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BIOCHEMICAL PARAMETERS OF *Saccharopolyspora erythraea* DURING FEEDING AMMONIUM SULPHATE IN ERYTHROMYCIN BIOSYNTHESIS PHASE

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The physiology of feeding ammonium sulphate in erythromycin biosynthesis phase of *Saccharopolyspora erythraea* on the regulation of erythromycin A (**Er-A**) biosynthesis was investigated in 50 L fermenter. At an optimal feeding ammonium sulphate rate of 0.03 g/L per h, the maximal Er-A production was 8281 U/mL at 174 h of growth, which was increased by 26.3% in comparison with the control (6557 U/mL at 173 h). Changes in cell metabolic response of actinomycete were observed, i.e. there was a drastic increase in the level of carbon dioxide evolution rate and oxygen consumption. Assays of the key enzyme activities and organic acids of *S. erythraea* and amino acids in culture broth revealed that cell metabolism was enhanced by ammonium assimilation, which might depend on the glutamate transamination pathway. The enhancement of cell metabolism induced an increase of the pool of TCA cycle and the metabolic flux of erythromycin biosynthesis. In general, ammonium assimilation in the erythromycin biosynthesis phase of *S. erythraea* exerted a significant impact on the carbon metabolism and formation of precursors of the process for dramatic regulation of secondary metabolites biosynthesis.

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Erythromycin is a polyketide antibiotic produced by *Saccharopolyspora erythraea* in submerged fermentation. Erythromycin A (Er-A) is the main active component of erythromycin products. Simultaneously, there exist two structurally similar by-products of eryhtromycin B (Er-B) and eryhtromycin C (Er-C) in the culture broth. In fermentation phase, improving Er-A production is an effective way to meet the market demands and reduce the production cost.

Ammonium salts are an available inorganic nitrogen source, which usually regarded to suppress the biosynthesis of antibiotics [1]. Addition of ammonium ions (5–30 mM) to *Penicillium urticae* shake-flask culture led to a strong repression of the enzyme activities of secondary metabolism [2]. However, in cephalosporin-C fermentation, ammonium sulphate as inorganic nitrogen source was suitable for higher yield of antibiotic [3]. In previous work, we first reported that feeding ammonium sulphate in erythromycin biosynthesis phase of *S. erythraea* could affect Er-A biosynthesis [4]. Nevertheless, a thorough understanding of the physiology mechanism of ammonium assimilation for enhancing Er-A production was still lacking.

As we know, there are close links between primary and secondary metabolism, both in terms of precursors formation and nutrient regulation. Er-A has 3 structural parts with the 14-member macrolide ring and 2 deoxysugars, in which the lactonic ring requires propionyl-CoA and 6 methylmalonyl-CoA molecules as precursors for its biosynthesis [5]. These compounds could have multiple metabolic origin including catabolism of odd-numbered fatty acids, reduction of acrylate, rearrangement of succinyl-coenzyme A and catabolism of Met, Thr or Val [6, 7]. Deeper knowledge of intermediary metabolism, especially of TCA cycle enzymes and precursors supply, may provide a better understanding of the link between primary and secondary metabolism for Er-A biosynthesis.

The aim of the study was to investigate some key enzymes and metabolites profiles in carbon metabolism, and amino acids utilization in culture broth of *S. erythraea* under the optimal feeding ammonium sulphate rate in erythromycin biosynthesis phase using 50 L fermenter. The results showed that cell metabolism was enhanced by ammonium assimilation, which induced an increase of the pool of TCA cycle and the metabolic flux of erythromycin biosynthesis. The information obtained in this work should be helpful for deeper understanding of ammonium assimilation in erythromycin biosynthesis phase for the regulation of Er-A biosynthesis in *S. erythraea*.

