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SHORT COMMUNICATION

Dai Sawada · Masatake Ohmasa · Masaki Fukuda
Kazuhiko Masuno · Hiroshi Koide · Shigeyuki Tsunoda
Kimiyoshi Nakamura

Disinfection of some pathogens of mushroom cultivation by photocatalytic treatment

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Abstract Photocatalytic disinfection of six bacteria and fungi, including pathogens of four mushroom diseases, *Trichoderma harzianum*, *Cladobotryum varium*, *Spicellum roseum*, and *Pseudomonas tolaasii*, and *Escherichia coli* and *Bacillus subtilis*, was studied. The photocatalyst reduced the number of viable microorganisms sufficiently by near-UV irradiation. Efficiency of disinfection was increased for *P. tolaasii* and *E. coli*, but not for *T. harzianum*, when the superhydrophilic properties of the photocatalyst were induced by 16 h irradiation of the photocatalyst by near-UV light just before treatment of microorganisms. Efficiency of disinfection was also affected by the state of the microorganisms, temperature, and the thickness of suspensions of organisms. Tests of disinfecting ability of the photocatalyst in mushroom growing rooms indicate that it can be used effectively for reducing numbers of environmental bacteria and fungi under black light, and that it was also effective under white light.

D. Sawada
Purchasing Department, Natori Co., Ltd., Tokyo, Japan

M. Ohmasa (✉)
Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan
Tel. +81-265-77-1620; Fax +81-265-77-1629
e-mail: ohmasa1@gipmc.shnshu-u.ac.jp

M. Fukuda
Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano, Japan

K. Masuno
Nagano Prefecture Forest Research Center, Shiojiri, Nagano, Japan

H. Koide
The former director of Division of Mushroom, Nagano Prefecture Forest Research Center, Shiojiri, Nagano, Japan

S. Tsunoda
Nagano Agricultural Research Center, Nagano, Japan

K. Nakamura
Nagano Vegetable and Ornamental Crops Experiment Station, Nagano, Japan

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In the cultivation of mushrooms, infection by bacteria and fungi living in the mushroom growing room is an important problem (Furukawa and Nobuchi 1986). Reducing the number of bacteria and fungi in the mushroom growing room will contribute to reducing damage from bacterial and fungal diseases. Usage of fungicides is restricted because of their residual toxicity. Reducing environmental pollutants and microorganisms by the titanium oxide (TiO_2) photocatalyst is a safe method that has been developed in many places, such as in hospitals and other public places (Tone et al. 1993; Watanabe 1995; Pham et al. 1995; Fujishima et al. 1997). In this study, we examined the possibility of using the technique in reducing numbers of bacteria and fungi pathogenic to mushrooms and those of environmental bacteria and fungi in the mushroom growing room.

The fungal and bacterial strains used in this study are listed in Table 1. For the preparation of suspensions of fungal spores or germinated fungal spores for photocatalytic experiments, fungi were inoculated to a potato dextrose agar (PDA; Eiken, Tokyo, Japan) slant media and cultured at 25°C for 1–3 weeks depending on the strain under light conditions. Spores of fungi were suspended in 3 ml dilution buffer for fungi (McIlvaine buffer, pH 5.5), and diluted to 1.0×10^5 spores/ml for the experiment. The spore number was determined with a hemacytometer. For the preparation of germinated spores, fungi were cultured in a PDA slant medium and spores were collected and suspended in 10 ml 2% malt extract medium in a test tube. After shake culture at 120 rpm at 25°C for 15–17 h, depending on the strain, the germinated spores were collected by centrifugation (150 *g*, 2 min), suspended in the dilution buffer, and washed twice by the same buffer. They were further diluted to an appropriate concentration and used for experiments. More than 90% of spores were germinated after the shake culture. For preparation of the bacterial

