Effect of Ferrous Ion on Mildiomycin Production by *Streptoverticillium rimofaciens*

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A specific regulatory effect of ferrous ion on a biosynthesis of mildiomycin by *Streptoverticillium rimofaciens* was investigated. The minimal concentration of ferrous ion necessary for the maximal production of mildiomycin was about 8 μg/ml. The physiological effects of ferrous ion on the mildiomycin production were examined in the media with and without the ferrous ion. Addition of ferrous ion to the culture medium increased both the mildiomycin production (10 times) and an assimilation of ammonium-nitrogen, activities of peptidase and protease.

Mildiomycin, a nucleoside antibiotic which is effective on powdery mildew diseases in many kinds of plant, is produced by *Streptoverticillium rimofaciens*.[1,2] During development of the fermentation process for the production of mildiomycin, we found that a ferrous ion played an important role in a regulatory mechanism of the mildiomycin production.

Mineral requirements of microorganisms for growth have been reviewed by E. D. WEINBERG.[3] In the field of actinomycete cultures, even if there have been reported on nutritional requirements, there have been only a few detailed investigation on the mineral requirements during a biosynthesis of antibiotics.[4~6] MAJUMDAR and MAJUMDAR[7] reported the effect of minerals on neomycin production by *Streptomyces fradiae*, and found that *S. fradiae* strain 3535 required a iron for neomycin production. On the other hand, ASAI and SHIMABARA[8] reported that the iron inhibited a production of streptomycin.

During the study on mildiomycin fermentation with corn steep liquor (CSL) as a nitrogen source, we noticed that mildiomycin production was remarkably stimulated by a presence of low concentration of a ferrous ion in CSL. Thus, it was found that the action of ferrous ion was related to an assimilation of ammonium-nitrogen and formations of peptidase and protease. This paper was aimed at investigating an effect of the ferrous ion on mildiomycin production by *S. rimofaciens*.

Materials and Methods

Microorganisms

Streptoverticillium rimofaciens strain C-257,[9] a high-producing mutant derived from strain B-98891,[1] was used throughout this work.

Culture Conditions and Media

Spores grown on slant agar medium (YKM) were inoculated into 30 ml of a seed medium in a 250-ml Erlenmeyer flask and grown at 28°C for 24 hours on a rotary shaker with an agitation rate of 200 rpm. One milliliter of seed culture was transferred into 10 ml of a production medium in a 250-ml Erlenmeyer flask. Cultures were carried out at 28°C for 8 days on the rotary shaker. YKM contained (%w/v) sucrose 2, soluble starch 0.5, NH_4NO_3 0.12, K_2HPO_4 0.25, $MgSO_4 \cdot 7H_2O$ 0.005, casamino acid 0.0025, yeast extract 0.0025 and agar 2. The composition (%w/v) of seed medium is glucose 3, CSL 3.5, $(NH_4)_2SO_4$ 0.1, $MgSO_4$ 0.05, and $CaCO_3$ 0.5. To investigate the effect of the ferrous ion on cell growth and mildiomycin production, a basal medium was used as following composition (%w/v): glucose 8, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 0.04, NaCl 0.5, $CaCO_3$ 1 and organic nitrogen sources 1. To investigate the mildiomycin production and activities of peptidase and protease, a production medium was used as following composition (%w/v): glucose 8, $(NH_4)_2SO_4$ 1, $CaCO_3$ 1, casein 3 and proflo 2.

Analytical Methods

Glucose concentration was determined by the method of WASHKO and RICE.[10] Ammonium-nitrogen concentration in the culture broth was measured by the method of MCCULLOUGH.[11] It was impossible to measure cell concentration, because of insoluble components contained the medium. Therefore, DNA concentration was measured instead of cell concentration. DNA extracted from cell by the method of SCHNEIDER,[12] and its concentration was determined by the method of BURTON.[13] Mildiomycin was assayed by a high per-

formance liquid chromatography (Model 633, Hitachi, Tokyo) under the following conditions: column (2.1 mm × 415 mm) packed Hitachi cation exchange resin 2610; solvent, 0.3 M borate buffer (pH 8.8 by lithium hydroxide); flow rate, 0.7 ml/minute; pressure, 120 kg/cm^2 G; detection wave, 254 nm.

