A Biodegradation Bench Study of Cork Wastewater using Gamma Radiation

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Abstract:

Wastewater from cork processing industry present high levels of organic compounds such as phenolics that must be degraded before discharge into the municipal sewer or into public water courses. The aim of this work was to find out if gamma radiation treatment could increase the biodegradability of recalcitrant compounds using a microbial consortium and a mixed solution of four phenolic acids as a model. Chemical and microbiological analyses were performed in non-irradiated (0 kGy) and irradiated (100 kGy) mixed phenolic acids cultures during incubation time. A preliminary HPLC and GC-MS analysis were performed to detect the major phenolic compounds in cork wastewater samples. Results indicated the presence of gallic, protocatechuic, vanillic and syringic acids in cork boiling water and gallic, protocatechuic and vanillic acids in sediment tank samples. The Total Phenolic content (TP) of mixed phenolic acids cultures during incubation time indicated a decrease of 38% for 100 kGy samples. The HPLC analysis suggested that the radiolytic products of syringic and vanillic acids are protocatechuic and gallic acids. The CFU counts pointed out to a decreasing tendency along the incubation time for phenolic acids cultures (0 kGy and 100 kGy) suggesting a non-degradation trend. The selected microbial consortium was not able to metabolize the phenolic compounds solutions at the used conditions. This could be due to the detected radiolytic degradation dynamics of the phenolic acids considering the antimicrobial activity of these compounds.

Keywords: Biodegradation, phenolic compounds, cork wastewater, gamma radiation.

Introduction

Contamination of water is an increasing problem caused by polluted effluents from municipalities and industry. Conventional technologies and others for wastewater purification do not solve all existing problems. The destruction of non-biodegradable organic compounds is one issue and the biological contamination of effluents is another. Particularly, wastewater from cork processing industry present high levels of organic compounds such as phenolics that must be degraded before discharge into the municipal sewer or into public water courses. In fact, these compounds present a low biodegradability and a significant toxicity (1) due to their recalcitrant and bioaccumulation potential, rending the phenolic acids as a significant environmental concern.

Biodegradation is considered as a sustainable process of wastewater treatment which, under appropriate conditions, can promote an efficient reduction of the organic matter content with minimal energy requirements and low costs (1). The organic compounds naturally produced could be used as carbon and energy source by some microorganisms. Thus, the simple biological treatment and/or combined with other processes could be an option to the wastewater treatment (1). Green Chemistry is one alternative, involving specially designed chemical processes and products that reduce or eliminate the use and generation of hazardous substances (2). Similarly, radiation processing, using electron beam accelerators and gamma sources, is an additive-free process that uses the short lived reactive species (formed by radiolysis and/or other reactions) for efficient decomposition of the hazard pollutants in the product. Ionizing radiation covering a high energy range that is safely kept below the radioactivity threshold proved to be a clean and environmental friendly technology with the potential to solve the problems left by the conventional

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methods and an alternative for some of the chemical treatments.

It was observed that gamma radiation induces both the degradation of compounds and the inactivation of microorganisms depending on the type of energy, dose rate, and absorbed dose (3). An alternative could be the use of gamma radiation as a complement process for cork wastewater treatment. One of the effects of the interaction of radiation with water is the production of highly reactive species, namely free radicals (4) that react with the compounds present in the matrix leading to its destruction and/or changes in its structure.

The purpose of this study was two-fold: a) to study the biodegradation of cork wastewater, after gamma irradiation, in order to assess its potential for biological treatment, and b) to find out if the radiolytic degradation of the phenolic acids in conjunction with microbial degradation could increase the treatment efficiency.

The radiolytic degradation of four phenolic acids (gallic acid, protocatechuic acid, vanillic acid and syringic acid) in a mixed solution was studied as a model, in order to find out if the gamma radiation treatment could increase the biodegradability of these recalcitrant compounds using a microbial consortium.

Experimental and Methods

Chemicals

Gallic acid, protocatechuic acid and syringic acid used in the study were purchased from Sigma (St Louis, MO, USA). Vanillic acid was obtained from Fluka (Buchs, Switzerland). Solvents and mobile phases used for HPLC, namely, acetonitrile (HPLC grade) and Formic acid were purchased from Sigma (St Louis, MO, USA) and from Panreac Química SA (Barcelona, Spain), respectively. The derivatising reagent, Bis(trimethylsilyl)trifluoroacetamide plus trimethylchlorosilane (BSTFA+TMCS, 99:1) ≥99.0% (GC grade) was purchased from Aldrich (Portugal). Ultrapure water (from Millipore® System) was used to prepare all solutions and buffers.

