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Effect of amino acids containing sulfur on dithiopyrrolone antibiotic productions by *Saccharothrix algeriensis* NRRL B-24137

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Keywords

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Abstract

Aims: To study the effect of sulfur-containing amino acids (L-cysteine, L-cystine, L-methionine and DL-ethionine) on the production of dithiopyrrolone antibiotics by *Saccharothrix algeriensis* NRRL B-24137.

Methods and Results: The production levels of dithiopyrrolones were investigated by using high performance liquid chromatography in a chemically semi-synthetic medium. The production of the studied antibiotics depends upon the nature, concentration and the time of addition of these sources in the culture medium. Both cysteine and cystine favoured the specific productions of dithiopyrrolones; iso-butyryl-pyrrothine (ISP) by cysteine, however butanoyl-pyrrothine, senecioid-pyrrothine and tigloyl-pyrrothine by cystine, when added initially to the culture medium. The maximum specific productions of dithiopyrrolones were observed in the presence of 5 mmol l⁻¹ cystine for thiolutin, 5 mmol l⁻¹ cysteine for ISP, and 10 mmol l⁻¹ cystine for others studied dithiopyrrolones as shown in Fig. 3. The production of these antibiotics was decreased when the concentrations of cysteine and cystine were in excess. All dithiopyrrolone specific productions were strongly inhibited by addition of methionine and ethionine, without inhibition of mycelial growth.

Conclusions: Among all studied amino acids, cystine and cysteine can be used as supplements for improvement the production of dithiopyrrolone antibiotics by *S. algeriensis* NRRL B-24137.

Significance and Impact of the Study: Dithiopyrrolone antibiotics have many important applications for employing them as medicaments, particularly in the treatment of human and animal cancers. In the present work, the influence of containing-sulfur amino acids on dithiopyrrolone antibiotic productions was studied. The obtained results can be employed for the optimization of the culture medium for the dithiopyrrolone productions in higher quantities.

Introduction

Dithiopyrrolones are members of the pyrrothine class of naturally occurring antibiotics that are characterized by the possession of a unique pyrrolinonodithiole nucleus (Celmer and Solomons 1955). They are the first recog-

nized examples of microbiologically active unsaturated lactams (Mizuhara 1962). This group of antibiotics included thiolutin, aureothricin, iso-butyryl-pyrrothine (ISP), butanoyl-pyrrothine (BUP), senecioid-pyrrothine (SEP), tigloyl-pyrrothine (TIP), propionyl-holothin, holomycin, xenorhabdins and thiomarinols (Yamagishi *et al.* 1971;

Okamura *et al.* 1977; McInerney *et al.* 1991; Shiozawa *et al.* 1997; Lamari *et al.* 2002b). Dithiolopyrrolone derivatives were previously identified from the culture broth of several actinomycetes belonging to genus *Streptomyces* (*Streptomyces thioluteus*, *Streptomyces albus*, *Streptomyces cyanoflavus*, *Streptomyces celluloflavus*, *Streptomyces kasugaensis*, *Streptomyces luteoreticuli* and *Streptomyces pimpriana*) (Yamagishi *et al.* 1971), and from other microorganisms such as symbiotic bacterium *Xenorhabdus* (*Xenorhabdus bovienii* and *Xenorhabdus nematophilus*) (McInerney *et al.* 1991; Paik *et al.* 2001), and marine bacterium *Alteromonas rava* (Shiozawa *et al.* 1997). Recently, *Saccharothrix algeriensis* NRRL B-24137 isolated from a south Algerian soil sample has been found to produce at least five dithiolopyrrolone antibiotics containing *N*-acyl derivatives of 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo [4,3-*b*] pyrrole: thiolutin, SEP, TIP, ISP and BUP (Lamari *et al.* 2002a; Zitouni *et al.* 2004) as in Fig. 1. Among actinomycetes, only *S. algeriensis* and *Streptomyces* spp. produced these antibiotics. Senecioid-pyrrothine and TIP antibiotics are produced only by *S. algeriensis* (Lamari *et al.* 2002b). Many authors reported that thiolutin and holomycin could be synthesized chemically (Stachel *et al.* 2003).

