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Heavy Metal Induced Antioxidant Defense System of Green Microalgae and its Effective Role in Phycoremediation of Tannery Effluent

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Abstract: Investigation of tannery effluent toxicology in green microalgae is of great importance from ecological point of view, because heavy metal has become a major contaminant in recent years. The present study determined the effect of various concentrations (0, 10, 25, 50, 75 and 100%) of heavy metals containing tannery effluent on cell growth and antioxidant defense system of two green microalgae. Treatment with effluent induced accumulation of Reactive Oxygen Species (ROS), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). Lower tannery effluent concentrations increased algal growth, whereas higher concentration suppressed the growth and photosynthetic content. Both strains of the microalgae had proven effective in removing heavy metals from aqueous solutions with the highest removal efficiency being near 100% and it can be used for phycoremediation of wastewater in large scale.

Key words: Tannery effluent, antixoidants, heavy metal removal, microalgae, phycoremediation

INTRODUCTION

Industrial activity and the appropriate disposal of residues have turned heavy metal pollution into serious environmental problem. Removal of heavy metals from polluted waters by the use of algae is called phycoremediation; is an expanding technology with several advantages over physical remediation methods (Salt *et al*., 1995). The world wide increase of human population and advanced living standards generates a high level of water pollution. Both rural and urban wastewater contains a massive amount of organic and inorganic pollutants (Bernhardt *et al*., 2008).

India is the third largest leather producers in the world, behind China and Italy. The states of Tamil Nadu, West Bengal and Uttarpradesh together have 88% of the tannery units of the country. Most of the tanneries are located in clusters in river basins. Tanners use a large number of chemicals during the process, discharging toxic wastes into rivers. The rivers in Vellore and the river sides there were reported as one of the most polluted places in the world by Blacksmith Institute in 2006 and they are heavily contaminated with salts and heavy metals especially Chromium. The most commonly occurring metals at these sites are lead (Pb), chromium (Cr), zinc (Zn), copper (Cu) and cadmium (Cd) (Baskar and Abdul Raheem, 2011). The nature and behaviour of Cr in wastewater depends on the physico-chemical conditions of the effluents originating from various industrial sources (Amezcua-Allieri *et al*., 2005).

The United States Environmental Protection Agency (USEPA, 2001) has established freshwater quality criteria for several toxic compounds. For Cr, Pb and Zn, the quality criteria is strictly dependent on the harness of the water, but EPA recommends that the concentrations for these metals in freshwater should be <0.017, 0.037 and 2.9 μM for Cr, Pb and Zn, respectively.

Sorption of the heavy metals, using microorganism as biosorbents offers a potential alternative to conventional processing methods, mainly because of their low cost, strong metal binding capacity, high efficiency in dilute effluents and environment friendliness (Gupta and Rastogi, 2008). *Chlorella* and *Scenedesmus* are photosynthetic, free living and unicellular microalgae that have been wildly used for the study of the biochemical mechanisms involved in the resistance of heavy metals (Cervantes *et al*., 2001; Einicker-Lamas *et al*., 2003).

The major goal of this research effort was thus to test the ability of the isolated *Chloerlla pyrenoidosa* and *Scenedesmus* sp. to remove heavy metals from tannery effluents and the influence of heavy metals on the antioxidant defense system of both microalgae.

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MATERIALS AND METHODS

Collection of tannery effluent: Samples were collected in February, 2012, at the wastewater outlets of tanneries located in the Vellore district of Tamilnadu. Tannery effluent samples were stored in polyethelene bottles in a cool box with plenty of synthetic ice for 48 h. On arrival at the laboratory, samples were processed immediately for analyses and bioassays. Some physico-chemical parameters of wastewater viz., temperature, pH and dissolved oxygen content were measured (APHA, 1998). Concentration of chromium (mg L^{-1}), copper (mg L^{-1}), zinc (mg L^{-1}) and lead (mg L^{-1}) were also measured from the wastewater samples.

Micro algae and culture medium: *Chloerlla pyrenoidosa* and *Scenedesmus* sp. were isolated from wastewater and grown in nutrient-rich BG11 medium under the conditions of 3000 lx in 12:12 h light/dark cycle at 27±2°C.

