Antibiotic production by *Streptomyces hygroscopicus* CH-7 in medium containing Schiff base complexes

Slavica B. Ilić[1], Sandra S. Konstantinović[1], Gordana D. Gojgić Cvijović[2], Vlada B. Veljković[1]

[1]*Faculty of Technology, University of Niš, Bulevar oslobođenja 124, 16000 Leskovac, Serbia*
[2]*Department of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia*

Abstract

Influence of a modified media on Hexaene H-85 and Azalomycine B production by *Streptomyces hygroscopicus* CH-7 was investigated. The amino acid L-tryptophan, as a nitrogen source, was replaced with isatin-3-thiosemicarbazone and its complexes with some divalent metal ions. Isatin-3-thiosemicarbazone was synthesized in crude glycerol obtained as a byproduct in biodiesel production from sunflower oil. The complexes were characterized by elemental microanalysis and magnetic susceptibility, as well as, by Atomic absorption(AA), Fourier-transform infrared (FTIR) and Ultraviolet–visible (UV/VIS) spectroscopic methods. The spectral studies indicated an octahedral geometry for the Mn(II), Fe(II) and Ni(II) complexes and a tetrahedral one for the Zn(II) complex. Comparing to the basal medium, isatin-3-thiosemicarbazone (ITC) and its metal complexes in the concentration of 0.5 g dm^{-3} showed better results in the antibiotics production. Use of medium supplemented with the Fe(II) complex resulted in the maximum Hexaene H-85 and Azalomycine B concentrations of 306 μg cm^{-3} and 127 μg cm^{-3}, respectively. Addition of ITC and its complexes changed the morphology of *S. hygroscopicus* CH-7 from filaments to pellets as a dominant shape in media resulting in higher antibiotic production.

Keywords: antibiotic production; isatin-3-thiosemicarbazone; metal complexes morphology; Streptomyces hygroscopicus

Dostupno na Internetu sa adrese časopisa: http://www.ache.org.rs/HI/

SCIENTIFIC PAPER

UDK: 579.2:604:615.3:54

Hem. Ind. **73 (2)** 93–101 (2019)

1. INTRODUCTION

Actinomycetes produce about three-quarters of known antibiotics with different chemical structures [1]. According to an estimate by Watve and co-workers, a tiny fraction of *Streptomyces genus* can produce about 100,000 antibiotics, so far [2]. Cultivation parameters like the pH value, carbon and nitrogen sources, temperature and metal ions play important role in the fermentation process [3]. Although, nutritive media contain small amounts of metals, their presence is very important and specific, since different microorganisms require different minerals. Many investigations have been carried out considering the impact of metals on the microorganisms' growth. The obtained results suggest significant impacts of different metals and metal ions on secondary metabolism in actinobacteria [4]. It is established that metals, such as K, Na, Mg, Ca, Mn, Fe, Co, Ni, Cu, Zn and Mo can interact with microbial cells inducing significant and selective impacts on cell growth and metabolism. Different studies have also shown that the amount of metal accumulation depends also on microbes type, and its concentrations used in media [5]. Although, microorganisms accumulate metals from natural organic and inorganic substances and water, sometimes, subsequent addition of metals to the nutritive medium is necessary. For *S. hygroscopicus*, trace elements play a very important role in the synthesis of antibiotics [6].

Salts, such as $MgCl_2$ and KNO_3 positively affect bioactive metabolite production by *Streptomyces* isolates, while $ZnCl_2$, $MnSO_4$ and $ZnSO_4$ have negative effects on bioactive metabolite production by *Streptomyces* isolates R5 and RUPA-08RP

Korespodencija: Slavica B. Ilić, Faculty of Technology, University of Niš, Bulevar oslobodjenja 124, 16000 Leskovac, Serbia, Tel: +381 16 247 203; Fax: +381 16 242 859
E-mail: ilicslavica@yahoo.com
Paper received: 16 October 2019
Paper accepted: 27 March 2019
https://doi.org/10.2298/HEMIND181016008I

[7-9]. Genus *Streptomyces* is capable of zinc(II) and copper(II) bioaccumulation from contaminated environments [10]. The strain used in the present study, *Streptomyces hygroscopicus* CH-7, was isolated from a soil sample in Vojvodina (Serbia), and during fermentation it produces three antibiotic compounds: polyenic Hexaene, polyetheric nygericine and macrodiolide Azalomycine B.

Hexaene H-85 is a polyene macrolide antibiotic and structurally similar to mediomycin, endomycin and hexafungin. Hexaene H-85 exhibits high activity against fungi and moderate activity against Gram-positive bacteria [11,12].

Azalomycine B (Elaiophylin) is a macrodiolide, and a monosaccharide derivative isolated from several bacteria including *Streptomyces hygroscopicus*CH-7 and other soil actinomycetes. It behaves as a bacterial metabolite, antifungal agent and autophagy inhibitor [13].

There are numerous investigations of different nitrogen and carbon sources, as well as different requirements of metal ions for growth of various microbes [3,14,15]. To increase the yield of antibiotics (Hexaene H-85 and Azalomycine B), the fermentation medium was redefined with different carbon and nitrogen sources. Previous investigations suggested that isatin derivatives, as nitrogen sources have different impacts on antibiotic production by *S. hygroscopicus* CH-7. The highest Hexaene H-85 concentration was achieved with 5-chloroisatin-3-hydrazone in medium, while isatin-3-hydrazone had the greatest impact on Azalomycine B production [16]. Also, addition of β-cyclodextrine inclusion complexes with isatin derivatives (as nitrogen sources) significantly influenced production of these two antibiotics by *S. hygroscopicus* CH-7 [17]. Similarly, derivatives of salicylaldehyde were shown to be good nitrogen sources in this system. The highest Hexaene H-85 and Azalomycine B concentrations were achieved in the medium with salicylaldehyde-thiosemicarbazide. Also, morphology of *S. hygroscopicus* CH-7 changed when derivatives of salicylaldehyde were added during the fermentation. In the medium with salicylaldehyde-phenylhydrazone and salicylaldehyde-thiosemicarbazone as nitrogen sources, *S. hygroscopicus* CH-7 grew in the form of large dispersive pellets with long twisted filaments that produced the highest antibiotics yield [18].

