EFFECTS OF PRECURSORS ON KITASAMYCIN PRODUCTION IN *Streptomyces kitasatoensis*

EFEITOS DOS PRECURSORES NA PRODUÇÃO DE KITASAMICINA EM Streptomyces kitasatoensis

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ABSTRACT: To improve kitasamycin biosynthesis by *Streptomyces kitasatoensis* Z-7, the addition of two precursors, sodium acetate and ethyl acetate, to the fermentation medium was evaluated. Ethyl acetate was the most effective precursor compared with control conditions; In a 15-L fermentor, the kitasamycin titer was 21% higher when 0.48% ethyl acetate was added compared to control conditions. Content of the A_5 component increased by 5.1%, and the A_4 content decreased slightly compared to that of the control. During kitasamycin synthesis, intracellular and extracellular concentrations of acetic acid were higher for *S. kitasatoensis* Z-7 supplemented with ethyl acetate than for the non-supplemented strain, and the activities of acyl-CoA synthetases, acyl-phosphotransferases, and acyl-kinases were also significantly increased, suggesting that increased acetyl-CoA levels can explain the high kitasamycin titer. These findings may improve the industrial-scale production of kitasamycin for clinical use, and the addition of 0.48% ethyl acetate as precursors in the medium at the beginning of cultivation was a new method to mitigate the negative influence on the cell growth of excess precursor.

KEYWORDS: *Streptomyces kitasatoensis*. Ethylacetate. Fermentation. Component. Kitasamycin

INTRODUCTION

Kitasamycin, also referred to as leucomycin, is a 16-membered polyketide macrolide antibiotic produced by *Streptomyces kitasatoensis* that is widely used as a broad-spectrum antibiotic in medical applications (HATA et al., 1953; VANDERHAEGHE; HOOGMARTENS, 1993). Kitasamycin is a multi-component antibiotic containing A_9, A_8, A_7, A_6, A_5, A_4, A_1, A_3, and A_{13} (WOODWARD, 1957). The components exhibit structural differences in the acyl substituent at position C-4″of the mycarose moiety and the alternation of a hydroxyl and an acetyl group at C-3 of the 16-membered lactone ring (VÉZINA et al., 1979). Its structure is described in the literature (ZHENG; GAO, 2015). A_1/A_3, A_4/A_5, A_6/A_7, A_8/A_9, A_{13} were very similar in structure, in each pair, the hydroxylated member is more active than its acetylated partner at C-3 of the 16-membered lactone ring. According to the Chinese Pharmacopoeia (2015 Edition), the main components of kitasamycin shall not total less than 85%, A_5 should comprise 35%–70%, A_4 should comprise 5%–25%. Among kitasamycin components, A_5 is the most clinically potent, with a butyryl group at position 4″ (R_2) (VANDERHAEGHE; HOOGMARTENS, 1993). Polyketides are assembled using several common biosynthetic precursors including acetyl-CoA, malonyl-CoA, and methylmalonyl-CoA (CHAN et al., 2009). The biosynthesis of the polyketide antibiotic kitasamycin, which consists of a 16-membered branched lactonic ring, requires five acetates, one propionate, one butyrate, and another unidentified two-carbon precursor (OMURA et al., 1975). Polymerization of activated acyl units to form the macrolide lactone ring is catalyzed by polyketide synthases in microorganisms by a mechanism similar to that utilized in fatty acid synthesis (HOPWOOD, 1997). Khaoua et al. (1992) showed that short-chain fatty acids increase spiramycin production by *Streptomyces ambofaciens* when cultivated on dextrins and ammonium chloride.

Based on these previous studies, short-chain fatty acids have the potential to increase kitasamycin titers. In general, the direct addition of these substances to the fermentation medium not only changes the medium pH but also influences the cell growth (ROYCE et al., 2013; TAN et al., 2016). Therefore, more research focusing on the addition of salt precursors, such as sodium acetate, sodium butyrate, and precursor analogs, to promote the biosynthesis of kitasamycin is needed, including analyses of the effects of excess precursor at the beginning of fermentation on cell growth. Ethyl acetate can mitigate this influence; similar to

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soybean oil, it has almost no effect on the cell growth. Ethyl acetate decomposes slowly, thereby minimizing the influence of excess precursor on the cell growth.

