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ANALYSES ON TOXICITY OF Pb^{2+} TOWARDS CHLOROPHYLL A, TOTAL SOLUBLE PROTEIN AND CASPASE-3-LIKE ENZYME ACTIVITY OF *Scenedesmus regularis*

(Analisa Kesan Ketoksikan Pb^{2+} Terhadap Klorofil A, Jumlah Protein Terlarut dan Aktiviti Enzim Bak Kaspase-3 *Scenedesmus regularis*)

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Abstract

Heavy metal pollutions, including lead (Pb), has become an increasing concern to humans due to their adverse effects and the fact that they are not easily degraded or destroyed. Microalgae are aquatic organisms that can be used in metal bioremediation since they can accumulate and detoxify metals. This study reported on the responses of a freshwater microalga, which was *Scenedesmus regularis*, when exposed to the inhibitory concentrations (IC) of Pb^{2+} at 25%, 50% and 75%. The tolerance level of *S. regularis* against Pb^{2+} at IC_{25}, IC_{50} and IC_{75} was determined to be 3.5 mg/L, 7.2 mg/L and 10.9 mg/L, respectively. Then, the microalga was treated with these inhibitory concentrations. The concentration of Chlorophyll A (Chl A) and total soluble protein (TSP), as well as caspase-3-like enzyme activity of the alga were analysed. It was observed that Chl A concentration of the alga significantly decreased as more cells were inhibited by Pb^{2+}. The highest concentration of Pb^{2+} significantly reduced the TSP concentration of the alga. However, no changes were observed amongst the concentrations of Pb^{2+}, which inhibited 25% and 50% of the alga population. Activity of caspase-3-like enzyme was significantly induced by more than 3-fold of control in IC_{25} of Pb^{2+}, while the activity of this enzyme was observed to be supressed in both the IC_{50} and IC_{75}. In conclusion, the alga has the potential to be a good indicator for Pb^{2+} toxicity and the Chl A concentration and caspase-3-like enzyme activity can be applied as biomarkers.

Keywords: lead(II) ion, metal toxicity, microalgae, biochemical analyses, inhibitory concentration

Abstrak

Pencemaran logam berat termasuk plumbum menjadi semakin menarik perhatian manusia akibat kesan buruknya dan hakikat bahawa ia tidak mudah diurai atau dimusnah. Mikroalga ialah organisma akuatik yang boleh digunakan dalam bioremediasi logam kerana ia boleh mengumpul dan menyahtoksik logam. Kajian ini melaporkan tentang tindak balas mikroalga air tawar, iaitu *Scenedesmus regularis* apabila didedahkan kepada ujian toksik Pb^{2+} pada kepekatan rencatan (IC) 25%, 50% dan 75%. Tahap toleransi *S. regularis* terhadap Pb^{2+} pada IC_{25}, IC_{50} dan IC_{75} ialah masing-masing pada 3.5 mg/L, 7.2 mg/L dan 10.9 mg/L. Kemudian mikroalga dirawat dengan kepekatan ini dan kepekatan Klorofil A. Jumlah protein terlarut (TSP) serta aktiviti enzim

bak kaspase-3 dianalisis. Didapati bahawa kepekatan Klorofil A alga berkurangan apabila sel-sel lebih banyak direncat oleh Pb^{2+}. Kepekatan tertinggi Pb^{2+} mengurangkan kepekatan TSP alga dengan ketara. Namun, tiada perubahan diperhatikan antara kepekatan Pb^{2+} yang merencat 25% dan 50% populasi alga. Aktiviti enzim bak kaspase-3 telah meningkat lebih daripada tiga kali ganda dalam IC_{25} Pb^{2+} berbanding kawalan, manakala aktiviti enzim ini berkurangan pada IC_{50} dan IC_{75}. Kesimpulannya, alga mempunyai potensi untuk menjadi petunjuk yang baik untuk ketoksikan Pb^{2+} manakala kepekatan Klorofil A dan aktiviti enzim bak kaspase-3 boleh digunakan sebagai biopenanda.

