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ANTIOXIDATIVE ENZYMES ACTIVITY OF *Chlorella vulgaris* (UMT-M1) IN RESPONSE TO IC_{50} OF Pb^{2+} AND Hg^{2+}

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ABSTRACT

Heavy metals accumulation in aquatic bodies will lead to many deleterious effects on organisms living in them. Furthermore, some organisms can accumulate metals and this will eventually reach the primary consumers. The application of microalgae as remediator and indicator of metals pollution has become an increasing interest to scientists. In this study, we reported on the defence responses of a marine microalga, *Chlorella vulgaris* (UMT-M1) against Pb^{2+} and Hg^{2+} in terms of three antioxidative enzymes activity: superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). The activity of the enzymes was measured in alga treated with 5.7 mg/L $PbCl_2$ and 0.18 mg/L $Hg(NO_3)_2$. These two concentrations were the IC_{50} values calculated using probit analysis. It was observed that Pb^{2+} and Hg^{2+} triggered a different strategy in counterattacking the metal's effects. While a significant induction of SOD and CAT activity was evident in alga treated with both metals, APX activity was significantly inhibited in Pb^{2+}-treated alga but was stimulated by more than 4-fold from the control in Hg^{2+}-treated alga. Higher SOD activity was observed in Pb^{2+}- compared to Hg^{2+}-treated alga. However, lower CAT activity was observed in Pb^{2+}- compared to Hg^{2+}-treated alga. Also, a significant reduction in total soluble protein (TSP) content was observed in alga treated with Hg^{2+}. Pb^{2+}, on the other hand, significantly increased the production of TSP. The changes observed in the antioxidative enzymes activity and TSP content in this alga are good potentials to be used as biomarkers for metal's bioindication tool.

Key words: Heavy metals, microalgae, superoxide dismutase, catalase, ascorbate peroxidase

INTRODUCTION

The impacts of heavy metals contamination in aquatic bodies have raised much concern for environmentalists worldwide. These metals can persist in the environment for millions of years. They cannot be easily degraded and to make matter worst, they can accumulate in the cells and magnifies in the ecosystems. Due to their ability to form complexes with molecules inside the cells, they can exert detrimental effects on living organisms. Lead (Pb) and mercury (Hg) are considered to be the most toxic heavy metals present in the environment. Both are nonessential elements in living organisms, thus, their toxic effects are more adverse even at a low amount, than other metals that are essential. Among the reported cases, Pb and Hg showed toxicity effects on the growth and photosynthetic processes of algae (Bakar *et al.*, 2015; Dao & Beardall, 2016), acetylcholinesterase activity of fish causing alterations in swimming patterns (Kim & Kang, 2017; Rodrigues *et al.*, 2018) and embryogenesis and early larval stages of clam (Fathallah *et al.*, 2013). However, the extent of impacts posed by these two metals may differ from one another.

Toxic effects of heavy metals appear to be partly related to the induction of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), inside the cell (Sytar *et al.*, 2013). Metal-induced oxidative stress has been strongly linked to early toxicity symptoms (Sharma & Dietz, 2009). These highly unstable reactive compounds can damage important biomolecules including proteins and interfere with many cellular metabolic processes. Also, they can act as important secondary messengers for inducing cellular defence mechanisms (Apel & Hirt, 2004; Tripathy &

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Oelmüller *et al.*, 2012). ROS along with autophagy has also been associated with cell death as part of cellular adaptation to stress (Pérez-Pérez *et al*., 2012). Cells are equipped with enzymatic and non-enzymatic defence mechanisms that can counteract the damages done by ROS. Enzymatic scavengers of ROS include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and peroxidase (POD) while non-enzymatic scavengers include glutathione, ascorbic acid, α-tocopherol, β-carotene, and organic acids (Mallick & Mohn, 2000). In this study, we focused on the enzymatic antioxidants, SOD, CAT and APX. SOD [EC1.15.1.1] is considered to act as the first line of defence against ROS within cells. It catalyses the conversion of superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2). CAT [EC 1.11.1.6] catalyses the dismutation of H_2O_2 into O_2 and H_2O. The enzyme occurs in all aerobic eukaryotes and its function is to remove the H_2O_2 generated in peroxisomes by oxidases. APX [EC 1.11.1.11], on the other hand, uses ascorbate as a hydrogen donor to break down H_2O_2 to form H_2O and monodehydro-ascorbate and performs this function in chloroplasts and cytosol of plant cells (Karuppanapandian *et al.*, 2011).

