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**Note**

# Ozone Inactivation of Lactic Acid Bacteria

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**Ozone inactivation of lactic acid bacteria in water was studied. The strains studied showed resistance to ozonated water in the following order: *Leuconostoc mesenteroides* IFO3426 > *Lactobacillus fructivorans* IFO13118 > *Lactobacillus plantarum* IFO3070 > *Weissella viridescence* IFO3949 > *Enterococcus faecium* IFO3128 > *Enteroccoccus feacalis* IFO12964. However, the survival ratio of *L. mesenteroides* IFO3426 was found to decrease by increasing the dissolved ozone concentration ranging from 0.5 to 5mg/*l* and the temperature in the range from 5 to 30℃.**

*Key words* : Ozone/Ozonated water/Lactic acid bacteria/*Leuconostoc mesenteroides*.

The effect of ozone on food related microorganisms has been studied and lethal dosages have been determined (Naito and Shiga, 1982; Restaino, et al., 1995). There is still, however, very little information available on the lethal dosage of ozonated water for lactic acid bacteria. Lactic acid bacteria have often been involved in food spoilage in the past decade because of their resistance to commonly used chemical sterilizers and their ability to cause serious food spoilage in heat sensitive food (Naito, 1999). In the present study, several strains of lactic acid bacteria were selected as the test organisms. *Lactobacillus fructivorans* IFO13118, *Lactobacillus plantarum* IFO3070, *Weissella viridescens* IFO3949, *Enterococcus feacalis* IFO12964, *Enterococcus faecium* IFO3128, and *Leuconostoc mesenteroides* IFO3426 were used as test microorganisms for estimating the ozone inactivation of such bacteria in water. Test bacteria were cultivated in a medium containing 0.5% (w/v) polypepton, 0.25% (w/v) yeast extract, and 0.1%(w/v) glucose (pH 7.0). After being incubated for 20−48h at 30℃, the cells were harvested by centrifuging at 1500×*g* for 10 min. The precipitate was washed by centrifugation at 5℃ three successive times with sterile distilled water, and was finally suspended in sterile distilled water. To 1ml of the suspension was added to make 100ml with sterile distilled water, yielding a bacterial concentration from $10^5$ to $10^9$ CFU/ml. These bacterial suspensions were diluted with ozonated water at the rate of 1:50. To assay the samples at set times, 1ml of the suspension was collected and put into 9ml Nutrient broth (Nissui Pharmaceuticals, Tokyo) to stop the ozone reaction and check the microbial counts. At the end of the experiment, 1ml of the mixture sample was poured onto a Tryptic Soy agar plate (Nissui Pharmaceuticals, Tokyo). The plates were incubated at 30℃ for 48h before colonies were counted. The ratio of the number of living cells after the treatment with ozonated water to those initially added was determined. Ozonated water was prepared as shown in Fig.1. Ozone was produced from pure oxygen by using a silent discharge ozone generator (NOR-2Z-WL-21-RKS-250, Nissui, Tokushima). The ozone was then circulated into deionized water at 20℃.

The concentration of dissolved ozone in water was measured using two types of polarographic ozonated monitors (Model 3600 Analyzer, Orbisphere Laboratories, Switzerland and OZ-20, TOA Electronics, Tokyo).

A reduction in bacterial concentration after ozonation was observed. A linear correlation between the logarithm of bacterial concentration and contact

