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DETECTION OF PHOSPHATASE ACTIVITY IN AQUATIC AND TERRESTRIAL CYANOBACTERIAL STRAINS

ABSTRACT: Cyanobacteria, as highly adaptable microorganisms, are characterized by an ability to survive in different environmental conditions, in which a significant role belongs to their enzymes. Phosphatases are enzymes produced by algae in relatively large quantities in response to a low orthophosphate concentration and their activity is significantly correlated with their primary production. The activity of these enzymes was investigated in 11 cyanobacterial strains in order to determine enzyme synthesis depending on taxonomic and ecological group of cyanobacteria. The study was conducted with 4 terrestrial cyanobacterial strains, which belong to *Nostoc* and *Anabaena* genera, and 7 filamentous water cyanobacteria of *Nostoc*, *Oscillatoria*, *Phormidium* and *Microcystis* genera. The obtained results showed that the activity of acid and alkaline phosphatases strongly depended on cyanobacterial strain and the environment from which the strain originated. Higher activity of alkaline phosphatases, ranging from 3.64 to 85.14 μmolpNP/s/dm^3, was recorded in terrestrial strains compared to the studied water strains (1.11-5.96 μmolpNP/s/dm^3). The activity of acid phosphatases was higher in most tested water strains (1.67-6.28 μmolpNP/s/dm^3) compared to the activity of alkaline phosphatases (1.11-5.96 μmolpNP/s/dm^3). Comparing enzyme activity of nitrogen fixing and non-nitrogen fixing cyanobacteria, it was found that most nitrogen fixing strains had a higher activity of alkaline phosphatases. The data obtained in this work indicate that activity of phosphatases is a strain specific property. The results further suggest that synthesis and activity of phosphatases depended on eco-physiological characteristics of the examined cyanobacterial strains. This can be of great importance for the further study of enzymes and mechanisms of their activity as a part of cyanobacterial survival strategy in environments with extreme conditions.

KEYWORDS: acid phosphatases, alkaline phosphatases, cyanobacteria, enzyme activity

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INTRODUCTION

Cyanobacteria, the oxygen-evolving photosynthetic prokaryotes originating about 3.5 billion years ago, occupy a credential position between pro- and eukaryotes (Atzenhofer et al., 2002). Cyanobacteria successfully colonise almost all kinds of terrestrial and aquatic ecosystems, due to their high ecological adaptability to different environmental conditions (Oren, 2000). They also play an important role in the global cycling of elements, such as carbon, nitrogen and phosphorus (Sanudo-Wilhelmy et al., 2001). Cyanobacteria represent productive and efficient biological system due to the fact that many cyanobacteria have ability to perform both photosynthesis and nitrogen fixation together with their efficient nutrient uptake mechanisms (Parveen and Pandey, 2011).

In the environment with widely fluctuating nutrient availability, cyanobacteria synthesize new proteins which contribute to survival of the organisms and become a part of their unique survival strategy. In order to deal with phosphate deprivation, cyanobacteria have devised a number of different measures (Pandey, 2006). Since inorganic phosphate is the only form of phosphorus that is directly used by cells in most ecosystems, there is a deficiency of phosphorus (Thingstad et al., 2005). Three main components involved in phosphorous metabolism in cyanobacteria include: inorganic phosphate (Pi) uptake, dissolved organic phosphorus (DOP) hydrolysis, and polyphosphate (polyp) biosynthesis and catabolism (Duncan, 2010). During short periods of phosphorous starvation, cyanobacteria use accumulated phosphate stored in the form of polyphosphate reserves for cellular metabolism (Bhaya et al., 2000). This enables cyanobacteria to propagate 3-4 cell divisions even when the dissolved phosphate is entirely depleted (Chorus and Mur, 1999). During long periods of phosphorus starvation, cyanobacteria produce extracellular phosphatases, extracting phosphate from a wide spectrum of organic compounds and converting it into biologically available inorganic phosphate and organic moiety (Stihl et al., 2001).

