STUDIES ON MIKAMYCIN B LACTONASE

V. METABOLIC CONTROL IN MIKAMYCIN B FERMENTATION

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In mikamycin B fermentation, some procedures were examined to remove the participation of mikamycin B lactonase, which reduces mikamycin B titers. Addition of enzyme inhibitors and control of pH resulted in the elimination of the enzyme activity, and in the stimulation of antibiotic production.

Mikamycins A and B[1~3] (abbreviated MK-A and -B) are the products of *Streptomyces mitakaensis*, which show typical synergistic activity against Gram-positive bacteria[4]. However, the fermentation course was complicated by the participation of another factor, MK-B lactonase, whose action was estimated to be the hydrolysis at the lactonic linkage of MK-B into the antimicrobially inactive MK-B acid: This enzyme reaction caused the depression of MK-B accumulation[5].

The lactonase, purified from the mycelial cells, has a molecular weight of 29,000, and catalyzes the hydrolytic degradation of MK-B and its analogues. The enzyme activity was maximal at pH 7.0 and 27°C for an incubation of 60 minutes; it was stimulated by the presence of Mg^{2+} ion, but inhibited by heavy metal ions such as Cu^{2+} and Ni^{2+} ions[6,7].

From the view point of antibiotic production, this type of interaction between two metabolites, *i.e.* production of a desired metabolite and its destruction by an enzyme produced by the same organism, is novel, but highly undesirable, hence the metabolic control or the selective elimination of this enzyme activity should be expected to enhance antibiotic production.

This paper deals with the factors involved in suppression of MK-B lactonase synthesis and activity, and the procedures that lead to increased production of MK-B.

Materials and Methods

Organism and Culture

S. mitakaensis K-1839 was cultivated in 100 ml of soybean meal - glycerol medium[5] in 500-ml Erlenmeyer flasks on a rotary shaker. 300 ml of 2-day-old culture was inoculated into 15 liters of the same medium in a 30-liter jar fermentor; incubation was at 27°C with an aeration rate of 15 liters/minutes and an agitation rate of 300 rpm.

Enzyme inhibitors were added at 24 hours of incubation and pH was adjusted from 20 hours by occasional additions of 20% NaOH or 20% HCl solution. When enzyme inhibition and pH adjustment were combined, pH control started at 20 hours and inhibitor solutions were injected to the medium at 40 hours of cultivation. The concentration of inhibitors were expressed in percentage of $CuSO_4 \cdot 5H_2O$ or $NiSO_4 \cdot 5H_2O$.

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Bioassay of MK-A and -B

The determination of MK-A and -B in the culture medium and in the enzyme reaction mixture was carried out according to the method described by WATANABE[4] using *Staphylococcus aureus* FDA 209P as a test organism.

MK-B Lactonase Activity

The enzyme activity of culture media was measured as follows. The mycelium was separated from the fermentation beer by centrifugation (5,200×*g*, 5 minutes), washed with water and suspended in 0.02 M phosphate buffer (pH 7.0, 5 ml/g of mycelium). The standard reaction mixture contained mycelial suspension (0.95 ml), 1 M $MgSO_4$ solution (0.05 ml) and MK-B solution (20 μg/ml, 0.25 ml); incubation was at 27°C for 60 minutes. The reaction was terminated by heating at 80°C for 10 minutes and the MK-B content in the reaction mixture was calculated from its antibacterial activity. One enzyme unit was defined as 1 μg/ml of MK-B acid converted from MK-B under the standard conditions above.

Chemicals

MK-A and -B were supplied by Kanegafuchi Chemical Industries Co., Ltd., Kobe, Japan. All other reagents used in this study were commercially available.

Results and Discussion

Mikamycin Fermentation

As reported in the previous paper[5], *S. mitakaensis* produces MK-A and -B together with MK-B lactonase which caused remarkable depression of MK-B titers, as shown in Fig. 1.

