0273-2289/01/91–93/0787/$11.00

The Effect of Additional Autopolyploidization in a Slow Growing Cellulase Hyperproducer of *Trichoderma*

Hideo Toyama* and Nobuo Toyama

Department of Food Science and Technology, Faculty of Horticulture, Minamikyushu University, Takanabe, Miyazaki 884-0003, Japan, E-mail: gaf00771@nifty.com

Abstract

M14-2 is a cellulase hyperproducer derived from *Trichderma reesei* QM 6a, but with a growth rate lower than that of the original strain. When M14-2 was autopolyploidized followed by haploidization and selection, the strain with both a higher cellulase productivity per mycelia and a higher growth rate could be obtained as M14-2B. This strain seemed to be constructed using gene sources amplified by additional autopolyploidization.

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

Introduction

Trichoderma reesei is a cellulolytic fungus and produces stable cellulase in high yield *(1)*. This fungus is widely utilized for industrial production of cellulase *(2)*. However, to expand the use of cellulase, the productivity of this fungus should be increased and the cost of cellulase should be reduced. Therefore, we attempted to develop a method for construction of cellulase hyperproducers for use in food processing using a model strain of *T. reesei* QM 6a without gene cloning techniques.

The M14-2 strain, a cellulase hyperproducer, was obtained from the conidia of M14 strain by chemical mutation *(3)*. The M14 strain is an autopolyploid and derived from the mycelial mat of *T. reesei* QM 6a treated with 0.1% (w/v) colchicine (Wako, Osaka, Japan) solution for 14 d at 26°C. The cellulase productivity per mycelia of M14-2 increased considerably, but the growth rate was lower than that of the original strain. Therefore, we attempted to improve the growth rate of M14-2 using haploidization and autopolyploidization techniques.

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

Methods, Results, and Discussion

M14-2 was incubated on a potato dextrose agar (PDA) medium (BBL, Cockeysville, USA) at 26°C and preserved at 4°C. A mycelial mat (2 mm × 2 mm) of M14-2 was directly incubated on the haploidizing medium for 3 wk at 26°C. PDA medium containing 0.6 µg/mL benomyl [1-(butylcarbamoyl)-2-benzimidazolecarbamate] (Sigma, St. Louis, MO) was used for haploidization *(4)*. During the incubation, fan-shaped sectors were produced from the colony. The selection of cellulase hyperproducers was carried out using those conidia generated on the colony. A Mandels's medium containing 1.0% (w/v) glucose (Wako, Osaka, Japan) and 0.5% (w/v) peptone (Difco, Detroit, MI) (pH 6.0) was used as the basic liquid medium *(5)*. For the selection of cellulase hyperproducers, conidia were added to 30 mL of Mandels's medium (bottom layer medium) containing 1.0% (w/v) Avicel (Funakoshi, Tokyo, Japan), 1.5% (w/v) agar (Difco, Detroit, MI), 0.5% peptone, and 0.1% (v/v) polyoxyethyleneoctylphenylether (Triton X-100) (Wako, Osaka, Japan) in a deep plate [6 cm (=depth) × 9 cm] and left for 1 h at 4°C to harden the agar. After hardening the agar, 200 mL of the same medium (upper layer medium) was poured on the bottom layer medium and left for 1 h at 4°C to harden the agar again. After 5 d of incubation, 16 colonies appeared on the surface of the selection medium and the estimation of cellulase productivity was carried out. Mandels's medium containing 1.0% (w/v) carboxymethylcellulose sodium salt (CMC-Na), degree of substitution 0.7 (Wako, Osaka, Japan), 0.5% peptone, 1.5% agar, and 0.1% Triton X-100 was used for the estimation of cellulase productivity (pH 6.0). The mycelial mat (2 mm × 2 mm) was put on the medium for estimation of cellulase productivity and incubated for 6 d at 26°C. After incubation, 0.1% (w/v) Congo red (Merck, Darmstadt, Germany) solution was poured on plates and left them for 1 h followed by washing with 1 *M* NaCl (Wako, Osaka, Japan) solution. After washing, a clear zone appeared around a colony by cellulase formation. Diameters of the clear zone and colony were measured by a digital caliper (Mitsutoyo, Kawasaki, Japan) and cellulase productivity was estimated using the ratio between the two diameters. After estimation of cellulase productivity, one colony was isolated as M14-2A. The growth rate of the strain, M14-2A, was almost the same with that of *T. reesei* QM6a but M14-2A lost hypercellulase productivity per mycelia.

Next, the second autopolyploidization of M14-2A was attempted. A mycelial mat (10 mm × 10 mm) was incubated stationarily in Mandels's medium containing 0.1% (w/v) colchicine (Wako, Osaka, Japan), 1.0% glucose, and 0.5% peptone (pH 6.0) for 35 d at 26°C. After incubation, haploidization was carried out on those colchicine-treated mycelial mats for 3 wk at 26°C, and fan-shaped sectors were also produced. The conidia were incubated in the selection medium mentioned above. After 4 d of incubation, 11 colonies appeared on the surface of the medium. After estimation of cellulase productivity, one colony was isolated as M14-2B. More-

Table 1
Cellulase Production on CMC-Na Plates

Strains	Diameter of clear zone (mm)[a]	Diameter of colony (mm)[a]	Ratio
T. reesei QM 6a	26.80 ± 0.62	16.47 ± 0.31	1.61
T. reesei Rut C-30	31.80 ± 0.65	14.30 ± 0.35	2.22
M14	29.90 ± 0.67	27.09 ± 0.36	1.10
M14-2	33.94 ± 0.65	9.71 ± 0.36	3.50
M14-2A	33.13 ± 0.63	20.98 ± 0.34	1.58
M14-2B	46.67 ± 0.63	15.05 ± 0.38	3.10

