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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Regulation of avilamycin biosynthesis in *Streptomyces viridochromogenes*: effects of glucose, ammonium ion, and inorganic phosphate

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Abstract Effects of glucose, ammonium ions and phosphate on avilamycin biosynthesis in *Streptomyces viridochromogenes AS4.126* were investigated. Twenty grams per liter of glucose, 10 mmol/L ammonium ions, and 10 mmol/L phosphate in the basal medium stimulated avilamycin biosynthesis. When the concentrations of glucose, ammonium ions, and phosphate in the basal medium exceeded 20 g/L, 10 mmol/L, and 10 mmol/L, respectively, avilamycin biosynthesis greatly decreased. When 20 g/L glucose was added at 32 h, avilamycin yield decreased by 70.2%. Avilamycin biosynthesis hardly continued when 2-deoxy-glucose was added into the basal medium at 32 h. There was little influence on avilamycin biosynthesis with the addition of the 3-methyl-glucose (20 g/L) at 32 h. In the presence of excess $(NH_4)_2SO_4$ (20 mmol/L), the activities of valine dehydrogenase and glucose-6-phosphate dehydrogenase were depressed 47.7 and 58.3%, respectively, of that of the control at 48 h. The activity of succinate dehydrogenase increased 49.5% compared to the control at 48 h. The intracellular adenosine triphosphate level and 6-phosphate glucose content of *S. viridochromogenes* were 128 and 129%, respectively, of that of the control at 48 h, with the addition of the 40 mmol/L of KH_2PO_4. As a result, high concentrations of glucose, ammonium ions, and inorganic phosphate all led to the absence of the precursors for avilamycin biosynthesis and affected antibiotic synthesis.

Keywords Regulation · Biosynthesis · Avilamycin · *Streptomyces viridochromogenes AS4.126*

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Introduction

Avilamycin is an oligosaccharide antimicrobial belonging to the orthosomycin group of antibiotics, which is produced by *Streptomyces viridochromogenes*. It inhibits the growth of Gram-positive bacteria effectively and is one of the antimicrobial agents approved for growth promotion in many countries (Buzzetti et al. 1968; Chauvin et al. 2005; Wright 1979). Structural studies revealed that the avilamycin molecule biosynthesis requires the formation of a polyketide moiety and its attachment to a heptasaccharide chain consisting of *d*-olivose, 2-deoxy-devalose, 4-*O*-methyl-*d*-fucose, 2,6-di-*O*-methyl-*d*-mannose, *l*-lyxose, and (methyl) eurekanate (Weitnauer et al. 2002). Recently, the complete biosynthetic gene cluster of avilamycin was reported and several genes were characterized successfully from *S. viridochromogenes* Tü57 (Gaisser et al. 1997; Weitnauer et al. 2001a,b, 2002; Treede et al. 2005; Hofmann et al. 2005; Mosbacher et al. 2005).

Secondary metabolites such as antibiotics are frequently inhibited by a rapidly utilized carbon source such as glucose (Lounes et al. 1996). The basic mechanism(s) of the phenomenon is not understood completely. Moreover, the biosynthesis of antibiotics is also inhibited by rapidly utilized nitrogen sources such as ammonium and regulated by inorganic phosphate (Juan and Arnold 1980; Lebrihi et al. 1992; Lounes et al. 1995; Chen et al. 2003; Juan 2004; Shikha et al. 2005; Hulya and Tarhan 2006).

Despite the excellent progress on the molecular and genetic level of avilamycin biosynthesis, little work has dealt specifically with the metabolic regulation of avilamycin biosynthesis on *S. viridochromogenes*. Up to now, the manner in which nitrogen source, carbon source, and phosphate influence the formation of the avilamycin is unknown. The aim of this work was to investigate the effects of glucose, ammonium, and phosphate on cell growth and avilamycin production and to understand their metabolic regulation on avilamycin biosynthesis in *S. viridochromogenes*.

Materials and methods

Microorganism

Streptomyces viridochromogenes AS4.126 was provided by the Institute of Microbiology, Chinese Academy of Sciences. The strain was maintained on solid Gause's synthetic medium (Du and Lu 1992) at 28 °C.

