INACTIVATION OF MICROORGANISMS BY PULSED HIGH VOLTAGE APPLICATION

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Abstract

As one of the applications of pulsed high voltage technology to the liquid, destruction of living cells in liquid has been investigated in attempts to sterilize drinking liquids.

The survivability of the cells and the energy efficiency of cell destruction for some species of cells such as "S. cerevisiae (yeast)", "E. coli" etc. have been measured against peak electric field, pulse width, pulse application number and conductivity of the liquid ("phosphate buffer solution"), using different electrode systems. Especially, in the electrode system of "Converged Electric Field type" which places an insulating plate with small holes between the parallel plate electrodes, it became clear that, the survivability of the cells decreased to $10^{-4}\sim10^{-6}$ when the energy input to unit volume of the liquid was about 30 cal/cm^3.

The results suggest a possibility of this technology to be used as a continuous, low temperature sterilization process.

In another method using an underwater arc discharge generated between the rod-rod electrode, "B. subtilis (spores)", which could never be destroyed by the high electric field method could be easily inactivated with much lower energy input. In this method, it was originally supposed that an intense shock wave generated by an underwater arc discharge destroyed the cells by mechanical force. However, it became obvious that what mainly attacked the cells was UV rays emitted from an arc discharge.

I. Introduction

Recent progress in the field of high voltage engineering enabled to provide a reliable pulse source [1] and pulsed high voltage technology having the following features is now being watched with keen interest.

(1) High density energy can be supplied by its high electric field and current. (Therefore, molecules are ionized and radicalized.)
(2) High electric field can be applied even in the liquid since electrolysis can be reduced by a short-width pulsed voltage.
(3) Input energy can be easily adjustable by change of peak voltage, pulse frequency and pulse width.

For the features described above, pulsed high voltage technology can be expected for its wide applications to various fields, especially to the fields of biotechnology and chemistry.

In this paper, as one of the applications of the pulsed high voltage technology to the liquid, test results on the cell destruction conducted are introduced below [2], [3], [4].

The technology, which has close connection with this study, is the "cell fusion technology" in which a pulsed voltage is applied to the cells in order to contact each other. The difference between cell fusion and sterilization is that, the former uses a reversible breakdown of the cell wall by relatively low voltage pulse, while in the latter, in order to promote the irreversible breakdown of the cells, a very high pulsed voltage of $10\sim30$kV/cm average electric field strength is applied.

Since one of the most important points to be investigated for industrial use is the energy efficiency, the survivability of the cells and the energy efficiency of the cell destruction were mainly measured for various conditions, using some electrode systems.

In the "Converged Electric Field type" electrode system, a high electric field formed in the hole of the insulating plate could effectively destroy the cells, while in the rod-rod electrode system, the cells seemed to be inactivated by not the shockwave, but UV rays emitted from an underwater arc discharge.

II. Experimental Apparatus

A. High Voltage Pulser

Fig.1 depicts a schematic drawing of experimental apparatus. In the high voltage pulser, a ceramic capacitor was charged by a dc high-voltage source and directly connected to the electrode system by a rotary spark gap. Negative polarity was used. The waveform of pulsed high voltage was recorded on the oscilloscope through the high voltage probe. Pulsewidth $\tau_{1/2}$ can be changed by adjusting the capacitance C. Conductivity of the sample liquid also affected the pulse-width. Pulse peak voltage can be adjusted by the charging dc high voltage. The pulse frequency was changed from 1 to 60pps by adjusting the rotation of a rotary spark gap by inverter.

B. Electrode System

Fig.2 shows the electrode configuration used in this experiment. Fig.2(a) shows a wire-cylinder electrode system. Inner diameter of the cylinder electrode was 20mm, and the wire diameter was 1mm. The wire electrode was held by plexiglas cap at one end at the center of the cylinder. The length of the electrode system was 110mm; 38cm^3 of liquid could be contained in this electrode system. This electrode system was mounted on the stirrer. The liquid in the cylinder was effectively agitated when a rotor of the stirrer rotated at the bottom of the cylinder.

Fig.2(b) shows a "Converged Electric Field type" electrode system. In this electrode system, insulating plate (teflon) with small holes was placed

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between the parallel disc electrodes. Sample liquid was continuously introduced into the vessel through the hole of the disc electrode. The inner diameter of the vessel was 20mm, and the distance between disc electrodes was also 20mm. Standard thickness of teflon plate was 10mm, and the diameter and number of the hole was varied. In this electrode system, since current is coverged into the small holes of the teflon plate, converged high electric field is formed in the part of these holes where the cell suspension flows.

