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Contributed Paper

Enhanced Production of Polysaccharides and Protein Content in Cyanobacterium, *Oscillatoria limnetica* as a Defense Mechanism Against Low pH and Pb^{2+}

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Abstract

Living biomass has better ability to remove Pb^{2+} from wastewaters as compare to dead biomass. But, living biomass also have some practical limitations, such as, they are not efficient at low pH, and at high concentrations of metal ions. In the present study living biomass of *Oscillatoria limnetica* was assessed for its metal sorption capacity. *O. limnetica* considerably sorbed a large amount of Pb^{2+} within pH range 3-7. The maximum Pb^{2+} sorption capacity of *O. limnetica* was 434.78 mg/g. As photochemical efficiency data depicted, Pb^{2+} exerted inhibitory effects on photosynthesis of the test cyanobacterium. However, the test cyanobacterium showed considerable tolerance to Pb^{2+} exposure as concentration increased from 0 to 5 mg /L. Defense mechanisms of *O. limnetica* under Pb^{2+} enriched condition is probably associated with greater accumulation of intracellular polysaccharides (IPS) and protein. Nonetheless, since Pb^{2+} binding capacity of *O. limnetica* is significantly high than that of several other biosorbents, it can be considered as a promising biomaterial for removal of Pb^{2+} from wastewaters.

Keywords: adsorption capacity, chlorophyll fluorescence, detoxification, living biosorbent, Pb^{2+} precipitation

1. Introduction

Lead (Pb^{2+}) contamination in natural water supplies has serious consequences. Various phytoremediation techniques have been developed to remove Pb^{2+} from contaminated water [1]. Living algae have potential to remove heavy metals and have several advantages over dead algae [2]. They have the ability of self-replenishment and continuous metabolic uptake of metals after physical adsorption [3]. Furthermore, the metals diffused into the cells during detoxification become bound to intracellular protein or chelation. The two major heavy metal-binding compounds in algae cells are the cyteine-based metal ligand systems, namely, phytochelatin peptides and metallothioneins. Phytochelatins specifically and strongly chelate several toxic heavy metals with the aid of cysteinyl thiols, and accumulate them in the cells [4]. Algal polysaccharides are the major components of mucilage (algal cell wall coat), which plays a role in the binding of heavy metals. The positively-charged heavy

metals can bind to negatively charged cell surfaces associated with poly-anions such as polysaccharides or polyphosphates on algal cells [5].

These processes are less risk of metals released back to the environment [6]. Nevertheless, there are significant practical limitations to bio-uptake by living algae, such as sensitivity of algae to extremes of pH and high metal concentration. These challenges can be met via strain selection. Resistant algae are expected to bind substantially more metals, usually because their growing cell, which in turn is a prerequisite for enhanced bioprecipitation and development of an efficient process [7].

Algae have a variety of mechanisms of metal-stress tolerance, including production of metal binding factors, such as polysaccharides and protein [8]. Generally, the synthesis of polysaccharides occurs inside the cells as intracellular polysaccharides (IPS). The completed polysaccharides chains are subsequently translocated through the cytoplasmic membrane to the cell surface and dissolved in medium solution. Such polysaccharides are known as exocellular polysaccharides (EPS) [9].

Cyanobacteria, or blue-green algae, are serving as one of the biomaterials with high capacity for removing Pb^{2+} from contaminated waters. Donglin et al. [10] reported that when cultured in 0.001 g/L of Pb^{2+} solution, the cyanobacterium *Synechocystis* sp. was able to remove 14.3-53.3% of Pb^{2+}. Live *Phormidium bigranulatum* mat successfully removed 88% of Pb^{2+} from 0.2 g/L of Pb^{2+} solution [11]. Pb^{2+} maximum adsorption capacity (q_{max}) of *Lyngbya majuscule* and *Spirulina subsalsa* was 20.96 and 16.72 mg/g [12].

Cyanobacterium, *Oscillatoria* sp. is ubiquitous in Thailand freshwaters it is readily available for local use, moreover, it has also been found in aquatic ecosystems polluted by heavy metals. Thus, we speculated that it could grow in Pb^{2+}-containing water and serve as one of the potential alternative biomaterials for removal of Pb^{2+} from contaminated water.

