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Effects of Mineral Salts on the Improvements of Sisomicin Yield

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무기질 염이 Sisomicin 발효 수율의 증가에 미치는 영향

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Effects of mineral salts on sisomicin fermentation were investigated. The optimal concentration of $CoCl_2$ for accomplishing a high antibiotic yield was found to be 16.8 μM at which it could function as a cofactor. At this level the other mineral salts tested had no effect.

On the other hand, at much higher concentration levels (above 1 mM), four mineral salts such as $ZnSO_4$, KH_2PO_4, $FeSO_4$ and $MgSO_4$ were used in order to liberate the intracellular sisomicin outside the cells, because the sisomicin accumulated mostly in cells and it was supposed to limit the improvement of antibiotic yield. $ZnSO_4$ and KH_2PO_4 had no effect at all, and $FeSO_4$ brought about some improvement. However, by keeping the concentration of $MgSO_4$ to be 25 mM or higher in culture broths, the antibiotic yield could be improved by more than 100%, partially due to the enhanced liberation of the intracellular antibiotic.

Mineral salts have been known to function as a cofactor for some antibiotic fermentations at relatively low levels (around 10 μM) in culture media. Cobalt ions have been reported to be a cofactor for the C-methylation steps of gentamicin- or sisomicin-synthesizing metabolism (1).

On the other hand, most of aminoglycoside antibiotics are excreted into the culture supernatant after they are formed in cells (2). However, some of them, such as gentamicin (3), sisomicin (4), verdamicin (5), etc., which are produced by the genus of *Micromonospora,* are mostly cell-bound and partly excreted into the culture supernatant. In the antibiotic fermentation where most of the antibiotic formed in cells remains inside the cells, a restriction on the maximal capacity of cellular volumes can result in relatively low yields. However, if the antibiotic formed in cells can be liberated outside the cells during the culture, higher yields may be achieved.

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In this study, various mineral salts were tested as a cofactor in sisomicin synthesis and their effect on the liberation of the intracellular sisomicin was also investigated.

Materials and Methods

Bacterial strains and cultures

Micromonospora inyoensis IFO 13156 was used in this study. The germination medium (6) consisted of 0.3% beef extract, 0.5% tryptone, 0.5% yeast extract, 0.1% dextrose, 2.4% potato starch, and 0.2% calcium carbonate. The fermentation medium (6) consisted of 5.0% potato starch, 3.5% soybean meal, and 0.2% calcium carbonate. These media were adjusted to pH 8.0 before sterilization.

In the germination stage, 20ml germination medium in a 250ml Erlenmeyer flask was inoculated from a slant of *M. inyoensis,* then cultured at 28°C for 3 days on a reciprocal shaker. In the fermentation

Table 1. Effects of various mineral salts as a cofactor on sisomicin production.

Mineral (3.4 μM)	X (g/l)	P (μg/ml)	P/X
None (DW)*	9.7	17	1.7
$CoCl_2$ (DW)	9.4	104	11.2
None	10.2	20	2.0
$CoCl_2$	9.6	110	11.4
$CuCl_2$	10.6	22	2.1
$FeSO_4$	9.9	18	1.8
$HgCl_2$	10.1	20	2.0
$MgSO_4$	10.7	20	1.9
$MnSO_4$	10.9	20	1.8
$ZnSO_4$	10.3	21	2.0

*Distilled water was used instead of tap water. Salts were added to the media at the beginning of the culture.

stage, 2.5ml germinated medium was transferred to a 500ml Erlenmeyer flask containing 50ml fermentation medium free of $CoCl_2$, followed by incubation at 28°C for 4 days on a reciprocal shaker (150rpm, 4cm stroke).

Extraction of sisomicin and antibiotic assay

Sisomicin was extracted with sulfuric acid from the culture broths according to Weinstein *et al.* (7), and oxalic acid was used to remove calcium ions. Antibiotic potencies were determined by means of cylinder cup agar diffusion assay using *Staphylococcus aureus* ATCC 6538P as the test organism (8).

Determination of cell concentration

After removal of insoluble soybean meal and calcium carbonate by centrifugation, the dry weight of cells was measured.