To increase the recovery of MK-B, selective elimination of the enzyme itself or its activity was examined by adding presumptive enzyme inhibitors to the fermentation system. Addition of SH reagents, such as phenylmercuric acetate and *p*-chloromercuric benzoate, into the fermentation was found to be harmful to the producing organism: The mycelial growth was markedly inhibited. How-

Fig. 1. Time course of mikamycin fermentation.
▲ Mikamycin A, ○ mikamycin B, □ pH, ● cell weight.

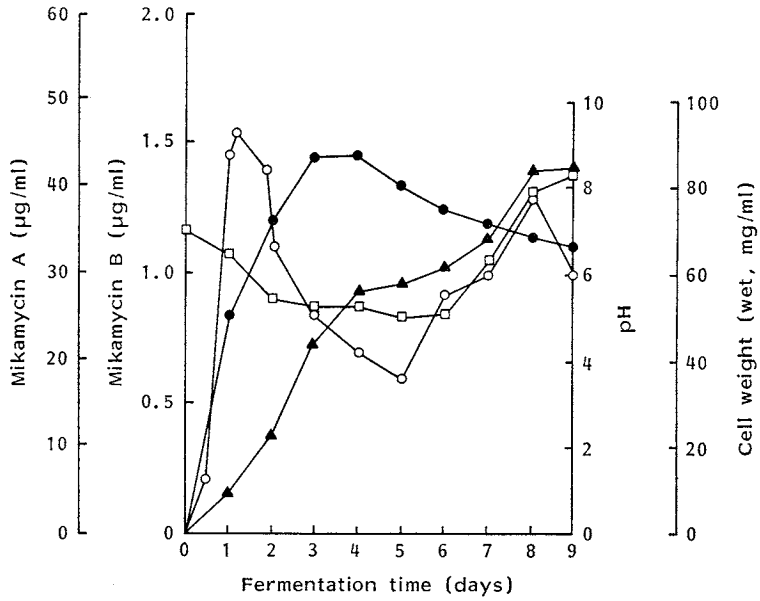
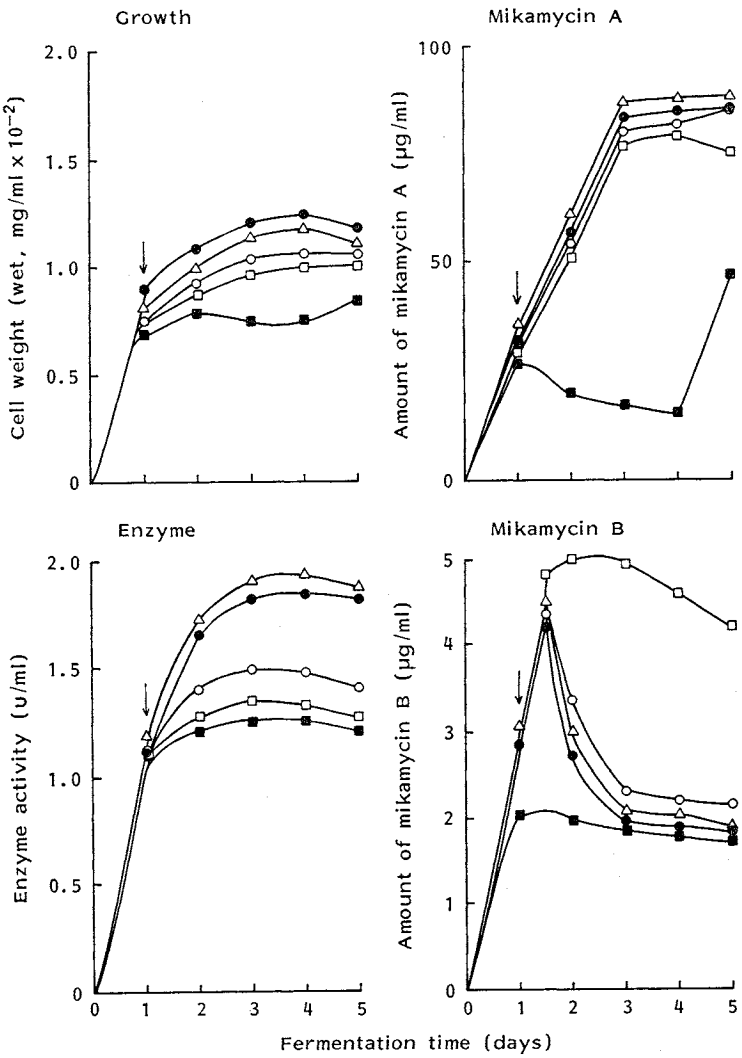


