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Removal of p-cresol using wash waters from lipopeptide production

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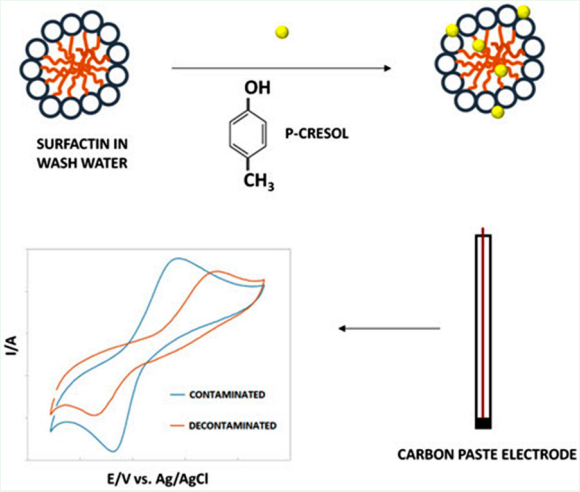
ABSTRACT

This work shows the efficiency of wash waters from lipopeptide production as a remediation strategy to treat urban water samples contaminated with p-cresol. The harvesting step in surfactin production involved a centrifugation step, generating a major soluble fraction and a fraction that is adsorbed to the biomass. The adsorbed fraction was recovered by washing steps. These wash waters containing lipopeptides (mostly surfactins), were successfully used to adsorb and solubilize p-cresol. The method of decontamination applied to an artificially contaminated natural water was monitored using a biosensor based on laccase/magnetic nanoparticles. Given the amount of surfactin within the wash water, the removal of p-cresol from artificially contaminated water was approximately 46.0%. This result confirms the successful and sustainable application of surfactin-rich wash waters to remove p-cresol from artificially contaminated natural water. The adsorption mechanism is potentially based on a multi-layer adsorption process, considering Langmuir and Freundlich adsorption isotherms.



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1. Introduction

Phenolic compounds are a class of aromatic pollutants that are the concern of public health [1]. These toxic organic pollutants are present in pharmaceutical and personal care products. Phenols are widely used as raw materials or as manufacturing by-products, especially in the resin, plastic, dye, paint and textile industries and in oil refineries [2,3]. In contact with skin or when absorbed by mucous membranes, they cause damage to various organs [4,5]. Among phenolic compounds of high toxicity, p-cresol (4-methylphenol) stands out, due to easy permeation through the skin and ability to undergo biotransformation [6,7]. Once detected in drinking water, the removal of p-cresol from the contaminated matrix is of paramount importance. Thus, the development of new methodologies aimed at sustainable remediation processes is of great interest [8–10].

Remediation involving adsorption processes combine the characteristics of sustainability and versatility, as various types of adsorbents can be used [11]. The successful association of low-cost adsorbent materials for

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decontamination of polluted waters may be even more advantageous [12]. For example, thermodynamic adsorption isotherms studies of nicotine, caffeine and amoxicillin were carried out by Anastopoulos et al., where production residues, as by-products of grape production, were used in the removal of those emerging pollutants [13].

The amphiphilic characteristics and the formation in water of supramolecular structures of lipopeptides, make such class of substances very efficient in the removal of organic contaminants in aqueous media [14,15].

The Lipopeptide surfactin finds application in various industry sectors due to its low critical micellar concentration, high ability to reduce surface tension and other properties such as antibiotic and antitumoral activities [16,17]. It can be synthesized by strains of *Bacillus sp.*, including *Bacillus velezensis* [18]. However, the production of lipopeptides is very expensive, so options to lower its cost have been proposed by Zanotto [19]. The first study considering circular bioeconomy to lower the production costs of biosurfactants by valorization of both microbial cells and their primary and secondary metabolites was successfully carried out by Etchegaray et al. [16], with the use of crude extract and biomass as adsorbents to remove p-xylene from contaminated water. In addition, the authors demonstrated the use of residual microbial biomass for the prodution of biodiesel. Similarly, sustainable and profitable production of surfactin was obtained by Carvalho et al. The co-production of biotechnological products with high added value as enzymes, such as arginase, was performed including the use of lipopeptide-rich wash waters to remove phenol for the first time [20]. It was made possible because the wash-waters obtained along with lipopeptide production are additional surfactin-rich sources. Therefore they can be useful in environmental decontamination processes.

