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Regulation of meilingmycin in *Streptomyces nanchangensis*: Effect of ammonium ion

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Abstract−Effects of different ammonium sulfate concentrations on meilingmycin biosynthesis were studied in this research. The results show that a lower concentration of ammonium ions stimulates the biosynthesis of meilingmycin, while a concentration higher than 5 mmol/L inhibits the mycelial growth and the biosynthesis of the products. However, increased sugar consumption rate with the elevated concentration of ammonium sulfate was observed during the fermentation process. On this basis, six enzymes, which are responsible for the meilingmycin biosynthesis and the glucose metabolism, were measured and analyzed during the bioprocess. The results suggest that glucose-6-phosphate dehydrogenase, citrate synthase, succinate dehydrogenase and fatty acid synthase are stimulated by higher concentration of ammonium ions, while valine dehydrogenase and methylmalonyl-CoA carboxyltransferase are inhibited. From the results it follows that the precursor supply was restricted with the higher concentration of ammonium ions, which results in the lower production of meilingmycin. Thus, the strategy of maintaining a low level of FAS activity is a critical factor for high yield of meilingmycin.

Key words: Meilingmycin, *Streptomyces nanchangensis*, Ammonium Ion

INTRODUCTION

The antiparasitic macrolide meilingmycin is a typical secondary metabolite produced by *Streptomyces nanchangensis* widely used in agriculture and veterinary medicine. It possesses a 16-membered macrolide, which owns the same structure as that in avermectin and milbenycin, with the exception of a different side chain. Meiligmycin is comprised of several components, in which MB1 is the major component, the structure of which is the identical with milbemycin α_{11} reported elsewhere [1]. Generally, it accounts for 40% of the whole ingredients. During the course of fermentation, another kind of antibiotic, nanchangmyicin (a polyether) is generated along with the production of meilingmycin. Because of its high efficiency, broad-spectrum insecticidal and anthelmintic activities, meilingmycin is therefore claimed to be superior in some aspects to that of avermectin [2,3], and has a promising market prospect in terms of novel bio-pesticide.

As a macrolide insecticide, the biosynthesis of meilingmycin is similar to the other antibiotics (insecticide) of the same group. Its macrolide is presumably synthesized through polymerization of 7 malonyl-CoA and 5 methylmalonyl-CoA one after another, head to tail to the primer acyl-CoA by polyketide synthetase [4]. The whole biosynthetic process is influenced by various factors, one of which is the supply of a nitrogen source. There are a few reports [5,6,8] concerning the effect of ammonium ion on the biosynthesis of other macrolides antibiotics, such as tylosine and avermectin, but no such effect on meilingmecin production has been reported. In this study, we began with the study of the effect of ammonium ion on the biosynthesis of meilingmycin, compared systematically the activities of the enzymes closely related to its biosynthesis, namely valine dehydrogenase (EC1.4.1.8), methylmalonyl-CoA carboxyltransferase (EC2.1.3.1), 6-phosphoglucose dehydrogenase (EC.1.1.49), citrate synthetase (EC4.1.3.7), succinate dehydrogenase (EC 1.3.5.1) and fatty acid synthetase (EC2.3.1.85), and elucidated the role of ammonium ion in each of these enzymes, so as to provide a rational basis for the high performance control of fermentation.

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MATERIALS AND METHODS

1. Microorganism

Streptomyces nanchangensis NS-6-10, which is an original strain provided by courtesy of Jiangxi Agriculture University, was improved through multi-mutagenesis.

2. Reagents and Media

The reagents and chemicals used were purchased from Fisher Scientific and Sigma (USA). Tryptone soy broth (TSB), beef extract, malt extract and casamino acids were from Oxoid (USA). Tryptone/peptone, yeast extract, Lennox broth (LB) and Bacto-casitone were from Difco.