Kata kunci: ion plumbum(II), ketoksikan logam, mikroalga, analisa biokimia, kepekatan rencatan

Introduction

Heavy metals, such as copper (Cu), zinc (Zn), cadmium (Cd), chromium (Cr), mercury (Hg) and lead (Pb), are major environmental pollutants particularly in industrial areas. When discharged into the aquatic ecosystem, they will pose threats to organisms that live in the ecosystem. This will become a serious concern since the metals can accumulate in organisms and eventually reach the primary consumers, which are humans. The metals are toxic to all organisms since they can exert adverse effects. They are not easily degraded nor destroyed. However, they can be removed from polluted areas through phytoremediation which include the application of microalgae [1].

Microalgae are ubiquitous organisms that can be found anywhere, whether in the terrestrial or aquatic ecosystems. Due to their photoautotrophic nature, microalgae are able to uptake metals into their cells and detoxify them. Therefore, they can be used as bioremediation tools to clean up metal contaminants. However, since some metals are non-essential and are not useful to them, these metals can exert toxicity effects to the algae. In fact Pb^{2+}, as one the non-essential and most toxic metals, was proven to affect amongst others, the cell growth [2, 3], photosynthetic rate [4, 5], pigment contents [6] and cell morphology [7] in algae. The non-redox active metal Pb^{2+} can also induce the generation of reactive oxygen species (ROS) which can lead to damages to important biomolecules such as proteins [8]. In addition, the metals may trigger some sort of intercellular defense mechanisms which can help the algae to protect themselves from the toxicity effect for survival [2, 3, 9].

This includes the trigger of cellular signalling pathways that can either be death or survival signals, leading to the programmed cell death (PCD) modes which are 'self-suicidal' apoptosis, 'self-eating' autophagy and necrosis [10]. Studies by many researchers have found several species of microalgae, for example, *Micrasterias denticulata* [11], *Dunaliella tertiolecta* [12] and *Karenia brevis* [13], which can undergo PCD coordinated by caspases, a family of cysteine proteases. According to Segovia and Berges [12], there is a link between cell death and ROS which involve caspase-like enzymes, as observed in the microalga, *Dunaliela tertiolecta*. All the above-mentioned responses may be used as biomarkers for Pb^{2+} contamination.

In this study, *Scenedesmus regularis* which is a freshwater microalga was treated with Pb^{2+} to determine the inhibitory concentration (IC) at 25%, 50% and 75% of the algae cell populations. *Scenedesmus* algae are known to have high biomass productivity and strong tolerability amongst green algae. Therefore, this genus of algae is a suitable material for toxicity test. The findings from this study can be cross-referenced against other experiments, whereby different species of *Scenedesmus* have been used as test algae. This study aims to assess the responses of the algae towards these three concentrations of Pb^{2+} in terms of its Chlorophyll A and total soluble protein (TSP) concentrations as well as the activity of caspase-3-like enzyme. From these observations, it can be concluded if the alga was suitable to be used as a bioindication tool for Pb^{2+} contamination in an aquatic ecosystem or not.

Materials and Methods

Culture of microalga sample

The algae stock culture of *Scenedesmus regularis* was provided by the Institute of Marine Biotechnology, Universiti Malaysia Terengganu. The algae were cultured in f/2 medium in filtered seawater at 5 ppt. The

algae were aerated at 25 °C and under the light intensity of 17 µmol photon m^{-2} s^{-1} until it reached the middle of the exponential growth phase, whereby they were harvested by centrifugation for treatment.