In the current study, the influence of precursor on the biosynthesis of kitasamycin and its components was further investigated by analyses of intracellular and extracellular short-chain fatty acids. At the enzymatic level, two enzyme systems were used to supply acetyl-CoA to form 16-membered lactone rings, i.e., an acyl-CoA synthetase system and an acyl-kinase system coupled with acyl-phosphotransferases.

MATERIAL AND METHODS

Microorganism and chemicals

S. kitasatoensis Z-7, a producer of kitasamycin, was obtained from Topfond Pharmaceutical Co., Ltd. (Henan, China). Sodium acetate, ethyl acetate and all other analytical-grade chemicals were from Meryer (Shanghai) Chemical Technology Co., Ltd. (Shanghai, China).

Culture conditions in 250-mL Erlenmeyer flasks

The seed medium contained the following (per liter): starch (20 g), cooked soybean meal (15 g), peptone (5 g), yeast extract (5 g), glucose (10 g), $(NH_4)_2SO_4$ (2.5 g), KH_2PO_4 (0.2 g), NaCl (4 g), $MgSO_4$ (0.25 g), $CaCO_3$ (3 g), pH 7.3.

The fermentation medium contained the following (per liter): starch (25 g), cooked soybean meal (25 g), glucose (10 g), silk fibroin powder (11 g), NH_4Cl (2 g), KH_2PO_4 (0.7 g), $ZnSO_4$ (0.06 g), $MnCl_2$ (0.5 g), $CaCO_3$ (3 g), soybean oil (40 mL), pH 7.3.

The mycelia of *S. kitasatoensis* were maintained in a 25% glycerol solution at -20°C. Then, 1 mL of the mycelia solution was inoculated into a 250-mL flask containing 40 mL of seed medium and cultivated at 28°C for 24 h. Then, 1 mL of the resulting seed culture was transferred to a 250-mL flask containing 25 mL of the desired fermentation medium and cultivated at 28°C for 112 h. All cultivations were performed on a rotatory shaker at 220 rpm.

Culture conditions in a 15-L fermentor

Preliminary seed medium contained the following (per liter): starch (20 g), cooked soybean meal (15 g), nitrogen ammonia powder (5 g), yeast extract (3 g), glucose (10 g), $(NH_4)_2SO_4$ (2.5 g), KH_2PO_4 (0.2 g), NaCl (4 g), $MgSO_4$ (0.25 g), $CaCO_3$ (3 g), soybean oil (5 mL), pH 7.1.

The secondary seed medium contained the following (per liter): starch (20 g), cooked soybean meal (25 g), silk fibroin powder (7 g), glucose (15 g), amylase (0.015 g), $ZnSO_4$ (0.06 g), $MnCl_2$ (0.5 g), $CaCO_3$ (2.5 g), soybean oil (6 mL), pH 7.1.

The fermentation medium contained the following (per liter): starch (18 g), cooked soybean meal (20 g), glucose (10 g), silk fibroin powder (7 g), amylase (0.03 g), NH_4Cl (1.5 g), KH_2PO_4 (0.7 g), $ZnSO_4$ (0.06 g), $MnCl_2$ (0.5 g), $CaCO_3$ (3 g), soybean oil (30 g), antifoam agent (0.2 mL), pH 7.3.

The mycelia solution (1 mL) was inoculated into a 250-mL flask containing 40 mL of preliminary seed medium and cultivated at 28°C for 26 h. 2.5mL of this preliminary seed culture was transferred to a 500-mL flask containing 50 mL of the secondary seed medium and cultivated at 28°C for 17 h. Finally, 1 L of the secondary seed culture was inoculated into a 15-L fermentor containing 10 L of fermentation medium and cultivated at 28°C for 112 h.

For the final fermentation, the dissolved oxygen concentration was controlled at 25–40% of air saturation by adjusting the agitation at 400-600rpm, and the pH was not controlled during the fermentation process. During the fermentation period, when the packed mycelia volume (PMV) was higher than 30%, a small amount of sterile water was added to the fermentor.

Fermentative parameter detection

PMV was calculated as the volume of precipitate/fermentation broth (CHEN et al., 2013). Total sugar and reducing sugar levels in the broth were assayed by the Fehling method and amino nitrogen levels were estimated by the formaldehyde titration method. The total protein in the culture supernatant was measured by the Bradford protein assay.

Assay of organic acids

To determine the intracellular and extracellular organic acids (acetic acid, butyric acid) affecting metabolism, the Shimadzu high-performance liquid chromatography system (LC-20AT; Kyoto, Japan) was used with a differential refractive index detector (RID-20A) and a Carbomix H-NP (10 μm, 7.8 × 250 mm) column. The flow rate was 0.6 mL/min and the mobile phase was a 2.5 mM H_2SO_4 solution. The column was operated at 55°C.

