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Efficiency of *Bacillus thuringiensis* biofilters on 17β-estradiol removal

Graziela Taís Schmitt[a*], Amanda Gonçalves Kieling[b], Marcelo Oliveira Caetano[a]

[a] *Programa de Pós-Graduação em Engenharia Civil da Universidade do Vale do Rio dos Sinos, São Leopoldo, 93022-750, Rio Grande do Sul, Brasil. *grazi_gts@hotmail.com*
[b] *Universidade do Vale do Rio dos Sinos, São Leopoldo, 93022-750, Rio Grande do Sul, Brasil.*

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Abstract

This study evaluated the potential of *Bacillus thuringiensis* biofilters and natural substrates for removal of the natural estrogen 17β-estradiol (E2) in aqueous solutions. Filters biologically activated with this bacterium and containing rice husk ash (RHA) or activated carbon (AC) were monitored for 20 days for biofilm formation. In all samples, E2 was quantified by SPE (solid phase extraction) and LC-MS/MS (liquid chromatography coupled to mass spectrometry). The results showed 83% removal for the biofilter containing RHA and 86% for AC, indicating the potential of the bacterium *Bacillus thuringiensis* to biodegrade E2. This study demonstrated that biofiltration can be a method used for E2 removal.

Keywords: Biodegradation, hormones, water treatment, adsorbents, *Bacillus thuringiensis.*

Eficiência de biofiltros com *Bacillus thuringiensis* para remoção de 17β-estradiol

Resumo

Este estudo avaliou o potencial de biofiltros com *Bacillus thuringiensis* e substratos naturais na remoção do estrogênio natural 17β-estradiol (E2) em soluções aquosas. Foi avaliado filtros ativados biologicamente com essa bactéria contendo como substrato a cinza de casca de arroz (CCA) ou carvão ativado (CA) foram monitorados durante 20 dias, quanto a formação de biofilme. A quantificação do E2 em todas as amostras foi realizada por extração em fase sólida (SPE) e determinação por cromatografia líquida acoplada ao espectro de massas (LC-MS/MS). Os resultados reportaram remoções de 83% para o biofiltro contendo a CCA e 86%, com o CA, indicando o potencial da bactéria *Bacillus thuringiensis* em biodegradar E2. Este estudo demonstrou que o uso da biofiltração pode ser um método utilizado para remoção de E2.

Palavras-chave: Biodegradação, hormônios, tratamento de água, adsorventes, *Bacillus thuringiensis*.

Introduction

The number of emerging contaminants and endocrine disruptors released into the aquatic environment is growing continuously and has been raising worldwide concerns, mainly due to the potential risks to human health and the environment. These compounds comprise natural and synthetic hormones, pharmaceutical and personal care products, pesticides, illicit drugs, and other industrial chemicals (Campos, Queiroz, & Roston, 2019; Riva *et al*., 2018). Among these substances, estrogens have received attention, being active compounds with concentrations expressed as $ng.L^{-1}$ and $\mu g.L^{-1}$. Although there are three forms of natural estrogen (estrone, 17β-estradiol, and estriol), 17β-estradiol or E2 has the highest biologically active estrogenic activity (Nazari & Suja, 2016).

This type of estrogen is excreted through feces and urine (human and animal), is persistent in the environment, and can accumulate in soil, sediment, sludge, and along the food chain. It is constantly detected in surface waters, sewage and water treatment plants, groundwater, and even drinking water, representing a serious risk to humans and animals (Vilela, Bassin, & Peixoto, 2018; Ying, Kookana, & Ru, 2002). Some studies have verified the presence of E2 in different environmental matrices in Brazil (Campanha *et al*., 2015; Montagner *et al*., 2019).

This compound is not removed by conventional drinking water and sewage treatments, therefore, it is necessary to adopt new technologies to remove emerging contaminants (Nazari & Suja, 2016).

In this scenario, an alternative technology is the use of biologically activated filters (biofilters). The biofilter works as a bioreactor in which two processes combine: adsorption and biodegradation (Borges, Minillo, Lemos, do Prado, & Tangerino, 2016). Together, these two mechanisms remove and degrade target compounds, thus increasing the potential of this technology for application as a tertiary water treatment (Sbardella, Comas, Fenu, Rodriguez-Roda, & Weemaes, 2018).