MATERIALS AND METHODS

Microorganism and culture conditions. *Saccharopolyspora erythraea* № 8, an erythromycin producer strain from Yidu HEC Biochem. Co. Ltd. (Hubei, China) was used, which was publicly available [9]. Agar slants were inoculated with spores and incubated at 32°C for 7 days, and then used for seed culture inoculation. For seed cultures, the medium composition was (g/L): starch – 30, soybean flour – 15, NaCl – 5, $(NH_4)_2SO_4$ – 2. The seed culture was grown in a 500 mL shake flask containing 50 mL of liquid medium and incubated at 32°C on a rotary shaker (220 rpm) for 7 days. The fermentation cultivation was inoculated with 10% (v/v) of the above seed culture medium and incubated at 33°C.

Feeding ammonium sulphate in erythromycin biosynthesis phase. The 50 L fermenter was manufactured by Shanghai Guoqiang Bioengineering Equipment Co., Ltd. (China) [8], which had a 30 L (working volume) agitated bioreactor with 3 turbine impellers and equipped with devices to monitor and control more than 14 on-line measurable parameters. The stirred reactor was aerated through a ring sparger. Dissolved oxygen (DO) level was set above 20% of air saturation and controlled by adjusting agitation speed and aeration rate during fermentation. DO concentration was detected using polarographic DO electrode (Mettler Toledo, Switzerland). The CO_2 off-gas from the fermenter was measured with the gas analyzer (Chongqing Hateman measuring instruments Co., Ltd, China). The cultivation temperature was kept at 33°C, and pH was controlled at 6.9–7.0 with feeding glucose concentration of 300 g/L during the whole of fermentation process. Three independent samples were taken every 8 h for the analyses of cell biomass, erythromycin production, NH_4^+ and total sugar concentration.

Feeding ammonium sulphate was commenced from 80 h to the end of fermentation in 50 L fermenter. The rate of the process was 0.03 g/L per h. Cultivation without feeding ammonium sulphate was used as the control.

Determination of cell biomass (packed mycelium volume, PMV). For the determination of cell biomass (PMV), 10 ml of culture broth was taken as sample, after removal of supernatant by centrifugation (4,000 × *g*, 10 min), PMV was calculated as the volume of precipitate/10 mL of culture broth.

Analysis of extra- and intracellular organic acids. For the determination of extra- and intracellular organic acids of metabolism, the HPLC system (Aligent 1200, USA) was equipped with AquaSep C8 column and a UV detector (210 nm). 0.6 mL/min mobile phase using 0.01mol/L H_3PO_4 solution was applied to the column. The column was operated at 30°C [9].

Analysis of amino acids. For the determination of free amino acids in culture broth, the amino acids were derived by automatic pre-column O-phthaldialdehyde (OPA) derivation methods. The HPLC was equipped with Agilent 1200 system, column ZORBAX Eclipse-AAA (4.6 mm × 150 mm, 5 μm, Agilent, USA). Mobile phase contained mixture of 0.04 M NaH_2PO_4, pH 7.0, as A phase and acetonitrile, methanol and distilled water (45 : 45 : 5, v/v) as B phase. Flow rate was 2 mL/min using UV detector at 238 nm.

Analysis of enzyme activities *in vitro*. *Preparation of cell extracts.* Enzyme activity was defined as μmol of substrate consumed per min and mg of protein (U/mg). In each case, reactor bulk liquid samples were withdrawn and centrifuged at 12,000 × *g* and 4°C for 15 min. The supernatant was removed and cells were resuspended with 50 mM potassium phosphate extraction buffer (pH 7.4), the ratio of buffer : cells was 10 : 1. Cells were lysed with addition of lysozyme (1 g/mL of buffer) at room temperature for 2 h. The extract was centrifuged at 12,000 × *g* and 4°C for 15 min to remove cell debris and the supernatant was used for subsequent activity measurements. Protein content was determined by Bradford assay method.

Pyruvate kinase. The activity of pyruvate kinase (PKS) was measured spectrophotometrically [10]. All compounds of the reaction mixture were pipetted into a colorimetric ware, and reactions initiated by adding the cell extract to give a final volume of 1 mL. The reaction mixture contained 80 mM Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 2 mM ADP, 10 mM phosphoenolpyruvate, 2 mM NADH, and 5 U lactate dehydrogenase (Shanghai Yuanju Biotechnology Co., Ltd., China).

