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Successive Construction of Cellulase Hyperproducers of *Trichoderma* Using Hyperpolyploids

Hideo Toyama* and Nobuo Toyama

Department of Food Science and Technology, Faculty of Horticulture, Minamikyushu University, Takanabe, Miyazaki 884-0003, Japan, E-mail: wonder@iris.dti.ne.jp

Abstract

When the swollen conidia of *Trichoderma reesei* QM 6a are treated with 0.1% (w/v) colchicine solution, huge autopolyploid nuclei can be formed in those swollen conidia. When a mycelial mat derived from such a conidum is treated with a haploidizing reagent, benomyl, many fan-shaped sectors are produced from the colony, and cellulase hyperproducers are selected from conidia on the colony. When colchicine and benomyl treatments are repeated on cellulase hyperproducers, new hyperproducers can be constructed successively and systematically. Moreover, when conidia derived from autopolyploids are treated with ethylmethanesulfonate solution, another type of cellulase hyperproducers (polyploids) can be obtained.

Index Entries: *Trichoderma*; cellulase; colchicine; cellulose; saccharification.

Introduction

Trichoderma reesei is a cellulolytic fungus whose conidium is mononucleate *(1)*. This fungus is known to produce cellulase more than any other fungi, and its cellulase is stable *(2)*. Therefore, *T. reesei* is utilized for industrial cellulase production *(3)*. It is also used for the development of cellulose saccharification for fuels *(4)*. Since *T. reesei* has these various advantages, several improvements have been made, e.g., mutation *(5)*, protoplast fusion *(6)*, genetic engineering *(7)*, autopolyploid formation *(8)*, micronuclei formation *(9)*, and polykaryon formation *(10)*. From the autopolyploid, we can construct cellulase hyperproducers and also strains with a more suitable ratio of cellulase components for substrates *(11,12)*. Since it was found that autopolyploids of *T. reesei* can contribute to the construction of cellulase hyperproducers, we introduce two methods in this article.

*Author to whom all correspondence and reprint requests should be addressed.

Materials and Methods

Microorganism and Media

T. reesei QM 6a was used for these experiments as a model strain. The microorganism was incubated on potato dextrose agar (PDA) (BBL, Cockeysville, MD) medium or Mandels' medium containing 1.0% (w/v) Avicel (Funakoshi, Tokyo, Japan), 0.5% (w/v) peptone (Difco, Detroit, MI), and 1.5% (w/v) agar (Difco) (pH 6.0) at 26°C and preserved at 4°C *(13)*.

For colchicine treatment, Mandels' medium containing 1.0% (w/v) glucose (Wako, Osaka, Japan), 0.5% peptone, and 0.1% (w/v) colchicine (Wako) (pH 6.0) was used.

For the selection medium of cellulase hyperproducers, 10 mL of Mandels' medium (bottom layer) containing 0.5% peptone, 0.1% (v/v) polyoxyethylene *(10)* octylphenylether (Triton X-100) (Wako), 1.5% agar, and conidia was overlayed with 200 mL of Mandels' medium (upper layer) containing 1.0% Avicel, 0.5% peptone, 0.1% Triton X-100, and 1.5% agar in a deep plate (9 × 6 cm).

As the medium for estimation of cellulase productivity, Mandels' medium containing 1.0% (w/v) carboxymethylcellulose sodium salt (CMC-Na) (Wako) (degree of substitution [D.S.] 0.3), 0.1% Triton X-100, 1.5% agar, and 0.5% peptone (pH 6.0) was used.

As the solid medium for the measurement of cellulose hydrolyzing activity, 7.5 g of wheat bran containing 7.5 mL of Mandels' medium with 4.5 g of peptone in a 100-mL Erlenmeyer flask was used.

Mandels' medium containing 1.0% Avicel and 0.5% peptone (pH 6.0) was used as the liquid medium for the measurement of cellulose hydrolyzing activity.

