Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
Journal of Inorganic Biochemistry 101 (2007) 817–823
Journal of Inorganic Biochemistry
www.elsevier.com/locate/jinorgbio

Catalytic sterilization of *Escherichia coli* K 12 on Ag/Al_2O_3 surface

Meixue Chen [a], Lizhu Yan [b], Hong He [b,*], Qingyun Chang [b], Yunbo Yu [b], Jiuhui Qu [a]

[a] *State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China*
[b] *State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China*

Received 13 April 2006; received in revised form 22 January 2007; accepted 24 January 2007
Available online 3 February 2007

Abstract

Bactericidal action of Al_2O_3, Ag/Al_2O_3 and $AgCl/Al_2O_3$ on pure culture of *Escherichia coli* K 12 was studied. Ag/Al_2O_3 and $AgCl/Al_2O_3$ demonstrated a stronger bactericidal activity than Al_2O_3. The colony-forming ability of *E. coli* was completely lost in 0.5 min on both of Ag/Al_2O_3 and $AgCl/Al_2O_3$ at room temperature in air. The configuration of the bacteria on the catalyst surface was observed using scanning electron microscopy (SEM). Reactive oxygen species (ROS) play an important role in the expression of the bactericidal activity on the surface of catalysts by assay with O_2/N_2 bubbling and scavenger for ROS. Furthermore, the formation of CO_2 as an oxidation product could be detected by diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and be deduced by total carbon analysis. These results strongly support that the bactericidal process on the surface of Ag/Al_2O_3 and $AgCl/Al_2O_3$ was caused by the catalytic oxidation.

Keywords: Silver; Alumina; Bactericidal activity; Catalytic oxidation; Reactive oxygen species; *E. coli*

1. Introduction

Sterilization is an important procedure to maintain a sanitary environment. For this purpose, many chemical germicides have been used. With these applications, the potential dangers of the compounds to human health and environment are concerned. TiO_2 photocatalysts applied in water disinfection process were noticeable, which provided an alternative to conventional chemical germicides [1–5]. However, this technology requires a relatively complex device and photo energy. Recently, we have reported the inactivation efficiency of Ag/Al_2O_3 and Cu/Al_2O_3 to SARS coronavirus, bacteria and yeast [6]. The microbes are completely inactivated in 5 min on Ag/Al_2O_3 surface at room temperature in air, and we suggest a catalytic oxidation mechanism in this disinfection process.

Even though the bactericidal effect of compounds containing silver has been investigated for a long time, its mechanism is still controversial. Some reports insisted that the antibacterial activity of silver-loaded compounds is depended on the cytotoxicity of Ag^+ eluted from the compounds into system containing the microorganisms [7,8]. At the same time, it has been suggested that the reactive oxygen species play an important role in the expression of the bactericidal activity in water [9,10].

To identify the inactivation mechanism of bactericidal process on the surface of Ag/Al_2O_3 in air, e.g. by catalytic oxidation or by toxicity of heavy metals, Ag/Al_2O_3 and $AgCl/Al_2O_3$ were used in this study since they have different amount of Ag^+ which may be eluted from the catalysts. In this study, we also report that reactive oxygen species (ROS) play an important role in the expression of the bactericidal activity on the surface of catalysts.

* Corresponding author. Tel.: +86 10 62849123; fax: +86 10 62923563.
E-mail address: honghe@rcees.ac.cn (H. He).