Kitasamycin titer and component assay

The biological titer was determined by the conventional disc method using *Bacillus subtilis* as

the test microorganism and a kitasamycin standard obtained from Topfond Pharmaceutical Co., Ltd. (Henan, China). The components of kitasamycin were determined using an HPLC system (Agilent 1260 Infinity; Agilent Technologies, Santa Clara, CA, USA), using a CAPCELL PAK C_{18} MGII column (150 mm × 4.6 mm, 5 μm) with a mobile phase mixture of 0.1 mol/L ammonium acetate (pH 4.5), methanol, and acetonitrile (40:55:5, v/v/v). The detection wavelength was 231 nm and the flow rate was 1 mL/min. The column was operated at 60°C.

Preparation of cell extracts

Mycelia were collected at various times by centrifugation at 10,626× *g* at 4°C and washed with 20mM Tris-HCl buffer (pH 8.0) containing 100mM NaCl. The mycelia were resuspended in a minimal volume of the same buffer and sonicated for 30 cycles with a working period of 3 s in each cycle of 6 s at 300W in an ice water bath using a sonicator (model JY92-II; Scientz Co., Ningbo, China). After cell lysis, the extract was centrifuged for 10 min at 10,626× *g* and 4°C to remove cell debris. The supernatant was used to determine enzyme activity and protein concentrations.

Enzyme assay

Acyl-CoA synthetase (AS) activity, acyl-kinase (AK) activity, and acyl phosphotransferase (APT) activity were assayed as described by Zeng et al. (2016). One unit (U) of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of each enzymatic reaction product per hour under the abovementioned conditions. The specific enzymatic activity is expressed in terms of U/mg of protein.

Statistical analysis

Each experiment was performed in triplicate and the data are presented as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Influence of precursors on kitasamycin titers in shake flasks

The effects on kitasamycin titers of adding to two precursors (sodium acetate and ethyl acetate) to the fermentation medium were examined. Each precursor was added to the culture medium at various concentrations at the beginning of cultivation. As shown in Table 1, the kitasamycin titer increased as the sodium acetate concentration increased in a certain concentration range. However, according to statistical analysis, the kitasamycin titer (11764 ± 351 U/mL) was merely 6.2% higher when 0.15% sodium acetate was added compared to control conditions(11077 ± 273 U/mL). Its effect on the kitasamycin titer was not significant, i.e., although sodium acetate was beneficial for kitasamycin biosynthesis, excess precursor also influenced the cell growth in the early phases (Figure 1).

Table 1. Effects of sodium acetate at various concentrations on the kitasamycin titer

Concentration (%)	Control	0.05	0.1	0.15	0.2
Titer (U/mL)	11077 ± 273	11220 ± 252	11435 ± 231	11764 ± 351	9210 ± 349

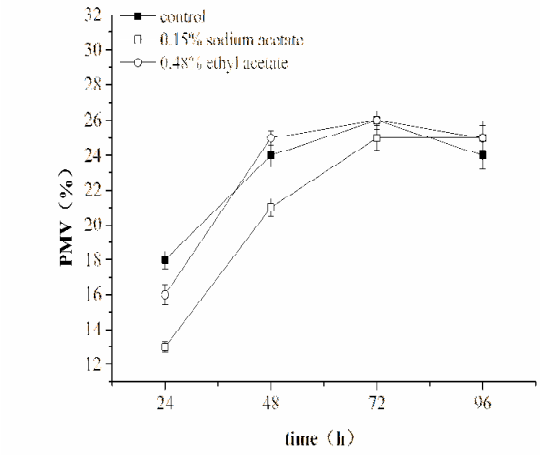

Figure 1. Effects of 0.15% sodium acetate and 0.48% ethyl acetate at various time (24h、48h、72h、96h) on PMV in shake flasks. Sodium acetate (□), ethyl acetate (○) and control (■).

