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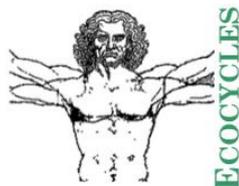
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ORIGINAL ARTICLE

Assessment of the biodegradation of doxycycline by biostimulation with addition of glucose, phenol or/and copper

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Abstract - Doxycycline, an antibiotic, is largely used in human and veterinary medicine. The conventional treatment with activated sludge is not very efficient. Laccase appeared to be the main enzyme secreted essentially by white rot fungi as *Trametes versicolor* and *Phlebia fascicularia* on the degradation of xenobiotic compounds from the pharmaceutical industry. The main purpose of this study was to enhance the biodegradation of doxycycline through activated sludge combined with addition of glucose as a carbon co-substrate to improve the growth of the microbial population present in the activated sludge, phenol as a laccase mediator, copper as a cofactor and inductor for laccase production. The enhancement of the biodegradation of doxycycline was 50, 90, 68 and 83% greater respectively with the addition of glucose, copper, phenol and with a mixture of the compounds after 14 days of treatment at 25°C. Compared with the biotic control (activated sludge alone), a 30% increase for the test with the addition of phenol was observed.

Keywords: doxycycline, micropollutant, wastewater, activated sludge, biostimulation

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INTRODUCTION

During the last few years, many studies have shown the importance of the treatment of micropollutants which are also called emerging contaminants. A huge amount of expanding formation of anthropogenic as well as natural substances has been observed. Among these, pharmaceutical substances, personal care products, hormones, industrial chemicals, pesticides, and many other emerging compounds can be found in wastewater in trace concentrations, from a few ngL⁻¹ to several µgL⁻¹. The 'low concentration' and diversity of micropollutants not only complicate the associated detection and analysis procedures but also create challenges for water and wastewater treatment processes (Luo et al, 2014). Micropollutants in wastewater, unless properly treated, pose substantial risks to human health and the environment (Boonnorat et al, 2019; Wolff et al, 2018).

Pharmaceuticals are used to cure, prevent or eliminate diseases in animals and humans. They are designed to resist biodegradation long enough to attain their beneficial effects, and this explains why they are resistant to biodegradation. Our dependency upon animal farming, for an increasing human population, also implies an increased medication used in this sector. Continuous input to the environment of these chemicals can lead to their accumulation in organisms and transfer of effects along natural food-chains (Corcoran et al, 2010). One main concern is that antibiotics promote the development and spread of resistant bacteria (Becker et al, 2016).

This research will focus on doxycycline's effect during wastewater treatment, this micropollutant is considered as a semisynthetic antimicrobial, which belongs to the

tetracycline class and it is usually obtained through the modification of oxytetracycline (Spina-Cruz et al, 2018).

Many treatments have been developed for the degradation of the pharmaceutical substances in wastewater, either biological or not, such as removal by nanoparticles (Xiong et al, 2019), electro-generated adsorbents (Zaidi et al, 2019), electro-coagulation coupled electro-flotation (Zaidi et al, 2015), oxidation processes (Spina-Cruz et al, 2018; Aboudalle et al, 2017; Anjali, 2019), biological system with sonochemical process (Serna-Galvis et al, 2019), catalytic decomposition (Payan et al, 2019), electro-coagulation (Baran et al, 2018), ozonation (Lakovides, et al, 2019). Although, these processes have a great efficiency it would be important to study their behavior as the toxicity is not always eliminated (Becker et al, 2016). Next to these treatments, we found the conventional biological treatment with activated sludge that is most commonly used for both domestic and industrial plants in the world (Wei et al, 2003). Activated sludge is a complex suspension of bacteria, fungi, protozoans and metazoans and some non-biological compounds that contain organic and inorganic particles (Failkowska et al, 2008; Pajdak-Stós, 2017).

