Photochemistry and Photobiology, 2014, 90: 609–614

Inactivation of *Bacillus subtilis* Spores Using Various Combinations of Ultraviolet Treatment with Addition of Hydrogen Peroxide

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Received 28 May 2013, accepted 4 November 2013, DOI: 10.1111/php.12210

ABSTRACT

This study aims at comparing the inactivation of *Bacillus subtilis* spores by various combinations of UV treatment and hydrogen peroxide (H_2O_2) addition. The combinations included sequential (UV–H_2O_2, H_2O_2–UV) and simultaneous (UV/H_2O_2) processes. Results showed that *B. subtilis* spores achieved a certain inactivation effect through UV treatment. However, hardly any inactivation effect by H_2O_2 alone was observed. H_2O_2 had a significant synergetic effect when combined with UV treatment, while high irradiance and H_2O_2 concentration both favored the reaction. When treated with 0.60 mM H_2O_2 and 113.0 μW/cm^2 UV irradiance for 6 min, the simultaneous UV/H_2O_2 treatment showed significantly improved disinfection effect (4.13 log) compared to that of UV–H_2O_2 (3.03 log) and H_2O_2–UV (2.88 log). The relationship between the inactivation effect and the exposure time followed a typical pseudo-first-order kinetics model. The pseudo-first-order rate constants were 0.478, 0.447 and 0.634 min^{-1}, for the UV-H_2O_2, H_2O_2–UV and UV/H_2O_2 processes, respectively, further confirming the optimal disinfection effect of the UV/H_2O_2 process. The disinfection could be ascribed to the OH radicals, as verified by the level of *para*-chlorobenzoic acid (*p*CBA).

INTRODUCTION

Ultraviolet (UV) treatment has been regarded as an alternative method to chlorine treatment, owing to its high effectiveness to a wide variety of waterborne microorganisms (1,2). This process leads to the inactivation of the microorganism by hindering the replication and multiplication of the cell or virus (3,4). Dai *et al.* (5,6) utilized UVC irradiation to prevent the *Candida albicans* infection and the central venous catheter-related infection. However, there are still some microorganisms that are resistant to conventional UV treatment. Therefore, high UV doses are required to achieve a certain inactivation credit (7). Ko *et al.* (8) reported that the adenovirus serotype 41 showed high UV resistance, in that a UV dose of ~222 mJ cm^{-2} was required for a 4-log reduction. In addition, as a physical process, UV treatment lacks the capability of continuous disinfection (9). As a result, it is important to develop innovative ways of optimizing UV treatment technology against microorganisms.

Recently, researchers have paid more attention to advanced oxidation processes (AOPs), for their capabilities to generate highly reactive hydroxyl radicals (·OH) (10). Among these AOPs, UV/H_2O_2 process has been widely studied due to its high oxidability to the nondegradable contaminants, such as disinfection by-products (DBPs) (11), natural organic matter (NOM) (12), herbicides (13) and pharmaceuticals (14) in drinking water and wastewater. UV/H_2O_2 has also been utilized as a suitable method to prevent biofilm formation in natural waters (15). Nevertheless, the effectiveness of this process in microorganism inactivation is still under debate. Mamane *et al.* (16) reported that UV/H_2O_2 did not exhibit any inactivation effect to *Bacillus subtilis* spores. However, Bounty *et al.* (17) believed that the UV/H_2O_2 process was much more efficient than UV alone, as the addition of 10 mg L^{-1} H_2O_2 reduced the required UV dose from 200 to 120 mJ cm^{-2} for a 4 log reduction of adenovirus. Teksoy *et al.* (18) demonstrated that UV/H_2O_2 was significantly effective in inactivating *Escherichia coli* and *Pseudomonas aeruginosa*; however, it exhibited low efficiency in *B. subtilis* spore inactivation. The differences may be concerned with various types of microorganisms, various kinds of UV sources, diverse UV doses and dissimilar microorganism initial concentrations (19).

