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# Effect of Low Doses of Guaiacol and Ethanol on Enzymatic Activity of Fungal Cultures

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## ABSTRACT

The influence of low doses of guaiacol and ethanol, the natural effectors of lignin and phenolics transformations, on laccase and peroxidase activities produced by two strains of Basidiomycetes, ***Pleurotus sajor-caju*** and ***Trametesversicolor,*** was evaluated. Fungal mycelia were grown for 2 weeks on liquid media containing serial dilutions of guaiacol or ethanol ranging from $100^{-1}$ to $100^{-20}$ mol/L. Laccase and peroxidase activities in the medium were measured at the end of 2 weeks. The effect of low doses of guaiacol and ethanol on enzyme activities was manifested in an oscillating manner. Similar response patterns were observed when pure enzymes were exposed to the same serial dilutions of guaiacol and ethanol. T. ***versicolor*** cultures enriched with 40 mmol guaiacol (simulating natural environmental conditions) also displayed oscillating enzyme activity patterns in response to serial dilutions of guaiacol, but the maximum enzyme activity values were increased compared to those observed in cultures not receiving 40 mmol gnaiacol. The differences between maxima and minima varied among the experimental groups and depended on the species of fungus, type of effector, and kind of enzyme. The results suggest the possibility of subtle regulation of enzymatic activity on the molecular level.

Key Words: laccase, peroxidase, ***Pleurolus sajor-caju, Trametes versicolor,*** ethanol, guaiacol, low doses.

## INTRODUCTION

The activities of enzymes catalyzing all metabolic pathways are in part regulated by low molecular effectors (Bonne 1997). Physiological events in cells are also regulated

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with the help of low molecular substances that influence the activity of respective receptors or enzymes in the nietabolic pathway and regulate the speed of substrate flow by these pathways. The concentration of effectors is very important for initiation of events such as the activation of chernoreceptors or cascade of apoptotic processes in humans (Calabrese 2001a,b,c), as well as mechanisms of biochemical immunization in plants (Manniger *et* al. 1998; Katay and Tyihak 1998; Tyihak et *al.* 2002).

Rapoport and Luebering (1951) reported that diphosphoglycerate phosphatase is strongly activated by low concentrations of potential metabolic poisons, Hg(II) or Ag(I) ions. In 1964 Dixon and Webb reported that some effectors can act as poisons for some enzymes and activators for others. More recently, bimodal types of regulation of tyrosine kinase by low doses of the inhibitor genistein (Morimoto and Bonavida 1992) and of protein kinase C by very low concentration of some antioxidants (Maltseva 2002) have been described.

Effectors are usually divided into activators and inhibitors, but according to the hormetic principle the concept of activation and inhibition is strongly dependent on the concentration of effectors and their time of action (Calabrese and Baldwin 2000 and 2001a).

Fungi were chosen as the experimental model because they can be grown on various substrates and produce large amounts of extracellular enzymes, such as laccase and peroxidase, which cause the white rot of wood and are involved with the process of enzymatic delignification (Leonowicz el al. 1999; Luterek et *al.* 1998; Malarczyk and Widenska 2002). These enzymes are sensitive to the presence of various small molecular effectors that regulate the reactions involving the biodegradation of lignin and transformation of natural phenolics (Molitoris, 2001; Malarczyk et *al.* 2001). Many aromatic substances, mainly alcohols, aldehydes and acids such as ferulic, vanillic, and anisic are substrates for laccase. The induction of laccase is also possible with the same natural aromatic activators or similar synthetic substances such as 2,5-xylidin (Kogalski and Leonowicz 1992). Peroxidase, which requires $H_2O_2$ as a co-substrate, is often activated by the same substances. This article describes the effects of very low doses of guaiacol and ethanol on the activity of extracellular laccase and peroxidase in the liquid cultures of two fungi, *Trametes versicolor* and *Pleurotus sajor-caju.*

## MATERIALS AND METHODS

### Pure Enzymes

*Cerrena unicolor* laccase was purified in the Biochemistry Department, MCS University in Lublin according to the procedure described by Luterek et al. (1998). Horseradish peroxidase was purchased from Sigma Chemical Company.

