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Effect of Magnesium Sulfate on Product Inhibition of Sisomicin Production

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Addition of 100mM $MgSO_4$ to a cell culture after 54 hours resulted in a 2.4-fold increase in the sisomicin titre compared to a control to which no $MgSO_4$ was added, and a considerable amount of intracellular sisomicin was liberated outside the cells. The occurrence of product inhibition in fermentation was confirmed by a reduction in net sisomicin production with increasing amounts of added sisomicin without addition of $MgSO_4$. All added sisomicin was bound to sisomicin-free cells in the absence of $MgSO_4$, whereas approximately 40% of added sisomicin was bound with the addition of 100mM $MgSO_4$. Under conditions of no enzmye synthesis, maintained by adding chloramphenicol to exclude product repression, sisomicin was produced in the presence of 100 mM $MgSO_4$ but little sisomicin was produced in the absence of $MgSO_4$.

In batchwise productions of secondary metabolites, the reason why production ceases can usually be considered in two respects: the first is product inhibition which retards the metabolic reactions necessary for product formation, and the second is decay or deactivation of the producing enzyme(s) (1). In the case of sisomicin fermentation where most of the metabolite produced during the fermentation accumulates inside the cells and is bound to the cell, the intracellular concentration reaches a high level causing a severe product inhibition (2, 5, 6). Addition of $MgSO_4$ in high concentrations (50 to 200mM) liberates a considerable amount of the bound intracellular sisomicin outside the cells, resulting in increased sisomicin yields (3). However, when high concentrations of $MgSO_4$ are maintained in fermentation broth, how the regulation of sisomicin production is affected by $MgSO_4$, i.e., an alleviation of product inhibition or a genetic change, is not clearly understood.

In this study the effects of added $MgSO_4$ on enhanced sisomicin production were investigated. The major points discussed include the alleviation of product inhibition by liberation of intracellular sisomicin by $MgSO_4$, product inhibition by added sisomicin, binding of added sisomicin to sisomicin-free cells in the presence or absence of $MgSO_4$, and sisomicin production under no growth conditions.

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MATERIALS AND METHODS

Strains (7, 9)

Micromonospora inyoensis IFO13156 was used in sisomicin fermentation, and *Staphylococcus aureus* ATCC 6538P was used as a test organism for sisomicin assay.

Media

The germination medium consisted of 0.3% beef extract, 0.5% tryptone, 0.5% yeast extract, 0.15% dextrose, 2.4% starch, and 0.2% calcium carbonate in distilled water. The fermentation medium contained 5% starch, 0.7% calcium carbonate, 3.5% soybean meal, and 16.8 μM $CoCl_2$ in tap water. The pH of both media was adjusted to 8.0 prior to sterilization. The medium for sisomicin assay using *S. aureus* was composed of 0.15% beef extract, 0.3% yeast extract, 0.4% casitone, 0.6% peptone, and 0.1% dextrose in distilled water. The pH was adjusted to 6.6 prior to sterilization.

Cultivation

For the germination culture 20 ml of germination medium in a 250 ml shake flask was sterilized, inoculated with *M. inyoensis*, and then cultivated at 28°C for 3 days on a rotary shaker at 150 rpm. Fermentation culture was performed in a 500 ml shake flask filled with 50 ml of fermentation medium at 28°C for 4 days on a rotary shaker at 150rpm. Various concentrations of $MgSO_4$ were added after 54 hours of cultivation.

Extraction and Assay of Sisomicin

Five ml of culture broth was treated with a 6 N H_2SO_4 solution to extract sisomicin (8). $MgSO_4$ was removed

from samples by precipitation with ammonium phosphate (2). The sisomicin content was measured by a cylinder method using *Staphylococcus aureus* (3, 4).

Measurement of Cell Mass

Five ml of culture broth was taken and centrifuged to separate cells from soybean meal. After the supernatants were centrifuged again, the precipitates were washed twice with distilled water to remove residual starch, then dry weight was measured.

Preparation of Sisomicin-free Cells

Cells cultivated in fermentation medium for 4 days were harvested by centrifugation and washed several times with TES buffer solutions (0.05M, pH7.3). The collected cells were suspended in a 250mM $MgSO_4$ containing TES buffer solution and incubated for 20 minutes, then centrifuged. Sisomicin-free cells were obtained after repeating the procedure three times.

Sisomicin Production Using Resting Cells

Cells cultivated in fermentation medium for 54 hours were harvested, centrifuged, and washed with TES buffer solution (0.05M, pH7.3). Then, the cells(6.9 g/l in final conc.) were suspended in TES buffer solution containing 40 g/l of maltose, 1 g/l of glucose, and 100 μg/ml of chloramphenicol. Parts of the solutions were treated with sisomicin(10 μg/ml) and/or magnesium sulfate(100 mM), and the remains were used as the control.

