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EFFECTS OF TEMPERATURE ON *SYNECHOCYSTIS* SP. R10 (CYANOPROKARYOTA) AT TWO IRRADIANCE LEVELS. I. EFFECT ON GROWTH, BIOCHEMICAL COMPOSITION AND DEFENSE ENZYME 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 growth, biochemical composition and enzymatic antioxidant defense of a newly isolated unicellular *Synechocystis* sp. strain R10 was studied. This photoautotrophic blue-green alga grew well in the temperature range 26-39°C under lower light intensity, but the cultures could grow, although slower, at 20°C and 44°C. Temperature, under higher irradiance level, did not significantly affect the growth, except for 44°C, where the growth was greatly reduced. *Synechocystis* sp. R10 responded to the unfavorable conditions by changes in its biochemical composition and enzyme activities. At 20°C and lower light, total carbohydrates content as well as the activities of superoxide dismutase, catalase, glutathione reductase and defined protease isoforms were increased. The response to the combination of high temperature (44°C) and lower light consisted in accumulation of carotenoids and C-phycocyanin, but not in the induction of antioxidant enzymes activity. A significant enhancement in carbohydrate content was observed at 44°C and higher light, simultaneously with the reduction in protein content and activity of all studied enzymes. The observed modulation of antioxidant enzyme and protease activities, and changes in carbohydrate and pigment contents could be a prerequisite for the thermal tolerance and ability of the cyanobacterium to adapt to different environment. Because of its fast growth rate, high phycobiliprotein, carbohydrate and protein contents, and wide adaptability, this strain is of biotechnological interest.

Key words: carbohydrates; catalase; cyanobacteria; glutathione reductase; growth; isoenzymes; phycobiliproteins; protease; superoxide dismutase; *Synechocystis*

Abbreviations: AOE – antioxidant enzymes; APC – allophycocyanin; C-PC – C-phycocyanin; CAT – catalase; DW – dry weight; EST – esterase; HL – high light intensity; GR – glutathione reductase; LL – low light intensity; PAGE – polyacrylamide gel electrophoresis; POD – peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase; *Synechocystis - Synechocystis* sp. strain R10

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INTRODUCTION

The cyanoprokaryotes (cyano-bacteria, blue-green algae) attract the attention of researchers as a rich source of biomass and valuable substances with various biological activities (Singh et al., 2005; Abed et al., 2009).

Although a large number of strains were screened for biological activities and there are numerous reports on isolated active compounds, the enormous cyanobacterial biodiversity is still largely unexplored. This determines the rising interest in isolation of new strains from the nature, searching for new biologically active substances, and finding opportunities to optimize the conditions for synthesis of cyanobacterial bioactive substances.

Light and temperature are the most important environmental factors that primarily affect the rate of photosynthesis and biomass productivity, but also influence the pattern and activity of cellular metabolism and thus dynamic cell composition. Cyanobacteria display considerable differences in their sensitivity, physiological and biochemical responses, and adaptive strategies to temperature and light intensity (Tang and Vincent, 1999; Bhandari and Sharma, 2006; Chaiklahan et al., 2007).

Recently, Martins et al. (2008) noted the abilities of marine cyanobacterial strains belonging to the genera *Synechocystis* to induce apoptosis in eukaryotic cells and cause growth inhibition on Gram-positive bacteria. Therefore, our investigation aimed to evaluate the potential of newly-isolated freshwater *Synechocystis* sp. strain R10 as a producer of biologically active substances. In this paper, we report on the effects of temperature and irradiance on the cyanobacterial growth, biochemical composition and defense enzyme activities. The understanding of how *Synechocystis* sp. R10 withstands adverse environmental conditions may facilitate its utilization for the production of useful components.

MATERIALS AND METHODS

Strain and growth conditions

Synechocystis sp. strain R10 (*Synechocystis*) was isolated from a shallow (40-50 cm) lake (42°C), formed by the leakage of thermal spring in the Rupite, southwest Bulgaria. Strain is deposited in the culture collection of the Institute of Plant Physiology and Genetics, BAS, Sofia, Bulgaria. Monospecific, non-axenic cultures were grown in mineral medium after Aiba and Ogawa (1977) using a block with a temperature gradient (Dilov, 1985). A carbon source was provided by bubbling sterile 2% (v/v) CO_2 in air through the cultures. Cool-white fluorescent lamps at two photon flux densities, 132 μmol m^{-2} s^{-1} unilateral (conditionally marked as LL – low light intensity) and 2×132 μmol m^{-2} s^{-1} bilateral (HL – high light intensity), measured at the surface of 200 ml flasks, provided a continuous illumination. Separate cultures were grown for each of the five applied temperatures (20, 26, 32, 39 and 44°C) at both light intensities. Culture, grown at 32°C, LL and standardised at a density of 1.1 mg mL^{-1} dry weight (DW) was used as inoculum in all experiments. The experimental cultures were harvested