3. Cultivation Method

Spores were stored in 30% glycerol at −70 °C. Also, cultures grown on seed medium (see below) were centrifuged and resuspended in 30% glycerol for storage at −70 °C in 1.5-mL aliquots. These stocks were used directly as inocula for the different cultivations. All the cultures were grown in 250 mL Erlenmeyer flasks containing 30 mL media. The seed medium was modified according to reference [7], which consists of corn meal (Penglai Dengfeng Fisheries Company, PR China) 15.0 g/L, maize starch 5.0 g/L, soybean meal 5.0 g/L, yeast meal (Jiangmen Biotechnology Base, PR China) 0.5 g/L, KH_2PO_4 0.5 g/L, KNO_3 0.5 g/L, NaCl 0.5 g/L, $CaCO_3$ 3.0 g/L. Inocula were grown for 30 h at 32 °C, 220 rpm on the seed medium. The pH of

the medium was adjusted to 8.0 with 1 M NaOH prior to autoclaving (121 °C, 30 min). Aliquots of 30 mL inocula and production medium were placed into 250 mL Erlenmeyer flasks and the amount of inocula used was about 10%. The components of the production medium are in the following (g/L): corn meal 44, maize starch 34, soybean meal 10, KH_2PO_4 0.5 , KNO_3 0.7, NaCl 0.5 , $CaCO_3$ 3.0, bean oil 6, pH 8.0.The experiments were performed in a rotary shaker at 30 °C and 220 rpm for 7 days. All experiments were repeated in tetrad.

4. Preparation of Cell-free Crude Extract

Biomass was collected by centrifuging at 4 °C under 4,000 rpm for 5 min, washed with saline twice, and the precipitate was suspended into a 5 mL-PED buffer (containing 75 mmol/L of KH_2PO_4, 1 mmol/L of EDTA and 2 mmol/L of dithiothreitol, pH 7.0). The biomass was disrupted at 4 °C by an ultrasonic disintegrator (model KS-250) for 150 s, and then centrifuged under 12,000 rpm for 30 min. The supernatant obtained was stored at −85 °C.

5. Analysis

Determination of biomass was carried out by nucleic acid UV absorption [8]. DNS method [9] was applied to determine the total sugar. The meilingmycin titer was quantified by using an Agilent 1100 series high pressure liquid chromatograph (HPLC) equipped with ultraviolet/visible wave detector (Model G1314A). The chromatographic column was an Agilent Eclipse XDB-C_8 (5 μm particle size, 4.6 mm×150 mm) maintained at 30 °C. The mobile phase was comprised of acetonitrile: water at a ratio of 75 : 25 (v/v) at a flow-rate of 1.2 mL/min. Detection was monitored at a wavelength of 238 nm.

5-1. Determination of Enzyme Activity

Activity determinations of valine dehydrogenase, methylmalonyl-CoA carboxyltransferase, 6-phosphoglucose dehydrogenase, citrate synthetase, succinate dehydrogenase and fatty acid synthetase was undertaken using the definition of enzyme activity according to the literature [10-15]. Specific enzyme activity is defined as enzyme unit per mg of protein. Enzyme activities were monitored real-time by spectrophotometer model UNICO UV-2102PC. Protein was determined by using Commassie Blue Fast Staining Solution G-250 method and bovine serum albumin was used to plot a standard curve.

RESULTS AND DISCUSSIONS

1. Effects of Ammonium Ion of the Biosynthesis of Meilingmycin

The optimized production medium, which contains 1% soybean meal, 0.07% KNO_3 as nitrogen source, was utilized for basal fermentation medium. Various amounts of $(NH_4)_2SO_4$ were added into the fermentation media to make up the final concentration as follows: 0, 2.5, 5, 7.5, 12.5, 20 and 25 mmol/L. After fermentation for 200 h, the content of total nucleic acid and meilingmycin MB1 was determined (Fig. 3).

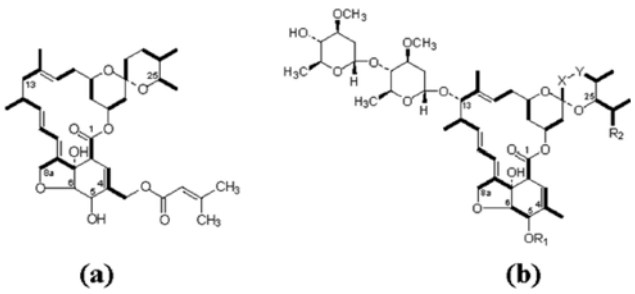

Fig. 1. Structure of meilingmycin(a) and avermectin (b).