Colchicine Treatment

Swollen conidia or a mycelial mat (10 × 10 mm) was added to 7–10 mL of Mandels' medium containing 1.0% glucose, 0.5% peptone, and 0.1% colchicine in a test tube or a 20-mL Erlenmeyer flask followed by stationary incubation at 26°C. Nuclear conditions in conidia or a mycelial mat were observed by nuclear staining. In the case of a mycelial mat, a small mycelial mat (2 × 2 mm) was cut out for nuclear staining each week.

Nuclear Staining and Calculation of Nuclear Diameters

Conidia were stained with Giemsa solution (Merck, Darmstadt, Germany) after successive treatments with 5, 3, and 1 *N* HCl at 60°C. In the case of mycelia, a small mycelial mat (2 × 2 mm) was stained with Giemsa solution, and photomicrographs were taken using a microscope (BH-2, Olympus, Tokyo, Japan) with an automatic exposure meter (PM-CBAD, Olympus) and a camera (C35AD, Olympus), and the photographs were enlarged two times *(14)*. The structures stained with Giemsa solution were also stained with 4,6'-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis,

MO) solution *(15)*. One hundred nuclei per sample were marked on the enlarged photomicrographs, and the nuclear diameters were measured using a digital caliper (Mitsutoyo, Kawasaki, Japan). From those measured values, the maximum, minimum, and most frequent nuclear diameters were calculated.

Measurement of DNA Content

DNA was extracted from a mycelial mat by the method of Herbert et al. *(16)* using perchloric acid (Wako) and measured by the method of Ceriotti *(17)* using indole (Wako). Mycelia were dried at 80°C for 17 h and weighed.

Benomyl Treatment

A small mycelial mat (2 × 2 mm) was put on Mandels' medium containing 0.4–0.8 μg/mL of benomyl (Sigma), 1.5% agar, and 0.5% peptone (pH 6.0), and incubated for 3 wk at 26°C to form fan-shaped sectors.

Chemical Mutation

Conidia generated on a colony were suspended in distilled water and filtrated with a glass filter (3G-2 type, Iwaki Glass, Funabashi, Japan) to remove mycelia. Collected conidia were washed three times with distilled water. Washed conidia were collected by a centrifuge and added to 9.5 mL of 0.2 *M* sodium phosphate buffer (pH 8.0), and then added to 0.5 mL of 4.0% glucose and 0.3 mL of ethylmethanesulfonate (EMS) (Wako). The suspension was incubated for 40 min at 26°C by a rotary shaker (5500*g*). The incubation time indicated 50% of lethality. The treated conidia were washed three times with distilled water.

Selection of Cellulase Hyperproducer

EMS-treated conidia were added to the bottom layer medium for selection of cellulase hyperproducers, and the upper layer medium was then overlayed followed by incubation at 26°C. After incubation, the first and second colonies that appeared on the surface were selected and incubated on Mandels' medium containing 1.0% Avicel, 0.5% peptone, and 1.5% agar at 26°C and preserved at 4°C.

Estimation of Cellulase Productivity

A small mycelial mat was put on the medium for estimation of cellulase productivity and incubated at 26°C for 6 d. After incubation, 0.1% (w/v) Congo red solution (Merck) was poured into plates and left for 1 h. Next, the surface of the medium was washed with 1.0 *M* NaCl (Wako) solution. After washing, the diameters of the clear zone and colony were measured with a digital caliper. Cellulase productivity was estimated using the ratio between the diameter of the clear zone and that of the colony.

Measurement of Cellulose Hydrolyzing Activity

As the substrates of enzyme reaction, 1.0% (w/v) of Avicel, CM-cellulose (Wako), or Salicin (Wako) was added to 100 mL of 0.1 *M* acetate buffer (pH 5.0). In the case of wheat bran culture, one loopful of conidia was added to flasks of the solid medium for the measurement of cellulose hydrolyzing activity and incubated at 26°C for 6 d. The flasks were 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. In the case of submerged culture, one loopful of conidia was added to the liquid medium for the measurement of cellulose hydrolyzing activity and incubated at 26°C for 6 d using a rotary shaker (TAITEC R-30 mini, Koshigaya, Japan). The agitation speed was 140 rpm. After incubation, the liquid medium was filtrated with filter paper (no. 2, Whatman, Maidstone, UK), and the filtrate was used as the enzyme solution. Zero point 3 mL of enzyme solution and 3.0 mL of substrate were mixed and incubated for 60 min at 40°C using a reciprocal shaker (TAITEC R-30 mini). Agitation speed was 125 strokes/min.