after 96 h of cultivation to avoid the effect of nutrient limitation. Cells were collected by centrifugation (5000×g, 20 min), rinsed three times with distilled water, frozen, and stored at -70°C. Both, cells and cell-free culture liquids were used for analyses. Cultures, grown at 32°C under LL for 14 days were analyzed as well.

Analytical methods

For biomass DW determination, algal suspensions (3×5 mL each) were filtered through Whatman GF/C glass filters (Whatman International Ltd, Maidstone, UK) and oven dried at 80°C to a constant weight. The growth of *Synechocystis* was evaluated gravimetrically by increase in biomass dry weight. The growth rate [μ] was calculated using the formula:

$$\mu = \frac{Ln(m_{t2}/m_{t1})}{t_2 - t_1}$$

(Levasseur et al., 1993)

where m_{t1} is the dry weight at the beginning of the cultivation period (t_1) and m_{t2} is the dry weight on the fourth day of cultivation (t_2).

Total protein content was determined by the method of Lowry et al. (1951), with BSA as a standard. Total carbohydrates were quantified as glucose equivalents by the phenol-sulfuric acid spectrophotometric method (Dubois et al., 1956). Chlorophyll *a* and carotenoid content in methanol extracts of cells were determined by measuring the absorbance at 665 and 460 nm, respectively (MacKinney, 1941). Phycobiliproteins were extracted in 0.01 M potassium phosphate buffer (pH 6.7) from homogenized cells (vibrations homogenisator VHG1, Germany, 4°C, 10 min), measured at 620 and 652 nm and calculated according to the equations of Siegelman and Kycia (1978). The absorption was measured on a T70 UV/Vis (PG Instruments Ltd, Leicester, UK) spectrophotometer at room temperature.

The experiments were done in triplicate. All data were expressed as the means±SD. The statistical significance between the treatments was evaluated by one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test using GraphPAD InStat, Software, USA. Values of $p < 0.05$ were considered significant.

Preparation of cell extracts, polyacrylamide gel electrophoresis (PAGE) and activity staining of antioxidant enzymes and esterases

The cells were homogenized in 60 mM TE (Tris-base with 0.1 mM EDTA) buffer (pH 6.8) and centrifuged at 13000×g for 15 min. Protein concentration in the supernatant (total soluble proteins) was determined by the method of Bradford (1976). Equal amounts (20 μg) of protein from cells exposed to different treatments were subjected to discontinuous PAGE under nondenaturing, nonreducing conditions essentially as described by Laemmli (1970), but without SDS. Electrophoretic separation was performed on 10% polyacrylamide gels, for 3–4 h with a constant current of 35 mA per gel. Upon completion of electrophoresis, separate gels were stained for the activities of the respective enzymes.

Localization of activity bands of superoxide dismutase (SOD, EC

1.15.1.1) on gels and identification of enzyme metalloforms were performed following the procedures of Azevedo et al. (1998). For catalase (CAT, EC 1.11.1.6) activity staining, the method described by Chandlee and Scandalios (1983) was used. Peroxidase (POD, EC 1.11.1.7) activity was visualized by staining the gel in benzidine/H_2O_2 (Rao et al., 1996). The in-gel activity staining of glutathione reductase (GR, EC 1.6.4.2) was performed after Anderson et al. (1995). Esterase (EST, EC 3.1.1.x) isoforms and activity were detected by staining with α-naphthyl acetate/Fast blue BB according to Murphy et al. (1996).

Detection of protease activities by gelatin–zymogram gel analysis

Substrate gel electrophoresis was performed according to the method of Lodemel et al. (2004) using 10% acrylamide – 0.25% gelatin (w/w) copolymerized gels.

Gel patterns were recorded immediately after staining using the *DigiGenius 1* gel documentation system (SynGene, UK). Image analysis of the gels was performed on a PC using Version 08-3d.3.SynGene (Synoptics Ltd, Cambridge, UK) software. The activity of each isoenzyme (band) resolved was measured as total integrated optical density (IOD), in arbitrary units. Some enzymes had multiple bands and the sum of their IOD values was considered as a total enzyme activity for a particular condition. Each value on the figures is given as mean±SD of three independent experiments. The data were analysed by one-way ANOVA followed by a Bonferroni post hoc test.

