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Isolation and Regeneration of Protoplast in *Streptomyces antibioticus*

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The present study has been performed to investigate the optimal conditions for protoplast formation and regeneration of oleandomycin-producing *Streptomyces antibioticus* (*S. antibioticus*) KCTC 1081. Mycelia were grown in YME medium containing 0.2% (w/v) glycine and converted into the protoplast by incubating at 35°C for 60 minutes in protoplast buffer (P buffer) containing 4 mg/ml lysozyme. The reversion of protoplasts to the normal filamentous state was examined by the growth on various synthetic agar media. A high reversion rate was obtained by incubating the protoplasts on a hypertonic agar medium containing 20 mM Mg^{++}, 5 mM Ca^{++} and 0.3 M sucrose at 28°C for 5 days. From these experiments, we established the improved regeneration medium and a protocol which supports higher and more consistent levels of regeneration of *S. antibioticus* protoplasts. The regenerant showed an increased antimicrobial activity compared with that of the initial strain.

KEY WORDS □ *Streptomyces antibioticus*, protoplast, oleandomycin

Streptomyces are a group of Gram-positive mycelial eubacteria. They are important as producers of secondary metabolites, including many antibiotics of medical (e.g., antimicrobial, antifungal, anticancer, antiparasitic and immunomodulatory agents), agricultural usage (e.g., herbicides, livestock growth promoters), and other biologically active substances (16). The study of *Streptomyces* has been advanced for increasing the yield of secondary metabolites and for producing useful new compounds. However, strain development and genetic analysis of *Streptomyces* strains have been hindered by the apparent lack of natural fertility in these strains. To establish the genetic recombination of *Streptomyces* strains, it is essential to form protoplasts from the mycelia and then regenerate the filamentous forms on a suitable hypertonic medium.

In order to obtain a good efficiency of regeneration freqeuncy, we investigated the conditions for the production and regeneration of protoplasts with higher viability, and developed a chemically defined hypertonic medium for use with an oleandomycin-producing strain of *S. antibioticus*. In addition oleandomycin-producing ability of the strain regenerated from protoplast was compared to that of original strain.

Materials and Methods

Bacterial strains and media

The strain used in this study was *Streptomyces antibioticus* KCTC 1081, and grown in liquid YME medium containing 0.4% yeast extract, 1% glucose and 1% malt extract. *Escherichia coli* (*E. coli*) ATCC 25922, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, *Staphylococcus aureus* subsp. *aureus* (*S. aureus*) ATCC 6538P, and *Micrococcus luteus* (*M. luteus*) ATCC 10240 were used for bioassay of antimicrobial activity. *E. coli*, *P. aeruginosa* and *S. aureus* were grown in nutrient broth (Difco Co.), and *M. luteus* was grown overnight at 37°C in trypticase soy broth (TSB; Difco Co.). The basal mineral-base medium (BMB; 0.1 M MOPS buffer, pH 7.5, 2 mM $MgSO_4$, 0.5 mM K_2HPO_4, 1 ml trace salt solution: $ZnCl_2$, 40 mg/L; $FeCl_3 \cdot 6H_2O$, 200 mg/L; $CuCl_2 \cdot 2H_2O$, 10 mg/L; $MgCl_2 \cdot 4H_2O$, 10 mg/L; $Na_2B_4O_7 \cdot 10H_2O$, 10 mg/L; $(NH_4)_6Mo_{24} \cdot 4H_2O$, 10 mg/L) was used for assaying the oleandomycin-producing activity. Protoplast (P) buffer (0.3 M sucrose, 0.25g K_2SO_4, 0.05g KH_2PO_4, 2.03g $MgCl_2 \cdot 6H_2O$, 3.68g $CaCl_2 \cdot 2H_2O$, 0.25 M TES buffer (pH 7.2), 2 ml trace element solution; per liter) was used for protoplast preparation. R1 (6), R2YE (6), R3 (6) medium, and International Streptomyces Project medium 4 (Difco Co.) supplemented with 0.3 M sucrose (ISPS4) were used for regeneration of protoplasts.

