Inactivation of *Escherichia coli* phage by pulsed electric field treatment and analysis of inactivation mechanism

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**Abstract.** Inactivation of bacteriophage by pulsed electric field (PEF) treatment, one of the effective procedures for bacteria nonthermal inactivation, was studied. Model phage particles *Escherichia coli* bacteriophages M13mp18 and λ phage, were successfully inactivated by PEF treatment. The survival ratios of both bacteriophages decreased depending on the PEF treatment time when applied peak voltage was 5 or 7 kV, and the survival ratios after 12 min PEF treatment were 10⁻⁴ - 10⁻⁵. Electrophoresis analyses of biological molecules of inactivated λ phage detected no degradation of total protein and genomic DNA. These results suggested that the factor of phage inactivation by PEF treatment was not based on the degradation of protein or DNA, but on the destruction of phage particle structure. Sensitivity of *E. coli* phage to PEF treatment was compared with that of *E. coli* cell. Phage and MV1184 cell were treated with same condition PEF at 5 kV, respectively. After 12 min treatment, the survival ratio of λ phage and MV1184 were 4.0×10⁻⁵ and 1.7×10⁻³, respectively. The survival ratio of phage was lower than that of MV1184. *E. coli* cell is more tolerant to inactivation with PEF treatment than *coli* phage.

1. **Introduction**

Fermentation by bacteria is one of the most important unit operations in the industry. In addition to the production of traditional foods, recently, enzymes, pharmaceuticals and chemicals production by fermentation using bacteria including genetic modified one has been applied to practical use. In these industrial scenes, infection with a bacteriophage to the fermentation bacteria would result in the decrease of the product amount and quality. Inactivation procedure of bacteriophages has been becoming increasingly important to prevent the contamination of the bacteriophage into the fermentation culture. Bacteria inactivation procedures, especially heat treatment, are also applied for inactivation of bacteriophage. The inactivation mechanism of heat treatment resulting from the biomolecules denaturation of bacteria and bacteriophage is secure. However, heat treatment also denatures and degrades other heat sensitive content such as vitamin, sugar, protein, flavour and color components of fermentation feedstock. To avoid these problems, application of nonthermal bacteria inactivation procedures for inactivation of bacteriophage has also been investigated [1-4].

As the one of nonthermal inactivation procedure of bacteria, pulsed electric treatment (PEF) has been attracting attention. PEF characterized by nanoseconds rising time and microseconds of half
bandwidth of applied voltage does not generate large amount of ohmic heat, and this characteristic phenomenon was applied for the liquid sterilization technology. The inactivation mechanism of microorganism in the liquid by PEF is the destruction of biological outer membrane structure of microorganism resulting from the electrical compression and fenestration [5, 6]. PEF dose not produce active species that react to components of fermentation feedstock.

In this study, inactivation of bacteriophage by PEF treatment was investigated as one of nonthermal inactivation procedure of bacteriophage. Escherichia coli and its bacteriophage were selected as the model host and bacteriophage. Inactivation profile of the E. coli bacteriophage by PEF was compared with inactivation profile of heat treatment. And damage of bacteriophage DNA and protein was analyzed by electrophoreses. Sensitivities for PEF of E. coli cell and bacteriophage were also compared using mixture solution of cell and bacteriophage.

2. Materials and methods

2.1. Strains and media

E. coli phage M13mp18 phage (Takara Bio Inc., Ohtsu, Japan) [7] and λ phage (NBRC 20016) were used as the inactivation target by the PEF treatment. E. coli MV1184 (Takara Bio) was used as the host strain of the E. coli phage infection, and cultivated in Luria-Bertani (LB) medium (1% (w/v) bacto peptone, 0.5% yeast extract and 0.5% sodium chloride) until OD_{660} = 0.5-0.8 at 35°C. Then the phages were inoculated into E. coli broth, respectively, and cultivated 4 h. The supernatants centrifuged for 10 min at 18,000 × g and 4°C were used as the phage stock solutions.