RESULTS

Temperature and light impact on the growth and biochemical composition of *Synechocystis* sp. R10

Cultures grew exponentially within the temperature range of 20-39°C. Growth was stimulated by 26 and 32°C, under LL (the end-point growth is presented in Fig. 1A). The temperature, under HL, did not significantly affect the growth, except for 44°C. The combined effect of the highest temperature studied and higher irradiance was particularly unfavorable for *Synechocystis* (Fig. 1A, B). The growth at 20 and 39°C was better in HL compared to LL. After 96 h of cultivation, maximum growth (3.9 mg mL^{-1} DW) was achieved at 39°C and HL. Optimal temperatures for accumulation of proteins were 39-44°C, under LL and 32°C, under HL (Fig. 1C). The level of irradiance had no significant effect on protein content, except for 44°C, where LL stimulated protein production by about 63% compared with HL. Carbohydrate content did not change significantly in the temperature range of 20-39°C, LL and 26-39°C, HL (Fig. 1D). However, total carbohydrates increased significantly at 20 and 44°C, HL and at 44°C, LL and reached the highest value (264.3 mg g^{-1} DW) at 44°C, HL. High light intensity significantly stimulated the accumulation of carbohydrates at 20 and 44°C, compared to LL.

Synechocystis synthesizes two accessory photosynthetic pigments, C-phycocyanin (C-PC) and allophycocyanin (APC), indicated by the absorption spectrum of the crude phycobiliprotein extract (data not shown). Their ratio varied in the

