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EFFECT OF HYDROGEN PEROXIDE ON BACILLUS SUBTILIS SPORE REMOVAL IN AN ELECTROPHOTOCATALYTIC SYSTEM

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ABSTRACT

Bacillus subtilis spores have been widely used in UV reactor validation as a possible surrogate for protozoa studies. UV light has become one of the most important methods of water disinfection. Hydrogen peroxide has been used as a strong oxidizing agent. The rate of disinfection of hydrogen peroxide is enhanced by UV and ozone. Bacillus subtilis spores are not sensitive to H2O2; thus, they have been the most important targets of peroxide sterilization. The goal of this study was to evaluate the potential of an electrophotocatalytic/hydrogen peroxide reactor for spore inactivation by studying this method with Bacillus subtilis spores. Contaminated water in an electrophotocatalytic reactor was prepared by adding 10²-10³ spores of B. subtilis to the water. The batch electrophotocatalytic reactor was a 360-ml glass vessel. The characteristics of the electrodes were as follows: An electrode of ZnO nanoparticles immobilized on zinc and a copper electrode. The studied variables were pH (6-8), the number of spore suspensions (10²-10³ spores / ml), the light emitting diodes (LED) UV-A lamps (2-4 W), time (5-40 min), layers of zinc oxide nanoparticles (1-3), the concentration of hydrogen peroxide (0.1-1000 mM), and voltage (10-20 V). The findings show that using H2O2 in the presence of UV-A irradiation (UV/H2O2) leads to an increase in spore removal efficiency. Optimal removal was obtained at pH 7, with a time of electrolysis of 5 minutes, 2 layers of nano ZnO, and a voltage of 10 V. This result suggests that this method is efficient for the enhanced disinfection of water.

KEYWORDS: Bacillus subtilis; spores; Electrophotocatalytic; Hydrogen peroxide.

1. INTRODUCTION

According to the WHO report [1], 1.1 billion people lack access to safe drinking water. Chlorination has become the most commonly accepted disinfection process for drinking water. The appearance of Cryptosporidium parvum oocysts, which is a chlorine-resistant microorganism and a diarrhea disease-causing waterborne parasite, has led to the use of free chlorine in many conventional drinking water treatment plants. These plants are faced with difficulties in reaching the required target goals for microbial inactivation [2]. The low metabolism of spores allows them to remain viable for large periods of time without requiring external nutrients or even water [3]. Due to several disadvantages of chlorination, studies on alternative disinfection processes, such as ozone, ultraviolet, chlorine dioxide, and chloramines have been investigated [2]. UV light has become one of the most important alternatives to chlorination for disinfection throughout the world [4]. Hydrogen peroxide is a weaker disinfectant than ozone [5]. The rate of activity of hydrogen peroxide is increased by UV. Hydrogen peroxide effectiveness increases when dissociated into hydroxyl free radicals (•OH). Thus, to improve its efficacy, H2O2 has been combined with germicidal UV-C radiation or ozone [6]. Hydrogen peroxide and UV irradiation are used in a variety of disinfection processes [7]. The most efficient hydroxyl radical yields are obtained when short wave UV wavelengths (200–280 nm) are used [8]. UV light and metal nanoparticles are effectively used for promoting free radical formation in hydrogen peroxide to increase effectiveness. The rate of hydrogen peroxide can be increased with the addition of peroxide activators, such as bicarbonate. In general, Bacillus subtilis spores are more resistant to H2O2 than are vegetative cells; therefore, they are generally the major targets of peroxide sterilization. Spore DNA is protected against peroxide and UV damage by small, acid-soluble, spore proteins (SASP), which bind to DNA and protect it [7]. Popham, Sengupta, and Setlow [9] have shown that SASP were more important to the survival of B. subtilis than water content. Hydrogen peroxide is an excellent source of singlet oxygen, superoxide radicals (O2•-) and hydroxyl radicals (•OH) that are highly reactive and very toxic for microorganisms [10]. The reactions of spore removal using UV / H2O2; and UV / ZnO under anodic potential processes can be showed by:

* Corresponding author
UV / H₂O₂ → 2’OH
Spore + ’OH → Mineralization
ZnO + UV → e⁻ CB (ZnO) + h⁺ VB (ZnO)
O₂ + 2H₂O + 4e⁻ → H₂O₂
e⁻ CB (ZnO) + H₂O₂ → ’OH + OH⁻ + ZnO
Spore + ’OH → Mineralization