Dithiolopyrrolone class of antibiotics has a broad-spectrum activity against a variety of micro-organisms including gram-positive and gram-negative bacteria, yeasts, ameboid parasites and phytopathogenic fungi (Celmer and Solomons 1955; Šturdíková *et al.* 1990; Lamari *et al.* 2002a). This class of antibiotic also exhibited protozoicidal, larvicidal and insecticidal activities which suppressed the growth of *Coccidioides mitis* (Seneca *et al.* 1952; Štur-

díková *et al.* 1990; Webster *et al.* 2002). Arnold *et al.* (1995) of the US National Cancer Institute postulated that dithiolopyrrolone derivatives have a chemopreventive activity in preventing the initiation of cell transformation from premalignant to malignant cancer in the rat tracheal epithelial cells. Dithiolopyrrolones have also strong anticancer activity against several human organs (colon, breast and cervical cancers) (Webster *et al.* 2000; Minamiguchi *et al.* 2001), and possess outstanding anti-allergic action (Stahl *et al.* 1988).

Nitrogen and sulfur sources play a major role in the biosynthesis of many secondary metabolites (Piret and Demain 1988). The biochemical steps involved in dithiolopyrrolones biosyntheses have not been completely elucidated yet.

The present study has been carried out with the objective of increasing the production of dithiolopyrrolones in *S. algeriensis* NRRL B-24137 using various amino acids containing sulfur. This is the first report on the use of amino acids as supplements to investigate dithiolopyrrolone productions by *S. algeriensis*.

Materials and methods

Micro-organism

Saccharothrix algeriensis NRRL B-24137 (=DSM 44581 = SA 233) was used throughout this study. It was maintained at 4°C on ISP 2 (International *Streptomyces* Project 2) medium slant composed of: 4 g glucose D(+) (Fisher Labosi), 10 g malt extract (Difco), 4 g yeast extract (Difco) and 18 g agar (Difco), in 1 l distilled water. The pH of the medium was adjusted to seven using 2 mol l⁻¹ NaOH before autoclaving for 20 min at 121°C.

Culture medium

The basal semi-synthetic medium (SSM), developed in our laboratory, was used for both preculture and production of antibiotics. This medium consisted of: 10 g glucose D(+) (Fisher Labosi), 2 g (NH₄)₂SO₄ (Prolabo), 2 g NaCl (Fisher Labosi), 0.5 g KH₂PO₄ (Acros), 1 g K₂HPO₄ (Acros), 0.2 g MgSO₄·7H₂O (Acros), 5 g CaCO₃ (Prolabo) and 2 g yeast extract (Difco), in 1 l distilled water. The initial pH of the medium was adjusted to seven using 2 mol l⁻¹ NaOH prior to autoclaving. The glucose was autoclaved separately and added aseptically to the medium before inoculation. Amino acids used [L-cysteine (Sigma, St. Louis, MI, USA), L-cystine (Fluka), L-methionine (Sigma) and DL-ethionine (Sigma)], were also autoclaved separately and added at the required concentrations for each experiment. The various concentrations

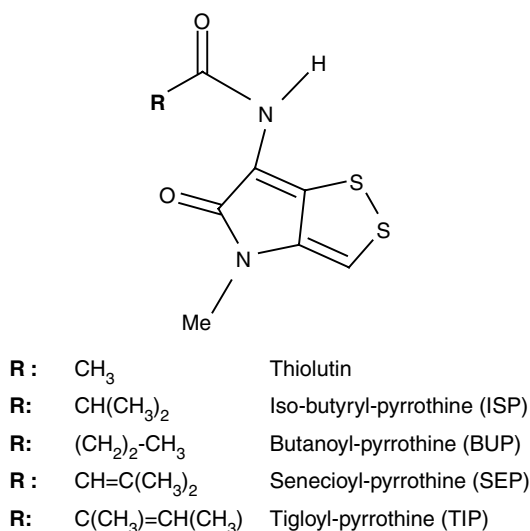


Figure 1 Structure of dithiolopyrrolone antibiotics.

of amino acids added to SSM, are as described subsequently in the text.

Culture conditions

The preculture (250 ml Erlenmeyer flask containing 50 ml of the medium) was incubated for 48 h on a rotary shaker (New Brunswick Scientific Co., NJ, USA) at 260 rev min⁻¹ and 30°C. The preculture was then homogenized and 5 ml was used to inoculate 100 ml of the same medium in 500 ml Erlenmeyer flask, under the same conditions. Each experiment was performed in triplicate.

