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THE EFFECT OF RIFAMYCIN AND CHLORAMPHENICOL ON ANTIBIOTIC AND PROTEASE PRODUCTION BY *Streptomyces hygroscopicus* CH-7

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Streptomycetes are common soil bacteria that grow in a filamentous form and produced spores. Secondary metabolites, particularly antibiotics, as well as extracellular enzymes produced by them have very significant role in the process of differentiation.

The *Streptomyces hygroscopicus* CH-7 strain produces polyketide antibiotics and an extracellular proteolytic complex. The aim of this work was to establish the influence of transcription and translation inhibitors on biosynthesis of polyene antibiotic and proteolytic enzymes. The inhibitors were added at inoculation and at specific time intervals of fermentation.

The production of hexaene H-85 is susceptible to rifamycin (transcription inhibitor) and chloramphenicol (translation inhibitor). Rifamycin reduces the production and trypsin like protease (TLP) and aminopeptidase (AP), while chloramphenicol acts only on TLP. Simultaneously, these inhibitors almost have no effect on strain growth.

Key words: Streptomyces, polyene antibiotic, proteolytic enzyme(s), rifamycin, chloramphenicol

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INTRODUCTION

Streptomycetes are gram positive soil bacteria which differentiate into substrate mycelium and spores. They produce a broad range of different metabolites, such as antibiotics, extracellular enzymes, enzyme inhibitors and pigments. For organisms producing them antibiotics appear to serve as competitive weapons used against other microorganisms, metal-transporting agents and differentiation effectors. Also proteolytic enzymes play certain role in growth cycle of actinomycetes. For example they are involved in aerial mycelium formation and sporulation (HERSHBERGER *et al.*, 1989; LANCINI *et al.*, 1993).

The *Streptomyces hygroscopicus* CH-7 strain was isolated from a soil sample originating from Vojvodina. From the fermentation broth of this strain three antibiotics of polyketide structure (polyene, polyether and macrodiolide) (KARADŽIĆ *et al.*, 1991), and an extracellular proteolytic complex (GOJGIĆ-CVIJOVIĆ *et al.*, 1996) were isolated. The effect of various carbon and nitrogen sources, as well as phosphate and sodium chloride content in medium on biosynthesis of antibiotics (VUČETIĆ, *et al.*, 1994) and proteases (GOJGIĆ-CVIJOVIĆ *et al.*, 1996) have been previosly reported.

The subject of present work was to determine the effect of transcription and translation inhibitors on biosynthesis of antibiotics and proteolytic enzymes.

MATERIAL AND METHODS

Microorganism : The *Streptomyces hygroscopicus* CH-7 belongs to the Collection of the Institute of Chemistry, Technology and Metallury, Department of Chemistry.

Fermentation: All fermentations were carried out in 0.5 L Erlenmayer flasks with 50 mL liquid medium at 28°C on a rotary shaker (Janke-Kunke KS 500) at 200 rev/min. Base medium composition : 1.5 % glucose, 1% soybean meal, 0.25 % NaCl, 0.2% $CaCO_3$ and tap water, pH 6.8. Antibiotics rifamycin and chloramphenicol were added as solutions in methanol to final concentration of 10 μg (rifamycin) and 30 μg (chloramphenicol) per mL culture broth. Addition was made at inoculation time (0 h) or at certain time intervals (12, 24, 36 and 48 h) of fermentation process.

Susceptibility to antibiotics: The susceptiility of *S. hygroscopicus* CH-7 to various antibiotics was determined using the disk diffusion method. The strain was cultivated on potato dextrose agar (PDA) at 28°C. Test supstances were antibiogram tablets, Torlak.

Determination of mycelial growth: The growth of the submerged culture was determined colorimetrically as an increase of cellular DNA using diphenylamine method (BURTON, 1968).

Polyene determination: The fermentation broth was extracted with *n*-butanol and content of hexaene H-85 determined spectrophotometrically at 364 nm (KARADŽIC *et al.*, 1991).

Assay of enzymes activities: Proteolytic activities were determined in supernatants obtained by centrifugation of the whole fermentation broth at 3000 rpm for 10 minutes.

