Brazilian Journal of Analytical Chemistry
2024, Volume 11, Issue 44, pp 59-75
doi: 10.30744/brjac.2179-3425.AR-45-2023

BrJAC

ARTICLE

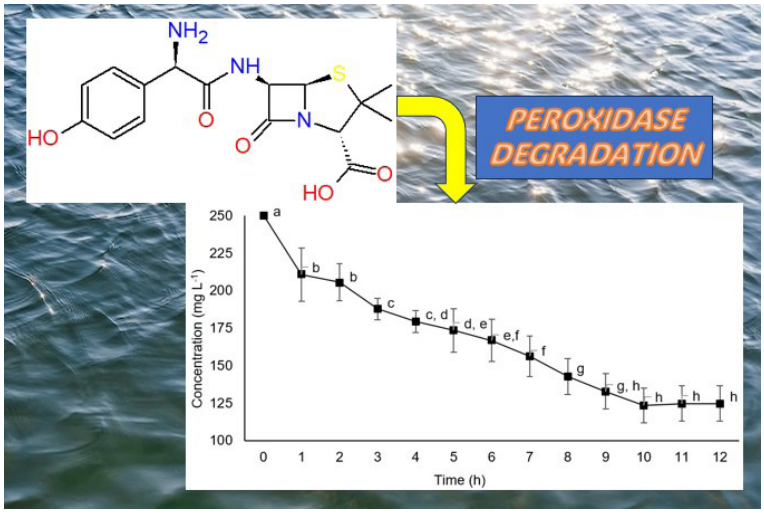
Degradation of the Micropollutant Amoxicillin using Enzymatic Treatment and Evaluation of Resulting Byproducts

Gabriela Vettorello[1], Lucas Schmidt[1], Daniel Kuhn[1], Bruno Eduardo da Silva[1], Ani Caroline Weber[1], Sabrina Grando Cordeiro[1], Guilherme Schwingel Henn[1], Bruna Costa[1], Jéssica Samara Herek dos Santos[1], Joana Elisa Willrich[1], Cristiano Aguiar Pereira[1], Alexandre Rieger[2], Clarice Steffens[3], Eduardo Miranda Ethur[1], Elisete Maria de Freitas[1], Lucélia Hoehne[1]*

[1]Universidade do Vale do Taquari, Programa de Pós-Graduação em Biotecnologia, Rua Avelino Talini, 171, Universitário, 95914-014, Lajeado, RS, Brazil

[2]Universidade de Santa Cruz do Sul, Departamento de Física e Química, Av. Independência, 2293, Universitário, 96815-900, Santa Cruz do Sul, RS, Brazil

[3]Universidade Regional Integrada do Alto Uruguai e das Missões. Programa de Pós-Graduação em Alimentos, Av. Sete de Setembro, 1621, Fátima, 99709-910, Erechim, RS, Brazil



Current Brazilian legislation for the treatment of drinking water does not require analysis for micropollutants such as the antibiotic amoxicillin. However, the presence of these compounds in the environment is directly linked to bacterial resistance, and the development of methodologies focusing on their removal is necessary. A few alternatives, such as Advanced Oxidative Processes, have already been proposed and, more recently, studies have shown that certain enzymes, like peroxidases, have the ability to degrade micropollutants in the presence of hydrogen peroxide (H_2O_2). In this sense, the present study aims to evaluate the enzymatic degradation of 25 mg L^{-1} amoxicillin using peroxidase. For the specific method, amoxicillin solutions were fed to a batch reactor and different concentrations of peroxidase combined with varying H_2O_2 concentrations (0.5, 1.0, and 2.5 mmol L^{-1}) were added. Reactions occurred for 9 hours. All samples were analyzed by liquid chromatography coupled with mass spectrometry, and the residual toxicity was assessed using *Daphnia magna*. The results showed around 50% degradation of the drug,

Cite: Vettorello, G.; Schmidt, L.; Kuhn, D.; da Silva, B. E.; Weber, A. C.; Cordeiro, S. G.; Henn, G. S.; Costa, B.; dos Santos, J. S. H.; Willrich, J. E.; Pereira, C. A.; Rieger, A.; Steffens, C.; Ethur, E. M.; de Freitas, E. M.; Hoehne, L. Degradation of the Micropollutant Amoxicillin using Enzymatic Treatment and Evaluation of Resulting Byproducts. *Braz. J. Anal. Chem.* 2024, *11* (44), pp 59-75. http://dx.doi.org/10.30744/brjac.2179-3425.AR-45-2023

Submitted 14 May 2023, Resubmitted 27 August 2023, Accepted 06 October 2023, Available online 03 January 2024.

This article was submitted to the BrJAC special issue dedicated to the 20th Brazilian Meeting of Analytical Chemistry (ENQA).

and byproducts originating from amoxicillin were identified. Toxicological tests indicated that the byproducts were minimally toxic to the microcrustacean, highlighting the importance of evaluating the safety of proposed treatments.

Keywords: antibiotic, amoxicillin, enzyme, peroxidase, toxicity

INTRODUCTION

Micropollutants are a class of pollutants defined as potentially toxic substances of which the effects and persistence in the environment are still unknown. These compounds are typically present in low concentrations ranging from nanograms per liter (ng L^{-1}) to milligrams per liter (mg L^{-1}). Examples include pesticides, cosmetics, polyhalogenated compounds, steroids, hormones, and pharmaceuticals in general and analytical methods for determination of pharmaceuticals and their metabolites in aqueous solutions must be developed.[1]

Especially in urban areas, several pharmaceutical, hormonal, and personal care substances are used daily by the general populace, and are mainly disposed of through wastewater systems, which creates the need to treat this effluent with more care.[2]

Regarding micropollutants, the growing use of pharmaceutical products and drugs has become an environmental problem.[3] In a literature review, authors reported on studies that indicated the presence of up to 22 μg L^{-1} of the anti-inflammatory ibuprofen, 21 μg L^{-1} of the analgesic acetylsalicylic acid, 8.3 μg L^{-1} of paracetamol, and 1.3 μg L^{-1} of the antibiotic amoxicillin in surface waters in Brazil.[4] Machado et al.[5] found caffeine and atrazine in 93% and 75% of all drinking water samples analyzed in their study, respectively. In China, Yao et al.[6] identified the antibiotic erythromycin in samples taken from surface waters, in concentrations ranging from 546 ng L^{-1} during the winter to 1600 ng L^{-1} during spring. Erickson (2002), as cited by Ahuja,[7] states that among all micropollutants, antibiotics are among the most concerning due to their relation with the emergence of microbial resistance.