Experimental set-up: Experiments were set up to investigate chromium, copper lead and zinc removal by growing two green microalgae *C. pyrenoidosa* and *Scenedesmus* sp. in various concentrations (0, 10, 25, 50, 75 and 100%) of Tannery Effluent (TE). The sample of precultivated microalgae was centrifuged at 9000 xg for 10 min and the supernatant was discarded. The microalgal cells were washed twice with sterile Milli-Q water and re-suspended in sterile Milli-Q water for inoculation into the growth medium. The initial microalgal densities of *C. pyrenoidosa* and *Scenedesmus* sp. were about 25×10^4 cells mL^{-1}. Culturing was performed in 250 mL Erlenmeyer flasks containing 150 mL culture volume at 150 rpm and 27±2°C. Light was provided by continuous cool white fluorescent lamps at 3,000 lx with a dark/light period of 12:12 h. The experiments were conducted for 12 days and all tests were carried out in triplicate.

Photosynthetic pigment determination: The centrifuged algal pellets were used for photosynthetic pigment determination according to Jeffrey and Humphrey (1975) and Strickland and Parsons (1972). The pelleted algal cells were frozen (-20°C) for 20 min and thawed (25°C) for 5 min and this procedure was repeated three times. Then, the algal cells were frozen (-20°C) overnight until the cell walls were broken. The processed algae were suspended in 80°C 95% acetone and heated for 2 min in a water bath. Then, photosynthetic pigment of each algal sample was extracted for 5 h at room temperature. After centrifugation of each extract solution for 10 min at 5,500 xg, the supernatant was analyzed spectrophotometrically.

Antioxidative enzymes assay: Algae were harvested by centrifugation and were broken by ultra-sonication in 1.5 mL of cold extraction buffer with 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM $MgCl_2$ and 1% w/w polyvinylpyrrolidone and 1 mM ascorbate (in case of APX assay). The homogenate was centrifuged at 15,000 xg for 20 min. The supernatant was used as the crude extract for the assay of enzyme activities.

Activity of superoxide dismutase (SOD, EC 1.1.5.1.1) (Beauchamp and Fridovich, 1971) was assayed by measuring the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT). 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 10 mM methionine, 1.17 mM riboflavin and 56 mM NBT and 50 μL enzyme extract. The absorbance of solution was measured at 560 nm. One unit of SOD was defined as the amount of enzyme causing half-maximal inhibition of the NBT reduction under the assay conditions. The activity was expressed as U mg $proteins^{-1}$.

Catalase (CAT, EC 1.11.1.6) activity was determined by consumption of H_2O_2 in absorbance at 240 nm according to method of (Aebi, 1984). 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 10 μL of enzyme extract. The activity was expressed as U mg $proteins^{-1}$.

Ascorbate peroxidase (APX, EC 1.11.1.1) activity was determined according to the method of Nakano and Asada (1981). 3 mL reactive solution contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2 and 10 μL of enzyme extracts. The decrease in absorbance at 290 nm was read. The activity was expressed as U mg $proteins^{-1}$.

Heavy metal analysis: The cultures were incubated for 12 days and from each flask (control and treated) 5 mL culture was taken out under sterilized conditions after 0 and 12 days, respectively. The cultures were spun down at 3000 rpm for 15 min and the supernatants were used for the estimation of different heavy metals by atomic absorption spectrophotometer (Varian, USA) at specific wavelength.

Statistical analysis: Statistical analysis was carried out using the SPSS 16.0 package. One way-ANOVA followed by Tukey's post hoc test was used to check the significance of treatments. Level of significance used was 5%. Data are presented as Mean±standard deviation.

RESULTS AND DISCUSSION

Algal growth: In order to set up and develop an efficient method to remove heavy metals from the tannery effluent,