Fig.2(c) shows a rod-rod electrode system, which was made to study the cell destruction by an intense shock wave generated by an underwater arc discharge. The rod electrode was screwed to the teflon chamber and held at the center of the chamber. The separation of the two rod electrodes was 3mm. The diameter of the rod electrode was 4mm. The shape of the rod end was semi-sphere. Sample liquids were separated not to mix up each other by thin nylon films (t=0.2mm) from the water where arc discharges generated. Nylon film was apart from the discharge point by 15mm, and the inner diameter and the length of the small vessel for sample liquid were 15mm respectively.

Fig.2(d) shows the apparatus to study the destruction of the cells in the stoppered sample tube held in the free space of water tank of 600mm cube. The rod electrodes were supported by the insulator. The cells in the sample tube was influenced by an underwater arc discharge indirectly, namely through the tube. The distance between sample tube and arc discharge point was adjusted. The separation of the two rod electrodes was 20mm. The diameter of the rod electrode was 3.2mm. The rod electrode had a cone-shaped tip with 40° angle. Sample tube was made of teflon. The inner diameter was 8mm and thickness was 0.35mm. Since effective length was 1.3cm, about $0.8cm^3$ of liquid could be contained in the sample tube. The apparatus described above was for batch process.

Fig.2(e) shows the apparatus for continuous process. Spiral tube in which the cell suspension continuously flows, was set around the rod electrodes. The specification of the tube is 4mm in the inner diameter, 1mm in thickness and 1250mm in length. The average diameter of the spiral was 80mm. The inner volume of the spiral tube was about $16cm^3$.

C. Experimental Procedure

S. cerevisiae ("yeast" cell) and E. coli were mainly used in the experiment. S. aurens and A. niger were also tested for comparison. And further, as a representative cell of the most tough microorganisms, B. subtilis (spores) was used. S. cerevisiae has an ellipsoidal shape, with length about 5∿8mm. E. coli has a cylindrical shape, with length of about 1∿2mm. B. subtilis (spores) is a egg shape with length of about 0.8∿1mm. (Refer to the photos Fig.14 for details). The cells were dispersed in phosphate buffer solution. The initial cell concentration was about 10^7 cells/cm^3. The survivability of the cell was measured by means of the cultivation method. A small amount of the liquid was sampled and implanted on the surface of malt agar culture medium in a Petri dish. The sampling and implantation was made inside a clean bench. The sample was incubated for 2 days at 30∿35°C, and a number of colonies was counted. The measurement was repeated 3 times in each experimental condition.

The survivability S was measured against the electrical energy input to unit volume of the liquid P.

For the batch process, P was calculated as follows.

$$P = 0.24 \cdot 1/2 \cdot CV_c^2 \cdot N \cdot (1/Vol) \quad (cal/cm^3)$$

where V_c = charging voltage of the capacitance C, N = pulse application number, Vol = volume of the chamber.

For the continuous flow process P was calculated as

$$P = 0.24 \cdot 1/2 \cdot CV_c^2 \cdot f_p \cdot (1/Q) \quad (cal/cm^3)$$

where f_p = pulse frequency, and Q = flow rate of the liquid.

According to the test results reported by Mizuno [2], the stored energy in the capacitor agreed with the energy consumed in the liquid within 10 percent error. This means that almost all the stored energy was consumed in the liquid, and that the energy loss at the spark gap and the pulse-feeding network was negligible.

Therefore, in the following experiments, the calculated capacitor energy described above is used as the energy input to the liquid.

III. Experimental Results

A. Pulsed-Voltage Waveform

Fig.3 shows the examples of pulsed high voltage waveform. Fig.3(a) and, 3(b) are the waveforms applied to wire-cylinder electrode system. Capacitance C was 10200pF. While Fig.3(a) is the waveform applied to the phosphate buffer solution with the conductivity of 500 μS/cm, Fig.3(b) is for of 5000 μS/cm. Fig.3(c) is the waveform applied to "Converged Electric Field" type electrode system. The conductivity of the solution in this case was also 5000 μS/cm. Capacitance C was 3400pF. The voltage decreased exponentially. The pulsewidth $\tau_{1/2}$ used in this paper is the pulse duration at half of its peak value. $\tau_{1/2}$ of the waveform in Fig.3(a) is 2.5 ns, Fig.3(b) is 250ns. The pulsewidth significantly according to the liquid conductivity. The pulsed voltage waveform Fig.3(c) in the "Converged Electric Field type" electrode system also showed the exponential decay, but pulsewidth became much wider in spite that small capacitance was used. Thus, using the newly proposed "Converged Electric Field type" electrode system, high voltage with wide pulsewidth could be applied even to the high conductivity liquid.