Medium and culture conditions

Two kinds of medium were used in this study: seed medium and basal fermentation medium. Seed medium composed of 10 g/L malt extract, 8 g/L yeast extract, 10 g/L glucose, and 0.1 g/L $CaCl_2$. The basal fermentation the medium contained 4 g/L yeast extract, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.5 g/L NaCl, 0.1 g/L $CaCl_2$, and 0.01 g/L $FeSO_4$. The concentrations of carbon, ammonium, and inorganic phosphate added to the basal fermentation medium are described in the text. The initial medium pH was adjusted to 7.2 prior to autoclaving at 121 °C for 20 min.

All liquid cultures were conducted in 250-mL flasks containing 50 mL medium at 28 °C with orbital shaking at 180 rpm. An amount of 1.5 mL of spore suspension (10^7–10^8) was inoculated into seed medium and incubated for 24 h. Then, 2.5 mL of the seed culture was inoculated into the fermentation medium and incubated for up to 80 h. Cells of different cultivation time were harvested by centrifugation at 6,500×*g* for 15 min at −20 °C for further analysis.

Measurements

For avilamycin analysis, cell were broken up by ultrasound (19 kHz, 200 W, 5 min) and suspended in acetone (1:10 w/v) for 6 h. Then the supernatant was collected by centrifugation at 6,500×*g* for 15 min. Avilamycin analysis was carried out by high-performance liquid chromatography (Zhang 2000) using a reversed-phase column (Waters 5C18-ms-II, 4.6×250 mm) and a gradient with acetonitrile in 0.2% mono ammonium phosphate solution (pH 3.0, and a flow rate of 2 mL/min). The detection was carried out at a wavelength of 214 nm. Detection and spectral characterization of peaks were accomplished with a 486-UV detector and M32 software (Waters).

For dry cell weight (DCW) determination, 3-mL fermentation culture samples were filtered through a preweighed filter paper (Xinhua no. 5) and washed four times with 20 ml distilled water. Wet filter papers were dried overnight at 90 °C and then weighed after cooling in a desiccator. The DCW in grams per liter of fermentation culture was determined.

Glucose and pH were measured using SBA-40C Microbial Sensor instrument and pHs-3CW pH meter, respectively (Academy of Shandong Agriculture Sciences and Shanghai Cany Precision Instrument). The concentrations of ammonium ion and phosphate in the medium were measured by Berthelot reaction (Xu et al. 1998) and phosphomolybdate blue spectrophotometry (Yuan et al. 1995), respectively.

Pyruvic acid was measured based on the method reported by Irschik and Reichenbach (1985). The measurement of cellular 6-phosphate glucose (6-P-G) was same as the measuring method of glucose-6-phosphate dehydrogenase (G-6-P DH). Adenosine triphosphate (ATP) was measured using ATP Assay Kit (Beyotime). For enzyme activity analysis, succinate dehydrogenase (SDH) was determined with a method described elsewhere (Levine et al. 2002; Noriteru et al. 2004). Valine dehydrogenase (VDH) and G-6-P DH activities were measured as described by Nigel and John (1989) and Kuby and Noltmann (1966), respectively.

Statistical analysis

All experiments were carried out in triplicate, and experimental data were analyzed using the Data Processing System (version 2.0).

Results

Metabolic regulation of glucose on avilamycin biosynthesis

In this study, glucose levels, DCW, variations of pH, and avilamycin production in the basal medium with different glucose concentrations (10–40 g/L) were investigated during the incubation period of *S. viridochromogenes AS4.126*. As shown in Fig. 1, the concentration of glucose in the medium, the residual glucose, and the DCW were greater after 48 h of incubation. These growth rates increased when the concentration of glucose was increased