Phosphatases (phosphomonoester hydrolases – PME) represent the group of phosphohydrolases which play an important role in phosphate release in aquatic environments (Matavulj and Flint, 1987; Chróst and Suida, 2002). Phosphatases represent inducible catabolic ectoenzymes and their expression is generally regulated by the external concentration of inorganic phosphate, but the internal N:P ratio may also play a role in this process (Hoppe, 2003). After the early phase of enzyme synthesis, phosphatases accumulate in the periplasmic space and at the later stage they are released outside the cell (Pandey, 2006). Their activity is modulated by different physicochemical factors like temperature, light, pH, micro and macronutrients, salinity and heavy metals (Singh et al., 2006).

Alkaline phosphatases (APA) include a group of inducible isoenzymes (Luo et al., 2010) which optimally react in pH ranging from 7.6 to 9.6 (Chróst and Suida, 2002). Their role is to catalyze the hydrolysis of a variety of phosphate esters and to liberate inorganic phosphate (Chróst and Suida, 2002). The active center of enzyme is conserved well, although the protein features of alkaline phosphatase are strongly divergent. Regulation of the APA synthesis is carried out through a repression-derepression mechanism and by competitive inhibition (Chróst and Suida, 2002). Phosphate causes repression of the enzyme activity in a concentration dependent manner while lower amounts of phosphate lead to derepression of the PMEase enzyme (Pandey, 2006).

Acid phosphatases represent a group of isoenzymes that optimally react in pH ranging from 4.0 to 5.5. Regulation of synthesis often takes place without any form of repression with inorganic phosphorus present in the environment (Chróst and Suida, 2002).

The importance of increased phosphorus loading in the process of eutrophication of water ecosystems was recognized at the end of the sixties (Matavulj *et al*., 1990; Pandey and Tiwari, 2003). The low TN:TP ratio, together with thermal stratification, reduces transparency and increases water temperature and pH, and frequently enhances the occurrence of cyanobacterial blooms (Mischke, 2003). Since phosphorus is an important factor in the growth of cyanobacteria and phosphorus concentration greater than 0,1 mg/L is sufficient to cause cyanobacterial blooms in aquatic ecosystems (Bartram et al., 1999), it is of great significance to test phosphatase enzyme activity in cyanobacterial strains.

The aim of the present study was to investigate the changes in PMEase activity of 11 cyanobacterial strains during the stationary phase of growth. The other objectives include comparison of PMEase activity in terrestrial and freshwater cyanobacterial strains as well as determining whether there is a difference in enzyme activity between nitrogen-fixing and non nitrogen-fixing strains.

MATERIALS AND METHODS

Cyanobacteria and culture conditions

Detection of phosphatase activity was performed in the cultures of 11 different cyanobacterial strains. Seven water cyanobacterial strains were isolated from surface waters in the region of Vojvodina (Simeunović, 2010) and four strains were isolated from different soil types in Vojvodina (Simeunović, 2005). All cyanobacterial strains that were examined belong to the Novi Sad Cyanobacterial Culture Collection-NSCCC. The studied terrestrial strains belong to the *Nostoc* and *Anabaena* genera, whereas the studied aquatic cyanobacterial strains belong to the *Microcystis, Nostoc, Phormidium* and *Oscillatoria* genera. Strain *Microcystis* PCC 7806 was purchased from Pasteur Culture Collection (http://www.pasteur.fr/bio/PCC). The cyanobacterial strains were grown in the laboratory conditions in liquid synthetic mineral medium BG-11 (Rippka et al., 1979), with or without nitrogen, depending on the ability of the strains to fix atmospheric nitrogen. The cultures were incubated photo-autrophically at 22–24ºC under illumination of cool white fluorescent light. Phosphatase activity was determined on the 21st day of incubation, during the stationary phase of growth.