Biofiltration is becoming an alternative technology for water treatment because it can use existing granular filter media and produce high quality water without additional chemicals. Many researchers use activated carbon (AC) as an adsorbent. However, industrial production of AC generates a high negative environmental impact. Therefore, studies on alternative adsorbents have been growing in recent years. Rice husk ash (RHA) is a residue originating from the burning of rice husk, showing high resistance, insolubility in water, and a granular and porous structure, presenting itself as an adsorbent material (Ngah & Hanafiah, 2008).

In view of these aspects, the present study evaluated the potential of *Bacillus thuringiensis* biofilters and adsorbent substrates for removal of the natural estrogen 17β-estradiol (E2) in aqueous solutions.

Material and Methods

This study was conducted in three distinct stages, namely: 1. Test of 17β-estradiol biodegradation by the bacterium *Bacillus thuringiensis* from a synthetic solution; 2. Making of two filters containing: i) rice husk ash, and ii) activated carbon. Both filters received the inoculum of the bacterium *Bacillus thuringiensis*, being called biofilters; 3. Biofiltration of a synthetic solution of 17β-estradiol in both biofilters.

Inoculation with bacteria

A strain containing the bacterium *Bacillus thuringiensis* was provided by the Liberato Salzano Vieira da Cunha Technical School Foundation (LIBERATO), Novo Hamburgo city, Rio Grande do Sul State, Brazil, where it was kept in glycerol medium in an ultrafreezer at -80 °C. We used Tryptic Soy Broth (TSB; Kasvi®) to reactivate the strain, following the manufacturer's instructions (30 g/L deionized water). The broth was homogenized until completely dissolved, and autoclaved at 121 ºC for 15 min. The strain was then inoculated into an Erlenmeyer flask containing 100 mL of TSB and incubated in an oven at 23 ºC for 24 h.

Biodegradation of 17β-estradiol

Initially, from the analytical standard of E2 (purity level > 98%, Sigma Aldrich Merck), fortified solutions were prepared at 100 μg.L^{-1} E2 in ultrapure water obtained by the ultrapure water system.

According to Spohr, Cirio, Pizzolato and Ruschel (2014), the bacterium *Bacillus thuringiensis* can consume hormones. Thus, we used this bacterium in biodegradation and biofiltration tests.

E2 biodegradation experiments were divided into three groups with different incubation times (24, 48, and 72h). For each group, six 250 mL Erlenmeyer flasks containing 180 mL of fortified E2 solution and 20 mL of 0.1% peptone water were used. In a laminar flow hood, the turbidity of the bacteria was adjusted to 0.5 McFarland (1.5 x 108 CFU/mL) in sterile saline (0.85% NaCl), and 200 μL of the bacterial suspension was added to each Erlenmeyer flask. All experiments were sterilized (120 °C for 15 min).

Erlenmeyer flasks were kept at 23 ºC, in a dark environment, during their respective incubation time. The solutions were centrifuged (Eppendorf centrifuge 5430 R) at 7800 rpm for 20 minutes and the supernatant was collected.

Quantification was performed by SPE (solid phase extraction) and LC-MS/MS (liquid chromatography coupled to mass spectrometry). Removal percentage (%Rem) was calculated by Equation 1, where: Ce is the final concentration of the compound (μg.L^{-1}) and Co is the initial concentration of the solution (μg.L^{-1}).

$$\%Rem = \frac{\Sigma(\mathrm{Co} - \mathrm{Ce})}{\Sigma \mathrm{Co}} * 100$$

Biologically activated filters

Two filters were made, according to the scheme illustrated in Figure 1. The filters were composed of 15 cm glass columns, with an internal diameter of 2.5 cm, and filled with a single layer of RHA and AC up to a height of 10 cm, totaling a filling volume of 24.5 cm³ for both filters. Considering that the apparent specific mass values of the materials are different, 5.07 g RHA and 10.4 g AC were used.

The substrates used in the filters were chosen based on a previous characterization (Table 1). The RHA used comes from the boiler combustion process of a company in Rio Grande do Sul that uses rice husk as a fuel source. The AC comes from the company Brascarbo Agroindustrial Ltda. and is derived from coconut shell.