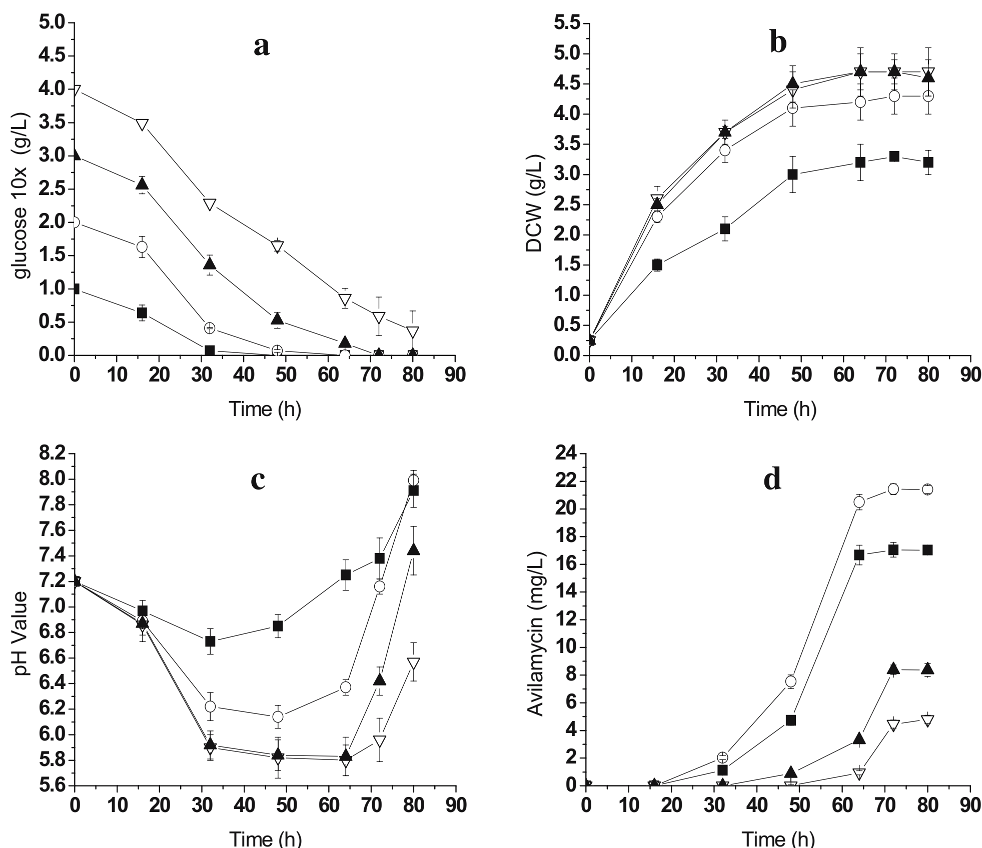

Fig. 1 Variation curve of glucose (**a**), dry cell weight (*DCW*) (**b**), pH value (**c**), and avilamycin yield (**d**) in the fermentation process under different glucose concentrations: 10, 20, 30, and 40 g/L glucose. All values are means of three measurements and expressed as mean±SD

to 30 g/L. When the concentration of glucose was above 30 g/L, growth rate did not increase significantly and indicated that carbon sources were no longer a growth-limiting factor for DCW. The pH value in broth decreased before 32 h of incubation and the decreases in pH values were correlated to the increase of initial glucose concentrations. Lower pH value was kept for a longer time when a higher concentration of glucose was used. Avilamycin yield heightened with the increase in glucose concentrations up to 20 g/L, and the avilamycin yield was the highest when the concentration of glucose was 20 g/L. Then, it decreased with the continuous increase in glucose concentrations from 20 to 40 g/L. The avilamycin yields in the basal medium with 30 and 40 g/L glucose were only 39.1 and 22.4% of that with 20 g/L glucose, respectively. This phenomenon indicated that the biosynthesis of avilamycin was inhibited by high glucose concentration.

As listed in Table 1, the avilamycin yield decreased by 70.2% and nearly 100% when glucose and 2-deoxy-glucose, respectively, were added at 32 h, and avilamycin yield was 2.05±0.12 g/L at this time. However, avilamycin yield changed little with the addition of the 3-methyl-glucose. On the other hand, DCW increased with the addition of glucose and 3-methyl-glucose, but only increased a little with the addition of 2-deoxy-glucose.

Metabolic regulation of ammonium ion on avilamycin biosynthesis

Experiments were performed by varying $(NH_4)_2SO_4$ concentrations (5–20 mmol/L) in basal medium with 20 g/L glucose to study the effects of ammonium ion on cell growth and avilamycin biosynthesis. Glucose, DCW, avilamycin, ammonium ion, pyruvic acid, and the key enzyme activities were assayed. The results are shown in Figs. 2 and 3.