Depending on their chemical structure or mechanism of action, antibiotics can be subdivided in β-lactams, quinolones, tetracyclines, macrolides, sulfonamides, and others.[8] β-lactams belong to the group of penicillins, which have in their basic composition (Figure 1) a thiazolidine ring (A) attached to a β-lactam ring (B) and a secondary amino group (RNH^-). The “R” substituents give rise to their variations. Penicillins and other β-lactams act on pathogens by interfering with the bacterial cell wall synthesis pathway, inhibiting the growth of bacteria.[9]

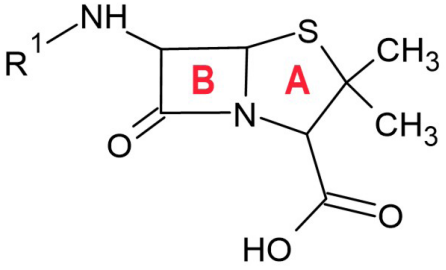


Figure 1. Basic structure of penicillins.

The increasing use of antibiotics for the treatment of humans and animals is a major factor in the emergence of resistant bacteria.[10] According to O’Neil (2014) cited by Carvalho and Santos,[11] it is estimated that 23,000 deaths in the United States and 25,000 deaths in Europe may occur each year due to infections caused by antibiotic-resistant bacteria. Globally, this number could reach 700,000 people, and if this problem is not addressed, 10 million people could be affected by 2050.[11]

Amoxicillin is a widely used antibiotic and is eliminated from the body in a non-metabolized form at levels around 70%,[12] a value that is also expressed in the medication’s package insert. Standard treatments using amoxicillin consists of three daily doses of up to 500 mg lasting at least seven days. In extreme

cases, the dosage may reach two daily doses of 3000 mg for the same period. This means that, during a common treatment for adults, the total non-metabolized excretion rate is 7.5 g, but this value may reach 29.4 g in extreme cases.[13] Park and Choi,[14] through their studies and literature review, proposed that the concentration at which amoxicillin is harmless is only up to 3.6 ng L^{-1}.

The processes used in water treatment plants to make it potable are effective for those parameters already mentioned in legislation. However, the degradation of micropollutants requires more specific studies, aimed at more effective treatments.[3] The treatment of antibiotic waste by enzymatic biodegradation processes is still relatively unexplored, but has shown promising results. The degradation of this type of drug by oxidative enzymes has already been shown to be effective for the substance sulfamethoxazole, reaching degradation rates up to 100%.[15]

Enzymatic degradation is a proposed alternative to supplement biological treatments. Among the enzyme classes used, so-called oxidoreductases stand out. This class of enzymes includes oxygenases, laccases, and peroxidases, which are proteins that carry out redox reactions on a wide range of substances, including micropollutants such as pesticides, dyes, and polycyclic aromatic hydrocarbons.[16]

Many enzymes are non-specific biocatalysts capable of converting a variety of substrates that share some structural similarity with their primary substrate. And it can be used in very low concentrations, and they speed up reactions without themselves being consumed during the reaction.[17]

In their review, Varga et al.[18] discuss how enzymatic treatments have proven to be viable solutions for the treatment of effluents in industries such as food, paper, and textile. Unlike activated sludge systems, enzymes are less susceptible to inhibition by substances that are toxic for living organisms. Compared to other chemically catalyzed processes, enzymatic systems consume fewer chemicals, less water and energy, and result in less waste, although the efficiency of micropollutant removal depends on the compound and operational circumstances. The authors highlight that comparative environmental assessments indicate that the use of enzymatic processes in industrial applications often mitigates environmental impacts. For example, the use of membrane filtration with enzymes for the treatment of drinking water had a lower negative environmental impact than adsorption with activated carbon.[18]

Peroxidase (POD) enzyme occur in animals, plants, and microorganisms. In plant cells, it is partially soluble in the cytoplasm and insoluble when bound to the cell wall. Soluble enzymes can be extracted from the tissue using a low ionic strength buffer, while ionically bound enzymes require ionic strength buffer. These enzymes catalyze four types of reactions: peroxidative, oxidative, catalytic, and hydroxylation.[19] In general, the reaction mechanism is as Equation 1.

$$ROOH + AH_2\ POD \rightarrow\ H_2O + ROH + A \qquad \text{Equation 1}$$

where R = -H, $-CH_3$, or $-C_2H_5$, AH_2 = hydrogen donor in reduced form, and A = hydrogen donor in oxidized form.

During the reaction, the following intermediates are formed, follow Equations 2, 3 and 4.

$$POD + H_2O_2 \rightarrow Compound\ I \qquad \text{Equation 2}$$

$$Compound\ I + AH_2 \rightarrow Compound\ II \qquad \text{Equation 3}$$

$$Compound\ II + AH \rightarrow POD + A \qquad \text{Equation 4}$$

Peroxidase enzymes are activated in the presence of H_2O_2, and are capable of catalyzing the oxidative cleavage of carbon-carbon bonds of various organic compounds.[20] However, there are still few reports in literature evaluating their efficiency in the biodegradation of antibiotics.

In this context, the present study aims to evaluate the degradation of amoxicillin in water using an enzymatic treatment (peroxidase in combination with H_2O_2), followed by analysis of byproduct formation and toxicity assessment, ensuring safe treatments to improve water quality.

MATERIALS AND METHODS

Preparation of samples and solutions

0.1 M phosphate buffer solution

According to Maciel, Gouvêa, and Pastore[21] a neutral pH between 6 and 7 at room temperature (25 - 35 ºC) are considered optimal for peroxidase activity. A 0.1 M phosphate buffer solution (pH 7) was prepared by dissolving 29 g of monobasic sodium phosphate and 51.6 g of dibasic sodium phosphate in 1000 mL of water in a volumetric flask.[22]

Synthetic amoxicillin sample

A 25 mg L^{-1} amoxicillin solution was prepared from a standard (≥ 900 µg mg^{-1}) (Sigma-Aldrich, Burlington, Massachusetts, USA), using a 0.1 mol L^{-1} phosphate buffer solution as the solvent.

Enzyme solution

Using the adapted methods of Al-Maqdi et al.[15] and Silva et al.,[23] a 30 U mL^{-1} solution was prepared by dissolving 5 mg of a commercial standard of horseradish peroxidase obtained from Sigma-Aldrich in lyophilized form (150-170 U mg^{-1}), then bringing it up to 25 mL with 0.1 M phosphate buffer solution (pH 7) using a volumetric flask.

Enzymatic reactor

The enzyme-based biodegradation tests were adapted from the methodology used by Silva et al.[23] The experiment was carried out in 250 mL Erlenmeyer flasks, containing 100 mL of amoxicillin solution in 0.1 M phosphate buffer at pH 7, with a concentration of 25 mg L^{-1}. Subsequently, 0.5, 1, and 2.5 mmol L^{-1} of H_2O_2 were added, and the solutions were kept in agitation using a shaker (Marconi, model MA830) at 100 rpm, and controlled temperature of 30 ºC.

