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Rapid Isolation of the *Trichoderma* Strain with Higher Degrading Ability of a Filter Paper and Superior Proliferation Characteristics Using Avicel Plates and the Double-Layer Selection Medium

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Abstract The cost of cellulase is still a problem for bioethanol production. As the cellulase of *Trichoderma reesei* is applicable for producing ethanol from cellulosic materials, the cellulase productivity of this fungus should be increased. Therefore, we attempted to develop a system to isolate the strain with higher degrading ability of a filter paper and superior proliferation characteristics among the conidia treated with the mitotic arrester, colchicine. When green mature conidia of *T. reesei* RUT C-30 were swollen, autopolyploidized, and incubated in the double-layer selection medium containing Avicel, colonies appeared on the surface earlier than the original strain. When such colonies and the original colony were incubated on the Avicel plates, strain B5, one of the colonies derived from the colchicine-treated conidia, showed superior proliferation characteristics. Moreover, when strain B5 and the original strain were compared in the filter paper degrading ability and the cellulose hydrolyzing activity, strain B5 was also superior to the original strain. It was suspected that superior proliferation characteristics of strain B5 reflects higher filter paper degrading ability. Thus, we concluded that the *Trichoderma* strain with higher degrading ability of a filter paper and superior proliferation characteristics can be isolated using Avicel plates and the double-layer selection medium.

Keywords Cellulase · Cellulose · Conidia · Nuclei · *Trichoderma* · Filter paper

The cellulolytic fungus *Trichoderma reesei* is well known to produce stable cellulase useful for saccharification of cellulose and is widely used for production of commercial cellulase [1, 2]. Fuel ethanol must be produced from cellulosic resources to prevent global warming [3]. The cellulase of this fungus is applicable for producing ethanol from cellulosic materials, but the cost is still a problem [4]. Thus, the cellulase productivity of this fungus

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must be increased. For this purpose, a system must be developed to rapidly isolate cellulase hyperproducers of this fungus. We earlier formed autopolyploids of this fungus using polyploidizer with swollen conidium [5]. Moreover, we could develop a double-layer selection medium that can rapidly isolate the strains with higher degrading ability of crystalline cellulose [6]. However, the proliferation characteristics of the selected strains varied.

In this study, we attempted to isolate the *Trichoderma* strain with higher degrading ability of a filter paper and superior proliferation characteristics using Avicel plates and the double-layer selection medium among the conidia treated with colchicine. *Trichoderma reesei* Rut C-30 (ATCC56765) was used as a model strain [7]. The strain was incubated on potato dextrose agar (PDA) medium (BBL, Cockeysville, MD, USA) at 28°C and preserved at 4°C. PDA medium was used as the medium for conidial formation. A mycelial block (2×2 mm^2) of the original strain was placed on the center of a PDA plate and incubated for 10 days 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, Tokyo, Japan) to remove hyphae. These conidia were collected by centrifugation at 5,510×*g* for conidial swelling.

The conidia were then added to the medium for conidial swelling and incubated for 6 h using a rotary shaker (TAITEC NR-30, Koshigaya, Japan) at 28°C. The agitation speed was 160 rpm. The Mandels' medium used for the basic medium consisted of $(NH_4)_2SO_4$ (Wako, Osaka, Japan), 1.4 g; KH_2PO_4 (Wako), 2.0 g; urea (Wako), 0.3 g; $CaCl_2$ (Wako), 0.3 g; $MgSO_4$ $7H_2O$ (Wako), 0.3 g; $FeSO_4$ $7H_2O$ (Wako), 0.005 g; $MnSO_4$ H_2O (Wako), 0.0016 g; $ZnSO_4$ H_2O (Wako), 0.0014 g; $CoCl_2$ (Wako), 0.0020 g; and distilled water, 1,000 mL (pH 6.0) [8]. Mandels' medium containing 1.0% (w/v) glucose (Wako) and 0.5% (w/v) peptone (Difco, Detroit, MI, USA) was used as the medium for conidial swelling.

After incubation, the swollen conidia were collected by centrifugation at 5,510×*g* and added to the medium for autopolyploidization followed by incubation for 7 days at 28°C. Mandels' medium containing 0.1% (w/v) colchicine (Wako), 1.0% (w/v) glucose, and 0.5% (w/v) peptone was used as the medium for autopolyploidization. After incubation, the medium was filtrated with a glass filter 3G-2. The treated swollen conidia in the filtrate were collected by centrifugation and washed with distilled water followed by preservation in distilled saline at 4°C.

