Sterilization of Pathogenic Bacteria Using Titanium Dioxide Photocatalyst

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Abstract. Titanium dioxide (TiO\textsubscript{2}) photocatalysts have attracted great attention as a material for photocatalytic sterilization in the food and environmental industry. This research aimed to design a new photobioreactor and its application to sterilize selected pathogenic bacteria, \textit{Salmonella choleraesuis} subsp., \textit{Vibrio parahaemolyticus}, \textit{Listeria monocytogenes}, and \textit{Pseudomonas sp}. The photocatalytic reaction was carried out with various TiO\textsubscript{2} concentrations, ultraviolet (UV) illumination time.

The bactericidal effect of TiO\textsubscript{2} under UV light irradiation on all bacterial suspension was much higher than that of without TiO\textsubscript{2}. As the concentration of TiO\textsubscript{2} increased to 1.0 mg/ ml, bactericidal effect increased. The complete killing time of \textit{Salmonella}, \textit{Vibrio and Pseudomonas} was achieved after 3 hr but \textit{Listeria} was more resistant. However, titanium dioxide powder could not be used satisfactorily for the sterilization of the bacteria due to its poor separation from the suspension. Therefore, in this study, chitosan bead coated with titanium dioxide was made by sol-gel method, and used for sterilization. The survival ratio of \textit{Salmonella} using chitosan bead coated with TiO\textsubscript{2} was greater than that of chitosan bead.

Keywords: Titanium dioxide, \textit{Salmonella}, \textit{Vibrio}, \textit{Listeria}, \textit{Pseudomonas}, chitosan.

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INTRODUCTION

TiO\textsubscript{2} (Titanium oxide) photocatalysts have attracted great attention as alternative materials to aids in the purification of water and air.\textsuperscript{1-3} TiO\textsubscript{2} photocatalysts generate strong oxidizing power when illuminated with UV light with wavelength of less than 385nm. Illuminated TiO\textsubscript{2} photocatalysts decompose organic compounds by oxidation, with holes (h\textsuperscript{+}) generated in the valence band and with conduction hydroxyl radical (OH\textsuperscript{-}) produced by the oxidation water.

Wastewater from hospital, food factories, and contaminated sites sometimes contains microorganisms, virus, and organic compounds. One of the typical sterilization method is the photocatalytic sterilization by illumination of UV light with the wavelength of 254nm, which provides a high rate of sterilization at room temperature. Alternately, it is well known that the TiO\textsubscript{2} in anatase form is capable of oxidizing and the decomposing various kinds of compounds.\textsuperscript{3-5}

\textit{Salmonella choleraesuis} are important component of the porcine respiratory disease complex (PRDC). The recognition of \textit{S. choleraesuis} as an important and common cause of swine respiratory disease and the emergence of Porcine reproduction and respiratory syndrom (PRRS) as a new swine disease have both occurred only relatively recently.\textsuperscript{6} \textit{Vibrio parahaemolyticus} is a prevalent food-borne pathogen in many Asian countries where marine foods are frequently consumed. Environmental strains of \textit{V. parahaemolyticus} are typically not human pathogens. However, these strains cause disease in shrimps,
oysters, mussels and other marine invertebrates and in fishes. *Listeria monocytogenes* is a Gram-positive facultative intracellular pathogen that is the causative agent of food borne listeriosis.\(^7\)

TiO\(_2\) particles catalyze the killing of bacteria and cancer cells\(^8\) by near-UV light, probably due to the generation of free radicals by photoexcited TiO\(_2\) particles. Reports have appeared concerning the bactericidal effects of TiO\(_2\) powder, often referring to OH\(^*\) as the toxic agent.\(^9\) In 1985, Matsunaga and coworkers reported that microbial cells in water could be killed by contact with a TiO\(_2\)-Pt catalyst upon illumination with near-UV light for 60 to 120 min. Since then, research work on TiO\(_2\) photocatalytic killing has been intensively conducted on a wide spectrum of organisms including viruses, bacteria, fungi, algae, and cancer cells.

For practical applications, however, there are some difficulties such as either filtration of fine TiO\(_2\) or fixation of catalyst particles. For these reasons, many scientists have been working to increase the efficiency of this process. One of direction is to improve efficiency of TiO\(_2\) by modification of its surface. TiO\(_2\) supported on the porous materials such as SiO\(_2\), ZrO\(_2\), zeolites and activated carbons were used to increase the efficiency. It was also reported that the addition of activated carbon to TiO\(_2\) slurry could increase decomposition of some organic compounds during the photocatalytic process.\(^10\) It has an additional benefit in reducing the recombination rate of electron with hole by increasing the energy of the band gap in TiO\(_2\). In other papers\(^11\), carbon coating of photocatalytic TiO\(_2\) particles was successfully performed without pronounced interruption of photoactivity. Much higher photocatalytic performance was obtained because of notable suppression of phase transformation to less photoactive rutile and also acceleration of crystal growth of anatase phase.

