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Polykaryon Formation Using a Swollen Conidium of *Trichoderma reesei*

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Abstract

The cellulolytic fungus, *Trichoderma* has oval and mononucleate conidia. When these conidia are incubated in a liquid medium, they begin to swell and their shape becomes spherical followed by an increase in inner space. In such swollen conidia, it is possible to produce a larger autopolyploid nucleus using a mitotic arrester compared with the case of the original conidia. In this study, polykaryon formation was attempted using these swollen conidia. Dried mature green conidia of *Trichoderma reesei* QM6a (IFO 31326) were incubated in Mandel's medium in order to swell. The swollen conidia were treated with a mitotic arrester, colchicine, for autopolyploidization. After autopolyploidization, polykaryon formation was carried out using the swollen conidia. After the treatment, multiple smaller nuclei whose diameter was almost the same as that of the original strain were generated from an autopolyploid nucleus in a swollen conidium. A cellulase hyperproducer without decrease in growth rate could be selected using such swollen conidia.

Index Entries: *Trichoderma reesei*; polykaryon; cellulase; colchicines; polyploid.

Introduction

We attempt to develop a new breeding system for *Trichoderma reesei* without gene-engineering techniques for the safety of food. As a result, we previously reported the new breeding techniques using colchicine and benomyl for this fungus *(1)*. A mitotic arrester, colchicine, causes autopolyploidization and benomyl causes deletion and recombination of chromosomes in an autopolyploid nucleus of this fungus *(2,3)*. When the new techniques were applied on this fungus, the cellulase productivity per mycelia increased but the growth rate tended to decrease *(1)*. The growth rate of such a low-growing strain of this fungus could be recovered by additional autopolyploidization, but it took substantial time *(4)*.

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We examined new conditions of these techniques in order to construct strains whose cellulase productivity increases and the growth rate does not decrease without additional autopolyploidization. At first, it was considered that cellulase genes are amplified in an autopolyploid nucleus of this fungus. However, the cellulase productivity of an autopolyploid strain did not increase (data not shown). In other words, the cellulase productivity of an autopolyploid strain seemed not to be directly related to the amplified cellulase genes. Thus, we investigated the change in cellulase productivity by altering the nuclear nature of an autopolyploid nucleus. For that purpose, we tried to produce smaller nuclei whose diameter is almost the same as that of the original strain from an autopolyploid nucleus in a swollen conidium of this fungus by polykaryon formation technique.

Materials and Methods

Microorganism and Medium

T. reesei QM6a (IFO 31326) was used as a model strain. This fungus was cultivated on potato dextrose agar (PDA) medium (Wako, Osaka, Japan) at 28°C and preserved at 4°C. For swelling of conidia, Mandels' medium containing 0.5% (w/v) peptone (Wako) and 1.0% (w/v) glucose (Wako) (pH 6.0) was used. Mandels' medium was composed of 1.4 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 0.3 g of urea, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.005 g of $FeSO_4 \cdot 7H_2O$, 0.0016 g of $MnSO_4 \cdot H_2O$, 0.0014 g of $ZnSO_4 \cdot H_2O$, 0.0020 g of $CoCl_2$ (all from Wako), and 1000 mL of distilled water: (pH 6.0) *(5)*.

As the medium for autopolyploidization, Mandels' medium containing 0.5% (w/v) peptone, 1.0% (w/v) glucose, and 0.1% (w/v) colchicine (Wako) was used (pH 6.0). As the medium for polykaryon formation, Mandels' medium containing 0.5% (w/v) peptone, 1.0% (w/v) glucose, and 0.5% or 2.0% (w/v) colchicine was used (pH 6.0).

For the selection medium, 96 mL of the Mandels' medium containing 1.0 g of Avicel (Funakoshi, Tokyo, Japan), 0.5 g of peptone, 1.0 g of wood powder (*Fagus crenata*), 0.1 mL of polyoxyethyleneoctylphenylether (Triton X-100) (Wako), 1.5 g of agar (Difco, Detroit, MI), and conidia (the bottom layer medium) was added to a deep glass plate (150-mm diameter and 60-mm depth) and left at 4°C in order to harden the agar *(6)*. After the agar hardened, 196 mL of Mandels' medium containing 1.0 g of Avicel, 0.5 g of peptone, 1.0 g of wood powder, 0.1 mL of Triton X-100, and 1.5 g of agar (the upper layer medium) was added to the bottom layer of medium and the agar was hardened at 4°C.