B. Survibability Measurement

1) Wire-Cylinder Electrode System:

Fig.4 shows the survivability of S. cerevisiae in the phosphate buffer solution having the conductivity of 500 μS/cm. The survivability was measured at V_c=20kV with the pulse frequency f_p=25pps. A capacitance of 10200pF was used. The initial cell concentration was about 10^7 cells/cm^3.

It became obvious from a solid line Ⓐ in Fig.5, that pulsed high voltage can destroy the yeast cell and the survivability decreases less than 1% in a short time. However, the destruction performance markedly deteriorated with decrease of survivability. In a sterilization method, so-called " law of logarithmic order of death " should be basically established.

To improve this performance deterioration, stirrer was used to promote the agitation of the liquid. By continuous movement of the cells to a high electric field region near the wire electrode,

the survivability linearly decreased to $10^{-4} \sim 10^{-5}$ with increase of energy input.

2) "Converged Electric Field type" Electrode System

In the wire-cylinder electrode system, the cell destruction performance was improved by agitation of cell suspension. However, inside the wire-cylinder electrode, electric field is essentially distributed. Namely, since electric field near the cylinder wall is much lower than that near the wire electrode, cell destruction performance in this area must be low. Therefore, if the high and uniform electric field can be formed in the electrode system, further improvement in performance can be expected.

For a continuous sterilization, the authors proposed the "Converged Electric Field type" electrode system. As shown in Fig.2(b), in this electrode system, an insulating plate having small holes was placed between plate-plate electrode to form a high electric field in the hole.

Equivalent circuit of this system is a series connection of R_1, R_2, and R_1, where R_1 is the resistance between the plate electrode and the insulating plate, and R_2 is the resistance of the insulating plate with holes (See Fig.6). Since the cross section area of the holes is very small, the applied voltage is mainly divided at the hole part, where the cell suspension flows.

Fig.6 shows the survivability of S. cerevisiae for different conductivity cell suspensions (κ =500, 5000 μS/cm), and two electrode systems were compared. A capacitance of 10200pF was used and average electric field was 20 kV/cm in both cases. As shown in Fig.6, compared to the wire-cylinder electrode, in the "Converged Electric Field type" electrode system, performance was improved and the survivability decreased especially for high conductivity (κ =5000 μS/cm) cell suspension. The higher performance of this electrode system may be attributed to the high electric field formed in the holes and the increase in the whole resistance of the electrode system as the load.

3) Effects of Electrical Conditions

Since the energy efficiency is considered one of the most important points to be investigated for industrial use of the cell destruction by pulse voltage as a new sterilization method, the survivability of the cells were investigated for the various electrical conditions in the converged electric field electrode system. S. cerevisiae and E. coli were used.

a) Electric field strength and cell species

The survivability of S. cerevisiae dispersed in the phosphate buffer solution (κ =500 μS/cm) was measured. The capacitance was C=5100pF. The pulse frequency was 20pps. The survivability decreased with the increase in peak electric field strength E_p and the pulse application number N, but the effect of E_p on the energy efficiency was rather small in the range of E_p=15∿30 kV/cm.

Fig.7 shows the relationship between E_p and $P(10^{-1})$ in the range of E_p less than 15 kV/cm. Here, $P(10^{-1})$ means the energy input/unit volume of the liquid necessary to reduce the survivability to 10^{-1} and was obtained from the average slope of the plots between the survivability and total energy input/unit volume of the liquid. The conductivity of the liquid was changed from 200 to 2000 μS/cm and therefore pulse width varied from 100 to 10 s.

At the range of $E_p \gtrsim 10$ kV/cm, the value of $P(10^{-1})$ reduces to 4∿5 cal/cm^3. Fig.8 shows the results of comparison test on the survivability of S. cerevisiae and E. coli for the conditions: C=1700pF, f=20pps, κ =500 μS/cm.

The survivability of the both cells decreased almost linearly to the level of $S=10^{-4} \sim 10^{-5}$ as a exponential function of input energy. In E. coli, compared to S. cerevisiae, E_p necessary for efficient cell destruction was much higher and $P(10^{-1})$ = 5∿6 cal/cm^3 was a little higher than that for S. cerevisiae.

