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The Analysis of Some Factors Involved in Sisomicin Fermentation Based on Temperature Effects

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Sisomicin 발효에 대한 온도 및 제반인자의 영향

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Effects of temperature on sisomicin fermentation were investigated. From the specific growth rates for logarithmic phase estimated at various temperatures, 8.2 kcal/g-mol was obtained as an activation energy for cell growth. It suggests that cell growth rate was limited by the internal diffusion layers for nutrients or oxygen caused by aggregated cells. Final antibiotic titer was decreased with increasing temperature, and it depended highly on the temperature to which cells were exposed during the logarithmic phase of growth. Temperature shifts during fermentation brought about an increase in antibiotic productivity.

Since most aminoglycoside antibiotics are extracellular (1), they accumulate mostly in culture supernatant. However, sisomicin which is produced by *Micromonospora inyoensis* is mostly cell-bound and partly excreted into culture broth (2, 3). In this case, antibiotic titers are limited by a restriction on the cell mass to be attained in fermentation broth.

On the other hand, this producing organism grows in a mycelial form so that cells can be aggregated as cell mass is increased in fermentation broth. As a result, there will exist internal diffusion layers which retard the diffusion of nutrients or oxygen onto the inside surfaces of aggregated cells.

In these experiments, the effects of temperature on sisomicin fermentation were investigated in terms of growth rate, growth stage, antibiotic titer, antibiotic productivity, diffusion problem, etc.

Materials and Methods

Strain

The producing microorganism used in these experments was *Micromonospora inyoensis* IFO 13156.

Media

Germination and fermentation media were prepared and adjusted to pH8.0 before sterilization (3, 4).

Experimental procedures

Twenty mililiters of germination medium in a 250 m*l* Erlenmeyer flask was inoculated from a slant of *M. inyoensis*. Then, it was cultured at 28 °C for 3 days in a reciprocal shaker. Two and half mililiters of germinated medium was transferred to a

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500 ml Erlenmeyer flask containing 50 ml of fermentation medium. And it was shaken at various temperatures for 4 days in a reciprocal shaker.

Extraction of sisomicin(5)

A certain amount of whole culture broth was taken and adjusted to pH2.0 with 6N sulfuric acid solution. The acidified broth was stirred for about 15 minutes and then, centrifuged at 3000 rpm for 10 minutes (Hanil Centrifuge-HA10). The supernatant was adjusted to pH7.0 with 6N ammonium hydroxide solution. To precipitate calcium ions, sufficient oxalic acid was added to the neutralized supernatant and then centrifuged. The supernatant was neutralized again with 6N ammonium hydroxide solution.

Antibiotic assay(3, 6)

Antibiotic potencies were determined by a cylinder cup agar diffusion assay using *Staphylococcus aureus* ATCC6538P as a test organism.

Cell concentration measurement(3)

After removal of insoluble soybean meal by centrifugation (2 minutes at 600 rpm, Hanil Centrifuge-HA10), the dry weight was measured.

Results and Discussion

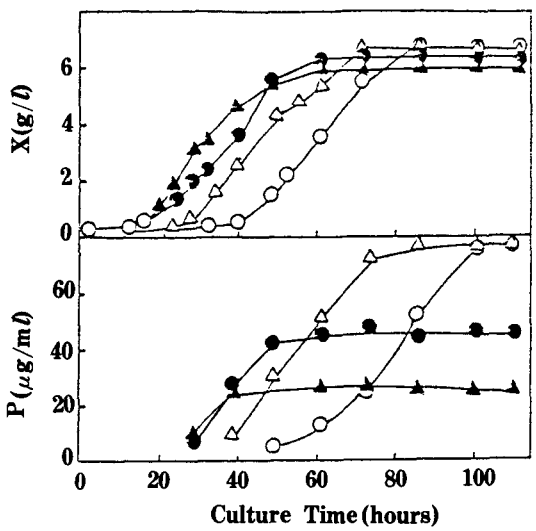

Fig. 1. Growth-production curves at various temperatures of sisomicin fermentation.