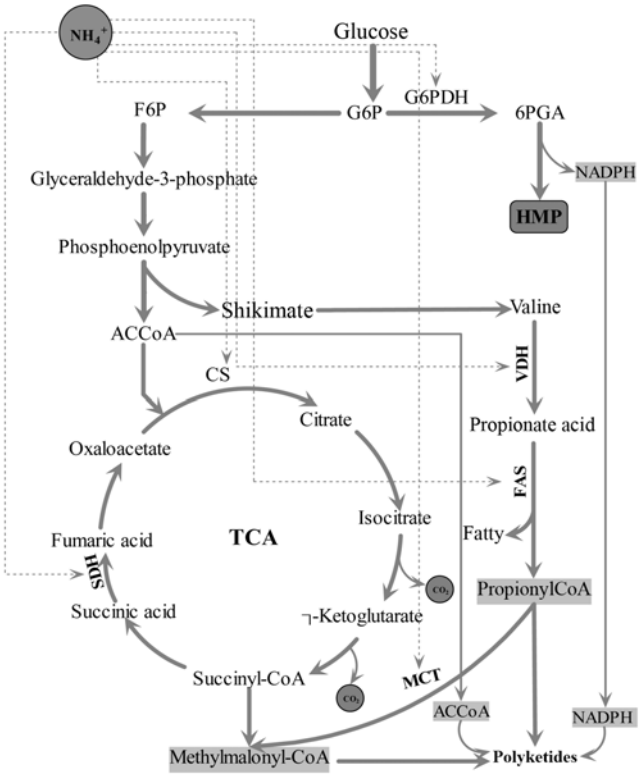

Fig. 2. Precursor requirements in the biosynthesis of MB lactone ring. CS: Citrate synthase; FAS: Fatty acid synthase; G6PDH: Glucose-6-phosphate hydrogenase; MCT: Methyl malonyl-CoA carboxyltransferase; SDH: Succinic aicd dehydrogenase; VDH: Valine dehydrogenase, HMP: Hexose monophosphate pathway, AcCoA: acetyl-CoA, 6PGA: 6-phosphogluconate.

Results (Fig. 3(a)) showed that the biosynthesis of meilingmycin was promoted when suitable amount of $(NH_4)_2SO_4$ (≤5 mmol/L) was added, whereas the production declined as the concentration of $(NH_4)_2SO_4$ increased to over 5 mmol/L, and became almost zero at the concentration of 25 mmol/L. The maximum total nucleic acid which characterized the growth was also attained at a 5 mmol/L.

Along with the increase of ammonium ion, although the growth was apparently repressed, the amount of sugar consumed increased continuously (Fig. 3(b)), which led to the formation of a large amount of intermediate products, such as pyruvic acid and the intermediates of TCA cycle with the decrease of pH of the fermentation broth, and the pH increased sharply when sugar was exhausted. This implies that ammonium ion in the range of experiment can promote greatly the primary metabolism of the culture.

2. Effects of Ammonium Ion on the Enzyme Activity Involved in Primary and Secondary Metabolism

2-1. Effect of Ammonium Ion on the Activity of Valine Dehydrogenase (EC1.4.1.8)

