INJURIES IN EXPONENTIAL PHASE CELLS OF *ESCHERICHIA COLI* SUSPENDED IN PHYSIOLOGICAL PHOSPHATE BUFFERED SALINE BY FAR-INFRARED IRRADIATION

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The pasteurization effect of far-infrared (FIR) radiation on *Escherichia coli* suspended in physiological phosphate buffered saline in the exponential growth phase (exponential phase) was studied. FIR irradiation decreased the number of viable cells in their exponential phase more rapidly than that in their stationary growth phase (stationary phase). By using four kinds of antibiotics as selective reagents to examine the injuries of bacteria, the damaged parts induced by FIR irradiation were specified on the basis of the changes in sensitivities to selective reagents. FIR irradiation damaged mainly RNA polymerase of *E. coli* in the exponential phase. Under the condition that the transient behavior of the bulk temperature of bacterial suspension irradiated by FIR was the same as that heated by thermal conduction, the pasteurization effect by FIR irradiation on the exponential phase cells was much greater than that by thermal conduction. However, both FIR irradiation and thermal conductive heating induced similar changes in the sensitivities to selective reagents for the exponential phase cells of *E. coli*. Under the same irradiation conditions, the changes in sensitivities to the selective reagents for the exponential phase cells were different from those for the stationary phase cells. The injuries of ribosome in the exponential phase cells were not as serious as those in the stationary phase cells. It was suggested that the mechanisms of death of the exponential phase cells and the stationary phase cells were different from each other.

Introduction

Recently, damage caused by microbial contamination is increasing and of serious harm to our living environment. The importance of biocontrol in food processing is being appreciated anew by these biodeteriorations, and developments of new technologies for biocontrol are desired. Since no heating medium is needed for far-infrared (FIR) radiative

heating, the application of FIR to thermal operations will improve the cleanliness of working environments. Especially, the application of FIR to pasteurization has attracted great interest, and fundamental studies of the influences of FIR irradiation on microorganisms are needed.

We have studied the pasteurization effect of FIR irradiation. Under the condition that the bulk temperature of bacterial suspension heated by FIR was the same as that heated by thermal conduction, FIR irradiation was more effective than thermal conductive heating for the pasteurization of *Escherichia coli* and *Staphylococcus aureus* (Hashimoto *et al.*, 1991). In the case of bacteria on or near the surface of the wet-solid medium, the pasteurization effect by FIR irradiation was higher than that by hot-air heating (Hashimoto *et al.*, 1992a). Also, FIR irradiation caused heat activation and death of spores

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of *Bacillus subtilis* in the range of temperature where thermal conductive heating exhibited no effects on the spores (Sawai *et al.*, 1997).

In the previous work (Sawai *et al.*, 1995a), we have proposed a simple method for the specification of the damaged parts in bacteria on the basis of the changes in sensitivities to antibiotics. It was suggested that FIR irradiation mainly damaged RNA polymerase and ribosomes in *E. coli* and led to death. The bacterial cells in the stationary growth phase (stationary phase) have been used as the test organism in our previous works. The growth phase of microorganisms is roughly divided into three parts: lag phase; exponential growth phase (exponential phase); and stationary phase. Microorganisms at different growth phases are introduced into food product in a series of processes. Especially, the generation time of the cells at the exponential phase is the shortest. This emphasizes the importance of avoiding contamination of food with microorganisms in the exponential phase. The character of cells at each growth phase is different. In particular, there are large differences in the sensitivities to stresses between the stationary and the exponential phase cells (Yanagida, 1980). Generally, vegetative cells of organisms in their exponential phase are the least resistant to lethal agencies, and they are more resistant in their stationary phase. It is necessary to grasp the behaviors of the exponential phase cells against FIR irradiation. The purpose of this work is to study the pasteurization effect of FIR on the exponential phase cells of *E. coli* and to examine the injuries by using the same method in the previous work (Sawai *et al.*, 1995a).