Table 1. Strains used in this study

Species	Strain no.	Origin
Fungi		
Trichoderma harzianum Rifai	SA320	From Forestry & Forest Products Research Institute
	SA321	Isolated from sawdust culture of *Lentinula edodes*
	SA328	IFO 31292
Cladobotryum varium Nees	SA329	From Vegetable & Ornamental Crops Experiment Station
	SA331	From Vegetable & Ornamental Crops Experiment Station
Spicellum roseum Nicot & Roquebert	SA332	From Vegetable & Ornamental Crops Experiment Station
	SA335	From Vegetable & Ornamental Crops Experiment Station
Bacteria		
Pseudomonas tolaasii Paine	SA409	Isolated from *Pleurotus ostreatus*
Escherichia coli (Migura) Castellani & Charmers	SA412	IFO 3301
Bacillus subtilis Marburg	SA478	IFO 3007

suspension, *Pseudomonas tolaasii* Paine, *Bacillus subtilis* Marburg, and *Escherichia coli* (Migura) Castellani & Charmers were cultured under dark conditions for 24 h on King B medium (Shirata et al. 1995), medium G (Gould 1971), or nutrient agar slant medium, respectively. *P. tolaasii* was cultured at 24°C, *B. subtilis* at 30°C, and *E. coli* at 31°C. The bacteria were susupended in the dilution buffer for bacteria (NaCl 0.8%, sodium phosphate 0.01 M, pH 7.0) and diluted with the same buffer to 1.2–4.0 $\times$ 10^4 colony-forming units (CFU)/ml for the experiment. Endospores of *B. subtilis* were prepared by the method described by Gould (1971) and suspended as bacterial cells. The yield of endospores was more than 80%.

TiO_2 photocatalyst was prepared by applying a photocatalytic coating reagent, "Bisutoreita" (Nihon Soda, Tokyo, Japan), to both sides of a slide glass (Micro Slide Glass, S-1111; Matsunami, Osaka, Japan) and by drying as indicated by the manufacturer. The slide glass was used as the photocatalyst for laboratory experiments. In experiments in mushroom growing rooms, one side of an acrylamide board, 265 $\times$ 230 mm, was coated similarly. The surface of portions of pipes made of stainless steel or of vinyl chloride of the shelves in the mushroom growing rooms was also treated by the same way.

The slide glass photocatalyst was washed with a neutral detergent (Lion, Tokyo, Japan), rinsed thoroughly with distilled water, dried, immersed in 70% ethanol for 30 s, dried, and used for experiments. One hundred microliters of the suspension of fungi or bacteria was placed on the slide glass photocatalyst and covered with a polypropylene film (21 $\times$ 21 mm, thickness 9 μm). The slide glass photocatalyst was set in a Pyrex Petri dish in which sterilized absorbent cotton balls containing sterile water were also set to prevent drying of samples.

The Petri dish was set in an irradiation box, in which two black-light blue fluorescent lamps (FL20S-BLB-A; Toshiba, Tokyo, Japan), which emited near-ultraviolet light, were set in parallel; the distance between the lamps and the Petridish could be changed. The irradiation box was set in an incubator. The irradiation temperature, which also could be changed, usually was set at 25° $\pm$ 1°C. The samples were irradiated at 390–555 $\pm$ 15 μW/cm^2. Intensity of ultraviolet radiation was measured with a radiometer (DRC-100x; Spectronics, New York, USA), with a DIX-365 sensor. The transmission factor of the lid of the Pyrex Petri dish combined with the polypropylene film was more than 90%. The irradiation experiment was performed in three replicates.

For experiments on the effect of superhydrophilic properties on the viability of microorganisms, the slide glass photocatalyst was irradiated in a sterile Pyrex Petri dish for 16 h at a radiation intensity of about 500 μW/cm^2 after washing, as already described. After this treatment, the slide glass photocatalyst was used immediately for photocatalytic experiments.

