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Evaluation of Heat Damage to Yeast Protoplasts by Dielectrophoresis

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Abstract: Heat stress has a lot of influence on human and animal cells because it damages the inside of the cells. In this work, we evaluate the heat stress toward yeast protoplasts using dielectrophoresis (DEP). We utilize the frequency-dependent property of cell viability in the DEP. For example, healthy yeast protoplasts can be trapped at about 10 kHz, while the frequency for trapping heat-damaged cells is about 50 kHz. In addition, the advantage of using the DEP is possible to observe the cells without damaging them. To apply this research for human and animal cells in general, since there is not cell wall on human and animal cells we eliminate cell wall on yeast cells by protoplastization. We observe yeast's activity by using fluorescence microscope.

Key words: *Dielectrophoresis, Fluorescence microscope, protoplast, yeast cell,*

INTRODUCTION

Heat stress affects the physiological responses of human and animal cells. [1] Therefore, it is very important to be able to easily evaluate heat stress on cell viability. Yeast cells construct with a strong cell wall, which is different from human and animal cells, which are covered with delicate cell membrane. In this experiment, yeast cells were transformed into yeast protoplasts, which have similar cell membrane as human and animal cells. Therefore, by studying yeast protoplasts, we can apply for human and animal cells. Cell membranes play a role of protecting the cells from external hazards. External temperature change may damage proteins inside the cells leading to the death of the cells. [2] In conventional study, dielectrophoresis (DEP) has been used for cell separation and trapping, as well as the distinction of cell's life and death. [3]

DEP was first introduced by Pohl, which is based on an important electrokinetic effect that emerges due to the application of inhomogeneous electric fields to polarizable particles. [4] DEP can easily trap biology cells by applying AC voltage on its two electrodes. Therefore, DEP is widely used for manipulation of cells in life science applications. In addition, a research has been done to arrange the carbon nanotubes on electrodes by DEP. [5] Also, DEP has been used for the distinction of the life and death of cells [6], dielectrophoretic force measurement [7], and cell manipulation [8]. Until now, the distinction of the life and death of cells often based on the method of using methylene blue and fluorescein diacetate (FDA) stain. However, the FDA stain affects negatively to the cells. In this paper, we did experiments to determine that the damage of protoplasts by heat can be evaluated by changing the frequency of applied voltage in DEP. This technique enables evaluating the damage of cells without causing harm to the cells. Therefore, it can be used for dealing with rare cells.

THEORY

A. Dielectrophoresis

DEP is a phenomenon that dielectric particle, such as biology cells, are subjected to force in a non-uniform electric field. The force is expressed by the following equation corresponding to the expression of Clausius-Mossotti.

$$\mathrm{F} = 2\,\pi\, r^3 \varepsilon_m Re\left[\frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}\right] \nabla E^2 \quad (1)$$

Here, r is the radius of the dielectric material, E is the applied electric field. ε_m and ε_p are the permittivity of solution and particles, respectively, and ε^* is complex permittivity defined as follows, where ω is angular frequency, σ is conductivity.

$$\varepsilon^* \equiv \varepsilon - i\frac{\sigma}{\omega} \quad (2)$$

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Fig. 1 shows the operation principle of DEP. In this work, we observed the specific frequency at which yeast protoplasts move in the direction as shown in Fig. 1.

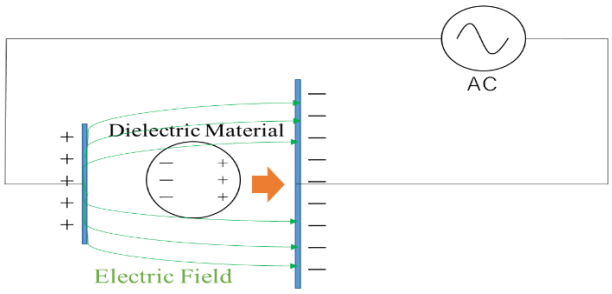


Figure 1. Simplified drawing of the experimental setup

B. *Yeast protoplasts*

Yeast cell is covered with the cell wall that composes mainly of polysaccharides, proteins, and lipids. Protoplast is the cell without the cell wall. The protoplast can synthesize DNA, RNA, and proteins, but its abilities of dividing and growing disappear. [9] Yeast cell can be transformed to yeast protoplast by using Zymolyase enzyme.

C. *Fluorescence microscope*

Fluorescence microscopy is an essential technique for investigation of the intracellular distribution of macromolecules and various organelles such as in yeast cells. Yeast protoplasts emit light when the fluorescein diacetate (FDA) reacts with enzymes in cells. The cells with low activity (damage or death) cannot emit light because the function of enzymes in cells are weakened. Based on this characteristic, the cell damage can be evaluated by fluorescence microscope image.