A great deal of attention has been focused in recent years on the development of nano semiconductor thin films to be used as a photocatalyst for drinking water treatment because of its oxidative degradation of microorganisms [11]. However, it has also been reported that aqueous solutions of H₂O₂ alone will not cause protein, lipid, or nucleic acid modifications without the presence of catalysts for radical formation [10]. Synergistic effects in integrated disinfection processes are beneficial because the retention time required for the same level of inactivation can be reduced. Cho and Yoon [2] reported that the inactivation of B. subtilis spores was significantly enhanced in a disinfection system consisting of O³ and H₂O₂ combined. Zuolian et al. [12] observed an 80% inactivation of E. coli by using of UVA LED after 45 min. Despite these findings, no investigation on coupled disinfection processes involving light emitted dynod UV-A lamps and hydrogen peroxide for microbial inactivation has been conducted. This study investigated the use of hydrogen peroxide in the presence of a light-emitted dynod UV-A lamp and immobilized ZnO semiconductor on a zinc electrode under anodic potential (an AOP-UV/H₂O₂) for the removal of B. subtilis spores as a surrogate of C. parvum oocysts. The coupling of a light-emitted dynod UV-A lamp, the immobilized ZnO semiconductor on a zinc electrode and hydrogen peroxide provides a new approach towards achieving a more efficient removal of B. subtilis spores as a model for C. parvum oocysts. A few methods are available for the removal of B. subtilis spores. The electrochemical properties and AOP were reported as antimicrobial methods, but there are no reports about using electrophotocatalytic processes for the removal of B. subtilis spores. The aim of this study was the removal of B. subtilis spores, which are considered an indicator microorganism for C. parvum oocysts in disinfection, from drinking water using a thin layer of electrophotocatalytic ZnO nanoparticles stabilized on zinc and hydrogen peroxide. It is clear that several additional variables should be explored in a subsequent study; the role of the water matrix and an eventual combination with other AOTs, particularly UVC radiation, will be important.

2. MATERIALS AND METHODS

2.1. Materials

The ZnO nanoparticles, with a special surface area of 50 m² g⁻¹ and a particle size of 20 nm, were supplied by Amohr Co. (Germany). The 1% Tween 80, nutrient agar culture media, trypytase soy broth media, manganese chloride, maniazium chloride, sodium chloride, potassium chloride, disodium monohydrogen phosphate, potassium dihydrogen phosphate, hydrogen peroxide, sodium bicarbonate, and nitric acid were purchased from Merck Co. (Germany). The nitric acid and sodium bicarbonate solutions (1 N) were used to adjust the pH.

2.2. Preparation of ZnO nanoparticles

Five grams of zinc oxide nanoparticles were placed into 100 ml of distilled water. The suspension was mixed with a magnetic stirrer for 30 min and then sonicated in an ultrasonic bath (MATR. N.B., Italy) at a frequency of 50 kHz for 22 min improve the dispersion of the ZnO in water. The weight of the zinc electrode was measured after hydroxylation and washing with distilled water.

2.3. Preparation of electrodes

The zinc electrode was used as the substrate for the immobilization of the ZnO nanoparticles. The zinc electrode was pre-treated by washing with detergent and a sodium hydroxide solution at 0/01 N to increase the number of OH groups.

2.4. Immobilization of ZnO nanoparticles

To prepare the ZnO films, dry methods were used [12, 13]. There are various supports for the immobilization of ZnO nanoparticles; in this study a Zn palate was used. After the pre-treatment, the zinc electrode was weighed, immersed in the colloidal solution, and dried in an oven at 35°C for 30 min. The coated particles were then calcined in a muffle furnace at 105 and 320°C for 60 min. The thermal treatment of the immobilized ZnO films acquires some properties, including a better electric connection among ZnO nanoparticles and the Zn plate, and good mechanical stability of the films. For the 2- and 3-layer coatings, the processes were repeated two and three times. They were washed with distilled water to remove any free ZnO particles.

