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EFFECT OF BRANCHED-CHAIN AMINO ACIDS, VALINE, ISOLEUCINE AND LEUCINE ON THE BIOSYTHESIS OF BITESPIRAMYCIN 4''-*O*-ACYLSPIRAMYCINS

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ABSTRACT

Bitespiramycin, a group of 4''-*O*-acylated spiramycins with 4''-*O*-isovalerylspiramycins as the major components, was produced by recombinant spiramycin-producing strain *Streptomyces spiramyceticus* harboring a 4''-*O*-acyltransferase gene. The experiment was initially performed in synthetic medium with 0.5 g l^{-1} Valine, Isoleucine or Leucine feeding at 36 h cultivation. When valine was fed, the biological titer of bitespiramycin was 45.3% higher than that of the control group, but the relative content of total isovalerylspiramycin components decreased by 22.5%. In the case of ilecine, the biological titer of bitespiramycin and the total isovalerylspiramycins alone were 85% and 72.1% of the control group, respectively. In contrast, the relative content of other acylated spiramycins increased by 54.41%. However, leucine feeding increased the relative content of total isovalerylspiramycins by 41.9% while the biological titer of bitespiramycin was nearly equal to that of the control group. The improvement effect of leucine on the biosynthesis of isovalerylspiramycins was further confirmed by feeding of 2.0 g l^{-1} leucine to the culture with complex medium. After batch feeding with a total amount of 2.0 g l^{-1} leucine to the culture from 70 h to 90 h, the biological titer of bitespiramycin was almost unreduced, and the final relative content of total isovalerylspiramycins increased from 31.1% to 46.9%.

Key words: bitespiramycin, valine, leucine, isoleucine, biosynthesis

INTRODUCTION

Bitespiramycin is a group of 4''-*O*-acylated spiramycins with 4''-*O*-isovalerylspiramycins as the major components (29). Although bitespiramycin has similar antibiotic activity with spiramycin *in vitro*, the former has superior pharmacokinetic properties compared with spiramycin *in vivo*. These properties are: quick oral absorption, high tissue permeability, wide distribution in the body. Long *in vivo* half-life owing to its high lipophilicity. Bitespiramycin is mainly used for the treatment of Gram-positive bacteria infectious diseases, such as upper respiratory tract and urinary system infections (19, 20, 21).

Bitespiramycin is produced by recombinant *Streptomyces spiramyceticus* F21 that harbores a 4''-*O*-acyltransferase gene from *S. mycarofaciens* 1748 (18). There are a total of 14 components in bitespiramycin owing to the relatively broad substrate specificity of the acyltransferase (10). Thus, during

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the fermentation process of bitespiramycin, the enhancement of the biosynthesis of 4"-*O*-isovalerylspiramycins is a distinctly significant step, as these are the main components responsible for the new pharmacokinetic properties of bitespiramycin.

Amino acids can be used as carbon or nitrogen source in the fermentation of antibiotics. Some amino acids such as valine (Val), isoleucine (Ile) or leucine (Leu) are precursors of some secondary metabolites, and the metabolites derived from them such as acetyl-CoA, propionyl-CoA, isovaleryl-CoA and butyryl-CoA are precursors of the biosynthesis of macrolide antibiotics (the biosynthesis pathway of bitespiramycin is illustrated in Fig. 1) (8,14,17,26,28).

