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THE IMPORTANCE OF EXTREMOPHILE CYANOBACTERIA IN THE PRODUCTION OF BIOLOGICALLY ACTIVE COMPOUNDS

ABSTRACT: Due to their ability to endure extreme conditions, terrestrial cyanobacteria belong to a group of organisms known as "extremophiles". Research so far has shown that these organisms posses a great capacity for producing biologically active compounds (BAC). The antibacterial and antifungal activities of methanol extracts of 21 cyanobacterial strains belonging to *Anabaena* and *Nostoc* genera, previously isolated from different soil types and water resources in Serbia, were evaluated. In general, larger number of cyanobacterial strains showed antifungal activity. In contrast to *Nostoc*, *Anabaena* strains showed greater diversity of antibacterial activity (mean value of percentages of sensitive targeted bacterial strains 3% and 25.9% respectively). Larger number of targeted fungi was sensitive to cultural liquid extract (CL), while crude cell extract (CE) affected more bacterial strains. According to this investigation, the higher biological activity of terrestrial strains as representatives of extremophiles may present them as significant BAC producers. This kind of investigation creates very general view of cyanobacterial possibility to produce biologically active compounds but it points out the necessity of exploring terrestrial cyanobacterial extremophiles as potentially excellent sources of these substances and reveals the most prospective strains for further investigations.

KEY WORDS: cyanobacteria, *Nostoc*, *Anabaena*, extremophiles, biologically active compounds

INTRODUCTION

At present, cyanobacteria generally remain as potential sources for further investigations as prospective and excellent sources of biologically active constituents produced during primary and especially secondary metabolism (Skulberg, 2000). Primary metabolites have been defined as low molecular weight compounds that are necessary for growth (Staley and Stanley, 1986). Thus, these compounds are produced by microorganisms during active growth, i. e., in the logarithmic growth phase. They include amino acids, nucleotides, coenzymes, organic acids and vitamins, as well, as intermediates in the biosynthetic pathways of these compounds. Secondary metabolites have been defined as low molecular weight compounds that are not necessary for microbial growth in pure culture continuously grown. These compounds are usually produced when the cyanobacterial culture enters the maximum stationary growth phase.

Among microorganisms, cyanobacteria are rapidly proving to be an extremely important source of biologically active secondary metabolites with potential benefits against human disease. They have been shown to produce a variety of antibacterial, antifungal, antilarval, antiprotozoan, antialgal, antihelmintic and generally cytotoxic secondary metabolites (Meeting and Pyne, 1986; Glombitza and Koch, 1989), some of which have potential for use as therapeutic or agrochemical agents (Moore, 1996). Among the commercially significant secondary metabolites are antibiotics and other pharmacologically active compounds, toxins and pigments (Staley and Stanley, 1986). Since they were first encountered, the toxins were always considered a threat to the health of living organisms.

Research of microalgae and cyanobacteria has mostly been directed towards examining water cultures. During the recent years, the emphasis has been shifted to organisms that do not primarily depend on aquatic surroundings, for not only survival but also reproduction. A large number of microalgae and cyanobacteria belong to the terrestrial types, which inhabit the soil (soil microalgae), rocks, tree bark, roofs, caves, city walls and other surfaces that are in contact with the atmosphere (Cvijan and Blaženčić, 1996; Petrovska, 1997). Due to their ability to endure extreme conditions (for algae and cyanobacteria, water represents the optimal environment), these organisms have become known as “extremophiles”. Extremophiles thrive on the edge of temperature, pH, pressure, hypersalinity, dryness and desiccation. Research so far has shown that these organisms posses a great capacity for producing biologically active compounds.

Significant biological activity *in vivo* and *in vitro* is found to happen in the cases of extracts of cyanobacterial strains isolated from freshwater and marine environments. It appears that extracts of terrestrial cyanobacteria have been found to possess even greater biological activity (Patterson et. al., 1994; Reisser, 2000). Because of their special growing conditions, terrestrial cyanobacteria posses survival and adaptation mechanisms not found in aquatic species. Some scientists believe that the more harsh and extreme conditions lead to a wider amplitude of metabolic extremities and possibilities, which causes the production of a most diverse range of, more or less, specific substances, reveling and pointing towards brilliant candidates for biotechnological application (Svirčev, 2005).

