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Factors affecting sisomicin production by *Micromonospora inyoensis*

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*Micromonospora inyoensis*에 의한 시소마이신 생산에 영향을 미치는 인자들

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The effects of cobalt chloride, methionine, and various carbon sources on the sisomicin production by *M. inyoensis* NRRL 3292 were investigated. It was found that both cobalt chloride and methionine exerted a greater stimulatory effect on sisomicin formation. Kinetic studies with various carbon sources revealed that polysaccharide such as starch or dextrin was found to be better than glucose for sisomicin production. Moreover, the relatively low concentration of dissolved carbon dioxide was one of the most important factors in accelerating sisomicin production during idiophase.

Sisomicin is an unsaturated aminoglycoside antibiotic produced by *Micromonospora inyoensis*.[1, 2] The antibiotic has shown a broad-spectrum of antibacterial activity, particularly against Pseudomonas.[3] Since the original development of sisomicin in 1970, another sisomicin producer *Micromonospora rosea* was isolated from a Hungarian soil sample.[4] Although the sisomicin titre by *M. rosea* (600-700 mcg/ml) was three times higher than that by *M. inyoensis*, the fermentation broth contained about 15 % of other accompanying antibiotics beside sisomicin.

In the present investigation, studies on fermentation medium were carried out in order to optimize the sisomicin production by *M. inyoensis*. With the view to obtaining high sisomicin titre, a relationship between dissolved carbon dioxide and sisomicin production was also examined.

Materials and Methods

Microorganism

M. inyoensis NRRL 3292 (ATCC 27600) was used throughout this study. The strain was grown at 32°C for 3 days under 180 rpm in a 250-ml corner-baffled flask containing 50 ml of the germination medium as illustrated in Table 1. The culture was then preserved as frozen cell state at -20°C. To grow fresh cultures, 5 ml of the frozen cell was added to 50 ml of fresh germination medium and incubated for 3 days at the same conditions described above.

Culture conditions

Unless otherwise specified, 5 ml of seed culture grown on germination medium for 3 days was transferred to 250 ml flask containing 50 ml of fermentation medium and incubated at 32°C for 6 days under 180 rpm.

A 30-l jar fermentor[5] (Marubishi, Japan) was used to investigate the effect of dissolved carbon dioxide on sisomicin production. A 5% (V/V) inoculum which was grown on germination medium in a 5-l flask was used. Fermentation was carried out at 32°C for 6 days. During the fermentation agitation was maintained at 200 rpm.

Key words: Sisomicin, *Micromonospora inyoensis*, dissolved carbondioxide, idiophase

Analytical methods

Total sugar was determined from 1 ml of fermentation broth using the phenol-sulfuric acid method.(6) Glucose was estimated on the supernatant after centrifugation (3000 rpm, 10 min) using a glucose oxidase-peroxidase method (Sigma diagnostic kit NO 510). DNA was measured by the method described by Herbert *et al.*(7) with minor modifications. The relative growth was determined from the absorbance measured at 600 nm against a control.

Sisomicin titre was estimated as follows: 0.1g of oxalic acid was added to the 15 ml of whole broth to precipitate calcium ions, and pH of the broth was further adjusted to 2 with 12N sulfuric acid and then shaked at 32°C for 30 min to release the antibiotic from mycelium. After centrifugation, the supernatant was neutralized with 14% ammonium hydroxide and then used for microbial assay. The assay procedure was similar to that developed by Oden *et al.*(8) and the assay was carried out with *S. aureus* ATCC 6538P as a test organism. Sisomicin sulfate (USP Reference Standard) having a potency of 625 mcg/mg was used for the microbial assay.

On-line estimation of dissolved carbon dioxide concentration was achieved by the use of a pCO_2 electrode and a monitor.(9) Dissolved oxygen tension was also monitored during the sisomicin fermentation.

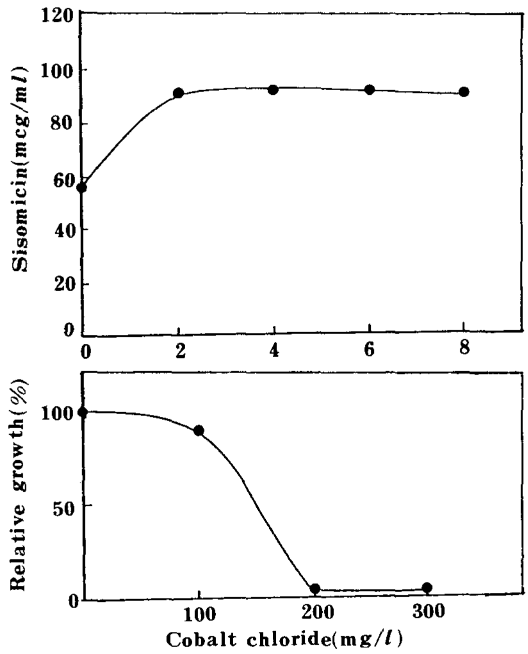

Fig. 1. Effect of cobalt chloride on growth and sisomicin production by *M. inyoensis*.