Since synthesis of the 16 membered-macrolide is the major pro-

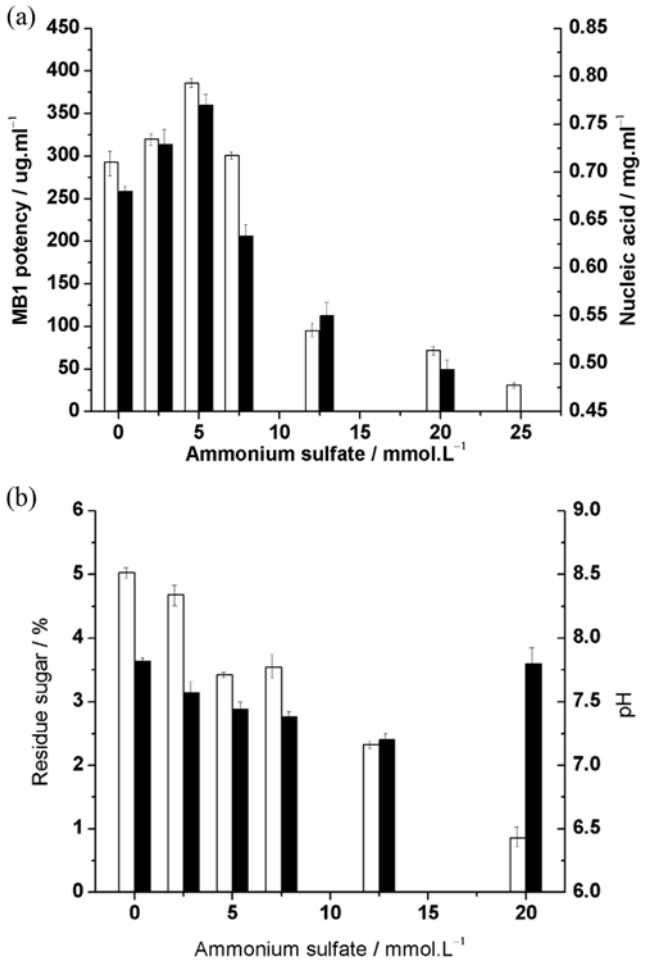

Fig. 3. (a) Effect of ammonium sulfate on mycelia growth and production of Meilingmycin MB1 (□-MB1 potency; ■-Nucleic acid concentration); (b) Residual sugar and pH value in shaking flask culture (191 hr) with different ammonium sulfate concentrations (□-Residue sugar; ■-pH value).

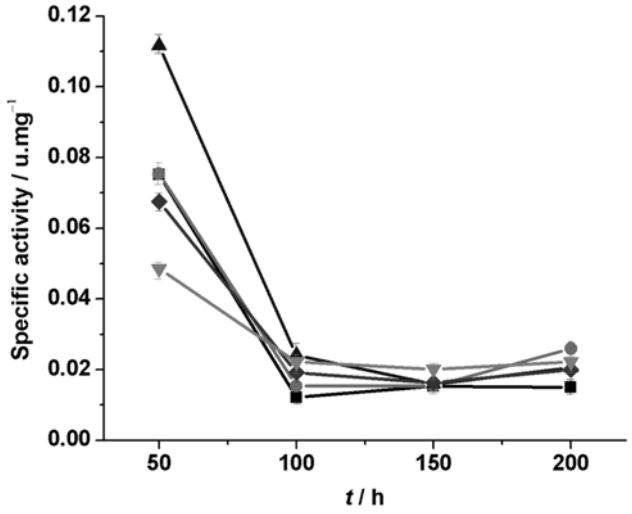

Fig. 4. The time course of VDH specific activity at different ammonium sulfate condition (■-control, ●-2.5 mmol/L, ▲-5 mmol/L, ◆-7.5 mmol/L, ▼-12.5 mM).

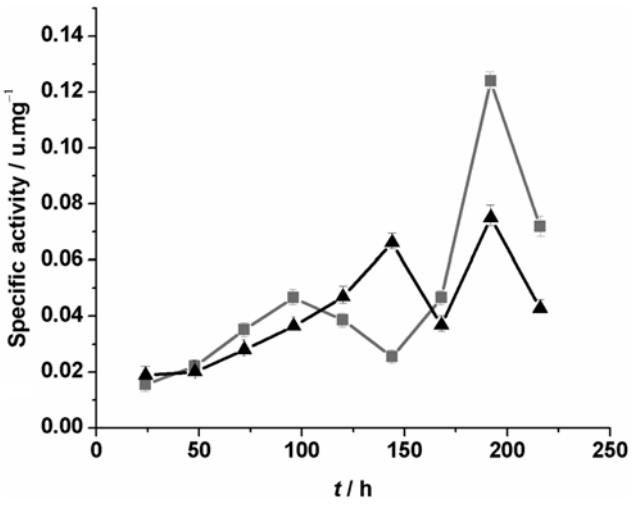

Fig. 5. The time course of MCT activity of *S. nanchangenesis* cultivated in shake flask culture with 5 mM (■) and 12.5 mM (▲) ammonium sulfate.