Culture conditions

The conditions used for vegetative growth, sporulation and germination of *S. antibioticus* have been described previously (6). Spores were scraped from the agar surface with a inoculation loop and resuspended in ice-cold deionized water. The crude suspension was filtered through glasswool to remove the mycelial fragments, the spores were pelletted by centrifugation, resuspended in 20% (w/v) glycerol and stored at −20°C until use. Inocula were prepared by inoculating 50 m*l* YME liquid medium in 250 m*l* Erlenmyer flask with 50 μ*l* spore suspension. After incubation at 28°C for 72 hrs in an orbital incubator with shaking at 150 rpm, growth of these cultures was estimated by measuring the optical density at 600 nm with spectrometer (Kontron Co.). The mycelia were harvested by low speed centrifugation (1500~2000 rpm) and washed with a solution of 0.3 M sucrose.

Preparation of protoplasts

Protoplasts were prepared by the procedure of Okanish *et al* (12). The strain was grown by shaking in YME medium containing 0.2% (w/v) glycine until reached the transition phase between the exponential and stationary growth phase. The mycelia were washed with 0.3 M sucrose and suspended in P buffer containing 4 mg/m*l* lysozyme. Incubation was carried out at 35°C for 60 min to 90 min and then P buffer was added to dilute the solution containing lysozyme. After filtering through glass wool, the protoplasts were collected by low speed centrifugation and washed 2~3 times with P buffer.

Regeneration of protoplast

Protoplasts were diluted in P buffer containing 0.3 M sucrose and 100 μ*l* portions of the samples (approximately 10^6 cells/m*l*) was spread onto R1, R2YE, R3, and ISPS4 plates, respectively. The plates were incubated for 5 to 7 days at 28°C to ensure that all the regenerating colonies developed. The regeneration frequency was measured by following equation (10);

$$\text{Regeneration frequency (\%)} = \frac{\text{No. of colony regenerated} - \text{No. of osmotic resistant colonies}}{\text{No. of protoplast plated}} \times 100$$

Determination of oleandomycin activity

To determine the oleandomycin activity, we used streaking plate method (16). *S. antibioticus* and its regenerant were plated on a ISPS4 agar plate and incubated at 28°C for 5 to 7 days. Thereafter, the test organisms were cross-streaking on the same ISPS4 agar plate, and the inhibition zone was observed after 24 hrs.

Result and Dissucussion

The protoplast formation of *Streptomyces*

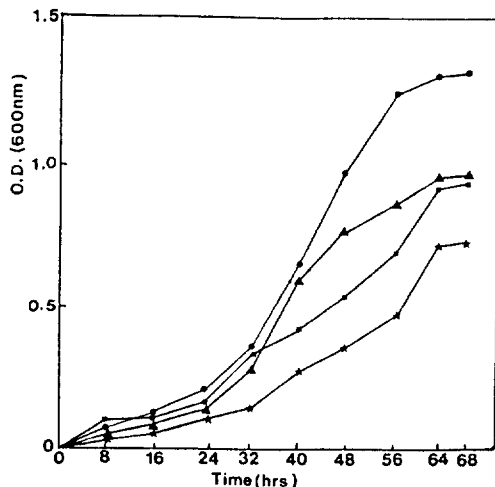

Fig. 1. *Growth curves of Streptomyces antibioticus. Cells were cultured in YME media containing various concentrations of glycine.* ■, *0.1% glycine;* ●, *0.2% glycine;* ▲, *0.4% glycine;* ★, *1.0% glycine*

Fig. 1 shows the growth kinetics of *S. antibioticus* in YME broth in the presence or absence of various concentrations of glycine. As shown in Fig. 1, the presence of 0.2% (w/v) glycine increased the cell doubling time, whereas more than 1% glycine (w/v) rather inhibited the cell growth.

To examine the efficiency of protoplasting of *S. antibioticus* various concentrations of lysozyme were treated according to the method described by Sagara *et al.* (13). Mycelia harvested from stationary phase culture were treated with various concentrations of lysozyme in solution of 0.3 M sucrose, 20 mM $MgCl_2$, 5 mM $CaCl_2$ (Figs., 2, 3, and 4). As indicated in the Figures, protoplasts were efficiently formed by 4 mg/m*l* of lysozyme at 35°C for 60 to 90 min.

