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Enzymatic process as a potential treatment technology to remove anticancer drugs from wastewater: Laccase-assisted degradation of etoposide

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O presente trabalho em nível de mestrado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Florianópolis, 2020.

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RESUMO EXPANDIDO

Introdução

Fármacos vêm sendo detectados em concentrações cada vez mais elevadas em efluentes hospitalares, domésticos e industriais. Esses compostos podem chegar ao meio ambiente majoritariamente através de excreções humanas, podendo também ser provenientes do descarte inadequado de medicamentos. Essas substâncias são consideradas poluentes emergentes devido ao potencial de risco que apresentam ao meio ambiente e à saúde humana, sendo em sua maioria recalcitrantes aos processos convencionais de tratamento de efluentes. Estações de tratamento de efluentes são projetadas para a remoção de matéria orgânica facilmente ou moderadamente biodegradável, não se sabendo ao certo como as mesmas são afetadas pela presença de poluentes emergentes. Assim como outros fármacos, os citostáticos (quimioterápicos) também estão sendo detectados no meio ambiente, não apenas em águas residuárias, mas também em águas superficiais. Quimioterápicos possuem efeitos carcinogênicos, teratogênicos e citotóxicos mesmo em baixas concentrações. Esses medicamentos são usados no tratamento de câncer e, com a estimativa de aumento na incidência de câncer no mundo, há uma preocupação quanto o aumento da concentração dos mesmos em efluentes. Estudos utilizando fungos da podridão branca (WRF, do inglês "White-rot Fungi") na degradação desses fármacos vêm sendo realizados. Apesar de serem estudos relativamente novos, sem aplicações industriais, os mesmos afirmam que a maior parte do processo de degradação dos fungos é atribuída à ação de enzimas excretadas pelo mesmo. Dentre elas está a lacase, que nos estudos investigados é a que apresenta a maior atividade enzimática. Até o momento, não há estudos publicados utilizando a lacase na degradação de fármacos anticâncer, sendo o presente estudo inovador tanto para aplicação em tratamentos de efluentes, como para potenciais usos da enzima. Neste estudo, avaliou-se a capacidade de degradação do fármaco anticâncer etoposido pela lacase. Este fármaco apresenta vários riscos ecotoxicológicos, como por exemplo, danos no DNA do verme aquático *Limnodrilus udekemianus* e dos microcrustáceos *Daphnia magna* e *Ceriodaphnia dubia* em concentrações numa escala de $\mu g \cdot L^{-1}$. Dado o risco apresentado, decidiu-se avaliar à degradação do mesmo em concentrações próximas a encontrada em efluentes, avaliando-se as condições de processo que melhor se aplicam em um tratamento de efluentes.

Objetivos

O objetivo principal desta pesquisa é avaliar a degradação do fármaco etoposido pela enzima lacase proveniente de *Trametes versicolor*, determinando a melhor concentração enzimática, melhor condição de pH e o efeito da concentração do etoposido na velocidade inicial da reação, visando a aplicação em condições encontradas em tratamento de efluentes.

Metodologia

Os ensaios de degradação do etoposido pela lacase foram realizados em placas de 24 poços, contendo um volume reacional de 1 mL em cada poço, e posteriormente fechadas com filme selante de PCR para evitar evaporação. Todos os experimentos foram realizados em tampão fosfato 0,01 $mol \cdot L^{-1}$, tendo amostras de controle do fármaco em tampão para garantir que a degradação ocorreu apenas pelo processo enzimático.

Os experimentos foram realizados em um agitador orbital a 180 rpm e 30 °C, com um tempo de reação de 6 horas. Durante as três primeiras horas, amostras de 0,8 mL foram retiradas a cada 20 minutos e, após esse tempo, retirou-se amostras a cada hora, sendo todas elas em duplicata. Para interromper a reação, adicionou-se 0,8 mL de acetonitrila fria nas amostras

(1:1 v/v) e centrifugou-se as mesmas a 10000 rpm e 4 °C durante 10 minutos. Posteriormente as amostras foram congeladas para análises de cromatografia líquida (HPLC-UV).
Visando-se a determinação da melhor concentração de enzima para a realização do processo, realizou-se a degradação do etoposido (500 $\mu g \cdot L^{-1}$) nas concentrações de lacase de 55, 110, 555 e 1100 $U \cdot L^{-1}$ em pH 6. Com o intuito de se observar a influência do pH, degradou-se o etoposido nos pHs 5, 6 e 7 em uma concentração enzimática de 55 $U \cdot L^{-1}$. Para avaliar o efeito da concentração de substrato, degradou-se o mesmo nas concentrações de 500, 1000, 1500, 2000, 5000, 7000, 10000, 15000 e 18000 $\mu g \cdot L^{-1}$ em uma concentração enzimática de 55 $U \cdot L^{-1}$ e pH 6. A velocidade inicial da reação foi utilizada como variável resposta dos experimentos.
Para quantificar-se a concentração do etoposido, utilizou-se a técnica de cromatografia líquida de alta eficiência (CLEA), tendo-se uma coluna C18 como fase estacionária, uma mistura acetonitrila:água (35/65) como fase móvel e um detector de absorbância UV. As amostras (20μL) previamente filtradas foram bombeadas pelo sistema com uma velocidade de 1.0 $mL \cdot min^{-1}$ e monitoradas a 254nm e 45°C. Uma curva padrão do etoposido em concentrações de 50 a 20000 $\mu g \cdot L^{-1}$ foi construída.

Resultados
A intenção de realizar a degradação em concentrações de $\mu g \cdot L^{-1}$ veio da necessidade de se aproximar ao máximo das concentrações em que o etoposido é encontrado em efluentes, dentro dos limites analíticos. Foi possível observar a degradação do etoposido em todas as condições testadas.
Quanto à concentração de catalisador, observou-se que a maior concentração (1100 $U \cdot L^{-1}$) degradou 100% do substrato em uma hora. Já a menor concentração (55 $U \cdot L^{-1}$) atingiu 86% de degradação em 6 horas de reação. Observou-se que mesmo reduzindo 20 vezes a concentração de catalisador, a velocidade inicial da reação diminuiu em 50% e que dobrando a concentração do mesmo, há um aumento de 20 a 30% nessa velocidade, mostrando que não há necessidade de altas concentrações enzimáticas para realização do processo. Tendo em vista a futura aplicação, realizaram-se os experimentos posteriores utilizando-se a concentração de catalisador de 55 $U \cdot L^{-1}$. Quanto ao melhor pH para o processo, sabe-se que estações de tratamento de efluentes operam em condições próximas a neutralidade, porém que as enzimas provenientes de fungos geralmente possuem pHs ótimos ácidos. Como esperado, o pH 5 alcançou a maior velocidade inicial de degradação, atingindo 100% em 160 minutos de reação, enquanto os pHs 6 e 7 alcançaram aproximadamente 80% de degradação em 180 minutos. Comparando com o pH 5, os pHs 6 e 7 exibiram uma redução de 33 e 66% na velocidade inicial da reação, respectivamente. Mesmo não sendo o pH ótimo de degradação, o pH 6 foi utilizado nos experimentos futuros por possuir uma boa velocidade de degradação e estar próximo a torno da neutralidade.
Quanto ao efeito da concentração de etoposido, observou-se uma relação linear entre a concentração do substrato e a velocidade inicial da reação, seguindo uma cinética de primeira ordem com uma constante K de 0,0477 min^{-1} ($r^2 = 0,995$). Mesmo na maior concentração de etoposido testada (18000 $\mu g \cdot L^{-1}$), não se observou inibição da lacase pelo substrato, mostrando a viabilidade de aplicação mesmo em concentrações bem acima das encontradas em efluentes.

Conclusões
A revisão da literatura exibiu os riscos ambientais da ocorrência de drogas anticâncer em águas residuais e águas superficiais. Pesquisas utilizando fungos da podridão branca (WRF)

mostraram o potencial de processos enzimáticos, especificamente a utilização da lacase, como um processo alternativo de degradação desses poluentes.
Este estudo demonstrou que a degradação do etoposido é tecnicamente viável através de um sistema catalisado por lacase proveniente de *Trametes versicolor*. A degradação do substrato ocorreu em todas as condições de concentração do catalisador, pH e concentração de fármaco testados. Em resumo, mesmo em concentrações de etoposido na escala $mg \cdot L^{-1}$, não ocorreu inibição da lacase pelo substrato, sendo escolhida a condição de pH 6, 55 $U \cdot L^{-1}$ e 30 ° C como a que exibiu o melhor resultado para a aplicação desejada, ocorrendo em pH em torno da neutralidade. Avaliou-se também o efeito do aumento da concentração de etoposido, tendo-se observado comportamento linear entre a concentração do fármaco e a velocidade inicial da reação, possuindo uma cinética de primeira ordem.