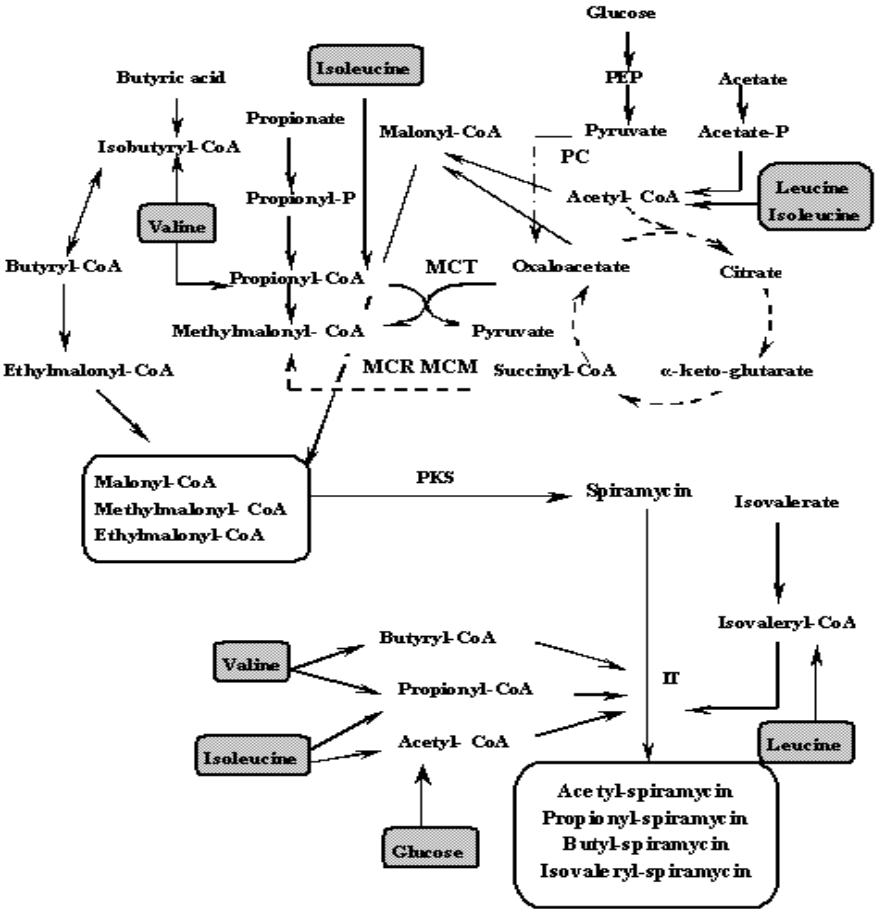


Figure 1. Central metabolic map of *S. spiramyceticus*.

MCT: Methylmalonyl- CoA Transcarboxylase; MCR: Methylmalonyl- CoA Racemase; MCM: Methylmalonyl-CoA Mutase; PC: Pyruvate Carboxylase; IT: Isovaleryl-CoA Transferase; PKS:Polyketide synthetase; CS: citrate synthase.

This paper focuses mainly on the effect of the supplementation of branched chain amino acids on the composition and biological titer of bitespiramycin. We evaluate whether supplementation with Val, Leu or Ile in the culture with synthetic media have different effects on the composition and biological titer of bitespiramycin, and discuss the mechanism of the three amino acids by analyzing the process data of organic acids and related enzyme activities. A comparison study of single and batch supplementation of Leu into the fermentation process with complex media is also made to verify the results derived from the culture with a defined medium.

MATERIALS AND METHODS

Microorganism and cultivation conditions

In all the experiments, a gene-engineered strain WSJ-1-195 constructed by integrating isovaleryltransferase gene from a carbomycin-producing strain *S. mycarofaciens* 1748 into a spiramycin-producing strain *Streptomyces spiramyceticus* F21 was used (18).

The primary seed culture and secondary seed culture used the same seed medium containing (g l^{-1}) glucose 1 g, corn starch 3 g, soybean meal 2 g, NaCl 0.4 g and $CaCO_3$ 0.5 g.

The synthetic medium contained (g l^{-1}): dextrin 50, NH_4NO_3 $2KH_2PO_4$ $0.65CoCl_2$ $0.0005MgSO_4$ 5.5NaCl 10 and $CaCO_3$ 5.

The fermentation medium contained (g l^{-1}): glucose 50, fish meal 23, starch 60, NH_4NO_3 6□KH_2PO_4 0.65 $MgSO_4$ 5.5 NaCl 10 and $CaCO_3$ 5.

The spore solution of recombinant Streptomyces spiramyceticus WSJ-1-195 in 20% glycerol was stored in -80 and the count was more than 5×10^6 spore ml^{-1}. For seed cultivation, one ml spore solution was inoculated into a primary 250-ml shake flask containing 50 ml seed medium. After 48 h of incubation at 28, 10 ml of primary seed culture was inoculated into a secondary 500-ml shake flask containing 100 ml seed medium. After 24 h of incubation at 28, 4 ml of secondary seed culture was inoculated into a 500-ml fermentation flask containing 50 ml fermentation medium, which was shaken at 220 revolutions per minute and 28 for 96 h of fermentation.

For the feeding method of the three amino acids, 0.5 g l^{-1} Val, Leu or Ile was treated with ultraviolet radiation for 12 h, and then the sterilized Val, Leu or Ile was supplemented into different fermentation flasks to obtain the final defined concentration of each of the amino acids.

Feeding method of the Leu in the culture with complex medium: (1) 2.0 g l^{-1} Leu was supplemented at one time at 72 h of fermentation; (2) at 72 h of fermentation, a total of 2.0 g l^{-1} Leu was fed at an interval of 6h (72 h, 80 h and 90 h of fermentation).

Total sugar, ammonium ion, dry cell weight and the biological titer determination

Total sugar concentrations in the medium were determined using dinitrosalicylic acid (DNS) method (15). The supernatant of the fermentation broth obtained after centrifugation was boiled with 6 N HCl for 10 min and neutralized by equal volumes of 6 N NaOH. The hydrolysate was analyzed using dinitrosalicylic acid (DNS) method. The ammonium ion in the defined medium was determined using a method described by Weatherburn (25), while NH_2-N in the complex medium was analyzed by Foreman's formaldehyde titration method. Determination of dry cell weight (DCW) was performed using the method described by Lebrihi (12). Biological titer was estimated by the conventional disc method using Bacillus pumilus as test microorganism and acetylspiramycin as the standard (1).

Components and organic acids measurements by HPLC

The fermentation broth of bitespiramycin was centrifuged at 3000 revolutions per minute for 10 min and the supernatant was stored at -20 for further use. The supernatant was centrifuged again at 12,000 revolutions per minite for 3 min and passed through the filtration membrane (0.45 μm) for the measurement of bitespiramycin components, acetic acid, propionic acid, butyric acid and isovaleric acid by high-performance liquid chromatography (HPLC) system (HP1100, Agilent Tech, USA).