### Biological Models and Culture Conditions

Two species of fungi from the fungi collection of the Biochemistry Department, M.C.S. University, Lublin, Poland (*i.e.*, strains *Trametes versicolor,* designated No. 20 and *Pleurotus sajor-caju,* designated No. 100 in the collection) were used as biological

sources of laccase and peroxidase. Fungal mycelia were inoculated on the Lindeberg medium according to the method described by Luterek *et al.* (1998). After 10 days, the mycelia were homogenized with glass beads. Small conical flasks containing 10 ml medium were inoculated with the homogenate (0.5 ml homogenate/flask). Twenty-one flasks (a control and 20 serial dilutions of enzyme) were used in each experiment. Each experiment was replicated three times.

### Preparation of Dilution and Stimulation Course

Serial dilutions (one part effector solution to 99 parts 75% ethanol) were prepared from solutions of guaiacol and ethanol (initial concentrations of 0.3 mol/L and 16 mol/L, respectively). The concentrations of the serial dilutions ranged from $100^{-1}$ to $100^{-20}$ mol/L [see Zenin's refractometric studies (Zenin 1999a,b) for an explanation of molecular changes that may occur at ultra high dilutions]. Twenty µl of serial dilutions were added to the cultures every second day, beginning on day 3 of the experiment (a total of 120 µl/flask over the course of the 14-day experiment). Control cultures received after 14 days a total amount 120 µl/flask of 75% ethanol only. For experiments with pure enzymes, 200 µl of serial dilutions were added to each flask containing 200 µl of purified laccase or peroxidase in 700 µl of proper buffer and enzyme activities were measured at the end of 1 hour.

### Enzyme Assays

Laccase activity was measured spectrophotometrically using syringaldazine (2.5 µ*M*) as the substrate in 0.1 Mcitrate-phosphate buffer, pH 5.2 according to the method of Leonowicz and Grzywnowicz (1981). Peroxidase activity was measured using 4 µM of odianisidine in methanol and 10 mmoles of $H_2O_2$ as the substrate and co-substrate, respectively, in 0.1 M acetate-Na buffer, pH 5.5 according to the method of Claiborne and Fridovich (1979). Enzyme activities were expressed in nkatals where one nkatal is defined as the enzymatic degradation of one nmole of substrate during 1 sec and one international unit of enzymatic activity correspond to 16.67 nkatals.

### Statistical Analysis

The results were analyzed by two-way ANOVA using the software package STATGRAPHICS version 2.6. Line regressing analyses were performed for the data from all 14 experiments presented in Table 1 and Figures 1 to 4. Trend lines are showed in all figures with proper equations. The values of maximal and minimal points (lying above or below the trend lines) were summarized and put in Figure 5 to illustrate how various dilutions of guaiacol and ethanol can work to activate and inhibit laccase and peroxidase activities (see also Javjock and Lewis, 2002).

## RESULTS

Oscillatory changes were observed in laccase and peroxidase activities in the media of both fungal species exposed to serial dilutions of guaiacol and ethanol.

