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Rapid Selection System of Strains with Higher Avicel Degrading Ability in a Cellulolytic Fungus, *Trichoderma*

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Abstract

We have attempted to develop an active selection system for strains that have a higher potential for Avicel degradation using haploidized conidia from colchicine-treated *Trichoderma reesei* Rut C-30 as a model strain. Avicel, absorbent cotton, and wood powder were used as substrates for selection. It appeared that the strains that degrade Avicel actively could be effectively selected when the solid medium containing the selection substrate and the liquid medium containing Avicel were used.

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

Introduction

The cellulolytic fungus *Trichoderma* is well known to have the potential for hyperproduction of cellulase *(1)*. The cellulase of this fungus is used for the textile industry, food processing, and waste paper utilization *(2,3)*. We have already reported the ability to increase cellulase production by *Trichoderma* without genetic engineering techniques *(4)*. In this breeding system, the gene sources of this fungus including cellulase genes can be amplified with the mitotic arrester, colchicine. Genetic recombination using such amplified gene sources can be caused by the haploidizing reagent Benomyl.

This breeding system was first developed for the production of cellulase for food processing. However, it may be possible for this system to construct cellulase hyperproducers for the bioconversion of cellulose sources. In this study, we investigated the possibiliy of active selection of

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the strains with the higher degrading ability of Avicel for the purpose of cellulose bioconversion. Experiments were designed to develop the active selection system of the strains with higher Avicel-degrading ability.

Materials and Methods

Microorganism and Media

Trichoderma reesei Rut C-30 (ATCC 56765) was used as a model strain. The basic medium was Mandels' medium containing 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 $MgSO_4{\cdot}7H_2O$, 0.0050 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 $CoCl_2$, and 1000 mL of distilled water (all from Wako, Osaka, Japan) *(5)*. The strain was incubated on 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) at 26°C and preserved at 4°C. The experiments were carried out using the strain within two generations from the dried conidia of the American Type Culture Collection.

The medium for autopolyploidization was the Mandels' medium containing 0.1% (w/v) colchicine (Wako), 0.5% peptone, and 1.0% (w/v) glucose (Wako) (pH 6.0). The medium for haploidization was the potato dextrose agar medium (BBL, Cockeysville, MD) containing 0.6 μg/mL of benomyl (Sigma, St. Louis, MO) (pH 6.0) *(6)*. For the measurement of cellulose hydrolyzing activity, 7.5 mL of distilled water was added to 7.5 g of wheat bran in a 100-mL Erlenmeyer flask.

For the medium for the primary selection, 96 mL of Mandels' medium containing 1.0% Avicel, 0.5% peptone, 1.0% (w/v) substrate, 0.1% (v/v) polyoxyethylene octylphenylther (Triton X-100) (Wako), 1.5% agar, and conidia (the bottom layer medium) was added to a deep glass plate (150 mm in diameter and 60 mm deep) and left at 4°C in order to harden the agar. After hardening the agar, 196 mL of Mandels' medium containing 1.0% Avicel, 0.5% peptone, 1.0% substrate, 0.1% Triton X-100, and 1.5% agar (the upper layer medium) was added on the bottom layer medium followed by hardening the agar at 4°C. As the substrates for selection, Avicel, wood powder (*Fagus crenata*), and absorbent cotton (for medical use) were used. As the Avicel liquid medium for the secondary selection, the Mandels' medium containing 1.0% (w/v) Avicel and 0.5% peptone was used (pH 5.0).

Autopolyploidization

A mycelial mat (10 × 20mm) was added to the medium for autopolyploidization in a glass test tube (16.5 × 165mm) with a Silico stopper and incubated stationarily for 35 d at 26°C. Autopolyploidization was confirmed by nuclear staining of mycelial mat using Giemsa solution (Merck, Darmstadt, Germany).

Haploidization

A mycelial mat (2 × 2 mm) was put on the medium for haploidization followed by incubation for 3 wk at 28°C. Haploidization was confirmed by formation of a fan-shaped sector.

Primary Selection

The conidia generated on the haploidized colony were incubated in the medium for primary selection for 6 d at 28°C. After incubation, the colonies that could break thorough the selection layer were used for the secondary selection.

Secondary Selection

A mycelial mat (2 × 2 mm) of the colonies selected by the primary selection was added to the medium for secondary selection and incubated using a rotary shaker (TAITEC NR-30), (80 rpm) at room temperature (16–23°C). After the Avicel liquid medium became transparent, the amount of Avicel sedimentation was observed by leaving it for 1 h.

Measurement Hydrolyzing Activity of Cellulose

A mycelial mat (2 × 2 mm) was added to flasks of the solid medium for the measurement of hydrolyzing activity of cellulose and incubated at 28°C for 6 days. 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. As the substrates of enzyme reaction, 1.0 g of Avicel, CM-cellulose (CMC) (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 60 min at 40°C using a reciprocal shaker (THOMASTAT T-22S). 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 a Glucose CII test (Wako). One international unit was based on the amount of enzyme-producing reducing sugar equivalent to 1 μmol of glucose/min.