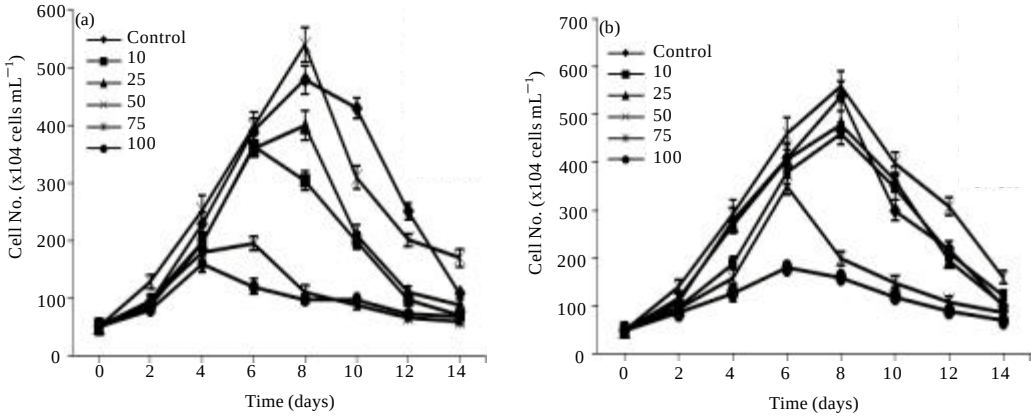

Fig. 1(a-b): Effect of various concentration of TE on the growth of (a) *C. pyrenoidosa* and (b)*Scenedesmus* sp.

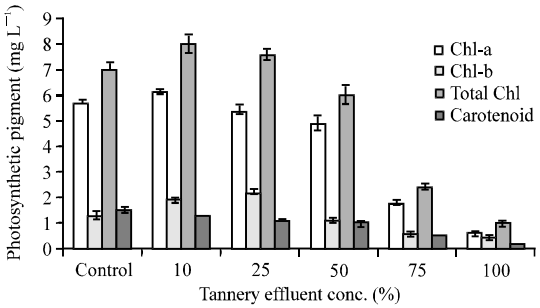

Fig. 2: Effect various concentration of TE on the photosynthetic pigment content of *C. pyrenoidosa* after 12 days treatment

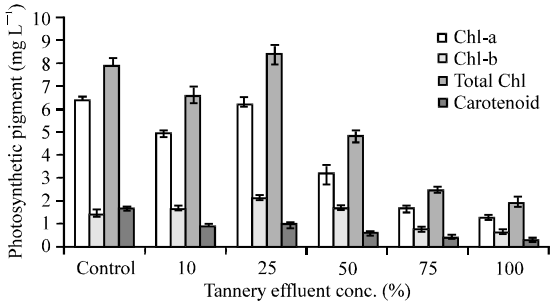

Fig. 3: Effect of various concentration of TE on the photosynthetic pigment content of *Scenedesmus* sp. after 12 days treatment

C. pyrenoidosa and *Scenedesmus* sp. were isolated from the wastewater. The ability of these strains to grow in tannery effluent was assessed and compared with control grown in synthetic mineral medium. Growth is one of the parameters used to determine toxicity induced by polluting agents on organisms and is based on cellular density (Doshi *et al*., 2008). In both algae species, growth was inhibited under higher concentrations (Fig. 1a, b). The growth of microalgae was decreased with increasing effluent concentrations. The maximum cell growth was attained at 8th day of incubation (Fig. 1). In the lower concentration of Tannery effluent (TE- 10, 25 and 50%) the growth of *C.pyrenoidosa* and *Scenedesmus* sp. exhibited an increased growth rate up to 8 days incubation than of the control.

Photosynthetic pigments: Another parameter used to evaluate adaptation capacity is pigment production, because it reflects the cellular viability associated with photosynthetic activity (Bezerra *et al*., 2008). Figure 2-3 show the effect of TE on the pigment production of *C. pyrenoidosa* and *Scenedesmus* sp. The photosynthetic of *C. pyrenoidosa* was not affected by low TE concentration but decreased significantly when culture was above 50% of TE. The highest chlorophyll-a content (6.2±0.11 mg L^{-1}) was observed in *C. pyrenoidosa* under 10% TE treatment. Whereas, highest chlorophyll-b content (2.2±0.12 mg L^{-1}) was observed in 25% TE treated culture. And inhibitory effects increased with increasing heavy metal concentrations. When its concentration increased above 50% all the cells were killed and had extreme low photosynthetic pigment contents (Fig. 2). The photosynthetic pigment of *Scenedesmus* sp. increased significantly at 25% of TE concentrations by the lower amount of heavy metal present in the effluent. But it decreased when increasing TE concentrations (Fig. 3). The carotenoid content was lower in all experiment than control (1.5±0.09 mg L^{-1}). The diminution of the photosynthetic activity by metal effect or other environmental polluting agents had reported in diverse microorganism (Perales-Vela *et al*., 2007). However, even