Isatin-3-thiosemicarbazone (ITC) can be easily coordinated with different metal ions and its complexes exhibit better antimicrobial activity than the ligand itself. Namely, the ITC complexes with metal ions show better antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Enterobacter* spp., *Proteus mirabilis*, *Bacillus anthracis*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* [29,30]. The authors suggested that this finding is probably the result of the chelation theory *i.e*. a decrease in metal polarizability induces lipophilicity of the complex [31].

In the present work, an extensive study has been made on isatin-3-thiosemicarbazone (synthesized in crude glycerol as a green solvent) and its Mn(II), Fe(II), Ni(II) and Zn(II) complexes as nitrogen sources in chemically defined media regarding the antibiotic production by *S. hygroscopicus* CH-7. According to our knowledge, this is the first study about the influence of ITC complexes with metal ions on the growth of this bacterial strain and its antibiotics production do not exist.

2. EXPERIMENTAL

2. 1. Chemicals

All chemicals, except crude glycerol, were of reagent grade and used without further purification. They were purchased from Sigma Aldrich, USA.

Crude glycerol, a by-product in the laboratory biodiesel production from sunflower oil, was obtained from the Laboratory for Chemical Engineering, the Faculty of Technology, University of Niš (Leskovac, Serbia).

2. 2. Characterization methods

Elemental analyses (C, H, N) were performed by standard micro methods using a microanalyzer (Model 1106, Carlo Erba, Devon, UK). Melting points were determined in a capillary melting point apparatus (Model Thomas-Hoover Unimelt, Philadelphia, USA) and were not corrected.

Chloride and metal contents were determined by the Mohr method and using a Virial AA-457 Double beam spectrometer (Perkin-Elmer, USA), respectively. Fourier-transform infrared (FTIR) spectra were obtained by using a spectrometer (Bomem MB-100, Model Hartmann & Braun, Canada), using the KBr pellet (1 mg/100 mg) technique. FTIR

spectra were recorded in the transmission mode (600 to 4000 cm^{-1}) with a resolution of 4 cm^{-1}. UV/VIS spectra of 10^{-5} mol dm^{-3} ethanol solution of synthesized compounds were recorded on a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer (Lambda 15, Perkin-Elmer, USA). Magnetic susceptibility measurements were made at room temperature using an MSB-MKI magnetic balance (Sherwood Scientific Ltd., UK). The data were corrected for diamagnetism.

2. 3. General procedure for preparation of isatin-3-thiosemicarbazone and metal complexes

Isatin-3-thiosemicarbazone (ITC) was prepared using crude glycerol as a green solvent. The excess of methanol was removed from the crude glycerol by distillation. Acidity of methanol-free crude glycerol was adjusted to pH 5 by addition of 85 % phosphoric acid [19]. Inorganic salts formed in this stage were removed by centrifugation (4000 rpm for 15 min).

Equimolar amounts of isatin and thiosemicarbazide were dissolved in crude glycerol and mixture was refluxed for 10 min at 70 °C. A dark-yellow product was filtered, washed with water and dried in Vacuum Dryer Vacuum Desiccator over $CaCl_2$.

Complexes were synthesized using a direct method between the ligand and the required metal(II) chloride (2:1 for Fe(II), Mn(II) and Ni(II)) and 1:1 molar ratio for Zn(II)). The solutions were heated under reflux for 3-5 h and the products were filtered, washed with ethanol and dried in Vacuum Dryer Vacuum Desiccator over $CaCl_2$[20]. The experiments were performed in triplicate.

2. 4. Characterization of complexes

ITC($C_9H_8N_4OS$). Yellow solid (solvent used for crystallization: ethanol); Yield: 95 %; m.p. 239–241 °C; Combustion analysis for $C_9H_8N_4OS$: Calcd.: C 49.08, H 3.66, N 25.46, S 14.53 %; found: C 49.11, H 3.62, N 25.40, S 14.52%; FTIR (cm^{-1}): 3400, 3295, 3255 ν(NH+NH_2), 1700 ν(C=O), 1606 ν(C=N), 1250, 854 ν (C=S); UV/Vis (ν(cm^{-1})/$\varepsilon\cdot10^3$ (dm^3 mol^{-1} cm^{-1})): 28.1/0.915 ($\pi\rightarrow\pi^*$), 27.3/1.345 ($\pi\rightarrow\pi^*$).

[Zn($C_9H_8N_4OS$)]Cl_2. Yellow solid (ethanol); Yield: 65 %; m.p. 258 °C; Combustion analysis for $C_9H_8N_8OSCl_2Zn$: Calcd.: C 30.31, H 2.26, N, 15.72, Cl 19.90, Zn 18.35 %; found: C 30.35, H 2.21, N 15.79, Cl 19.89, Zn 18.25 %, FTIR (cm^{-1}): 3400, 3265, 3250 ν(NH+NH_2), 1704 ν(C=O), 1575 ν(C=N), 1225 835 ν (C=S), UV/Vis (ν(cm^{-1})/$\varepsilon\cdot10^3$ (dm^3 mol^{-1} cm^{-1})): 18.1/0.125 (CT), 16.9/0.105 ($d\rightarrow d^*$); Mol. cond. (S cm^2 mol^{-1}): 10.

