 7) from 0.451563 PU/ml*10⁻³ to 0.1263 PU/ml*10⁻³, V4 (pH 5) from 0.403125 PU/ml*10⁻³ to 0.061875 PU/ml*10⁻³, V8 (pH 9) from 0.2625PU/ml*10⁻³ to 0.1625PU/ml*10⁻³, V7 (pH 8) from 0.160938 PU/ml*10⁻³ to 0.143516 PU/ml*10⁻³ and V1 from 0.207813 PU/ml*10⁻³ to 0.037813 PU/ml*10⁻³.

CONCLUSIONS

The analysis of experimental results obtained in determinations of pH dependence on the cultural medium activity in the fungus *Monilinia laxa* on oxidoreductase enzymes, revealed some differences in the catalase activity in fungus mycelium and in liquid culture, but also between variations in time of the extracellular and endocellular catalase activity.

In mycelium, at 7 days from inoculation, catalase activity recorded at an optimum pH value of 3 and 4, being inhibited at pH 8. At 14 days, optimum activity of catalase in the mycelium was detected at pH5; at the rest of the variants ,enzyme activity ranging almost uniformly, stimulated by pH 4 and pH 7.

In liquid culture in the age of 7 days, pH 4, pH 5 and pH 3 stimulated the extracellular catalase activity and in the medium with pH 8, enzyme activity is inhibited. At 14 days after inoculation, the strongest inductive effect on the activity extracellular catalase had pH 5. Inhibition of enzyme variants occurred at culture medium pH 7 and pH 9.

Peroxidase activity at 7 days, in mycelium, was stimulated by the pH 9, and was inhibited at neutral pH. Aging culture enhanced peroxidase activity of the mycelium at pH 5, pH 3 and pH 4, complete inhibition of the enzyme took place at pH 2.

In culture liquid in the age 7 days, pH 6 and pH 7 stimulated activity of peroxidase activity and pH 4 and pH 8 inhibited the peroxidase activity. Aging culture did not reveal significant variations compared with the control of extracellular peroxidase activity.

REFERENCES

- Artenie V., Ungureanu E., Negura Anca Mihaela, 2008.** *Metode de investigare a metabolismului glucidic si lipidic.* Editura PIM, Iași, p. 182.
- Bacigálová , K., Zemek , J., Lačok , P., Kuniak · L.2008.** *Enzymatic Activity of the Genus Monilia Pers., Journal of Phytopathology*, Volume 96 Issue 3, Pages 251 – 257
- Batra, L.R.** 1991. World species of *Monilinia* (Fungi): Their ecology, biosystematics and control. *Mycol. Mem.* 16: 1-246.
- Böckle,B., Martínez, M.J., F Guillén,F., Martínez, A.T. 1999.** Mechanism of Peroxidase Inactivation in Liquid Cultures of the Ligninolytic Fungus *Pleurotus pulmonarius*, *Applied and Environmental Microbiology*, March 1999, p. 923-928, Vol. 65, No.
- Bradford M.M. (1976)** *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* *Anal Biochem*, 72: 248-254
- Byrde, R.J., Willets, H.J. 1977.** *The brown rot fungi of fruit, Their Biology and control*, Pergamon Press, Oxford, 171 pp.
- Cojocar D.C., 2007.** *Enzimologie generală*, Editura Tehnopress, Iași, pag.25- 30
- Cojocar, D. C., 2009.** *Enzimologie practică*. Editura Tehnopress, Iași, p. 422, 458
- Fiedurek J, Gromada A, Słomka A, Kornilowicz-Kowalska T, Kurek E, Melke J. 2003.** *Catalase activity in microfungi grown at different temperatures.* *Acta Biol Hung.* 54(1):105-12.
- Glodeanu, E., Babeanu, C., Marinescu, G., Ciobanu G., 2003.** *Specific biochemical markers for identification of Monilinia laxa and Monilinia fructigena fungus species*, *Annals of the University of Craiova, The Chemistry Series*, XXXII, p 62.
- Herranz,S., Rodríguez, J.M., Bussink, H.J., Sánchez-Ferrero, J.C., Arst, H.N., Peñalva, M.A., Vincent, O. 2005.** *Arrestin-related proteins mediate pH signaling in fungi*, Edited by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC
- Maehly A, Chance B (1954).** "The assay of catalases and peroxidases". *Methods Biochem Anal* 1: 357–424
- Moller K.M., Ottolenghi, P. (1966)** *The oxidation o-dianisidine by H₂O₂ and peroxidase at neutral pH.* *Compts. Rend. Trav. Lab.*, 35, 369-389.
- Mordue, J.E.M., 1979.** *Sclerotinia laxa*. No 619 in *Descripton of Pathogenic Fungi and Bacteria*, Commonw. Mycol. Inst., Assoc. Appl.Biol., Kew, Surrey, England.
- Porta,A., Ramon,A.M., Fonzi , W.A. 1999.** *PRR1, a Homolog of Aspergillus nidulans palF, Controls pH-Dependent Gene Expression and Filamentation in Candida albicans*, *J Bacteriol.* 1999 December; 181(24): 7516–7523.
- Wilson, E.E.; Ogawa, J.M. (1979)** *Fungal, bacterial and certain non-parasitic diseases of fruit and nut crops in California.* Californian Agricultural Science Publications, Berkeley, California, USA.

¹ Biological Research Institute, Iași.

² "Alexandru Ioan Cuza" University of Iași, Faculty of Biology.

* alexandru.manoliu@uaic.ro