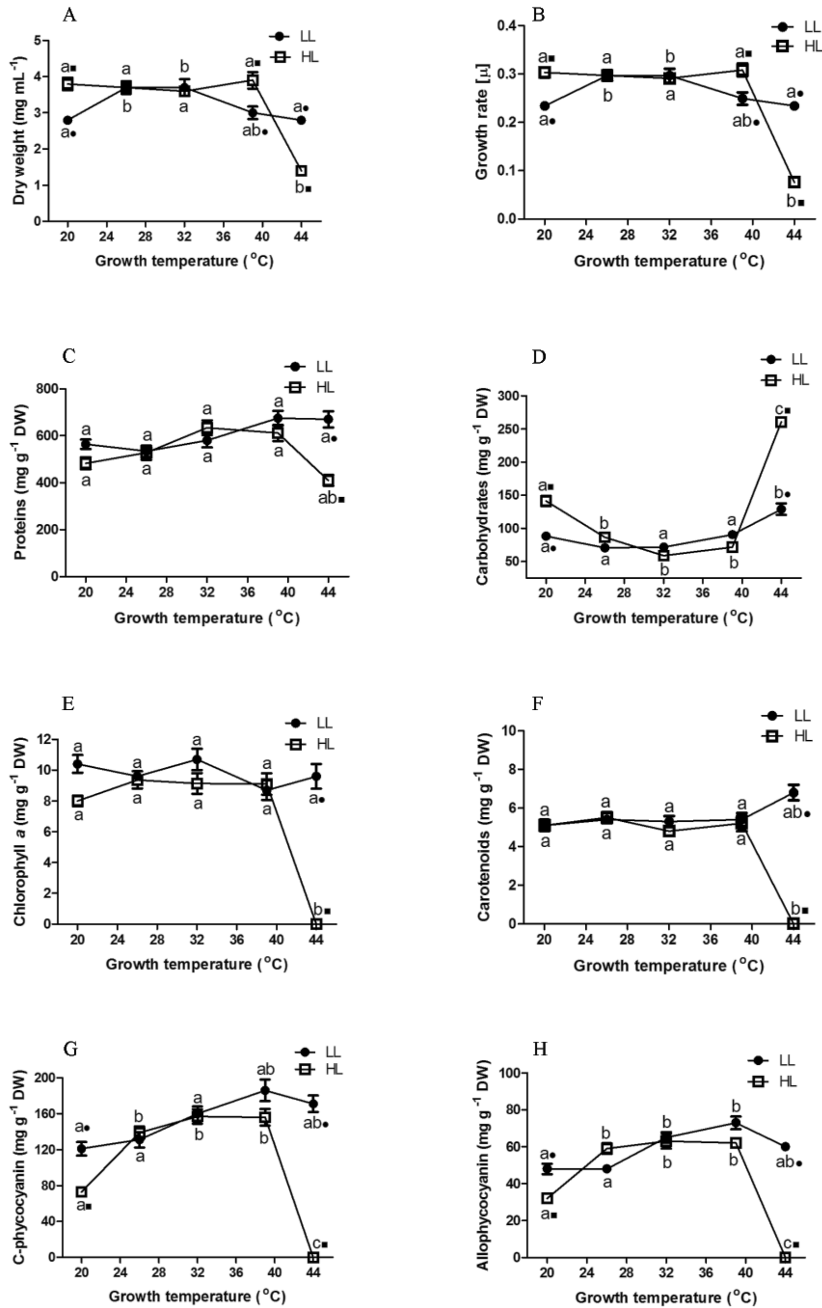

Figure 1. Changes in the end-point growth (A), growth rate (B) and the content of total proteins (C), carbohydrates (D) and pigments (E, F, G, H) in *Synechocystis* sp. R10 in dependence of the cultivation temperature and irradiance levels. LL, 132 µmol photons m^{-2} s^{-1}; HL, 2×132 µmol photons m^{-2} s^{-1}. Bars indicate SD (n=3). Means for a particular parameter with different letters are significantly different ($p<0.05$) between temperatures for a specific light intensity. Squares and circles indicate significant differences for each parameter for a specific temperature between LL and HL treatments.

range of 2.3-2.8:1, depending on the cultivation temperature and irradiance. The synthesis of both pigments, especially of C-PC, was enhanced with increasing the temperature from 20 to 39°C, regardless of irradiance (Fig. 1G, H). The contents of C-PC and APC were slightly decreased at 44°C and LL, while at 44°C and HL, no pigments were registered and cell bleaching was observed. The low light intensity stimulated the synthesis of C-phycocyanin and APC from 119 to 165% and from 118 to 150%, respectively. The phycobiliprotein content reached the highest values at 39°C and LL - 187 mg g^{-1} DW for CPC and 73 mg g^{-1} DW for APC. The content of C-PC (109 mg g^{-1} DW) and APC (41 mg g^{-1} DW), after prolonged cultivation (14 days) at 32°C and LL, was lower than that of cells cultivated for 4 days, possibly due to the depletion of nitrogen in the medium (Boussiba and Richmond, 1980; Da Silva et al., 2009).

Behaviour of defense enzymes of *Synechocystis* sp. R10, grown at different temperatures under two light intensities

Three bands of SOD activity (designated SOD1-3 and arranged according to their increase in migration mobility) were resolved (Fig. 2A) in crude enzyme extracts from *Synechocystis* cultured under different temperature and irradiation conditions. All bands were identified as FeSOD isoenzymes according to their insensitivity to cyanide inhibition and their sensitivity to inactivation by H_2O_2. The relative total SOD activity under LL was highest at 20°C and 26°C, moderate at 32°C and 39°C and very low at 44°C. In contrast, the total enzyme activity under HL did not change significantly within the temperature range of 20–39°C, while the highest temperature (44°C) had a considerable decreasing effect. Six bands of CAT activity were found after native PAGE (Fig. 3A). The relative total CAT activity was highest at

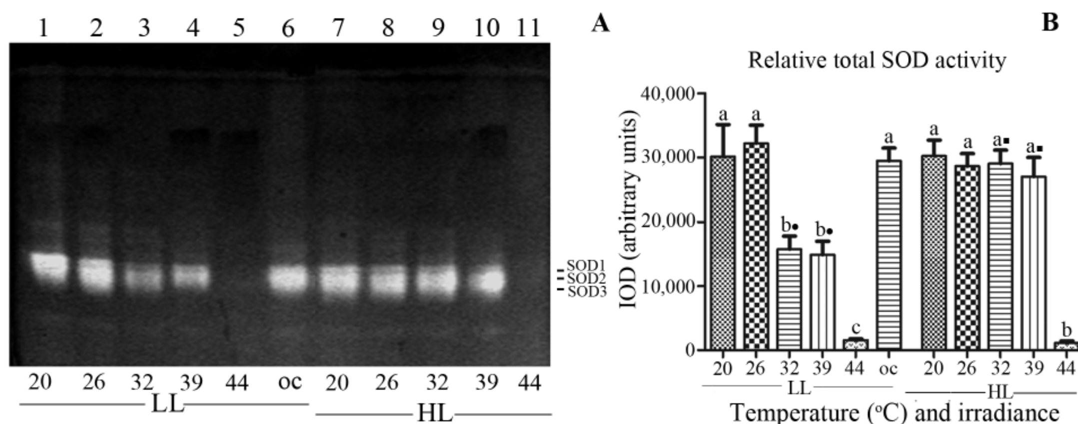

Figure 2. Isoenzyme patterns (A) and activity (B) of superoxide dismutase (SOD) in *Synechocystis* sp. grown at different temperatures and irradiance levels. Equal amounts of protein (20 µg) were loaded per well. Oc, old culture (32°C, LL, 14 days of cultivation).