As a kind of Gram-positive bacterium (20), *B. subtilis* spores can survive under extreme unfavorable environments (21). Compared with its vegetative cells, *B. subtilis* spores were reported 5–10 times more resistant to UV treatment (22). This study aimed at comparing the effectiveness of UV and H_2O_2, used individually and with various combinations. Meanwhile, the contribution of the OH radical, as well as the relationship between the inactivation effect and the concentration–time product (CT) values was investigated.

MATERIALS AND METHODS

Experimental apparatus. The apparatus used in the experiments was a collimated beam apparatus containing a 40 W low-pressure (LP) mercury lamp (Philips, The Netherlands), as illustrated in Fig. 1. The monochromatic UV radiation emitting by this lamp was directed to the surface of the test samples. By virtue of a UV-M radiometer (Beijing Normal University Experiment Co., China), the average irradiance at the solution surface was examined (1). In addition, as UV irradiance depends on several parameters such as solution volume and solution absorbance, these factors were taken into account following the separate Excel spreadsheets, which is available at www.iuva.org. The irradiance at the solution surface in this study was 113.0, 56.5 and 28.3 μW cm^{-2}, respectively.

Materials. Reagent grade *para*-chlorobenzoic acid (*p*CBA) was supplied by Sigma Company (Germany), and all the other reagents were

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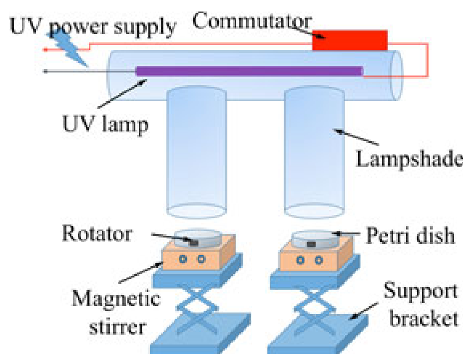


Figure 1. Schematic diagram of collimated beam apparatus.

provided by Sinopharm Chemical Reagent Company Limited (China). Distilled water for analytical use was from Direct-Q3 (MilliPore, USA).

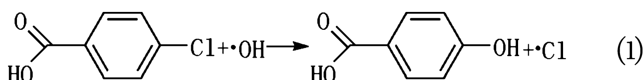
B. subtilis spores culture and enumeration. Pure cultured *B. subtilis* spores (ATCC 9372), provided by China General Microbiological Culture Collection Center, were rehydrated aseptically with Nutrient Broth (peptone 10 g L^{-1}, sodium chloride 5 g L^{-1} and beef extract 3 g L^{-1}). Broth cultures were incubated in a shaker at 37°C for 24 h and then in sporulation medium (yeast extract 0.7 g L^{-1}, glucose 1 g L^{-1}, peptone 1 g L^{-1}, magnesium sulfate heptahydrate 0.2 g L^{-1} and vitriolic 0.2 g L^{-1}) at 37°C for 48 h. Almost 90% of the vegetative cells with spores were centrifuged (2700 ***g***, 10 min) and redissolved in physiological salt solution. Then the bacterial suspension received heat shocked (80°C, 10 min) to kill the remaining vegetative cells. Cell densities of 10^6–10^7 colony-forming units per milliliter (CFU mL^{-1}) were harvested.

After disinfection, the spores were serially diluted depending on the order of magnitudes. Then 0.1 mL of the suspension was injected onto nutrient agar medium, and incubated with nutrient agar medium (37°C, 24 h) to enumerate the spores, using a pour plate method (23). Each experiment was carried out in triplicate and all experimental steps were carried out in an aseptic manipulation room, to prevent *B. subtilis* spores from being exposed to air.

Different combination modes of UV and H_2O_2. Petri dishes (90 mm diameter) with 40 mL samples were exposed to the UV in the collimated beam apparatus and stirred gently by a magnetic stirring apparatus. H_2O_2 (30%) was diluted and added to the samples to achieve final concentrations of 0.15 and 0.60 mM. The concentration was decided referring to some published article (16,17,24), which used UV/H_2O_2 process to inactivate microorganism. As soon as the time was finished, 1 mL sodium thiosulfate solution of 1 g L^{-1} was added into samples to quench the remaining H_2O_2 and cease further oxidation process.