Palavras-chave: Quimioterápicos. Águas residuárias. Lacase. Etoposido.

ABSTRACT

Anticancer drugs exhibited recalcitrant behavior to conventional wastewater treatments and have been found in domestic, industrial, and hospital wastewater. They present environmental risks, as well as cytotoxic and carcinogenic effects in higher organisms, even at low concentrations. Studies were developed looking for new processes to meet this demand, among them, biological treatments using white-rot fungi (WRF), with degradations attributed mainly to laccase activity. Laccase was tested in etoposide anticancer drug degradation. Tests were carried out with 55, 110, 555, and 1100 $U \cdot L^{-1}$ of laccase activity, and, in all of them, the etoposide degradation was achieved. The pH conditions were also varied, using pH 5, 6, and 7. The highest initial reaction rate was obtained at pH 5; however, pH 6 was chosen due to application in environmental conditions (pH around neutrality). Etoposide concentration variations were performed in the range of 500-18000 $\mu g \cdot L^{-1}$ and exhibited a linear behavior with an increasing initial reaction rate, with degradation kinetics of 0.0477 min^{-1} ($r^2 = 0.995$).

Keywords: Anticancer drugs. Wastewater. Laccase. Etoposide.

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ABTS - 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

ACN – Acetonitrile

EC_{50} - Half maximal effective concentration

e-Biotech - Environmental Biotechnology Laboratory

d - Cell path length

HWW - Hospital wastewater

HRT - Hydraulic retention time

MEC - Measured environmental concentration

pH - Hydrogen potential

PNEC - Predicted no-effect concentration

RP - Removal percentage

RCR - Risk characterization rate

WRF - White-rot Fungi

WWTP - Wastewater treatment plants

t - Reaction time (min).

V - Reactional volume.

v - Laccase solution volume.

Δabs - Absorbance variation.

ε - ABTS molar extinction (3.6×10^4 $M^{-1} \cdot cm^{-1}$).

SUMÁRIO

CHAPTER 1

In this chapter, we have a brief introduction to the research developed and its general and specific objectives.

INTRODUCTION

Pharmaceuticals compounds first occurrence in the environment was reported in 1976. Since then, those substances have been detected in treated and non-treated wastewaters and freshwaters around the globe (EBELE; ABOU-ELWAFA ABDALLAH; HARRAD, 2017). They are considered as emerging pollutants due to their growing occurrence in the environment and the potential risk to human health (LI, 2014).

Those compounds can reach the environment through several pathways, such as hospital, domestic and industrial effluents, wastewater treatment plants, and disposal of expired and unworn drugs (ČELIĆ *et al*., 2018). Sewage is the largest source of various types of drugs and their metabolites, which can reach aquatic environments (GAW; THOMAS; HUTCHINSON, 2014; ISIDORI *et al*., 2016).

Wastewater treatment plants (WWTP) are not projected to deal with pharmaceuticals compounds (LUO *et al*., 2014). Usually, WWTP are designated to remove organic matter, and others easily and moderately biodegradable compounds, and microorganisms.

Some studies report adverse effects of drugs to marine organisms, which varies according to each mechanism of action (GAW; THOMAS; HUTCHINSON, 2014). Drugs with anti-depressant action have neurobehavioral effects, while antibiotics can cause antibiotic resistance in marine bacteria, fish, and seabirds (ROSE *et al*., 2009). Furthermore, anticancer drugs have adverse effects on the development and survival of some organisms, such as mutagenic effects (BOSSUS *et al*., 2014; TOOLARAM; KÜMMERER; SCHNEIDER, 2014).

Anticancer drugs are currently used for the treatment of cancer and have been detected in hospital wastewaters, WWTPs and river water samples in concentrations ranging from ng to $\mu g \cdot L^{-1}$ (BIEL-MAESO *et al*., 2018; CRISTÓVÃO *et al*., 2019). Most of these compounds are mutagenic, carcinogenic, genotoxic, and teratogenic to aquatic organisms, mainly upon chronic exposures (NOVAK *et al*., 2017; TOOLARAM; KÜMMERER; SCHNEIDER, 2014).

Some researchers demonstrated that these compounds are hardly biodegradable and not satisfactorily removed by conventional wastewater treatment processes (FERRANDO-CLIMENT; RODRIGUEZ-MOZAZ; BARCELÓ, 2014). There is much to be investigated about the potential risk of anticancer drugs. Moreover, there is a demand for new technologies to remove them from effluents, such as advanced oxidative processes, membranes, and new

biological processes, such as enzymatic treatment (FRANQUET-GRIELL *et al*., 2017; HAMON *et al*., 2018).

In recent years, the Environmental Biotechnology (e-biotech) Laboratory at the Federal University of Santa Catarina (UFSC) has developed research on emerging pollutant influence in aerobic and anaerobic wastewater treatment microorganisms. Bressan (2012) analyzed colistin sulfate toxicity, an antibiotic used for therapeutic and growth purposes in swine, on nitrifying and methanogenic bacteria cultures, toxicity indicators in aerobic and anaerobic effluent treatment systems, respectively. Perazzoli (2015) evaluated two iron oxide nanoparticles types effect on a mixed bacterial culture enriched with ammonia-oxidizing bacteria, which are part of the aerobic effluent treatment system. Michels (2016) investigated the inhibitory effect of silver nanoparticles on ammonia-oxidizing bacteria. Steinmetz (2016) analyzed the veterinary drug effect on the specific biogas production from agricultural substrates. Langbehn (2018) evaluated the inhibitory effect of tetracycline and oxytetracycline in a nitrifying bacteria mixed culture.

Following this group research field, this study also worked on emerging pollutants, specifically with anticancer drugs, focusing on this micropollutant removal, using enzymatic processes, since there are no known works with this purpose.

1.1 OBJECTIVES

1.1.1 General objective

This work aims to evaluate the potential of etoposide degradation by commercial laccase from *Trametes versicolor*.

1.1.2 Specific objectives

- Determine the best laccase concentration to apply to wastewater treatment.
- Determine the pH condition that is better applied to wastewater treatment.
- Determine the effect of etoposide concentration on the etoposide degradation reaction rate.

CHAPTER 2

This chapter presented a literature review of researches that uses fungi to degrade anticancer drugs, this degradation being attributed mainly to laccase, and that this enzyme has the potential to be used on the degradation of these compounds. The literature review presented in this chapter was published in the journal Applied Microbiology and Biotechnology in November of 2019 (doi.org/ 10.1007/s00253-019-10229-y).

2 POTENTIAL OF ENZYMATIC PROCESS AS AN INNOVATIVE TECHNOLOGY TO REMOVE ANTICANCER DRUGS IN WASTEWATER

Abstract

Anticancer drugs are a class of pharmaceuticals compounds that have been found in the hospital, domestic and industrial wastewaters, and also in surface waters. They have been showing recalcitrance to conventional wastewater treatment technologies and present a potential risk to the environment and human health, since they exhibit cytotoxic, teratogenic, and carcinogenic among other effects in higher organisms, even at low concentrations. The presence of these compounds in the environment is a recent challenge for wastewater treatment, and some alternative strategies to remove them were already studied, such as White-rot Fungi (WRF) technologies. Despite promising results, processes involving fungi are complex, have high reaction times, and require nutrients addition for fungus growth and maintenance. Due to this potential, strategies to make the technology feasible were studied, such as the possibility for direct application of enzymes secreted by WRF. Enzymatic processes were studied in the removal of other pharmaceuticals such as antibiotics, anti-inflammatory, and steroid hormones; however, to the best of our knowledge, there is a gap in the literature about their direct action on anticancer drugs.

2.1 INTRODUCTION

Emerging pollutants are compounds that are not investigated in routine monitoring of wastewater treatment plants (WWTP), but are candidates for future legislation due to the risks to human health and persistence on the environment (NORMAN, 2017). It is not entirely known about how they can affect the efficiency of the WWTP (LI, 2014), by inhibiting the activity of the microorganisms responsible for the treatment, such as hindering nitrogen removal (MICHELS; PERAZZOLI; M. SOARES, 2017). Pharmaceuticals products are on the list of emerging pollutants due to the fact they were detected in trace concentrations in domestic, hospital, and agroindustry wastewaters and may have adverse effects on the environment and human health (BRESSAN *et al*., 2013; MIR-TUTUSAUS *et al*., 2018). The occurrence of these compounds in wastewaters and surface waters is a recent challenge for wastewater treatment plants' operation.