Caseinolytic activity was determined by the casein digestion method and expressed as PUK (HASHIMOTO *et al.*, 1963)

Trypsin like activity (TPL) was measured using N^{α} -benzoyl-L-arginine-4-nitroanilide hydrochoride (BAPA) Merck, as a substrate. 1U catalyses the transformation of 1μmol of substrate per minute under test conditions (EBELING *et al.*, 1974).

Aminopeptidase activity (AP) was determined on L-leucine-4-nitroanilide hydrochloride, Sigma, as a substrate. 1U catalyses the transformation of 1 μmol of substrate per minute under test conditions (PFLEIDERER *et al.*, 1964).

RESULTS AND DISCUSSION

In the course of investigations on biochemical characteristics of *S.hygroscopicus* CH-7 it was found that the tested strain is very sensitive to aminoglycoside antibiotics streptomycin and neomycin as well as to polypeptide antibiotic bacitracin, moderate sensitive to tetracycline and erythromycin and insensitive to rifamycin, chloramphenicol and β-lactam antibiotics (Table 1). Antibiotics rifamycin and chloramphenicol in concentration applied didn't show any lethal effect on *S. hygroscopicus* and were therefore selected for our inhibition studies.

Table 1. - Susceptibility of Streptomyces hygroscopicus CH-7 to antibiotics

Antibiotic	Concentration	Diameter* of inhibition zone, mm
Streptomycin	30 IJ	33.6
Neomycin	30 IJ	28.6
Tetracycline	30 IJ	14.6
Penicillin G	6 μg	-
Chloramphenicol	30 μg	11.5
Erythromycin	15 μg	18.1
Rifamycin	5 μg	-
Bacitracin	10 IJ	32.4
Methicillin	5 μg	-

* mean value calculated from three independent experiments

Rifamycin is transcription inhibitor and binds to β-subunit of bacterial DNA- dependent RNA polymerase and inhibits the chain initiation but not elongation. The bonding is not covalent and it has been suggested that there may be interactions between the fused aromatic ring system of antibiotics and aromatic amino acids of the enzyme.

Translation inhibitor chloramphenicol inhibits the peptidyltransferase reaction in the bacterial protein synthesis by reversibly binding to the 50S subunit of the 70S ribosome to a maximum extent of one molecule per subunit. This attachment prevents the attachment of amino acid-containing end of the amino acyl-transfer RNA complex to the ribosome, thereby inhibiting the formation of a peptide bond (STRATTON, 1996).

Effect of rifamycin

Figure 1 shows the growth curve of *S hygroscopicus* on the base medium and media with addition of rifamycin. In the base medium maximum growth is reached at 1st day of fermentations. By the addition of rifamycin at 0 and 12 h the growth is a little diminished (84% as compared to the control) while the addition of antibiotic at other time intervals has no effects. When rifamycin is added in later phases the differences mainly reflect in subsequent and prolonged synthesis. Even the curve labeling addition of antibiotic at 48 h of fermentation is almost identical to the control. These findings confirm susceptibility test shown in Table 1.

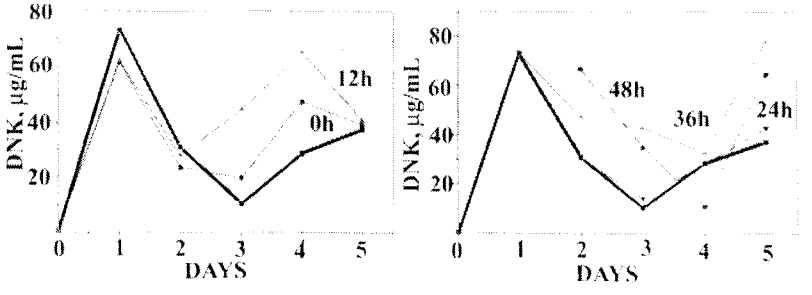

Figure 1. The effect of rifamycin on *S. hygroscopicus* growth. Growth in the base medium without antibiotic addition represents the control shown with a thick line.