In addition to the samples, three types of blank solutions were prepared, each devoid of one of the reagents: H_2O_2, enzyme, or amoxicillin. The preparation of blanks under these conditions aims to individually evaluate the influence of each of the reagents on the degradation of the antibiotic in solution. The amoxicillin-free solution was used as reference for the evaluation of the chromatograms obtained in SCAN mode, identifying only the substances related to the micropollutant degradation process.

Initially, aliquots of the treated solutions were collected every 60 min, to evaluate the reaction time during which the enzyme remained active in the medium. After this period had been determined, the subsequent tests were carried out under the same parameters.

Degradation assessment

The evaluation of the decrease in antibiotic concentration during treatment was performed through high performance liquid chromatography coupled with mass spectrometry (HPLC-MS), quantified by constructing a calibration curve ranging from 10 to 500 µg L^{-1} (equation of the line = 10701.094x-21882.400; with R^2 = 0.999864) using an amoxicillin standard obtained from Sigma-Aldrich. The HPLC-MS equipment was a Triple Quadrupole LCM-8040 from Shimadzu®, equipped with an Agilent® Poroshell 120 EC-C18 column (4.6x50 mm, 2.7 µm at 30 ºC). Mobile phase A consisted of ultrapure water, and mobile phase B was HPLC-grade acetonitrile (J. T. Baker), both acidified with 0.1% formic acid obtained from Merck. The flow rate was 0.4 mL min^{-1}, with a gradient starting at 5% of B, gradually increasing to 15%, then to 60% for 2 min, and reduced to 5% for 3 min, remaining at this proportion until the end of the run at 5 min. The oven temperature was 30 ºC and injection volume was 10 µL. The triple-quadrupole conditions were as follows: the electrospray was used in positive mode, the drying gas was

nitrogen flowing at 10 L min^{-1}, and the collision gas was argon. Data was controlled and processed using LabSolutions software. The *m/z* ratios used to identify amoxicillin are presented in Table I.

Table I. Mass spectrometer settings for the detection of amoxicillin, including precursor ion, product ion, dwell time (analysis time of the spectrometer), and collision energy (CE)

Antibiotic	Precursor ion *m/z*	Product ion *m/z*	Dwell time (mseg)	Q1 (eV)	CE (V)	Q3 (eV)
Amoxicillin	366.1	349.1	100	-26.0	-8.0	-27.0
	366.1	208.0	50	-20.0	-13.0	-15.0
	366.1	114.0	50	-29.0	-21.0	-24.0

Identification of degradation products

The degradation products were identified through the LC-MS methodology, using the same chromatographic conditions as in the degradation evaluation step. The total ion scan method was employed with monitoring of the mass-to-charge ratios (*m/z*) in the range of 100 to 800, allowing for comparison of the compounds identified in the three treatment proposals and the elaboration of a possible degradation pathway for each method.

Final toxicity analysis

After evaluating the best micropollutant degradation process in each condition, toxicity tests were conducted with the microcrustacean *Daphnia magna*. The proportions of affected and unaffected individuals were counted, and the median effective concentration (EC_{50}) was determined, classifying the confidence interval and its toxicity.[24]

Cultivation and exposure

The toxicity evaluation was performed using the test organism *Daphnia magna* Strauss, 1820. *D. magna* neonates between 2 and 26 hours old were used in the tests in accordance with the Brazilian Technical Standard 12713.[25] Individuals were cultivated in reconstituted water with a photoperiod of 16 hours of light and 8 hours of darkness (20 ºC ± 2 ºC). Organisms were fed daily with *Desmodesmus subspicatus* algae. Before subjecting *D. magna* neonates to the ecotoxicological tests, a sample of the batch was submitted to sensitivity testing with exposure to potassium dichromate. The sample showed sensitivity and was cleared to be used in tests according to the recommendations of ABNT NBR 12713.[25]

For the ecotoxicological tests with *D. magna*, acute exposure of neonates was performed for 48 hours, and surviving individuals were counted at the end of the exposure period. The tests consisted of exposing 10 neonates to 25 mL of each sample, which had their pH adjusted to 7.0 previously. Five sample concentrations with a ½ dilution factor were tested, ranging from 100% to 6.25%. Dilutions were made with reconstituted water, and all tests were performed in duplicate. For each exposure, a negative control group (NC) consisting of only reconstituted water was used.

Statistical analysis

Statistical analysis tests were performed using Analysis of Variance (ANOVA) followed by Tukey's test with a significance level of 95% ($p \leq 0,05$), with the help of BioEstat 5.3 software.

RESULTS AND DISCUSSION

The concentration of amoxicillin result was calculated from the quantification of the chromatogram area obtained through the monitoring of characteristic product ions from the antibiotic structure (Figures 2-A and 2-B).