The determination of bitespiramycin components was conducted using a C_{18} column (150 mm ×4.6 mm, 5 μm) at 25. The mobile phase was the mixture solution of 0.083 mol L^{-1} phosphoric acid water solution and methanol (53:47) at a flow rate of 1 ml min^{-1} and detected at 234 nm. The standard of bitespiramycin was provided by the Institute of Medicinal Biotechnology (IMB) of the Chinese Academy of Medical Sciences.

The determination of acetic acid and propionic acid in the broth was conducted using a C_8 column (250×4.6 mm, 5 μm) at 30. The mobile phase was the the mixture solution of 0.01 mol l^{-1} phosphoric acid water solution (adjusted to pH 2.3±0.1 with NaH_2PO_4) and methanol (98.5:1.5) at the flow rate of 1ml min^{-1}, and detected at 210 nm.

The determination of butyric acid and isovaleric acid in the broth was conducted using a C_{18} column (150×4.6 mm, 5 μm) at 30. The mobile phase was the the mixture solution of 0.01 mol l^{-1} phosphoric acid water solution (adjusted to pH 2.3±0.1 with NaH_2PO_4) and methanol (60:40) at the flow rate of 1ml min^{-1}, and detected at 210 nm. The standard of acetic acid, propionic acid, butyric acid and isovaleric acid was purchased from Sigma.

Determination of intracellular pyruvate acid by HPLC

The mycelia were centrifuged at 4 and 4000 r min^{-1} for 5 min, washed with 0.88% (m v^{-1}) normal saline twice, and then resuspended in 5 ml of 0.01 mol l^{-1} phosphoric acid water solution. The mixture solution was disrupted with French Pressure (Xinzhi, Shanghai) at 200 Mpa, after which the lysate was centrifuged at 4 and 12000 r min^{-1} for 30 min. The supernatant was used for determining intracellular pyruvate acid. The measurement conditions for HPLC were the same as those for the extracellular acetic acid and propionic acid described above.

The activities of pyruvate carboxylase (PC) and citrate synthase (CS) measurement

The mycelia were collected by freeze centrifugation at 4 and 4000 r min^{-1} for 5 min, washed with 10% (m v^{-1}) sucrose solution for two times, and then resuspended in 5 ml of 0.05 mol l^{-1} Tris-HCl buffer solution (pH7.4) containing 20% glycerol. The mixture solution was disrupted with French Pressure (Xinzhi, Shanghai) at 200 Mpa, after which the lysate was centrifuged at 4 and 12000 r min^{-1} for 30 min. The supernatant was used for the assay of enzymatic activity.

The activities of PC and CS were measured according to the reference papers (6, 13). One unit (U) of a specific enzyme was defined as 1 μmol of catalysate produced per min. The protein content was determined by Coomassie brilliant blue G250 staining method, and calculated according to the standard curve of bovine serum albumin.

RESULTS

The effects of Val, Ile or Leu on the yield and the composition of bitespiramycin

The supplementation of Val, Ile or Leu during the fermentation process of bitespiramycin influenced the growth of microorganism and the biosynthesis of bitespiramycin. After supplementation with amino acids, the biomass was a little lower than that of the control group, the increase in the titer of bitespiramycin was nearly stopped (48 h), and the inter-group differences increased with the ongoing fermentation process. In the culture supplemented with Val, the titer of bitespiramycin increased significantly after 12 h adaptation, higher than that of the control group by 45.3% at the end of fermentation. As for Ile, the titer increased slowly, and towards the end of the fermentation process, was only 85% of the control group. For Leu, the titer of bitespiramycin was almost equal to that of the control group.

Table 1 shows that after supplementation with Leu, the contents of isovalerylspiramycin II and isovalerylspiramycin III increased to 141.2% and 150.4% of the control group, respectively, and the content of total isovalerylspiramycins increased by 42.3%. The content of spiramycins decreased significantly, and the contents of other minor acylated components decreased as well.

After supplementation with Ile, the relative content of propionylspiramycin III, acetylspiramycin III and isobutyrylspiramycin II increased by 126.1%, 53% and 296%, respectively, while the contents of spiramycins and isovalerylspiramycins decreased to only 66.2% and 72.1% of that of the control group, respectively.

After supplementation with Val, the relative content of spiramycins and butyrylspiramycin increased, the most

apparent changes being the increase of the relative content of spiramycin I and spiramycin III. The relative contents of total isovalerylspiramycins and other acylated components decreased by 22.5% and 27.5% while the relative contents of total spiramycins increased by 54.5%, respectively.