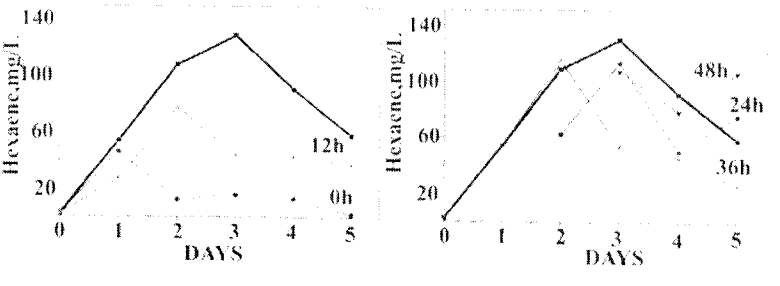

Figure 2. The effect of rifamycin on polyene production

Changes in hexaene production when rifamycin was added during fermentation are presented in Fig 2. The addition of rifamycin at inoculation time (0h) almost stopped the polyene synthesis. Maximum value in 1st day of fermentation is 40 μg /mL and then polyene concentration decreases bellow 10 μg/mL. Probably this initial value (40 μg/mL) could orginate from inoculum. By the addition of inhibitor at 12h of fermentation, biosynthesis of polyene is decreased to 60%. Rifamycin added in later stages of fermentation only slighty decreases the hexaene production.

It could be seen from Fig. 3.a,b that addition of rifamycin at 0, 12 and 24 h of fermentation markedly decreased caseinolytic activity. Maximum values are only 30% as compared to the control. Addition of inhibitor in later stages of fermentation decreases the total proteolytic activity too, but in somewhat lesser extent.

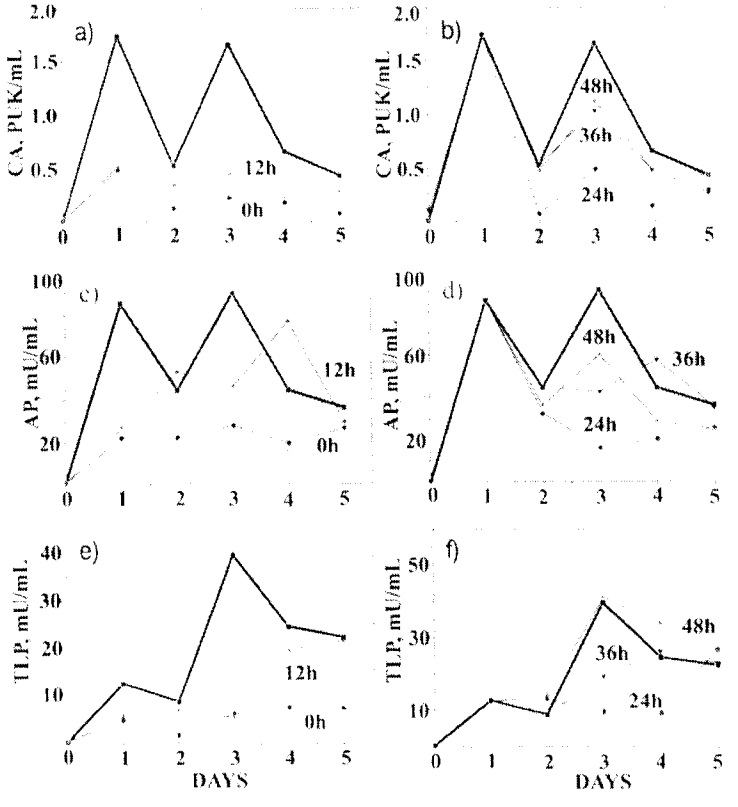

Figure 3. The effect of rifamycin on production of proteolytic enzymes (CA- caseinolytic activity, AP-aminopeptidase activity, TLP-trypsin like protease activity)

Addition of rifamycin at the beginning of fermentation causes maximum decrease of aminopeptidase activity (Fig 3.c,d). Maximum values are 28 mU/mL and 32.7 /mL representing 31 , 36 % of the control respectively. By the addition of inhibitor at 12 h of fermentation on AP curve two peaks could be seen corresponding to 2nd and 4th day of fermentation, which means that there is some shifting for 24 h in respect to the control. Decrease in aminopeptidase activity is 15-25 %.