cess in the production of macrolide antibiotics [8], the source of its precursors, namely acetate and propionate, is critical. It was found that addition of valine during fermentation would promote the production of macrolide antibiotics [20]. The metabolic pathway of valine is also one of the major pathways for the synthesis of methylmalonyl-CoA and malonyl-CoA, and valine dehydrogenase (VDH) as the first enzyme involved in valine metabolism; its enzyme activity has a great positive influence on the production of the macrolides. Consequently, it is presumed that the degradation pathway of amino acids might be the target of the regulation of ammonium ion on secondary metabolite biosynthesis [8]. This is why VDH activity was our first object for the study of meilingmycin fermentation.

From the VDH activity profile, as shown in Fig. 4, it can be seen that no matter how much the ammonium ion was added, VDH activity manifests itself on a higher level in the early phase of fermentation, and maintains a low level thereafter. At 50 h of fermentation, VDH activity was promoted at 2.5 and 5 mmol/L, and inhibited at 7.5 mmol/L of ammonium sulfate, respectively. This is in accordance with the variation of the fermentation titer of meilingmycin. This shows that ammonium ion affects VDH activity, which in turn influences the supply of macrolide precursor. It can also be hypothesized that the formation of the precursor is synthesized essentially during the early phase of fermentation.

2-2. Effect of Ammonium Ion on the Activity of Methylmalonyl-CoA Carboxyltransferase (EC2.1.3.1)

Methylmalonyl-CoA carboxyltransferase (MCT) is an important precursor for the biosynthesis of meilingmycin, of which there are at least three pathways [21] hitherto claimed to be responsible for its synthesis and two of them are taken to play the major role in the biosynthesis of the precursor [21,22]. In this study, MCT activity was determined. Results in Fig. 5 show the time course of MCT activities with media containing 5 mmol/L and 12.5 mmol/L of ammonium sulfate.

Under two circumstances, two peak values occurred in MCT activity profiles: in one circumstance with 5 mmol/L ammonium sulfate, its first and second peak values appeared at 96 h and 192 h,

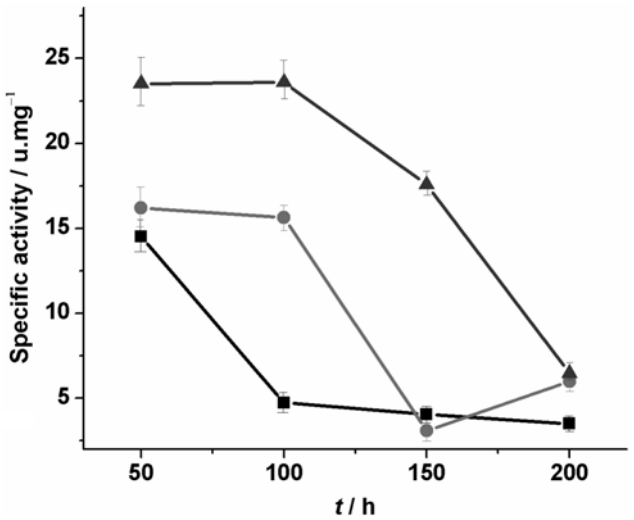

Fig. 6. The time course of specific activity of Glucose-6-phosphate dehydrogenase in shake flask culture with different ammonium sulfate concentrations, ■-control, ●-5 mmol/L, ▲-12.5 mmol/L.

respectively; while in the other circumstance, the first and second peak values occurred at 144 h and 192 h of fermentation, respectively. This means that the delay of the first peak level is attributed to the inhibition of MCT by high concentration of ammonium ion, which resulted in promoting the primary metabolism and delaying the secondary metabolism of the culture. The enzyme peak level that emerged at 192 h reflects the activity of the meilingmycin biosynthetic pathway at its peak.