However, the cells of B. subtilis (spores) could never be destroyed by the pulsed high electric field method.

b) Pulse application number

Fig.9 shows the survivability of S. cerevisiae against pulse application number. Experiment was done at the condition of Ep=15 kV/cm, C=10200pF, f_p=6pps, κ =500 μS/cm and $\tau_{1/2}$=80μs. The survivability S decreased with the increase in the pulse application number N. Especially around N > 7, survivability drastically decreased to 10^{-6} and $P(10^{-1})$ markedly reduced to about 4 cal/cm^3. This result suggests that minimum pulse number N_{min} exists for effective irreversible breakdown of the cell, though N_{min} may increase with decrease of E_p.

Therefore, in order to conduct efficient cell destruction, it seems to be desirable that electrical conditions should be adjusted so that the target on survivability may be achieved with 10∿30 pulses.

c) Pulse width and conductivity of the liquid

Effect of the pulse width on the degree of kill of S. cerevisiae was tested for conditions: E_p=30 kV/cm, κ=500 μS/cm, f_p=20pps. Pulse width was set in a range of 1-2.5 μs, 4-10 μs, 20-30 μs by change of capacitance from 170pF to 5100pF. Among these three ranges, pulse width of 4-10 μs was most energy efficient.

Fig.10 shows the energy input $P(10^{-1})$ necessary for the 10^{-1} survivability against the conductivity of the liquid κ. Test was conducted with E. coli for conditions: E_p=30 kV/cm, C=1700pF, N=20 (shots). $P(10^{-1})$ depended upon the liquid conductivity or the pulse width and decreases with increase of liquid conductivity up to 5000 μS/cm. In the case of E. coli, at around κ =500 μS/cm, $P(10^{-1})$ had a minimum value of 5 cal/cm^3.

Despite the reduction of the pulsewidth less than 10 μs, the cells could be destroyed. This may be attributed to the increase in the peak pulse current due to the increase in the liquid conductivity.

On the other hand, in the case of S. cerevisiae tested for conditions: E_p=15kV/cm, C=5100pF, N=12 (shots), at the low conductivity range of κ < 500 μS/cm, similar tendency was observed while at the high conductivity range of κ > 2000 S/cm, $P(10^{-1})$ increased or became energy inefficient. However, even in these cases, $P(10^{-1})$ could be improved to the similar level of $P(10^{-1})$=5 at κ =500 μS/cm by widening pulse width by changing insulating plate or capacitance.

From the test results this time, the effect of the pulse width and the liquid conductivity on the cell destruction efficiency could not become clear. This seems to be influenced by

various, completed factors such as the rise time, the peak voltage and the operating time (pulse width) of the pulsed high voltage working directly in the cell wall.

4) Method using underwater arc discharge

Using the electrode system shown in Fig.2(c), effect of an underwater arc discharge on the survivability of S. cerevisiae, E. coli and B. subtilis (spores) was preliminarily investigated for the conditions: V_c=26kV, C=40000pF (energy per one pulse P_p=13.5 J/pulse), f_p=1pps, κ=1000 μS/cm. The original concept of this method was that a very intense shock wave generated by an underwater arc discharge destroys the cells by its mechanical force.

As shown in Fig.11, the survivability S of three species of cells decreased with the increase in the pulse application number N. What should be noted above all is that B. subtilis (spores), which could never be destroyed by pulsed high voltage, was easily inactivated by this method in the similar tendency to S. cerevisiae and E. coli.

According to the waveform of the pulsed pressure at the wall measured with piezosensor, the peak value and the pulse width were about 170kg/cm^{2}G and 20μs respectively.

Before conducting a continuous sterilization test with the apparatus shown in Fig.2(e), fundamental study on the effect of underwater arc discharge on the survivability of various cells was performed by batch process with the method using sample tube shown in Fig.2(d). Fig.12 shows the survivability of B. subtilis cells dispersed in a phosphate buffer solution for the conditions: V_c=40kV, C=150000pF, f_p=1pps, κ=1000 μS/cm, peak value of pulsed pressure P_p=60-550 kg/cm^{2}G. The pulse width was about 5 μs. Tests were done by changing the distance D between arc discharge point and sample tube. As the distance D increased, peak value of the pulsed pressure and cell destruction performance decreased. The parenthesized figures in Fig.12 express the peak value of the pulsed pressure.

Fig.13 shows the effect of underwater arc discharge on the survivability of various species of cells for the conditions: V_c=40kV, C=150000pF, f_p=1pps, P_p=550 kg/cm^{2}G (D=30mm). Though the effect differed for cell species, survivability in every case decreased to the level less than 10^{-4}.