Enzyme assay

Phosphatase activity (PA) in the cyanobacterial cultures was measured using the spectrophotometric method and enzyme activity was measured as the rate of hydrolysis of the phosphatase substrate p-nitrophenylphosphate (p-NPP, Sigma Aldrich), by detecting the released product, p-nitrophenol (Matavulj, 1986). Activities of alkaline and acid phosphatases of tested strains were determined at pH values of the appropriate sterile buffer (pH5 and pH9) as their potential activities at a temperature of 30°C. Phosphatase activities were determined by adding 0.3 ml of 5% p-nitrophenylphosphate into 2.4 ml of sample. After one hour of incubation, the reaction was interrupted directly by adding 10 M NaOH and the result of enzymatic reaction was a yellow product, para-nitrophenol (pNP). The intensity of the yellow color was proportional to the level of phosphatase activity of the sample and therefore the sample absorbance was measured at 420 nm using a spectrophotometer (Beckman 25). The calculation of enzyme activity was performed according to Matavulj (1986). Sterile distilled water was used as a control. All enzyme assays were done in triplicate and the results are expressed as mean values.

RESULTS

In order to determine the possible relationship between phosphatase activity and taxonomic and ecological background of the studied cyanobacteria, comparison of the enzyme activity was made during the stationary phase of the growth between water and terrestrial strains, as well as between nitrogen-fixing and non-nitrogen-fixing strains.