Measurement of DCW and pH

Culture broth samples were taken every 24 h of fermentation. The pH value of fermentation broth was measured with a pH meter (Consort C 832, Consort, NY, USA). For the dry cell weights (DCWs), we used the method of Pfefferle *et al.* (2000) with slight modifications. The DCWs were determined by centrifuging (Microlitre Centrifuges, Heraeus instruments, Biofuge) 4 ml of homogenized culture broth in preweighed Eppendorf tubes for 10 min at 16 000 g. The pellet was washed three times with HCl (0.35 mol l⁻¹), followed by distilled water. The Eppendorf tubes containing pellet were dried at 105°C for 24 h, cooled in a desiccator, and weighed. The results were expressed as gram per litre.

High performance liquid chromatography (HPLC) analysis

After centrifuging the samples, the supernatant was kept for further analysis. Four millilitre of the supernatant were extracted with an equal volume of dichloromethane. The organic phase (containing antibiotics) was collected and dried with anhydrous sodium sulfate. The extracts were concentrated to dryness under vacuum on Rotavapor (Laborata 4000, Heidolph, France), dissolved in 1 ml of MeOH as crude extract. The analysis of dithiolopyrrolone antibiotics was carried out by HPLC, Bio-Tek Instruments. The equipment consisted of variable wavelength detector (u.v.-vis 545 V diode detector array), pump system 525, column thermostat 582 and auto-sampler 465. The acquisition was carried out by computer system (Kroma 3000). The stationary phase consisted of C₁₈ reverse phase column (Zorbax SB, 150 × 4.6 mm, 5 μm particles). The samples were analysed by linear gradient elution using acetonitrile as solvent A and water as solvent B. The separation gradient starting with 0% solvent A and 100% solvent B, reaching 30% solvent A and 70% solvent B in 5 min, con-

tinuing from 30 to 100% solvent A in 25 min, using a flow rate of 0.8 ml min⁻¹. The injection volume was 60 μl. The detection of these antibiotics was carried out at 390 nm. The retention times were recorded at 11.03, 13.42, 14.02, 15.39 and 16.32 min for thiolutin, ISP, BUP, TIP and senecieryl-pyrrothine, respectively. Quantification of antibiotic was performed using thiolutin standard calibration curve. The ε of thiolutin is nearly the same for all others dithiolopyrrolones (ε₃₉₀ = 8317–9333 l mol⁻¹ cm⁻¹) as described by Lamari *et al.* (2002b).

Statistical analysis

Analysis of variance was performed using SPSS, Version 11.5.1, for windows. Significant differences in the mean values were reported at *P* < 0.05.

Results

Kinetics of growth and dithiolopyrrolone productions in *Saccharothrix algeriensis*

The samples were taken for pH, biomass, and antibiotic analysis at different time intervals every 12 h during 96 h (Fig. 2). The micro-organism revealed a good specific growth rate (0.091 h⁻¹). In SSM broth, the DCW reached a maximum at 24 h (0.360 g l⁻¹) and then decreased after 36 h of fermentation to reach a value of

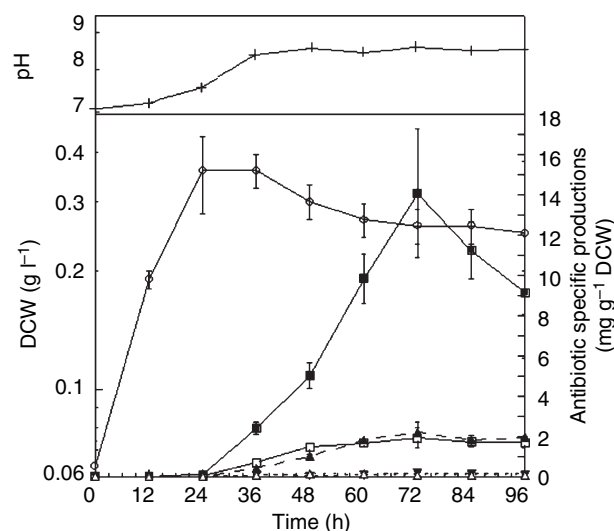


Figure 2 (■) Kinetic evolutions of specific productions of thiolutin; (□) senecieryl-pyrrothine; (▲) tigloyl-pyrrothine; (▼) iso-butyryl-pyrrothine and (△) butanoyl-pyrrothine; DCW (○) dry cell weight and (+) pH value, in *Saccharothrix algeriensis* on the basal culture medium (SSM) containing 10 g l⁻¹ glucose as sole carbon source at 30°C.

