AN IMPROVED METHOD FOR THE PREPARATION OF STREPTOMYCETES AND *MICROMONOSPORA* PROTOPLASTS

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In 1974, OKANISHI[1] reported on culture media and conditions for preparing stable protoplasts of Streptomycetes, and the procedures for regeneration to normal mycelia. This method, sometimes with minor modification, has been employed by many researchers for protoplast fusion[2~7] or genetic transformation in Streptomycetes. However, it has been found that protoplast formation and regeneration frequency varies according to the species used. In 1981, SHIRAHAMA *et al.*[8] reported a new regeneration method, however we found that it was very difficult to obtain protoplasts and regenerated cells from protoplasts in high yield among several economically important *Streptomyces* and *Micromonospora*, for instance, antibiotic producers. A more general means, genetic recombination in *Streptomyces* and *Micromonospora* would be quite useful for strain improvement and genetic analysis in economically important strains. Therefore, we have worked out new protoplast methodology which be applied to some mutants or species which are hard to form protoplasts. This is the subject of this communication.

Streptomycetes protoplast formation was carried out as follows: cells from a slant culture or lyophilized mycelia were shake-cultured at 28°C for 24~48 hours in medium S-1 consisting of 2.0% soluble starch, 1.0% peptone, 0.3% meat extract, 0.05% K_2HPO_4, pH 7.0. The culture was transferred with 2% inoculum size to medium S-2 consisting of 2.0% glucose, 0.4% yeast extract, 1.0% malt extract, 0.3% glycine, pH 7.0, and shake-cultured for 16~24 hours.

In case of *Micromonospora*, cells from a slant culture or lyophilized mycelia were shake-cultured at 28°C for 48 hours in medium S-3 consisting of 2.0% soluble starch, 2.5% wheat germ, 0.15% NaCl, 0.00025% $CoCl_2 \cdot 6H_2O$, 0.3% $CaCO_3$, pH 7.0. The culture was transferred with 5% inoculum size to medium S-2, and shake-cultured for 20~24 hours. Cells from 8 ml culture were harvested by centrifugation, washed with 0.5 M sucrose solution and then with medium P3[8] solution. The washed cells were suspended in 4.8 ml of medium P3. Then, 0.6 ml of lysozyme (7,500 μg/ml) and 0.6 ml of achromopeptidase (TBL-1, Wako Pure Chemical Industries, LTD., 5,000 μg/ml) were added, followed by incubation at 37°C for 60~90 minutes. The lysozyme achromopeptidase-treated cell suspension was centrifugated at 700 r.p.m. for 5 minutes, then filtered through cotton to remove the intact cells. The protoplast suspension was gently diluted with medium P3 and plated on the regeneration medium R4 consisting of 0.4 M sucrose, 1.5% KCl, 0.025% K_2SO_4, 0.2% trace element solution[1], 0.005% KH_2PO_4, 1.01% $MgCl_2 \cdot 6H_2O$, 1.0% glucose, 0.2% L-asparagine, 0.05% Casamino Acids, 0.74% $CaCl_2$, 3.0% corn steep liquor (supernatant of 12 v/v % corn steep liquor pH 7.0), 0.025 M TES buffer pH 7.2, 0.75% agar, or medium MR consisting of 0.2 M sucrose, 0.025% K_2SO_4, 0.007% KH_2PO_4, 1.01% $MgCl_2 \cdot 6H_2O$, 1.0% glucose, 0.3% L-proline, 0.1% Casamino Acids, 0.15% $CaCl_2$, 3.0% corn steep liquor (supernatant of 12 v/v % corn steep liquor pH 7.0), 0.25% yeast extract, 0.05% $CaCO_3$, 0.025 M TES buffer pH 7.2, 0.75% agar.

As shown in Fig. 1, the addition of achromo-

Fig. 1. Effect of lytic enzymes on the protoplasts formation of *Streptomyces hygroscopicus* ATCC 21705.

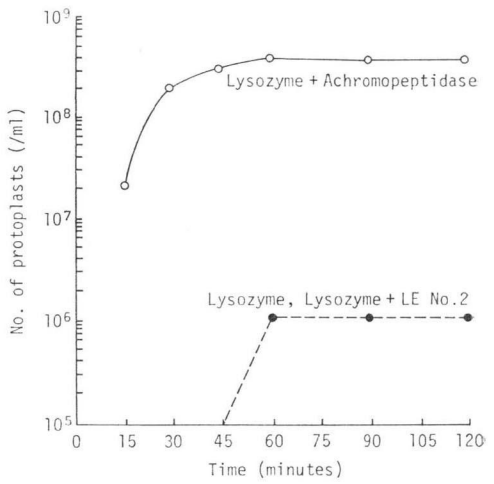

Table 1. Protoplasts formation and regeneration of Streptomycetes and *Micromonospora*.

