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ANTIMICROBIAL AGENTS PRODUCTION BY *Streptomyces* sp.

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Abstract: *The aim of this research was to investigate the possibility of antimicrobial substances production in a medium containing different carbon and nitrogen sources using Streptomyces hygroscopicus. Screening of antimicrobial activity included 6 test microorganisms: Aspergillus niger, Candida albicans, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa. The cultivation was carried out under aerobic conditions at 27°C for 168 h. The diffusion-disc method was employed to determine the antimicrobial activity of cultivation broths. According to the results, Streptomyces hygroscopicus has showed ability to produce antimicrobial substances against the examined fungi and Gram-positive bacteria in applied experimental conditions.*

Key Words: ***Streptomyces hygroscopicus/ antimicrobial activity/ secondary metabolites/ diffusion-disc method/***

1. INTRODUCTION

The increasing problem of antibiotic resistance presents a challenge for many research groups in the way of not only finding and exploring the potential of new bioactive substances, but also developing rational approaches for their production. The results of extensive studies led to discovery of about 4000 antibiotic substances produced by bacteria and fungi. Great amount of them are produced by streptomycetes, including aminoglycosides, macrolides, peptides, polyenes, polyether, tetracyclines, etc [1]. Extracellular metabolites from *Streptomyces* isolates are of high economical and biotechnological value due to importance of their superior antifungal and antibacterial activity [2,3]. Potential to produce more than one antifungal agent is in accordance with activity against fungal strains that belong to different taxonomic groups [4]. Additionaly, significant amount of the commercially and medically used antibiotics, around 75%, are produced by filamentous bacteria, while about 60% are used in agriculture [2].

When it comes to the quality and quantitaty of secondary metabolites production, it is clear that they depend on the strains and species of microorganisms used, and also on their nutritional and cultural conditions [5]. Different carbon and nitrogen sources used in the cultivation medium have a great influence on synthesis of chemically distinct biological metabolites. Medium content and production conditions should stimulate the synthesis of secondary metabolites [6,7]. On the other hand, there is also a focus on possibility to find and utilize some cheaper nutrients, which could replace the traditional ones, and significantly decrease production costs [8].

Aspergillus niger presents one of the the most important fungiused in biotechnology, but also contaminant commonly present in food. It has a potential to produce two different groups of mycotoxins: fumonisins and ochratoxins. It is important to mention that it can cause various plant disorders and post-harvest contamination of some agricultural products [9,10].

Although *Candida albicans* normally resides in the human body, combination of different factors can lead to even potentially life-threatening systemic infections. Pathogenic potential of *Candida albicans* is based on different virulence factors and a number of atributes, like effective nutrient acquisition systems, metabolic adaptation, polymorphism, ability to form biofilms, environmental stress response and many others [11].

Staphylococcus aureus is Gram-positive bacterium, with a great resistance against all classes of antibiotics that can be explained by two different mechanisms: gene mutation and horizontal gene transfer. Infections caused by *Staphyloccocus aureus* may be the result of direct infection or the production of bacterial toxins [12].

Enterococcus faecalis is Gram-positive bacterium, well-known as a normal member of the human gastrointestinal tract. Also, it is associated with the majority of human enterococcal infections [13].

Escherichia coli is Gram-negative bacterium and one of the most thoroughly studied bacterial species. While most isolates are common inhabitants of the gastrointestinal tract, some strains have developed

pathogenic mechanisms and ability to cause diseases in humans and animals [14].

Pseudomonas aeruginosa is Gram-negative microorganism, medically significant for its potential to cause a broad spectrum of opportunistic infections. It has abillity to grow in a medium with minimal nutritional requierements, and it is difficult to control with antibiotics [15].

The principal aim of this research was to examine the possibility of antimicrobial substances production in a medium containing different carbon and nitrogen sources using *Streptomyces hygroscopicus*. This study inolved the following test microorganisms: *Staphylococcus aureus* and *Enterococcus faecalis* as representatives from Gram-positive bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* as representatives from Gram-negative bacteria. Antifungal activity was examined using *Aspergillus niger* and *Candida albicans*.

