Removal of Phenanthrene in an Aqueous Matrix by Entrapped Crude Enzymes on Alginate Beads Combined with TiO_2-C-Ag Coated Fiberglass

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***Abstract*—The polycyclic aromatic hydrocarbons (PAHs) are classified as a public health hazard, and priority pollutants to be eliminated from water. The degradation of PAHs is abetted by its low water solubility, yet countered by its attached to organic matter complicating the PAHs' removal from wastewater. In this study, the use of two different types of mechanisms to overcome the drawbacks of the presence of dissolved organic matter was evaluated in PHE (phenanthrene) removal. The combination of crude enzymes entrapped in alginate gels with heterogeneous photocatalysis adds the catalytic activity of the enzymes to PHE treatment without requiring special conditions. The application of photocatalysis techniques for water recuperation is subject to photon incidence over the catalyst, which affects the efficiency of the process. The combined system removed 94.3 ± 2.0% of PHE in the presence of polyoxyethylene sorbitan monooleate and dimethyl sulfoxide. The alginate gels were able to maintain the catalytic activity of the enzymes, while the TiO_2-C-Ag was activated under low-energy UV light (365-465 nm) with the Ag islands acting as electron donors. The latter was confirmed in the surface analysis by X-ray photoelectron spectroscopy, which presented an increase in carbonyl groups in both materials and a change from Ag^0 to Ag^{3+} after 13 h of treatment. The combination of both treatments improved removal in a single TiO_2-C-Ag treatment.**

***Index Terms*—Biocatalyst, coupled treatment, heterogeneous photocatalysis, hydrocarbons.**

I. Introduction

The recovery of wastewater for human consumption requires the fulfillment of regulations and standards for water safety. The polycyclic aromatic hydrocarbons (PAHs) are a group of persistent organic pollutants (POPs), which are present in wastewater and are recognized as carcinogenic, teratogenic and mutagenic by the WHO [1]. The EPAs from the EU and the US have included the PAHs in the list of priority pollutants to be eliminated from water. The PAHs are found attached to dissolved organic matter due to their non-polar hydrophobic nature [2].

The phenanthrene (PHE) is a PAH which have been used as a model for the development and evaluation of wastewater treatments. The maximum solubility of PHE in water is 1.1 mg/L. The presence of solubilizing agents in wastewater allows its mobility and incorporation into the environment [3]. Substances like anthropogenic and naturally occurring organic acids and surfactants are solubilizing agents present in dissolved organic matter. The dissolved organic matter takes part in the transport mechanism of PAHs [4].

The current wastewater treatments are ineffective in the removal of PAHs. The incorporation of Advanced Oxidation Processes (AOPs) in wastewater treatment is a promising solution because of their constant evolution. Among the AOPs, heterogeneous photocatalysis has been effective against PAHs as a result of its strong oxidizing agent production [2]. The combination of heterogeneous photocatalysis with biological, chemical or physical operations enhances the efficiency of the process and reduces the operational costs. The use of biological processes in combination with heterogeneous photocatalysis does not interfere with the photocatalyst mechanism [5].

The combination of anaerobic sludge blanket reactor and $TiO_2/UV/H_2O_2$ has been successfully applied in the removal of 2,4 dichlorophenol by [6], where the sequential treatment improves the performance of the biological treatment and reduce the treatment time. However, the excessive presence of oxidizing agents (H_2O_2, O_3, metals, metal oxides, and metal salts) is toxic to microorganisms affecting the efficiency of the combined system [7]. The use of enzymes overcomes the limitations of microorganisms. However, the application of enzymes in industrial process is limited by cost, availability, and instability of the enzymes [8].