Terrestrial collections have frequently provided too little material for isolation and identification of the active agents (Barch et. al., 1983). Among 976 cyanobacterial isolates, we managed to culture a great number of terrestrial cyanobacterial strains which display significant growth rate (higher than 2g/l). On this basis, we have been engaged in a search for biologically active constituents in cyanobacteria isolated from the soil samples. In this work, we present the significance of cyanobacterial extremophiles in the production of biologically active compounds.

MATERIALS AND METHODS

Cyanobacteria and culture conditions

Cyanobacterial strains used for antibacterial and antifungal screening tests were cultivated in Novi Sad Cyanobacterial Culture Collection (NSCCC), Department of Biology, University of Novi Sad, Serbia. They were previously isolated from different soil types (S, SB, C, CB, LC, LCB, 3, 4, 14) and water resources (L, D, W, G) in Serbia. Isolates used to belong to genus *Anabaena*: *2S6B, 2S7, S8, C2, C3, C5, LC1B, LC2, 4, L, D*, and genus *Nostoc*: *S1, S2, 2S3B, S9B, 2S9, 2C1B, 3, 14, W, G.* All the strains were cultivated in eight Erlenmeyer flasks of 500ml volume, at the temperature of 26°C and fluorescent light intensity of 50 $\mu molm^{-2}s^{-1}$, for thirty-five days. They were grown in BG11 liquid nutritive medium (R i p p k a et al., 1979), without supplemented nitrogen.

Methanol extracts preparation
(Modified method by Ø s t e n s v i k, 1998)

After incubation, microalgal biomass was separated from supernatant by centrifugation, at 3500 g/min and than freeze-dried. Final dry biomass was precisely measured. 15ml of solvent MeOH/EtOAc (ratio 10:1) was added to each biomass sample. The dissolving process was improved by using the ultrasonificator (10x10 sec). The mixtures were kept over night at 4°C, in the dark. After extraction, extracts were filtrated through sterile filter paper to eliminate the cells and left at 37°C, in the dark for 48 hours to evaporate to dry residue. In supernatant fluid, the same volume of MeOH/EtOAc was added as solvent and all the fluid was evaporated to dry residue. Evaporated cell-free extracts and dry supernatant residue were dissolved in 1% (v/v) MeOH/EtOAc in 0.9% NaCl and sterilized by filtration through ∅ 0.22 μm filter. Finally, the extracts of biomass and supernatants were administrated to obtain different dilution rates.

Antibacterial-antifungal screening in agar plates

Bacterial strains *Staph. oxfordii, Staph. aureus, Str. pyogenes, E. coli* and nine bacterial strains isolated from the river Danube water were used in the investigation. The following fungal isolates, as part of the Department of Biology, University of Novi Sad collection, were used in the antifungal screening: yeasts *Saccharomyces* sp., *Schizosaccharomyces* sp. and *Candida albicans* and molds *Trichoderma* sp., *Cladosporium* sp., *Penicillium* sp., *Aspergillus* sp. and *Rhizopus* sp. Bacterial or fungal suspensions were added in sterile plates with

MPA medium. Using the opposite end of sterile pipette, 4 mm wells were made. In two different experiments, 100 μl volume of methanol extracts of a) cell-free supernatant (CE) and b) supernatant residue (CL) of all 21 cyanobacterial strains were added in the wells. After 24h of incubation at 37°C, the inhibition zones were measured (distance in [mm] from the edge of the well to the point of normal colony size of the test bacteria).

RESULTS AND DISCUSSION

Among all cyanobacterial strains tested, some representatives of *Anabaena* and *Nostoc* genus showed no biological activity (19%), some strains detected less or more significant activity against different number of bacterial (19%) and fungal (29%) species, but the most of the strains with detected biological activity, influenced both group of targeted organisms, bacteria and fungi (Tab. 1, Fig. 1). *Anabaena* strains, however, showed greater diversity of antibacterial activity (mean value 25.9%), while *Nostoc* strains produced active compounds against less number of bacterial strains tested (mean value 3%) (Fig. 2). According to activity shown against the fungi, approximately the same number of fungal species was sensitive to *Nostoc* and *Anabaena* active compounds (mean values 15.3%: 16.9% respectively).