For the double-layer selection medium, the upper-layer medium containing 100 mL of Mandels' medium containing 3.0 g Avicel (Funakoshi), 0.5 g peptone, 0.3 mL polyoxyethylene (10) octylphenylther (Triton X-100) (Wako), and 3.0 g agar (pH 6.0) was overlayed on the bottom-layer medium, which contained 100 mL of Mandels' medium containing 3.0 g Avicel, 0.5 g peptone, 0.3 mL Triton X-100, 3.0 g agar (Difco), and conidia in a deep glass plate (150 mm in diameter and 60 mm in depth) (pH 6.0), followed by incubation. Colchicine-treated swollen 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 treated-swollen conidia were then incubated at 28°C. The colony appearance was observed during incubation. Colonies began to appear on the surface of the medium after 4 days of incubation. After 6 days of incubation, there were many colonies on the surface, but the colony diameters varied.

Five larger colonies were selected as B1, B2, B3, B4, and B5 from the colonies appearing on the surface of the selection medium, and their growth characteristics were compared with the original strain using the Avicel medium. Two Avicel plates were used for a strain. Mandels' medium containing 1.0% (w/v) Avicel, 0.5% (w/v) peptone, 3.0% (w/v) agar, and

0.1% (v/v) Triton X-100 was used as the Avicel medium. A mycelial block (2×2 mm^2) of the original strain or the selected strains was placed on the Avicel medium and incubated for 13 days at 28°C. The colony diameter was measured by a digital caliper (Mitsutoyo, Koshigaya, Japan) to calculate the average value. It was found that the colony diameter of strain B5 was 1.56 times larger than that of the original strain, as shown in Table 1. Figure 1 shows the superior proliferation characteristics of strain B5 compared with the original strain.

The five selected strains and the original strain were compared in filter paper degrading ability. A mycelial block (2×2 mm^2) of the selected strains or the original strain was added to 50 mL of the medium for enzyme production in a 100-mL Erlenmeyer flask, followed by incubation for 5 days using a rotary shaker (TAITEC NR-30) at 28°C. The agitation speed was 160 rpm. Two flasks were used for a strain. Mandels' medium containing 1.0% (w/v) Avicel and 0.5% (w/v) peptone was used as the medium for enzyme production. 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 0.1 N HCl, and the filter paper degrading ability of the filtrate was evaluated. The addition of 0.1 N HCl did not collapse the filter paper. 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) at an agitation speed of 75 strokes per minute. The time of collapse of the filter paper was then measured using a digital stopwatch (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 another filter paper (no. 2 Whatman), and the amount of reducing sugar in the filtrate was measured using 3,5-dinitrosalicylic acid (Wako) [9]. As shown in Table 2, strain B5 collapsed the filter paper within 15 min, but the original strain took 25 min. Moreover, the amount of reducing sugar in the L-type glass tube of strain B5 was over four times larger than that of the original strain. These results indicate that the filter paper degrading ability of strain B5 is over four times greater than that of the original strain.

From the above results, the cellulose hydrolyzing activity of strain B5 was compared with that of the original strain. As the substrates of enzyme reaction, 1.0 g of Avicel, 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,

Table 1 The colonial diameter of the original strain and the selected strains, B1–B5.

Strains	Colonial diameter (mm)
T. reesei Rut C-30	28.32±1.03
B1	41.25±1.23
B2	34.03±1.18
B3	36.23±1.38
B4	35.41±1.05
B5	44.24±1.15

A mycelial mat (2×2 mm^2) was incubated on the Avicel medium for 13 days at 28°C. The colonial diameter was measured by a digital caliper. Two plates were used per strain.

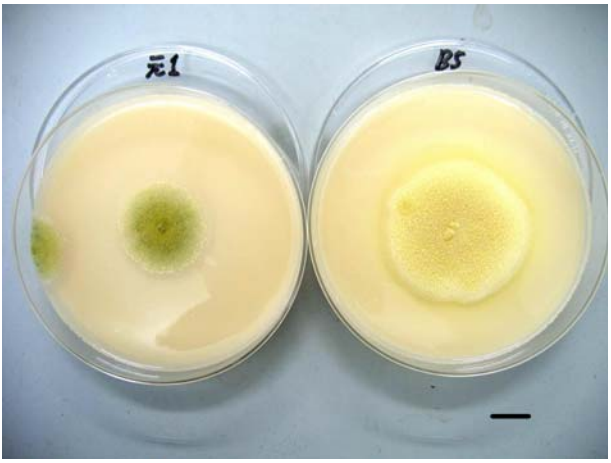

Fig. 1 Colonies of the original strain and strain B5. *Left*: *T. reesei* Rut C-30. *Right*: strain B5. Mycelial blocks (2×2 mm^2) of the original strain and strain B5 were incubated on Avicel plates for 13 days at 28°C. *Bar* indicates 1.0 cm

Japan) for 1 h at 50°C at an agitation speed of 125 strokes per minute. The glass tubes containing the Avicel substrate were tilted on the shaker and shaken by hand every 30 min to avoid the precipitation of Avicel. The reaction mixture was filtered with filter paper (Whatman, no. 2), and the amount of reducing sugar was measured using 3,5-dinitrosalicylic acid. Activity was defined as the amount of enzyme producing reducing sugar equivalent to 1 μmol of glucose per minute. Consequently, the Avicel and Salicin hydrolyzing activity of strain B5 increased by a factor of 1.4 times and 3.0 over the original strain, respectively, as shown in Table 3. The CMC-Na hydrolyzing activity of strain B5, however, was almost the same as that of the original strain. Moreover, the mycelial amount of strain B5 decreased less than that of the original strain.