It has been proved that the production of some enzymes has the possibility to help in the removal of some pollutants, in this case the antibiotics, especially from native enzymes from activated sludge, which are able to transform micropollutants through biodegradation (Krah et al, 2016). Several studies focused on the biodegradation of organic micropollutants with free or immobilized enzymatic processes, the results were promising. Treatment with whole-cell fungi showed superior performance for many compounds due to the synergetic effects of intracellular and extracellular enzymes coupled with sorption onto fungal biomass (Naghdi et al, 2018). Laccase is an oxidoreductase enzyme, catalyzes the oxidation of certain aromatic compounds, particularly phenolic compounds, using molecular oxygen as the terminal electron acceptor. Laccase has four copper atoms divided into three types at the catalytic center of each monomer (Naghdi et al, 2018). They are present in plants, in a large number of fungi (degradation of lignin) as well as in some bacteria. Some authors showed that addition of CuSO_4 to culture media improved the increase in the total laccase activity (Giardina et al, 1999). For its maximal effect copper had to be supplemented during the exponential phase of growth (Galhaup et al, 2001). Ding et al, (2016) have indicated that laccases have enormous potential in the remediation and treatment of contaminated water of several pharmaceutical personal care products. These observations were previously showed by some studies in this topic (Hata et al, 2010; Suda et al, 2012). However, for some authors the treatment cost is very high, which is explained by the cost of the use pure enzyme and the gaps between laboratory and field-scale research need to be overcome in order to assess the viability for real application (Stadlmair et al, 2018; Morsi et al, 2020). So, it's very important to enhance the production of laccase, for example by activated sludge, the biostimulation process is the main method to succeed. Semrany et al, (2012) explained that it's necessary to enhance the growth of the bacteria by

addition of a carbon source or/and to stimulate the production of the enzyme by addition of mediator or cofactor and inductor for enzyme production.

The main purpose of this study was to investigate the improvement of the doxycycline degradation by activated sludge. It's about testing the addition in the growth medium of glucose as a conventional carbon source, phenol as laccase mediator and copper as cofactor and inductor for laccase enzyme production.

MATERIALS AND METHODS

Chemicals

Doxycycline hyclate (DC) ($\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_8 \cdot \text{HCl} \cdot 0.5\text{H}_2\text{O} \cdot 0.5\text{C}_2\text{H}_6\text{O}$, 99%) was obtained from Sigma-Aldrich (France). All of the other chemicals used were of the high purity grade (>97%) and were bought from VWR. The chemical structure for doxycycline is given in Fig. 1.

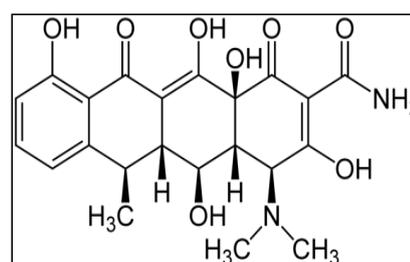


Figure 1. Chemical structure of doxycycline

Activated sludge

Mixed liquor (ML) a mixture of activated sludge (AS) and wastewater was taken from the aerobic tank of the wastewater treatment plant (WWTP) of Beaurade, Rennes, France which receives predominantly domestic wastewater. Mixed liquor was transported to the laboratory within 20 minutes and immediately used for the experiments. The washed activated sludge was used for the doxycycline biosorption and degradation experiments. To eliminate any additional nutrient to those contained in the culture medium, the activated sludge was washed four times with osmosis water as follows: 25 mL of mixed liquor were centrifuged (*HERAUS MEGAFUGE 16R centrifuge*) at 4 000 rpm for 10 min, at 4°C and the supernatant was removed. The pellet was resuspended with 25 mL osmosis water and after vigorous agitation with vortex, the tube was centrifuged again. These steps were repeated three times. Finally, the pellet was resuspended in 2 mL of osmosis water and added to the culture media after vigorous agitation. Flasks were incubated under constant shaking at 200 rpm on an orbital shaker, at 25°C (Orbital shaker IKA KS 4000 i control).

Biosorption experiments

The biosorption experiments were carried out in order to evaluate the sorption of the doxycycline onto activated sludge. 5 mL of a suspension of mixed liquor was placed in a 500 mL Erlenmeyer flask containing 200 mL of 100 mg L⁻¹ doxycycline solution. The test was carried out over a period of 240 min with continuous stirring at room temperature. Samples were taken every 30 min, then centrifuged at 4 000

rpm for 10 min and filtered on a 0.45 µm needle filter to measure the absorbance.

