Removal of triclosan in municipal wastewater by using an enzymatic method

S Lugo-Bueno¹, J E Becerril-Bravo², J A Barrios-Perez², A Cano¹,² and N Ornelas-Soto¹

¹Escuela de Ingeniería y Ciencias, Tecnológico de Monterrey, Ave. Eugenio Garza Sada 2501, Monterrey, NL 64849, México
²Treatment and Reuse Group, Instituto de Ingeniería UNAM, Circuito Escolar, Ciudad Universitaria, 04510, México, D.F., México
E-mail: ornel@tec.mx

Abstract. Currently, wastewater treatment has received attention as one of the most relevant activities to ensure environmental sustainability. This is due to the increased consumption of chemicals as many of them enter directly or indirectly into the environment through effluent discharge, causing pollution of water bodies, which consequently have negative effects in humans. Herein, in this study was focused on the elimination of triclosan (Contaminant of Emerging Concern, CEC) in wastewater, by using immobilized lignicolous enzymes (laccases). Moreover, an electrochemical oxidation pretreatment was applied over the effluents in order to improve the biocatalytic removal performance of Triclosan. Laccase from *P. sanguineus* CS43 was covalently bonded onto titanium dioxide (TiO₂) nanoparticles whose surface was previously functionalized with (3-aminopropyl)-triethoxysilane (APTES) and glutaraldehyde. The optimized parameters used in the electrooxidation for pH, current density and treatment time were 2, 10 mA/cm², and 76 min, respectively. The monitoring of triclosan concentration was carried out by means of gas chromatography coupled to mass spectroscopy (GC-MS).

1. Introduction
The growing demand for water and the continued discovery of new potentially hazardous contaminants, referred to as Contaminants of Emerging Concern (CEC), make clear the need for further research in all areas that can contribute to protecting human health and the environment, achieving a sustainable use of water [1]. CEC’s involve a wide range of chemical compounds that include everyday products with both domestic and industrial applications [2]. These contaminants are known to enter the environment through different pathways such as domestic and industrial wastewater from treatment plants [3,4], which contain CEC’s that are produced at different concentrations, therefore, conventional wastewater treatment plants are not designed to dispose of them [5]. Enzymatic-based processes have been shown to be effective in eliminating persistent organic compounds, generating increasing interest due to their outstanding advantages such as high catalytic activity on a wide range of substrates and the production of less toxic biotransformation by-products [6,7]. Within these, laccase enzymes (EC 1.10.3.2) belonging to the family of multi-copper oxidases that catalyze the oxidation of several aromatic substrates, have been shown to have a high potential in the removal of organic contaminants in water, reaching high percentages in short treatment times [8]. Even though, these enzymes have excellent biocatalytic properties, the presence of inhibitory compounds, such as dissolved organic matter, can reduce considerably its activity. One way to overcome this problem is by performing enzymatic immobilization, which has been shown that it can...
greatly increase enzyme stability and performance [9,10]. In addition to the above mentioned, another way to improve the biocatalytic efficiency of enzymes is by performing a pretreatment of wastewater, by means of a conventional method, to remove interferences that may hinder the biocatalytic process. Within the alternative methods for wastewater treatment, electrooxidation (EO) is an electrochemical process that has been studied and used in the removal of different contaminants [11,12]. This process consists in the use of electrodes of different materials that allow the generation of highly oxidant species, such as hydroxyl radicals (•OH); that in general, have prove capable of eliminating most organic compounds found in high concentrations in wastewater, organic compounds are completely oxidized when exposed to hydroxyl radicals because they are electrophiles and react quickly with electron-rich organic compounds [13]. Therefore, in this work we propose an EO-pretreatment followed by an alternative enzymatic treatment in order to facilitate and enhance the elimination of the persistent Triclosan found in wastewater.

2. Experimental section

2.1. Reagents

High purity standards were used: triclosan (TCS) and titanium oxide nanoparticles (TiO2) (Degussa, P25) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), dibasic sodium phosphate (Na2HPO4) and citric acid salt (C6H5O7·H2O) were purchased from Aldrich, USA. 3-aminopropyl triethoxysilane (APTES) was purchased from Sigma. Glutaraldehyde (GLU) was purchased from Merck Millpore. Supel™-Select HLB SPE tube extraction cartridges (bed wt. 200 mg, volume 6 ml) were purchased from SUPELCO. Laccases from P. sanguineus CS43 were obtained from a tomato medium as described in our previous work [14].