Table 1. Effects of Val, Ile or Leu on the components of bitespiramycin

Component	Content (%)			
	Valine	**Isoleucine**	**Leucine**	**Control**
Isovalerylspiramycin	8.22±0.21	3.26±0.22	2.78±0.12	2.92±0.11
Isovalerylspiramycin	5.61±0.28	5.90±0.25	14.12±0.67	10.00±0.54
IsovalerylspiramycinIII	10.25±0.86	13.25±0.71	27.34±0.92	18.18±0.84
Total isovalerylspiramycins	24.08±0.45	22.41±0.45	44.24±0.64	31.09±0.75
Acetylspiramycin III	8.30±0.42	18.40±0.63	8.71±0.54	12.02±0.43
Propionylspiramycin	2.68±0.12	3.65±0.27	3.12±0.14	4.06±0.12
Propionylspiramycin III	5.57±0.26	23.67±0.65	9.87±0.25	10.47±0.24
Butylspiramycin	6.79±0.23	8.69±0.65	2.16±0.18	2.17±0.18
Other acyl-Spiramycin	23.34±0.37	54.41±1.54	23.86±0.95	28.72±0.83
Total spiramycin	48.92±0.69	20.97±0.52	23.95±0.89	31.67±0.66

A total of 0.5 g l^{-1} Val, Ile or Leu was fed at 36 h of bitespiramycin fermentation. The culture without amino acid feedings was used as control. The content of other acylated spiramycins was calculated by (100-Total spiramycins-Total isovalerylspiramycins).

After supplementation with Val, the relative content of spiramycins and butyrylspiramycin increased, the most apparent changes being the increase of the relative content of spiramycin I and spiramycin III. The relative contents of total isovalerylspiramycins and other acylated components decreased by 22.5% and 27.5% while the relative contents of total spiramycins increased by 54.5%, respectively.

The effects of Val, Ile or Leu on glycometabolism

Fig. 2A shows that the pH of the culture broth did not increase after supplementation with Val, but was 0.1-0.2 pH higher than that of the control group for Leu and Ile. Fig. 2B shows that the concentrations of ammonium ion in the fermentation broth 12 h after supplementation with Ile and Leu were 4.0 mmol l^{-1} and 3.5 mmol l^{-1}, respectively, which eventually decreased slightly. However, after supplementation with Val, the concentration of ammonium ion in the fermentation broth became almost the same as that of the control group, i.e., maintained at about 1.0 mmol l^{-1}. These results indicate that *Streptomyces spiramyceticus* utilized and metabolized the three amino acids in different ways.

Fig. 3A shows that after supplementation with Val, the glucose consumption rate increased, the residual total sugar in the fermentation broth decreasing rapidly from 23.4 g l^{-1} to 11.4 g l^{-1} from 48 h to 60 h, at this phase the glucose consumption rate reached the highest level of 1.0 g $l^{-1}h^{-1}$. After supplementation with Ile, the glucose consumption rate was only 0.1-0.2 g $l^{-1}h^{-1}$, which was apparently lower than that of the control group, whereas after supplementation with Leu, the glucose consumption rate was 0.3-0.5 g $l^{-1}h^{-1}$, which was similar to that of the control group. It is obvious that the glucose consumption rate is closely correlated to the titer of bitespiramycin.

It is shown in Fig. 3B that after supplemention with Val, Ile or Leu, the concentration of intracellular pyruvic acid was in the range of 2.7-4.3 mmol l^{-1}, 0.5-1.0 mmol l^{-1}, and 0.9-2.4 mmol l^{-1}, respectively, which was higher, lower and roughly equal to that of the control group (1.0-2.3 mmol l^{-1}). This implies that the concentration of intracellular pyruvic acid is reflective of the utilization status of glucose to some extent (5).

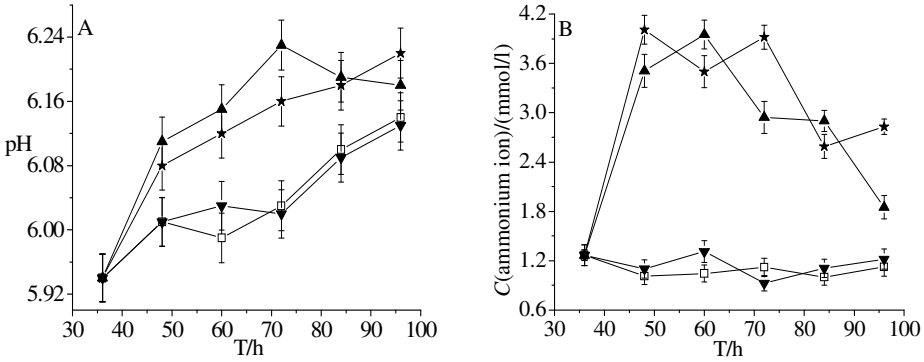


Figure 2. Profiles of pH and ammonium concentration in bitespiramycin fermentation with 0.5 g l^{-1} Val, Ile or Leu supplemented at 36 h.

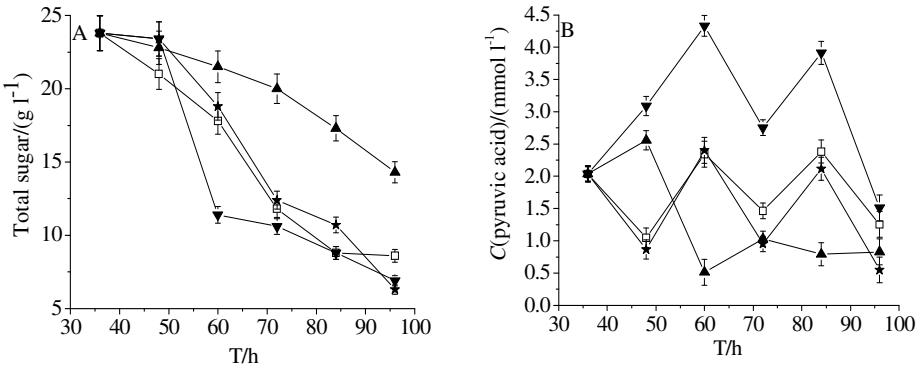


Figure 3. Profiles of total sugar and intracellular pyruvic acid concentration in bitespiramycin fermentation with 0.5 g l^{-1} Val, Ile or Leu supplemented at 36 h.