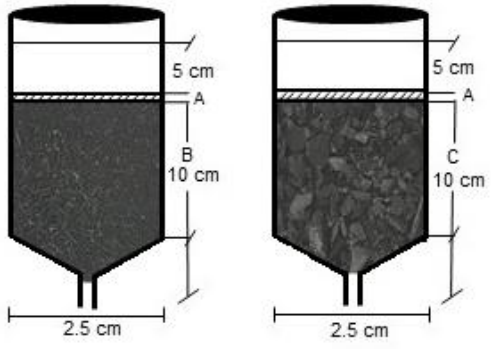

Figure 1. Schematic representation of filters composed of rice husk ash (RHA) and activated carbon (AC). Legend: A - biofilm layer; B - RHA layer; C - AC layer.

Table 1. Physical characteristics of the adsorbents used in the filters: rice husk ash (RHA) and activated carbon (AC).

Parameter	Rice Husk Ash	Activated Carbon
Particle size distribution (mm)	0.05 and 3.35	0.05 and 3.35*
Specific surface area (m^2/g)	43.006	573.462
Volume of micropores (cm^3/g)	12.378	185.014
Volume of mesopores (cm^3/g)	9.025	34.083
Actual Specific Mass (g/cm^3)	2.16	2.08

Source: Adapted from Kieling (2016). * with 50% of particles between 0.22 and 0.56 mm.

For biological activation of the filters, 50 mL of peptone water and 200 μL of the bacterial suspension adjusted to the

0.5 McFarland scale were added to both filters. After this biological activation process, the filters were called biofilters in this study. The biofilters and biofilm growth were monitored for 20 days.

17β-estradiol removal by biofiltration

After 20 days, both the biofilter containing RHA and the one containing AC as a substrate were exposed to fortified E2 solution under continuous flow. Then, 1 L of the fortified solution was biofiltrated at a flow rate of 10 $mL.min^{-1}$. Quantification was performed by SPE and LC-MS/MS.

Sample preparation and chromatographic analysis

Extractions were performed using a Tecnal® vacuum pump and a Supelco® manifold system with capacity for 12 simultaneous extractions. Silica extraction cartridges type C8 (130 mg, 3 mL, Agilent) were used, which were conditioned with 7 mL acetonitrile, 5 mL methanol, followed by 5 mL of ultrapure water. Then, 1 L of sample was percolated in the cartridge, under vacuum. In the clean-up step, 5 mL of ultrapure water was added and the cartridges were dried for 5 min under vacuum. The analyte was then eluted in 10 mL of acetonitrile and evaporated to dryness in a dry bath, being reconstituted in 1 mL of methanol.

Moreover, E2 was determined using an Agilent 1260 liquid chromatograph coupled to a single quadrupole Agilent 6120 mass spectrometer. Analyses were performed with a Zorbax XDB-C18 column (2.1 x 150 mm x 5 μm). The mobile phase consisted of a 5 mM ammonium formate solution (Vetec®, Rio de Janeiro, Brazil) and 0.1% glacial acetic acid (Synth®, Brazil), both with analytical reagent grade and HPLC grade acetonitrile.

Mass spectrometry was performed in positive polarity mode for E2. The samples were read in triplicate with a running time of 25 minutes. The analytical curve was plotted by an analytical standard addition from a stock solution.

Results and Discussion

The biodegradation of 17β-estradiol by the bacterium *Bacillus thuringiensis* after 24, 48 and 72h were (mean ± standard deviation), respectively: 20.31% ± 0.65, 25.71% ± 2.13, 62.05% ± 2.44. The removal differed for the different bacterium-hormone contact times used in this study.

The 20.31% removal after 24 hours of incubation, indicating that the bacteria are consuming E2 as a source of carbon and energy, enabling its degradation. After 72 hours, removal was 62%. This fact may be related both to the half-life of E2 and its consumption by bacteria. According to the literature, the half-life of E2 is about 2 to 3 days (Adeel, Song, Wang, Francis, & Yang, 2017; Ghiselli & Jardim, 2007). Thus, the removal percentages observed at incubation times 48 h and 72 h may be associated with the natural disintegration of the compound.