The irradiation period was set from 30 min to 12 h. The effect of irradiation period on survival rate was examined for all microorganisms shown in Table 1, especially for germinated and ungerminated spores of *T. harzianum*, *Cladobotyrum varium*, *Spicellum roseum*, and *B. subtilis*. In the experiment of the effect of temperature, the temperature was set at 10°, 15°, 20°, 25°, or 30° $\pm$ 1°C. Thickness of the layer of the suspension of microorganisms was varied by changing the volume of the suspension. When the volume was 100, 50, or 25 μl, the thickness was 226.8, 113.4, and 56.7 μm, respectively.

After irradiation, each of the irradiated samples of the fungal spores or germinated fungal spores was suspended in 9.9 ml dilution buffer for fungi in a sterilized Petri dish. Two hundred microliters of the suspension was spread over an glucose-malt extract-yeast extract agar medium in a Petri dish and incubated at 25°C in the dark. Nongerminated spores of *Trichoderma harzianum* Rifai were incubated for 40–45 h and those of *Cladobotryum varium* Nees and *Spicellum roseum* Nicot & Roquebert for 60–65 h; then, the number of colonies that appeared was counted. Germinated spores of *T. harzianum* were incubated for 24 h and those of *C. varium* and *S. roseum* for 40 h. The irradiated bacteria

and bacterial spores were diluted in a similar way in the dilution buffer for bacteria. Then, 500 µl of the suspension of *P. tolaasii* was placed on a King B agar medium in a Petri dish, and 10 ml of the molten medium kept at 41°C was poured, mixed, and incubated at 24°C in the dark for 40–45 h; 500 µl of the suspension of bacteria of *E. coli* or bacteria or endospores of *B. subtilis* was taken on nutrient agar medium in a Petri dish, and 10 ml of the molten medium kept at 41°C was poured, mixed, and incubated at 31°C in the dark. *E. coli* was incubated for 24–28 h and *B. subtilis* for 36–40 h. Survival rates of bacteria and fungi after irradiation were determined by counting the numbers of colonies of microorganisms treated with the slide glass photocatalyst and those not treated.

An experiment on the effect of TiO_2 coating on the number of microorganisms in a practical cultivation room was performed in mushroom growing rooms of two research institutions: one, the Nagano Prefecture Forest Research Center, and the other, the Nagano Vegetable and Ornamental Crops Experiment Station. In the former installation, three acrylamide boards coated with the photocatalytic coating reagent and three acrylamide boards of the same size but not coated with the reagent were prepared; a pair of coated and uncoated boards was set on a surface of the wall, and two pairs were set on a mushroom growing shelf in the mushroom growing room. The surface of four shelf pipes made of stainless steel was also coated with the photocatalytic coating reagent. One pair of the acrylamide boards and two pairs of the coated and uncoated shelf pipes were irradiated with the 40-W black-light blue fluorescent lamp at distances from 300 to 500 mm. Another pair of the acrylamide boards and another two pairs of the shelf pipes were irradiated with a 40-W fluorescent lamp emitting white light, also at distances from 300 to 500 mm. A pair of the acrylamide boards set on the surface of the wall were irradiated with the 20-W black-light blue fluorescent lamp at distances of 70–150 mm.

In the latter installation, three pairs of the acrylamide boards, coated with the photocatalyst and not coated, were set on the surface of the wall of the room at distances of 300–2100 mm from the 20-W black-light blue fluorescent lamp. These boards were also irradiated with 40-W fluorescent lamps, emitting white light in 30-min intervals, at a distance of about 400 mm. Two vinyl chloride pipes of the mushroom growing shelf were also coated with the photocatalyst, which were irradiated by the 20-W black-light blue fluorescent lamp and by the 40-W fluorescent lamp.