Fig. 4 shows the change of activities of PC and CS after supplementation with the three amino acids. PC converts pyruvic acid into oxaloacetate, while CS is the key enzyme of TCA cycle, and the activity of CS may reflect the intensity of TCA cycle (11), which is directly correlated to the primary metabolism of microorganisms. As displayed in Fig. 4A, at 48 h and 60 h, the activity of PC in the culture with Val increased to 120% and 195.5% of that of the control group,

respectively. After supplementation with Ile, the activity of PC decreased to 62.6% and 72.5%, respectively. For Leu, the activity of PC did not manifest obvious changes. As shown in Fig. 4B, at 48 h and 60 h, the activity of CS in the culture with Val decreased to 42% and 23.2% of that of the control group, respectively. After supplementation with Ile, the activity of CS decreased to 47.3% and 51.7%, respectively. For Leu, the activity of CS did not manifest obvious changes.

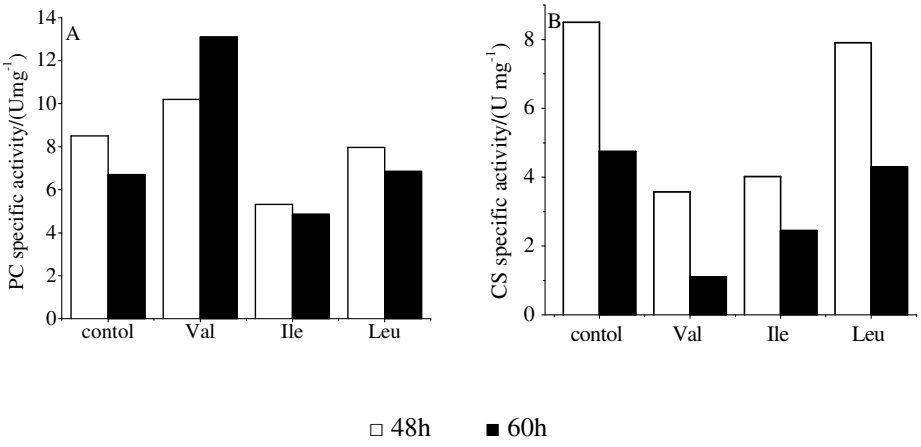


Figure 4. Effect of 0.5 g l^{-1} Val, Ile or Leu supplemented at 36 h on the pyruvate carboxylase (PC) and citrate synthase (CS) activities at 48 h and 60 h, respectively

The above results indicate that after supplementation with Val, both the activity of PC and concentration of intracellular pyruvic acid increased substantially. Intracellular pyruvic acid is mainly converted into acetyl-CoA and oxaloacetic acid (which is one of the main substrates of the biosynthesis of malonyl-CoA and methylmalonyl-CoA). The increase in the activity of PC and the concentration of intracellular pyruvic acid may have resulted in more sufficient direct precursors pool for the biosynthesis of macro ring, thus the increase in the titer of bitespiramycin.

After supplementation with Ile, both PC and CS activity decreased, and the concentration of intracellular pyruvic acid decreased at the same time, implying that both the primary and secondary metabolism of the biomass became weaker. This resulted in the reduction of biomass and titer of bitespiramycin. However, after supplementation with Leu, activities of both PC and CS were almost the same as those of the control group, resulting in the absence of notable decreases in bitespiramycin titers, but a great increase in the amount of total isovalerylspiramycin components (especially isovalerylspiramycin III) was observed.

The effects of Val, Ile or Leu on the extracellular short-chain fatty acids

As shown in Fig. 5A, after supplementation with Val and Ile, the concentration of extracellular acetic acid increased rapidly and displayed a constant upward trend. But after supplementation with Leu, the concentration of extracellular acetic acid did not obviously change, and the accumulation of extracellular acetic acid did not occur until 72 h later.

A comparison of the changes in extracellular propionic acid was shown in Fig. 5B. The concentration of extracellular propionic acid was almost equal to that of the control group after supplementation with Leu while it was higher than that of the control after Val or Ile supplementation.

The change trend of isovaleric acid is depicted in Fig. 5C. After supplementation with Leu, the concentration of extracellular isovaleric acid increased rapidly to the highest

level at 1.66 mol l^{-1} at 84 h, and then decreased to 0.9 mmol l^{-1}. But after supplementation with Val or Ile, the concentration of extracellular isovaleric acid became slightly lower than that of the control group.

The behavior of extracellular butyric acid is shown in Fig. 5D. After supplementation with Val, the concentration of extracellular butyric acid increased to 14 mmol l^{-1} at 48 h, then decreased quickly, and reached a plateau within the range of 6.2-8.6 mmol l^{-1}. After supplementation with Ile, the concentration of extracellular butyric acid became consistently higher than that of the control group, and was maintained within the range of 13-19 mmol l^{-1}. The change in the trend of the concentration of extracellular butyric acid after supplementation with Leu was similar to that of the control group.

