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A System Development of the Protoplast Fusion of *Streptomyces coelicolor*

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*Streptomyces coelicolor*의 Protoplast Fusion 방법개발

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ABSTRACT

Attempts were made to optimise protoplast formation and regeneration methods to improve the protoplast fusion frequencies of *Streptomyces coelicolor*.

The yields of protoplast formation and regeneration were varied with different growth phase of the culture. Maximum yields were obtained when cells were taken from the late logarithmic phase. Protoplast formation reached almost its maximum with lysozyme treatment at a concentration of 2mg/ml without any other lytic enzyme.

A high frequency of protoplast regeneration was accomplished by overlay method: the method gave 14% recovery of regenerated protoplast versus 1.8% recovery for monolay method.

A recombinant frequency of 1.8×10^{-2} was obtained by protoplast fusion using PEG 1000(50% w/v).

INTRODUCTION

Streptomyces species produce many of the known antibiotics and have thus attracted widespread interest.

In spite of the widespread interspecific conjugation, strain improvement and genetic analysis have been hindered from the low frequency of recombination (10^{-6}) obtained by this method of genetic recombination, (D.A. Hopwood and M.J. Merrick, 1977).

Recently, it was demonstrated that high frequencies of recombination could be obtained with *Streptomyces* by fusion of artificially induces protoplasts, even in the absence of known sex factors (R.H. Baltz, 1978).

Hopwood *et al.* were the poineering group who described the protoplast fusion of *Streptomyces spp.* (D.A. Hopwood and H.M. Wright, 1979). Their method gave poor regeneration frequency and the preparation of spore suspension for inoculum was difficult.

In this experiment, a overlay method was used to regenerate colonies and sonicated mycelia were inoculated for the protoplast preparation. Modified methods were established to achieve higher frequencies of protoplast regeneration and fusion.

METHODS and MATERIAL

1. Bacterial strains

S. coelicolor arg$^-$, *S. coelicolor his*$^-$ *met*$^-$

2. Selection of auxotrophic mutants and antibiotics-nonproducing strains.

Spore solution was washed with 0.1M TM buffer by centrifugation at 3,000 rpm for 15 minute. The spore pellets were suspended in the same buffer containing 1mg/ml MNNG (N-methyl-N′-nitro-N-nitrosoguanidine) and mixed

by a stirrer(V. Delic, D.A. Hopwood and E.J. Friend, 1970).

This mixture was incubated in a 30°C water bath for 50 min., centrifuged and decanted. The Spore were suspended in the minimal medium containing pentachlorophenol(P.C.P) (40 mg/ml) and continued incubation of the culture with shaking for 5 hours (S. Sermonti, 1969).

The spore suspension was filtered through cotton wool and centrifuged. The spore pellet was washed with 0.1M sodium phosphate buffer and spread on complete agar media (D.A. Hopwood, 1967).

Antibiotics-nonproducing strains after treatment with acriflavin plus high temperature were isolated from wild strain and the arginine auxotroph strain.

In order to cure the plasmids from the mycelia, each culture was grown in a complete medium, which was composed of glucose 1%, peptone 0.4%. yeast extract 0.4%, KH_2PO_4 0.2% and K_2HPO_4 0.4%, at 30°C, for 48 hour in a reciprocal shaker at 140 strokes/min.

Full grown mycelia were sonicated for 60 seconds at 48W, inoculated(4% (v/v)) into a new medium of the same composition containing 15mg ml acrifiavin and shake-cultured for 5~7 days at 39°C. A control without acriflavin was cultured in the same way.

After the incubation, the cells were transferred to complete media (D.A. Hopwood, 1967) and cultured at 30°C, 7 day, for spore formation.

In order to select antibiotics-nonproducing strains, spores were transferred on to complete agar medium and incubated for 7 days at 30°C.

The antibiotic productivity of each colony grown on this medium was assayed by soft agar over overlay technique (Kim, 1983). *Bacillus subtilis* was used as test organism for methylenomycin production.