In order to study the feasibility of using *O. limnetica* as a living biosorbent for Pb^{2+} removal and to know the defensive ability of this cyanobacterium, the objectives are as follows: (1) to study Pb^{2+} removal by this cyanobacterium; and, (2) to investigate the toxic effect of Pb^{2+} on photosynthesis, growth, polysaccharides and protein contents of *O. limnetica* under low pH conditions.

2. MATERIALS AND METHODS

2.1 Isolation and Cultivation of Cyanobacterial Biomass

Cyanobacterium *Oscillatoria limnetica* isolated from Klong Pravatebureerom which located close to an industrial wastewater reservoir in Bangkok, Thailand, was employed in this study. Stock culture was grown in BG-11 medium (without EDTA- to avoid precipitation of Pb^{2+}) under continuous illumination with daylight fluorescent tube lamps at 200 mmol photon/m^2/s with constant bubbling of air at 25 °C.

2.2 Pb^{2+} Removal by Living Filaments of *O. limnetica*

Living cyanobacterial filaments were added to Milli-Q water with 1-300 mg/L of Pb^{2+}. They were shaken at 25 °C for 120 min, equilibrium point was determined from the preliminary test. Cyanobacterial filaments were then harvested and the residual Pb^{2+} concentrations were determined. The adsorption characteristics of *O. limnetica* were described by Langmuir (Eq. 1), Freundlich (Eq. 2) [13], Temkin (Eq. 3) [14] and Dubinin-Radushkevich adsorption isotherms (Eq. 4) [15].

$$q_{eq} = q_{max} bC_{eq} / 1 + bC_{eq} \quad \text{(Eq. 1)}$$

$$q_{eq} = K_f C_{eq}^{1/} \quad \text{(Eq. 2)}$$

$$q_{eq} = B \ln A_T + B \ln C_{eq} \quad \text{(Eq. 3)}$$

$$\ln q_{eq} = \ln q_{max} - a\ln^2 (1+1/C_{eq}) \quad \text{(Eq. 4)}$$

where q_{eq} is the amount Pb^{2+} adsorbed by *O. limnetica* (mg/g) at equilibrium, q_{max} is the

maximum Pb^{2+} uptake by *O. limnetica* (mg/g), *b* is a coefficient related to the affinity between the *O. limnetica* and Pb^{2+} (L/mg), C_{eq} is the equilibrium concentration of Pb^{2+} (mg/L), K_f and *n* are parameters of the Freundlich isotherms, *B* is a constant related to heat of sorption (J/mol), A_T is a Temkin isotherm equilibrium binding constant (L/g).

2.3 Effects of Pb^{2+} on Photosynthesis

Exponentially grown filaments of *O. limnetica* were cultivated in BG-11 medium (without EDTA) containing 0-20 mg/L of Pb^{2+}, which is the maximum concentration range of Pb^{2+} found in industrial wastewater (Unpublished data from our preliminary study in tertiary treatment pond of the wastewater treatment plant of Ladkrabang industrial estate). Pb^{2+} solutions were prepared from $Pb(NO_3)_2$ stock solution in Milli-Q water.

To study the role of nutrients in medium in the Pb^{2+} tolerance mechanism of cyanobacterium, the cyanobacterial filaments immersed in Milli-Q water with similar Pb^{2+} concentrations were compared. To avoid the precipitation of Pb^{2+}, the pH of medium and Milli-Q water were adjusted to 5. The filaments were incubated under these conditions with 12:12 L/D illumination of 200 mmol photon/m^2/s at 25 ^oC. The initial and residual Pb^{2+} concentrations in medium and Milli-Q water were determined.

After 48 h exposure, samples were taken for chlorophyll fluorescence analysis. Samples of *O. limnetica* filaments were uniformly placed on a 45 mm glass fiber filter. All samples were dark-adapted for 20 min before measurements by using a pulse -amplitude - modulated (PAM) fluorescence monitoring system (Phyto-PAM, Canada) with an halogen actinic light of 2,000 and 5,000 mmol photon/m^2/s flash light with a peak wavelength of 660 nm focused on the sample surface. After measuring the initial fluorescence (Fo), maximal fluorescence (Fm) was determined. The variable fluorescence (Fv) was calculated from the formula, Fv=Fm-Fo. The maximum photochemical efficiency of PSII was calculated as Fv/Fm [16].