Fig. 2. Effect of Cu^{2+} concentration on mikamycin fermentation.
△ Control, ● 0.001% Cu^{2+}, ○ 0.005% Cu^{2+}, □ 0.01% Cu^{2+}, ■ 0.02% Cu^{2+}.
Arrow indicates the time of Cu^{2+} addition.



ever, heavy metal ions, such as Cu^{2+} and Ni^{2+}, behaved as non-competitive inhibitors of the lactonase† at concentrations which did not inhibit growth. Their application to the MK-B fermentation were expected to suppress enzyme activity, but not to affect mycelial growth and antibiotic production.

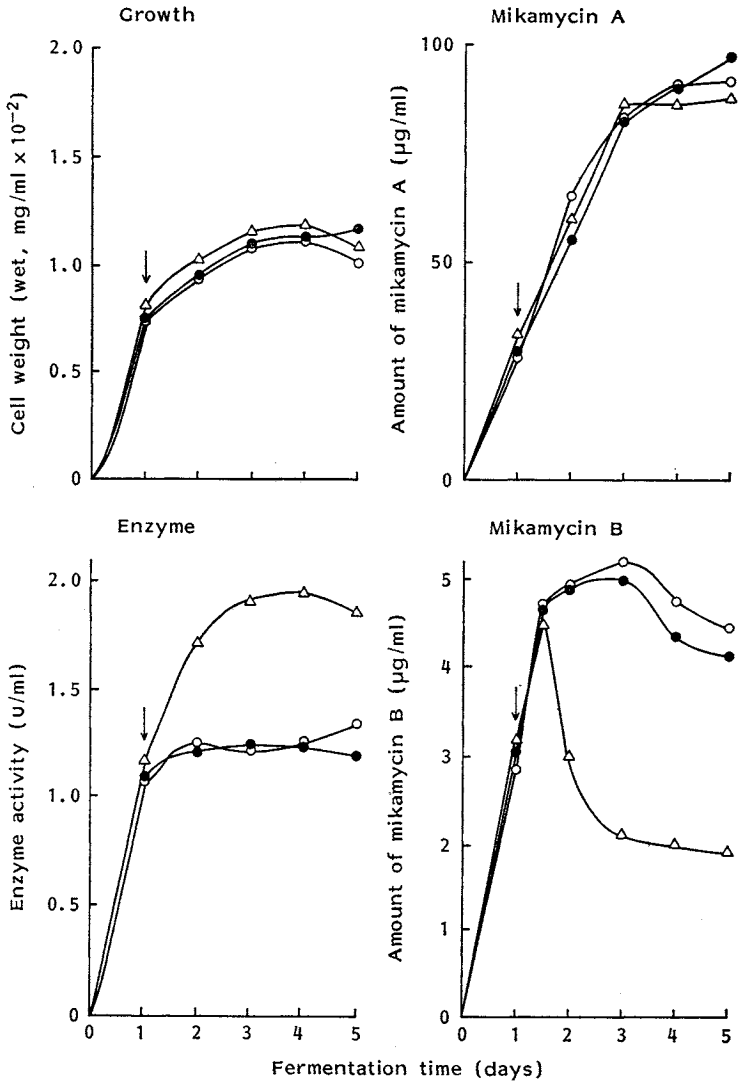
Effect of Enzyme Inhibitors

Various amounts of Cu^{2+}, the strongest inhibitor of the lactonase, were added at 0 and 24 hours of the fermentation medium. Zero time addition showed some undesirable influence on the microbial growth, but addition at a later stage was satisfactory.

Fig. 2 shows the time course of the fermentation with or without addition of copper ion. The addition of 0.02% of $CuSO_4 \cdot 5H_2O$ to the medium significantly inhibited the organism and caused

† Kinetic studies on mikamycin B lactonase will be published elsewhere.