In this study, sterilization experiments for *S. choleraesuis* subsp., *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Pseudomonas sp* were conducted in a batch type photocatalytic reactor in terms of UV illumination time, TiO\(_2\) concentration. And TiO\(_2\) was coated on chitosan bead for the easy recovery of the photocatalyst after photocatalytic-reaction.

### MATERIALS AND METHODS

Two crystalline forms of TiO\(_2\) have photocatalytic activity, anatase and rutile. Anatase has a band gap of 3.2 eV and for rutile it is 3.0 eV. Anatase has been found to be the most active form. The action spectrum for anatase shows a sharp decrease in activity above about 385 nm. The photocatalytic process includes chemical steps that produce reactive species that in principal can cause fatal damage to microorganisms. The reactive oxygen species (ROS) may disrupt or damage various cell or viral functions or structures. The preponderance of evidence on photocatalytic chemistry in aqueous solution suggests that the hydroxyl radical formed by hole transfer does not diffuse from the surface of the TiO\(_2\) into bulk aqueous phase\(^13\). For a cell or virus in contact with the titanium dioxide surface there also be direct electron or hole transfer to the organism or one of its components. If titanium dioxide particles are of small size, they may penetrate into the cell and these processes could in the interior. Since light is an essential component of the photocatalytic system, there can also be direct photochemistry as there would be from any UV source. Mechanism of a photocatalytic process on irradiated titanium dioxide:

**Electron-Hole Pair Formation**

\[
\text{TiO}_2 + \text{hv} \rightarrow \text{TiO}_2^- + \text{OH}^* \quad (\text{or TiO}_2^+) \\
\text{[conduction band electron and valence band hole]}
\]

(1)
Electron removal from the conduction band

TiO$_2^-$ + O$_2$ + H$^+$ $\rightarrow$ TiO$_2$ + HO$_2$ \hspace{1cm} (2)

TiO$_2^-$ + H$_2$O$_2$ + H$^+$ $\rightarrow$ TiO$_2$ + H$_2$O + OH$^-$ \hspace{1cm} (3)

TiO$_2^-$ + H$^+$ $\rightarrow$ TiO$_2$ + •H$_2$ \hspace{1cm} (4)

Oxidation of organic compounds

OH$^-$ + O$_2$ + C$_n$O$_m$H$_{2(n-2m+2)}$ $\rightarrow$ nCO$_2$ + (n-m+1)H$_2$O \hspace{1cm} (5)

Nonproductive Radical Reactions

TiO$_2^-$ + OH$^-$ + H$^+$ $\rightarrow$ TiO$_2$ + H$_2$O [recombination] \hspace{1cm} (6)

2OH$^-$ $\rightarrow$ H$_2$O$_2$ \hspace{1cm} (7)

2HO$_2^-$ $\rightarrow$ H$_2$O$_2$ + O$_2$ \hspace{1cm} (8)

OH$^-$ + H$_2$O$_2$ $\rightarrow$ H$_2$O + O$_2$ \hspace{1cm} (9)

OH$^-$ + HCO$_3^-$ $\rightarrow$ CO$_3^-$ + H$_2$O \hspace{1cm} (10)

Hydroxyl radicals are highly reactive and therefore short-lived. Superoxide ions are more long-lived; however, due to the negative charge they cannot penetrate the cell membrane. Upon their production on the TiO$_2$ surface, both hydroxyl radicals and superoxide would have to interact immediately with the outer surface of an organism unless the TiO$_2$ particle has penetrated into the cell.

Compared to hydroxyl radicals and superoxide ions, hydrogen peroxide is less detrimental. However, hydrogen peroxide can enter the cell and be activated by ferrous ion via the Fenton reaction.

Fe$^{+2}$ + H$_2$O$_2$ $\rightarrow$ OH$^-$ + OH$^+$ + Fe$^{+3}$ \hspace{1cm} (11)

The ability of bacteria, such as E. coli, to sequester iron is well documented. Iron levels on the cell surface, in the periplasmic space or inside the cell, either as iron clusters or in iron storage proteins (such as ferritin) are significant and can serve as a source of ferrous ion. Therefore, while the TiO$_2$ is being illuminated to produce H$_2$O$_2$, the Fenton reaction may take place in vivo and produce the more damaging hydroxyl radicals. When the light is turned off, any residual hydroxyl radicals through the Fenton reaction. When both H$_2$O$_2$ and superoxide ion are present, the iron-catalyzed Haber-Weiss reaction can provide a second pathway to form additional hydroxyl radicals.