[Ni($C_9H_8N_4OS$)]$_2Cl_2$. Brown solid (ethanol); yield: 51 %; m.p. 297 °C; Combustion analysis for $C_{18}H_{16}N_8O_2S_2Cl_2Ni$: Calcd:.C 37.92, H 2.83, N 19.66; Cl 12.45, Ni 10.30 %, found: C 37.98, H 2.80, N 19.58, Cl 12.41, Ni 10.26 %; FTIR (cm^{-1}): 3415, 3290, 3255, ν(NH+NH_2), 1655 ν(C=O), 1559 ν(C=N), 1224, 815(C=S); UV/Vis (ν(cm^{-1})/$\varepsilon\cdot10^3$ (dm^3 mol^{-1} cm^{-1})): 22.5/1.209 ($^3A_{2g}\rightarrow{}^3T_{1g}$(P)), 18.1/0.351 ($^3A_{2g}\rightarrow{}^3T_{1g}$(F)), 13.9/0.02 ($^3A_{2g}\rightarrow{}^3T_{2g}$(F)); μ_{eff}= 3.35 μB; Mol. cond. (S cm^2 mol^{-1}): 102.

[Mn($C_9H_8N_4OS$)]$_2Cl_2$. Light-brown solid (ethanol); Yield: 49%; m.p. 295 °C. Combustion analysis for $C_{18}H_{16}N_8O_2S_2$ Cl_2Mn: Calcd.: C 38.17, H 2.85, N 19.79, Cl 12.53, Mn 9.71%, found: C 38.21, H 2.80, N 19.85, Cl 12.51, Mn 9.76%; FTIR (cm^{-1}): 3410, 3315, 3221 ν(NH+NH_2), 1651 ν(C=O), 1552 ν(C=N), 1221, 820 ν(C=S); UV/Vis (ν(cm^{-1})/$\varepsilon\cdot10^3$ (dm^3 mol^{-1} cm^{-1})): 38.5/0.656 ($^6A_{1g}\rightarrow{}^4E_g$ (G)), 27.5/0.455 ($^6A_{1g}\rightarrow{}^4T_{1g}$(P), 23.2/0.358 ($^6A_{1g}\rightarrow{}^4T_{1g}$(D), 18.2/0.295 ($^6A_{1g}\rightarrow{}^4T_{1g}$(G)), μ_{eff} = 5.85 μB; Mol. cond. (S cm^2 mol^{-1}): 105.

[Fe($C_9H_8N_4OS$)]$_2Cl_2$. Dark-brown solid (ethanol); Yield: 55 %; m.p. 298 °C. Combustion analysis for $C_{18}H_{16}Cl_2N_8O_2S_2Fe$: Calcd.: C 38.11, H 2.84, N 19.79, Cl 12.51, Fe 9.85 %; found: C 38.15, H 2.89, N 19.85, Cl 12.45, Fe 9.86 %; FTIR (cm^{-1}): 3415, 3315, 3225 ν(NH+NH_2), 1654 ν(C=O), 1554 ν(C=N), 1220, 823(C=S); UV/Vis (ν(cm^{-1})/$\varepsilon\cdot10^3$ (dm^3 mol^{-1} cm^{-1})): 17.2/0.295 ($T_{2g}\rightarrow{}^5E_g$); μ_{eff} = 4.91 μB; Mol. cond.(S cm^2 mol^{-1}): 121.

2. 5. The bacterial strain, media and growth conditions

The bacterial strain *S. hygroscopicus* CH-7 (NCAIM(P) B-001336) was obtained from the Microbial collection at the Faculty of Chemistry and the Institute of Chemistry, Technology and Metallurgy, Belgrade, Serbia [12,21]. *S. hygroscopicus* CH-7 was maintained in spore and mycelia suspensions in sterile glycerol (20 % w/v) prepared as described elsewhere [22,23]. Fermentation media were inoculated with a 48 h preculture (5 % v/v) and incubated at 28 °C for 168 h under standard conditions of aeration and agitation (220 rpm, rotary shaker, PSU-20i, Biosan, EU, Latvia). The fermentation basal medium had the following composition: 15 g dm^{-3} glycerol, 3 g dm^{-3} $CaCO_3$, 3 g dm^{-3} NaCl,

0.5 g dm^{-3} $MgSO_4 \times 7\ H_2O$, 0.5 g dm^{-3} $(NH_4)_2HPO_4$, 0.5 g dm^{-3} K_2HPO_4, and 10 g dm^{-3} *L*-tryptophan. After the fermentation, antibiotics were extracted by using 1-butanol and ethyl acetate [12, 21].

Concentrations of the antibiotics were determined by measuring the absorbance at λ_{max} = 364 nm for Hexaene H-85 and at λ_{max} = 252 nm for Azalomycine B by using a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer (Lambda 15, Perkin-Elmer, USA) [12,21,24].

Bacterial growth was determined by measuring dry cell weights. The broth was centrifuged at 4000 rpm for 15 min to separate the mycelial biomass, which was then dried at 105 °C to a constant weight and weighed.

During the fermentation, samples were periodically taken, and microscopic photographs were obtained by Leica (Microsystem, Heerbrugg, Germany) using the Leica application Suite program (ver 2.5.0, Leica microsystem, Switzerland). All experiments were performed in triplicate.

3. RESULTS AND DISCUSSIONS

3. 1. Chemistry

All synthesized complexes are air-stable and soluble in hot 95 % ethanol, dimethylformamide (DMF) and dimethyl-sulfoxide (DMSO). Elemental microanalysis showed two kinds of stoichiometries in complexes, $M(ITC)_2Cl_2$ (M = Mn, Fe, Ni) and $M(ITC)Cl_2$ (M = Zn). Molar conductance measurements indicated electrolytic behavior of $M(ITC)_2Cl_2$ since chloride ions are present in the outer space of compounds, which is not characteristic for Zn(II) complex.

Although isatin-3-thiosemicarbazone (ITC) can exhibit thione-thiol tautomerism, the absence of 2500–2600 cm^{-1} vibration in the spectra of all compounds suggests the thione form of ITC in all coordination compounds. The band assigned to *ν*(C=O) vibration is in the similar position in spectra of ITC and its Zn(II) complex, which exclude this group in the complex formation. Data not shown

In the spectra of Mn(II), Fe(II) and Ni(II) complexes, this band is shifted to lower frequencies (Δ = 45 - 50 cm^{-1}), which denotes the carbonyl group as a coordination site of the ligand. The absorption bands of *ν*(C=N) and *ν*(C=S) undergo a negative shift in all complexes suggesting azomethine and thione group to be the second and third coordination sites of the ligand. The obtained results indicate N,S bidentate in the Zn(II) complex (with tetra coordination around the central ion) and O,N,S tridentate ligand behavior in Mn(II), Fe(II) and Ni(II) complexes (with octahedral coordination around the central ion) [20,25,26].