After enzyme reaction, two drops of 0.1 *N* HCl (Wako) solution were then added to the mixture to stop the reaction. The reaction mixture was filtrated with filter paper (no. 2, Whatman). The amount of reducing sugar in the reaction mixture was measured using 3,5-dinitrosalicylic acid (Wako) *(18)*. One international unit (IU) equals the enzyme amount that produces reducing sugar equivalent to 1 μmol of glucose per minute.

Results

Colchicine Treatment of Conidia

When resting conidia were stationarily incubated in 10 mL of the liquid medium without colchicine in a 20-mL Erlenmeyer flask for 240 h at 26°C, the diameters of nuclei in the conidia ranged from 0.55 to 0.85 μm. These were called *normal nuclei*. Nuclei whose diameter was >0.86 μm were called *larger nuclei*. The resting conidia were stationarily incubated in 10 mL of the liquid medium (pH 7.0) containing 0.1% (w/v) colchicine in a 20-mL Erlenmeyer flask for 240 h at 26°C. The proportions of larger nuclei and normal nuclei in the conidia were monitored every 24 h. The proportion of larger nuclei increased. Since the inner volume of resting conidia did not change during the colchicine treatment, the diameter of nuclei subsequently ranged from 1.36 to 1.85 μm.

One nucleus enlarged but multinucleated later in the resting conidia. When five loopfuls of the resting conidia were incubated in 50 mL of the liquid medium in a 100-mL Erlenmeyer flask using a rotary shaker (140 rpm) at 26°C, conidia began to swell almost simultaneously after 3 h of incubation, and germination began after 10 h. These morphological changes in

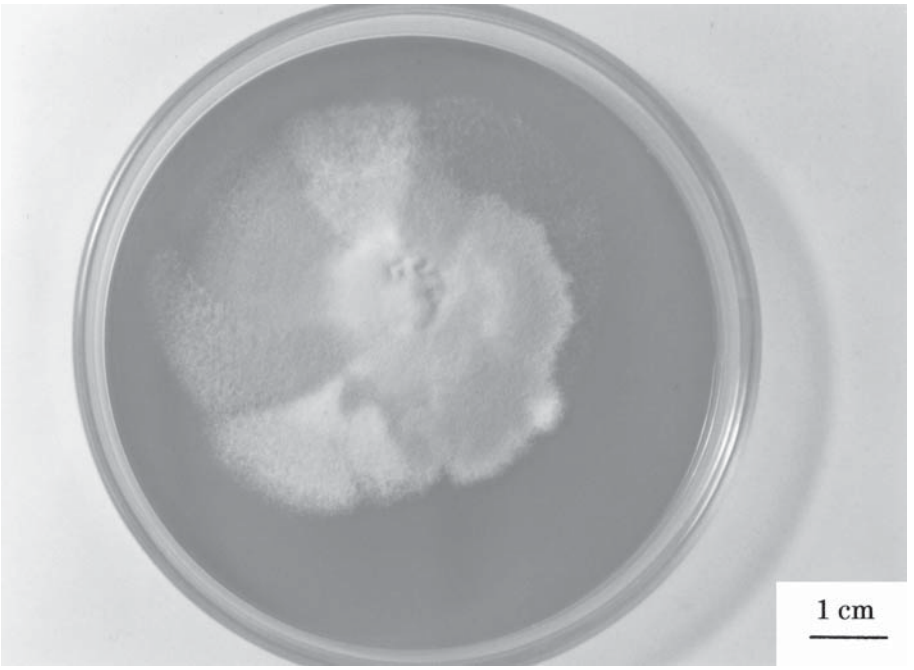

Fig. 1. Formation of fan-shaped sectors. A mycelial mat of strain B1 was put on the solid medium containing benomyl and incubated for 3 wk at 26°C. After the incubation, fan-shaped sectors were generated from the colony.