Results and Discussion

Role of cobalt ions as a cofactor

Various kinds of mineral salts were first tested at a low concentration (3.4 μM) during sisomicin fermentation. As shown in Table 1, sisomicin yield was remarkably enhanced only by cobalt chloride. However, there was practically no difference in cell concentration with addition of these mineral salts. This result suggested that cobalt ions could be used as a cofactor for this anitibiotic fermentation. When tap water was used in the culture media instead of distilled water, no significant difference was observed

Table 2. Cell growth and sisomicin yield at the different concentrations of cobalt chloride.

$CoCl_2$ (μM)	X (g/l)	P (μg/ml)	P/X
0	9.1	18	2.0
0.08	9.1	37	4.1
0.4	9.0	73	8.1
3.4	9.1	85	9.4
16.8	9.1	105	11.5
84.0	7.8	90	11.4
420	4.8	55	11.4

*$CoCl_2$ was added to the media at the beginning of the culture.

in the fermentation yield. From this, it was concluded that the other mineral salts but cobalt chloride were not important for the sisomicin synthesis. The optimal concentration of cobalt chloride in fermentation media was found to be around 16.8 μM (Table 2). At higher concentrations cell growth was inhibited, the specific antibiotic-producing activities (P/X) were kept to be constant.

At different fermentation stages cobalt chloride was added to the fermentation media. As shown in

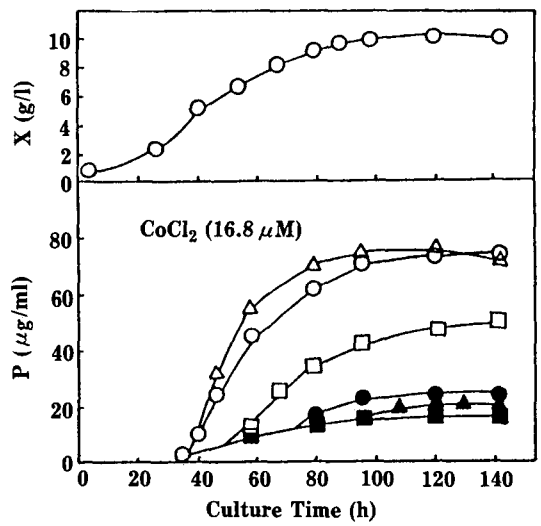


Fig. 1. Effect of cobalt chloride addltion at different growth stages.

○ - ○ : added at the beginning of culture
△ - △ : added after one day
□ - □ : added after two days
● - ● : added after three days
▲ - ▲ : added after four days, ■ - ■ : not added

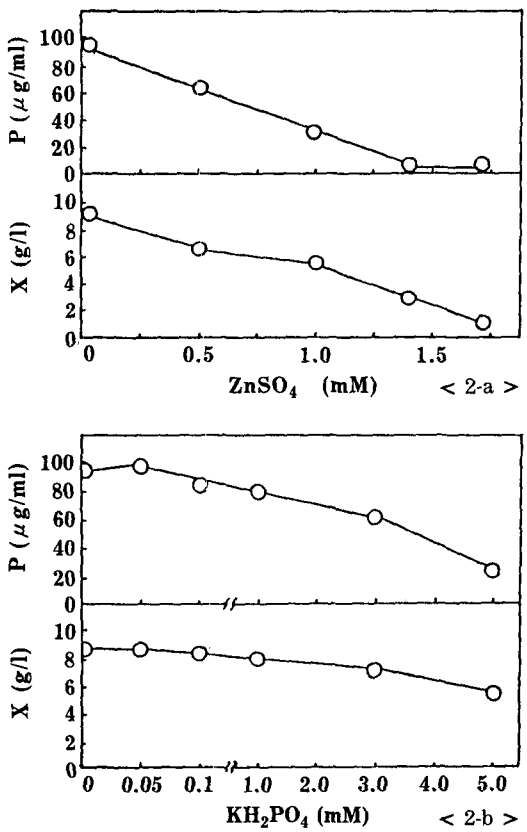
Table 3. Effect of vitamin B_{12} substituted for cobalt chloride on sisomicin yield.

Source	Concentration (μM)	X (g/l)	P (μg/ml)	P/X
$CoCl_2$	16.8	7.9	80	10.1
Vitamin B_{12}	0	9.4	16	1.7
	0.07	9.1	31	3.4
	0.37	8.8	65	7.4
	0.74	8.0	80	10.0
	7.4	7.2	78	10.9
	74	7.6	71	9.3

*$CoCl_2$ and vitamin B_{12} were added to the media at the beginning of the culture.