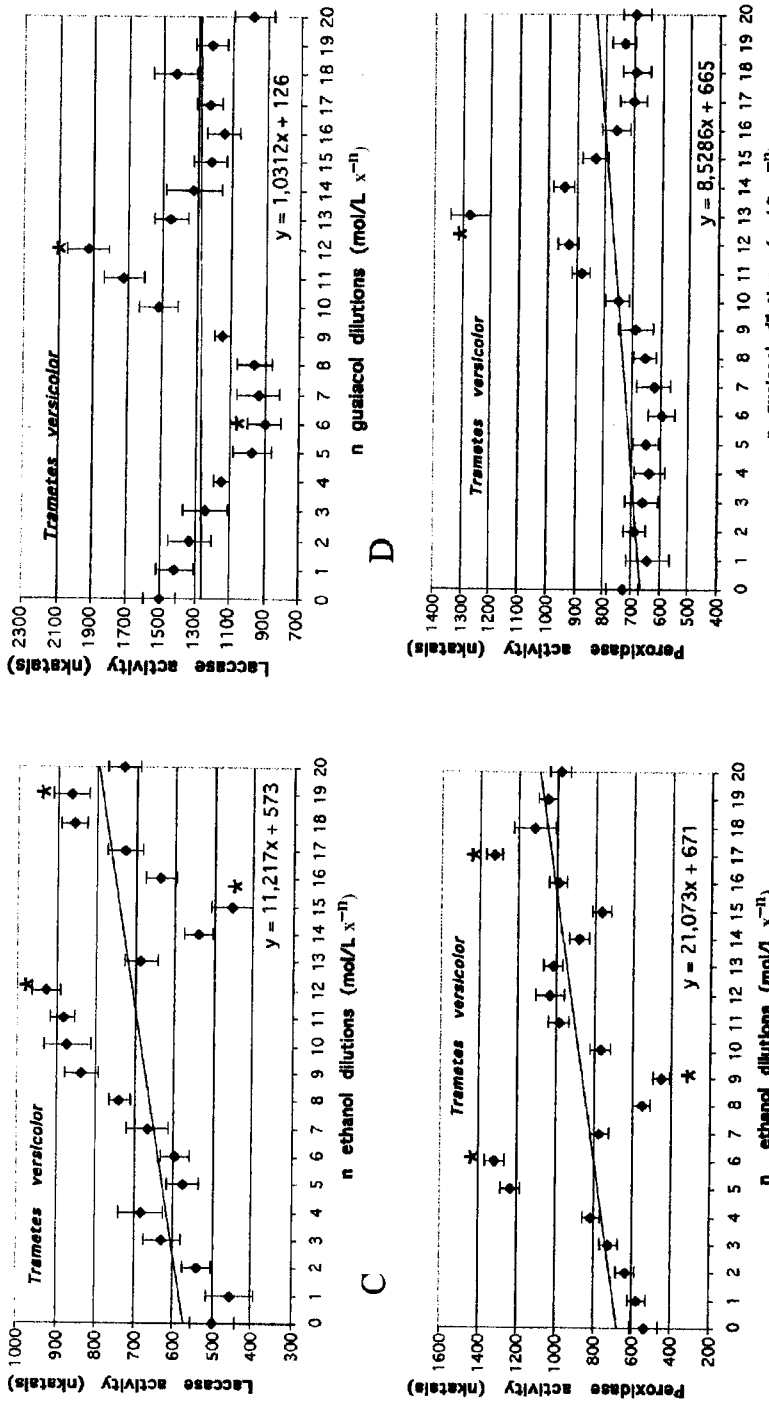


**Figure 1.** Laccase activities measured on day 15 in the media of *Trametes versicolor* cultures grown in serial dilutions of ethanol and guaiacol, respectively (A and B). Peroxidase activities measured on day 15 in the media of *Trametes versicolor* cultures grown in serial dilutions of ethanol and guaiacol, respectively (C and D). Lines represent the results of trend analysis. The error bars mean the values (+/- SD) counted from the data of three replicate experiments. Mean values followed by asterisk (*) differ significantly ($p=0.05$) from control value according to results from STATGRAPHICS program.