conidia were consistent with those reported by Rosen et al. *(1)*. Swollen conidia were stationarily incubated in 10 mL of the liquid medium containing 0.1% (w/v) colchicine in a 20-mL Erlenmeyer flask for 240 h at 26°C. Many nuclei >1.86 μm in diameter appeared. The larger nuclei (>1.36 μm) were named *hyperpolyploid nuclei* to distinguish them from the larger nuclei whose diameter ranged from 0.86 to 1.35 μm in swollen conidia. The mycelia derived from such conidia were called *hyperpolyploids*.

Selection of Hyperpolyploids

Isolation of hyperpolyploids was attempted using the swollen conidia incubated stationarily in 10 mL of the liquid medium containing 0.1% colchicine in a 20-mL Erlenmeyer flask for 240 h at 26°C. The swollen conidia treated for 240 h were called *conidia B*. Treated swollen conidia were incubated on a PDA medium containing 0.1% Triton X-100 for 7 d at 26°C. After incubation, some colonies produced yellow pigment in large quantities and generated enlarged conidia. Larger nuclei were seen in the mycelia of such colonies after nuclear staining. Haploidization was carried out on the mycelia (2 × 2 mm) derived from conidia B. After haploidization, almost all the colonies produced many sectors, as shown in Fig. 1. A colony derived from conidia B was called *strain B1*. Larger nuclei also existed in the mycelia of the B1 strain.

Selection of Cellulase Hyperproducer (1)

The earliest colony to appear on the upper layer medium for selection of cellulase hyperproducers was selected from the whole conidia (about 1.4×10^5) of the benomyl-treated B1 strain. Strain B2 was isolated from the whole conidia of the benomyl-treated B1 strain after 3 d of incubation.

Repetition of Benomyl Treatment

When strain B2 was haploidized using the haploidizing medium, strain B2 produced new sectors. One strain, B3, was selected from the whole conidia of the benomyl-treated strain B2 using the medium for selection of cellulase hyperproducers.

Estimation of Cellulase Productivity on Solid Medium (1)

The ratios between the diameter of a clear zone and that of the colony (strains B1, B2, and B3) during incubation were compared with those of the original strain and *T. reesei* QM 9414 using the medium containing CMC-Na. The ratios derived from strains B2 (2.07) and B3 (2.21) were apparently higher than that of the original strain (1.33). In particular, strain B3 showed the highest ratio after 6 d of incubation, as shown in Fig. 2. The ratios of strains B2 and B3 were also higher than that of *T. reesei* QM 9414 (1.94).

Measurement of Cellulose Hydrolyzing Activity (1)

The cellulose hydrolyzing activities (IU/mL) of strains B1, B2, and B3 were compared with those of the original strain using wheat bran culture. In strain B2, Avicel, cellulose powder, CMC-Na, and Salicin hydrolyzing activities were 1.9, 1.6, 2.2, and 4.4 times higher, respectively, than those of *T. reesei* QM 6a. Especially in strain B3, Avicel, cellulose powder, CMC-Na, and Salicin hydrolyzing activities were 2.2, 1.7, 2.6, and 4.8 times higher, respectively, than those of the original strain. Strains B2 and B3 were ascertained as cellulase hyperproducers because all cellulose hydrolyzing activities were higher than those of *T. reesei* QM 6a.

Measurement of DNA Content

When the average DNA contents of conidia derived from strain B1 was compared with that of the original strain, they were found to be 8 and 13 times greater, respectively. DNA contents in conidia derived from strains B2 and B3 were measured and compared with that of the original strain. The DNA contents of strains B2 and B3 were about 1.6 and 1.3 times greater, respectively, than that of the original conidia. Thus, strains B2 and B3 were regarded as haploids or aneuploids.