Various concentrations of ethyl acetate were added to the fermentation medium (0.32%, 0.48%, 0.64%, 0.8%, and 0.96%) to determine the optimum initial ethyl acetate concentration for maximizing kitasamycin titers. The results are summarized in Table 2. The highest kitasamycin titer, 12,758 U/mL, was obtained using an initial ethyl acetate concentration of 0.48%, which resulted in an increase in kitasamycin biosynthesis by *S. kitasatoensis Z-7* of approximately 18.8% compared with biosynthesis in the control conditions (i.e., no precursor added). A high concentration of ethyl acetate did not inhibit the biosynthesis of kitasamycin in a certain concentration range, and the favorable effect of ethyl acetate on kitasamycin titer did not further improve for ethyl acetate concentrations above 0.64%. This asymptote in titer can be explained by the lack of other short-chain fatty acids related to lactonic ring construction or restricted metabolic flux.

Table 2. Effects of ethyl acetate at various concentrations on the kitasamycin titer

Concentration (%)	Control	0.32	0.48	0.64	0.8	0.96
Titer (U/mL)	10734 ± 307	11633 ± 324	12758 ± 357	11994 ± 454	12407 ± 423	12242 ± 242

These results indicate that ethyl acetate is better than sodium acetate for enhancement of kitasamycin production by *S. kitasatoensis* Z-7. Kitasamycin synthesis was not enhanced by addition of sodium acetate to the medium, which influenced the cell growth of *S. kitasatoensis Z-7*. Ethyl acetate had almost no effect on cell growth (Figure 1), it was slowly decomposed during the process of fermentation, minimizing the potential influence of excess precursor on *S. kitasatoensis Z-7*.

Influence of ethyl acetate on kitasamycin titers and components in a 15-L fermentor

To further investigate the effect of ethyl acetate on the biosynthesis of kitasamycin, fermentation experiments were performed in a 15-L fermentor. When ethyl acetate was added, the kitasamycin titer was 10546 U/mL at 102 h, which was 21% higher than that of the control (Figure 2).

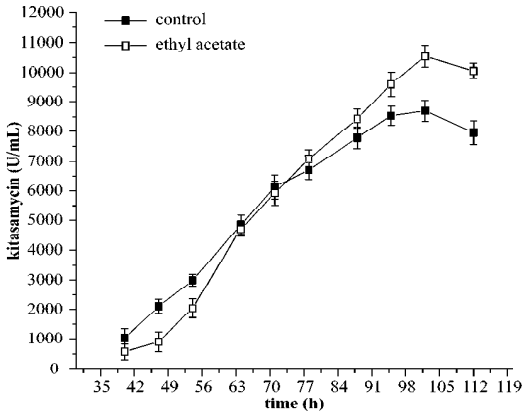

Figure 2. Effects of ethyl acetate on kitasamycin titers in a 15-L fermentor. Ethyl acetate (□) and control (■).

When the kitasamycin titer reached its maximum, the content of the A_5 component was 5.1% higher for medium with ethyl acetate than for the control. The content of the A_4 component was slightly lower for medium with ethyl acetate than the control (Figure 3).

The 3-acetylated A_4 component is derived from its 3-hydroxylated partner A_5. It has been speculated that C-3 acetylation is repressed by butyrate (KITAO et al., 1979). After 78 h, the concentrations of intracellular and extracellular butyric acid were higher with ethyl acetate than without ethyl acetate during kitasamycin biosynthesis (Figure 4). These data suggest that ethyl acetate plays a significant role in increasing the A_5 content and reducing the conversion of A_5 to A_4.

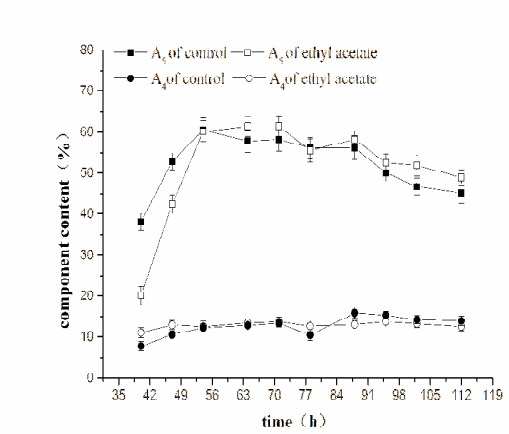

Figure 3. Time course analysis of components upon addition of ethyl acetate in a 15-L fermentor. Ethyl acetate (□,○) and control (■,●)