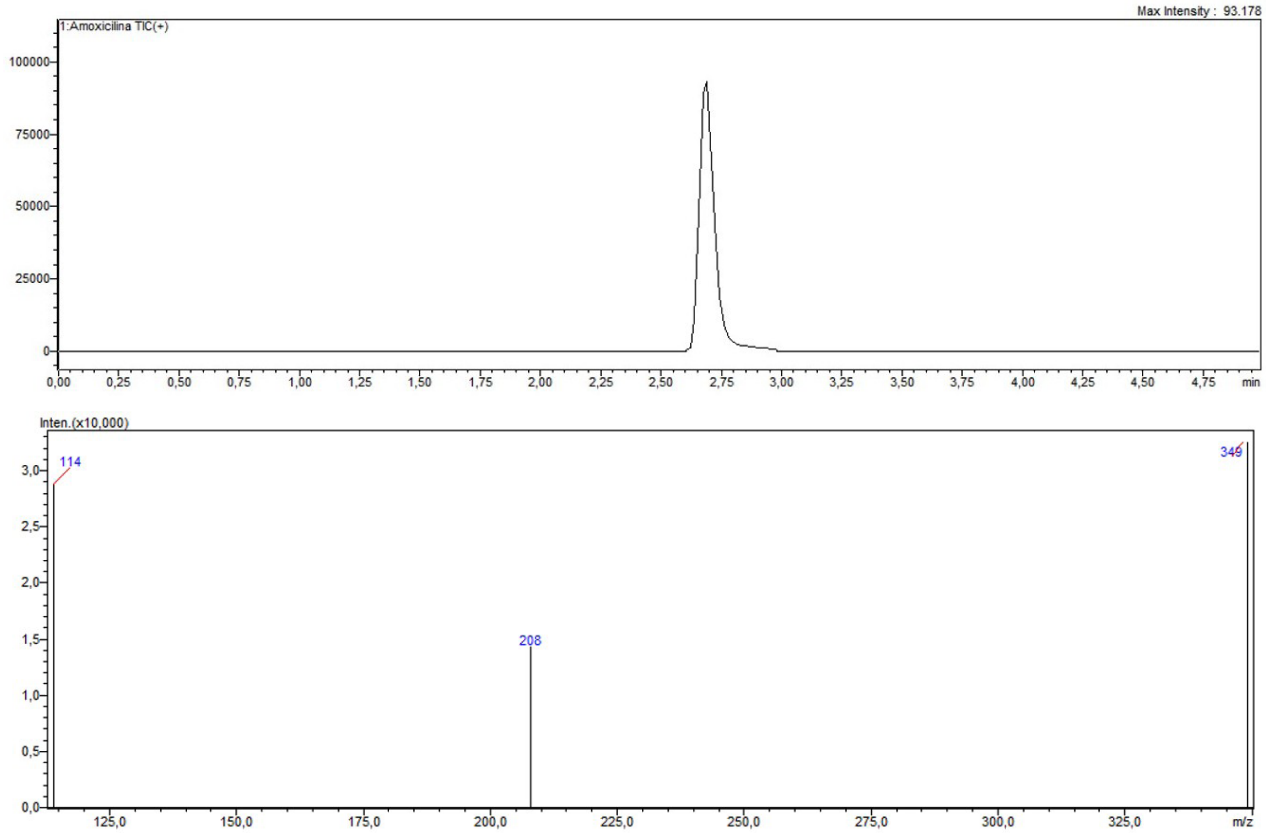


Figure 2. (A) Chromatogram of total ions of the 25 mg L^{-1} amoxicillin standard (diluted 100x) and (B) ions monitored for the analysis of amoxicillin concentration (Shimadzu LCMS-8040).

Construction of the bioreactor

Peroxidase enzymes have a greater oxidation-reduction potential than laccase enzymes. However, peroxidases can undergo permanent deactivation in the presence of high concentration of H_2O_2 due to the oxidation of amino acids in their structure.[26] This highlights the importance of designing a degradation system that uses sufficient levels of H_2O_2 to promote the oxidation reaction, without causing enzymatic deactivation or resulting in high residual concentrations.

Initially, tests involving varying concentrations of amoxicillin, enzyme, and H_2O_2, as well as the analytical technique adopted, were conducted. No other studies using peroxidase enzymatic action for the degradation of amoxicillin were found in the consulted literature. The composition of the biotechnological reactor was adapted from Silva et al.,[23] who investigated the discoloration of textile effluents using peroxidase enzymes extracted from turnips.

The preliminary enzymatic tests were performed in a single replicate using 250 mL Erlenmeyer flasks, with the addition of the following reactants, respectively:

a) 50 mL of the 25 mg L^{-1} sample solution in 0.1 phosphate buffer (pH 7);
b) 2 mL of synthetic 30 U mL^{-1} enzyme solution;
c) 0.5, 1, and 2.5 mmol L^{-1} of H_2O_2 (2, 4, and 10 µL of 30% H_2O_2, respectively).

The Erlenmeyer flasks were kept under agitation in a horizontal shaker (Marconi, model MA 830), at 120 rpm and 30 ºC for 5 hours. The results are displayed in Table II.

Table II. Results of the tests for the determination of the minimum H_2O_2 concentration

Peroxide concentration (mmol L^{-1})	Initial solution (mg L^{-1})	Enzyme-free solution after 5 h (mg L^{-1})	Enzyme solution after 5 h (mg L^{-1})
0.5	25.0	19.5	7.8
1.0	25.0	12.6	4.6
2.5	25.0	7.7	2.7

The addition of exceeding H_2O_2 acts in the degradation of the antibiotic, even in the absence of enzyme. However, the presence of residual peroxide is not advantageous. The permanence of this reagent influences in important parameters for the disposal of effluents, such as in the Chemical Oxygen Demand (COD), and interferes with the determination of the real efficiency of the process.[27]

Moreover, high concentrations of H_2O_2 can inhibit the photodegradation process reacting with hydroxyl radicals, acting as a remover of •OH ions, in accordance with Equations 5 and 6.

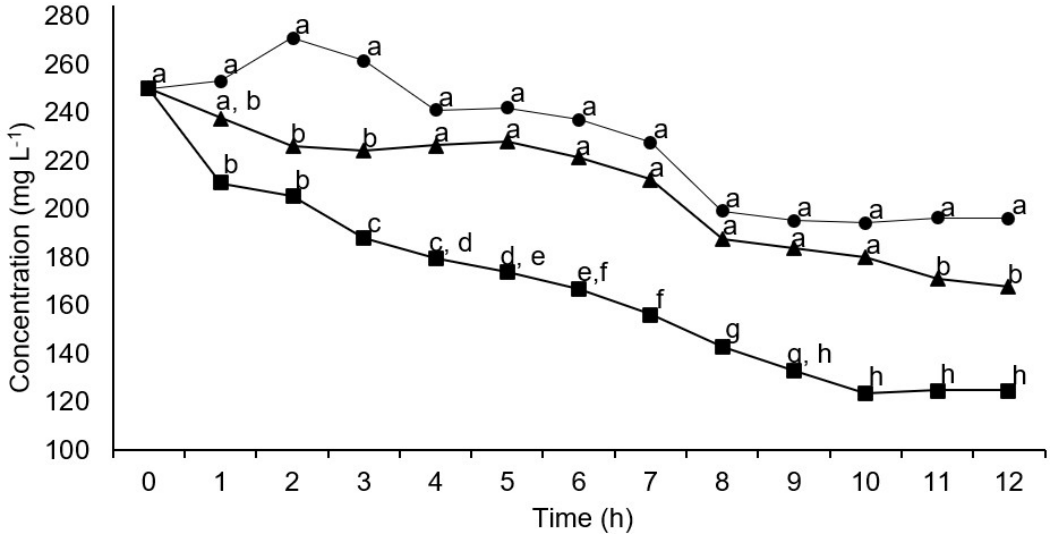
$$H_2O_2 + \bullet OH \rightarrow HO_2\bullet + H_2O \qquad \text{Equation 5}$$

$$HO_2\bullet + \bullet OH \rightarrow H_2O + O_2 \qquad \text{Equation 6}$$

Therefore, the study progressed using the lowest concentration of H_2O_2 (0.5 mmol L^{-1}).

Enzymatic degradation of amoxicillin

After determining the optimal condition, degradation tests were carried out in triplicate for 12 hours. Aliquots were collected every hour to verify the period required for degradation until enzymatic activity had stabilized. Additionally, three other samples were prepared as blank controls, each without one of the reagents, in order to evaluate the individual influence of each reagent. The blank tests (Figure 3) showed that, when the reagents are added separately, these can influence the concentration of amoxicillin in the medium.



