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Exploring the Potential of Bacterial Cellulose and Biosurfactant as Eco-Friendly Biosorbent for Pharmaceutical Contaminants

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Abstract: The contamination of aquatic environments by pharmaceutical products consistently takes the attention of researchers due to the compounds' toxicity even at low concentrations. In response, we have developed an ecologically sustainable biosurfactant derived from a microorganism and incorporated into the bacterial cellulose. This bioproduct, along with the bacterial cellulose itself, was employed as a sorbent for pharmaceuticals (hormones and paracetamol) present in water. Bacterial cellulose membranes were generated through the cultivation of *Gluconacetobacter xylinus* ATCC 53582. The biosurfactant was produced by pre-inoculating *Bacillus subtilis* in a synthetic medium, followed by immersing the bacterial cellulose membranes in the biosurfactant solution, for incorporation. Tests were conducted using water experimentally contaminated with paracetamol and 17α-ethinylestradiol (EE2), evaluating the biosurfactant's effect on bacterial cellulose sorption. Paracetamol levels were analyzed using spectrophotometry, and EE2 levels were assessed using high-performance liquid chromatography. In summary, bacterial cellulose exhibited superior adsorption for EE2 compared to paracetamol. The incorporation of biosurfactant onto bacterial cellulose reduced hormone adsorption but enhanced paracetamol sorption. Our findings indicated that adsorption is more effective with bacterial cellulose in its original and freeze-dried forms, without the incorporation of biosurfactant. Notably, we achieved more promising results in remediating the hormone EE2 compared to the paracetamol.

Keywords: biosorption; bacterial cellulose; biosurfactant; hormones; micropollutants; drug

1. Introduction

Human activities and rapid industrial advancement have increased pollution in water resources, and monitoring of micropollutants in the environment has gained substantial focus due to compelling evidence of aquatic toxicity, endocrine disruption, induction of antimicrobial resistance, and other adverse effects [1,2]. This category of pollutants covers a variety of pharmaceutical products from different classes, including analgesics, antibiotics, anti-inflammatories, and contraceptives. Additionally, it includes natural and synthetic hormones excreted by humans and other animals, such as estrone, estradiol, estriol, and ethinyl estradiol [3,4].

Currently, there is a lack of consensus or adequate evidence regarding the safety limits necessary to prevent the adverse effects of these substances on the environment or human health. Consequently, pharmaceutical products, especially those with endocrine-disrupting characteristics such as the hormones in contraceptives, have become a focal point of investigation [5]. Notably, 17α-estradiol (EE2) is a prominent estrogenic steroid capable of causing harmful effects (endocrine disruption) even at extremely low concentrations [6].

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Another pharmaceutical compound that deserves to be highlighted is paracetamol, also known as acetaminophen, a potent analgesic and antipyretic most used both over the counter and prescribed for pain and fever [7,8], Capable of inducing gastrointestinal, renal, vascular side effects and liver damage [9,10]. However, toxicity is typically not acute, making it difficult to establish a clear link between the toxic agent and its long-term effects [11].

Sorption is a phenomenon in which a substance dissolved in a fluid infiltrates the solid part of a porous medium and this process can occur through absorption or adsorption [12]. Representing a highly effective method for recovering toxic compounds from aqueous solutions, various biomaterials, such as bacteria, yeast, fungi, and algae, have demonstrated success as biosorbents. Biosorption is an ecologically friendly approach used to remove metals [13,14]

Studies have demonstrated that the immobilization of microorganisms has been used as biocatalysts in situations of environmental pollution [15–17]. However, research on the immobilization of bioproducts produced by microorganisms for the same purpose is limited. In recent years, research on adsorption using surfactant mixtures has received considerable attention. This is due to the fascinating interactions within these mixtures, resulting in remarkable interfacial effects, characterized by modifications in adsorption and surface charge density [18]. Thus, a biosurfactant produced by a microorganism will be immobilized in bacterial cellulose, and may be an effective, economically and environmentally favorable option as drug adsorbents.