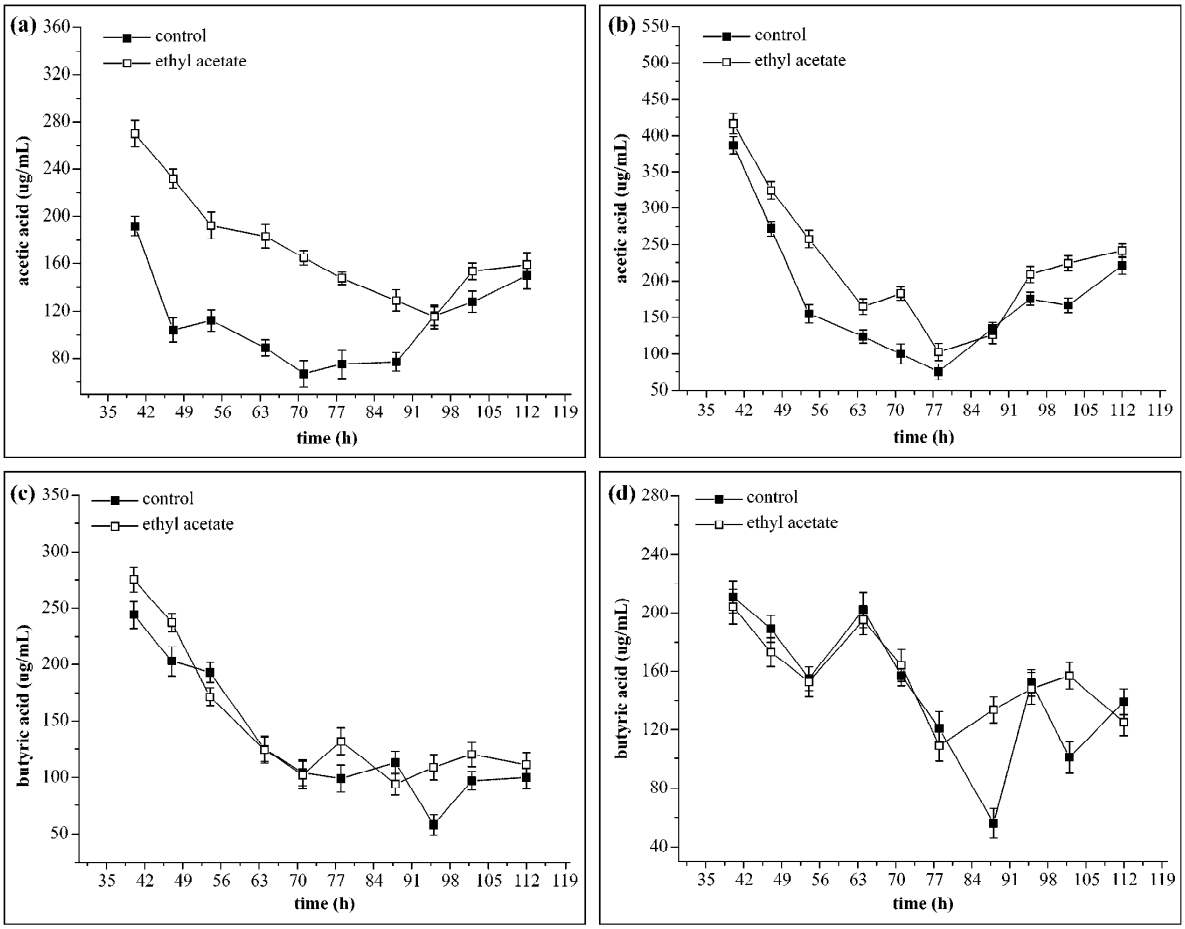

Figure 4. Time course analyses of intracellular organic acid (a, c) and extracellular organic acid (b, d) levels with and without the addition of ethyl acetate in a 15-L fermentor. Ethyl acetate (□) and control (■).

Effects of ethyl acetate on fermentation parameters

The PMV in the fermentor with ethyl acetate was lower than that in the control conditions within 78 h, but then increased sharply and was higher than that of the control. These results indicate that addition of ethyl acetate at an early stage had a negative effect on cell growth; as ethyl acetate was slowly broken down and consumed, the negative effects gradually weakened. After the PMV reached

approximately 30%, it declined rapidly owing to the addition of sterile water. In the early phase of fermentation, the pH value for both treatments began to increase; after 54 h, the pH decreased rapidly because soybean oil began to rapidly decompose, producing various acidic materials. The minimum pH values were observed at 112 h when ethyl acetate was added to the medium (pH 4.62) and at 95 h without ethyl acetate (pH 4.97) (Figure 5).