Citrate synthetase. The activity of citrate synthetase (CS) was detected by monitoring the disappearance of NADH during reaction [11]. The assay conditions were the following: 2 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.5 mM Tris-HCl buffer (pH 7.4), 0.2 mM acetyl-CoA, and 0.15 mM oxaloacetic acid.

Glutamine synthetase. The activity of glutamine synthetase (GS) was determined by measuring the increase of glutamylhydroxamate at 540 nm [12]. The reaction mixture contained 30 mM glutamate, 0.4 mM ADP, 20 mM sodium dihydrogen arsenate, 60 mM hydroxylamine, 3 mM $MnCl_2$ in 40 mM imidazole buffer (pH 7.0) and cell extract in a total volume of 1 mL.

Methylmalonyl coenzyme A mutase. Methylmalonyl coenzyme A mutase (MCM) was assayed as reported by Bermudez et al. [6] with minor modifications. The activity was detected by measuring the decreased succinyl-CoA by HPLC. The reaction mixture contained 1 mM dithioerythritol, 60 μM coenzyme B_{12}, 50 μM succinyl-CoA in 100 mM Tris-buffer (pH 8.0) and cell extract in a total volume of 1 mL. The reaction was initiated by addition of succinyl-CoA and incubated in

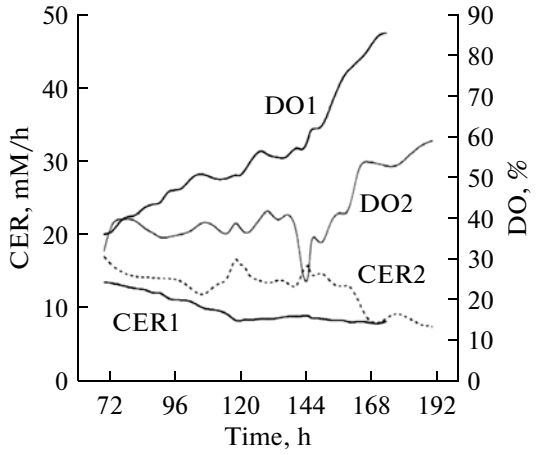

Fig. 1. The changes of the CER and DO level during growth of *S. erythraea* with feeding ammonium sulphate. CER_1 and DO_1 – control; CER_2 and DO_2 – feeding ammonium sulphate.

the dark for 30 min at 30°C. It was stopped by the addition of 20 μl perchloric acid. Precipitated protein was removed by centrifugation at 15,000 × *g* for 20 min, and the supernatant was assayed by HPLC chromatography. Activity was expressed as picomols of succinyl-CoA decreased per min per mg protein. The HPLC was equipped with Agilent 1200 system, column Hypersil BDS C18 (4.6 mm × 250 mm, 5 μm, Elite, China). Mobile phase contained mixture of phosphate buffer (pH 7.0) and methanol (95 : 5, v/v). Flow rate was 1.2 mL/min using UV detector at 259 nm.

Protease activity. Protease activity was determined as described by Shimogaki et al. [13], using casein as the substrate. Enzyme solution (1 mL) was added to 1 mL of casein (20 g/L), and the mixture was incubated at 40°C for 10 min. The reaction was stopped by adding 2 mL of trichloroacetic acid and kept at room temperature for 10 min followed by filtration through Whatman filter paper № 1 (USA). The supernatant (1 mL) was mixtured with 5 mL of sodium carbonate and 1 mL of Folin reagent, and kept at 40°C for 20 min. The absorbance of the filtrate was measured at 680 nm. One unit of protease activity was defined as the amount of enzyme required to produce one μg of Tyr per min under the conditions described above.