○—○: 28°C ; △—△ : 31°C ; ●—●: 34°C ; ▲—▲: 37°C

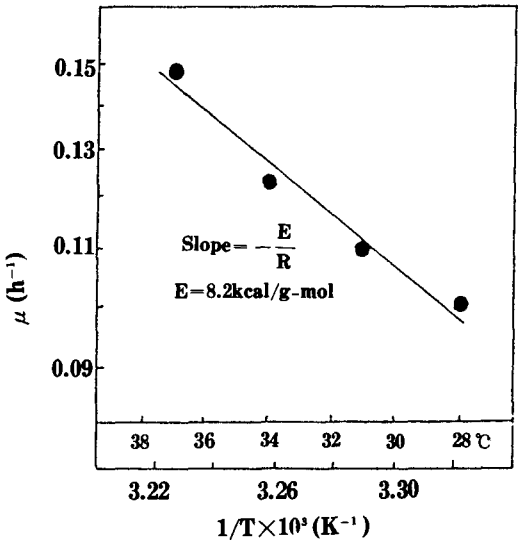

Fig. 2. Estimation of an activation energy for cell growth in sisomicin fermentation.

μ: specific growth rate for logarithmic phase, h^{-1}
T: absolute temperature, °K
E: activation energy, kcal/g-mol
R: gas constant, 1. 987 cal/g-mol/ °K

Sisomicin fermentations were carried out batchwise at various temperatures (28, 31, 34 & 37 °C). The growth-production curves were prepared from them (Fig. 1). As shown in Fig. 1, the lag phase for cell growth was shortened with increasing temperature and the growth was early finished under the condition. From the growth curves shown in Fig. 1, the specific growth rate (μ) for logarithmic phase was estimated at each temperature, respectively. And the values were plotted semi-logarithmically by using the Arrhenius equations of [1] and [2] (Fig. 2).

$$\mu = A \exp(-E/RT) \quad [1]$$

$$\ln(\mu) = \ln(A) - (E/R)(1/T) \quad [2]$$

As shown in Fig. 2, specific growth rate was increased with increasing temperature, and a straight line was constructed from the relationship between reciprocal temperature and logarithmic value of the corresponding specific growth rate. From the slope, the activation energy for growth was estimated to be 8.2 kcal/g-mol. It suggests that the overall rate of cell growth be determined by the internal diffusion rate of nutrients or oxygen onto the surfaces of

cells (7). Since the microorganism which was used in this experiment grew in a mycelial form, they were easily aggregated as fermentation proceeded. The aggregated cells can form internal diffusion layers which cause the slow diffusion of nutrients or oxygen onto the inside surfaces in them.

Final concentrations of cell and sisomicin were plotted again from the growth-production curves of Fig. 1 (Fig. 3). Cell concentration was decreased a little with increasing temperature, and sisomicin titiers (or concentrations) were not changed significantly at temperatures of 28 °C and 31 °C. However, they were sharply decreased at 34 °C and 37 °C as compared to those of 28 °C and 31 °C.

In the next experiment, 34 °C and 31 °C as fermentation temperature were chosen and temperature shifts were used (Fig. 4). Final antibiotic titer was the lowest at 34 °C and the highest at 31 °C. Among the cultures to be temperature-shifted at 34 °C & 31 °C, the shift by one day brought about the highest final antibiotic titer. It was the case in

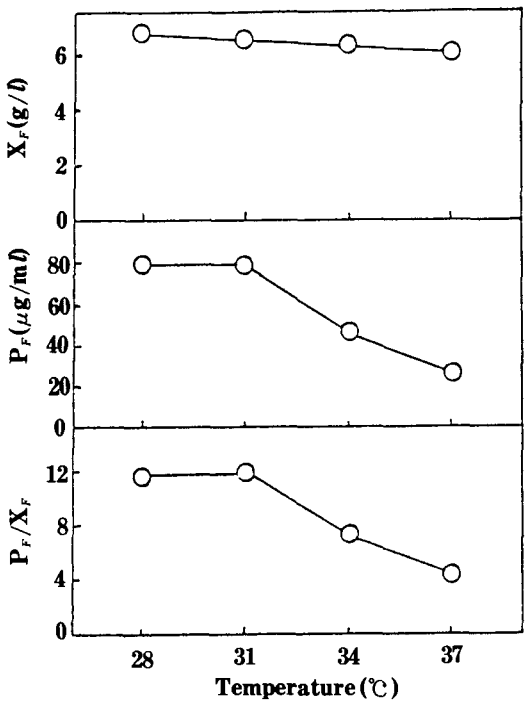

Fig. 3. Final concentrations of cell and antibiotic obtained at various temperatures.
X_F: final cell concentration, g/l
P_F: final antibiotic concentration, μg/ml