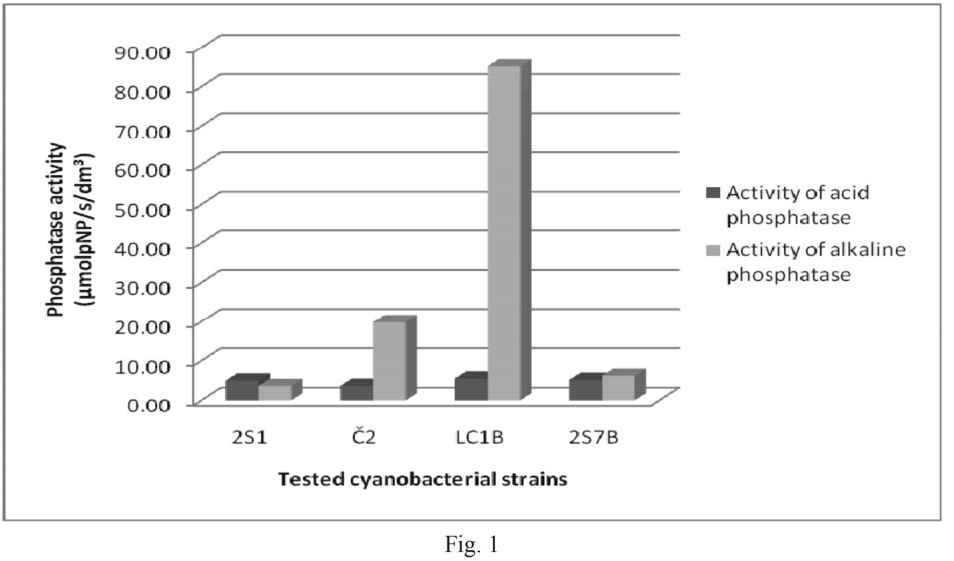

Fig. 1

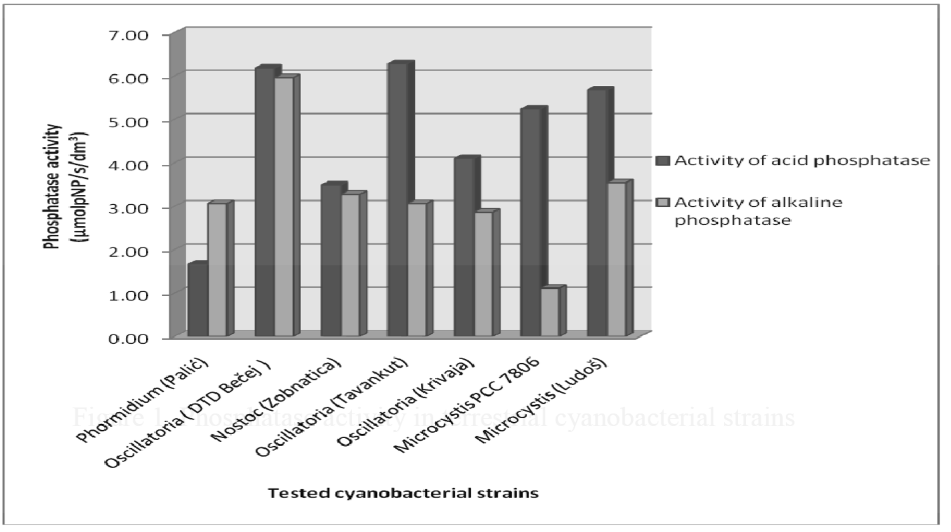

Fig. 2

When analyzing the phosphatase activity in terrestrial strains, it was observed that the activity of alkaline phosphatase ranged from 3.64 to 85.14 μmolpNP/s/dm^3, while the activity of acid phosphatase ranged from 3.66 to 5.48 μmolpNP/s/dm^3, indicating a higher activity of the enzyme alkaline phosphatase (Figure 1). The highest AP activity was detected only in *Anabaena* strain LC_1B (85.14 μmolpNP/s/dm^3) compared to the other terrestrial strains, and the lowest activity was recorded in *Nostoc* strain $2S_1$ (3.64 μmolpNP/s/dm^3). Acid phosphatase was less active in the studied terrestrial strains, ranging from 3.66 to 5.48 μmolpNP/s/dm^3. The exception was the strain $2S_1$ and in case of this strain an increased activity of acid phosphatase was observed (5.01 μmolpNP/s/dm^3) in comparison with the alkaline phosphatase (3.64 μmolpNP/s/dm^3).

Unlike terrestrial strains, most of the water strains (86%) were characterized by a higher activity of the acid phosphatase in comparison to alkaline phosphatases (Figure 2). An exception was the strain *Phormidium* (Palić) in which acid phosphatase showed a lower activity than the alkaline phosphatase. The activity of alkaline phosphatase ranged from 1.11 to 5.96 μmolpNP/s/dm^3, while the acid phosphatase activity ranged from 1.67 to 6.28 μmolpNP/s/dm^3. Acid phosphatase in the examined strains was almost equally active in strains *Oscillatoria* (DTD Bečej) and *Oscillatoria* (Tavankut) with values of 6.18 μmolpNP/s/dm^3 and 6.28 μmolpNP/s/dm^3, respectively (which also represent the highest recorded value of acid phosphatase activity in the examined aquatic strains). The strain with the lowest activity of acid phosphatase was strain *Phormidium* (Palić) (1.67 μmolpNP/s/dm^3).

In case of nitrogen fixing cyanobacteria, the activity of alkaline phosphatase ranged from 3.27 to 85.14 μmolpNP/s/dm^3, while the range of acid phosphatase enzyme activity was lower, with values between 3.49 and 5.48 μmolpNP/s/dm^3 (Figure 3). Thus, in most nitrogen-fixing strains (60%) a higher

