The Relation of \textit{E. coli} Growth Phase and Low-Temperature He Plasma Jet Exposure

T. Nakajima, H. Kurita, H. Yasuda, K. Takashima, and A. Mizuno
Department of Environmental and Life Sciences, Toyohashi University of Technology, Japan

Abstract—The relationship between the resistance to plasma inactivation of \textit{Escherichia coli} (\textit{E. coli}) and its incubation conditions was evaluated. The effects of the growth phase and incubation temperature were separately investigated. Low temperature He plasma jet was used as plasma source. The effect of the growth phase was investigated by harvesting the \textit{E. coli}, at regular time intervals, from a culture under incubation and then applying plasma inactivation to the harvested samples. Our results show that the resistance to plasma treatment increases around early exponential phase and at transition from exponential phase to stationary phase. These effects most likely occur due to cell adaptation to the changed cell environment. To investigate the effect of temperature, the \textit{E. coli} was cultivated at 19\degree C, 28\degree C, 37\degree C, 40\degree C and 43\degree C, then the investigations to determine the resistance to plasma were carried out. The resistance to plasma treatment is evidently impaired by incubation temperatures lower or higher than 37\degree C.

Keywords—plasma jet, plasma inactivation, \textit{E. coli} growth phases, temperature inactivation

I. INTRODUCTION

The studies and applications of low-temperature atmospheric pressure plasma jets in biomedicine and biodecontamination have been increasing [1-5]. Specific evidence of successfully using low-temperature plasma jet [6] for \textit{Escherichia coli} (\textit{E. coli}) inactivation has previously been reported [7].

Breaking down cells by chemical activity and electric field can induce lethal effect. In particular, the reactive species with high oxidation power are considered as the most important factor in cell sterilization [8].

Since it is convenient for practical use, understanding in detail the characteristics of plasma inactivation is important. In our present study, the plasma bacterial inactivation effect occurred as results of changing the incubation condition of \textit{E. coli} was investigated.

The properties of microbes change depending on cell environment conditions such as incubation time and temperature [9-14]. Adaptation of cell response to nutrient depletion caused by cell growth, suboptimum temperature, etc. determines changes in bacterial properties. \textit{E. coli} growth phase and incubation temperature was separately investigated.

Typical cell growth phases consist of a lag time at the start of growth, rapid growth, conservation at saturated concentration for a period of time, and rapid death. These stages are called lag phase, exponential phase, stationary phase, and death phase [15-17]. This four-phase model of the bacterial growth has been described in the microbiology literature for some decades. [16-17] However, the effect of non-thermal plasma on the \textit{E. coli} in the growth phase has not been studied thus far.

In most cases, microbes designated for research are prepared by incubation in liquid medium. For incubation of microbes, the culture is kept at optimum temperature for cell growth. Based on the growth phase model, results on \textit{E. coli} phase relation to low levels of ultraviolet light exposure have been reported [12-14]. Relevance between incubation temperature and survival rate after heat inactivation has also been investigated and reported [9-11]. For applications of low temperature plasma, the understanding of its inactivation effect on microbe growth under a variety of condition can be highly beneficial.

Cells such as \textit{E. coli} conduct its adaptation by control of gene expression. Both environmental change and non-lethal damage act as cellular stress. \textit{E. coli} genome contains about 4000 genes. Among them, dozens to hundreds of genes contribute recovery from and adaptation to stresses. Though the answering mechanisms are remaining incompletely defined, it is reported that different set of genes answer to each type of stress such as nutritional depletion, oxidation, and heat etc.

In the present paper, we report results on the \textit{E. coli} growth phase effect and cultivation temperature effect under low-temperature plasma jet exposure, separately.

In a separate experiment, aimed to evaluate the effect of the incubation temperature, the \textit{E. coli} culture was incubated at 19\degree C, 28\degree C, 37\degree C, 40\degree C, and 43\degree C. After the incubation, the \textit{E. coli} bacteria were treated with plasma jet for 5 min, and a comparison among their survival rate was carried out. Treatment by heat instead by plasma jet was also carried out with the aim of a comparison to another stress source, heat treatment.