Fe$^{+3}$ + O$_2^-$ $\rightarrow$ Fe$^{+2}$ + O$_2$ \hspace{1cm} (12)

Fe$^{+2}$ + H$_2$O$_2$ $\rightarrow$ Fe$^{+3}$ + OH$^-$ + OH$^+$ \hspace{1cm} (13)

Since the initial actions of these reactive oxygen species (ROS) target the outer surface of a cell, the rigidity and chemical arrangements of their surface structure will determine how effectively the TiO$_2$ photocatalytic disinfection process functions.

TiO$_2$

The photocatalyst TiO$_2$ (anatase) was obtained from Aldrich Co.. The physical properties of TiO$_2$ were characterized by BET analysis. The specific surface area, pore size and pore volume were 12.02 m$^2$/g, 57.48 Å and 0.0172 cm$^3$/pore, respectively. Prior to use, this compound was sealed and stored at
room temperature.

**CHTOSAN BEAD COATED WITH TiO₂**

The supports for TiO₂ powder used in this study were chitosan, manufactured by Sehwa Co. (South Korea). Chitosan was obtained as flaked type from Sehwa Co., without further purification. Flaked chitosan was milled to pass through a 180-μm sieve. The degree of deacetylation of chitosan flakes was determined to be 85%. The average molecular weight of the chitosan was measured to be $8.2 \times 10^5$ by the Mark–Houwink equation from viscosity measurements at different concentrations. When used as a support for TiO₂ powder, the powdered chitosan was dissolved in a 2 wt% acetic acid solution to produce a viscous solution with approx. 2 wt% chitosan. TiO₂ powders (5 g) were dispersed into 100 ml of the chitosan solution during 24 h under continuous stirring. Thereafter, the mixed solution was cast into beads by a phase-inversion technique using 2 M NaOH. The physical properties of the prepared chitosan bead and TiO₂-coated chitosan beads are given in Tables 1.

<table>
<thead>
<tr>
<th>Unit</th>
<th>Chitosan bead</th>
<th>Chitosan beads coated with TiO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle diameter</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Multi point BET</td>
<td>350</td>
<td>79</td>
</tr>
<tr>
<td>Average pore diameter</td>
<td>856</td>
<td>97.4</td>
</tr>
</tbody>
</table>

**MICROORGANISMS AND CULTURE CONDITIONS**

Microorganisms, *Salmonella choleraesuis subsp.*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Pseudomonas sp.* were purchased from KCCM (Korean Culture Collection of Microorganisms, Seoul, Korea). The culture medium for stock cultures of *S. choleraesuis subsp.*, *V. parahaemolyticus*, *L. monocytogenes*, *Pseudomonas sp.* was typtic soy broth (TSB, Merck, Germany), TSB with 3% (w/v) NaCl, and a brain heart infusion (BHI, Becton Dickinson, USA) respectively. The working culture was prepared by transferring each bacterium from stock culture to 300 ml conical glass flasks containing 50 ml of medium. Culture flasks were aerobically incubated on a rotary shaker at 33°C for 24 h. Planktonic cell preparation *S. choleraesuis subsp.*, *L. monocytogenes* *V. parahaemolyticus* and *Pseudomonas sp.* cells grown from working culture were harvested by centrifugation of them using a centrifuge (Hanshin, medical, Co., LTD., Korea) at 4000 rpm for 15 min. *S. choleraesuis subsp.* *L. monocytogenes* and *Pseudomonas sp.* were washed twice using a sterile phosphate-buffer saline (PBS, pH 7.2) and resuspended with PBS. The cells of *V. parahaemolyticus* was washed twice in sterile deionized water containing 3% (w/v) NaCl. All the washed cells in solution was counted and stored at 5°C until use.
PHOTOCATALYTIC REACTION

The photocatalytic reactor consisted of UV lamp (A), sampling tube (B), magnetic stirrer (C), air filter (D), thermo-circulator (E), peristatic pump (F), sampling tube (G), and temperature controller (H) (Fig. 1). The reaction was performed at 1000 ml working volume of batch reactor.

UV lamp was centered to illuminate properly the inner of vessel while the mixture of cells and TiO₂ were agitated continuously. Temperature was controlled 25°C using the thermo-circulator during the photocatalytic reaction. The initial concentration of cells of *S. choleraesuis* subsp., *V. parahaemolyticus*, *L. monocytogenes* and *Pseudomonas sp.* was adjusted to about 10⁸ cfu/ml. To investigate the optimal dose of TiO₂, photocatalytic reactions were carried out with TiO₂ concentrations ranging from 0.25 to 1.25 mg/ml. The control was treated either without TiO₂ or with UV only. The illumination time was 3 hr for *S. choleraesuis* subsp., *V. parahaemolyticus*, *L. monocytogenes* and *Pseudomonas sp.* For vial cell counting, the media of *S. choleraesuis* subsp., *V. parahaemolyticus*, *L. monocytogenes* and *Pseudomonas sp.* was a TSA respectively. Bacterial cells were sampled in triplicates at every 30 min and 1 hr for 3 hr. All plates were incubated at 33°C for 24 h.