The use of co-cultures enables the segregation of valuable enzymes as a result of the stimuli derived from the presence of other microorganisms and given to the regulatory network involving multiple proteins and complexes of the secondary metabolism gene clusters [9]. Among the enzymes, the lignin modifying enzymes, especially laccase and manganese-dependent peroxidase (MnP), are of great interest as they are low specific for the substrate, non-stereo selective, and strongly oxidative. Such characteristics enable the transformation of a wide variety of recalcitrant molecules. In continuous operations, the entrapment of enzymes is preferred over other methods due to its easy preparation, low

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cost and stable derivative formed [8].

The gel entrapment is one of the most used techniques in which the enzymes are surrounded by a semipermeable membrane [10]. The immobilization results in the association of the enzyme with an insoluble matrix via ionic interactions, Van der Waals forces and hydrogen bonds retaining a proper reactor geometry for its economic reuse under stabilized conditions [8]. The gels of natural polymers are non-toxic and biodegradable. Therefore, until now they have been used mainly in medical applications [11].

The alginate gels are homopolymeric networks of polysaccharides of physical interactions (ionic interactions, hydrogen bonds, or hydrophobic interactions). The term alginate describes a family of polysaccharides, specifically linear polymers of 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G), from brown algae and bacteria [11]. The two types of constituting saccharides are arranged in blockwise patterns composing homopolymeric regions indicated as M-blocks and G-blocks, and copolymeric regions of MG -blocks [12].

On the other hand, heterogeneous photocatalysis processes make use of high-energy ultraviolet radiation (254 nm) in order to generate electron-hole pairs in the catalyst surface, which produce highly oxidant radicals capable of removing complex chemicals from water. One of the most studied photocatalyst is TiO_2 due to its chemical stability, catalytic activity, and low cost. The incorporation of impurities such as metallic and non-metallic elements in the structure of TiO_2 allows the reduction of the energy necessary to generate the electron-hole pairs, improving its photocatalytic activity [13]. However, the presence of dissolved organic matter is a detrimental factor for the photocatalytic activity. These substances deposit in the active sites of the catalyst and interfere in photon incidence on the TiO_2 surface [14].

The objective of this research was the removal of PHE by the combination of crude enzymes entrapped in alginate with a TiO_2-C-Ag coated fiberglass photocatalysis treatment. So far, the proposed system configuration has not been reported in the available scientific literature. It consists of the use of the entrapped enzymes as pretreatment followed by the TiO_2-C-Ag under low-energy ultraviolet radiation (365 to 465 nm). The treatment was evaluated in the presence of the solubilizing agents polyoxyethylene sorbitan monooleate (TW80) and dimethyl sulfoxide (DMSO), which hinder photocatalytic operations and are present in wastewaters.

II. Materials and Methods

A. Production of Crude Extract

The strains of *Trametes maxima* (GenBank HF947516, *T. maxima*) and *Paecelomices carneous* (GenBank HF947521, *P. carneous*) were donated by the Institute of Ecology strain collection in Mexico. The culture medium used to induce the enzymes was modified from [15], using 20 g/L of glycerol (99.5%, J. T. Baker) as the carbon source. The mixed culture was obtained by inoculating 4 disks of 5-mm diameter of *T. maxima* strain growth in PDA plates (BD Bioxon), and the same proportion of *P. carneous* into 240 mL of culture medium. The crude enzyme extract was obtained after 13 days of incubation of the mixed culture. The biomass was separated by filtration, followed by addition of protease inhibitor cOmpletemini EDTA-free (Roche). The protein precipitation was made using acetone in a proportion of 83.3%, according to [16]. The protein pellet was reconstituted in buffer acetate 50 mM pH 4.5 [17].