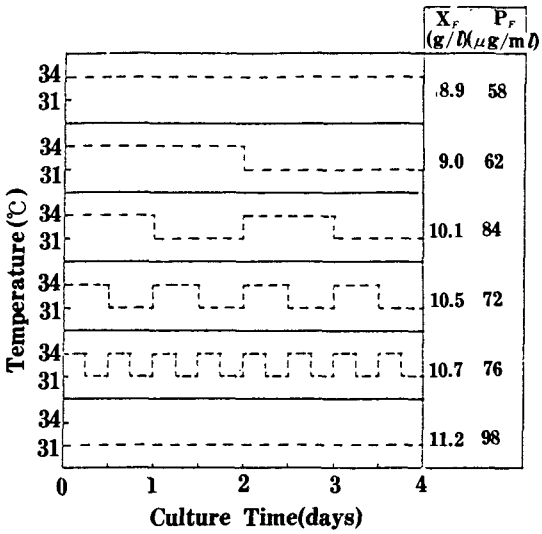

Fig. 4. Final concentrations of cell and antibiotic obtained for various temperature-shifts between 34°C and 31°C.
X_F: final cell concentration, g/l
P_F: final antibiotic concentration, μg/ml

which culture temperature was maintained at 31 °C for 24 hours on the second and the fourth day (Fig. 4). During the time (the second day), cell growth belonged to the logarithmic phase for which it was the most active and the most of cell mass was formed. The third and fourth highest titers of antibiotic were attained for the case that cells were cultured at 31 °C for 12 hours of logarithmic phase. When temperature was kept at 34 °C for the logarithmic phase and at 31 °C later, the final antibiotic titer was pretty close to that for the case that temperature was kept to be 34 °C all the time. These results suggest that final sisomicin titer depended highly on the temperature to which cells were exposed during the logarithmic phase of growth.

In another experiment, temperature shift of 34 °C to 31 °C was only once carried out respectively at an internal of 12 hours (Fig. 5). And the final concentrations of cell and antibiotic, which were obtained at the end of fermentation, were plotted in Fig. 6-I. Fig. 6-II shows the growth curve of cells grown at 34 °C. In case fermentation temperature was kept initially at 34 °C for 0, 12 or 24 hours respectively and then kept at 31 °C, the final titers of sisomicin were almost same. However, when tem-

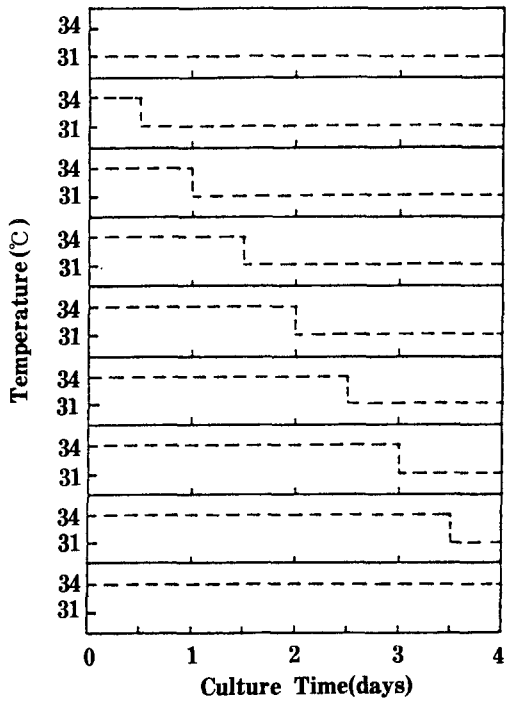

Fig. 5. One step of temperature-shifts between 34°C and 31°C.

perature shifts of 34 °C to 31 °C were carried out at the time between 24 and 48 hours, the final antibiotic titers were reduced remarkably. Although the final titers of the antibiotic had no differences for the cultures in which temperature shifts were done beyond 48 hours, they were reduced by more than 40% as compared to those for the cases to be temperature-shifted within 24 hours. These results confirm again that the final titers of antibiotic were affected significantly by the temperature to which cells were exposed during the logarithmic phase.