Results

Autopolyploidization

A mycelial mat of *T. reesei* Rut C-30 was incubated in the medium for autopolyploidization for 35 d at 28°C. After treatment with colchicine, it appeared that autopolyploid nuclei were produced in the mycelia after

nuclear staining. The nuclei varied in size because those nuclei were not synchronized. Those autopolyploid nuclei existed stably in the mycelia for at least five generations.

A small piece (3 × 3 mm) of the colchicine-treated mycelial mat was incubated on Mandels' medium containing 1.0% Avicel, 1.5% agar, and 0.5% peptone at 26°C and preserved at 4°C before conidial formation.

Haploidization

A mycelial mat of the colchicine-treated mycelial mat was incubated on the medium for haploidization. After incubation, fan-shaped sectors were produced. The conidia generated on the benomyl-treated colony were regarded as genetic recombinants.

Primary Selection

Ten loopfuls of the conidia generated on the haploidized colony were incubated in the medium for primary selection for 6 d at 28°C. The colonies that could break through the selection layer were used for the secondary selection. Ten colonies were selected on the selection medium containing Avicel, four on the selection medium containing absorbent cotton, and four on the selection medium containing wood powder.

Secondary Selection

A small mycelial piece of the strain was added to the Avicel liquid medium and incubated using a rotary shaker for 6 d at room temperature. *T. reesei* Rut C-30 took 6 d to make the Avicel liquid medium transparent whereas strains selected by Avicel, AV-1 and AV-2, took 4 d to make the medium perfectly transparent, as shown in Fig. 1. The strain selected by absorbent cotton, AB-1, and those selected by wood powder, BU-1 and BU-2, could also make the medium transparent after 4 d of incubation.

Measurement of Hydrolyzing Activity of Cellulose

All of the selected strains were compared with *T. reesei* Rut C-30 for hydrolyzing activity of cellulose by wheat bran culture. Although only the hydrolyzing activity of Avicel increased in AV-1 selected by Avicel, all Avicel, CMC, and Salicin hydrolyzing activity increased in AV-2 as shown in Table 1. In the strain AB-1 selected by absorbent cotton, Avicel hydrolyzing activity increased especially. In the strains BU-1 and BU-2 selected by wood powder, all Avicel, CMC, and Salicin hydrolyzing activity increased.

Discussion

At first, we considered how the strains with higher Avicel-degrading ability could be selected by the medium for primary selection containing Avicel. That medium has a selection layer (10 mm depth) containing 1% Avicel. If the strains lacked the ability for Avicel degradation, they could