2. EXPERIMENTAL

2.1. Producing microorganism

The producing microorganism used for experiments was *Streptomyces hygroscopicus*, isolated from soil samples collected from various locations from the territory of Novi Sad, Serbia and stored in the Microbial Culture of the Faculty of Technology Novi Sad, Serbia. According to the Bergey's Manual of Systematic Bacteriology, the isolate was identified as *Streptomyces hygroscopicus,* by analysing morphological, biochemical and physiological properties.

2.2. Cultivation medium

The medium for the growth of producing microorganisms contained following components (g/L): glucose (15.0), soybean meal (10.0), $CaCO_3$, (3.0), NaCl, (3.0), $MgSO_4$, (0.5), $(NH_4)_2HPO_4$, (0.5), K_2HPO_4, (1.0). Medium for biosynthesis of antimicrobial substances consisted of the different combinations of carbon and nitrogen sources. The amount of carbon sources used in experiments (maltose, fructose, lactose, glycerol and starch) was 15.0 g/L. Nitrogen source (yeast extract, soybean meal, peptone, $NaNO_3$) amount was calculated to be equal to nitrogen quantity present in 10.0 g/L of soybean meal. The cultivation medium was inoculated with 10% (v/v) of a preculture grown for 48 h. Production was carried out during the next 168 h, at 27°C under standard conditions of aeration, with agitation using rotary shaker at 150 rpm. In the end of bioprocess, after 7 days, cultivation broth samples were centrifuged at 10 000 g (Eppendorf Centrifuge 5804th) for 10 min. After the separation of solid and liquid phase, supernatant was evaporated to achieve 1/10 of the initial volume, using rotary vacuum evaporator (Rova-100, MRC, London, UK) and used for the further analysis.

2.1. Analytical methods

The antimicrobial activity of the cultivation medium was determined using test microorganisms by diffusion - disc method. After seeding Petri dishes with test organisms, sterile discs (HiMedia, India) were impregnated with previously concentrated supernatant, dried and placed onto them. After incubation at 30°C for 48 hours, the inhibition zone diameters were measured by a special ruler (HiAntibiotic ZoneScale, Himedia ®).

3. RESULTS AND DISCUSSION

Depending on nutritional and cultural conditions, *Streptomyces hygroscopicus* has ability to produce a great number of secondary metabolites which have a wide spectrum of biological activities. The chemical diversity of substances with antibacterial and antifungal activity is fueled by the emergence of different biosynthetic pathways in secondary metabolism [16].

Two-way ANOVA was employed to determine whether there are significant differences between inhibition zone diameters obtained by assaying *Streptomyces* sp. against examined bacteria and fungi, as a result of using different test microorganisms and different carbon and nitrogen sources in cultivation medium. ANOVA results showed that effects of carbon and organic nitrogen sources, test microorganisms, as well as their interaction, were significant (*p*-values less than 0.05) (Table 1).

Table 1. *Univariate tests of significance for inhibition zone diameters [mm]*

Effect	SS	DF	MS	F	*p* - value
Intercept	20850.70	1	20850.70	22044.80	*0.0000
C and N	1582.17	29	54.56	57.68	*0.0000
Test microrganism	48.15	3	16.05	16.97	*0.0000
C and N · Test microrganism	1216.48	87	13.98	14.78	*0.0000
Error	113.50	120	0.95		

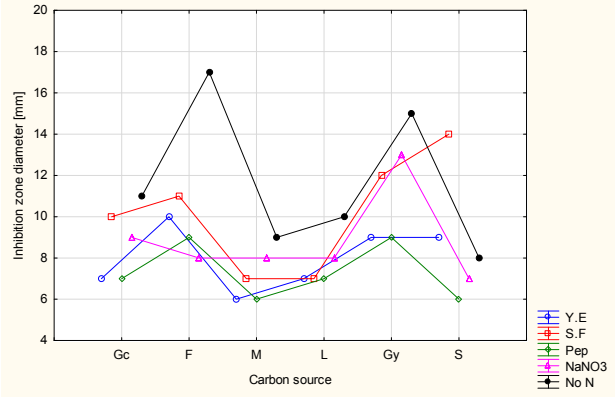
C – carbon source; N – nitrogen source; SS – sum of squares; DF – degree of freedom; MS – mean square
*statistically significant difference

The obtained results clearly indicated opposed susceptibility of Gram-positive, Gram-negative bacteria and fungi to *Streptomyces hygroscopicus* antimicrobal substances, which can be explained by the existing morphological differences between these microorganisms. Gram-negative bacteria cell walls create an impermeable barrier consisting of thin peptidoglycan layer, surrounded by a thick membrane. Results of this study have revealed that antimicrobial substances produced by *Streptomyces hygroscopicus* hadn't showed any activity against tested Gram-negative bacteria. On the other hand, Gram-positive bacteria have only one layer of peptidoglycan, which is not sufficient to protect them from the produced antimicrobial substances. Similar to that, cell walls of fungi are made of glycoproteins and polysaccharides, thus making them more sensitive to antifungal substances activity [1,17].