EXPERIMENTAL SET UP

A. *Method of making DEP electrodes*

We made electrodes used for the DEP by coating gold on a glass substrate using an ion coater. Fig. 2 shows the electrodes that used for our experiment. The electrodes were connected to a function generator using two strips of conductive tape. The distance between the electrodes was measured using a Wray Spect. The distance between the electrodes was 148.82 pixels. Since 1 pixel = 158 nm, the actual distance between the electrodes was approximately 23.5 μm.

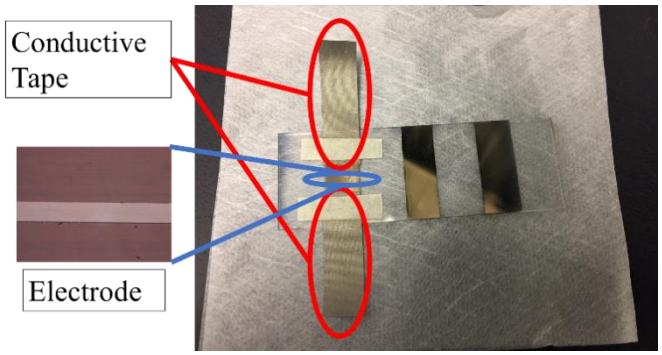


Figure 2. Electrodes used for DEP

B. *Method of making yeast protoplasts*

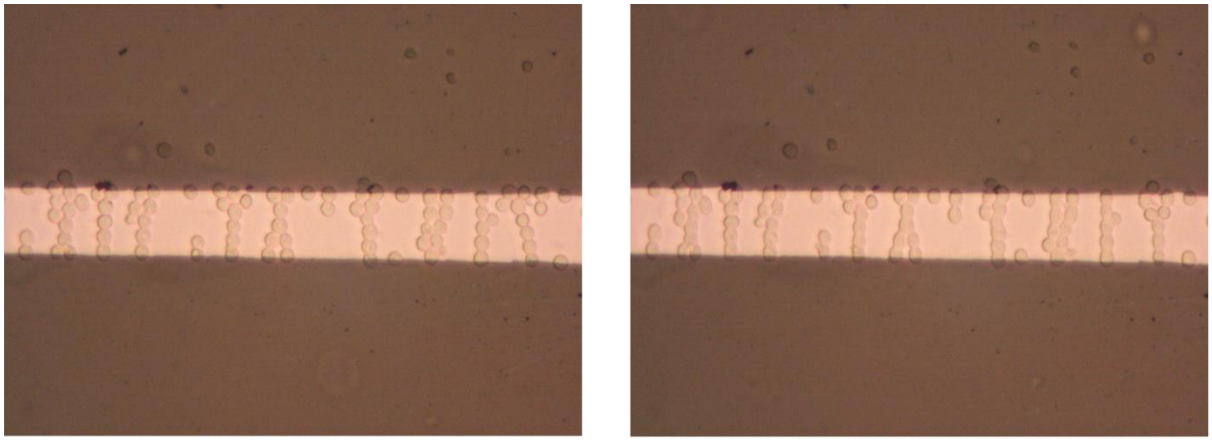
To make yeast protoplasts from yeat cells, we used Zymolyase enzyme solution. Table. 1 shows the composition of the enzyme solution. The enzyme solution is adjusted to obtain pH of 7.5. [10] Yeast cells were incubated in the enzyme solution for 60 minutes at 30°C to make yeast protoplasts. We used sorbitol as osmotic stabilizers for the yeast protoplasts. Table 2 shows the composition of buffer solution used for the enzyme solution. Fig. 3 shows yeast protoplasts manipulated by the DEP. When we increased the voltage from 2V to 7V, we could observe the chains of yeast protoplasts.

Table 1: Composition of the enzyme solution

Sorbitol	1.82	g
Zymolyase	0.01	g
2-Mercaptoethanol	20	μl
Phosphate Buffer	10	ml

Table 2: Composition of the buffer solution

Water	70	ml
Disodium Hydrogenphosphate	1.79	g
Sodium Dihydrogenphosphate	0.312	g



(a) the DEP at voltage 2 V of the yeast protoplasts

(b) the DEP at voltage 5 V of the yeast protoplasts

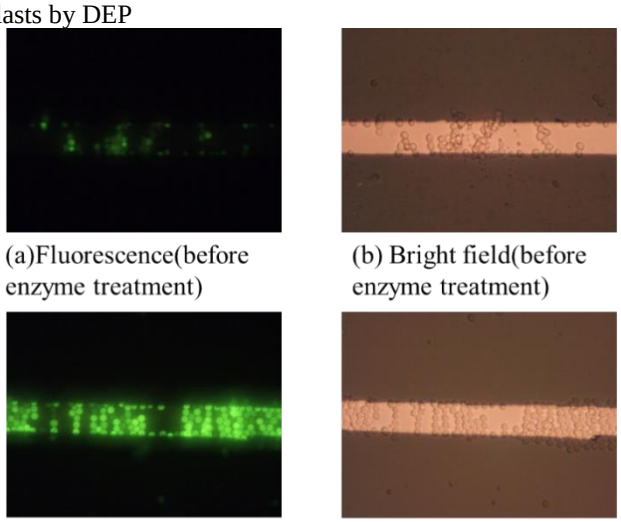
Figure 3. Separation of protoplasts by DEP