Pharmaceuticals products arrive at WWTP mainly through human excretions, either urine or feces. After administered, some of them are metabolized, and others remain intact, being excreted in both forms (BIEL-MAESO *et al*., 2018). WWTP is not designed to remove pharmaceuticals. Usually, they focus on the removal of easily or moderately biodegradable carbon, nitrogen, and phosphorus compounds and biomass, which regularly are present in WWTP at concentrations to the order of $mg \cdot L^{-1}$ (VERLICCHI; AL AUKIDY; ZAMBELLO, 2012). In another order of magnitude, emergent pollutants belong to diverse chemical classes and are typically detected at trace levels ($ng \cdot L^{-1}$ to $mg \cdot L^{-1}$) in wastewaters (VERLICCHI; AL AUKIDY; ZAMBELLO, 2012), and surface and subsurface waters (BAI *et al*., 2018).

There is no global consensus on the anticancer drugs emissions control in the environment. However, the European Commission (2015) established the Commission Implementing Decision (EU) 2015/495 that implemented a watch list of substances for monitoring surface waters, which regulates some emerging pollutants, including the anti-inflammatory diclofenac, macrolide antibiotics, and steroid hormones. Many countries have no legislation that includes these drugs in the routine of the WWTP monitoring list. Due to the absence of legislation about the limits of pharmaceutical drug discharge on wastewater, there is no control of these compounds on conventional WWTP removal effectiveness. Once released to the environment, these contaminants can reach rivers, lakes and other water reservoirs in an urban area that are typically used for drinking water and recreation activities, being the most significant routes for human exposure (BAI *et al*., 2018).

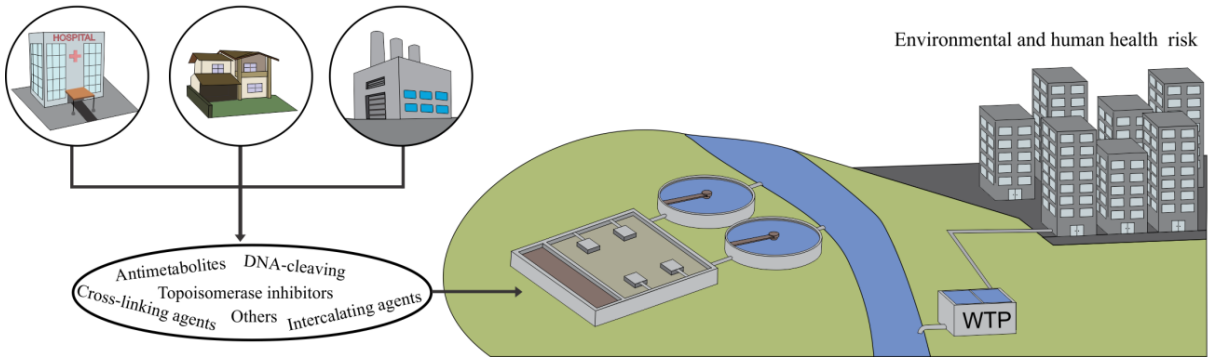
On the list of pharmaceuticals, there are anticancer drugs. With the increasing number of cancer cases, they are the subject of concern since they have potential risks for human health and the environment (BALCERZAK; REZKA, 2014). Anticancer drugs were found in concentrations lower than other classes of drugs in aquatic systems (RUSSO *et al*., 2018). Nevertheless, a cytotoxic, mutagenic, embryotoxic, teratogenic and carcinogenic effect and low biodegradability in WWTP of anticancer drugs in small concentrations (MATER *et al*., 2014) were reported (KOSJEK; HEATH, 2011). In this paper, the problem of anticancer drugs in the environment is discussed, and approaches on the use of non-conventional biological alternatives for bioremediation are presented.

2.2 OCCURRENCE AND POTENTIAL RISKS OF ANTICANCER DRUGS IN THE ENVIRONMENT

The number of people diagnosed with cancer increased significantly in recent years. Only in 2018 more than 18 million new cancer cases were diagnosed around the world, and almost 560 thousand in Brazil (INTERNATIONAL AGENCY FOR RESEARCH ON CANCER, 2018), which promotes the production and consumption of large quantities of anticancer drugs (FRANQUET-GRIELL *et al*., 2017). As other pharmaceuticals, anticancer drugs can be excreted as human metabolites or in its original form through urine or feces after being administered, some of them are metabolized and the residual stay at the original form (SANTANA-VIERA *et al*., 2016).

Focusing on patient comfort, some cancer treatments can be made at home and, with the increase of this trend, hospital effluents are not the only concern, as they can be present on domestic and industrial effluents (Fig.1.) (BESSE; LATOUR; GARRIC, 2012; HABIBZADEH; CHAIBAKHSH; NAEEMI, 2018). Each type of cancer requires specific drugs, with specifics action mechanisms that can be used isolated or combined, generating effluents with a complex composition (GÓMEZ-CANELA *et al*., 2014).

Figure 1 - Risk potential of anticancer drugs: since effluents generation through wastewater and water treatment plant to environmental and human exposition.



Source: from author.

Countries such as Spain, Switzerland, Portugal, and Japan detected anticancer drugs in environment, at concentrations ranging from $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$ (AZUMA, 2018; BALCERZAK; REZKA, 2014; KÜMMERER, 2001; NEGREIRA; DE ALDA; BARCELÓ,

2014a). In Spain, the occurrence of anticancer drugs was traced from the hospital effluent, through the WWTP, until the release into water bodies (rivers). Cyclophosphamide was found at the concentration range of 3089 to 14826 $ng \cdot L^{-1}$ in hospital effluent, but at the influent of the treatment plant, by dilution effects, this range reduced from 1172 to 1558 $ng \cdot L^{-1}$. Another unsettling fact is their occurrence on surface waters, with a concentration between 103 and 147 $ng \cdot L^{-1}$, which shows they are already present in the natural environment (FERRANDO-CLIMENT; RODRIGUEZ-MOZAZ; BARCELÓ, 2014).

Tamoxifen, mainly used in hormonal treatment for breast cancer in both pre- and post-menopausal women (CRISCITIELLO *et al*., 2011), was found at concentrations ranging from 178 to 181 $ng \cdot L^{-1}$ in the influent and from 102 to 147 $ng \cdot L^{-1}$ in the municipal WWTP effluent in Spain (NEGREIRA; DE ALDA; BARCELÓ, 2014a), exhibiting recalcitrance to conventional sewage treatment. Other drugs, as ciprofloxacin and methotrexate, showed similar recalcitrance to conventional sewage treatment (FERRANDO-CLIMENT; RODRIGUEZ-MOZAZ; BARCELÓ, 2014).

Anticancer drugs showed recalcitrance not only to conventional WWTP but also to some advanced and unconventional technologies, as hydrolysis and UV radiation. Seventy percent (70%) of etoposide, ifosfamide and, cyclophosphamide remained unaltered after 8.33 hours activated sludge treatment (FRANQUET-GRIELL *et al*., 2017).

Anticancer drugs can do an additional toxicological effect in the environment (MATER *et al*., 2014). They showed high impact to organisms with carcinogenic, endocrine disruptor, teratogenic, genotoxic, mutagenic and cytotoxic effects, since they were developed to disrupt or prevent cellular proliferation (KÜMMERER *et al*., 2016; NEGREIRA; DE ALDA; BARCELÓ, 2014b; NOVAK *et al*., 2017).

Conventional treatments for drinking water and wastewater have a disinfection stage that typically uses a chlorination process. Studies analyzed degradation with free chlorine of anticancer drugs and its metabolites and toxicity of the final effluent. Tamoxifen (and its metabolites) and etoposide were exposed to free chlorine at various conditions in a synthetic and real wastewater. Disinfection by-products showed until 110-fold more potential aquatic toxicity compared to the parent compound, exhibiting an increase in final effluent toxicity (NEGREIRA *et al*., 2015). Etoposide reacted quickly with free chlorine and formed a major by-product 3′-O-desmethyl etoposide (NEGREIRA; DE ALDA; BARCELÓ, 2015) that, according to literature, exhibit a cytotoxic activity by (TERNES; JOSS, 2015) inactivating a biologically active DNA (STREMETZNE; JAEHDE; SCHUNACK, 1997).

One way to measure the environmental risk associated with a drug is through the risk characterization rate (RCR), which is the ratio between the predicted no-effect concentration (PNEC) and, the measured environmental concentration (MEC). RCR values above 1 indicate that the drug is found in the environment at concentrations may cause an effect in a given organism. Ciprofloxacin and tamoxifen exhibited RCR values between 1 and 10 to many organisms, showing a potential impact on the aquatic environment (FERRANDO-CLIMENT; RODRIGUEZ-MOZAZ; BARCELÓ, 2014).

Araujo *et al*. (2019) exposed *Lithobates catesbeianus* tadpoles at various concentrations levels of cyclophosphamide and 5-fluorouracil. The authors reported that all the animals exhibited some intestinal abnormalities, color change, and variations in the distance interocular, which results can lead to genomic instability, cell death, and cancer.