Biodegradation experiments

Biodegradation experiments were performed in 500 mL Erlenmeyer flasks clogged with a plug containing 200 mL of appropriate medium contained per liter: KH_2PO_4 85 mg L⁻¹; K_2HPO_4 208 mg L⁻¹, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 154.4 mg L⁻¹, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 22.6 mg L⁻¹, CaCl_2 27.6 mg L⁻¹, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.26 mg L⁻¹, NH_4Cl 74 mg L⁻¹ and the pH was adjusted to 7 with KOH 1 M. DC was added into the flasks to give the desired final concentration of 100 mg L⁻¹. Four different systems were evaluated: **A**- Biotic control: without addition of carbon source or cofactor (control), **B**- biostimulation test with addition of glucose (2 g L⁻¹) as conventional carbon source, **C**- biostimulation test with addition of copper (160 mg L⁻¹) as cofactor and inductor for laccase enzyme production, **D**- biostimulation test with addition of phenol (0.5 g L⁻¹) as laccase mediator, **E**- biostimulation test with addition of glucose (2 g L⁻¹), phenol (0.5 g L⁻¹) and copper (160 mg L⁻¹). The flasks were incubated under constant shaking at 200 rpm on an orbital shaker, at 25°C (New Brunswick Scientific INNOVA 40). Inoculation was carried out in order to achieve an initial biomass concentration in the aqueous phase at 0.5 g L⁻¹. All experiments were conducted at least in duplicate, samples were taken at 0, 7 and 14 days.

Analytical methods

For pH (pH-meter WTW 315i) the measures were done directly in the mixed liquor. For other measures, samples of 5 mL were periodically taken. Immediately after sampling homogeneous suspension of the culture medium, cell growth was monitored by measuring the turbidity (HACH 2100Q) and Biomass dry weights (MLSS) which were obtained by centrifuging 5 mL of cell suspension at 4000 rpm and 4°C for 15 minutes, the pellet was dried at 105 °C (VWR DRY-Line) for 48 hours. The DC analysis was performed by the lecture of the absorbance at 346 nm, Absorbance = 0.0223 [DC] + 0.0321 with $R^2 = 0.99$ for a concentration between 10 and 100 mg L⁻¹ in DC. The Chemical Oxygen Demand (COD) was measured using the HACH method (25–1500 mg/L range) in COD vials (CSB-Kuvettentest, Merck, Darmstadt, Germany), which involves heating at 150°C for 2 hours and then spectrophotometric reading (spectrophotometer Secomam, Ales, France).

Biodegradation efficiency (η)

For a better visualization of the results, the concentrations observed in biodegradation were expressed as a percentage, considering the initial concentration as 100%. The removal efficiency of doxycycline (%) was calculated according to Equation 1:

$$\eta (\%) = [(C_0 - C)/C_0] \times 100\% \quad \text{Equation 1}$$

When C_0 and C are the DC concentration at initial and at time t , respectively.

RESULTS AND DISCUSSION

Biosorption experiments

The elimination of DC for 250 min is lower than 20 %, this shows the negligible DC biosorption on the activated sludge (Fig. 2), this can be explained by the value of the log K_{ow} of DC (log $K_{ow}=3.5$), since for these values, the biosorption was reported to be very low (Naghdi et al, 2018). The effect of biosorption was negligible compared to the biodegradation. This result was also observed by Ferrag-Siagh et al, (2014) on the tylosine and Aboudalle et al, (2018) on the metronidazole.

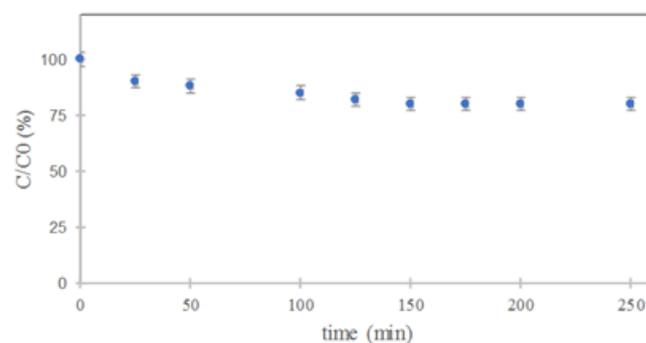


Figure 2. Time-course of biosorption on activated sludge of the doxycycline (100 mg L⁻¹ initial concentration) at 25°C, pH 7 for 2 hours.