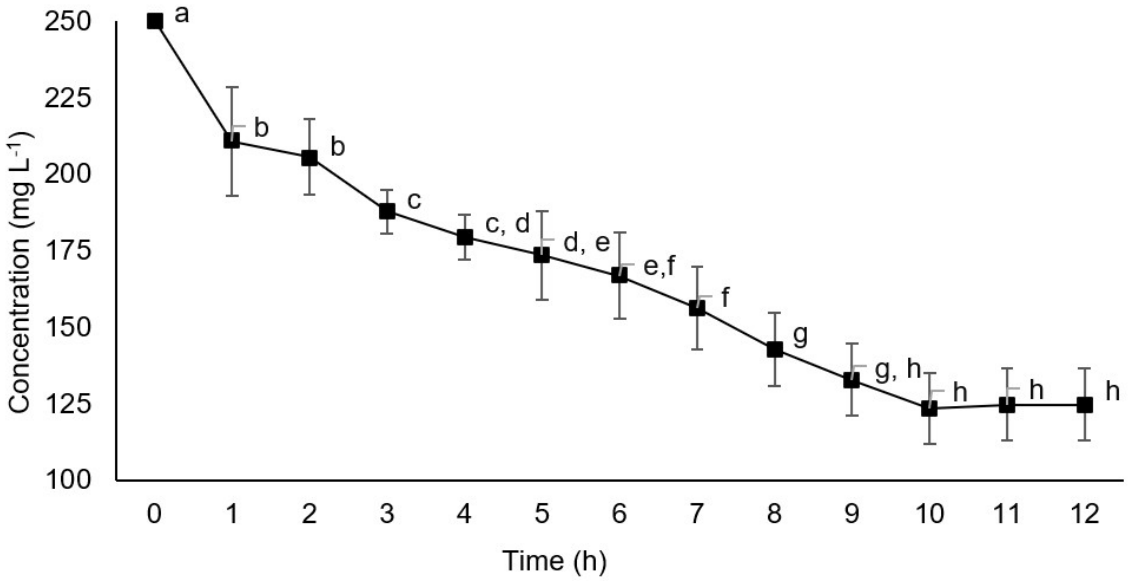
● Amoxicillin + Enzyme: 25 mg L^{-1} of amoxicillin + 2 mL of 30 U mL^{-1} enzyme solution in 0.1 M phosphate buffer (pH 7); ▲ Amoxicillin + H_2O_2: 25 mg L^{-1} of amoxicillin + 0.5 mmol L^{-1} of H_2O_2; ■ Amoxicillin + Enzyme + H_2O_2: 25 mg L^{-1} of amoxicillin + 2 mL of 30 U mL^{-1} enzyme solution in 0.1 mol L^{-1} phosphate buffer (pH 7) + 0.5 mmol L^{-1} of H_2O_2. Different letters for the same time point indicate significant differences ($p<0.05$) in degradation under different conditions.

Figure 3. Influence of each reagent on amoxicillin degradation.

The biodegradation tests involving the addition of peroxidase and H_2O_2 showed initial results like those of a process that could still be optimized, but already demonstrate a potential for amoxicillin degradation (Figure 5). Guardado et al.[28] also identified a decrease in amoxicillin concentration, even in blank tests without the addition of enzymes. The class of β-lactam antibiotics is known for its instability due to hydrolysis in aqueous solutions. Nonetheless, the degradation potential resulting from the activity of peroxidase and H_2O_2 is considerably higher than the self-degradation of the molecule.

In the present study, under controlled conditions, a 51% amoxicillin degradation was achieved in 9 hours through enzymatic action in the presence of H_2O_2. These results are consistent with those obtained by other authors that have investigated the performance of similar processes. Guardado et al.[28] achieved the removal of 72% of initial amoxicillin after 24 hours of incubation with three laccase enzymes. Wen, Jia, and Li[29] obtained degradation rates of 72.5% and 84.3% for tetracycline and oxytetracycline, respectively, using manganese peroxidase and 0.4 mM H_2O_2 after 4 hours of reaction. Similar to the analyzed process, other authors also identified the degradation of amoxicillin using different types and sources of oxidoreductase enzymes. Lueangjaroenkit et al.[30] achieved 100% inactivation of amoxicillin by exposing the antibiotic to the activity of a type of Mn peroxidase extracted from white rot fungi. Another Mn peroxidase from the same source achieved 45% inactivation of the substance, while an extracted laccase resulted in a reduction of 25%.

Figure 4 indicates that there were no significant differences between results after 9 hours of reaction, suggesting that enzymatic activity stabilizes and no longer reduces the concentration of amoxicillin in solution.



Different letters indicate significant difference ($p<0.05$) in the concentration in relation to degradation time.

Figure 4. Amoxicillin degradation by enzymatic process.

Although the application of enzymatic treatments may seem insufficient, enzyme-catalyzed oxidation can be used as a pre-treatment for bioreactors, improving the biodegradability of persistent compounds. However, this is still a poorly studied application. In Brazil, 75% of industrially produced enzymes are concentrated in the detergent (37%), textile (12%), starch (11%), baking (8%), and animal feed (6%) industries. Meanwhile, this percentage is still insignificant in the wastewater treatment sector and is not yet accounted for in statistics.[31]

Laccases are one option for the degradation of amoxicillin. These enzymes are known for their interactions with small to medium-sized molecules containing phenolic and phenolic-substituted residues

such as methoxy or amine, which are constituents capable of fitting in the active site of the enzyme and are found in the structure of this antibiotic.[32]

Besides H_2O_2, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is also an alternative additive that can be used in conjunction with peroxidase, with the purpose of enhancing the oxidative action of organic compounds. As evidenced by the studies of Leng et al.,[33] the association of HRP and ABTS shows better tetracycline degradation potential than pure enzymes or laccases, for example, as well as providing higher stability against variations in pH and temperature.

Degradation products

An initial scan was proposed to monitor all the compounds present in the *m/z* range between 100 and 800. Due to the high levels of background noise, it was not possible to identify with precision the compounds generated from the treatment methods. Therefore, a bibliographical survey of the main byproducts was performed, providing some sensitivity to the analytical method. The studies by Nägele and Moritz,[34] Gozlan et al.,[35] Liu et al.,[36] Lamm et al.,[37] and Dogan and Kidak[38] were used as references for the monitoring of the structures.