The increasing number of new cases of cancer, which increases the amount of treatments and consequently the concentration of anticancer drugs in hospital and domestic effluents, combined with the environmental and human health risk, even in low levels, make the occurrence of these compounds in natural environments a challenge for conventional water and sewage treatment processes. Nowadays, such compounds are generally found in surface waters at concentrations in $ng \cdot l^{-1}$; however, there is an expectation of growing this value and consequently, their potential risk. The treatment process needs to be prepared to prevent the anticancer drug's impact on the environment.

According to recent researches, conventional processes cannot satisfactorily meet the demand for removal of anticancer drugs in domestic and hospital effluents. The traditional technologies used for biological wastewater treatment are based on bacteria metabolism degrading these compounds as substrates. Therefore, alternative and clean biological treatment technologies need to be developed shortly, such as fungi or enzymatic processes (CASTELLET-ROVIRA *et al*., 2018; DA ROSA *et al*., 2019; FRANQUET-GRIELL *et al*., 2017).

2.3 ANTICANCER DRUGS REMOVAL FROM THE ENVIRONMENT BY FUNGAL TREATMENT

WRF is a group of fungi species that can degrade lignin. They can secrete lignin modifying enzymes, such as manganese peroxidases, lignin peroxidases, versatile peroxidases, and laccase, all extracellular enzymes (ŁEBKOWSKA; ZAŁESKA-

RADZIWIŁŁ, 2014). These enzymes have a characteristic of being non-specificity due to the action through the generation of radicals. This allows them to be capable of degrading a wide range of compounds, such as dyes, pesticides, and some pharmaceuticals (MIR-TUTUSAUS *et al*., 2018).

Pleurotus ostreatus, *Phanerochaete chrysosporium*, *Trametes versicolor*, *Ganoderma lucidum,* and *Irpex lacteus* are some relevant species of WRF (MIR-TUTUSAUS *et al*., 2018) and they were used for many pollutants degradation such as pharmaceutical compounds, pesticides, industrial chemicals, and endocrine disruptors (CRUZ-MORATÓ *et al*., 2014; LUCAS *et al*., 2016; MARCO-URREA *et al*., 2010; MIR-TUTUSAUS *et al*., 2018).

Fungi require readily biodegradable carbon sources to grow and degrade drugs or other micropollutants co-metabolically. This means that carbon sources of the micropollutants are not enough to maintain the fungi, making the pharmaceuticals a part of these microorganisms' secondary metabolism (WEN *et al*., 2011). This feature is interesting from wastewaters treatment since the hospital and domestic effluents consist of easily or moderately biodegradable organic material that can serve as a carbon source for fungus growth. At the same time, the micropollutants are co-metabolized (MIR-TUTUSAUS *et al*., 2018).

Recently, WRF was tested on the removal of anticancer drugs from effluents, showing promising results in some cases (Table 1). Tests using *Trametes versicolor* fungus showed its potential for degradation and removal of these drugs, reaching total removal for etoposide and azathioprine (FERRANDO-CLIMENT *et al*., 2015).

Trametes versicolor, *Ganoderma lucidum*, *Irpex lacteus*, *Stropharia rugosoannulata*, *Gymnopilus luteofolius,* and *Agrocybe erebia*, all WRF, had their ability to degrade some pharmaceuticals compounds in a synthetic solution tested. Cyclophosphamide and ifosfamide proved to be recalcitrant with most fungi tested, exhibiting low removal rates, under 40% (CASTELLET-ROVIRA *et al*., 2018). Other studies, using different WRF, showed similar behavior corroborating with their recalcitrance (FERRANDO-CLIMENT *et al*., 2015; HAROUNE *et al*., 2014).

Ciprofloxacin occurring in hospital wastewater (HWW) was treated with *Trametes versicolor* which showed good removal percentages, reaching almost 100% (CRUZ-MORATÓ *et al*., 2014; FERRANDO-CLIMENT *et al*., 2015; PRIETO *et al*., 2011) removal, except for those found by Cruz-Morató *et al* (2013) and Mir-Tutusaus *et al* (2017), that reached 35 and 47.1% removal, respectively. This fact can be explained due to the difference

in enzymatic activity, since, in the first two cases, activity values between 100 and 1002 $U \cdot L^{-1}$ were observed. In the case of Mir-Tutusaus *et al* (2017), the enzymatic activity was negligible at first 29 days and from days 30-56 showed values below 80 $U \cdot L^{-1}$. A similar event occurred in the experiment of Cruz-Morató *et al* (2013), where the maximum enzymatic activity was 70 $U \cdot L^{-1}$ during the 7 days.

In a real sample of HWW, tamoxifen (45 $ng \cdot L^{-1}$) was partiality removed (48%), but, when it was tested in a synthetic solution with a concentration of 0.3 $mg \cdot L^{-1}$ was 92% removed in 1 hour and removed after 9 days. The removal levels were attributed to two processes, the sorption in fungal biomass with posterior biodegradation by intracellular enzymes and biodegradation with extracellular ligninolytic enzymes, characteristic of WRF (FERRANDO-CLIMENT *et al*., 2015). Even with the potential showed from WRF, their use in the treatment of micropollutants is still a challenge (MIR-TUTUSAUS *et al*., 2018). As expected, in the experiments with the synthetic medium, it was required to add nutrients to promote fungus growth and, as a secondary route, micropollutants degradation. In most cases, glucose, ammonium tartrate, malt extract (CASTELLET-ROVIRA *et al*., 2018), and yeast extract (HAROUNE *et al*., 2014) were added to the reaction medium. However, when real effluent samples were used as the substrate, the addition of nutrients was still necessary, bringing a complicating factor to the process, since nutrients addition in WWTP increases operational cost. Glucose and ammonium tartrate was used as nutrients with real wastewater in all works reviewed (CRUZ-MORATÓ *et al*., 2013, 2014; FERRANDO-CLIMENT *et al*., 2015; MIR-TUTUSAUS *et al*., 2017).

Most WRF optimal pH growth is acid, generally in a range of 3 to 5, which generates an additional cost in the process to control the pH and equipment that supports this condition. Acidic pH is important for the process efficiency, it prevents the growth of most bacteria, avoiding substrate competition between fungi and bacteria (MIR-TUTUSAUS *et al*., 2018). As seen in Table 1, processes with fungi need high reaction times, in the order of days, to be able to degrade anticancer drugs. This feature requires high hydraulic retention time (HRT) in bioreactors and, consequently, large reactor volume is needed, making technology implementation more expensive. Since fungal processes have high reaction time for growth and produce their metabolites, the use of enzymes extracts may be advantageous to reduce the HRT in bioreactors.

Table 1 - Removal percentage (RP) of fungus treatment for anticancer drugs.

Anticancer Drug	[C] (μg/L)	Matrix	Fungus	Reaction Time (Days)	Analyzed Enzyme	Maximum Enzymatic Activity ($U \cdot L^{-1}$)	RP (%)[a]	Reference
Azathioprine	0.055	Non-sterilized HWW	*T. versicolor*	8	Laccase	100	100	(FERRANDO-CLIMENT *et al*., 2015)
Ciprofloxacin	2000	Synthetic Solution	*T. versicolor*	7	Laccase	1002	>90	(PRIETO *et al*., 2011)
	84.71	Non-sterilized urban wastewater	*T. versicolor*	8	Laccase	70	35	(CRUZ-MORATÓ *et al*., 2013)
	2.179	Non-sterilized HWW	*T. versicolor*	8	Laccase	100	97	(FERRANDO-CLIMENT *et al*., 2015)
	13.04	Non-sterilized HWW	*T. versicolor*	8	Laccase	320	>99	(CRUZ-MORATÓ *et al*., 2014)
	0.3664	Flocculated HWW	*T. versicolor*	56	Laccase	-	47.1	(MIR-TUTUSAUS *et al*., 2017)
Cyclophosphamide	~60	Synthetic Solution	*T. versicolor, I. lacteus, G. lucidum*	6	Laccase	130	<40	(CASTELLET-ROVIRA *et al*., 2018)
	0.02/0.1/0.5	Synthetic Solution	*T. hirsute*	*5*	Laccase	70	<40	(HAROUNE *et al*., 2014)

	10000	Synthetic Solution	*T. versicolor*	*9*	Laccase	100	-	(FERRANDO-CLIMENT *et al*., 2015)
Etoposide	198	Non-sterilized HWW	*T. versicolor*	8	Laccase	100	100	(FERRANDO-CLIMENT *et al*., 2015)
Ifosfamide	~60	Synthetic Solution	*T. versicolor, I. lacteus, G. lucidum*	6	Laccase	130	<40	(CASTELLET-ROVIRA *et al*., 2018)
	77	Non-sterilized HWW	*T. versicolor*	8	Laccase	100	61	(FERRANDO-CLIMENT *et al*., 2015)
	20/100/500	Synthetic Solution	*T. hirsute*	5	Laccase	70	<40	(HAROUNE *et al*., 2014)
Tamoxifen	45	Non-sterilized HWW	*T. versicolor*	8	Laccase	100	48	(FERRANDO-CLIMENT *et al*., 2015)
	300	Synthetic Solution	*T. versicolor*	9	Laccase	350	99	(FERRANDO-CLIMENT *et al*., 2015)

[a] Removal Percentage

2.4 POTENTIAL OF ENZYMATIC PROCESS FOR REMOVAL OF ANTICANCER DRUGS IN THE ENVIRONMENT

Enzymatic processes of micropollutants biotransformation have attracted attention due to high activity in a wide range of substrates and less toxic transformation products. Studies have shown that enzymes in the isolated form can be used in bioremediation processes to remove dyes, drugs, hormones, and other pollutants from domestic and industrial effluents (BARRIOS-ESTRADA *et al*., 2018; CATHERINE; PENNINCKX; FRÉDÉRIC, 2016; FARIAS *et al*., 2017; HOELSCHER *et al*., 2018).

