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Photocatalytic bactericidal effect of TiO_2 on *Enterobacter cloacae* Comparative study with other Gram (−) bacteria

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Abstract

The bactericidal action of heterogeneous photocatalysis (UV-A/TiO_2) has been tested on *Enterobacter cloacae*, a microorganism very resistant to UV-A irradiation. Results have been compared with other representative strains of Gram (−) bacilli of different photosensitivity like *E. coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*. The TiO_2 photocatalytic technology can inactivate bacteria resistant to oxidative membrane damage caused by direct UV irradiation, like *E. cloacae*, a common soil and aquatic microorganism, which normally is not affected by low UV-A irradiation intensity. In all cases, sublethal UV-A doses provoked an important lethality in the presence of TiO_2. Inactivation rates of the microorganisms are compared and some clues on the mechanism of bacteria destruction are discussed.

Keywords: Heterogeneous photocatalysis; TiO_2; Disinfection; *Enterobacter cloacae*; Gram (−) bacilli

1. Introduction

Ultraviolet radiation deleterious effects on bacterial cells have been long recognized and its applications on antimicrobial process have received great attention. The most energetic fraction of the ultraviolet spectra, corresponding to the UV-C range (200–290 nm), is commonly used as an antibacterial agent in water and air treatments, allowing effective disinfection rates by the employment of germicidal lamps (254 nm). Furthermore, photo-induced bacterial inactivation caused by UV-A (320–400 nm) is well known and its lethal and sublethal effects have been studied by several workers [1–5]. Heterogeneous photocatalysis, an Advanced Oxidation Technology that uses UV and TiO_2, has emerged in last years as an innovative method for water treatment. The potential applications of the technology include organic matter degradation, abatement of metal toxic ions and water disinfection ([6–12] and references therein). Moreover, UV/TiO_2 has been proposed as one of the best disinfection technologies, because no dangerous (carcinogenic or mutagenic) or malodorous halogenated compounds are formed, in contrast with other disinfection techniques, e.g. those that use halogenated reagents.

The antimicrobial activity of UV/TiO_2 has been essayed in several bacteria and viruses including *Escherichia coli* [10,16,17], *Lactobacillus acidophilus* [13], *Serratia marcescens* [10,16], *Pseudomonas aeruginosa* [16], *Pseudomonas stutzeri* [18], *Bacillus pumilus* [19], *Streptococus mutans*, *Streptococus rattus* and *Streptococus cricetus* (references in [12]), *Streptococus sobrinus AHT* [20], *Deinococcus radiophilus* [13,21], yeasts as *Saccharomyces cerevisiae* [13], algae as *Chlorella vulgaris* [13], and viruses such as phage MS2 [13,21,22], *B. fragilis bacteriophage* [13,21] and *Poliovirus 1* [23]. Transparent TiO_2 films [24], TiO_2 immobilized in acetylcellulose membranes [14] and entrapment of TiO_2 into sol–gel prepared pellets [21] have been tested, and use of optical fibers [25] or intermittent and variable irradiation [21] have been also recommended to improve the application. Municipal wastewaters have been also treated with relatively good efficiency [26,27] and total and fecal coliforms and viruses present in secondary wastewater effluents have been successfully removed [23]. The technology can even be applied to destroy bioaerosols in air [28,29]. As TiO_2 photocatalysis can make use of the UV part of the solar spectrum, it becomes promising to potabilize waters in developing tropical countries

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with scarce hydric resources and high availability of solar irradiation [17,26,27].

In this paper, the lethal efficiency of UV/TiO_2 on *Enterobacter cloacae*, a common soil and aquatic microorganism, which has been found previously a microorganism very resistant to UV-A exposure [30], has been studied. We compare the bactericidal capability of the technology with that on already studied microorganisms of different photosensitivity, such as the model *E. coli* and other bacteria, i.e. *P. aeruginosa* and *Salmonella typhimurium*.

2. Experimental

2.1. Materials

TiO_2 (Degussa P-25) was a gift from Degussa A.G. (Germany). The sample used in this particular work contained 80% anatase and 20% rutile, as determined by DRX. All other reagents were at least of reagent grade and used without further purification. Water was double distilled in a quartz apparatus. All solutions and materials were sterilized by autoclaving.

2.2. Bacterial strains

E. coli K-12 ATCC 15153, *E. cloacae* 29C/M-A4 K.F. Mayer UCL Berkeley, *P. aeruginosa* ATCC 27853 and *S. typhimurium* LT-2 were used. All strains were maintained on nutrient agar slants, and stock cultures were transferred at monthly intervals.