To measure the metal ions in the CSL and culture broth, samples were dried at 500°C and 160°C, respectively for 6 hours. The ashes were dissolved in a suitable amount of concentrated 0.1 N HCl and then their supernatants were injected to an atomic adsorption spectrophotometer (Polarized Zeeman Z-6100 Hitachi, Tokyo).

To measure free amino acids in a culture broth, the culture broth was centrifuged, and the resulting supernatant was diluted to an appropriate concentration with 0.2 N citrate buffer (pH 2.2). Its concentration was analyzed by a modified method of ROTH[14] using a high performance liquid chromatography (Model RF-10A, Shimadzu, Kyoto). To measure intracellular activities of protease and peptidase, harvested cells were suspended in a 0.05 M tris-HCl (pH 7.5) buffer and sonicated for 20 minutes with a sonifier (M-200, Kubota, Tokyo). The resulting cell debris were removed and then, the supernatant fluid was stored as a crude enzyme. Protease activity was assayed employing a Henkel laboratory method.[15] Peptidase activity was assayed by measuring released *p*-nitroaniline libration from the mixture of enzyme solution and substrates.[16]

Results and Discussion

Effect of Ferrous Ion on Mildiomycin Production

In the study on mildiomycin fermentation with among five kinds of organic nitrogen sources, the mildiomycin production increased drastically at the concentration of 1% CSL, as shown in Fig. 1. Soybean meal, casein and proflo was good nitrogen sources for cell growth, but not for mildiomycin production. This suggests that the CSL should contain an effective components for the production of mildiomycin. The CSL usually contains

Fig. 1. Effect of organic nitrogen sources on cell growth and mildiomycin production in the basal medium containing 1% nitrogen sources.

Open and closed bars denote cell growth and mildiomycin concentration, respectively.

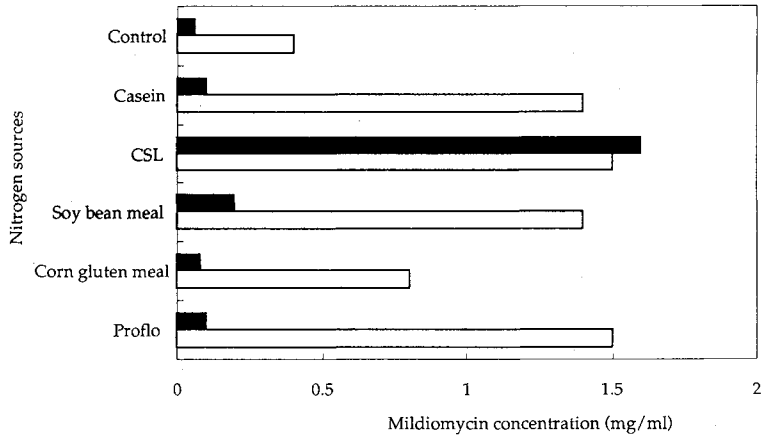
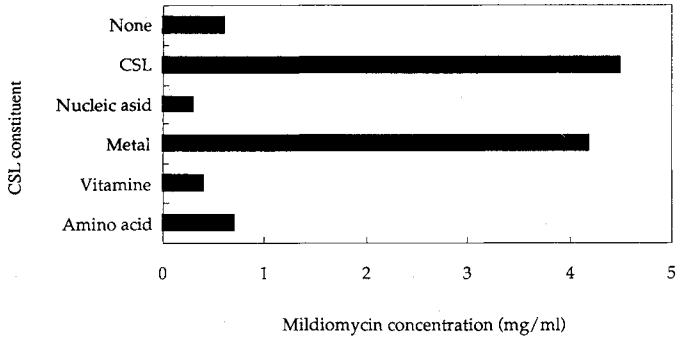


Fig. 2. Effect of CSL component on mildiomycin production in the basal medium containing 3.5% of CSL.