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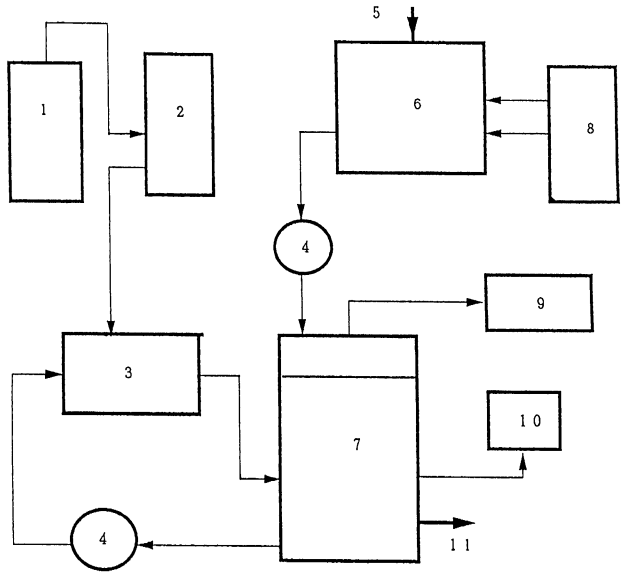


**FIG. 1**. Diagram of ozonated water manufacturing machine. 1, Ozone cylinder; 2, ozone generator; 3, ozone dissolution vessel; 4, pump; 5, water; 6, water vessel; 7, dissolution vessel; 8, chiller; 9, ozone decomposition catalyst; 10, ozone monitor, and 11, ozonated water.

time was found in all cases. The linear correlation coefficient was shown to be at the level of significance ($\alpha = 0.05$) in all experiments. Under experimental conditions, ozone inactivation followed first order kinetics with respect to the bacterial concentration.

The first order inactivation rate constants ($k_1$) obtained from 6 lactic acid bacteria were shown to be linearly dependent on the initial dissolved ozone concentration. Table 1 shows the relationships of initial dissolved ozone concentration in water and the $k_1$. The $k_1$ value is a quantitative measurement of the resistance of microorganisms to ozone. According to the $k_1$ values obtained, the strains showed ozone resistance in the order: *L. mesenteroides* > *L. fructivorans* > *L. plantarum* > *W. viridescens* > *E. faecium* > *E. faecalis*.

The lethal action induced by the ozone decomposition in water was evaluated. Ozone decomposition rates are shown in a plot of the logarithm of a fraction of the original ozone concentration in the aqueous solution at time $t$ (Wickramanayaka and Sproul, 1984).

Earlier kinetic experiments of ozone decomposition in aqueous solution were carried out spectrophotometrically and in the some cases iodometrically.

Alder and Hill (1950) reported that the decomposi-

**TABLE 1**. First order rate constant in ozone inactivation of lactic acid bacteria.

| Bacterial strain | $O_3$ (mg/l)[a] | $k_1$ ($min^{-1}$)[b] |
|---|---|---|
| *Lactobacillus fructivorans* IFO13118 | 0.5 | 0.41 |
| | 1.0 | 1.25 |
| | 3.0 | 2.53 |
| | 5.0 | 4.40 |
| *Lactobacillus plantarum* IFO3070 | 0.5 | 0.59 |
| | 1.0 | 1.38 |
| | 3.0 | 2.83 |
| | 5.0 | 4.76 |
| *Weissella viridescence* IFO33949 | 0.5 | 0.63 |
| | 1.0 | 1.53 |
| | 3.0 | 2.94 |
| | 5.0 | 4.49 |
| *Enterococcus faecalis* IFO12964 | 0.5 | 0.72 |
| | 1.0 | 2.05 |
| | 3.0 | 3.74 |
| | 5.0 | 6.10 |
| *Enterococcus faecium* IFO3128 | 0.5 | 0.69 |
| | 1.0 | 1.85 |
| | 3.0 | 3.29 |
| | 5.0 | 5.38 |
| *Leuconostoc mesenteroides* IFO3426 | 0.5 | 0.32 |
| | 1.0 | 1.05 |
| | 3.0 | 2.08 |
| | 5.0 | 3.65 |

[a]Initial dissolved ozone concentration.
[b]First order inactivation rate constant.
The correlation coefficient was 0.90 to 0.94 with a significance of 0.05.

