0273-2289/1559–0291 (Online)/07/136–140/155–160/$30.00

Filter Paper Degrading Ability of a *Trichoderma* Strain With Multinucleate Conidia

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Abstract

The multinucleate conidia were produced from the green mature conidia of *Trichoderma reesei* Rut C-30 strain by colchicine treatment. The strain with higher Filter paper degrading ability was selected among those conidia using a double layer selection medium. The selected strain, JS-2 was able to collapse the filter paper within 15 min but the original strain took 25 min to collapse it completely. Moreover, the amount of reducing sugar in the L-type glass tube of the strain, JS-2, was greater than that of the original strain. The Avicel, CMC-Na, and Salicin hydrolyzing activity of the strain, JS-2, increased 2.1 times, 1.2 times, and 3.6 times higher than that of the original strain.

Index Entries: Cellulase; cellulose; conidia; nuclei; *Trichoderma*; filter paper.

Introduction

Trichoderma reesei is a cellulolytic fungus commonly used for the production of commercial cellulases *(1)*. Colchicine has been shown to induce polyploidy in plants by inhibiting mitosis *(2,3)*. Colchicine has also been shown to induce polyploidy in fungi and Basidiomycetes *(4,5)*. When fungal conidia are incubated in a liquid medium containing colchicine, the diameter of nuclei in conidia gradually increase and generate polyploid nuclei. Multinucleation occurs when multiple smaller nuclei are generated from a polyploid nucleus in a conidium after long-term colchicine treatment *(6)*. Mycelia derived from such a multinucleated conidia contain a larger number of nuclei compared with that of the original strain. The nuclear diameter of the mycelia of multinucleated conidium does not increase, although the DNA content of the mycelia increases. In this report, we selected a strain with a higher filter paper degrading ability from the multinucleated conidia of *T. reesei*.

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T. reesei Rut C-30 American Type Culture Collection (ATCC56765) was used as a model strain *(7)*. The strain was incubated on potato dextrose agar (PDA) medium (BBL, Cockeysville, USA) at 28°C and preserved at 4°C. A mycelial block (2×2 mm^2) was placed on the center of a PDA plate and incubated at 28°C to generate green mature conidia. The conidia were suspended in distilled water and filtered with a glass filter (3G-2 type, Iwaki Glass, Funakoshi, Japan) to remove hyphae. The conidia were collected by centrifugation at 5510*g* and desiccated to prepare dried mature green conidia. Conidia that were stained with Giemsa solution (Merck, Darmstadt, Germany) after treatment with 5 *N* HCl (Wako, Osaka, Japan) for 30 min at 50°C were found to be mononucleate *(8)*. Similar results were obtained when the conidia were stained with 4,6′-diamidino-2-phenylindole (Sigma, St. Louis, MO) solution after HCl treatment.

As the basic medium, Mandels' medium $(NH_4)_2SO_4$ (Wako); 1.4 g, KH_2PO_4 (Wako); 2.0 g, urea (Wako); 0.3 g, $CaCl_2$ (Wako); 0.3 g, $MgSO_4 \cdot 7H_2O$ (Wako); 0.3 g, $FeSO_4 \cdot 7H_2O$ (Wako); 0.005 g, $MnSO_4 \cdot H_2O$ (Wako); 0.0016 g, $ZnSO_4 \cdot H_2O$ (Wako); 0.0014 g, $CoCl_2$; (Wako) 0.0020 g, and distilled water: 1000 mL was used (pH 6.0) *(9)*. When dried green mature conidia were added to 25 mL of Mandels' medium containing 0.025 g colchicine, 0.25 g glucose, and 0.125 g peptone (Difco, Detroit, USA) in a 50 mL-Erlenmeyer flask and incubated statically for 3 wk at 28°C, multiple smaller nuclei were produced.

These multinucleated conidia were incubated using a double layer selection medium in order to select the strain that has higher degrading ability of a filter paper. The bottom layer medium contained 100 mL of Mandels' medium containing 1.0 g glucose, 0.5 g peptone, 0.3 mL polyoxyethylene (10), octylphenylther (Triton X-100) (Wako), and 3.0 g agar (Difco) in a deep glass plate (150 mm in diameter and 60 mm in depth) (pH 6.0). The upper layer medium contained 100 mL of Mandels' medium containing 1.0 g Avicel (Funakoshi, Tokyo, Japan), 0.5 g peptone, 0.1 mL Triton X-100, and 3.0 g agar (pH 6.0). The multinucleated conidia were added to the bottom layer and left for 30 min at 4°C to harden the agar. After the agar hardened, the upper layer selection medium was overlaid and left for 30 min at 4°C to allow the agar to harden. The conidia were then incubated at 28°C. After 3 d of incubation, colonies began to appear and the largest colony on the surface was selected as strain JS-2 after 6 d of incubation. When the same experiments were carried out using conidia untreated with colchicine, colonies began to appear on the surface after four days of incubation. The colony of strain JS-2 was incubated on a PDA medium and preserved at 4°C.