2.3. Culture conditions

Bacterial cells were incubated at 37 °C in a shaking incubator in Luria–Bertani (LB) broth. The overnight culture was harvested by centrifugation, washed, and diluted in bidistilled water to give a cell concentration of approximately 10^6–10^7 colony-forming units (cfu)/ml.

2.4. Irradiation source

A high intensity long-wave (highest emission at 365 nm) ultraviolet lamp (model B-100 A, Ultraviolet Products, San Gabriel, CA) was employed. The incident photon flux, measured with a NJ 9811-58 radiometer (Cole-Parmer Instruments Co., Chicago, IL), was 5.5 mW/cm^2 except for *P. aeruginosa* cells, which were exposed to 1.4 mW/cm^2.

2.5. Irradiation procedure

A TiO_2 aqueous suspension was ultrasonicated with a TEST LAB sonicator at 40 kHz for 7 min. This ultrasonicated suspension was added to the bacterial suspension immediately prior to the reaction in order to have a final 0.1 g/l TiO_2 concentration. Forty milliliter of this suspension in a vessel (4 cm diameter, 3.2 cm liquid height), placed in an ice bath and open to the air, was irradiated with the UV-A lamp from above, while keeping constant and gentle magnetic stirring. The initial pH was the natural pH of the suspensions, 5.8–6.0 in all cases. A bacterial control without TiO_2 was also run, while another TiO_2 control was kept in the dark. Samples (0.1 ml) were withdrawn periodically, and the number of viable cells was determined by plating appropriate dilutions (in 0.9% NaCl) of control and treated cells on Bacto nutrient agar. Immediately after spreading, triplicate plates were incubated at 37 °C and the colonies counted after 24 h. Photocatalytic experiments were performed at least by triplicate, with good reproducibility of results.

3. Results and discussion

It is well known that direct UV-A irradiation produces deleterious effects in bacteria cells, with different sensitivity to the radiation depending on the type of bacteria and amount of light doses ([1–3] and references therein). It has been found, for example, that UV-A irradiation causes cell death in *P. aeruginosa* at doses at which *E. coli* or *E. cloacae* cell viability is not affected [31]. In the present work, irradiation in the presence of TiO_2 induced, under sublethal doses, important values in loss of viability, showing the high bactericide capability of the procedure.

In order to evaluate the activity of the UV/TiO_2 disinfection technology, Gram (−) rods were exposed to UV-A (365 nm) in the presence of 0.1 g/l of the photocatalyst. This amount of TiO_2 is a generally recognized optimum value for *E. coli* inactivation that avoids TiO_2 interference effects with cells [10,17]. Although this amount could be different for the other microorganisms, the reaction has been performed at the same catalyst concentration for the sake of comparison. Initial pH, which varied between 5.8–6.0, was not adjusted, as it is a normal value of real wastewaters and in the range reported in other papers [10,17,23,24]. Without exposure to UV-A, TiO_2 has no deleterious effects on the bacterial cells, as usually reported [10,25,26]. Blanks in the absence of TiO_2 revealed that cells were not directly affected by light under the present irradiation conditions (see however *P. aeruginosa* case).

In all the studied strains, the viability loss was higher than 99.9% after 40 min of irradiation, indicating an almost total cell inactivation (Table 1).

E. coli, as said before, has been thoroughly studied as the model microorganism to test the loss of cell viability when submitted to the photocatalytic treatment. We confirmed this phenomenon, shown as a very important decrease (5 orders of magnitude in 40 min) in viable cell counts when exposed to a 5.5 mW/cm^2 UV-A photon flux in the presence of TiO_2 (Fig. 1). Similar results (more than 4 orders of magnitude in 40 min) were obtained when *S. typhimurium* was exposed to the same conditions (Fig. 2).

Table 1
Percentage of viability loss after 40 min of irradiation and first-order rate constants for the lethality of the cells submitted to UV-A light in the presence of TiO_2

Bacterial strain	%Viability loss	k (min^{-1})
E. coli K-12	99.999	0.29
S. typhimurium LT-2	99.996	0.29
P. aeruginosa ATCC 27853	99.943	0.23
E. cloacae 29C/M-A4	99.974	0.23

Conditions: $[TiO_2] = 0.1$ g/l, photon flux $= 5.5$ mW/cm^2, except for *P. aeruginosa* cells, which were exposed to 1.4 mW/cm^2.