2-3. Effect of Ammonium Ion on the Activity of 6-Phosphoglucose Dehydrogenase (EC1.1.1.49)

In the course of macrolide antibiotic synthesis, a great deal of NADPH is necessary for the formation of the lactonic ring, and so if NADPH is insufficient, the synthesis of the macrolides will be restricted. Since NADPH is essentially derived from the hexose monophosphate pathway (HMP pathway), the activity of the enzymes involved has direct influence on the supply of NADPH. In this study, the 6-phosphoglucose dehydrogenase involved in HMP pathway is thus determined.

The activity of 6-phosphoglucose dehydrogenase is greatly influenced by ammonium ion concentration. Under low concentration of ammonium ion, the activity of 6-phosphoglucose dehydrogenase is maintained at a low level in middle and late phase of fermentation, with the production of meilingmycin at high level. All existing evidence suggests that the supply of NADPH is sufficient and does not become a limiting factor in aglycone formation. In the range of our experiment, ammonium ion promoted the activity of 6-phosphoglucose dehydrogenase, and resulted in the intensification of HMP pathway and the growth of the culture.

2-4. Effect of Ammonium Ion on the Activities of Citrate Synthetase (EC4.1.3.7) and Succinate Dehydrogenase (EC1.3.5.1)

Citrate synthetase (CS) is a key enzyme in TCA cycle, where the level of its activity reflects the potency of TCA cycle. The fate of succinate, an intermediate of the TCA cycle, is of critical importance in secondary metabolism. Succinate dehydrogenase (SDH) catalyzes the transformation of succinate into fumarate. When SDH

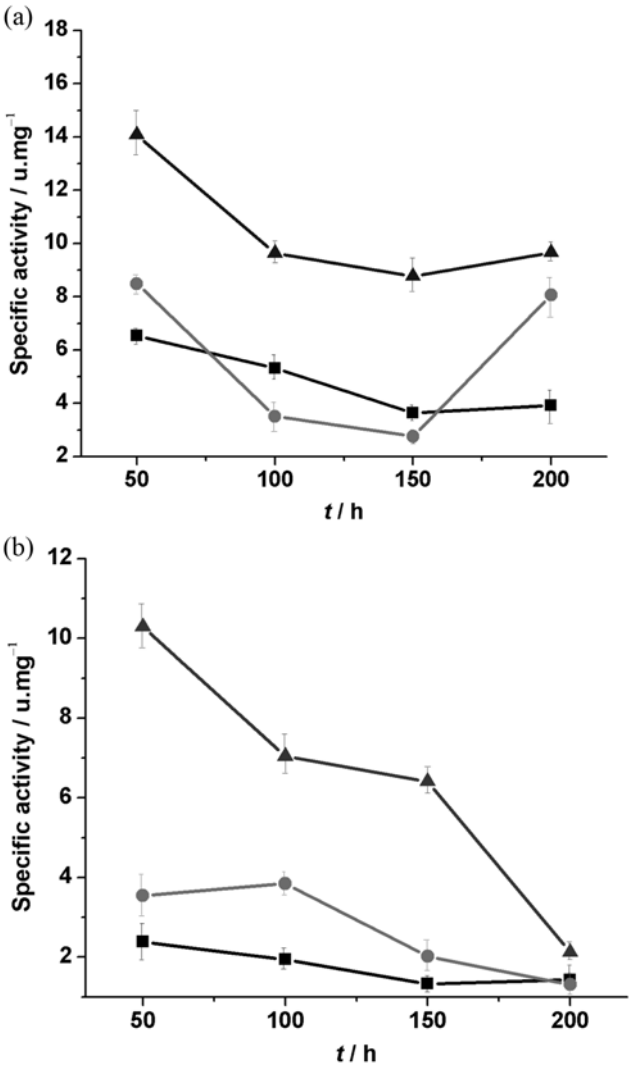

Fig. 7. (a) The time course of Citrate syntheses in medium with different ammonium sulfate concentration (■-control, ●-5 mM, ▲-12.5 mM); (b) The time course of SDH in medium with different ammonium sulfate concentration (■-control, ●-5 mM, ▲-12.5 mM).

is at high level, succinate is metabolized to various intermediates in the TCA cycle, and the generation of macrolides precursor, 2-methylmalonyl-CoA by isomerization of succinyl-CoA is restricted [23].