The combination process was performed based on UV disinfection, with the addition of H_2O_2, including sequential disinfection UV–H_2O_2 (UV followed by H_2O_2), H_2O_2–UV (H_2O_2 followed by UV) and simultaneous treatment UV/H_2O_2. In the UV–H_2O_2 process, petri dishes containing spores were initially exposed to UV irradiation. When the designed exposure time (1 up to 10 min) was finished, the petri dishes were kept in dark place and injected with H_2O_2. The H_2O_2 contact time was equated to the corresponding UV exposure time. The H_2O_2–UV process was carried out by revising the order of disinfectant, namely, H_2O_2 as the pretreatment and UV irradiation as the secondary disinfectant. The UV exposure time was equivalent to corresponding H_2O_2 contact time (1 up to 10 min) as well. In the UV/H_2O_2 process, H_2O_2 was injected with simultaneous UV irradiation into the petri dish containing the spores. The designed reaction time was 1 up to 10 min.

Determination of the OH radical concentration. *p*CBA has been used as an OH radical probe, as it can react rapidly with OH radicals (25), and slowly with H_2O_2 (16,17). The chemical equation was shown as Eq. (1)



By means of determining the *p*CBA degradation rate (26,27), the OH radical steady-state concentration ($[OH]_{ss}$, M) was determined indirectly according to Eq. (2) (25):

$$-d[pCBA]/dt = k_{OH,pCBA}[pCBA][OH]_{ss} \quad (2)$$

where $k_{OH,pCBA}$(=5 × 10^9 M^{-1} s^{-1}) refers to the rate constant of *p*CBA with OH radical.

Rearranging and integrating Eq. (2) (17), the expression becomes

$$[OH]_{ss} = k_{exp}/k_{OH,pCBA} \quad (3)$$

where k_{exp} (s^{-1}) refers to the decay rate slope of *p*CBA, namely, dln ($[pCBA]_0/[pCBA]$)/d*t*.

Analytical methods. The concentration of *p*CBA was detected via an ultrahigh-performance liquid chromatography system (ACQUITY UPLC H-Class) equipped with a UV detector at 240 nm and a C-18 (1.7 µm, 100 mm × 2.1 mm,) reversed-phase column (ACQUITY UPLC BEH), with an injection volume of 2 mL and an initial concentration of 1 mg L^{-1} (28). A solvent mixture of acetonitrile and ultrapure water (70:30) was applied as the mobile phase at the flow rate of 0.5 mL min^{-1}. The initial concentration of H_2O_2 was detected by the iodide method (29).

Data presentation. The level of disinfection is usually analyzed by the inactivation effect *vs*. the exposure time (7). Both the shoulder and the tail region were not observed due to the chosen time internal in this study. Therefore, a linear relationship between the effect and the time is based on the first-order model, described as follows:

$$\log_{10}(N_0/N) = kt \quad (4)$$

where, N_0 and N are microbial concentrations (CFU mL^{-1}) before and after disinfection. *k*, the slope of the line, is the pseudo-first-order rate constant (min^{-1}).

RESULTS AND DISCUSSION

Effect of UV treatment alone

Figure 2 shows the response curve on *B. subtilis* spores inactivation by UV treatment alone. As illustrated, the inactivation effect of *B. subtilis* spores increased with the increasing irradiance. About 3.85 log was achieved at irradiance of 113.0 µW cm^{-2} after 10 min. Meanwhile, the inactivation effect resulted in a 1.28-log reduction with UV irradiance decreasing to 28.3 µW cm^{-2}, indicating that high irradiance was imperative in UV disinfection process.