Since the fact that the cell having high heating resistance like B. subtilis was easily inactivated at the low temperature range, seemed to be a new finding, the effect of arc discharge method tried to be checked in the commercialized drinking liquids such as milk, coffee, tea, etc. The result was that performance markedly decreased especially for the drinking liquid of deep color. Further, authors noticed that the order of the survivability of four species of cells in Fig.13 seems to agree with that of the resistance for UV rays of each cell. Therefore, in order to investigate the effect of UV rays, the survivability test was conducted using the sample tube wrapped with SC filter (SC-37) which shields UV rays. Thickness of the filter was 90 μm. As shown in Fig.13, under this condition, B. subtilis cells were hardly destroyed. From the test results described above, it became obvious that, what mainly attacks the cell in arc discharge method is unexpectedly, not the shock wave, but UV rays emitted from arc discharge. Though further study on the effect of pulsed UV rays may be necessary, it is generally said that UV rays is undesirable for sterilization of drinking liquids and foods because they are finally oxidized.

Ⅳ. Discussion

A. Observation of the destroyed cell

Using an electron microscope, the destroyed cells were observed. A pair of photos in Fig.14 for three species of cells which were taken before and after pulsed high voltage treatment with converged electric field type electrode system for the conditions: E_p=30kV/cm, C=1700pF, κ=500 μS/cm, $\tau_{1/2}$=12 μs, N=25, P=36 cal/cm^3. The liquid was phosphate buffer solution. The upper photos were the photo taken before treatment. The surface of S. cerevisiae cell is originally smooth, as shown in the upper photo of Fig.14(a).

The lower photo of Fig.14(a) shows destroyed cells. Survivability at this condition was less than 10^{-4}, and almost all the cells observed should have been destroyed. Since energy input was about 35 cal/cm^3, destruction by heat should be negligible. Almost all the cells did not change the ellipsoidal shape, but their surface became very rough. Something like fine debris were observed to attach on the cell surface.

Among the destroyed cells, a deformed cell having a hole at its center could be rarely observed. The hole may be formed by the strong breakdown of the cell membrane where pulsed current flowed through. The debris may be discharged from these breakdown points.

For the E. coli cells, as shown in Fig.14(b), similar change could be also observed. Namely, their shape was not basically changed, but the size became rather small and the surface became rough. Further, on the cell surface something like fine debris were also observed. Fig.14(c) were the photos of B. subtilis (spores) which showed no change in survivability between before and after treatment.

B. Configuration of insulating plate in converged electric field type electrode system

Effect of the configuration of the insulating plate on the survivability of the cell was investigated. Thickness of the insulating plate was 2.5mm, 5mm and 10mm. Combination of the diameter and number of the hole through the plate was 4mmφ × 1, 2mmφ × 4, 1mmφ × 16 and 0.5mmφ × 64, each having the same open cross section area. From the investigation, it became obvious that, in order to conduct a reliable, energy efficient cell destruction, high electric field should be formed in the holes and to put it concretely, the configuration such as thickness, diameter and number of the hole of the plate should be selected so that R_1/R_2 may be larger than 20 at the least. R_1 is the ratio of the resistance of the insulating plate and R_2 is the resistance between the plate electrode and the insulating plate R_2 (See Fig.6).

C. Necessary conditions for sterilization

From the test results described above, it can be suggested that in the pulsed high voltage method, the necessary conditions for sterilization are the followings:

1) electric field strength $E_p > 10$ kV/cm
2) pulse width $\tau_{1/2} > 2$μs
3) pulse application number $N > 10$ (shots)

Further, it became obvious that, for the liquids having the conductivity of 200 ∿ 5000 μS/cm, the survivability of the cells could be reduced to 10^{-4} ∿ 10^{-6} and energy input necessary for effective sterilization could be minimized to $P(10^{-1})$ = 4 ∿ 6

cal/cm^3 by adjusting the electrical conditions and the shape of the insulating plate.

V. Conclusion

Effective destruction of living cells in liquid by high-voltage pulse application has been confirmed. The survivability of the cells, and the energy efficiency of the cell destruction for various kinds of cells have been measured using different electrode geometries. Especially, using the electrode system of "Converged Electric Field" type, a reliable, energy efficient cell destruction can be realized even for the high conductivity liquid. On the survivability of the cells, "law of logarithmic order of death" basically established, namely the survivability decreased as an exponential function of the total energy input. The energy input P necessary to achieve the survivability of 10^{-6} could be estimated about 25-35 cal/cm^3 with some scatter for the cell species. Though further studies especially on the change in taste and fragrance of drinking liquids, the value suggests that pulsed high voltage method can be a low-temperature sterilization process.

