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Construction of cellulase hyper-producers of *Trichoderma reesei* Rut C-30 for utilization of waste paper using colchicine and benomyl

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Abstract

Autopolyploidization and haploidization techniques were used in an attempt to obtain cellulase hyper-producers for the utilization of waste paper. The mycelial mat of *Trichoderma reesei* Rut C-30 treated with 0.1% colchicine solution for 35 days was haploidized by benomyl. Cellulase hyper-producers were selected from conidia generated on the haploidized colonies using the primary selection medium containing 1.0% (w/v) waste paper powder and the secondary selection medium containing 1.0% (w/v) Avicel. The hydrolyzing activities of the selected strain, WP-1, toward Avicel, CMC, and Salicin were 1.9, 2.0, and 3.5 times higher than those of the original strain, respectively. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cellulase; *Trichoderma reesei*; Colchicine; Benomyl; Waste paper

1. Introduction

The filamentous fungus, *Trichoderma reesei*, is a cellulolytic fungus and its cellulase is stable [1]. Moreover, the cellulase has all components necessary for cellulose hydrolysis. So, this fungus is widely utilized for industrial production of cellulase [2]. Waste paper is regarded as one of the wastes which can be recycled [3]. One of the means of recycling it is bioconversion by enzyme.

We reported that a mitotic arrester, colchicine, can produce autopolyploid nuclei, and that a haploidizing reagent, benomyl, can cause genetic recombination using amplified gene sources in *Trichoderma* [4]. This breeding method was developed as a breeding system without genetic engineering techniques in order to produce cellulase for food processing. In this report, we attempted to investigate the possibility of constructing cellulase hyper-producers for the utilization of waste paper.

2. Experimental

2.1. Microorganism and media

T. reesei Rut C-30 (ATCC 56765) was used as a model strain. The experiments were carried out using the strain within two generations from the dried conidia of the American Type Culture Collection. The basic medium was Mandels' medium, containing 1.4 g $(NH_4)_2SO_4$ (Wako, Osaka, Japan), 2.0 g KH_2PO_4 (Wako), 0.3 g urea (Wako), 0.3 g $CaCl_2$ (Wako), 0.3 g

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$MgSO_4 \cdot 7H_2O$ (Wako), 5.0 mg $FeSO_4 \cdot 7H_2O$ (Wako), 1.6 mg $MnSO_4 \cdot H_2O$ (Wako), 1.4 mg $ZnSO_4 \cdot H_2O$ (Wako), 2.0 mg $CoCl_2$ (Wako) in 1000 ml distilled water [5]. The strain was incubated on Mandels' medium containing 1.0% (w/v) Avicel (Funakoshi, Tokyo, Japan), 0.5% (w/v) peptone (Difco, MI, USA), and 1.5% (w/v) agar (Difco) at 26 °C and preserved at 4 °C. For colchicine treatment, Mandels' medium containing 0.1% (w/v) colchicine (Wako), 0.5% (w/v) peptone, and 1.0% (w/v) glucose (Wako) (pH 6.0) was used. The medium for haploidization was the potato dextrose agar (PDA) medium (BBL, MD, USA) containing 0.6 μg/ml benomyl (Sigma, MO, USA) (pH 6.0). For the primary selection, 100 ml of Mandels' medium containing 1.0 g waste paper powder, 0.5 g peptone, 0.1 ml (v/v) polyoxyethylene(10)octylphenylether (Triton X-100) (Wako), 1.5 g agar, and conidia (the bottom layer medium) was added to a deep glass plate (150 mm in diameter and 60 mm in depth) and left at 4 °C in order to harden the agar. After the hardening, 200 ml of Mandels' medium containing 2.0 g waste paper powder, 1.0 g peptone, 1.0 g substrate, 0.1 ml Triton X-100, and 1.5 g agar (the upper layer medium) was added to the bottom layer medium followed by hardening of agar at 4 °C. As the substrate for selection, powder of ground waste paper was used. The ground waste paper was obtained from Prof. Hideki Fukuda (Kobe University). For the secondary selection, 50 ml of Mandels' medium (containing 0.5 g Avicel and 0.25 g peptone) was added to a 100 ml Erlenmeyer flask with a Silico stopper. For the measurement of cellulose hydrolyzing activity, 7.5 ml distilled water was added to 7.5 g of wheat bran in a 100 ml Erlenmeyer flask with a Silico stopper.

2.2. Colchicine treatment

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

2.3. Benomyl treatment

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

2.4. Primary selection

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

2.5. Secondary selection

A mycelial mat (2 mm × 2 mm) of the colonies, selected by the primary selection, was added to the medium for secondary selection and incubated for 6 days by a rotary shaker (TAITEC NR-30, Koshigaya, Japan) (80 rpm) at room temperature (16–24 °C; average, 20 °C). After the Avicel liquid medium became transparent, the amount of Avicel sedimented was observed after leaving it to stand for 1 h.

2.6. Cellulose hydrolyzing activity

A mycelial mat (2 mm × 2 mm) was inoculated to a flask containing the wheat bran-solid medium for the measurement of cellulose hydrolyzing activity and incubated at 28 °C for 6 days. The flask was 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 supernatant was used as the enzyme solution. As the substrate of enzyme reaction, 1.0 g of Avicel, CM-cellulose (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, Tokyo, Japan). 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 Glucose CII test (Wako). One unit (U) was defined as the activity that produced reducing sugar equivalent to 1 μmol of glucose per minute.