Corresponding author: Akira Mizuno
e-mail address: mizuno@ens.tut.ac.jp
II. METHODOLOGY

A. Plasma jet generator and experimental set-up

We employed a 40 mm, helium (He) plasma jet. The schematic of the plasma jet generator and experimental set-up are shown in Fig. 1. The plasma jet generator consists of a quartz glass tube, and a 10 mm width aluminum tape. The aluminum tape was wrapped at 20 mm from the tip of the glass tube and used as the high voltage electrode. Dielectric barrier discharge (DBD) [18] was generated inside the glass tube, using a pulsed power supply (ECG-KOKUSAI, PPS-8000).

The applied voltage was 15 kV, the frequency was 3 kHz, and the pulse width was 2.8 µs. The measured discharge power was 8 W. A grounded aluminum plate was placed at 40 mm below the tip of the generator. The helium flow rate was 2.0 l/min. These parameters were fixed for all experiments.

The applied voltage and the resulted current waveform were simultaneously measured through an HV probe (Tektronix, model P6015A, 100 MΩ, 3 pF) and a current monitor (Pearson Electronics Inc., model 2877, 1 Volt/Ampere +1/-0%, 200 MHz) respectively. Both probes were connected to a digital oscilloscope (Tektronix, model DPO 2024, 200 MHz, 1 GS/s). The electric field was also evaluated through an HV probe and oscilloscope measurement in preparation to the cell exposure to plasma.

Temperature of sample immediately after plasma treatment was measured with infrared thermometer (THERMO HUNTER, OPTEX CO., LTD; PT-S80). The measurement was carried out to confirm absence of heat effect in inactivation, and its possible applicability to body. As a result, temperature rise was not occurred in experimental conditions of this paper.

B. Evaluation of the effect of growth phase

E. coli strain ATCC13706 was used for the experiment. E. coli colony on LB agar medium was mixed into 20 ml LB liquid medium and pre-incubated. 500 µl of the bacterial suspension in stationary phase was added to 100 ml fresh LB nutrient broth in a 1 L flask. This new culture was incubated at 37°C while shaking for aeration.

Once every hour, a portion of the bacterial suspension was harvested. Data was collected this way for 8 hours. Before plasma jet treatment, the bacterial cells were collected by centrifugation, and resuspended into phosphate buffer saline (PBS). The cell density was adjusted to 10⁸ cell/ml at each plotted time. At each cultivation time, the harvested bacterial suspension was exposed to plasma jet treatment for 5 minutes. After that, viable cell concentration was measured by standard plate count. The survival rates after the plasma jet treatment were calculated and plotted against the cultivation time. The cell concentrations of the culture before without the adjustment were also shown, for observation of growth phase.

C. The effect of the cultivation temperature and comparison with the effect of heat treatment

19°C, 28°C, 37°C, 40°C, or 43°C was investigated as E. coli cultivation temperature. Pre-cultivation was carried out similarly to the experiment regarding growth phase. A colony of E. coli ATCC13706 on LB agar medium was mixed into 20 ml LB liquid medium and incubated at 37°C until stationary phase. 500 µl of the bacterial suspension was added to 100 ml fresh LB nutrient broth in a 1 L flask. The new culture was cultivated at each of temperatures listed above. Respective cultures were treated by plasma jet or heat after preparation, as below.

Harvesting was carried out based on optical density at 595nm (OD₅₉₅), because growth rate was varied by cultivation temperature. The cultures were harvested around OD₅₉₅ = 0.5, corresponding to 2 × 10⁷ cells/ml. The bacterial cells were collected by centrifugation and resuspended into PBS.

In plasma jet treatment, E. coli cell concentration was adjusted to 10⁸ cell/ml. 400 µl of the suspension was put in a cylindrical well of a 24-well tray (BD Falcon™, Becton, Dickinson and Company; 35-3047). The well was 15 mm in depth and 2 cm² in well base, therefore sample depth was around 2 mm. The distance between the plasma generator and the sample surface was about 35 mm.

For heat treatment, cell concentration was adjusted to 2 × 10⁷ cell/ml, and 380 µl of PBS was of pre-warmed 55°C in heat block. 20 µl of the cell suspension was added to the PBS, to cell concentration 10⁶ cell/ml.