C. Evaluation of yeast protoplasts' activity by fluorescence microscope

We used FDA as a staining solution. The FDA hydrolyzes living organisms that have the esterase and the phosphatase, it emits green fluorescence under blue light excitation around 488 nm wavelength. [11] However, the FDA is difficult to fluoresce the cell with the presence of cell wall, because the esterase and the phosphatase cannot come out of the cell. Therefore, we can use the FDA staining solution to separate yeast cells and yeast protoplasts. Table 3 shows the composition of FDA staining solution. We used a fluorescence microscope TC-5500 (Mage Techno). Fig. 4 shows the fluorescence observation of yeast cells and yeast protoplasts by fluorescence microscopy before and after enzyme treatment.

Table 3 Quantity of FDA staining solution

Acetone	1	ml
Fluorescein Diacetate	5	mg



(a)Fluorescence(before enzyme treatment)

(b) Bright field(before enzyme treatment)

(c)Fluorescence(after enzyme treatment)

(d) Bright field(after enzyme treatment)

Figure 4. Confirming the yeast protoplasts using the fluorescence microscope

D. Experiment of heat stress by dielectrophoresis and fluorescence microscope

D-1 *The method of applying heat damage to the yeast*

We used a heat pool as shown in Fig. 5. The heat pool is a device that can adjust water temperature from

30°C to 100°C. In this experiment, the water was set at 80°C. Yeast protoplasts contained in microtubes immersed in water at 80°C. After about 2 minutes, the yeast protoplasts died. Therefore, to evaluate the effect of heat stress on the yeast protoplasts, we treated them for 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 seconds, respectively.

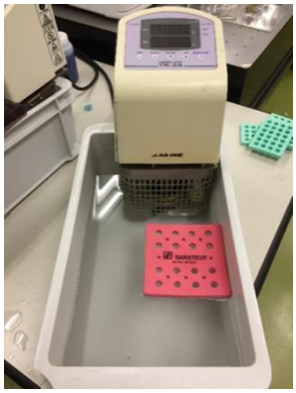


Figure 5. The image of thermostatic chamber used for our experiment

D-2 *The damage observation by the fluorescence microscope*

In fluorescence microscopy, the heat damage is observed by the level of fluorescence. The method of evaluation of the heat damage was by using equation (3).

$$\frac{X}{W} \times 100 = V\ [\%] \quad (3)$$

Where, X is the number of yeast protoplasts that emitted light by the FDA counted in fluorescence state and W is the number of yeast protoplasts counted in the bright field state. V is the proportion of yeast protoplasts emitting light. In this study, the viability of protoplasts was evaluated based on the proportion V.

D-3 *The damage observation by DEP*

A function generator was used for applying an alternating voltage to the two electrodes of DEP. We changed the frequency of the applying voltage from 1 kHz to 15 MHz to characterize the frequency-dependent property of protoplasts. The voltage was fixed at 1 V. After putting a drop of the solution containing yeast protoplasts on the electrodes, the frequency was increased from 1 kHz to 15MHz. We recorded the frequency at which most the yeast protoplasts trapped to the electrodes. In this experiment, we used the yeast protoplasts which were damaged by heat stress of the conditions indicated in the D-1 section.

RESULT and DISSUSION

A. The experimental results by fluorescence observation

Fig. 6 (a) and (b) shows the experimental results of fluorescent observation of yeast protoplasts treated by heat stress at 80℃ for 0 second (healthy protoplasts) and 30 seconds (damaged protoplasts). Obviously, when the cells are subjected to thermal stress for 30 seconds, the level of fluorescence was low (fig. 6 (b)). That meant the viability of the protoplasts significantly decreased by heat stress at 80℃.