Concentration of nucleic acid, metal ions, vitamins and free amino acids were corresponded to the constituent of the CSL of 3.5%.

four kinds of the component such as nucleic acid, metals, vitamin and amino acids. To investigate the effect of the CSL on the mildiomycin production, the four kinds of the components were used in the culture instead of the CSL. The concentrations of vitamins, trace metals, amino acids, and nucleic acids in the CSL were measured according to Traders guide to fermentation media formulation.[17] Each concentration of the constituents added was corresponded to that in 3.5% of the CSL. The results were shown in Fig. 2. Nucleic acid, vitamin and amino acid did not affect to the mildiomycin production, which was similar to that of control culture. The mildiomycin production drastically increased due to addition of metal ions and its concentration was similar to that in the case of CSL addition. This indicates that the mildiomycin production is depended upon only metal compounds in the CSL. The metallic components contained in the CSL were measured by an atomic absorption spectrophotometer and shown in Table 1. Seven kinds of metal ions were detected, and among them large amount of ferrous and mangane ions were contained in the CSL. Therefore, we investigated the effect of these metal ions on the mildiomycin production, and the ferrous ion was the most effective on the mildiomycin production; the effect of the mangane ion was negligible (data not shown).

Table 1. Metal components in CSL.

Concentration (μg/ml)						
Fe^{2+}	Mg^{2+}	Zn^{2+}	Mn^{2+}	Cu^{2+}	Ni^{2+}	Co^{2+}
692.2	7,231	151.2	31.9	8.9	0.0063	0.00033

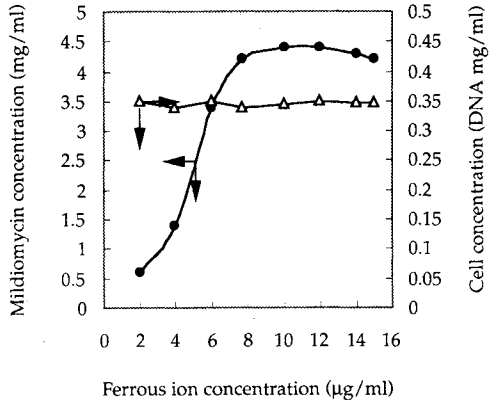
Data were averaged 10 samples of CSL.

Fig. 3 showed the effects of ferrous ion on cell growth and the mildiomycin concentration. The mildiomycin concentration reached maximal at the above 8 μg/ml of ferrous ion, but cell concentration was hardly affected. This shows that the CSL essentially acts as an iron donor in the mildiomycin fermentation and can be completely replaced with ferrous sulfate in the medium.

Comparison of Fermentation Time Course with (Fe+) and without Ferrous Sulfate (Fe−)

The mildiomycin productions in the Fe+ and Fe− production media were compared with each other. As shown in Fig. 4, cell growth and glucose consumption were hardly affected by the ferrous ion. However, the most remarkable feature between with the Fe+ and Fe− media was the mildiomycin production and ammonium-nitrogen concentration. In the case of Fe+ cultivation, an assimilation of ammonium-nitrogen was stimulated,

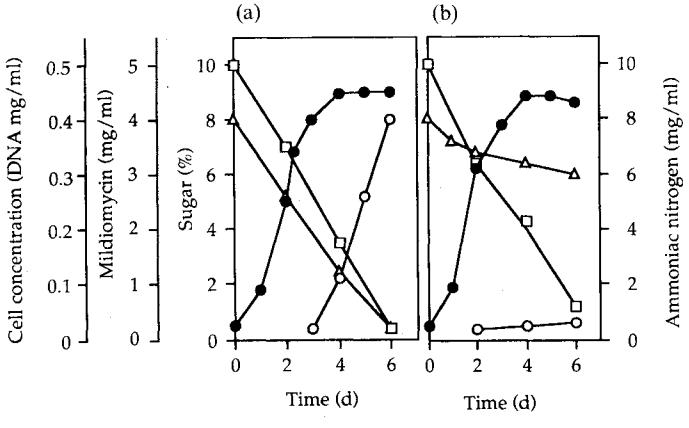
Fig. 3. Effect of ferrous ion on cell growth (△) and mildiomycin production (●) in the Fe+ production medium.