As shown in the Fig. 2, the rate of glucose utilization (p>0.05) and cell growth rate were enhanced with the

Table 1 Effects of glucose derivative added at 32 h on avilamycin biosynthesis

	Control (none)	Glucose (20 g/L)	2-Deoxy-glucose (20 g/L)	3-Methyl-gulcose (20 g/L)
DCW (g/L)	4.22±0.22	4.74±0.25	4.29±0.27	4.55±0.21
Avilamycin (mg/L)	21.40±0.29	7.82±0.25	2.09±0.13	21.48±0.24

All values are means of three measurements and expressed as mean±SD.

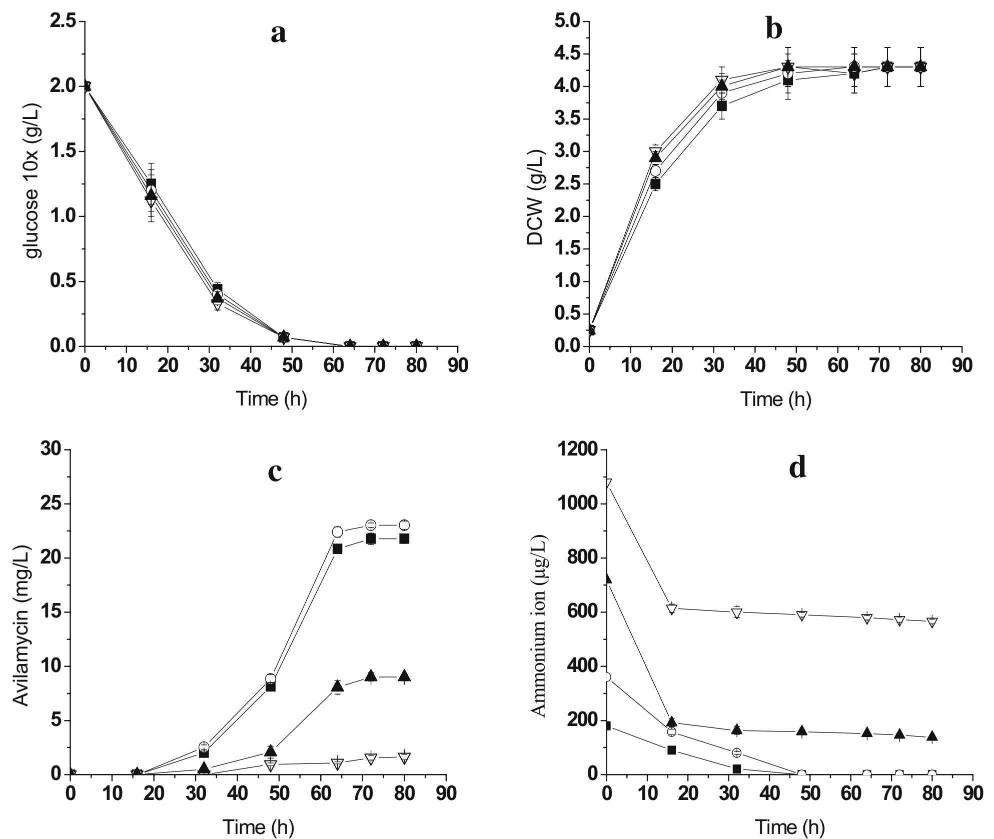

Fig. 2 Variation curve of glucose (**a**), dry cell weight (*DCW*) (**b**), avilamycin yield (**c**), and ammonium (**d**) in the fermentation process under different $(NH_4)_2SO_4$ concentrations: 5, 10, 15, and 20 mmol/L $(NH_4)_2SO_4$. All values are means of three measurements and expressed as mean±SD

increases in $(NH_4)_2SO_4$ concentrations. Avilamycin yield also increased with ammonium ion concentration up to 10 mmol/L. But, when $(NH_4)_2SO_4$ concentration was above 10 mmol/L, avilamycin yield decreased with the increases in $(NH_4)_2SO_4$ concentration. The avilamycin yield was improved by the optimal $(NH_4)_2SO_4$ concentration (10 mmol/L). High $(NH_4)_2SO_4$ concentration in the medium promoted cell growth but inhibited the avilamycin biosynthesis. During the 16-h fermentation period, the concentration of ammonium ion in the broth descended, and then the decreasing rate of ammonium ion lowered. Moreover, the concentration of ammonium ion was all maintained in a certain range when its concentration was above 10 mmol.