2.2. Sampling of wastewater

Wastewater from the secondary clarification part prior to the disinfection process was obtained from wastewater treatment plant in Iztapalapa located in Mexico City. Samples were collected in glass containers and stored at 4°C before transportation to a certified laboratory for characterization and treatment. Wastewater was characterized by pH, conductivity, color, and spectral absorbance.

2.3. Laccase immobilization onto TiO2 nanoparticles

Laccases (P. sanguineus CS43) were obtained from a tomato medium described in Ramirez-Cavazos, et al., 2014 [14]. Immobilized Laccases (TiO2-Lac) were obtained as described in our previous work [15]. Briefly, 400 mg of TiO2 were dispersed in anhydrous ethanol and disaggregated by bath sonication, afterwards the corresponding amount of APTES for 5 %wt. was added drop wise to the TiO2–ethanol suspension, this mixture was placed under reflux during 24 h at 65°C. After that, the nanoparticles were separated by filtration and washed with anhydrous ethanol, and dried under vacuum at room temperature. For GLU functionalization, 50 mg of TiO2-nanoparticles functionalized with APTES were dispersed in a solution of 4% (v:v) of GLU previously dispersed in a 200 mM phosphate buffer solution (pH 7) and left to react for 12 h, unreacted GLU was removed by centrifugation and re-dispersed in phosphate buffer three times. For enzyme immobilization, functionalized nanoparticles with GLU were suspended in 10 ml of laccase solution (2000 U/L) for 72 h at 20 °C (TiO2-Lac). Afterwards, the unreacted laccase was removed by centrifugation and re-dispersed in phosphate buffer solution, repeating this washing step three times.

2.4. Electrooxidation (EO) procedure

EO pretreatment was performed in a Diaclean® electrochemical cell (WaterDiam, Switzerland) using boron-doped diamond electrodes (surface area: 70 cm2; electrode gap: 2 mm). 1 L of wastewater samples were continuously mixed in a glass reactor at a low speed (250 rpm). The system worked in total recirculation mode (2.8 L/min), with a peristaltic pump (JP Selecta Percom N-M328) at constant temperature (20°C). Power was supplied by a Delta Elektronika ES030-10 power source [16]. For wastewater treatment the pH, current density (j) and treatment time were adjusted to 2, 10 mA/cm2,
and 76 min, respectively. 15 ml of sample were withdrawn every certain time from the system and analyzed for pH, conductivity, color Pt/Co, turbidity and UV–Vis absorbance.

2.5. Evaluation of enzymatic activity in previously treated wastewater by EO

Laccase activity was determined by monitoring the oxidation rate of ABTS (probe molecule) to ABTS+ at 420 nm. For this, 50 mg of TiO$_2$-Lac were dispersed in 190 ml of pretreated wastewater by EO, then 10 mL of 5 mM ABTS solution were added in order to initiate the reaction, taking an aliquot every 30s. Each aliquot was rapidly filtered through a PTFE 0.2 μm syringe filter to remove TiO$_2$-Lac and its absorbance was measured in a HACH DR 5000. A control sample was also analyzed by using a pH 3 phosphate–citrate buffer solution as matrix instead of the pretreated wastewater. One unit (U) of laccase activity was defined as the amount of laccase forming 1 μmol of ABTS+ per minute. To evaluate the catalytic capacity of the enzyme, the relative activity was determined for the treated wastewater sample with respect to the control sample (Ec 1).

$$\text{Relative activity (\%) = \frac{\text{Laccase activity in treated wastewater}}{\text{Laccase activity in control sample}} \times 100}$$ (1)

2.6. Bioenzymatic treatment

A volume of 1.25 L raw wastewater was spiked with Triclosan (TCS, 1 mg/L): After EO pretreatment, appropriate amount of TiO$_2$-Lac nanoparticles was dispersed to the resulting treated wastewater to obtain an activity of 100U/L. Then, mixture was placed into beaker and protected from light to avoid TiO$_2$ photo-degradation and allowing it to react for 6 h under magnetic stirring. 250 mL aliquots were withdrawn in order to analyze the pollutant concentration at each stage of the treatment, i.e., at the beginning, after electrochemical process and every 2h during the enzymatic treatment (until 6h). The removal percentage was calculated with equation (2),

$$\text{Removal (\%) = } \frac{C_0 - C_i}{C_0} \times 100$$ (2)

where $C_0$ is the initial pollutant concentration in mg/L in the raw doped wastewater and $C_i$ is the pollutant concentration at each stage of treatment