The bacteria and fungi on the surface of acrylamide boards were examined at 2-week intervals, using two kinds of commercial agar media for measuring environmental bacteria and fungi (Petan Check; Eiken Kizai, Tokyo, Japan). The media for measuring fungi included chloramphenicol. The surface of the plate of the agar medium was pressed to the surface of the acrylamide boards to examine the number of bacteria or fungi; then, the media for bacteria were incubated for 24–48 h at 30°C and the media for fungi for 2–3 days at 25°C. After incubation, the number of colonies that appeared was counted. Two plates of each media were used for each acrylamide board. Bacteria on the surface of the stainless steel pipe and the vinyl chloride pipe were examined by wiping their surfaces with sterilized wet cotton balls for 10 cm. Each cotton ball was suspended in 5 ml sterile water, and 100 µl suspension was spread on the surface of nutrient agar media prepared in our laboratory and incubated for 24–48 h at 30°C. After incubation, the number of colonies was counted in three replicates. After sampling, the surface of acrylamide boards was wiped and cleaned to eliminate stains on the surface.

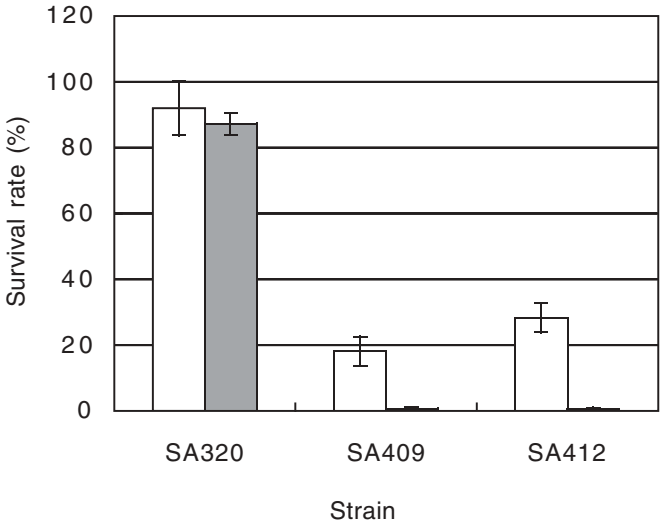

Fig. 1. Effects of superhydrophilic state on the survival rate of *Pseudomonas tolaasii* (*SA409*), *Escherchia coli* (*SA412*), and spores of *Trichoderma harzianum* (*SA320*). Irradiation period was 3 h. *Open bars*, nonsuperhydrophilic state, *closed bars*, superhydrophilic state; *error bars* show standard error in this and other figures

After irradiating by black light for 16 h at an irradiation rate of about 500 µW/cm^2, a water drop on the slide glass photocatalyst spread widely, whereas on the photocatalyst not irradiated with black light, a water drop did not spread so widely. This result showed that the photocatalyst became more hydrophilic. Koizumi et al. (1998) reported that TiO_2 became more hydrophilic after 20 h irradiation and very hydrophilic after 200 h irradiation by near-UV light at 10 µW/cm^2. Because our irradiation rate was about 50 times stronger than this value, our photocatalyst samples were concluded to possess superhydrophilic properties.

Figure 1 shows the effect of the superhydrophilic properties of the photocatalyst on the survival rate of *P. tolaasii*, *E. coli*, and spores of *T. harzianum*. The result shows that the superhydrophilic photocatalyst decreases the survival rate of bacteria significantly but not that of *Trichoderma* spores. Active oxygen produced in the process of photocatalytic reaction is considered to be the causal agent for breaking substances and killing microorganisms on the photocatalyst during irradiation with near UV-light (Fujishima and Honda 1972; Watanabe 1995). Therefore, the results shown in Fig. 1 seemed to indicate that, by induction of superhydrophilic properties, conditions for the interaction between active oxygen and cells have changed for *P. tolaasii* or *E. coli* but not for *T. harzianum*.