Tab. 1. — The percentage of bacterial and fungal strains affected by cyanobacterial extracts (CE — crude cell extract, CL — cultural liquid extract)

BIOASSAY	ANTIBACTERIAL		ANTIFUNGAL	
STRAINS	CE	CL	CE	CL
Anabaena				
2S6B	26.8	13.4	5.9	5.9
2S7	60.3	53.6	5.9	11.8
S8	26.8	13.4	0	0
C2	80.4	33.5	0	0
C3	6.7	6.7	0	0
C5	0	0	11.8	23.6
LC1B	53.6	33.5	23.6	29.5
LC2	46.9	33.5	47.2	64.9
4	53.6	26.8	64.9	64.9
L	0	0	5.9	5.9
D	0	0	0	0
Nostoc				
S1	0	0	0	0
S2	13.4	6.7	11.8	11.8
2S3B	13.4	6.7	29.5	29.5
S9B	0	0	11.8	41.3
2S9	0	0	53.1	41.3
2C1B	0	0	0	0
3	13.4	6.7	0	0
14	0	0	0	0
W	0	0	29.5	35.4
G	0	0	5.9	5.9

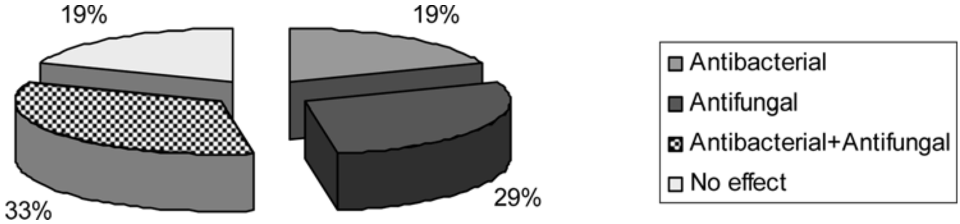

Fig. 1. — The percentage of cyanobacterial strains with only antibacterial, only antifungal, both antibacterial and antifungal effect, and without influence on the targeted organisms