Markedly decrease of TLP activity (Fig. 3.e,f) is evident only by the addition of inhibitor at inoculation. Maximum value during all five days of fermentation didn't exceed 20% related to the control. Some shifting of maximum is observed on curves shown inhibitor addition at 12-36h of fermentation. With rifamycin addition at 48h the change of TPL activity in fermentation broth is almost parallel to the contol curve.

It can be concluded that rifamycin as transcription inhibitor blocked the polyene synthesis by addition in medium at the beginning and at 12 h of fermentation. Protease production is markedly decreased only by addition of rifamycin at inoculation. In later stages protease production is also decreased but depending on type of activity somewhat lesser.

Effect of chloramphenicol

Changes in growth of *S. hygroscopicus* caused by the addition of chloramphenicol at various stages of fermentation are showed in Fig. 4. In the growth curve in the base medium two maxima could be seen in 1st and 4th day. By the addition of chloramphenicol at 0 and 12 h of fermentation only one peak occurs in 3rd day while the maximum appeared in 5th day by inhibitor addition at 24 and 36 h of fermentation. The curve corresponding to addition of inhibitor at 48 h of fermentation almost coincides with the control curve. It is evident that chloramphenicol slowed down certain process in the cells in the way that some shift and accumulation of particular phases occured, but didn't stop the growth.

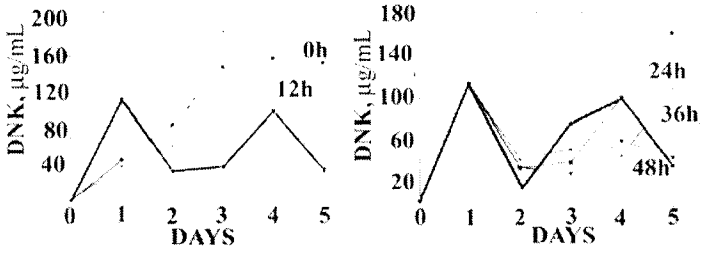

Figure 4. The effect of chloramphenicol on *S. hygroscopicus* growth

Synthesis of antibiotic is the most sensitive process (Fig. 5.) Chloramphenicol added in medium at 0-36 h of fermentation totally inhibits biosynthesis of hexaene - during all fermentation the concentration of antibiotic is retained at 20 μg level (only 15 % as compared to the control). Only when inhibitor is added at 48h of fermentation there is no change.

Addition of inhibitor decreases caseinolytic activity (Fig.6a,b) of fermentation broth in various degrees depending on time of addition. Maximum inhibition of enzyme is observed with chloramphenicol addition at 36, 0 and 12 h (45.3, 60.7, 60.7 % respectively).

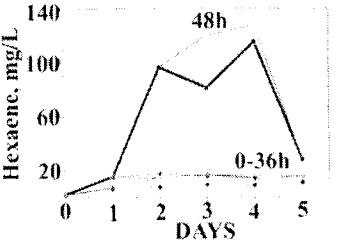

Figure 5. The effect of chloramphenicol on antibiotic production

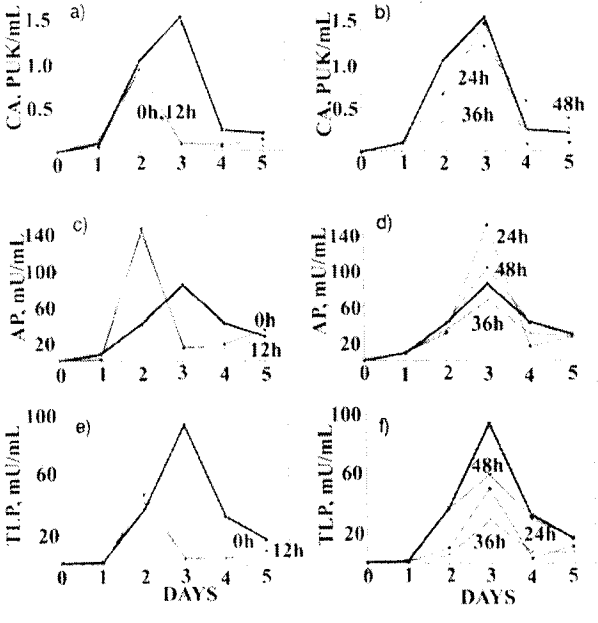

Figure 6. The effect of chloramphenicol on production of proteolytic enzymes

Aminopeptidase activity (Fig.6.c,d) is decreased only by inhibitor addition at 36 h of fermentation. Addition in other stages causes an increase of AP.

