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Research Article

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Inactivation of *Escherichia coli* phage by pulsed electric field treatment

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ABSTRACT

Inactivation of bacteriophages by a non-thermal bacteria inactivation procedure--pulsed electric field (PEF) has been investigated. Model phage particles, λ phage and ΦX174 phage, were successfully inactivated by PEF treatment. The survival ratios of the two bacteriophages decreased significantly depending on treatment time when electric field intensity set between 1 to 10kV, and pulse width set between 1 to 10μs, and after 200 pulses the survival ratios were lower than 4- log. Sensitivity of phages to PEF treatment was compared with that of E.coli MV1184 cell. Phage and MV1184 cell mixtures were treated by PEF at 6kV/cm with 100 pulses, the survival ratio of λ phage and MV1184 were 5-log and 2-log. Bacteriophage is more sensitive to PEF treatment than E.coli cell, and its survival ratio was lower than that of MV1184. The results showed that phage inactivation by PEF treatment was based on destruction of phage particle structure. These finding indicated that PEF treatment inactivates E.coli phage in high-efficiency. PEF treatment could be a valuable application in food and chemical industry for inactivation of contaminated bacteriophages in fermentation culture.

Keywords: bacteriophage, *Escherichia coli*, inactivation, pulsed electric field

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INTRODUCTION

Bacteria are the most important units in the fermentation industry. With the rapid development of molecular biology and cell engineering technology, many kind of genetic modified bacteria have been applied in industrial fermentation [1][2]. Bacteriophage is a kind of virus that infects and replicates within bacteria. In the industrial case, bacteriophage contamination of the fermentation bacteria could reduce the amount and quality of fermentation production. Other bacteria inactivation procedures, such as heat and high pressure chemical disinfection treatment, are also applied in inactivation of bacteriophage [3][4]. High-temperature heating can effectively inactivate phage and bacterial contamination, but it also denatures and degrades heat sensitive content, such as vitamin, carbohydrate, protein and color components; while chemical disinfection may cause secondary pollution of the product [5]. It is important to develop a new procedure to inactive bacteriophages in fermentation industry.

As a most popular non-thermal sterilization technology, pulsed electric field (PEF) treatment has been attracting attentions. The most widely accepted mechanism of microbial inactivation by PEF has been attributed to the direct interaction between the cell membrane and an external electric field, which results in the destruction of the membrane structure [6][7]. PEF is one of the non-thermal technologies being developed for the preservation of foods as an alternative to traditional thermal methods [8][9].

In this paper, inactivation of bacteriophages by PEF treatment was investigated as one of non-thermal inactivation procedure. λ phage and ΦX174 phage were used as model particles, and *E.coli* MV1184 was used as host bacterial. We used different electrical pulse parameters protocols, including electric field intensity, pulse number and pulse width, systematically changing only one parameter at a time, to ascertain the parameters that crucially contribute to the inactivation results. *E.coli* cell and bacteriophage were also compared using mixture to determine sensitivities for PEF treatment.

EXPERIMENTAL SECTION

Strains and culture: λ phage and ΦX174 phage were used as the model particles by the PEF treatment. *E.coli* MV 1184 was used as the host strain of the phage infection and cultivated in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride, pH7.0). *E.coli* MV1184 was inculcated at 37ºC until OD_{600}=0.5~0.6, then the phage suspensions were added respectively. After shaking cultivation for 6h, a few drops of chloroform were added to the mixture. The mixture was centrifuged for 20min at 18,000×g and the upper supernatant were collected as the phage stock solutions.

Phage titer measurement: Phage titer was measured by the plaque forming units (PFU) methods. In brief, the diluted phage suspension and *E.coli* cells were mixed well and seeded onto LB medium plate containing 1.5% agar. Then 3ml upper cultured agar (0.6%) were poured on the underlying culture agar, inverted cultured for 24h at 37ºC. the phage titer was calculated from the number of the plaques formatted on plate. Each dilution inoculated on three plates.

PEF generation and treatment: A bench scale continuous high-intensity PEF system with square-wave pulses was used in this study. The output voltage and pulse width reaches 1~100kV and 1μs~10μs, respectively. Treatment chamber consisted of two parallel stainless plate electrodes. The distance between the electrodes varied within 1-10cm which set to 1cm in this study. The volume of sample treatment chamber is 1mL, and the pulse frequency is 100Hz here. The phage solutions and *E.coli* cells were resuspended to 10^7PFU/ml (phage) or 10^7CFU/ml (*E.coli*) in sterile phosphate buffer (10mM, pH7.0) before treatment. The phage titer of the treated phage solution was measured as described above. *E.coli* cell number was measured by colony counting. The mixture of *E.coli* cell and phage was inoculated into LB medium immediately after PEF treatment at 37ºC. The turbidity (optical density at 600nm: OD_{600}) was measured by the time course. Samples without PEF treatment were used as a negative control.