Inhibitory concentrations and treatments of Pb^{2+} on *S. regularis*

Tolerance assay was done to determine the inhibitory concentration (IC) based on number of cells response for *S.regularis* against Pb^{2+} in the form of lead(II) nitrate, $Pb(NO_3)_2$ toxification. A 24-well plate was filled with f/2 medium at initial cell density of 10^6 and six different $Pb(NO_3)_2$ concentrations (i.e. ranging from 0 mg/L to 30 mg/L). Treatment was done for 24 hours under light intensity of 17 µmol photon m^{-2} s^{-1} and 25°C, and the number of live cells were counted at 0, 12 and 24 hours by using a haemocytometer. To differentiate between live and dead cells, trypan blue was used to stain the dead cells. The concentrations of Pb^{2+} which inhibited 25% (IC_{25}), 50% (IC_{50}) and 75% (IC_{75}) of *S. regularis* cells were then calculated based on probit analysis [14].

S. regularis was then treated with the $IC_{25,}$ $IC_{50,}$ and IC_{75} of Pb^{2+}, as determined above. Treatments were done for 24h at an initial cell density of 10^6 in similar conditions as the tolerance assay. The number of cells was counted at the end of treatment by using a haemocytometer (Merck, BLAUBRAND® Neubauer). The treated algae aliquots were pelleted in falcon tubes via centrifugation at 15000 xg and 4°C for 15 min. The pellets were then stored at -80 °C before further analyses.

Parameters analysed

After treatments, the algae Chlorophyll A (Chl A) concentration, total soluble protein (TSP) concentrations and activity of caspase-3-like enzyme were studied. The Chl A was extracted following a method by Ritchie [15] with some modifications. The pellet obtained earlier was suspended in acetone and homogenised for 10 s. The mixture was then left overnight in the dark at -20 °C. Prior to the absorbance reading, the extract was centrifuged at 15000 xg and 4 °C for 10 minutes. Absorbance was then read at 630 nm, 647 nm, 664 nm, and 691 nm by using a UV-Vis spectrophotometer (Shimadzu, UV-1800)) and the content of Chl A was calculated by using this formula (Equation 1) [15]:

$$\text{Chl A content (mg/mL)} = [(-0.3319*A_{630nm}) - (1.7485*A_{647nm}) + (11.9442*A_{664nm}) - (1.4306*A_{691nm})] \quad (1)$$

Extraction of proteins was done according to a method described by Faurobert et al. [16] with some modifications. The pellet obtained earlier was ground with liquid nitrogen into fine powder. The powder was added to a cold extraction buffer (i.e. stored at 4 °C), which contained 500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, 2% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 0.001 mg/mL protease inhibitor cocktail at pH 8.5. The extract was then vortexed and incubated on ice for 10 minutes. Next, an equal volume of Tris-saturated phenol was added. Samples were vortexed for 10 minutes at room temperature and then centrifuged at 13000 xg and 4 °C for 20 minutes. The phenolic phase was recovered and reextracted with the same volume of extraction buffer. Subsequently, centrifugation was repeated and acetone was added to the recovered phenolic phase. Proteins were allowed to precipitate overnight. After centrifugating for 20 minutes at 13000 xg and 4 °C, the pellet was washed three times with the precipitation solution and once with acetone. Each washing step was followed by 5 minutes of centrifugation, as described above. After being dried under vacuum, the pellet was resuspended in lysis buffer which contained 7 M urea, 2 M thiourea, 2% CHAPS, 2% Triton X, 1% DTT and 0.001 mg/ml of protease inhibitor cocktail at pH 8.0. Total soluble protein (TSP) content of the extract was then determined by using the method reported by Bradford [17].

To quantify the caspase-3-like activity, Enzchek® Caspase-3 Assay Kit #2 (Invitrogen, USA) was used

following the method by Bouchard and Yamasaki [18]. The protein extract obtained above was mixed with 2x reaction buffer (Caspase 3 Assay Kit #2, Invitrogen) and Z-DEVD-R110 substrate. Sample was then incubated in the dark at room temperature for 4 hours. The fluorescence was detected by using Varioskan™ Flash Multimode Reader (Thermo Scientific). The excitation and detection wavelength used were 496 nm and 520 nm, respectively. Rhodamine 110 (R110) was used to generate a standard curve in the range of 0.002 µM to 0.01 µM to quantify the caspase activity.