2.2. Phage titer measurement

A phage titer (plaque formation unit: PFU) was measured by the plaque counting method. In Brief, the E. coli cells cultivated for 12 h were collected by centrifugation for 5 min at 1,000 × g and suspended into sterilized distilled water. An appropriate diluted phage stock solution, E. coli solution and LB medium containing 0.6% agar, which was autoclaved and cooled down to 45°C, were mixed well and overlaid onto LB medium plate containing 1.5% agar. The phage titer was calculated from the number of the plaques formatted on plate after 12 h cultivation at 37°C.

2.3. PEF generation and PEF treatment apparatus

A high-voltage pulse generator with a rotary spark gap described in our previous report was used [8]. The capacitor capacity, pulse frequency and peak applied voltage were 8 nF, 50 Hz and 5 and 7 kV, respectively. The schematic diagram of the treatment chamber used in this study is shown in figure 1. Treatment chamber consisted of two parallel acrylic plates that have plate stainless steel electrodes facing each other with a gap of 31 mm and an acrylic cylinder. The phage solution diluted to 10^7 PFU/ml was poured into the treatment chamber, and pulsed voltage was applied for 0-12 min. The phage titer of the treated phage solution was measured as described above. E. coli cell suspension was also treated by PEF, and viable cell number (colony formation unit: CFU) was measured by colony counting. The mixture of E. coli cell (10^7 CFU/mL) and phage (10^7 PFU/mL) was treated by PEF at 5 kV, and then treated sample was inoculated into LB medium. The mixture was cultivated at 35°C and the time course of culture turbidity (optical density at 660 nm: OD_{660}) was measured.

The capsid proteins of phage were concentrated by ultrafiltration (30 kDa cut-off) and applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gel. The proteins separated on the gel were stained with Silver stain II kit Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The phage DNA was purified form the proteins by phenol-chloroform extraction and concentrated by ethanol precipitation. The purified and concentrated DNA was applied to electrophoresis using 0.7% agarose gel and stained by ethidium bromide.
3. Results

3.1. Inactivation of bacteriophage by PEF treatment

The effect of PEF treatment on inactivation of *E. coli* bacteriophage was confirmed using M13mp18 phage at room temperature. Figure 2 shows the time course of phage survival ratio in the PEF treatment at 5 and 7 kV. The survival ratio of M13mp18 phage was decreased with the increase of treatment time. The survival ratio of M13mp18 phage after 12 min PEF treatment at 5 and 7 kV were decreased to $9.0 \times 10^{-5}$ and $2.0 \times 10^{-6}$, respectively. Increase of PEF applied voltage improved the inactivation efficiency of M13mp18 phage. The temperature of phage solution after 12 min PEF treatment at 5 and 7 kV reached to 48 and 71 °C, respectively. It was also confirmed that the survival ratio of m13mp18 phage did not decreased by heat treatment at 80 °C for 12 min (data not shown). From these results, it was confirmed that *E. coli* bacteriophage could be inactivated by PEF treatment.

Inactivation of λ phage, which has different structure of the phage particle from M13mp18 phage, by PEF treatment, was also studied. The decrease of λ phage survival ratio with the increase of the treatment time was also confirmed (figure 3). In addition to M13mp18 phage, PEF treatment could inactivate λ phage. The survival ratio of λ phage in the PEF treatment at 5 and 7 kV were almost same until 9 min. The survival ratio of λ phage after 12 min PEF treatment at 5 kV was decreased to $4.0 \times 10^{-5}$. Temperatures of phage solution after 12 min PEF treatment at 5 and 7 kV were 51 and 68 °C, respectively, and no inactivation by heat treatment at 70 °C for 12 min were also confirmed. Combination of PEF applied voltage and solution temperature above some threshold might increase efficiency of λ phage inactivation.