B. Entrapment of Crude Extract on Alginate Hydrogel Beads

The entrapment of the reconstituted pellet was made in a 3% (w/v) solution of alginic acid sodium salt from brown algae (Sigma-Aldrich) prepared in buffer acetate 50 mM pH 4.5, and mixed with a 2-mL aliquot of the protein precipitate according to [18]. The mixture was dripped into a solution of 0.15 M $CaCl_2$ (96%, J. T. Baker) in buffer acetate 10 mM pH 5. The beads were kept at 4 ℃ in the $CaCl_2$ solution for 24 h. The beads were washed with buffer Tris 0.05 M pH 7 previous crosslinking with a 1% glutaraldehyde (50% wt. in H_2O, Sigma-Aldrich) solution for 90 min [19]. The beads were washed with buffer acetate 50 mM pH 4.5, and freeze-dried after being frozen with liquid nitrogen. In order to determine the content of proteins, the beads were dissolved in buffer phosphates 0.1 M pH 6.0. The protein analysis and laccase activity study were made as described by [18], while MnP activity was assayed with the phenol red technique [20].

C. Synthesis of TiO_2 Doped with C and Ag

The TiO_2 synthesis was modified as in that the reported by [21]. The doping precursors 6×10^{-3} mol/L of TW80 (average mol wt 1310, Sigma-Aldrich) and 6.8×10^{-3} mol/L of $AgNO_3$ (99.7%, J. T. Baker) were mixed with 0.7 mol/L of acetic acid (Ac ac) (97%, J. T. Baker) and 10.9 mol/L of ethanol (EtOH) (97%, J. T. Baker). The hydrolysis reaction was started by adding a mixture of 0.3 mol/L titanium tetrabutoxide (TTB) (97%, Sigma-Aldrich) and 10.6 mol/L of EtOH by drip into the Ac ac/EtOH/TW80/$AgNO_3$ solution. The solution was mixed for 1 h at room temperature in the dark. Afterwards, the fiberglass without resin was coated directly with the solution and kept in the dark for 24 h, as described by [22]. The coated material was dried at 80 ℃ for 24 h, and annealed at 500 ℃ for 2 h.

D. Characterization of the Materials

The material's structure was determined using scanning electronic microscopy (SEM, JEOL JSM-5900LV, 200 kV, at resolution 2.2\AA with a microanalysis detector) and transmission electron microscopy (TEM, JEOL JEM 2010H, 200 kV). The composition was identified using X-ray photoelectron spectroscopy (XPS, K-Alpha Thermo Fisher Scientific, Al Kα=1486.6 eV). The water content in the hydrogels and TiO_2-C-Ag content on the fiberglass were determined by dry weight difference. The Origin Pro 8.1 SR3 v8.1.34.90 software was employed for the XPS.

E. Removal of PHE in the Combined System

The fixed-bed packed column was used as pretreatment and implemented before the photocatalytic reactor, as indicated in the flowchart of Fig. 1a. The lyophilized alginate hydrogels were packed to a height of 40 mm in a glass column (25 mm × 100 mm) to have a length-to-particle-diameter ratio of 21. The packing was

made of two sections of 30 mm of glass beads and fiberglass, and the hydrogels were placed in the middle as observed in Fig. 1b. The reactor was operated in an up-flow mode. The photochemistry reactor FOTO Q200 ESVE of 0.27 L with two external-lamps of fluorescent light in the range of 365 to 465 nm (UVA light irradiation) was used as indicated in Fig. 1c. The system was fed with 1 L of a mixture of 28.0 ± 0.3 mg/L of PHE (90%, Sigma-Aldrich), 0.01 mg/L of DMSO (99.5%, Sigma-Aldrich), and 10 mg/L of TW80 using a peristaltic pump in multiple-pass with feed recycle at a velocity of 14.7 mL/min. After the first 2 h of circulating the PHE/DMSO/TW80 mixture in the system, 5 min pulses of 0.125 mL/min of O_2 from an oxygen concentrator (MARK 5 Nuvo lite 115 V unit, Nidek Medical Products, Inc) were administered to the reactor vessel and the photocatalyst was irradiated with UVA light. All the experiments were done in triplicate taking samples every hour and obtaining their UV-VIS absorbance spectra in a spectrometer, Shimadzu model UV-1800.

