Kor. J. Appl. Microbiol. Bioeng.
Vol. 16, No. 2, 163—167 (1988)

Intraspecific Protoplast Fusion of Cellulolytic Fungus, *Penicillium verruculosum*

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*Penicillium verruculosum*의 종내 원형질체 융합

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The conditions for the protoplast fusion of auxotrophic mutants of *Penicillium verruculosum* were determined. A preparation of commercial enzyme Novozym 234 was used to successfully isolate protoplast from the 20hr old mycelium of *P. verruculosum*. Under optimal condition, the protoplast yield ranged from 2.4×10^7 to 3.0×10^7 protoplasts from 400mg of damp mycelia of various auxotrophic mutant strains. The regeneration frequency ranged from 26.6 to 42.4% and the spontaneous reversion frequency of the protoplasts on the regeneration minimal medium was less than 10^{-7}. The optimal concentration of PEG 6000 was 20%, and exposure of protoplasts to PEG for 10 min was found to be sufficient for protoplast fusion. Optimal pH of fusion mixture was determined as 5.5 and 10mM of calcium chloride in fusion mixture effectively enhanced the protoplast fusion frequency. Under optimal condition, the fusion frequency between various auxotrophs ranged from 1.8×10^{-3} to 3.5×10^{-3}.

Protoplast fusion has been used successfully for genetic study and improvement of fungi and yeasts, and a substantial progress has been made in intraspecific as well as interspecific fusion experiments. The new fusion technology has particular usefulness in between species and between genus interactions in that the process offers a unique way to bypass the natural barriers to genetic exchange in poorly fertile or nonfertile microorganisms(1,2).

Recently, efficient protoplast fusion techniques have been developed in edible fungi(3) and cellulolytic *Trichoderma*(4,5,6), which should facilitate genetic analysis and gene manipulation. However, any protoplast work with *P. verruculosum* has not been yet reported. In our previous work we demonstrated that the optimal conditions for the protoplasts formation and regeneration of *P. verruculosum*(7).

In this paper we report a procedure for the intraspecific protoplast fusion of *P. verruculosum* F-3.

Materials and Methods

Organisms

P. verruculosum F-3 (IFO 31136) and its auxotrophs PV 1(met), PV 2 (phe), and PV 3 (cys) were used throughout the experiment.

Media

Potato dextrose agar(PDA) medium and modified Mandels' medium(8) containing 1% glucose were used as the complete(CM) and minimal medium(MM), respectively. The regeneration complete medium(RCM) and regeneration minimal medium(RMM) were prepared by adding 0.6M of magnesium sulfate to CM and MM, respec-

Key words: *Penicillium verruculosum*, protoplast fusion, heterokaryon formation
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Table 1. A list of auxotrophic mutants derived from ultraviolet light mutagenesis.

Strain	Genotype*)	Phenotype
P. verruculosum	prototype	Dark green color conidia. Reverse red colored.
PV 1	met	Light green color conidia.
PV 2	phe	Dark green color conidia. Reverse red colored.
PV 3	cys	Green color conidia.

*) Abbreviation: met (methionine)
phe (phenylalanine)
cys (cystine)

tively. In preparing regeneration media, 2% agar was autoclaved separately to avoid acid hydrolysis of agar.

Isolation of auxotrophic mutants

Conidia suspension(2×10^7/m*l*) was irradiated with UV-light (Sankyo GL-15(15W) lamp) at a distance of 45cm for 19 min, and it resulted in 0.2% survival rate. For mutant enrichment a filtration technique was employed(9). Nutritional mutants were identified by means of standard methods(10). A list of auxotrophic mutants is shown in Table 1.

Protoplast preparation and regeneration

The preparation and regeneration of protoplast derived from the mycelium of *P. verruculosum* were performed by the methods described in the previous report(7). That is, 400mg(wet weight) of 20hr-old mycelium grown in liquid potato dextrose yeast extract medium was digested with 1m*l* of filter-sterilized 0.5% (w/v) Novozym 234(Novo Biolabs, Denmark) solution containing 0.7M sorbitol and 50mM citrate buffer(pH 5.6). The mycelium was treated in the enzyme solution for 1hr at 30 °C with gentle shaking. Protoplasts were purified from the residual mycelial debris by filtration through the sintered glass filter(porosity; 40-60 μ m) and washed twice with stabilizer by centrifugation (1,000 $\times g$, 10 min). The washed protoplasts were resuspended in the stabilizer and plated on the RCM or RMM for regeneration. The number of protoplasts was counted by using a haemacytometer.