![PEF reactor](image)

**Figure 1.** Schematic illustration of the PEF reactor used in this study.
3.2. Analysis of biological molecules of inactivated phage

Total proteins and DNA extracted from λ phage inactivated by PEF treatment were analyzed by the SDS-PAGE. Figure 4 shows the result of SDS-PAGE of PEF inactivated λ phage total protein. Some bands indicating capsid proteins were detected in the extracted samples from all PEF treated phage. Comparison between the band patterns of PEF untreated (lane 1) and PEF treated λ phages (lanes 2-5) showed no significant change and increase of small protein band generated by capsid protein digestion is not confirmed though survival ratios of λ phage were quite different each other (figure 3). From these results, it was confirmed that PEF treatment did not cause the protein degradation of λ phage during the inactivation process. In the agarose gel electrophoretic analysis of total DNA extracted from PEF treated λ phage, only single DNA band indicating genomic DNA of λ phage and no smear band were detected (figure 5). Meanwhile, purified bare λ phage genomic DNA was degraded by PEF treatment [9]. From these results, it was also confirmed that genomic DNA was not released from the phage particle and PEF had not degraded the genome DNA in the phage particle in the inactivation process.

From our electrophoresis analysis, degradation of biological molecules, which were capsid protein and phage DNA, of PEF inactivated λ phage was not detected. Inactivation of λ phage by PEF treatment might result from the denaturation of the protein or phage particle structure change.

3.3. Inactivation of E. coli phage in E. coli cell suspension

Sensitivity of E. coli phage to PEF treatment was compared with that of E. coli MV1184 cell. λ phage and MV1184 cell were treated by same PEF treatment at 5 kV, respectively (figure 6). The survival ratio of λ phage was lower than that of MV1184 at any treatment time, the survival ration of λ phage and MV1184 were 4.0×10⁻⁵ and 1.7×10⁻³, respectively, after 12 min treatment. This result shows that λ phage is more sensitive to the PEF treatment than MV1184, i.e. E. coli cell is more tolerant to inactivation of PEF treatment than E. coli phage. This difference of sensitivity (tolerance) to PEF treatment might allow preferential inactivation of E. coli phage in the mixture of E. coli phage and E. coli cell.
Figure 4. Picture of SDS-PAFE electrophoresis with total protein of native λ phage (lane 1) and PEF treated λ phage at 7 kV for 3 (lane 2), 6 (lane 3), 9 (lane 4) and 12 (lane 5) min.

Figure 5. Picture of agarose gel electrophoresis with λ phage genomic DNA extracted from native λ phage (lane 1) and PEF treated λ phage at 7 kV for 3 (lane 2), 6 (lane 3), 9 (lane 4) and 12 (lane 5) min.

Figure 6. Time courses of E. coli MV1184 (closed circles) and λ phage (open squares) survival ratio in PEF treatment.

The mixture of λ phage and MV1184 was treated by PEF at 5 kV. And then, treated suspensions were inoculated into LB medium and cultivated at 35 °C. Figure 7 shows the time course of culture turbidity (OD₆₆₀) of each treated and cultivated suspension. In the cultivation of the phage and cell mixture without PEF treatment, the turbidity of culture reached peak value (0.36) at 2 h, and then decreased due to the cell lysis result from the infection of phage. In the cultivation of the mixture suspension treated by PEF for 3, 6, and 9 min, the time required to reach peak value of culture turbidity increased with the PEF treatment time. The culture turbidity of mixture suspension treated by PEF for 3, 6 and 9 min reached at 3, 4 and 6 h cultivation, respectively. This elongation of time resulted from the decrease of viable cell number existed in the mixture suspension used for the inoculum by the PEF treatment. However, the peak value of culture turbidity increased with the PEF
treatment time. The peak values of culture turbidity of mixture suspension treated by PEF for 3, 6 and 9 min were 0.55, 0.68 and 0.74, respectively. These increases of attainable cell growth indicated the inactivation of phage in the phage and cell mixture suspension by PEF treatment. In the cultivation of mixture suspension treated by PEF for 12 min, significant cell growth did not detected until 7 h. However, cell growth reached to the high value as same as that of pure \textit{E. coli} cell cultivation and no decrease by the cell lysis was determined. This result shows that phage in the mixture suspension was successfully inactivated by PEF treatment without complete inactivation of \textit{E. coli} cell.