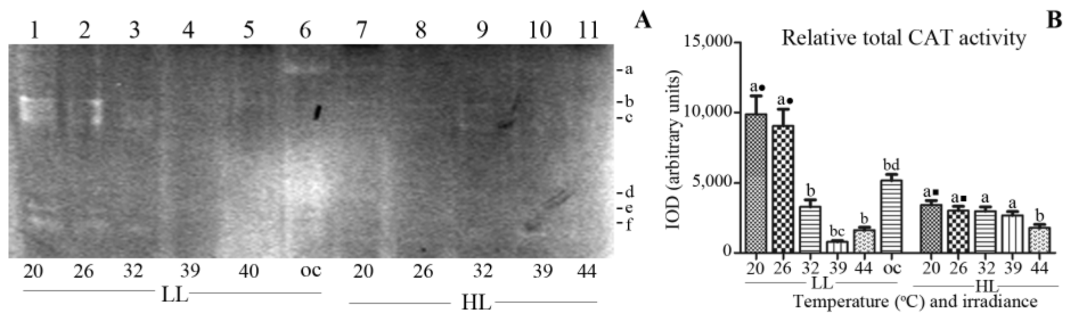

Figure 3. Changes in catalase (CAT) activity in *Synechocystis* sp. in response to different temperatures and irradiance levels. A, isoenzyme patterns. Letters (a-f) indicate the bands of CAT activity in order of increasing electorphoretic mobility. B, relative total CAT activity.

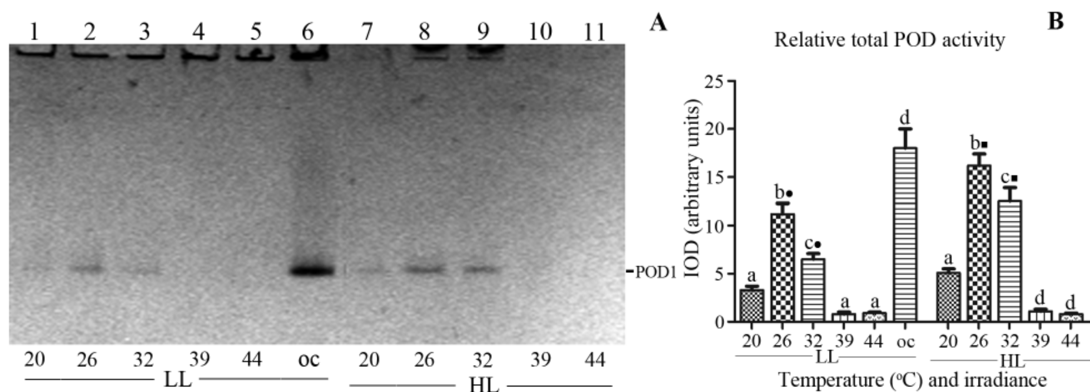

Figure 4. In gel activity staining (A) and relative total activity (B) of benzidine-specific peroxidase (POD) in *Synechocystis* sp. in response to different temperatures and irradiance levels.

20°C, LL. A gradual decrease in enzyme activity was observed with the rise of the temperature under LL (Fig. 3B). The temperature range of 20-39°C under HL maintained similar, low activity levels, which were further reduced at 44°C. Gels stained for POD activity revealed one band (Fig. 4A) in cultures grown under 20°C, 26°C and 32°C and both light intensities. This band was almost invisible at higher temperatures. The band was least intensive at 20°C and most intensive at 26°C. The relative POD activity was about 1.5–2 times higher in cells grown at HL than at LL (Fig. 4B). Eight main bands of GR (GRa-h) were detected in *Synechocystis* under all growth conditions (Fig. 5A). The intensity of the bands varied between treatments and led to changes in total enzyme activity. Cells cultured at 32°C under LL showed a relatively moderate level of GR activity (Fig. 5B). Growth at lower temperatures enhanced the activity of GR, while at higher temperatures, especially 44°C, a significant decreasing effect was observed. The relative total GR activity did not show significant changes within the temperature range of 20–39°C and HL, but the activity was reduced approximately 3 times at 44°C. Gelatin zymograms revealed