SEM analysis: Culture fluids were centrifuged for 5min at 3000×g. The cell pellet was suspended and washed in sterile phosphate buffer (10mM, pH7.0) and 1% osmium tetroxide treatment for 4h, and then fixed overnight with 5% glutaraldehyde. The material was dehydrated by a series of graded ethanol solutions and critical-point dried using liquid $C0_2$, and the preparation was coated with gold in a polaron sputter-coater and examined with a gold field emission scanning electron microscopy (FESEM, HITACHISU-70, JAPAN).

Statistical analysis: All experiments were repeated at least three times. Statistical analysis was applied to all results and a paired *t*-test was used on results obtained from the same sample and on those normalized results if they were obtained from different samples. All the data was analyzed by Statgraphics5.1 software (Statistical Graphics Corporation, Rockville, MD, USA).

RESULTS

Inactivation of bacteriophage by PEF treatment: As shown in figure1, λ phage and ΦX174 phage survival ratio significantly decreased by PEF treatment at room temperature while the parameters of PEF set at the following range: electric field intensity at 1~10kv, pulse width in 1~10μs, number of pulses within a range of 50 to 300. The survival ratio of λ phage and ΦX174 phage after PEF treatment at 6kV were decreased to 4.7-log and 5.2-log ($P>0.05$).

The effect of different electric field intensity and pulse number on the inactivation curve was showed in figure 1a and 1b. Increasing the intensity from 2kV to 10kV caused an almost 3-log reduction. For electric field intensity 6kV and pulse width 5μs, almost 2-log reduction was observed while increasing the pulse number from 50 to 300. The experimental results showed no significantly difference on sensitivity to PEF treatment between λ phage and ΦX174 phage.

The temperature of phage solution after treatment by PEF at 2kV and 10kV reached to 42°C and 56°C, respectively. And the solution temperature reached 36°C and 68°C respectively after PEF treatment by 50 and 300 pulses. These results indicated that *E.coli* phage could be inactivated by PEF treatment but not decreased by heat treatment. In practical applications, it should be used by reducing the pulse frequency or cold cycle to keep the lower temperature of the objective.

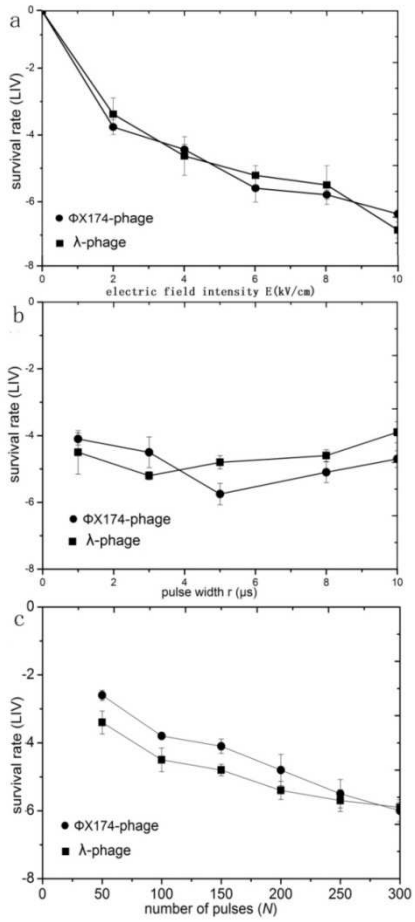

Figure 1. Survival rate curves of *λ* phage andΦX174 phage by PEF (f=100Hz). (a) Under different electric field intensity; (b) With different pulse width; (c) with different number of pulses

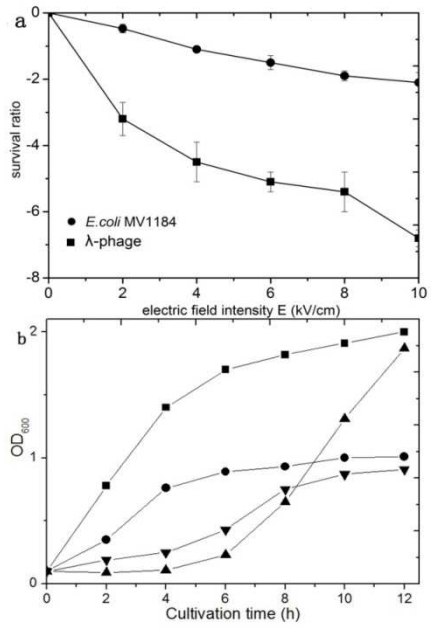

Figure 2. (a)Survival rate curves of *λ* phage and *E.coli* MV1184 in the mixture liquid by PEF at electric field intensity courses with r=5μs, N=100. (b) OD_{600} of cell and phage mixture culture in time courses. ■*E.coli* MV1184 cell without PEF treatment; ●*E.coli* MV1184 and λ phage mixture without PEF treatment, ▼*E.coli* MV1184 and λ phage mixture treated by PEF at 2kV, 5μs, 1min;▲*E.coli* MV1184 and λ phage mixture treated by PEF at 10kV, 5μs, 1min

Figure 1b showed the results obtained by treating phage suspensions with a ranging pulse width from 1μs to 10μs. It was able to achieve an almost 4-log reduction ranging from 1μs to 10μs. The inactivation efficiency reached higher

at 3μs and 5μs for λ phage and ΦX174 phage, respectively. The inactivation profile of λ phage was a little different from that of ΦX174 phage. It means the structural difference of phage particles have an impact on the efficiency for PEF treatment.