Using an intense underwater arc discharge in the rod-rod electrode system, even B. subtilis (spores), which could never be destroyed by electric field method, can easily be inactivated and P markedly decreased less than 5 cal/cm^3. However, it became obvious that, what mainly attacks the cell in this method is, unexpectedly, not the shock wave, but UV rays.

Acknowledgement

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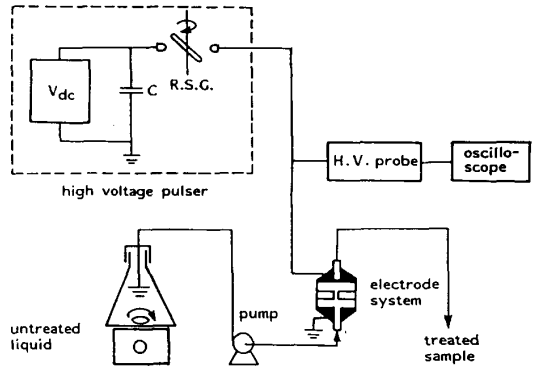
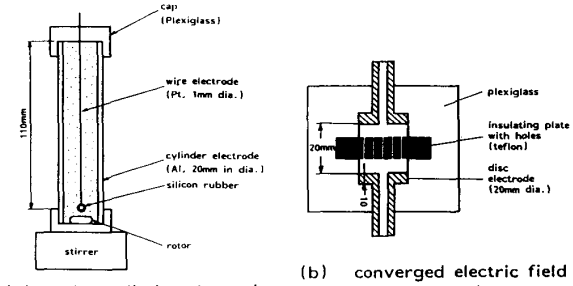
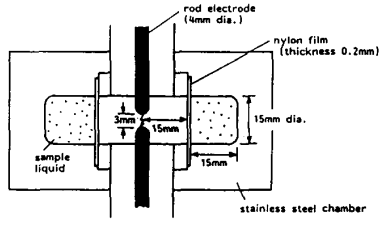


Fig.1 Schematic diagram of experimental set-up

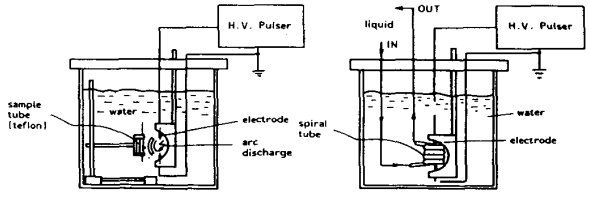
V_{dc} : DC high-voltage source, C : ceramic capacitor, R.S.G. : rotary spark gap



(a) wire-cylinder electrode (b) converged electric field type electrode system



(c) rod-rod electrode system



(d) for batch process (e) for continuous process

Fig.2 Electrode systems

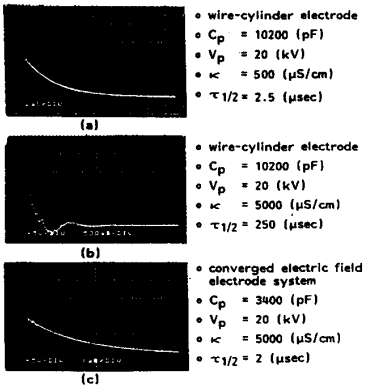


Fig.3 Examples of pulsed voltage waveforms using wire-cylinder and converged electric field electrode system