3. Bacterial growth

Mycelia of *S. coelicolor* fully grown in S-medium (M. Okanish, K. suzuki and H. Umezawa, 1974) were sonicated for 60 seconds at 48W and used to inoculate (4% v/v) S-medium containg 1% glycine and incubated in an reciprocal shaker at 30°C for 3days. The mycelia grew as pellets in the siliconised flasks. Cells were collected by centrifugation and either used immediately or suspended in 20% glycerol and stored at-20°C. Growth rate was measured by dry cell weight.

4. Determination of the frequencies of protoplast formation, regeneration, and fusion.

10 ml of harvested cell were centrifuged, washed two times with 0.3M sucrose solution, suspended in 1.5ml of lysozyme solution (2mg/ml P-medium) (M. Okanish, K. Suzuki and H. Umezawa, 1974) and incubated at 32°C for 90 minutes. 5ml of P-medium was added and filtered through cotton wool. Protoplasts were counted by a haemacytometer under a light microscope.

Regeneration frequency of protoplasts was estimated by colony counts on the regeneration plates prepared from the protoplasts. Regeneration frequency waw calculated as follows:

$$\frac{\text{The number of regenerated colony}}{\text{The number of protoplasts formed}} \times 100(\%)$$

For protoplast fusion, equal volumes (0.5~1.0ml containing about 2×10^8 protoplasts) of protoplast suspensions of two auxothic strains were mixed, and after centrifugation the supernatant was decanted. After dispersion of the pellet in the minute volume of the remaining liquid by stirring, 1.8ml of 50% (w/v) PEG was added. Control pellets were resuspended in 1.8ml of P-medium. After incubation at 30°C for 3 min with mild stirring, 4ml of P-medium was added, centrifuged and the supernatant was discarded. The pellet was resuspended in P-medium and 0.1ml of appropriate dilutions were plated on the regeneration agar media with or without corresponding nutritional requirements. They were incubated at 32°C for 7 to 10 days unitl no more colonies appeared. Freqency of prototrophic re-

combinants was defined as the ratio of prototrophic colonies appering on the minimal regeneration agar medium to the total number of colonies appearing on the supplemented regeneration agar medium (M. Okanish, K. Suzuki and H. Umezawa 1974).

RESULTS AND DISCUSSION

1. Mutation

Pentachlorophenol (P.C.P.) was used to enrich auxotrophs in this spore-forming test organism, instead of penicillin used generally in bacteria. Spore grown in minimal medium containing 40mg/ml of P.C.P and the number of auxotroph increased by ten times after 5 hour incubation.

Table 1. Effect of pentachlorophenol to concentrate the mutant

	without pentachlorophenol treatment	with pentachlorophenol treatment
Mutant frequency	4.1×10^{-4}	3.02×10^{-3}

Table 1 showed the effect of pentachlorophenol treatment on enrichment of mutants.

2. Protoplast formation.

The yield of protoplast formation of *S. coelicolor* varied greatly with different growth phase of the culture. Maximum yield of protoplasts were obtained when cells were taken from the late logarithmic phase. Fig. 1 shows the effect of different culture ages on the yield of protoplast formation.

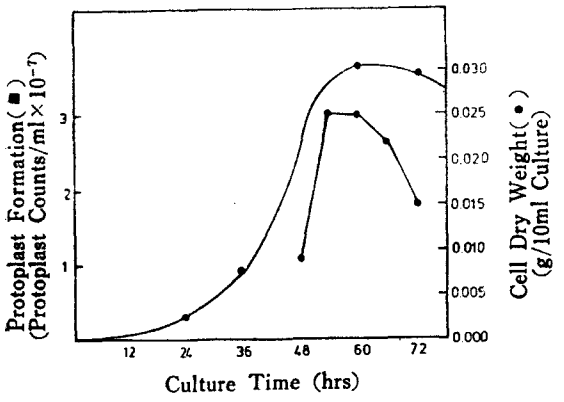

Fig. 1. Effect of culture time on protoplast and growth.

3. Regeneration.

The most difficult step in the protoplast fusion technique was the regeneration of fused protoplasts.