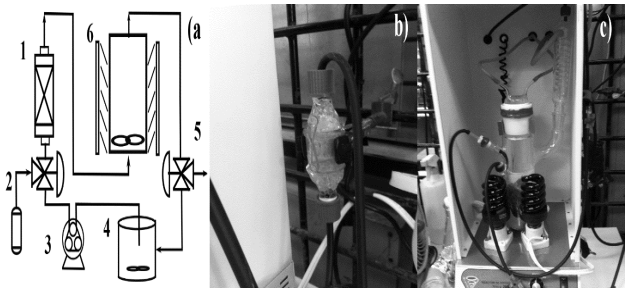

Fig. 1. Diagram of the process: a) 1 column, 2 oxygen concentrator, 3 peristaltic pump, 4 mixing tank, 5 sampling well, and 6 the photocatalytic reactor, b) fixed-bed packed column, and c) photocatalytic reactor.

The samples were extracted using solid phase extraction (SPE) cartridges (ENVI-18 Supelco bed wt. 500 mg of 6 mL). The cartridges were conditioned with HPLC grade solvents (Honeywell): dichloromethane (DCM), acetonitrile (ACN), methanol, and deionized water (10 mL each). The elution was made with 10 mL of DCM. The 0.22 μm filter extract was analyzed by ultra-performance liquid chromatography in tandem with Mass Spectrometry (UPLC-MS) techniques. The UPLC-MS analysis was in a gradient elution program (the analysis began with 40:60 H_2O/ACN, which was followed by a gradient from 10:90 H_2O/ACN for 1 min, 40:60 H_2O/ACN for 1.1 min and 40:60 H_2O/ACN for 2.5 min) with an injection volume of 3 μL and flow rate of 0.5 mL/min at 20 ℃ using a column UPLC BEC C18 (1.7μm, 2.1 mm × 50 mm) in a Waters UPLC-MS Class H system.

III. Results and Discussion

A. Characterization of the Materials

The alginate hydrogels were freeze-dried prior to usage in order to keep the enzymatic activity from depleting. The pellets had an ovoid shape with a length of the major axis of 1.9 ± 0.2 mm, and a porous core; as seen in Fig. 2a-b. The porousness (> 100 μm) is the result of the collapsing of the walls due to loss of water (the beads had had a water content of 95.8 ± 1.0%) from freeze-drying. The entrapment preserved 85% of the content of proteins in the crude extract (5.8 ± 1.7 g/L) after dissolving the lyophilized beads. The remaining percentage of the crude extract´s enzymatic activity on the surface of the beads was 0.3% and 4.3% for laccase and MnP, respectively. The poor precipitation of acetone a difference of salting out is the result of electrostatic repulsion between proteins [16]. However, the use of acetone allowed the direct entrapment without further steps. It was observed that the use of glutaraldehyde as a crosslinking agent enhanced the MnP activity.

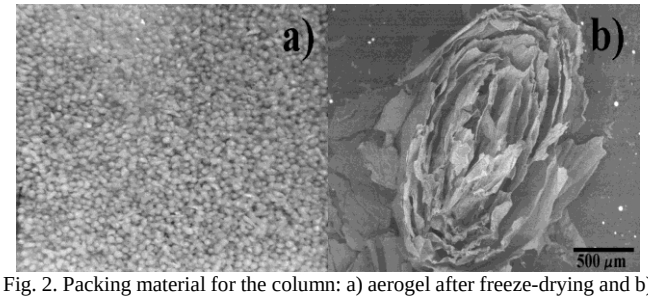

Fig. 2. Packing material for the column: a) aerogel after freeze-drying and b) SEM micrograph at 20 kV.