Table 1. The changes of erythromycin components in the culture medium of *S. erythraea* during feeding ammonium sulfate

Time, h	Erythromycin, U/mL			Er-A:Er-C
	A	B	C	
78	3593	201	307	11.70:1
102	5096	204	429	11.87:1
126	6185	213	580	10.66:1
150	7422	250	690	10.75:1
174	8281	235	727	11.39:1
184	8240	199	777	10.60:1
Control (173 h)	6557	199	1483	4.42:1

Assay of erythromycin production and its components. The concentration of total erythromycin production was measured by the modified colorimetric method [14]. After removing the biomass and insoluble ingredients, the fermentation broth was extracted with butyl acetate. Extracted erythromycin was mixed with the 0.1 M hydrochloric acid. The aqueous phase fraction was separated with great care, and further mixed with sulfuric acid for 3 min. Its absorbance was measured at 498 nm. To confirm the production of erythromycin, the fermentation broth samples at the end of fermentation were further bioassayed against *Bacillus pumilus* CMCC (B) 63202 using cylinder plate assay method [9].

The components of erythromycin (A, B and C) were determined by HPLC method [4] with Hypersil BDS-C18 column (4 mm × 250 mm, 5 μm, Elite, China). Mobile phase contained mixture of acetonitrile and 0.025 M potassium hydrogen phosphate (60 : 40, v/v). Flow rate was 0.9 mL/min using UV detector at 215 nm.

RESULTS

Physiology of feeding ammonium suplhate in erythromycin biosynthesis phase. Based on our previous data of control the cell growth in erythromycin biosynthesis phase of *S. erythraea*, an appropriate ammonium sulphate feeding rate of 0.03 g/L per h [4] was applied in this work. The changes of erythromycin components in the culture medium with feeding ammonium sulphate were shown in Table 1. The Er-A production and the rate of Er-A to Er-C were obviously enhanced compared with the control. The highest Er-A production of 8281 U/mL at 174 h obtained was 26.3% higher than in the control (6557 U/mL at 173 h). The ratio of Er-A to Er-C was 11.39 : 1, which was increased remarkably with respect to the control (4.42 : 1).

The changes of cell metabolic response with feeding ammonium sulphate were shown in Fig. 1. In comparison with the control, the level of carbon dioxide evolution rate (CER) was remarkably enhanced after feeding ammonium sulphate in erythromycin biosynthesis phase. Correspondingly, DO concentration was