The electronic spectrum of the diamagnetic and tetrahedral zinc(II) complex exhibits two bands assigned to charge transfer and $d \rightarrow d^*$ transitions.

The electronic spectrum of the nickel(II) complex shows three distinct bands at 22.500 ($^3A_{2g} \rightarrow {}^3T_{1g}$), 18.100 ($^3A_{2g} \rightarrow {}^3T_{1g}$) and 13.900 ($^3A_{2g} \rightarrow {}^3T_{2g}$). The electronic spectrum of the Mn(II) complex exhibits bands belonging to $^6A_{1g} \rightarrow {}^4E_g$, $^6A_{1g} \rightarrow {}^4T_{1g}$, $^6A_{1g} \rightarrow {}^4T_{1g}$ and $^6A_{1g} \rightarrow {}^4T_{1g}$, while the spectrum of the Fe(II) complex has a band at 17.200 characteristic for $T_{2g} \rightarrow {}^5E_g$ transition.

Electronic spectra data and magnetic moments of Mn(II), Fe(II)and Ni(II) complexes suggest octahedral geometry for those coordination compounds [27,28].

Previous investigations have shown that the antibiotic production by *S. hygroscopicus* CH-7 could be significantly improved, by replacement of some amino acids (alanine, arginine, phenylalanine, tryptophan or valine) with ITC as a nitrogen source [3,22]. In the present study the effects of $Fe(ITC)_2Cl_2$, $Zn(ITC)Cl_2$, Mn(ITC)2Cl2 and $Ni(ITC)_2Cl_2$ complexes as nitrogen sources were tested. As the referent nitrogen source in basal media, L-tryptophan was used (10 g dm^{-3}) due to its similar structure with ITC, while glycerol was used as the carbon source [18,23]. ITC and its metal complexes were added at concentrations of 0.5, 1.0 and 1.5 g dm^{-3} to the basal medium. The maximal concentrations of Hexaene H-85, Azalomycine B and dry biomass, achieved during the fermentation in the basal and modified media are presented in Table 1, while their variations with the progress of the fermentation are shown in Figure 1.

The results show that the optimal concentration of ITC and its complexes in the chemically defined media for the production of the antibiotics is 0.5 g dm^{-3}.

Table 1. The impact of ITC and its complexes on maximal concentrations of dry biomass (X_{max}) and antibiotics (c_{max}) achieved in fermentations by S. hygroscopicus CH-7

Compound	Concentration, g dm^{-3}	Dry biomass		Hexaene H-85		Azalomycine B	
		X_{max} / g dm^{-3}	SD, %	c^H_{max} / μg cm^{-3}	SD, %	c^A_{max} / μg cm^{-3}	SD, %
L- tryptophan	0.5	8.9	± 4.67	148	± 2.07	64	± 1.43
	1	9.2	±2.13	153	± 3.58	56	± 4.17
	1.5	8.8	±3.84	136	± 3.01	43	± 1.51
ITC	0.5	9.6	±3.06	272	± 2.29	98	± 3.79
	1	9.4	± 3.43	253	± 3.71	82	± 3.75
	1.5	9.2	± 4.82	241	± 3.73	65	± 2.42
$Fe(ITC)_2Cl_2$	0.5	9.3	±1.74	306	±1.78	127	±1.08
	1	8.9	±4.40	215	±4.36	103	±3.61
	1.5	8.5	±3.12	186	±4.30	91	±4.01
$Ni(ITC)_2Cl_2$	0.5	9.1	±2.98	173	±2.50	87	±4.45
	1	8.7	±2.35	167	±3.87	62	±4.13
	1.5	9.0	±4.51	152	±3.75	54	±3.81
$Mn(ITC)_2Cl_2$	0.5	8.6	±3.67	283	±2.77	112	±2.49
	1	8.4	±4.76	177	±3.27	76	±4.30
	1.5	8.2	±4.32	152	±4.15	58	±3.76
$Zn(ITC)Cl_2$	0.5	8.3	±1.18	291	±2.26	118	±1.01
	1	8.6	±3.08	197	±3.06	99	±3.84
	1.5	9.3	±3.68	183	±4.45	84	±3.31

SD - Standard deviation

As it can be seen, production of Hexaene H-85 with ITC in the medium reached the maximum at the third day of fermentation (Fig. 1a), while the concentration of Azalomycine B reached a maximum at the fifth day of fermentation (Fig. 1b). In the chemically modified media with added complexes, both Hexaene H-85 (Fig. 1a) and Azalomycine B (Fig. 1b) reached the maximal production at the fourth day of fermentation. Influence of tested compounds decreased in the order:

$Fe(ITC)_2Cl_2$ > $Zn(ITC)Cl_2$ > $Mn(ITC)_2Cl_2$ > ITC > $Ni(ITC)_2Cl_2$ > basal media

It can be also seen that media with ITC or $Fe(ITC)_2Cl_2$, $Zn(ITC)Cl_2$ and $Mn(ITC)_2Cl_2$ complexes enhanced Hexaene H-85 and Azalomycine B production from the very beginning of fermentation.