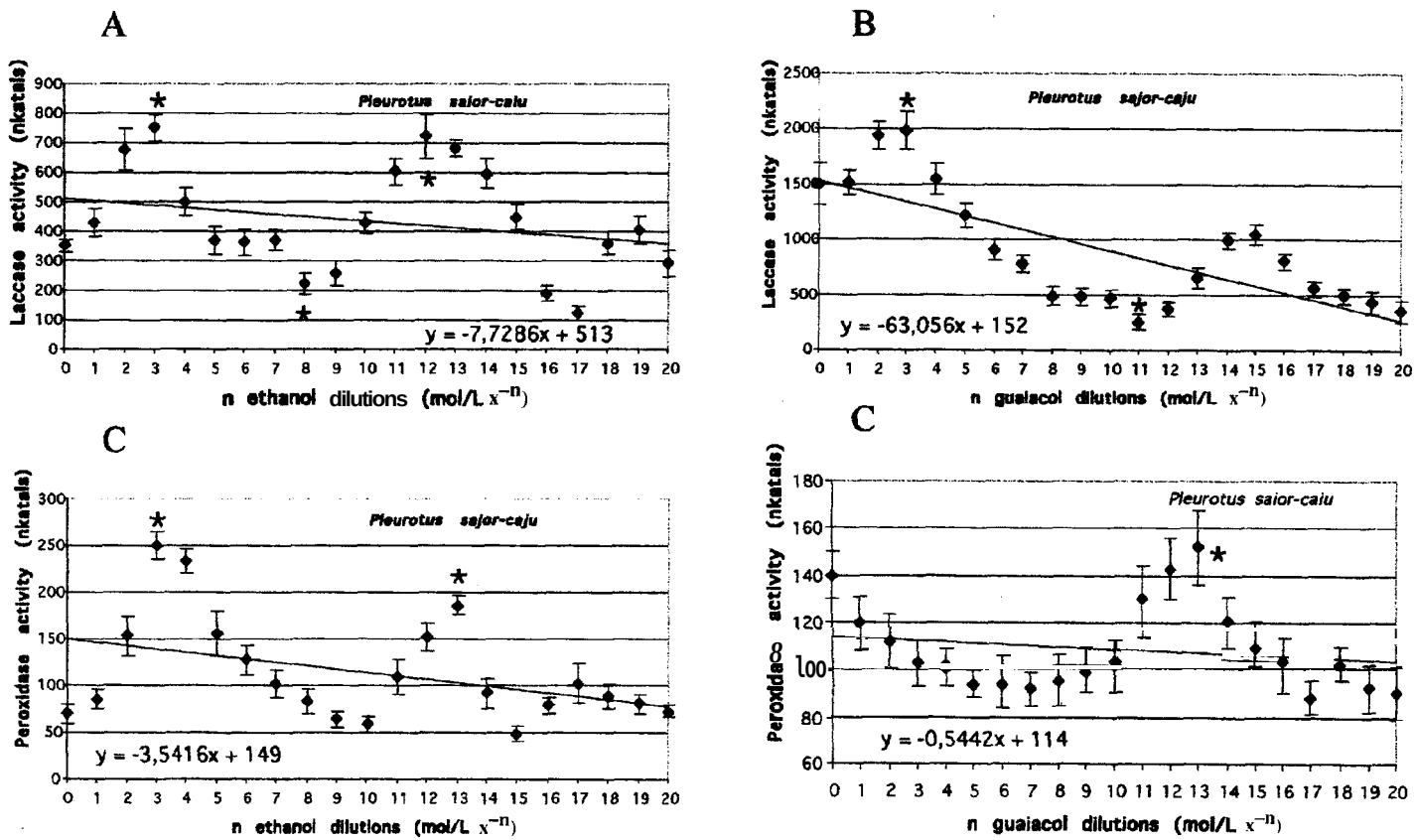


**Figure 2.** Laccase activities measured on day 15 in the media of *Pleurotus sajor-caju* cultures grown in serial dilutions of ethanol and guaiacol, respectively (A and B). Peroxidase activities measured on day 15 in the media of *Pleurotus sajor-caju* cultures grown in serial dilutions of ethanol and guaiacol, respectively (C and D). Lines represent the results of trend analysis. The error bars mean the values (+/- SD) counted from the data of three replicate experiments. Mean values followed by asterisk (*) differ significantly (p=0.05) from control value according, to results from STATSGRAPHICS program.