Inactivation sensitivity of *E.coli* MV1184 and λ phage to PEF treatment: The mixture of *E.coli* MV1184 and λ phage was treated by PEF on different electric field intensity. The survival ratio of λ phage was lower than that of *E.coli* MV1184 at any treatment intensity. The survival ratio of λ phage and *E.coli* MV1184 were 3-log and 47% reduction, respectively after 2kV treatment. This result shows that λ phage is more sensitive to the PEF treatment than *E.coli* MV1184.

The mixture of *E.coli* MV1184 and λ phage was treated by PEF at 2kV and 10kV with 5μs and 1min. Treated suspensions were inoculated into LB medium and cultivated at 37ºC. Figure 2b showed the OD_{600} of the suspension in time courses. The OD_{600} reached 2.0 after 12h for pure *E.coli* MV1184 cell, and OD_{600} reached only 1.0 after 12h for the mixture of *E.coli* MV1184 and λ phage due to phage lyses *E.coli* cells. For the cultivation of the mixture suspension treated by PEF for 2kV, peak value reached 0.97 after 12h, but for 10kV the peak value could reach 1.86. This result showed that phage in the mixture suspension was successfully inactivated without complete inactivation of *E.coli* cell by 10kV PEF treatment, but the cell phage still existed in the suspension which treated by 2kV PEF.

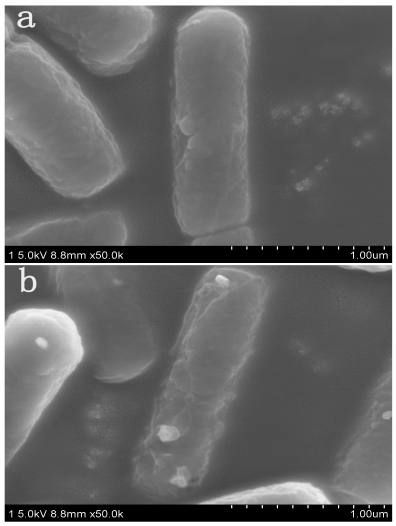

Figure 3. The scanning electron micrograph (FESEM) of disrupted cell envelopes by PEF (U=2kV, τ=5μs, N=100). (a)*Escherichia coli* MV1184; (b)*Escherichia coli* cells with *λ* phage particles

Pure *E.coli* MV1184 and the mixture were observed by field emission scanning electron microscopy (FESEM) after treatment by PEF at 2kV (Figure 3). λ phage particles showed low density with irregular structure on the *E.coli* surface and electroporation formed on *E.coli* cell membrane.

DISCUSSION

In this study, PEF was successfully used to inactive λ phage and ΦX174 phage. No significant difference of survival ratio was detected between two different phages which might indicated that PEF treatment inactivates bacteriophages independence of particle structures. The peak inactivation efficiency reached at 3μs and 5μs for λ phage and ΦX174 phage, respectively. The two phages have different particle structures [1]. That means the structural difference of phage particles have an impact on the inactivation efficiency for PEF treatment.

Survival ratios of *E.coli* cell and phage in PEF treatment in the mixture were detected and compared in this study. The result showed that λ phage is more sensitive to the PEF treatment than *E.coli* MV1184. It suggested that PEF treatment could be applied for the phage-free fermentation process in food and chemical industry.

Electroporation is one of various theoretical models that have been proposed to explain the PEF treatment on bacteria cells. When cells are exposed to an external electric field with sufficient amplitude and duration, the cell membrane is electroporated [10][11]. Aqueous pores are assumed to be induced in the cell membrane and the pores increase in size and number with pulse intensity and duration [7]. It was reported that PEF had a stabilizing effect on the secondary structure of insulin chain-B [12], which restricting the inherent flexibility that is crucial for its biological activity. PEF could also destroy the secondary structures of papain, pepsin, peroxidase and polyphenol

oxidase [13]-[16], and caused conformation of disulfide linkage of protein enzymes [5][16]. The inactivation of phage under PEF treatment should be caused by the secondary structure disruption of coating proteins. The hydrophobic and sulfydryl groups lead to a disulfide cross linkage of phage proteins. Future study should be remains on the mechanism of phage inactivation.

In conclusion, this study demonstrated that PEF treatment could inactivate *E.coli* phage in high efficiency at room temperature. PEF treatment could be applied for the phage-free fermentation process in food and chemical industry.

Acknowledgments
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