0.250 g l⁻¹ at 96 h. All antibiotic productions started after 24 h, and continued to increase to 72 h. After this period of fermentation, the specific productions were reduced. The production of thiolutin was the highest one among the other studied antibiotics. The maximum thiolutin specific production (14.07 mg g⁻¹ DCW) was reached at 72 h and then reduced to 9.14 mg g⁻¹ DCW at the end of fermentation (96 h). Furthermore, the maximal SEP and TIP specific productions (1.93 and 2.19 mg g⁻¹ DCW, respectively) were nearly the same, and reached a maximum at 72 h. The two later antibiotics represented percentage ranged from 13 to 16% of the specific production of thiolutin. ISP and BUP specific productions (0.18 and 0.13 mg g⁻¹ DCW, respectively) were poor (lower than 2% of the thiolutin specific production). Moreover, the final pH of the culture medium was slightly changed during the fermentation; it varied between 7 and 8.5.

Effect of amino acids containing sulfur at different times of addition on growth and dithiolopyrrolone productions in *Saccharothrix algeriensis*

Amino acids containing sulfur (L-cysteine, L-cystine, L-methionine and DL-ethionine) at a concentration of 10 mmol l⁻¹ were added at 0 h and during the exponential phase (at 12 h) to the SSM. The effects of these amino acids on growth and dithiolopyrrolone specific productions were summarized in Table 1. The growth of *S. algeriensis* was influenced by sulfur-containing amino acids. Among all the amino acids tested, only cysteine (0 h and 12 h) exhibited significant negative effect on the growth of *S. algeriensis*. While, in the other conditions, slightly effects were observed. The higher value of DCW_{max} was observed in the presence of 10 mmol l⁻¹ of ethionine added at 12 h (1.125 g l⁻¹), the second higher value was 0.850 g l⁻¹ with cystine added at 0 h and methionine added at 12 h. The minimum DCW_{max} was 0.375 g l⁻¹ with addition of methionine at 0 h as compared to 0.525 g l⁻¹ with control. The lower value of DCW_f (at the end of fermentation) was observed in the presence of methionine (0.200–0.275 g l⁻¹) and ethionine (0.200 g l⁻¹) added at 12 h as compared to the control (0.400 g l⁻¹). The production of thiolutin was reduced by addition of all sulfur-containing amino acids whatever the addition time. The specific production of thiolutin was decreased three to fivefold when the cysteine and cystine were supplemented at the two different stages of addition. The specific productions of ISP and BUP were increased by addition of cysteine and cystine, specially, by the addition of cystine (at 0 h) for BUP production. Our results showed that only the addition

Table 1 Effect of amino acids containing sulfur at different times of addition on mycelial growth, pH and dithiolopyrrolone specific productions by *Saccharothrix algeriensis* NRRL B-24137