2.5. Batch reactor

The batch reactor was a 360-ml glass vessel (10×6× 6 cm) (Figure 1). The characteristics of the electrodes were as follows: two electrodes of thin layer zinc oxide nanoparticles immobilized on zinc (anode), and a copper electrode (cathode). The area of each electrode was 36 cm² (9×4×0.1 cm). The distance between the bottom of the reactor and the electrodes was 1 cm, and the distance between the UV lamp and the Zn-ZnO electrode was adjusted to 2-3.5 cm. The AC electrical source had an electrical energy production equal to 1-5A, and a maximum electrical power of 60 W. The LED UV-A lamp had an electrical power of 1 W, radiation intensity of 120 mW cm⁻², a wavelength of 395 nm, and a voltage of 3.4 V. To evaluate the effect of the anodic potential, catalysts, hydrogen peroxide, and UV light on the disinfection process, samples underwent UV-A lamp treatments (at 2, 3, and 4 W), with an electrode of thin layer zinc oxide nanoparticles immobilized on zinc (5%, 10%, and 15%) and at different voltages (10 and 20 V), and different times (1, 5, 10, 20, and 40 min). A magnetic
stirrer was used for the homogeneous mixing of the contaminated water samples. The percentage spore reduction was calculated according to the following equation [14]:

$$R (%) = \frac{1-A}{B} \times 100$$

Where R was the percentage of spore reduction, and B and A were the average number of live bacterial spores per milliliter before and after treatment.

2.6. Preparation of *B. subtilis* spores

Suspensions of *B. subtilis* spores (ATCC 6501) in water were obtained following the technique proposed by other researchers [10]. *B. subtilis* was reactivated from a frozen stock (15% glycerinated trypticase soy broth) in a 100-ml Erlenmeyer flask containing 50 ml of trypticase soy broth (Merck). The sample was incubated at 37°C for 24 h under aerobic conditions. Afterwards, dilutions were prepared using phosphate buffer containing MnCl₂ (20 mg/l). The surface of the culture medium was seeded with 1 ml of the 10⁻² dilution in preparation for sporulation (trypticase soy agar [TSA] enriched with 0.25 mg/l MgSO₄) and then subjected to 37°C for 10 days for sporulation. To obtain the spore inocula, the surface of the TSA was rinsed with sterile water and scraped with a spatula. Three rinsing procedures were carried out in 50-ml tubes: the first rinse was done with 1% Tween 80, and the next two were made with distilled water at 12000 rpm for 5 min. A quantity of spore suspension (100 ml) was poured into sterile Erlenmeyer flasks and placed in a water bath at 80°C for 15 min to eliminate vegetative cells. Afterwards, they were kept at 4°C for subsequent bottle inoculation. The optical density of the cell suspension was measured with a spectrophotometer at 546 nm. Sporulation was confirmed by optical microscopy using malachite green staining to confirm the presence of spores and counter staining with safranin to show the presence of vegetative cells [15]. After each round of the study, 1 ml of reactor water was picked and cultured on nutrient agar plates to evaluate the efficiency of the removal process. After incubation at 37°C for 48 h, the number of spores formed on the agar plate was counted, and the results were expressed as the mean number of spores per milliliter.

3. RESULTS AND DISCUSSION

3.1. Spore removal under UV-A light

The effect of the light was investigated using the initial spore concentration in the range of 10² to 10³ spores per ml (Figure 2). The removal percentage for *B. subtilis* spores (10² ml) increased from 60 to 90% as the UV-A power increased from 2 to 4 W, with 40 min of radiation and pH 7. Exposure to UV-A can result in formation of reactive oxygen species, such as O₂⁻, H₂O₂, •OH, which can cause cell mutations. This is in agreement with Kühn et al. [16], who reported that *B. subtilis* spores were resistant to 60 min of UV-A. In the treatment with UV-A, a slightly faster removal rate was observed at a higher pH and light power. This effect was attributed to an increase in the •OH concentration at a higher pH and light power. This observation was not consistent with the absence of the pH effect for *B. subtilis* spore inactivation in a pH range of 5.6-8.2 as reported by Min et al. [15].