Protoplast fusion

Protoplast fusion was carried out by the methods of Hong *et al.*(9). Protoplasts of the two parental strains were mixed at 5.0×10^6 protoplasts each, and sedimented by centrifugation at 700 $\times g$ for 10 min. The pelleted protoplasts were resuspended in a minute volume of the remaining stabilizer by gentle shaking, and the protoplasts were treated with 1 m*l* of prewarmed polyethylene glycol 6000(PEG, Sigma) containing calcium chloride at 30 °C. To examine the factors affecting protoplast fusion, the concentration of PEG(ranged from 10 to 60%, w/v), the concentration of calcium chloride(ranged from 10 to 50mM), the length of exposure time to PEG(ranged from 10 to 60 min), and the pH of PEG solution(ranged from 4.5 to 10.0) were tested. After incubation, the fusion mixture was serially diluted and spread on the RCM, and then regeneration frequency was determined from the number of colonies appeared on the RCM and the number of protoplasts inoculated. Fusion frequency was determined from the number of colonies appeared on the RMM and RCM plates.

Table 2. Formation, regeneration and reversion frequency of protoplasts from auxotrophic mutants.

Strain	Genotype	Yield of protoplast formation. ($\times 10^7$)[a]	Regeneration frequency on RCM. (%)	Reversion frequency on RMM.
P. verruculosum	prototype	3.2	27.8	NT[b]
PV1	met	3.0	36.6	1.0×10^{-7}
PV 2	phe	2.7	42.4	1.3×10^{-7}
PV 3	cys	2.4	26.6	2.1×10^{-7}

[a] 1.0×10^7 protoplasts per 400mg as wet weight of mycelium.
[b] Not tested.

Results

Formation and regeneration of protoplasts

Protoplast were produced and regenerated as described previously(7). Under optimal condition, the protoplast yield ranged from 2.4×10^7 to 3.0×10^7 protoplasts from 400mg of damp mycelia of auxotrophic mutant strains. The regeneration frequency ranged from 26.6 to 42.4%, and the spontaneous reversion frequency of protoplasts on the RMM was in the order of 10^{-7} (Table 2).

Effect of PEG concentration on the fusion frequency

PEG and calcium chloride ions are widely used to stimulate fusion of protoplasts of various organisms(11,12). The protoplasts from the mutant strain PV 1 (met) and PV 3 (cys) were mixed, treated for 10 min with varied concentration of PEG solution containing 10mM $CaCl_2$ in 50mM citrate buffer(pH 5.6), serially diluted, and spread on RCM and RMM. After four days of incubation, the number of colonies developed on RCM and RMM was counted. Nutritionally complementing protoplasts regenerated on RMM as heterokaryons were identified by the criteria of Pontecorvo and Sermonti(13). As shown in Fig. 1, the highest fusion frequency was obtained when the concentration of PEG was 20%.

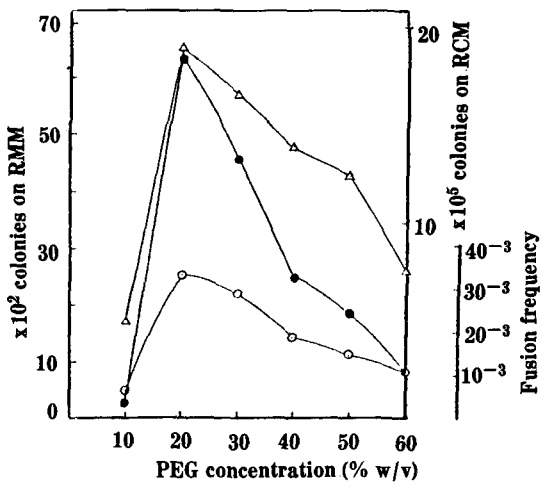

Fig. 1. Effect of polyethylene glycol (PEG) concentration on the fusion frequency (○ - ○) of *P. verruculosum* protoplasts.

5.0×10^6 protoplasts of each auxotrophic mutant were treated with PEG at different concentrations, dissolved in 0.05M citrate buffer(pH 5.6) with 0.01M $CaCl_2$ (30 °C, 10 min).

● - ● ; the number of colonies on regeneration minimal agar medium(RMM),

△ - △ ; the number of colonies on regeneration complete agar medium(RCM).

