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ENZYMATIC TREATMENT AS A TECHNOLOGY TO MITIGATE ANTICANCER DRUGS IMPACT IN THE ENVIRONMENT: LACCASE-ASSISTED DEGRADATION OF ETOPOSIDE

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ABSTRACT

Emerging contaminants are substances produced by human activities, such as personal care products and pharmaceuticals, which enter rivers, lakes, and groundwater through wastewater, posing potential threats to human health and the environment. Furthermore, they are not yet regulated by any legislation. Anticancer drugs, a subset of pharmaceuticals, exhibit resistance to conventional wastewater treatment methods. This study investigates the enzymatic degradation of etoposide by different laccase activities and pH levels. The results showed that laccase activity at 1100 $U \cdot L^{-1}$ completely remove etoposide within 60 minutes, while a laccase activity of 55 $U \cdot L^{-1}$ removed 86% of etoposide after 360 minutes. Laccase was effective at all tested pH levels, including those conditions found in wastewater treatment plants (pH 6 and 7). These findings suggest that laccase has the potential to remove anticancer drugs from wastewater in treatment plants.

Keywords: Antineoplastic drugs. Oxidoreductases. Degradation.

1 INTRODUCTION

Emerging pollutants (EPs) are substances not typically monitored in wastewater treatment plants (WWTPs). However, they are likely to be subject to future regulations due to their potential risks to human health and environmental persistence (NORMAN, 2017). Pharmaceutical substances (PS) fall under the category of EPs and have been found in trace amounts in domestic, hospital, and agro-industrial wastewater. They potentially cause harmful effects on the environment and human health. Anticancer drugs, an PS, can adversely affect healthy cells, presenting considerable risks even at low concentrations due to their carcinogenic, mutagenic, and teratogenic properties and are found at concentrations up to $\mu g \cdot L^{-1}$ in effluents (PARRELLA *et al.*, 2014; RUSSO *et al.*, 2018).

Several innovative strategies have been implemented to reduce the environmental impact of EPs, including advanced oxidation processes (FRANQUET-GRIELL *et al.*, 2017), and biological treatments (MIR-TUTUSAUS *et al.*, 2017). Fungal treatments have been extensively researched for their ability to degrade EPs, particularly anticancer drugs in wastewater. Fungi produce extracellular ligninolytic enzymes, such as laccase and versatile peroxidase, which facilitate their metabolism and are credited with the degradation of anticancer drugs. Therefore, research has highlighted the potential of using these enzymes as biocatalysts to eliminate anticancer pharmaceuticals (PEREIRA, 2020).

Laccase (LC) is an enzyme classified as an oxidoreductase, which catalyzes the oxidation of a variety of organic molecules by employing oxygen as the electron acceptor, thereby producing water as a by-product (CAÑAS; CAMARERO, 2010). Recent studies have shown that LC can successfully degrade the anticancer drug doxorubicin. With an enzyme activity of 450, 900, and 1800 $U \cdot L^{-1}$, LC was able to degrade doxorubicin at concentrations between 50 and 500 $\mu g \cdot L^{-1}$ within a 12-hour period. Furthermore, cytotoxicity assessments using the standard cell line L-929 revealed that the degradation products were less toxic compared to the original doxorubicin solution (KELBERT *et al.*, 2023). I this sense, this work proposes de degradation of the anticancer drug etoposide by laccase at different enzymes concentrations and pH.

2 MATERIAL & METHODS

Determination of LC Activity

LC activity measurement was carried out according to the method described by García-Morales *et al.*, (2018). One unit of LC activity ($U \cdot L^{-1}$) was defined as the enzyme amount that catalyzes the oxidation of 1 mM of ABTS per min at 25 °C and pH 6.

ETO Measurement

The ETO measurement was carried out in HPLC (Shimadzu, LC-20A Prominence series, with UV spectrophotometer detector and Supelcosil™ LC-18 column (size 250 mm ×4.6 mm, 5 µm). The method was adapted from Solano *et al.*, (2012) in isocratic mode. The mobile phase composition was acetonitrile:water (35:65, v/v) at 1.0 $mL \cdot min^{-1}$ of flow rate and 45 °C. The injection volume was 20 µL, and detection was performed at 254 nm. The ETO retention time for this method was 5.6 min. ETO standard line was made in a concentration range from 50 to 20,000 $\mu g \cdot L^{-1}$. All samples and mobile phase were filtered with a 0.22-µm filter before the injection.