![FIGURE 2 - Efficiency of UV-A lamp in *B. subtilis* spore removal from contaminated water (spore number 10² CFU/ml) at radiation time 40 minute, distance between the UV-A lamp and water surface 2 cm, different pHs, and different power](image)

3.2. Spore removal under H₂O₂

Control experiments were performed to test the effect of H₂O₂ alone on *B. subtilis* spores (in the range of 10² to 10³ per ml) removal (Table 1). Results show no removal of spores at a H₂O₂ dose of up to 0.5 mM, with a 40 min contact time and different pH values. These results were in agreement with previously published data. Mamen et al. did not find any influence of H₂O₂ on *B. subtilis* spore inactivation at a dose of 20 mg/l H₂O₂ and a contact time of 60 min [8].
The effect of H$_2$O$_2$ concentration on the removal percentage of B. subtilis spores was investigated. Zn electrodes, with a catalyst amount in the range of 5-15%, were used to study the electrophotocatalytic / H$_2$O$_2$ removal of B. subtilis. The efficiency of B. subtilis spore removal was greatly increased in the presence of H$_2$O$_2$ (Tables 2 and 3). Strategies for inhibiting e$^-$/h$^+$ recombination were to add irreversible electron acceptors (such as H$_2$O$_2$) and electrical potential to the reactor. For better results, these additives led to the formation of $\cdot$OH and other oxidizing agents, thereby decreasing the reactor dimensions. Due to its electron acceptor nature, it reacted with conduction band electrons to generate hydroxyl radicals. The effect depended on the H$_2$O$_2$ concentration. At higher concentrations, the improvement started to lessen. Whereas, this beneficial effect could be explained in terms of the prevention of electron/hole recombination and the additional $\cdot$OH production, inhibition could be explained in terms of the ZnO/Zn surface modification by H$_2$O$_2$ adsorption, the scavenging of photoproduced holes, and the reaction with hydroxyl radicals. If the spore number was less than the H$_2$O$_2$ concentration, adsorption decreased because of adsorption of the hydrogen peroxide. When the spore number was higher than the H$_2$O$_2$ concentration, radicals reacted more easily. At a high optimal ratio (H$_2$O$_2$/spore number), an inhibition effect would be expected because the unfavorable reactions become more pronounced. All of these observations may be summarized as follows:

- The removal rate; and, second, if the ratio of H$_2$O$_2$ to spore number was high, the same was true. Similar results were demonstrated by Poulios et al. [17], who have reported an optimal molar ratio (H$_2$O$_2$/ contaminant) of 10-100. The efficiency of the disinfection process increased when the number of thin layers of ZnO nanoparticles on the zinc electrode increased to two, and then decreased when the number of thin layers of the nano catalyst on the zinc electrode increased to three. The efficiency of the disinfection process increased when the number of thin layers of ZnO nanoparticles on the zinc electrode increased to two, and then, H$_2$O$_2$ concentration decreased (Tables 2 and 3). The optimum number of thin layers of the zinc oxide catalyst nanoparticles on the zinc electrode for the complete removal of spores was two. It was found that the efficiency of B. subtilis spore removal increased as the number of ZnO nanoparticle layers increased to two, and this could be attributed to an increase in the photon absorption by the ZnO, in addition to greater ROS production at the outer layer of the ZnO film, and an increase in the surface area for inactivation of the spore. We also found that the efficiency of B. subtilis spore removal increased in the presence of zinc oxide photocatalyst nanoparticles and UV-A/ H$_2$O$_2$, due to the production of hydroxyl radicals, as shown in Tables 2 and 3. Hydroxyl radicals led to the progressive photocatalytic degradation of the compounds in the spore coat at its point of contact with the photocatalytic surface, which did not cause lethal injuries to the spores. The loss of spore viability leading to inactive spores could be due both to a loss of coat integrity, core damage.

### Table 1 - Exposure of B. subtilis spore (spore number $10^2$-$10^3$ CFU/ml) to hydrogen peroxide concentration 5-500 mg l$^{-1}$at contact time 40 minute, and different pHs with drinking water.

<table>
<thead>
<tr>
<th>Spore no. (CFU)</th>
<th>Hydrogen peroxide concentration (mg l$^{-1}$)</th>
<th>Result of cultured microbial at pH 6</th>
<th>Result of cultured microbial at pH 7</th>
<th>Result of cultured microbial at pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^2$</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>+</td>
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<tr>
<td>50</td>
<td></td>
<td>+</td>
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<td>+</td>
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<td>100</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>500</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>$10^3$</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>5</td>
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<tr>
<td>100</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

### Table 2 - Exposure of B. subtilis spore (spore number $10^3$ CFU/ml) to hydrogen peroxide concentration 5-500 mg l$^{-1}$at radiation time 5 minute, and distance between the UV lamp and Zn/ZnO electrode 2 cm for completed spore removal, different concentration of zinc oxide nanoparticles, LED UVA lamp power, voltage and pHs