2.4 Effects of pH on Photosynthesis

Our preliminary test indicates that *O. limnetica* could not grow at a pH lower than 3. Hence, the pH range chosen for this study was 3 to 7. *O. limnetica* filaments were added to BG-11 medium (without EDTA) that was adjusted to pH 3-7 by using 0.03 N HNO_3. The filaments were incubated with 12:12 L/D illumination of 200 mmol photon/m^2/s at 25 ^oC for 48 h. The pH of the culture medium was measured at 24 h intervals using a digital pH meter (HANNA HI 8424, Japan). After each measuring, the pH was then adjusted to the initial value. Samples were taken for chlorophyll fluorescence analysis after 24 and 48 h exposure.

2.5 Effects of Low pH and Pb^{2+} on Growth, Polysaccharides and Protein Content

To investigate the inhibitory effect of Pb^{2+} on growth, and protection mechanism of *O. limnetica* under low pH conditions, *O. limnetica* were cultivated in BG-11 medium without EDTA and with 0, 2.5, and 5 mg/L concentrations of Pb^{2+} as treatment B, C and D, respectively. The experiments were performed at pH5. A control treatment was also done without Pb^{2+} at pH 7 (treatment A).

Since pH was changed after a period of cultivation, the pH of the medium was adjusted to the initial pH at 24 h intervals. Pb^{2+} was added to the medium only at the initial stage of each experiment and Pb^{2+} concentrations were measured at the 5^{th}, 10^{th}, 20^{th} and 30^{th} day of cultivation. Pb^{2+} concentration on cyanobacterial filaments was determined at the 30^{th} day of cultivation.

The effects of Pb^{2+} and pH on growth of *O. limnetica* were investigated by determining its growth phase, which was based on chlorophyll *a* content and dry weight. The total polysaccharides and protein of the filaments were also measured since they are related to heavy metal removal and detoxification mechanisms [3].

2.6 Chemical Analysis and Statistical Analysis

Chlorophyll *a* content was measured as described by Becker [17]. *O. limnetica* filaments were harvested via centrifuging for 10 min at 3000 rpm for measuring IPS and EPS. IPS were measured from pelleted filaments and EPS were measured from supernatant by the phenol sulfuric acid method [18]. Protein contents were determined according to the Lowry method [19]. Pb^{2+} concentration was analyzed using an atomic absorption spectrophotometer (GBC Avanta, Australia). All of the experiments were conducted in four replicates. Significant differences were determined by using analysis of variance (ANOVA) with 95% confidence.