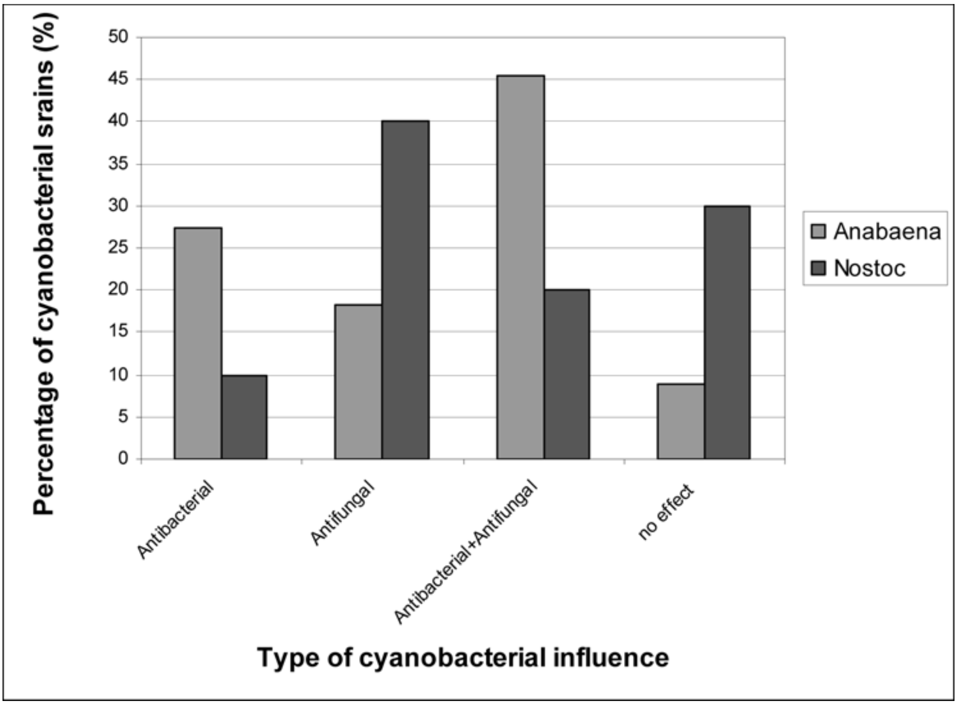

Fig. 2. — Mean values of percentages of targeted bacterial and fungal strains sensitive to extracts obtained from *Anabaena* or *Nostoc* strains

We can notice that tested *Anabaena* strains detected greater antibacterial then antifungal activity, but most strains were producing both antibacterial and antifungal substances (Fig. 2). It is opposite with *Nostoc* strains, which exhibit mostly fungicidal influence (only 3% of the investigated bacterial strains were sensitive to cyanobacterial extracts comparing to 15.3% of sensitive fungal strains). Investigations of 50 cyanobacterial isolates, belonging to *Nostoc* genus, showed that more than 80% of the strains had significant biological activity, mostly antifungal. Antibacterial activity was less frequent (only 8 of 50 strains) (P i c c a r d i et al., 2000). According to other research, less then 10% of 255 strains of microalgae showed fungicide or fungistatic activity (N e -

m e t h and Ö r d ö g, 2000). Different results of various investigations conducted as primary screening for production of biologically active compounds (S v i r č e v, 2005), could lead us to a conclusion that this capability is not genus dependent but vary from strain to strain, and the obtained differences are only the result of small number of tested strains. It is also obvious that biological activity can be directed towards one target, but can also refer to more then one secondary metabolite, which is also depending on the cyanobacterial strain (Tab. 1, Fig. 1). For example, C2 strain showed great antibacterial and no antifungal effect, while S2 strain had similar biological activity on both bacteria and fungi. At the same time, S1 strain showed neither antibacterial nor antifungal effect.

In general, larger number of cyanobacterial strains showed antifungal activity (Fig. 1). It was detected with 13 of 21 cyanobacterial strains. In all cases but one (2S9 strain), cultural liquid extract affected exact or larger number of targeted fungi then crude cell extract (Fig. 3). 12 of 21 investigated cyanobacterial strains showed antibacterial influence. All of those, except C3 strain, affected larger number of targeted bacteria using CE then when CL was used in experiment. This is most obvious with C2 strain, where CE: CL ratio is 80.4%: 33.5%. Similar results were obtained in some previous researches where terrestrial strains were included (S v i r č e v, 2005). Investigations of 50