Irradiation Studies

The irradiations were performed in a Co-60 irradiation equipment (model Precisa 22, Graviner Lda, UK 1971 with 5.21 kCi in May 2013), located at Centro de Ciências e Tecnologias Nucleares, Instituto Superior Técnico, Portugal. Samples were irradiated at 100 kGy with a dose rate of 2 kGy h^{-1}. Absorbed doses were measured by routine dosimeters (5). The dose rate was previously determined by Fricke reference dosimeter (6).

High Performance Liquid Chromatography (HPLC-DAD) Analysis

The concentration of studied phenolic compounds in both sampling sites and time-aliquots from bio-degradation experiments was determined using a HPLC (Prominence CBM 20-A, Shimadzu) with a diode array detector (DAD) at 280 nm. As stationary phase, a Merck Purospher STAR RP-18e (5 µm, 250 x 4.0 mm) was used, with a mobile phase consisted on: A (90% (v/v) ultra-pure water with 0.1% formic acid) and B (10% acetonitrile solution), with a flow of 1 $mL.min^{-1}$. The column temperature was maintained at 25 ºC and the injection volume was 20 µL. For quantification purposes, a calibration plot (50-300 mg L^{-1}) was performed under the instrumental conditions used.

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Derivatisation of phenolic analytes was performed in BSTFA+TMCS (99:1) solvent according to the following procedure. A 30 µL aliquot of BSTFA+ TMCS (99:1) was added to 200 µL of wastewater samples and standard solutions of the phenolic compounds previously dried with a N_2 stream. The reaction took place for 60 min at 60 ◦C prior to injection in the GC–MS. GC–MS analysis was performed using an Agilent 6850 GC fitted with a 5975 VL MSD (Triple Axis Detector) Agilent mass spectrometric detector. A DB-50 5% Phenyl-/95% dimethylpolysiloxane capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) from Agilent was used, with helium as carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was maintained at 60 °C for 3 min and programmed to 350 °C. Compound identification was based on matching the measured electron impact ionization (EI) mass spectra with those from MSD ChemStation software (Agilent), followed by manual interpretation.

Chemical Oxygen Demand (COD) and Total Phenolic Compounds (TP)

Chemical Oxygen Demand (COD) was measured using the Titrimetric method accordingly to the Standard Methods for the Examination of Water and Wastewater (7).

Total Phenolic Compounds (TP) method was based on Singleton et al. method (8) using Gallic acid as standard. Absorbance was measured at 765nm with a spectrophotometer (UV 1800, Shimadzu).

Culture Media

A minimal phenolics acids media [170.90 mg L^{-1} gallic acid, GA; 154.35 mg L^{-1} protocatechuic acid,