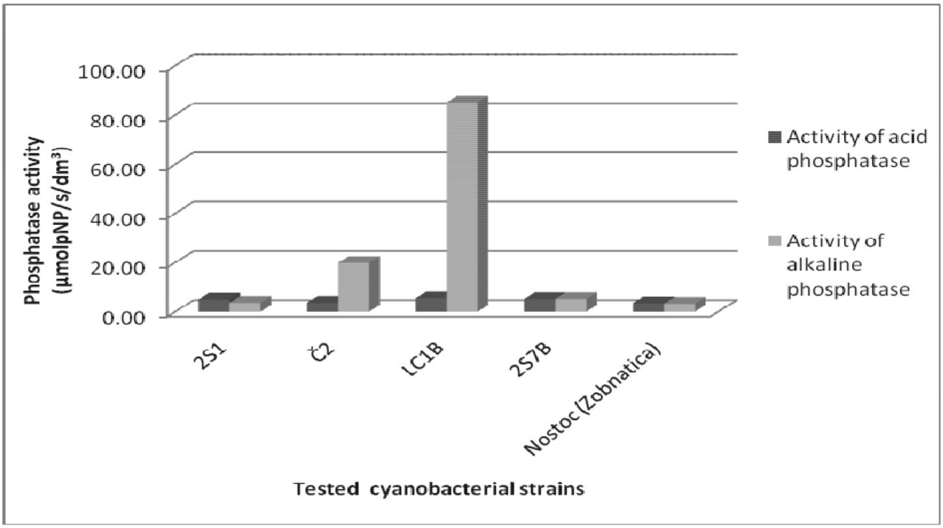

Fig. 3

activity of alkaline phosphatases was observed. The exceptions were strains $2S_1$ and *Nostoc* (Zobnatica) in which a higher activity of acid phosphatase was detected. The highest AP activity, reaching the value of 85.14 µmolpNP/s/dm^3, was detected in cyanobacterial strain *Anabaena* LC_1B.

The results obtained for non-nitrogen-fixing strains showed dominant activity of acid phosphatase, and the values ranged from 1.67 to 6.28 µmolpNP/s/dm^3 (Figure 4). Alkaline phosphatase in these strains showed a lower activity than the acid phosphatase, with values between 1.11 and 5.98

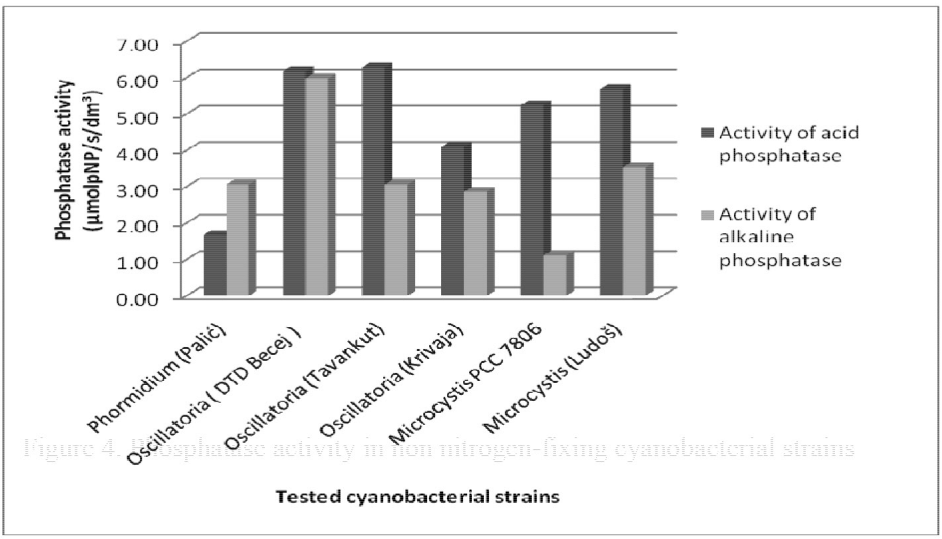

Fig. 4

μmolpNP/s/dm^3. The exception was the strain *Phormidium* (Palić) which was characterized by increased activity of alkaline phosphatase (3.06 μmolpNP/s/dm^3) compared to acid phosphatase (1.67 μmolpNP/s/dm^3).

DISCUSION

Based on the results obtained by examining cyanobacterial strains, it can be noticed that the phosphatase activity is a strain specific property. The results suggest that during growth under laboratory conditions each strain reacts differently to the environmental conditions; some strains are characterized by the dominance of alkaline phosphatase, while in the others there was a higher activity of acid phosphatase. This leads to a conclusion that an organism of a given genotype is a very much product of its environment (Tempest and Neigssel, 1978). Singh et al. (2007) have collected similar data indicating that the phosphatase activity is a species-specific property. The results of Tetu et al. (2009) indicate that different species and even different strains of the same species are likely to react quite differently to phosphate deficiency. The physiological manifestation of P stress, nutrient requirements and uptake capacity are complex and variable among cyanobacterial species and strains (Schreiter et al., 2001).

