The impact of isatin derivatives on antibiotic production by *Streptomyces hygroscopicus* CH-7

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Abstract

The effect of isatin derivatives as a nitrogen source on antibiotic (hexaene H-85 and azalomycine B) production by *Streptomyces hygroscopicus* CH-7 was studied. Isatin-3-hydrazone, 5-chloroisatin-3-hydrazone, isatin-3-tosylhydrazone, 5-chloroisatin-3-tosylhydrazone, isatin-3-(4`-hidroxy)benzoilhydrazone and 5-chloroisatin-3-(4'-hidroxy)benzoylhydrazone were synthesized in a crude glycerol, obtained during the biodiesel production from edible sunflower oil. The highest concentration of Hexaene H-85 is achieved with 5-chloroisatin-3-hydrazone (197 μg/cm^3) in medium, while isatin-3-hydrazone has the greatest impact on azalomycine B production (72 μg/cm^3).

Keywords: isatin derivatives, *Streptomyces hygroscopicus*, hexaene H-85, azalomycine.

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A large number of bacteria and fungi have the ability to produce secondary metabolites. Microorganisms are the main sources of bioactive components, of which more than 60% are produced by Actinomycetales, 28% by moulds and about 11% by nonfilamentous organisms. Antibiotics are the most important secondary metabolites [1,2], and about three-quarters of known antibiotics with different chemical structures are produced by Actinomycetales [3,4]. Species of the genus Streptomyces are known as one of the best antibiotic producers [5], whereby some strains can produce more than 180 different secondary metabolites [6].

Streptomyces hygroscopicus CH-7 produces antibiotics such as hexaene H-85, nigericin and azalomycine B. By changing the conditions of fermentation process and the composition of the nutrient medium at an early stage of trial, it is possible to increase the yield of antibiotics [7,8].

The production of antibiotics by *Streptomyces* species depends on the growth phase. The secondary metabolism occurs when growth is limited, when nutrients are worn-out or their availability is reduced. The nature of limiting nutrient is very important, and essential ingredients of substrate are carbon, nitrogen and phosphorus. Other nutrients, such as mineral substances, have an impact on production, but their absence is not essential [9,10].

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Isatin derivatives possess different biological activity, such as antimicrobial, anticonvulsative, anticancer, antiHIV, etc. [11,12]. The usage of some isatin derivatives, such as isatin-3-thiosemicarbazone, isatin-3--semicarbazone and isatin-3-phenylhydrazone as a nitrogen source for antibiotic production of *S. hygroscopicus* CH-7 significantly increased hexaene H-85 and azalomycine B production [9,13,14]. Since those isatin derivatives were synthesized in crude glycerol as a green solvent, and similar compounds have a positive effect on antibiotic production, the idea was to replace a part of tryptophan with isatin products and gained even better results in hexaene H-85 and azalomycine B production.

MATERIALS AND METHODS

Organism, media and growth conditions

A strain *Streptomyces hygroscopicus* CH-7 (NCAIM (P) B-001336) was gained from the Microbial Collection at Faculty of Chemistry and Institute of Chemistry, Technology and Metallurgy in Belgrade, Serbia [15,16]. The culture was stored at 4 °C at soybean medium containing the following: 15 g/dm^3 glucose; 10 g/dm^3 soybean; 3 g/dm^3 $CaCO_3$; 3 g/ dm^3 NaCl; 2 g/ dm^3 agar (pH 7.2). Flasks (250 ml) that contained 50 ml of this media were inoculated with 0.1 ml of spore suspension and incubated at 30 °C with shaking at 200 rpm. The fermentation media were inoculated with 5 vol.% of a preculture after 48 h growth and incubated at 30 °C for 240 h under the standard condition of aeration and agitation (200 rpm). The composition of media used for fermentation were: basal medium (M_1, 15 g/dm^3 glucose; 10 g/dm^3 soybean; 5 g/dm^3 yeast extract; 3 g/dm^3 $CaCO_3$; 3 g/dm^3 NaCl; 0.5 g/dm^3 $MgSO_4 \cdot 7H_2O$; 0.5

g/dm^3 $(NH_4)_2HPO_4$; 1 g/dm^3 K_2HPO_4); medium with tryptophan (M_2, 15 g/dm^3 glucose; 15 g/dm^3 tryptophan; 3 g/dm^3 $CaCO_3$; 3 g/dm^3 NaCl; 0.5 g/dm^3 $MgSO_4 \times 7H_2O$; 0.5 g/dm^3 $(NH_4)_2HPO_4$; 1 g/dm^3 K_2HPO_4) and media with isatin derivatives (15 g/dm^3 glucose; 10 g/dm^3 isatin derivatives; 5 g/dm^3 tryptophan; 3 g/dm^3 $CaCO_3$; 3 g/dm^3 NaCl; 0.5 g/dm^3 $MgSO_4 \times 7H_2O$; 0.5 g/dm^3 $(NH_4)_2HPO_4$; 1 g/dm^3 K_2HPO_4).