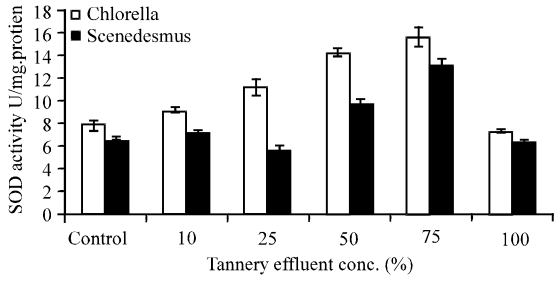

Fig. 4: Effect of TE concentration on the activities of superoxide dismutase (SOD) in *C. pyrenoidosa* and *Scenedesmus* sp after 12 days treatment

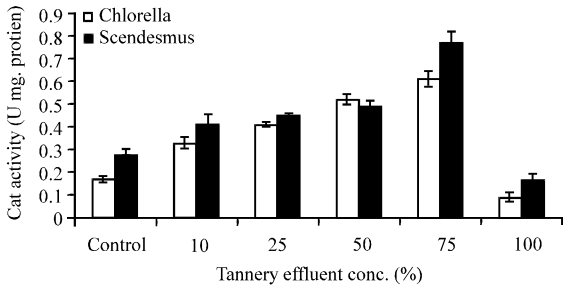

Fig. 5: Effect of TE concentration on the activities of catalase (CAT) in *C. pyrenoidosa* and *Scenedesmus* sp after 12 days treatment

though the metal inhibited pigment production, the levels of this parameter still indicated cellular viability, demonstrating the adaptability of these algae cultures exposed to the metal (Tripathi *et al*., 2006; Takagi *et al*., 2006).

Effect of TE on the antioxidant enzyme activity: Activities of antioxidant enzymes in microalgae species substantially changed when the cells were exposed to heavy metal contaminated water. The SOD activity in algae progressively increased with TE concentrations 25 to 75%. However, increasing concentration of TE above 75% led to the reduced activity (Fig. 4). With respect to the algae, SOD activity could be increased by different metals (Cr, Pb, Cu and Zn) in the *C. pyrenoidosa* and *Scenedesmus* sp. (Rijstenbil *et al*., 1994; Okamoto *et al*., 1996). Heavy metal induced ROS accumulation is counteracted by intrinsic antioxidant system in plants including enzymatic scavengers such as SOD, CAT, APX or Other antioxidant enzymes. Within cells, SOD constitutes the first line of defense against $O_2^{-\bullet}$ by rapidly converting $O_2^{-\bullet}$ to O_2 and H_2O_2 (Alscher *et al*., 2002). The highest level of SOD activity in *C.pyrenoidosa* (15.7±0.9 U mg protein^{-1}) and

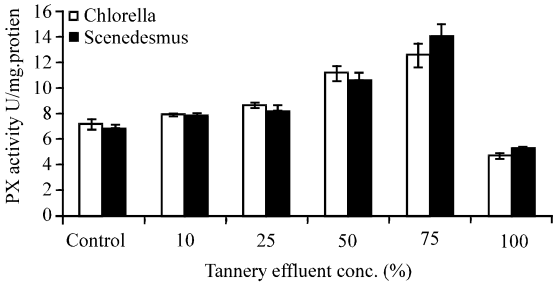

Fig. 6: Effect of TE concentration on the activities of ascorbate peroxidase (APX) in *C. pyrenoidosa* and *Scenedesmus* sp after 12 days treatment

Scenedesmus sp (13.2±0.6 U mg protein^{-1}) were observed in 75% of TE treated culture (Fig. 4). The results indicated that activities of SOD were enhanced in algae exposed to TE concentrations of 25 to 75%. The increased SOD activity might be attributed to the enhanced production of super oxides, thus resulting in activation of existing enzyme groups.