For the solid medium for the estimation of cellulase productivity, Mandels' medium containing 1.0% (w/v) carboxymethylcellulose sodium salt (CMC-Na) (degree of substitution [D.S.] 0.7) (Wako), 0.5% peptone, 1.5% (w/v) agar, and 0.1% (v/v) Triton X-100 was used (pH 6.0). For measurement of cellulose hydrolyzing activity, 7.5 g of wheat bran was added to 7.5 mL of Mandels' medium in a 100-mL Erlenmeyer flask.

Preparation of Dried Mature Green Conidia

A mycelial mat (3×3 mm) was put on the PDA medium and incubated for 2 wk at 28°C in order to generate mature green conidia. Conidia generated on the PDA medium were added to 50 mL of sterilized distilled water in a 100-mL Erlenmeyer flask and suspended well. The conidial suspension was filtrated with a glass filter 3G-2 in order to remove hyphae. Conidia were collected by centrifugating at 5510*g*. The collected conidia were dried in a desiccator containing silica gel (Wako). These conidia were used for experiments as dried mature green conidia *(7)*.

Nuclear Staining of Conidia

Conidia were fixed on a slide glass by heating and treated with 5 *N* HCl (Wako) for 40 min followed by washing with tap water. After washing, the treated conidia were immersed in Giemsa solution (Merck, Darmstadt, Germany) for 30 min followed by washing with tap water for observation with a microscope (BH-2; Olympus, Tokyo, Japan) *(8)*. The HCl-treated conidia were also stained with 0.1 µg/mL 4.6'-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) solution followed by observation using a fluorescent microscope (BX-50; Olympus) *(9)*. Photomicrographs were taken using a microscope with an automatic exposure meter (PM-CBAD; Olympus) and a camera (C35AD; Olympus), and the photographs were then enlarged. The diameter and number of conidia were measured using a digital caliper (Mitsutoyo, Koshigaya, Japan) on enlarged photographs.

Preparation of Swollen Conidia

Dried mature green conidia were added to 50 mL of the medium for swelling in a 100-mL Erlenmeyer flask and incubated for 6 h at 28°C using a rotary shaker (TAITEC R-30 mini; Taitec, Koshigaya, Japan) (160 rpm). The changes in conidium and nucleus were then observed using a microscope (BH-2, BX-50; Olympus) with or without nuclear staining.

Autopolyploidization

Swollen conidia were added to 25 mL of the medium for autopolyploidization in a 50-mL Erlenmeyer flask and incubated statically for 7 d at 28°C. The nuclear changes were then observed by nuclear staining using microscopes.

Polykaryon Formation

The swollen conidia containing autopolyploid nuclei were added to 25 mL of the medium for polykaryon formation in a 50-mL Erlenmeyer flask and incubated statically for 3 or 7 d at 28°C. The nuclear changes were then observed by nuclear staining using microscopes.

Selection of Cellulase Hyperproducer

After polykaryon formation, the treated swollen conidia containing multiple smaller nuclei were added to the bottom layer of selection medium and left for 60 min at 4°C to harden the agar. The upper layer selection medium was then overlayed and left for 60 min at 4°C in order to harden the agar, and the conidia were incubated at 28°C.

Comparison of Cellulase Productivity on Solid Medium

A mycelial mat (2 × 2 mm) was put on the center of the solid medium for estimation of cellulase productivity and incubated for 6 d at 28°C. Then, 0.1% (w/v) Congo red solution (Wako) was added to the plate and left for 1 h, followed by washing with 1 *M* NaCl (Wako). The diameter of a clear zone around a colony and the diameter of the colony were measured using a digital caliper (Mitsutoyo).