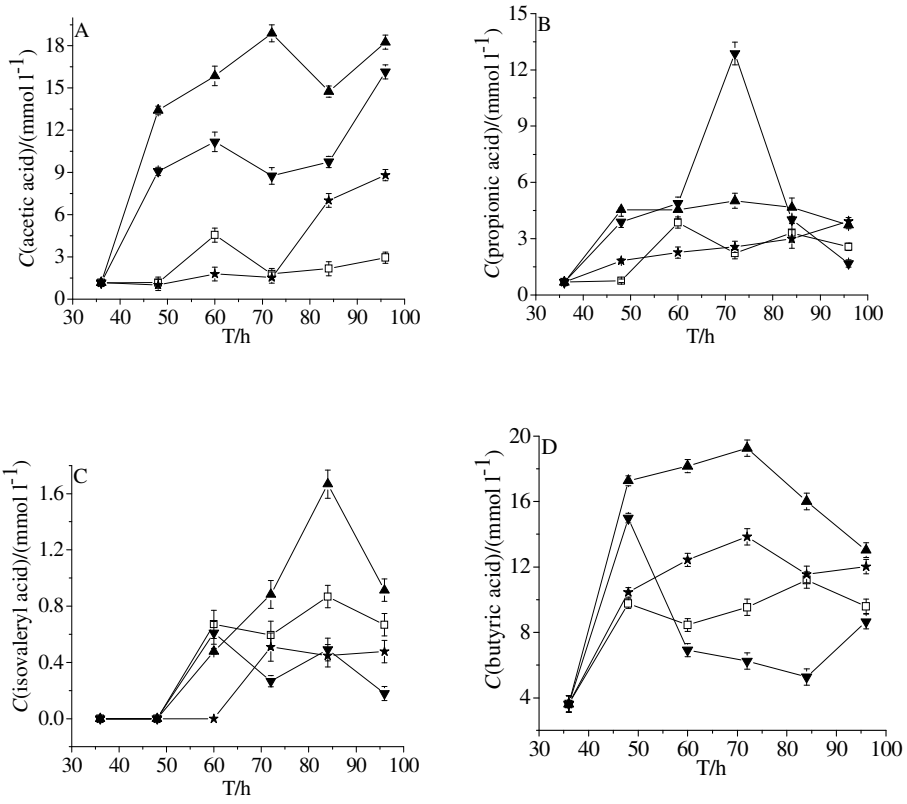


Figure 5. Profile of short-chain fatty acid concentrations in bitespiramycin fermentation with 0.5 g l^{-1} Val, Ile or Leu supplemented at 36 h

After supplementation with Val, the glucose consumption rate, acetic acid, and propionic acid increased. The increase in the activity of PC showed that more intracellular pyruvic acid can be converted into oxaloacetic acid. Oxaloacetic acid is involved in the biosynthesis of malonyl-CoA and methylmalonyl-CoA, which provide the direct precursors for the biosynthesis of lactone ring, thus improving the biological titer of bitespiramycin.

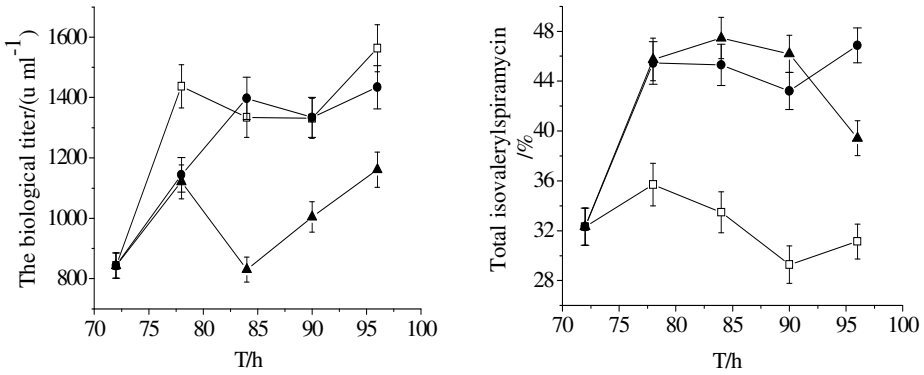
After supplementation with Leu, butyric acid accumulated slightly, while the change trends in the concentrations of acetic acid and propionic acid were not significantly affected. The biological titer of bitespiramycin

did not obviously vary, either. The relative content of isovalerylspiramycin components increased significantly, indicating that isovaleryl-CoA produced by the catabolism of Leu can be introduced at the 4 position of spiramycins.

Effecst of Leu feeding on the composition of bitespiramycin in the culture with complex medium

Fig. 6 shows that both single and multiple batch supplements of Leu obviously improved the composition of bitespiramycin. At 96 h post inoculation (at the end of fermentation), the content of total isovalerylspiramycins was 46.87% and 39.42% by batch or single modes compared to 31.14% in the culture without Leu. After single supplementation with Leu, the biological titer of bitespiramycin was 74% compared to that of the control group. But the biological titer of bitespiramycin in the group with batch supplementation with Leu was similar to that of the control group, reaching 91% of the control at the end of fermentation.