Even though the toxic metals are not easy to be degraded, they are, fortunately, can be removed from polluted areas through phytoremediation which includes the application of microalgae (Kumar *et al.*, 2015). Microalgae are ubiquitous organisms that can be found anywhere, whether in the terrestrial or aquatic ecosystems. Microalgae like any other photoautotrophs required inorganic nutrients for growth. The fast-growth rate of some microalgae species can account for rapid nutrient removal from water bodies. Most of them can immobilize the metals to make them less toxic (Sánchez-Rodriguez *et al.*, 2001). They also can absorb and metabolize trace metals due to their large surface: volume ratios; the presence of high-affinity, metal-binding groups on their cell surfaces; and, efficient metal uptake and storage systems (Rajamani *et al.*, 2007). Also, microalgae play a major role in marine ecosystems. As the first organism in marine food chains, they not only provide nutrients and energy but also shelter and habitat for many coastal animals. Thus, it is important to study what happened inside the microalgae to obtain more information on how they can survive and adapt to the harsh environmental conditions.

Given this, the present study was designed to investigate the extent of Pb^{2+}- and Hg^{2+}-induced oxidative stress at 50% population of *Chlorella vulgaris*. The dominant type of green alga, *C. vulgaris* was chosen in this study due to its suitability for toxicity tests, its simple growth conditions and strong tolerability (Ouyang *et al.*, 2012). The effects of Pb^{2+} and Hg^{2+} on the algal total soluble proteins and antioxidative enzymes short-term response were analysed. It is aimed to further explore the mechanism of metal toxicity to algae and the mechanism of resistance of the algae towards the heavy metals.

MATERIALS AND METHODS

Culture of *C. vulgaris* (UMT-M1) and treatments with IC_{50} of Pb^{2+} and Hg^{2+}

A stock culture of the microalga, *C. vulgaris* (UMT-M1) was maintained in F2 medium (Guillard *et al.*, 1975) under the light intensity of ~80 µmol m^{-2} at 25°C with 24 hr constant light. The cell growth was monitored for 1 week and harvested for treatments when the growth reached the log phase.

The harvested microalgae (i.e. a cell density of 10^6) were then treated with a series of $PbCl_2$ (i.e. 0.05 – 50 mg/L) and $Hg(NO_3)_2$ (i.e. 0.002 – 2.0 mg/L) concentrations individually. About 250 mL of treated cultures were kept in 500 mL flasks for 48 hr in similar conditions as the culture. After 48 hr, the IC_{50} value of $Pb(Cl_2)$ and $Hg(NO_3)_2$ against the microalga was determined from a dose-response curve. The microalga was than further treated with these concentrations individually for 8 hr at similar conditions of medium, light intensity and temperature as the culture. Each treatment was done in triplicates while treatments without the $Pb(Cl_2)$ and $Hg(NO_3)_2$ were set as control. After 8 hr, the treated microalgae were harvested and their total soluble proteins (TSP) and antioxidative enzymes activity were analysed.

Total soluble proteins extraction and quantification

For extraction of total soluble proteins (TSP), treated *C. vulgaris* was first broken down by ultra-sonication in 1.5 mL cold extraction buffer containing 50 mm Tris-HCl (pH 7.8), 1 mm EDTA, 1 mm $MgCl_2$, 1% (w/w) polyvinylpyrrolidone and 1 mm ascorbate (Ajayan & Selvaraju, 2012). The homogenate was then centrifuged at 15000 × *g* for 20 min and the supernatant was collected for TSP quantification. This supernatant was also used for antioxidative enzymes assay as stated below.