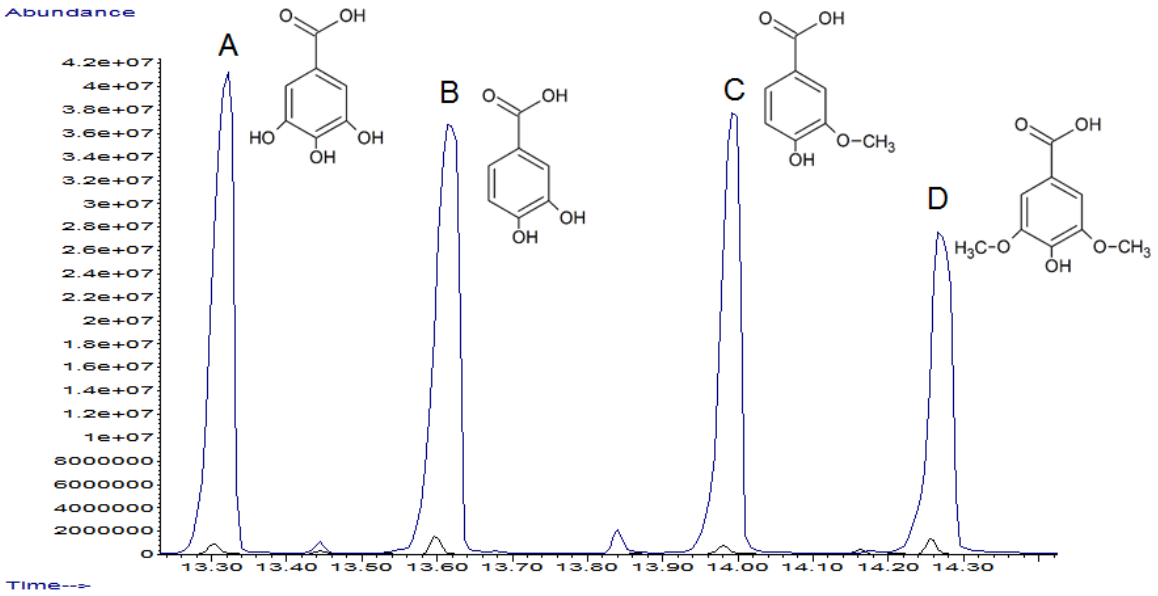

Figure 1 HPLC chromatogram of cork wastewater (black line) and standard mixed phenolic (gallic acid – A; protocatechuic acid – B; vanillic acid – C and syringic acid - D) solution (blue line).

PA; 168.53 mg L^{-1} vanillic acid, VA and 198.50 mg L^{-1} syringic acid, SA; plus 1% salt solution: 8.05 g L^{-1} $CaCl_2$, 608.8 mg L^{-1} $FeCl_3.6H_2O$, 1.00 g L^{-1} $MnSO_4.H_2O$, 1.00 g L^{-1} $MgSO_4.7H_2O$, 5.01 g L^{-1} K_2HPO_4, 13.03 g L^{-1} KH_2PO_4] was used as a model. A non-irradiated minimal phenolic acid media (0 kGy) and an irradiated minimal phenolic acid media (100 kGy) were used in the batch biodegradation experiments in order to assess the biodegradability of the phenolic acids radiolytic products.

Batch Biodegradation Experiments

An autochthonous mixed microculture of four bacterial strains naturally present in cork wastewater sedimentation tank samples was selected, due to its adaptation to this environment and its potential metabolizing capacity. The morphological characterization of the four isolates was carried out by conventional bacteriological techniques (e.g. cell morphology; gram staining; biochemical tests) (9). The biodegradation experiments were performed into 250 mL erlenmeyer flasks containing: the minimal phenolic acids media (0 kGy and 100 kGy), TSB solution (as positive medium control; C+) and ultrapure water with concentrated salt solution (as negative medium control; C-). The selected microbial consorptium was inoculated in all culture media at a concentration of 10^5 CFU/ml (Colony Forming Units – CFU) and incubated at 30 ºC in an orbital shaker (150 rpm). Sample aliquots for each culture assay were withdrawn at regular time intervals (time-aliquots) and analyzed for total viable biomass (CFU/ml counts), TP and HPLC analysis. CFU counts were performed by direct plating into Tryptic Soy Agar of triplicate aliquots of serial decimal dilutions of culture assays. A period until the residual concentration of phenolic compounds and the amount of biomass in flask had reached asymptotic values in time was carried out for each experiment. Results obtained from the same set of triplicate experiments were averaged and are reported.

Results and Discussion

HPLC and GC-MS analysis

HPLC and GC-MS analysis were performed in order to select the most abundant phenolic compounds in cork wastewater to be used as model in the degradation studies. HPLC results indicated that gallic acid, protocatechuic acid, vanillic acid and syringic acid were the major phenolic compounds in cork boiling water, while in sediment tank, the major compounds observed were gallic acid, protocatechuic acid and vanillic acid (Figure 1). These phenolic compounds were previously reported in literature as cork components (10, 11). In GC-MS the fragmentation pattern characteristic of the chemical derivatives formed was then used as a mass spectral fingerprint to confirm the identity of the phenolic compounds. The predominant phenolics in sediment tank samples were gallic acid, protocatechuic acid and vanillic acid. In cork boiling water, the major compounds identified were the previous ones and the syringic acid which are in agreement with HPLC analysis.