1. Materials and Methods

1.1 Test bacteria

E. coli 745, which was stored at Tokyo Metropolitan Research Laboratory of Public Health, was used as a test bacterium. *E. coli* were incubated with Brain Heart Infusion broth (Difco). The growth curve of *E. coli* 745 in the broth is shown in Fig. 1. After a certain period of the lag phase, the cell numbers increased continuously at a constant rate (exponential phase). When the bacterial concentration increased to 1.0×10^9 CFU/dm³ (CFU: Colony Forming Unit), *E. coli* 745 reached the stationary phase. In this work, *E. coli* incubated for 2 h and 24 h were used as the exponential phase cells and the stationary phase cells, respectively.

1.2 FIR irradiation

The FIR irradiation apparatus was the same as that used in the previous works (Hashimoto *et al.*, 1991, 1992a, b). The culture of *E. coli* 745 was suspended in 0.05 M physiological phosphate-buffered saline (pH 7.0) to yield a final concentration of approximately 10^9 CFU/dm³. The bacterial

Fig. 1 Growth curve of *E. coli*

suspension of 2.0×10^{-2} dm³ was dispensed in a petri dish made of stainless steel (14.5×88.5 mmφ), and irradiation was carried out. The depth of the bacterial suspension was about 3 mm. The power irradiated on the suspension was from 1.8 to 3.2 kW/m². Irradiation power was obtained experimentally from the evaporation rate (Hirota *et al.*, 1991). After irradiation, the petri dish was rapidly cooled in iced water.

1.3 Conductive heating

The conductive heating apparatus and the heating method were the same as those used in the previous works (Hashimoto *et al.*, 1991). By varying electric power supplied to a submerged heater, the transient behavior of the bacterial suspension was controlled to be the same as that irradiated by FIR.

1.4 Finding of damaged parts in *E. coli*

Four kinds of antibiotics were used as the selective reagents in order to specify the damaged parts in *E. coli* (Table 1). Their primary inhibitory actions are also shown in Table 1. The method was the same as that used in the previous work (Sawai *et al.*, 1995a). After FIR irradiation or conductive heating, the cooled suspension was diluted with sterilized physiological saline. The diluent was pour-plated with agar medium. Sensitivity Disk Agar-N (Nissui Seiyaku Co., Ltd.) was used as the non-selective medium. Sensitivity Disk Agar-N containing the selective reagent was used as the selective medium. The colonies were enumerated after incubation at 310 K for 48 h, and the damaged parts in bacteria were specified on the basis of the changes in sensitivities to the selective reagents. The concen-

Table 1 Selective reagents and their C_{MAX} for log phase cells of *E.coli*

Reagent	Primary inhibitory action	C_{MAX} [mg/l]
Penicillin G (PCG)* ¹	synthesis of cell wall	14.8
Chloramphenicol (CP)* ¹	synthesis of protein	1.5
Nalidixic acid (NA)* ²	synthesis of DNA	0.5
Rifampicin (RFP)* ²	synthesis of RNA	0.4

*¹ Sigma Chemical Company*² Daiichi Pharmaceutical Co. Ltd. **Fig. 2** Transient behavior of temperatures of bacterial suspension irradiated by FIR **Fig. 3** Effect of FIR irradiation power on survival ratio of *E. coli* in exponential phase

trations of the selective reagents in the agar medium for the exponential phase of *E. coli* were determined by the same method used in the previous work (Sawai *et al.*, 1995a).

2. Results and Discussion

2.1 Pasteurization effect of FIR on *E. coli* cells in exponential phase

The transient temperature behaviors of the bacterial suspensions irradiated by FIR were shown in **Fig. 2**. **Figure 3** shows the death curves of the exponential phase cells of *E. coli* irradiated by FIR. An increase in irradiation power markedly enhanced the pasteurization effect of FIR on the exponential phase cells. **Figure 4** exhibited the pasteurization effects of FIR irradiation on the cells in the exponential and stationary phases at an irradiation power of 3.2 kW/m². The rate of decrease in the number of the exponential phase cells was much faster than that of the stationary phase cells. This means that *E. coli* at the exponential phase was more sensitive to FIR to that than the stationary phase.

Fig. 4 Effect of FIR irradiation on *E. coli* in exponential and stationary phase

2.2 Injury in *E. coli* at exponential phase by FIR irradiation

The damaged parts in the exponential phase cells of *E. coli* irradiated by FIR were examined. In the previous work (Sawai *et al.*, 1995a), it was supposed that sublethally injured cells induced by stresses become more sensitive than intact cells to the agent which had the inhibitory action on the damaged parts of the bacterial cells. When the cells injured by stresses were incubated with the agar media containing several kinds of antibiotics, the damaged parts could be specified by comparing the changes in sensitivities to the antibiotics.