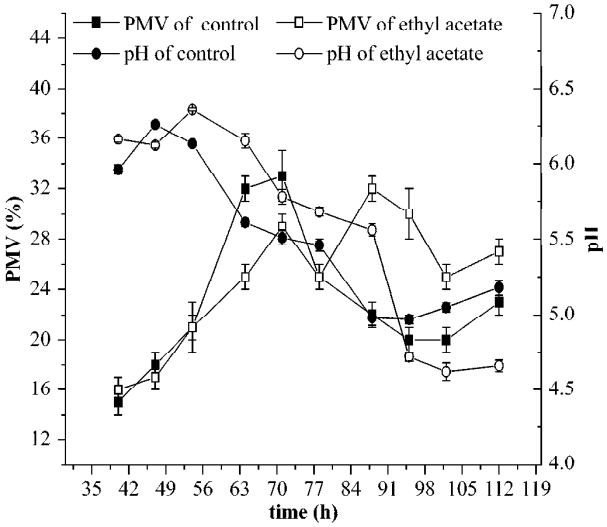

Figure 5. Effects of ethyl acetate on PMV and pH in a 15-L fermentor. Ethyl acetate (□,○) and control (■,●).

When ethyl acetate was added to the medium, the residual amino nitrogen, total sugar, and reducing sugar contents were obviously higher than those of the control at early time points. These results indicate that at the early stage, ethyl acetate had an effect on the metabolic capability of *S. kitasatoensis Z-7*, in accordance with the reduction in the PMV.

Amino nitrogen was rapidly consumed in both conditions during the biosynthesis of kitasamycin. From 40 to 102 h, the consumption rate with the addition of ethyl acetate was 17.4 mg/L·h, which was higher than that of the control (0.6 mg/L·h). However, at 102 h, amino nitrogen content did not differ significantly between conditions (Figure 6(a)).

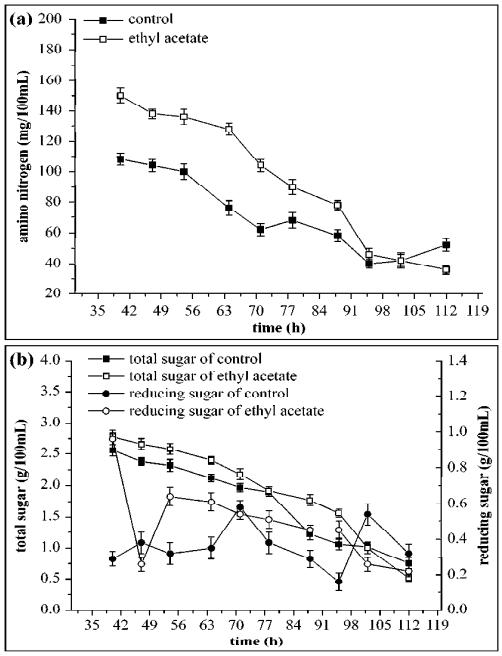

Figure 6. Time course analysis of amino nitrogen (a), reducing sugar, and total sugar (b) upon addition of ethyl acetate in a 15-L fermentor. Ethyl acetate (□,○) and control (■,●)

Time course analyses of total sugar and reducing sugar are summarized in Figure 6(b). From 40 to 102 h, total sugar was continuously consumed in both cases, and the rate of consumption of total sugar was 0.36 g/L·h, which was obviously higher than that observed in the control condition (0.29 g/L·h). From 40 to 47 h, reducing sugar was rapidly consumed with the addition of ethyl acetate; however, the reducing sugar content was only 0.26 g/100 mL at 40 h in the control condition. The consumption of reducing sugar occurred comparatively later with addition of ethyl acetate than in the control conditions. When the reducing sugar content is too low, starch decomposition provides the reducing sugar in the fermentation process, enabling cell growth. Accordingly, the reducing sugar content was maintained in a certain range in both conditions in the production phase.

Changes in organic acid content in a 15-L fermentor

Short-chain fatty acids are precursors in the biosynthesis of kitasamycin; accordingly, they may explain the higher kitasamycin titer observed in the ethyl acetate treatment compared to the control and clarify the role of ethyl acetate in kitasamycin biosynthesis, as shown in Figure 4(a) and Figure 4(b). After addition of ethyl acetate, the increase in kitasamycin production corresponded with higher intracellular and extracellular acetic acid concentrations compared to the control. These results indicate that more acetic acid accumulated as a raw material for kitasamycin lactone ring biosynthesis. After 78 h, acetic acid levels began to increase, probably owing to the reduced demand for acetic acid, the decomposition of ethyl acetate, and the utilization of soybean oil. From 78 h to 102 h, the accumulation of intracellular and extracellular butyric acid was higher for the ethyl acetate treatment than in the control, and the observed increase in butyric acid content repressed C-3 acetylation, reducing conversion of A_5 to A_4. Two peaks for extracellular butyric acid were detected at 64 h and 95–102 h for both conditions (Figure 4(c) and Figure 4(d)).

