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Isolation and Characterization of Intraspecific Complementing Fusants of *Penicillium verruculosum*

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*Penicillium verruculosum*의 종내원형질 융합체의 분리 및 특성

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The possibility of strain improvement of cellulolytic fungus, *Penicillium verruculosum* via protoplast fusion was investigated. The cellulolytic activities of the six fusants, finally selected for their hyper-cellulolytics were 2 times of those of wild type and 1.2 to 4.4 times of those parental auxotrophs. It was confirmed that the nuclear fusion occurred in fusants by their DNA contents and nuclear staining with Giemsa. It was also found that the fusants were aneuploids, and their genetic stability was demonstrated from the subculture for four months.

Protoplast fusion technique has proved to be a very efficient procedure for generating genetic recombination through intra-and interspecific transfer of nuclear genes in fungi(1-4).

In intraspecific fusion of filamentous fungi, heterokaryon formation is the basic and most frequent events leading to complementation, occasionally followed by the production of transient or stable diploids. Heterokaryosis, as the only known means of complementation after protoplast fusion, was reported with *Phycomyces blakesleeanus*(5) and *Mucor racemosus*(6). Similarly, in *Cephalosporium acremonium,* protoplast fusion of complementing auxotrophic mutant strains resulted in heterokaryons(7). Diploidization also occurred, but this stage proved very unstable, yielding haploid recombinants. In this way, strain improvement for higher cephalosporin production was also achived(8). Recently, complementing heterokaryons were formed by the protoplast fusion of auxotrophic mutants of celluloytic fungus, *Trichoderma reesei* and *T. koningii*(9-11).

Penicillium verruculosum F-3 has been assessed as one of the most promising candidates for microbial conversion of cellulosic biomass(12). We have already reported on the conditions for the preparation, regeneration and fusion of protoplasts from the mycelium of *P. verruculosum*(13,14). Here we report heterokaryon formation through fusion of protoplasts of *P. verruculosum* and discuss the possibility of applicating this technique to enhancement of cellulase productivity. Some characteristics of the complementing intraspecific fusants were also described.

Materials and Methods

Organisms and media

P. verruculosum F-3(IFO 31136) and its auxotrophic mutants(14) PV 1(met), PV 2(phe), and PV 3(cys) were used in this experiments. Potato dextrose agar(PDA) medium and modified Mandels' medium(15) containing 1% glucose were used as the complete(CM) and minimal medium

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(MM), respectively. As regeneration complete medium(RCM) and regeneration minimal medium (RMM), 0.6M magnesium sulfate was supplemented to CM and MM, respectively. In preparing regeneration media, 2% agar was autoclaved separately to avoid acid hydrolysis of agar.

Preparation and fusion of protoplasts

The preparation and fusion of protoplasts were performed by the methods described in the previous report(14).

Selection of hyper-cellulolytic fusants

One loopful of conidia was inoculated to a test tube (0 1.8 × 20cm) with the 5m*l* of the A-3 medium (12). A filter paper strip(Whatman No. 1, 1 × 10cm) was supplied as carbon source instead of Avicel. The cultures were incubated at 30°C for 7-12 days on a reciprocal shaker (rpm 110, 2.5cm). The fusants having high-filter paper degradation activity than wild and parental strains were selected. The fusants that showed high enzyme productivity were continuously selected during subculture for 4 months. The selected hyper-cellulolytic fusants was cultured on the KC-M-W medium(16) at 30°C for 2-14 days. The resulting supernatant of the culture was again assayed for filter paper activity.

Assay for filter paper degradation activity

Filter paper degradation activity was assayed as the method described in Mandels *et al.*(17). One unit of the enzyme activity was defined as the amount of enzyme which released 1 μ mol of reducing sugar as glucose per min under the assay conditions.

Genetic stability

Genetic stability of the fusants having high-enzyme productivity was tested by the methods of Seu *et al.*(18). That is, conidiospores of the fusants were suspended in saline solution(2×10^7 spores/m*l*) and diluted to about 70-100 colonies per petridish. The spores were plated on CM and incubated at 30°C for 5-6 days. When the colonies appeared on CM, they were plated with replica on CM and MM, and then segregants that appeared on CM but not on MM were isolated. Frequency of auxotrophic segregation was estimated by the following equation.

$$\text{Frequency of auxotrophic segregation}(\%) = \left(1 - \frac{\text{Number of colonies on MM}}{\text{Number of colonies on CM}}\right) \times 100$$

Determination of DNA content in the conidia

Each strain was grown at 30°C on CM and MM. After 4 to 5 days of incubation, the conidia were suspended in sterilized water at 10^7 spores/m*l*, and the DNA fraction was prepared by the method of Steward(19) and the DNA content was determined spectrophotometically by the method of Burton (20). Herring sperm DNA(Sigma) was used as standard.