Measurement of Cellulose Hydrolyzing Activity

A mycelial mat (2 × 2 mm) was added to the medium for the measurement of cellulose hydrolyzing activity in a 100-mL Erlenmeyer flask and incubated at 28°C for 5 d. Three flasks were used for one strain and shaken once a day. After incubation, 15 mL of 0.1 *M* acetate buffer (pH 5.0) was added, stirred using a glass rod, and left to stand for 1 h. The enzyme solution was then extracted from the wheat bran culture using a nylon cloth. The extracts were centrifuged at 5510*g*, and the top clear portion was used as the enzyme solution. As the substrates of enzyme reaction, 1.0 g of Avicel (Funakoshi), CM-cellulose (Wako), or Salicin (Wako) was added to 99 mL of 0.1 *M* acetate buffer (pH 5.0). Then 0.2 mL of enzyme solution and 4.0 mL of substrate were mixed and incubated for 120 min at 40°C using a reciprocal shaker (Thomastat T-22S; Thomaskagaku, Tokyo, Japan). The agitation speed was 125 strokes/min. The reaction mixture was filtrated with filter paper (no. 2; Whatman, Maidstone, UK). The amount of reducing sugar in the reaction mixture was measured using dinitrosalicylic acid (DNS) (Wako) *(10)*. One unit (IU) was defined as the amount of enzyme-producing reducing sugar equivalent to 1 μmol of glucose/min.

Results

Formation of Swollen Conidia

Dried mature green conidia, which are mononucleate, were incubated in the medium for swelling of conidia for 6 h at 28°C using a rotary shaker. After incubation, the conidia became spherical and the inner volume increased. However, the mononucleate nature of *Trichoderma* was maintained and the diameter was unchanged *(7)*. Such conidia, called swollen conidia, were used for the experiments.

Autopolyploidization

Swollen conidia were incubated statically in the medium for autopolyploidization for 7 d at 28°C. After incubation, it appeared that a larger

Table 1
Changes in Number and Diameter of Nucleus in Swollen Conidia

Nuclear number	Proportion (%)	Nuclear diameter (μm)	Proportion (%)
Swollen conidia containing autopolyploid nuclei			
1	100	0.10–0.49	0
2	0	0.50–0.89[a]	0
3	0	0.90–1.29	0
4	0	1.30–1.69	0
5	0	1.70–2.09	4
6	0	2.10–2.49[b]	96
Swollen conidia treated with 0.5% colchicine for 3 d			
1	18	0.10–0.49	0
2	26	0.50–0.89[a]	0
3	44	0.90–1.29	5
4	12	1.30–1.69	10
5	0	1.70–2.09	12
6	0	2.10–2.49[b]	73
Swollen conidia treated with 2.0% colchicine for 3 d			
1	63	0.10–0.49	2
2	21	0.50–0.89[a]	33
3	12	0.90–1.29	5
4	4	1.30–1.69	10
5	0	1.70–2.09	8
6	0	2.10–2.49[b]	42
Swollen conidia treated with 0.5% colchicine for 7 d			
1	0	0.10–0.49	0
2	8	0.50–0.89[a]	15
3	34	0.90–1.29	49
4	41	1.30–1.69	36
5	17	1.70–2.09	0
6	0	2.10–2.49[b]	0

[a] Nuclear diameter of original strain.
[b] Diameter of autopolyploid nucleus.

nucleus was produced in one swollen conidium by nuclear staining using Giemsa solution or DAPI solution, as shown in Table 1 and Fig. 1. This larger nucleus was regarded as an autopolyploid nucleus.

