SUMMARY

of the doctoral thesis titled:

BIOTECHNOLOGICAL IMPLICATIONS OF SOME MICROBIAN ORIGIN OXIDO-REDUCTASES

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Oxidoreductases are a class of enzymes that catalyze redox reactions occurring in living organisms. Laccase belongs to the polyphenol oxidase family and is very important for removing environmental pollutants due to its structural and functional properties. Recently, the ability of laccase to oxidize phenolic and non-phenolic substances has been considered by many researchers. Although filamentous fungi produce large amounts of laccase, industrial-scale production still faces many challenges. Currently, researchers are trying to increase the efficiency and productivity of laccase and reduce its final cost by finding suitable microorganisms and improving the production and purification process of laccase.

Given the above aspects, the main objective of this work was to select microorganisms producing oxidoreductase enzymes, especially laccase, to optimize the enzyme production process, characterize it, and test its various applications.

The bibliographic part is structured into three chapters, presenting oxidoreductases as a major group of enzymes, focusing on laccase, manganese peroxidase, and lignin peroxidase, complemented by the latest information on microorganisms reported to produce oxidoreductases/laccase, methods of obtaining and purifying them, as well as applications of laccases in various industries (food, pharmaceutical, bioremediation).

The experimental part focused on four major objectives, structured into five chapters, complemented by one of conclusions and perspectives.

The first experimental chapter (Chapter 4) presents the results of the selection of some micromycete and macromycete strains potentially producing laccase enzymes, correlated with the optimization of the plate screening method by adding guaiacol as an inducer. The screening was performed on fungal strains belonging to the genera *Aspergillus, Botrytis, Neurospora, Penicillium, and Trichoderma*, as well as on macromycete strains belonging to the species *Agaricus bisporus, Pleurotus sp., Ganoderma lucidum, and Laetiporus sulphureus*.

As a result of this research, we identified two strains belonging to the micromycete group (filamentous fungi) as positive for laccase production, namely *Trichoderma* sp. MI2 and *Trichoderma* sp. CP. Both *Trichoderma* spp. strains showed high laccase activity at a concentration of 0.025% guaiacol. A high concentration of guaiacol solution (0.1% to 1%) can inhibit the development of micromycetes. It was concluded that when performing plate screening, it is recommended to use lower guaiacol concentrations, starting from 0.01% to 0.075%.

Based on the results obtained in this study, it was concluded that the *Pleurotus* strain, originating from supermarket waste, is a producer of laccase enzymes. When cultivated on PDA medium supplemented with guaiacol solution, the optimal concentration for the *Pleurotus* strain was 0.025%. Within the first 24 hours, the strain demonstrated high laccase activity, as evidenced by the immediate appearance of a reddish-brown halo after inoculation.

The next chapter (Chapter 5) presents the identification of the two previously selected *Trichoderma* strains at the species level using molecular biology techniques. The molecular approach for identifying the two strains involved amplifying the conserved ITS1-ITS4 fragments, followed by the application of the PCR-ITS-RFLP technique, correlated with sequencing results. After amplifying the bacterial DNA with the ITS1/ITS4 primer pair, a single band of approximately 600 bp was obtained.

For the identification and discrimination of *Trichoderma* strains, a combination of three endonucleases (*SduI, HinfI, and HhaI*) was used. The PCR products were not cleaved with the *SduI* restriction enzyme. Both strains showed the same restriction profile: three restriction fragments were obtained with the *HinfI* enzyme, and four restriction fragments were obtained with the *HhaI* enzyme, corresponding to the theoretical restriction profile for *Trichoderma* sp. The strains were identified by sequencing the 5.8S-ITS region as *Trichoderma asperellum*, with a 100% identity match to numerous *T. asperellum* sequences in the database.

The next chapter (Chapter 6), one of the most substantial, includes a series of preliminary tests and optimization of oxidoreductase activity under laboratory conditions, in liquid medium, in the presence or absence of inducers. Several experimental variants were conducted. In a preliminary stage, the influence of pH on enzymatic activity in the presence of guaiacol, as well as the influence of $CuSO_4$ as an inducer, was tested. Subsequently, the optimization of enzymatic activity in the presence of $CuSO_4$ as an inducer was carried out, followed by the optimization of manganese peroxidase and lignin peroxidase activity by including $MnSO_4$ and veratryl alcohol in the medium.

Preliminary testing revealed that the highest oxidoreductase activity (laccase, lignin peroxidase, and manganese peroxidase) was recorded in cultures with an initial pH of 8; the addition of guaiacol as an inducer slightly increased enzymatic activity. The presence of $CuSO_4$ inducer at a concentration of 1 mM in M7 medium and 2 mM in PDB medium increased laccase activity.

During optimization, the presence of $MnSO_4 \times 7H_2O$ inducer at a concentration of 1 mM resulted in manganese peroxidase activity of over 1 U/mL after 72 hours of incubation for both Trichoderma strains. The use of veratryl alcohol at a concentration of 2 mM resulted in lignin peroxidase activity of over 1 U/mL after 96 hours of incubation for both Trichoderma strains.

The following two chapters address practical applications of using oxidoreductase enzyme complexes. Chapter 7 presents a study in which two commercial laccase strains (*Trametes versicolor and Trametes hirsuta*) were tested on the HT-29 cell line, a human cancer cell line derived from colorectal adenocarcinoma with typical epithelial morphology. To determine the working dose, the viability of HT-29 cells was evaluated using the quantitative spectrophotometric MTT assay after 24 hours of exposure to 11 dilutions of commercial laccases. It was observed that the highest enzyme concentration (1 mg/mL) inhibited tumor cell development compared to the control, with no significant differences between the two laccases. Thus, commercial laccases inhibit tumor cell viability by 25-40%.
The final experimental chapter (Chapter 8) aimed to test the effect of laccase on the total polyphenol content in apple juice over a 7-day refrigeration period, correlated with the evolution of antioxidant activity. Polyphenols are known for their antioxidant effect, but they are also the main factors involved in the

degradation of fruit juices, causing turbidity, color intensification, taste and aroma alterations, and sediment formation, affecting the shelf life of products and consumer perception.

The tests showed an accelerated decrease in the total polyphenol content in the apple juice samples treated with laccase, compared to the control sample, which had a slower decrease. The apple juice samples treated with commercial laccase at different concentrations (LC 0.5%, LC 1%, and LC 1.5%) showed a significant reduction in total polyphenol content, reaching 80% less than the initial amount after 3 days of storage.

Laboratory enzyme complexes obtained with *Trichoderma* TdMI2 and *Trichoderma* TdCP at a concentration of 1.5% showed a 56-61% reduction in polyphenol content after 7 days compared to the initial amount. A negative effect of using laccase in apple juice treatment is browning, caused by the formation of oligomers through polyphenol denaturation, which could be removed however by subsequent centrifugation.

Regarding novel elements, *Trichoderma* species have been very little studied in relation to obtaining oxidoreductases, especially laccase. For the first time, *Trichoderma asperellum* strains have been reported as potential laccase producers. These strains were isolated and preserved by the research team at the Faculty of Biotechnology, USAMV Bucharest.

In terms of optimizing enzymatic activity, the use of inducers such as $MnSO_4$ and veratryl alcohol has not been previously reported for inducing oxidoreductase activity (laccase, lignin peroxidase, and manganese peroxidase) in *Trichoderma asperellum* species.

In line with current global research trends, the first national test of colon adenocarcinoma (HT29) tumor line inhibition using microbial laccase was conducted, yielding positive and encouraging results.

For the first time, an enzyme complex from *Trichoderma asperellum* with high potential for use in apple juice clarification has been reported.