In the last years, enzymes from WRF have been studied for the removal of pharmaceutical drugs wastewaters (STADLMAIR *et al*., 2018). Data on this subject are compiled in Table 2. Batch experiments using a crude extract of versatile peroxidase from *Bjerkandera adusta* exhibited a 100% degradation of diclofenac and some steroid hormones, and 80% of antibiotic sulfamethoxazole and anti-inflammatory naproxen (EIBES; DEBERNARDI; LEMA, 2011).

The enzyme chloroperoxidase from *Caldariomyces fumago* exhibited the potential for antibiotic norfloxacin degradation using artificial wastewater, showing the degradation of 82.18% within 25 minutes. Ecotoxicological tests were performed in *Chlorella pyrenoidosa*, freshwater green algae, submitted to the initial solution, and after treatment with enzymes. Analyzes indicated algae sensitivity to norfloxacin, exhibiting a concentration of 0.0293 $mg \cdot L^{-1}$ to reach half of the toxic effect (EC_{50}). When the tests were carried out in the effluent after treatment, EC_{50} value increased from 0.0293 to 0.3668 $mg \cdot L^{-1}$, exhibiting lower toxicity. Thereby, ecotoxicological tests revealed that effluent after enzymatic treatment is less toxic than the norfloxacin degradation products (ZHAO *et al*., 2017).

A study with lignin peroxidase from *Phanerochaete chrysosporium* showed total degradation of the anti-inflammatory diclofenac within 2 hours (ZHANG; GEISSEN, 2010). Partial degradation of the tetracycline and oxytetracycline drugs, both antibiotics, was achieved with the enzyme manganese peroxidase within 4 hours, reaching 72.5 and 84.3% of degradation, respectively (WEN; JIA; LI, 2010).

Laccase, produced by a variety of fungi, have been tested to degrade different types of drugs due to its low substrate specificity and its ability to catalyze the electron transfer from substrate to molecular oxygen, which is then reduced to water (DURÁN *et al*., 2002; SPINA *et al*., 2015). Some studies with laccase from *Trametes versicolor* found significant results for

the degradation of several pharmaceutical compounds. Total removal of steroid hormones present in environmental concentration could be removed with this enzyme within 1 hour (AURIOL *et al*., 2007).

Sulfadimethoxine and sulfapyridine were partially degraded by laccase, however in 360 hours (15 days), which is a relatively high reaction time when compared with the results obtained using other drugs (SCHWARZ; AUST; THIELE-BRUHN, 2010), such as tetracycline, chlortetracycline, doxycycline, and oxytetracycline, that were degraded by the same enzyme within 1 hour (SUDA *et al*., 2012).

Degradation in the range of 72.5 and 79.3% was achieved for ketoprofen, diclofenac, salicylic acid, and naproxen, using laccase from *Trametes pubescens* MUT 2400 in real samples from municipal WWTP within 24 hours. However, the authors found a significant irreversible loss of enzymatic activity, which was attributed to the interaction of the enzyme with other components present in real effluent, causing inhibition or instability in its active sites (SPINA *et al*., 2015).

Cell-free enzymes in solution are poorly stable, are hard to recover, and their active sites can be inhibited by organic matter present in wastewater. This increases the cost associated with this technology since a batch of enzymes is used only once (KÜES, 2015). As a strategy, following the process conditions, enzymatic treatment can be applied as a polishing process in WWTP effluent, avoiding direct contact with high concentrations of organic matter and a consequent drop in enzyme activity and drug degradation efficiency. Another alternative may be the use of the immobilized enzyme in support as membranes or nanoparticles that, in most cases, increases the stability of the protein and facilitates its recovery (DANTAS *et al*., 2019). Besides, the immobilized enzyme enables the use of continuous reactors, avoiding biocatalyst loss (KÜES, 2015; SHARMA; DANGI; SHUKLA, 2018).

WRF are the only ones able to secrete ligninolytic enzymes, most of them are manganese peroxidase, lignin peroxidase, and laccase (SONGULASHVILI *et al*., 2012), but fungal treatments are complex and have several associated complications, such as high reaction times. Considering their potential for anticancer drug degradation or removal in wastewater, the application of the direct enzyme secreted by these fungi can be one alternative to get around these problems. They already have shorter reaction times, and there is a possibility to form less toxic products compared to an original compound.

Table 2 - WRF enzymes and their potential application for the removal of pharmaceutical compounds from wastewaters.

Enzyme	Producing Microorganisms	Pharmaceutical	[C]	Maximum Enzymatic Activity ($U \cdot L^{-1}$)	Reaction Time (h)	Degradation (%)	Reference
Chloroperoxidase	*Caldariomyces fumago*	Norfloxacin	80.2 $\mu mol.L^{-1}$	3152 $x10^{3}$	0.352	82.18	(ZHAO *et al*., 2017)
Lignin peroxidase	*Phanerochaete chrysosporium*	Diclofenac	5 $mg.L^{-1}$	180	2	100	(ZHANG; GEISSEN, 2010)
Laccase	*Trametes versicolor*	Estrone	33.15 $ng.L^{-1}$	150	1	100	(AURIOL *et al*., 2007)
		Estriol	6.2 $ng.L^{-1}$	150	1	100	
		17β-estradiol	25.3 $ng.L^{-1}$	150	1	100	
		17α-ethinylestradiol	6.25 $ng.L^{-1}$	150	1	100	
		Sulfanilamide	1 $mmol.L^{-1}$	48	360	10	(SCHWARZ; AUST;
		Sulfadimethoxine	1 $mmol.L^{-1}$	48	360	75	THIELE-BRUHN, 2010)
		Sulfapyridine	1 $mmol.L^{-1}$	48	360	95	
		Tetracycline	0.1 $mmol.L^{-1}$	600	0.25	100	(SUDA *et al*., 2012)
		Chlortetracycline	0.1 $mmol.L^{-1}$	600	0.25	100	
		Doxycycline	0.1 $mmol.L^{-1}$	600	1	100	
		Oxytetracycline	0.1 $mmol.L^{-1}$	600	1	100	
		Diclofenac	20 $mg.L^{-1}$	730	6	100	(MARGOT *et al*., 2013)
		Mefenamic acid	20 $mg.L^{-1}$	730	4	100	
		Diclofenac	10 $\mu g.L^{-1}$	1500	12	100	(TRAN; URASE;
		Naproxen	10 $\mu g.L^{-1}$	1500	12	100	KUSAKABE, 2010)
		Indomethacin	10 $\mu g.L^{-1}$	1500	12	100	

	Trametes pubescens MUT 2400	Salicylic acid	9.5 $\mu g.L^{-1}$	100	24	75	(SPINA *et al*., 2015)
		Naproxen	15.3 $\mu g.L^{-1}$	100	24	79.3	
		Diclofenac	3.5 $\mu g.L^{-1}$	100	24	76.8	
		Ketoprofen	2.8 $\mu g.L^{-1}$	100	24	72.5	
	Not specified	Acetaminophen	50 $\mu mol.L^{-1}$	1000	1.56	100	(LU; HUANG; MAO, 2009)
	Myceliophthora thermophila	Estrone	5 $mg.L^{-1}$	2000	8	100	(LLORET *et al*., 2010)
		17b-estradiol	5 $mg.L^{-1}$	2000	0.5	100	
		17a-ethinyl-estradiol	5 $mg.L^{-1}$	2000	0.5	100	
		Naproxen	5 $mg.L^{-1}$	2000	8	60	
		Diclofenac	5 $mg.L^{-1}$	2000	8	100	
Manganese peroxidase	*Phanerochaete chrysosporium*	Tetracycline	50 $mg.L^{-1}$	40	4	72.5	(WEN; JIA; LI, 2010)
		Oxytetracycline	50 $mg.L^{-1}$	40	4	84.3	
Versatile peroxidase	*Bjerkandera adusta*	Diclofenac	4 $mg.L^{-1}$	10	0.42	100	(EIBES; DEBERNARDI; LEMA, 2011)
		Estrone	5 $mg.L^{-1}$	10	0.17	100	
		17β-estradiol	5 $mg.L^{-1}$	55	0.08	100	
		17α-ethinylestradiol	5 $mg.L^{-1}$	10	0.17	100	
		Sulfamethoxazole	25 $mg.L^{-1}$	200	7	80	
		Naproxen	4 $mg.L^{-1}$	1110	7	80	