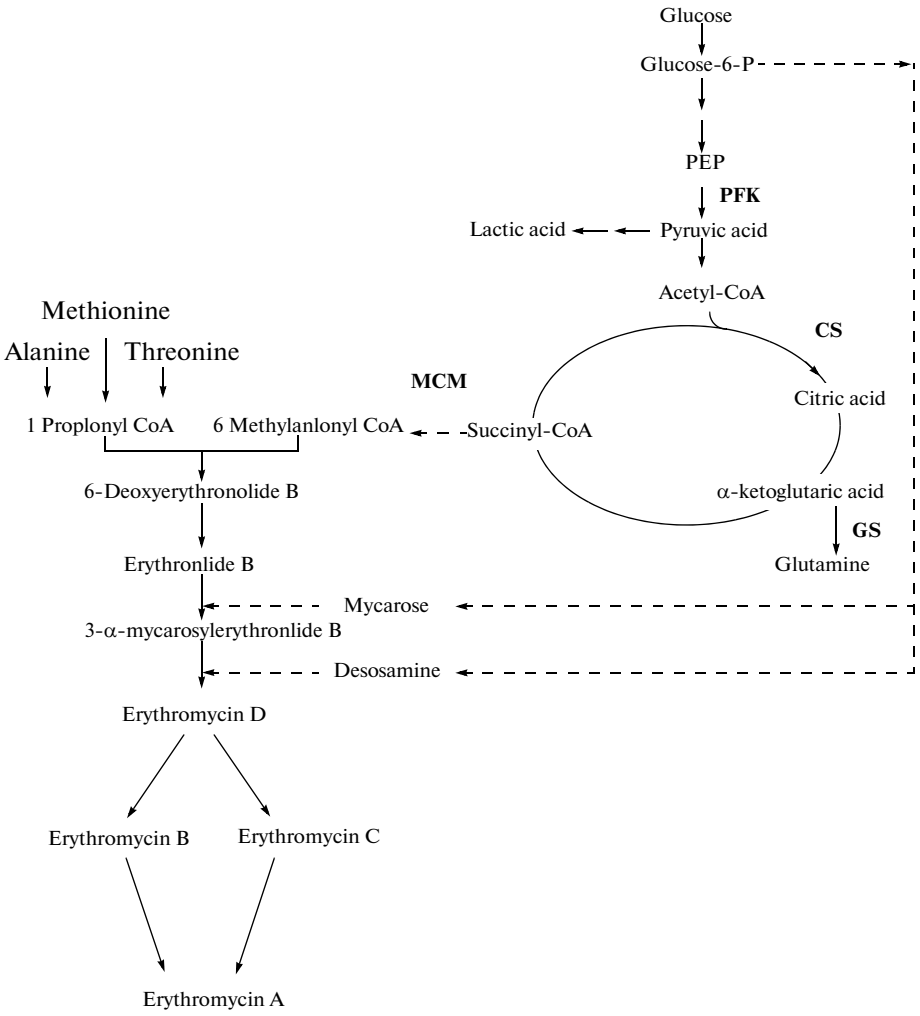

Fig. 2. The schematic representation of key metabolic pathway in erythromycin A biosynthesis [5]. The *S. erythraea* enzyme activities detected in the study shown in bold.

decreased compared with the control. These results indicated that feeding ammonium sulphate enhanced the activity of microbial metabolism.

Variation of the intra- and extracellular metabolites level and key enzymes activity. The lactonic ring of erythromycin requires propionyl-CoA and 6 methylmalonyl-CoA molecules as precursors for its biosynthesis (Fig. 2). The changes of key enzyme activities of *S. erythraea* during feeding ammonium sulphate were further investigated as shown in Fig. 3. It was revealed that the activity of PKS was similar to the control, while the activities of CS and GS were higher compared with the control. Methylmalonyl-CoA was used as precursor for erythromycin biosynthesis and its synthesis from succinyl-CoA depended mainly on the activity of MCM [5]. Fig. 3d indicated that the activity of this enzyme was higher during feeding ammonium sulphate of *S. erythraea* than that of the control. Moreover, the changes of organic acids in the process were analyzed as shown in Fig. 4. It is obvious that the accumulation of intracellular pyruvic, citric, and lactic acids was higher compared with the control. Propionic acid was the precursor for propionyl-CoA biosynthesis, and the accumulation of this compound in the *S. erythraea* cells was more than that of the control.

Variation in utilization of amino acids and protease activity. Protease activity reflected the ability of protein broken down into amino acids. Some amino acids including Ala, Thr or Met were provided for propionyl-CoA biosynthesis as erythromycin precursors [6, 7]. As shown in Fig. 5 after feeding of actinomycete with ammonium sulphate, the protease activity was higher than in the control, which indicated that more protein was hydrolysed into amino acids. The contents of amino acids in culture broth was presented in Table 2, the total contents of amino acids were definitely lower than those of the control. The contents of Ala, Thr and Met in culture broth revealed that the level these