A strategy for increasing antibiotic productivity was considered by using only two different temperatures of 34 °C and 31 °C. The antibiotic titer and cell concentration for the culture exposed to 34 °C for first 24 hours and then 31 °C up to the end were compared with those of the culture which was kept to be 31 °C all the time (Fig. 7). Although final antibiotic titers for both cases were about same, the product formation from the temperature-shifted (34→31) culture was faster than that from being constantly kept at 31 °C due to the faster growth of

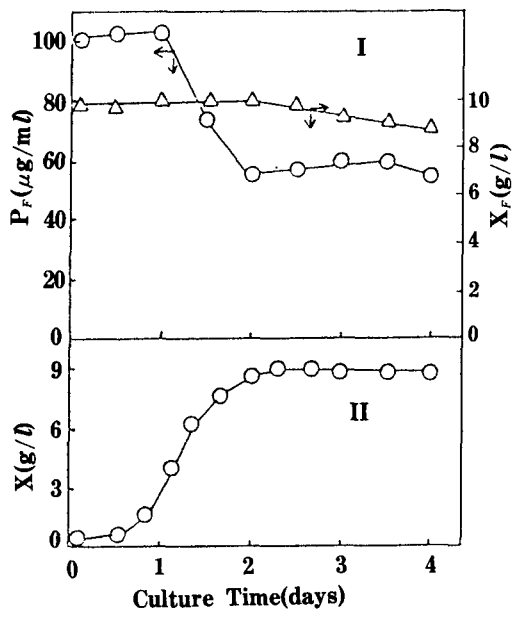

Fig. 6. Final concentrations of cell and antibiotic obtained for one step of temperature-shifts between 34°C and 31°C at intervals of 12 hours.
X_F: final cell concentration, g/l
P_F: final antibiotic concentration, μg/ml

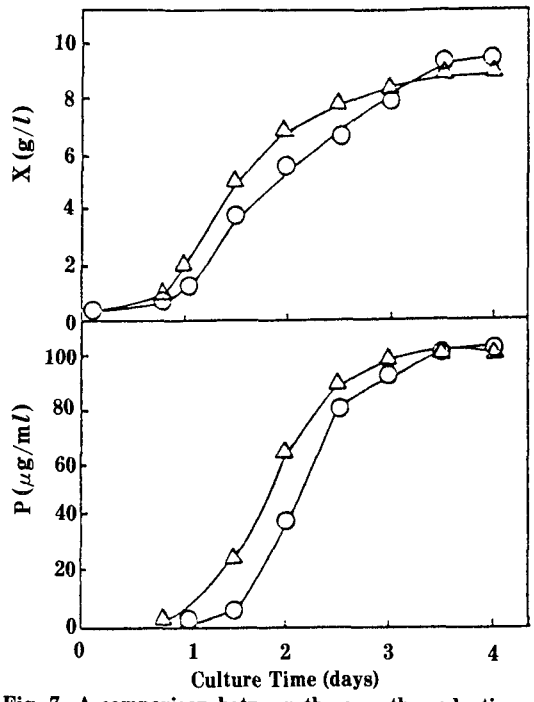

Fig. 7. A comparison between the growth-productions for control and temperature-shift.
○—○ : 31→; △—△ : 34→31→

the cells exposed to the temperature-shift. When 90 μg/ml as a final antibiotic titer was considered, the fermentation times needed for the control and the temperature-shifted culture were about 3 and 2.5 days, respectively. Thus, temperature-shift can reduce fermentation time by 12 hours. It corresponds to about 17% increase in antibiotic productivity.

요 약

Sisomicin 발효에 대한 온도의 영향을 살펴보았다. 각각의 온도에 대하여 균체의 비생육속도로 부터 8.2 kcal/g-mol의 activation energy를 얻었다. 이 값은 발효중 균체가 균사체를 이루어 배지성분이나 산소에 대한 internal diffusion layer를 형성하는 것을 시사해 주고 있다. 한편 최종 항생물질농도는 온도가 증가함에 따라 감소하였으며, 온도의 변화를 주는 실험으로 부터 대수증식기에 유지되는 배양온도가 최종 항생물질의 농도에 심한 영향을 주는 것으로 나타났다. 배양온도를 변화시켜 항생물질의 생산성을 증가시키는 방법에 대해 생각하여 보았다.

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