The TiO_2-C-Ag film obtained had a heterogeneous distribution on the fiberglass, as observed in Fig. 3a. The TiO_2-C-Ag coated fiberglass enables recuperation from the aqueous media preventing nanoparticles' liberation into the environment. It is known that free nanoparticles act as colloid-facilitated contaminant transporters giving stability to pollutants and enabling their penetration into the soil layers to groundwater [23]. The synthesized material presented a crystalline structure as confirmed in the diffractogram of Fig. 3b. The presence of Ag on the TiO_2 surface was observed by TEM, in which the Ag nanoparticles were identified as black dots on the TiO_2 surface; as observed in the Fig. 3c. The impurities incorporate into the TiO_2 structure through different mechanisms such as structural hydroxyl groups, extended defects, surface, and charge-compensated solid solutions. Doping changes the degree of structural metastability and the activation energy by affecting the number and distribution of surface sites suitable for nucleation as well as the rate of coarsening in fine crystalline materials [13].

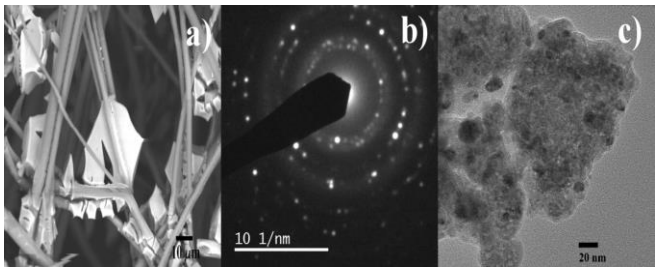

Fig. 3. TiO_2-C-Ag film on fiberglass: a) SEM micrograph at 20 kV of TiO_2-C-Ag coated fiberglass, b) TEM diffractogram at 200 kV, and c) TEM micrograph at 200 kV of the composite.

B. Removal of PHE in the Combined System

The first step in the treatment of 28.0 ± 0.3 mg/L of PHE with the solubilizing agents (0.01 mg/L of DMSO, and 10 mg/L of TW80) was the fixed bed packed column with 0.43 ± 0.04 g of beads followed by the photocatalytic reactor with 0.51 ± 0.18 g of TiO_2-C-Ag per g of fiberglass. During the first two hours of treatment with the combined system, the

mixture was circulated without O_2 and UVA light irradiation. In this homogenization phase, the PHE was deposited on the surface of the TiO_2-C-Ag film and the alginate hydrogels reaching 47.4 ± 9.9% of removal. A hydrogel is a three-dimensional cross-linked polymeric network which retains a significant fraction of water within its structure but whose substrate diffusion is restricted by a low porosity [11]. In the homogenization phase, the removal is presumed to be the result of accumulation onto the surface of the alginate beads and the coated fiberglass due to the system's lack of O_2 which is required for enzymatic and TiO_2-C-Ag catalysis. The presence of carbon in TiO_2 nanoparticles reported by [24] resulted in an enhancement of PHE sorption. The PHE removal reached 94.3 ±2.0% as observed in Fig. 4, after 11 h of treatment adding O_2 in the packed column and UVA light irradiation on the TiO_2-C-Ag catalyst. The removal at the end of the single treatments was 90.9 ±5.6% for TiO_2-C-Ag, 94.3 ± 0.7% for the hydrogel with enzymes and 45.3 ± 23.9% without enzymes.

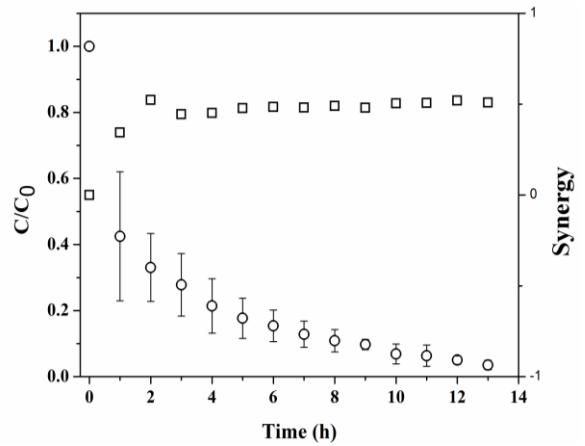

Fig. 4. Removal of PHE ○ and synergy □ of the combined system, n=3.

