STANDARD DEHYDRATION OF REGENERATION
PLATE RESULTS IN MARKED DECREASE
IN PROTOPLAST REGENERATION FREQUENCY
IN Streptomyces STRAINS WITH
OSMOTIC INSTABILITY ?

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Protoplast regeneration is currently an essential procedure for genetic transformation of Streptomyces strains. The technique was first developed by Okanishi *et al.*¹⁾, and a variety of parameters influencing protoplast regeneration frequency have been characterized²⁻⁴⁾. The most important factors are growth phase of the mycelium, the incubation temperature of the mycelium and regenerating protoplasts, the relative dehydration of regeneration plates and medium composition. Thus, with some modifications, conditions established by Okanishi *et al.*¹⁾ and Thompson *et al.*⁴⁾ have been employed successfully in heterologous Streptomyces strains. However, we sometimes encounter Streptomyces strains which do not form protoplasts and/or can not be regenerated under the conditions reported so far. Streptomyces sp. 60-1 and 74-4, which were isolated from soils collected in Korea, are examples of such strains. In this report, we describe conditions for

protoplast formation and regeneration of these two strains. The most critical factor we observed was dehydration of the regeneration medium. These strains are inhibited by excessive dehydration for regeneration. This might be due to osmotic instability of the protoplasts.

Conditions for protoplast regeneration were essentially similar to those established by Thompson *et al.*⁴⁾. Under the conditions shown in Table 1, S. lividans TK21 readily forms protoplasts and regenerates at 30-50 % frequency. Thus, strain TK21 regenerates most successfully at a level of 15-20 % dehydration of regeneration plate. Under the same conditions, we find that protoplasts of heterologous Streptomyces strains regenerate at frequencies ranging 5-50 % (data not shown). However, neither conditions optimized for S. lividans nor other standard procedures were satisfactory for protoplast regeneration of strains 60-1 and 74-4.

Strains 60-1 and 74-4, which produce unidentified antibiotics active against Xanthomonas campestris pv. oryzae and other bacteria, did not grow well in YEME medium but in Tryptic Soy Broth (TSB; Difco). For protoplast formation, strains 60-1 and 74-4 were grown at 28°C in TSB supplemented with glycine. Strain 60-1 was grown 45-48 h and strain 74-4 was grown 15-20 h. Subsequent treatment with a mixture of lysozyme and achromopeptidase of the mycelia resulted in good protoplast formation as high as $1-1.5 \times 10^{10}$ protoplasts per 20 ml culture. R2YE medium was used for protoplast regeneration. When regeneration plates were dried normally in a clean bench, we noticed that protoplast regeneration frequency fluctuated drastically depending on location of the

plates in the clean bench. When about 1,300 protoplasts were plated, about 400 colonies appeared on some plates, while no colonies appeared on other plates. Measurement of dehydration of these plates suggested that regeneration frequency might correlate with the degree of dehydration. Therefore, effect of dehydration of regeneration plate was examined. As shown in Fig. 1, regeneration frequencies were relatively high at 10 % dehydration or less and declined linearly above 10 % dehydration. Thus, 10 % or less dehydration resulted in good regeneration frequencies (15-30 %

for strain 60-1 and 2-5 % for strain 74-4). Both strains exhibited an osmotic instability, although their taxonomic properties were quite different from each other (strain 74-4 has taxonomic properties similar to those of *S. aureofaciens*). They did not grow in YEME medium containing 34 % sucrose, while they grew well when sucrose was omitted from the medium. In addition, when they were incubated in ISP No.4 medium, supplementation of the medium to 3 % NaCl resulted in a markedly retarded growth and no growth occurred at 5 % NaCl. In contrast, *S. lividans* grew

Table 1. Protoplast regeneration under the conditions established.

Process	Strains		
	TK-21	60-1	74-4
[Cultivation]			
Medium	YEME	TSB	TSB
Glycine(%)	0.5	0.7	1.4
Time(hr)	45	45	15-20
[Protoplasting]			
Lysozyme(mg/ml)	3	10	3
Achromopeptidase(mg/ml)	-	0.5	0.5
Temperature(*C)	28	28	28
Time(min)	60	60	60
Protoplast($\times 10^{10}$)*	1	1-1.5	1-1.5
[Regeneration]			
Medium	R2YE	R2YE	R2YE
Dehydration(%)			
Bottom layer	15-20	5-10	5-10
Top layer	15-20	5	5
Temperature(*C)	28	28	28
Time(day)	3-5	3-5	5-7
Frequency(%)	30-50	15-30	2-5

* Protoplasts formed per 20 ml culture.

Fig. 1. Effect of dehydration of regeneration plate on regeneration frequency.
●:60-1, ▲:74-4

normally in the presence of 5 % NaCl. Their low NaCl tolerance might also be due to osmotic instability. Thus, because of their osmotic instability, our standard procedure (15-20 % dehydration) for drying regeneration plates, R2YE medium containing 10.3 % sucrose, resulted in marked decreases in regene-

ration frequencies of strains 60-1 and 74-4. We propose that other strains that are sensitive to NaCl concentrations such as *S. aureofaciens* may also require careful attention to the level of dehydration of regeneration plates in order to achieve successful protoplast regeneration.

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