0162-0134/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2007.01.008

2. Experimental

2.1. Catalysts

Ag/Al_2O_3 and $AgCl/Al_2O_3$ (Ag 5 wt%) were prepared by impregnation and precipitation methods respectively. After Al_2O_3 powder was introduced into an appropriate amount of silver nitrate aqueous solution, the mixture was stirred at room temperature. As for $AgCl/Al_2O_3$, an appropriate amount of ammonium chloride aqueous solution was added into the mixture at a rate of 0.5 ml/min, while the Ag/Al_2O_3 need not this step. Both of the wet samples were dried at 120 °C for 12 h, and then calcined in air at 600 °C for 3 h [11]. Before using, Al_2O_3, Ag/Al_2O_3 and $AgCl/Al_2O_3$ powders were pressed into wafers of ca. 20 mg/cm^2. XRD is used to investigate the silver phase on γ-Al_2O_3. In the case of Ag/Al_2O_3, only the γ-Al_2O_3 phase was detected when the silver loading was 5 wt%, and the absence of diffraction lines of silver phase on 5 wt% Ag/Al_2O_3 catalyst indicates that silver is at a very high dispersion degree. To 5 wt% $AgCl/Al_2O_3$, however, the peaks attributable to AgCl phase was observed at 2θ of 27.76°, 32.24°, 55.86°, 57.34° and 76.54° (PDF-ICDD 85-1355, PDF-ICDD 31-1238). The absence of diffraction lines of silver phase on 5 wt% Ag/Al_2O_3 catalyst indicates that silver is at a very high dispersion degree. TEM was also used to investigate the silver phase on γ-Al_2O_3. As for 5 wt% Ag/Al_2O_3, the silver particle (ranging from 15 to 20 nm) at a very high dispersion degree was observed. In the case of 5 wt% $AgCl/Al_2O_3$, the silver particle was greater than that of 5 wt% Ag/Al_2O_3 (ranging from 20 to 50 nm).

2.2. Culture of micro-organisms

E. coli K 12 strain ATCC23716 was inoculated into LB broth (Fluka Co. 61748) and grew aerobically for 24 h at 37 °C with constant agitation. Aliquots of the culture were inoculated into fresh medium and incubated at 37 °C for 4–5 h until reaching an exponential growth phase. Bacterial cells were collected using centrifugation at 10,000 rpm for 10 min, and the bacterial pellet was washed with sterile water. Finally, bacterial cells were suspended in the water then diluted to the required cell density corresponding to 10^8 colony forming units per milliliter (CFU/ml).

2.3. Bactericidal experiment and analysis method

A 20 μl aliquot of the *E. coli* suspension was applied onto the wafer surface of catalyst, and the contacting times (refers to the bacteria contacting time with the wafers of catalyst before sampling) were for 0.5, 2, 5, 10 and 20 min at room temperature. The survival cells were washed off from the catalyst wafers with sterile water. 0.5 ml eluate was immediately injected into 4.5 ml 0.9% NaCl aqueous solution to eliminate the effect of Ag^+ [12], and then plated on LB agar (Fluka Co.61746) plates. The plates were incubated at 37 °C for 24 h before counting. All experiments were repeated three times. The wafers of catalysts were investigated using SEM during the bactericidal process. Before SEM measurement, the *E. coli* on the wafers were fixed with glutaraldehyde and osmium tetroxide, drained with ethanol/water with the concentrations of ethanol increasing. The absolute ethanol was replaced by dimethoxymethane, and the samples underwent critical point drying with CO_2. The wafers were glued onto stages with conductive silver and metallized with gold. The samples were microscoped and photographed with a scanning electron microscope (Fei QUANTA 200).

The bactericidal activity was measured under either aerobic or anaerobic conditions, which was achieved by oxygen or nitrogen bubbling to investigate the effect of dissolved oxygen. Superoxide dismutase (SOD, Sigma) was used to investigate the effect of ROS on bactericidal activity. Fifty unit per ml and 100 unit/ml of SOD solution was made, respectively. The solution was added to the *E. coli* K12 suspension and mixed sufficiently. A 20 μl of the mixture of *E. coli* suspension and SOD solution was applied onto the wafer surface of catalyst, and the sampling times were for 0.5, 2, 5, 10 and 20 min at room temperature.

In situ diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) method was used to investigate the dynamic changes of IR spectra on Ag/Al_2O_3 surface during the bactericidal process, and DRIFTS spectra were recorded on a Nexus 670 (Thermo Nicolet) FT-IR, equipped with an in situ diffuse reflection chamber and a high sensitivity MCT detector. After *E. coli* suspension and catalyst powder were mixed completely, the mixed sample was placed in a ceramic crucible in the in situ chamber. Total carbon (TC) content of mixture of *E. coli* and catalysts is determined using Apollo 9000 Analyzer (Terkmar Dohmann) during the bactericidal process. It was observed that TC decreased during the catalytic oxidation process, which implied that TC of *E. coli* had converted to CO_2 and released from the catalysts. The formation of CO_2 during this process can be calculated using the following equation:

$$\text{formation of } CO_2/\% = \frac{TC_0 - TC_i}{TC_0} \times 100\%,$$

where TC_0 signifies initial TC and TC_i is TC at different times during this process.