Polykaryon Formation

The swollen conidia containing autopolyploid nuclei were incubated statically in the medium for polykaryon formation containing 0.5 or 2.0% (w/v) colchicine for 3 or 7 d at 28°C. When these swollen conidia were treated with 2.0% colchicine for 3 d, micronucleation occurred, as shown

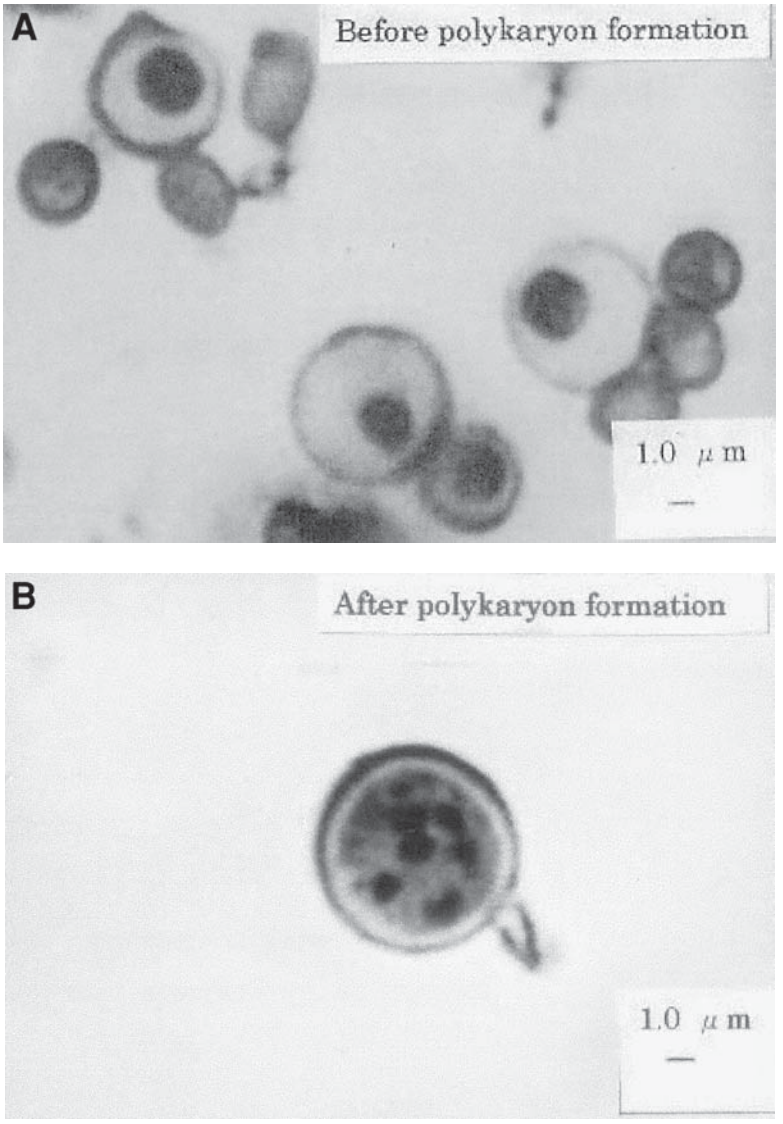

Fig. 1. **(A)** Autopolyploid nuclei produced with 0.1% (w/v) colchicine solution. **(B)** Nuclei after treatment with 0.5% (w/v) colchicine.

in Table 1. This phenomenon was also seen when the swollen conidia were treated with 2.0% colchicine for 7 d (data not shown). However, when the swollen conidia were treated with 0.5% colchicine for 7 d, multiple smaller nuclei whose diameter was almost the same as that of the original nucleus were generated from one autopolyploid nucleus, and the autopolyploid nucleus disappeared, as shown in Table 1 and Fig. 1. Thus, we used 0.5% colchicine in order to cause polykaryon formation.