Amino acids*	Dithiolopyrrolone specific productions (mg g ⁻¹ DCW)§								
	Biomass (g l ⁻¹)†	Final biomass (g l ⁻¹)‡	Final pH	μ _{max} (h ⁻¹)‡	Thiolutin	ISP	BUP	SEP	TIP
Control	0.525 ± 0.143	0.400 ± 0.130	8.12 ± 0.01	0.088 ± 0.013	19.92 ± 5.89	0.25 ± 0.19	0.19 ± 0.11	2.74 ± 1.66	2.87 ± 1.73
L-cysteine at 0 h	0.525 ± 0.045	0.525 ± 0.047	8.45 ± 0.03	0.050 ± 0.002	4.76 ± 0.39	1.08 ± 0.47	2.70 ± 0.99	0.55 ± 0.21	0.20 ± 0.05
L-cysteine at 12 h	0.475 ± 0.045	0.475 ± 0.043	8.57 ± 0.01	0.038 ± 0.004	4.87 ± 0.22	0.65 ± 0.03	1.15 ± 0.05	0.74 ± 0.03	0.29 ± 0.03
L-cystine at 0 h	0.850 ± 0.070	0.850 ± 0.078	8.66 ± 0.02	0.091 ± 0.006	6.53 ± 1.27	0.30 ± 0.14	10.25 ± 0.53	11.44 ± 4.58	27.13 ± 2.43
L-cystine at 12 h	0.525 ± 0.035	0.525 ± 0.039	8.73 ± 0.02	0.076 ± 0.004	7.36 ± 0.36	1.04 ± 0.05	2.22 ± 0.09	1.79 ± 0.09	0.53 ± 0.03
L-methionine at 0 h	0.375 ± 0.035	0.200 ± 0.020	8.71 ± 0.02	0.074 ± 0.004	3.56 ± 0.14	0.03 ± 0.00	0.06 ± 0.01	0.47 ± 0.01	0.13 ± 0.01
L-methionine at 12 h	0.850 ± 0.078	0.275 ± 0.025	8.74 ± 0.03	0.108 ± 0.008	0.12 ± 0.01	0	0	0.24 ± 0.01	0.07 ± 0.01
DL-ethionine at 0 h	0.650 ± 0.046	0.400 ± 0.036	8.77 ± 0.07	0.097 ± 0.007	0.89 ± 0.04	0	0	0.33 ± 0.01	0.26 ± 0.04
DL-ethionine at 12 h	1.125 ± 0.105	0.200 ± 0.020	8.65 ± 0.05	0.119 ± 0.019	0.66 ± 0.02	0.01 ± 0.00	0	0.27 ± 0.01	0.05 ± 0.01
LSD	0.129	0.101	0.0664	0.0154	3.463	0.305	0.652	2.795	1.711

*Each amino acid containing sulfur was added to the medium at a final concentration of 10 mmol l⁻¹.

†Mycelial biomass is given as g of dry weight per litre of culture broth at the time of maximal value during 96 h of fermentation.

‡μ_{max} is the maximum growth rate.

§§Specific dithiolopyrrolone productions are given as milligram per gram of biomass at the time of maximal productions during 96 h of fermentation. Each value represents the average of three measurements ±SD. LSD: Fisher's Least Significantly Difference at probability level (P < 0.05).

of cystine at 0 h, stimulated the SEP and TIP productions (four and ninefold, respectively). The specific production of all studied antibiotics were strongly inhibited by addition of methionine and ethionine whatever the time of addition. We observed that the final pH (from 8 to 9) did not change during different fermentations.

Effect of different concentrations of cysteine and cystine on dithiolopyrrolone productions by *Saccharothrix algeriensis*

Cysteine and cystine were added independently at concentrations of 2, 5, 10, 20 and 40 mmol l⁻¹ at 0 h to SSM in order to study their influences on the dithiolopyrrolone productions. The specific production of thiolutin was increased proportionally with the concentration of cystine to reach a maximum at 5 mmol l⁻¹ (325.09 mg g⁻¹ DCW) and then reduced sharply at 10 mmol l⁻¹ (10.91 mg g⁻¹ DCW) (Fig. 3a). In contrast, when the concentration of cysteine ranged from 0 to 40 mmol l⁻¹, a significant decrease was shown in the thiolutin production from 19.84 to 0.34 mg g⁻¹ DCW. We noticed that cysteine at 5 mmol l⁻¹ and cystine at 10 mmol l⁻¹ exhibited the best specific productions of SEP and TIP (Fig. 3b,c), ISP and BUP (Fig. 3d,e). Furthermore, cysteine yielded more production of ISP than cystine (Fig. 3d). In contrast, the maximum specific productions of thiolutin, SEP, TIP and BUP were higher with cystine than with cysteine. In general, specific productions of antibiotics were strongly reduced in the presence of excess of cysteine or cystine.