The amounts of cobalt chloride illustrated in the fermentation medium were varied.

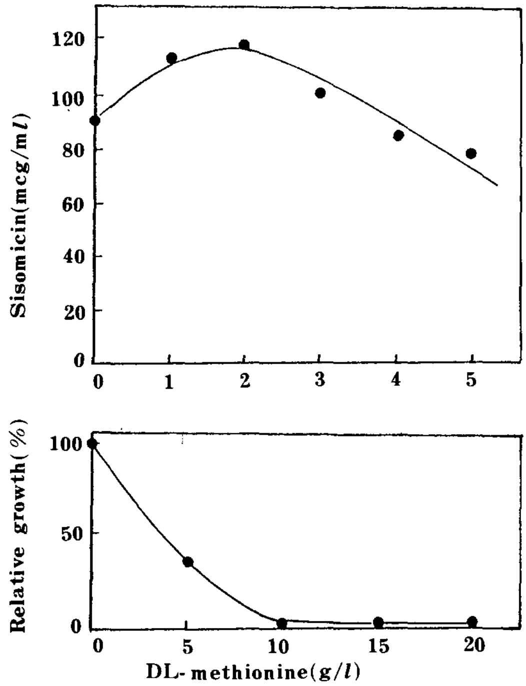

Fig. 2. Effect of methionine on growth and sisomicin production by *M. inyoensis*.

The amounts of DL-methionine illustrated in the fermentation medium were varied.

Results and Discussion

Effect of cobalt chloride on growth and sisomicin production

As Co^{++} may be required when the methylation reactions involved in sisomicin biosynthesis are conducted,(10) the effects of cobalt chloride on the growth and sisomicin production by *M. inyoensis* NRRL 3292 were investigated as shown in Fig. 1. When 2 mg/*l* $CoCl_2$ was added to the fermentation medium, the sisomicin titre was increased significantly up to 90 mcg/ml. However, the sisomicin titre was not increased further with increased addition of $CoCl_2$ up to 30 mg/l.

With regard to the effect of $CoCl_2$ on growth, the growth of *M. inyoensis* NRRL 3292 was slightly decreased when 0.1 g/l $CoCl_2$ was supplemented to the fermentation medium. However, growth was blocked completely at higher concentration beyond 0.2 g/l. It is interesting to note that the growth was largely unaffected when other minerals such as $FeCl_3$, $MnCl_2$, NaCl or $MgCl_2$ were supplemented up to 0.3 g/l (data not shown).

Table 1. Compositions of germination and fermentation media.

Germination		Fermentation[a]	
Beef extract	3g/l	Potato dextrin	50g/l
Tryptone	5g/l	Soybean flour	35g/l
Dextrose	1g/l	Corn Steep Liquor	5g/l
Potato starch	24g/l	Calcium carbonate	5g/l
Yeast extract	5g/l	Cobalt chloride	4mg/l
Calcium carbonate	2g/l	DL-methionine	2g/l

a: pH was adjusted to 7.1 after sterilization.

Effect of methionine on growth and sisomicin production

It is well known that methionine exerts a greater stimulatory effect on cephalosporin C biosynthesis.(11, 12) Lee *et al.* reported that methionine was an excellent methyl donor for gentamicins.(13) In Fig. 2, the effects of DL-methionine on the growth and sisomicin production are shown. As can be seen from Fig. 2, the sisomicin production was slightly stimulated at lower concentration of DL-methionine, reaching its maximum between 1-2 g/l. However, the sisomicin production was decreased at higher DL-methionine concentration beyond this range.

Also shown in Fig. 2 is the effects of DL-methionine on growth. It is clear that increases in DL-methionine supplementation to the medium resulted in the decreased rate of the growth. Furthermore, no growth was occurred above 10 g DL-methionine/l.

Comparison of sisomicin production on various carbon sources

In Table 2, the effect of various carbon sources on the sisomicin production is illustrated. It is clear that with 5% initial glucose the sisomicin production was significantly suppressed although the most extensive growth was supported. Similar results have been observed in many antibiotic fermentations due to carbon catabolite regulation.(14, 15, 16)

Table 2. Effect of various carbon sources on sisomicin production by *M. inyoensis* NRRL3292.