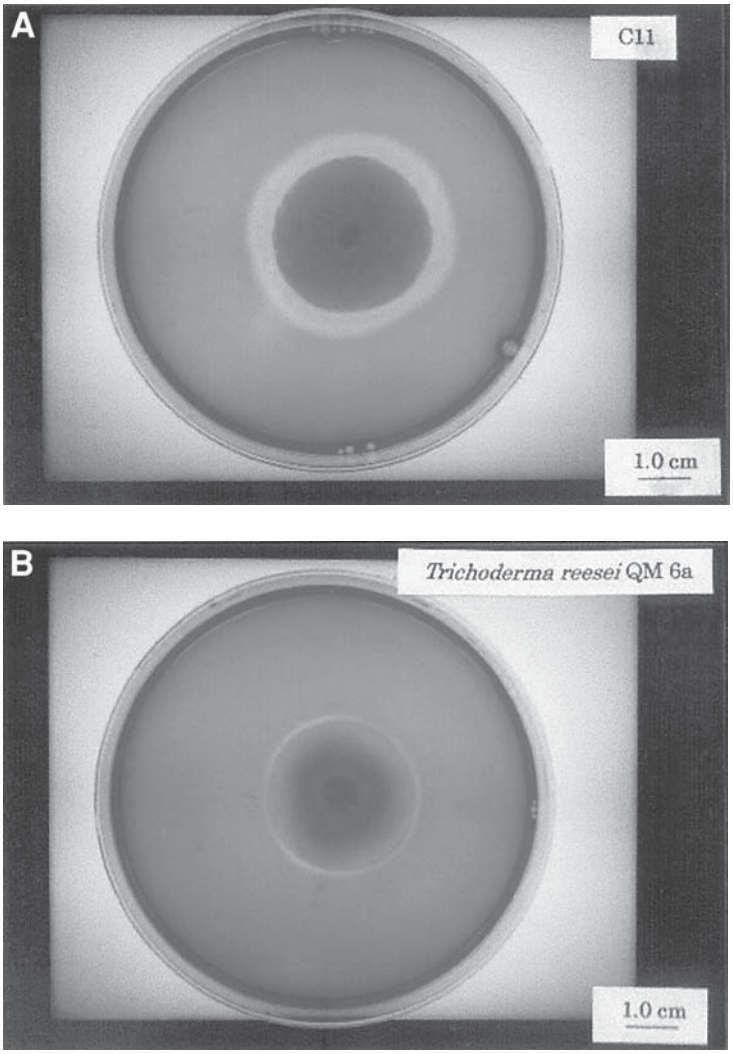

Fig. 2. Cellulase production of *T. reesei* QM6a and strain C11. A mycelial mat (2 × 2 mm) was incubated on the medium to estimate cellulase productivity for 6 d at 28°C. After incubation, 0.1% (w/v) Congo red solution was added to the plate and left for 1 h, followed by washing with 1 *M* NaCl. After washing, the diameter of a clear zone around a colony and the diameter of the colony were measured using a digital caliper.

Selection of Cellulase Hyperproducer

The swollen conidia treated with 0.5% colchicine for 7 d after auto-polyploidization were incubated in the double layer selection medium for 10 d at 28°C in order to select cellulase hyperproducers. After incubation, 35 colonies appeared on the surface of the selection medium. Those colonies were used for estimation of cellulase productivity on the solid medium.

Table 2
Cellulase Productivity of *T. reesei* QM6a and Strain C11 on Solid Medium[a]

Strain	Diameter of clear zone	Diameter of colony (mm)
T. reesei QM6a	32.54 ± 0.81	30.76 ± 0.64
C11	42.32 ± 0.95	32.65 ± 1.01

[a]A mycelial mat (2 × 2 mm) was incubated on the medium for estimation of cellulase productivity for 6 d at 28°C. After incubation, the diameter of a clear zone and the diameter of a colony were measured with a digital caliper. Those values are average of nine plates in each strain.

Estimation of Cellulase Productivity on Solid Medium

Mycelial mats of the selected 35 strains and the original strain were incubated on the medium for estimation of cellulase productivity for 6 d at 28°C in order to compare cellulase productivity. After Congo red staining, strain C11, showed the highest cellulase productivity. Moreover, the cellulase productivity of strain C11 was compared with that of the original strain using 18 agar plates of the medium for estimation of cellulase productivity. After incubation, strain C11 showed superior cellulase productivity, as shown in Fig. 2 and Table 2. Thus, strain C11 was selected as a cellulase hyperproducer.