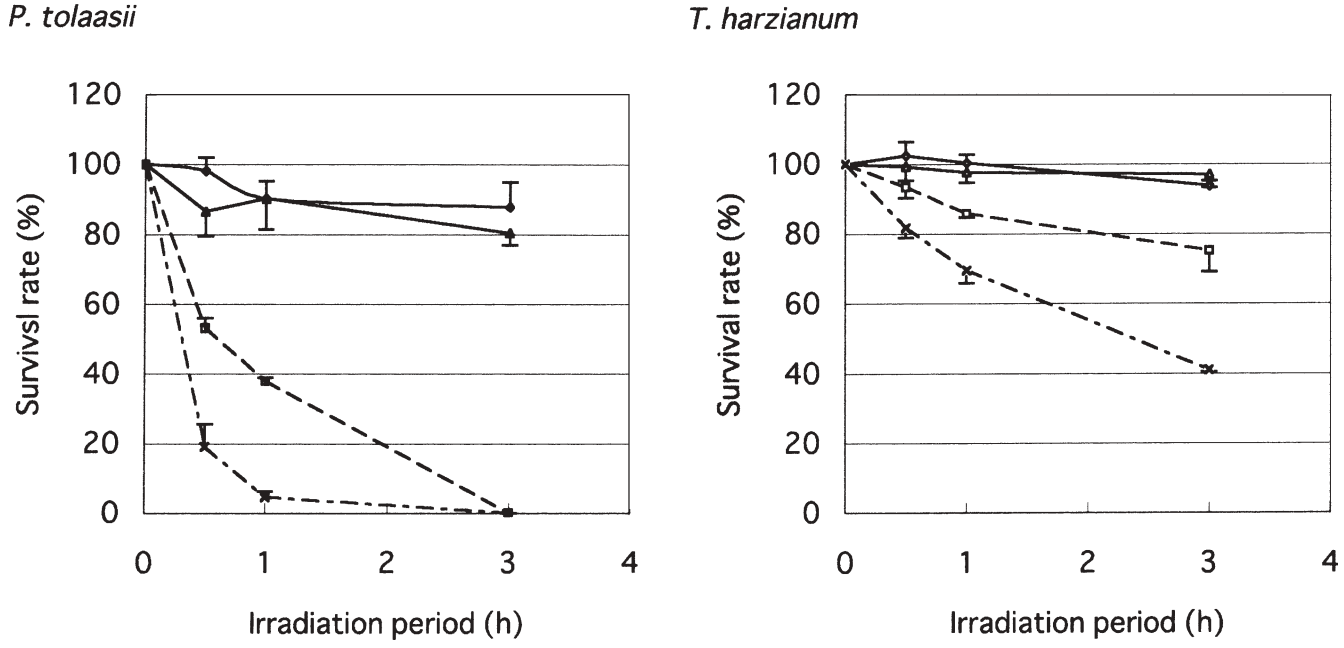

Fig. 2. Effects of titanium oxide photocatalyst on survival rates of *P. tolaasii* (SA409) and spores of *T. harzianum* (SA328) after irradiation with near-UV light: ◇, noncoated, no irradiation; □, noncoated, irradiation; △, coated, no irradiation; ×, coated, irradiation