The above result shows that batch supplementation with Leu is superior to single supplementation with Leu for the improvement of the composition of bitespiramycin. Specifically, batch supplementation with Leu compensated for the loss of the biological titer of bitespiramycin that occurred after single supplementation with Leu.



□□—control —▲— Leu fed by single addition —●— Leu fed by multiple batch addition

Figure 6. Effect of Leu feeding mode on bitespiramycin fermentation with complex medium. Single addition: 2.0 g l^{-1} Leu was supplemented at one time at 72 h of fermentation. Multiple batch addition: at 72 h of fermentation, a total of 2.0 g l^{-1} Leu was fed at an interval of 6 h (72 h, 80 h and 90 h of fermentation).

Fig. 7 shows that single supplementation with 2.0 g l^{-1} Leu at 72 h of fermentation slowed down the utilization of sugar, with the glucose consumption rate being 1.1 g $l^{-1}h^{-1}$ from 72 h to 84 h, lower than that (1.6 g $l^{-1}h^{-1}$) of the control group. In addition, the pH level and amino nitrogen increased. But after batch supplementation of Leu, the utilization profile of total sugar became similar to that of the control group. The amino nitrogen did not accumulate, and the pH became higher than that of the control group but lower than that of the group with single supplementation of Leu. The pyruvic acid concentration after single and multiple batch supplementation of Leu increased from 4.7 mmol l^{-1} to 9.8 mmol l^{-1} and 12.82 mmol l^{-1}, respectively, but the pyruvic acid concentration of the control group increased to 16.23 mmol l^{-1}.

By comparing the metabolism parameter changes after single and batch supplementation with Leu, it has been found that after single supplementation with Leu, the utilization of

glucose was inhibited to some extent, the amino nitrogen obviously increased, and the pH had a corresponding sharp increase. After batch supplementation with Leu, the utilization rate of glucose and amino nitrogen did not change greatly. The reason may be that after single supplementation with Leu, large amount of ammonium ion produced by deamination of Leu brought about a notable increase in amino nitrogen, resulting in the sharp change in pH and the inhibition of the utilization of glucose, thus the decrease in biological titer.

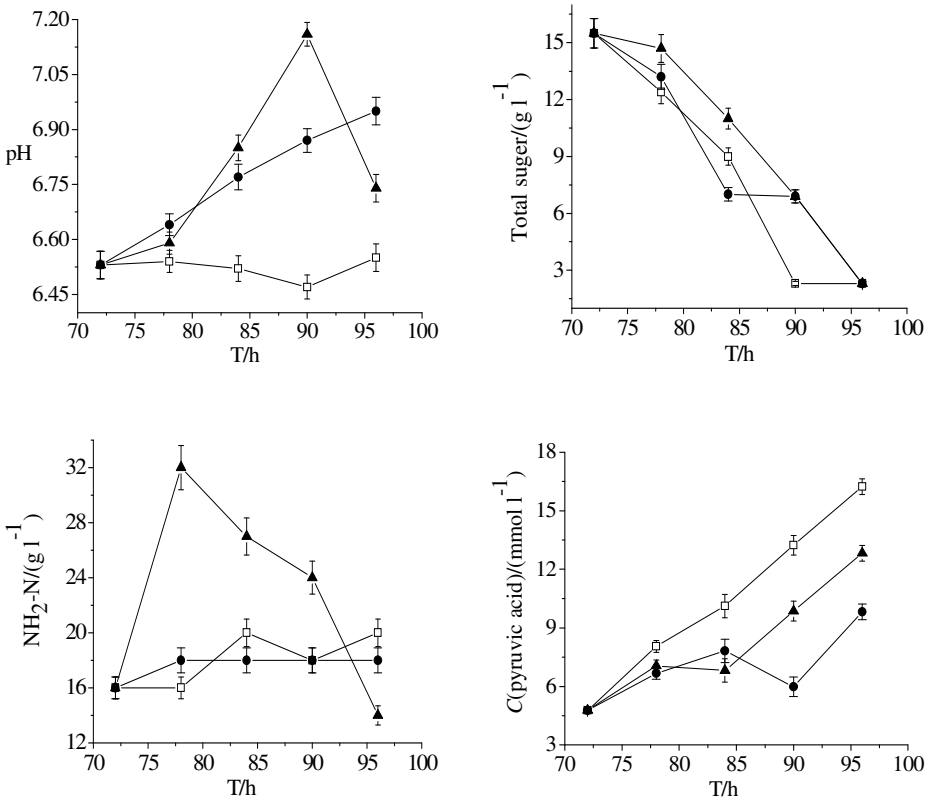


Figure 7. Profiles of pH, total sugar, NH_2-N and intracellular pyruvate acid during bitespiramycin fermentation with different leucine feeding modes. Single addition: 2.0 g l^{-1} Leu was supplemented once at 72 h of fermentation. Multiple batch addition: at 72 h of fermentation, a total of 2.0 g l^{-1} Leu was fed at an interval of 6 h (72 h, 80 h and 90 h of fermentation).