Since many enzymes require metal ions for activation, the obtained results suggest that $Fe(ITC)_2Cl_2$, $Zn(ITC)Cl_2$ and $Mn(ITC)_2Cl_2$ may be involved in the enzyme system utilized at some stages of antibiotic production. Maximal biomass as well as Hexaene H-85 and Azalomycineproduction can be achieved by addition of $Fe(ITC)_2Cl_2$ complex at the concentration of 0.5 g dm^{-3} into the basal medium. Higher concentrations of this complex inhibited the bacterial growth and antibiotics production so that the lowestvalues of these parameters were obtained at the $Fe(ITC)_2Cl_2$ concentration of 1.5 g dm^{-3}. Previous research has shown that lower concentrations of $FeSO_4$ increased *Streptomyces clavuligerus* DSM 738 growth, while higher concentrations had an inhibitory effect [32]. Schrader and Blevins [5] reported that increasing concentration of iron inhibited biomass accumulation and geosmin production by *Streptomyceshalstedii*. Maximal geosmin production occurred with 0,2 μM iron, while increasing concentration of manganese inhibited geosmin production [5].

In the present study, presence of the $Mn(ITC)_2Cl_2$ complex had negligible impact on biomass production, while higher concentrations of this additive inhibited the antibiotic production. Likewise, maximal antibiotic production in the medium supplemented with the $Zn(ITC)Cl_2$ complex was achieved at the concentration of 0.5 g dm^{-3}. On the other hand, maximal biomass accumulation while lower antibiotics production was obtained at the concentration of this additive of 1.5 g dm^{-3}.

Similar investigations suggested importance of ferrous ions for the growth and antibiotic production by *Streptoverticillium rimofaciens* and manganese ions for the growth and granaticin production by *Streptomyces violaceolatus* [33,34]. Addition of Mn in the form of compound $MnCl_2 \times 4H_2O$ at the concentration of 1 μg dm^{-3} to the nutrient medium enhanced the biomass of *S. hygroscopicus* DA 15, while further increaseing the Mn in theform of

compound $MnCl_2 \times 4\ H_2O$ concentration from 3, 5 to 7 μg dm^{-3} gradually decreased the biomass as compared to the control. The same trend was also reported for Zn ions. Namely, addition of Zn in the form of compound $ZnSO_4 \times 7\ H_2O$ at the concentration of 3 μg dm^{-3} enhanced accumulation of *S. hygroscopicus* while further increase in Zn in the form of compound $ZnSO_4 \times 7H_2O$ concentration from 5,7 to 10 μg dm^{-3} induced a gradual decrease in the biomass as compared to the control [6]. Also, iron and manganese could play important roles in antibiotic production by *Streptomyces violates* [9].

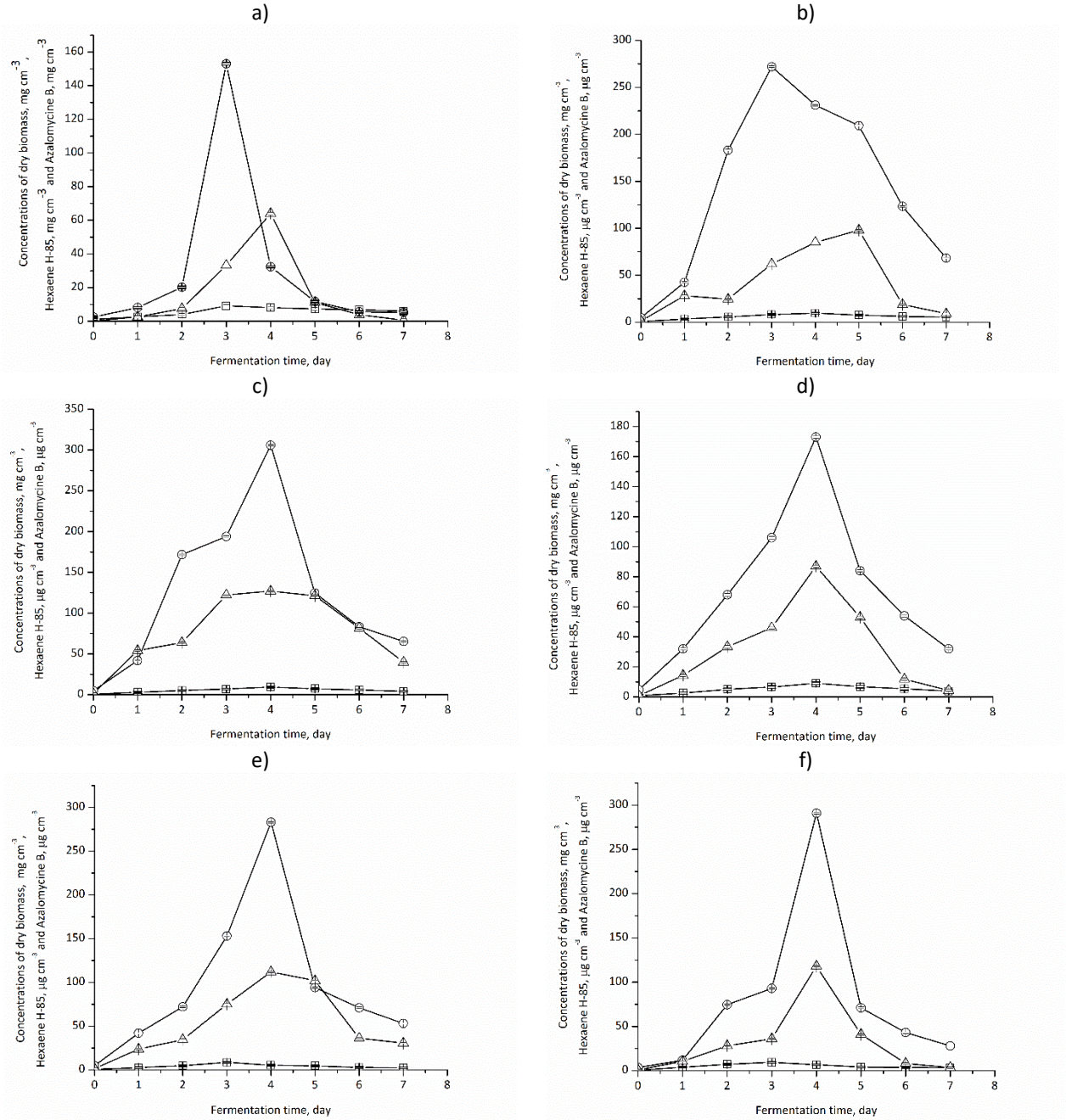

Fig. 1. Concentrations of dry biomass - (□) - Hexaene H-85 - (o) - and Azalomycine B - (Δ) - during fermentation by S. hygroscopicus *CH-7 in: a) basal medium, b) medium with 0.5 g dm^{-3}ITC, c) medium with 0.5 g dm^{-3} $Fe(ITC)_2Cl_2$, d) medium with 0.5 g dm^{-3} $Ni(ITC)_2Cl_2$, e) medium with 0.5 g dm^{-3} $(ITC)_2Cl_2$, f) medium with 0.5 g dm^{-3} $(ITC)Cl_2$ (data are average of n = 3)*