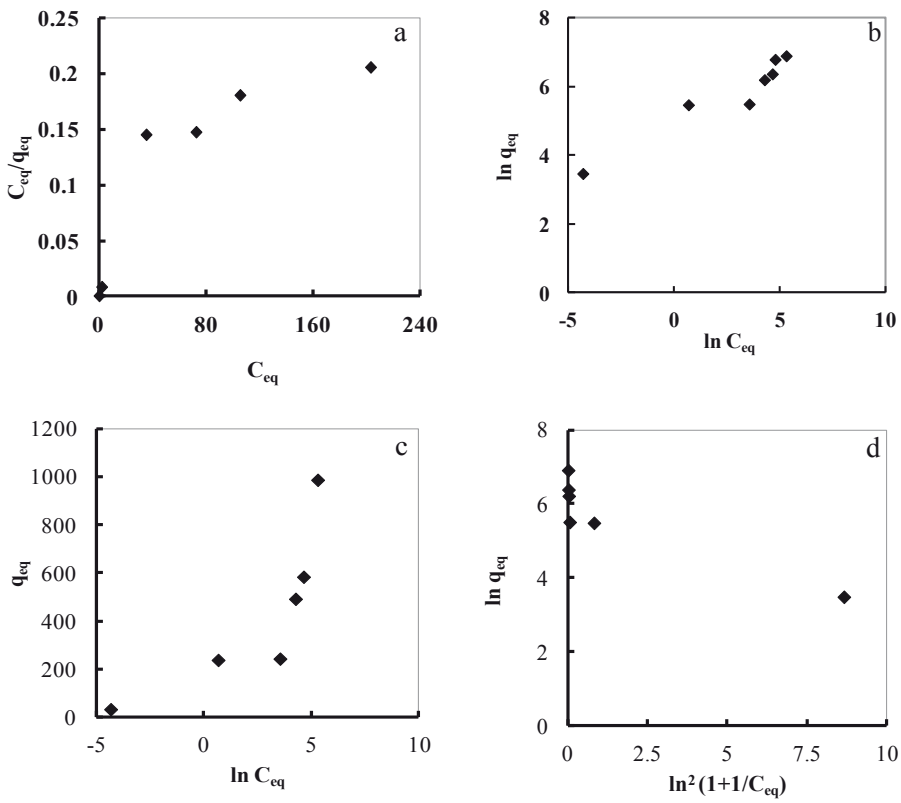

Figure 1. Lead adsorption isotherms by *O. limnetica* (initial pH 5.0, T = 25 °C, biomass concentration 0.1 g dry wt/L, initial Pb^{2+} 1-300 mg/L), Langmuir (a), Freundlich (b), Temkin (c) and Dubinin-Radushkevich Isotherms (d).

Table 1. Langmuir, Freundlich, Temkin and Dubinin-Radushkevich parameters for adsorption of Pb^{2+} by cyanobacterium *O. limnetica* (initial pH 5.0, T = 25 °C).

Adsorption Isotherm							
Langmuir		**Freundlich**		**Temkin**		**Dubinin-Radushkevich**	
q_{max} (mg/g)	434	K_f	83,714	K_T (L/g)	33	q_{max} (mg/g)	468
b (L/mg)	5.75	n	3.05	b_T	33.88		
r^2	0.986	r^2	0.924	r^2	0.627	r^2	0.832

3. RESULTS AND DISCUSSION

3.1 Pb^{2+} Adsorption Capacity of *O. limnetica*

The Langmuir isotherm best fits the experimental data over the experimental range studied, for it presents the greater coefficient of correlation (r^2). Therefore, the mechanisms involved in Pb^{2+} removal by *O. limnetica* were discussed based on the Langmuir isotherm parameters. The maximum Pb^{2+} adsorption (q_{max}) by *O. limnetica* was 434.78 mg/g dry weight (Figure 1., Table 1.), it was much higher than those of other types of cyanobacteria reported earlier [10-12, 20-22]. Thus, study to assess feasibility of the test cyanobacterium as a living Pb^{2+}, particularly at low pHs seems a necessary task.

3.2 Effects of Pb^{2+} on Photosynthesis

Impact of Pb^{2+} concentrations on the Fv/Fm are presented in Figure 2a. When *O. limnetica* was cultured in BG-11 medium and Milli-Q water with increasing Pb^{2+}, the Fv/Fm decreased. The Fv/Fm of *O. limnetica* both cultured in BG-11 medium and Milli-Q water in all Pb^{2+} concentrations were significantly different from the control (0 mg/L). In BG-11 medium, Fv/Fm decreased by 15, 13, 28 and 39% at Pb^{2+} concentrations of 5, 10, 15 and 20 mg/L, respectively.

The obtained value of Fv/Fm in *O. limnetica* cultured in medium that were significantly higher than those cultured in Milli-Q water