The main mechanism involved in the degradation of the amoxicillin molecule occurs via the opening of the β-lactam ring.[39] This process can lead to the binding of a water molecule resulting in the formation of a free carboxylic acid group, and the protonated compound acquires a *m/z* ratio of 384 (Figure 5).[34]

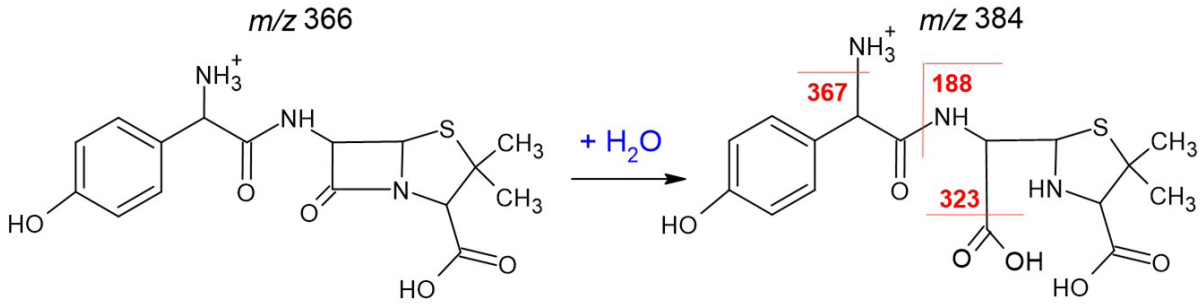
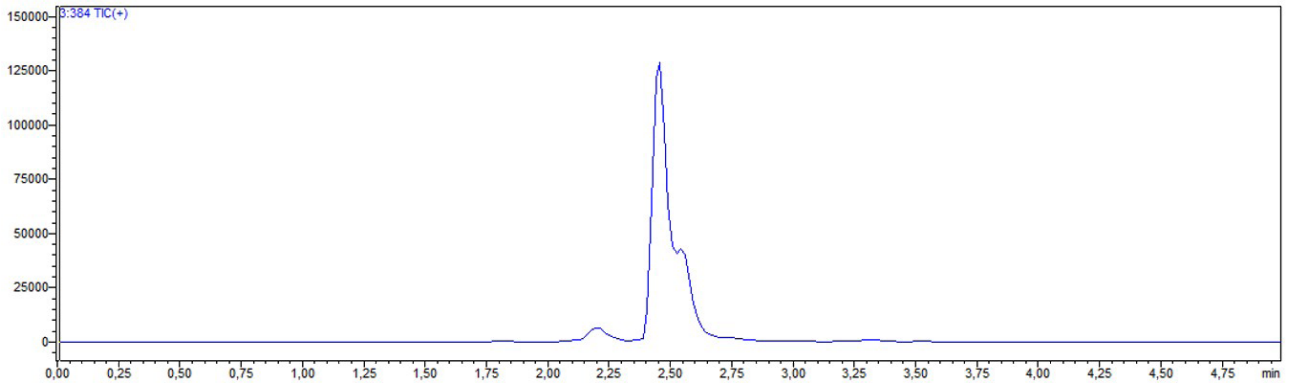


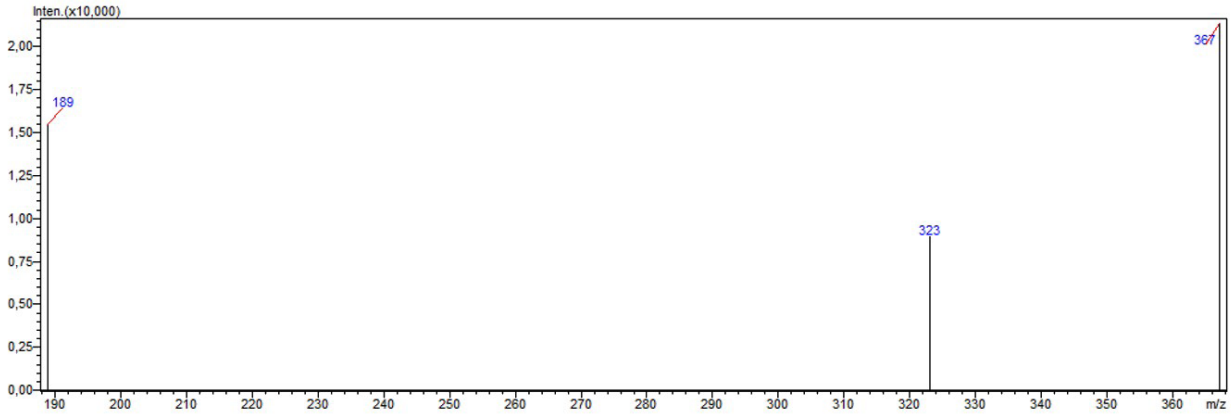
Figure 5. Formation of the *m/z* 384 byproduct from the initial amoxicillin molecule.

As the solutions are in an aqueous environment, the high presence of free Waters facilitates the addition of a water molecule to the already destabilized structure of the antibiotic under study. The presence of a free carboxylic acid group increases the polarity of the substance, meaning the compound is eluted earlier (retention time: 2.50 min) than the amoxicillin molecule in its original form, as shown by Figure 6 (retention time: 2.75 min).[34]



(A)

Figure 6. (A) Chromatogram of the degradation product *m/z* 384 and (B) Mass spectrum of the degradation product *m/z* 384. (continues on the next page)



(B)

Figure 6 (continuation). (A) Chromatogram of the degradation product *m/z* 384 and (B) Mass spectrum of the degradation product *m/z* 384.

And it was possible the *m/z* 340 byproduct from the *m/z* 384 intermediate (Figure 7).

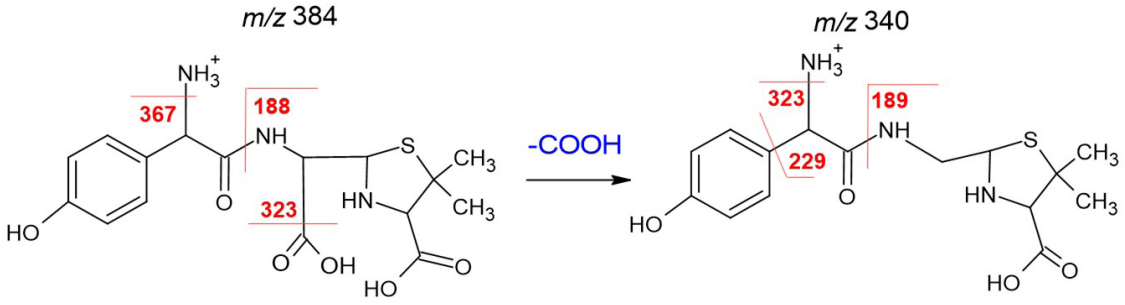
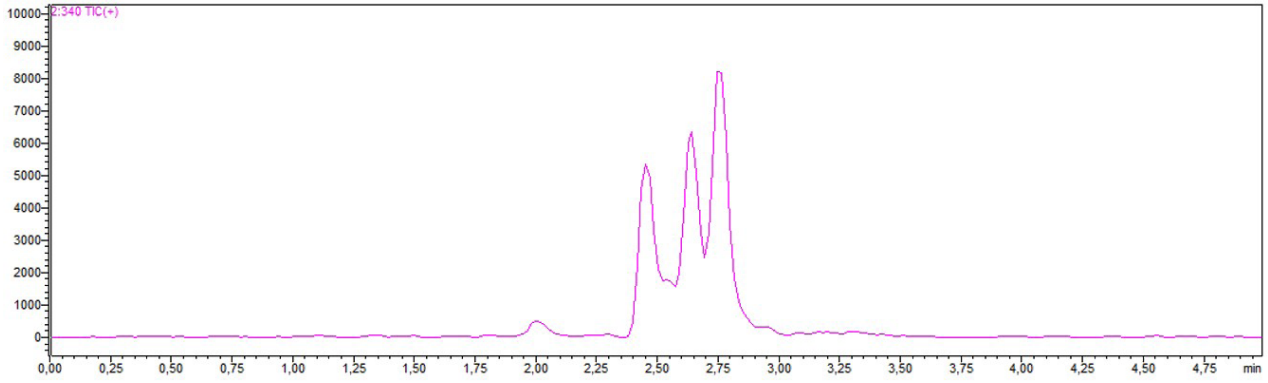