[a]The diameter of clear zone and the diameter of colony were measured by a digital caliper after 6 d incubation at 26°C. The ratio between the diameter of clear zone and the diameter of colony was used for estimation of cellulase productivity.

over, cellulase productivities of *T. reesei* QM 6a, *T. reesei* Rut C-30 (ATCC 56765), M14, M14-2, M14-2A, and M14-2B were estimated using the medium for estimation of cellulase productivity. From these results, it appeared that the growth rate of M14-2B increased extremely compared with that of M14-2 and cellulase productivity was also maintained as shown in Table 1. Cellulose hydrolyzing activities of *T. reesei* QM 6a, M14, M14-2, M14-2A, and M14-2B were measured using the wheat bran culture. For the measurement of cellulose hydrolyzing activities, 7.5 g of wheat bran was used containing 7.5 mL of Mandels's medium and 4.5 g of peptone in a 100 mL Erlenmeyer flask. One loopful of conidia was added to a flask of the solid medium for the measurement of cellulose hydrolyzing activity and incubated at 26°C for 6 d. Those 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. As the substrates of enzyme reaction, 1.0% (w/v) of Avicel, CM-cellulose (Wako, Osaka, Japan), or Salicin (Wako, Osaka, Japan) was added to 100 mL of 0.1 *M* acetate buffer (pH 5.0). Three tenths of a milliliter of enzyme solution and 3.0 mL of substrate were mixed and incubated for 60 min at 40°C using a reciprocal shaker (Thomastat T-22S, Tokyo, Japan). The agitation speed was 125 strokes/min. After enzyme reaction, two drops of 0.1 *N* HCl (Wako, Osaka, Japan) solution were then added to the mixture in order to stop the reaction. 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 3,5-dinitrosalicylic acid (DNS) (Wako, Osaka, Japan) *(6)*. IU was based on the amount of enzyme producing reducing sugar equivalent to 1 µmol of glucose per minute. As shown in Table 2, it appeared that the hydrolyzing activities of Avicel, CMC, and Salicin in M14-2B were about 2, 3, and 3 times higher, respectively, than those of *T. reesei* QM 6a, and 1.4, 2, and 2 times higher than those of M14-2. The proliferation of M14-2B on the wheat bran medium was superior to that

Table 2
Cellulose Hydrolyzing Activities in Wheat Bran Culture

Strains	Hydrolyzing activities (IU/mL) of[a]			Growth
	Avicel	CMC	Salicin	
T. reesei QM 6a	53	45	26	++
M14	68	55	36	++
M14-2	88	74	44	+
M14-2A	93	88	51	++
M14-2B	120	136	70	++

[a]For the measurement of cellulose hydrolyzing activities, 7.5 g of wheat bran was used containing 7.5 mL of Mandels's medium and 4.5 g of peptone in a 100 mL Erlenmeyer flask. One loopful of conidia was added to a flask and incubated at 26°C for 6 d. The amount of reducing sugar in the reaction mixture was measured using 3,5-dinitrosalicylic acid (DNS) (Wako, Osaka, Japan) *(6)*. IU was based on the amount of enzyme producing reducing sugar equivalent to 1 μmol of glucose per minute.

of M14-2. The distribution of nuclear diameters of *T. reesei* QM 6a, M14, M14-2, and M14-2B were compared. A mycelial mat was directly stained by Giemsa solution (Merck, Darmstadt, Germany) and photomicrographs were taken using a microscope (BH-2, Olympus, Tokyo, Japan) with an automatic exposure meter (PM-CBAD, Olympus, Tokyo, Japan) and a camera (C35AD, Olympus, Tokyo, Japan). Those photomicrographs were enlarged and the nuclear diameter of 100 nuclei per sample was measured by a digital caliper. The distribution of nuclear diameter per sample was investigated from those measured values. From the results, M14-2B was found to be still polyploid in addition to M14 and M14-2. We suspected that M14-2B seemed to be constructed through chromosomal recombination from the colchicine-treated M14-2 (autopolyploid). From the above results, it was concluded that a cellulase hyperproducer which has higher growth rate and higher cellulase productivity per mycelia, M14-2B, might be constructed using gene sources amplified by additional autopolyploidization from a low growing cellulase hyperproducer, M14-2.

Acknowledgments

We would like to thank Yakult Pharmaceutical Industry Co. Ltd., and Tsukishima Kikai Co. Ltd., for useful discussions and financial support.

References

1. Evans, E. T., Wales, D. S., Sagar, B. F., and Bratt, R. P. (1992), *J. Gen. Microbial.* **138,** 1639–1646.
2. Nieves, R. A., Ehrman, C. I., Adney, W. S., Elander, R. T., and Himmel, M. E. (1998), *World J. Microbiol. Biotechnol.* **14,** 301–304.
3. Toyama, H. and Toyama, N. (1999), *Microbios* **100,** 7–18.
4. Peterbauer, C. K., Heidenreich, E., Kubicek, C. P., and Baker, R. T. (1992), *Can. J. Microbiol.* **38,** 1292–1297.
5. Mandels, M. and Sternberg, D. (1976), *J. Ferment. Technol.* **54,** 267–286.
6. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.