The concentrations of the selective reagents for the exponential phase cells of *E. coli* were determined. The concentration of the selective reagent in the agar medium was defined within the range of concen-

trations where the reagent exhibited no effects on colony formation of intact cells of *E. coli* at the exponential phase. **Figure 5** illustrates the effect of CP on colony formation of the intact exponential phase cells of *E. coli*. N_i/N_0 represents the survival ratio and the ratio of the number of colonies on the agar medium containing CP to that on non-selective agar medium. The survival ratio was unity at CP concentration less than 1.5 mg/l and declined linearly over that concentration. The maximum concentration (C_{MAX}) of CP in the agar medium was determined to be 1.5 mg/l. Incubation with the selective agar medium at C_{MAX} can enable detection even for slight injuries to the bacterial cells. Cells not forming colonies at much lower concentrations than C_{MAX} are considered to be seriously injured. The values of C_{MAX} of four kinds of selective reagents were summarized in Table 1.

Figure 6 shows the changes in sensitivities in *E. coli* at the exponential phase to the selective reagents by FIR irradiation. The abscissa, C/C_{MAX} , represents the ratio of a specified concentration (C) to the maximum concentration of the selective agar medium (C_{MAX}) given in Table 1. At an irradiation time of 4 min, there were no decreases in survival ratios and little change in sensitivities to the selective reagents (Fig. 6(A)). At 4.5 min, the survival ratio decreased to 2.0×10^{-2} on non-selective agar medium. Furthermore, only the survival ratio on the selective medium containing RFP remarkably decreased with increasing concentration of the selective reagents (Figs. 6(B) and 6(C)). This latter decrease in the survival ratio indicates the increase in sensitivity of *E. coli* in the exponential phase to RFP. RFP acts on RNA polymerase and inhibits initiation of DNA-dependent RNA synthesis (Hartmann *et al.*, 1967;

Fig. 5 Effect of CP concentration on colony formation of *E. coli* in exponential phase

Fig. 6 Changes in sensitivity in *E. coli* in exponential phase to selective reagents by FIR irradiation: (A) 4 min, (B) 4.5 min, (C) 5 min

Tanaka and Nakamura, 1984; Zillig *et al.*, 1970). The function of RNA polymerase in cells is one of the most sensitive components to extracellular environmental changes. It was reported that some stresses caused increase in the surface hydrophobicity of *E. coli* and in the sensitivities to hydrophobic antibiotics (Mackey, 1983). The validity of this methodology was proved by applications to the treatments of some kinds of physical and chemical stresses, such as UV irradiation and alkaline treatment (Sawai *et al.*, 1995a, 1995b, 1996). Also, Considering that the concentrations of antibiotics used here are much lower than those in the literature or minimal inhibitory concentrations (MIC), the increase in the sensitivity to RFP is a consequence of the injury of RNA polymerase, and not changes in the permeability of cell membrane. Therefore, the results in this work suggested that the exponential phase cells of *E. coli* irradiated by FIR mainly incurred damage to their RNA polymerase. This tendency in injuries of *E. coli* by FIR irradiation did not vary with irradiation power (data not shown).

2.3 Comparison with conductive heating

The injuries by FIR irradiation were compared with those by conductive heating for the exponential phase cells of *E. coli* (Fig. 7). The transient temperature of bacterial suspension heated by thermal conduction was the same as that heated by FIR irradiation at irradiation power of 3.2 kW/m². The survival ratio rapidly decreased over 4 min. Considering that the death of *E. coli* over 4 min is logarithmic, the death rate constants for FIR irradiation (k_{FIR}) and thermal conduction ($k_{\text{Cond.}}$) were $1.39 \times 10^{-1} \text{ s}^{-1}$ and $6.51 \times 10^{-2} \text{ s}^{-1}$, respectively. This indicated that the pasteurization effect of FIR irradiation was much higher than that of conductive heating on the exponential phase cells under the same condition of the bulk temperatures of bacterial suspensions. Similar results were obtained in the case of the stationary phase cells of *E. coli* (Sawai *et al.*, 1995a).