TSP content was quantified according to the method of Bradford (1976). Bradford reagent was first prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol. Concentrated phosphoric acid was then added to the mixture. The solution was filtered and stored at room temperature in a light-proof bottle before use. A total of 100 µL of TSP was added to a 3 mL Bradford reagent and the absorbance of the sample was measured after 10 min at 595 nm using a UV-VIS spectrophotometer. TSP content was determined

from a standard curve constructed using a series of bovine serum albumin (BSA) concentrations (i.e. 0 – 2 mg/mL).

Antioxidative enzymes assays

The antioxidative enzymes analysed were superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). The activity of SOD was assayed according to a method by Beauchamp and Fridovich (1971) with slight modifications. SOD activity was based on the ability of the enzyme to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). A reaction mixture containing 50 mm sodium phosphate buffer (pH 7.8), 10 M methionine, 1.17 mm riboflavin and 56 mm NBT was first prepared. To start the reaction, about 50 µL of TSP extract containing the enzyme was added to the mixture. The absorbance of the mixture was then monitored at 560 nm for 3 min. One unit of SOD was defined as the amount of protein inhibits the rate of NBT reduction by 50%.

CAT activity was determined according to a method of Aebi (1984). To start the reaction, 10 µL of TSP extract was added to a 3 mL reaction mixture containing 50 mm sodium phosphate buffer (pH 7.0) and 10 mm H_2O_2. The absorbance of the mixture was then monitored at 240 nm for 3 min. The activity of CAT was calculated based on the disappearance of H_2O_2 using the extinction coefficient for H_2O_2 of 0.0398 mm^{-1} cm^{-1}. APX activity was determined according to the method by Nakano and Asada (1981) with slight modifications. To start the reaction, 10 µL of TSP extract was added to a 3 mL reaction mixture containing 50 mm sodium phosphate buffer (pH 7.0), 0.5 mm ascorbate and 0.1 mm H_2O_2. The absorbance of the mixture was then monitored at 290 nm for 3 min. The activity of CAT was calculated based on the disappearance of ascorbate using extinction coefficient for ascorbate of 2.8 mm^{-1} cm^{-1}.

Statistical analyses

Values of all the parameters tested were related to 100% of controls for better comparison. Mean values and standard error were determined from three replicates of each treatment. The statistically significant differences between the treatments were analysed using a one-way ANOVA followed by Tukey HSD post-hoc test at a probability level of 0.05. The statistical software used was Daniel's XL Toolbox v. 7.2.13 Add-in for Microsoft Excel.

RESULTS AND DISCUSSION

From the dose-response curve, it was determined that the concentration of Pb^{2+} and Hg^{2+} needed to inhibit half of the population of *C. vulgaris* was 5.7 mg/L and 0.18 mg/L, respectively. In a similar study on a microalga, *Scenedesmus regularis*, it was observed that the IC_{50} of Pb^{2+} and Hg^{2+} were 7.2 mg/L and 0.42 mg/L, respectively (Bayani *et al.*, 2017). Hg^{2+} was also found to exert higher toxicity effects than Pb^{2+} in some organisms including *Dunaliella* alga (Imani *et al.*, 2011) and *Fucus vesiculosus* (Henriques *et al.*, 2017). Inhibition concentration (IC) value is often used to determine the effectiveness of any substance in inhibiting a specific biological or biochemical function. It also can indicate how well the organism can tolerate any foreign substances that they encounter in their cells. In this study, the presence of Pb^{2+} and Hg^{2+} interferes with the growth or survival of *C. vulgaris* and low level of these metal ions can indeed become toxic to the alga. Since both Pb^{2+} and Hg^{2+} are nonessential metals to the microalgae, these metals can inhibit the physiology and biochemistry processes of, for examples, *S. regularis* (Bayani *et al.*, 2017), *Cladophora* (Cao *et al.*, 2015), *Acutodesmus obliquus* (Piotrowska-Niczyporuk *et al.*, 2015) and *Gracilaria manilaensis* (Hazlina & Shuhanija, 2013).