3. Results

3.1. Colchicine treatment

After the colchicine treatment, autopolyploid nuclei were produced in the mycelia. The nuclei varied in size. Those autopolyploid nuclei existed stably in the mycelia for at least five generations. A small piece (3 mm × 3 mm) of the colchicine-treated mycelial mat was incubated on Mandels' medium containing 1.0% (w/v) Avicel, 1.5% (w/v) agar, and 0.5% (w/v) peptone at 26 °C and preserved at 4 °C.

3.2. Benomyl treatment

After the incubation on the medium for benomyl treatment, colonies with fan-shaped sectors appeared. The conidia generated on the benomyl-treated colony were regarded as genetic recombinants.

3.3. The primary selection

Ten loopfuls of the conidia generated on the haploidized colony were incubated in the medium for primary selection for 6 days at 28 °C. The colonies which could break through the selection layer were used for the secondary selection. Four larger colonies were selected on the selection medium containing waste paper powder.

3.4. Secondary selection

A small piece of the strain was added to the medium for secondary selection and incubated by a rotary shaker at room temperature (16–24 °C; average, 20 °C). The result is shown in Fig. 1. *T. reesei* Rut C-30 took 6 days to make the Avicel liquid medium transparent, whereas strains selected by waste paper, WP-1, took 4 days to make the medium perfectly transparent. WP-2, WP-3, and WP-4 could make the medium perfectly transparent within 5 days.

3.5. Measurement of cellulose hydrolyzing activity

The hydrolyzing activities of WP-1 toward Avicel, CMC, and Salicin were 1.9, 2.0, and 3.5 times higher than those of C-30, respectively (Table 1). In the strain WP-2, only the CMC hydrolyzing activity was 1.4 times higher than that of C-30. The CMC and Salicin hydrolyzing activities of WP-3 and WP-4 were higher than those of C-30.

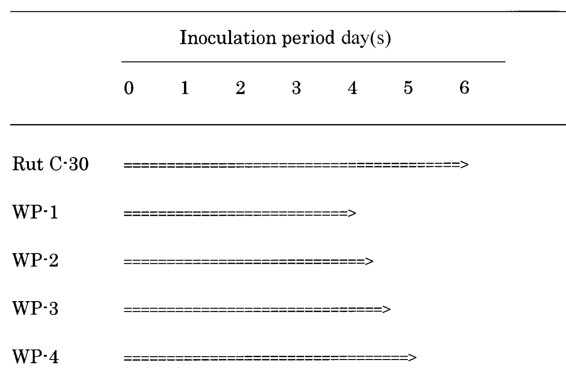

Fig. 1. Results of Avicel liquid medium test. A mycelial mat (2 mm × 2 mm) of the colonies, selected by the primary selection, was added to the medium for secondary selection and incubated by a rotary shaker at room temperature (average 20 °C) until the Avicel liquid medium becomes transparent. Arrow heads show that the Avicel liquid medium became transparent.

Table 1
Cellulose hydrolyzing activities of the strains selected by screening on medium containing waste paper

Activity (U/ml)

Strain	Avicel	CMC	Salicin
Rut C-30	35	28	20
WP-1	67	57	69
WP-2	34	38	22
WP-3	22	34	31
WP-4	20	40	34

A mycelial mat (2 mm × 2 mm) was added to flasks of the solid medium for the measurement of cellulose hydrolyzing activity and incubated at 28 °C for 6 days. As the substrate of enzyme reaction, 1.0 g of Avicel, CM-cellulose, or Salicin 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.

4. Discussion

When the mycelial mat was treated with colchicine, autopolyploid nuclei were produced, as shown in our previous paper [6]. In these experiments, the mycelial

mat treated for 35 days with 0.1% colchicine solution was used. The nuclear diameter in the mycelia, as well as the DNA content of the mycelial mat increased (data not shown). Therefore, the colchicine-treated mycelial mat was regarded as autopolyploids. Benomyl is known to delete chromosomes from polyploid nuclei through chromosomal recombination [7]. The conidia generated on the benomyl-treated colony were, therefore, regarded as genetic recombinants.

At first, we discussed why such cellulase hyper-producers could be generated. When colchicine-treated mycelial mat was treated by benomyl, color mutants and colonies with fan-shaped sectors appeared (data not shown). These strains are genetically different from the original one, showing that they were produced through genetic recombination in haploidization. We considered from these results that cellulase hyper-producers were also produced through genetic recombination.

Next, we considered the possibility that these cellulase hyper-producers were generated by mutation by colchicine or benomyl. If such mutation occurred in the colchicine-treated mycelia or benomyl-treated mycelia, all conidia of the colchicine-treated mycelial mat or benomyl-treated mycelial mat should have higher cellulase productivity. But, such tendency was not observed in these experiments. Moreover, such mutation tended to decrease cellulase productivity (data not shown). Therefore, we suspected these cellulase hyper-producers were not produced by mutation.

We considered the strains carrying higher Avicel-degrading ability could be selected by the medium for primary selection containing Avicel. The medium has a selection layer (depth = 10 mm) containing waste paper powder. If the strains lacked the ability for waste paper degradation, they could not break through the waste paper layer and could not have produced colonies on the surface of the medium within 6 days. Thus, only the strains with higher degrading ability of waste paper were effectively selected.

We also considered why the cellulase hyper-producers could be selected by the secondary selection. It was found that *Trichoderma* had the ability to make Avicel liquid medium transparent when the fungus was incubated by slow rotary shaking (data not shown). As the results reflected the cellulase productivity in each strain, this method was used for selection of cellulase hyper-producers. The secondary selection of cellulase hyper-producers using waste paper instead of Avicel should be carried out for further investigation.

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