Results of this study also confirmed that utilization of different carbon and nitrogen sources in cultivation medium reflects on the intensity of the antimicrobial effect on test microorganisms, probably caused by diversity of produced metabolites. Production of different metabolites arises from intracellular intermediates, which transform into more complex structures through specified biochemical pathways. The source of nitrogen is often crucial factor due to the fact

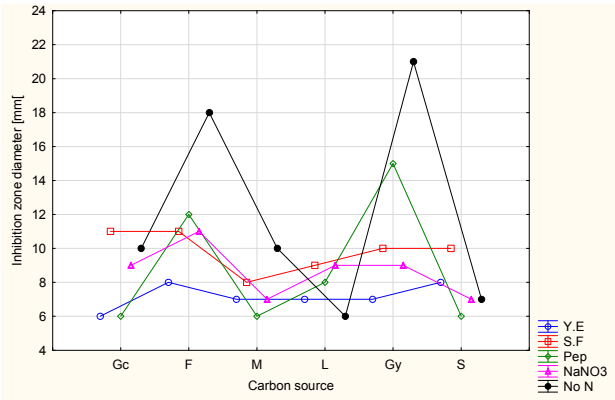
that it strongly influences the metabolites formation. Beside the type of nutrient, the synthesis is influenced by their concentration and availability in the culture media, which also affect the biomass growth [16,18].

Figures 1-4 were generated in order to determine the optimal combination of carbon and nitrogen sources for production of antimicrobial substances effective against tested microorganisms. In applied experimental conditions, production of antifungal metabolites is increased by the presence of fructose or glycerol in cultivation medium that doesn't contain nitrogen. Considering economical aspect, beside the fact that nitrogen is not necessary for production, possibility to utilize glycerol in a cultivation media preparation is a significant benefit. It is a cheap by-product of transesterification in biodesel production, with a great potential to be used in fermentation as a carbon source [19]. Also, significantly large inhibition zone diameters for *Aspergillus niger* were obtained using cultivation medium that contains starch and soybean meal, while in case of *Candida albicans* it was combination of glycerol and peptone.



GC – glucose; F – fructose; M – maltose; L – lactose; Gy – glycerol; S – starch; Y.E – yeast extract; S.F – soybean meal; Pep – peptone

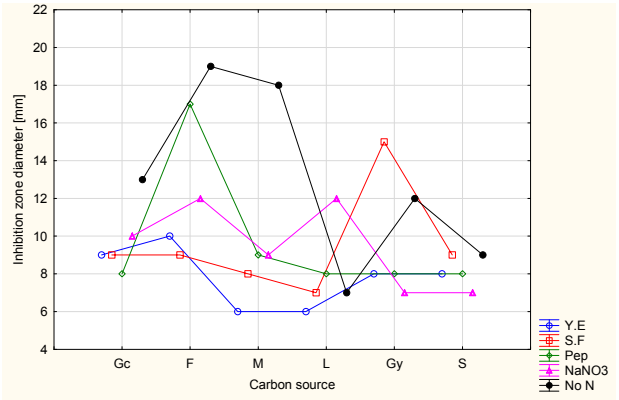
Fig. 1. *Inhibition zone diameters obtained using different carbon and nitrogen sources in cultivation medium for Streptomyces hygroscopicus against Aspergillus niger*



GC – glucose; F – fructose; M – maltose; L – lactose; Gy – glycerol; S – starch; Y.E – yeast extract; S.F – soybean meal; Pep – peptone