2.5 CONCLUSIONS AND FUTURE OUTLOOK

The potential risk of anticancer drugs and its recalcitrance to conventional treatments were shown, but information about how they can affect biological processes and their environmental risk is still needed. In addition to that, this fact increases the demand for an environmentally friendly process, economically viable and socially acceptable, to prevent or reduce their environmental impact. However, it is not known as a process that can be used in large-scale treatment. For some years now, fungal treatment has been presenting promising results in the removal of anticancer drugs from real effluents. Nevertheless, there is a need to investigate this process since it demands a high HRT deeply, needs nutrients addition, and handles the bacteria competition with the substrate, even when applied to real effluents. In this sense, enzymatic treatments appear to be a better alternative, since they have the potential to degrade anticancer drugs at reduced reaction time. There are still no studies in the literature using an enzymatic treatment for real or synthetic wastewaters containing anticancer drugs, indicating to be a potential innovative process to promote the removal of these compounds in domestic and industrial effluents, preventing their release in the environment.

CHAPTER 3

In this chapter, the degradation of etoposide by the laccase from *Trametes versicolor* was evaluated. The best experimental conditions of enzyme concentration, pH values, etoposide concentration for the enzymatic degradation of etoposide were established.

3 LACCASE-ASSISTED DEGRADATION OF ETOPOSIDE

3.1 INTRODUCTION

Etoposide (4'-Demethyl-epipodophyllotoxin 9-[4,6-O-(R)-ethylidene-beta-D-glucopyranoside]) is an epipodophyllotoxin, podophyllotoxins semisynthetic derivative. It induces DNA double-strands breaks by inhibiting DNA topoisomerase II (AISNER; LEE, 1991; RUSSO *et al*., 2018). It is generally administered for testicular carcinoma and small cell lung cancer treatment. Lung cancer contributed to 15.5% of new cancer cases diagnosed in 2018, is the most common type of cancer in men, according to the International Agency For Research on Cancer (IARC) (2018).

Etoposide was detected at levels from 110 to 600 $ng \cdot L^{-1}$ in hospital wastewaters (FERRANDO-CLIMENT *et al*., 2015). Nevertheless, it is considered carcinogen (class 1) to humans for IARC and as group 1 for the National Institute for Occupational Safety and Health (NIOSH), which may cause risks to humans at low concentrations (NIOSH, 2016).

Etoposide reacts quickly with free chlorine and forms a by-product 3′-O-desmethyl etoposide that inactivate a biologically active DNA (NEGREIRA; DE ALDA; BARCELÓ, 2015; STREMETZNE; JAEHDE; SCHUNACK, 1997; TERNES; JOSS, 2015). This by-product was reported in various rivers water and raw wastewater samples from Catalonia (Spain), at 34 $ng \cdot L^{-1}$ and 14-31 $ng \cdot L^{-1}$ concentrations, respectively (NEGREIRA; DE ALDA; BARCELÓ, 2014b).

This anticancer drug showed risks to some aquatic organisms. It induced an increase in DNA damage at 23.5 $\mu g \cdot L^{-1}$ to *Limnodrilus udekemianus* (aquatic worm) (KRAČUN-KOLAREVIĆ *et al*., 2015), and in a range from 0.01 to 2 $\mu g \cdot L^{-1}$ to microcrustaceans *Daphnia magna* and *Ceriodaphnia dubia* (PARRELLA *et al*., 2015).

Given the potential risk, even at low concentrations and its presence in wastewater and surface water, strategies should be used to treat these effluents before it reaches water bodies (FERRANDO-CLIMENT; RODRIGUEZ-MOZAZ; BARCELÓ, 2014). Enzymatic processes can be used to remove drugs present in wastewater, since degradation processes involving WRF are attributed to enzymatic processes, specifically to laccase (PEREIRA *et al*., 2020).

Laccase is an oxidoreductase enzyme that catalyzes several organic compounds oxidation by using oxygen as an electron acceptor and producing water as a by-product (CAÑAS; CAMARERO, 2010). They are able to degrade various types of emerging pollutants, such as pesticides, dyes, and some pharmaceutical products (ALCALDE *et al*., 2006; RAO *et al*., 2014). This enzyme is produced by several types of bacteria, plants, and fungi; however, those produced by fungi have attracted attention due to the low substrate specificity, which allows the degradation of various compounds (GASSER *et al*., 2014). Another advantage is its extracellular production by fungi, which facilitates its extraction and purification process(MAJEAU; BRAR; TYAGI, 2010; SHARMA; DANGI; SHUKLA, 2018).

In this study, the etoposide degradation by laccase was evaluated. The effects of the catalyst concentration variation, as well as the best pH condition for the process and the effect of etoposide concentration variation in the degradation rate, were analyzed in this research.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Laccase (EC 1.10.3.2) from *Trametes versicolor*, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, >98%) and etoposide (>98%) were purchased from Sigma-Aldrich. Monobasic sodium phosphate and bibasic sodium phosphate were purchased from Neon (Brazil) with purities higher than 98%. Acetonitrile (ACN) HPLC grade was obtained from Panreac. ACN and water HPLC-MS grade were purchased from Riedel. Etoposide stock solution (20 $mg \cdot L^{-1}$) were prepared in ultrapure water (Millipore) and stored at 4 °C.

3.2.2 Enzyme activity assay

Laccase activity assay was performed according to the method described by García-Morales *et al*. (2018). The tests were performed in a quartz cuvette (3.5 mL) where was added 2.4 mL of sodium phosphate buffer 0.1 $mmol \cdot L^{-1}$ (pH 6), 0.3 mL of ABTS 5 $mmol \cdot L^{-1}$ and 0.3 $mg \cdot L^{-1}$ of laccase solution. Therefore, the reaction medium consisted of 0.01 $mol \cdot L^{-1}$ sodium phosphate buffer pH 6, 0.5 $mmol.L^{-1}$ ABTS, and 0.1 $mg \cdot L^{-1}$ laccase solution. The mixture was incubated at 25 ºC, and the absorbance increase at 420 nm was followed for 2 minutes in a HACH DR 5000 spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the oxidation of 1 mmol of ABTS per min at 25 ºC and pH 6. The assays were performed in triplicate.

The enzyme activity was calculated according to Equation (1).

$$(U \cdot L^{-1}) = \frac{\Delta abs \,.\, V}{\varepsilon \,.\, d \,.\, v \,.\, t} \quad (1)$$

Where:

Δabs = absorbance variation;

V = reactional volume;

d = cell path length;

v = laccase solution volume;

t = reaction time;

ε = ABTS molar extinction ($3.6x10^4$ $M^{-1}\cdot cm^{-1}$) (CHILDS; BARDSLEY, 1975).

3.2.3 Enzymatic treatment

The laccase solution was prepared immediately before each experiment. The etoposide stock solution was prepared at a maximum concentration of 20 $mg\cdot L^{-1}$ stored at 4 ºC protected from light. Suitable dilutions of etoposide stock solutions were made on 0.01 $mol\cdot L^{-1}$ phosphate buffer. Laccase-assisted etoposide degradation assays (optimum enzyme concentration, optimum pH determination, and etoposide concentration variation effect, as described below) were performed in 24-weel plates covered with PCR plate sealing film to avoid evaporation, with 1 mL of reactional volume per well for 6 hours. Control samples with etoposide in 0.01 $mol\cdot L^{-1}$ phosphate buffer were conducted to certify the stability of the drug under the experimental conditions.

Assays were made in an orbital Quimis Q816M20 (Brazil), at 30 °C, and 180 rpm. In the first 3 hours, 0.8 mL samples were taken every 20 minutes, and then, they were taken every hour until the end of the reaction. To interrupt the reaction, 0.8 ml of cold acetonitrile (ACN) was added to the sample (1:1 v/v). Furtherly, they were centrifuged for 10 minutes at 10000 rpm and 4 °C to improve laccase precipitation. The supernatant was separated and kept frozen until etoposide HPLC-UV chromatography analysis.