The great sensitivity of *P. aeruginosa* to UV-A was reported previously [31]. For this reason, suspensions of these bacteria were exposed to a lower UV-A intensity than the other bacteria, i.e. 1.4 mW/cm^2, assuring in this way the absence of cellular death due only to irradiation effects. The results show a rapid decrease in the colony-forming ability with time (more than 3 orders of magnitude after 40 min) in the presence of TiO_2. The control without TiO_2 confirmed the absence of lethality (Fig. 3).

As it was reported, *E. cloacae* are more resistant to UV-A effects than other Gram (−) bacilli [30]. However, when *E. cloacae* was exposed to a 5.5 mW/cm^2 of UV-A photon flux in the presence of TiO_2, a very important bacterial cell lethality was detected, reaching a reduction of almost 4 orders of magnitude after 40 min exposure (Fig. 4).

One distinctive feature of all profiles is the exponential decrease of viability with time, already observed in other cases [19,23]. From the plots, first-order constants for the four cases have been obtained, with rather good correlation coefficients, which are presented in Table 1. The results indicate that *E. coli* and *S. typhimurium* present similar lethality rates, and that *P. aeruginosa* is a very sensitive microorganism, because it presents a similar rate when submitted to a four-fold lower irradiation intensity than the other bacteria. Concerning *E. cloacae*, it can be observed that the resistance

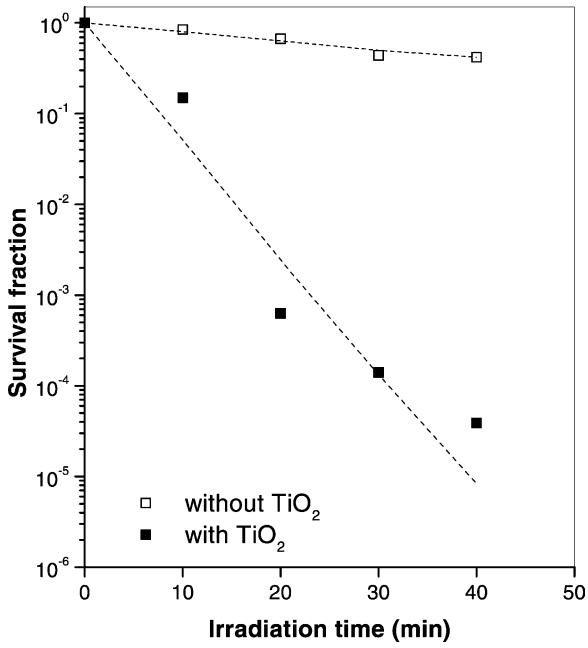

Fig. 2. Survival curves for *S. typhimurium* LT-2, exposed to UV-A irradiation (365 nm) in the presence of TiO_2. Conditions: $[TiO_2] = 0.1$ g/l, photon flux $= 5.5$ mW/cm^2. Dashed lines are first-order fittings.

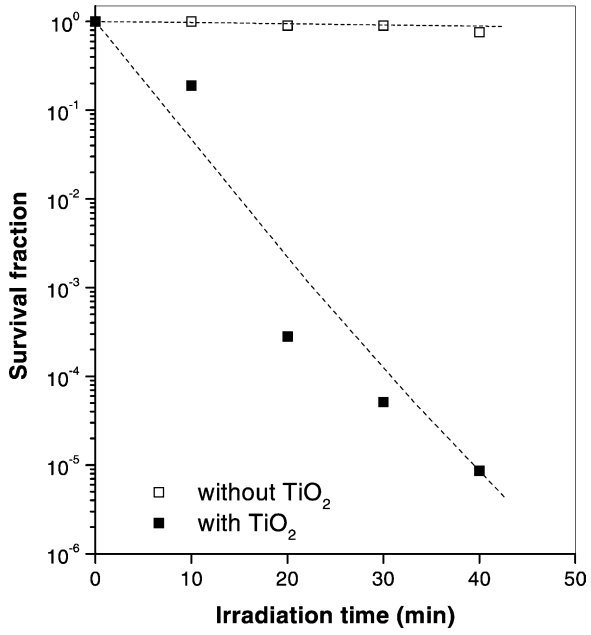

Fig. 1. Survival curves for *E. coli* K-12, exposed to UV-A irradiation (365 nm) with and without TiO_2. Conditions: $[TiO_2] = 0.1$ g/l, photon flux $= 5.5$ mW/cm^2. Dashed lines are first-order fittings.