Nuclear staining

Nucleus of the conidial protoplasts was stained with Giemsa stain by the method of Hong *et al.*(21). The specimen was examined under a multipurpose light microscope and photographed (Nikon Nippon Kogaku K.K., Japan).

Results and Discussion

Selection of hyper-cellulolytic fusants

Intraspecific complementing fusants were successfully produced through fusion of protoplasts from the mycelium of different auxotrophic mutants of *P. verruculosum* F-3. The intraspecific fusion frequency ranged 1.8×10^{-3} to 3.5×10^{-3} (14).

To elucidate the possibility of strain improvement in the cellulolytic fungus, *P. verruculosum* via protoplast fusion, extracellular cellulase activities of the fusants were measured. The fusants having high-filter paper degradation activity than wild and parental strains were selected. Finally, six strains that showed high cellulase productivity continuously in subculture for four months were again selected as hyper-cellulolytics out of the 165 fusants. As shown in table 1, the cellulase activities of the 6 fusants were 2 times of those of wild type and 1.2 to 4.4 times of those of parental auxotrophs.

Table 1. Extracellular cellulase productivity of various parental strains and their fusants obtained from intraspecific protoplast fusion.

Strain	FP activity (U/ml)
P. verruculosum	0.20
PV 1(met)	0.35
PV 2(phe)	0.10
PV 3(cys)	0.36
FPV 1201[a]	0.44
FPV 1213[b]	0.42
FPV 1367[c]	0.45
FPV 2301[d]	0.42
FPV 2313[e]	0.43
FPV 2316[f]	0.42

[a] and [b]: PV 1(met) × PV 2(phe), [c]: PV 1(met) × PV 3(cys), [d,e,] and [f]: PV 2(phe) × PV 3(cys).

Recently, Toyama *et al.*(9) reported that they produced the fusants of auxotrophic mutants of *T. reesei* having twice the carboxymethyl cellulose hydrolyzing activity of the parents. Park *et al.*(11) also reported that the interspecific hybrids of *Trichoderma* revealed partially enhanced cellulolytic activities.

Genetic stability

After fusion induction, the first stage of complementation, if auxotrophic nuclear markers are employed, is heterokaryon formation. The heterokaryotic state can be either permanent or transient if the fusants are kept under the selective pressure of a minimal medium, but transient in complete media. The heterokaryons were unstable in nutrionally complete medium and readily dissociated into the parental mutants. Diploids arose from the haploid heterokaryons, and somatic segregants and recombinants could be obtained from the diploids.

The six fusants, finally selected for their hypercellulolytic activity were checked for their genetic stability over twelve generations. As shown in Table 2, they were very stable genetically with segregation rate less than 1% through the subculture for four months.

Table 2. Genetic stability of fusants.

Strain	Colonies on		% of segregants
	CM	MM	
FPV 1201	1.25×10^7	1.25×10^7	0
FPV 1213	1.10×10^7	1.10×10^7	0
FPV 1367	1.09×10^7	1.08×10^7	0.9
FPV 2301	1.18×10^7	1.18×10^7	0
FPV 2313	1.11×10^7	1.11×10^7	0
FPV 2316	1.38×10^7	1.38×10^7	0

Determination of DNA contents in the conidia

The conidial DNA fractions of the fusants were prepared and the DNA content was determined spectrophotometically. Karyotypes of the six fusants were considered to be aneuploid on the basis of DNA content(Table 3).

Nuclear straining

The nucleus of the protoplast was easily observed when the glutaraldehyde-fixd protoplasts were allowed to soak in 5N HCl for 10 min at 60 °C and stained with Giemsa solution for 1hr, and washed with 95% ethanol. The results of nuclear staining with Giemsa also showed that fusants had larger nuclei than the parents(Fig. 1).

Segregation in the progeny, measurement of DNA content, and nuclear staining with Giemsa strongly indicated that heterokaryons formed first and then nuclear fusion followed. It was concluded

Table 3. DNA contents of parental strains and fusants obtained from intraspecific protoplast fusion.

Strain	DNA/10^7 conidia (ng)	Ploidy (n)*)
P. verruculosum	121.1 ± 0.19	1
PV 1(met)	127.5 ± 0.33	1
PV 2(phe)	119.3 ± 0.26	1
PV 3(cys)	120.2 ± 0.12	1
FPV 1201	155.7 ± 0.12	1.26
FPV 1213	195.8 ± 0.14	1.59
FPV 1367	204.0 ± 0.12	1.65
FPV 2301	171.2 ± 0.13	1.43
FPV 2313	201.3 ± 0.39	1.68
FPV 2316	166.7 ± 0.13	1.39

*) For fusants, haploid(n) value based on average of parents PV 1, PV 2 and PV 3.

