Applied Catalysis B: Environmental 93 (2010) 212–216

Contents lists available at ScienceDirect

Applied Catalysis B: Environmental

journal homepage: www.elsevier.com/locate/apcatb

Enhanced inactivation of bacterial spores by atmospheric pressure plasma with catalyst TiO_2

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ARTICLE INFO

Article history:
Received 28 May 2009
Received in revised form 17 September 2009
Accepted 23 September 2009
Available online 1 October 2009

Keywords:
TiO_2
Atomspheric pressure plasma
Bacterial spore
Sterilization
Bacillus subtilis
Plasma catalysis
Oxygen vacancy

ABSTRACT

Both atmospheric pressure plasma and photo-catalyst metal oxide titanium dioxide (TiO_2) are well known for their microorganism inactivation and chemical material decomposition abilities. In this work, radio-frequency atmospheric pressure plasma and TiO_2 are used together to inactivate *Bacillus subtilis* spores that have a very high degree of environmental resistance to ultra-violet (UV) photons and heat. The combinational use of the plasma and TiO_2 demonstrates an enhanced performance of *B. subtilis* spore inactivation by showing a decrease in the decimal reduction time of as large as 40% compared with the use of plasma alone. A significant increase of hydroxyl (OH) and excited oxygen atomic emission line (O I) intensities in the presence of TiO_2 suggests that the atmospheric pressure plasma assisted by TiO_2 is very effective at generating reactive oxygen radicals, which is known to be a dominant factor in bacterial spore inactivation. Possible TiO_2 activation mechanism by the plasma is investigated.

1. Introduction

Over the years, a considerable number of studies have been performed on the inactivation of microorganisms [1–8]. One of the conventional inactivation methods is thermal treatment using dry heat or an autoclave, but this may cause thermal damage to some treated objects and usually takes a long time [1,2]. Ultra-violet (UV) photons can kill microorganisms without direct contact to materials, but bacterial spores usually have a high degree of UV resistance. Since there are also screening problems, some new approaches have been attempted [1,3].

One of the alternative methods is atmospheric pressure plasma. Plasma is a capable sterilizing method to inactivate a wide range of microorganisms [4–7]. Among many physical inactivation sources such as heat, UV photons, charged particles, and reactive species, the reactive oxidation species have been known as the most effective sterilization source [4–6]. Therefore, oxygen gas is often additionally added to the supply gas to enhance the sterilization effect. In the presence of too much oxygen, however, the plasma characteristics tend to become altered to show higher breakdown voltage and gas temperature causing the plasma to be less stable [9]. Hence, development of a new means for enhancing the inactivation efficiency without affecting the plasma characteristics is imperative. On the other hand, as a non-plasma method, photo-catalytic oxidation inactivation using metal oxides such as titanium dioxide (TiO_2) has offered a number of positive features. The photo-catalytic inactivation of Gram-negative *Escherichia coli* and Gram-positive *Lactobacillus helveticus* by TiO_2 with 365 nm UV photons is one of many examples [8]. However, this method takes a long time to inactivate microbes and contaminated materials.

Recently, TiO_2 has shown synergetic effects with the plasma, i.e., enhanced destruction of benzene and toluene was achieved by combining the plasma and the photo-catalytic metal oxides, compared to the use of plasma or the catalysis alone [10]. Although prior studies have been made on the destruction of chemical and pharmaceutical materials by simultaneous use of plasma and catalysis [10], little is known about the inactivation of microbes such as bacterial spores. In this work, experimental results are presented showing the enhanced inactivation of *Bacillus subtilis* spores by the catalyst TiO_2 utilized together with atmospheric pressure plasma; the results are compared with the plasma only treatment case. This result may be easily applicable to inactivating various microorganisms including *Bacillus anthracis* spores, which have the potential to be used in bioterrorism. The TiO_2 concentration effects are also shown along with brief discussions of the mechanism of the TiO_2 plasma-catalysis.

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doi:10.1016/j.apcatb.2009.09.031

2. Experimental

2.1. Bacterial spore suspension and catalyst preparation

Based on the method of Nicholson and Setlow [11], *B. subtilis* spore samples were prepared and cultured in a medium containing 1 g Bacto nutrient broth (Difco), 10 ml 10% (w/v) KCl, 10 ml 1.2% (w/v) $MgSO_4 \cdot 7H_2O$, 1 ml 1 M $Ca(NO_3)_2 \cdot 4H_2O$, 1 ml 0.01 M $MnCl_2 \cdot 4H_2O$, and 1 ml 1 mM $FeSO_4 \cdot 7H_2O$ in 1 l of double distilled water (ddH_2O). The number density of the spore suspension was approximately 2×10^8 spores/ml.

The catalyst TiO_2 powder (JUNSEI, extra pure anatase form, 150 nm typical size) of a certain mass concentration was added to the *B. subtilis* spore suspensions, keeping the same spore number density. To prepare a uniform suspension, the same ratios of spores and TiO_2 particles were mixed using a vortex mixer (SI-0236, Scientific Industries).

The treatment sample was prepared by applying 10 μl (5 droplet × 2 μl, $N_0 \sim 2 \times 10^6$ spores/ml) of the spore suspension to a slide glass and letting it dry for about 1 h. After the treatments by plasma, UV, or heat, the spores were taken off the slide glass and submerged in 50 ml ddH_2O. Then, the treated spores were cultured on a nutrient agar at 37 °C for 24 h for quantification of the treatment results. The nutrient agar was prepared by mixing 8 g Bacto nutrient broth (Difco) and 15 g Agar powder (JUNSEI) in 1 l of ddH_2O.

