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Dielectrophoresis Conditions for Pearl Chain Formation and Effect of Pulse Field Strength on Protoplast Breakdown of *Hericium erinaceum*

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The fusion method using PEG as a fusogenic agent has a number of disadvantages in comparison with the newly developed electrical fusion method. However, there has been little application yet of this electrical fusion method to the fusion of protoplasts of mushrooms. In this report, we describe the pearl chain formation and effect of pulse field strength on protoplast breakdown of protoplasts prepared from *Hericium erinaceum*. The protoplasts density favorable for pearl chain formation was about 10^8 protoplasts/ml, and the yield of single pairs was maximum(30%)after 60 sec dielectrophoresis at 100 V/cm and 1 MHz. The field intensity required to decompose half of the protoplast from *H. erinaceum* was 8 kV/cm in a 0.7M mannitol solution.

Introduction

Although the fusion method using PEG as a fusogenic agent has been long used for the fusion of mushroom[1), 2)], yeast[3), 4)], streptomycetes[5), 6)], plant[7), 8)]and animal[9), 10)]cells, it has a number of disadvantages in comparison with the newly developed electrical fusion method. However, there has been little application yet of this electrical fusion method to the fusion of protoplasts of mushrooms.

In this report, we describe the pearl chain formation and effect of pulse field strength on protoplast breakdown of protoplasts prepared from *Hericium erinaceum*. To monitor the pearl chain formation of the *H. erinaceum* protoplasts, a fusion microchamber, constructed from a slide glass for microscope and aluminum foil, was developed.

Materials and Methods

Organisms

H. erinaceum was obtained from Nagano Keizairen.

Medium

Malt extract medium, for the cultivation of cells to obtain protoplasts, comprised 2% malt extract(pH 5.6).

Preparation of protoplasts

From a stock culture, one loopful of mycelia was transferred to a test tube containing 5ml of malt extract medium and some glass beads(ϕ

4mm)to prevent the mycelia from clumping, and then incubated at 25℃ with shaking for the preculture. After the precultivation(3 days), 1ml of the culture was transferred to the malt extract medium(5ml)and incubated further for 3 days on a shaker.

Protoplasts were prepared according to the procedure of Abe *et al.*[1]. The mycelia harvested by centrifugation were ground in a teflon pestle tissue grinder, washed twice with 0.7M mannitol solution and then suspended in a appropriate volume of 0.7M mannitol solution. To the mycelial suspension, 0.5% cellulase Onozuka R-10(Kinki Yakult Mfg.Co.), 0.5% Zymolyase 5000(Kirin Brewery Co.)and 1.5% β-glucuronidase(Sigma Co.), were added, followed by incubation at 30℃ for 2 hours in a microtube.

The protoplasts formed were separated with a membrane filter from intact cells and mycelial debris, harvested by centrifugation, washed with 0.7M mannitol solution and suspended in the same solution. The concentration of protoplasts was regulated by direct counting with a Thoma hemacytometer under an Olympus phase contrast microscope BHS-323(Olympus Co., Japan).

Fusion equipment

A Shimadzu Somatic Hybridizer SSH-2 (Shimadzu Co., Japan)was used as a fusion apparatus and the pearl chain formation was observed under an Olympus phase contrast microscope BHS-323, equipped with an Olympus camera OM-1. In order to observe the pearl chain formation of mushroom protoplasts, a fusion microchamber, which was constructed from a slide glass with two parallel(1mm apart)aluminum foil(15μm thick)electrodes, was used.

Results and Discussion

Dielectrophoresis conditions for pearl chain formation

The process of electrofusion can be divided into 3 stages, namely: close contact of protoplasts due to dielectrophoresis in an alternating electric field; temporary breakdown of plasma membranes at the contact zone due to a unidirectional DC pulse of high intensity; and rearrangement of the lipid bilayer leading to the fusion of apposed protoplasts. The dielectrophoresis induces the formation of a chain of cells(pearl chain)of variable length, because of the attractive forces between the dipoles of adjacent cells.

The dielectrophoretic behaviors of the protoplasts prepared from *H. erinaceum* were very similar to protoplasts(ϕ 1−3μm) preparation of *streptomyces*, regardless of their difference in size[5), 6)]. The conditions preferable for the formation of a two-celled pearl chain(single pair)were sought. The experimental results obtained can be summarized as follows:(1)The protoplasts density favorable for pearl chain formation was about 10^8 protoplasts/ml, and no essential differences in the pearl chain formation were observed in the concentration range of 0.5 X 10^8 to 5 X 10^8 protoplasts/ml(Fig.1). (2)The yield of single pairs was maximum(30%)after 60 sec dielectrophoresis at 100 V/cm and 1 MHz, and then decreased gradually as more multi-celled pearl chains formed(Fig.2).

Effect of pulse field strength on protoplast breakdown

For protoplast fusion, the breakdown of the plasma membrane through electric stimulation at the contract zone with an apposed protoplast is essential. Therefore, the electrical breakdown of *H. erinaceum* protoplasts by field pulses of various intensities was measured by counting the remaining protoplasts. Figure 3 shows that the amount of protoplasts remaining unbroken decreased with increasing pulse intensity. The survival rate of protoplasts was determined by direct counting under a microscope. The field intensity required to decompose half of the *H. erinaceum* was 8 kV/cm in a 0.7M mannitol solution.