The accumulation of short-chain fatty acids was directly associated with biosynthesis of kitasamycin. In summary, the accumulation of intracellular and extracellular acetic acid was obviously higher with ethyl acetate than without ethyl acetate. More acetic acid could provide more precursors for kitasamycin. The addition of ethyl acetate could meet the high demand for acetic acid, thereby increasing kitasamycin titers.

Effects of ethyl acetate on the specific activity of enzymes

To further determine the mechanism underlying high kitasamycin production, the specific activities of AS, AK, and APT were investigated in both conditions. AS and AK coupled with acyl-phosphotransferase are two enzymatic systems that supply acetyl-CoA for macrolide lactonic ring formation, leading to kitasamycin biosynthesis (KHAOUA et al., 1992). As shown in Figure 7(a), the specific activity of AS with ethyl acetate addition reached a maximum of 17.7 U/mg at 47 h. The control showed a higher specific activity of 15.2 U/mg at 47 h. Subsequently, acetyl-CoA activity in both cases declined rapidly, and from 78 to 112 h, it remained relatively constant until the termination of fermentation. These results indicate that AS was primarily important during the early phase of kitasamycin biosynthesis.

Compared with the control, two peaks of AK specific activity emerged later in the fermentation period with the addition of ethyl acetate. There was no difference in AK specific activity between the ethyl acetate conditions and the control condition with respect to the first peak. However, the second peak for the ethyl acetate condition showed an AK specific activity of 765 U/mg protein, which was significantly higher than that of the control (Figure 7(b)), indicating that more acetate was phosphorylated to generate acetyl-phosphate, which provided more substrates for the synthesis of acetyl-CoA.

The APT-specific activity curves for the two conditions showed a similar trend, with two peaks in the fermentation process. A peak of 22.4 U/mg in the APT specific activity emerged at 64 h and a second peak of 25.3 U/mg emerged at 88 h for the ethyl acetate treatment; both peaks were higher than those of the control (Figure 7(c)). These results indicate that the effect of APT was more remarkable in the middle and later periods, in which more acetyl-CoA was present to promote the biosynthesis of kitasamycin.