The present study has also shown that the chemically defined medium supplemented with the $Ni(ITC)_2Cl_2$ complex induced better antibiotic productivity then the basal medium, but this effect is lesser than that of ITC. This result

suggests that probably this metal ion may be involved in inhibition of the enzyme system at some stages of the antibiotic production.

Addition of ITC and $Fe(ITC)_2Cl_2$, $Zn(ITC)Cl_2$, $Mn(ITC)_2Cl_2$ and $Ni(ITC)_2Cl_2$ complexes to the medium had different effects on the growth of *S. hygroscopicus* CH-7. In the media with $Fe(ITC)_2Cl_2$ and $Ni(ITC)_2Cl_2$ complexes, the growth was slightly higher than that in the basal medium whilethe maximal dry biomass concentration (9.6 g dm^{-3}) was achieved in the medium supplemented with ITC (Table 1, Fig. 1c).

Also, Fe(II) as $FeSO_4x\ 7H_2O$ and Mn(II) as $MnCl_2x4H_2O$, stimulated production of a nonpolyenic macrolide antibiotic and niphimycin derivatives of *S. hygroscopicus* AK-111-81 in media with $FeSO_4\times7H_2O$ (300–400 mg dm^{-3}) and $MnCl_2\times4H_2O$ (5-10 mg cm^{-3}), whereas Azalomycin B was present only in traces [35].

3. 2. Strain morphology

Addition of isatin-3-thiosemicarbazone metal complexes had an effect on morphology of *S. hygroscopicus* CH-7. In the basal medium, the strain grew in the form of short and twisted filaments (Fig. 2a), while in the medium supplemented with ITC the strain took the form of some small as well as single large pellets (Fig. 2b).

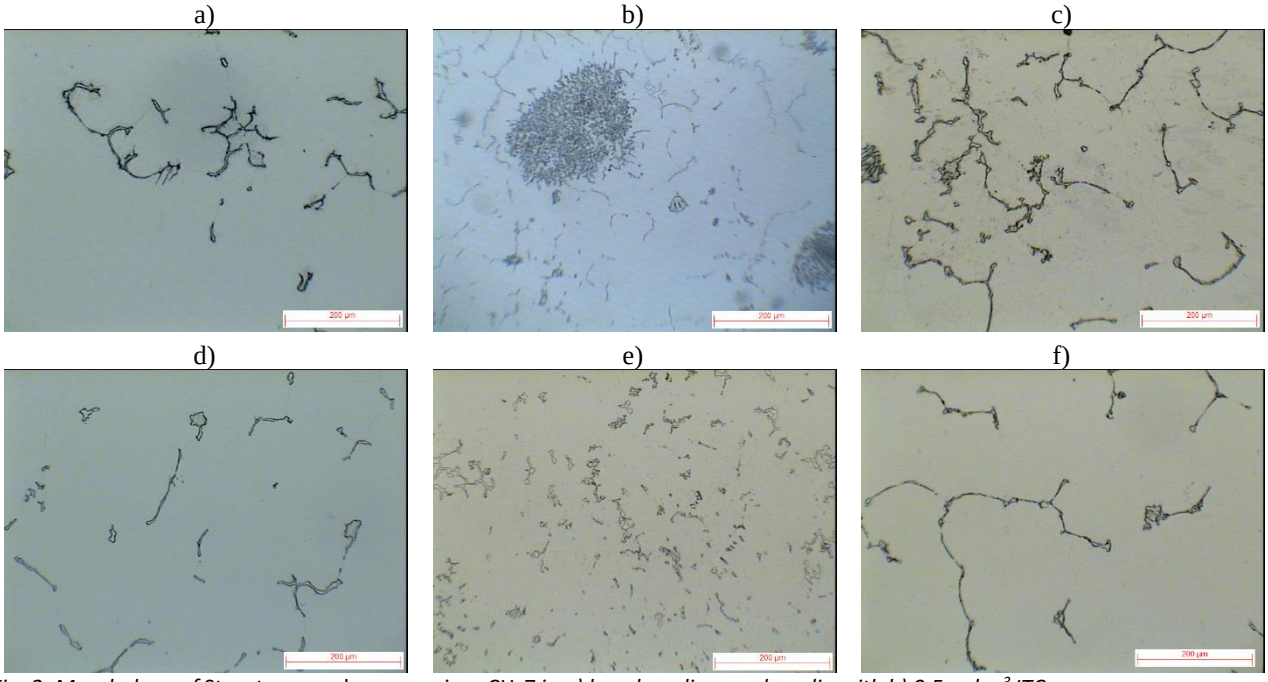

Fig. 2. Morphology of Streptomyces hygroscopicus CH-*7 in a) basal medium and media with b) 0.5 g dm^{-3} ITC, c) 0.5 g dm^{-3} $Zn(ITC)Cl_2$, d) 0.5 g dm^{-3} $Mn(ITC)_2Cl_2$ e) 0.5 g dm^{-3} $Fe(ITC)_2Cl_2$ and f) 0.5 g dm^{-3} $Ni(ITC)_2Cl_2$ (Magnification: 100×)*

In the medium supplemented with the Zn(II) complex, the strain grew in forms of branched filaments and small pellets (Fig. 2c), while in the medium supplemented with the Ni(II) complex it was in the form of long filaments (Fig. 2d). *S. hygroscopicus* CH-7 in the medium containing the Fe(II) complex grew in the form of small pellets (Fig. 2e), while in the medium supplemented with the Mn(II) complex the characteristic bacterial shape was small pellets with short filaments (Fig. 2f).

