Cross Gradient Plate Method for Differential Isolation of Actinomycetes with Varied Physiological Properties

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Isolation of wide varieties of actinomycetes has been, and still is, important not only for searching for bioactive secondary metabolites but also for investigating differentiation and evolution. In the natural environment, it has been believed that there exists varieties of microorganisms including actinomycetes that have resisted isolation effort. Therefore, various methods have been innovatively established and successfully used for isolation of actinomycetes as such. These methods involve the use of specific substrates such as humic acid¹⁻²⁾ and intact plant leaves³⁾, and specific selective pressures such as antibiotics¹⁻⁶⁾.

The innovative isolation methods were generally established on the basis of some physiological or ecological properties that are common and unique to specific group of genera and antibiotic producers. Indeed, these specific properties have been revealed by physiological and ecological characterization of actinomycete strains¹⁻⁶⁾. We have been running projects that involve isolation and characterization of actinomycetes in terms of antibiotic resistance, particularly resistance to aminoglycoside antibiotics (AGs). Consequently, we demonstrated that AG producers exhibit individual and multiple AG resistance⁷⁾, and that the use of varieties of AGs was advantageous for efficient isolation of varieties of AG producers⁸⁾.

In the present report, we describe the use of a new isolation method termed the cross gradient plate method which was established by modifying the gradient plate method described by Szybalski and Bryson⁹⁾. In the usual isolation methods using antibiotics as the selective pressures, microbiologists have used only one concentration of antibiotics that should have restricted the growth of microorganisms with relatively low levels of resistance.

Therefore, we have thought that it should be necessary and useful to establish more sophisticated, but simple, isolation method for actinomycetes with wider levels of resistance to selective pressures because they may include ones with unique morphology and secondary metabolism. For this purpose, the gradient plate method described by Szybalski and Bryson, that is often used for characterization of antibiotic resistance in the genetic and biochemical field of actinomycetes, seemed to be obviously useful. In their method, the concentration gradient plate containing an antibiotic was made by the use of a regular size Petri dish. This should actually be advantageous to isolate varieties of microorganisms. However, we have thought that it should be more advantageous to use the cross gradient plate using two different antibiotics and larger size plate.

Figure 1 illustrates the basic principle of the cross gradient agar plate established. The plate was made of the two tapered-agar layers in a rectangular plastic dish (7.5 cm x 22.5 cm Sterile Square Schale No.1; Eiken Instruments, Tokyo). The half-strength (1/2 x) Inorganic salt-Starch agar medium (ISP No. 4; Difco) supplemented with cycloheximide (50 µg/ml) and nystatin (25 µg/ml) was used as the basal medium. One antibiotic was added to 40 ml of the basal medium which was then poured into a plastic dish slanted just sufficiently to cover the entire bottom (lower layer). After hardening, the plate was placed in the horizontal position and poured with another 40 ml of the basal medium added with the second antibiotic. After hardening, the agar plates were placed in a refrigerator overnight to let the added reagents diffuse so as to form a good concentration gradient. Concentration of the antibiotics added was varied. The slope of concentration gradient in the medium is steep with relatively high concentration of antibiotics added and is gentle with the low concentration of antibiotics added. One can expect the single effect of one antibiotic at the both end of the plate and the associative effect of both antibiotics at the internal regions (Fig. 1-a).

Photograph 1-D represents a typical case that provided well-localized growth of actinomycete colonies with varied levels of resistance to the selective antibiotics (25 µg/ml), kanamycin and astromi-

cin when a soil suspension was plated. Numbers of colonies appeared were obviously large at the both terminals of the plate and small at the center region where there should exist a concerted effect of kanamycin and astromicin. There was a obvious difference in colony profile among the regions where they grew. Thus it was easy to distinguish different colonies with different resistance from each other on the cross gradient plate. This was a contrast with colonies on the control plate without addition of antibiotics (not shown) where they could not be distinguishable from each other except for ones with clear morphological difference. Thus the advantage of the cross gradient plate method was verified.

Additional idea that struck us was the combination of antibiotic effect with pH effect. Based on this idea, a pH gradient plate (pH about 4 to 9 with 1/15 M phosphate buffer) was established by adding 2/15 M of KH_2PO_4 and Na_2HPO_4 (20ml each; autoclaved separately) to 20 ml each of ISP No. 4 medium for A and B layers, respectively. As results, Photo. 1-B and C represent the effect of the pH gradient plate containing the same concentration (25 $\mu\text{g/ml}$) of gentamicin C complex and astromicin,

respectively. With the pH gradient plate we could expect a concerted effect of pH gradient and antibiotic activity gradient, since AG antibiotics exhibit the peak activity at pH around 8 and the decreasing activity at lower or higher pH ranges than pH8 as illustrated in Fig. 1-c. On a simple pH gradient plate as the control (Photo. 1-A), a well-dispersed colony growth of actinomycetes was observed. Effect of pH on the growth was obvious because the size of the actinomycete colonies at alkaline pH region was smaller than that in neutral and acidic pH regions. However, there was no clear difference in colony profile in the different pH regions. Photograph 1-B shows the colonies appeared on the pH gradient plate containing gentamicin C complex. On this plate, only bacterial colonies appeared only in the acidic pH region, showing the strong antibiotic effect of gentamicin in neutral to alkaline pH regions. Photograph 1-C is another pH gradient plate containing astromicin. In this case, actinomycete colonies grew, reflecting its weaker inhibitory effect than that of gentamicin. There was a clear difference in colony profile at each region of the plate.

In summary, the cross concentration gradient plate method and the pH gradient plate method have

Fig. 1. Cross, pH and activity gradient agar plates.
dashed line : pH, solid line : antibiotic activity

Photo. 1. Colony profiles on cross, pH and activity gradient plates. A: pH gradient (pH 4 at the left to pH 9 at the right), B: Activity gradient of gentamicin (25 $\mu\text{g}/\text{ml}$; pH 4 at the left to pH 9 at the right), C: Activity gradient of astromicin (25 $\mu\text{g}/\text{ml}$; pH 4 at the left to pH 9 at the right), D: Cross concentration gradient with kanamycin (left; 25 $\mu\text{g}/\text{ml}$) and astromicin (right; 25 $\mu\text{g}/\text{ml}$). 0.2 ml of a soil suspension (1 g soil in 20 ml 0.85% NaCl) was spread on each plate.

the following advantage in isolating actinomycete strains. 1) Well-localized colony growth may be expected, that makes it easy to distinguish strains with difference in physiology or resistance. 2) Because of the concerted effect of two factors and the use of large size plate, one can expect well-dispersed and well-isolated colony growth even from a heavy inoculum. 3) One can have varieties of choices of selective pressures with which varied variety of colony growth can be expected.

With these advantages of the method established, we were actually successful to obtain wide varieties of actinomycete strains including rare ones and producers of novel bioactive secondary metabolites such as isoxazole-4-carboxylic acid¹⁰⁾. In the present study, we used ISP No. 4 medium as the basal medium. If one use different media allowing the growth of rare actinomycetes that hardly grow on ISP No. 4 medium, the isolation of wider varieties of actinomycete strains will be undoubtedly possible. Thus, the use of varieties of the cross gradient plates would be of help in isolating microorganisms that have resisted isolation efforts.

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