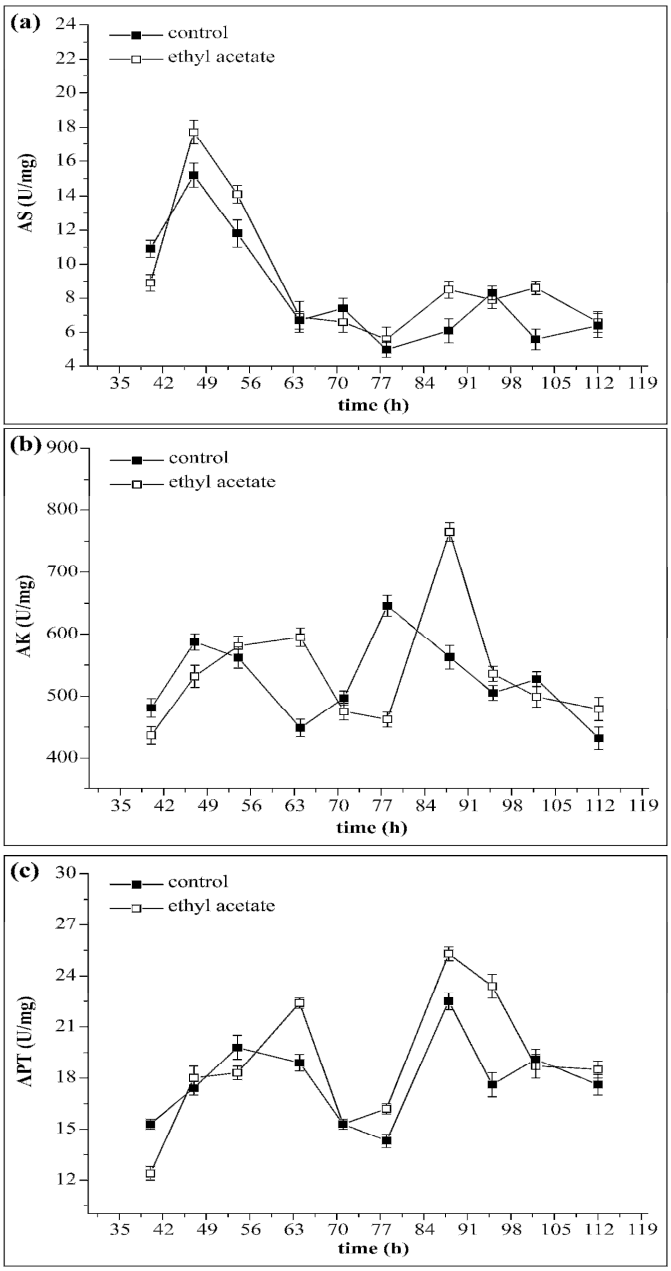

Figure 7. Influence of ethyl acetate on the specific activity of kitasamycin acetyl-CoA synthetase (a), acetate kinase (b), and acyl-phosphotransferase (c). Ethyl acetate (□) and control (■).

DISCUSSION

Ethyl acetate was more effective than sodium acetate with respect to the enhancement of kitasamycin titers. When ethyl acetate was added, the kitasamycin titer increased by 21% and the A_5 content increased by 5.1% in a 15-L fermentor. The A_5 fraction had the highest content and had extremely high antimicrobial activity among all main components of kitasamycin. The A_5 component increased with supplementation of ethyl acetate, which resulted in remarkable advantages in the culture broth. However, the components of kitasamycin are various and complex, and the detailed pathways of A_5 to A_4 and relevant acetylation genes are not clear. Further studies should thus identify acetylation inhibitors of C-3 of the 16-membered lactone ring to optimize kitasamycin production. Inhibition of the expression of relevant acetylation genes for A_5 to A_4 might also enhance kitasamycin production (ZHENG; GAO, 2015).

Acetic acid and butyric acid are precursors for kitasamycin biosynthesis (OMURA et al., 1975). The addition of ethyl acetate not only supplied the precursors for kitasamycin biosynthesis but also induced the synthesis of AS, AK and APT, which activated short-chain fatty acids. The presumed

function of AS, AK and APT was to increase the availability of acylthioesters C2, C3, C4 for aglycone synthesis, especially the increased AK and APT were made during the antibiotic production phase (KHAOUA et al., 1992), it was known from kitasamycin structure that aglycone was necessary parts for kitasamycin (OMURA et al., 1975). Our results show that AS, AK, and APT were all activated and enhanced the production of kitasamycin during the synthesis phase. As can be seen, the addition of 0.48% ethyl acetate as precursors in the medium at the beginning of cultivation was favorable to improve kitasamycin biosynthesis.

CONCLUSION

Overall, using ethyl acetate as precursor was a new strategy in the field of antibiotic biosynthesis. This slow decomposition of ethyl acetate has favorable practical value with respect to precursor addition and reduces the inhibitory effects of excessive precursors.

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RESUMO: Para melhorar a biossíntese de kitasamicina por Streptomyces kitasatoensis Z-7, a adição de dois precursores, acetato de sódio e acetato de etila, ao meio de fermentação foi avaliada. O acetato de etila foi o precursor mais efetivo em comparação com as condições de controle; Em um fermentador de 15 L, o título de kitasamicina foi 21% maior quando 0,48% de acetato de etila foi adicionado em comparação com as condições de controle. O conteúdo do componente A5 aumentou 5,1%, e o conteúdo A4 diminuiu ligeiramente em comparação com o do controle. Durante a síntese de kitasamicina, as concentrações intracelulares e extracelulares de ácido acético foram maiores para S. kitasatoensis Z-7 suplementado com acetato de etila do que para a cepa não suplementada, e as atividades de acil-CoA sintetases, acil-fosfotransferases e acil-cinases também foram significativamente aumentadas, sugerindo que níveis aumentados de acetil-CoA podem explicar o alto título de kitasamicina. Esses achados podem melhorar a produção em escala industrial da kitasamicina para uso clínico, e a adição de 0,48% de acetato de etila como precursores no meio no início do cultivo foi um novo método para mitigar a influência negativa no crescimento celular do excesso de precursor.

PALAVRAS-CHAVES: *Streptomyces kitasatoensis*. Acetato etílico. Fermentação. Componente. Kitasamicina.

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