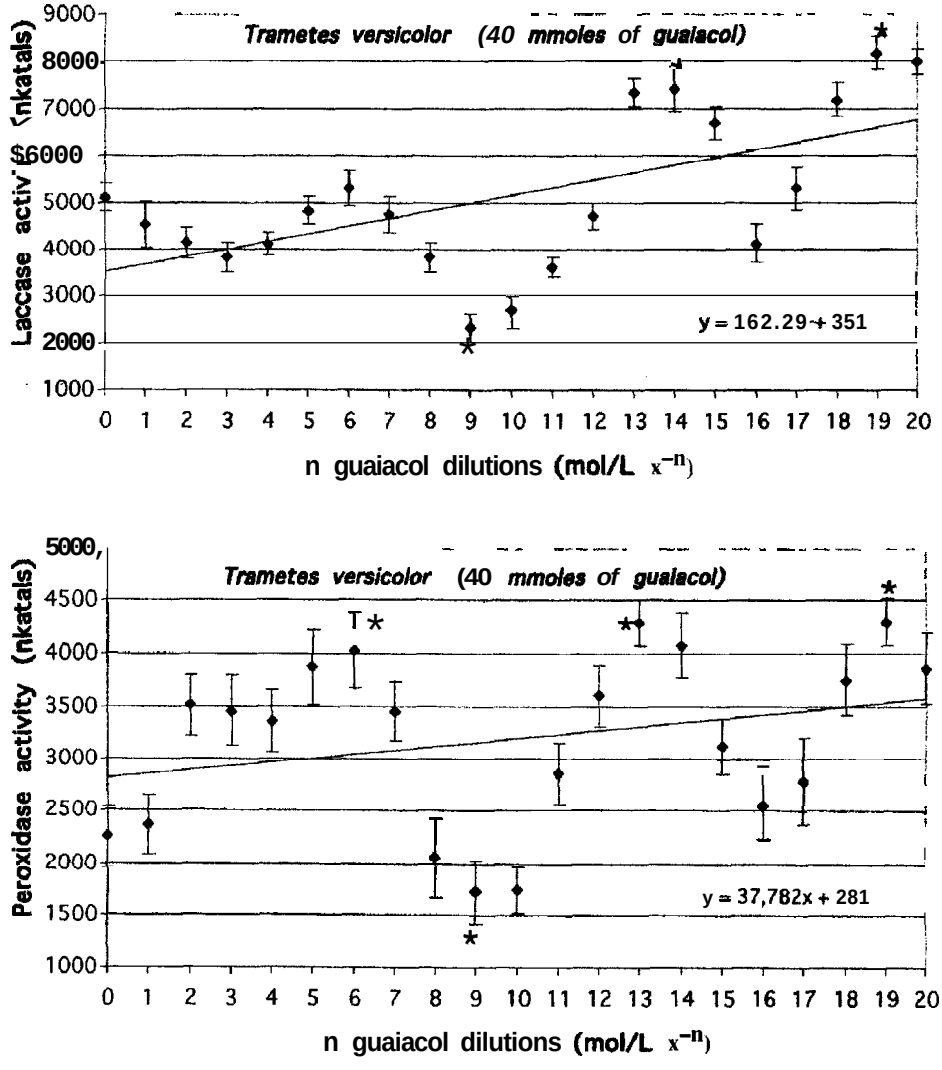


**Figure 3.** Laccase and peroxidase activities measured on day 15 in the media of *Trametes versicolor* cultures enriched with 40 mmol of guaiacol and exposed to serial dilutions of guaiacol. Lines represent the results of trend analysis. The error bars mean the values (+/- SD), which were calculated from the data of three replicate experiments. Mean values followed by asterisk (*) differ significantly (p=0.05) from control value according to results from STATSGRAPHICS program.