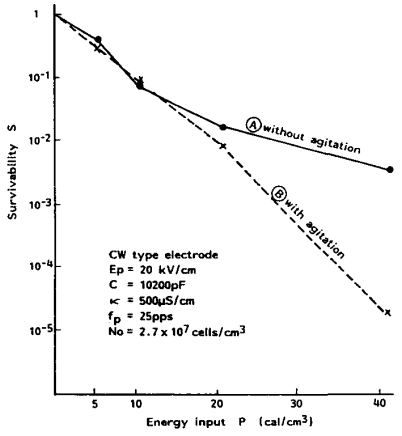


Fig.4 Effect of agitation on the survivability of S. cerevisiae

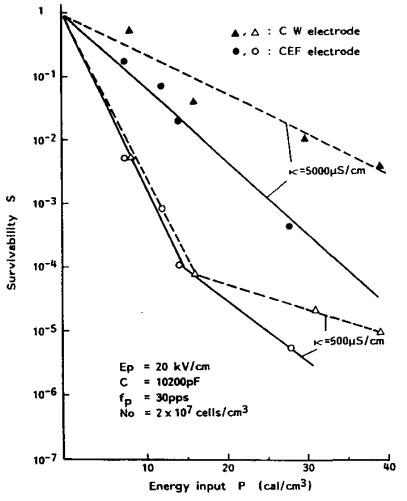


Fig.5 Electrode type and survivability of S. cerevisiae

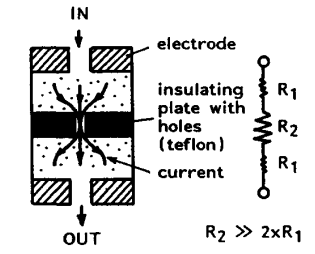


Fig.6 Equivalent circuit of converged electric field type electrode system

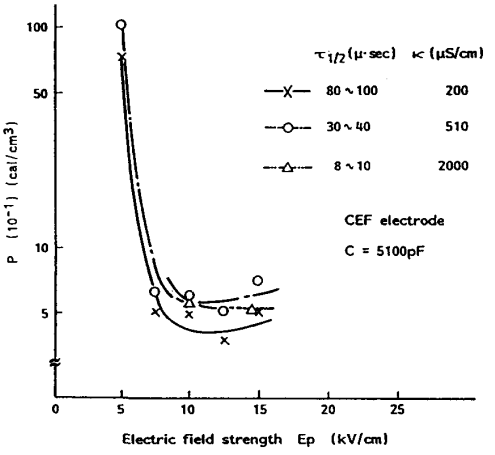


Fig.7 Electric field strength and energy efficiency (S. cerevisiae)

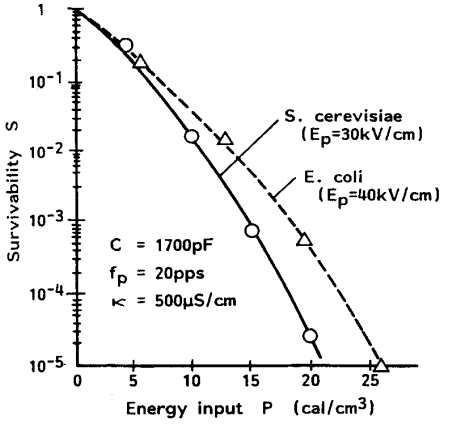


Fig.8 Survivability of S. cerevisiae and E. coli using converged electric field electrode system

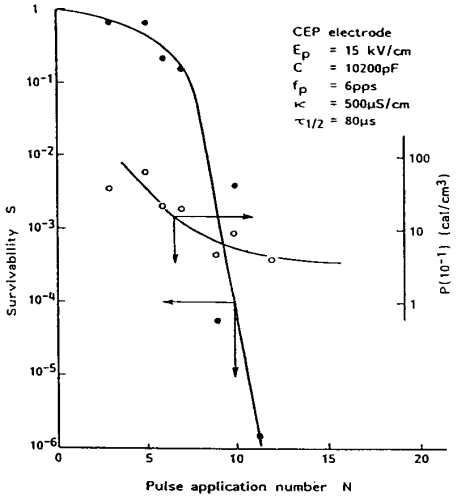


Fig. 9 Effect of pulse application number on the survivability of S. cerevisiae

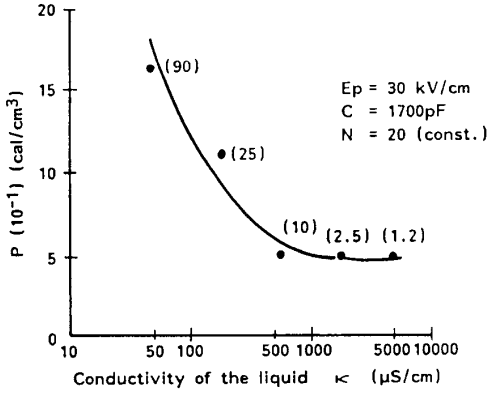


Fig. 10 Effect of the liquid conductivity on the sterilization efficiency of E. coli using converged electric field type electrode system