Pyruvic acid is a key component in the Embden–Meyerhof–Parnas (EMP) pathway, G-6-P DH and SDH are the key enzymes of the pentose phosphate (PP) pathway and the tricarbocylic acid (TCA) cycle, respectively (Chen et al. 2003). VDH is assumed to be the main enzyme responsible for the utilization of branched-chain amino acids that could provide large numbers of precursors for the synthesis of polyketide in many *Streptomyces* spp. (Hyun et al. 2000). As shown in the Fig. 3, optimal concentration of $(NH_4)_2SO_4$ (10 mmol/L) could enhance the activity of VDH and G-6-P DH and the content of pyruvic acid. The addition of $(NH_4)_2SO_4$ (20 mmol/L) inhibited the activity of VDH and G-6-P DH and the formation of pyruvic acid but enhanced the activity of SDH. When $(NH_4)_2SO_4$ (20 mmol/L) was added into the basal medium without $(NH_4)_2SO_4$, the activities of VDH and G-6-P DH were 52.3 and 41.7%, respectively, of that of the control at 48 h. The activity of SDH was 149.5% of that of the control at 48 h. So, the data in Figs. 2 and 3 indicate $(NH_4)_2SO_4$ (20 mmol/L) TCA cycle was enhanced, EMP and PP pathways were weakened, and the catabolism of branched-chain amino acids was repressed. Consequently, the precursors and nicotinamide adenine dinucleotide phosphate (NADPH) for the synthesis of avilamycin were reduced and the synthesis of avilamycin was inhibited.

Metabolic regulation of phosphate on avilamycin biosynthesis

Experiments were performed by varying KH_2PO_4 concentrations (5–40 mmol/L) at basal medium with 20 g/L glucose and the results were shown in Fig. 4. DCW increased with the increase of KH_2PO_4 concentration at the same sampling time, while pH decreased corresponding KH_2PO_4 concentration enhancement at the same sampling time. Avilamycin biosynthesis in *S. viridochromogenes AS4.126* was stimulat-