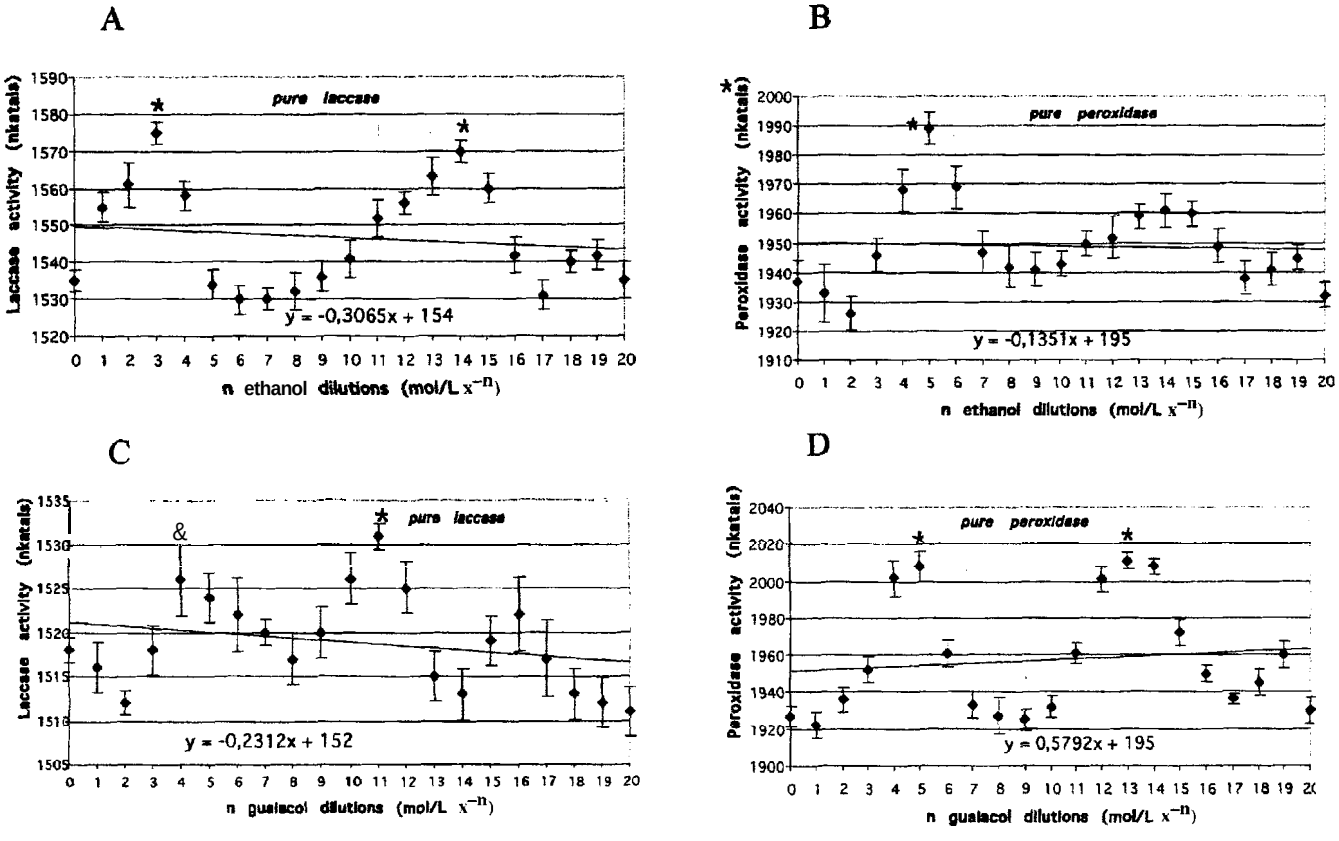


**Figure 4.** Purified laccase (A and B) and pure peroxidase (C and D) activities measured after one hour of incubation with guaiacol or ethanol dilutions. Lines represent the results of trend analysis. The error bars mean the values (+/- SD) counted from the data of three replicate experiments. Mean values followed by asterisk (*) differ significantly (p=0.05) from control value according to results from STATSGRAPHICS program.

Table 1. Summary of data from the 14 experiments.