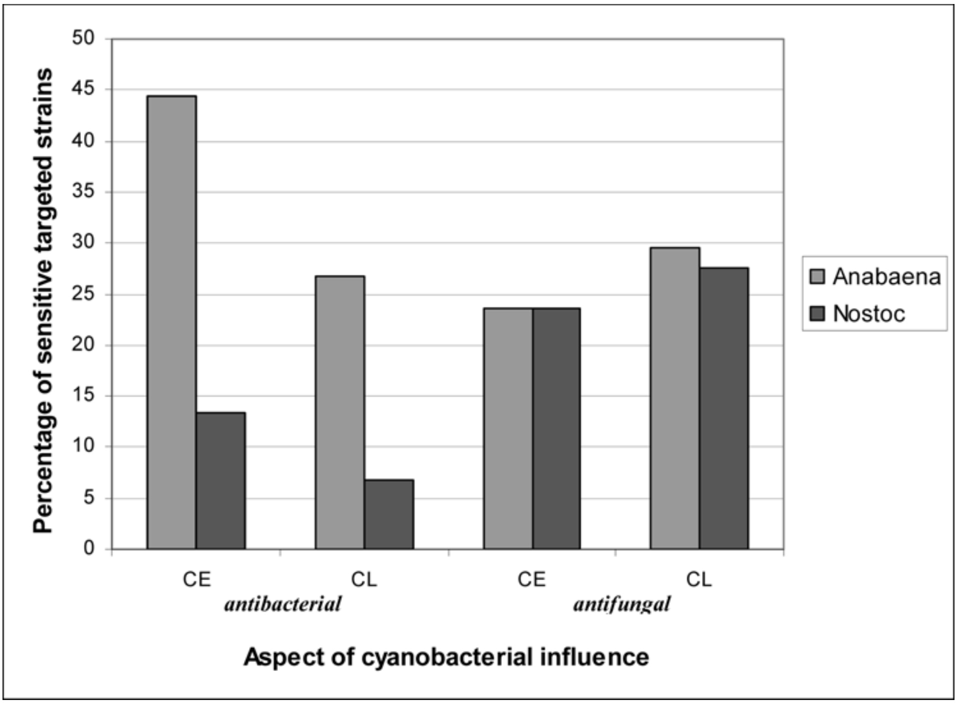

Fig. 3. — Mean values of percentages of targeted bacterial and fungal strains sensitive to different aspects of cyanobacterial extracts: crude cell extract (CE) and cultural liquid extract (CL); cyanobacterial strains with no detected influence excluded

Nostoc strains showed that the bioactivity is equally distributed between lipophilic and hydrophilic extracts of lyophilized cyanobacterial biomass. Methanolic extracts of *Nostoc* strain ATCC 53789, a known cryptophycin producer, obtained from both thawed biomass (BE) and thawing water (WE) were active against 9 of 12 fungi tested, but higher concentrations of WE were necessary to obtain activity of this extract (B i o n d i et al., 2004). Various investigations of different aspects of cyanobacterial influence pointed that antifungal substances are mostly not excreted out of the cell, but stay within the biomass of cyanobacteria observed. Nevertheless, screening of larger number of cyanobacterial strains for antifungal activities showed significant influence of excreted bioactive compounds (K u l i k, 1995). Various results from different investigations points that type of extract, as well as method of extraction, are an important factor in intensity of BAC activity.

Aquatic strains used in this experiment showed no antibacterial influence and less significant activity against fungal species then terrestrial strains (Fig. 4). According to these results, terrestrial strains, as representatives of extremophiles, should be taken into consideration as potent BAC producers more seriously. Some scientists believe that revealing new capabilities and possibilities of terrestrial strains presents a beginning of new era in biotechnology of microalgae (R e i s e r, 2000, S v i r č e v, 2005).