Reversion of protoplast

Most of current techniques for genetic manipulation of *Streptomyces* are dependent upon the efficient isolation and regeneration of protoplasts. Therefore, it is necessary to regenerate mycelial forms efficiently in a suitable hypertonic medium. Previous experiments showed that the regeneration effiencies of protoplasts in *Streptomyces* were depending on various regeneration media (6, 11, 12). In this study the efficiencies of protoplast regeneration were examined in R1, R2YE, R3, and ISPS4 media, respectively. As shown in Table 1, ISPS4 medium was found to be the most suitable medium for regeneration. Levret'eva *et al.* (7) reported that repeated protoplasting and regeneration resulted in a very low regeneration frequency, i.e. 0.0002%. In this study, however, protoplasting with subsequent reversion to a

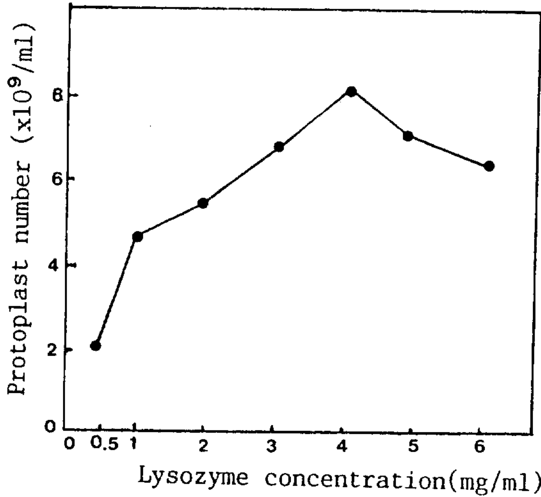

Fig. 2. *Effect of lysozyme concentration on the formation of protoplast*

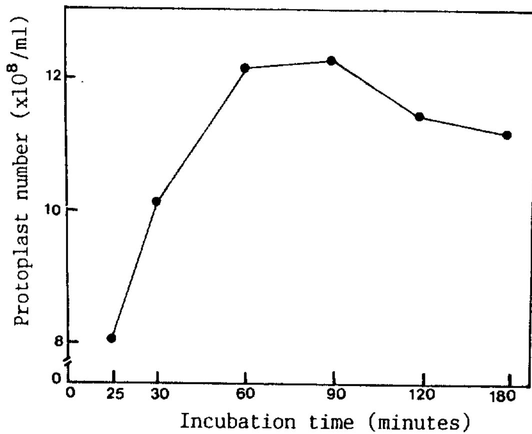

Fig. 3. *Formation of protoplast of Streptomyces antibioticus with the lapse of time of lysozyme (4 mg/ml) treatment.*

cellular form of *S. antibioticus* could efficiently monitored in the ISPS4 medium. It may therefore be useful for the culture of protoplast of *S. antibioticus.* Even though the ISPS4 medium improved protoplast regeneration frequencies, it may not possible to obtain the very high frequencies of regeneration with other streptomycete strains.

On the other hand, it was reported that protoplast formation and regeneration are affected by osmotic stabilizer of streptomycetes (11). Weibull (19) suggested that the effect of Mg^{++} on the stabilization of spheroplasts might be related to the prevention of release of lipid residues from the plasma membrane. Subsequently, Mg^{++} has been employed by many researchers for spheroplasting or protoplasting (8).

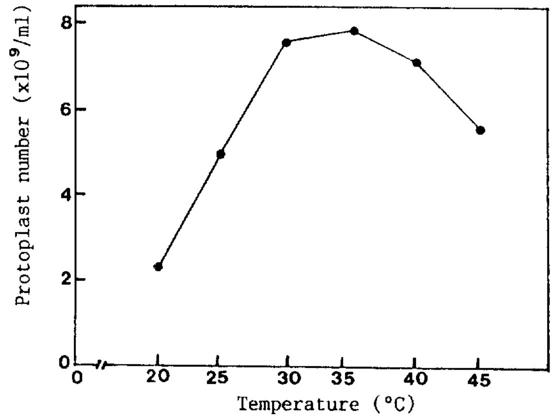

Fig. 4. *Effect of temperature on the formation of Streptomyces antibioticus protoplasts*

Table 1. *Protoplast regeneration efficiency in various regeneration media.*

Regeneration media	Osmotic stabilizer	Mg^{++}	Ca^{++}	Effiency (%)
R1	Sucrose(0.3 M)	20mM	50mM	0.002
R2YE	Sucrose(0.3 M)	50mM	20mM	0.015
R3	di-sodium succinate(0.3 M)	50mM	20mM	$<10^{-3}$
ISPS4	Sucrosee(0.3 M)	20mM	5mM	0.25

To examine the effect of osmotic stabilizer on regeneration, protoplasts from *S. antibioticus* was tested in a regeneration medium containing different concentrations of Mg^{++} and Ca^{++}. Table 1 shows that 20 mM Mg^{++} and 5 mM Ca^{++} are optimal for regeneration in the presence of 0.3 M sucrose. But the regeneration efficiency decreased as Ca^{++} concentration increased. Above results suggested that high Mg^{++} and low Ca^{++} concentrations are essential for good regeneration of *S. antibioticus.* The regenerated colonies were appeared after 3 days of incubation and most of them shown after 5 to 7 days at 28°C. The process of cell regeneration from *S. antibioticus* was generally slow and asynchronous.