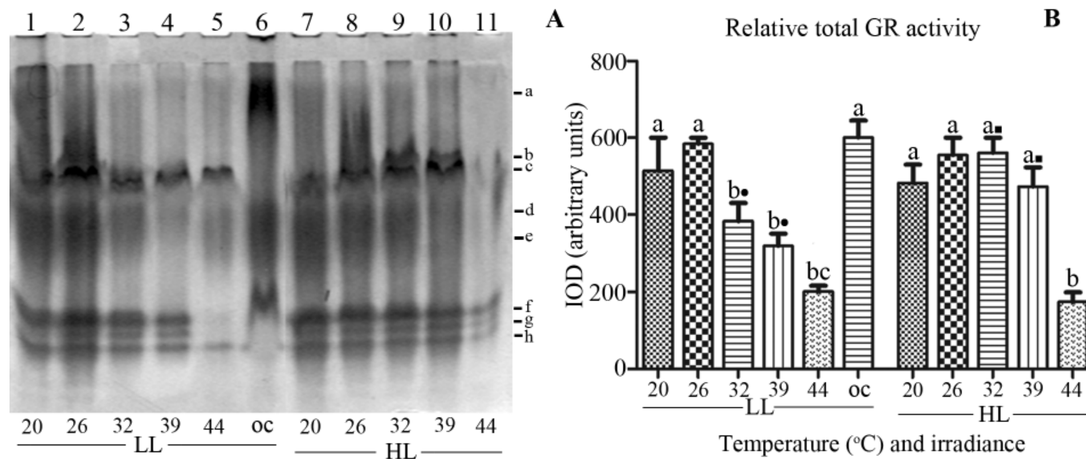

Figure 5. Changes in glutathione reductase (GR) activity in *Synechocystis* sp. in response to different temperatures and irradiance levels. A, isoenzyme patterns. Letters (a-h) indicate the bands of GR activity in order of increasing electorphoretic mobility. B, relative total GR activity.

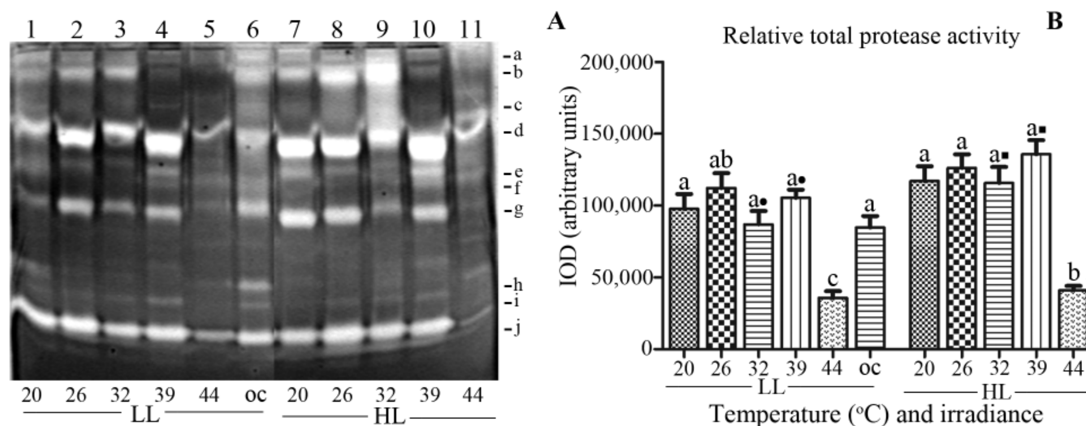

Figure 6. Changes in intracellular proteases in *Synechocystis* sp. in response to different temperatures and irradiance levels. A, gelatin zymograms of protease activities. Letters (a-j) indicate the bands of protease activity in order of increasing electorphoretic mobility. B, relative total protease activity.

the presence of ten major proteases (Fig. 6A, a-j). Although the relative total protease activity at 20–39°C was similar at the respective irradiance level (Fig. 6B), the intensities (activities) of individual bands changed according to temperature. The most responsive to temperature treatment were proteases b, d, g and j. Total gelatinolytic activity significantly decreased under 44°C and both light intensities.