3. Results and discussion

3.1. Bactericidal effects of the catalysts

The bactericidal activity of Al_2O_3, Ag/Al_2O_3 and $AgCl/Al_2O_3$ in air was investigated. Fig. 1 shows the time course changes with the viable cell count of *E. coli*. The process where Al_2O_3 used as host compounds (Fig. 1a) shows that the cell concentration decreased from 2.8×10^8 CFU/ml to 1×10^6 CFU/ml within 20 min after addition. Obviously,

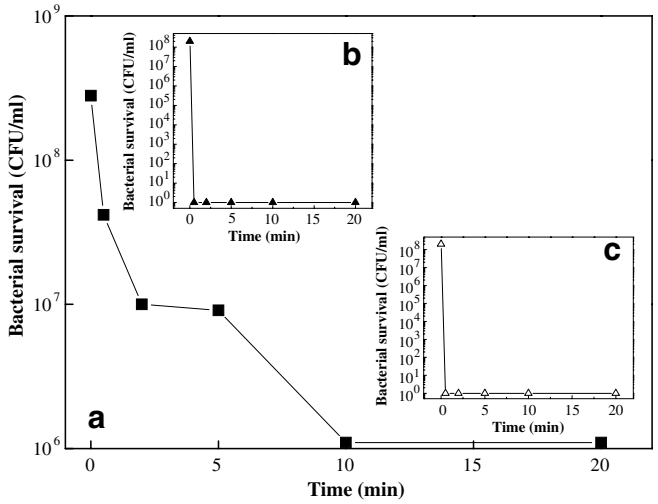

Fig. 1. *E. coli* K 12 inactivation on (a) Al_2O_3, (b) $AgCl/Al_2O_3$ and (c) Ag/Al_2O_3 surface. Initial concentration of bacteria: 2.8×10^8 CFU/ml.

the processes with Ag/Al_2O_3 (Fig. 1b) and $AgCl/Al_2O_3$ (Fig. 1c) show a higher bactericidal effect on *E. coli* than Al_2O_3. The bacteria lost their colony forming ability in 0.5 min after applied onto the wafer of Ag/Al_2O_3 and $AgCl/Al_2O_3$. The spoiling of bacteria was also observed by SEM photographs as shown in Fig. 2.

Fig. 2a and b show the SEM images of *E. coli* adhered on Al_2O_3 at room temperature for 0.5 min and 24 h, respectively. Although some cells were damaged after *E. coli* exposured to the wafer of Al_2O_3 for 0.5 min, the viable cells were still detectable using plate counting method. Some cells collapsed and led to release of intracellular constituents, however, their membrane and intracellular constituents covered the surface even after 24 h. Fig. 2c–e and f–h show the SEM images of *E. coli* adhered on $AgCl/Al_2O_3$ and Ag/Al_2O_3 at room temperature for 0.5 min, 0.5 and 1 h, respectively. As shown in Fig. 1, it is obvious that Ag/Al_2O_3 and $AgCl/Al_2O_3$ gave a higher bactericidal effect on *E. coli* than Al_2O_3. Although $AgCl/Al_2O_3$ exhibited a similar bactericidal ability comparing with Ag/Al_2O_3, the SEM images indicate that different mechanisms are involved in the bactericidal action of $AgCl/Al_2O_3$ and Ag/Al_2O_3. The cell membrane damage mainly started at one end of *E. coli* on the surface of $AgCl/Al_2O_3$ (Fig. 2c), and intracellular constituents were released from the damaged site. After exposure time of 0.5 h, the cell was further spoiled (Fig. 2d). After 1 h, the clean catalyst surface reappeared (Fig. 2e). The damage of bacteria on the surface of Ag/Al_2O_3 was more serious than that of $AgCl/Al_2O_3$ (Fig. 2f–h). Even though after 30 s on $AgCl/Al_2O_3$, a great deal of intracellular constituents spouted from damaged sites on the cell membrane (Fig. 2f). After exposure time of 0.5 h, *E. coli* cells shrank and the catalyst surface covered by the cells reappeared (Fig. 2g, h).