Degradation Condition and Sample Processing

For all assays, LC-assisted ETO degradation (C_0 = 500 µg·L^{-1}) was performed in 24-weel plates covered with sealing film to avoid evaporation, with 1 mL of total volume per well. In addition, control assays without the addition of LC were conducted to certify the stability of the drug under experimental conditions. The LC solution was prepared immediately before each experiment, and suitable dilutions of ETO stock solutions were made on phosphate buffer 0.01 M. Assays were performed in an orbital shaker (Quimis Q816M20—Brazil) at 30 °C and 180 rpm. In the first 3 h, samples were taken every 20 min and taken every hour until the end of the assay (6 h). After, cold acetonitrile was added to the samples (1:1 v/v) to interrupt the enzymatic catalysis. Furtherly, they were centrifuged for 10 min, at 10,000 rpm and 4 °C, to promote the LC precipitation. The supernatant was separated and kept frozen until chromatography analysis.

Optimum Enzyme Concentration and optimum pH

To define an appropriate amount of LC, assays were performed with four different enzyme activities (55, 110, 555, and 1100 U·L^{-1}). The LC activities were chosen based on previous studies (KELBERT et al., 2021). Thus, ETO was used at a concentration of 500 µg·L^{-1}. The degradation assays were performed at pH 6 and 30 °C. The influence of pH on the ETO degradation rate by LC was carried out in phosphate buffer (0.01 M) at pH 5, 6, and 7. The degradations were performed as described with an ETO concentration of 500 µg·L^{-1} and LC activity found in previous assay.

3 RESULTS & DISCUSSION

Optimum LC Concentration to Degrade ETO

ETO degradation at diferent enzyme concentrations was measured to select the enzyme activity that could achieve a satisfactory substrate degradation rate. As expected, a higher enzyme activity implied a higher initial reaction rate (Table 1) and, consequently, a shorter degradation time. After 6 h, the lowest LC concentration (55 U·L^{-1}) reached ETO degradation above 86% (Figure 1-A). The complete ETO degradation could be achieved after 2 h using the LC activity of 555 U·L^{-1}. Meanwhile, the highest LC activity (1110 U·L^{-1}) yielded the fastest degradation achieving 100% removal within 1 h. Alongside, assays without LC addition (control) were carried out under conditions, and no degradation could be seen (Figure 1-A). The initial reaction rate was calculated for each added enzyme activity (Table 1). Even reducing LC concentration 20-fold, the reaction rate dropped only to half of the value, showing no need to use a large amount of enzyme. Thus, to degrade ETO, a high amount of LC is unnecessary. Aiming the application in WWTP, costs must be minimized without a high loss of efciency, which happens in 55 U·L^{-1}.

Table 1 Initial reaction rates for degradation of etoposide by laccase (C_0=500 µg·L^{-1}) at pH 6 and 30 °C.

Laccase activity (U·L^{-1})	Initial reaction rate (µg·L^{-1}·min^{-1})
55	10.94
110	14.14
555	18.00
1100	21.65

Efect of pH on ETO Degradation by LC

According to Figure 1-B, at pH 5, 100% of ETO degradation was achieved after 160 min. Meanwhile, at pH 6 and 7, it reached around 80% degradation at the same time. The pH directly interferes with the enzyme conformation and stability, which can delay or facilitate ETO access to the active the enzyme sites(DARONCH *et al.*, 2020). Usually, to white-rot fungi enzymes, stability is achieved at acid pH, reaching an optimum between 4 and 5 and decreasing at higher pHs (FRASCONI *et al.*, 2010). This tendency corroborates with the initial specifc degradation rate found for ETO degradation by LC, obtained for each pH condition (Table 2). Among the tested parameters, pH 5 showed the highest degradation rate. As a control, the ETO stability was evaluated in all assays, showing that hydrolysis did not degrade the molecule in these conditions (Figure 1). Furthermore, it is already reported that ETO is stable in diferent bufers and pHs in an aqueous solution at pH between 4 and 5 (BEIJNEN *et al.*, 1988). In this sense, ETO degradation can be attributed to enzymatic action, whereas the highest initial reaction rate (pH 5) occurred in the most stable ETO condition.