Spohr *et al*. (2014) evaluated the biodegradation of E2 using *Bacillus thuringiensis* for 8, 16, and 72 hours. For these incubation times, removals of 3.2%, 56.07%, and 64.3% were achieved, respectively. According to the authors, the bacterium *Bacillus thuringiensis* needs more than 8 hours in contact with E2 for high removals.

Authors such as Borges *et al*. (2016) and Ferreira, Rosales, Danko, Sanromán, & Pazos (2016) analyzed the ability of the bacterium *Bacillus thuringiensis* to biodegrade other compounds. However, none of these studies assessed the half-life of the pollutant, which can influence degradation efficiency.

Borges *et al*. (2016) carried out a biodegradation experiment through a batch system using biofilm from biological carbon filters. Biodegradation values were close to 90% for diclofenac and naproxen, and 99% for ibuprofen. The authors found dominance of bacteria of the genus *Bacillus* and report that this genus is composed of microorganisms considered ubiquitous, which can be isolated from soil, water (fresh and salt), and food. In addition, the authors found biodegradation values higher than those found here. We emphasize the need to evaluate the time of contact with the bacteria or biofilm present, as well as the half-life of the compound under study, to know the portion actually biodegraded by the microorganisms.

The same fact was observed in the study by Ferreira, Rosales, Danko, Sanromán, & Pazos (2016), where the authors isolated bacteria present in marine sediment and identified them as *Bacillus thuringiensis* based on their morphological characterization and phylogenetic characteristics. The authors evidenced the ability of this bacterium to biodegrade Phenanthrene (PHE), a polycyclic aromatic hydrocarbon, and Imidacloprid (IMI), a pesticide, showing an almost complete removal for PHE (97.3%) after 10 days, and 78% removal for IMI after 11 days.

Czajka & Londry (2006) highlight other parameters to be considered to understand the biodegradation and destination of estrogens. These include assessing the potential for transformation or mineralization of estrogens under aerobic and anaerobic conditions, and assessing the effects of environmental conditions on biodegradation rates. In the present study, E2 biodegradation was verified without considering environmental effects, only the half-life of the compound.

Monitoring of the biofilm composed by bacteria in the biofilters began after knowing the isolated behavior of the bacterium *Bacillus thuringiensis* and E2. There was rapid development (17 days), indicating that the characteristics of the substrates and the contact time were satisfactory for biofilm formation. Borges *et al*. (2016) emphasize that cell-substrate contact time is a limiting factor for biofilm formation, as well as the influence of the adhesion surface, material porosity, and cell morphology.

According to Westphalen, Corção, & Benetti (2016), the biofilm formed in biofilters must be thick so that biodegradation can occur and biofilter clogging can be avoided. Fu, Shen, Zhang, Ge, & Chen (2019) verified the presence of biofilm in AC beds, and report that the removal of target compounds may be due to bioadsorption and biodegradation of biofilm.

Rice husk ash (RHA) has a much smaller specific surface area (43.006 m^2/g, Table 1) when compared to activated carbon (AC) (573.462 m^2/g, Table 1). Even with this

difference, the biofilters had not been clogged by the adsorbents nor by the biofilm.

Activated carbon (AC) is a porous carbonaceous material with highly developed internal surface area and porosity. It is noteworthy that RHA also has a granular and porous structure (Kieling, 2016; Ngah & Hanafiah, 2008), indicating that both materials have good morphological characteristics for the immobilization of bacteria and growth of biofilm. Regarding biofiltration in biofilters, Table 2 shows the final E2 concentration and the removal percentage for both adsorbents used in the biofilters.

Table 2. Final E2 concentraion (μg. L^{-1}) and removal (%) with an initial concentration of 100 μg L^{-1} after biofiltration with rice husk ash (RHA) e activated carbon (AC) as adsorbent.

Adsorbent	Final concentration	Removal
RHA	17.26 ± 2.59	83.74% ± 2.59
AC	14.28 ± 10.58	86.72% ± 10.58

The AC biofilter showed a higher standard deviation when compared to the RHA biofilter. This fact may be associated with the biofiltration process, where the solution introduced in the biofilter may have followed preferential paths, causing differences between triplicate readings. In addition, the system contains microorganisms, which can generate greater discrepancies between analyses.