As mentioned above, the bactericidal mechanism of Ag containing compounds is still controversial [8–11]. During the process of the *E. coli* suspension contacted with Ag/Al_2O_3 and $AgCl/Al_2O_3$, Ag^+ released unavoidablely. In order to confirm the bactericidal effect of Ag^+, the catalyst wafers were dip in sterile water for 10 min and the quantitative analysis of Ag^+ in the eluate was carried out with an atomic adsorption spectrophotometer. The bactericidal effect of a series of $AgNO_3$ (10^{-9}, 10^{-8}, 10^{-5}, 10^{-4}, 10^{-3} M) contacting with *E. coli* suspension for 10 min were determined. The correlation between the concentration of Ag^+ and the quantity of survival cells was reflected in a curve, so the effect of Ag^+ released from the catalyst can be confirmed. As shown in Fig. 3, the colony-forming ability of *E. coli* lost in Ag^+ containing solution when the concentration of Ag^+ is more than 10^{-4} M. The concentrations of Ag^+ eluted from Ag/Al_2O_3 and $AgCl/Al_2O_3$ were less than 2.9×10^{-4} M and 4.7×10^{-7} M under such experimental conditions, respectively. According to this result, Ag^+ was released from the catalysts, and the bactericidal ability of the Ag/Al_2O_3 would be related to the amount of Ag^+ dissolved in a certain extent. However the amount of Ag^+ eluted from $AgCl/Al_2O_3$ is too little to cause any bactericidal effect against *E. coli*.

3.2. Effect of oxygen and nitrogen gas bubbling

The effect of oxygen and nitrogen gas bubbling on the bactericidal activity was also investigated. This investigation was performed to obtain information about the role of oxygen in the bactericidal activity of Ag/Al_2O_3 and $AgCl/Al_2O_3$. 0.5 g Al_2O_3, Ag/Al_2O_3 and $AgCl/Al_2O_3$ powder was added into 40 ml sterilized water respectively, and then the aerobic and anaerobic condition was achieved by oxygen and nitrogen gas bubbling for 40 min before piping the bacteria into the system. The bubbling was continued throughout the treatment period. The bactericidal activity of Al_2O_3 under the same condition was studied as the control experiment. The results are shown in Fig. 4. Under the anaerobic condition, the bactericidal activity of Ag/Al_2O_3 sharply decreased compared to that in the aerobic condition. Though the bactericidal activity of $AgCl/Al_2O_3$ was lower than Ag/Al_2O_3, it can also be obviously observed that the bactericidal activity in aerobic condition is better than that in anaerobic condition. These results suggest that nitrogen gas bubbling drastically suppresses the bactericidal activity of Ag/Al_2O_3, and indicate that the presence of oxygen is necessary for the bactericidal activity by Ag/Al_2O_3 and $AgCl/Al_2O_3$.

3.3. Effect of ROS on the surface of catalysts in the bactericidal process

From above experimental results, we consider that oxygen can be activated to ROS by the catalyst, which play an important role in the bactericidal activity. The ROS might contain superoxide anions, hydrogen peroxide and hydroxyl radicals. SOD is a scavenger for superoxide anions and hydroxyl radicals. The effect of addition of SOD on the bactericidal activity was investigated (Fig. 5).