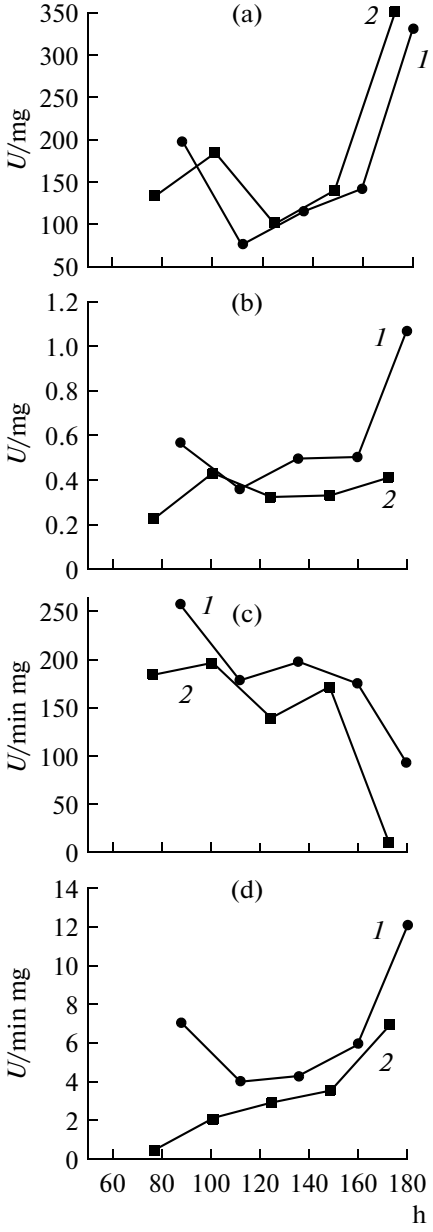

Fig. 3. The key enzymes activities (a – PFK; b – CS; c – GS; d – MCM) during growth of *S. erythraea* with feeding ammonium sulphate. *1* – feeding ammonium sulphate; *2* – the control.

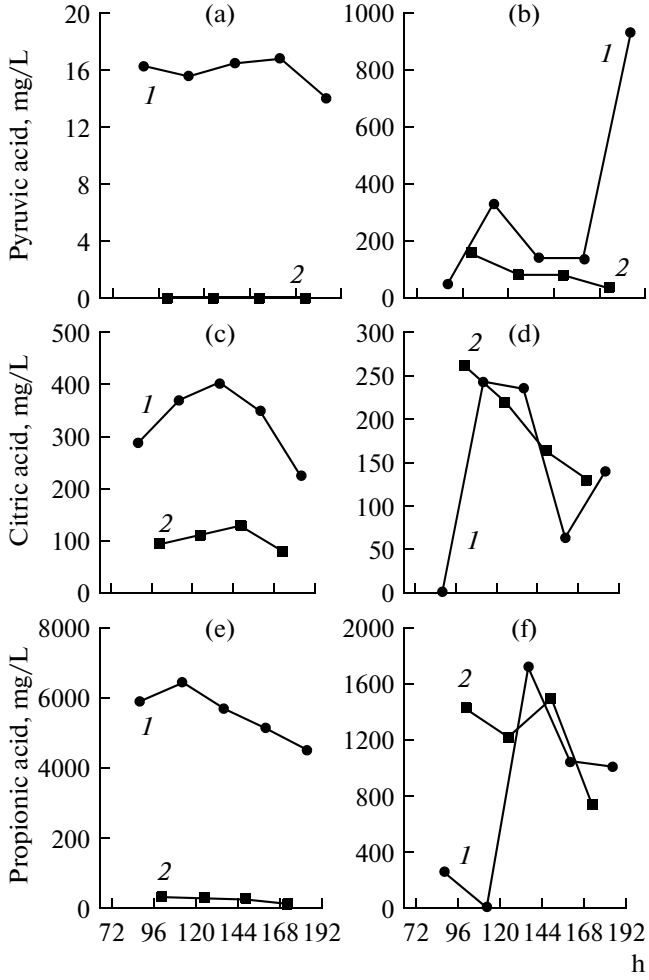

Fig. 4. Time course of the intra- (a, c, e) and extracellular (b, d, f) organic acids levels of *S. erythraea* during feeding ammonium sulphate. *1* – feeding ammonium sulphate; *2* – the control.

3 amino acids were obviously lower compared with the control. The results implied that there were more amino acids consumed as precursors supply for erythromycin biosynthesis under high activity of protease.

DISCUSSION

Although some researchers reported that antibiotics biosynthesis may be inhibited or repressed by ammonia [15], the most striking characteristics of the regulation of this process were its diversity and complexity. In this study, the physiology of feeding ammonium sulphate in erythromycin biosynthesis phase of *S. erythraea* on the regulation of Er-A biosynthesis was investigated. At an appropriate feeding rate, the maximal Er-A production was 8281 U/mL at 174 h, which was increased by 26.3% with respect to the control (6557 U/mL at 173 h). Cell metabolism revealed by the aid of some physiology parameters on bioreactor scale was enhanced by ammonium assimilation (Fig. 1).