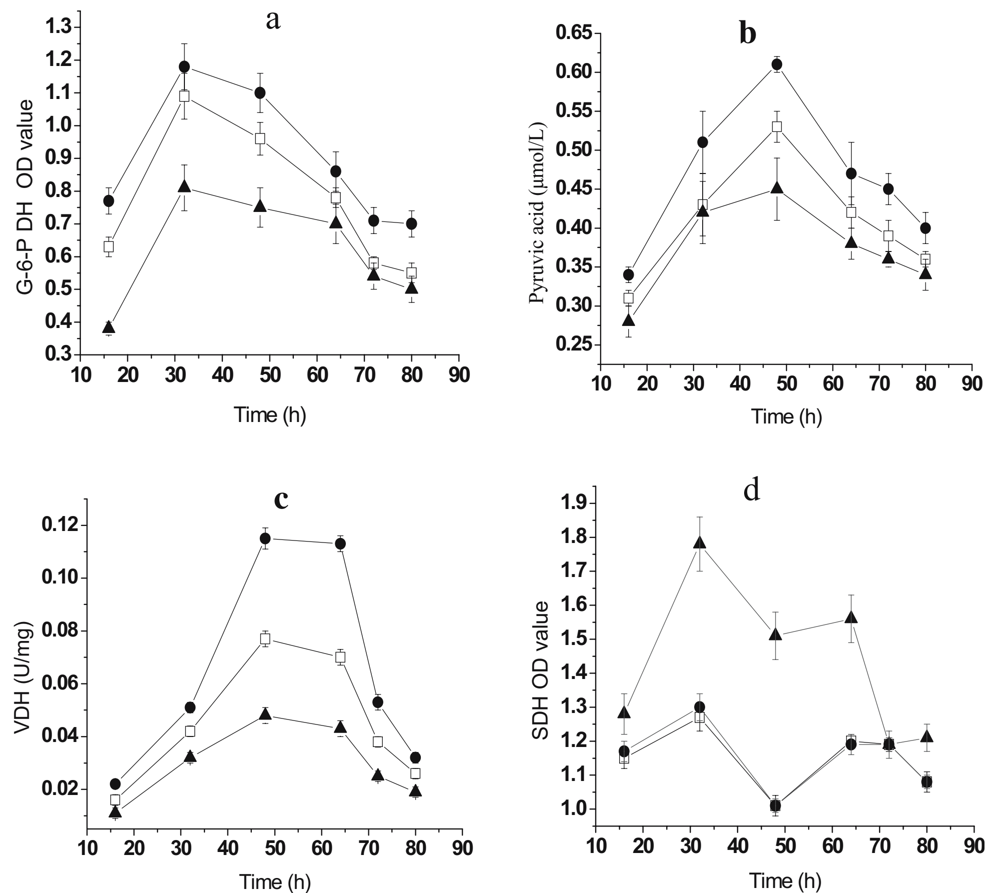

Fig. 3 Effects of ammonium ion concentration on key enzyme activities [glucose-6-phosphate dehydrogenase (*G-6-P DH*) (**a**), content of pyruvic acid (**b**), valine dehydrogenase (*VDH*) (**c**), and succinate dehydrogenase (*SDH*) (**d**)] in avilamycin biosynthesis process. Control; 10 mmol/L; 20 mmol/L $(NH_4)_2SO_4$. All values are means of three measurements and expressed as mean±SD

ed at 10 mmol/L KH_2PO_4, and avilamycin biosynthesis was repressed under higher inorganic phosphate concentrations (above 10 mmol/L). If 40 mmol/L KH_2PO_4 was added at the start of fermentation, extracellular phosphate would not be depleted and avilamycin synthesis would not have occurred. So, the avilamycin biosynthesis was triggered when KH_2PO_4 was nearly exhausted.

As shown in Fig. 5, the intracellular ATP level and 6-P-G content of *S. viridochromogenes* were much higher at 40 mmol/L KH_2PO_4, which were 128 and 129%, respectively, of that of the control. From Figs. 4 and 5, a higher ATP level resulted from the higher phosphate-inhibited (40 mmol/L) avilamycin biosynthesis, but when ATP was maintained in a certain range, there existed no influence on avilamycin biosynthesis. A high ATP level inhibited the activity of phosphofructokinase in the EMP pathway and consequentially resulted in the accumulation of 6-P-G and weakened the EMP pathway (Wang et al. 2002). Therefore, the avilamycin biosynthesis was inhibited.

Discussion

Streptomycetes and related actinomycetes are important sources of many secondary metabolites with a range of biological activities, particularly antibiotic. Antibiotic biosynthesis in streptomycetes appears to be regulated in response to nutritional status (Keqian et al. 1995). Many antibiotic biosyntheses were inhibited or repressed by glucose, ammonium, and phosphate in streptomycetes (Juan and Arnold 1980; Lebrihi et al. 1992; Lounes et al. 1995; Juan 2004). One of the most striking characteristics of the regulation of antibiotic biosynthesis in *streptomycetes* is its diversity and complexity. Generally, no defined regulation types have been suitable for all strains.

Glucose is an excellent carbon source for growth, but a high concentration of glucose usually inhibits the biosynthesis of antibiotics (Juan and Arnold 1980; Chu and Li 2002; Cao 2003). Avilamycin biosynthesis was also inhibited by the high concentration of glucose. When the concentration of glucose was at a lower level, *S. viridochromogenes AS4.126* began to synthesize the antibiotic (Fig. 1). Several mechanisms for carbon catabolite regulation have been reported in many microorganisms (Saier 1998). For instance, the catabolite regulation of glucose is brought into effect by phosphorylation. In most bacteria, 2-deoxy-glucose can be preformed into the 2-deoxy-phosphate glucose by phosphorylation, but it cannot be further metabolized. On the contrary, 3-methyl-glucose is transportable but cannot be phosphorylated (Mardy and