Ferrous sulfate heptahydrate was used as the ferrous ion source.

Fig. 4. Time course of mildiomycin production in the Fe+ (a) and Fe− cultivations (b).

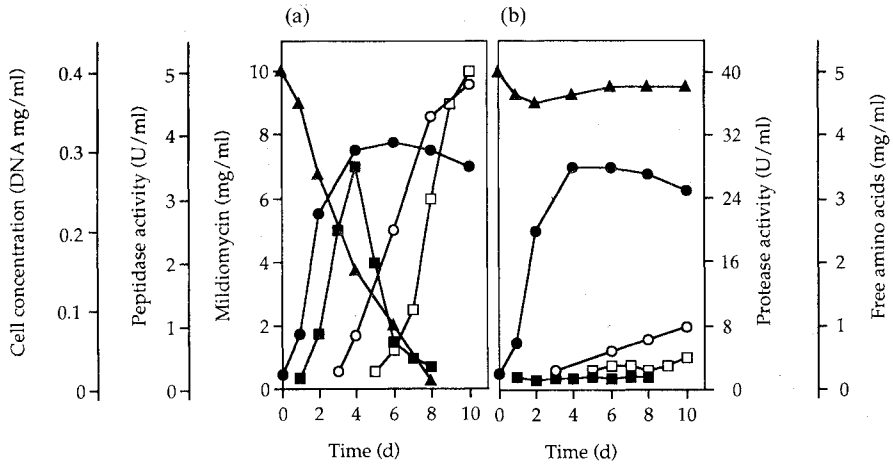
●, Cell concentration; □, glucose concentration; △, ammonium-nitrogen concentration; ○, mildiomycin concentration.



The ferrous ion (10 μg/ml) was added into the basal medium.

Fig. 5. Time courses of mildiomycin production in the Fe+ (a) and Fe− cultivations (b) in the basal medium containing 2% proflo and 3% casein as an organic nitrogen source.

●, Cell concentration; ○, mildiomycin concentration; ▲, amino acid concentration; □, protease activity; ■, peptidase activity.



and then the production of mildiomycin (Fig. 4a). It suggests that since nitrogen content of the mildiomycin molecule is 21% by weight, a large amount of nitrogen source might be needed to synthesize the mildiomycin. This indicates that the nitrogen metabolism plays an important role on the mildiomycin production.

When 3% of casein and 2% of proflo as organic nitrogen sources were used with the addition of 10 μg/ml of the ferrous ion, the mildiomycin production was also markedly stimulated (Fig. 5). In the case of Fe+ cultivation, the mildiomycin concentration was 4-fold higher than that of Fe− cultivation. In the presence of ferrous ion, free amino acid concentration in the culture was completely consumed after culture time of 8 days. Moreover, an extracellular peptidase activity increased during the cell growth and became the maximal value at the culture time of 4 days, and after decreased rapidly. On the other hand, after cell growth protease activity increased by the ferrous ion in the culture. These enzyme activities in the presence of the ferrous ion might explain the assimilation of the ammonium-nitrogen.

The most remarkable thing in the mildiomycin production by *S. rimofaciens* is that there is a close relationship between the ferrous ion and the mildiomycin production. The ferrous ion stimulated the iron-dependent assimilation of ammonium-nitrogen, proteolytic enzyme activity and the production of mildiomycin. This indicates that the ferrous ion plays an important role in amino acid metabolism to facilitate the mildiomycin biosynthesis.

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