The injuries of the exponential phase cells of *E. coli* heated by thermal conduction were examined (Fig. 8). There were minor increases in sensitivities to selective reagents at 4 min (Fig. 8(A)). The sensitivities to RFP, PCG and CP increased with heating time (Figs. 8(B) and 8(C)). In particular, the increase in the sensitivity to RFP was the largest. Therefore, conductive heating also mainly damaged RNA polymerase in the exponential phase cells of *E. coli*.

From evaluations of injuries of bacteria by using four kinds of selective reagents, the action of FIR against the exponential phase cells of *E. coli* was found to be a thermal effect, and the pasteurization effect of FIR was found to be due to the temperature of bacterial suspension. Similar results were obtained

Fig. 7 Effect of FIR irradiation and thermal conductive heating on *E. coli* in exponential phase

in the case of the stationary phase cells of *E. coli* (Sawai *et al.*, 1995a). The power irradiated on the bacterial suspension at 3.22 kW/m² was reduced to 10% within a layer of 40 μm thickness (Hashimoto *et al.*, 1991). The absorbed energy was converted into heat energy and raised the temperature of bacterial suspension. By estimating the temperature distribution near the surface of the suspension, it was suggested that the temperature near the surface was much higher than the bulk temperature measured by thermocouples (Hashimoto *et al.*, 1992a). Therefore, the difference in the pasteurization effect between FIR irradiation and conductive heating originated from the effect that the bacteria receive near the surface of the suspension. In practice, the depth of media affects the pasteurization effect of FIR irradiation. Since the rate of increase in the temperature of suspension irradiated by FIR varies with the depth of suspension, it is difficult to assess exactly only the effect of the depth. However, the pasteurization effect of FIR irradiation is higher than that calculated from the death rate constant determined in the experiments of thermal conductive heating, and the effect increased as the depth of the suspension decreased (Hashimoto *et al.*, 1991). In addition, even when the bulk temperature of the suspension irradiated by FIR was kept below the lethal temperature of bacteria, *E. coli* and *S. aureus* were injured and killed. The decrease in the depth of the suspension enhanced the injury and death of the bacteria (Hashimoto *et al.*, 1992b, 1993). For solid

Fig. 8 Changes in sensitivity of *E. coli* in exponential phase to selective reagents by thermal conductive heating (A) 4 min, (B) 5 min, (C) 6 min

media, the pasteurization effect of FIR irradiation depends on a place where bacteria exist in the media. In the experiments using the agar medium as a wet-solid food model, the pasteurization effect against bacteria present within the depth of 1 mm from the surface was much higher than the calculated results. However, for bacteria within the agar medium deeper than 1 mm, FIR irradiation gave only the pasteurization effects corresponding to those obtained by conductive heating (Hashimoto *et al.*, 1992a). FIR pasteurization will thus be applicable to film foods, sheet foods and the surface disinfection of solid foods.

2.4 Comparison with stationary phase

As shown in Fig. 4, FIR irradiation rapidly decreased the number of exponential phase cells of *E. coli* compared to the stationary phase cells. This shows that the exponential phase cells of *E. coli* were more sensitive to FIR irradiation than the stationary phase cells. In the case of the stationary phase, FIR irradiation markedly increased the sensitivities to RFP and CP. CP acts on ribosomes and inhibits protein synthesis (Fernandez-Muñoz *et al.*, 1971; Nierhaus and Nierhaus, 1973; Pestka, 1969; Pongs *et al.*, 1973). The ribosome is an enzyme-RNA (ribosomal RNA) complex. It was suggested that FIR irradiation mainly damaged both RNA polymerase and ribosome in *E. coli* at the stationary phase cells and led to death (Sawai *et al.*, 1995a). To examine the differences in the injuries between the exponential and the stationary phase cells, the following equation is defined.

$$R_{IN} = \frac{N_i(C/C_{MAX} = 0) - N_i(C/C_{MAX})}{N_i(C/C_{MAX} = 0)} \\ = 1 - \frac{N_i(C/C_{MAX})}{N_i(C/C_{MAX} = 0)} \quad (1)$$

R_{IN} is the ratio of the number of injured cells to that of viable cells.