The catalytic activity of the enzyme is affected by the limitations of internal and external mass transfer, and blocking of active sites by the matrix [8], [10]. The contact time is inversely proportional to substrate low rate due to the increase in the amount of substrate supplied to the enzyme until saturation [10]. The use of a concentration of 0.5 mg/mL of MnP enzyme by [25] resulted in the predomination of the biodegradation mechanism for reactive dyes. The alginate beads with entrapped MnP retained their activity after several decolourization cycles [25]. In this work, the system was operated in multiple-pass with feed recycle. In single and combined treatments, there was a difference in removal between the crude enzyme entrapped in the alginate beads and the beads alone. The synergy analysis of the combined treatments was made according to [26], where a value above 1 indicates an additive effect, while a value lower than -1 indicates an antagonistic effect. The interaction of both treatments showed no effect from the combination during the 13 h of treatment, as shown in Fig. 4. The latter indicates that both catalysts were active during the treatment, that the diffusion of the pollutant did not affect the enzymatic activity in the biological treatment, nor was the catalyst inactivated in the photocatalytic treatment even in the presence of photon competitors (TW80 and DMSO).

The presence of by-products was not detected in the UPLC-MS analysis. The split of endocyclic C-C bonds in cyclic hydrocarbons is much slower than those of aliphatics due to the tendency of the alkenyl cation (formed by β-scission of a cycloalkyl cation) to recycle, or because of a lower β-scission rate in cycloalkyl cation ions caused by an unfavorable orientation of the π-orbital at the positively charged carbon atom and the β-bond to be broken [27]. The presence of TW80, which acts as a photon competitor, might interfere with the orientation of PHE molecule. It is known that surfactants have a preferential orientation in the air/water interface [28]. The PHE molecule absorbs strongly at 290 nm, and requires high energy (< 315 nm) to experience photocatalytic reactions [29], [30]. The energy gap between PHE's highest occupied molecular orbital and lowest unoccupied molecular orbital is 8.2, therefore requiring a high intensity to make modifications in its molecule [30]. This PHE removal was made under low intensity UV irradiation (365-465 nm) in the combined system.

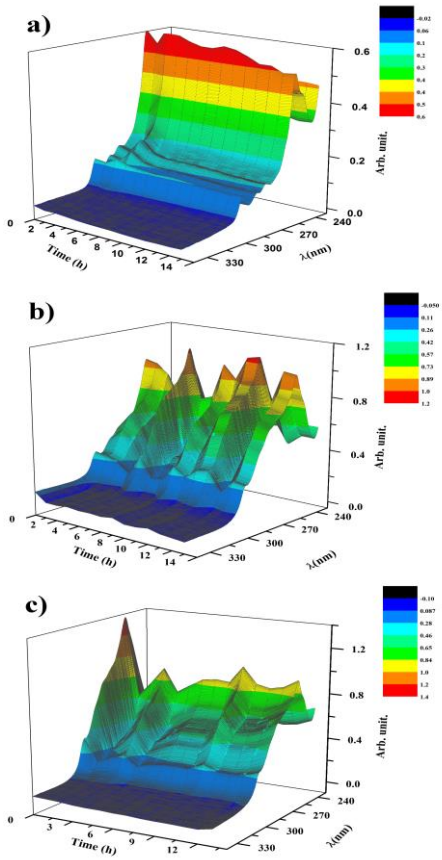

Fig. 5. UV-Vis absorbance spectrum of the treatments: a) hydrogel with enzymes, b) TiO_2-C-Ag, and c) hydrogel with enzymes/TiO_2-C-Ag.

