

Changes in antioxidant systems and biomass in response to selenate in blue-green microalgae *Spirulina platensis*, Cyanophyta

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Abstract:

The effects of selenium (Se) on growth and antioxidant activity in *Spirulina platensis* were investigated. *S. platensis* was grown in zarrouk medium containing three different concentrations of selenate (Na_2SeO_4) (0 as a control, 5 and 10 mg l^{-1}) for 7 days. Selenate, at the concentration of 5 mg l^{-1} was found to improve *S. platensis* growth. However, selenate was toxic to *S. platensis* at the concentration of 10 mg l^{-1} . Selenium increased the activity of enzymatic (peroxidase and catalase) and non-enzymatic (carotenoids) antioxidants when Se was applied to the microalgae at low concentration (5 mg l^{-1}). Phenolic compounds were significantly increased in Se-treated samples. The contents of chlorophyll a and carotenoids in Se-treated *S. platensis* did not significantly change as compared to control. The FTIR analysis clearly indicated that the spectra of cells grown on different Se concentrations did not significantly change as compared to control. In addition, the variety of different compounds such as aromatic, phosphine, alcohols and carboxylic acids were synthesized in all examined treatments. The present results suggested that Se at low concentration had suitable effects on the physiology of *S. platensis*.

Key words: FTIR, Growth, Heavy metals, Nutrition, Selenium, *Spirulina platensis*.

Introduction:

Selenium (Se) is an essential element for microalgae growth and reproduction. Some algal species need it in low levels (Li *et al.*, 2003). However, a high concentration of Se, as selenite and selenate, could affect microalgae as toxic element (Li *et al.*, 2003).

Selenium toxicity and its deficiency depends upon how much it is available in the environment. In aquatic environments, Se is found mainly in two valence types, selenite, Se^{3+} , and Selenate, Se^{6+} . Aquatic organisms tend to take in much more selenate than selenite due to its high solubility.

Selenium can improve microalgae growth at nanomolar concentrations as described for *Emiliania huxleyi* (Danbara and Shiraiwa, 1999; Araie and Shiraiwa, 2009; Morlon *et al.*, 2005). However, at micromolar concentrations, Se is toxic (Fournier *et al.*, 2010; Morlon *et al.*, 2005).

Microalgae assimilate selenite and selenate efficiently into selenoprotein, volatile compounds and Se-amino acids (Neumann *et al.*, 2003), especially Selenomethionine and Selenocysteine, which is probably due to their reductive metabolism.

Selenocysteine (Secys) co-transnationally integrated into protein at the site of UGA codon when Secys insertion sequence is located in the 3'-untranslated of selenoprotein mRNAs. The specific Secys-tRNA with the help of Secys-specific elongation factor and Secys insertion sequence binding protein 2 implicated in

insertion Secys into the protein (Araie and Shiraiwa, 2009). The most abundant selenoprotein, EhSEP2, is known as a protein disulfide isomerase-like protein, PDI. PDI controls conformational status and folding of the protein in the endoplasmic reticulum via influencing protein disulfide bonds (Araie and Shiraiwa, 2009). The replacement of cysteine by a Secys in the active site of PDI, located in the N-terminal region, leads to an increase in PDI activity (Araie and Shiraiwa, 2009).

The second abundant selenoprotein is known as thioredoxin reductase 1, TR1, which regulates enzyme activity by oxidizing or reducing the disulfide bond (Araie *et al.*, 2008).

Selenomethionine is considered as a bioavailable Se form for humans, which cannot be produced by the humans (Schrauzer, 2000). It is likely that natural Se supplements are safer than inorganic form (Umysova *et al.*, 2009).

S. platensis is used as animal- algal food or human supplements owing to a wide variety of compounds such as lipids, proteins, pigments, antioxidants, and vitamins (Pulz and Gross, 2004; Becker, 2007; Stengel *et al.*, 2011).