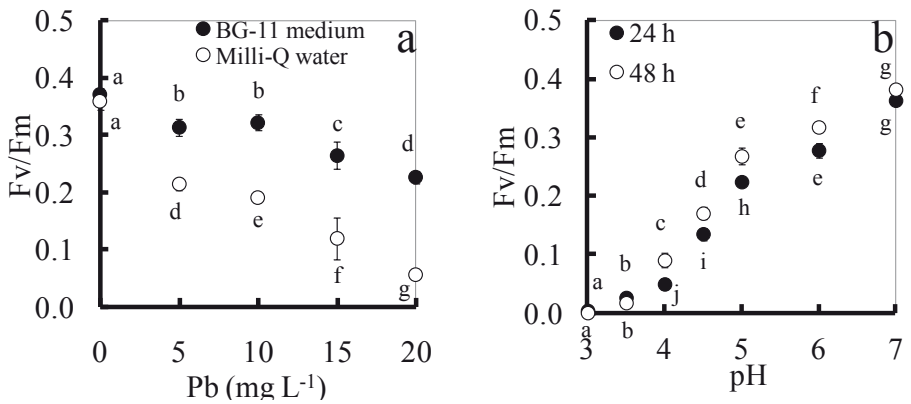

Figure 2. The changes of maximum photochemical efficiency of PSII (Fv/Fm) of *O. limnetica* that was exposed to various Pb^{2+} concentrations (a), and to media of varying pH (b). Different small letters on the lines indicate significant difference (p<0.05). Error bars represent ± standard deviation with n =4.

when compared in the same Pb^{2+} concentration ($p<0.05$). The possible explanation is nutrients in medium may induce defense mechanisms in *O. limnetica* to reduce toxicity of Pb^{2+} inside their cells. This emphasize that the cyanobacterium is ideal candidate to be a living biosorbent for wastewater contaminated by Pb^{2+} and other organic waste.

3.3 Effect of pH on Photosynthesis

The Fv/Fm of *O. limnetica*, which was cultured in pH 3-6 for 24 and 48 h, was significantly lower than observed for the control (pH 7) (Figure 2b.). At pH 4 to 6, the longer exposure yielded higher Fv/Fm, comparing 24 with 48 h exposure, but was not significantly different at pH 3 and 3.5. Decreasing of Fv/Fm values were found at pH 4.5 (55%), 5 (30%), and 6 (17%) with 48 h exposure.

High H^+ concentration (pH 4.5 and lower) has a severe effect on the photosynthesis of *O. limnetica*. When the pH of cultured solution was decreased, the photosynthetic inhibition tends to increase. But this inhibition could be recovered (at pH 4-6) after 48 h of exposure period. This indicated an adaptive mechanism of *O. limnetica* under high H^+ concentration. Rai et al. [23] explained that the mechanisms of algae to protect their cells from low pH include reduction of cation uptake coupled with an accelerated uptake of anions. Therefore, their cytoplasmic pH is maintained at relatively constant level even after exposure to the low pH condition.

3.4 Effect of Low pH and Pb^{2+} on Growth

The results from previous steps (3.2 and 3.3) indicated that Pb^{2+} concentration lower than 10 mg/L and pH 6 have little effect (< 20%, compared with control) on the Fv/Fm of *O. limnetica*. In addition, we determined the concentration of heavy metal in industrial wastewater, where data showed that the average initial Pb^{2+} concentration in wastewater of the tertiary treatment system was about 4.9±0.0 mg/L. Thus, in this experiment we study co-effect of Pb^{2+} and pH on *O. limnetica* growth at 0-5 mg/L of Pb^{2+} and pH 5, which Pb^{2+} is in the ionized form.

The stress conditions, pH 5 and Pb^{2+} contamination, were able to reduce the time of growth cycle and the maximum biomass. Growth of *O. limnetica* in treatment A went to the dead phase after 27 days of cultivation, while the dead phases of treatments B, C and D were at 18, 18 and 15 days of cultivation,