In this context, considering these applications for the residues from biotechnological processes, a deeper study involving the remediation process with wash waters obtained from surfactin production is presented. To check for decontamination, water monitoring is required before and after remediation, using a viable, low-cost electrochemical biosensor, which is highly selective, sensitive and easy to handle [21,22].

The aim of this work was the use of residues from the biotechnological production of surfactin for the removal of p-cresol from artificially contaminated natural water. This was made possible by monitoring effluent treatment, using a biosensor based on laccase/magnetic iron oxide nanoparticles. Optimum remediation conditions for pH and mixing time were obtained using the experimental design.

2. Methods

2.1. Reagents

All the analytical grade reagents were purchased from Sigma-Aldrich. Phosphate buffer was prepared by mixing solutions of 0.1 mol L^{-1} Na_2HPO_4 and 0.1 mol L^{-1} NaH_2PO_4 in deionized water (>18 MΩ cm, Milli-Q, Millipore). A natural water sample was collected from a lake in Campinas, state of São Paulo (Brazil). Sampling was carried out in February 2019 (24/02/2019) and the sample was fortified by the addition of p-cresol.

2.2. Biosensor preparation

Iron magnetic nanoparticles (Fe_3O_4) were synthesized according to the literature [23]. A suspension of 50 µL of nanoparticles was used and the pellet was collected using a magnet. The pellet was mixed with a solution containing laccase (3.0 mg L^{-1}), the mixture was stirred manually and refrigerated for 15 min. The supernatant was discarded. The nanoparticles modified with enzyme were washed 5 times with phosphate buffer and mixed sequentially for 5 min with graphite powder (0.175 g) and Nujol®, using pestle and mortar. The resulting carbon paste was transferred to a glass tube, making electrical contact with a Ni/Cu wire. This system was placed, together with the Ag/AgCl reference electrode and a platinum counter electrode, in a 5 mL electrochemical cell.

2.3. Electrochemical measurements

Electrochemical experiments were carried out using cyclic voltammetry in 0.1 mol L^{-1} phosphate buffer pH 8.5 within the range of −400 to 400 mV at 50 mV s^{-1} in a 5 mL electrochemical cell equipped with a Teflon® cap. All experiments were carried out in an AUTOLAB micropotentiostat (Metrohm) PGSTAT 101.

2.4. Wash waters from surfactin production

The strain *Bacillus velezensis* 0G was cultivated in LB growth medium as previously described by Heuson and coworkers [24] with modifications. A pre-culture was inoculated, thus 1 mL of inoculum to 50 mL of LB was distributed in eight 500 mL flasks [16]. These were incubated at 37°C and 250 rpm for 72 h. The broth was collected and centrifuged 9000 g for 10 min. This step yields soluble surfactin in the supernatant and a fraction of surfactin that coprecipitates with the biomass. Quantification was carried out as described by Heuson et al. [24]. To recover surfactin from biomass, a sequence

of 3 resuspensions in 10 mL of phosphate buffer, and centrifugation steps (9000 g for 10 min) was carried out, generating three wash waters. The first wash water was used to treat water samples containing p-cresol as described below.