Carbon source[a]	Sisomicin (mcg/ml)[b]	DNA (mg/ml)	Residual sugar (%)	Final pH
Glucose	17	0.15	4.1	7.1
Maltose	103	0.12	2.8	7.1
Maltotriose	117	0.11	2.7	7.1
Dextrin	100	0.10	2.0	8.0
Starch	92	0.10	2.5	8.0

a: 5% Initial sugar content was used.
b: Fermentations were carried out at 32℃ for 6 days under 180 rpm in 250 ml corner-baffled flasks each containing 50 ml medium.

As can be seen from Table 2, either maltose or maltotriose was found to be good substrate for the sisomicin production and sisomicin titre in the range 103-117 mcg/ml was achieved. However, it appears from Table 2 that polysaccharides such as dextrin and starch are also considered to be good carbon sources for the sisomicin production because these raw materials are relatively inexpensive and readily available.

It is worthwhile noting that with regard to the comparison of sisomicin production on various nitrogen sources (Proflo, Pharmamedia, soybean flour, and yeast extract were tested), a soybean flour was found to be one of the best nitrogen sources.

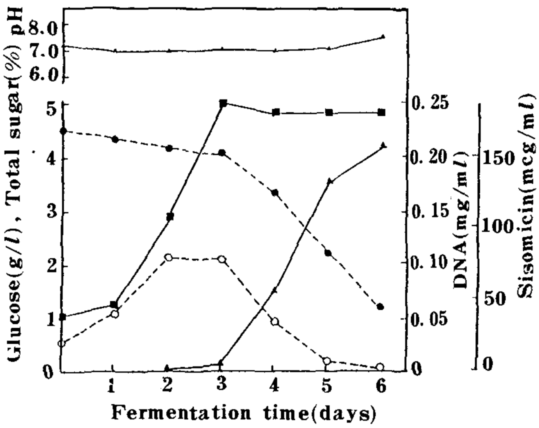

Fig. 3. A typical time course of sisomicin fermentation in 30-l jar fermentor. ● total sugar; ○ glucose; ▲ sisomicin; ■ DNA

Effect of dissolved carbon dioxide on sisomicin production

A typical time course of sisomicin fermentation in 30-l jar fermentor is shown in Fig. 3. During the early period of fermentation (2 day), there was an accumulation of glucose up to 2 g/l. Following this initial period, the glucose concentration declined toward the end of fermentation. As can be seen from Fig. 3, the distinction between trophophase and idiophase is clear. During the period of trophophase only 0.5% of total sugar was consumed, while significant changes in total sugar concentration was evident during the idio-

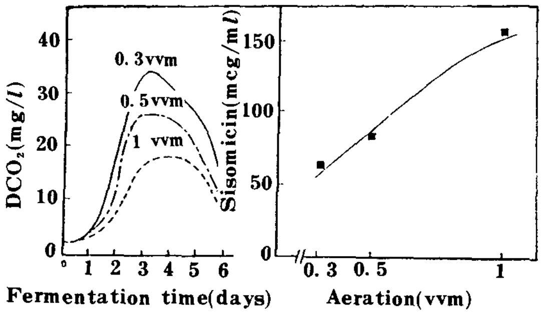

Fig. 4. Effect of aeration on dissolved carbon dioxide and sisomicin production by *M. inyoensis*.

phase.

In Fig. 4, the effect of aeration rate on the dissolved carbon dioxide concentration (DCO_2) together with its effect on the sisomicin titre is shown. It was observed that increases in aeration rate from 0.3 vvm to 1.0 vvm resulted in decreases in DCO_2 during the course of fermentation. However, dissolved oxygen tension was affected slightly by the increased aeration rate. Therefore, it is clear from Fig. 4 that DCO_2 does affect the sisomicin production significantly. Similar negative influence of carbon dioxide on penicillin,[17] inosine[18] and sisomicin biosynthesis[10] have been reported. From this result and others obtained by other workers, it appears that there is a close relationship between carbon dioxide and antibiotic production in many fermentation processes.

요 약

M. inyoensis NRRL 3292에 의한 시소마이신 생산에 영향을 미치는 인자들에 관해 조사한 결과 cobalt chloride 및 methionine 모두가 시소마이신 생산을 현저히 촉진하였으며, 탄소원으로서는 전분 또는 덱스트린이 포도당에 비해 시소마이신 생성에 적합하였다. 특히 시소마이신 발효공정중 항생물질 생성시기에 발효액내의 용존 이산화탄소 농도가 낮을수록 시소마이신 생성 농도가 증가하였다.

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