Toxicity effect of Hg^{2+} was evident from the reduction in total soluble proteins (TSP) of the alga as shown in Figure 1 (p=0.003). Contrastingly, Pb^{2+} significantly increases the TSP content by more than 2-fold of controls (p=0.03). In comparison, in a study by Bayani *et al.* (2017) both metals exhibited negative effects on the TSP content of the green alga *S. regularis*. This shows that similar metals may have triggered different pathways inside cells and that different algae may adopt different ways to detoxify the metals. Heavy metals affect proteins either by forming a complex with functional side chains group (Chrestensen *et al.*, 2000; Wu *et al.*, 2016) or by displacing essential metal ions in metalloproteins (Wu *et al.*, 2016; Dudev *et al.*, 2018). Also, certain heavy metals and metalloids have been found to inhibit the *in vitro* refolding of chemically denatured proteins, to interfere with protein folding *in vivo* and to cause aggregation of nascent proteins in living cells (Tamás *et al.*, 2014). Metal-induced stress may also affect the important biomolecules by way of generating ROS, which can lead to damages or impairment of functions (Møller *et al.*, 2007; Choudhury *et al.*, 2017). Among the many adverse effects of Hg on proteins, Hg can bind the sulfhydryl group of proteins, rendering the proteins inactive (Ynalvez *et al.*, 2016). It can also down-regulates gene expression of proteins (Beauvais-Flück *et al.*, 2017) and displaces essential metals required by the protein, thus, the protein will become dysfunctional (Zampino *et al.*, 2014).

Increase in the TSP content as shown by Pb^{2+} may indicate the overexpression of genes controlling the synthesis of proteins associated with

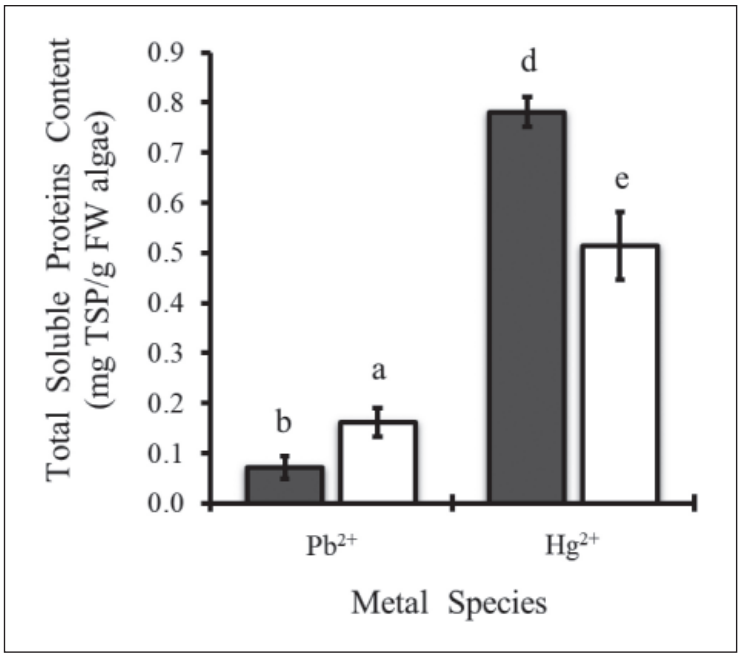

Fig. 1. Total soluble proteins content of *C. vulgaris* exposed to Pb^{2+} and Hg^{2+}. Different letters indicate statistically significant differences between treatments exposed to similar metals (*a-b for* Pb^{2+}, *e-d for* Hg^{2+}) at $p<0.05$.

tolerance and detoxification of Pb^{2+} in *C. vulgaris*. This may also explain why this alga is more tolerant against Pb^{2+} than Hg^{2+} since it needs a higher concentration of Pb^{2+} than Hg^{2+} to inhibit 50% of the algal population. Pb was reported to trigger the synthesis of heat shock proteins in chloroplasts to protect photosynthetic machineries (Heckathorn *et al.*, 2004). Accumulation of Pb correlated with synthesis of metallothionein, a Cys-rich protein, which plays a role in in the survival of the alga (Cao *et al.*, 2015) and phytochelatin, a chelating protein which bound and compartmentalized metals for detoxification was also synthesized in the presence of excess Pb (Fischer *et al.*, 2014). In addition to the above proteins, other types of proteins such as glutathione and chaperones may also be stimulated as a response to the toxicity of metals such as Pb (Jain *et al.*, 2018).