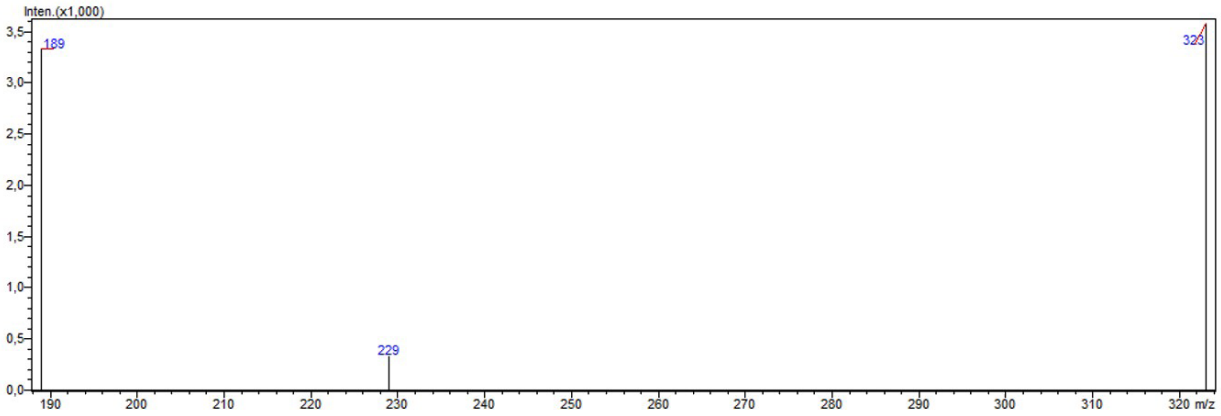
Figure 7. Formation of *m/z* 340 byproduct from the *m/z* 384 intermediate.

Figures 8-A and 8-B show that it was observed the *m/z* 340 byproduct from the *m/z* 384 intermediate.



(A)

Figure 8. (A) Chromatogram of the degradation product *m/z* 340 and (B) Mass spectrum of the degradation byproduct fragment with *m/z* 340. (continues on the next page)



(B)

Figure 8 (continuation). (A) Chromatogram of the degradation product *m/z* 340 and (B) Mass spectrum of the degradation byproduct fragment with *m/z* 340.

Another byproduct identified in literature is amoxicillin diketopiperazine, originating from penicilloic acid after the loss of a water molecule and the rearranging of the atoms in its structure.[36]

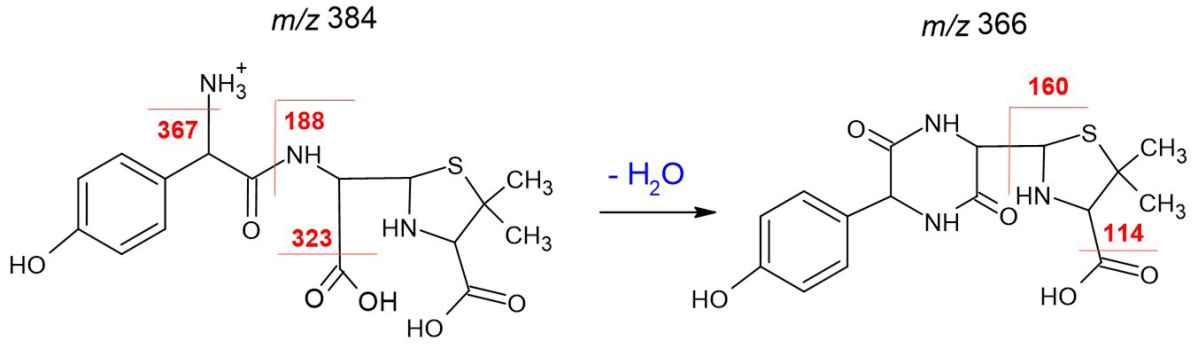
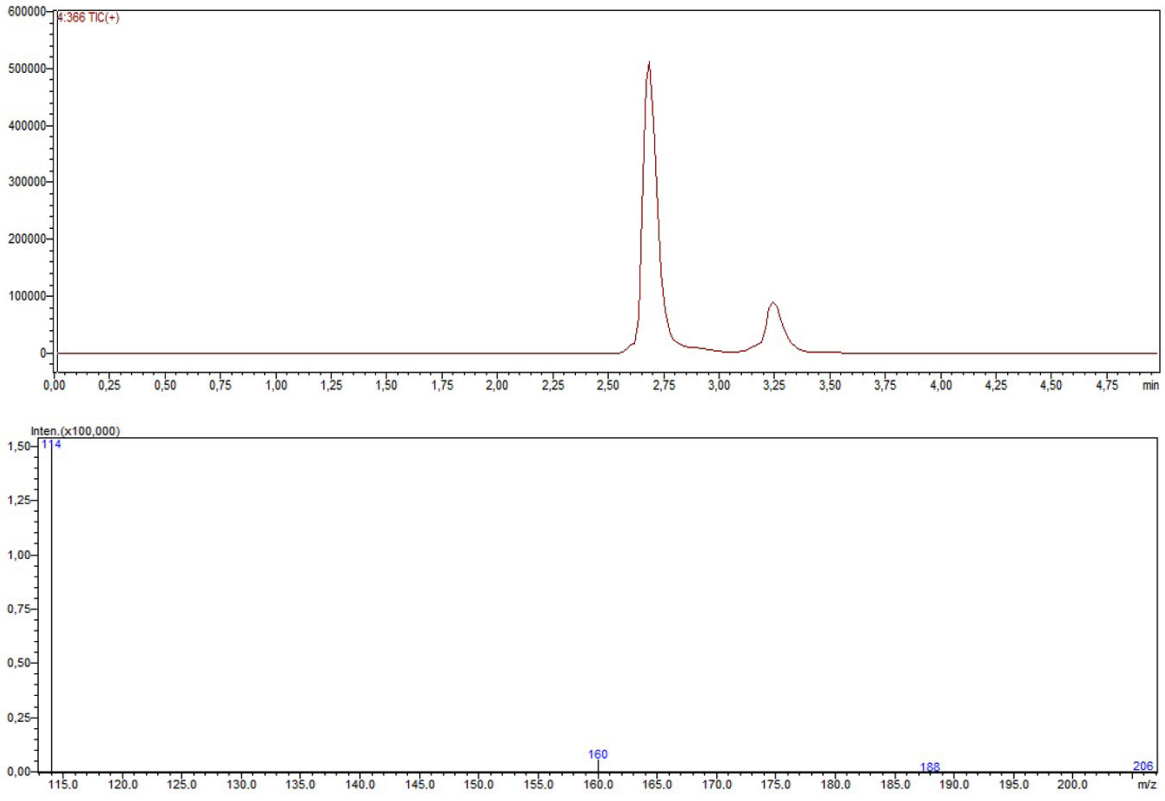


Figure 9. Formation of byproduct *m/z* 366 from the *m/z* 384 intermediate.