RESULTS AND DISCUSSION

Various concentrations of $MgSO_4$ were added to the fermentation media in the range of 0 to 200 mM after 54 hours of cultivation, and the effects were examined. As shown in Fig. 1, there was little difference in cell growth due to different concentrations of $MgSO_4$ because a considerable cell growth had already occurred prior to addition of $MgSO_4$. The antibiotic titre at the end of fermentation increased with higher $MgSO_4$ concentrations, up to 100 mM, compared to the control to which no $MgSO_4$ was added. However, at concentrations higher than 100 mM the titre decreased. The maximum titre obtained at 100 mM $MgSO_4$ was approximately 2.4 times greater than the control. The amount of extracellular antibiotic liberated outside the cells increased remarkably as the $MgSO_4$ concentration increased. At 200 mM most of the intracellular antibiotic was liberated. Thus, enhancement of the antibiotic titre by added $MgSO_4$ was considered due to the alleviation of a product inhibition which occurred in the biosynthetic metabolism of sisomicin.

In order to determine if a significant product inhibition exists in sisomicin fermentation, various concentrations (0 to 40 μg/ml) of sisomicin were added to fermentation broth after 54 hours of cultivation. At this culture period product inhibition, rather than product repression, is expected to dominate. As shown in Fig. 2, the final antibiotic concentration reached approximately 60 to 65 μg/ml, indicating that net sisomicin production was decreased by the presence of extracellular sisomicin, supposedly affected by product inhibition. This may suggest that the presence of sisomicin, regardless of whether it is added or produced, regulates itself its biosynthetic metabolism. Considering that the amount of sisomicin liberated upon the addition of $MgSO_4$ was related to the increased antibiotic yields, as shown in Fig.1, the sisomicin in the extracellular state, i.e., not

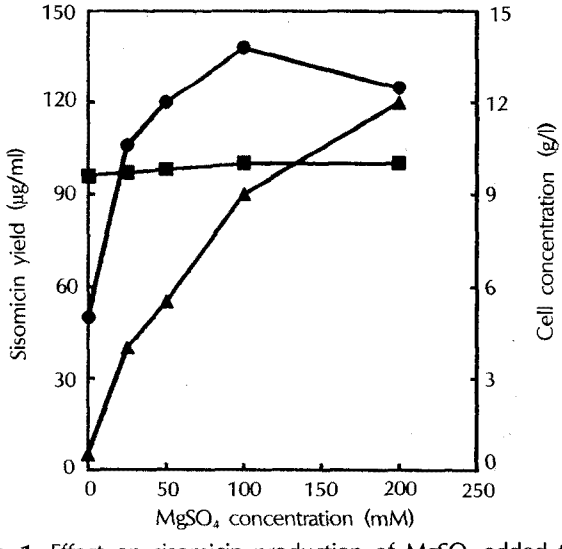

Fig. 1. Effect on sisomicin production of $MgSO_4$ added to fermentation media after 54 hours of cultivation.
Total sisomicin(●), extracellular sisomicin(▲) and cell growth(■).

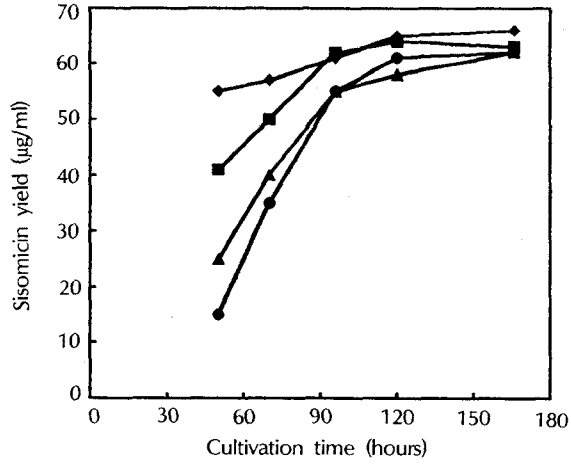

Fig. 2. Effect on sisomicin production of sisomicin added to fermentation media after 54 hours of cultivation.
The sisomicin contents(μg/ml) added to fermentation media: 0μg/ml (●), 10μg/ml(▲), 25μg/ml(■), 40μg/ml(◆).