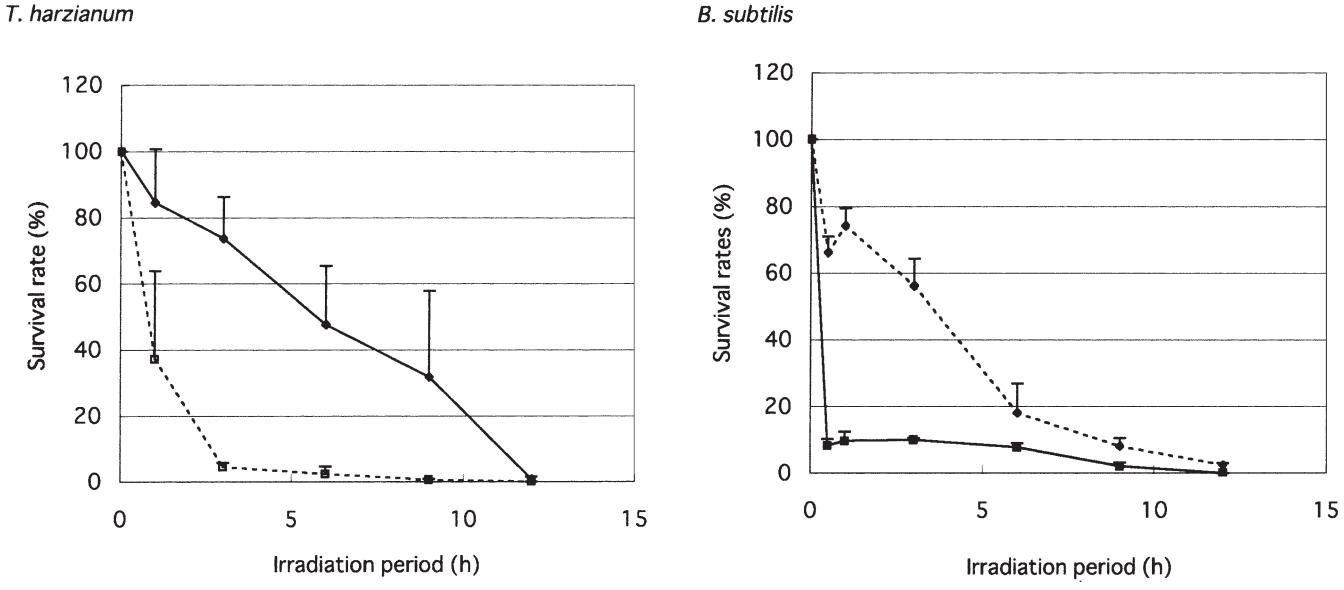

Fig. 3. Difference in sensitivity to photocatalytic treatment between spores and germinated spores of *T. harzianum* (SA320) and endospores and cells of *Bacillus subtilis* (SA478): ◇, spores; □, germinated spores; ◆, endospores; ■, bacterial cells

Figure 2 shows the effect of the slide glass photocatalyst on the survival rate of *P. tolaasii* and *T. harzianum* after irradiation with black light. The black-light irradiation lowered the survival rate for the slide glasses with or without the photocatalyst compared to those without irradiation. The survival rate for the photocatalyst with irradiation, however, was significantly lower than that without irradiation. Similar results were observed for all strains of bacteria and fungi listed in Table 1. This fact shows that the photocatalyst is effective in lowering the survival rate of these microorganisms. The effect of the photocatalyst was more prominent for bacteria than for germinated fungal spores. There were variations in effect among species of fungi and also among different strains of the same species (data not shown).

Figure 3 shows the difference in sensitivity to photocatalytic treatment between spores and germinated spores of *T. harzianum* or endospores and cells of *B. subtilis*. For all cases, the survival rate for spores are significantly higher than that for germinated spores or nonspore bacterial cells.