In both plasma jet and heat treatment, exposure times were 5 minutes, and viable cell concentrations were measured by standard plate count. Data were shown as survival rates, with viable cell concentration of control being 100%.
III. RESULTS

A. Electrical measurements

Before and during each plasma treatment experiment, the applied voltage amplitude, frequency and the DBD discharge current are closely monitored, and fixed. Fig. 2 illustrates the displayed voltage pulse and discharge current waveforms.

To calculate the power consumption, we used the oscilloscope capability to concomitantly measure the RMS voltage and the RMS current. As these values per cycle can vary, the power was calculated using the RMS values over a length of approximately 10 voltage cycles. The obtained value for the power consumption in our setup is 8 W.

B. Evaluation of effect of growth phase

In practice, growth phases are determined by cell concentration. Substantially, the cell growth is caused by adaptation of cell to environment. In lag phase, microbes adapt themselves to new the environment. If the change in the cell environment is small, this phase becomes short, as in our case, and hard to investigate. In the exponential phase, cells continue division in its doubling time.

Therefore, the growth rate is proportional to the cell concentration at the time. The logarithmic plot of cell concentration in this phase is a linear function of the incubation time. The proportionality constant of the function indicates the doubling time. The transition to the stationary phase occurs by restriction of growth through nutrient depletion, accumulation of waste material, and congestion.

Previously, the stationary phase was considered as equilibrium between proliferation and death of cell. In recent years, controlled transition of gene expression is demonstrated by metabolomic analysis. This changed the interpretation of stationary phase to the phase including the controlled adaptation to high stress environment factor.

The experimental results are shown in Fig. 3. From cell density plots, the early exponential phase to early stationary phase growth segment was investigated in this experiment. Lag phase, namely in the first hour, was not observed for the aforementioned reason. Two distinct peaks can be observed on the survival rate graph. The first peak is after around 1 hour of cultivation and the second peak is at 6 hours of cultivation time. At 1 hour cultivation, the survival rate was around 90%, though it dropped to about 20% at 3 hours, 4 hours, and 5 hours. At 6 hour cultivation, the cell viability rose to 30%, then dropped again to about 15% after 8 hours.

These peaks of plasma resistance coincidently correspond to two of the known state transitions of E. coli, mentioned in the introduction. Specifically, from Fig. 3, the 1 hour cultivation time and the 6 hour cultivation time are considered as corresponding to the transition from the lag phase to the early exponential phase, and the exponential phase to the early stationary phase respectively.

C. Evaluation of effect of cultivation temperature

Results are shown in Fig. 4. Survival rate was highest at 37°C cultivation temperature. Higher or lower cultivation temperatures impaired the plasma resistance by about 20% in average.

The standard deviation (STDV) variability with cultivation temperature is higher for temperatures different from 37°C. For example, the STDV is 5.7 for 19°C and around 3.5 for 28°C and 43°C, while the STDV for 37°C is very low (0.8).

The standard deviation of our plasma treatment data varied between 2 for 37°C and around 21.7 for 19°C. In one set of experiments, the cell viability after plasma treatment for cells cultivated at 40°C and 43°C was around 24.6% and 22.8% respectively. This data was not
take into account in the average and STDV calculation of the results shown in Fig. 4.

Change of resistance occurred by suboptimum cultivation temperature. This was observed in both plasma jet and heat treatment, though trend in two treatments were notably different. Contrary to the case of the plasma jet treatment, the survival rate at 37°C cultivation temperature was the lowest for the heat treatment. The cell viability after the heat treatment was around 2.4%. For cultivation at 40°C and 43°C, the survival rate rose to around 13.0% and 28.0%, respectively. An increase in survival rate was also observed at lower cultivation temperatures of the heat treatment. These survival rates were 30.4% at 19°C and and 18.0% at 28°C, respectively.

IV. DISCUSSION

A. Evaluation of the effect of growth phase

As seen in Fig. 3, the resistance of E. coli to plasma treatment increased at the transitions of growth phase. Cell adaptation during these transitions is considered as the reason. Generally, when cells confront stresses, such as change of environment, they express genes for repair from occurred damage, protection, and adaption. This can result in increase of resistance to other stressors. For example, reports show that proteins with the highest rate of synthesis during the lag phase are not detectably produced at 37°C [11].