Colchicine Treatment of Mycelial Mats

The mycelial mat treated with 0.1% colchicine solution for 14 d was called M14. When the mycelia of M14 were stained with Giemsa solution,

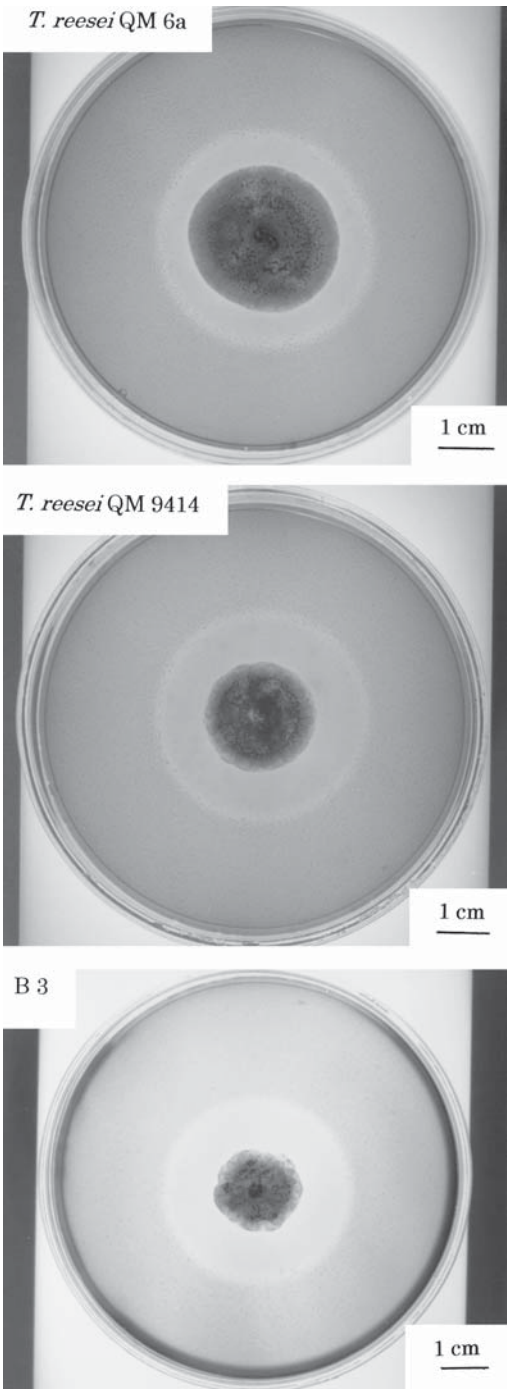

Fig. 2. Cellulase production of *T. reesei* QM 6a, QM 9414, and B3 on the medium containing CMC-Na. A mycelial mat was put on the center of the medium for estimation of cellulase productivity and incubated for 6 d at 26°C. After incubation, 0.1% (w/v) Congo red solution was poured into the plate and left for 1 h. Next, the surface of the medium was washed with 1 *M* NaCl. A clear zone appeared around a colony after washing. The diameters of the clear zone and colony were measured by a digital caliper.

Table 1
DNA Content of Original Strain,
Colchicine-Treated Strain (M14),
and Cellulase Hyperproducers (M14-1 and M14-2)[a]

Strain	DNA content (μg/mg mycelia)
T. reesei QM 6a	33.3
M14	347.8
M14-1	275.4
M14-2	331.3

[a]DNA was extracted from a mycelial mat by the method of Herbert et al. *(16)*, using perchloric acid and measured by the method of Ceriotti using indole *(17)*. Mycelia were dried for 17 h at 80°C and weighed.