Effect of length of exposure time to PEG on the fusion frequency

Effect of the length of PEG treatment on protoplast fusion was examined. Protoplasts of the mutant strains were treated with PEG(20%) containing 10mM $CaCl_2$ at various durations, and then the protoplasts were plated under the same condition as described in Fig. 1. As shown in Fig. 2, it was found that the highest fusion frequency was obtained with the treatment for 10 min. This indicates that 10 min is optimal for coating of the protoplasts by PEG or for the reaction of all the fusogenic factors in the incubation mixture with the protoplast surface(14).

Effect of calcium chloride concentration on the fusion frequency

The optimal calcium chloride concentration for protoplast fusion was examined by using 20% PEG 6000 solution containing varied concentrations of

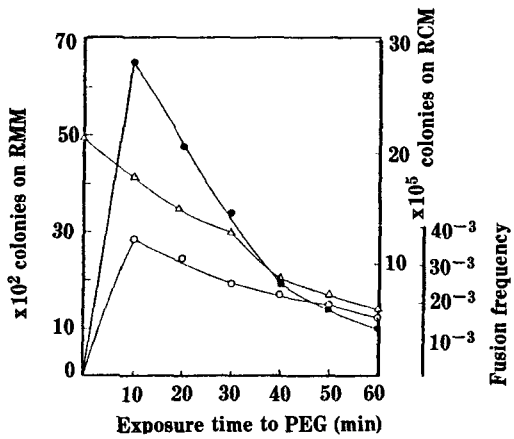

Fig. 2. Effect of exposure time to polyethylene glycol on the fusion frequency (○ - ○) of *P. verruculosum* protoplasts.

● - ● ; the number of colonies on regeneration minimal agar medium(RMM),

△ - △ ; the number of colonies on regeneration complete agar medium(RCM).

calcium chloride ranging from 0 to 50mM. As shown in Fig. 3, the highest level of fusion was observed when calcium chloride concentration was 10mM.

Effect of pH on the fusion frequency

To determine the optimal pH for protoplast fusion, the 20% PEG 6000 solution containing 10mM calcium chloride was prepared in various buffer systems. The fusion mixture buffered with 50mM citrate(pH 5.5) resulted in the highest fusion frequency(Fig. 4). Consequently, the fusion mixture buffered with 50mM citrate was used throughout the following experiments.

Complementation of auxotrophic mutants by protoplast fusion

Treatment of protoplasts with 20% PEG 6000 and 10mM calcium chloride in 50mM citrate buffer (pH 5.5) led to the fusion of protoplasts. The fusion frequencies for complementing heterokaryons between strain PV 1 (met) and PV 2 (phe), PV 1 (met) and PV 3 (cys), and PV 2 (phe) and PV 3 (cys) were 1.8×10^{-3}, 3.5×10^{-3}, and 2.6×10^{-3}, respectively (Table 3).

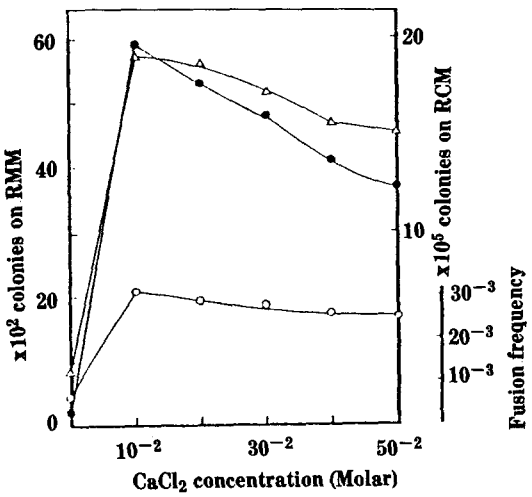

Fig. 3. Effect of $CaCl_2$ concentration on the fusion frequency (○ - ○) of *P. verruculosum* protoplasts.

● - ● the number of colonies on regeneration minimal agar medium(RMM),

△ - △; the number of colonies on regeneration complete agar medium(RCM).