2. Results

2.1. Biomaterials characterization

The comparison between biosurfactant (BS) produced and standard surfactin is presented in Figure 1, that represents the characterization run by Fourier Transform Infrared Spectroscopy - FTIR, using transmittance modes. The bands were associated with the main chemical groups, as compared to the standard. The bands that appeared in the spectrum, which pertains to the resuspension of the lyophilized powder in methanol, remain similar to those found in the literature and to the surfactin standard that was analyzed.

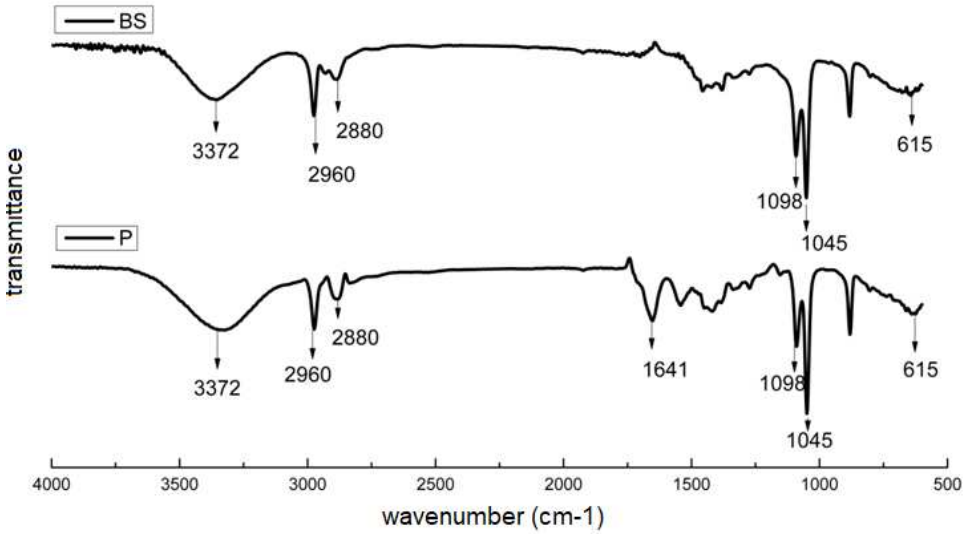

Figure 1. Fourier Transform Infrared Spectroscopy (FTIR) transmittance spectra of both the biosurfactant produced (BS), in freeze-dried powder form, and the Surfactin pattern (P), used as a standard.

The incorporation of the biosurfactant into bacterial cellulose was also analyzed using FTIR. As observed in Figure 2, the peaks characterizing the biosurfactant (BS) incorporated into the cellulose

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membrane are present, even after the membrane was subjected to washing. The analysis of the solubilized BS also showed these same bands.