As it can be seen, the dominant shape is small pellets, which is in correlation with all media, in which the antibiotic production is enhanced as compared to that in the basal medium.

4. CONCLUSION

The results obtained in this study have clearly shown dependence of the antibiotic synthesis on the type of nitrogen source included into the fermentation medium. In this respect the optimal concentration of ITC and its complexes in the chemically defined medium is 0.5 g dm^{-3}. The improved medium supplemented with the $Fe(ITC)_2Cl_2$ complex resulted

in the maximal antibiotics concentration of 306 and 127 μg cm^{-3} for Hexaene H-85 and Azalomycine B, respectively. Addition of isatin-3-thiosemicarbazone complexes influenced morphology of the *S. hygroscopicus* CH-7. The maximum concentration of antibiotics was correlates with the formation of small reproducible pellets of *S. hygroscopicus* CH-7.

Acknowledgements: *This work has been funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project III 45001).*

LITERATURA

[1] Berdy J. Bioactive Microbial Metabolites. *J Antibiot.* 2005; *58*: 1-26.

[2] Watve MG, Tickoo R, Jog MM, Bhole BD. How many antibiotics are produced by the genus Streptomyces?.*Arch Microbiol.* 2001; *176*: 386-390.

[3] Ilić SB, The effect of composition and rheology of nutrition medium on kinetic of antibiotic production by bacteria *Streptomyces hygroscopicus* CH-7, Ph.D. Thesis, Faculty of Technology, University of Nis, Leskovac, 2010.

[4] Haferburg G, Kothe E. Microbes and metals: interactions in the environment. *J Basic Microbiol*. 2007; 47: 453-467.

[5] Schrader KK, Blevins WT. Effects of carbon source, phosphours concentration, and several micronutrients on biomass and geosmin production by *Streptomyces halstedii*. *J Ind Microb Biotech.* 2001; 26: 241-247.

[6] Thanh HL, Nga TV, Thuy TH, Tam BKN, Van HTD. Effects of some microelements on antifungal activity and biomass of the *Actinomyces* producing Validamycin-A. *J Viet Env*. 2018; 9:128-131.

[7] Bundale S, Begde D, Nashikkar N, Kadam T, Upadhyay A. Optimization of Culture Conditions for Production of Bioactive Metabolites by *Streptomyces spp*. Isolated from Soil. *Adv Microb*. 2015; 5: 441-451.

[8] Ripa FA, Nikkon F, Zaman S, Khondkar P. Optimal Conditions for Antimicrobial Metabolites Production from a New *Streptomyces sp*. RUPA-08PR Isolated from Bangladeshi Soil. *Mycobiology.* 2009; 37: 211-214.

[9] Hassan MA, El-Naggar MY, Said WY. Physiological factors affecting the production of an antimicrobial substance by *Streptomyces violatus* in batch cultures. *Egypt J Biology*. 2001; 3: 1-10.

[10] Majzlik P, Strasky A, Adam V, Nemec M, Trnkova L, ZehnalekHubalek J, Provaznik I, KizekR.Influence of zinc (II) and copper (II) ions on *Streptomyces* bacteria revealed by electrochemistry. *Int J ElectrochemSci.* 2011; 6: 2171-2191.

[11] Cai P, Kong F, Fink P, Ruppen ME, Williamson RT, Keiko T. Polyene antibiotics from *Streptomyces mediocidicus. J Nat Prod*. 2007; 70:215–219.

[12] Karadzic I, Gojgic-Cvijovic G, VučetićJ.Hexaene H-85, A hexaene H-85 macrolide complex. *J Antibiot*. 1991; 12: 1452-1453.

[13] Kaiser H, Keller-Schierlein W. Metabolites of microorganisms. Part 202. Structure elucidation of elaiophylin:spectroscopic studies and degradation. *HelvChim Acta*. 1981; 64:407-424.

[14] Banga J, Praveen V, Singh V, Tripathi CKM, Bihari V. Studies on medium optimization for the production of antifungal and antibacterial antibiotics from a bioactive soil actinomycete. *Med Chem Res*. 2008; 17: 425-436.

[15] Mahesh M, Meenakshi N. Effect of carbon and nitrogen source for the production of tetracycline analysis by using HPLC. *Int J Adv Biotechnol Res.* 2013; 4: 218–223.

[16] Ćirić JT, Konstantinović SS, Ilić SB, Gojgić-Cvijović G, Savić DS, Veljković VB. The impact of isatin derivatives on antibiotic production by *Streptomyces hygroscopicus* CH-7. *Hem Ind*. 2016; 70: 123-128.

[17] Ilić SB,KonstantinovićSS,Gojgić-Cvijović G, Savić DS, Veljković VB. The influence of Schiff base inclusion complexes with β-cyclodextrine on antibiotic production by *Streptomyces hygroscopicus* CH-7. *Hem Ind*. 2015; 69:9-15.

[18] Konstantinović SS, Zlatković MZ, ĆirićJT,Ilić SB, Gojgić-Cvijović G, Veljković VB. The use salicylaldehyde derivatives as a nitrogen sources for antibiotic production by *Streptomyces hygroscopicus* CH-7. *Hem Ind*. 2017; 71:487-494.

[19] Isahak WN, Ismail M, Yarmo YA, JahimJM, SalimonJ.Purification of Crude Glycerol from Transesterification RBD Palm over Homogeneous and Heterogeneus Catalysts for the Biolubricant Preparation. *J App Sci.* 2010; 10: 2590-2595.

[20] Konstantinovic SS, Radovanovic BC, Krkljes A. Thermal behaviour of Co(II), Ni(II), Cu(II), Zn(II), Hg(II) and Pd(II) complexes with isatin-β-thiosemicarbazone. *J Therm Anal Calorim.* 2007; 90: 525–531.