2.7. Determination of TCS by GC-MS

The GC–MS analysis of raw doped and treated wastewater was conducted. Raw doped (500 mL) and treated (250 mL) wastewater was pre-filtered through a 1.2 μm glass fiber filter. Afterwards the samples were acidified to pH 2 with sulfuric acid (98%). For each sample, a Supelco HLB cartridge (200 mg) was conditioned with acetone (2x5 mL) followed by water (5 mL). The samples were then passed through the cartridge at a rate of approximately 10 mL/min. On completion of the extraction, the cartridges were washed with HPLC grade water (5 mL) before being eluted with 10 mL of acetone. The acetone fraction was evaporated to approximately 200 μL then 1 mL of ethyl acetate was added. Water was removed by addition of anhydrous sodium sulfate. The sample was evaporated to dryness under a stream of nitrogen at room temperature. Pyridine (15 μL) and BSTFA (35 μL) were added to the wastewaters [17]. N-Trimethylsilyl derivatives were produced by reaction at 60°C for 30 min before analysis by GC–MS. The GC-MS measurements were done in a HP 6890 gas chromatograph, fitted with a 30 m HP5-MS fused silica capillary column (Agilent 30 m x 0.25 mm, 0.25 μm film thickness), and connected to an HP 5973 mass selective detector. The carrier gas was helium at a constant flow rate of 1.0 mL/min. A split less injection technique was used to inject 1 μL of sample at the injection port temperature of 250°C. For the analysis of all compounds the oven program was as follows: 100 °C for 1 min, 20 °C/min to 280°C, 280°C for 10 min. The detector was used predominantly in selected ion mode (SIM). The electron impact source temperature was 230°C with electron energy of 70 eV. The instrument was tuned on perfluorotributylamine. Quantification and confirmation ions monitored are detailed in Table 1. A scan range of 50–500 m/z was used for full scan analysis of selected samples.
Table 1. Characteristic ions and retention time for TCS by using GC-MS conditions described.

| Compound  | Retention time (min) | Characteristic ions (m/z) |
|-----------|----------------------|---------------------------|
| Triclosan | 9.43                 | 200 360 362               |

3. Results and discussion

3.1. Sequential electrochemical-bioenzymatic treatment

Once the electrochemical process was optimized, the following conditions were selected: pH=2, \( j = 10 \) mA/cm\(^2\) and time= 76 min. The combined treatment was applied to remove Triclosan. Figure 1 shows the concentrations of the target pollutant, by means of the technique of Gas Chromatography coupled to mass spectrometry (GC-MS) in each stage of the combined treatment. From these results, it can be observed that Triclosan shows a good degradation yield of 94% after applying the electrochemical/bioenzymatic treatment. As shown in Fig. 1, the elimination of TCS consists of two steps, the first one (from point a to point b) corresponds to the electrooxidation of TCS where it was possible to oxidize 85%. The second step (from point b to point e) corresponds to the enzymatic treatment. It is important to note that during the 6 h of the enzymatic process, the concentration of TCS presents a decrease of only the 9% of the total concentration; such low enzymatic activity may be due to the interference of by-products that were formed during the electrooxidation step, as it has been proved that such by-products can have a negative effect as they can cause the deactivation or even denaturation of the enzymes [18]. Nonetheless, the overall elimination of TCS after the complete treatment reached 94%, which is a successful elimination rate, indicating that the combination of both, the electrooxidation and the enzymatic processes, is a promising coupled method to be used in the elimination of CEC.

4. Conclusions

An enzymatic method was developed for the elimination of the persistent organic compound Triclosan in municipal wastewater. A pretreatment was previously carried out by using a conventional electrooxidation process. Immobilized laccase enzymes from \( P. \) *sanguineus* CS43 into TiO\(_2\)
nanoparticles were used in the enzymatic treatment. Triclosan concentrations were determined at each stage of the combined treatment by using GC-MS technique, demonstrating a high level of removal (93%). These results suggest that the use of a conventional method as pretreatment, e.g., electrooxidation, followed by novel enzymatic treatments can represent an effective integral process for removing persistent organic pollutants in aqueous media.

5. References

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