Strain	Antibiotics	No. of protoplasts formed (/ml)			No. of protoplasts after regeneration (/ml) and frequencies (%)			Media for protoplasts regeneration
		A	B	C	A	B	C	
S. hygroscopicus ATCC 21705	AMPBA*	1.0×10^{8}	1.0×10^{8}	4.0×10^{8}	1.3×10^{5} (13.0%)	1.3×10^{5} (13.0%)	6.0×10^{7} (15.0%)	R4
S. viridochromogenes 3–9	Fosfomycin	4.5×10^{7}	6.9×10^{7}	8.0×10^{8}	9.0×10^{5} (20.0%)	1.5×10^{7} (21.7%)	2.6×10^{8} (32.5%)	R4
S. wedmonensis 209–97	Fosfomycin	8.5×10^{7}	8.5×10^{7}	8.0×10^{8}	4.0×10^{7} (47.1%)	4.2×10^{7} (52.5%)	3.9×10^{8} (48.8%)	R4
S. kanamyceticus 4–480	Kanamycin	2.8×10^{8}	2.0×10^{7}	2.0×10^{8}	4.0×10^{5} (15.4%)	4.0×10^{6} (20.0%)	5.0×10^{7} (25.0%)	R4
S. mycarofaciens No. 6	Midecamycin	3.5×10^{8}	4.0×10^{8}	7.5×10^{7}	9.0×10^{4} (2.6%)	1.5×10^{5} (3.8%)	3.6×10^{6} (4.8%)	R4
M. purpurea	Gentamicin	$<10^{5}$	$<10^{5}$	3.0×10^{8}	—	—	1.1×10^{8} (36.7%)	MR
Micromonospora sp. SF-1854	Dactimicin	$<10^{5}$	$<10^{5}$	1.1×10^{8}	—	—	2.0×10^{7} (18.2%)	MR

A: Cells were treated with lysozyme (1,000 μg/ml).
B: Cells were treated with lysozyme (1,000 μg/ml)+lytic enzyme No. 2 (100 μg/ml).
C: Cells were treated with lysozyme (750 μg/ml)+achromopeptidase (500 μg/ml).
* 2-Amino-4-(hydroxy) (methyl)phosphinoyl-butyryl-alanylalanine.

peptidase caused marked stimulation of protoplasts formation compared with lysozyme or the combined use of lysozyme and lytic enzyme No. 2 (Kyowa Hakko Kogyo). When *Streptomyces hygroscopicus* ATCC 21705 was treated with lysozyme in combination with achromopeptidase, the yield of protoplasts was about 400 times as compared with those of lysozyme only or the combined use of lysozyme and lytic enzyme No. 2, and the yield of protoplasts exceeded 4×10^{8}/ml. (Table 1). As shown in Table 1, when *S. viridochromogenes, S. wedmonensis, S. kanamyceticus* or *S. mycarofaciens* was treated with lysozyme in combination with achromopeptidase, the yield of protoplasts was about 10 to 100 times as compared with those of lysozyme only or the combined use of lysozyme and lytic enzyme No. 2.

When *Micromonospora purpurea* or *Micromonospora* sp. SF-1854 was treated with lysozyme only or mixture of lysozyme and lytic enzyme No. 2, mycelia were fragmented, but protoplasts were not formed. When a mixture of lysozyme and achromopeptidase was used, $1.1\sim3.0\times10^{8}$/ml protoplasts were formed.

OKANISHI *et al.*[1] and SHIRAHAMA *et al.*[8] pointed out that protoplasts formation by using lysozyme is significantly affected by the conditions of their growth medium and cultural age, in Streptomycetes. However, combination of lysozyme and achromopeptidase allowed all of the species tested to form the protoplasts with high frequency under the normal growth medium used.

Streptomycetes and *Micromonospora* protoplasts were plated on the medium R4 and MR for regeneration, respectively. A suspension of protoplasts was diluted with medium P3, and 0.5 ml of the diluted solution was plated on the agar plate R4 or MR. Medium R4 and MR contained 0.75% agar, and so it was not necessary to overlay the soft agar medium. Since there was no marked difference in the regeneration frequency among the methods evaluated, the use of lysozyme and achromopeptidase results in a remarkable increase in the number of regenerated colonies from protoplasts. This protoplast formation and regeneration methodology should be useful for protoplast fusion or genetic analysis in a wider range of Streptomycete and *Micromonospora* species.

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