| No | Fungus or pure enzyme | Kind of enzymatic activity | Specific activity (nkatals/mg prot.) | Effector | $\bar{*}$ (Maximum – Minimum) (nkatals) | Quantity of transits with J / U shapes |
|---|---|---|---|---|---|---|
| 1 | *P. s-c.* | Peroxidase. | 460 | ethanol | 200 | 4 / 2 |
| 2 | *P. s-c.* | Peroxidase. | | guaiacol | 60 | 2 / 1 |
| 3 | *P. s-c.* | Laccase | 4 155 | ethanol | 626 | 4 / 2 |
| 4 | *P. s-c.* | Laccase | | guaiacol | 1724 | 3 / 1 |
| 5 | *Tr. vers.* | Peroxidase. | 548 | ethanol | 664 | 3 / 1 |
| 6 | *Tr. vers.* | Peroxidase. | | guaiacol | 680 | 2 / 2 |
| 7 | *Tr. vers.* | Laccase | 5 020 | ethanol | 471 | 3 / 2 |
| 8 | *Tr. vers.* | Laccase | | guaiacol | 1030 | 5 / 2 |
| 9 | *Tr. vers.*** | Peroxidase. | 1 000 | guaiacol | 2570 | 5 / 3 |
| 10 | *Tr. vers.*** | Laccase | 45 000 | guaiacol | 5870 | 3 / 2 |
| 11 | Peroxidase (pure) | Peroxidase. | 2 720 | guaiacol | 88 | 4 / 2 |
| 12 | Peroxidase (pure) | Peroxidase. | | ethanol | 65 | 2 / 0 |
| 13 | Laccase (pure) | Laccase | 600 000 | guaiacol | 20 | 6 / 4 |
| 14 | Laccase (pure) | Laccase | | ethanol | 40 | 4 / 2 |

$\bar{*}$ - distance between extreme maximal and minimal values of enzyme activities calculated from Fig. 1 – 4