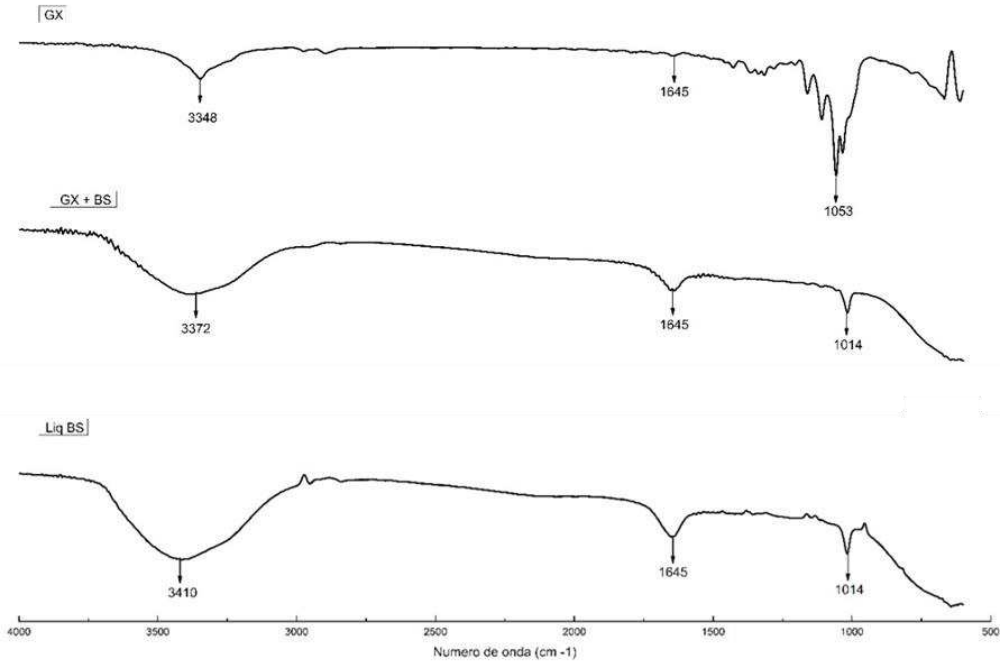

Figure 2. FTIR Transmittance Spectra of biosurfactant (BS) incorporation: 'GX' for bacterial cellulose from Gluconacetobacter xylinus, 'GX+ BS' for bacterial cellulose with incorporated BS, for washed bacterial cellulose with incorporated BS, and 'Liq BS' for BS in solution.

2.2. Adsorption kinetics

Figure 3 presents the analysis of paracetamol adsorption in three different samples. Figure 3A depicts the rate of paracetamol adsorption on bacterial cellulose, revealing limited efficiency of the technique. Paracetamol remains in high concentrations in the medium, reaching its peak adsorption between 45 and 60 minutes, with an adsorption of 7.4% and 4.1%.

Figure 3B illustrates the kinetics of paracetamol adsorption on bacterial cellulose with the addition of BS, indicating intermediate performance of the technique. Paracetamol maintains significant concentrations in the medium, with peak adsorption occurring between 60 and 120 minutes, resulting in 19.1% and 16.5% adsorption.

For lyophilized and pulverized bacterial cellulose, a shorter contact time was chosen, as it had been observed that desorption occurred after 100 minutes. Figure 3C demonstrates the kinetics of paracetamol adsorption in this sample, showing good performance of the technique. Paracetamol still remains in high concentrations in the medium, reaching its peak adsorption between 30 and 45 minutes, with 31.8% and 13.11% of adsorption.

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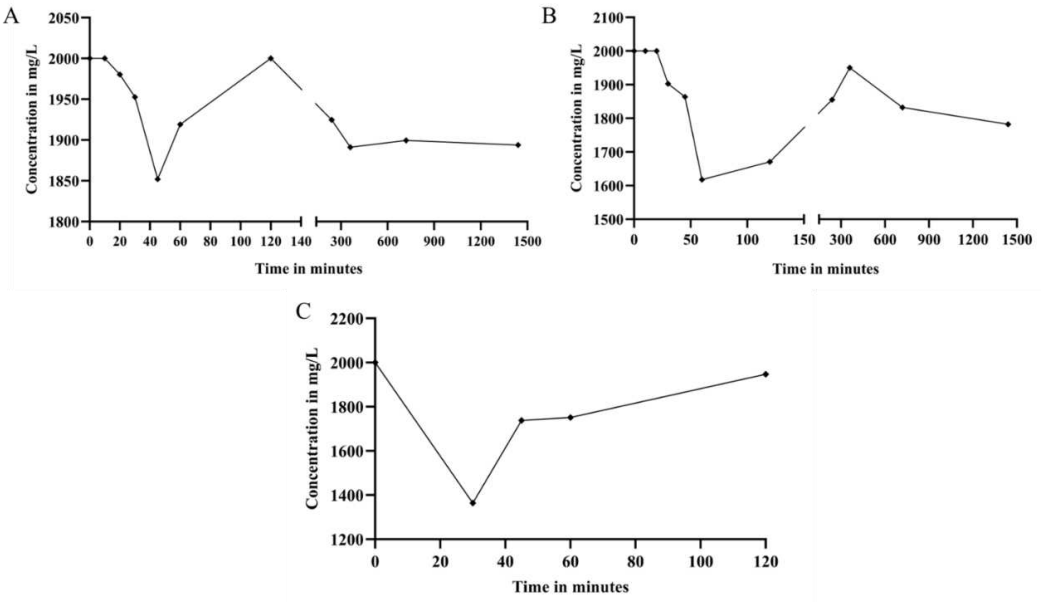