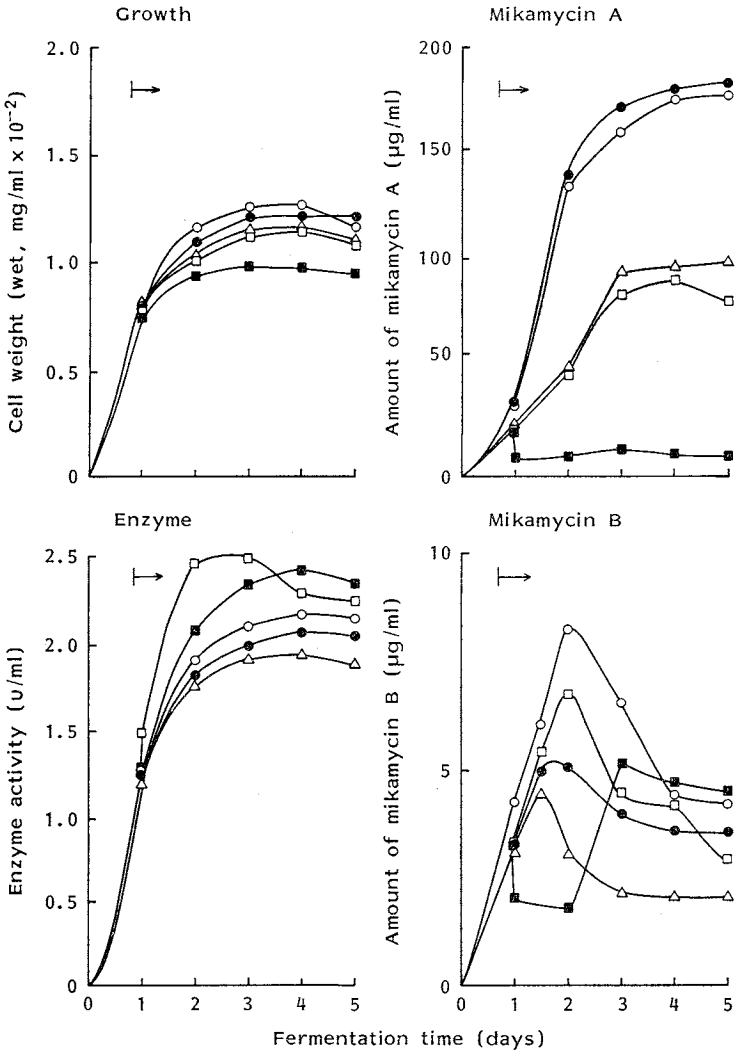
Fig. 3. Effect of Ni^{2+} on mikamycin fermentation.
△ Control, ● 0.01% Ni^{2+}, ○ 0.005% Ni^{2+} + 0.005% Cu^{2+}.
Arrow indicated the time of Ni^{2+} addition.



low yield of mycelium and low titers of MK-A and -B. The addition of 0.001 ~ 0.005% did not inhibit mycelial growth but was not sufficient to inhibit lactonase; therefore, no beneficial effect on MK-B could be observed. An intermediate concentration was found (0.01% $CuSO_4 \cdot 5H_2O$), which had no deleterious effect on mycelial growth and MK-A production, but which was sufficient to inactivate the lactonase and to prevent the hydrolysis of MK-B.

A similar effect was observed by adding $NiSO_4 \cdot 5H_2O$ (0.01%) (Fig. 3). In these experiments, the Ni^{2+} ion seemed to be slightly less toxic than the Cu^{2+} ion to the producer *Streptomyces*; the most desirable effect was obtained by a combination of both ions (0.005% each). However, no treatment inhibited the lactonase completely, and the net amount of MK-B at 30 hours of incubation was not increased.

Fig. 4. Effect of pH on mikamycin fermentation.
△ Control (no pH adjustment), ● pH 6.0, ○ pH 7.0, □ pH 8.0, ■ pH 9.0.
Arrow indicates the time of pH adjustment.



Effect of pH

MK-B lactonase is most active at pH 7.0~8.0[7]. Therefore, maintaining the pH at values below 7 might inhibit enzyme activity further without influencing antibiotic biosynthesis adversely.