The significantly increased CAT activities were observed at 10 and 75% of TE concentration. When increasing the concentrations of tannery effluent the CAT activity was decreased to the level below the control (Fig. 5). The pattern of APX activities was similar to that CAT in both algae under TE concentrations. Higher activity of APX was observed in *Scenedesmus* sp. treated with 75% of TE than *C. pyrenoidosa* (Fig. 6). SOD dismutates $O_2^{-\bullet}$ and requires copper and zinc for its activity. Copper ions appear to have a functional role in the reaction by undergoing alternate oxidation, whereas zinc ions seem to stabilize the enzyme (Halliwell and Gutteridge, 1989).

In the present study, SOD, CAT and APX activities of both algae were extremely significantly promoted ($p<0.05$) in almost all treatment groups after 12 days with TE of different concentrations. Although the responses were irregular, CAT activity *Scenedesmus* sp. showed higher sensitivities (0.28±0.02 to 0.77±0.04 U mg protein^{-1}) to heavy metal aqueous solution than that of *C. pyrenoidosa* (0.17±0.01 to 0.61±0.03 U mg protein^{-1}). However, quite to the contrary, SOD activity of *C. pyrenoidosa* was more susceptible. Similar results were obtained in chronic toxic test of the two algae. CAT and APX are considered as the most dominant enzymes that catalyse H_2O_2 to H_2O or other non toxic products (Nakano and Asada 1981; Del Rio *et al*., 2006). CAT is a heme- containing enzyme that catalyses the dismutation of H_2O_2 in to oxygen and water.

Table 1: The initial and final concentration of heavy metal present in the TE by phycoremediation process

			Heavy metal present in the Tannery Effluent (mg L^{-1})			
Treatment	Conc. (%)	Microalgae	Cr	Cu	Pb	Zn
Control	Initial		ND	0.008	ND	0.003
	Final	*C.pyrenoidosa*	ND	0.000	ND	0.000
		Scenedesmus sp.	ND	0.000	ND	0.000
10	Initial		1.40	0.130	0.042	0.520
	Final	*C.pyrenoidosa*	0.00	0.000	0.00	0.000
		Scenedesmus sp.	0.00	0.000	0.00	0.000
25	Initial		3.50	0.460	0.085	2.300
	Final	*C.pyrenoidosa*	0.60	0.110	0.02	0.300
		Scenedesmus sp.	0.45	0.120	0.03	0.800
50	Initial		6.70	0.900	0.92	4.800
	Final	*C.pyrenoidosa*	1.80	0.250	0.23	0.900
		Scenedesmus sp.	2.10	0.130	0.35	1.050
75	Initial		9.60	2.300	2.00	6.200
	Final	*C.pyrenoidosa*	3.60	0.570	1.00	1.640
	Final	*Scenedesmus* sp.	3.81	0.820	0.80	1.830
100	Initial		12.5	4.800	3.20	7.400
	Final	*C.pyrenoidosa*	5.90	1.100	1.80	2.300
		Scenedesmus sp.	6.00	1.000	1.67	2.520

ND: Not determined

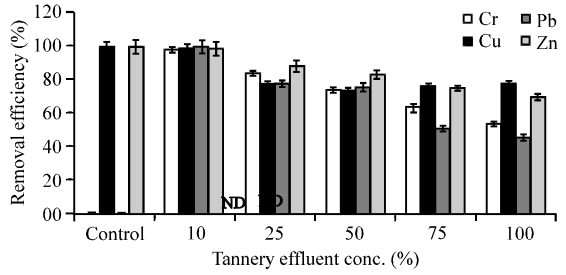

Fig. 7: Heavy metal removal efficiency of *C.pyrenoidosa* grown under Tannery effluent

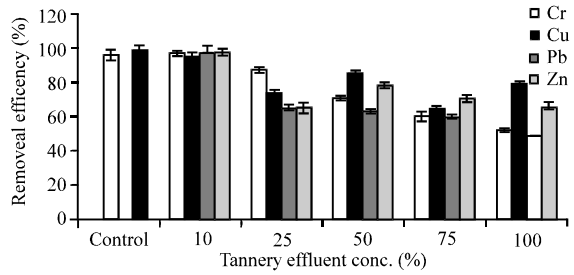

Fig. 8: Heavy metal removal efficiency of *Scenedesmus* sp grown under Tannery effluent

In chloroplast, APX removes H_2O_2 using ascorbate as an electron donor. Recent studies have demonstrated that heavy metals such as Cr and Cu affect the ascorbate-glutathione pathway in the primary leaves of plants (Palma *et al*., 2002). The present study result also indicated that APX was activated by the presence of Cr, Cu, Pb and Zn.