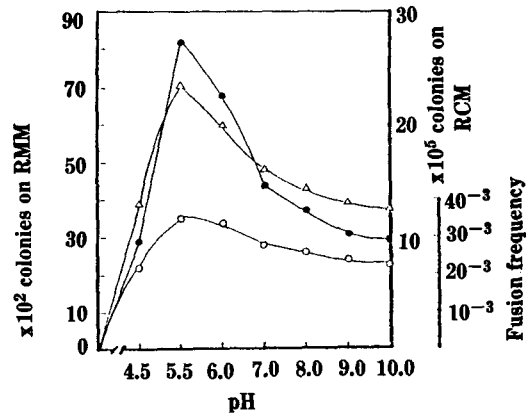

Fig. 4. Effect of pH on the fusion frequency (○ - ○) of *P. verruculosum* protoplasts.

● - ● ; the number of colonies on regeneration minimal agar medium(RMM),

△ - △; the number of colonies on regeneration complete agar medium(RCM).

pH 4.0-6.0; 0.05M citrate buffer, pH 7.0; 0.05M phosphate buffer, pH 8.0; 0.05M Tris-HCl buffer, pH 9.0-10.0; 0.05M Glycine-NaOH buffer.

Discussion

P. verruculosum F-3, identified as a new potential producer of cellulase complex(15), may be one of the most promising candidates for microbial conversion of cellulosic biomass. Although much effort has gone into the production of cellulase and strain improvement by induced mutation of this fungus, the knowledge of its genetic background is still poor. To our knowledge this is the first report on the protoplast fusion of *P. verruculosum*.

In the previous reports, we described the formation and regeneration of protoplasts from the mycelium of *P. verruculosum* F-3(7). In the present report, we have described the optimal conditions

Table 3. Fusion frequency of the crosses between protoplasts from various auxotrophic mutants.

Cross (genotype*)	Fusion frequency
PV 1(met) × PV 2(phe)	1.8×10^{-3}
PV 1(met) × PV 3(cys)	3.5×10^{-3}
PV 2(phe) × PV 3(cys)	2.6×10^{-3}

*) Abbreviation: cys;cystine, met;methionine, phe; phenylalanine.

for protoplast fusion of *P. verruculosum*. Protoplasts were successfully produced from the mycelium of *P. verruculosum* by using Novozym 234 as a protoplasting agent.

The maximal heterokaryon formation and viable colony formation were obtained when the PEG concentration was 20%. As reported Anne and Peberdy(14), at least 20%(w/v) of PEG was needed to stabilize most of the protoplasts and the concentrations lower than this failed to stabilize and resulted in protoplasts burst. The frequency of protoplast fusion and viable colony formation decreased with higher PEG concentration or prolonged incubation time. Fusion of protoplasts was enhanced by $CaCl_2$, and the optimal pH of fusion mixture for heterokaryon formation was 5.5. These results were similar to those with *T. koningii*(9). It is shown in Table 3 that complementing heterokaryons could be obtained by the protoplast fusion. Since the spontaneous reversion frequency of protoplasts of various auxotrophic mutants was less than 2.1×10^{-7}, the possibility of the complementing colonies resulted by spontaneous reversions is excluded.

The results reported here show that the protoplast fusion technique would be a very effective tool for the genetic studies of cellulolytic fungus *P. verruculosum*.

Isolation and genetic analysis of the stable complementing hybrids produced by the intraspecific protoplast fusion of *P. verruculosum* have been done in this laboratory, and the results will be soon reported.

요 약

*Penicillium verruculosum*으로부터 유도된 영양요구성 돌연변이주간의 원형질체 융합을 위한 조건을 검토하였다. 20시간 배양한 *P. verruculosum*의 균사체에 Novozym 234를 처리하여 원형질체를 추출할 수 있었다. 원형질체 생성 최적조건하에서의 원형질체 생성량은 각 영양요구성 변이주의 균사체 400 mg당 $2.4 \sim 3.0 \times 10^7$ 수준이었고, 재생율은 26.6~42.4%, RMM에서의 환원율은 10^{-7} 수준이었다. 원형질체 융합을 위한 PEG 6000의 최적농도는 20%였고, PEG 최적처리시간은 10분, $CaCl_2$ 최적첨가농도는 10 mM, 최적 pH는 5.5였으며, 융합 최적조건하에서의 융합율은 $1.8 \times 10^{-3} \sim 3.5 \times 10^{-3}$ 수준으로 나타났다.

Acknowledgement

This report is a part of the result of the research project supported by a research grant of the Korea Research Foundation (1984-1986). We would like to express our thanks to Dr. Chang-Soon Ahn for the critical review of the manuscript.

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(Received March 10, 1988)