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EFFECTS OF TEMPERATURE ON *SYNECHOCYSTIS* SP. R10 (CYANOPROKARYOTA) AT TWO IRRADIANCE LEVELS. II. EFFECT ON ANTIBACTERIAL, ANTIFUNGAL AND CYTOTOXIC ACTIVITIES

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Summary. The influence of five different temperatures (20, 26, 32, 39 and 44°C) under two irradiance levels (132 µmol photons m^{-2} s^{-1}, unilateral and 2×132 µmol photons m^{-2} s^{-1}, bilateral) on the biological activity of a newly-isolated unicellular cyanobacterium *Synechocystis* sp. strain R10 was studied. Water cellular extracts, fatty acids, culture liquids and extracellular polysaccharides of *Synechocystis* sp. were found to have activities against bacteria, fungus and tumor cells. The exopolysaccharides showed the most potent antibacterial and antifungal activities (MIC=0.25 mg mL^{-1}). The mixture of substances, excreted in the culture medium, inhibited a broad range of target pathogens. The cyanobacterial fatty acids had the strongest growth inhibitory effect on HeLa cells (IC_{50}<15 µg mL^{-1}). The cultivation conditions (temperature, light intensity) and the culture age had considerable and specific effects on the activity of the different groups of metabolites tested. Because of the pronounced potential of *Synechocystis* sp. R10 to produce various biologically active substances and the possibility to increase the activity of its intracellular and extracellular metabolites through manipulation of the cultivation conditions, this strain is of undoubted biotechnological interest.

Key words: antimicrobial activity; blue-green algae; *Candida albicans*; cellular extracts; culture liquids; cytotoxicity; extracellular polysaccharides; fatty acids; Gram-negative bacteria; HeLa cells; *Synechocystis*

Abbreviations: EPS – extracellular polysaccharides; HL – high light intensity; IC_{50} – the concentration required for 50% inhibition; LL – low light intensity; MIC – minimum inhibitory concentration

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INTRODUCTION

Recent screening programs for discovery of bioactive compounds from microalgae have shown that cyanobacteria represent an untapped bioresource for metabolites, some of them unique, which show a diverse range of activities such as antibacterial, antifungal, antiviral, antitumor, antiinflammatory, enzyme-inhibitory, immunopotentiating (El-Sheekh et al., 2006; Gharaei-Fathabad et al., 2007; Lopes et al., 2010; Bechelli et al., 2011). The cyanobacterial bioactive substances include peptides, lipopeptides, bromophenols, alkaloids, fatty acids, alcohols, polysaccharides, phycobiliproteins (Singh et al., 2005; Tan, 2007).

Cultivation temperature and light intensity not only control the photoautotrophic growth and metabolism of cyanobacteria, but have also been found to impact strain-specifically the synthesis of bioactive agents (Song et al., 1998; Issa, 1999; Ame et al., 2003; Griffiths and Saker, 2003; Preubel et al., 2009).

Recently, *Synechocystis* sp. strain R10, isolated from a shallow lake in the Rupite, Bulgaria, was cultivated under laboratory conditions. The cyanobacterium was found to respond to different cultivation temperatures and irradiance levels by changes in the growth, biochemical composition, and defense enzymes activity (Gigova et al., 2012). Based on these results, we hypothesized that the main environmental factors would have impact on the expression of its biological activity, if any. To test this assumption, in the present study we examined the antibacterial, antifungal and cytotoxic activities of intracellular and extracellular compounds produced by *Synechocystis* sp. R10 and assessed the effect of temperature, irradiance and cultivation period on these activities.

MATERIALS AND METHODS

Water extracts preparation

The cell pellet was suspended in 60 mmol L^{-1} TE (Tris-base with 0.1 mmol L^{-1} EDTA) buffer (pH 6.8) and cells were homogenized. After centrifugation (10000×g, 20 min) the supernatant (water extract) was freeze-dried. The dry residues were resuspended in double distilled water at a concentration of 25 mg mL^{-1} and sterilized by filtration through a 0.22 μm filter (Sartorius, Goettingen, Germany).