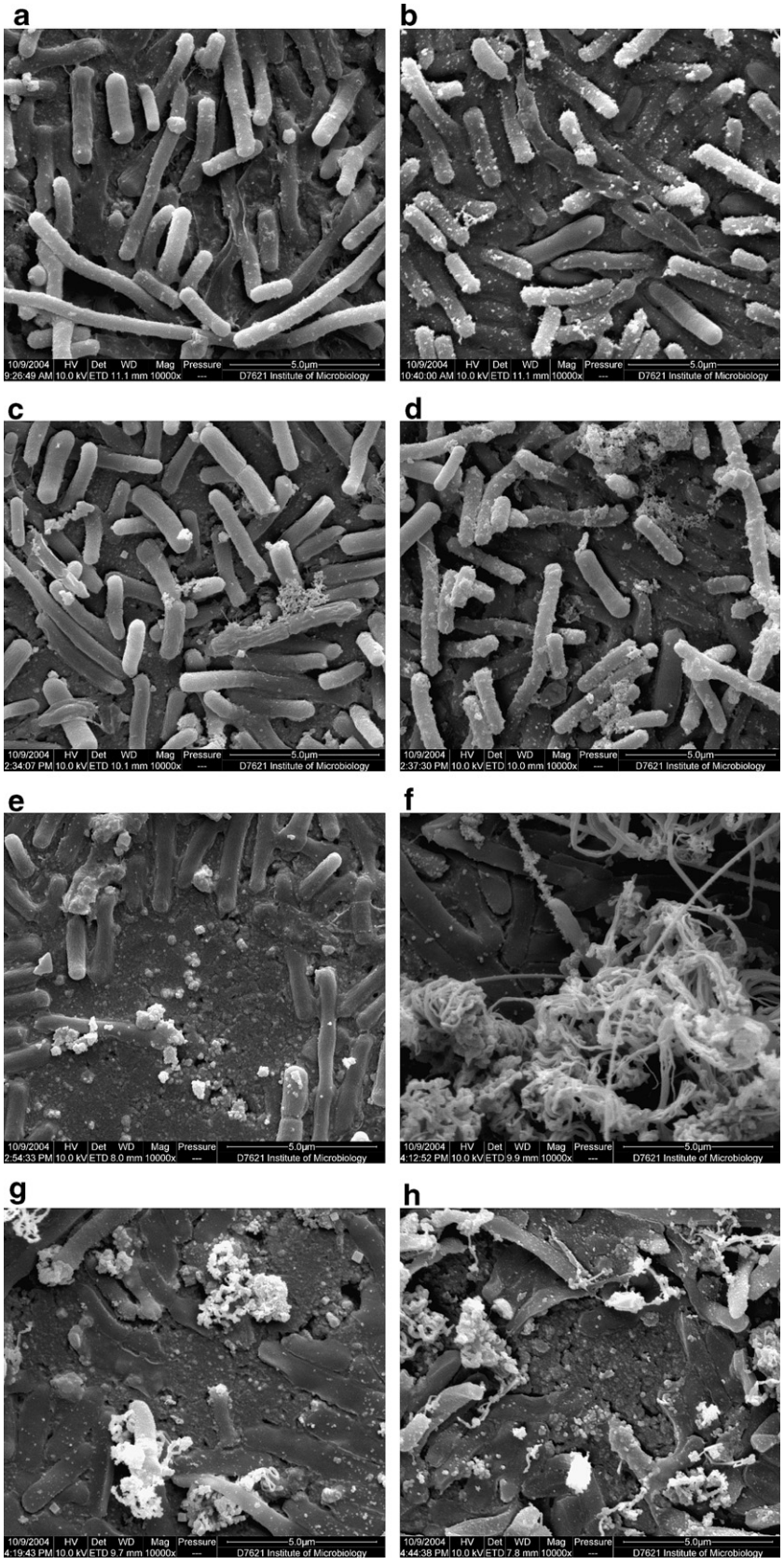

Fig. 2. SEM images of *E. coli* adsorbed on (a) Al_2O_3 at room temperature for 0.5 min and (b) 24 h, on (c) $AgCl/Al_2O_3$ for 0.5 min, (d) for 0.5 h, (e) for 1 h and on (f) Ag/Al_2O_3 for 0.5 min, (g) for 0.5 h and (h) for 1 h.

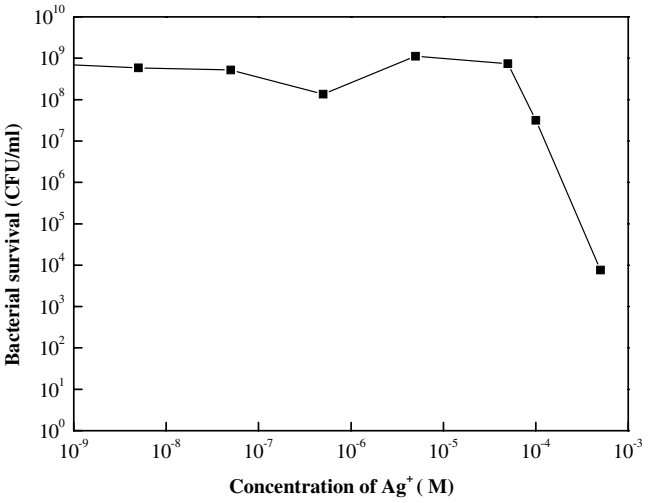

Fig. 3. Effect of the concentration of Ag^{+} on the colony-forming ability of *E. coli*.