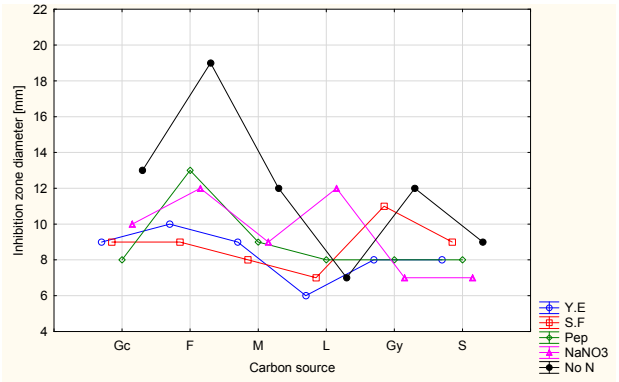
Fig. 2. *Inhibition zone diameters obtained using different carbon and nitrogen sources in cultivation medium for Streptomyces hygroscopicus against Candida albicans*

The results presented in Figures 3 and 4 show that production of antibacterial substances against Gram-positive bacteria was the highest in a medium also containing fructose, and without nitrogen source. Large inhibiton zone diamater was obtained in the case of *Staphylococcus aureus* in the presence of peptone as a nitrogen source in combination with fructose. Also, cultivation in the medium without nitrogen source, but with maltose as a carbon source, stimulated production of metabolites with antibacterial activity against *Staphylococcus aureus.*



GC – glucose; F – fructose; M – maltose; L – lactose; Gy – glycerol; S – starch; Y.E – yeast extract; S.F – soybean meal; Pep – peptone

Fig. 3. *Inhibition zone diameters obtained using different carbon and nitrogen sources in cultivation medium for Streptomyces hygroscopicus against Staphylococcus aureus*



GC – glucose; F – fructose; M – maltose; L – lactose; Gy – glycerol; S – starch; Y.E – yeast extract; S.F – soybean meal; Pep – peptone

Fig. 4. *Inhibition zone diameters obtained using different carbon and nitrogen sources in cultivation medium for Streptomyces hygroscopicus against Enterococcus faecalis*

4. CONCLUSION

The results of this study have pointed out that *Streptomyces hygroscopicus* has ability to produce antimicrobial substances effective against the examined representatives from Gram-positive bacteria and fungi using different carbon and nitrogen sources.The medium content that provided the best results of antimicrobial metabolites production contained fructose as a carbon source, and no nitrogen source. Also, this study

confirmed the necessity to investigate the medium composition and further analysis should involve optimization of nutrients content in order to obtain highly efficient antimicrobial substances.

5. REFERENCES

[1] S. Parthasarathi, S. Sathya, G. Bupesh, M. Manikandan, C. J. Kim, T. Manikandan, and K. Balakrishnan, "Isolation, Characterization and Extraction of Antimicrobial Compound from Marine Actinomycete *Streptomyces hygroscopicus* BDUS 49", *Res. J. Biotechnol*., 2013, Vol. 8, No. 3, pp. 40-48.

[2] M. E. El-Safey, M. H. Atta and K. M. AlJaralah, "Antibiotic Production by *Streptomyces hygroscopicus*, M 121 Isolated from Kingdom of Saudi Arabia", *Life Sci. J*., 2013, Vol. 10, No. 2, pp 1157-1163.

[3] J. A. Grahovac, M. Grahovac, J. M. Dodić, B. Ž. Bajić, J. S. Balaž, "Optimization of Cultivation Medium for Enhanced Production of Antifungal Metabolites by *Streptomyces hygroscopicus*", *Crop Prot.,* 2014, Vol 65, pp. 143-152.

[4] I. Tadijan, J. Grahovac, J. Dodić, M. Grahovac, S. Dodić, "*Effect of Cultivation Time on Production of AntifungalMetabolite(s) by Streptomyces hygroscopicus in Laboratory-Scale Bioreactor*," *J Phytopathol.,* 2015, Vol. 164, No. 5, pp. 310-317.

[5] N. Singh, and V. Rai, "Improved Antimicrobial Compound Production by a New Isolate *Streptomyces hygroscopicus* MTCC 4003 using Plackett-Burman design and Response Surface Methodology", *Bioinformation,* 2012, Vol. 21, No. 8, pp. 1021-1025.

[6] J. M. Dodić, J. A. Grahovac, I. Ž. Tadijan, S. N. Dodić, D. G. Vučurović, S. D. Popov, "Screening of antibiotics production by *Streptomyces* spp. Strains isolated from the enviroment", *Proc. of The Forth Joint UNS - PSU International Conference on BioScience: Biotechnology and Biodiversity*, Jun 18-20, 2012, Novi Sad, Serbia, pp. 145-149.