Alternatively, a structure with a *m/z* ratio of 366 can also originate from the loss of an ammonia molecule from the *m/z* 384 compound, which would maintain the same product ions (*m/z* 160 and 114) as observed in the respective mass spectrum (Figures 10-A and 10-B). The loss of NH_3 in amoxicillin degradation process has been reported in the studies of Jung et al.,[40] who analyzed the concentration of ammonium ion (NH_4^+) throughout UV and UV/H_2O_2 treatment processes. As the concentration of the antibiotic decreased, the concentration of NH_4^+ increased and continued to grow even after its complete degradation, indicating that the degradation of intermediate byproducts also occurs during the process involving the loss of nitrogen atoms.



(A)

(B)

Figure 10. (A) Chromatogram of the degradation byproduct and (B) Mass spectrum of the degradation byproduct *m/z* 366.

The intensities of the peaks of each mass/charge ratios are displayed semi-quantitatively in Table III.

Table III. Semi-quantitative peak intensity of the degradation products

Conditions	*m/z* ratios			
	340	**366**	**384**	**731**
Enzyme	1.064	54.963	21.328	-
Enzyme + H_2O_2	1.244	42.828	25.142	-
H_2O_2	964	83.878	8.824	-

The carboxyl, amide, thiazole, and phenol groups present in the amoxicillin molecule are stable and oxidation resistant, but indicate the formation of similar degradation products even for different treatments, since there is a tendency for the molecule to undergo cleavage in bonds with higher electronegativity difference between atoms due to weaker bond energies in these locations.[41]

Although many studies use the term “degradation products” when referring to the products of enzymatic reactions, the mineralization of organic micropollutants cannot be achieved by enzymatic treatment alone. Products of enzymatic treatments with oxidoreductases are mainly oligomers that can form polymers and/or insoluble compounds; therefore, “transformation products” can be considered a more accurate term. Nevertheless, in some cases, the bond cleavage during enzymatic reactions can produce compounds with lower molecular weight, but information about specific reaction products is scarce, even for experiments conducted with synthetic solutions like in the present study. To confirm the suggested structures formed,

subsequent analyses will be performed by the use of liquid/gas chromatography with mass spectrometry where the identification and their relation to structural alterations originating from the proposed treatments, as demonstrated in the literature.[18]

Ideally, the identification and quantification of the generated products would only be achieved by comparison with a standard of each of the substances generated, which would render the cost of the study rather unfeasible.

Toxicity

Toxicity was determined by calculating the half maximal effective concentration (EC_{50}) using the Trimmed Spearman-Karber method.[42] A toxicity scale based on the distribution of calculated EC_{50} percentiles was used, in accordance with Lobo et al.,[43] where an EC_{50} (%) below 25 is considered extremely toxic, 25-50 is highly toxic, 50-75 is moderately toxic and over 75 is considered low toxic.

The results of the toxicity analysis for each proposed methodology are presented in Table IV.

Table IV. Toxicity results of the treated solutions

Treated solution sample	EC_{50} ($IC_{95\%}$)	Toxicity[b]
Initial amoxicillin (25 mg L^{-1})	-	Toxic
25 mL water + 2 mL enzyme (30 U mL^{-1}) + 0.5 mmol L^{-1} H_2O_2	55.5 (47.1-65.3)	Moderately toxic
25 mL amoxicillin (25 mg L^{-1}) + 0.5 mmol L^{-1} H_2O_2	93.3 (70.6-123.2)	Low toxic
25 mL amoxicillin (25 mg L^{-1}) + 2 mL enzyme (30 U mL^{-1})	75.2[a]	Low toxic
25 mL amoxicillin (25 mg L^{-1}) + 2 mL enzyme (30 U mL^{-1}) + 0.5 mmol L^{-1} H_2O_2	93.9[a]	Low toxic
25 mL amoxicillin (25 mg L^{-1}) + UV radiation (10 min)	NC	Non-toxic
25 mL amoxicillin (25 mg L^{-1}) + 0.5 mmol L^{-1} H_2O_2 + UV radiation (10 min)	NC	Non-toxic

NC: Not calculable; [a]Confidence interval cannot be calculated; [b]Toxicity scale[43]

Al-Maqdi et al.[15,16] presented in their studies proposals for the treatment of sulfamethoxazole and thiazole using enzymatic reactions. The authors found different results for the toxicity of each substance after treatments. Using peroxidase from soybeans, the authors found that the products generated by enzymatic degradation were toxic against seeds of *Lactuca sativa*. This confirms the importance of studies on various classes of micropollutants, and warns that not all treatments may be suitable for every substance.

The toxicity results found for the solutions which underwent enzymatic treatments may be related to the presence of the β-lactam ring in the amoxicillin molecular structure, which is responsible for its antibacterial activity.[40] In the present work, approximately 49% of the initial concentration of amoxicillin is still present in the medium in an undegraded form even after treatment.

Based on the results obtained, it is necessary to carry out toxicity analyses involving other methodologies and trophic levels of control in order to confirm the different results found in literature and in the present study.

CONCLUSION

The present work is a precursor study in the application of peroxidase enzymes in the treatment of amoxicillin. Therefore, further studies are still necessary to optimize the process and its implementation viability, since the initial results presented demonstrate that its oxidative activity in the presence of H_2O_2 is capable of degrading the drug.

A few proposals should still be evaluated to enable enzymatic treatment, which is still a recent approach with respect to micropollutants. Immobilization techniques are also considered efficient strategies in comparison to the usage of free biodegrading agents due to the ease of separating the biomass from the liquid medium, protection against harsh external conditions, and low susceptibility to contamination by undesired organisms. Enzyme immobilization technology increases the recycling efficiency, storage stability, and reusability of enzymes in continuous processes. Immobilized enzymes are rendered more stable against variations in pH and severe thermal and pressure conditions in comparison to free enzymes, and this technology represents a simple and efficient method to improve the catalytic properties of enzymes. Therefore, the application of enzymatic degradation for the treatment or removal of micropollutants is still a field that should be explored further, with several conditions that can be optimized to ensure the quality and safety of the final effluent before it is released into water bodies.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This study was financed in part by the Coordination for the Improvement of Higher Education Personnel – Brazil (CAPES), Finance Code 001, National Council for Scientific and Technological Development (CNPq) and University of Vale do Taquari – Univates.

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