Compared to pH 5, the initial reaction rate decreased by 33% at pH 6 and 66% at pH 7 (Table 2). Thus, this indicates that pH 5 has a higher degradation rate of ETO, and the relation between the increasing pH and the initial degradation rate is inversely proportional. Similar behavior was observed in norfoxacin degradation by LC, where reaction rates reached a maximum value at pH 5 and dropped at pH 6 (ZHAO *et al.*, 2017). Moreover, enzymatic treatments using LC as a biocatalyst showed that, generally, the optimum pH ranges from 4.5 to 6 (GASSER *et al.*, 2014).

Table 2 Initial reaction rates for degradation of etoposide by laccase (C_0=500 µg·L^{-1}) at pH 6 and 30 °C.

pH	Initial reaction rate (µg·L^{-1}·min^{-1})
5	15.25
6	10.94
7	5.40

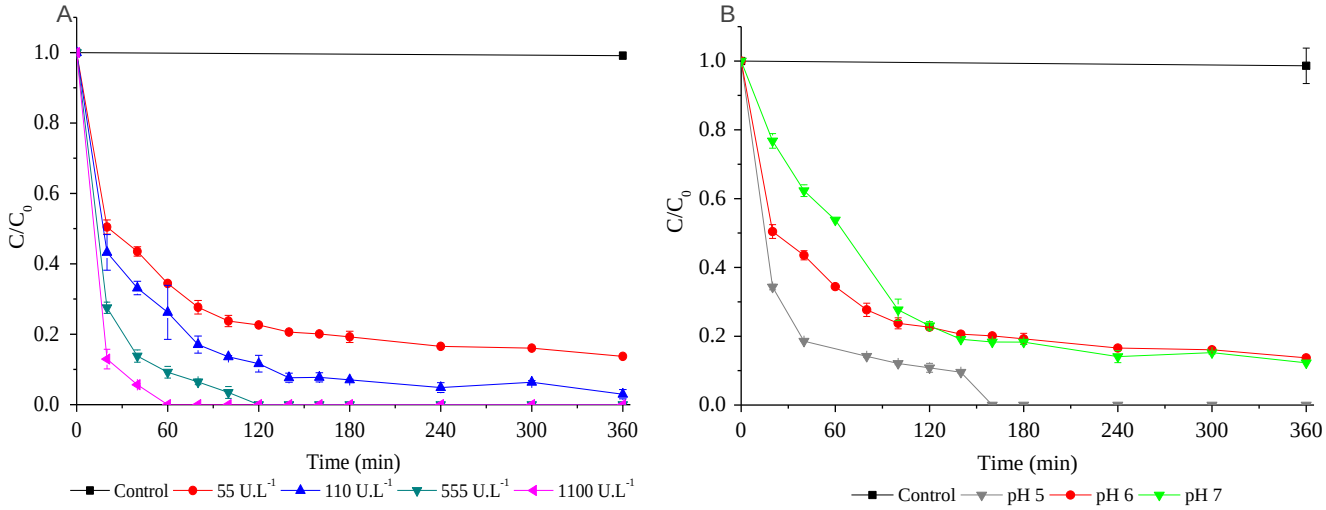

Figure 1 A) Etoposide degradation (C_0=500 µg·L^{-1}) with 55, 110, 555, and 1100 U·L^{-1}, at pH 6 and 30 ºC and B) Etoposide degradation (C_0=500 µg·L^{-1}) 55 U.L^{-1} at 30 ºC, and different pH 5,6 e 7.