tion of ozone in aqueous solution follows the first order law. The ozone decomposition rate in water was then estimated by the method of Alder and Hill. Figure 2 shows the relationship of ozone decomposition $(1+\log [O_3]/ [O_3]_i)$ and the reaction time by polarographic data. The ozone decomposition rate as the ozone concentration in water increased, so the ozone decomposition rate depended on the initial ozone concentration in water. When the initial ozone concentration in water is known, the ozone decomposition rate can be found from this graph, and the residual ozone concentration in water can be predicted for different reaction times. It has been well documented that the ozone concentration and temperature of the applied ozonated water are the dominant factors affecting the degree of ozone inactivation of food related microorganisms (Naito and Shiga, 1982; Yang and Chen, 1979). A typical set of survival curves for *L. mesenteroides* with the dissolved ozone concentration ranging from 0.5 to 5mg /*l* at 20℃ and pH 7 is shown in Fig. 3. Similar sets of curves were obtained at temperatures of 5, 10 and 30℃.

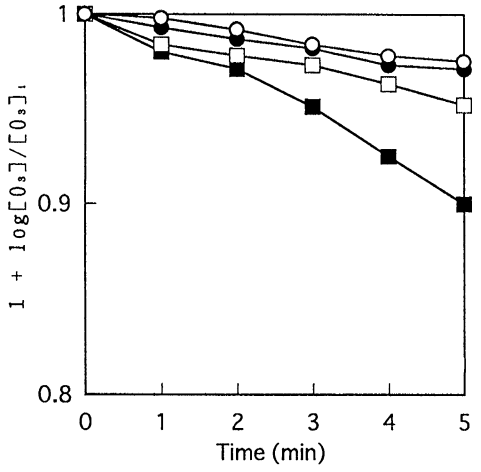


**FIG. 2**. Relationship of ozone decomposition and dissolved ozone concentration. Ozone decomposition rate measured with initial dissolved ozone concentration of 0.5 ( ○ ), 1.0 ( ● ), 3.0 ( □ ), and 5.0mg/*l* ( ■ ) at 20℃ and pH 7.0.

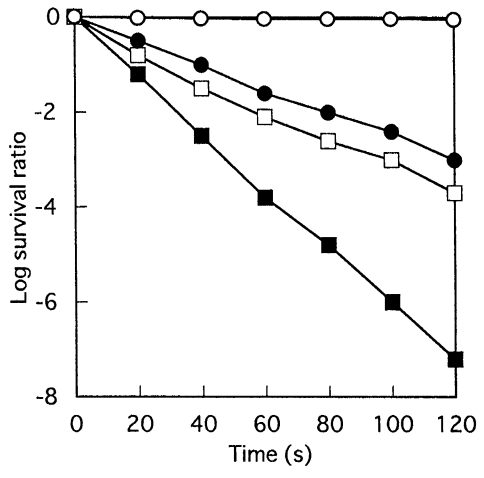


**FIG. 3**. Survival curves for *Leuconostoc mesenteroides* IFO3426 exposed to ozonated water. Ozone inactivation of cells in water was measured with initial dissolved ozone concentration ranging from 0.5 ( ○ ),1.0 ( ● ), 3.0 ( □ ) to 5.0mg/*l* ( ■ ) at 20℃ and pH 7.0.