Preparation of fatty acid mixtures for bioactivity analyses

Cyanobacterial biomass was extracted with hot ethanol 3 times for 30 min under reflux. The ethanol was evaporated *in vacuo*. The lipophilic samples were converted to K-salts of fatty acids by saponification with 5% KOH in 96% ethanol for 2 h under reflux. The K-salts of fatty acids were extracted with distilled water and converted to free fatty acids by H_2SO_4. Free fatty acids were extracted with n-hexane. The free fatty acid samples were evaporated and re-dissolved in dimethyl sulfoxide (DMSO, Sigma) at a concentration of 20 mg mL^{-1} for screening of antimicrobial activity, or were converted to water soluble Na-salts (0.5 mg mL^{-1}) by $NaHCO_3$ treatment for cytotoxicity tests.

Extracellular sample preparation

Twenty ml of each cell-free culture liquid were freeze-dried. The dry residues were resuspended in double distilled water at a concentration of 25 mg mL^{-1} and sterilized by filtration through a 0.22 μm filter (Sartorius, Goettingen, Germany).

Exopolysaccharide isolation and estimation

The extracellular polysaccharides (EPS) from the cell-free culture liquids were precipitated with 99% ethanol in a ratio 1:3 (v/v) and pelleted at 10000×g for 10 min. The pellets were washed three times with 65% ethanol to remove any contaminants, dried at 37°C and dissolved in sterile double distilled water. Total carbohydrates were quantified as glucose equivalents by the phenol-sulfuric acid spectrophotometric method (Dubois et al., 1956).

Cytotoxicity screening

The *in vitro* cytotoxic activity was tested on human cervix epitheloid carcinoma cell line, HeLa. Cells were cultured in DMEM (Gibco BRL, UK) medium supplemented with 10% fetal bovine serum, penicillin (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Tumor cells were seeded in a 96-well microtiter plate at a concentration of 2×10^4 cells per well. After 24 hours, when a confluent monolayer was formed, the supernatant was replaced by 100 μl fresh medium containing cyanobacterial samples at various concentrations and cells were incubated for another 24 h. The antutumor drug doxorubicin hydrochloride (DOX, Sigma-Aldrich) at a final concentration of 10 μg mL^{-1} was used as a positive control. Cells cultured only in medium were used as a negative control. Four replicates were performed for each treatment and controls. After the treatment, MTT cell viability assay was performed as described previously (Mossmann, 1983). This test is based on the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to purple formazan in the functional mitochondria during cellular metabolitic electrochemical processes. The formazan crystals were solubilized in DMSO/EtOH (1:1, v/v) and the absorbance was measured at 570 nm on a microplate reader (TECAN, SunriseTM, Grödig/Sazburg, Austria). Cell viability (%) was calculated as follows: Cell viability (%) = OD_{570} (experimental)/OD_{570} (control) × 100. The IC_{50} value for each sample was interpolated from the concentration response curve.

Antibacterial and antifungal screening

Eight human pathogens (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Escherichia coli, Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Salmonella typhimurium* and *Candida albicans*) were used to assess the antibacterial and antifungal activities of *Synechocystis* sp. Broth microdilution method (Andrews, 2001) was followed for determination of minimum inhibitory concentration (MIC) values. Serial 2-fold dilutions were prepared from sample stock solutions and 50 μL of each test strain suspension (10^5 CFU mL^{-1}) was added to an equal volume of each sample dilution in a 96-

well microplate. Untreated bacterial and fungal suspensions were used as negative controls. The MIC was recorded as the lowest concentration of the respective sample showing no visible growth of the test strains after 24 h incubation at 37°C for bacteria and 30°C for fungus.

RESULTS

Antimicrobial and cytotoxic activities of aqueous cell extract, culture liquid, mixture of fatty acids, and extracellular polysaccharides from *Synechocystis* sp. cultured for 4 days under different temperatures and irradiance levels were analysed. Cultures grown for 14 days at 32°C, LL were also studied for biological activity.

Effect of temperature and light on the antibacterial and antifungal activities of *Synechocystis* sp. R10