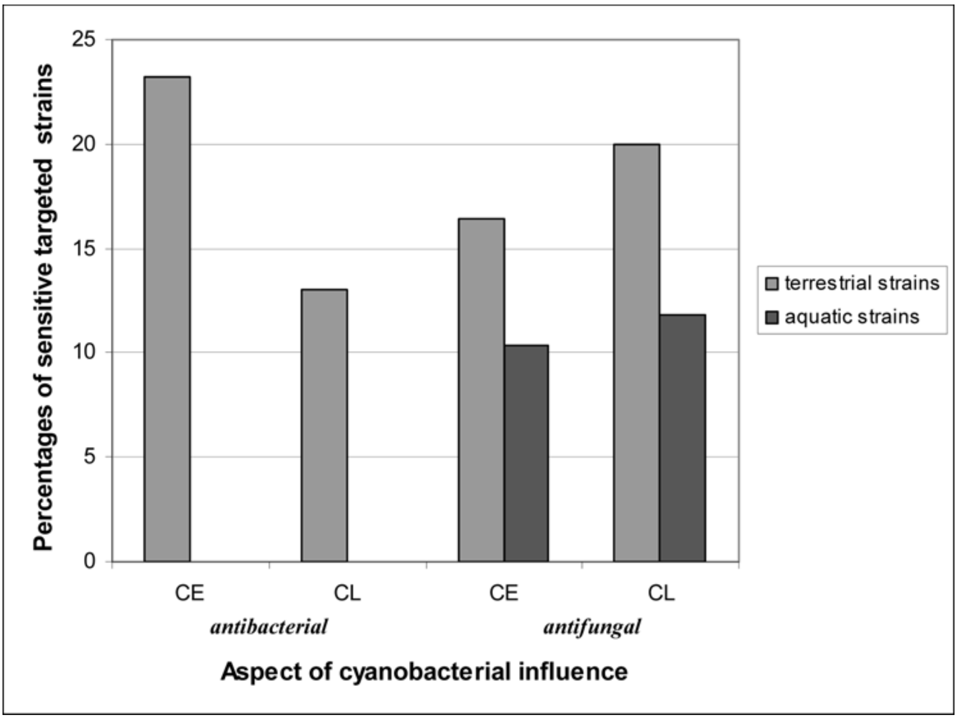

Fig. 4. — Mean values of percentages of targeted bacterial and fungal strains sensitive to extracts obtained from terrestrial or aquatic strains

This kind of investigation creates very general view of cyanobacterial possibility to produce biologically active compounds but it points out the necessity of exploring terrestrial cyanobacterial extremophiles as potentially excellent sources of these substances and reveals the most prospective strains for further investigations. The action spectrum and the potency of different cyanobacterial extracts is strain dependent. Therefore, the screening type of investigations, with as many cyanobacterial strains and targeted organisms as possible, are necessary to provide a large scale of information for each strain, which could help in further researches toward possible application in biotechnology.

CONCLUSION

In general, larger number of cyanobacterial strains showed antifungal activity. *Anabaena* strains, however, showed greater diversity of antibacterial activity (mean value 25.9%), while *Nostoc* strains produced active compounds against less number of bacterial strains tested (mean value 3%). According to activity shown against the fungi, approximately the same number of fungal species was sensitive to *Nostoc* and *Anabaena* active compounds (mean values 15.3%: 16.9% respectively). Tested *Anabaena* strains detected greater antibacterial then antifungal activity, but most strains were producing both antibacterial and antifungal substances. In contrast, *Nostoc* strains exhibit mostly fungicidal influence. Cultural liquid extract (CL) affected exact or larger number of targeted fungi then crude cell extract (CE), while more targeted bacterial strains were affected by CE then CL used in experiment. According to these investigation, the higher biological activity of terrestrial strains as representatives of extremophiles may present them as significant BAC producers.

This kind of investigation creates very general view of cyanobacterial possibility to produce biologically active compounds but it points out the necessity of exploring terrestrial cyanobacterial extremophiles as potentially excellent sources of these substances and reveals the most prospective strains for further investigations.

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ЗНАЧАЈ ЕКСТРЕМОФИЛНИХ ЦИЈАНОБАКТЕРИЈА У ПРОДУКЦИЈИ БИОЛОШКИ АКТИВНИХ МАТЕРИЈА

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Резиме

Земљишне цијанобактерије припадају групи организама под називом „екстремофили", захваљујући способности да опстају у staништима са екстремним условима средине. Досадашња истраживања су показала да ови организми имају велик капацитет продукције биолошки активних материја (БАМ). Посматране су антибактеријске и антифунгалне активности метанолских екстраката двадесет и

једног цијанобактеријског соја сврстаних у родове *Anabaena* или *Nostoc*, а претходно изолованих из различитих типова земљишта и водених станишта Србије. Већи број свих испитиваних цијанобактерија показао је антифунгалну активност. За разлику од *Nostoc*, сојеви из рода *Anabaena* показали су већи диверзитет антибактеријске активности (средња вредност процента бактеријских сојева на које је испољено дејство екстраката била је 3% и 25.9% редом). Екстракт фугата (CL) је испољио дејство на већи број испитиваних гљива него бактеријских сојева, док је код ћелијског екстракта (CE) био обрнут случај. Према овим истраживањима, већа биолошка активност земљишних сојева као представника екстремофила у односу на водене сојеве може их истаћи као значајне продуценте биолошки активних материја. Овакав вид истраживања даје уопштену слику могућности цијанобактерија да продукују БАМ, али такође истиче неопходност проучавања земљишних цијанобактеријских екстремофила као потенцијално веома важног извора у добијању ових материја, и указује на најперспективније сојеве за даља истраживања.