S. mitakaensis was cultivated at pH 5, 6, 7, 8 and 9 by occasional additions of 20% NaOH or 20% HCl from 20 hours after inoculation.

As shown in Fig. 4, maintenance of pH at 9 decreased antibiotic MK-A and -B production and stimulated lactonase production. Adjustment at pH 5 led to complete inhibition of growth (not shown in Fig. 4).

At pH 6, the enzyme is less active[7], but no clear effect was observed on MK-B accumulation; however, MK-A production was unexpectedly enhanced to afford a 2-fold increase in yields over

the control (no pH adjustment). The same effect on MK-A production was observed by maintaining the pH at 7; moreover, MK-B yield were doubled. Under these conditions, the lactonase activity was still noticeable and MK-B yields were depressed, but hydrolysis was retarded by 1 day. This time difference might be explained by the relative amounts of MK-B and its lactonase produced; at pH 7, MK-B biosynthesis was favored over lactonase biosynthesis.

Cultivation at pH 8 showed intermediate effects. Mycelial growth was not affected, but MK-A production, and MK-B lactonase production were stimulated.

Generally, high pH (~9) stimulated enzyme biosynthesis and depressed mycelial growth and the MK-A production, whereas low pH (6~7) stimulated mycelial growth and antibiotic production, but slightly depressed enzyme biosynthesis:

Fig. 5. Effect of metal ions on mikamycin fermentation at pH 7.0.
△ Control, ● 0.01% Cu^{2+}, ○ 0.01% Ni^{2+}, □ 0.005% Cu^{2+} + 0.005% Ni^{2+}.
* adjustment to pH 7.0, ** addition of metal ions.

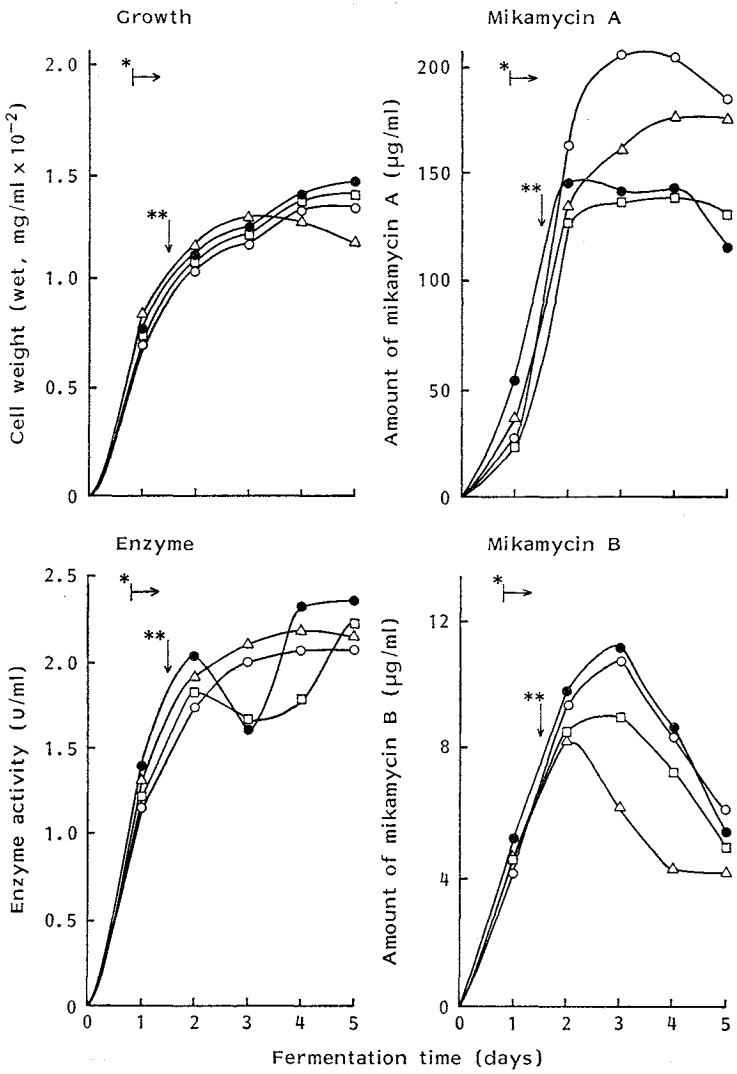
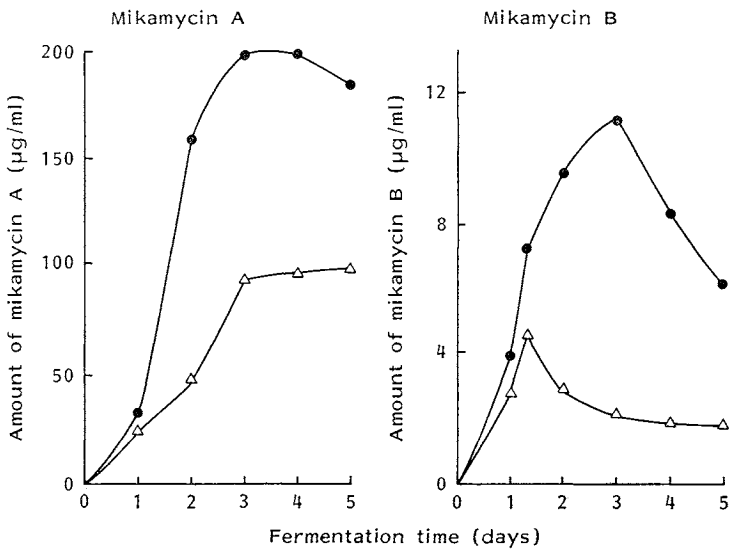