Fig. 1, the effect of cobalt chloride addition on the final antibiotic yield was sufficiently exerted at the beginning or after one day of culture. However, its addition after two or three days was much less effective. From these results, it could be concluded that the addition of cobalt ions should be made before the onset of the antibiotic formation or mid-logarithmic phase of the cell growth.

Instead of cobalt chloride, vitamin B_{12} was tried as an organic cobalt source (Table 3). The optimal concentration of vitamin B_{12} in the fermentation



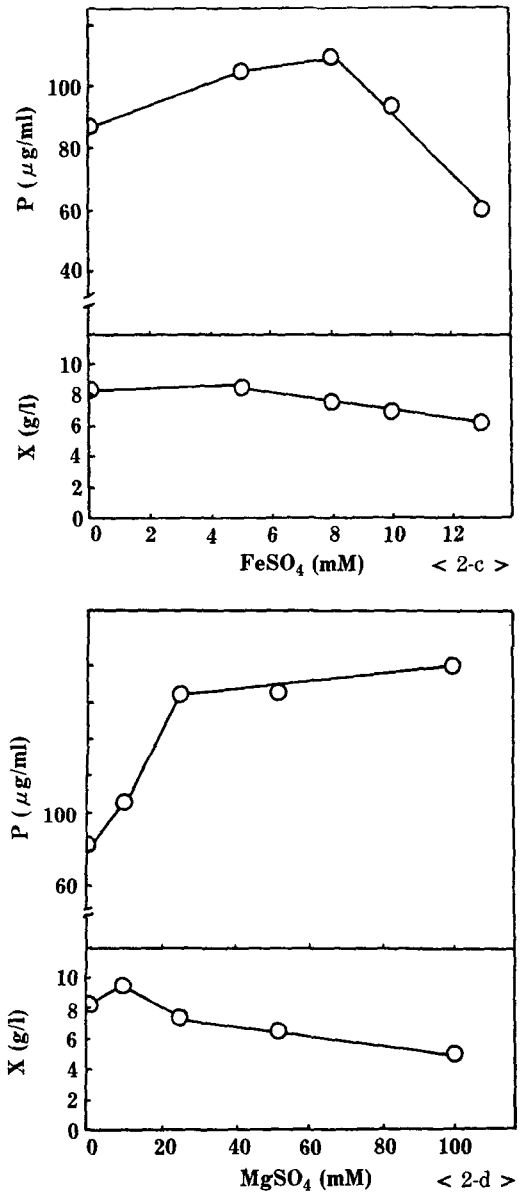


Fig. 2. Effects of various mineral salts at the high levels of concentration in media on cell growth and antibiotic yield.

*Salts were added to the media at the beginning of the culture.

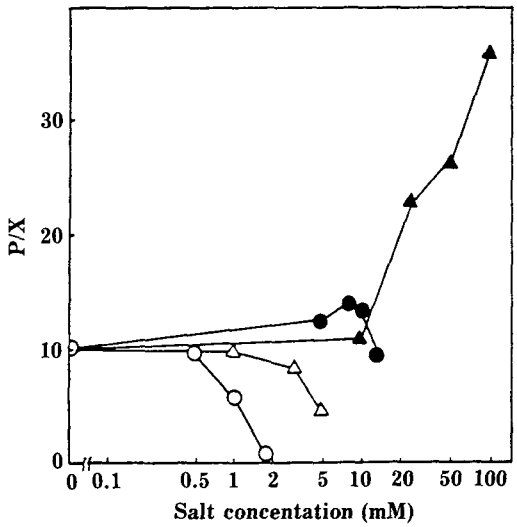


Fig. 3. Antibiotic-producing actvities per unit cell mass at the different concentrations of various mineral salts.
○ - ○ : $ZnSO_4$ △ - △ : KH_2PO_4
● - ● : $FeSO_4$ ▲ - ▲ : $MgSO_4$

media appeared to be 0.74 μM, and the final antibiotic yield was almost the same as that with cobalt chloride addition.