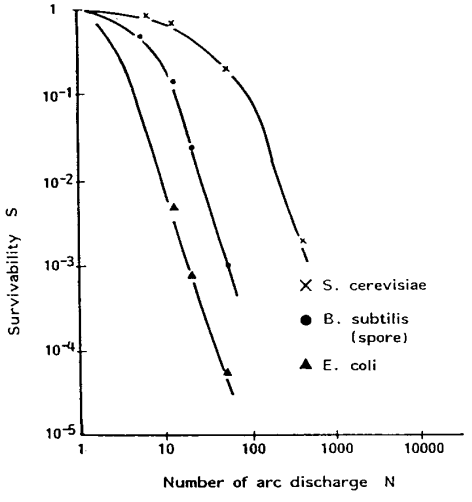


Fig. 11 Difference in survivability for various species of cells using rod-rod electrode system (cell destruction by underwater arc discharge)

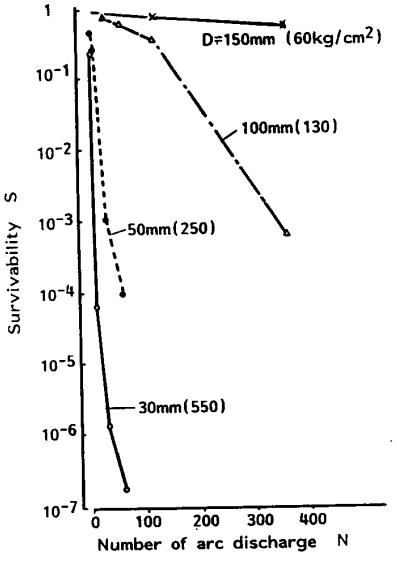


Fig. 12 Survivability of B. subtilis with change of distance from arc discharge point

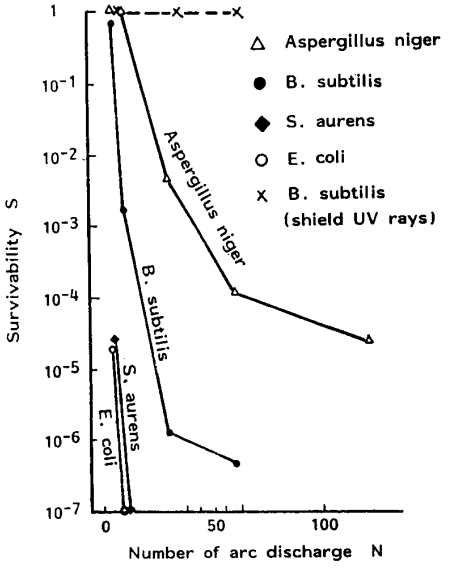
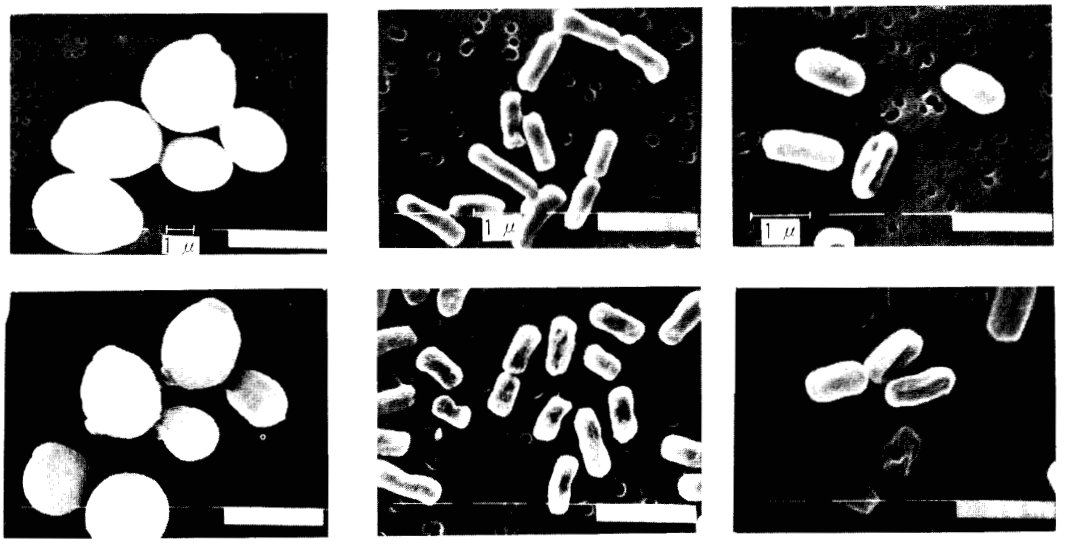


Fig. 13 Difference in survivability for various species of cells using rod-rod electrode system (cell destruction by underwater arc discharge)



a) S. cerevisiae b) E. coli c) B. subtilis (spores)

Fig.14 Cells before and after treatment using the converged electric field type electrode system (upper photo: before treatment, lower: after treatment)