The isoenzyme pattern and changes in the esterase activity of *Synechocystis* under different growth conditions are shown in Fig. 7. Four clearly visible bands and eight weaker bands of EST

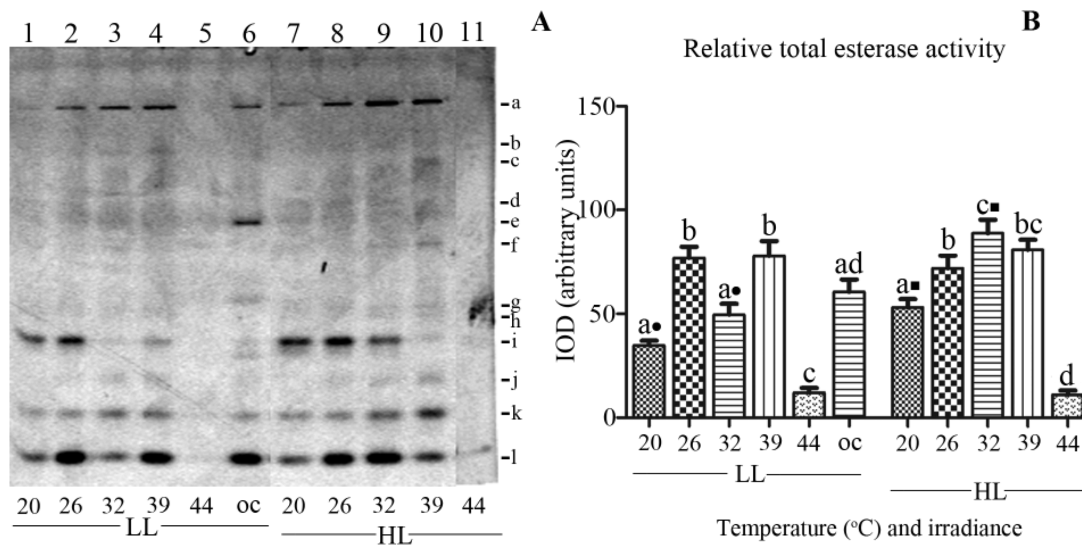

Figure 7. Isoenzyme patterns (A) and activity (B) of esterases in *Synechocystis* sp. in response to different temperatures and irradiance levels. In A, letters (a-l) indicate the bands of esterase activity in order of increasing electorphoretic mobility.

activity were detected by gels analysis, indicated in sequence by *letters* (Fig. 7A, a-l) starting from the anode, according to the increase in the negative charge. Under LL, the relative total esterase activity (Fig. 7B) was highest at 26°C and 39°C, mainly due to the strongest intensity of the ESTi and/or ESTl. Total esterase activity in the cells, grown at 20°C, and especially at 44°C, was significantly reduced ($p<0.05$ and $p<0.001$, respectively) compared to cells grown at 32°C. Under HL, the cells maintained a relatively high level of esterase activity within the temperature range of 26–39°C, while at 20°C and 44°C the activity was significantly lower. The most responsive to the temperature treatments under HL were ESTa, i, k and l.

Prolonged growth of cells (14-days) at 32°C and LL led to an enhancement of the activities of all studied antioxidant enzymes, while the relative total protease and esterase activities did not change significantly.

DISCUSSION

Synechocystis sp. strain R10 demonstrated the ability to grow in a wide range of temperatures (20–44°C) at irradiance levels of 132 and 2×132 µmol photons m^{-2} s^{-1}. Among all tested, 39°C and 2×132 µmol photons m^{-2} s^{-1} were the conditions at which maximum growth was achieved. Only the combinations of 20°C and LL, 44°C and LL, and especially 44°C and HL were unfavorable. The cyanobacterium responded to the unfavorable conditions by changes in its biochemical composition and enzyme activities. At the lowest temperature tested (20°C), *Synechocystis* accumulated total carbohydrates under both light intensities. A significant enhancement

in carbohydrate content was observed at 44°C and HL, simultaneously with a reduction in protein content. The high levels of cellular carbohydrates, which are an effective source of stored energy, could help survival of cells until improvement of the growth conditions. *Synechocystis* regulated also its phycobiliprotein content in response to temperature and light intensity (Fig. 1G, H). Several authors (Bhandari and Sharma, 2006; Jodłowska and Latala, 2010) have described the reduction in the content of light-harvesting pigments with increasing illumination and increased pigment content at low light intensity, which is in accordance with our results. It has long been considered that under light-limiting conditions cells increase their pigmentation, thereby enhancing the efficiency of light harvesting as an important adaptive response (Falkowski and LaRoche, 1991). The optimum temperature for pigment synthesis depends on the strain, but typically, extremely high and low temperatures significantly decrease the amount of cyanobacterial pigments (Chaneva et al., 2007; Hemlata, 2009). Low temperature-induced reduction could be an acclimation response expressed in the adjustment of light harvesting to the reduced metabolic activity of the cell (Huner et al., 1998). The high temperature-related decrease could be at least partially due to the pigment degradation, especially in the case of a simultaneous impact of high temperature and high light intensity.