FTIR spectroscopy use intact microorganisms; the resulting spectra indicated the whole biochemical compound of the cells such as protein, lipid, and carbohydrate (Giordano *et al.*, 2001).

Nowadays, there are many interests to make Se-enriched products. Therefore, the elucidation of exact

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process contributed to the reactions of *S. platensis* to Se, especially antioxidant activity and biomass, has great importance. Meanwhile, in order to produce Se-enriched *S. platensis* in a large scale, the impact of different concentrations of selenate on the metabolic process of *Spirulina* need to be assessed. The aim of this study was to investigate the effects of selenate on the growth of *S. platensis* and to thoroughly characterize changes in the composition of the *S. platensis* cells during the course of the experiment.

Material and Methods:

Stock preparation and cultivation: *S. platensis* stock was prepared from Shahid Beheshti University, Tehran, Iran, that previously cultured on Zarrouk medium. *S. platensis* was cultured in 1-L Erlenmeyer flasks containing 0.5 L medium. Sodium selenate was added in the three concentrations 0 (as control) (C), 5 and 10 mg l⁻¹ to Zarrouk medium at the beginning of cultivation (Li *et al.*, 2003). 100 ml homogenous suspensions of *S. platensis* were placed in each flask, which was covered by cotton. The cultures were aerated with air, using an aquarium pump, and incubated at the temperature of 30±2°C. The cultures were illuminated by fluorescent lamps saving 2500 lux at the surface of flasks and grown at alternating light and dark periods (14h light:10 h dark). All experiments involving the transfer of *S. platensis* in the zarrouk medium were done under the aseptic condition in laminar flow. The experiments were run in three replicates for all analyses and measurements. The 7-day old cultured samples were analyzed.

Determination of *S. platensis* biomass dry weight: Biomass dry weight was obtained every 48 hours, where 10 ml homogenous suspensions of *Spirulina* samples were filtered through preweighed filter paper, rinsed with distilled water to remove loosely bound selenate and oven dried at 75°C for 24 hours. The difference between the initial and final weight was considered as the dry weight and expressed in g l⁻¹.

Determination of *S. platensis* cell concentrations: *S. platensis* cell concentration was determined every 48 hours by measuring the optical density of 10 ml homogenous suspensions of *Spirulina* at a wavelength of 560 nm using a UV/Vis spectrophotometer.

Measurement of photosynthetic pigments: Chlorophyll a (Chl a) and carotenoids were measured according to Lichtenthaler, 1987. Two ml of culture centrifuged at 10,000 rpm for 5 min. Then, 2 ml of acetone was added to the pellet and incubated at 45°C for 24 hours in the dark. The total pigment was measured according to following Equations:

$$\text{Chlorophyll a (}\mu\text{g/ml)} = 16.72 A_{665.2} - 9.16 A_{652.4}$$

$$\text{Chlorophyll b (}\mu\text{g/ml)} = 34.09 A_{652.4} - 15.28 A_{665.2}$$

$$\text{Carotenoids} = (1000 A_{470} - 1.63 \text{ Chla} - 104.9 \text{ Chlb})/221$$

Determination of phenolic compounds: Soluble phenolic acids were extracted from control and Se-exposed *S. platensis* (1 g) using pure methanol. The

extracts were centrifuged at 3000×g for 40 min at 4 °C. Total phenols were assessed according to (Arnaldos *et al.*, 2001). 75 µL of Folin–Ciocalteu reagent (Sigma–Aldrich) and 1 mL of 2% Na₂CO₃ were mixed with 100µL of phenolic extract. Then, it was incubated for 15 min at 25 °C in the dark and the absorbance was measured at 725 nm. Gallic acid was used as a standard

Assay of Antioxidant Enzymes: Enzymes were extracted at 4 °C by crushing samples in a mortar in the presence of phosphate buffer, 0.1 M pH 7.5 containing Na₂-EDTA 0.5 mM and ascorbic acid 0.5 mM, as an extraction buffer. The extracts were centrifuged for 15 min at 4°C and the supernatant was used as enzyme extract.