Presence of excess metals typically stimulates the production of reactive oxygen species (ROS) which can interfere with the biochemistry of cells. The build-up of ROS in cells initiate signalling response to induce gene expression of antioxidative enzymes (Sewelam *et al.*, 2016). For example, expression of the genes for SOD, CAT and APX was observed to be activated and increased in the algae, *Closterium ehrenbergii* (Wang & Ki, 2020), *Ulva compressa* (Laporte *et al.*, 2016) and *C. reinhardtii* (Elbaz *et al.*, 2010). The response of these anti-oxidant enzymes to metal stress, however, varies among plant species and the metals involved (Mazhoudi *et al.*, 1997). Figure 2 shows the activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) in the alga after 8 hr treatment with Pb^{2+} and Hg^{2+}. It was observed that Pb^{2+} significantly triggered the highest activity of CAT in the alga while Hg^{2+} significantly triggered the highest activity of CAT and APX. Also, the activity of APX was observed to be suppressed by Pb^{2+} to 8% compared to controls (p=0.01). Hg^{2+}, on the other hand, did not show any significant changes in the activity of SOD (p=0.063).

Potential important sources of ROS in photo-synthetic cells include over-reduction of PSII, the Mehler reaction and photorespiration (Foyer & Noctor, 2005). Over-reduction of PSII occurs during environmental stress due to repression of carbon assimilation. PSII will become progressively reduced which leads to oxidative stress through the generation of $^1O_2$ or $O_2^{\bullet-}$. The Mehler reaction as well as photorespiration can function as alternative routes to de-energizing photosystems and thus preventing the over-reduction of PSII. In both the reactions, $O_2^{\bullet-}$ is also converted to H_2O_2. This reaction is catalysed by SOD (Reddy *et al.*, 2005). Since H_2O_2 is itself toxic, it must be flushed out from the system. The principal H_2O_2-scavenging enzyme in plants and algae are CAT, which is primarily located in peroxisomes and APX, which is primarily found in the cytosol and chloroplasts (Asada *et al.*, 2006). In the Mehler reaction, O_2 is reduced first to $O_2^{\bullet-}$ and then to H_2O_2. This H_2O_2 is subsequently converted to H_2O by APX, thus generating a pseudocyclic electron flow, in which electrons from

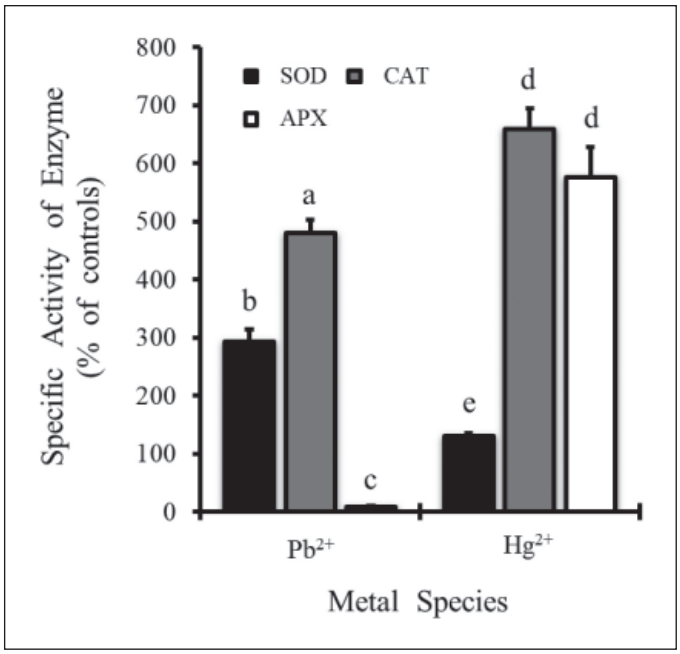

Fig. 2. Antioxidative enzymes (superoxide dismutase, SOD; catalase, CAT; and, ascorbate peroxidase, APX) activity of *C. vulgaris* exposed to Pb^{2+} and Hg^{2+}. Different letters indicate statistically significant differences between antioxidative enzymes exposed to similar metals (*a-c for* Pb^{2+}, *e-d for* Hg^{2+}) at $p<0.05$.