Discussion

The production levels of dithiolopyrrolone derivatives varied from species of micro-organisms to another and according to the culture conditions. In the present study, we observed that *S. algeriensis* produced about 75% of thiolutin, 9% of SEP, 9% of TIP, 1% of ISP and 1% of BUP in SSM. On the other hand, it was reported that about 70% of aureothricin, 15% of thiolutin and 10% of ISP were produced by *Streptoverticillium thioluteum* (*S. thioluteus*) (Stahl *et al.* 1988). The production of the studied dithiolopyrrolones started during the idiophase (between 24 and 36 h). Additionally, the growth and the

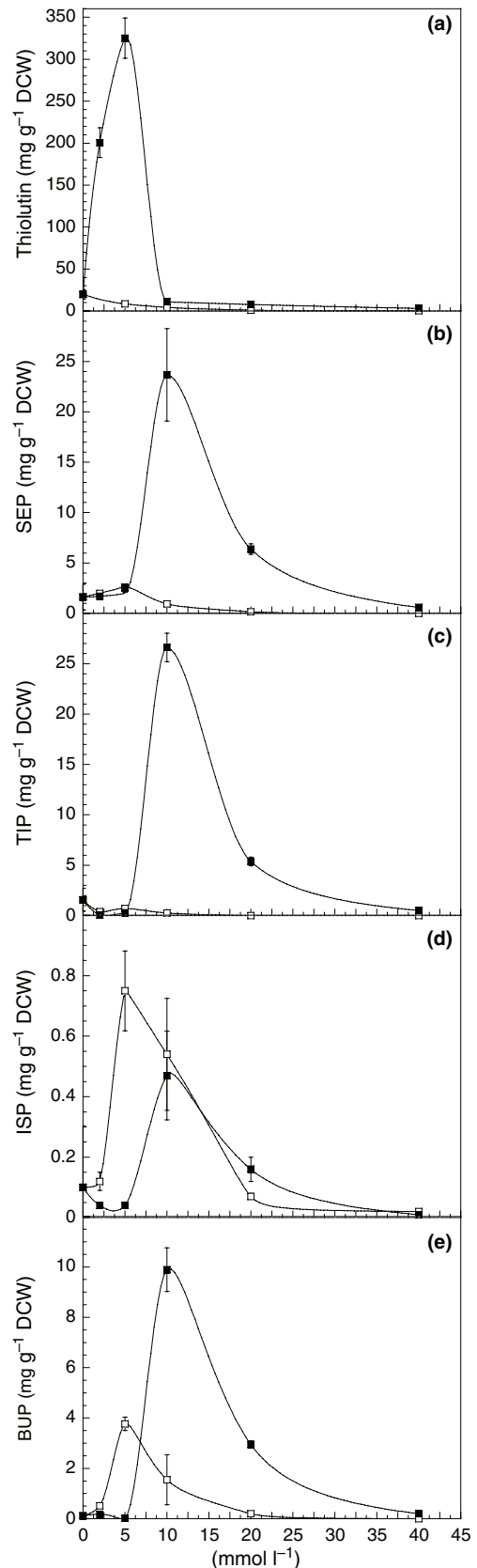


Figure 3 Effect of cysteine (□) and cystine (■) addition at different concentrations on specific production of thiolutin (a), senecioly-pyrrothine (b), tigloyl-pyrrothine (c), iso-butyl-pyrrothine (d) and butanoyl-pyrrothine (e). Specific dithiolopyrrolone productions are given as mg per g of biomass at the time of maximal productions during 96 h of fermentation.

antibiotic productions were dissociated. The same results were obtained by many species such as *Streptomyces clavuligerus* (Aharonowitz and Demain 1979) and *Streptomyces fradiae* (Gray and Bhuwapathanapum 1980) in the case of β -lactam and tylosin productions, respectively.

The biosynthetic pathways leading to the production of secondary metabolites such as antibiotics are often connected to and influenced by the pathways of primary metabolism (Drew and Demain 1977). For example, an end product or an intermediate of a primary pathway frequently serves as a precursor for the antibiotic molecule.

In the strains producing β -lactam antibiotics, the cysteine may be synthesized by two different pathways (Martín and Aharonowitz 1983). The first one, the reduction of sulfate (SO_4) to hydrogen sulfide (H_2S), which was incorporated to *O*-acetyl-serine using *O*-acetylserine sulphydrase enzyme. The second way, the conversion of methionine to cysteine by reverse transsulfuration (Suzuki *et al.* 1980). The use of one of the two pathways depended upon the genetic characteristics of different microorganisms and the regulated sulfur metabolism, which controlled the desired way.