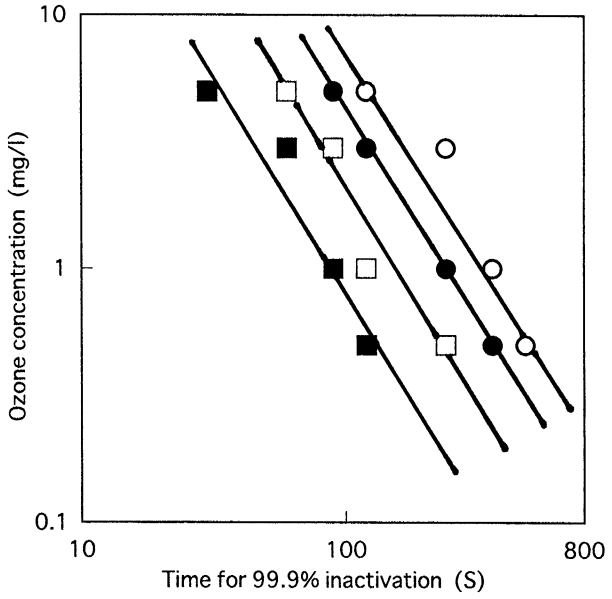


**FIG. 4**. Ozone concentration-time for 3 log of ozone inactivation of *Leuconostoc mesenteroides* IFO3426 at various temperatures. Cells were treated with various concentration of dissolved ozone at 5 ( ○ ),10 ( ● ), 20 ( □ ) and 30 ℃ ( ■ ).

The ozonated water-contact time required for three order reduction (99.9%) of ozone concentration in the inactivation of *L. mesenteroides* were examined at different temperatures. The results in Fig. 4 show that the curves for 99.9% ozone inactivation were approximately linear when the log of contact time was plotted at each indicated temperature. Curves for one order reduction (90%) of ozone concentration in the inactivation at 20℃ of *L. mesenteroides* also was linear when the log of the dissolved ozone concentration was plotted against the logarithm of contact time, as described by Watson's law (Wickramanayake and Sproul, 1984).

When the reaction temperature is given, the rate constant can be calculated from the survival curves, and the ozone concentration can be predicted for different reaction times. The ozone inactivation of *L. mesenteroides* was found to be enhanced by

increasing the ozone concentration ranging from 0.5 to 5 mg/$l$ and the temperatures in the range of 5 to 30℃.

A recent study showed that the direct reaction between molecular ozone and bacterial cells is very slow, and therefore, ozone decomposition is due to initial reactions with hydroxyl ion ($OH^-$) and transformation of the molecular ozone to the OH radical by reactions with the chain carriers hydroperoxyl ion ($HO_2^-$) and superoxide anion ($O_2^-$) (Elovitz and Gunten, 1999). Ozone decomposition is best represented by a one-half order dependence on the $OH^-$ concentration (Alder and Hill, 1950). Effect of pH on the decomposition of ozone was examined and then compared in terms of its ability to elevate the ozone inactivation of *L. mesenteroides*. When the total of the ozonated water treatment time was identical, operation with pHs 7 and 8 was usually more effective for the ozone inactivation of *L. mesenteroides* than that with pHs 3 and 4.

The decomposition of ozone in alkaline solution was initiated by $OH^-$ and accelerated by a chain reaction, in which the OH radical acts as a chain carrier.

The OH radical yield from decomposed ozone in alkaline solution can be considered essentially independent of pH and corresponds to approximately 50% of the decomposed ozone (Hoigne and Bader, 1979). In the pH range of 7 to 8, only a small fraction 1 to10% of the oxidation compounds takes part in reactions with molecular ozone (Gunten and Hoigne, 1994). In ozone treatment, microbial cell walls and cytoplasmic membranes are thought to be gradually oxidized and destroyed (Heath and Frederic, 1979).

When the ozonated water applied is then treated, the absorbed ozone will rapidly or slowly decompose on cell surfaces. Ozone can react with microorganisms by itself or indirectly as free radicals derived from ozone such as the OH and $HO_2$ radical. With this mechanism, the death of a target microorganism is therefore believed to occur mainly due to the oxidation of same component of cell walls and cytoplasmic membrane (Perrich, 1975). Our present results with *L. mesenteroides* however, suggest that the above free radicals may play a significant role in microbial reduction in a short time such as a 120s treatment with 5mg/$l$ ozonated water, as Baxendale (1964) also pointed out. As shown in Fig. 4, when the total time of the ozonated water treatment was identical, rapid ozone decomposition at higher temperatures was usually more effective inactivation against the *L. mesenteroides* than a slow ozone decomposition at low temperatures. These results suggest that the OH and $HO_2$ radicals derived from ozone might be the species responsible for the strong bactericidal activity of ozonated water in short contact times such as 120s.

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