The peroxidase (POX) activity was measured as previously described by Abeles and Biles, 1991. The reaction mixture included acetate buffer (0.2 M, pH 4.8), 3 % H₂O₂ and 0.04 M benzidine in 50 % methanol. The reaction began by adding an enzyme extract. The increase in absorbance of the solution was continuously recorded at 530 nm for 1 min.

Catalase (CAT) activity was assessed as absorbance decrease at 240 nm for 1 min. (Pereira *et al.*, 2002) and expressed in ΔA min⁻¹ g⁻¹ FW.

Spectroscopic Methods by FT-IR: *S. platensis* biomass was washed 2 times with distilled water. After blotting, the biomass was dried in an oven at 70°C until dryness. 0.1mg of powder was mixed and crushed with KBr at a weight ratio of 1:100. then, 60 mg of the mixture was pressed to a tablet. The functional groups were analyzed using FTIR. The absorbance spectra were between 4000 and 400 cm⁻¹

Statistical Analysis: Data were subjected to analysis of variance (ANOVA) and differences between groups were assessed by Duncan's multiple-range test using SPSS software (Ardebili *et al.*, 2014).

Results:

The results showed that concentration of 5 mg l⁻¹ Se improved the growth rate as compared to the control. Although, at 10 mg l⁻¹ Se, *Spirulina* was able to grow but the growth rate decreased significantly (figure 1 and 2). The changes of Chl a with different concentrations of Se is shown in Figure 3. The addition of Se had no significant effect on the contents of Chl an in comparison with the control. The carotenoid quantification was performed in *S. platensis* after 7 days from beginning of the experiment (Figure 4). No significant changes in carotenoid contents were observed in the *S. platensis* exposed to 5 mg l⁻¹ Se as compared to the control.

In order to investigate antioxidant enzymes in *S. platensis* under Se treatment, we examined POX and CAT activity in the cells (Figure 5 and 6). The results indicated that the activities of POX and CAT increased markedly when the *S. platensis* were exposed to 5mg l⁻¹ Se, whereas the activities of the two mentioned antioxidant enzymes in 10 mg l⁻¹ Se- supplemented plants were lower than 5mg l⁻¹ Se. The effect of Se on soluble phenol contents was shown in Figure 7.

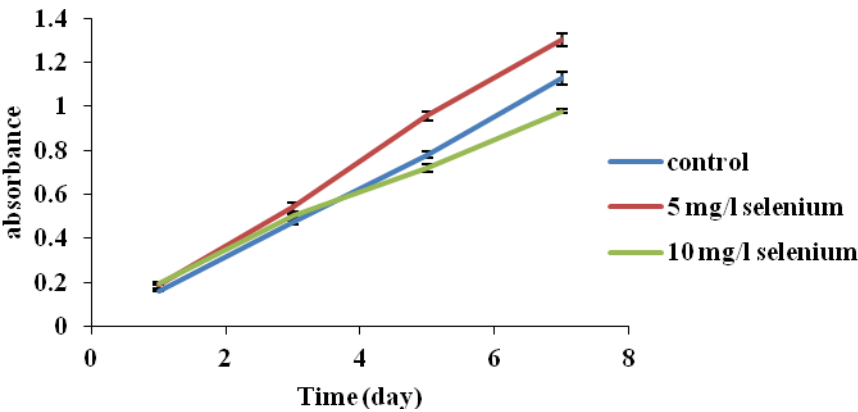


Figure1. Effect of different Se concentrations on the absorbance rates. Data are presented as means \pm SE of three replications.