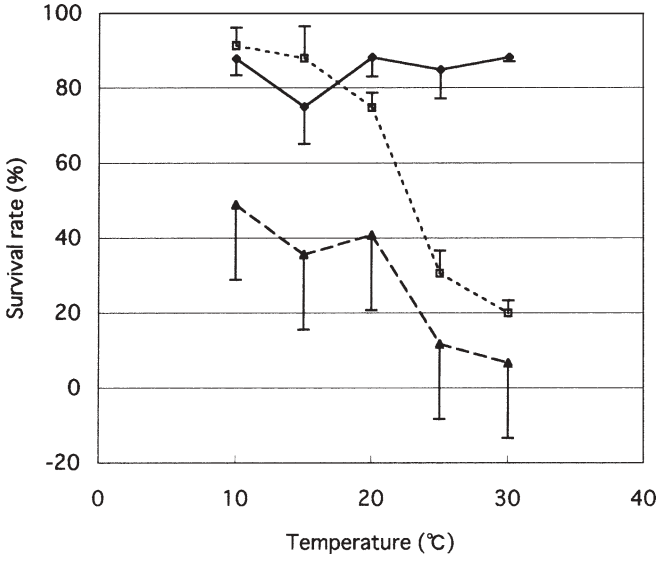

Fig. 4. Effect of temperature of samples during photocatalytic treatment on survival rates of spores of *T. harzianum* (SA320) and cultured cells of *P. tolaasii* (SA409) and *E. coli* (SA412) for irradiation period of 3 h: ◇, *T. harzianum*; □, *P. tolaasii*; △, *E. coli*

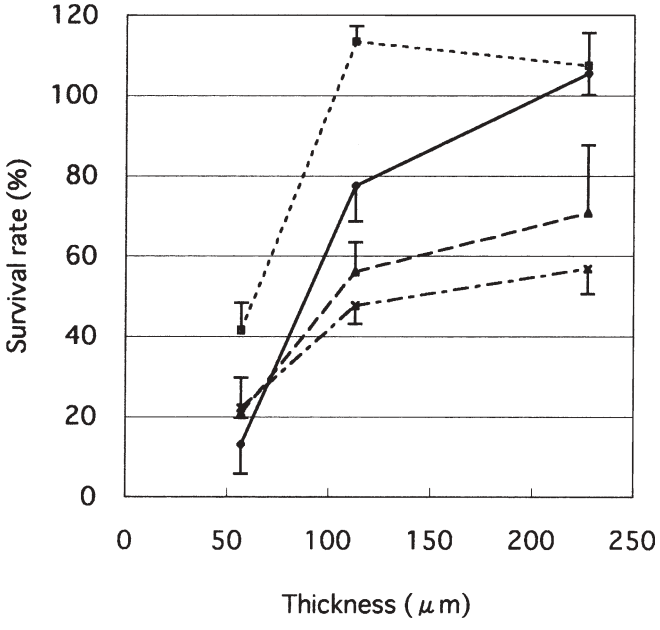

Fig. 5. Effect of thickness of suspensions of spores during photocatalytic treatment on survival rates of spores of *T. harzianum* (SA320), *C. varium* (SA329), *S. roseum* (SA335), and *B. subtilis* SA478 for irradiation period of 4 h: ◇, *T. harzianum*; □, *Cladobotyrum varium*; △, *Spicellum roseum*; ×, *B. subtilis*

A similar effect was observed for all other strains of fungi listed in Table 1.

Figure 4 shows the effect of temperature of samples during photocatalytic treatment on the survival rate for cells of *P. tolaasii* and *E. coli* and spores of *T. harzianum*. The survival rate decreased as the temperature increased for the bacteria, but no clear temperature-dependent effect was observed for spores of *T. harzianum*, for which the survival rate was high for all temperatures tested.

Figure 5 shows the result of the experiment on the effect of the thickness of samples for three fungi and *B. subtilis*. In all cases tested, the survival rate decreased as the thickness was decreased from 226.8 to 56.7 μm; especially, the survival rate was clearly low for 56.7 μm.

Saito et al. (1992) reported that phosphate ion decreased the effect of photocatalyst for streptococci, and Sjogren and Sierka (1994) reported that the activity of photocatalyst is increased in the presence of ferrous ions. We tested effects of phosphate, ferrous, and ferric ions on the survival rates of two bacteria and a fungus, *P. tolaasii* (SA409), *E. coli* (SA412), and *T. harzianum* (SA320). Effects of phosphate, ferrous, and ferric ions, however, were not so evident (data not shown). The effect of pH of the suspension buffer in the photocatalytic treatment was examined for *P. tolaasii* (SA409) and *T. harzianum* (SA320), but the effect was not significant between pH 5 and 8.