From these considerations, the first peak in cell viability (Fig. 3) for plasma treatment after one hour of cultivation is likely caused by the transition of E. coli from the lag phase (not shown) to the exponential phase. The second peak, at the transition to stationary phase, can be due to an increase of cyclopropane fatty acids (CFA) content occurred in the cell membrane. [19] Cell membrane of E. coli is composed of phospholipid bilayer. The composition of the fatty acid chains in phospholipids is one of the factors that affect the properties of the membrane.

CFA synthesis in E. coli is related to the gene transcription factor RpoS. RpoS plays the dominant role at the beginning of the stationary phase. CFA concentration in cell membrane has its peak at the transition between the exponential phase to the stationary phase. Increase of CFA in cell membrane reduces the fluidity of the lipid bilayer, i.e. the fluidity of the cell membrane. This leads to a decrease in membrane permeability, as a defense mechanism to cell environmental shock. This cell hardening mechanism has been previously explained in detail. [19]

B. Evaluation of effect of cultivation temperature and comparison with the effect of resistance to heat treatment

The gaps of examined low cultivation temperatures between optimum temperature was three times larger than it of high temperature side. Though, in both lower and higher temperature side, effect to survival rates were similar level. It can be said that stress of higher cultivation temperature is larger than lower cultivation temperature.

Generally, microbes soon after suffering from a stress have resistance to the stress. This phenomenon is called “tolerance”. It is induced by expression of a set of stress genes for the type of stress. Additionally, some stress genes can induce some resistance to another type of stress. It is called “cross protection.” Heat shock protein (HSP) can be an influential factor in the cell adaptation to suboptimum temperature. When cells are exposed to cold shock, elevated heat or other stressors, the HSP initiate or increase their expression. HSP contain functional proteins that help folding of other proteins, called chaperone protein, and dispose irreversibly damaged proteins etc. From the studies of HSP proteins resulted from temperature as stressor on E. coli, it is then known that different proteins are produced this way for different stressors. Moreover, reports show that many types of proteins, from tens in regular conditions to thousands, depending on the stress type and condition, can be expressed. Increase of survival rate after heat treatment in higher cultivation temperature appears like the tolerance. Though, survival rate also increased in lower temperature side. HSP which is expressed at high temperature is different from it in low temperature. Therefore, increase of heat resistance at low cultivation temperature can rather be considered as a cross protection. In plasma jet treatment, resistance decreased in suboptimum cultivation temperature. The trend was nearly opposite to heat treatment. It means cross protection was not occurred between stress of suboptimum cultivation temperature and plasma jet treatment. It is shown that gene sets of E. coli for these stresses are namely different. As mentioned in
introduction, the reactive oxidative species (ROS) are considered as the main cell inactivation factor in low temperature plasma treatment. Therefore, the treatment can broadly be compared to the effect of a cocktail of oxidative reactive stressors. Based on previous studies involving \(E.\ coli\) as model for response to oxidative stress [20–21], antioxidant enzymes are responsible for preventing free radical modification of cellular components due to plasma treatment. It can be considered that \(E.\ coli\) grown in suboptimum temperature had lessened oxidative damage resistance, because of dealing with temperature stress, such as synthesis of HSP.

Not only the change in defense against stressors, but also changes in recovery ability through cell adaptation can have effect on survival rate. The cultivation temperature also has effect on the composition of fatty acid chains in cell membrane. It is reported that when incubated at low temperature, the \(E.\ coli\) automatically increases the proportion of unsaturated fatty acid in its cell membrane. This increase is considered as an adaptive mechanism for maintaining the optimum fluidity of cell membrane [9]. The increase in the fatty acid chain of the membrane lipid bilayer causes the increase of permeability of the membrane, which can be considered as one reason for the cell behavior cultivated at these temperatures.

V. CONCLUSIONS

The effect of the cultivation conditions on the resistance of \(E.\ coli\) to non-thermal plasma treatment was investigated. The effect of the plasma treatment with growth phase stages, and the cultivation temperature were separately evaluated.

1) In early exponential phase and early stationary phase, the \(E.\ coli\) resistance to plasma treatment increased.

2) Cultivation temperatures both lower and higher than around 37°C decreased the \(E.\ coli\) resistance to plasma. This characteristic was different from the resistance to heat.

3) These effects can be attributed to change of fatty acid composition, as a cell adaptation mechanism.

REFERENCES