Figure 3. Paracetamol adsorption kinetics. In A) paracetamol adsorption on bacterial cellulose; In B) adsorption of paracetamol on bacterial cellulose + BS; In C) adsorption of paracetamol on crushed and lyophilized bacterial cellulose without BS.

Figure 4 displays the results of the analysis of EE2 adsorption on different bacterial cellulose samples. The evaluation was carried out using high performance liquid chromatography (HPLC). The kinetic analysis of EE2 adsorption on bacterial cellulose, depicted in Figure 4A, highlights the remarkable effectiveness of this approach. The highest adsorption rate of EE2 occurs in the range of 10 to 60 minutes, with both points reaching an adsorption rate of 74%.

The adsorption kinetics of EE2 on bacterial cellulose in combination with the BS adsorbent, as illustrated in Figure 4B, shows a positive performance of this technique. The peak adsorption rate of EE2 is observed between 10 and 20 minutes, with adsorption levels reaching 38% and 42%, respectively. The analysis of the adsorption kinetics of EE2 on whole lyophilized bacterial cellulose, presented in Figure 4C, reveals an exceptional performance of this technique. The peak adsorption of EE2 occurs between 20 and 60 minutes, resulting in adsorption rates of 88% and 82%, respectively.

Additionally, the analysis of the adsorption kinetics of EE2 on lyophilized and pulverized bacterial cellulose, shown in Figure 4D, points to a satisfactory performance of this technique. The peak adsorption of EE2 is recorded between 20 and 60 minutes, with adsorption rates reaching 52.5% and 39%, respectively.

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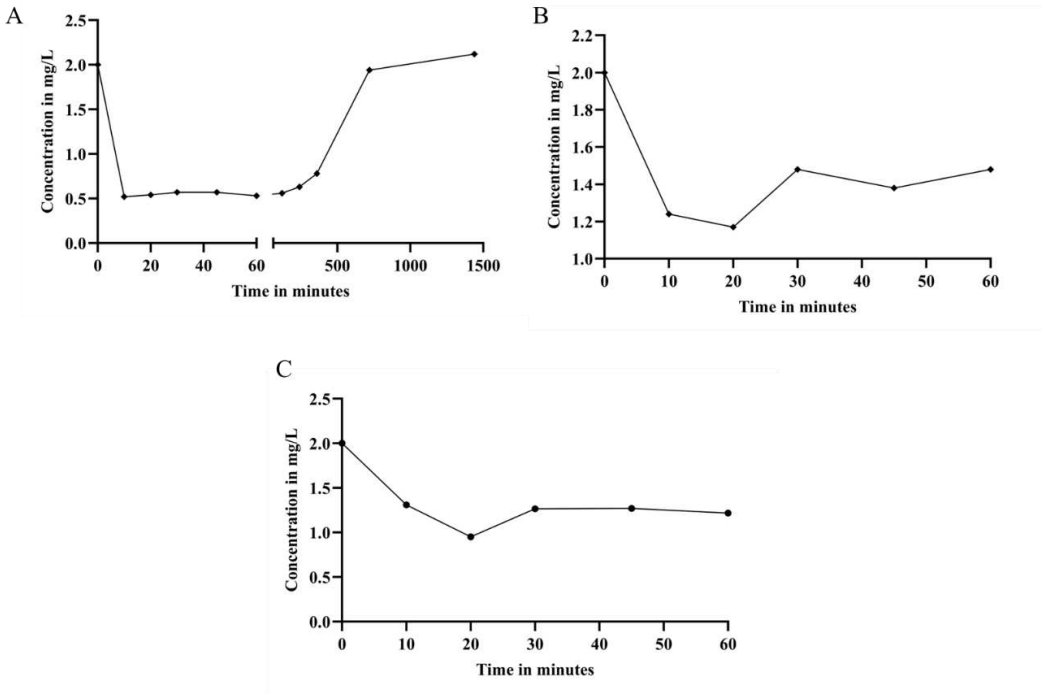