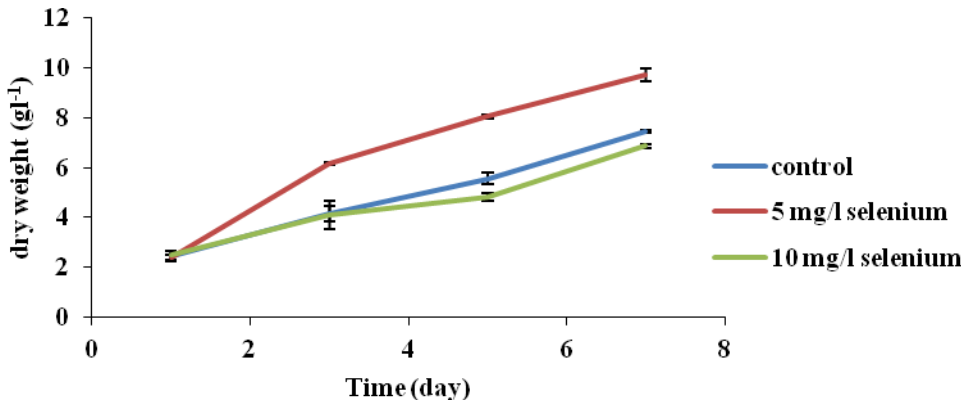


Figure 2. Effect of different Se concentrations on the dry weight of *S. platensis*. Data are presented as means \pm SE of three replications.

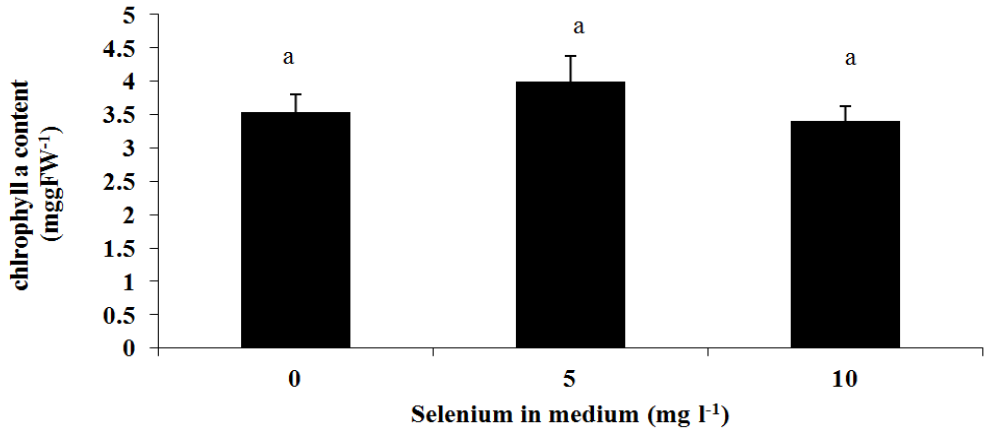


Figure 3. The changes in chlorophyll a content induced by Se. Values showed with the same letter are not significantly different, as measured using a Duncan test ($P < 0.05$). Data are presented as means \pm SE of three replications.

Selenium increased phenolic compounds significantly ($P < 0.05$) compared to the control. The soluble phenol contents were 4.294, 3.817 and 3.326 in 5 mg l⁻¹ Se, 10 mg l⁻¹ Se, and control, respectively. In this study, FTIR spectra of the control and Se- treated samples of *S. platensis* are shown in figure 8. The presence of peaks in the region of 1655.1, 1656 and 1654 cm⁻¹ for amide I was seen in control, 5 mg l⁻¹ Se and 10 mg l⁻¹ Se- treated samples, which were due to C=O stretching vibration. The peaks in the region of 1585(C), 1589 (5 mg l⁻¹ Se)

and 1585 cm⁻¹ (10 mg l⁻¹ Se) for amide II may be ascribed to N-H and C-H stretching. Display of peaks in the region of 2929.87 (C), 2930 (5 mg l⁻¹ Se) and 2932.70 (10 mg l⁻¹ Se), because of vibration of the C-H, which indicated the lipid present. Carbohydrates were identified by vibration C-O-C of polysaccharides at 1051.71(C), 1034.86 (5 mg l⁻¹ Se) and 1053.19 (10 mg l⁻¹ Se). However, some other compounds (nucleic acids) possessed functional groups that absorb spectrum in the same region. The C-H bending for aromatic group