Heavy metal removal: The initial and final concentrations of heavy metal found in the TE are presented in Table 1. Cr, Cu, Pb and Zn were found to be removed very effectively, with removal rates ranging from 60 to 100% (Table 1). Microalgae were reported to be more efficient in sequestering metal species from solution than bacterial and fungal biomass (Khosmanesh *et al*., 1996). The mechanism of the effectiveness in removing heavy metals from wastewater by microalgae is related to their large surface area and high binding affinity (Ajayan *et al*., 2011). The Cr, Zn removal efficiency increased rapidly in the lower concentrations and thereafter remained nearly constant throughout the experiment. After 12 days of culturing, the alga *C. pyrenoidosa* and *Scenedesmus* sp. removed 72.8- 97 , 81.2- 96% (Fig. 7) and 70- 98, 65.6-98% of Cr and Zn, respectively in lower concentrations (Fig. 8). The copper and lead removal efficiency by *C. pyrenoidosa* increased gradually during the first 6 days and thereafter remained nearly constant throughout the experiment. However, chromium, copper and lead removal efficiency by *Scenedesmus* increased gradually during the whole experiment period. After 12 days of culturing, the algae *C. pyrenoidosa* and *Scenedesmus* removed 72.6-98.3, 75.3- 99% (Fig. 7) and 73.2-98.1, 64.1-96% of Cu and Pb respectively (Fig. 8). Different algal species have different size, shape and cell wall composition, which affect their metal binding efficiency (Tam *et al*., 1997) and the cell wall, in particular is the main binding site for metals (Wehrheim and Wettern, 1994).

The effect of metal on algal growth depended on the algae species and the metal concentration in the medium (Folgar *et al*., 2009). The results of the present study indicated that *Scenedesmus* sp. was more resistant to the toxicity of heavy metal than *C. pyrenoidosa*. It is generally accepted that smaller algae cells have relatively larger surface area for binding metal ions than those of larger algae cells (Yan and Pan, 2002). As known, the cell

of chlorella is smaller than that of *Scenedesmus*. Several previous studies about heavy metal removal by microalgae had been reported (Ahluwalia and Goyal, 2007; Solisio *et al*., 2006), it is difficult to compare data of two algal species used in the current study and other algae species in the previous reported study because of different experimental conditions used in those studies. From the present study reveals that *C.pyrenoidosa* and *Scenedesmus* showed good results in terms of removal of Cr, Cu, Pb and Zn, with highest removal efficiency being near 100%. In the present study Cr and Cu uptake by both algae followed an initial rapid phase of uptaking during the culture period, reached maximum and thereafter, followed decrease stabilization. A similar pattern in removal of metals by *Scenedesmus subspicatus*, *S. quadricauda* and *chlorella vulgaris* was also observed (Knauer *et al*., 1997). This time course can be explained by the binding of metal ions by functional groups on the cell surface. However it took at least 2 days for *chlorella* for Cr and Cu uptake to reach the maximum and thus this process was slower than its uptake for Pb and Zn. In the present study, both algae, especially *Scenedesmus* formed thicker cell walls to improve their adaptation to the stress caused by high concentrations of heavy metal present in the Tannery effluent.

CONCLUSION

In conclusion, it is evident that the feasibility of cultivating *Chlorella* and *Scenedesmus* prevails in tannery effluent. Both algae could endure certain concentrations of heavy metal present in the TE and exhibited a high removal efficiency of Cr, Cu, Pb and Zn ions, hence supporting their potential application in the treatment of tannery effluent polluted by metals. After 12 days of culturing, >75% removal efficiency was found by both algae for all metal ions, with the highest removal efficiency being near 100%. TE used as a nutrient medium has a direct effect on the production or scavenging of the ROS. The present results show that higher concentrations may promote oxidative damage to the membrane. To deal with the TE heavy metal induced oxidative stress, both microalgae activated a variety of antioxidative enzymes such as SOD, CAT and APX to diminish the ROS. These biological responses can be interpreted as a tolerant mechanism.

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