The amount of phycobiliproteins (26% DW) synthesized by *Synechocystis* sp. R10 at 39°C, LL was similar to that reported for *Arthronema africanum* (30% DW) under optimal growth conditions (Chaneva et al., 2007), and was about 2-2.5 times greater than that found for *Spirulina* (Belay et al., 1993) and *Plectonema boryanum* (Chaneva et al., 1997).

Ten proteases were detected in *Synechocystis* under optimal growth conditions, probably involved in normal protein turnover. The highest growth temperature (44°C) decreased their activity suggesting that proteolysis was not essential in high temperature stress. The observed enhancement in the relative intensity of proteases a, e, h, and j (under LL) and proteases b, and g (under HL) at 20°C, however, indicated the involvement of these probably substrate-specific proteases in the low temperature response. Similarly, the exposure of *Synechococcus* sp. PCC 7942 to low temperature (from 37°C to 25°C) resulted in an induction of ClpP1 protease and a mutant with inactivated clpP1 gene does not properly acclimate to cold conditions (Porankiewicz et al., 1998). At the same time, ClpP1 induction is not essential for high temperature tolerance of *Synechococcus* sp. PCC 7942 (Clarke et al., 1998).

Brookes et al. (2000) have shown that the esterase activity (measured by the rate of FDA conversion to fluorescein) correlates with photosynthesis and nutrient-limited growth, which validates the use of this assay to assess the metabolic activity of phytoplankton cells. In the present study, it was possible to evaluate the changes in metabolic activity of *Synechocystis* in response to different conditions using gel staining for esterase activity. The observed reduction in esterase levels in cells grown at the lowest and highest temperatures under

both light intensities (Fig. 7B) proved a marked reduction of metabolic activity under these conditions. Unfavorable environmental conditions disturb the delicate cellular redox balance leading to a significant accumulation of harmful reactive oxygen species (ROS) (Suzuki and Mittler, 2006). The importance of antioxidant enzymes (AOE) is in their ability to scavenge ROS and prevent oxidative damage. Superoxide radicals are converted to H_2O_2 by the action of SOD, and the accumulation of H_2O_2 is prevented by the activities of CAT, PODs and GR. Although most of the cyanobacteria contain more SOD isoforms with different metal cofactors (Mn, CuZn, Ni or Fe), *Synechocystis* R10 contains only FeSOD. Nefedova et al. (2003) have reported the key role of the single cytoplasmic FeSOD for *Synechocystis* sp. PCC 6803. In the present study, *Synechocystis* R10 maintained high levels of FeSOD in all tested cultivation conditions, except for 44°C (Fig. 2B). An increase in FeSOD activity relative to control level (32°C) was observed only at 20°C, LL simultaneously with an elevation of CAT and GR activities (Figs 3B and 5B). Although the cyanobacterium exhibited sensitivity to the combination of high temperature (44°C) and LL, it was able to acclimate to these conditions. The acclimation involved only accumulation of carotenoids (Fig. 1F) and C-phycocyanin (Fig. 1G) but not an induction of ROS-scavenging enzymes activity. Carotenoids provide an effective elimination of specific ROS and their accumulation is a common response of the cyanobacterial cells to stress (Burton and Ingold, 1984; Kelman et al., 2009). Under extreme stress conditions (44°C and HL) *Synechocystis* R10 showed bleaching (Fig. 1E-H) and decreased activity of all investigated AOE, which was related to a significantly reduced, but not ceased growth (Fig. 1A, B). Thus, *Synechocystis* R10 showed the ability to employ different mechanisms at low temperature, LL, relative to high temperature, LL and high temperature, HL, each mechanism being effective in helping cells to overcome the respective adverse environmental conditions.

The results of the present study showed that the newly-isolated *Synechocystis* sp. R10 was a fast growing, thermotolerant strain, and a valuable source of biomass rich in carbohydrates, protein, and especially phycobiliproteins. The changes in the studied physiological factors were associated with alterations in cellular metabolism, leading to a significant difference in the quantity of the synthesized useful products. These findings may be used as a biotechnological tool to manipulate the production of valuable metabolites by adjusting the growth conditions of *Synechocystis* sp. R10.

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