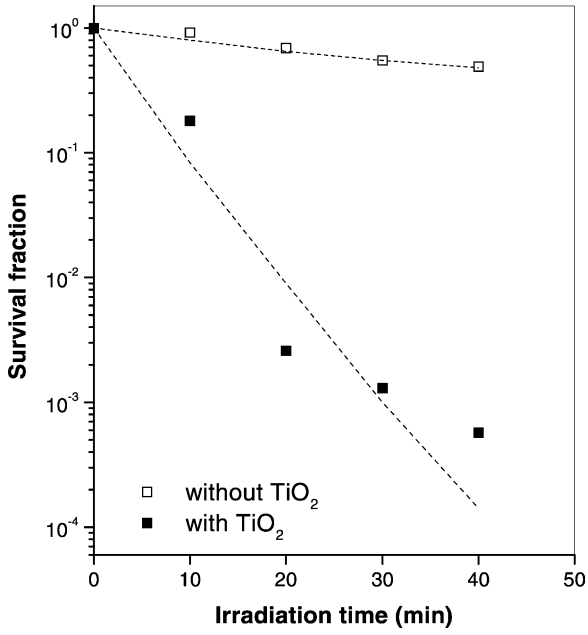

Fig. 3. Survival curves for *P. aeruginosa* ATCC 27853, exposed to UV-A irradiation (365 nm) in the presence of TiO_2. Conditions: $[TiO_2] = 0.1$ g/l, photon flux $= 1.4$ mW/cm^2. Dashed lines are first-order fittings.

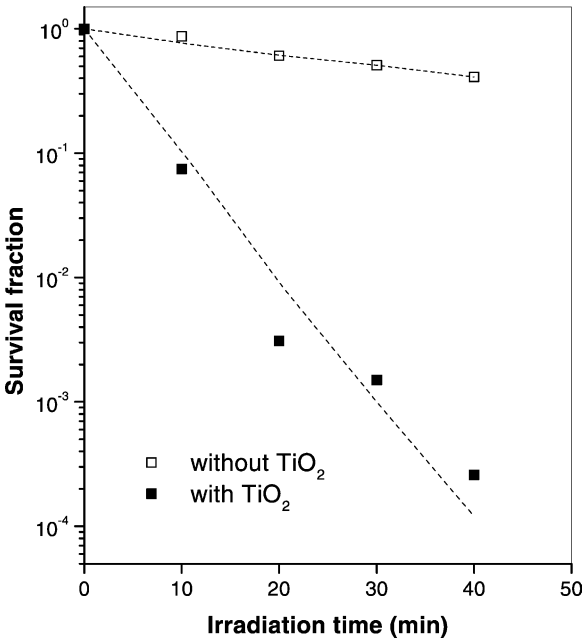

Fig. 4. Survival curves for *E. cloacae* 29C/M-A4, exposed to UV-A irradiation (365 nm) in the presence of TiO_2. Conditions: $[TiO_2] = 0.1$ g/l, photon flux = 5.5 mW/cm^2. Dashed lines are first-order fittings.

to the UV-A irradiation is overcome in the presence of TiO_2, with a similar lethality rate as in the case of the other cells. First-order kinetics has been also reported by Wei et al. [32] for *E. coli*, but the assayed strain, the experimental setup, the light intensity and other experimental conditions in that paper are very different from ours to compare the absolute rate values.