Figure 4. Adsorption kinetics of 17α-ethinylestradiol. In A) adsorption of EE2 on bacterial cellulose; In B) adsorption of EE2 on bacterial cellulose + BS; In C) EE2 adsorption on crushed and lyophilized bacterial cellulose without BS.

3. Discussion

Biosurfactants, a category of natural substances manufactured by microorganisms, have garnered growing recognition owing to their significant role in a variety of scientific disciplines and industrial sectors [19]. These surface-active agents of biological origin play a fundamental role in the solubilization of water-insoluble substances, in reducing the surface tension of liquids and in stabilizing emulsions [20].

Moreover, their impressive biodegradability and reduced ecological footprint in contrast to conventional chemical surfactants position biosurfactants as a hopeful substitute in a spectrum of applications spanning from remediating pollutants to sectors such as petroleum, agriculture, food, cosmetics, and pharmaceuticals [21].

In our research, mirroring the approach of Vedaraman and Venkatesh (2011) [22], we encountered certain challenges during the laboratory-scale biosurfactant production process, which encompassed pre-cultivation and cultivation in Erlenmeyer flasks. This procedure entailed three sequential centrifugation steps: the first aimed at cell removal, the second, akin to the method described by Das et al. (2008) [23], involved acid extraction to obtain a pre-purified biosurfactant. Lastly, the process featured a liquid-liquid extraction via centrifugation, followed by the maintenance of the precipitate until the complete evaporation of any solvents, culminating in freeze-drying.

As indicated in Figure 1, the peak bands between 3340 and 3365 cm^{-1} can be attributed to possible axial deformations of OH [24]. Furthermore, other bands observed in both the produced BS and the surfactin standard include the bands at 2973 cm^{-1} and 1045 cm^{-1}, related to methyl CH stretching [25,26], the band at 2883 cm^{-1} referring to CH2 [27], and the band at 1092 cm^{-1} referring to OC in the molecule [28].

The cellulose used in this study was obtained from the bacterium *Gluconacetobacter xylinus* (ATCC 53582), following the same approach as Jozala and colleagues [29]. Bacterial cellulose is recognized as a highly effective polymer due to its inertness, high crystallinity, hydrophilicity, permeability and remarkable mechanical resistance, as mentioned by Popa (2022) [30]. FTIR analysis, in line with Pinto's (2013) [31] observations, reveals peaks characteristic of bacterial cellulose. This

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can be clearly observed in Figure 2, where the band at 3300 cm^{-1} represents the OH group, the band at 1600 cm-1 is attributed to the CH2 deformation and the band at 1000 cm^{-1} is related to CO/CC.

After integration of the BS bioproduct, the kinetic tests usually applied in the biosorption of metals [13; 14] were carried out. In our study, focused on the drugs acetaminophen (paracetamol) and the hormone 17α-ethinylestradiol, the adsorption rates by the bioproduct in relation to these drugs were analyzed, considering the interaction of the mixtures and the interfacial effects generated. This includes the evaluation of parameters such as surface charge density and porosity, as highlighted by Ayoub (2021) [32].

Singh et al. (2007) [33] observed that both chemical and biological surfactants can have variable effects on the speed of pollutant bioremediation, without the ability to accurately predict the results, emphasizing the need for empirical confirmation. Pacwa-Płociniczak et al. (2011) [34] discussed the ability of biosurfactants to form micelles that can offer protection to contaminants, potentially inhibiting degradation. Furthermore, Guo et al. (2019) [35] demonstrated that the concentration of rhamnolipids, a type of biosurfactant, can influence the mobility and dissociation of the contaminant. This finding allows us to draw parallels with the rapid desorption observed in analyses involving bacterial cellulose containing biosurfactant.