2.5. Optimizing the conditions for remediation using wash waters

Factorial design with central and axial points was used for the optimization of pH and mixing time. A volume of 5 mL phosphate buffer 0.1 mol L^{-1} was adjusted to the desired pH and contaminated with p-cresol to a final concentration of 76.95 µmol L^{-1}. Each sample was analysed with the biosensor for the presence of phenol, before and after treating with 1.0 mL of the lipopeptide first wash water. Upon treatment and before measurements, the mixture was agitated for different time intervals. The remediation was determined using the biosensor and it was estimated from the difference between the electrical current obtained from non-treated (control) and treated sample. The higher levels of pH and time were respectively 60 min and pH 10. Minimum levels were 5 min and pH 2. The central point corresponded to 30 min and pH 6, respectively. Data was processed using Design Expert® version 11. Finally, the biosensor was tested for real conditions, by monitoring a sample of natural water (5 mL) that was adjusted to the optimized pH and contaminated with p-cresol to a final concentration of 76.95 µmol L^{-1}.

2.6. Surfactin adsorption isotherms

The wash waters were pooled and mixed with the cell-free supernatant making a surfactin-rich mixture. This extract was partially purified by precipitation with HCl using a 1.2 mol L^{-1} to reach pH 2.0. Partially purified surfactin was collected by centrifugation, dried in an oven at 60°C for 24 h and kept in a desiccator until use.

For the adsorption studies, a set of four p-cresol solutions of different concentrations was prepared. These were placed in four separate 1.5 mL tubes containing 15 mg of solid surfactin, prepared as described above. The volume was completed to 1.5 mL with 0.1 mol L^{-1} phosphate buffer, using pH 10, indicated by the design of experiments as the one with the highest adsorption. It was then kept at a constant temperature of 4.0°C in the refrigerator for one week. The p-cresol solutions were analysed using the biosensor. Measurements were performed at room temperature (25°C) before and after mixing with surfactin.

3. Results and discussion

3.1. Analytical parameters of the biosensor

The repeatability of the biosensor for the analysis of p-cresol was not evaluated due to interference given by oxidation products that passivate the electrode. The phenolic compound oxidation products tend to encrust onto the electrode surface, with the consequent loss of signal after the successive voltametric cycles. As it was used a biosensor constructed by carbon paste electrode for measurements, this inconvenience was easily corrected by polishing the electrode surface after use with clean paper. Thus, only the biosensor construction reproducibility study was performed. The obtained Relative Standard Deviation was 4.07% (n=4). This was considered adequate, indicating that the method used in the assembly of the biosensor is reproducible.

A calibration curve for p-cresol was prepared. The signals obtained at different p-cresol concentrations are presented in Figure 1. The calibration curve indicates that the biosensor showed linearity for p-cresol from 22.9 to 131 µmol L^{-1} (n=6). The equation $y\ (\mu A) = 1 \times 10^{-7} x\ (\mu mol\ L^{-1}) + 2 \times 10^{-6}$ ($R^2 = 0.9995$) was obtained. The calculated limit of detection for p-cresol using the biosensor was 2.5 µmol L^{-1} (LOD = 3 SD/slope).

The biosensor's reliability for real samples was verified by testing samples of natural water from a lake. For this, 5.0 mL of the sample was artificially contaminated with 76.95 µmol L^{-1} p-cresol and tested with the biosensor. This study was performed in triplicate and the average concentration found was 78.32 µmol L^{-1} with a relative error of 1.41%, in relation to the expected concentration. It is considered a satisfactory result, indicating the biosensor's response is not significantly affected by other species present in the water.

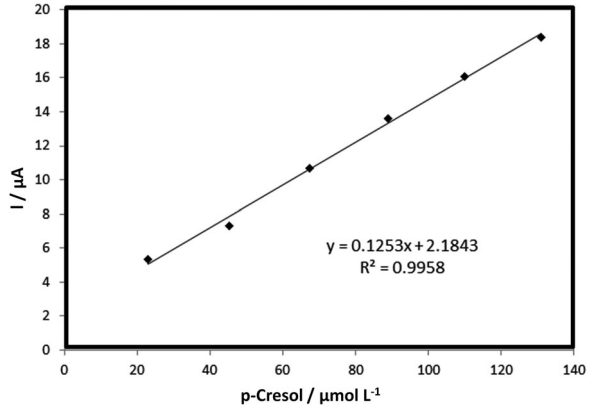

Figure 1. Calibration curve obtained using the biosensor after successive additions of p–cresol in 0.1 mol L^{-1} phosphate buffer pH 8.5 within the range of −400 to 400 mV at 50 mV s^{-1}.