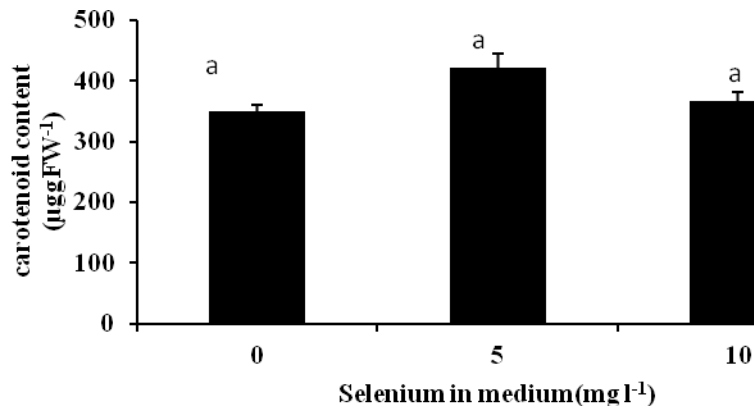


Figure 4. The changes in carotenoid content induced by Se. Values showed with the same letter are not significantly different, as measured using a Duncan test ($P<0.05$). Data are presented as means \pm SE of three replications.

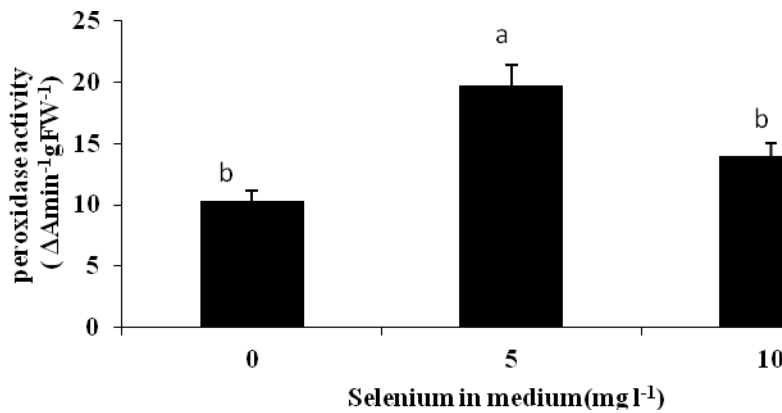


Figure 5. The change in peroxidase activity (means \pm SE) induced by Se. Values showed with the same letter are not significantly different, as measured using a Duncan test ($P<0.05$). Data are presented as means \pm SE of three replications.

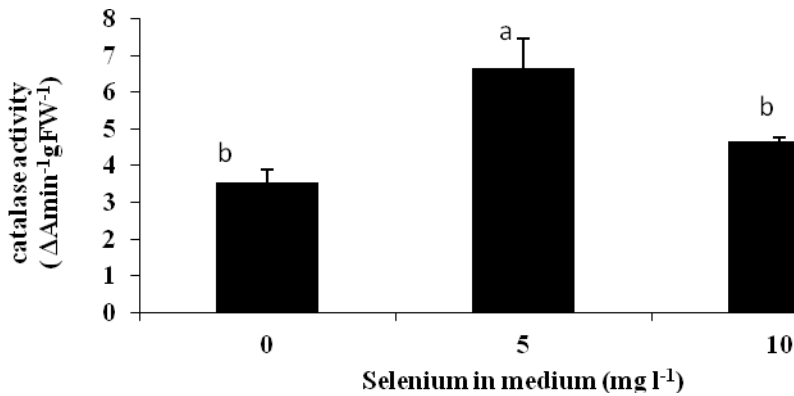


Figure 6. The change in catalase activity (means \pm SE) induced by Se. Values showed with the same letter are not significantly different, as measured using a Duncan test ($P<0.05$). Data are presented as means \pm SE of three replications.