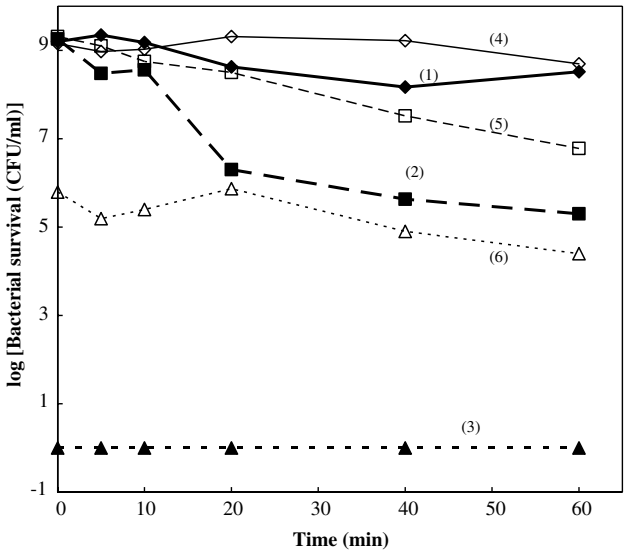

Fig. 4. Effect of O_2 and N_2 bubbling on the bactericidal activity of Ag/Al_2O_3 and $AgCl/Al_2O_3$: (1) Al_2O_3, (2) $AgCl/Al_2O_3$, (3) Ag/Al_2O_3 in aerated condition; (4) Al_2O_3, (5) $AgCl/Al_2O_3$, (6) Ag/Al_2O_3 in nonaerated condition.

Compared with the situation without SOD, in the presence of SOD, the bacterial survival apparently increased on the surface of Ag/Al_2O_3 and $AgCl/Al_2O_3$, respectively. The addition of 100 unit/ml SOD drastically suppressed the bactericidal activity. The survival bacterial was kept more than 1×10^6 CFU/ml when 100 unit/ml of SOD added (The survival bacteria were less than 5×10^5 CFU/ml when 50 unit/ml of SOD added). This result indicates that the formation of superoxide anions and hydroxyl radicals contributes to the bactericidal activity.

3.4. Formation of CO_2 during bactericidal process

As shown in Fig. 6, Ag/Al_2O_3 added with *E. coli* was investigated using DRIFTS during exposure of the sample

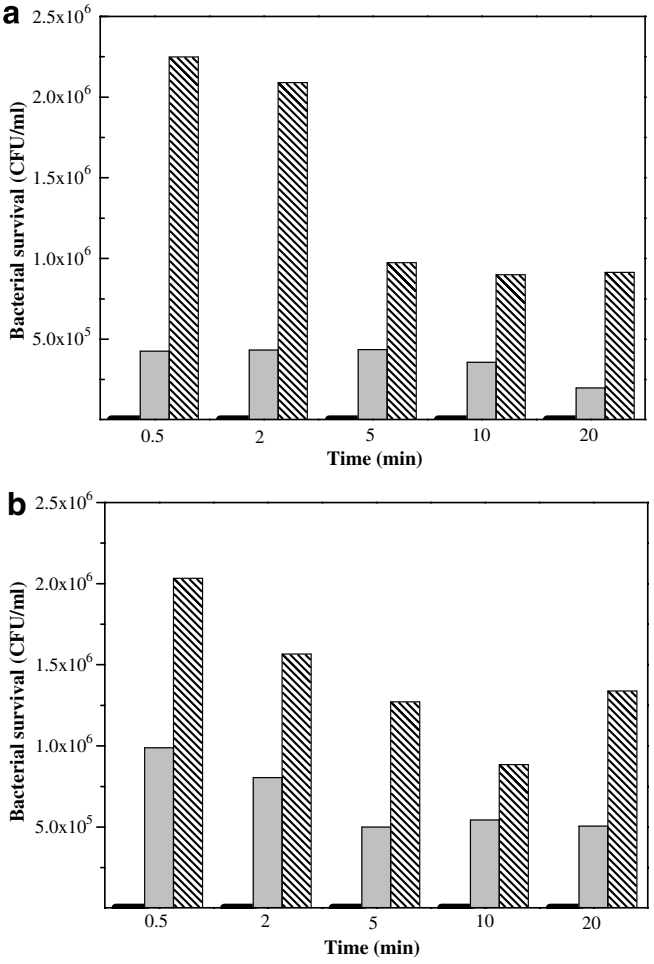

Fig. 5. Effect of SOD against ROS on the bactericidal activity of (a) Ag/Al_2O_3 and (b) $AgCl/Al_2O_3$, respectively. ▬ 0 unit/ml of SOD (The bactericidal survival in the case of 0 unit/ml of SOD reached 0.0E + 00 CFU/ml); ▭ 50 unit/ml of SOD; ▨ 100 unit/ml of SOD.