** cultivated with 40 mmols of guaiacol in medium

**Hormetic Effect of Guaiacol and Ethanol on Enzymatic Activity of Fungi**

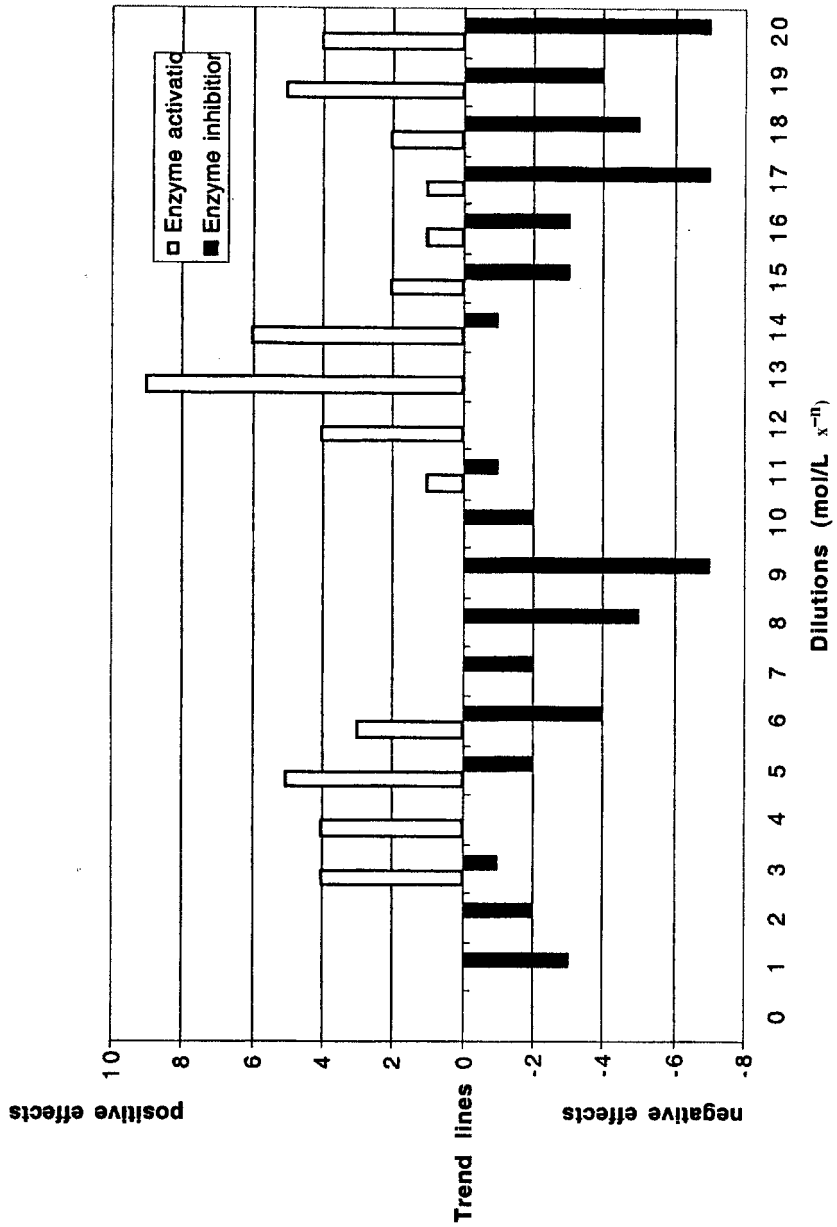


**Figure 5.** Comparative analysis for the data from all 14 experiments presented in Table 1 and Figures 1 to 4. Trend lines are showed in all these figures and the values of maximal and minimal points (lying above or below the trend lines) were summarized to illustrate the activating or inhibiting possibilities of various dilutions of guaiacol and ethanol against laccase and peroxidase activities.

The oscillations were characterized by a similar number of maximal and minimal levels of activity (Figures 1 and 2, Table 1 no. 1 to 8).

Similar oscillatory changes were observed in 40 mmol guaiacolenriched cultures of *T. versicolor* exposed to serial dilutions of guaiacol. However, the distance between the maximal and minimal activity values increased more dramatically. For peroxidase it reached approximately 2570 nkatals (Figure 3, Table 1 no. 9) and for laccase it reached up to 5870 nkatals (Figure 3, Table 1 no. 10). The corresponding values for non-activated cultures of *T. versicolor* were approximately 680 nkatals for peroxidase and 1030 nkatals for laccase.

Oscillatory changes were also observed in solutions of purified laccase and peroxidase, although the distance between the maximal and minimal activity values were much lower compared to those obtained from the fungal culture media (*i.e.*, for peroxidase the distance between maximum and minimum did not exceed 90 nkatals (Figure 4 C and D, Table 1 no. 11 and 12) and for laccase the distance did not exceed 40 nkatals (Figure 4 A and B, Table 1 no. 13 and 14). The frequency of oscillations in enzymatic activity, however, was similar to that observed for the enzymes in fungal cultures.

Figure 5 represents an assessment of the combined data for the 14 different experiments and indicates the most likely probability of observing a maximal enzymatic activity.

## DISCUSSION

Guaiacol and ethanol are found in the natural environment and essential for the metabolism of the fungi *Trametes* and *Pleurotus*. Increasing dilutions of both effectors showed the exponential dependence between the enzymatic protein reaction and the effector's concentration. The oscillatory character of activity changes was observed for both enzymes in showing systematically repeated frequencies (Figure 5), indicating the tendency of some effector's dilutions to stimulate the enzyme activity while other dilutions inhibit.

It should be emphasized that in all experimental conditions the oscillatory patterns were similar. However, the distance between maximum and minimum of activity did vary among the experimental groups and depended on the species of fungus, type of effector and kind of enzyme. Generally, the laccase activity level in the *Trametes* cultures was always higher than that in the *Pleurotus* cultures, which had an effect on the distance between the maximum and minimum activity values. Guaiacol showed a greater stimulation of laccase activity in both *Trametes* and *Pleurotus* cultures, whereas ethanol showed a greater stimultaion of peroxidase activity in the *Pleurotus* cultures.

In the case of *Trametes* cultures enriched with 40 mmol of guaiacol, statistically significant ($p=0.05$; see Figure 3) increases in the activities of both enzymes were observed in response to serial dilutions of guaiacol. Maximum laccase activity increased to approximately 8000 nkatals compared with 504 nkatals in the control

cultures. Maximum peroxidase activity increased to 4250 nkatals cornpared to 54 nkatals in the control cultures. It is conceivable that this activation can take place in nature in in *vivo* conditions during fungal growth. Low concentrations of guaiacol may be generated during fungal metabolism of various phenol substances.

Changes in activity of both enzymes (Table 1) assume the shape of the letters J or U described as characteristic of hormesis (Calabrese 2001). The occurrence of oscillatory patterns of enzyme activation/inhibition in response to effectors should be examined for other species of fungi and other enzymes. The findings reported here suggest the possibility of subtle regulation of enzymatic activity on the molecular level and clarification of these processes should be the focus of future research.

## ACKNOWLEDGMENT

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