The changes of the key enzyme activities and organic acids participating in metabolism of *S. erythraea* confirmed that ammonium assimilation might depend on the glutamate transamination pathway for enhancing the activity of microbial metabolism (Figs. 3 and 4). Ammonium assimilation promoted the cell growth, which induced the increase of the pool of TCA cycle and the metabolic flux of erythromycin biosynthesis.

In previous work, we also found that adding ammonium ions in culture medium was favorable to primary metabolism of actinomycete in meilingmycin biosynthesis [16].

Biosynthesis of antibiotics was markedly affected by compounds containing amino acids [1]. As shown in Table 2, the consumption of amino acids (Ala, Thr and Met) was obviously elevated under high activity of protease (Fig. 5), which indicated that more precursors were supplied for erythromycin biosynthesis. In our previous work, overexpression of S-adenosylmethionine synthetase which catalyzed the biosynthesis of S-adenosylmethionine (SAM) from ATP and Met was induced to erythromycin A increased by 132% and erythromycin B decreased by 30% [17]. Compared with the level of Ala and Thr in culture broth, the content of Met was lower (Table 2). We suppose that more Met used for the biosynthesis of SAM, which enhanced the biotransformation from Er-C to Er-A in actinomycete by increasing the substrate concentration of the methylation reaction, and further increased the Er-A production and the ratio of Er-A to Er-C.

In this work, it seemed that adding ammonium ions in erythromycin biosynthesis phase had no effect on the activities of secondary enzymes. Instead, in patulin biosynthesis, adding ammonium ions during appearance of secondary enzymes caused a rapid decrease in specific activity [2]. In conclusion, the present study should be helpful for better understanding ammonium assimilation on the regulation of erythromycin biosynthesis. Moreover, it can also help us to establish novel strategy for regulation the central

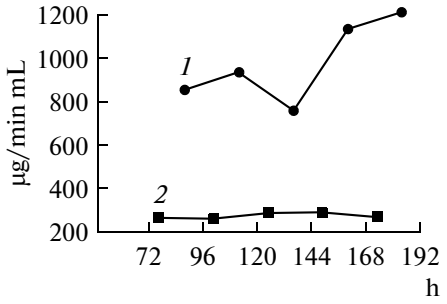

Fig. 5. Protease activity of *S. erythraea* during feeding ammonium sulphate. *1* – feeding ammonium sulphate; *2* – the control.

Table 2. Variation of the amino acids contents (mg/L) in the culture broth during feeding of *S. erythraea* with ammonium sulphate and the control at the later stage of fermentation

Amino acids	Control, mg/L			Feeding ammonium sulphate, mg/L			
	101 h	125 h	149 h	88 h	112 h	136 h	160 h
Asp	24.87	30.08	28.48	0.00	6.00	5.57	13.71
Glu	55.21	90.12	117.72	20.79	36.69	82.34	339.16
Ser	11.74	10.52	15.41	4.88	7.43	8.59	17.19
His	12.23	11.62	12.84	0.00	7.97	11.39	17.65
Arg	16.49	21.53	20.16	5.79	8.69	8.69	33.09
Gly	5.24	7.62	9.05	3.23	3.39	4.00	10.62
Thr	15.10	13.96	18.23	10.09	10.37	11.49	21.85
Ala	14.07	20.36	22.21	8.59	10.87	16.31	39.28
Met	30.16	23.62	34.88	4.16	7.81	10.41	25.77
Phe	11.39	14.45	18.39	0.00	16.11	29.74	40.89
Ile	27.06	22.65	145.30	0.00	3.49	5.23	14.82
Leu	79.07	77.46	179.04	38.74	42.12	61.87	69.93
Lys	10.03	13.14	28.35	6.61	6.61	8.35	33.40
Total	413.64	482.13	799.08	190.88	279.53	399.98	837.35

metabolism in antibiotics biosynthesis phase to improve the secondary metabolites biosynthesis.

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