to O_2 for 42 h at 44 °C. After exposure of the sample to pure O_2 for 18 h, peaks at 2339 and 2362 cm^{-1} assigned to gas phase CO_2 were observed, and the intensity of CO_2 peaks increased with time increasing. The appearance of gas phase CO_2 strongly supports that the bactericidal process on Ag/Al_2O_3 not only leads to the cell death, but also causes the completely catalytic oxidation of bacteria and other intermediates.

Furthermore, the formation of CO_2 was investigated by TC analysis as shown in Fig. 7. About 49% and 28% of TC of *E. coli* were converted to CO_2 in air at 44 °C after the *E. coli* cells were added to the surfaces of Ag/Al_2O_3 and $AgCl/Al_2O_3$ for 125 h, respectively.

It should be noticed that the time of surface reappearance is different from the time producing CO_2. In the SEM images, we can only see the surface uncovered with bacteria reappeared, but the surface might not as clean as the fresh surface, where small molecules might adsorb. The small molecules might have not been oxygenated completely into CO_2, but they already could not be seen using SEM. In addition, the catalyst could adsorb a mass of CO_2,

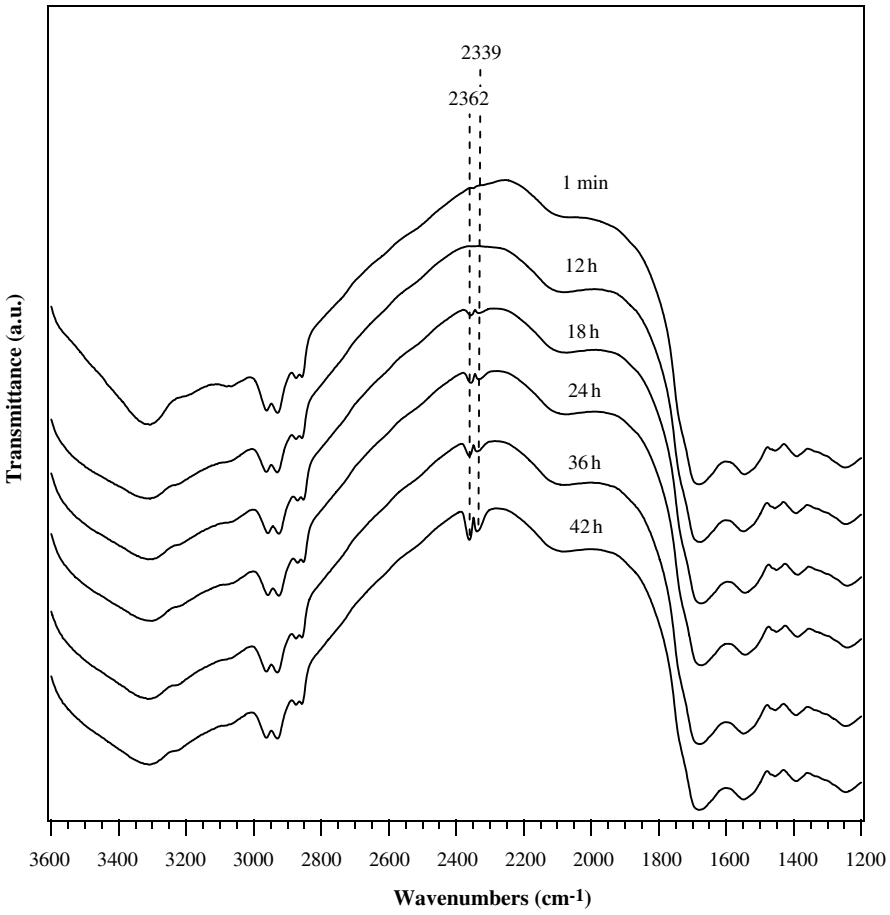

Fig. 6. Dynamic changes of in situ DRIFTS spectra of Ag/Al_2O_3 as a function of time during exposure of the mixed sample to pure O_2 at 44 °C. Before measurement, the catalyst powder was mixed with *E. coli* suspension.