Mean values and standard errors were determined from three replicates of each treatment. The statistically significant differences between treatments were analyzed by using a one-way ANOVA followed by Tukey HSD post-hoc test at probability level of 0.05. The statistical software used was Daniel's XL Toolbox v. 6.60 Add-in for Microsoft Excel.

Results and Discussion

From Figure 1, it was determined that the concentrations of Pb^{2+} needed to inhibit 25%, 50% and 75% of *S. regularis* populations were 3.5 mg/L, 7.2 mg/L and 10.9 mg/L, respectively. Inhibitory concentration (IC) value is often used to determine the effectiveness of any substance in inhibiting a specific biological or biochemical function. It also can also indicate how well the organism can tolerate any foreign substances that they encounter in their cells. In this study, presence of Pb^{2+} in the form of $Pb(NO_3)_2$ interfered with the survivality of *S. regularis* and low level of Pb^{2+} indeed can become toxic to this alga as 10.9 mg/L was needed to reduce the population of the alga by 75%. This was expected as Pb^{2+} is known to inhibit the growth of many organisms, including three green unicellular algae, namely *Cladophora* [2], *Acutodesmus obliquus* [3] and *Chlorella sorokiniana* [7].

Presence of Pb^{2+} did not only affect the growth of *S. regularis*, but also significantly reduced the algae Chl A concentration when the concentration of Pb^{2+} increased the reduction was found to be the highest in IC_{75} by about 70% (Figure 2a). Chl A is the primary pigment found mainly in Photosystem I and Photosystems II of the photosynthetic machinery. Therefore, the reduction in Chl A concentration of *S. regularis* after Pb^{2+} exposure can suggests that there are detrimental effects on the core complexes of the photosystems. This will lead to the inhibition of photosynthetic processes and eventually affects the growth and development of the alga. Pb^{2+} can also affect the synthesis and degradation of chlorophylls through various mechanisms. For example, Pb^{2+} affects the synthesis of chlorophylls by inhibiting the activity of essential enzymes involved in the pathway [19] or through the substitution of the central Mg ion [20]. In addition, metals affect chlorophylls degradation via the synthesis of reactive oxygen species (ROS) [21] or by enhancing activity of the enzyme chlorophyllase which triggers the breakdown of chlorophylls [22]. Furthermore, the colour of the algae culture was observed to change from bright green in the control to colourless in IC_{75} Pb^{2+}. This phenomenon is normal to metal-stressed photosynthetic organisms, whereby metals can cause chlorosis in them [23].

Total soluble protein (TSP) content of *S. regularis* was also observed to be significantly reduced in Pb^{2+} (Figure 2b). However, the impact was in smaller scale as compared to Chl A. While IC_{75} Pb^{2+} decreased the TSP content to 85%, there was a 10-11% reduction in IC_{25} and IC_{50} with no significant changes observed among these two concentrations. The reduction in TSP observed in the alga may be explained by several factors. Firstly, exposure to heavy metals stress, including that of non redox active metal Pb^{2+}, can induce the generation of cellular ROS [8]. According to Villiers et al. [24], excess ROS level alters the protein structure by inducing oxidation of both protein backbone and amino acid side chain residues. Secondly, Pb^{2+} has the ability to react with the N and S elements in amino acids [5] and can render the proteins dysfunction. Thirdly, Pb can also inhibit the activity of proteins at cellular level by reacting with their sulfhydryl groups [23]. Lastly, to ensure cell growth during the metal-stress, the alga used the proteins as emergency sources of N and S and to supply carbon skeletons which resulted from the low photosynthetic rate [27].