3.2.3.1 Optimum enzyme concentration and optimum pH determination

Assays were performed with four different enzyme activities to define an appropriate laccase concentration to degrade the substrate. Etoposide degradation (500 $\mu g\cdot L^{-1}$) was performed with 55, 110, 555, and 1100 $U\cdot L^{-1}$ of laccase at pH 6 and 30 °C.

The influence of pH on the degradation rate was analyzed by adding 500 $\mu g\cdot L^{-1}$ of etoposide in 0.01 $mol\cdot L^{-1}$ phosphate buffer and degrading at pH 5, 6, and 7 with 55 $U\cdot L^{-1}$ of laccase solution at 30 °C.

3.2.3.2 Etoposide concentration variation effect

Experiments were performed at etoposide concentrations of 500, 1000, 1500, 2000, 5000, 7000, 10000, 15000 and 18000 $\mu g \cdot L^{-1}$ with 55 $U \cdot L^{-1}$ laccase solution, at pH 6 and 30 °C. The etoposide concentration effect was evaluated by the initial degradation rate. Graphic of etoposide concentration versus initial reaction rate was plotted, thus calculating the constant K, which externalizes the effect of etoposide concentration on the initial reaction rate.

3.2.4 HPLC-UV quantitative analysis

The HPLC analysis was performed on a Shimadzu LC-20A Prominence series HPLC-UV machine, with an adapted method from Solano *et al*. (2012). Samples were filtered with a 0.22 μm syringe-filter. They were analyzed by injecting 20 μL of samples with a mobile phase of ACN: water (35:65, v/v) pumped through the Supelcosil LC-18 column (size 250 mm x 4.6 mm, 5 μm, at a flow rate of 1.0 $mL \cdot min^{-1}$. The etoposide retention time for this method was 5.6 minutes, monitored at 254 nm and 45 °C.

Etoposide standard curve was made in a concentration ranging from 50 to 20000 $\mu g \cdot L^{-1}$ (Appendix A).

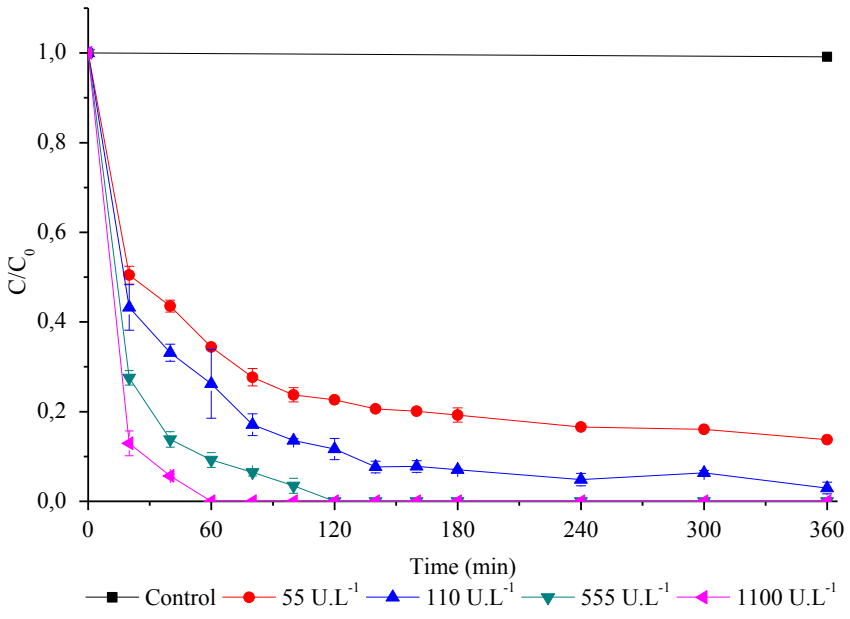
3.3 RESULTS AND DISCUSSION

3.3.1 Optimal laccase concentration

Etoposide degradation at different enzyme activities was measured to choose a catalyst quantity that could reach a satisfactory substrate degradation rate. In literature, the acetaminophen degradation by laccase was evaluated and calculated the reaction rates for each catalyst concentration (LU; HUANG; MAO, 2009). Bearing the mind the application, the smaller the catalyst amount needed to carry out the reactions, the lower cost to the future process development. The intention to degrade the pharmaceutical drug in $\mu g.L^{-1}$ concentrations range came from the magnitude, which is generally found in wastewaters (110-600 $ng.L^{-1}$).

Due to the etoposide minimum detection limit (50 $\mu g \cdot L^{-1}$) on chromatographic analyses, reactions were executed at, at least, 500 $\mu g \cdot L^{-1}$ (C_0). Enzymatic reactions are generally fast when compared with the conventional microbiological process used to treat wastewater, and then smaller substrate quantities would difficult reaction monitoring. Figure 2 shows assays reactions with 55, 110, 555, and 1100 $U \cdot L^{-1}$. The residual etoposide concentration was represented by the C/C_0 ratio, where C is the etoposide concentration during the reaction and C_0 is the etoposide initial concentration.

Figure 2 - Etoposide degradation (C_0=500 $\mu g \cdot L^{-1}$) with 55, 110, 555 and 1100 $U \cdot L^{-1}$, at pH 6 and 30 ºC.



As expected, in all tested concentrations, higher enzyme activity implied a higher initial reaction rate and, consequently, shorter degradation time. After 6 hours (360 min) of the experiment, laccase lowest concentration (55 $U \cdot L^{-1}$) reached etoposide degradation above 86%. The highest laccase activity (1110 $U \cdot L^{-1}$) yielded the fastest degradation, reaching 100% removal within 1 hour. To compare, the initial reaction rate was calculated for each enzyme activity condition (Table 3).

Table 3 - Initial reaction rates of laccase degradation of etoposide (C_0=500 $\mu g \cdot L^{-1}$) at pH 6 and 30 °C.

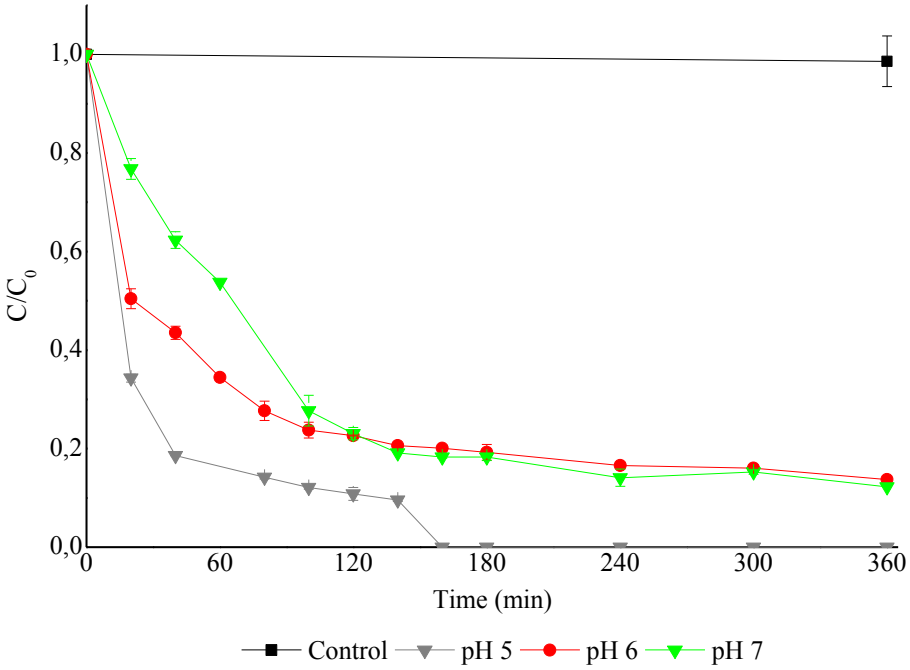
Laccase activity ($U \cdot L^{-1}$)	**Initial reaction rate ($\mu g \cdot L^{-1} \cdot min^{-1}$)**
55	10.94
110	14.14
555	18.00
1110	21.65

Even reducing laccase concentration 20-fold, the reaction rate dropped only to half of the value, showing that there is no need to use large catalyst concentrations. By doubling the enzyme activity, a 20 to 30% increase in the initial reaction rate was achieved. With these results, it was decided to continue the assays using 55 $U \cdot L^{-1}$ of enzymatic activity. Bearing in mind the application in wastewater treatment, costs must be minimized without a high loss of efficiency, which happens in 55 $U \cdot L^{-1}$.

