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Short communication

Efficient sterilization using reactive oxygen species generated by a radical vapor reactor

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ABSTRACT

Numerous sterilization techniques using ozone have been proposed. However, singlet oxygen and hydroxyl radicals show stronger oxidation activity than ozone, and therefore exert a greater sterilization effect against microorganisms. We recently developed a radical vapor reactor (RVR) as a powerful tool for microorganism sterilization, which is based on the efficient generation and exposure of reactive oxygen species (ROS) such as singlet oxygen and hydroxyl radicals including ozone. Therefore, effective sterilization via ROS generation is expected by optimizing the RVR conditions. In this study, the generations of ROS was measured under various RVR conditions using spin-trapping electron spin resonance analysis and ozone monitoring. We demonstrated the effective sterilization of *Escherichia coli* and *Bacillus subtilis* by exposure to ozone, singlet oxygen, and hydroxyl radicals whose production levels were successfully controlled.

1. Introduction

Sterilization is an important hygiene process in the medical and healthcare fields. Currently, ethylene oxide gas (EOG) sterilization is one of the most widely used sterilization methods [1]. However, the applications of EOG are limited given that it is a highly toxic chemical to humans [2]. Gamma-ray sterilization is another widely used sterilization method; however, this method requires a radiation facility and can only be used with specific materials [3]. Similarly, the main disadvantage of ultraviolet (UV) irradiation-based sterilization is that its application is limited to materials of specific shapes and sizes because of the potential for degradation. Steam autoclaving is also only appropriate for certain materials because of the high temperature and pressure required. Therefore, non-toxic and low-temperature sterilization methods are required in the medical and healthcare fields. Ozone-based sterilization techniques have been actively investigated, because this approach has many advantages such as an economical system, ability to operate at a standard temperature and pressure, it is relatively safe, and has low toxicity due to its degradation to oxygen.

In this study, we investigated the sterilization effect induced by singlet oxygen and hydroxyl radicals that tend to show stronger oxidation activity than ozone. The main mechanism contributing to the sterilization effect of singlet oxygen and hydroxyl radicals has been suggested to be cell membrane breakdown by oxidation [4]. Therefore, the use of reactive oxygen species (ROS) with stronger oxidation potential than ozone is expected to result in more effective sterilization. These ROS are well known to be easily generated by ozone, water, and UV irradiation [5,6]. Additionally, these residual ROS may self-decompose into non-toxic substances. We previously successfully developed a radical vapor reactor (RVR) that allowed for the effective generation of ROS [7]. Many studies have shown that these ROS (i.e., ozone, hydrogen peroxide, singlet oxygen, and hydroxyl radicals) were effective for sterilization [8–11].

The RVR is expected to allow for the appropriate control of ROS generation by modification of the operating conditions. In this study, the generated ROS were measured by the spin-trap technique using electron spin resonance (ESR) under several operating conditions of the RVR. Accordingly, we showed that the generation of ROS could be manipulated according to the mode of the RVR system employed. We demonstrated the effective sterilization of *Escherichia coli* and *Bacillus subtilis* under various RVR conditions.

