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Photocatalytic inactivation of bacteria in a fixed-bed reactor: Mechanistic insights by epifluorescence microscopy

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ABSTRACT

The photocatalytic inactivation of *Escherichia coli* bacteria with titanium dioxide immobilised in a fixed-bed reactor has been studied and compared with the results obtained using aqueous titania suspensions. In both cases, the photocatalytic inactivation has been successfully achieved, reaching a 6-log decrease in the concentration of bacteria. The slurry system shows a higher inactivation rate at the beginning of the reaction that decreases progressively, whereas the fixed-bed reactor increases the inactivation rate as the irradiation time increases, leading both catalytic systems to comparable irradiation times for the total inactivation of bacteria, i.e. concentration below the detection limit. This opposite trend seems to be related to differences in the bacteria–TiO_2 interaction, being also observed that the fixed-bed catalytic system is less affected by the competition for the oxidant species of byproducts released after the bacterial cell lysis. Epifluorescence microscopy pictures taken after a dual DAPI/PI staining membrane permeability assay provide mechanistic insights into this different behaviour, showing for the fixed-bed experiments an increase in the damaged bacteria from the very beginning of the reaction, whereas for the slurry system they were not observed until longer irradiation exposition is achieved. These results suggest as hypothesis that although in absolute terms the number of hydroxyl radical attacks could be even lower in the immobilised systems, the damages are more concentrated on the area in which the bacteria–TiO_2 interaction takes place. Although the fixed-bed reactor requires comparable irradiation time for total inactivation of bacteria than that corresponding to the slurry system, it shows minor deactivation and allows the continuous operation without the need of particles removal, making this immobilised system a good alternative to successfully scale-up the photocatalytic disinfection technology.

1. Introduction

Chlorination processes have been traditionally used for disinfection of both drinking water and tertiary treatment of wastewater effluents, involving the expenses related to the dosing of chemical compounds but even more important, the formation of carcinogenic and mutagenic chloro-organic disinfection byproducts [1]. As a consequence, new technologies have to be developed to overcome the drawbacks of the present disinfection processes.

Heterogeneous photocatalysis using TiO_2 as catalyst has been long studied due to the advantages over other advanced oxidation processes (AOPs), such as operation under ambient temperature and pressure, no requirements of pH adjustment or additional oxidant chemicals apart from air, and also the possibility of using solar light as radiation source. Since the early work of Matsunaga et al. [2], titania-based photocatalytic processes have been reported by many research groups [3,4] as alternative technology to inactivate microorganisms successfully.

Most of the reports about photocatalytic disinfection of water sources use TiO_2 slurries [5,6], reaching a high efficiency to inactivate bacteria. Some efforts have been also focused on using immobilised TiO_2 systems [7–12], usually showing to be less active and require more irradiation time compared with TiO_2 slurries due to several problems such as mass transfer limitations and lower titania surface area. Moreover, the immobilised catalysts must keep the stability, avoiding the possible leaching of TiO_2 particles to the solution [13]. However, from an engineering viewpoint, the real application of this technology has to avoid the separation step for a feasible continuous water treatment.

Different reactor configurations of the catalytic material inside the reactor can lead to changes in the bacteria–catalyst interaction and consequently, not only yielding different absolute activities in the photocatalytic inactivation of bacteria at long irradiation time [14], but also variations in the mechanism of bacterial damages generation that could explain the differences in the shape of the inactivation profiles reported in the literature for immo-

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bilised and slurry TiO_2 [7,9]. As certain amount of damages is necessary to cause the irreversible inactivation of the bacteria [15], plots of viable bacteria calculated by counting colony forming units (CFU) on agar plates (commonly used to follow bacterial inactivation experiments) could mask significant changes in the damaging mechanism, which makes interesting the possibility to discriminate the concentration of bacteria with intermediate levels of damage.

This work deals with the photocatalytic inactivation of *Escherichia coli* with TiO_2 in slurry and in a fixed-bed reactor, focusing the study on the novel application of epifluorescence microscopy techniques after a dual DAPI/PI staining membrane permeability assay to provide mechanistic insights into the possible differences between both photocatalytic systems concerning bacteria–TiO_2 interaction.

2. Experimental

2.1. Photoreactor and catalysts

Photocatalytic experiments of bacteria inactivation were carried out using two different catalytic systems: (i) a slurry reactor, using 0.1 g L^{-1} of Degussa P25 TiO_2 in suspension, and (ii) a fixed-bed reactor with Degussa P25 TiO_2 immobilised onto 6 mm × 6 mm glass Raschig rings. The illumination source was a Philips TL 6 W black light lamp placed in the axis of the annular photoreactor. The UV-A incident photon flow, determined by ferrioxalate actinometry, was 2.8×10^{-6} Einstein s^{-1} with a maximum emission peak centred at 365 nm. More details of the experimental setup, the immobilisation procedure and the optimisation of the reactor system can be found elsewhere [16].