Cellulase production was carried out using the original strain and strain JS-2. A mycelial mat (2×2 mm^2) of each strain was added to 50 mL of Mandels' medium containing 0.5 g Avicel and 0.25 g peptone in a 100-mL Erlenmeyer flask followed by incubation using a rotary shaker (TAITEC BR-12FH, Koshigaya, Japan) for 6 d at 28°C. The agitation speed

Table 1
Collapse Time of a Filter Paper and the Amount of Reducing Sugar in an L-type Glass Tube After Enzymatic Reaction

Strain	Collapse time (min)	Amount of reducing sugar (mg/mL)
C30	25	0.74
JS-2	15	1.86

Filter paper (10×10 mm^2) was added to the enzyme solution in an L-type glass tube followed by incubation for 30 min at 50°C using a Monod shaker. Collapse time of filter paper was measured using a digital stop-watch. After the reaction, the amount of reducing sugar in an L-type glass tube was measured using 3,5-dinitrosalicylic acid.

was 160 rpm at 0.59*g*. After incubation, the medium was filtered with a glass filter (3G-2 type) to remove hyphae. The filtrate was used as the enzyme solution. The pH of the enzyme solution was adjusted to 5.0 using 1 *N* HCl. Five milliliters of the enzyme solution and a filter paper (10×10 mm^2) (Whatman, No. 2, Maidstone, UK) were added to an L-type glass tube (120×68 mm) and incubated for 30 min at 50°C using a Monod shaker (TAITEC MONOD SHAKER PERSONAL-11, Koshigaya, Japan) at an agitation speed of 75 strokes/min. The time of collapse of the filter paper was then measured using a digital stop-watch (CITIZEN, Tokyo, Japan). The term "collapse," was defined as the condition when the reaction mixture contained only fibers without fragments of a filter paper. After collapse, the reaction mixture was filtered with a filter paper (No. 2, Whatman) and the amount of reducing sugar in the filtrate was measured using 3,5-dinitrosalicylic acid (Wako) *(10)*. After examination, it appeared that strain JS-2 was able to collapse the filter paper within 15 min but the original strain, *T. reesei* Rut C-30, took 25 min to collapse it completely as shown in Table 1. After measurement, the amount of reducing sugar in the L-type glass tube of strain JS-2 was more than that of the original strain.

Next, cellulose hydrolyzing activity of the enzyme solution was measured. As the substrates of enzyme reaction, 1.0 g of Avicel, carboxymethylcellulose CMC-Na (D.S. 0.7–0.8) (Wako), or Salicin (Wako) was added to 100 mL of 0.1 *M* acetate buffer (pH 5.0). Two milliliters of the enzyme solution was added to 4 mL of substrate in a glass tube (185×18.5 mm) and incubated by a reciprocal shaker (THOMASTAT T-22S, Tokyo, Japan) for 1 h at 50°C at an agitation speed of 125 strokes/min. The glass tubes containing the Avicel substrate were tilted on the shaker and shaken by hand every 30 min in order to avoid the precipitation of Avicel. The reaction mixture was filtered with filter paper (Whatman, No. 2) and the amount of reducing sugar measured using 3,5-dinitrosalicylic acid. Activity was defined as the amount of enzyme producing reducing sugar equivalent to 1 μmol of glucose/min. The cellulase production was carried out twice using two flasks per strain.