Figures 9(A) and 9(B) exhibit the values of R_{IN} for the exponential phase and the stationary phase of *E. coli* irradiated by FIR ($q_{ir} = 3.2 \text{ kW/m}^2$) in the case where C/C_{MAX} is equal to unity. The abscissa is the bulk temperature of the bacterial suspension. Figure 9 also proves that the exponential phase cells received injuries more easily than the stationary phase cells. For the exponential phase cells, FIR irradiation especially increased the sensitivity to RFP and damaged RNA polymerase of the exponential phase cells. On the other hand, the injuries of ribosomes in the exponential phase cells were not as serious as those in the stationary phase cells. It was reported that the ribosome itself would be a sensor of temperature (VanBogelen and Neidhardt, 1990). For the exponential phase cells, the temperature where the cells began to die might be lower than the temperature where the ribosomes receive serious damages.

In addition, the exponential phase cells were more sensitive to PCG than the stationary phase cells. The action mode of PCG is the inhibition of the synthesis of cell wall (Araki *et al.*, 1966; Blumberg and Strominger, 1971; Tipper and Strominger, 1965; Wise and Park, 1965). The increase in sensitivity to PCG indicates the injuries of the cell envelope such as cell membrane and cell wall. During the exponential phase, the rate of multiplication is most rapid, and new cell envelopes are also synthesizing energetically. Therefore, it was considered that the injury of the cell envelope in the exponential phase cells was more serious than that in the stationary phase cells because the cell envelopes in the exponential phase cells were unstable. For the studies on the injuries of bacterial cell envelopes induced by heat treatment, the

Fig. 9 Injuries of *E. coli* induced by FIR irradiation and thermal conductive heating, (A) FIR irradiation: stationary phase, (B) FIR irradiation: exponential phase, (C) Thermal conductive heating: exponential phase

exponential phase cells have been mainly used as test organisms. There are many reports of functional and morphological injuries of cell envelopes of *E. coli* by heat treatment (Hitchener and Egan, 1977; Mackey, 1983; Tuchido and Takano, 1988). The increase in sensitivity to PCG should correspond to the functional and morphological injuries of cell envelopes. However, the increase in sensitivity of the exponential phase cells to PCG disappeared when the survival ratio decreased to approximately 3.0×10^{-4} . This phenomenon was observed for the exponential phase cells of *E. coli* heated by thermal conduction as well (Fig. 9(C)), but not for the stationary phase cells (Figs. 9(A)). Since the age of cells during the exponential phase is distributed widely, the mechanisms of their response to heat stress might be different from each other.

Little increase in the sensitivity to NA was

observed for both the phase cells. The action mode of NA is DNA-synthesizing systems (Gellert *et al.*, 1976, 1977; Pedrini *et al.*, 1972; Schaller and Perent, 1972). Though there are many reports of DNA injury induced by heat treatment, the results in this work suggested that the bacterial cells incurred little injury to their DNA.

From the results of changes in sensitivities to the selective reagents, it was found that the mechanisms of death of the exponential phase cells were different from those of the stationary phase cells.

Conclusion

The effect of FIR irradiation on *E. coli* in the exponential phase was studied. The following results and conclusions were obtained.

1) The pasteurization effect of FIR irradiation on the exponential phase cells was much higher than that on the stationary phase cells.

2) FIR irradiation damaged mainly RNA polymerase of *E. coli* in the exponential phase.

3) The pasteurization effect of FIR irradiation on the exponential phase cells was much greater than that of thermal conduction. However, both FIR irradiation and thermal conductive heating induced similar changes in the sensitivities to the selective reagents.

4) Under the same irradiation conditions, the changes in sensitivities to the selective reagents for the exponential phase cells were different from those for the stationary phase cells. It was suggested that the mechanisms of death of the exponential phase cells and the stationary phase cells differed from each other.

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Nomenclature

C	= concentration of selective reagent in agar medium	$[\text{mg} \cdot \text{l}^{-1}]$
N_i	= the number of colonies formed on agar medium	$[\text{CFU} \cdot \text{l}^{-1}]$
q_{ir}	= irradiation power	$[\text{W} \cdot \text{m}^{-2}]$
t_h	= heating time	$[\text{s}]$
T_i	= temperature of bacterial suspension	$[\text{K}]$

<Subscript>

MAX	= maximum
0	= initial state

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