3.2. Multivariate optimization of experimental remediation conditions

Two important parameters for remediation refer to the pH and interaction time between wash waters and p-cresol. To examine these two effects simultaneously, we used a factorial design 2^2 with central and axial points.

Table 1 shows the variation in current (ΔI) for each assay considering the measurements of electric current before and after the addition of wash water.

Response surface curves were obtained using the Design Expert® software based on the data presented in Table 1. The model was studied by analysis of variance (ANOVA). The best model, with significant regression and a lack of non-significant fit, was the quartic polynomial model, as shown in Table 2. The response surface (Figure 2) was obtained from the data presented in Table 2. The current values correspond to the difference (ΔI) between the measurement in the presence of p-cresol before and after its removal by wash water treatment.

Figure 2 shows that the condition that gives the highest value for ΔI correspond to extreme values: higher pH and longer agitation time (red area of the graph). From the chemical point of view, this result suggests that the best interaction of p-cresol with surfactin micelles occurs with longer mixing time. Thus, considering the alkaline environment that contributes to the solubility of surfactin micelles, and, in order to use the shortest possible analysis time, the best conditions selected were: x1 (time) of 1.15 (coded variable) and x2 (pH) of 1.41 (coded variable). Under these conditions, the mixing time of 55 min and pH 10 were chosen. Therefore, these conditions were adopted for the remediation tests whose results are presented below.

Table 1. Influence of the reaction pH and interaction time between wash waters / contaminated water in the p-cresol remediation process. Measurements correspond to differences in the current (ΔI) measured (μA) based on experimental conditions designed by factorial design 2^2 with central and axial points.

Assay	Time (min)	pH	ΔI_1 (μA)	ΔI_2	ΔI_3
1	−1	−1	2.27	1.83	-
2	+1	−1	2.98	3.14	-
3	−1	+1	4.10	4.16	-
4	+1	+1	7.61	8.06	-
5	0	0	1.98	1.97	2.46
6	−1.41	0	2.64	3.07	-
7	0	+1.41	1.67	1.49	-
8	+1.41	0	3.25	3.40	-
9	0	−1.41	6.01	5.89	-

3.3. Wash waters performance in real sample remediation test

From this study, it was possible to verify the wash waters remediation efficiency for p-cresol in a sample originated from environmental matrices. It was performed in triplicate using a natural water sample. Using the calibration curve, the corresponding pollutant concentrations were calculated and the respective remediation percentage was obtained. The results are shown in Table 3.

The average remediation value was 46.83 (±1.55) % and Relative Standard Deviation, 3.31%, considering the capacity of the first wash water used in this study. Other remediation techniques showed the following efficiency: surfactant micelles in Emulsion Liquid Membrane (ELM, Span 80 in NaOH) remediate 98% of phenol in aqueous media [25], advanced oxidation processes with UV light (UV/H_2O_2) could remove 90% of phenolic compounds of olive mill wastewater [4] and a mix of Triton X-100 and laccase enzyme itself, at concentration of 1155 U L^{-1} could remove nearly 90% of aqueous phenol, under optimum conditions [26]. Thus, the wash waters obtained in the surfactin biotechnological production process can be used for decontamination of industrial effluents or even urban waters contaminated with phenolic compounds. It is noteworthy that the wash waters are industrial waste and would be discarded. However, as it still contains adsorbed surfactin, it can be successfully used in processes that require adsorption, such as decontamination. Therefore, it is shown that the proposed remediation is of low cost,

Table 2. Analysis ANOVA for Quartic model, obtained from data of factorial design 2^2 with central and axial points.