Three Gram-positive (*Staphylococcus aureus*, *Streptococcus pyogenes, Bacillus cereus*), four Gram-negative (*Escherichia coli, Yersinia enterocolitica*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*) bacteria and fungus *Candida albicans* were selected for the assessment of antibacterial and antifungal activities. Only samples isolated after culturing of *Synechocystis* sp. at 26, 32 and 39°C, LL, among the aqueous extracts, showed antibacterial activity against *P. aeruginosa* (Table 1). It was suggested that under these conditions the algal cells synthesized substances able to inhibit the growth of this Gram-negative bacterium. The minimum inhibitory concentration of the three samples was the same (12.5 mg mL^{-1}), indicating that the temperature did not affect the synthesis of active substances. A longer cultivation at 32°C, LL led to manifestation of a broader antimicrobial spectrum and an increased activity. The aqueous extract from the older culture was active against *S. aureus* and *B. cereus* (MIC=6.25 mg mL^{-1}) as well as against *Y. enterocolitica* and *C. albicans* (MIC=1.56 mg mL^{-1}). Lyophilized culture media inhibited the growth of all tested organisms, with MIC values ranging from 12.5 to 3.12 mg mL^{-1} for various pathogens and between some samples, but no clear trend was found (Table 1). Most susceptible was the Gram-positive bacterium *S. aureus*, with the lowest MIC values of culture medium from *Synechocystis* sp. grown at 26, 32 and 39°C, LL. The broad antipathogenic spectrum and the difference in activity levels are an indication of synthesis and secretion in the culture medium of different quantities and/or various bioactive compounds. The effect of fatty acid mixtures, isolated from *Synechocystis* grown at 20, 32 and 39°C under both LL and HL was studied on some of the test organisms. All fatty acid samples showed a strong inhibitory effect (MIC of 1.25 or 2.5 mg mL^{-1}) (Table 1). Only the sample isolated after cultivation at 39°C, LL was not active against *S. pyogenes* and *E. coli*. Among the investigated cyanobacterial compounds, the extracellular polysaccharides had the highest activity (with MIC values ranging from 1 to 0.25 mg mL^{-1}). *Candida albicans*, *Y. enterocolitica* and *P. aeruginosa* were most sensitive to their action (Table 1). The EPS from the culture grown at 26°C, LL inhibited the growth of all tested pathogens, except for *S. pyogenes*, which was resistant to

Table 1. Effect of temperature, light intensity, and cultivation period on the antibacterial and antifungal activities of the culture liquids, exopolysaccharides, aqueous cell extracts and fatty acids from *Synechocystis* sp. R10.

Sample/Test pathogen	*S. aureus*	*S. pyogenes*	*B. cereus*	*E. coli*	*P. aeruginosa*	*Y. enterocolitica*	*S. typhimurium*	*C. albicans*
Culture liquid								
20ºC, LL	6.25[a]	6.25	12.50	12.50	12.50	0	12.50	6.25
26ºC, LL	3.12	6.25	6.25	12.50	12.50	6.25	12.50	6.25
32ºC, LL	3.12	6.25	6.25	12.50	12.50	6.25	12.50	6.25
39ºC, LL	3.12	6.25	6.25	12.50	12.50	6.25	12.50	6.25
44ºC, LL	12.50	12.50	6.25	6.25	12.50	6.25	6.25	12.50
32ºC, LL oc	6.25	0	12.50	12.50	12.50	6.25	12.50	12.50
20ºC, HL	6.25	12.50	6.25	6.25	12.50	6.25	6.25	12.50
26ºC, HL	6.25	12.50	12.50	12.50	12.50	6.25	12.50	6.25
32ºC, HL	6.25	12.50	6.25	12.50	12.50	6.25	12.50	6.25
39ºC, HL	6.25	12.50	6.25	6.25	12.50	6.25	12.50	6.25
44ºC, HL	6.25	12.50	6.25	12.50	12.50	6.25	0	12.50
Exopolysaccharides								
20ºC, LL	0	0	0	0	1.00	0	0	0
26ºC, LL	0.50	0	1.00	1.00	1.00	0.50	1.00	0.25
32ºC, LL	0	0	0	0	0	0.50	0	1.00
39ºC, LL	0	0	0	0	0	0	0	1.00
44ºC, LL	0	0	0	0	0	0	0	0.50
20ºC, HL	0	0	0	0	1.00	0	0	1.00
26ºC, HL	0	0	0	0	1.00	1.00	0	0.25
32ºC, HL	1.00	0	0	0	1.00	1.00	0	1.00
39ºC, HL	0	0	0	0	0	0	0	0
44ºC, HL	0	0	0	0	0	0.25	0	0
Water extract								
26ºC, LL	0	0	0	0	12.50	0	0	0
32ºC, LL	0	0	0	0	12.50	0	0	0
39ºC, LL	0	0	0	0	12.50	0	0	0
32ºC, LL oc	6.25	0	6.25	0	12.50	1.56	0	1.56
Fatty acids								
20ºC, LL	1.25	2.50	-	-	-	-	-	-
32ºC, LL	1.25	2.50	-	-	-	-	-	1.25
39ºC, LL	2.50	0	-	0	-	-	-	-
32ºC, LL oc	1.25	1.25	2.50	1.25	-	-	-	1.25
20ºC, HL	1.25	2.50	-	-	-	2.50	-	-
32ºC, HL	1.25	2.50	-	-	-	-	-	1.25
39ºC, HL	10.00	1.25	-	1.25	-	-		1.25