We discuss here why strain B5 showed superior proliferation on an Avicel medium. Strain B5 is one of the colonies that appeared earlier on the double-layer selection medium. This suggested that strain B5 could rapidly saccharify the Avicel in the selection medium. Hence, we suspected that strain B5 possesses higher Avicel hydrolyzing ability, and the results of the measurement of enzyme activity confirmed this. Thus, we considered that strain B5 could quickly break through the selection medium containing Avicel and show superior proliferation on an Avicel medium because of its higher Avicel and Salicin

Table 2 Evaluation of degrading ability of a filter paper.

Strains	Collapse time of a filter paper (min)	Amount of reducing sugar (mg)
T. reesei Rut C-30	25	0.425
B1	22	0.975
B2	24	0.774
B3	24	0.652
B4	25	0.682
B5	15	1.730

A filter paper (10×10 mm^2) was added to 5 ml of enzyme solution in an L-type glass tube and incubated at 50°C using a Monod shaker. After 15 min of shaking, the amount of reducing sugar was measured using 3,5-dinitrosalycilic acid.

Table 3 Measurement of cellulose hydrolyzing activity.

Strains	Avicel hydrolyzing activity	CMC-Na hydrolyzing activity	Salicin hydrolyzing activity (IU/ml)	Mycelial weight (mg)
T. reesei Rut C-30	19	68	2	320
B5	26	67	6	283

A mycelial mat (2×2 mm^2) was incubated in the medium for enzyme production in a 100-ml Erlenemyer flask for 5 days at 28°C using a rotary shaker. The amount of reducing sugar was measured using 3, 5-dinitrosalicylic acid. Two flasks were used for a strain. These values are average.

hydrolyzing ability. The higher filter paper degrading ability of strain B5 also seems to be owed to its higher Avicel and Salicin hydrolyzing ability.

Next, we discuss why Avicel and Salicin hydrolyzing activity increased more than those of the original strain. In this study, unsynchronized green mature conidia were used. When such conidia were treated with colchicine, their nuclear diameter and number varied (data not shown). We consider that the cellulose hydrolyzing ability of such conidia also varies. We therefore think that strain B5 possessing higher Avicel and Salicin hydrolyzing ability was selected through the selection using the double-layer selection medium among such conidia.

The nuclear diameter and number of conidia of strain B5 were compared with those of the original strain. The diameter and number of conidia were observed using Giemsa staining after 5 N HCl treatment for 40 min at 60°C, after which, microphotographs were taken [10]. The conidia of strain B5 was mononucleate similar to the conidia of the original strain shown in Fig. 2 [11]. However, the nuclear diameter of strain B5 enlarged more than that of the original strain. From these results, strain B5 seemed to be polyploid or aneuploid. When strain B5 was incubated on an Avicel medium, no sector was segregated, which means that this strain is genetically stable and polyploid. Therefore, the increase of Avicel and Salicin hydrolyzing ability of strain B5 is suspected to be related with autopolyploidization. From these results, we conclude that the strain with higher filter paper degrading ability and superior proliferation characteristics can be rapidly selected using Avicel plates and the double-layer selection medium.

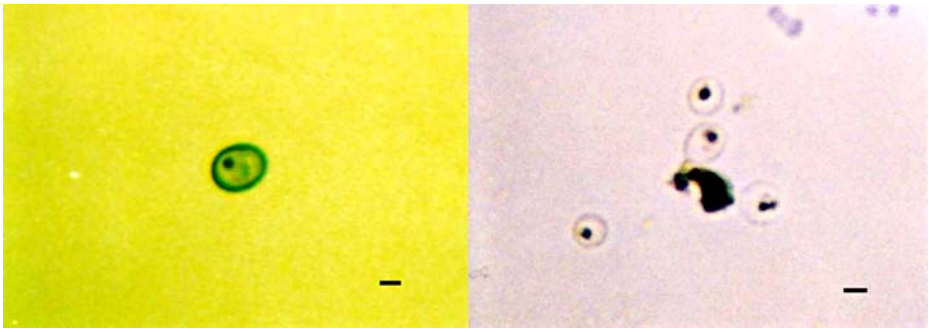

Fig. 2 Nuclear staining of the conidia derived from the original strain and strain B5. *Left*: *T. reesei* Rut C-30. *Right*: strain B5. Conidia were fixed on a slide glass by heating and treated with HCl at 60°C. After HCl treatment, conidia were washed with water followed by nuclear staining using Giemsa solution. *Bar* indicates 1.0 μm

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