Source	Sum of squares	df	Mean square	F-value	*p*-value	
Model	38.05	8	4.76	101.04	0.0002	Significant
A-Time	0.1861	1	0.1861	3.95	0.1177	
B-pH	9.42	1	9.42	200.08	0.0001	
AB	1.96	1	1.96	41.64	0.0030	
A^2	1.09	1	1.09	23.13	0.0086	
B^2	4.47	1	4.47	94.87	0.0086	
A^2B	19.84	1	19.84	421.45	<0.0001	
AB^2	1.41	1	1.41	29.93	0.0054	
A^3	0.0000	0				
B^3	0.0000	0				
A^2B^2	1.44	1	1.44	30.52	0.0052	
A^3B	0.0000	0				
AB^3	0.0000	0				
A^4	0.0000	0				
B^4	0.0000	0				
Pure Error	0.1883	4	0.0471			
Cor Total	38.24	12				

Final equation in terms of coded factors:

Current = +2.07 + 0.2157*A − 1.53*B + 0.7*AB+ 0.4365*A^2 +0.884*B^2 +3.15*A^2B +0.8393*AB^2 + 0.8475*A^2B^2.

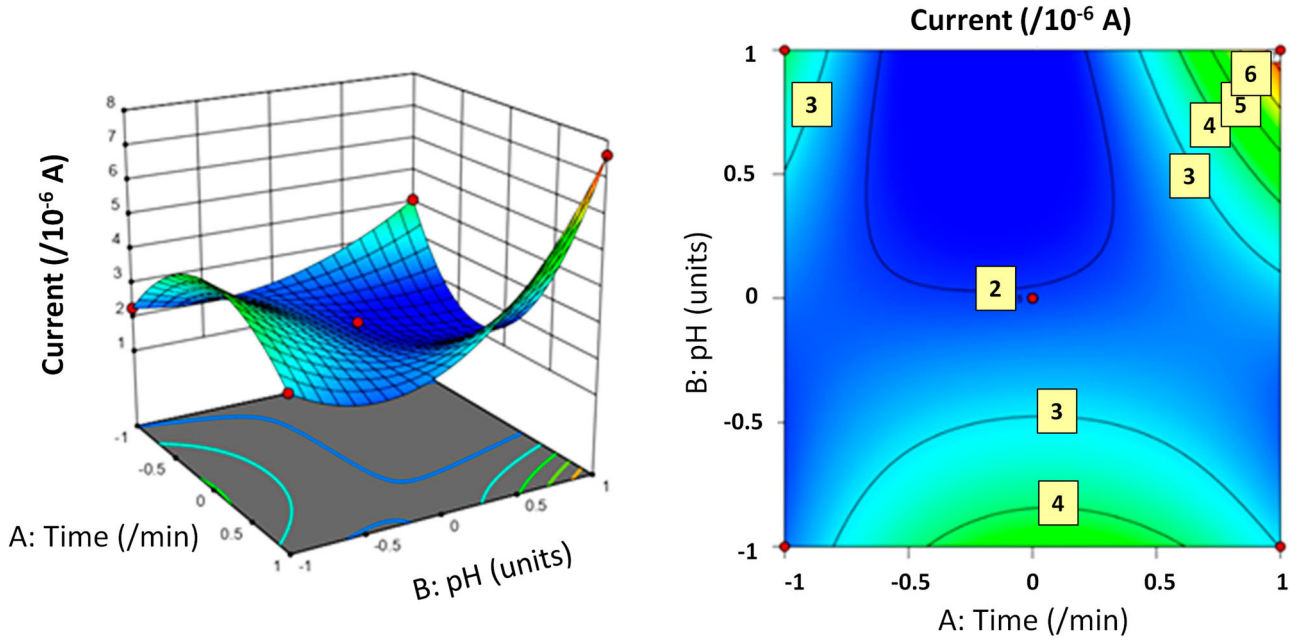

Figure 2. Response surface curves were obtained using Factorial Design 2^2 with central and axial points, corresponding to the experimental design conditions. The values of current in the best conditions of coded variables *Time* and *pH* were found as 1.15 and 1.41, respectively, i.e. interaction time 55 min and pH 10. These conditions were adopted for the remediation in the subsequent studies.

especially when compared to activated charcoal, and it is carried out sustainably.