After model solution irradiation at 100 kGy, the performed HPLC chromatograms confirmed the degradation of phenolic compounds (Figure 2). As

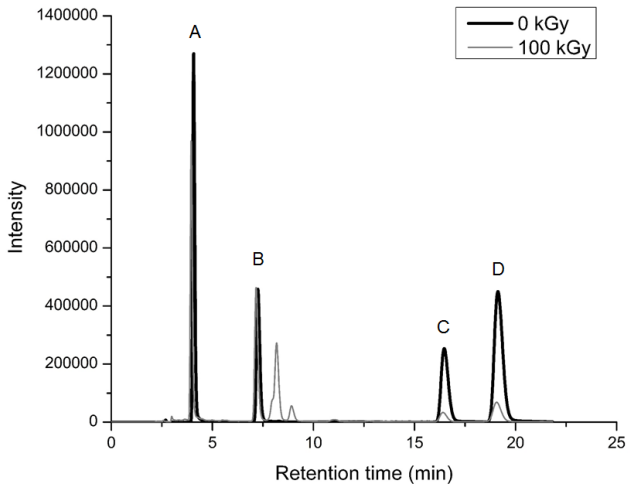

Figure 2. HPLC chromatograms of non-irradiated (0 kGy) and irradiated (100 kGy) mixed solutions of phenolic compounds (gallic acid – A; protocatechuic acid – B; vanillic acid – C and syringic acid - D).

presented in Figure 2, the radiolytic degradation intermediates were predominantly the phenolic compounds. However, a further study on radiolytic products identification will be performed.

Chemical Oxygen Demand (COD) and Total Phenolic Content (TP)

COD is a routine parameter that reflects the amount of oxidizable organic matter that can be due to a large variety of compounds including the phenolic acids. The results show that the non-irradiated sample presented an average of 1866 ± 247 mgO_2 L^{-1}. After irradiation at 100 kGy, there was a decrease of approximately 20% in oxidizable organic matter (average of 1488 ± 247 mgO_2 L^{-1}). Other authors (12, 13) also reported that the COD decreases with gamma irradiation in industrial wastewaters, indicating a chemical degradation of organic matter (14).

The total phenolic content gives a measurement of the phenolic groups present in the sample. For the non-irradiated sample, it was observed an average of 642 ± 31 mg GAE L^{-1} and after irradiation at 100 kGy there was a decrease of approximately 11% (average of 570 ± 8 mg GAE L^{-1}). These results are also in accordance with described by others authors (1). Thus, the standard mixture solution can be used as a representative model of industrial wastewater for biodegradation studies.

Batch Biodegradation Experiments

An autochthonous mixed microculture of four bacterial strains naturally present in cork wastewater sedimentation tank samples was selected, due to its adaptation to this environment and its potential metabolizing capacity. A non-irradiated minimal phenolic acid media (0 kGy) and an irradiated minimal phenolic acid media (100 kGy) were used in the batch biodegradation experiments in order to assess the biodegradability of the phenolic acids radiolytic products. The dose of 100 kGy was selected for these assays based on the results obtained from preliminary analyses where reduced degradation (<20%) of mixed target phenolic compounds was verified for doses bellow 75 kGy (data not shown).

TP will reflect all groups present even if some of them are in the same molecule and the behavior is shown in Figure 3a) for the non-irradiated and irradiated (at 100 kGy) samples of mixed phenolic acids cultures during incubation time. In order to validate the TP results, HPLC analysis of 0 kGy and 100 kGy culture assays aliquots were carried out. The results are shown in Figure 3b). The results indicated that there is almost no variation of TP for 0 kGy culture, while for 100 kGy culture, this parameter decreases 38%. These results could be an indicator of partial biodegradation.

Regarding the HPLC analysis, the results point out to an initial (time 0) chemical degradation of approximately 90% of vanillic acid and syringic acid with gamma radiation. Moreover, it is evident that protocatechuic acid concentration increase at 100 kGy. This fact could be due to the radiolytic by-products of vanillic and syringic acids since these products seem to be similar compounds of protocatechuic acid. To confirm this hypothesis, it was performed an analogous HPLC study for each phenolic acid using 100 kGy irradiated solutions. The results suggested that the radiolytic by-products for syringic and vanillic acids are protocatechuic and gallic acid. Along the incubation time, there was no detected degradation tendency of the analyzed phenolic acids until the end of the assays.

The assessment of microbial growth during incubation time was performed by CFU counts as presented in Figure 4. As expected, the CFU counts for the positive control (C+) increased during the initial incubation time, indicating the adequacy of methods to evaluate the growth of tested bacteria.

A rapidly bacteria growth along time is seen to C+ (in two days is reached exponential phase) so is our comparison parameter.