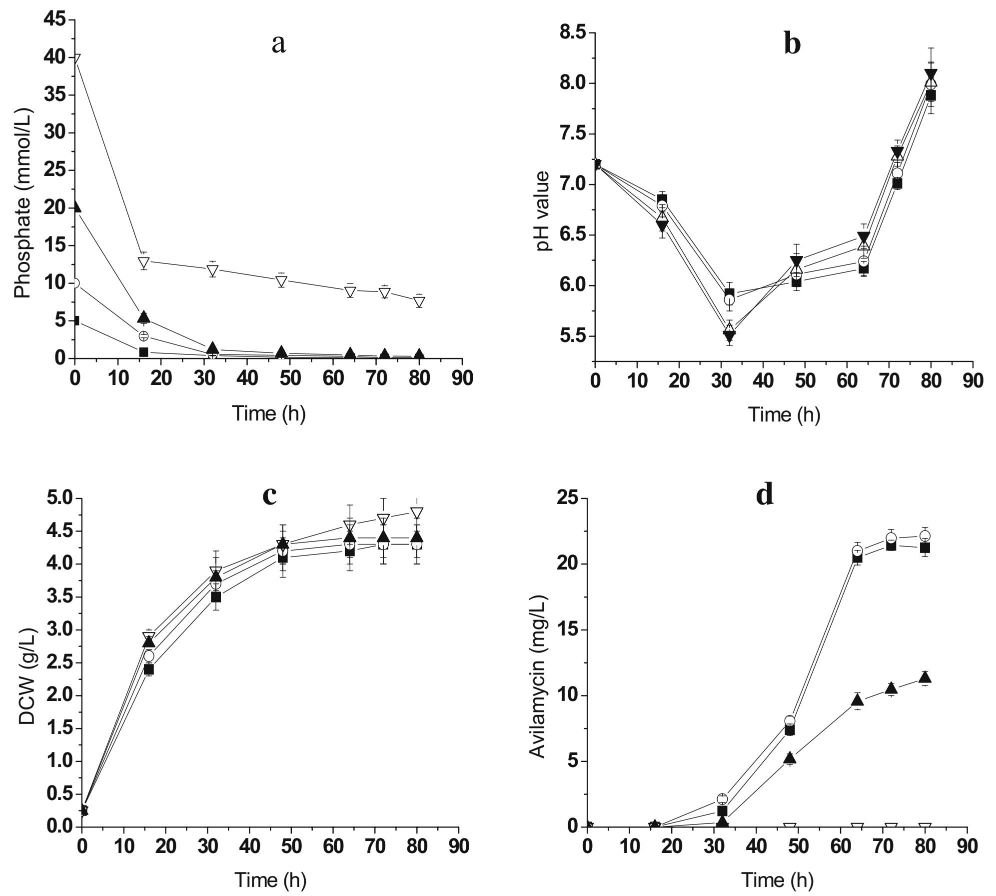

Fig. 4 Variation curve of phosphate (**a**), pH value (**b**), dry cell weight (*DCW*) (**c**), and avilamycin yield (**d**) in the fermentation process under different KH_2PO_4 concentrations: 5, 10, 20, and 40 mmol/L KH_2PO_4. All values are means of three measurements and expressed as mean±SD

Sprinkmeyer 1979; Lounes et al. 1996; Chen et al. 2003). In *S. viridochromogenes*, it is not known whether glucose or a glucose metabolite exerted this repressive effect. To expound the inhibition mechanism of avilamycin biosynthesis in the medium with high glucose concentration, *S. viridochromogenes AS4.126* was first cultured in basal medium with 20 g/L glucose for 32 h. Then, 2-deoxy-glucose (20 g/L), 3-methyl-glucose (20 g/L), and glucose (20 g/L) were added into the flasks, respectively, and cultured for up to 80 h. The results (Table 1) indicate that avilamycin biosynthesis regulation of glucose is in effect through its phosphorylation in *S. viridochromogenes AS4.126*.

Some researchers reported that antibiotic biosynthesis may be inhibited or repressed by ammonia and other rapidly utilized nitrogen sources (Juan and Arnold 1980; Lebrihi et al. 1992; Lounes et al. 1995; Novak et al. 1992; Wang et al. 2005). The analysis of results of some key enzymes and the intermediate contents in TCA-cycle, EMP pathway, and PP pathway indicated that the EMP and PP pathways were inhibited or weakened and the TCA cycle was stimulated by ammonium ion. Consequently, the precursors and