LL, 132 μmol photons m^{-2} s^{-1}; HL, 2×132 μmol photons m^{-2} s^{-1}; oc old culture (14-day cultivation); 0 absence of bioactivity, - not tested

[a]Antimicrobial activity of the samples was determined using the broth microdilution assay and expressed as minimum inhibitory concentration (MIC, milligram per milliliter)

all EPS samples. Samples produced at 26°C and 32°C, HL were active against 3 and 4 tested species, respectively.

Effect of temperature and irradiance on the cytotoxicity of *Synechocystis* sp. R10

The *in vitro* antiproliferative activity of *Synechocystis* sp. was tested on HeLa cells. Water extracts and culture media from cultures grown at different conditions were applied at two concentrations – 500 and 1250 μg mL^{-1}. The lower concentration of all water extracts enhanced the growth of HeLa cell (Fig. 1). The inhibitory effect of the higher concentration of the mixtures of intracellular metabolites under HL, increased with increasing the temperature of cultivation (except for 39°C, where a slight stimulation was noticed), reaching an 80% inhibition (IC_{50}=895 μg mL^{-1}) at 44°C. In contrast, only the extract (1250 μg mL^{-1}) obtained from cells grown at 32°C under LL significantly decreased the viability of tumor cells. Prolonged cultivation of *Synechocystis* sp. at 32°C, LL resulted in the production of a more active sample (32°C vs. 32°C oc, the inhibition rate increased from 23.7% to 60%) (Fig. 1). Unlike cell extracts, all culture liquids of *Synechocystis* inhibited the proliferation of HeLa cells and this effect was dose-dependent (Fig. 2). The inhibitory ability

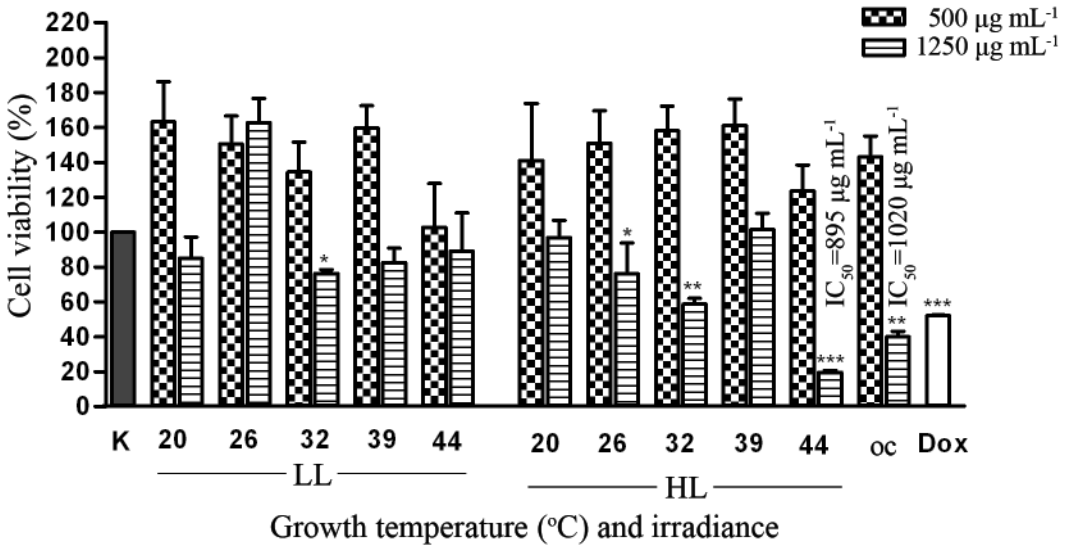

Figure 1. Effect of growth temperature, light intensity, and incubation period on the toxicity of aqueous cell extract from *Synechocystis* sp. R10 on HeLa cells. The inhibition of HeLa cell viability after treatment with two concentrations of the extract for 24 h related to untreated control cells (K, 100% viability) was assessed by the MTT method. The antitumor drug doxorubicin hydrochloride (Dox, 10 μg mL^{-1}) was used as a positive control. LL, 132 μmol photons m^{-2} s^{-1}; HL, 2×132 μmol photons m^{-2} s^{-1}. oc means 32°C, LL, 14 days of cultivation. The concentration required for a 50% inhibition (IC_{50}), interpolated from the concentration response curve, is indicated.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant differences from untreated control values.