For the negative control (C-) it was not observed substantial variations in growth during incubation time. As presented in Figure 4, the results point out to a decrease in CFU counts in phenolic acids cultures. Therefore, the radiolytic by-products seem to have a negative effect on the growth of the selected microbial community. One of the products of water radiolysis is hydrogen peroxide. Despite the apparent contradiction

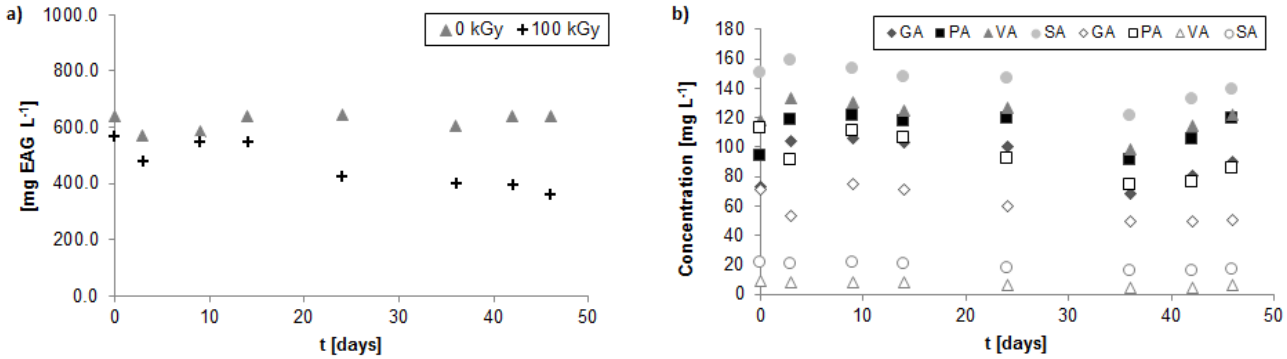

Figure 3. a) Total Phenolic Compounds concentration for 0 kGy and 100 kGy culture assays, and b) HPLC analysis of phenolic compounds (0 kGy samples are represented by filled markers and 100 kGy samples by empty markers).

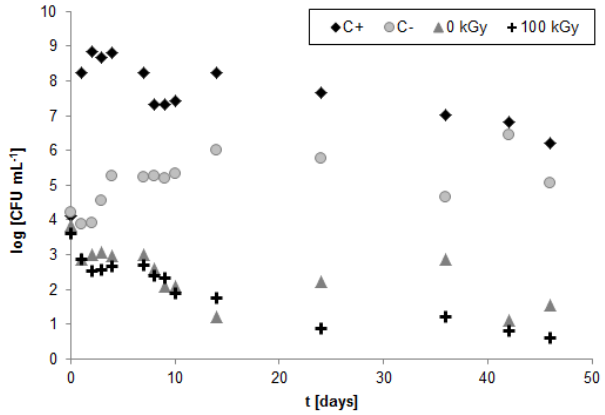

Figure 4. Log Colony Forming Units counts (CFU) for culture assays and controls during incubation time.

of using a potential toxicant to promote biological degradation, bioremediation with H_2O_2 has proceeded on the premise that H_2O_2 toxicity would not impair the biodegradation process (15). According to a study on the detoxification of phenolic compounds commonly found in industrial wastewaters using a combination of biological and advanced oxidation processes (AOPs), the sample toxicity increased at higher concentrations due to the generated intermediates (16). The anti-microbial activity of target phenolic acids to the selected microbial consortium must be evaluated. The antimicrobial activity of phenolic acids has been demonstrated against to a wide range of micro-organisms (17). In fact, the HPLC analysis of individually and mixed phenolic acids solutions suggested that the radiolytic by-products were pre-dominantly the phenolic compounds. As so, the initial approach that the gamma radiation will turn these recalcitrant compounds into simpler ones more bio-degradable was not achieved for the tested conditions. Although, must be noticed that microbial consortium (or part of) was maintained viable (with countable colonies) in phenolic acids cultures until the end of the experiments (Table 1).

The morphological characterization of the isolated colonies indicated a microbial population dynamics along incubation time, with the predominance of two morphotypes at the end of experiments for 0 kGy samples, and only one type for 100 kGy samples (Table 1).

There are few studies that been published on the treatment of real industrial wastewater regarding the vast variety of compounds and their different toxicities. Among these, there are a reduced number of reports on the potential benefits of the combination of AOPs and biological treatments for similar waters containing herbicides or pesticides or so-called emerging pollutants (18-20). Other investigators reported degradation of persistent organic matter by an electron beam irradiation using radiation doses up to 75 kGy (21). This type of ionizing radiation equipment could be technically more feasible for real scale applications, and is already applied in Korea for wastewater treatment. However, to our knowledge, this study represents a unique approach that tries to associate the metabolic capacities of cork wastewater microbial consortium along with a radiolytic process, in order to promote the recalcitrant compounds degradation. Moreover, this bench scale approach has raised some issues to investigate, namely a basic study of mechanistic insights of radiolytic dynamics of phenolic acids; and an applied research on the valorization of cork wastewater by extraction of phenolic compounds by adsorption onto activated carbon.