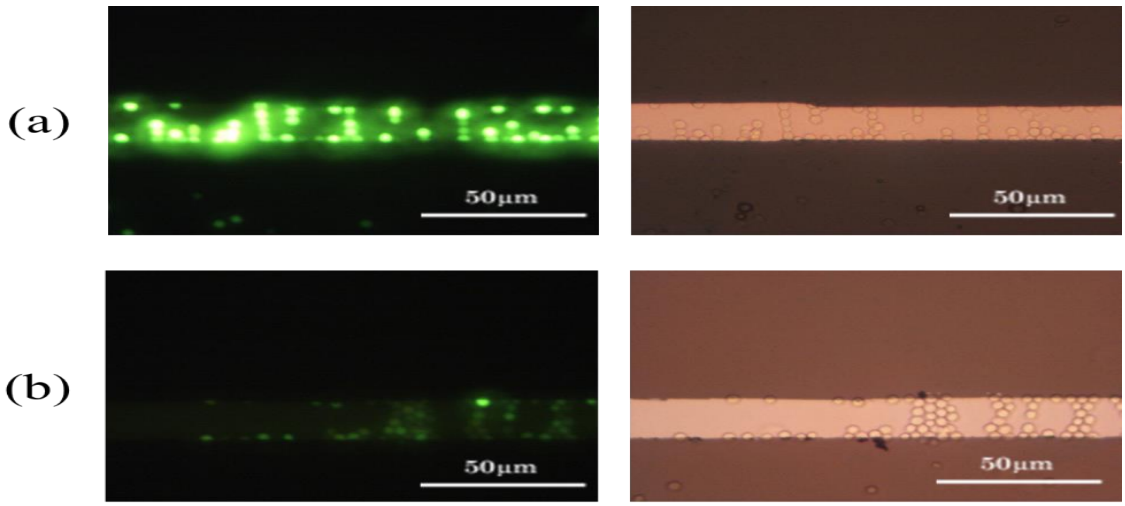
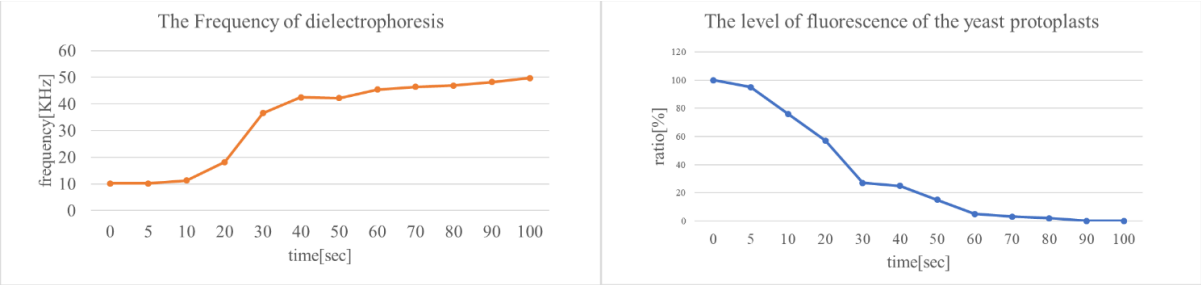


Figure 6. The experimental results of fluorescent observation of yeast protoplasts treated by heat stress at 80℃ for (a) 0 second and (b) 30 seconds

B. The evaluation of heat damage by DEP



(a) (b)

Figure 7 (a) The frequencies at which the heat-treated yeast protoplasts could be trapped at the electrodes of DEP as a function of heat-treated time. (b) V [%] ratio (Eq. (3)) as a function of heat-treated time.

Fig. 7 (a) shows the frequencies at which the heat-treated yeast protoplasts could be trapped at the electrodes of DEP as a function of heat-treated time. Also, the viability evaluation factor V [%] ratio (Eq. (3)) as a function of heat-treated time is shown in Fig. 7 (b). It can be seen that, to trap the protoplasts, the applied frequency increased as the heat-treated time increased (Fig. 7 (a)). In contrast, the V factor decreased as the heat-treated time increased. As V decreased, the number of viable protoplasts decreased. In addition, V decreased fast as heat-treated time in the time frame of 5-30 seconds. As a result, the applied frequency increased fast in such time frame in order to trap the damaged protoplasts because as protoplasts got damaged by heat stress, the dielectric constant of the cells decreased .

CONCLUSION

In this study focused on evaluating heat damage to yeast protoplasts by DEP and by fluorescence observation. First, we demonstrated on fluorescent microscope how much the heat damage at 80°C affects to the yeast protoplasts. Next, the heat-damaged protoplasts are trapped by DEP. Comparing the two results of the DEP and the fluorescence observation, we found the correlation between the increase in frequency and the decrease in cell viability. The reason for the increase in frequency is considered that the dielectric constant of the cells decreased as the damage increases. A problem of DEP is that the frequency at which the cells can be trapped changes as the dielectric constant of the solution around the cells varies. In order to solve this problem, we need to know the dielectric constant of the solution. In the future, we will try to develop a sensor that can conveniently measure the damage of the cells.

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