EFFECT OF UV ILLUMINATION TIME AND TiO₂ CONCENTRATION
The photoreactor employed in batch process was designed for the mixture of cells and TiO$_2$ to contact continuously or be close to the surface of UV lamp as much as possible. In all cases, the inactivation of cells was evaluated by counting the viable cells with regular sampling. The feasible synergistic effect by UV and TiO$_2$ after 1 h or more treatments was appeared in all three strains. (Fig. 2: S. choleraesuis subsp., Fig. 3: V. paraaemolyticus, Fig. 4: L. monocytogenes, Fig. 5: Pseudomonas sp.). These Figures showed the changes in the cells viabilities of S. choleraesuis subsp. (Fig. 2), V. paraaemolyticus (Fig. 3), L. monocytogenes (Fig. 4) and Pseudomonas sp.(Fig. 5), respectively during constant UV illumination at various TiO$_2$ concentrations ranging from 0 to 1.25 mg/ml. After 30 min of irradiation with UV light in the presence of 1 mg TiO2/ml, 62% of S. choleraesuis subsp. lost survival. However, when the concentration of TiO$_2$ was decreased to 0.25 mg/ml, killing efficiency was decreased to 55% of bacteria. TiO$_2$ concentrations greater than 1 mg/ml resulted in a decrease in killing efficiency. We supposed that probably this trend related to be so many TiO$_2$ particles that the intensity of UV illumination became weak. As a result, the most effective TiO$_2$ concentration in the present study was 1 mg/ml. In the case of 1 mg/ml TiO$_2$ concentration, complete killing time was observed at 3 h and more than 99% of the cells lost their viability. Almost complete killing was achieved after 2 h of illumination. After 30 min of irradiation with UV light in the presence of 1 mg TiO2/ml, more than 80% of V. paraaemolyticus lost viability (Fig. 3B). When the concentration of TiO$_2$ was decreased to 0.25 mg/ml, killing efficiency was a decrease to 35% of bacteria. Similar as S. choleraesuis subsp., TiO$_2$ concentrations greater than 1 mg/ml resulted in a decrease in killing efficiency. As a result, the most effective TiO$_2$concentration in the present study was 1 mg/ml. In the case of 1 mg/ml TiO$_2$ concentration, complete killing time was observed at about 2 h and more than 99% of the cells lost their viability. The survival curve of L. monocytogenes in Fig. 3B indicated that killing activity was much lower than that of the other bacteria. At 30 min illumination, only 35% of cells were killed and their complete killing did not appear until 4 h reaction. In this study, a sensitive approach using TiO$_2$ has been adapted successfully to examine TiO$_2$-mediated damages and their contribution to the deactivation of viable cells.
Data from Figs. 2–5 suggest that TiO$_2$ particles remaining in the slurry may still retain their bactericidal activity. Another explanation is that certain lethal reactions would continue to propagate even after the UV illumination stops. This effect may be masked by the standard viable count procedure involving serial dilutions in buffered saline, as it allows cells to form colonies on rich nutrient media, which in turn allows the injured cell to recover. It is not surprising that cells would lose viability even after the removal of TiO$_2$ and/or UV-light.

Fig. 6 shows the bactericidal effect of S. choleraesuis subsp for chitosan bead and TiO$_2$ coated on chitosan bead. As can been seen in this figure, the survival ratio of S. choleraesuis subsp onto chitosan bead is lower than that of TiO$_2$ coated on chitosan bead. Furthermore, the similar results were obtained when we processed the experiment with recycled TiO$_2$-coated chitosan bead. We have examined the possibility of use chitosan bead as a support material for TiO$_2$ through this experiment.

Titanium dioxide (TiO$_2$) powder could not have been used satisfactorily due to its poor recovery in water contain bacteria. However, TiO$_2$ coated on chitosan bead can be applied to environment purification.

CONCLUSIONS

In the study of the photocatalytic bactericidal effect of TiO$_2$ on food pathogenic bacteria, it was confirmed that both near-UV illumination time and TiO$_2$ concentrations influenced the cell killing
activity. We founded that optimal TiO$_2$ concentration was 1mg TiO$_2$/ml. After 30 min near-UV illumination, killing activity of V. parahaemolyticus cells was higher than other bacteria, however, L. monocytogenes cells showed a lower killing activity than that of other bacteria.

Killing activity of _S.choleresuis_ subsp for TiO$_2$ coated on chitosan bead was more efficient than chitosan bead. Furthermore, the similar results were obtained when we processed the experiment with recycled TiO$_2$-coated chitosan bead. We have examined the possibility of use chitosan bead as a support material for TiO$_2$ through this experiment.

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