Nuclear Characteristics in Conidium of Strain C11

The nuclear nature of the conidia of strain C11 was observed. For this purpose, a mycelial mat of strain C11 was incubated on a PDA plates for 7 d at 28°C in order to generate green conidia. After nuclear staining of these conidia using Giemsa solution or DAPI solution, it appeared the number of nuclei in the conidia of strain C11 ranged from one to four although the original conidia were mononucleate, as shown in Table 3. Furthermore, the mycelia of strain C11 were observed by nuclear staining. As a result, a larger number of nuclei were observed in the mycelia of strain C11 compared with that of the original strain (data not shown). Thus, the genetic conditions of the mycelia of strain C11 were regarded as polykaryon.

Measurement of Cellulose Hydrolyzing Activity

The cellulose hydrolyzing activity of strain C11 and the original strain was compared using the medium for measurement of cellulose hydrolyzing activity. As a result, the Avicel and CMC hydrolyzing activity of strain C11 was increased more than that of the original strain, as shown in Table 4. The Salicin hydrolyzing activity of strain C11 was almost the same as that of the original strain.

Table 3
Nuclear Diameter and Number of Conidia of Strain C11 Derived from Swollen Conidia Treated with 0.5% Colchicine for 7 d

Nuclear number	Proportion (%)	Nuclear diameter (μm)	Proportion (%)
1	11	0.10–0.49	17
2	55	0.50–0.89[a]	47
3	32	0.90–1.29	36
4	2	1.30–1.69	0
5	0	1.70–2.09	0
6	0	2.10–2.49[b]	0

[a] Nuclear diameter of original strain.
[b] Diameter of autopolyploid nucleus.

Table 4
Cellulose Hydrolyzing Activity in *T. reesei* QM6a and Strain C11[a]

	Hydrolyzing activity		
Strain	Avicel	CMC	Salicin (IU/mL)
T. reesei QM6a	46 ± 7	46 ± 0	37 ± 6
C11	53 ± 6	70 ± 6	38 ± 8

[a] A mycelial mat (2 × 2 mm) was incubated in wheat bran medium for 6 d at 28°C. Three Erlenmeyer flasks were used for estimation of one strain. The enzyme solution and the substrate were incubated for 2 h at 4°C using a reciprocal shaker (125 strokes/min). The amount of reducing sugar was measured using DNS.

Discussion

The question arises: Why does this polykaryon formation occur? In animal cells, the phenomenon of micronucleation is known to occur *(11)*. It is suspected that this is owing to abnormal nuclear division. We previously reported that micronucleation occurs in the fungus *T. reesei* when this fungus is treated with higher concentrations of colchicine, although autopolyploidization occurs when this fungus is treated with 0.1% (w/v) colchicine *(12,13)*. In the present study, it was suspected that 2.0% (w/v) colchicine (higher concentration) caused abnormal nuclear division followed by micronucleation from one autopolyploid nucleus in a swollen conidium. However, 0.5% (w/v) colchicine could produce multiple smaller nuclei whose diameter was almost the same as that of the original nucleus from an autopolyploid nucleus. Such smaller nuclei matched with our experimental purpose. This phenomenon also seems to be owing to abnormal nuclear division.

We discussed how the multinucleate conidia were produced. In the original conidia of this fungus, nuclear division was carried out in the first conidium and only one nucleus was distributed into one conidium correctly *(7)*. However, there were an excessive number of nucleus in the swollen conidium after polykaryon formation, and there were a larger number of nuclei in the mycelial mat derived from such a swollen conidium (data not shown). When many nuclei began to divide in a swollen conidium and to distribute into conidia through mycelia, an excessive number of nuclei seemed to be transported into a conidium by the nuclear transfer mechanism of this fungus. The genetic stability of such polykaryons will be the next target of investigation.

Finally, we discussed why cellulase productivity increased. The cellulase productivity of the autopolyploid strain was almost the same as that of the original strain or decreased (data not shown). However cellulase productivity increased after polykaryon formation. Thus, it is likely that the changes in nuclear nature are related to the changes in cellulase productivity. Changes in the nature of autopolyploid nucleus might cause the expression of amplified genes in an autopolyploid nucleus. But, further investigation is necessary to elucidate this phenomenon clearly.

Based on our study's results, we believe that the discussed techniques can be used to increase cellulase productivity in cellulase hyperproducers produced by chemical mutation, genetic engineering, and protein engineering.

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