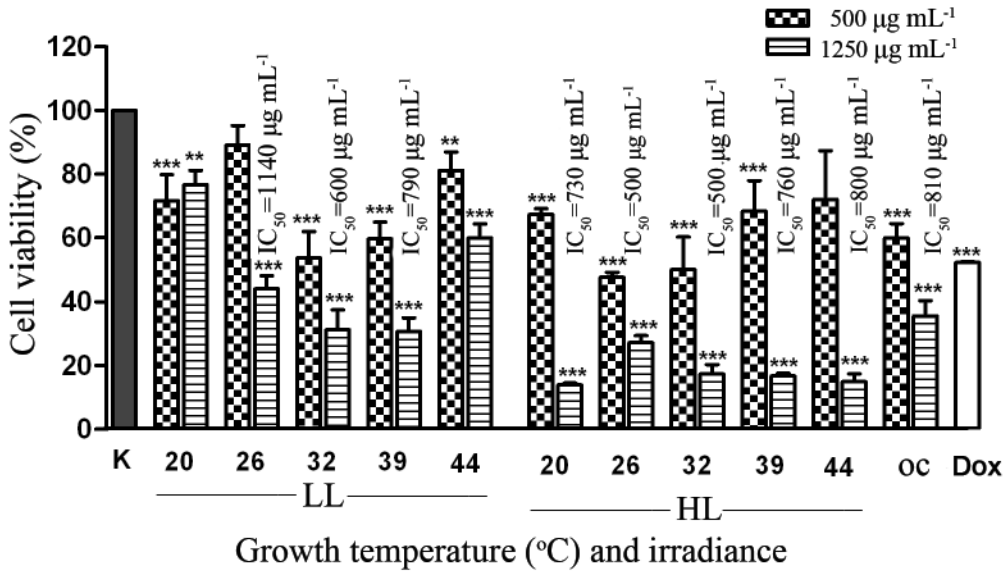

Figure 2. Effect of temperature, light intensity, and incubation period on the growth inhibitory activity of culture liquid of *Synechocystis* sp. R10 on HeLa cells, assessed by the MTT assay. LL, 132 μmol photons m^{-2} s^{-1}; HL, 2×132 μmol photons m^{-2} s^{-1}; oc, 32°C, LL, 14 days of cultivation; IC_{50}, concentration required for 50% inhibition. $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant differences from untreated control values.

of culture liquids (at a concentration of 1250 μg mL^{-1}) increased from 23% to 69% with the rise of cultivation temperature in the range 20-39°C under LL, with the lowest IC_{50} (600 μg mL^{-1}) for the sample obtained at 32°C. Longer cultivation at 32°C, LL did not affect positively the activity of culture liquid against HeLa. The HL stimulated the production and/or diversity of extracellular substances with an inhibitory effect, which was reflected in lower IC_{50} values compared to LL. The most active among the samples, under HL, were those obtained at growth temperatures 26°C and 32°C. Extracellular polysaccharides of *Synechocystis* applied at concentrations of 20, 40 and 60 μg mL^{-1} showed a weak inhibitory effect (Fig. 3). The activity of the samples isolated from cyanobacterium, cultured at 26°C, HL and at 39°C, LL was significant, with IC_{50} values of 55.6 and 58 μg mL^{-1}, respectively, despite of the relatively low concentrations. All tested fatty acid mixtures of *Synechocystis* showed a strong suppression effect on the growth of HeLa cells (Fig. 4). The IC_{50} values ranged from less than 15 μg mL^{-1} to 23.8 μg mL^{-1} depending on the cultivation temperature and irradiance. The fatty acids synthesized at 20°C and 32°C under LL exibited a higher activity compared to HL. The samples obtained from cultures grown at 32°C, LL for 4 days and for 14 days had the strongest reducing effect on the viability of tumor cells (IC_{50}<15 μg mL^{-1}).