The exposure of bacteria to UV-A radiation can cause severe alterations to the membrane structure including changes in membrane-bound enzyme activities, metabolic pathways, transport systems and permeability alterations leading to bacterial cell death [4,5,31]. In the present work, very low sublethal UV-A intensities were used, which do not produce ordinarily this type of alterations. However, the presence of TiO_2 during the irradiation caused a very important bacterial inactivation in all Gram (−) bacilli assayed. The antimicrobial photobiological activity of TiO_2 on bacterial cells using UV-A irradiation in an oxygen atmosphere has been attributed to the generation of very active free radical species called ROS (reactive oxygen species), but the nature of these species remains controversial [33]. In fact, hydroxyl radicals ($HO^\bullet$) and superoxide anions ($O_2^{\bullet -}$) are considered the main generated species in the anodic and cathodic pathways, respectively, of photocatalytic processes in the presence of oxygen [12,15,19,24], both species known to be highly reactive with biological samples. Other oxygen reactive species have been also proposed, including hydrogen peroxide (H_2O_2), hydroperoxyl radical ($HO_2^\bullet$) and singlet oxygen ($^1O_2$) [12,24,34]. Chromosomal aberration by DNA lesion caused by photoexcited TiO_2 was also reported [35]. Although a thorough study has not been made and the mechanism of the process is still unknown [12], there are some suggestions and evidences of the steps leading to cell inactivation. In the earliest papers, the bactericide activity was attributed to the inhibition of respiration by decrease of Coenzyme A and formation of its dimer [13]. Later, photo-induced alterations, caused by ROS and inducing significant disorder in cell membranes (e.g. in streptococci [20]), were demonstrated by rapid leakage of potassium ions and slow release of protein and DNA, leading ultimately to cell wall breakdown and complete cell death. Kikuchi et al. [24] suggested that hydroxyl radicals are not the only species responsible for the bactericidal effect, but that also the cooperative action of hydrogen peroxide together with superoxide radical might be important. The authors propose that long-range interactions between the active species and the cells, due to the larger bacteria size are necessary for the occurrence of photocatalytic processes in bacterial systems.

Recent works attribute the TiO_2 photocatalytic action to promoted peroxidation of phospholipid components of the lipid membrane, inducing cell membrane disorder, followed by loss of essential functions as respiratory activity and cell death [32,33,36]. The most direct evidence of membrane damage was described by Sunada et al. [37], based on the simultaneous photocatalytic destruction of the endotoxin produced by *E. coli* cells. In any case, as a large extent of mineralization could be measured through the amount of CO_2 produced during the photocatalytic process, a strong action of $HO^\bullet$ on the microorganism is suggested, with SEM and ^{14}C radioisotope labeling experiments confirming this hypothesis [38]. Other investigations on *E. coli* also revealed cell mineralization through TOC measurements of the bacteria suspension before and after the treatment [27].

The results here obtained indicate the high efficiency of the procedure, particularly in the inactivation of the highest resistant bacteria. As mentioned above, *E. cloacae* cells are very resistant to the oxidative membrane damage when exposed to UV-A, and this observation was attributable to the existence of an efficient antioxidative defense system [30]. However, the photocatalytic UV/TiO_2 treatment produced a great increase in bacterial death even under sublethal UV-A intensities. The results here informed reinforce the hypothesis of the participation of very active species as $HO^\bullet$ radicals, which are generating an important oxidative potential able to overcome the antioxidative response of *E. cloacae*. The fact that under our conditions three different microorganisms (we do not include here the sensitive *P. aeruginosa*) present very similar lethality constant rates is indicative that non selective oxidative species as $HO^\bullet$ are responsible for cell attack, no matter the inherent traits of each microorganism. This seems however to be not a general feature, as other even less sensitive microorganisms like *D. radiophilus* show kinetic inactivation delay and need oxygen supplementation to attain good destruction yields [13].

In spite of the fact of the largely demonstrated ability of the UV/TiO_2 technology, some considerations should be

kept in mind for the successful application of the process. The inherent traits of wild microorganisms must be taken into account as much as the influence of the environmental conditions in their sensitivity or resistance to stressing agents such as UV radiation [39]. Moreover, although compounds toxic for bacteria may be not initially present in waters, photocatalytic processes can give rise to new noxious products. Also, the presence of inorganic-radical scavengers or organic matter competing with cells by the oxidative photogenerated species, can inhibit cell lethality [10,15,22], provoking light-filter effect or even acting as nutrients [27]. In addition, regrowth of bacteria has been observed after crude water treatment, showing the lack of residual effect of the TiO_2 photocatalytic process [40]. The possibility of reducing bacterial recovery and enhancing disinfection rates by application of photoelectrocatalysis, i.e. with an electrical bias to the working electrode, has been recently considered [36].

4. Conclusions

The photocatalytic UV/TiO_2 system was tested for its bactericide action against representative strains of indicative bacilli of bacterial contamination (*E. coli* and *S. typhimurium*) and/or common soils and aquatic rods (*E. cloacae* and *P. aeruginosa*) cells. High efficiency has been found in the case the studied microorganisms, particularly for the very resistant *E. cloacae*, which cannot be inactivated in the absence of TiO_2. Here, exposure to sublethal UV-A doses provoked an important lethality for all tested bacilli, with similar efficiencies independently of the inherent microorganism features. Not only a high effect in the first minutes of irradiation was observed, but also the overall ability was increased.

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