Experimental results (Fig. 7(a)) showed that in the early phase of fermentation, CS activity was rather high, and declined to a minimum around 150 h, and increased afterwards, whereas SDH activity decreased continuously (Fig. 7(b)). It was found that ammonium ion could promote the activities of CS and SDH. Between 150 to 200 h of fermentation, CS activity still increased, while SDH activity maintained at low level, suggesting that the flux of succinyl-CoA to methylmalonyl-CoA had increased greatly, and therefore presumably improved meilingmycin titer.

2-5. Effect of Ammonium Ion on the Activities of Fatty Acid Synthetase (EC2.3.1.85)

The synthesis of the macrolides by polyketide synthetases is similar to the synthesis of long chain fatty acids [1]. During their synthesis macrolides and fatty acid compete for the same acyl-CoA

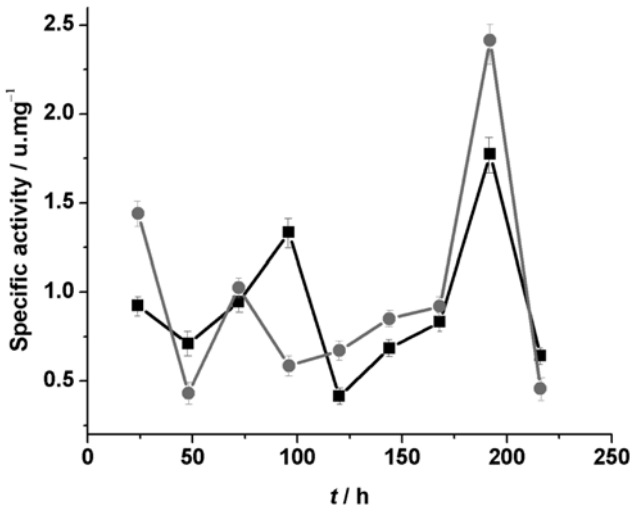

Fig. 8. The time course of FAS in medium with different ammonium sulfate concentration (●-5 mM, ■-12.5 mmol/L).

precursor in terms of quantity and time. To clarify the flux direction of the precursor, the fatty acid synthetase (FAS) activity was determined with media containing 5 mmol/L or 12.5 mmol/L of ammonium sulfate, which has a favorable or adverse effect on the production of MB1, respectively.

Results (Fig. 8) show that FAS activity appears to have three peak levels in the course of fermentation. The first one emerged at 24 h in the exponential phase, suggesting that the biosynthesis of fatty acids is necessary for the growth; the second one appeared around 80 h, when the production started; and the last one occurred in the late phase of fermentation, when product synthesis was culminated. The emergence of the last two peak values might be attributed to the induction effect resulting from the accumulation of precursor. Usually, FAS activity is promoted by high level of ammonium ion during the early and late phase of fermentation, though the level of enzyme activity in middle phase of fermentation is unstable (data not shown). Therefore, during the production phase, a high level of ammonium ion is favorable to the enhancement of FAS activity, which leads to the increase of the synthesis of fatty acid, and eventually results in low yield of meilingmycin because of a lack of precursor.

CONCLUSION

The activities of 6-phospho-glucose dehydrogenase, citrate synthetase and succinate dehydrogenase are upregulated by ammonium ion. The corresponding HMP pathway and TCA cycle are thus intensified with the increase of ammonium ion. With the increase of the consumption of sugar, the primary metabolism of *Streptomyces nanchangensis* is greatly enhanced. However, high concentration of ammonium ion inhibits the activities of VDH and MCT, which leads to the restriction of the supply of the precursor necessary for the synthesis of meilingmycin macrolide. These two factors account for the low yield. Whereas, the synthesis of fatty acid by FAS and the macrolide competes for the same precursor, and ammonium ion is beneficial to the synthesis of FAS, which leads to the increase of fatty acid, and the loss of precursor for meilingmycin production. Consequently, the strategy of maintaining a low level of FAS activity is the critical factor for high yield of meilingmycin.

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