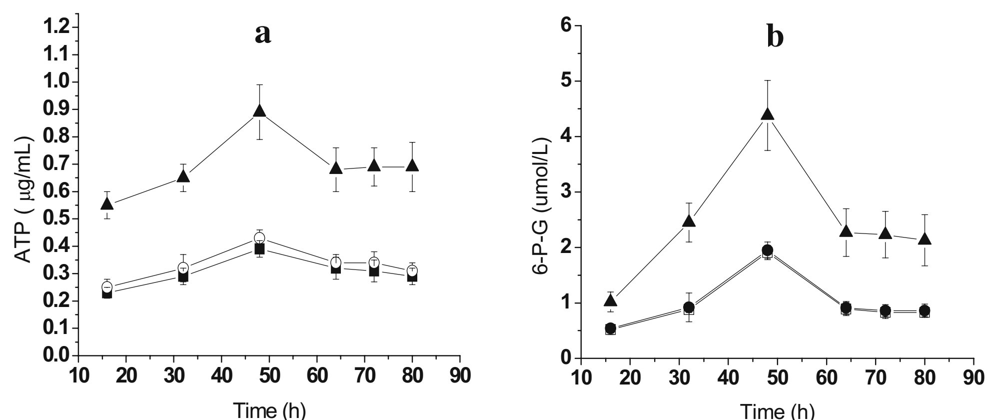

Fig. 5 Effects of KH_2PO_4 concentration on adenosine triphosphate (*ATP*) (**a**) and the content of cellular 6-phosphate glucose (*6-P-G*) (**b**) in avilamycin biosynthesis process. Control; 10 mmol/L;40 mmol/L KH_2PO_4. All values are means of three measurements and expressed as mean±SD

NADPH for the synthesis of antibiotics were reduced and the synthesis of avilamycin was inhibited (Chen et al. 2003; Wang et al. 2005). High ammonium ion concentration had a negative effect on macrolide production, and a strong correlation existed between macrolide production and the level of VDH (Omura et al. 1984a,b; Li et al. 1994; Lebrihi et al. 1992; Hyun et al. 2000; Fabrizio et al. 2004). The high activity of VDH enhanced the catabolism of branched-chain amino acid and was in favor of producing important sources of the macrolide building blocks (Omura et al. 1983, 1984a,b; Lebrihi et al. 1992; Wang et al. 2005). This regulation mechanism of ammonium ion was also proved in *S. viridochromogenes AS4.126* (Figs. 2 and 3).

Phosphate is well known for its important effects on the antibiotic synthesis and is a crucial growth-limiting nutrient in antibiotic fermentation (Juan 2004). Our results were in agreement with those evidences, which showed that high-concentration phosphate had a negative effect on the antibiotic biosynthesis (such as controlstreptomycin, oxytetracycline, clavulanic acid, tylosin, echinomycin, cephalosporin, cephamycin C, thienamycin, and antibiotic AGPM biosynthesis) (Chen et al. 2003; Juan 2004). Several mechanisms have been proposed to explain the effect of phosphate on the antibiotic biosynthesis, such as phosphate promoting primary metabolism, phosphate transferring carbohydrate catabolic pathways, and phosphate inhibiting the formation of antibiotic precursors (Lounes et al. 1996; Juan 2004). But how the nutritional message is transmitted to control the expression of antibiotic production genes is still not clear. Is phosphate the ultimate effector or does it simply regulate the level of an intracellular effector that in turn controls antibiotic synthesis? It was reported that in several other antibiotic biosyntheses, ATPs may be the intracellular effectors of phosphate that control antibiotic synthesis. Moreover, the regulatory effect of phosphate on antibiotic synthesis was put into practice by the regulatory action of the intracellular 6-P-G (Mardy and Sprinkmeyer 1979; Juan and Arnold 1980; Lounes et al. 1996; Orduna and Theobald 2000; Chen et al. 2003). But there is no report about the regulation mechanism of phosphate on avilamycin biosynthesis in *S. viridochromogenes*. To understand the regulatory effect of phosphate, experiments were designed as follows: the basal medium with 20 g/L glucose was the control. Ten and 40 mmol/L KH_2PO_4 were added to the basal medium. In this work, a similar effect of phosphate on avilamycin biosynthesis was also approved in *S. viridochromogenes AS4.126*.

Summary

By now, the genetic aspects of *S. viridochromogenes* for producing avilamycin have been very well studied, but less attention has been focused upon metabolic regulation of avilamycin biosynthesis. The understanding of the regulation of avilamycin synthesis would eventually lead to improvements in antibiotic yield. In this work, the metabolic regulation of glucose, ammonium ion, and inorganic phosphate for avilamycin biosynthesis in *S. viridochromogenes AS4.126* was investigated. The results should lead to a better understanding of secondary metabolism in *S. viridochromogenes AS4.126*. Moreover, it can also help us to screen a high avilamycin-producing strain and optimally control the process of avilmycin fermentation.

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