the oxygen-splitting complex pass through the photosynthetic electron carriers back to O_2. Photorespiration, on the other hand, recycles carbon that is used by oxygenation of ribulose-1,5-bisphosphate and produces H_2O_2 in the peroxisomes through the enzyme glycolate oxidase. The subcellular distribution of these enzymes suggests that chloroplastic APX removes H_2O_2 produced during the Mehler reaction and other chloroplastic processes, whereas CAT scavenges photorespiratory H_2O_2. All these three antioxidative enzymes work together in combination to minimize the damages done by the toxic ROS.

Therefore, from the results obtained in this study, an induction in the antioxidative enzymes observed when the alga was exposed to IC_{50} of Pb^{2+} and Hg^{2+} can be correlated to an increase in $O_2^{\bullet-}$ and H_2O_2. However, a no change in the activity of SOD in Hg^{2+}-treated algae may mean that Hg^{2+} did not trigger the gene expression of SOD. The high content of H_2O_2 in these algae as shown by induction in CAT and APX activities may be due to spontaneous dismutation of $O_2^{\bullet-}$ to H_2O_2 without the help of SOD (Sewelam *et al.*, 2016). High induction in CAT activity as shown by Pb^{2+} may be due to the production of H_2O_2 was high in peroxisomes generated through the Mehler reaction or photorespiration. In comparison, both CAT and APX activities were observed to be the highest in the presence of Hg^{2+} and this may be due to high production of H_2O_2 in both the peroxisomes and chloroplasts generated through the inhibition of photosynthesis. Also, CAT can scavenge H_2O_2 generated during mitochondrial electron transport and β-oxidation of fatty acids (Karuppanapandian *et al.*, 2011). Thus, it can also be said that Hg^{2+} may also affect photosynthesis, respiratory as well as lipid metabolic processes of the alga while damage by Pb^{2+} may restrict to the respiratory and lipid metabolic processes. The results of the present study also suggested that the induction in antioxidant responses could be occurring as an adaptive mechanism to the oxidative potential of Pb^{2+} and Hg^{2+} accumulation. For example, the reduction in the number of cells to half of the population after treatment may be due to the activation of cell survival and/or cell death processes such as autophagy and apoptosis activated by ROS (Redza-Dutordoir & Averill-Bates, 2016) including H_2O_2. H_2O_2 is the most stable, can diffuse across the membrane and is recognized as important signalling molecules which can activate the mechanisms leading to autophagy or apoptosis (Peréz-Peréz *et al.*, 2012). Both Pb^{2+} and Hg^{2+} have been implicated in the autophagic and apoptotic activities in some organisms (Vergilio *et al.*, 2015; Bayani *et al.*, 2017).

CONCLUSION

In conclusion, Pb^{2+} and Hg^{2+} at IC_{50} concentration, exert different responses in *C. vulgaris*. At this concentration, Pb^{2+} induces the synthesis of soluble proteins while Hg^{2+} denatured the available proteins and inhibits the synthesis of new proteins, leading to a reduction in the content of soluble proteins. Superoxide radicals and hydrogen peroxide are two major ROS that is produced as observed in the changes in antioxidative enzymes activity. Pb^{2+} stimulates the activity of SOD and CAT but inhibits the activity of APX. Hg^{2+}, on the other hand, did not affect the activity of SOD but both CAT and APX activity was stimulated. The above responses can be used as biomarkers to monitor the level of Pb^{2+} and Hg^{2+} contamination in the aquatic bodies.

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