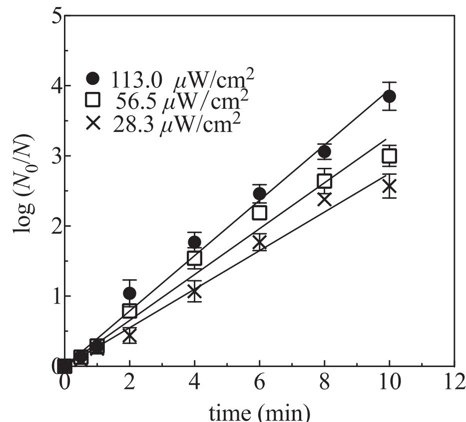


Figure 2. Effect of UV irradiance on *B. subtilis* spores inactivation by UV treatment alone. Error bars represent the standard deviations. UV irradiances were 113.0, 56.5 and 28.3 µW cm^{-2}.

Table 1. Effect of contact time on *B. subtilis* spores inactivation by H_2O_2 treatment alone. H_2O_2 concentration was 0.60 mM.

Contact time (min)	1	2	4	6	8	10	15	20
Inactivation effect (log)	0.014	0.037	0.128	−0.027	0.089	0.085	0.175	0.067

The effect of UV irradiance has been reported in microcystin-LR removal (30), DNA photorepair of *E. coli* (31) and dimethyl phthalate (DMP) degradation (32) by UV/H_2O_2. Benabbou *et al.* (33) reported that the inactivation effect decreased with the irradiance in *E. coli* inactivation by UV treatment and UV/TiO_2. Wu *et al.* (34) found the same tendency in *Dunaliella salina* inactivation using UV/Ag-TiO_2/O_3 process. This phenomenon may be explained by the increasing amount of radiation photon, which was brought about by higher UV irradiance. Accordingly, it resulted in less possibility to repair the injured enzymes for bacteria (35,36).

Effect of H_2O_2 alone in the dark

The effect of H_2O_2 alone in the dark on *B. subtilis* spores inactivation was investigated at final concentration of 0.60 mM for 1 up to 20 min. The results showed that almost no inactivation effect was observed during the process (Table 1). Similarly, this result was confirmed by several other studies. Mamane *et al.* (16) showed that H_2O_2 dose of 25 mg L^{-1} at a contact time of 60 min did not exhibit any inactivation effect on *E. coli, B. subtilis* spores and MS-2, T4 and T7 phage. Meanwhile, none of total coliforms, fecal coliforms or *E. coli* performed any inactivation effect when exposed to H_2O_2 for 30 min at a concentration of 20 mg L^{-1}, as described by Bianchini *et al.* (24).

Effect of different combination modes of UV and H_2O_2

The effects of UV–H_2O_2 H_2O_2–UV and UV/H_2O_2 were investigated and compared, at UV irradiance of 113.0 μW cm^{-2}. As illustrated in Fig. 3a, the inactivation effect of *B. subtilis* spores achieved a certain augment through UV–H_2O_2 than UV treatment alone. For instance, the inactivation effect after 10 min at 0.15 and 0.60 mM H_2O_2 was 4.22 and 4.40 log, namely, 0.37 and 0.55 log higher than UV treatment alone. However, only a slight increase in inactivation effect was observed by H_2O_2–UV (Fig. 3b). The inactivation effect after 10 min at 0.15 and 0.60 mM H_2O_2 was 4.07 and 4.18 log, namely, only 0.22 and 0.33 log higher than UV treatment alone. According to Fig. 3c, the inactivation effect increased statistically significant by UV/H_2O_2 simultaneous disinfection. Take contact time of 10 min for instance, the inactivation effect with 0.15 and 0.60 mM H_2O_2 increased remarkably to 4.92 and 5.85 log, namely, 1.07 and 2.0 log higher than UV treatment alone. All the three processes followed the tendency that higher inactivation effect was received at higher H_2O_2 concentration.

On the basis of Fig. 3a–c, the profiles of the experimental data were all fitted to pseudo-first-order kinetics model. To make a striking comparison, the pseudo-first-order rate constants (k) as well as the correlation coefficient (r^2) of each combination modes were exhibited, at UV irradiance of 113.0 μW cm^{-2} (Table 2). As presented, rate constants varied at different combination modes. For example, at 0.60 mM H_2O_2, the rate constants were 0.476, 0.445 and 0.631 min^{-1}, for UV–H_2O_2, H_2O_2–UV and UV/H_2O_2 process, respectively. Clearly, the rate constants followed the order: UV/H_2O_2 > UV–H_2O_2 > H_2O_2–UV > UV.