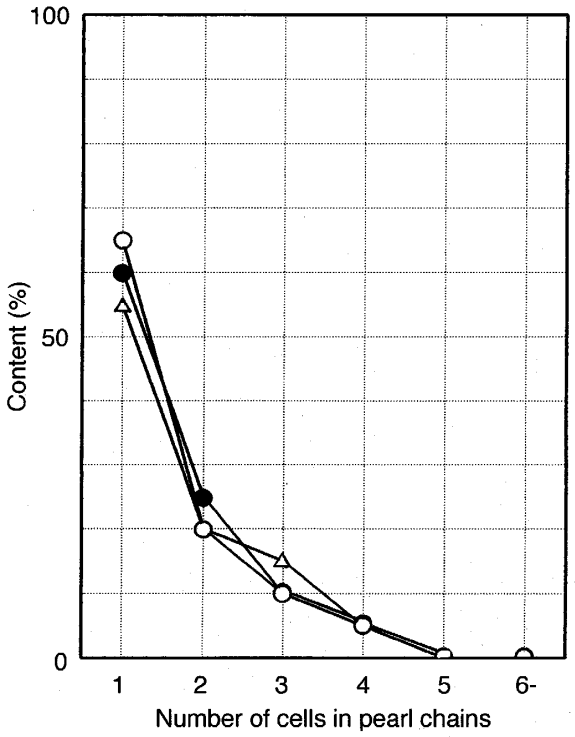

Fig.1 Effect of protoplast concentration on the pearl chain formation.

H. erinaceum protoplasts were suspended in a 0.7M mannitol solution and then subjected to dielectrophoresis in a field of 100 V/cm and 1 MHz. Then the pearl chains formed were counted photomicrographically. The duration of dielectrophoresis was 30 sec.

○, 0.5 X 10^8; ●, 1 X 10^8; and △, 5 X 10^8 protoplasts/ml.

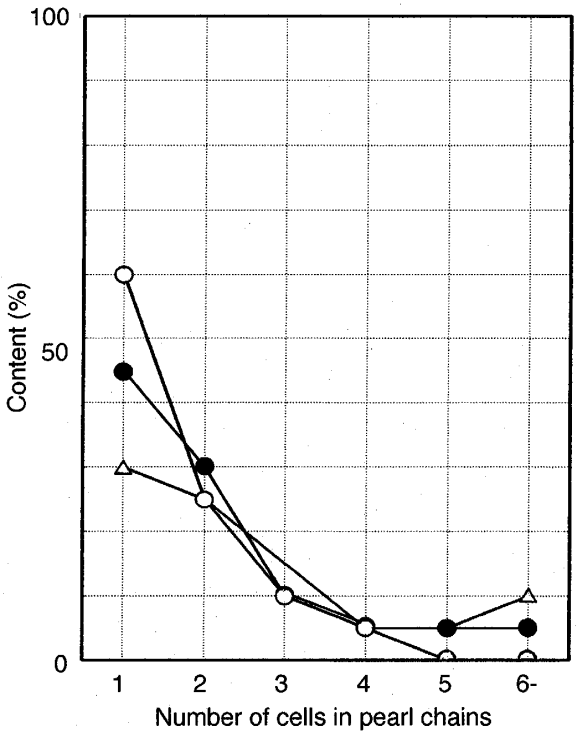

Fig.2 Effect of duration of dielectrophoresis on the pearl chain formation.

A protoplast suspension(1 X 10^8 protoplasts/ml) was subjected to dielectrophresis for 30 sec (○), 60 sec(●)and 120 sec(△).

Therefore, on the basis of the experimental results described above, the concentration of protoplasts, the intensity of the alternating electric field and the time for dielectrophoresis were fixed at 1 X 10^8 protoplasts/ml, 100 V/cm strength, 1MHz frequency and 60 sec, respectively. And the field pulse intensity required to break 50% of the protoplasts was 8 kV/cm.

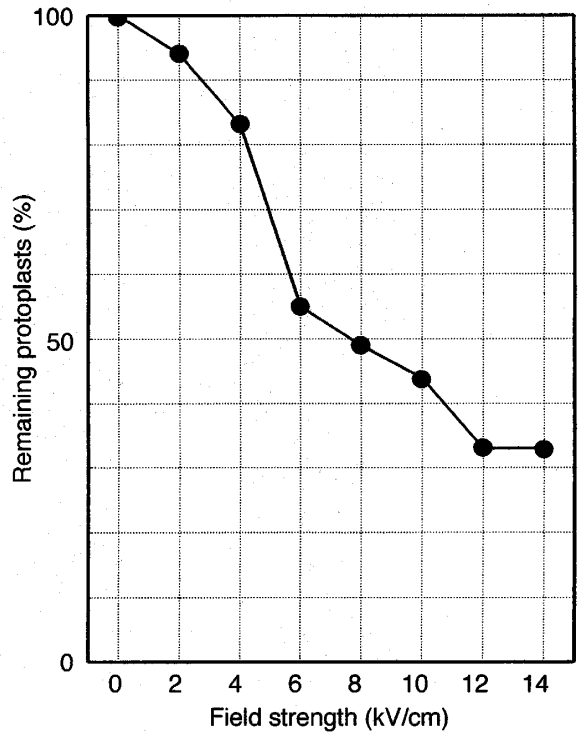

Fig.3 Effect of pulse strength on the breakdown of protoplasts.

A protoplasts suspension(1 X 10^8 protoplasts/ml) was exposed to DC fields of various strengths for 100μsec. Then, the number of remaining protoplasts were determined by direct counting under a microscope.

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