observed in the region 831, 833.8 and 833.9 cm^{-1} in all treated samples. The display of P-H stretching for phosphine group was shown in 2368.42 (C), 2380.91, 2320.91 (5 mg l^{-1} Se), 2368.34 and 2338.3 cm^{-1} in 10 mg l^{-1} Se. The peaks at 3292.7, 3072.6 (C), 3292.86, 3082.1 (5 mg l^{-1} Se), 3287 and 3072.6 (10 mg l^{-1} Se) were caused due to O-H stretching for H₂O and N-H stretching for protein. The peaks at 1461, 1461, 1456.2 indicated that bending of methyl for protein and lipid in

control, 5 mg l^{-1} Se, and 10 mg l^{-1} Se - treated samples, respectively. In the present study, multiple absorption bands were observed in *S. platensis* with and without treatment.

Discussion:

In the green algae, it is more likely that the selenate might be transferred into the cells via sulfate transport system. In this study, the dry mass of *S. platensis* reduced with more than 5 mg l^{-1} Se. This probably was

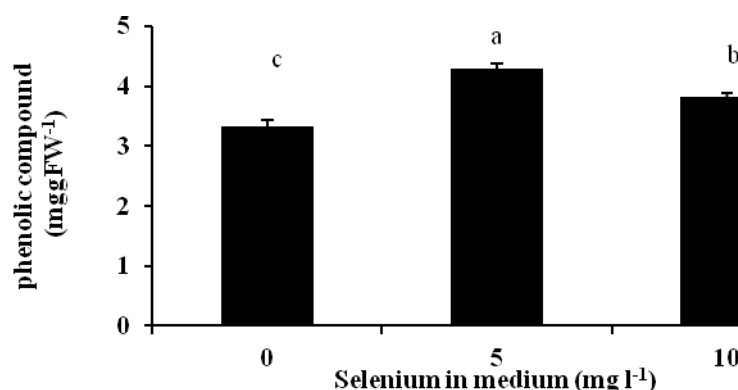


Figure 7. The changes in phenolic compounds (means \pm SE) induced by Se. Values marked with the different letter are statistically significant, as measured using a Duncan test ($P < 0.05$). Data are presented as means \pm SE of three replications.

owing to an influence of Se on cell division in microalgae (Geoffroy *et al.*, 2007). In microalgae, Se toxicity is evaluated in terms of reduction in the exponential growth rate (Bennett, 1988). The maximum growth rate is used as a key indicator of Se toxicity, which will reduce if any metabolic reaction is affected by Se in cells (Gojkovic *et al.*, 2015).

The concentration of 5mg l⁻¹ Se as the selenate improved the growth rate as compared to the control. Therefore, selenate at low concentration exerts a favorable effect on *Spirulina* by enhancing the dry weight. The present findings are consistent with the previous results reported by Umysová *et al.*, 2009. Similarly, other authors have found that Se at the low level has a positive effect on plants (Hartikainen, 2005; Pilon-Smits *et al.*, 2009; Schiavon *et al.*, 2012; Ardebili *et al.*, 2014). (Li *et al.*, 2003) have suggested that the promoting growth of *S. platensis* in the presence of selenite may result from the increased level of enzyme leading to the efficient scavenging of free radical by which declining the reduction rate of algal cells.

We postulate that increase in *S. platensis* dry mass may be attributed to the increase in antioxidant activity, scavenging toxic oxidants, and consequently an increased efficiency of metabolic processes like photosynthesis.

At 10mg l⁻¹ Se, *Spirulina* was able to grow but the growth rate decreased dramatically. Maximal population density and the growth rate of *Chlamydomonas reinhardtii* declined with an increase in selenate concentration, which is in accordance with our results (Geoffroy *et al.*, 2007; Fournier *et al.*, 2010).

Selenium toxicity has been mainly attributed to main mechanisms as follows: excessive Se (Fournier *et al.*, 2010; Geoffroy *et al.*, 2007; Pastierová *et al.*, 2009), stronger bioaccumulation of microalgae compared to macroalgae (Schiavon *et al.*, 2012), inactivation of enzymes containing cysteine (Zhong *et al.*, 2015) and inhibitory impacts on photosynthetic electron transport chain (Gojkovic *et al.*, 2015), thereby reducing photosynthesis yield, metabolism disorder and ultimately limiting growth rates.