The behavior of the C=C bonds in PHE was followed due to the π bonds in PAHs that present a strong absorbance in the ultraviolet region of the solar spectrum [30]. The behavior of the solution was influenced by the interference in degradation of the cosolvent DMSO under UVA light irradiation. As confirmed by the absence of alterations in the UV-Vis spectra in the treatment with only hydrogels with enzymes, as shown in Fig. 5a. The cosolvent originated a hyperchromic effect (250 to 300 nm) in the individual and combined treatments of TiO_2-C-Ag, as observed in Fig. 5b and c. DMSO is part of a sulphur cycle and undergoes a photolysis reaction under UV light [31]. The absorption

spectra of the treatments (Fig. 5) suggests that there was no increase in aromaticity in the PHE. In the study by [29], a PHE solution was irradiated in a solar simulator in the presence of O_2 without finding any sign of fluorescent products or increased aromaticity conjugation. In the absorption spectrum of the treatments there were no red shifts.

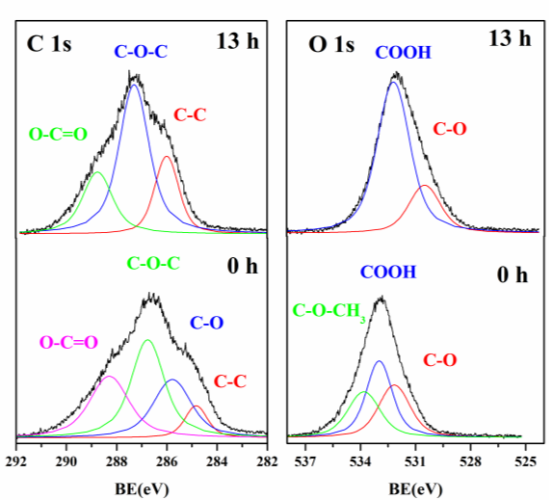

Fig. 6. Surface analysis of hydrogel with enzymes before and after the PHE treatment.

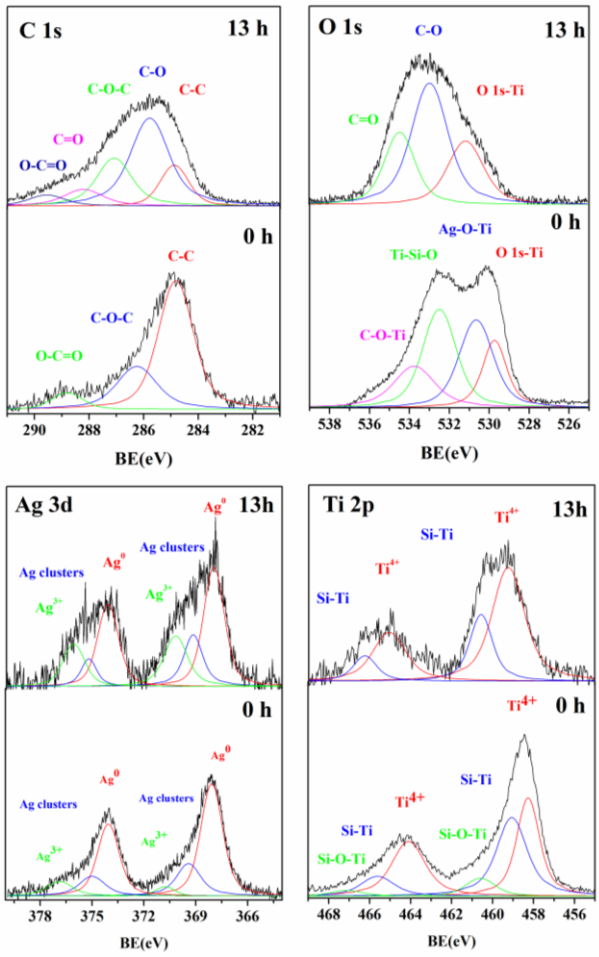

Fig. 7. XPS surface analysis of TiO_2-C-Ag supported in fiberglass before and after the PHE treatment.

The compositional surface analysis by XPS in Fig. 6 and 7 was made by the static method with a Gaussian-Lorentzian fitting, and a Shirley-type baseline adjustment with a charge correction to adventitious C at 284.8 eV. The data obtained was identified using the NIST database, version 4.1 for XPS [32].