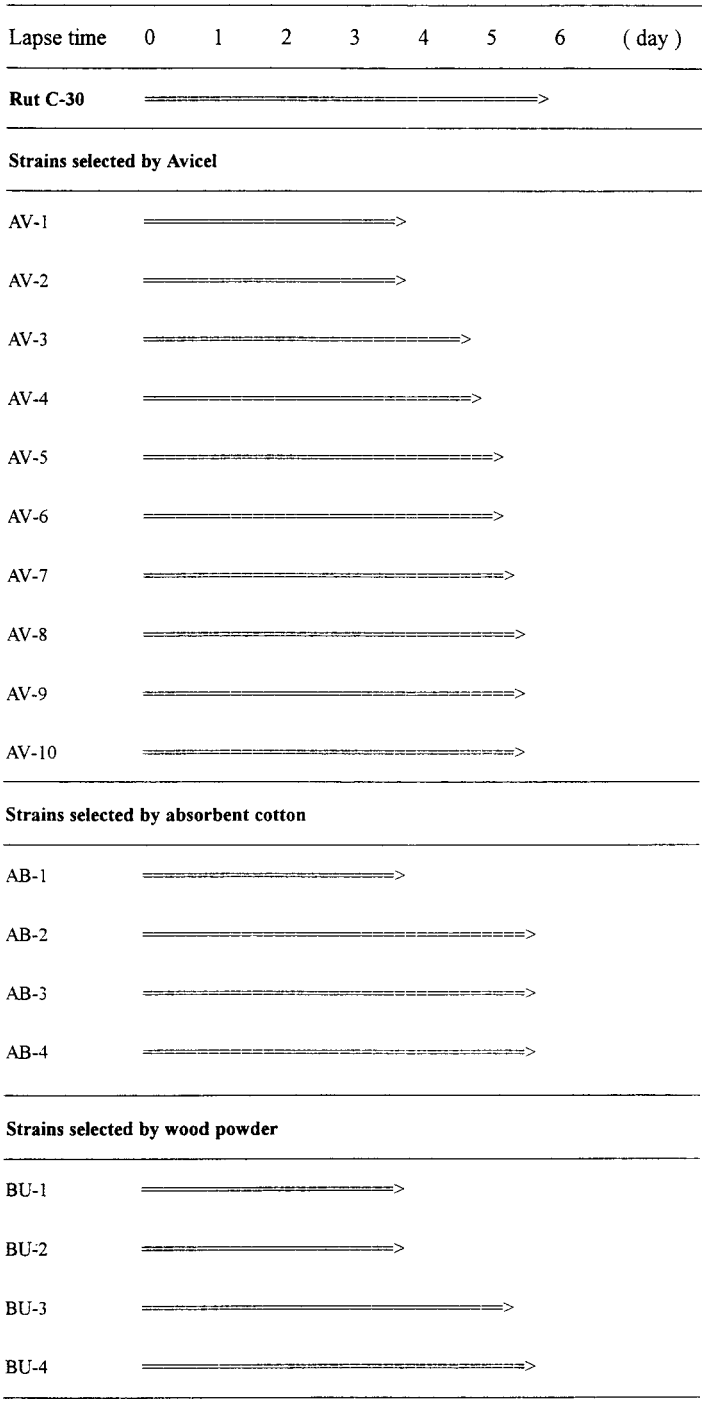

Fig. 1. Results of secondary selection, Avicel liquid medium test. A mycelial mat (2 × 2 mm) was added to the liquid Mandels' medium containing 1.0% (w/v) Avicel and 0.5% (w/v) peptone (pH 5.0) and incubated for 6 d at room temperature (16–23°C) using a rotary shaker (80 rpm). The degree of transparency of the medium and the amount of Avicel sedimentation were compared every day.

Table 1
Hydrolyzing Activity of Cellulose of Strains
Selected by Avicel, Absorbent Cotton, and Wood Powder[a]

	Avicel (IU/mL)	CMC (IU/mL)	Salilcin (IU/mL)
Rut C-30	35.4	28.3	20.1
Strains selected by Avicel			
AV-1	74.8	29.5	21.6
AV-2	49.2	47.2	57.1
AV-3	47.2	39.4	35.4
AV-4	37.4	41.3	39.4
AV-5	31.5	55.1	21.6
AV-6	29.5	11.8	45.2
AV-7	29.5	23.6	49.2
AV-8	27.6	21.6	15.7
AV-9	23.6	45.3	20.0
AV-10	15.7	31.5	25.9
Strains selected by absorbent cotton			
AB-1	53.1	27.6	29.5
AB-2	39.4	25.6	25.6
AB-3	35.7	22.0	21.8
AB-4	33.9	15.7	20.0
Strains selected by wood powder			
BU-1	74.8	57.1	34.0
BU-2	66.9	61.0	66.9
BU-3	39.7	21.6	30.0
BU-4	27.9	23.8	23.3

[a] AV lines were selected by Avicel, AB lines were selected by absorbent cotton, and BU lines were selected by wood powder.

not break through the Avicel layer and could not have produced colonies on the surface of the medium within 6 d. Therefore, we suspected that only the strains with higher Avicel-degrading ability were selected and could break through the selection layer, followed by colony formation.

Next, we considered why the strains with higher Avicel-degrading ability could be estimated by the secondary selection. It was found that *Trichoderma* has the ability to make Avicel liquid medium transparent when the fungus is incubated by slow rotary shaking (data not shown). For the purpose of estimating the degree of transparency, the amount of Avicel sedimentation and the transparency of the medium were compared after incubation. Since the time for making the medium transparent apparently differed in each strain, we concluded that this secondary selection can estimate the strains with higher Avicel-degrading ability.

Only the hydrolyzing activity of Avicel increased in the strain selected by absorbent cotton, AB1. Since the strains with higher Avicel, CMC, and

Salicin hydrolyzing activity could be selected using the selection medium containing wood powder or Avicel, the selection possibility of such strains seemed not to be lower in the selection medium containing absorbent cotton. In the strains selected by wood powder, BU-1 and BU-2, all of the hydrolyzing activity of cellulose was higher. Because wood powder is a lignin-cellulose complex, such structure seemed to contribute to the selection of such strains *(7)*. In other words, the higher hydrolyzing activity of Avicel, CMC, and Salicin might be needed in order to break through the selection layer containing wood powder. Moreover, we suspected that the strains that had only a higher hydrolyzing activity of Avicel took a longer time to appear on the surface of the selection medium.

In the primary selection, the strains with lower hydrolyzing activity were also obtained. It was suspected that such strains were derived from the conidia contaminated in the selection layer. It was concluded that the strains with higher Avicel-degrading ability could be selected actively by our selection system. Further investigations should be conducted using various types and concentrations of substrates.

Acknowledgments

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