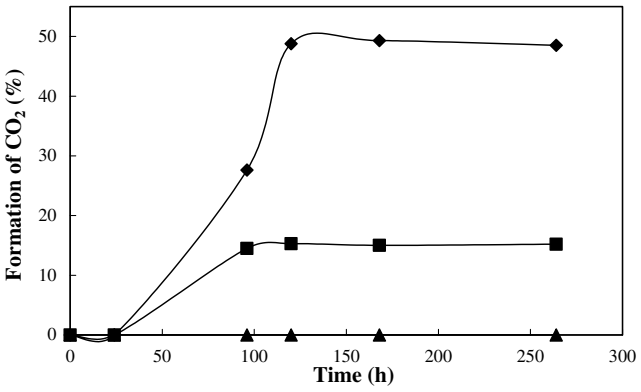

Fig. 7. Detection of CO_2 produced during the bactericidal process at 44 °C by TC analysis: (◆) Ag/Al_2O_3; (■) $AgCl/Al_2O_3$; (▲) Al_2O_3.

because of its large specific surface area. Therefore the detection of CO_2 desorbed from the catalyst will be delayed.

4. Conclusion

Ag/Al_2O_3 and $AgCl/Al_2O_3$ demonstrated a stronger bactericidal activity than Al_2O_3. The colony-forming ability of *E. coli* was completely lost in 0.5 min on both of Ag/Al_2O_3 and $AgCl/Al_2O_3$ at room temperature in air. Relying on SEM investigation, the different bactericidal mechanisms were identified on the surfaces of Ag/Al_2O_3 and $AgCl/Al_2O_3$. The role of Ag^+ eluted from $AgCl/Al_2O_3$ can be ignored in our experiments. Oxygen, which can be activated to ROS by catalyst, is essential for the expression of bactericidal activity. The strong catalytic oxidation ability was expressed on the Ag/Al_2O_3 surface, and CO_2 as the ultimate product was observed during the bactericidal process. All of these results indicate that the catalytic oxidation is the essential mechanism in bactericidal process. Further work is needed to clarify the details of these actions.

5. Abbreviations

ROS	reactive oxygen species
SEM	scanning electron microscopy
SARS	severe acute respiratory syndrome
XRD	X-ray polycrystalline diffraction
TEM	transmission electron microscopy
CFU/ml	colony forming units per milliliter
SOD	superoxide dismutase

DRIFTS diffuse reflectance infrared Fourier transform spectroscopy
TC total carbon

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (50621804, 50538090).

References

[1] J.C. Yu, W. Ho, J. Lin, H. Yip, P. Wong, Environ. Sci. Technol. 37 (2003) 2296–2301.
[2] A. Rincón, C. Pulgarin, Appl. Catal. B 51 (2004) 283–302.
[3] A. Rincón, C. Pulgarin, Appl. Catal. B 49 (2004) 99–112.
[4] Z. Huang, P. Maness, D.M. Blake, E.J. Wolfrum, S.L. Smolinski, W.A. Jacoby, J. Photochem. Photobio. A 130 (2000) 163–170.
[5] T. Tatsuma, S. Takeda, S. Saitoh, Y. Ohko, A. Fujishima, Electrochem. Commun. 5 (2003) 793–796.
[6] H. He, X. Dong, M. Yang, Q. Yang, S. Duan, Y. Yu, J. Han, C. Zhang, L. Chen, X. Yang, Catal. Commun. 5 (2004) 170–172.
[7] P. Dowling, A.J. Betts, C. Pope, M.L. McConnell, R. Eloy, M.N. Arnaud, Surf. Coat. Tech. 163–164 (2003) 637–640.
[8] M. Sökmen, F. Candan, Z. Sümer, J. Photoch. Photobio A: Chemistry 143 (2001) 241–244.
[9] Y. Inoue, M. Hoshino, H. Takahashi, T. Noguchi, T. Murata, Y. Kanzaki, H. Hamashima, M. Sasatsu J. Inorg. Biochem. 92 (2002) 37–42.
[10] H. Le Pape, F. Solano-Serena, P. Contini, C. Devillers, A. Maftah, P. Leprat, J. Inorg. Biochem. 98 (2004) 1054–1060.
[11] H. He, C.B. Zhang, Y.B. Yu, Catal. Today 90 (2004) 191–197.
[12] Yoshinobu Matsumura et al., Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate, Appl. Environ. Microbiol. 69 (2003) 4278–4281.