Activity of TLP (Fig.6.e,f) is markedly reduced by addition of chloramphenicol at all stages of fermentation. Some shifts of maxima observed at inhibitor addition takes place at 0 and 12 h of fermentation.

Finally, on the basis of presented results it can be concluded that the addition of chloramphenicol markedly decreased biosynthesis of polyene antibiotic. The production of caseinolytic activity and TPL protease is decreased too. There is no effect of chloramphenicol on aminopeptidase activity.

Similar effect of rifamycin and chloramphenicol on biosynthesis of macrolide antibiotic tylosin is reported (STRATTON, 1996; MARDY *et al.*,1981) – rifamycin causes only a slight inhibition, whereas chloramphenicol can almost block antibiotic biosynhesis. A continuos synthesis of proteins involved in polyketide synthesis (synthetases, enzymes of fatty acid catabolism and regulatory proteins) appears to be necessary for a continous synthesis of antibiotics. It is supposed the existence of stable mRNA coding for antibiotic formation proteins, formed only during a short time interval, over a continuos transcription.

The difference in sensitivity of aminopeptidase and TLP to chloramphenicol is probably due to specific role of these enzymes in growth cycle. In culture filtrates of *S. albidoflavius* SMF301 and *S. exfolliatus* three protease, namely chimotrypsin-like protease (CTP), trypsin-like (TLP) and metalloprotease (MTP) were identified (KANG *et al.*, 1997; KANG *et al.*, 1995; KIM *et al.*,1996). The role of the proteases in morphological differentiation were deduced to be as follows : CTP is essential for hydrolysis the proteinaceous nitrogen source for mycelium growth, TLP plays a role in the formation of aerial mycelium, MTP may participate in the maturation of spores.

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DEJSTVO RIFAMICINA I HLORAMFENIKOLA NA PRODUKCIJU ANTIBIOTIKA I PROTEAZA SOJA *Streptomyces hygroscopicus* CH-7

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Streptomicete su gram pozitivne bakterije čije je najvažnije stanište zemlja. Pri rastu na čvrstim podlogama i u submerznim uslovima grade supstratni i vazdušni micelijum. Morfološka diferencijacija je praćena produkcijom sekundarnih metabolita kao što su antibiotici, a i različitih hidrolitičkih enzima. Za mikroorganizme koji ih proizvode antibiotici predstavljaju kompetitivno oružje, agense za transport metala kao i efektore diferencijacije. Proteolitički enzimi, takodje, imaju odredjenu ulogu u razvojnom ciklusu aktinomiceta. Na primer, uključeni su u procese gradjenja vazdušnog micelija i sporulaciju.

Soj *Streptomyces hygroscopicus* CH-7 pri fermentaciji proizvodi antibiotike poliketidne strukture i smesu ekstracelularnih proteolitičkih enzima. U ovom radu je ispitivan uticaj antibiotika rifamicina i hloramfenikola, inhibitora transkripcije i translacije, na produkciju polienskog antibiotika heksaena H-85 i proteolitičkih enzima. Inhibitori su dodavani pri inokulaciji (0 h) i u različitim vremenskim intervalima fermentacije (12-48 h).

Dejstvom antibiotika inhibitora transkripcije i translacije utvrdjeno je da se enzim polien sintetaza transkribuje do 12. časa, a translacija teče do 48. časa fermentacije. Dodatak rifamicina pri zasejavanju drastično snižava proteolitičku aktivnost, a u kasnijim fazama izaziva smanjenje (36-80%). Hloramfenikol smanjuje produkciju tripsinu slične proteaze, ali nema dejstvo na aminopeptidaznu aktivnost.

Rifamicin i hloramfenikol ne pokazuju značajno inhibitorno dejstvo na rast soja.

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