The increase in carbonyl groups in the C1s region in Fig. 6 is the result of the attraction of PHE to the surface of alginate. After the treatment, the predominant species at the hydrogel with crude enzymes were C-O-C (286.3 eV) and C-O=O (288.08 eV), whereas at the beginning of the treatment, they were C-O (285.7 eV), C-O-C (286.7 eV), and O-C=O (288.2 eV). There were no traces of sulphur, which indicates low affinity of DMSO for alginate. The O 1s region after the treatment had a predominance of the carboxyl group in the species C-O (532.1 eV), COOH (532.9 eV), and C-O-CH_3 (533.8 eV) that effect was associated with surface modification due to oxidant agents [33]. The latter indicates that there was a transformation of PHE on the surface of the hydrogel.

The TiO_2-C-Ag fiberglass film also increased the carbonyl groups in the C 1s region, as observed in Fig. 7. At the beginning of the treatment the prevailing species were C-O-C (286.2 eV) and O-C=O (288.8 eV). After 13 h of treatment, C-O (286.7 eV), C-O-C (287.08 eV), C=O (288.2 eV), and O-C=O (289.5 eV) were found on the surface of the catalyst. The O 1s region before the treatment had O 1s-Ti (529.7 eV), Ag-O-Ti (530.6 eV), Ti-Si-O (532.4 eV), and C-O-Ti (533.7 eV). Such species were species related to the fiberglass support and TiO_2 doping agent's. The reaction between Ti and Si-OH has been reported in the nucleation of TiO_2 clusters over fiberglass by [34]. The Ag was incorporated as a mixture of Ag^0 and Ag^{3+}, after the treatment there was an increase in Ag^{3+} species with respect to Ag^0 as observed in their spin orbit splitting at $3d_{3/2}$ (374.05 eV for Ag^0 and 376.8 eV for Ag^{3+}). The species at the binding energies (BE) of 369.4 and 369.1 eV at 0 and 13 h, respectively for Ag $3d_{3/2}$ were associating with the presence of Ag clusters smaller than 4 nm as previously described in the work of [35]. After the treatment, the species in the O 1s region were Ti-O 1s at 531.1 eV and the carbonyl groups C-O (532.9 eV) and C=O (539.4 eV). In Ag 3d region, there was an increase in Ag^{3+} (376.09 eV at Ag $3d_{3/2}$). Such changes were the result of electron donation on the surface of the TiO_2-C-Ag fiberglass film. The Ti 2p had no changes in the oxidation state, as observed in its spin orbit splitting Ti $2p_{1/2}$ at the beginning (464.1 eV for Ti^{4+}, 465.6 eV for Ti-Si, and 467.1 eV for Si-O-Ti) and end (465.06 eV for Ti^{4+} and 466.2 eV for Ti-Si) of the treatment, as observed in Fig. 7. The latter suggests that Ag was an active oxidizing agent on the surface of TiO_2.

IV. Conclusion

The combined treatment of phenanthrene by the entrapped enzymes in alginate gels and fiberglass coated TiO_2-C-Ag resulted in a removal of 94.3 ± 2.0%. The presence of carbonyl groups on the surface of the hydrogels indicated that the enzymes were active, and there were oxidative reactions on the surface. The Ag was an active reducing agent in the TiO_2-C-Ag film. Both materials are capable of removing phenanthrene, but their combination of both improves the removal in a single photocatalytic treatment. The presence of the solubilizing agents did not affect the performance of the

combined treatment. The latter indicates that the development of continuous combined treatments in which biological and chemical catalysts are involved overcomes the presence of dissolved organic matter in the removal of a target pollutant. In order to attain application of such treatments in real wastewater, further toxicity studies and optimization of thermodynamic and kinetic parameters will help in developing efficient systems for PAHs removal.

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