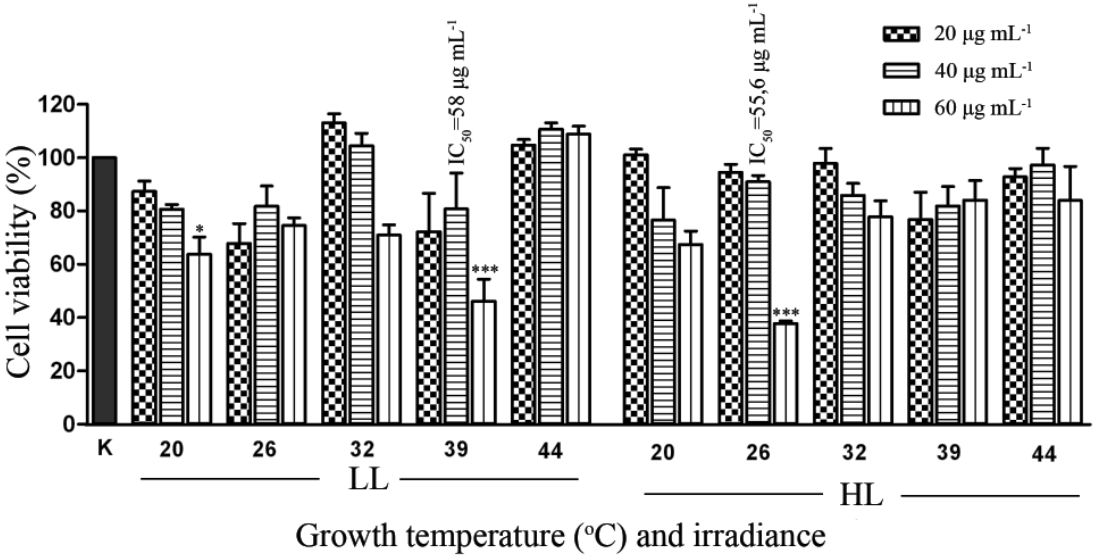

Figure 3. Effect of growth temperature, light intensity, and incubation period on the toxicity of exopolysaccharides of *Synechocystis* sp. R10 on HeLa cells.

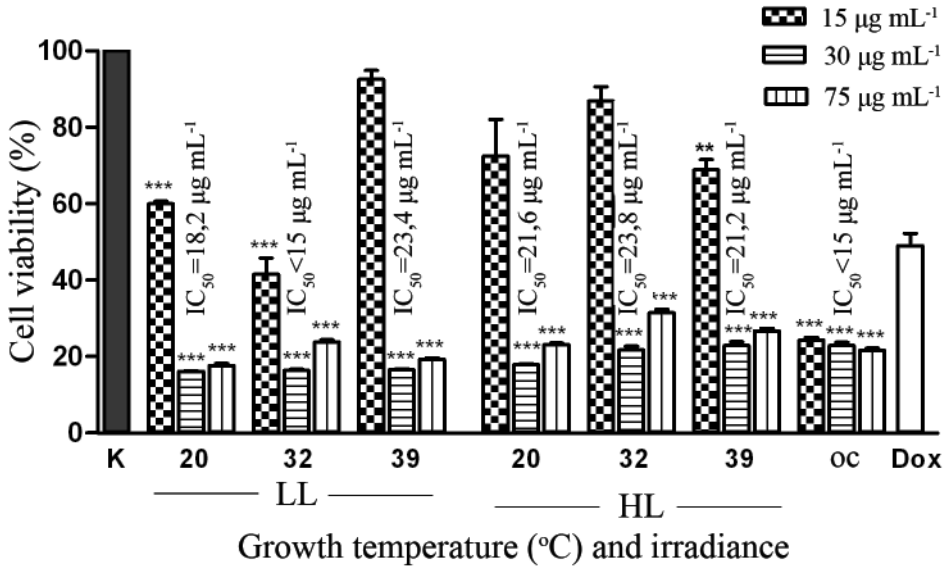

Figure 4. Growth inhibitory activity of fatty acid mixtures of *Synechocystis* sp. R10 on HeLa cells. Fatty acids were isolated from the cyanobacterium cultured at indicated temperatures (°C) and light intensities (LL, 132 μmol photons m^{-2} s^{-1}; HL, 2×132 μmol photons m^{-2} s^{-1}). HeLa cells were exposed to various sample concentrations for 24 h, and cell viability was determined using the MTT assay. $^{***}p < 0.001$, $^{**}p < 0.01$ indicate significant differences from untreated control values.