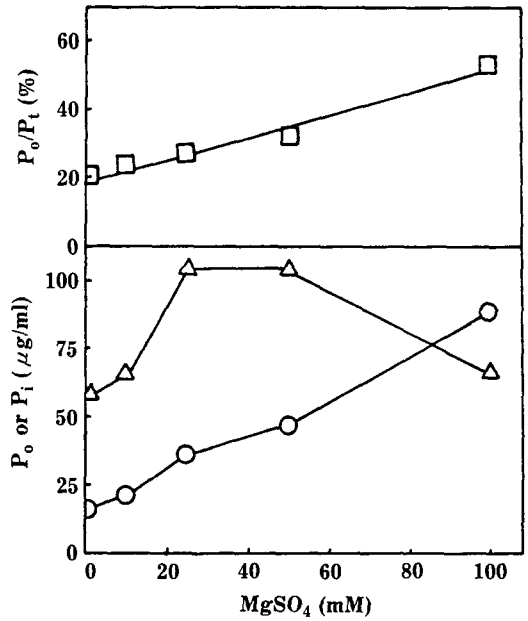


Fig. 4. Liberation of intracellular sisomicin at the different concentrations of $MgSO_4$.
Po (○ - ○): outside sisomicin concentration
Pi (△ - △): inside sisomicin concentration
Pt : total sisomicin concentration

Effects of various mineral salts on mycelial growth and antibiotic-producing activity

Since most of the sisomicin produced during the fermentation accumulates inside the cells, Shin *et al.* (9) added sodium chloride to the fermentation media during the culture processes in order to liberate the intracellular antibiotic molecules outside the cells. In this study, at the beginning of culture four different salts such as $ZnSO_4$, KH_2PO_4, $FeSO_4$ and $MgSO_4$ were added to the fermentation media containing 16.8 μM $CoCl_2$, and their effects on cell growth and antibiotic yield were observed (Fig. 2). And the salt concentrations used in these experiments were much higher than those used previously.

At a concentration of 0.5 mM, $ZnSO_4$ inhibited the cell growth and antibiotic synthesis. Moreover, at higher concentrations, the growth and antibiotic production were severely inhibited (Fig. 2-a). Although KH_2PO_4 was less inhibitive for the growth and antibiotic production, no improvement could be observed (Fig. 2-b). On the other hand, $FeSO_4$ did not inhibit the cell growth up to 5 mM and a maximal antibiotic yield was obtained around 8 mM (Fig. 2-c). As the concentration increased to higher than 10 mM, the growth inhibition and reduction in antibiotic yield had gradually appeared. In case of $MgSO_4$, the growth was observed to be stimulated up to 10 mM. However, a relatively high cell density was still maintained up to 100 mM which was pretty higher than those for the other mineral salts (Fig. 2-d). On the other hand, the final antibiotic yield was remarkably promoted up to 25 mM $MgSO_4$, and even above that it was still stimulated. Its maximal antibiotic yield was more than double the control.

In order to analyze the enhan cing-effect of $MgSO_4$ on the antibiotic yield, the amount of antibiotic formed per unit cell mass (P/X) was estimated from the previous results for four different salts (Fig. 3). As shown in Fig. 3, the specific antibiotic-producing activities (P/X) were not improved with addition of $ZnSO_4$ or KH_2PO_4, and rather decreased at higher than 1 mM. However, they were to some extent stimulated in a range of 5 to 10 mM of $FeSO_4$. On the other hand, the addition of $MgSO_4$ in a range of 10 to 100 mM brought about a sharp increase in P/X. Beyond that range, despite mycelial growth was slightly inhibited, antibiotic-producing activities were kept high enough so that the overall antibiotic yield

could be greatly improved. It was partly due to the enhanced liberation of the intracellular antibiotic which resulted from the high concentrations of $MgSO_4$ in culture broths (Fig. 4).

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요 약

여러가지 염이 sisomicin 발효에 미치는 영향에 대해 살펴본 결과, $CoCl_2$만이 항생물질 생성의 대사반응에 cofactor로서 작용하였으며 16.8 μM에서 최대 항생물질의 수율이 얻어졌다.

한편, 앞의 경우보다 훨씬 높은 염농도에서 $ZnSO_4$, KH_2PO_4, $FeSO_4$ 그리고 $MgSO_4$가 각각 발효배지에 첨가되었다. $ZnSO_4$와 KH_2PO_4는 전혀 효과가 없었으나 $FeSO_4$는 약간 항생물질 수율의 향상을 가져왔다. 그러나 $MgSO_4$의 경우, 매우 높은 염농도에서도 균체생육의 저해가 약간 일어났으며, 최종 항생물질의 수율은 100% 이상 증가되었다. 이러한 결과는 부분적으로 발효 중 균체내에 생성된 항생물질이 균체외로 유출되는 효과가 증진된 것에 기인한다.

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