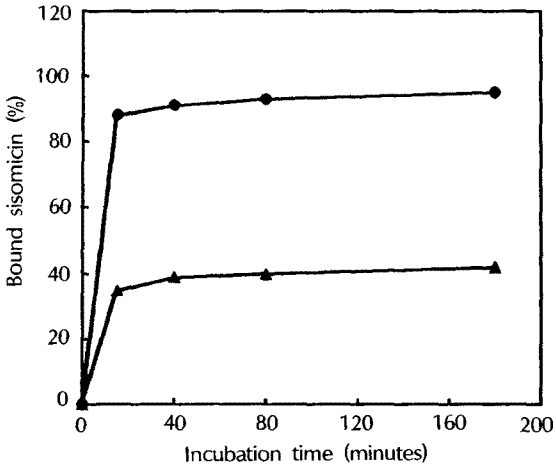

Fig. 3. Binding of sisomicin added to sisomicin-free cells.
A(●): sisomicin 50μg/ml, TES buffer 0.05M (pH7.3) and washed cells 6.3g/ml; B(▲): same as A, plus $MgSO_4$ 100mM

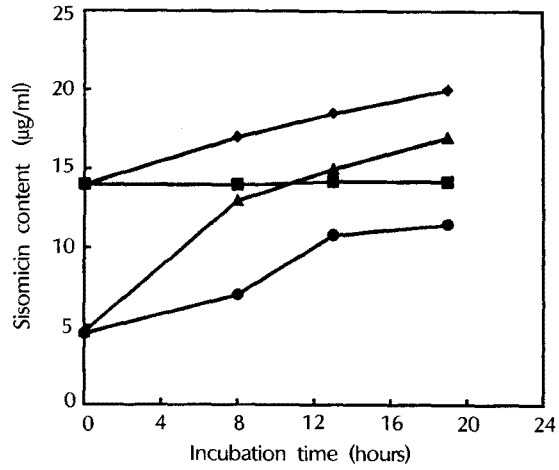

Fig. 4. Effect of $MgSO_4$ on sisomicin production by resting cells in maltose-glucose containing buffer solutions.
A(●): maltose 40g/l, glucose 1g/l, TES buffer 0.05M (pH7.3), washed cells 6.9g/l, and chloramphenicol 100 μg/ml; B(▲): same as A, plus $MgSO_4$ 100mM; C(■): same as A, plus sisomicin 10 μg/ml; D(◆): same as A, plus $MgSO_4$ 100 mM and sisomicin 10 μg/ml

bound to the cells in the presence of high $MgSO_4$ concentrations, is probably not responsible for product inhibition. If this is true, sisomicin added to the culture broth can be bound to the cells and inhibit sisomicin production. As a mechanism to inhibit antibiotic production, certain enzymatic reactions in the metabolic pathway must be considered.

To elucidate the binding mechanism of sisomicin to cells, followings were tested. Cells cultivated for 4 days were collected, and sisomicin-free cells were prepared. The cells were suspended in two different sisomicin-buffer solutions; TES buffer and 100 mM $MgSO_4$ containing TES buffer. As shown in Fig. 3, in the TES buffer containing only 50 μg/ml of sisomicin, most of the suspended sisomicin was bound to the sisomicin-free cells after 10 minutes of incubation, whereas approximately 40% of the sisomicin was bound in the TES buffer containing 100 mM $MgSO_4$ and 50 μg/ml of sisomicin. These results indicate that binding sites for the sisomicin molecules exist on the cell surface, and sisomicin molecules can be reversibly bound to or liberated from these sites.

Since cell growth also proceeded after 54 hours of cultivation, at which $MgSO_4$ was added to the fermentation broth, it is not clear whether the enhanced antibiotic production by added $MgSO_4$ was directly due to an alleviation of product inhibition, or any possible genetic changes on expression or synthesis of sisomicin-producing enzymes, or both. It is necessary to separate the effect alleviation of product inhibition from other possible mechanisms. To investigate the effect of alleviation of product inhibition by $MgSO_4$, enzyme reactions should proceed in the absence of protein synthesis, including enzymes engaged in sisomicin biosynthetic pathway. Cells harvested after 54 hours of cultivation were washed with buffer to remove the sisomicin fractions stuck to the cell surface, and resuspended in buffer solutions containing 100 g/ml of chloramphenicol. Chloramphenicol acts to inhibit protein synthesis to exclude product repression. The buffer solutions were supplemented with 40 g/l of maltose and 1 g/l of glucose, and incubated at 28°C and 150 rpm on a rotary shaker. To the solutions 100 mM $MgSO_4$ was added right before the incubation.

As shown in Fig. 4, the net amounts of sisomicin produced in 20 hours of incubation were 12 μg/ml and 6 μg/ml, with and without 100 mM $MgSO_4$, respectively. On the other hand, in the case where 10 μg/ml of sisomicin was added to the reaction solution, extra sisomicin was not produced in the case of no addition of $MgSO_4$, whereas 5 μg/ml of sisomicin was produced in the presence of 100 mM $MgSO_4$. In the absence of $MgSO_4$ most sisomicin was bound to the cells, whereas the net sisomicin fraction which was increased in the presence of $MgSO_4$, was liberated outside the cells. From the result, it is concluded that sisomicin production is enhanced by addition of $MgSO_4$ because product inhibition is alleviated by liberation of the intracellular sisomicin facilitated by $MgSO_4$.

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