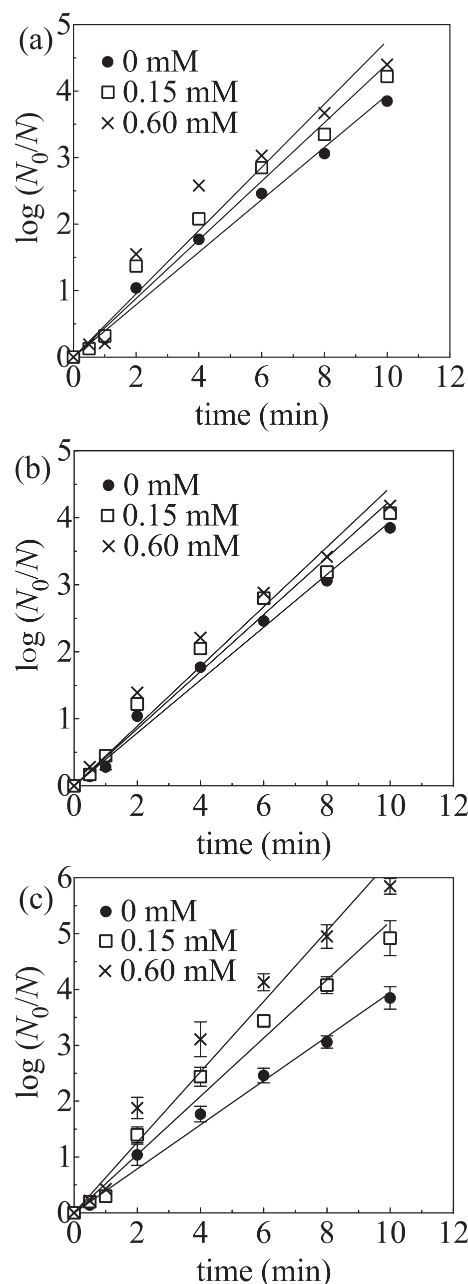


Figure 3. Effect on *B. subtilis* spores inactivation by: (a) UV–H_2O_2, (b) H_2O_2–UV and (c) UV/H_2O_2. UV irradiance was 113.0 μW cm^{-2}. H_2O_2 concentrations were 0.15 and 0.60 mM. Error bars represent the standard deviations.

In addition, the rate constant k increased with the increasing H_2O_2 concentration. Take UV/H_2O_2 process, for example, with H_2O_2 concentration increased from 0.15 to 0.60 mM, k jumped accordingly from 0.522 to 0.631 min^{-1}. The limited inactivation efficiency was attributed to low H_2O_2 concentration produced inadequate OH radicals in the reactions.

Table 2. Fitting parameters of pseudo-first-order kinetics model on *B. subtilis* spores inactivation under different combination modes of UV and H_2O_2. UV irradiance was 113.0 μW/cm^2.

Process	$[H_2O_2]$ (mM)	Fitted equation	r^2
UV	0	$y = 0.394x$	0.990
UV–H_2O_2	0.15	$y = 0.441x$	0.974
H_2O_2–UV	0.15	$y = 0.426x$	0.975
UV/H_2O_2	0.15	$y = 0.522x$	0.981
UV–H_2O_2	0.60	$y = 0.476x$	0.948
H_2O_2–UV	0.60	$y = 0.445x$	0.966
UV/H_2O_2	0.60	$y = 0.631x$	0.969

The reasons for the increase effect of different combination modes can be explained as follows.

For H_2O_2–UV process, despite a limited inactivation effect, H_2O_2 was able to remove protein from spore coats and cause damages of cell structure. Khadre and Yousef (37) found that H_2O_2 resulted in extraction of spore coat material and finally disrupted the spore coats. Zdolsek *et al.* (38) also indicated that H_2O_2 led to an evident and rapid reduce in cell viability and destructed the lysosomal integrity. The injured bacteria became vulnerable when treated with UV irradiation afterward (39). The difference between H_2O_2–UV and UV was not significant, indicating that applying H_2O_2 as a pretreatment did not apparently favor the secondary treatment.