The results were obtained by measuring absorbance at λ_{max} = 364 nm (Hexaene H-85) and λ_{max} = 252 nm (azalomycine B) with Perkin-Elmer Lambda 15 UV/Vis spectrophotometer [15,16]. Microbial growth was determined by measuring dry weights of cells [16].

Synthesis of isatin derivatives in the crude glycerol

All chemicals, except crude glycerol, were of analytical grade and used without further purification. They were purchased from Sigma Aldrich. The crude glycerol, a by-product in the production of biodiesel from sunflower oil, was obtained from the Laboratory for Chemical Engineering, Faculty of Technology, Leskovac. The excess of methanol was removed from the crude glycerol by distillation. After distillation, the acidity of crude glycerol was adjusted to pH 5 by addition of 85% phosphoric acid. The inorganic salts formed in this stage were then removed by centrifugation at 400 rpm for 15 min.

Isatin derivatives were synthesized by the reaction of equimolar amounts of isatin and amine components in the crude glycerol as a green solvent [17]. The mixture was refluxed at 80 °C. The products, precipitated as a colored solid, were filtered and washed out with water.

RESULTS AND DISCUSSION

To achieve better concentration of antibiotics, soybean and yeast extract in basal medium were replaced with tryptophan (15 g/dm^3) and mixtures of tryptophan (5 g/dm^3) and isatin derivatives (10 g/dm^3). Amino acids are known as a good nitrogen source [18,19], as well as tryptophan, which was already used for antibiotic production by *Streptomyces hygroscopicus* CH-7 [14]. Tryptophan is similar to the isatin (indole moiety is constitutional part of their structure), and therefore, in this paper, isatin derivatives were used as a nitrogen sources for antibiotic production by *S. hygroscopicus* CH-7. Isatin derivatives (Fig. 1) were synthesized by using „green method“ in a crude glycerol obtained as a by-product in biodiesel production.

Table 1 shows the effect of tryptophan and isatin-3--hydrazone, 5-chloroisatin-3-hydrazone, isatin-3-tosylhydrazone, 5-chloroisatin-3-tosylhydrazone, isatin-3--(4'-hidroxy)benzoylhydrazone and 5-chloroisatin-3-(4'--hidroxy)benzoilhydrazone on concentration of dry biomass and antibiotics, while the kinetic of fermentation is shown in Figures 2–4.

Figure 2 shows the variation of dry biomass during the fermentation. Independently of nitrogen source, the concentration of dry biomass increased during the first 72 h of fermentation, after which it began to decrease. The highest concentration of dry biomass was