Westphalen *et al*. (2016) clarifies that the biofiltration process involves two removal mechanisms: adsorption on the active sites of the adsorbent and biodegradation. The authors also mention that organisms grown in biofilters can degrade organic compounds such as some emerging contaminants. This fact was verified in this study, where removal occurred through adsorption and biodegradation by microorganisms present in a biofilm.

Studies such as those of Spohr *et al*. (2014) and Ferreira *et al*. (2016) indicate the potential of the bacterium *Bacillus thuringiensis* to biodegrade organic compounds in isolation, that is, without being associated with a biofilm as in the present study. Biofiltration research was conducted using activated carbon as a substrate.

Borges *et al*. (2016) used biofilters to remove emerging contaminants such as pharmaceuticals, reaching values greater than 80% through activated carbon biofiltration. For biological filtration, the filters used in the study by Borges *et al*. (2016) received biofilm inoculum from the filter bed of a polishing column containing activated carbon from the Pilot-Scale Multistep Filtration Installation of the Civil Engineering Department (DEC) of FEIS-UNESP.

Bundy, Doucette, McNeill, & Ericson (2007) performed activated carbon filtration with an initial E2 concentration of 1 μg.L^{-1}, reaching removals of 94% for caffeine, 95% for trovafloxacin, and 93-95% for estradiol, with a filtration time of 22 minutes. The present study did not analyze the composition of the formed biofilm.

Regarding the reduction of active pharmaceutical compounds in effluents from sewage treatment plants, Sbardella *et al*. (2018) reached removals of 78, 89, 83, and 79% for antibiotics, betablockers, psychiatric drugs, and a mixture of other therapeutic groups, respectively. The authors used activated carbon based on coconut shell and report that biofilters can be used as an independent tertiary treatment.

Li *et al*. (2019) built a laboratory-scale treatment system consisting of a wetland-stabilization tank, an activated carbon filter, and an outlet tank, which were connected in series. Using this system, the authors reported good removals, above 90% for caffeine, DEET (insect repellent), paracetamol, and triclosan, with adsorption and biodegradation being considered the main removal mechanisms.

These studies show the potential of using biofilters to remove emerging contaminants. This removal aims at total degradation of the compound, eliminating it without generating waste. This happens when using only the adsorption process, where the pollutant is transferred from the liquid phase (water) to the solid phase (adsorbent), thus contaminating the adsorbent material.

Aquino, Brandt, & Chernicharo (2013) report that natural hormones can be removed with efficiencies of >80% due to their great tendency to sorption (high logKow values), being classified as hydrophobic organic compounds.

Therefore, hormones tend to be adsorbed onto substrates. When E2 is present in the pores of RHA and AC, the microorganisms present there have the function of degrading it, thus increasing the useful life of these biofilters. This fact was also reported by Fu *et al*. (2019), who mentions that biological activation processes lead mainly to the adsorption of compounds with low solubility in water, such as E2.

The complete biodegradation of hormones depends on phenolic ring rupture, which may lead to the formation of metabolites (Simpson, 2008). Moreover, depending on the oxidizing or reducing conditions of the medium, one hormone can convert to another. In this study, the formation of metabolites was not verified, but Machado (2010) mentions that this phenomenon may have occurred in his study, as there was an increase in estrone concentration with decreased E2 concentration.

In the present study, high removals were achieved from a fortified solution. In addition, this study presents the possibility of isolating a specific microorganism to biodegrade hormones, which has also been proposed by Borges *et al*. (2016).

Conclusion

From the conditions employed in the present study, it was possible to analyze the biological activation potential of RHA and AC, using the bacterium *Bacillus thuringiensis* as a 17β-estradiol biodegradable agent. We demonstrated that biofiltration can be a method used in the removal of hormones and other contaminants.

Furthermore, using RHA as an adsorbent stands as a recycling alternative for this residue in Rio Grande do Sul. This application aims to reduce the use of activated carbon, which is a material with high production costs and a negative environmental impact regarding its manufacturing and activation.

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