For UV–H_2O_2 process, there are several different possibilities accounting for the mechanism. To begin with, UV treatment was able to destroy the cell membrane, which was the outmost layer and firstly exposed to UV irradiation, as confirmed by Liu *et al.* (40) and Wu *et al.* (41). In addition, UV irradiation may damage the DNA in spores by generating a lethal spore photoproduct, methylene-bridged thymine dimer (5-thyminyl-5,6-dihydrothymine) (42). Wang *et al.* (19) proposed that the spore photoproduct resulted in the inactivation of the spores under UV irradiation. Moreover, the catalase activity was likely to be considerably lessened under UV irradiation (43,44), giving rise to difficulties in protecting the spores from H_2O_2 damage (45). Thus, when treated with H_2O_2 afterward, it was convenient for H_2O_2 to transfer through the spore coat and to make massive destruction in the inner spore (46). In conclusion, the inactivation effect of UV–H_2O_2 exhibited a slight improvement compared to H_2O_2–UV, indicating that UV irradiation as the pretreatment made more contribution than H_2O_2 did. Since it was still uncertain that which factor played the vital role, further work in this area should be carried out to figure out a more convincing mechanism.

As to UV/H_2O_2 process, UV primarily reacts with H_2O_2 to create OH radical rather than inactivating spores directly (Eq. (5)) (47). The high efficiency of UV/H_2O_2 relied heavily on the OH radical generating in the reaction (16). As the standard reduction potential of OH radical (2.70 V) is higher than H_2O_2 (1.77 V), it is easier for OH radicals to oxidize and damage the spore outer coat. Therefore, the OH radicals were able to slowly eat away the spore protective wall and reach the protoplast and membrane, leading to the lethality of spore ultimately (48):

$$H_2O_2 \xrightarrow{hv} 2 \cdot OH \quad (5)$$

It has been reported that OH radical generation plays a significant role as an antimicrobial technique. Cross *et al.* (49) developed an aqueous modified Fenton reagent treatment to kill *Baccilus globigii* spores. With the help of catalyst, aqueous dissolved oxygen was utilized to convert into OH radicals and then perform the killing action. This approach was operative in biological systems, as no additional strong oxidizer such as H_2O_2 was needed. Zacarőas *et al.* (48) found that UV/TiO_2 was much better than UV irradiation alone in *B. subtilis* spores inactivation because of the OH radical generating by photocatalysis. Jung *et al.* (50) investigated the synergistic effect of UV/O_3 to inactivate *B. subtilis* spores.

In conclusion, UV–H_2O_2 exhibited higher efficacy than did H_2O_2–UV, whereas simultaneous UV/H_2O_2 exhibited the optimal inactivation effect on *B. subtilis* spores inactivation.

The role of OH radical

The *p*CBA concentrations were measured to indirectly determine the concentrations of OH radical during UV/H_2O_2 process, at UV irradiance of 113.0 μW cm^{-2} (51). Figure 4 depicts the kinetic data for the photolytic oxidation of *p*CBA. The degradation of *p*CBA abided to pseudo-first-order, whereas k_{exp} at H2O2 concentration of 0.15 and 0.60 mM was equal to 0.0081 and 0.0294 min^{-1}, respectively. According to Eq. (3), the steady-state OH radical concentrations ($[OH]_{ss}$) were 2.7×10^{-14} and 9.8×10^{-14} mM at H_2O_2 concentration of 0.15 and 0.60 mM.

Figure 5 presents the relationship between the inactivation effect of *B. subtilis* spores due to OH radical exposure and contact time of OH radical in the UV/H_2O_2 system. The log inactivation here was computed as the gap between the inactivation of UV/H_2O_2 and UV treatment alone at H_2O_2 concentration of 0.15 and 0.60 mM. The inactivation effect at 0.15 mM H_2O_2 showed a second-order polynomial relationship with exposure time: $y = -0.0122x^2 + 0.2369x - 0.0697$ ($r^2 = 0.981$), while that of 0.60 mM H_2O_2 followed: $y = -0.0224x^2 + 0.4268x - 0.0518$ ($r^2 = 0.986$).