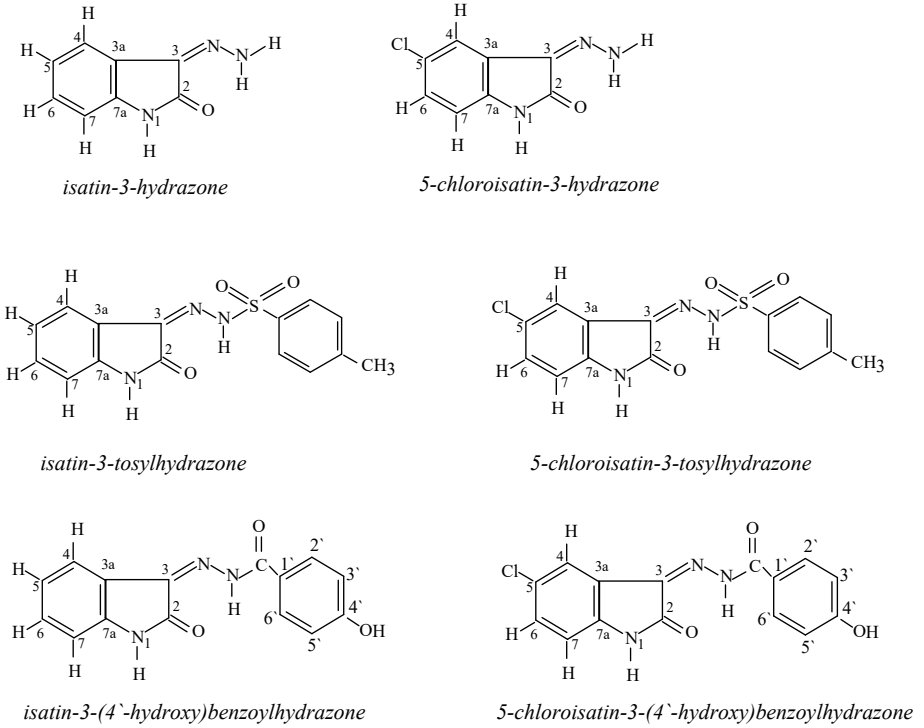

Fig. 1. Chemical structure of isatin derivatives synthesized in the crude glycerol as a green solvent.

Table 1. The effect of tryptophan and isatin derivatives as a nitrogen source on the maximum dry biomass concentration (X_{max}) and the maximum antibiotic concentration (c_{max})

Nitrogen source	Dry biomass X_{max} / g dm^{-3}	Hexaene H-85 c_{Hmax} / μg cm^{-3}	Azalomycine B c_{Emax} / μg cm^{-3}
M_1	9.0	114	36
M_2	8.3	156	48
5-chloroisatin-3-hydrazone + tryptophan (10 g/dm^3+5 g/dm^3)	8.6	197	61
5-chloroisatin-3-tosylhydrazone + tryptophan (10g/dm^3+5g/dm^3)	8.0	172	54
Isatin-3-tosylhydrazone + tryptophan (10g/dm^3+5g/dm^3)	8.1	165	67
Isatin-3-hydrazone + tryptophan (10g/dm^3+5g/dm^3)	9.0	183	72
Isatin-3-(4`-hidroxy)benzoylhydrazone + tryptophan (10g/dm^3+5g/dm^3)	8.2	145	49
5-chloroisatin-3-(4`-hidroxy)benzoilhydrazone + tryptophan (10g/dm^3+5g/dm^3)	7.9	162	51

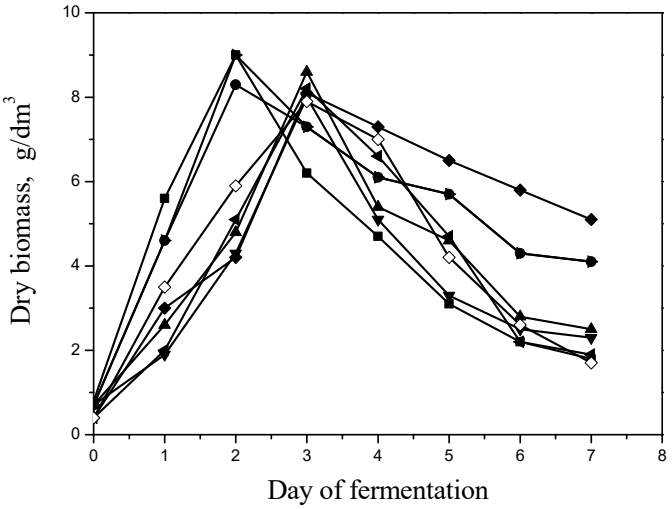

Fig. 2. Variation of dry biomass concentration during the fermentation of Streptomyces hygroscopicus CH-7 in basal medium (■) and media with tryptophan (●); 5-chloroisatin-3-hydrazone (▲); 5-chloroisatin-3-tosylhydrazone (▼); isatin-3-tosylhydrazone (♦); isatin-3-hydrazone (►); isatin-3-(4`-hidroxy)benzoilhydrazone (◄); 5-chloroisatin-3-(4`-hidroxy)benzoilhydrazone (◊).

achieved in medium M_1 and medium modified with isatin-3-hydrazone, after 48 h (9.0 g/dm^3). Comparing to all tested media, the lowest value of dry biomass was achieved with 5-chloroisatin-3-tosylhydrazone (8.0 g/dm^3).