Doxycycline degradation by activated sludge

Table 1 shows some parameters (Turbidity, pH, removal of doxycycline and COD) for the monitoring of the biodegradation of DC by activated sludge at 25°C. It was observed that in the experimental conditions after 14 days of biotreatment, a decrease of the target compound by 60%. Nevertheless, at the same time, the COD value didn't evolve. It means that despite the action of the activated sludge on the removal of the doxycycline, the by-products formed from initial doxycycline were not eliminated by activated sludge.

Table 1: Turbidity and pH of culture medium, doxycycline removal, COD after 7 and 14 days of culture with AS at 25°C

Time-course (days)	0	7	14
Turbidity (NTU)	140	156	200
pH	6.5	6.4	6.3
Doxycycline removal η (%)	-	49	60
Chemical Oxygen Demand (mg O ₂ L ⁻¹)	90	90	90

Biostimulation of activated sludge for the doxycycline removal by activated sludge

The main aim of this part was to verify the relevance of stimulating the activity of the enzymes responsible for the degradation of the doxycycline by biostimulation of activated sludge with glucose, phenol and copper.

Biostimulation of activated sludge by addition of glucose

The use of easily biodegradable co-substrate (glucose) is the main way to enhance the growth of bacteria from activated sludge (Semrany et al, 2012). The turbidity values obtained after 14 days of treatment were 200 and 600 NTU respectively for assays without and with glucose. The addition of glucose as co-substrate allowed the growth of activated sludge' microbial population (Fig. 3). Concerning the pH evolution, pH decreased from 6.6 to 3.8 in presence of glucose and no evolution was observed in the assay without glucose (Fig. 4). These results clearly illustrate the significant effect of the addition of glucose on bacteria growth and pH. The metabolism of glucose by microorganisms is accompanied by a release of proton.

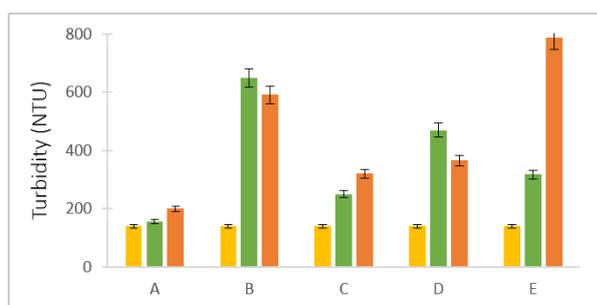


Figure 3. Time-course of turbidity of culture medium at 0 ■, 7 ■ and 14 ■ days

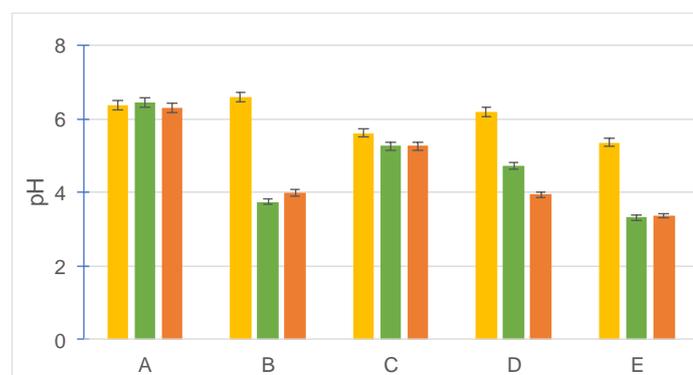


Figure 4. Time-course of pH of culture medium at 0 ■, 7 ■ and 14 ■ days

However, doxycycline removal was not improved by the addition of glucose, bacteria choose to oxidize glucose at the expense of the target compound (Fig. 5).

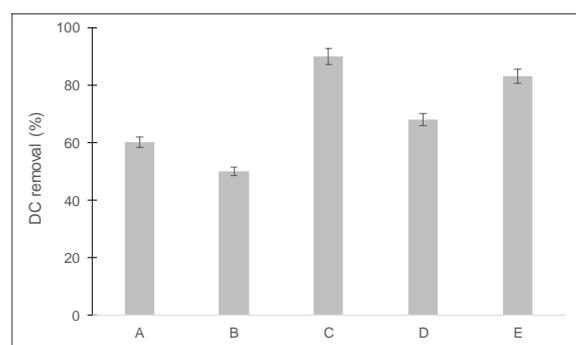


Figure 5. Time-course of absorbance of culture medium at 14 days

Biostimulation of activated sludge by addition of copper

After 7 and 14 days of culture with addition of copper in culture medium, the turbidity increase by 44% and 56.3% respectively, these results are lower than those with addition of glucose but higher than control assay (Fig. 3). pH profile was quite similar than control (Fig. 4). However, the addition of copper increased doxycycline removal (90%), which made an increase of 30% compared to the control (Fig. 5).