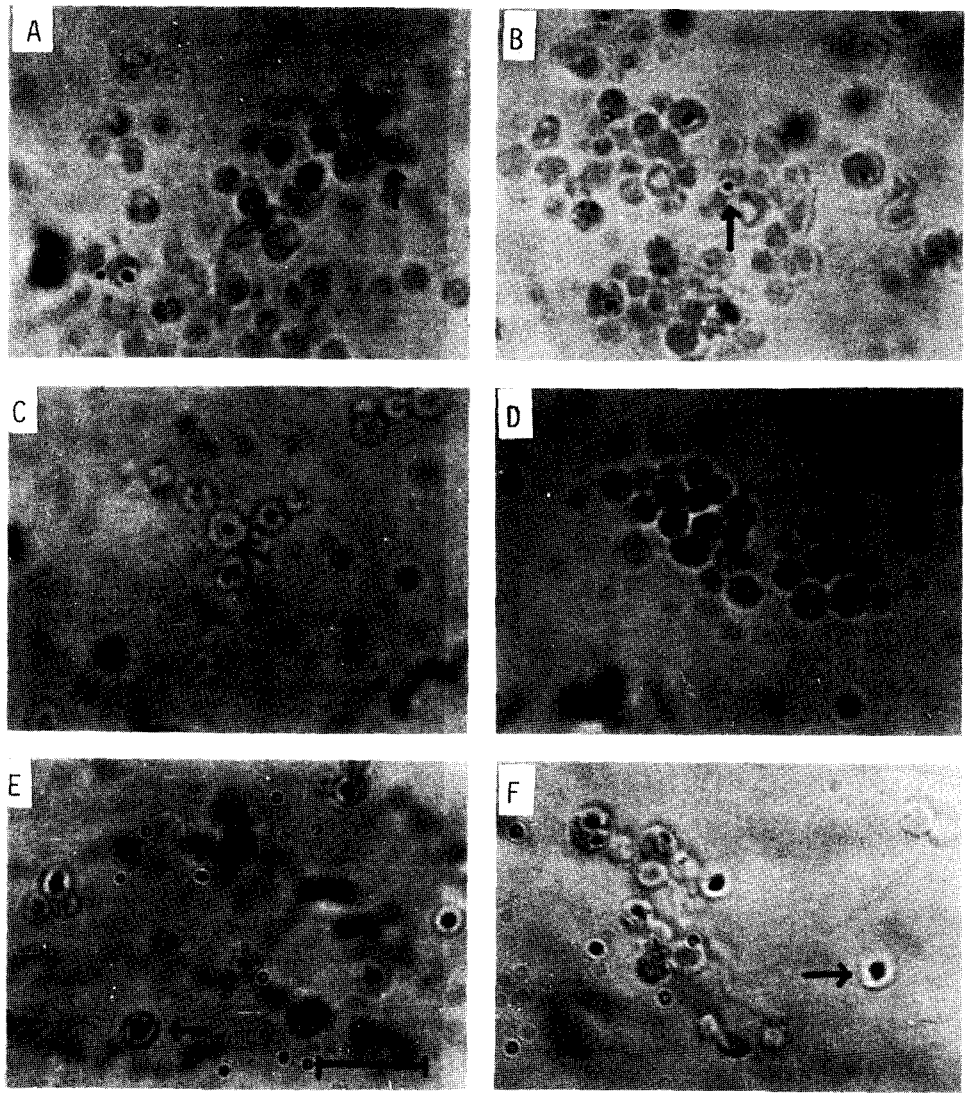

Fig. 1. Photomicrography of nuclei of wild type, parentals and fusion hybrids.
A: Wild type, B: PV 1(met), C: PV 2(phe), D: PV 3(cys), E: FPV 1367, F: FPV 2313.
Arrow indicate nucleus, Bar equals 10μm.

that protoplast fusion technique could be effectively used for genetic study and improvement of cellulolytic *Penicillium*.

요 약

원형질체 융합기술에 의한 *Penicillium verruculosum*의 균주개량 가능성을 검토하였다. 영양요구변이주간의 원형질 융합체 165주중 cellulase 고생산 융합체로 선별된 6주의 cellulase 활성은 야생주에 비해 2배이상, 모균주에 비해 1.2-4.4배의 증가를 나타냈다. Cellulase 고생산 융합체는 유전적 안정성 조사, DNA 함량 및 핵염색 비교검토를 통해 핵융합이 일어난 것으로 확인되었고, 핵형이 이수체이며 4개월 이상의 계대배양 후에도 자연형질 분리가 거의 일어나지 않는 것으로 보아 이들 융합체의 안정성이 매우 높은 것으로 인정되었다.

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