The results obtained during the ferementation show that isatin derivatives have different impact on antibiotic production by *Streptomyces hygroscopicus* CH-7 (Table 1 and Fig. 3). The concentration of hexaene H-85 increases in first 48 h and reaches the highest values (197 μg/cm^3) with 5-chloroisatin-3-hydrazone as a nitrogen source in 4th day of fermentation. This is 72% higher, while the yield of hexaene H-85 in medium with 5-chloroisatin-3-tosylhydrazone is higher for 51% than value for medium M_1, actually 26 and 11% higher than medium M_2. Higher values for antibiotic concentration, comparing to basal medium and medium with tryptophan were also obtained in media with isatin-3--tosylhydrazone (165 μg/cm^3) and isatin-3-hydrazone (183 μg/cm^3). The highest concentration of hexaene H-85 in a medium with tryptophan is achieved during the 72 h of fermentation (156 μg/cm^3), which is 36% higher than in medium with soybean and yeast extract.

The variation of azalomycine B during the fermentation is given in a Fig. 4. The highest concentration of azalomycine B in basal medium was reached after 72 h of fermentation (36 μg/cm^3) and in the media with tryptophan and isatin derivatives during 72–96 h. The increase of azalomycine B concentration in the medium with tryptophan is 33% higher than basal medium (Table 1). The addition of 5-chloroisatin-3-hydrazone and 5-chloroisatin-3-tosylhydrazone stimulates azalomycine B production, with maximum 61 and 54 μg/cm^3, respectively. The higher yield was achieved in media with isatin-3-tosylhydrazone (67 μg/cm^3) and isatin-3--hydrazone (72 μg/cm^3).

It`s very difficult to find a connection between the structure of isatin derivatives and antibiotic production. The results show that isatin-3-hydrazone and 5-chloroisatin-3-hydrazone have greater influence on hexaene H-85 production. The main structure of those compounds is identical, and the only difference is in sub-

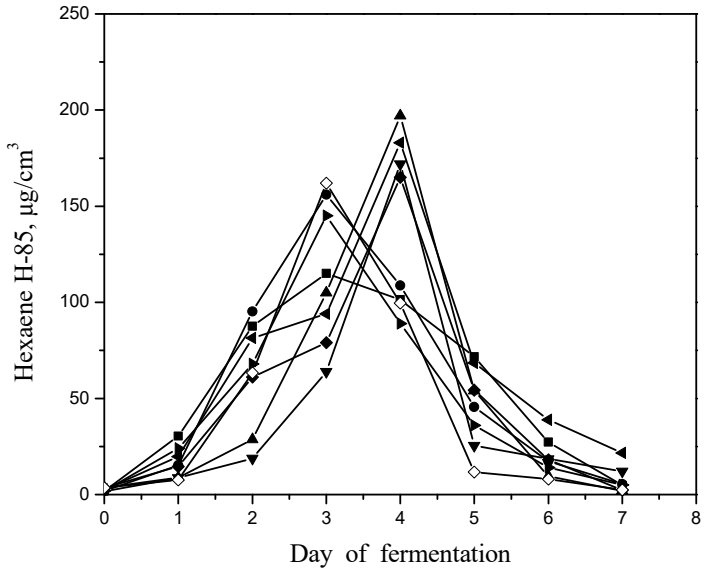

Fig. 3. Variation of Hexaene H-85 concentration during the fermentation in basal medium (■) and media with tryptophan (●); 5-chloroisatin-3-hydrazone (▲); 5-chloroisatin-3-tosylhydrazone (▼); isatin-3-tosylhydrazone (♦); isatin-3-hydrazone (►); isatin-3-(4`-hidroxy)benzoilhydrazone (◄); 5-chloroisatin-3-(4`-hidroxy)benzoilhydrazone (◊).