Zhong *et al.*, (2015) showed that the microalgae

absorbed Se quickly during the first three days of exposure and more than 70% of Se was assimilated into the organic form.

Selenium toxicity mainly depends on its bioaccumulation in algae and the amount of biotransformation of inorganic Se form. In microalgae that convert inorganic Se into reduced form, detoxification is related to the extent of reduced Se forms, leading to less toxicity when a large amount of Se is biotransformed to Se-amino acids (Gojkovic *et al.*, 2015).

The results showed that the addition of Se did not have any significant impact on the contents of Chl *a* and carotenoids in comparison with the control. Chlorophyll contents in *Ulva* sp., exposed to 2.5-100μM selenate did not change in comparison with the control. Likewise, in *Chlorella vulgaris* and *Chlorella sorokiniana* cultivated in the presence of selenate no significant differences in chlorophyll and carotenoid contents were found as compared to the control (Gojkovic *et al.*, 2014; Simmons and Emery, 2011). However, Formation of hydroxide peroxide and concomitant synthesis of carotenoids was observed in *Ulva* exposed to Se (Schiavon *et al.*, 2012). (Gojkovic *et al.*, 2015) suggest that Se is more effective on photosynthetic electron transport chain, due to its substitution in Cyt b6f complex, than influencing the pigment production in microalgae.

Carotenoids, in particular, play vital role in the protection of chloroplast membrane from damage produced by reactive oxygen species (ROS) (Schiavon *et al.*, 2012).

The results showed that the concentration of 5mg l⁻¹ Se, POX, and CAT activity was promoted significantly in the *S. platensis*, contrasted to the highest applied Se level, 10mg l⁻¹. These findings indicated that Se at suitable concentrations had desirable effects on antioxidant system and growth rates, in contrast to excessive Se levels.

It seems that the increased activity of antioxidant enzymes in the presence of Se is properly due to the action of Selenoproteins (PDI and TR1); such selenoproteins play vital roles in regulating enzyme activity by oxidizing or reducing the disulfide bond.

Excess Se might affect enzyme through reacting with cysteine in the catalytic subunits of the enzyme including a protein kinase, which oxidize the sulphydryl groups to disulfide linkage. It, therefore, inactivates the enzyme. Both production of ROS and inactivation of the enzyme may trigger cell damage in the presence of Se (Zhong *et al.*, 2015).

The increase of total phenol contents observed in *Spirulina* supplied with 5 and 10 mg l⁻¹ Se is in agreement with a study by Schiavon *et al.*, 2013, who reported the stimulation of phenolic compound by Se in tomato. In our research, a linear correlation between Se concentrations and total phenol applied to *spirulina* was not observed. One of the non-enzymatic substances with antioxidant activity is phenolic compounds which are necessary for pigmentation and growth. They are also considered as signaling agents and metal chelators (Schiavon *et al.*, 2013). Selenium-enhanced phenol accumulation in basil plant led to increased capacity to conquer stress conditions (Ardebili *et al.*, 2015).

The FTIR spectroscopy is one of the advanced methods for analysis of the whole organism, which

includes the assessment of infrared absorption concerning a range of molecular vibrational modes. Each peak allocates to a functional group. The spectra of cells grown in the presence of different Se concentrations were similar. The FTIR analysis of dried *S. platensis* clearly indicated varieties of the compound such as aromatic, phosphine, alcohols and carboxylic acids were synthesized.

Conclusion:

We concluded that the addition of Se into the culture of *S. platensis* can improve or reduce growth rate mainly via affecting non-enzymatic and enzymatic antioxidants, modifying secondary metabolism, and influencing photosynthesis yield. More precise studies are needed to find the exact physiological and molecular mechanisms invalved in plant reactions to Se.

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