Biostimulation of activated sludge by addition of phenol

Mediators are low weight molecular compounds that are stable but reactive and facilitate the oxidizing process and improve the transformation rates of micropollutants (Varga et al, 2019). In this study, the phenol is used as mediator to enhance the removal of doxycycline, phenol can also serve as co-substrate for bacteria growth. In previous study, we observed that addition of phenol with a concentration higher than 0.5 g L⁻¹ inhibited the growth of activated sludge (data not shown). The addition of phenol in the culture conditions allowed the growth of microbial population by 70% and 62% after 7 and 14 days respectively with comparison with the control assay. The profile of the pH decreased by 6.2 to 4.7 after 7 days and to 3.9 after 14 days of culture. It was the same profile with the assay with addition of glucose as co-substrate (Fig. 4). The doxycycline removal reached 62% after 14 days of culture (Fig. 5). In this case, phenol does not serve only as a primary source of energy but induce catabolic enzymes which were involved in biodegradation of the doxycycline, this result was also mentioned by Stadlmair et al, (2018).

Biostimulation of activated sludge by addition of glucose, phenol and copper

In the aim to improve the diminution of doxycycline the last assay was conducted with addition of glucose, phenol and copper. After 14 days of culture, the turbidity reached 787 NTU, i.e. an increase of the biomass by 82.2% (Fig.3). The profile of the pH followed those of assays with addition of glucose or phenol (Fig. 4). The doxycycline removal reached 82% after 14 days of culture (Fig. 5). The mixture of the three compounds, certainly allowed the increase of the biomass production but did not improve the doxycycline removal after 7 or 14 days of culture (Fig. 5).

Dry weight values were correlated with turbidity values (Figure 2 and Table 2), however, the ratio (doxycycline removal/dry weight) showed that the biomass production was not correlated with the doxycycline removal. Addition of glucose as co-substrate certainly improved the biomass production but did not allow higher remove of doxycycline. Addition of easily biodegradable co-substrate, inhibited the production of enzymes which were able to degrade doxycycline as carbon substrate for biomass growth. Addition of copper in the culture medium, allowed a better yield of doxycycline removal by the biomass (Table 2) and a better removal efficiency of doxycycline (Figure 3). Biostimulation by addition of copper was a possible way to enhance the doxycycline removal.

As indicated in Table 2, not all glucose or phenol was used by microbial population. Furthermore, COD values showed

that doxycycline was removed but not biodegraded. Indeed, doxycycline concentration decreased during the treatment, but not the COD, the target compounds was degraded to some compounds but not mineralized (Table 2).

Table 2: Dry weight, Doxycycline removal, Chemical Oxygen Demand after 14 days of treatment with activated sludge at 25°C

Assays	A	B	C	D	E
Dry weight (mg L ⁻¹)	116	519	310	342	956
Doxycycline removal (mg L ⁻¹)	60	50	90	68	83
Doxycycline removal/dry weight	0.51	0.10	0.29	0.20	0.09
Chemical Oxygen Demand (mg O ₂ L ⁻¹)	90	238	90	145	268
Chemical Oxygen Demand removal (%)	0	88.6	0	85.9	92.8

CONCLUSION

Doxycycline a pharmaceutical and recalcitrant compound, is the target compound in this study. The aim of this study, is the enhancement of its biodegradation after stimulation of the microbial population of activated sludge. Glucose, copper and phenol were added to the medium culture in presence of doxycycline and activated sludge. According to the biological tests, the relevance of the biostimulation of activated sludge's microbial population was confirmed. Indeed, the enhancement of the biodegradation of doxycycline was 50, 90, 68 and 83% greater respectively with the addition of glucose, copper, phenol and with a mixture of the compounds after 14 days of treatment at 25°C with comparison with the control.

In the study conditions, doxycycline was not an inhibitor of the growth of activated sludge's microbial population although the microbial population could evolve in time and in the presence of doxycycline. The enzymes involved in doxycycline degradation were not inactivated. However, supplementary tests are needed in the future to check the presence and the activity of laccase and search other enzymes involved in doxycycline removal.

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