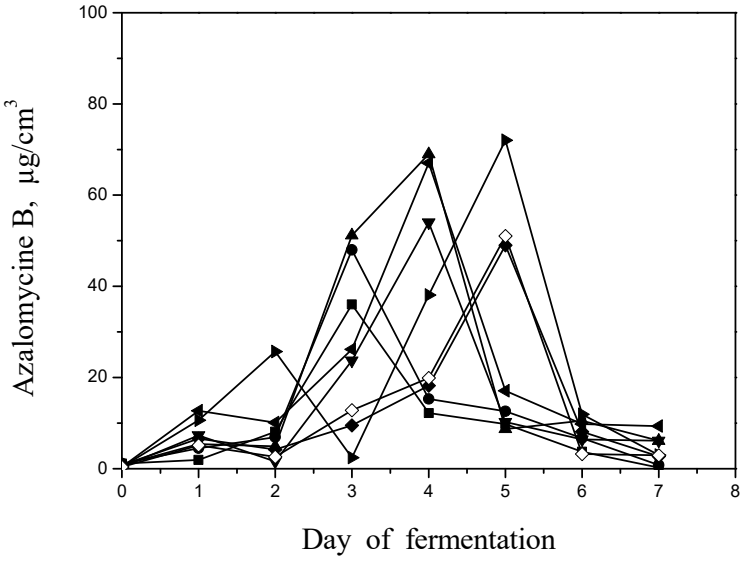

Fig. 4. Variation of Azalomycine B concentration during the fermentation in basal medium (■) and media with tryptophan (●); 5-chloroisatin-3-hydrazone (▲); 5-chloroisatin-3-tosylhydrazone (▼); isatin-3-tosylhydrazone (♦); isatin-3-hydrazone (►); isatin-3-(4`-hidroxy)benzoilhydrazone (◄); 5-chloroisatin-3-(4`-hidroxy)benzoilhydrazone (◊).

stituent at position 5, which means that chloro-ion does not have a negative effect on antibiotic production.

On the other hand, isatin derivatives with no chloro substituent in position 5 (isatin-3-tosylhydrazone and isatin-3-hydrazone), have a better influence on azalomycine B production. Isatin-3-hydrazone has the best impact on azalomycine B production. It`s main difference with isatin-3-tosylhydrazone is in SO_2 group and aromatic moiety, which means that those groups reduce azalomycine B production.

CONCLUSIONS

Comparing to the results obtained for similar nitrogen sources [13,14] the impact of isatin-3-hydrazone, 5-chloroisatin-3-hydrazone, isatin-3-tosylhydrazone, 5-chloroisatin-3-tosylhydrazone, isatin-3-(4'-hidroxy)-benzoylhydrazone and 5-chloroisatin-3-(4'-hidroxy)benzoylhydrazone on antibiotic production is lower than those achieved for isatin-3-thiosemicarbazone, isatin-3--semicarbazone and isatin-3-phenylhydrazone [13,14]. Those were expected, especially with isatin-3-thiosemi-

carbazone and isatin-3-semicarbazone**,** since their structure is the most similar with tryptophan.

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IZVOD

UTICAJ DERIVATA IZATINA NA PRODUKCIJU ANTIBIOTIKA POMOĆU *Streptomyces hygroscopicus* CH-7

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(Naučni rad)

Intenzivna istraživanja poslednjih godina vrše se na polju poboljšanja produkcije sekundarnih metabolita sa različitom aktivnošću i primenom u biotehnologiji. Streptomiceta *Steptomyces hygroscopicus* raste i produkuje sekundarne metabolite na podlogama različitog sastava. Izvor azota značajno utiče kako na primarni tako i na sekundarni metabolizam, odnosno na rast, razvoj i produkciju sekundarnih metabolita. Pri fermentaciji u tečnoj podlozi, ovaj soj proizvodi smešu antibiotika. U ovom radu je proučavan uticaj derivata izatina, kao izvora azota na produkciju antibiotika heksaena H-85 i azalomicina B pomoću soja *Streptomyces hygroscopicus* CH-7. Derivati izatina poseduju različite biološke aktivnost i dosadašnjim istraživanjima je ustanovljeno da imaju stimulativno dejstvo kako na primarni tako i na sekundarni metabolizam. Izatin-3-tozilhidrazon, izatin-3-hidrazon, izatin-3-(4`-hidroksi)benzoilhidrazon**,** 5-hloroizatin-3-(4`-hidroksi)benzoilhidrazon, 5-hloroizatin-3-tozilhidrazon i 5-hloroizatin-3-hidrazon su sintetisani u sirovom glicerolu dobijenom tokom procesa proizvodnje biodizela od jestivog suncokretovog ulja. Najviša koncentracija Heksaena H-85 je postignuta u podlozi sa 5-hloroizatin-3-hidrazonom (197 μg/cm^3) dok je izatin-3-hidrazon imao najveći uticaj na produkciju azalomicina B (72 μg/cm^3).

Ključne reči: Derivati izatina • *Streptomyces hygroscopicus* • Heksaena H-85 • Azalomicina B