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Table 1. Microbial population dynamics for non-irradiated (0 kGy) and irradiated (100 kGy) culture assays during incubation time.

Strain	Morphotype	Phenolic acids cultures															
		0 kGy								100 kGy							
		Incubation time [days]								Incubation time [days]							
		0	3	9	14	24	36	42	46	0	3	9	14	24	36	42	46
A	Gram-positive, oxidase-negative, rods	+	+	-	-	-	-	-	-	+	+	-	-	+	-	-	-
B	Gram-negative, catalase-positive, cocci	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-
C	Gram-positive, oxidase negative, rods	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
D	Gram-negative, Catalase-positive cocci	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

References

(1) Dias-Machado, M.; Madeira, L.M.; Nogales, B.; Nunes, O.C.; Manaia, C.M. *Chemosphere* **2006**, *64*(3), 455-461.

(2) Tundo, P.; Anastas, P. *Green Chemistry: Challenging Perspectives*; Oxford University Press: United Kingdom, 2000.

(3) Melo, R.; Cabo Verde, S.; Branco, J.; Botelho, M.L. *Radiat Phys Chem.* **2008**, *77*, 98-100.

(4) Getoff, N. *Radiat Phys Chem.* **1996**, *47*(4), 581-593.

(5) Whittaker, B.; Watts, M. *Radiat Phys Chem.* **2001**, *60*, 101-110.

(6) American Society for Testing and Materials (ASTM). Practice for using the Fricke reference standard dosimetry system. Pennsylvania: vol. 12.02, 1992.

(7) American Public Health Association (APHA), American Water Works Association (AWWA), Water Pollution Control Federation (WPCF). Standard Methods for the Examination of water and wastewater (20th edition). Washington DC, 1999.

(8) Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. *Methods Enzymol.* **1999**, *299*, 152-178.

(9) Holt J. Bergey's Manual of Determinative Bacteriology (9th edition). Baltimore: Williams & Wilkins, 1994.

(10) Fernandes, A.; Sousa, A.; Mateus, N.; Cabral, M.; Freitas, V. *Food Chem.* **2011**, *125*, 1398-1405.

(11) Santos, S.A.O.; Pinto, P.C.R.O.; Silvestre, A.J.D.; Neto, C.P. *Ind Crops Prod*. **2010**, *31*, 521-526.

(12) Madureira, J.; Melo, R.; Botelho, M.L.; Leal, J.P.; Fonseca, I.M. *Water Sci Technol*. **2013**, *67*(2), 374-379.

(13) Melo, R.; Leal, J.P.; Botelho, M.L. *Rapid Commun Mass Spectrom* **2011**, *25*, 218-222.

(14) Kurucz, C.N.; Waite, T.D.; Otaño, S.E.; Cooper, W.; Nickelsen, M.G. *Radiat Phys Chem*. **2002**, *65* (4/5), 367-378.

(15) Fiorenza, S., Ward, C. H. *J Ind Microbiol Biot* **1997**, *18*, 140-151.

(16) Ortega Méndez, J.A.; Herrera Melián, J.A.; Araña, J.; Doña Rodríguez, J.M.; González Díaz, O.; Pérez Peña, *J. Appl Catal B* **2015**, *163*, 63-73.

(17) Alves, M.J.; Ferreira, I.C.F.R.; Froufe, H.J.C.; Abreu, R.M.V.; Martins, A.; Pintado, M. *J Appl Microbiol* **2013**, *115*(2), 346-357.

(18) Kyriacou, A.; Lasaridi, K.E.; Kotsou, M.; Balis, C.; Pilidis, G. *Process Biochem* **2005**, *40*, 1401-1408.

(19) Ballesteros Martín, M.M.; Sánchez Pérez, J.A.; Casas López, J.L.; Oller, I.; Malato Rodríguez, S. Water Res **2009**, *43*(3), 653-660.

(20) Oller, I.; Malato, S.; Sánchez-Pérez, J.A. *Sci Total Environ* **2011**, *409*, 4141-4166.

(21) Lim, S. J., Kim, T-H. *Sep Purif Technol* **2015**, *146*, 42-49.

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