According to Żółtowska-Aksamitowska et al. (2018) [36], the sorption capacity of chitin/lignin increases with the amount of solvent (paracetamol), being proportional to this amount. On the other hand, studies with bacterial cellulose showed different results, showing minimal adsorption over short periods of time. Ferandin Honorio et al. (2018) [37], when investigating the adsorption of 17β-estradiol on rice husk, they observed that the ideal period for adsorption was 120 minutes. For 17α-ethinylestradiol, the best adsorption time using bacterial cellulose was 20 minutes. Additionally, Ferandin Honorio et al. (2018) [37], analyzed the adsorption process of the hormone 17β-estradiol by rice husk and soybean husk directly in pig manure. These animals receive hormonal supplementation.

Silva et al. (2018) [38] highlighted that cellulose modified with phthalic anhydride (used as an adsorption matrix) favors hydrogen bonds and electrostatic interactions with dyes, enabling the comparison of these interactions with bacterial cellulose in the context of EE2. On the other hand, Debs et al. (2019) [39], who studied biosorption by yeasts in the ethanol industry, also mentioned the hypothesis that sorption may increase due to electrostatic effects.

4. Materials and Methods

4.1. Bacterial Cellulose Production

From the strain *Gluconacetobacter xylinus* ATCC 53582, bacterial cellulose was cultivated in synthetic Hestrin & Schramm medium (20 g/L glucose, 5 g/L bacteriological peptone, 5 g/L yeast extract, 2.7 g/L Anhydrous sodium phosphate; 1.5 g/L citric acid monohydrate). Cultivation was carried out in cell culture plates with 24 wells, containing 1 mL of inoculum per well. The plates were maintained for 7 days in static culture at 30 ºC. After growth, the membranes were washed in running water and immersed in a 1M NaOH solution, under agitation at 60 ºC for 1h 30 min. Subsequently, the membranes were washed until reaching neutral pH, thus being autoclaved at 121 ºC for 15 minutes in MilliQ water, and stored at 4 ºC, a technique adapted from Jozala et al. (2015) [29].

After Following this procedure, a portion of the cellulose was crushed and stored in a biofreezer set at -80ºC (REVCO® ULT-1386-3-D) for approximately 24 hours. Subsequently, the frozen samples underwent freeze-drying using a Thermo Savant Freeze Dryer, LK-40, for about 48 hours.

4.2. Biosurfactant Production

To produce biosurfactant (BS), 1 mL of the microorganism pre-inoculum was used *Bacillus subtilis* Erlenmeyer flasks (125 mL), containing Tryptone Soy Broth (TSB) cultivation medium. To obtain this pre-culture, it was kept shaking at 150 rpm/ 35 ºC/ 24 hours. After finishing the pre-cultivation time, 5 mL of the volume was inoculated into new Erlenmeyer containing 45 mL of TSB, kept under agitation at 150 rpm/ 35 ºC/ 96 hours. The culture media from all Erlenmeyer were

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centrifuged at 4 ºC and 5000 rpm for 30 min to remove the cell suspension. The pH of the culture medium was adjusted to 2.0 to undergo acid extraction, being centrifuged at 5ºC at 8000 rpm for 20 min, keeping only the precipitate. This precipitate passes into a liquid-liquid suspension, containing chloroform and methanol, and is centrifuged at 25ºC and 5500 rpm for 10 min. After separation, the formed pellet was lyophilized.

4.3. Incorporation

The resuspension 0.1 grams of freeze-dried biosurfactant were reconstituted in 10 milliliters of methanol to submerge both the bacterial celluloses and the crushed, freeze-dried bacterial cellulose. The test was conducted in 24-well plates, with each well containing cellulose and 1 mL of the biosurfactant solution. The plates were agitated at 25 ºC at a speed of 100 rpm for a duration of 24 hours.

All bacterial cellulose sample with or without the added biosurfactant and the biosurfactant resuspension were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) analysis, in the wavelength range of 4000 to 500 cm^{-1}.