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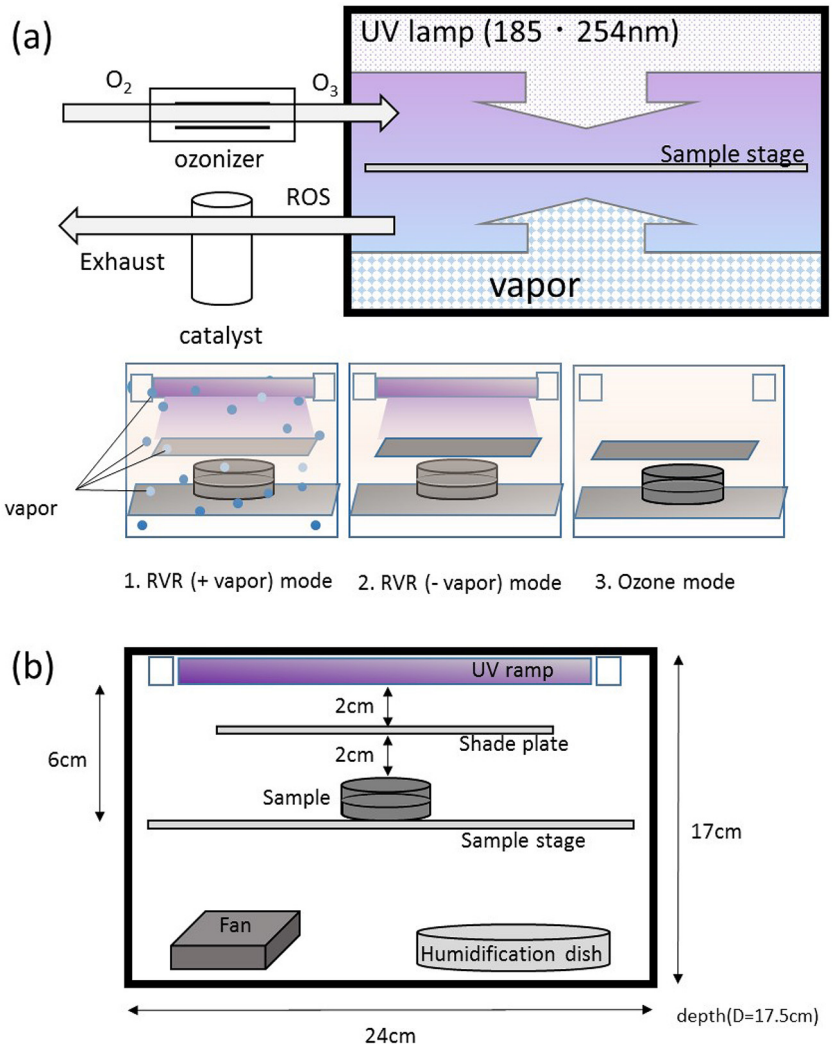

Fig. 1. Schematic illustration of (a) RVR treatment and modes and (b) the reaction system including their positions and distances.

2. Materials and methods

2.1. Chemicals and materials

2,2,5,5-Tetramethyl-3-pyrroline-3-carboxamide (TPC) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and LABOTEC (Tokyo, Japan) respectively. The chemical solutions were prepared using ultrapure water. For ozone generation, an ozonizer was used (RMO-50AC, REGAL JOINT CO., LTD., Kanagawa, Japan). The catalyst for exhaust gas treatment was NC004A (EBARA JITSUGYO Co., LTD., Tokyo, Japan), whose main components are silicon dioxide and carbon.

2.2. RVR treatment and mode

In brief, the RVR protocol involved the following two steps. The detailed conditions and protocol are described in our previous report [7]. In the first step, the RVR chamber was heated at 40 °C with or without vapor. In the second step, ozone was injected for 1 min at 4 L/min and then irradiated with UV light (185 and 254 nm). The UV strength was an average of 2.64 mW/cm^2 at a distance of 5 cm. In case of blinding by the shade plate, the UV light strength was 0 mW/cm^2. There were three modes of RVR. The RVR (+vapor) mode involves ozone injection with vapor and UV irradiation. The RVR (−vapor) mode involves ozone injection with UV irradiation and without vapor. The ozone mode involves only ozone injection. An illustration of each mode is shown Fig. 1(a) and the positions and distances in the RVR system are shown Fig. 1(b).

2.3. ROS analysis with the spin-trapping technique by ESR

The solution of the spin trap reagent, TPC or DMPO, was prepared to reach a concentration of 0.5 M by ultrapure water in a glass dish. Five hundred microliters of the prepared reagent solution in the dish was placed on the sample stage in the RVR and reacted with ROS that were generated by the RVR when run under several modes. After the solution was reacted with the ROS in the RVR, it was analyzed according to ESR spectrometry (JES-FA-100, JEOL, Tokyo, Japan). In the experiment, UV light was blocked by a shade plate.

2.4. Cultivation of E. coli and B. subtilis

In pre-cultivation, *E. coli* and *B. subtilis* were incubated in 3 mL of lysogenic broth (LB) liquid medium at 37 °C for 24 h. After the pre-culture, *E. coli* and *B. subtilis* were incubated in 5 mL of LB liquid medium at 37 °C with a cell number of 10^5 that was adjusted to an optical density at 600 nm of approximately 0.5 based on the adsorption measurements on a spectrometer (Ultrospec 3300 pro, GE Healthcare, USA). To simplify colony counting, the LB liquid