The results of experiments in the mushroom growing room in Nagano Prefecture Forest Research Center are shown in Table 2. The following observations can be made: (1) the TiO_2 coating is effective for reducing the number of bacteria and fungi on the acrylamide board and the stainless steel pipes of a shelf under black-light illumination; (2) the TiO_2 coating is also effective under white fluorescent light; and (3) black light alone is fairly effective but less effective than photocatalysts. The second point is interesting, because although white fluorescent light emitted a rather small amount of near-ultraviolet light, the surface of the photocatalyst illuminated with the white fluorescent lamp also became cleaned. On the surface of the acrylamide board, we could not detect the near-ultraviolet radiation with the radiometer we used. Watanabe (1995) reported that the efficiency of the photocatalyst was increased as the intensity of near-UV light was decreased. The fact may be related to our result.

The result of the experiment in the Nagano Vegetable and Ornamental Crops Experiment Station also showed that the photocatalyst was effective under black light and also under white light (data not shown). The mist was much greater in this room than in the growing room in the Nagano Prefecture Forest Research Center, which indicated the photocatalyst was effective in the condition with much mist.

Results obtained in the two mushroom growing rooms indicate that the photocatalyst is effectively used for reducing the number of bacteria and fungi in the mushroom growing room if large part of walls and shelves of mushroom growing room are coated with photocatalyst under condition of illumination with black light or white fluorescent light. In practical application, several results obtained in our laboratory experiments can reinforce the effectiveness of the photocatalyst.

Acknowledgments We thank Nihon Soda Co. for providing us with the photocatalytic coating reagent "Bisutoreita."

Table 2. Colony numbers of bacteria and fungi collected from the acrylamide boards or coated stainless steel pipes of shelves in the mushroom growing room of Nagano Prefecture Forest Research Center

Type of photocatalyst	Acrylamide board on wall surface[a]			Acrylamide board on shelf[a]			Acrylamide board on shelf[a]			Coated on surface of stainless pipes of shelves[b]			Coated on surface of stainless pipes of shelves[c]		
	Black light			Black light			White light[d]			Black light			White light[d]		
Irradiation Period (weeks)	Coated with TiO_2	Control	Ratio[e]	Coated with TiO_2	Control	Survival rate[f]	Coated with TiO_2	Control	Survival rate[f]	Coated with TiO_2	Control	Survival rate[f]	Coated with TiO_2	Control	Survival rate[f]
2	0.5	1.5	0.33	5	24.5	0.047	13.5	106	0.127	7	164.3	0.002	19	3300	0.006
	2	2	1	7.5	45	0.083	68	90	0.756	27	128.6	0.006	7.3	4400	0.002
4	0	9	0	54	150	0.034	38	1600	0.024	0[g]		0	0	270	0
	1	22	0.05	22.5	134.5	0.035	22.5	650	0.035	1[g]		0.001	1	819	0.001
6	0.5	3	0.17	3	87	0.003	33.5	1200	0.028	0.5	3	0.003	1.5	169	0.009
	14	31	0.45	7	135	0.048	59	147	0.401	3.5	14	0.01	131	366	0.358
8	ND[h]	ND	ND	ND	ND	ND	ND	ND	ND	1.5	26.5	0.002	1	832	0.001
										0.5	70	0.001	18.5	476	0.039

[a] For each week, upper values show colony numbers of bacteria and lower values those of fungi
[b] For each week, upper values show colony numbers of bacteria collected from first pair of shelves and lower values those collected from second pair of shelves
[c] For each week, upper values show colony numbers of bacteria collected from third pair of shelves and lower values those collected from fourth pair of shelves
[d] White light means white fluorescent light
[e] Values for coated with TiO_2 was divided by the values for control of black light
[f] Values for coated with TiO_2 was divided by the values for control of white light
[g] Bacteria grew over the whole of the media in Petri dish
[h] Not determined

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