[21] Vucetic J, Karadzic I, Gojgic-Cvijovic G, Radovanovic E. Improving hexaene H-85 production by *Streptomyces hygroscopicus*. *J Serb Chem Soc*. 1994; 59: 973-980.

[22] Ilic SB, Konstantinovic SS, Savic DS, Veljkovic VB, Gojgic-Cvijovic G. The impact of Schiff bases on antibiotic production by *Streptomyces hygroscopicus*. *Med Chem Res.* 2010; 19: 690-697.

[23] Ilic SB, Konstantinovic SS, Gojgic-Cvijovic G, SavicDS,Veljkovic VB. The impact of glycerol and some carbohydrates on antibiotic production by *Streptomyces hygroscopicus* CH-7. *Med Chem Res*. 2013; 22: 934-937.

[24] Arai M. Azalomycins B and F. Two new antibiotics. *J Antibio Ser A*. 1960; 13: 51-56.

[25] Temperini MLA, dos Santos MR, Paoli Monteiro VR.Spectroscopic study of the isomerization of Z- to E-pyridine-2-formyl thiosemicarbazone. *Spectrochim Acta, Part* A, 1995; 51: 1517-1524.

[26] Abdulghani AJ, Hussain RK. Synthesis and Characterization of Schiff Base Metal Complexes Derived from Cefotaxime with 1H-indole-2,3-dione (Isatin) and 4-N,N-dimethyl-aminobenzaldehyde. *Open J Inor Chem*. 2015; 5: 83-101.

[27] Ferrari MB, Pelizzi C, Pelosi G, Rodrigez-Arguelles MC. Preparation, characterization and X-ray structures of 1-methylisatin 3-thiosemicarbazone copper, nickel and cobalt complexes. *Polyhedron.* 2002; 21: 2593–2599.
[28] Mohanan K, Murakan B. Complexes of Mn(II), Fe(II), Co(II), Ni(II), Cu(II) and Zn(II) with a bishydrazone. *Synth React Inorg Met Org Chem*. 2005; 35: 837–844.
[29] Konstantinovic SS, Synthesis, structure and antimicrobial activity of coordination compounds of isatin Schiff base, Ph. D. Thesis, Faculty of Mathematics and Natural Science, University of Nis, October 2007.
[30] Konstantinovic SS, Radovanovic BC, Sovilj SP, Stanojevic SS. Antimicrobial activity of some isatin-3-thiosemicarbazone complexes. *J Serb Chem Soc*. 2008; 72: 7-13.
[31] Podunavac-Kuzmanović SO, Leovac VM, Perišić-Janjić NU, Rogan J, Balaž G. Complexes of Cobalt(II), Zinc(II) and Copper(II) with Some Newly Synthesized Benzimidazole Derivatives and Their Antibacterial Activity. *J Serb Chem Soc*. 1999; 64: 381-388.
[32] Hamedi J, Nobary SG. Effect of single additive bivalent metal ions on the growth of *Streptomyces clavuligerus* and clavulanic acid production. *Prog Biolog Sci.* 2012; 3: 24-30.
[33] Kishimoto K, Park YS, Okabe M, Akiyama S. Effect of ferrous ion on amino acid metabolism in mildiomycin production by *Streptoverticilliumrimofaciens*. *J Antib*. 1997; 50: 206-211.
[34] Mansour FA, El-Shirbiny SA, El-Metwaly NA. Demethyltetracycline biosynthesis by *Streptomyces aureofaciens* Sub-species viridulans as influenced by medium composition. *Egypt J Microbiol*. 1996; 31: 221-235.
[35] Gesheva V, Ivanova V, Gesheva R. Effects of nutrients on the production of AK-111-811 macrolide antibiotic by *Streptomyces hygroscopicus*. *Microbiol Res*. 2005; 160: 243-248.

САЖЕТАК

Продукција антибиотика помоћу *Streptomyces hygroscopicus* CH-7 у хранљивим подлогама са комплексима шифових база

Славица Б. Илић[1], Сандра С. Константиновић[1], Гордана Д. Гојгић Цвијовић[2], Влада Б. Вељковић[1]

[1]*Универзитет у Нишу, Технолошки факултет, Лесковац*
[2]*Универзитет у Београду, Институт за хемију, технологију и металургију, Београд, Србија*

(Научни рад)

У раду је испитиван утицај модификованих хранљивих подлога на производњу антибиотика Hexaene H-85 и Azalomycine B помоћу *Streptomyces hygroscopicus* CH-7. Аминокиселина L-триптофан, као извор азота, замењена је изатин-3-тиосемикарбазоном (синтетисаним у сировом глицеролу који се добија као нуспродукт у производњи биодизела из сунцокретовог уља) и његовим комплексима са неким двоалентним металним јонима. Комплекси су карактерисани елементарном микроанализом, магнетном сусцептибилношћу, атомском аспорпцијом (A), инфрацрвеним (IR) и ултравиолетним (UV/VIS) спектроскопским методама. Спектралне анализе показале су октахедралну геометрију комплекса Mn(II), Fe(II) и Ni(II) и тетраедарску за комплекс Zn(II). Изатин-3-тиосемикарбазон и његови комплекси са металима (0,5 g dm^{-3}) утичу на повећање продукције антибиотика у поређењу са основном подлогом. Највеће концентрације антибиотика Hexaene H-85 и Azalomycine B су остварене у хранљивој подлози са Fe(II) комплексом (306 и 127 μg cm^{-3}, редом). Комплекси ИТС и изатин 3-тиосемикарбазон утичу и на морфологију тестираног соја. *Streptomyces hygroscopicus* CH-7 расте у облику филамената и пелета, у подлогама у којима је остварена највећа продукција антибиотика.

Кључне рачи: продукција антибиотика, изатин-3-тиосемикарбазон, комплекси метала, морфологија, *Streptomyces hygroscopicus*