In this study, activity of caspase-3-like enzyme was observed to be highly induced in alga treated with IC_{25}

of Pb^{2+} (Figure 2c). The activity significantly rose to more than 3-fold of that from the control, which indicated that apoptosis was triggered by Pb^{2+} in the alga since caspase-3 enzyme played a central role in mediating the progress of apoptosis [25]. In fact, Pb was proven to trigger the activation of caspase-3, which led to the induction of apoptosis in a study by Wan Bayani et al. [26] in *Chlorella vulgaris* and Yedjou et al. [27] on human leukemia cells. However, the activity of this enzyme suddenly slowed down to below that of the control in algae treated with IC_{50} and IC_{75} of Pb^{2+} (Figure 2c). Caspases can inhibit the non-apoptotic signaling events such as autophagy by cleaving and destroying pro-autophagic activity of pro-autophagy related proteins [28]. Both apoptosis and autophagy are two important catabolic processes which contribute to the maintenance of cellular and tissue homeostasis and they are highly interconnected [28]. When one is activated, the other will be inhibited. Several studies have also indicated that autophagy itself may be a mechanism of caspase- independent and apoptosis-independent cell death [29, 30].

Furthermore, Sui et al. [9] stated that cells protect themselves from Pb toxicity by promoting cellular autophagy. Autophagy can prevent cells from undergoing apoptosis,and thus ensure their survivality [31]. Therefore, from this study it can be suggested that at IC_{25} Pb^{2+} level, the algae cells triggered the cell death through apoptosis by inducing the production of caspase-3-like enzyme but at the IC_{50} and IC_{75} levels, the algae protected the remaining cells from death by inducing autophagy. Following either apoptosis or autophagy, necrosis may occur as the secondary events of cell death [32]. However, further analyses are needed to prove this theory.

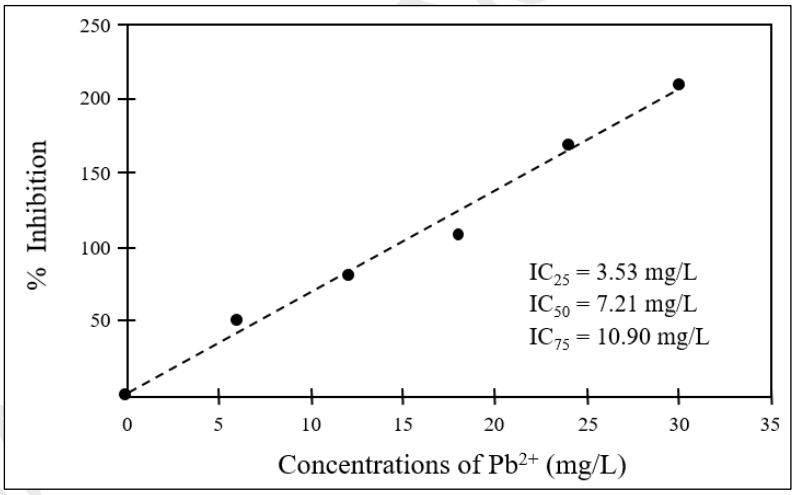

Figure 1. Percentage of *S. regularis* cells inhibited by Pb^{2+} to determine the inhibitory concentration