many larger nuclei were seen. When the same mycelia were stained with DAPI solution, the larger nuclei were fluorescent. Therefore, it appeared that many larger nuclei were formed in the mycelia of M14 by the colchicine treatment. The nuclear diameter of 100 nuclei in each sample was measured by a digital caliper and the maximum, minimum, and most frequent diameters were calculated. The diameter of the original strain ranged from 0.50 to 0.89 μm, and the most frequent nuclear diameter ranged from 0.60 to 0.69 μm. After colchicine treatment, the nuclear diameter increased, ranging from 0.60 to 0.99 μm. The most frequent nuclear diameter ranged from 0.70 to 0.79 μm. DNA content was measured and compared, as shown in Table 1. The results indicated that the DNA content of M14 was higher than that of the original strain. The aforementioned results suggest that autopolyploidization occurred in the mycelia of M14. The distribution of nuclear diameters in the conidia of M14 was similar to that in the mycelia of M14.

Mutation and Selection for Cellulase Hyperproducers

When conidia of M14 treated with EMS solution were incubated in the selection medium for cellulase hyperproducers, two colonies appeared on the surface after 4 d. The first and second colonies to appear were called M14-1 and M14-2, respectively. Those colonies were incubated on Mandels' medium containing 1.0% Avicel, 0.5% peptone, and 1.5% agar (pH 6.0) at 26°C and preserved at 4°C.

Estimation of Cellulase Productivity on Solid Medium (2)

Each small mycelial mat (2 × 2 mm) from the original strain, M14-1, and M14-2 was incubated on the medium for estimation of cellulase productivity at 26°C for 6 d. After incubation, 0.1% Congo red solution was poured on the plates and washed with 1 *M* NaCl solution to form clear zones around colonies, as shown in Fig. 3.

The diameters of the clear zones and colonies were measured by a digital caliper. It appeared that the cellulase production per mycelia of

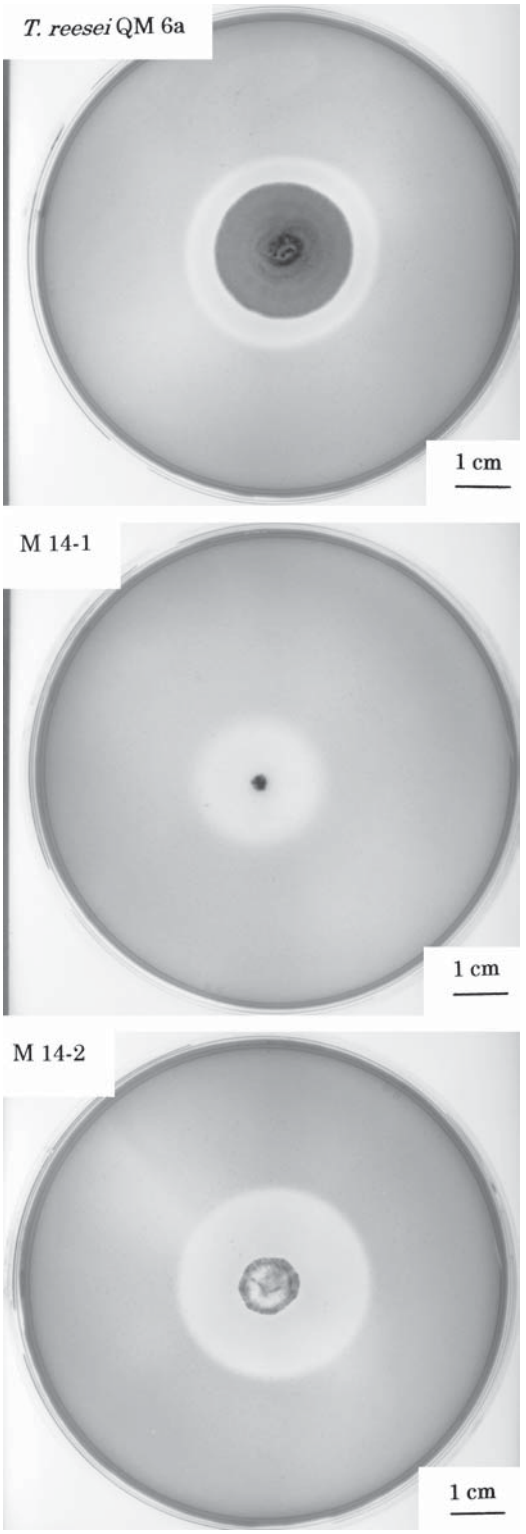

Fig. 3. Cellulase production of *T. reesei* QM 6a, M14-1, and M14-2 on the medium containing CMC-Na. See the legend to Fig. 2 for additional details.