2.2. Photocatalytic experiments

E. coli K12 provided by the Colección Española de Cultivos Tipo (CECT 4624, corresponding to ATCC 23631) was used as model microorganism. *E. coli* is frequently used as a model organism in microbiology studies and it is a common faecal contamination indicator to evaluate the microbiological quality of water. K12 is an *E. coli* strain well-adapted to the laboratory environment, and, unlike wild type strains, has lost its ability to thrive in the intestine, making safer the experimental work. Fresh liquid cultures with a stationary concentration around 10^9 colony forming units (CFU) mL^{-1} were prepared by inoculation in a Luria–Bertani nutrient medium (Miller's LB Broth, Scharlab) and incubation at 37 °C for 24 h under constant stirring on a rotary shaker, being diluted to the initial concentration of bacteria required for the experiments. Two different kinds of water have been used: (i) deionised water; and (ii) synthetic municipal wastewater [17] diluted to a total organic carbon value of 15 mg L^{-1} to simulate the effluents of a wastewater treatment plant. The analysis of the samples along the reaction was carried out following the concentration of viable bacteria through a standard serial dilution procedure and agar plating, using eight independent measurements of each sample to obtain statistically significant data. Additionally, key experiments were repeated three times to test the reproducibility of the disinfection results. More details of the procedure followed to prepare the cultures, the initial reaction suspension and the bacterial quantification can be found elsewhere [16].

2.3. DAPI/PI staining permeability assay

The physiological bacterial state was examined by a permeability assay using two nucleic acid stains, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodide, (PI) (Sigma–Aldrich). DAPI is used as specific DNA fluorochrome to stain all DNA bacteria cells (viable and non viable) since it is able to cross uncompromised cell membranes and bind DNA. In contrast, PI is an indicator of the membrane integrity since it can not cross the cell membrane unless it has been damaged or compromised. If the cell membrane is damaged, PI enters the cell and binds to the nucleic acids [18–22]. Therefore, the combination of both stains assess the physiological state of the bacteria by establishing a relationship between staining and membrane integrity. DAPI stock solution was made up to a concentration of 5 mg/5 mL, in sterile deionised water and 0.5 mL of 2.5% glutaraldehyde. PI stock solution was made up to a concentration of 25 mg/25 mL in sterile deionised water. Both of them were stored at 3–5 °C in the dark.

A volume of 80 μL of DAPI and PI (1 mg mL^{-1}) was added to 4 mL of the suspension sample during the photocatalytic experiment, incubated in the dark at room temperature for 10 min and filtered onto 0.22 μm black polycarbonate filters (Millipore). The filters were placed on glass slides, covered with a cover slip and visualised under immersion oil with a 100× objective on a Leica DMI 4000B microscope fitted with a fluorescence attachment ebq-100mc-L and coupled with a Canon Power Shot S80 digital camera. For each sample, three different areas from the inner part of the filter (to avoid edge effects) were randomly chosen, taking two images in each of them using a filter with excitation at 340–380 nm and suppression at 425 nm, and a filter with excitation at 515–560 nm and suppression at 590 nm to visualise DAPI-stained cells and PI-stained cells, respectively. Counting of the cells has been carried out with the help of image processing software (ImageJ 1.42, National Institutes of Health, http://www.nih.gov), averaging the three independent bacteria counting measurements to obtained statistically significant data.

3. Results

3.1. Photocatalytic disinfection experiments

Fig. 1 shows the comparison between the photocatalytic inactivation of aqueous suspensions of increasing concentration of *E. coli* in deionised water using a slurry reactor with TiO_2 in suspension and the immobilised fixed-bed reactor, both catalytic systems using the optimal TiO_2 loading determined previously [16]. TiO_2 suspensions lead to higher inactivation activities for short reaction time, decreasing the rates for longer irradiation time, probably due to the competition for the hydroxyl radical of the compounds released after the bacterial cell lysis. In contrast, the inactivation curves obtained with fixed-bed reactor show a longer initial delay, but for longer irradiation time, the bacterial inactivation is accelerated, reaching a 6-log decay in *E. coli* concentration after an irradiation time comparable to that of the TiO_2 slurry. This somewhat unexpected high activity of the immobilised system might be due to a relatively high active surface given titania supported on the Raschig rings placed in the whole photoreactor volume, in comparison with other immobilised systems such as wall reactors [7,16].

As the initial concentration of *E. coli* increases, more irradiation time is required to reach the total inactivation below the bacterial detection limit, in agreement with other authors [23–25]. These results were expected, as a higher concentration of bacteria obviously requires a higher number of •OH radical attacks to be inactivated. However, it is observed that an increase in the initial concentration of bacteria does not lead to a proportional increase in the irradiation time required to reach a complete inactivation. Therefore, there is not a linear correlation between the amount of inactivated bacteria and the amount of hydroxyl radicals generated, which can be assumed to be proportional to irradiation time. In fact, plots of inactivation profiles in relative terms with respect to the initial concentration of bacteria (insets of Fig. 1) clearly overlap,