3.3.2 Optimal pH degradation

Numerous factors can interfere in enzymatic reactions, such as substrate properties, enzyme specificities, and process conditions (pH, temperature) (STADLMAIR *et al*., 2018). Enzymatic treatments using laccase as a biocatalyst showed that generally, the optimum pH is ranging from 4.5 to 6 (GASSER *et al*., 2014). According to the manufacturer, pH 6 is optimal, considering the standard substrate (catechol). Effluent treatment processes usually operate at a pH range close to neutrality (SPINA *et al*., 2015). Therefore, degradation tests were performed at pH 5, 6, and 7. Figure 3 shows etoposide (C_0=500 $\mu g \cdot L^{-1}$) degradation with different pH at 30 °C.

Figure 3 - Etoposide degradation (C_0=500 µg·L^{-1}) with 55 U.L^{-1} at 30 ºC and different pH.



At pH 5, after 160 min, 100% of etoposide degradation was achieved, while at pH 6 and 7, it reached around 80% degradation at this time. The etoposide concentration barely changes after 180 minutes of reaction at pH 6 and 7, which can be explained by enzymatic stability, since laccase stability is usually higher at pH 4 and 5, decreasing in higher pHs (FRASCONI *et al.*, 2010; MAJEAU; BRAR; TYAGI, 2010). For better comparison, the initial reaction rate was calculated for each pH condition (Table 4).

Table 4 - Initial reaction rates comparison among pH 5, 6, and 7 and 30 °C.

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

Among the tested conditions, pH 5 showed the highest degradation rate. Compared to pH 5, pH 6 and 7 showed a decrease in the initial reaction rate of 33% and 66%, respectively. Similar behavior was observed to norfloxacin degradation by laccase, where reaction rates reached a maximum value at pH 5 and dropped at pH 6

(ZHAO *et al*., 2017). Thus, when compared to the best condition, pH 7 showed a substantial drop in the reaction rate, an intermediate pH was chosen for further experiments (pH 6).

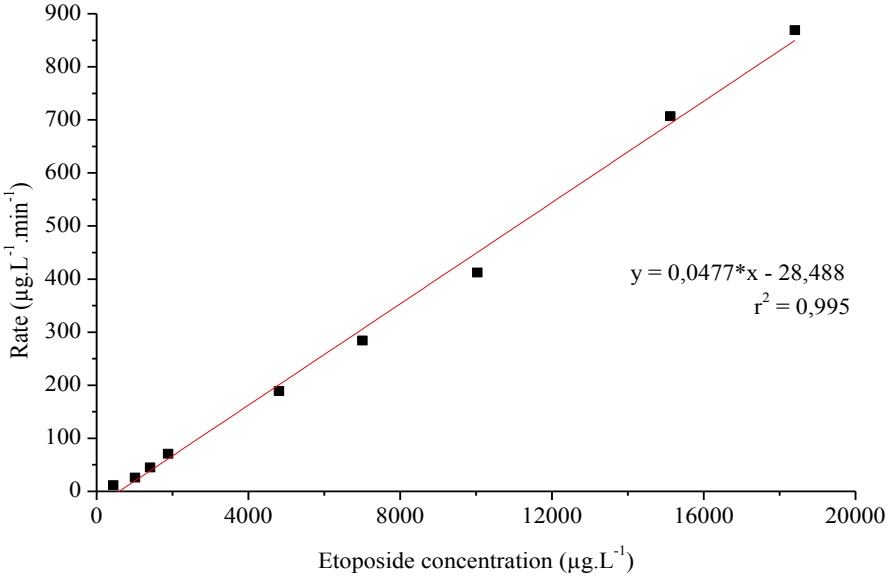
A study evaluated the stability of etoposide in various buffers and pH, demonstrating that it is most stable between pH 4 and 5 (BEIJNEN *et al*., 1988). Therefore, degradation can be attributed to enzymatic action, whereas the highest initial reaction rate (pH 5) occurred in the most stable etoposide condition.

Despite not having the highest initial reaction rate, pH 6 was chosen considering the future process application, which would be in environmental conditions, around neutrality. The same strategy was used to degrade steroid hormones present in wastewater, laccase degradation was carried out at neutral pH, although the optimum pH is acidic (AURIOL *et al*., 2007).

3.3.3 Effect of etoposide concentration on initial reaction rate

The etoposide concentration variation effect was evaluated by plotting the initial reaction rate versus the initial concentration to values from 500 to 18000 $\mu g \cdot L^{-1}$ (Figure 4). The aim was to find the maximum initial reaction rate value where the degradation constant k is zero, in other words, when an increase in etoposide concentration does not affect the initial reaction rate. However, such behavior was not observed in this range of concentrations.

Figure 4 - Effect of etoposide concentration effect on the initial reaction rate at pH 6 and 30 °C



As seen in Figure 4, the relation between the substrate and the initial rate was linear, following first-order kinetics with the substrate and degradation kinetics constant of k=0.0477 min^{-1} (r^2 = 0.995). When etoposide biodegradation with activated sludge was tested, it remained up to 70% after 8 hours, with degradation kinetics of 0.0082 min^{-1} (SANTOS *et al.*, 2017), almost 6 times lower than that reached by laccase degradation.

Even at an etoposide concentration of 18 $mg \cdot L^{-1}$, there is no laccase substrate inhibition. The anticancer drugs concentration in wastewater and WTTP is generally on a scale from $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$, thus, even in specific cases where these drugs can reach an $mg \cdot L^{-1}$ range, that process would still be well applied.

3.4 POTENTIAL APPLICATION AND FUTURE PROSPECTS

As seen in items 3.3.1 and 3.3.2, the condition of 55 $U \cdot L^{-1}$, pH 6, and 30 °C were better for application in wastewater treatment. However, when applied directly in the process, laccases can be inactivated due to raw wastewater unfavorable conditions (pH, temperature), denaturing agents, and high salt concentration (MAJEAU; BRAR; TYAGI, 2010). An alternative to overcome such circumstances would be enzymatic process use as an advanced/tertiary treatment. Storage and operational stability, catalyst recovery, and high activity enzymatic properties are important enzyme proprieties to

treatment success (RAO *et al*., 2014). These characteristics allow several catalyst treatment cycles and can be improved by immobilizing enzymes on supports. Increasing the laccase cycle reuse reduces catalyst costs (GASSER *et al*., 2014; STADLMAIR *et al*., 2018).

One strategy for laccase uses improvement would be its use as a polishing process for decentralized treatment plants. The enzymatic process would be operated in continuous, using immobilized laccase on appropriate support (fixed or fluidized bed bioreactors). These alternatives allow catalyst reuse, avoids laccase contact with raw wastewater and its potential inactivating/denaturing agents, thus increasing its operational stability. Keeping in mind the laccase non-specificity with the substrate, in contact with raw wastewater, it can degrade other organic compounds easily degraded by conventional WWTPs. Therefore, as a polishing process, the laccase amount required for the procedure would also be reduced since it would be used to degrade just recalcitrant compounds.

3.5 CONCLUSIONS

This study demonstrated that etoposide degradation is technically feasible by a catalyzed system with laccase from *Trametes versicolor*. Substrate degradation occurred in all conditions of catalyst concentration, pH, and pollutant level tested. All catalyst concentration tested showed to be capable of promoting etoposide degradation. To summarize, even at concentrations of etoposide in $mg.L^{-1}$ scale, there was no laccase inhibition and chosen condition of pH 6, 55 $U \cdot L^{-1}$, and 30 °C exhibited a satisfactory result for the desired application, occurring at pH close to neutrality.

CHAPTER 4

In this chapter, consideration remarks and suggestions for future work are presented.

4 CONCLUDING REMARKS

The literature review showed the environmental risks of anticancer drugs occurrence in wastewaters and water surface. Research using WRF showed the potential of enzymatic processes, specifically the fungal laccase.

Etoposide degradation by laccase was achieved in all tested conditions, reaching 100% degradation in less than 1 hour with 1100 $U \cdot L^{-1}$ of enzymatic activity and in 2 hours and 40 minutes with 55 $U \cdot L^{-1}$ at pH 5. However, bearing the mind the application in tertiary/advanced wastewater treatment, the best condition was pH 6, 55 $U \cdot L^{-1}$, and 30 °C.

4.1 SUGGESTIONS FOR FUTURE STUDIES

- Test other pH conditions to better observe primary pH effects in the enzymatic reaction.
- Test temperature variation effect to observe climate change interference.
- Perform etoposide degradation of etoposide at higher concentrations to reach maximum speed and calculate the Michaelis-Menten kinetic parameters.
- Identify etoposide by-products with LC-MS.
- Perform ecotoxicological tests of by-products.

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6 APPENDIX A

6.1 ETOPOSIDE CALIBRATION CURVE FOR DEGRADATION TESTS BY LACCASE FROM *Trametes versicolor* (ITEM 3.2.3)

Figure 5 - Etoposide calibration curve