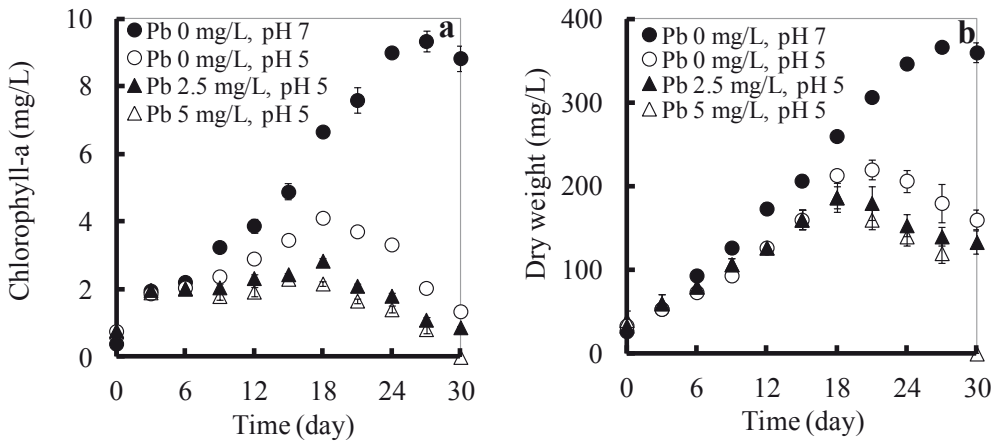

Figure 3. The changes of chlorophyll *a* content (a), and dry weight (b) during the growth of *O. limnetica* under various Pb^{2+} concentrations and pH. Data are presented in means ± standard deviation with $n = 4$.

respectively (Figure 3a., b.). Decreasing of chlorophyll *a* and dry weight indicated that elevated Pb^{2+} concentration suppressed growth of *O. limnetica*. The maximum chlorophyll *a* in treatments B, C, and D decreased by 43, 57, and 67% compared to the control at the 18th day. Changes in dry weight had a similar pattern as data for chlorophyll *a*.

3.5 Effect of Low pH and Pb^{2+} on Polysaccharides and Protein Production

The maximum IPS for treatment A was lowest, 0.80±0.04 mg/g at the 30th day of cultivation, with increasing amounts found in treatments B (0.91±0.13 mg/g at the 27th day), C (0.94±0.19 mg/g at the 30th day), and D (1.01±0.17 mg/g at the 27th day) (Figure 4a.). When Pb^{2+} concentrations were increased and pH were decreased, *O. limnetica* synthesized significantly higher amounts of IPS than those of the control, which obviously shown in treatment B-D after the 6th day of cultivation. As polysaccharides served as energy sources for the cells [24], it was believed that the increase of IPS in *O. limnetica* could increase its Pb^{2+} and H^+ detoxification ability, because higher IPS mean greater energy to repulse excessive cations. The production of EPS in