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Concentration of zinc oxide nanoparticles (%)</th>
<th>Hydrogen peroxide concentration at pH 6</th>
<th>Hydrogen peroxide concentration at pH 7</th>
<th>Hydrogen peroxide concentration at pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>lamp power (W)</td>
<td>lamp power (W)</td>
<td>lamp power (W)</td>
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<td>10</td>
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<tr>
<td>5</td>
<td>1.8</td>
<td>1.4</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>10</td>
<td>1.4</td>
<td>1.4</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>15</td>
<td>1.4</td>
<td>1.0</td>
<td>0.6</td>
<td>1.2</td>
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<tr>
<td>20</td>
<td>5</td>
<td>1.2</td>
<td>0.8</td>
<td>0.4</td>
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<td></td>
<td>0.8</td>
<td>0.4</td>
<td>0.2</td>
<td>0.6</td>
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<tr>
<td>15</td>
<td>0.8</td>
<td>0.4</td>
<td>0.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>
or both processes [18]. The super oxide radical anion, hydroperoxyl radical and hydrogen peroxide formed by the reduction of dissolved oxygen in the anode can also feed into the photocatalytic disinfection mechanism. These species could contribute to the spore inactivation

3.4. Effect of electrical current

The efficiency of *B. subtilis* spore removal increased as the electrolysis voltage increased. Higher electrical current and lamp power increased the efficiency of *B. subtilis* spore removal and could shorten the H₂O₂/spore number. The optimum electrical current for complete removal of the spore was 20 V. The experimental results show that the voltage electrode increased the resulting gradient separated electron–hole, thereby decreasing its recombination rate, decreasing the H₂O₂/spore number, increasing the photocurrent rate, and eventually accelerating the spore removal as shown in Tables 2 and 3. Moreover, under higher applied voltages, the external electric field could also improve the direct and indirect electro-oxidation reactions at the anode. The biocidal efficiency was proportional to the specific surface area of the photocatalysts and the quantum yield of photocatalytic system because the number of OH• was proportional to the specific surface area and inversely proportional to the electron-hole recombination rate [18, 19]. Additionally, the photoelectrocatalytic activity increased the mass transfer by electro-migration of negatively charged bacteria spores towards the electrode [20-22]. The viability of *B. subtilis* spores decreased significantly and inactivation increased with the electrolysis voltage and time irradiation [3]. At the beginning of the process, all the spores on the photocatalytic electrode were intact, but after the irradiation process started, the organic compounds of the spore’s coat were progressively oxidized, and the core began to be oxidized as well. The damage in the coat and in the core was cumulative, and the initially intact spores evolved through states of increasing degradation. When this damage reached a level that could not be repaired or reversed by the spores during germination, the spore became inactive.

### 4. CONCLUSIONS

The experimental results suggest that zinc oxide thin layer nanoparticles immobilized on zinc in an electrophotocatalytic/H₂O₂ process is a promising method for the disinfection of water against *B. subtilis* spores. Spore removal was affected by pH, UV-A lamp electrical power, H₂O₂ concentration, voltage, the number of layers the nanoparticle catalyst, and the number of spores. The electrophotocatalytic/H₂O₂ treatments were capable of spore removal at the pH values investigated (6-8), with an electrolysis time of less than 5 minutes. Enhanced spore removal was obtained with an increase in the H₂O₂ concentration, voltage, lamp electrical power, and pH. An increase in H₂O₂ concentration led to faster removal up to an optimum value; at a higher optimum value, the spore removal became slower. Although a suite of microorganisms have been removed using the electrophotocatalytic process in this study, the major organism of public health concern, *C. parvum* oocysts, has not been examined to date. Further research is required to understand the pathways and mechanisms of destruction for cells exposed to electrophotocatalyst/H₂O₂.

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[5] Fernando, W.J.N.and Othman, R. (2006) Relevance of diffusion through bacterial spore coats / membranes and the associated concentration boundary layers in the initial lag phase of inactivation: A case study for *Bacillus subtilis* spores. Spore removal and could shorten the H₂O₂/spore number. The optimum electrical current for complete removal of the spore was 20 V. The experimental results show that the voltage electrode increased the resulting gradient separated
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