3.4. Surfactin adsorption isotherms

General analysis for adsorption process requires the equilibrium of the reaction, i.e. when the concentration of solute in the liquid phase (C_e) remains constant and the adsorption capacity of the adsorbent (q_e) is determined. Graphs involving q_e versus C_e are called *Adsorption isotherms* and inform us about the type of adsorption mechanism. They show changes in equilibrium concentration between adsorbent mass with partial pressure or liquid phase concentration at a given temperature [27,28]. A favourable isotherm indicates that the adsorbed mass retained per unit mass of adsorbent is high given a low equilibrium concentration of adsorbate in the liquid phase, while the unfavourable one indicates that the adsorbed mass retained per mass unit of adsorbent is independent of the concentration of adsorbent balance. A linear fit indicates the adsorbed mass retained per unit mass of the adsorbent is proportional to the equilibrium concentration.

Table 4 presents the experimental results obtained, and the corresponding parameters for the Langmuir and Freundlich adsorption isotherms for the p-cresol/surfactin system. The concentration values were obtained from the biosensor analytical curve, as in section 3.1.

Figure 3(a and b) shows the isotherms obtained for Langmuir and Freundlich model, respectively.

According to Figure 3(a), by the line equation presented by the Langmuir isotherm, we have that q_{max} 1.2943 is and K_L is 0.0068. However, in Figure 3(b), the line equation presented by Freundlich isotherm, n is 1.2268 and K_f is 0.2894. Given that the factor R^2 of the Freundlich linear approximation is higher, it is therefore considered that the Freundlich adsorption model fits better the studied system. Thus, we tend to believe that p-cresol adsorption by surfactin aggregates occurs in multilayers, that is, chemisorption and physisorption may occur simultaneously. In addition, the value of 1/n parameter was 0.8151 and the lower the value of 1/n (the higher the value of n), so physical adsorption is favoured.

Despite the simplicity of the remediation method, the adsorption capability of wash waters, containing

Table 3. Results of the remediation experiment of natural water that was artificially contaminated with *p*-cresol. Treatment was carried out using the first wash water from the biomass obtained in the production of surfactin.

Assay	Initial current (μA)	Final current (μA)	Initial concentration (μM/L)	Final concentration (μmol/L)	Remediation (%)
1	15.5	9.2	134.8	72.4	46.3
2	16.7	10.7	147.5	80.8	45.2
3	14.8	8.6	128.2	65.5	48.9

Table 4. Construction parameters for the Langmuir and Freundlich adsorption isotherms for the system containing solid surfactin as adsorbent and *p*-cresol as the adsorbate.

	x (Δ mass of *p*-cresol)	m (surfactin mass)/g	x/m	Log (x/m)	c / (x/m)	Log (concentration)
1	$6.1\ 10^{-6}$	0.015	$4.0\ 10^{-4}$	−3.4	$7.56\ 10^{-1}$	−3.5
2	$1.3\ 10^{-5}$	0.015	$8.7\ 10^{-4}$	−3.1	1.01	−3.1
3	$3.0\ 10^{-5}$	0.015	$2.0\ 10^{-3}$	−2.7	$9.96\ 10^{-1}$	−2.7
4	$3.7\ 10^{-5}$	0.015	$2.4\ 10^{-3}$	−2.6	1.23	−2.5

surfactin, for p-cresol remediation (1.29 mg/g at 298 K) is comparable to other complex materials used as adsorbates, as the organic emergent contaminants adsorbed by graphene nanoplatelets: aspirin (12.98 mg/g), acetaminophen (18.07 mg/g) and caffeine (19.72 mg/g) at 296 K [3]. Some modified nanoparticles, as ZnO covered with cetyltrimethyl ammonium bromide (CTAB) could go even further and adsorb 89.9 mg/g of phenolic pesticides [29].