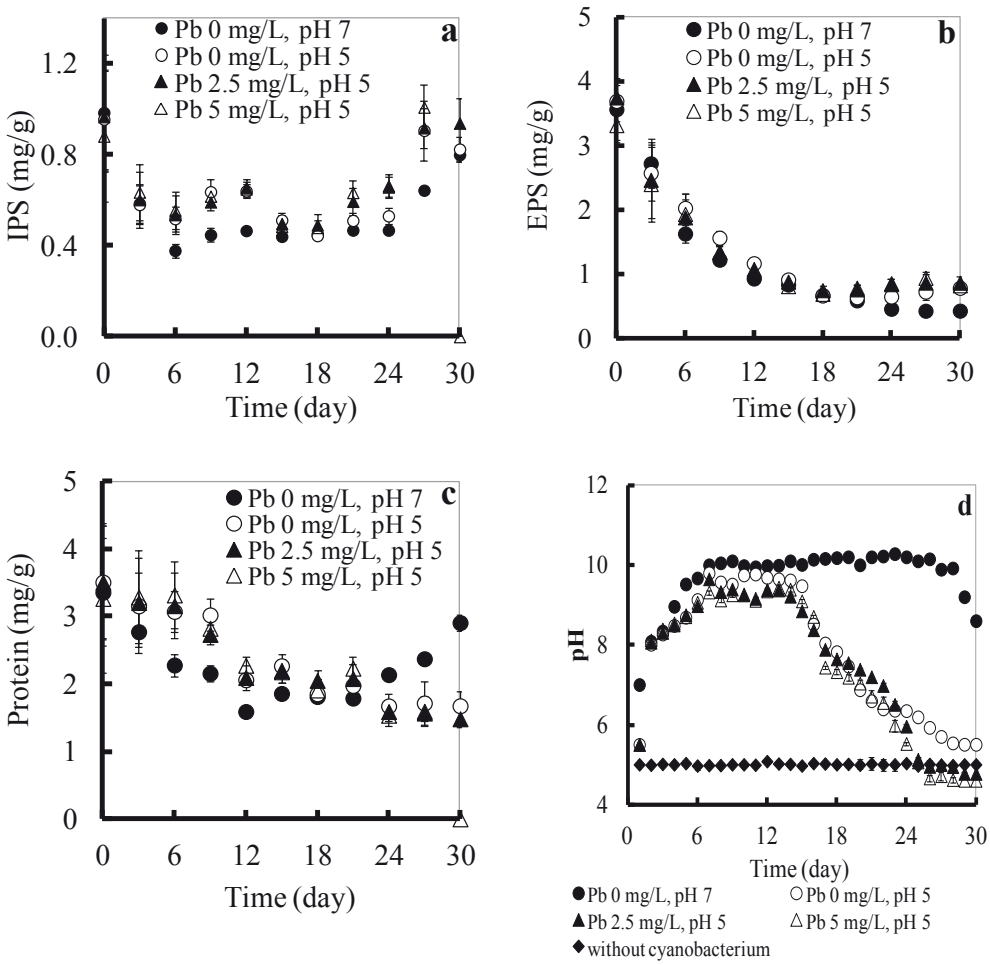

Figure 4. The changes of polysaccharides IPS (a), EPS (b), protein (c), and pH (d) during growth of *O. limnetica*, cultured under various Pb^{2+} concentrations and pH. Data are presented in means ± standard deviation with *n =4*.

all treatments decreased from 3.31-3.74 mg/g at the beginning, to 0.42-0.86 mg/g after 30 days of cultivation (Figure 4b.).

During days 6-12 of cultivation, the amounts of protein in treatments B-D were significantly higher than the control, while no differences were observed among treatments B, C, and D (Figure 4c.). The importance of protein is that they can bind with metals as a means of inactivating them within the cell [3]. From this study *O. limnetica* increased its intracellular protein content when exposed to Pb^{2+} with low pH. This could indicate a mechanism to reduce Pb^{2+} through the induction of protective protein synthesis. The production of protein in all treatments decreased during 18 days of cultivation. Possibly *O. limnetica* reduced the accumulation of protein in cells and used protein as energy source during the exponential growth phase.

After the 24th day of cultivation, the protein content of control was continuously increased and significantly higher than treatments B-D, which related to growth phase (Figure 3a., b.). Simon [25] also reported that cyanobacterium, *Anabaena cylindrica* culture in normal condition was able to increase the amount of phycocyanin pigment (reserve protein) and soluble protein during exponential growth but falls when the cells enter the stationary phase.

3.6 Changes of pH and Pb^{2+} Concentrations During the Growth of *O. limnetica*

Figure 4d. showed that after addition of cyanobacterial filaments into the media, the pH changed following the growth of cyanobacterium. *O. limnetica* in treatment B-D were able to increase pH of the medium from the 1st until the 14th day of cultivation where growth was in the exponential phase, after that the pH gradually decreased, likely because the culture reached a dead phase which had low photosynthetic activity.

After 14 days of cultivation, pH of treatments A-D increased from 7, 5, 5, and 5 at the beginning to about 10.2, 9.6, 9.2 and 9.4, respectively. When the pH of medium was increased above 5 (from the photosynthesis mechanism which releases OH^-), it could lead to the change of Pb speciation in solution, from Pb^{2+} to $PbOH^+$, $Pb(OH)_2$ and $Pb(OH_2)^-$, resulting in the precipitation of Pb [26].

During *O. limnetica* cultivation in Pb^{2+}-containing medium, the concentration of Pb^{2+} decreased as experimental time increased. About 88 and 71% of Pb^{2+} were removed on the 5th day of treatments C and D, and 100% of Pb^{2+} was removed after 10 days of cultivation from both treatments. At the end of study Pb^{2+} concentration containing in cyanobacterial filament indicated that Pb^{2+} removed from the medium by cyanobacterial filament was 94.5 and 88% in treatment C and D. Thus the other concentration of Pb^{2+} was assumed to have been removed through precipitation.

4. Conclusion

The results from this work showed that *O. limnetica* could grow in Pb^{2+} contaminated water with acidic pH, the indicators of resistance and detoxification mechanisms was increased like IPS and protein. Living filaments of *O. limnetica* could remove Pb^{2+} by directly sorb to their filaments and indirectly increase the pH of the medium which resulted in Pb^{2+} precipitation. These results are useful to assign the conditions that are most suitable and are most tolerable for use of this cyanobacterium as a living biosorbent.

Acknowledgments

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