In this study the enzymatic activity was compared between aquatic and terrestrial strains, as well as between nitrogen-fixing and non-nitrogen-fixing strains. It was observed that the phosphatase activity was clearly during the stationary phase of growth differs between terrestrial and water strains, as well as between nitrogen-fixing and non-nitrogen-fixing strains. In 3 out of 4 terrestrial strains alkaline phosphatase had greater activity than acid phosphatases. Unlike terrestrial strains, 6 of 7 water strains were characterized by high activity of the enzyme acid phosphatase during the stationary phase of growth. The results indicate that there is a connection between enzymatic activity and the processes of cell differentiation (sporulation, formation of heterocyst) in cyanobacteria. This may explain the higher activity of alkaline phosphatase in the majority of nitrogen-fixing cyanobacterial strains examined in this study, because during their life-cycle they form heterocysts and often permanent spores. Pandey et al. (1991) had similar results and they observed a higher activity of alkaline phosphatase in a wild type *Anabaena dolium* during sporulation which suggests that the enzyme activity is related to sporulation rather than the phosphate starvation. The assumption that the induction of the APase activity during P-stress may be considered as an early biochemical event preceding sporulation is strengthened by the observation that excess phosphate inhibits both sporulation and alkaline phosphatase activity (Pandey, 2006). Banerjee and John (2005) showed that the phase of rapidly increasing phosphatase activities correlates with the gradual loss of ability to form hormogonia in some examined *Rivularia* strains.

The results of this study show different activity of acid and alkaline phosphatases depending on their origin and eco-physiological characteristics. On the basis of these results, we may assume that there could be a connection between enzymatic activity and the taxonomic and ecological groups of cyanobacteria.

Whitton et al. (1998) indicate that the taxonomic affiliation and origin of cyanobacterial strains play an important role in the phosphatase activity in cyanobacteria, and their results suggest that representatives of family *Rivulariacae* have a greater activity in comparison with the strains which do not belong to this family.

Enzymatic activity is modulated by macro- and microelements present in the medium and in the cell (Pandey, 2006). The examined cyanobacterial strains were grown in mineral medium containing EDTA complex and Mg^{2+} and Zn^{2+} ions. Therefore, there is a possibility that the mineral medium could influence the enzyme activity in some strains. In this study enzymatic activity was measured during the stationary phase of growth, when concentration of nutrients in the medium is considerably reduced, which could also affect the activation of certain types of phosphatase enzymes. Pandey (2006) reported the requirement for Mg^{2+} in the APase activity in four diazotrophic cyanobacterial strains. There are several reports of enhanced phosphatase activity in cyanobacterial strains in response to elevated calcium (Whitton et al., 2005). Relatively low level (1 μM) of all micronutrients (Mn^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+}) enhanced the activity of PMEase or kept it stable (Pandey, 2006). Ions such as Na^{+}, K^{+}, Fe^{3+} and Zn^{2+} at moderate concentrations had a stimulating effect on phosphatase activity in *Lyngbya majuscula* (Al-Shehri, 2006). Singh et al. (2007) show that salinity (NaCl) significantly stimulated phosphate uptake which is followed by a greater P-accumulation in the cells. Therefore, the availability and concentration of certain nutrients may play an important role in regulation of phosphatase synthesis in cyanobacteria. Liu et al. (2011) showed that the lower concentrations of inorganic phosphate led to inhibition of the cell growth rather than cell death. Pandey (2006) registered a decrease in phosphatase activity during incubation, which correlates with a gradual increase in internal phosphorus content. In cyanobacterial cells internal phosphate pool regulates the synthesis of repressible phosphatases (Fitzgerald and Nelson, 1996). Thus, the concentration of phosphate initially supplied in the medium and the cellular phosphate level significantly affect the time required for the expression of phosphatase (Kumar et al., 1992). Banerjee (2007) found the presence of significant phosphatase activity in cyanobacteria strain *Calothrix anomala* 182 even when the concentration of P in the medium was high. Besides this, physical factors like temperature and light significantly affect the enzymatic activity. In case of cyanobacteria *Anabaena oryzae* enzymatic activity was greatly reduced in cells that were incubated in the dark, compared with cells incubated in light conditions, which indicates that photo energy is required for the synthesis of APases (Singh and Tiwari, 2000). From this point of view, further study of the influence of different factors on enzyme phosphatases in the examined cyanobacterial strains would be of great importance.