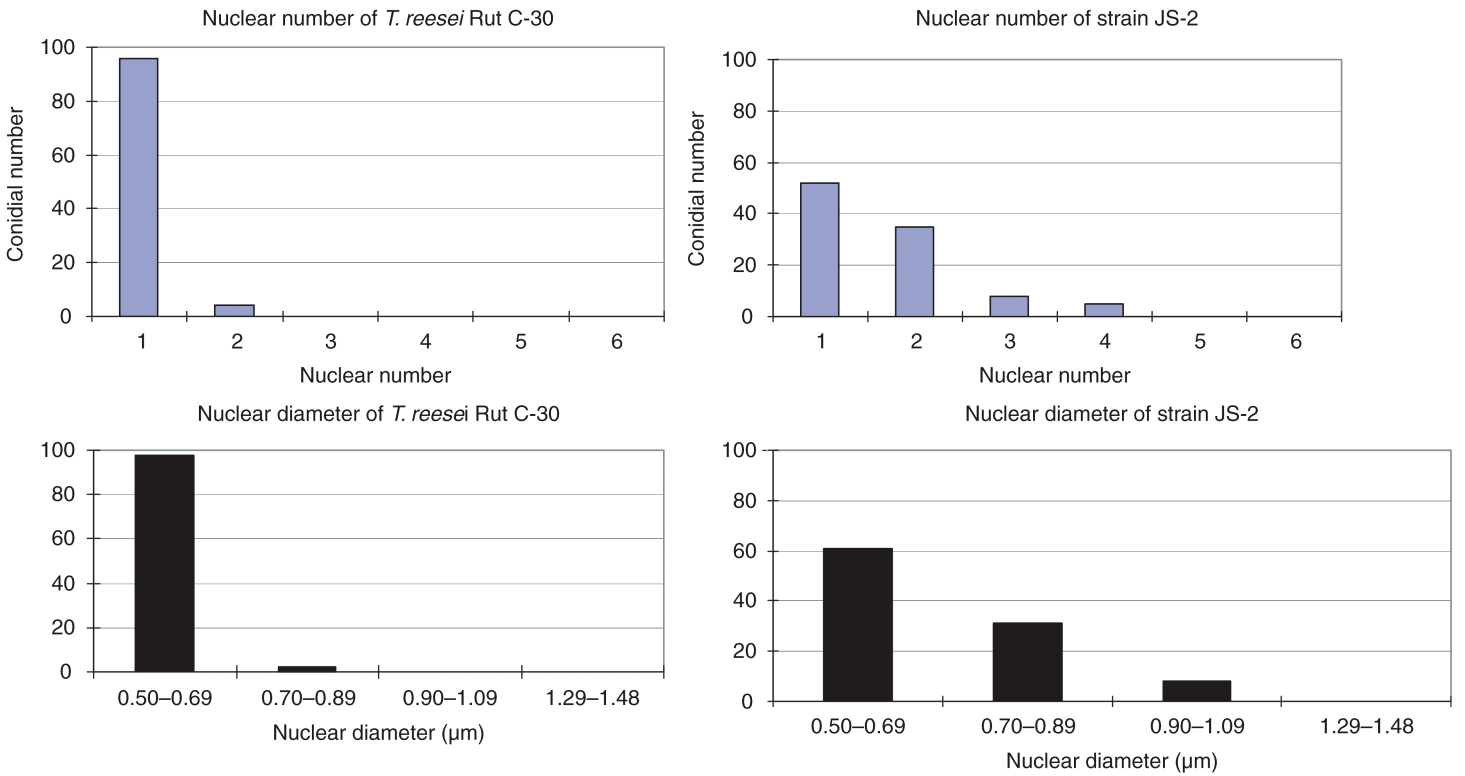

Fig. 1. Genetic conditions of the conidia derived from *T. reesei* Rut C-30 and the strain, JS-2.

Table 2
Cellulose Hydrolyzing Activity of the Strain, JS-2
and the Original Strain, *T. reesei* Rut C-30

	Hydrolyzing activity (IU/mL)		
Strain	Avicel	CMC-Na	Salicin
C30	62	141	10
JS-2	130	172	36

Enzyme production was carried out twice using two flasks per strain. Two milliliters of the enzyme solution were added to 4 mL of the substrate in a glass tube incubated by a reciprocal shaker for 1 h at 50°C. The amount of reducing sugar in the reaction mixture was measured using 3,5-dinitrosalicylic acid. The enzyme activity shows average values from two measurements.

Consequently, it was found that the Avicel, CMC-Na, and salicin hydrolyzing activity of strain JS-2 increased by a factor of 2.1, 1.2, and 3.6 over the original strain, respectively, as shown in Table 2.

The diameter and number of the conidia of strain JS-2 were compared with those of *T. reesei* Rut C30 using Giemsa staining after HCl treatment. The nuclear diameter of 100 conidia was measured using a digital caliper (Mitsutoyo, Koshigaya, Japan) on enlarged photomicrographs. The diameter of the original nuclei in an oval conidium ranged from 0.50 to 0.89 μm, whereas the diameter of the nuclei of strain JS-2 ranged from 0.50 to 1.09 μm as shown in Fig. 1.

The nuclear number of the same 100 conidia was counted on the photograph. From the results, the nuclear number of the conidia of strain JS-2 ranged from 1 to 4 and almost half of the conidia were multinucleate. When the mycelia of strain JS-2 were stained with Giemsa solution, it appeared that a larger number of nuclei existed in the mycelia compared with that of the original strain. Therefore, we propose that the higher degrading ability of a filter paper of strain JS-2 may be related to the multinucleated conidia. However, further investigation is necessary to clear the mechanism, which enhances a filter paper degrading ability in strain JS-2.

Acknowledgments

We wish to thank Tsukishima Kikai Co., Ltd. and Yakult Pharmaceutical Ind. Co., Ltd. for their useful suggestions.

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