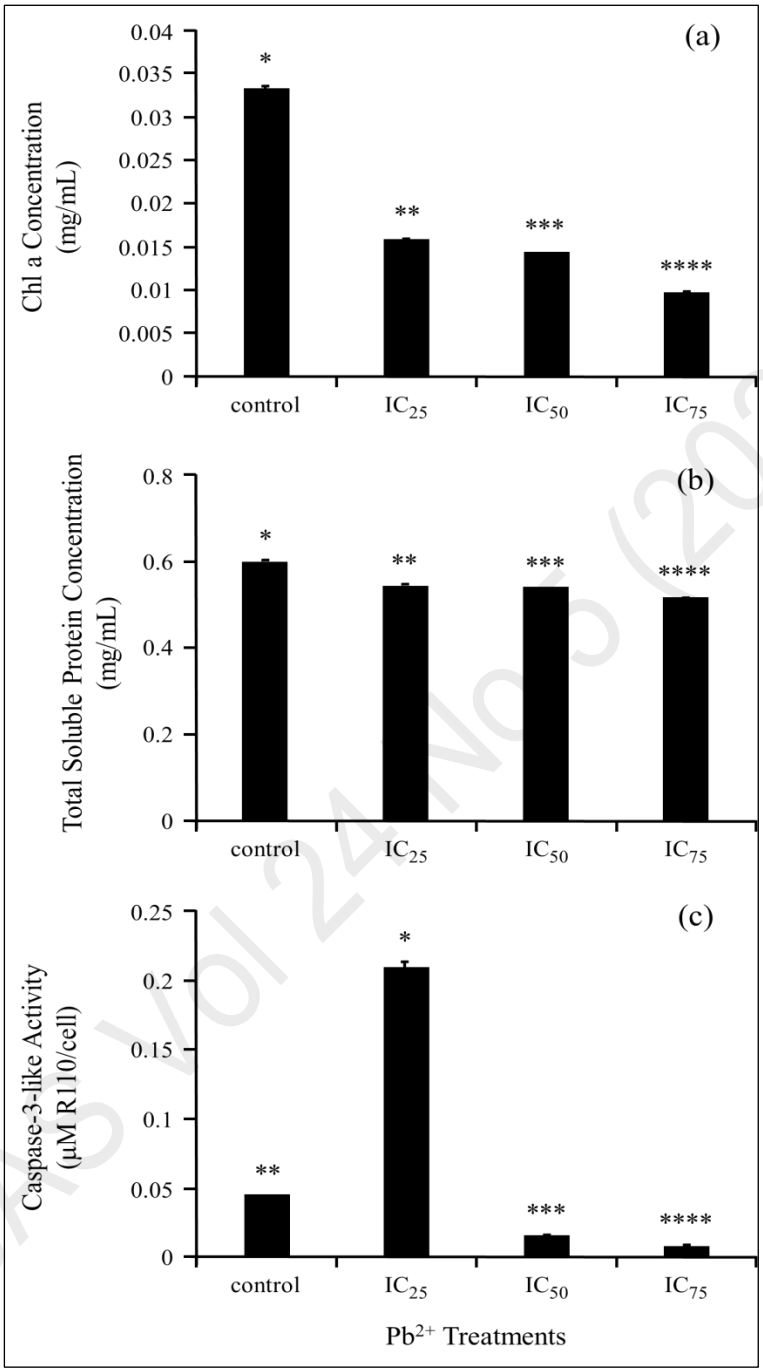

Figure 2. (a) Chlorophyll a concentration, (b) total soluble protein concentration and, (c)caspase-3-like activityof *S. regularis* after 24 h in IC_{25}, IC_{50} and IC_{75} of Pb^{2+} as compared to the control. Asterisks above barsindicate statistically significant differences at $p < 0.05$

Conclusion

Pb^{2+} exerts toxic effects on *Scenedesmus regularis* by reducing the algae Chlorophyll A (Chl A) and TSP content. At low level of Pb^{2+}, the algae induced apoptosis, whereas at high level of Pb^{2+}, the algae induced autophagy as mode of cell death. This is shown by the high activity of caspase-3-like enzyme observed at low Pb^{2+}, but a reduction below the control was seen in cells at high Pb^{2+}. In general, changes observed in this alga, as shown by its Chl A content and caspase-3-like enzyme activity, can be used as biomarkers for Pb^{2+} toxicity. However, further analyses should be done in other areas of toxicity to fully explore the potential of *S. regularis* as heavy metals bioremediation and bioindication tools.

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