Table 2
Cellulase Production of *T. reesei* QM 6a, M14, M14-1, and M14-2[a]

Strain	Diameter of clear zone (mm)	Diameter of colony (mm)	Diameter of clear zone divided by diameter of colony (mm)
T. reesei QM 6a	26.80	16.47	1.63
M14	29.90	27.09	1.10
M14-1	19.99	2.58	7.75
M14-2	33.94	9.71	3.50

[a]*See* Materials and Methods.

Table 3
Cellulose Hydrolyzing Activity of *T. reesei* QM 6a, M14, M14-1, and M14-2[a]

	Hydrolyzing activity (IU/mL)			
Strain	Avicel	CMC	Salicin	Mycelial weight (mg)
T. reesei QM 6a	133.6	120.4	85.4	466
M14	167.7	153.2	95.0	542
M14-1	198.0	191.3	105.5	138
M14-2	264.4	235.1	186.7	226

[a]*See* Materials and Methods. The strains were incubated in Mandels' medium containing 1.0% Avicel and 0.5% peptone (pH 5.0) at 26°C for 6 d using a rotary shaker (TAITEC R-30 mini) (140 rpm).

M14-1 and M14-2 increased remarkably more than that of the original strain, as shown in Table 2.

Measurement of Cellulose Hydrolyzing Activity (2)

One loopful of conidia of the original strain, M14, M14-1, or M14-2 was added to the liquid medium for the measurement of cellulose hydrolyzing activity and incubated for 6 d at 26°C using a rotary shaker (TAITEC R-30 mini) (140 rpm). After incubation, the hydrolyzing activities of Avicel, CM-cellulose, and Salicin were measured. As shown in Table 3, production of cellulose hydrolyzing activity per mycelia in M14-1 and M14-2 was significantly increased compared with the original strain.

Discussion

Autopolyploidization occurred in *T. reesei* by colchicine treatment. This indicates that autopolyploid nuclei can be produced even in fungus by colchicine treatment. It was suspected that the mechanism of autopolyploidization in *T. reesei* is almost the same as that in plants.

Autopolyploid nuclei are produced only by stationary incubation. When colchicine treatment was carried out actively using a rotary or reciprocal shaker, micronucleation occurred. This indicates that moderate condi-

tions for nuclear division are necessary in order to form autopolyploid nuclei *(9)*.

The haploidizing reagent, benomyl, is known to delete chromosome(s) from polyploid nuclei, and some chromosomal reconstruction, e.g., recombination, seems to occur *(19)*. It is suspected that the cellulase hyperproducers, B2 and B3, are constructed through such chromosomal reconstruction.

The cellulase productivity of M14 was not much higher than that of the original strain. We suspected that cellulase productivity increased because chromosomes (or chromosome) containing cellulase genes were also amplified by autopolyploidization in M14, but our results showed otherwise. Therefore, it was suspected that some type of inhibition might occur in cellulase production in M14. The mechanism of the inhibition is unknown.

Next, we discussed the reason that the cellulase productivity of M14-1 and M14-2 increased after chemical mutation. We suspected that this might be owing to an "unlocking" of the inhibition after chemical mutation, but the mechanism remains unknown. M14-1 and M14-2 were genetically stable through 10 generations (data not shown). It was concluded that cellulase hyperproducers can be constructed successively and systematically from autopolyploids of *T. reesei* by these methods. We consider these to be the advantages of the aforementioned methods.

Acknowledgments

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