Fig. 6. Production of mikamycin under optimal conditions of pH and Ni^{2+} concentration.
△ Control, ● pH 7.0, 0.01% Ni^{2+}.



Mycelial growth	pH 6<7>8>9
Production of MK-A	6≧7≫8≫9
Production of MK-B (at maximum)	6<7>8>9
Production of MK-B lactonase	6<7≪8>9

In conclusion, pH adjustment alone (7.0) was effective in increasing MK-A and -B titers, but not in decreasing sufficiently enzyme biosynthesis.

Combination of pH Adjustment and Enzyme Inhibition

In the preceding experiments, it was established that the addition of enzyme inhibitors to the MK-B fermentation was effective in preventing MK-B from degradation by MK-B lactonase, and that pH adjustment (7.0) was effective in stimulating antibiotic production. The combination of these treatments was therefore warranted.

Fermentation was run in a jar fermentor; pH was maintained at 7.0 by the occasional addition of NaOH or HCl starting 20 hours after inoculation, and by the addition of inhibitor solutions 20 hours later.

As shown in Fig. 5, no treatment resulted in the inhibition of mycelial growth, but copper containing solutions (Cu^{2+}, Cu^{2+} + Ni^{2+}) were found to suppress MK-A production. This might suggest that Cu^{2+} exerts toxicity on both the enzyme systems involved in MK-A biosynthesis and the MK-B lactonase. Addition of Ni^{2+} ion did not show this effect, and the accumulation of MK-A exceeded that of control culture (pH 7.0, no inhibitor).

The addition of Cu^{2+} or Ni^{2+} at 0.01% was more beneficial than that of the combination (0.005% each) on MK-B production which was prolonged until 72 hours of cultivation. At this time, accumulation of MK-A and -B reached their maximum.

Fig. 6 shows the summary of these experiments. *S. mitakaensis* in soybean meal - glycerol medium (control) yielded 90 μg/ml of MK-A and 2 μg/ml of MK-B after 72 hours of cultivation. Adjustment of pH to 7 starting 20 hours and addition of an enzyme inhibitor, especially Ni^{2+} ion (0.01%), in-

creased the yields to 200 μg/ml of MK-A and 12 μg/ml of MK-B at 72 hours of cultivation, a 2- and a 6-fold increase over the control.

In conclusion, the properties of the MK-B lactonase have been exploited successfully to improve MK-B production and to prevent its degradation; yields were increased 6-fold.

Acknowledgment

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