It was important to use cells from the growth phase which gave the highest frequency of protoplast regeneration. We have found that cells from the late exponential phase just before the stationary phase gave the highest regeneration frequencies (Figure 2)

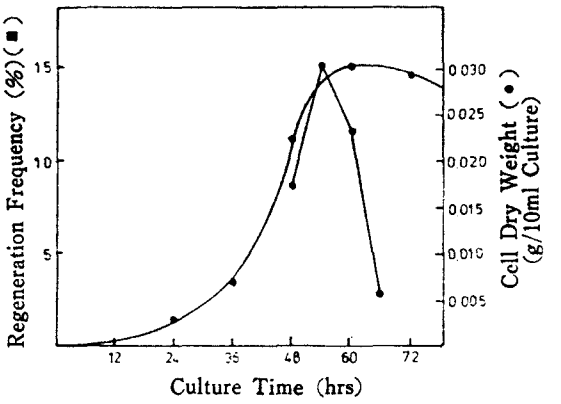

Fig. 2. Effect of culture time on the regeneration.

In order to differentiate between the colonies from protoplast regeneration and those from remaining unprotoplasted cells, a portion of the fused protoplast preparation was diluted with distilled water or the Hopwood's medium and plated, by taking advantage of the fact that protoplasts would not be regenerated in water or the Hopwood's medium. The number of colonies appeared on the R-medium and the Hopwood's medium (D.A. Hopwood, 1967) were compared.

In order to increase the regeneration frequencies, as was done in ***Bacillus subtilis***, the overlay method was used (T. Schirahama, T. Furumai and M. Okanishi, 1981).

In this method, the fused protoplast was spread on 2.2% agar R-medium and then the plate was overlaid with 0.6% agar solution

containing the same medium.

Alternatively the fused protoplast preparation was suspended in the 0.6% agar solution containing the R-medium and overlaid over the plate of 2.2% R-medium. No difference in the regeneration frequencies between the two method were found.

Great difference in regeneration frequency was noticed between the overlay and monolay method (Table 2). The overlay method gave higher recovery of regenerated cells from the input protoplasts, than the monolay method.

Table 2. Comparison of regeneration efficiency between monolay method and overlay method

	Regeneration efficiency
Monolay method	1.8%
Overlay method	14 %

4. Protoplast fusion.

Fusion was carried out by mixing protoplasts and treating them (30°C, 3min, slowly shaking) with 1.8ml of a 50% solution of polyethylene glycol (PEG) 1000.

Recombination frequency of fused protoplasts and that of standard crossing were estimated and the results are shown in Table 3. The effect of PEG treatment on the recombinant frequency is shown in Table 4.

Table 3. Genetic recombination of multiple marker genotype by protoplast fusion and standard crossing

Cross (genotype)	Recombinant Frequency (*arg*$^+$ *his*$^+$ *met*$^+$)	
	Standard crossing	Fusion
JS 105(*arg*$^-$) × JS 110(*his*$^-$ *met*$^-$)	5×10^{-6}	2.8×10^{-2}

Table 4. Recombinant frequency by protoplast fusion

Cross (genotype)	Recombinant frequency (*agr*$^+$ *his*$^+$ *met*$^+$)	
	Without PEG	With PEG1000 (50% w/v)
JS 105(*arg*$^-$) × JS 110(*his*$^-$ *met*$^-$)	6×10^{-3}	1.8×10^{-2}

적 요

*Streptomyces*속 균주개발의 수단으로서 이용할 목적으로 원형질체 융합방법의 확립을 시도하였다. 특히 융합빈도를 높이고 실험을 간편화하는데 역점을 두었다.

원형질체의 형성 및 재생빈도는 균의 배양시간에 따라 변하였는데 대수기에서 수확한 균체로부터 가장 높은 빈도의 수율을 얻었다.

원형질체의 형성은 다른 용균효소를 사용하지 않고 Lysozyme 단독처리 만으로도 충분히 가능하였고 원형질체의 세포막 재생은 Monolay법 보다는 Overlay법이 훨씬 좋은 결과를 주었다. Monolay법은 1.8%, Overly법은 14%의 재생빈도를 나타냈다.

본 실험에서 PEG1000 (50% W/V)를 사용한 원형질체 융합방법으로 얻은 *S. coelicolor*의 재조합체의 빈도는 1.8×10^{-2}이었다.

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