In our experiments, we found that the most studied dithiopyrrolones antibiotics were induced by addition of cysteine and cystine. These probably account for the relationship between amino acids containing sulfur (as precursors) and the dithiopyrrolone biosyntheses. Cystine may be transformed to the studied dithiopyrrolones through an intermediate (pyrrothine) by a sequence of dehydrogenation, decarboxylation and cyclization. These are in agreement with Furumai *et al.* (1982), which reported that cystine may be the precursor of aureothricin biosynthesis in *S. kasugaensis*.

We found that the addition of 10 mmol l^{-1} cysteine or cystine exhibited a negative effect on thiolutin production. Šturdíková *et al.* (1990) conducted a similar study on thiolutin and aureothricin productions using *S. kasugaensis*. They showed that the addition of cysteine to the culture medium at 0.1% (equivalent of 8.25 mmol l^{-1}) did not affect the yield of thiolutin and aureothricin. Cysteine and cystine can be used as precursors for the formation of pyrrothine nucleus (Furumai *et al.* 1982; Šturdíková *et al.* 1990), although these amino acids have not the same effect on different dithiopyrrolone productions. These are probably due to an inhibitory or inducing effect of these amino acids on the enzyme system necessary for the dithiopyrrolone biosyntheses after the formation of pyrrothine nucleus.

The maximum specific productions were observed in the presence of 5 mmol l^{-1} cysteine for ISP, and 10 mmol l^{-1} cystine for SEP, TIP and BUP. While, thiolutin specific production was maximum by addition of 5 mmol l^{-1} cystine and without any addition of cysteine.

The antibiotic productions were favoured at low concentrations of sulfur-containing amino acids. Wherever, if their concentrations increased in the culture medium, the productions of these antibiotics were significantly reduced, probably, due to the excretion of excess ammonium ions in the medium (Drew and Demain 1977) or direct inhibition of some enzymatic systems. The higher concentrations of ammonium ions affected the antibiotic productions by inhibition or repression of certain enzymes of amino acid catabolism such as glutamate dehydrogenase, glutamine synthetase, valine deshydrogenase, threonin deshydratase and alanine deshydrogenase (Aharonowitz and Demain 1979; Lebrihi *et al.* 1992; Lounès *et al.* 1995a). Secondary metabolites in actinomycetes were usually delayed or reduced by an excess of readily available nitrogen (Shapiro *et al.* 1989). The ammonium ions suppressed many antibiotic productions such as tylosin by *S. fradiae* (Tanaka *et al.* 1986) and cephamycin by *S. clavuligerus* (Aharonowitz and Demain 1979). Omura *et al.* (1984) reported that the ammonium ions reduced considerably the production of protylonolide, precursor produced by amino acids catabolism and responsible for tylosin production, and the conversion of protylonolide to tylosin antibiotic in *S. fradiae*.

The addition of methionine or ethionine at 10 mmol l^{-1} to a culture medium, appeared to exert a catabolite repression or inhibition. The later amino acids supported the growth rate but not antibiotic productions. These coincide with Šturdíková *et al.* (1990) which reported that the effect of methionine and ethionine on thiolutin and aureothricin productions by *S. kasugaensis* showed the same inhibitory effects. Many authors observed the negative effect of methionine on secondary metabolites biosynthesis such as ochratoxin A (Lisker and Paster 1983). The significant negative effect of methionine on the biosynthesis of antibiotics containing methyl group derived from methionine has been observed for many years, but has remained unexplained (Kim *et al.* 2000). These effects have been postulated to be due to either an interaction of the thiol group of methionine (or its reactive metabolites) with the antibiotic intermediates (Gairola and Hurley 1976) or due to an inhibition of a methyltransferase (Uyeda and Demain 1988). The similar inhibitory effect was observed with ethionine. In the case of methionine and ethionine, an increase of growth rate could repress key enzymes of antibiotic biosyntheses (Gray and Bhuwapathanapum 1980).

In conclusion, it is suggested that sulfur-containing amino acids can serve as a tool for studying the mechanism of dithiopyrrolones biosynthesis in *S. algeriensis*. Further studies on the regulation of dithiopyrrolone productions by *S. algeriensis* on different culture conditions are further investigated.

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