DISCUSSION

The intracellular (fatty acids, water extract) and extracellular (exopolysaccharides, culture liquid) compounds produced by *Synechocystis* sp. R10 were found to have pronounced antibacterial and antifungal activities as well as a growth inhibitory activity towards HeLa cells. The results on the antifungal activity and the activity against Gram-negative bacteria of the culture media, exopolysaccharides, and fatty acids (Table 1) were particularly interesting and promising. The expression of such activities occurred less frequently compared to the effect of cyanoprokaryotes against Gram-positive bacteria. Moore et al. (1988) found that among 1000 strains, only 90 strains (9%) inhibited fungal growth. Activity against Gram-positive bacteria but not against fungi was reported by Martins et al. (2008). Gram-positive bacteria were sensitive to extracts from 22 freshwater and soil blue-green algae, while Gram-negative bacteria were resistant (Pandey and Pandey, 2002). These differences in the antimicrobial effects of the extracts may be related to differences in permeability to the cyanobacterial compounds (Martins et al., 2008). Nevertheless, an ability to inhibit the growth of Gram-negative bacteria has been reported for some cyanoprokaryotes (Gasemi et al., 2007; Plaza et al., 2010).

The strong antiproliferative activity towards HeLa cells of exopolysaccharides (IC_{50} values of 55-58 μg mL^{-1}), and especially of fatty acids (with IC_{50} values less than 15 μg mL^{-1}) from *Synechocystis* sp. R10 was also an usefull finding. The extracts of 25 from 48 antarctic cyanobacteria were cytotoxic to HeLa (Biondi et al., 2008) in concentrations similar to or higher than the applied in the present study. Two out of six terrestrial cyanobacterial strains from Serbia inhibited the growth of HeLa and MCF7 (human breast adenocarcinoma) cell lines (Svircev et al., 2008). Li et al. (2011) reported that EPS produced by *Nostoc sphaeroides* had cytotoxic effects on four human cancer cell lines with inhibition rates (ranging from 34% to 68%) similar to those obtained in our study, but at a concentration of 2 mg mL^{-1}. In another study carried out by Pugh et al. (2001), polysaccharides of *Spirulina platensis* and *Aphanizomenon flos-aquae* have been found to possess potent immunostimulatory activity (with IC_{50} values between 20 and 110 ng mL^{-1}).

The results of the present study showed that both irradiance and temperature were important factors contributing to the expression of biological activities of *Synechocystis* sp. R10. In general, *Synechocystis* cultured at 26°C and 32°C exhibited the highest biological activity. The low light intensity enhanced the activity of cell extracts and culture media of the cyanobacterium to some pathogens, as well as the growth inhibiting effect of fatty acids on HeLa cells. In contrast, the antiproliferative activity of aqueous extracts and culture media on HeLa cells was higher after cultivation at HL compared to LL. The biological activity of *Synechocystis* sp. R10 was found also to change with culture age. The intracellular metabolites in the water extract after prolonged cultivation (14 days) showed a broader antimicrobial spectrum and higher activity against microbes and HeLa cells, while the

extracellular substances in the culture medium had less antimicrobial and antiproliferative potential in comparison to samples from the younger culture. The effect of the studied physiological factors might be associated with alterations in cellular metabolism leading to a significant difference in the quantity and/or diversity of the synthesized bioactive substances. Considering the tolerance of *Synechocystis* sp. to a wide range of temperatures under the two light regimes, some of the produced bioactive compounds could provide benefits in the adaptation response to changing environmental factors.

In conclusion, the newly-isolated thermotolerant, fast growing *Synechocystis* sp. strain R10 proved to be a rich source of valuable biomass, phycobiliproteins and biologically active substances. The extracellular polysaccharides of *Synechocystis* sp. had the highest antibacterial and antifungal activities (MIC=0.25 mg mL^{-1}) among all tested components, while the mixture of substances in the culture liquid exhibited the broadest range of antipathogenic activity. The cyanobacterial fatty acids had the strongest inhibitory effect on HeLa cells (IC_{50}<15 μg mL^{-1}). The opportunity to increase the activity of its intracellular (fatty acids and aqueous extract) and extracellular (polysaccharides, culture liquid) substances only through appropriate cultivation conditions, emphasizes the biotechnological potential of *Synechocystis* sp. R10.

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