CONCLUSION

Analysis of phosphatase activity in water and terrestrial cyanobacterial strains provided the evidence that there is a strong connection between enzymatic activity and the taxonomic and ecological groups of cyanobacteria. Activity of alkaline phosphatases was dominant in most of the examined terrestrial and nitrogen-fixing cyanobacteria during the stationary phase of growth. On the other hand, acid phosphatases showed a higher activity in the largest number of water and non nitrogen-fixing cyanobacteria. The results suggest that synthesis and activity of these enzymes are the specific property of every cyanobacterial strain. The obtained results are significant for the study of cyanobacterial metabolism and their responses to environment conditions. In that respect it is very important to understand how different factors affect phosphatase activity of cyanobacteria, which requires further investigations.

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ДЕТЕКЦИЈА ФОСФАТАЗНЕ АКТИВНОСТИ КОД ВОДЕНИХ И ЗЕМЉИШНИХ СОЈЕВА ЦИЈАНОБАКТЕРИЈА

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РЕЗИМЕ: Цијанобактерије се као врло адаптибилни микроорганизми одликују способношћу преживљавања у различитим неповољним условима спољашње средине у чему значајна улога припада њиховим ензимима. Фосфатазе (фосфомоноестеразе) представљају ензиме које микроорганизми, укључујући и микроалге, продукују у релативно великим количинама као одговор на ниску концентрацију неорганског фосфора. Активност две групе фосфатаза (киселих и алкалних) испитивана је код 11 филаментозних сојева цијанобактерија у циљу одређивања синтезе и активности ензима у зависности од таксономске и еколошке групе цијанобактерија. Испитивања су вршена са 4 азотофиксирајућа земљишна соја цијанобактерија који припадају родовима *Nostoc* и *Anabaena*, као и са 7 водених сојева који су представници родова *Nostoc, Oscillatoria, Phormidium* и *Microcystis.* Резултати испитивања указали су на доминантну активност алкалних фосфатаза код већине испитиваних земљишних сојева цијанобактерија (75%) при чему се активност кретала од 3,64 до 85,14 μmolpNP/s/dm^3. Нижа активност алкалних фосфатаза (1,11 до 5,96 μmolpNP/s/dm^3) констатована је код већине водених сојева у поређењу са земљишним сојевима. Киселе фосфатазе су показале значајно већу активност код већине водених сојева (86%), при чему су се детектоване вредности кретале од 1,67 до 6,28 μmolpNP/s/dm^3. Резултати испитивања су указали на то да је активност ензима фосфатаза својство специфично за сваки цијанобактеријски сој (сој-специфично својство) и да значајно зависи од њиховог порекла. Поредећи активност ензима фосфатаза између азотофиксирајућих и неазотофиксирајућих сојева, констатовано је да је већа активност алкалних фосфатаза била карактеристична за већину испитиваних азотофиксирајућих сојева (60%), док је код већине неазотофиксатора забележена доминација активности киселих фосфатаза (83%). Добијени резултати иду у прилог томе да активност ових ензима значајно зависи и од екофизиолошких карактеристика тестираних цијанобактеријских сојева. Свакако би од великог значаја било спровести даља испитивања активности ових ензима у зависности од различитих фактора спољашње средине и механизама њиховог деловања као дела стратегије преживљавања цијанобактерија у неповољним условима спољашње средине.

КЉУЧНЕ РЕЧИ: киселе фосфатазе, цијанобактерија, алкалне фосфатазе, ензимска активност