\textbf{Figure 7.} Time courses of turbidity of cell and phage mixture culture. Symbols show pure cell (closed circles), cell and phage mixture (open circles) and mixture treated by PEF at 5 kV for 3 (open triangles), 6 (open squares), 9 (open reverse-triangles) and 12 (open diamonds) min.

\section*{4. Discussion}

In this study, the inactivation of the bacteriophage by PEF treatment was investigated using \textit{E. coli} bacteriophage M13mp18 and \textit{\lambda} as the model bacteriophage. The survival ratio of M13mp18 phage treated by PEF decreased with the increase of treatment time and applied voltage (figure 2). The heat treatment at 80 °C for 12 min did not decrease the survival ratio of M13mp18 phage (data not shown). Phage inactivation ability of PEF treatment was confirmed from these results, \textit{\lambda} phage was also successfully inactivated by PEF treatment (Figure 3). M13mp18 phage particle is simple liner form (filamentous) bacteriophage harboring cyclic single strand genomic DNA surrounded by capsid protein [10-12]. \textit{\lambda} phage particle is complicated form bacteriophage harboring double strand genomic DNA in the polyhedral head structure [12, 13]. The successful inactivation of both \textit{E. coli} bacteriophages by PEF treatment demonstrated that PEF treatment could inactivate bacteriophage without dependence of bacteriophage particle form. However, inactivation profile of \textit{\lambda} phage was a little different from that of M13mp18 phage. Increase of applied voltage of PEF did not affect the decrease of survival ratio of \textit{\lambda} phage though M13mp18 phage inactivation was depended on the applied voltage. The difference of the dependence on the applied voltage for \textit{\lambda} phage and M13mp18 inactivation was not clear from our results. The structural difference of phage particle seems to affect the dependence on the applied voltage for PEF inactivation. Further study should be necessary to discuss the dependence on the applied voltage.

Electrophoretic analysis of biological molecules, total protein and genomic DNA, of inactivated \textit{\lambda} phage were carried out to investigate inactivation mechanism. Significant degradations of protein (figure 4) and DNA (figure 5) were not detected. In our previous study, it was reported that PEF treatment degrades the genomic DNA [9]. These results indicated that phage particle was not broken down by PEF treatment and genomic DNA was not release to the solution. Therefore, inactivation of
phage might result form the disruption of phage function. Because the PEF treatment could denature several proteins [14, 15], part of capsid protein might be denatured, then phage particle lost infection ability to the cell.

The comparison of survival ratio of E. coli cell and phage in the PEF treatment showed that E. coli phage exhibited more sensitive behavior to the PEF treatment (figure 6). Meanwhile, in the heat treatment at the temperature below 70 °C, decrease of survival ratio of E. coli cell was larger than that of phage (data not shown). As the application of this difference, inactivation of E. coli phage in the mixture suspension of E. coli cell and phage without complete inactivation of E. coli cell was investigated (figure 7). The time required to detect significant cell growth was increased due to the cell death by the PEF treatment, however, the peak value of the culture turbidity before the cell lysis start was increased with the PEF treatment time. In the cultivation of the 12 min PEF treated suspension; no cell lysis by phage infection was detected. This result demonstrated that the inactivation of E. coli phage below the level to exhibit an influence for cell growth by PEF treatment without complete inactivation of cell. This phenomenon with PEF treatment could be applicable for the phage-free fermentation process because the phage could be inactivated completely in the suspension of bacteria and phage.

In conclusion, we demonstrated that PEF treatment could inactivate E. coli phage without dependence on the phage form. Unlike the heat treatment, E. coli phage was inactivated easier than E. coli cell during the PEF treatment. These finding could be available for inactivation of contaminated E. coli phage in the culture of gene modified E. coli to produce useful protein. Furthermore, it could be useful for other bacteria used in the food and chemical industry if this phenomenon used by PEF treatment could be confirmed in other phage-host system.

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