Although the use of surfactin for remediation of hydrocarbon is well known [15], based on the previous results one can infer that the mechanism of adsorption of p-cresol by surfactin aggregates is relatively complex. The physical state of the adsorbent material is important to describe the adsorption processes, e.g. 'pore filling' mechanism occurs for solid particles presenting pores as interaction sites with soluble contaminants [30]. In this study, this is not the case. In fact, the pH 10 used as an optimum condition for remediation, enables the formation of surfactin aggregates that are negatively charged, solubilized in micellar state, which is characterized by a fluid matrix malleability and susceptibility to shape modifications.

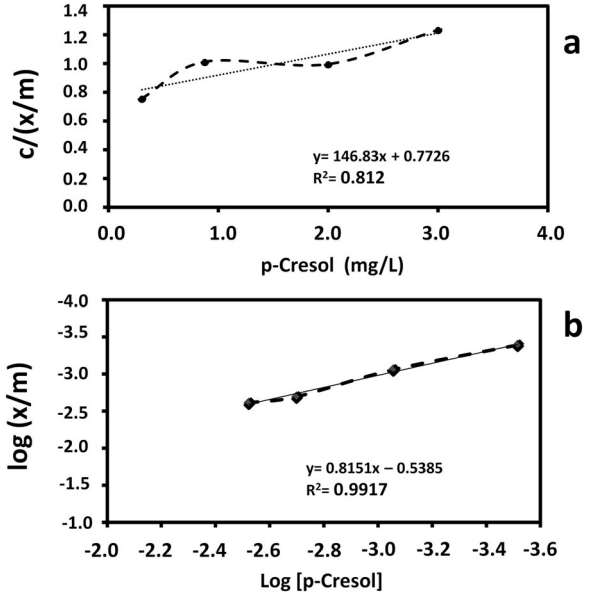

Figure 3. (a) Langmuir adsorption isotherm for p-cresol and surfactin aggregates (adsorbent); (b) Freundlich adsorption isotherm for p-cresol and surfactin aggregates (adsorbent).

Thus, to tentatively describe the remediation mechanism with this very malleable sorbent, we consider the aggregate characteristics separately. Therefore, its charged outer interface and its amphiphilic characteristic. The aggregation number of nearly 101 molecules in an aqueous solution is described for surfactin. In addition, its negative charge improves the adsorption of cations in water, but the presence of counter-ions and other charged species can change the shape and area of the surfactin aggregates [31]. For the latter, the amphiphilic nature of the surfactin molecules can also provide interaction with cyclic structures, such as tetracycline antibiotics [32].

Therefore, considering both effects, a charge sensitive malleable surfactin aggregate can accommodate p-cresol molecules on its surface (as the Langmuir isotherm suggests). However, this process is overwhelmed by another capture process, therefore also employing the aggregate's interior, in resemblance to a multi-layer adsorption, indicated by the best fitting of Freundlich isotherm. Other sorption mechanisms could also be considered, as the internal particle diffusion model [30].

4. Conclusion

Tests using wash water from surfactin production demonstrate that this lipopeptide-rich extract can be used for the remediation of organic wastes. We successfully removed approximately 46% of p-cresol, from artificially contaminated water using the obtained lipopeptide extract in the form of wash water. This finding is important as it demonstrates the potential applications of an industrial waste for remediation. Therefore, it can be considered as potentially cheap and sustainable. The carbon paste biosensor, using laccase, was shown to be reliable and useful to monitor water decontamination. In connection with flow-cells, it may be possible to monitor and control large effluents and their treatment.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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