4 CONCLUSION

This study demonstrated that ETO degradation is technically feasible by a catalyzed system with LC from *Trametes versicolor*. Substrate degradation occurred in all conditions of catalyst concentration and pH reaching 100% degradation in less than 1 h with 1100 U·L^{-1} of enzymatic activity and in 2 h and 40 min with 55 U·L^{-1} at pH 5. The chosen condition of pH 6, 55 U·L^{-1}, and 30 °C exhibited a satisfactory degradation of etoposide, occurring at pH close to neutrality, which is good for application in effluents treatment.

REFERENCES

BEIJNEN, J. H. *et al.* Degradation kinetics of etoposide in aqueous solution. **International Journal of Pharmaceutics**, [*s. l.*], v. 41, n. 1–2, p. 169–178, 1988.

CAÑAS, Ana I.; CAMARERO, Susana. Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. **Biotechnology Advances**, [*s. l.*], v. 28, n. 6, p. 694–705, 2010.

DARONCH, Naionara Ariete *et al.* Elucidating the choice for a precise matrix for laccase immobilization: A review. **Chemical Engineering Journal**, [*s. l.*], v. 397, p. 125506, 2020.

FRANQUET-GRIELL, Helena *et al.* Biological and photochemical degradation of cytostatic drugs under laboratory conditions. **Journal of Hazardous Materials**, [*s. l.*], v. 323, p. 319–328, 2017.

FRASCONI, Marco *et al.* Kinetic and biochemical properties of high and low redox potential laccases from fungal and plant origin. **Biochimica et Biophysica Acta - Proteins and Proteomics**, [*s. l.*], v. 1804, n. 4, p. 899–908, 2010.

GARCÍA-MORALES, Raul *et al.* Biotransformation of emerging pollutants in groundwater by laccase from P. sanguineus CS43 immobilized onto titania nanoparticles. **Journal of Environmental Chemical Engineering**, [*s. l.*], v. 6, n. 1, p. 710–717, 2018.

GASSER, Christoph A. *et al.* Laccases to take on the challenge of emerging organic contaminants in wastewater. **Applied Microbiology and Biotechnology**, [*s. l.*], v. 98, n. 24, p. 9931–9952, 2014.

KELBERT, Maikon *et al.* Inhibitory impact of the anticancer drug doxorubicin on anaerobic microbial community. **Aquatic Toxicology**, [*s. l.*], v. 264, p. 106706, 2023.

KELBERT, Maikon *et al.* Laccase as an efficacious approach to remove anticancer drugs: A study of doxorubicin degradation, kinetic parameters, and toxicity assessment. **Journal of Hazardous Materials**, [*s. l.*], v. 409, p. 124520, 2021.

MIR-TUTUSAUS, J. A. *et al.* Pharmaceuticals removal and microbial community assessment in a continuous fungal treatment of non-sterile real hospital wastewater after a coagulation-flocculation pretreatment. **Water Research**, [*s. l.*], v. 116, p. 65–75, 2017.

NORMAN. **Glossary of terms**. [*S. l.: s. n.*], 2017.

PARRELLA, Alfredo *et al.* Toxicity of exposure to binary mixtures of four anti-neoplastic drugs in Daphnia magna and Ceriodaphnia dubia. **Aquatic Toxicology**, [*s. l.*], v. 157, p. 41–46, 2014..

PEREIRA, Camila Senna. **Enzymatic process as a potential treatment technology to remove anticancer drugs from wastewater: Laccase-assisted degradation of etoposide**. 1–67 f. 2020. [*s. l.*], 2020.

RUSSO, Chiara *et al.* Toxicogenomic responses of low level anticancer drug exposures in Daphnia magna. **Aquatic Toxicology**, [*s. l.*], v. 203, p. 40–50, 2018.

SOLANO, Ana Gabriela Reis *et al.* Development and validation of a high performance liquid chromatographic method for determination of etoposide in biodegradable polymeric implants. **Quimica Nova**, [*s. l.*], v. 35, n. 6, p. 1239–1243, 2012.

ZHAO, Ruinan *et al.* Efficient enzymatic degradation used as pre-stage treatment for norfloxacin removal by activated sludge. **Bioprocess and Biosystems Engineering**, [*s. l.*], v. 40, n. 8, p. 1261–1270, 2017.

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