DISCUSSION

Short-chain organic acids such as acetic acid, propionic acid and butyric acid, which are essential in the biosynthesis of lactone ring of macrolide antibiotics, are produced mainly by the EMP pathway, TCA cycle, and metabolism of fatty acids and amino acids. They play an important role in the primary and secondary metabolism of microorganism producing macrolide antibiotics, i.e., acting as a bridge between primary and secondary metabolisms. The results of this study indicate that the supplement of Val, Ile or Leu not only provided direct precursors for the biosynthesis of bitespiramycin, but also had various effects on the metabolism of microorganisms.

The microorganism has different utilization patterns for each of the three amino acids, resulting in different effects on glycometabolism. It was observed that the pH of the culture medium did not increase after supplementation with Val, while it was higher than that of the control group after supplementation with Leu or Ile, and the concentration of ammonium ion in the fermentation broth had a marked increase after supplementation with Ile or Leu (Fig. 3A). During the metabolism process of Leu and Ile, the amino group was excreted in the culture broth after deamination reaction, and the remaining carbon skeleton was directly used by the bacteria cells. However, after supplementation with Val the concentration of ammonium ion in the fermentation broth became almost equal to that of the control group. A similar phenomenon was also observed by Voelker *et al* (23). They proposed that the amino group from the adsorbed Val was further used by mycerlia, and the remaining carbon skeleton, i.e., α-ketoisovaleric acid, was temporarily excreted out and then re-adsorbed into the mycerlia for further use after the intracellular ammonium ions were exhausted.

It is reported that the transamination reaction of Val activates the enzymatic activity of glycometabolism (24), resulting in the increase of the glucose consumption rate. Meanwhile, the amino group transamination reaction of Val provides a N atom for the glycoside side chain of lactone ring of spiramycin (4). The change rule of the activity of PC and the content of intracellular pyruvic acid imply that supplementation of Val promotes the conversion of intracellular pyruvic acid into oxaloacetic acid, which is involved in the biosynthesis of malonyl-CoA and methylmalonyl-CoA. The above reaction not only provides direct precursors for the biosynthesis of lactone ring but also relieves the substrate inhibition effect, which converts redundant short-chain fatty acids such as acetyl-CoA and propionyl-CoA into malonyl-CoA and methylmalonyl-CoA via the catalysis of acyl carboxylases (4). Thus the biosynthesis of macro ring is promoted and the biological titer of bitespiramycin increases. In addition, after the supplementation of Val, the 4”-*O*-butyryl group increased to some extent. Vezina *et al* (22) also observed that the biosynthesis of 4”-*O*-butyrylated components of kitasamycin (A4/A5) increased after Val feeding.

After supplementation of Ile, the glucose consumption rate and the biological titer of bitespiramycin decreased; however, the relative content of propionylspiramycin III, acetylspiramycin II and isobutyrylspiramycin increased significantly. Zhang *et al* (27) observed that because of the substrate inhibition effect of Ile on methylmalonyl-CoA racemase and allomerase, succinyl-CoA produced by TCA cycle was mainly excreted to the broth in the form of succinic acid, and could not be converted to the direct precursors of lactone ring. Ivan *et al* (9) demonstrated that the re-adsorption and reutilization of acetic acid and propionic acid were positively correlated to the activity of propionyl-CoA carboxylase. Therefore, although the carbon skeleton of Ile was adsorbed into the bacteria cells and then catabolized to produce abundant acetyl-CoA and propionyl-CoA, these acyl CoAs may not be converted to the direct precursors of lactone ring to produce bitespiramycin. In contrast, after supplementation with Ile, the relative content of propionylspiramycin III, acetylspiramycin II and isobutyrylspiramycin increased significantly, which means that short-chain organic acid-CoAs from Ile catabolism were transferred to the 4-OH of mycarose and the 3-OH of macro ring (16).

The supplementation of Leu to the defined medium had no obvious effects on the biological titer of bitespiramycin, but resulted in the remarkable increase in the content of isovalerylspiramycins, indicating that isovaleryl-CoA derived from Leu can be used as the precursor of the 4□side chain of spiramycins. Vezina *et al* (22) also showed that the isovaleryl group of the A1/A3 component of kitasamycin came from leucine, and it was demonstrated that the isovaleryl side chain of carbomycin also came from Leu (3,7). Leu catabolism can also provide an isovaleryl side chain for the biosynthesis of 3-*O*-acetyl-4”-*O*-isovaleryltylosin, when a tylosin-producing strain *Streptomyces fradine* was transformed with macrolide 4”-*O*-acyltransferase gene (2). The improvement effect of

Leu on the biosynthesis of 4”-*O*-isovalerylspiramycins was further confirmed by supplementation with 2.0 g l^{-1} Leu to the complex media, and the result shows that after batch supplementation of Leu the relative content of total isovalerylspiramycins increased from 31.4% to 46.9%. This indicates that even with cultivation in complex media, the supply of isovaleryl-CoA is still a restriction factor significantly influencing isovaleryl spiramycin biosynthesis.

Although Val, Ile and Leu all belong to the group of branched-chain amino acids, they have different catabolism pathways within the microorganism, resulting in different effects on primary and secondary metabolism, and providing the possibility to regulate the composition and biological titer of bitespiramycin, a product of combinational biosynthesis, during the fermentation process.

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