2.2. Experimental set-up for plasma treatment

The schematic of the atmospheric pressure plasma source and three types of *B. subtilis* spore samples are illustrated in Fig. 1. Each sample has the same initial spore density (2×10^6 spores/ml). A detailed description of the plasma characteristics is found in our previous report [4]. The atmospheric pressure plasma was generated at 13.56 MHz radio-frequency (rf) in ambient air with either helium or argon supply of 6 lpm. The plasma has a relatively large area (110 mm × 15 mm) and low discharge current and gas temperature, applicable to thermally sensitive materials. The gap distance between the powered electrode and the spore sample was fixed at 2 mm to satisfy the sufficient uniform discharge generation condition.

2.3. Photo-catalyst analyses

The characteristic changes in the plasma-treated TiO_2 particles were analyzed by X-ray diffraction (XRD, RIGAKU, D/MAX-RB 12 kW), UV–vis absorbance spectrum (JASCO, V-570), high resolution dispersive Raman microscope (Horiba Jobin Yvon, LabRAM HR UV/vis/NIR), and photoluminescence (PL) spectrum. The XRD diffraction pattern was measured at an angle of 2θ, of which the angle was scanned from 20° to 60° with a speed of 1°/min. The Raman spectrum was obtained using an Ar ion laser of 514.5 nm wavelength with an output power of 10 mW as an exciting source. The UV–vis absorbance spectra of the original and the treated TiO_2 samples were measured for the wavelength range of 300–700 nm. The PL spectrum was analyzed using a He–Cd laser of 325 nm wavelength with 17 mW output power. The TiO_2 mass concentration was fixed at 1 mg/ml.

3. Results and discussion

3.1. Survival curves in various plasma conditions with and without TiO_2

Fig. 2(a) depicts survival curves of the *B. subtilis* spores treated by helium and argon plasmas with and without TiO_2 (1 mg/ml) addition [as depicted in samples (a) and (b) of Fig. 1]. The decimal reduction time (D-value) for inactivating 90% of the microbial population in the sample is plotted in Fig. 2(b). As shown in the figure, the helium plasma (gray) is much less efficient than the argon plasma (blue and red), which agrees with the results of other works [5]; the inactivation speed is enhanced by the increase of the input power, as expected. At 100 W, the D-value is decreased from 435 to 333 s by the helium plasma and decreased from 26 to 15 s by the argon plasma, each with TiO_2 added, which demonstrates more than 40% inactivation enhancement compared with the use of plasma alone. One interesting thing to note is that in contrast to the conventional notion, in which the treatment sample should be in contact with the catalyst to expect inactivation, it is found that there are still catalyst effects even when the spore suspensions and the catalyst TiO_2 are not mixed but spatially separated on the slide glass [as depicted in Fig. 1 sample (c)]. When this sample was

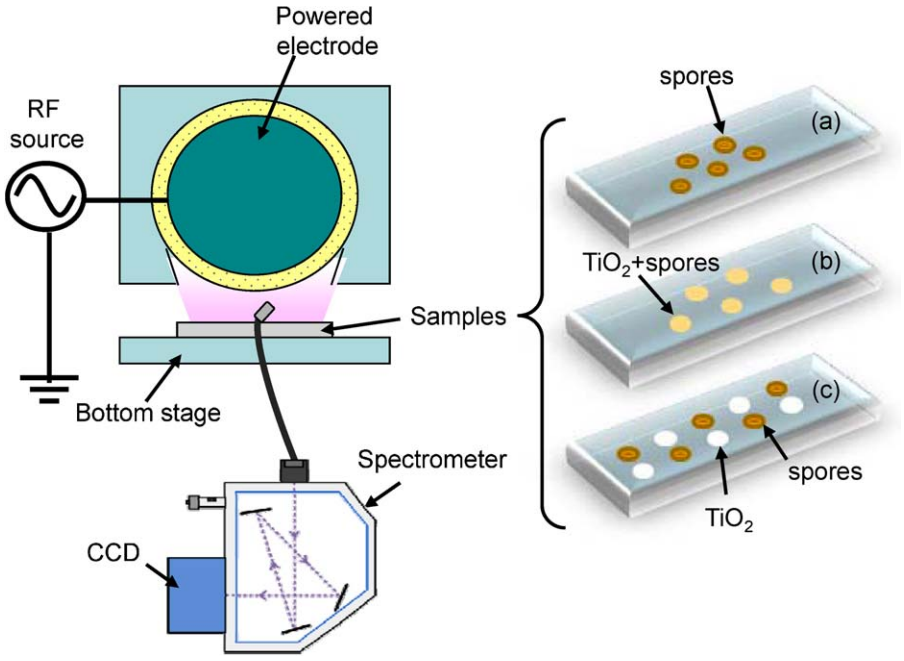

Fig. 1. Schematic of the experimental set-up and the treatment samples ($N_0 \sim 10^6$ spores/ml) of (a) bacterial spore only, (b) bacterial spore mixed with TiO_2 particles (1 mg/ml), and (c) bacterial spore with TiO_2 particles separated.