4.4. Adsorption kinetics

To evaluate the biosorbent capacity, 0.5 g samples of whole bacterial cellulose membranes without surface water with and without BS were added to 60 mL of 2 g/L paracetamol and 17α-ethylestradiol (EE2) solutions. The systems were kept shaking, and samples were taken at point in time of 10, 20, 30, 45, 60, 120, 240, 360, 720 and 1440 minutes. Samples of whole and lyophilized and pulverized cellulose without BS were taken at times of 10, 20, 30, 45, 60 and 120 min. The samples were filtered and then analyzed using either a UV-Visible Spectrophotometer or High Performance Liquid Chromatography.

4.5. Evaluation of Acetaminophen by UV-Visible Spectrophotometer

Acetaminophen was analyzed by spectrophotometry, following a methodology adapted from Shihana et al., 2010.To achieve this, it was necessary to standardize the medication dosage, using a calibration curve (represented by the equation y = 0.0233x - 0.0019, with a coefficient of determination (R^2) of 1). Sodium nitrite (NaNO2 10g%) and hydrochloric acid (HCl 6M) were added to the water samples, leading to nitration. In basic medium, added with sodium hydroxide (NaOH 50%) and ammonium sulfate ((NH4)2SO4 15%), the solution turned yellow (azo dye), with absorption at a wavelength of 430 nm. To treat the samples taken at times established above, it was necessary to carry out deprotenization, using trichloroacetic acid (C2HCL3O2 15%), followed by nitration, adding 10g% NaNO2 and 6M HCl to the samples (forming the compound 2-nitro-5-acetaminophenol). After showing a slight yellow color, 15% (NH4)2SO4 and 50% NaOH were added, the reading was taken on a spectrophotometer, at a wavelength of 430 nm.

4.6. Evaluation of Ethinylestradiol by High Performance Liquid Chromatography

EE2 analysis was performed using High Performance Liquid Chromatography (HPLC). For this, it was necessary standardization for medication dosage, through a calibration curve, with concentrations from 0.025 to 2 mg/L, thus measuring the area of the peaks in the HPLC program. The data were adjusted to a linear equation represented by y = 115131x - 420 and it presented a coefficient of determination (R^2) of 0.9991.

The samples taken at the times mentioned were read on the Liquid Shimadzu chromatograph – Model Class-VP, using a C18 column 125 mm high, 4.60 mm in diameter and filled with 5 µm (Thermo Scientific) at 37 °C in the oven. The results were collected with a 20 µL injection and a run time of 6 minutes with detection at 202 nm. The mobile phase was composed of 70% HPLC standard acetonitrile (sigma-Aldrich) and 30% ultrapure water – Milli-Q, in an isocratic system (Unruh, 2011). The equipment's software gives us the peak area, in a chromatogram, and then the EE2 concentration is calculated from the straight-line equation.

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4.7. Data analysis

Data were expressed as absolute results or percentage of sorption. The adsorption capacity (AC) of bioproducts and the percentage of removal (%R) were determined by the equations:

$$\frac{CA = (Co - Ce)\ x\ V}{mpb}$$

and

$$\%R = \frac{Co - Ce)\ x\ 100}{Co}$$

where Co is the initial concentration, Ce is the concentration found after contact with the bioproduct, V is the total volume of the solution and mbp is the mass of the bioproduct. The results were analyzed with the help of the program Oringin 8 and GraphPad Prima®.

5. Conclusions

Our findings affirm the successful production of biosurfactant and its seamless integration into bacterial cellulose. Adsorption experiments demonstrated that freeze-dried bacterial cellulose exhibited the highest adsorption efficiency for the contaminant EE2, while bacterial cellulose without surface water showed proficiency in paracetamol adsorption. The addition of biosurfactant improved the adsorption of paracetamol but decreased the adsorption of EE2. To conclude, it is plausible that bacterial cellulose, particularly in its freeze-dried state, holds promise as an environmentally friendly biosorbent for the extraction of certain pharmaceuticals from polluted waters.

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