Conducted from the data, the CT of OH radical for 1 log *B. subtilis* spores inactivation was approximately calculated to be 2×10^{-13} mM·min, which indicated that the OH radical played a significant role in the disinfection (52). There is no doubt that the OH radical was much more effective than other chemical disinfectants, such as monochloramine (290 mM·min) (53), O_3 (0.13 mM·min) (53), chlorine (1.1 mM·min) (54), chlorine dioxide

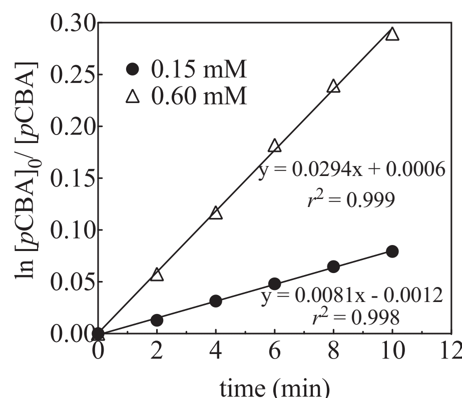


Figure 4. Pseudo-first-order rate plots of kinetic data for the photolytic oxidation of *p*CBA. Initial concentration of *p*CBA was 1 mg L^{-1}.

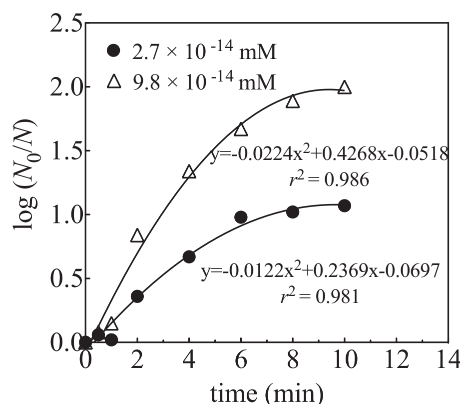


Figure 5. Relationship between the inactivation effect of *B. subtilis* spores due to ·OH exposure and contact time of ·OH. $[OH]_{ss}$ was 2.7×10^{-14} and 9.8×10^{-14} mM.

Table 3. Concentration–time (CT) values of various chemical disinfectants correspond to 1 − log inactivation of *B. subtilis* spores.

Chemical disinfectants	CT values (mM·min)	Reference
Monochloramine	290	Larson and Mariñas (53)
Chlorine dioxide	4.4	Barbeau *et al.* (54)
Chlorine	1.1	Barbeau *et al.* (54)
Fe(VI)	0.54	Makky *et al.* (55)
O_3	0.13	Larson and Mariñas (53)
·OH	2×10^{-13}	–

(4.4 mM·min) (54), Fe(VI) (0.54 mM·min) (55), for *B. subtilis* spores inactivation (Table 3).

CONCLUSIONS

B. subtilis spores could be inactivated under UV irradiation and the inactivation effect performed better at higher irradiance. Meanwhile, the efficiency could be significantly improved upon adding H_2O_2, owing to its synergetic effect with UV treatment. Higher dose of H_2O_2 further favored the inactivation effect. Both the pseudo-first-order rate constants rates and the inactivation effect indicated that UV–H_2O_2 exhibited higher efficacy than did H_2O_2–UV, whereas simultaneous UV/H_2O_2 exhibited the optimal inactivation effect on *B. subtilis* spores inactivation. The highly reactive OH radical played a significant role in UV/H_2O_2 disinfection. The CT of OH radical for 1 log *B. subtilis* spores inactivation was approximately calculated to be 2×10^{-13} mM·min, which was much more effective than other chemical disinfectants.

Acknowledgements—This work was supported by the National Natural Science Foundation of China (Grant 51178323, 51108329), China Postdoctoral Science Foundation funded project (grant 2012T50413) and the Fundamental Research Funds for the Central Universities (grant 0400219205).

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