Bioassay of oleandomycin-producing activity

Levret'eva *et al* (7) was reported that the use of protoplasting with subsequent reversion to the cellular form in improvement of oleandomycin-producing organism provides a 110% increase in the range of culture varation with respect to the antibiotic property. Demain (3) was also reported that antibiotics were important compounds in the transition from vegetative cells to spores. In the present study, we also observed that the

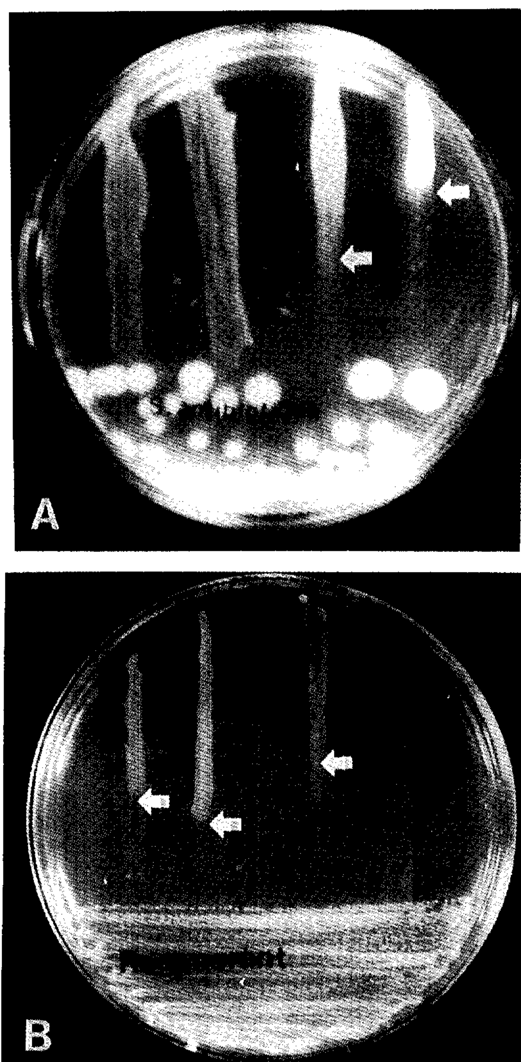

Fig. 5. *Antimicrobial activity of Streptomyces antibioticus.*
A, *Streptomyces antibioticus*; B, regenerant
Arrows indicated the inhibition zone.

regeneration colonies of *S. antibioticus* showed higher antimicrobial activity to the test oragnisms, such as *E. coli, P. aeruginosa, S. aureus*, and *M. luteus*. As shown in Fig. 5, the regenerated *S. antibioticus* exhibited the increased antibacterial activity against the test organisms, especially to *M. luteus*.

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초 록: *Streptomyces antibioticus*의 원형질체 분리와 재생

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본 연구는 oleandomycin을 생산하는 *Streptomyces antibioticus* (*S. antibioticus*) KCTC1081 균주에서 원형질체 형성 및 재생의 최적 조건을 조사하였다. 균사체는 0.2% glycine을 포함하는 YME 배지에서 배양하였으며, 이 균사체를 원형질체로 전환시키기 위해 4 mg/m*l* lysozyme을 포함한 P 완충용액에서 60분간, 35℃ 로 배양하였다. 여러 가지 합성배지에서 원형질체의 정상적인 균사체 상태로의 전환을 관찰하였다. 20 mM Mg^{++}, 5 mM Ca^{++} 그리고 0.3 M sucrose를 함유한 배지에서 5일간 28°C로 원형질체를 배양한 결과 높은 재생빈도를 얻을 수 있었다. 이러한 실험 결과로 *S. antibioticus*의 원형질체 재생 정도가 향상되고 안정화된 개량된 재생 배지 및 실험 방법을 확립하였다. 원 균주와 비교해 볼 때 재생균주의 항생제 활성도 또한 증가함을 확인하였다.