[7] J. M. Dodić, J. A. Grahovac, N. D. Kalajdžija, S. Z. Kovačević, L. R. Jevrić, Sanja O. Podunavac Kuzmanović, "Chemometric approach to prediction of antibacterial agents production by *Streptomyces hygroscopicus*", *Appl. Biochem. Biotechnol*., 2014, Vol. 174, No. 2, pp. 534-541

[8] J. A. Grahovac, B. Ž. Bajić, J. M. Dodić, Z. Z. Rončević, A.I. Jokić, "Optimization of Cultivation Medium for the Production of Antibacterial Agents", *APTEFF*, 2013, Vol. 44, No 1, pp. 217-227.

[9] J. C. Frisvad, T. O. Larsen, U. Thrane, M. Meijer, J. Varga, R. A. Samson, and K. F. Nielsen,, "Fumonisin and ochratoxin production in industrial *Aspergillus niger* Strains," *PLoS One*, Vol. 6, No. 8, pp. e23496, 2011.

[10] S. Kocsubé, J. Várga, G. Szigeti, N. Baranyi, K. Suri, B. Tóth, É. Toldi, T. Bartók, and A. Mesterházy, "*Aspergillus* Species as Mycotoxin Producers in Agricultural Products in Central Europe," *Jour. Nat. Sci. Matica Srpska Novi Sad*, 2013, No. 124, pp. 13-25.

[11] F. L. Mayer, D. Wilson, and B. Hube, "*Candida Albicans* Pathogenicity Mechanisms", *Virulence*, 2013, Vol. 4, No. 2, pp. 119–128.

[12] H. Grundmann, M. Aires-De-Sousa, J. Boyce, and E. Tiemersma, "Emergence and Resurgence of Meticillin-resistant *Staphylococcus aureus* as a Public-health Threat", *Lancet*, 2006, Vol. 368, No. 9538, pp. 874-885.

[13] H. C. Vebo, M. Solheim, L. Snipen, I. F. Nes, and D. A. Brede, "Comparative Genomic Analysis of Pathogenic and Probiotic *Enterococcus faecalis* Isolates, and Their Transcriptional Responses to Growth in Human Urine", *Plos One*, 2010, Vol. 5, No. 8, pp. e12489.

[14] A. Clements, J. C. Young, N. Constantinou, and G. Frankel, "Infection Strategies of Enteric Pathogenic *Escherichia coli*", *Gut Microbes*, 2012, Vol. 3, No. 2, pp. 71-87.

[15] R. E. Vance, S. Hong, K. Gronert, C. N. Serhan, and J. J. Mekalanos, "The Opportunistic Pathogen *Pseudomonas aeruginosa* Carries a Secretable Arachidonate 15-lipoxygenase," *Proc. Natl. Acad. Sci. USA*, 2004, Vol. 101, No. 7, pp. 2135-2139.

[16] V. A. Mariadhas, T. S. Rejiniemon, N. A. Al-Dhabi, V. Duraipandiyan, S. Ignacimuthu, P. Agastian, S. Kim, V. A. J. Huxley, K. D. Lee, and K. C. Choi, "Nutritional Requirements for the Production of Antimicrobial Metabolites from *Streptomyces*" *Afr. J. Microbiol. Res*., 2014, Vol. 8, No. 8, pp. 750-758.

[17] S. M. Bowman, and S. J. Free, "The Structure and Synthesis of the Fungal Cell Wall", *Bioessays*, 2006, Vol. 28, No. 8, pp. 799-808.

[18] S. Sanchez, A. Chavez, A. Forero, Y. Garcia-Huante, A. Romero, M. Sanchez, D. Rocha, B. Sanchez, M. Avalos, S. Guzman-Trampe, R. Rodriguez-Sanoja, E. Langley, and B. Ruiz, "Carbon Source Regulation of Antibiotic Production", *J. Antibiot. (Tokyo)*, 2010, Vol. 63, No. 8, pp. 442-459.

[19] G. P. da Silva, M. Mack, and J. Contiero, "Glycerol: a Promising and Abundant Carbon Source for Industrial Microbiology", *Biotechnol. Adv*., 2009, Vol. 27, No. 1, pp. 30-39.