Available online at www.sciencedirect.com

ScienceDirect

APCBEE Procedia 10 (2014) 269 – 274

Procedia APCBEE

www.elsevier.com/locate/procedia

ICESD 2014: February 19-21, Singapore

Effect of Lead and γ-Polyglutamic Acid Produced from *Bacillus subtilis* on Growth of *Brassica chinensis* L.

O. Chunhachart[a,b], N. Kotabin[a,b], N. Yadee[a], Y. Tahara[c] and K. Issakul[b,d*]

[a]*Department of Microbiology, Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Sean campus, Nakorn Pathom 73140, Thailand*
[b]*Center of Advanced Studies in Tropical Natural Resources, Kasetsart University, Bangkok, 10900, Thailand*
[c]*Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan*
[d]*Department of Environmental Science, School of Energy and Environment, University of Phayao, Phayao 56000, Thailand*

Abstract

γ-Polyglutamic acid (γ-PGA) has been reported to be an effective biosorbent for metal ions. *In vitro* binding of lead (II) (Pb) by γ-PGA produced from *Bacillus subtilis* NBRC16449 was examined and the effect of Pb and γ-PGA on *Brassica chinensis* L. grown in the laboratory was investigated. The results revealed that the optimal pH for Pb adsorption was 5.0. γ-PGA was stable at 35-50°C and pH 5-7. The maximum removal efficiency was 87.9%. *Brassica chinensis* L. seeds were germinated and grown at 28±1°C on filter paper soaked with Pb solution at 0, 50, 100 and 250 μM and γ-PGA at 0, 100, 500 and 1,000 mg/L for 7 days. The results indicated that Pb markedly inhibited growth of roots by reducing root length ($P<0.05$). However; the addition of 500 mg/L γ-PGA significantly protected seedlings from the adverse effects of Pb ($P<0.05$). Thus, γ-PGA has high potential as a biopolymer to be used for alleviation of Pb toxicities in plants.

Selection and peer review under responsibility of Asia-Pacific Chemical, Biological & Environmental Engineering Society

Keywords: γ-polyglutamic acid; biosorption; lead; *Brassica chinensis*; *Bacillus subtilis*

1. Introduction

Lead (II) (Pb) is one of toxic heavy metals released by industry and motor vehicles. It is a potential

* Corresponding author.
E-mail address: kissakul@gmail.com

Selection and peer review under responsibility of Asia-Pacific Chemical, Biological & Environmental Engineering Society
doi:10.1016/j.apcbee.2014.10.051

pollutant that persists in the soil and is widespread in the urban environment. Although Pb is not an essential nutrient for plants, it is easily taken up by them from the soil and accumulated in edible parts. Excess Pb in plants causes several toxicity symptoms associated with the retardation of growth, inhibition of root elongation, appearance of chlorosis and inhibition of enzyme activities [1]. Vegetables (Brassicaceae) cultivated in land close to traffic roads and highways in Thailand contain Pb levels between 1.0 and 1.6 mg/kg FW [2] while the maximum permissible level of Pb in vegetables set by Codex Alimentarius (2001) is 0.3 mg/kg FW [3]. Thus, the consumption of edible plant parts might cause adverse health effects in humans. Recently, microbial cells and biopolymers derived from microorganisms have been emerging as an alternative treatment for scavenging heavy metals from soil and aqueous systems. γ-Polyglutamic acid (γ-PGA) is a naturally occurring anionic polymer, and is biodegradable, edible and nontoxic to humans and is benign to the environment. PGA consists of D- and L-isomers of glutamic acid linked by amide bonds between the α-amino and carboxyl groups [4]. In the past few years, there has been interest in various applications of γ-PGA in thickeners, humectants, drug carriers, biological adhesives, foods, cosmetics, medicines, water absorbents, bioflocculants and wastewater treatment [4]. Moreover, it has been reported to be an effective biosorbent for cationic dyes [5] and several metal ions: Ni (II), Cu(II), Mn(II), Al(III) and Cr(III) [6], Hg(II) [7] and Cd (II) [8] in water treatment. Thus, it is of interest to consider whether γ-PGA can be used as a Cd biosorbent with plants and to reduce Pb toxicity in plants. This study investigated the optimal conditions for *in vitro* Pb adsorption by γ-PGA and the effects of Pb and γ-PGA on *Brassica chinensis* L. seedlings grown in the laboratory.

2. Experimental Method

2.1. Production and purification of γ-PGA

γ-PGA from *Bacillus subtilis* NBRC16449 was produced in a PGA-production medium and purified by the prescribed method [9].

2.2. In vitro Pb(II) binding experiments

The reaction was carried out by taking 50 mL of 500 μM of lead(II) chloride ($PbCl_2$) (Sigma, USA) solution and adjusting to pH 5.0. Next, 10 ml of 1,000 mg/L γ-PGA was added and the mixture was stirred at 150 rpm for 10 min at 37°C. Then, samples were taken at different time intervals to determine the residual Pb concentration (no further absorption of Pb). Each sample solution was filtered through 0.2-μm membrane filter and the filtrate was analysed for Pb concentration by using a flam atomic absorption spectrophotometer (AAS) (spectrAA 220 F.S. model, Australia) with an oxidizing flame of an air-acetylene mixture at a ratio of 7:1. The optimum pH for Pb adsorption was determined at pH 3-7. For pH stability, γ-PGA was kept at pH 3-9 at 37°C for 2 h. Temperature stability was performed at pH 7.0 at 30-50°C for 2 h. Finally, Pb adsorption was carried out at the optimal conditions and the residual Pb concentration was analysed by AAS.

2.3. Effect of γ-PGA on lead uptake by Brassica chinensis L.

The healthy *B. chinensis* L. seeds were surface sterilised in 10% sodium hypochlorite for 10 min, then rinsed four times with deionised water. The seeds were soaked in deionised water for 30 min. A filter paper was placed on a Petri dish (9 cm in diameter) and moistened with 5 ml of the Pb and γ-PGA mixture in different series of concentrations (Pb; 0, 50, 100, 250 μM and γ-PGA; 0, 50, 100, 500, 1,000 mg/L). Ten seeds were placed on a Petri dish and incubated in a growth chamber with 12 h light and 12 h darkness at 28ºC±1ºC for 7 days. Three replications were conducted for each treatment. Physical parameters of the rice seedling (the shoot and root lengths, the fresh and dry weights, the percentage of germination and the vigour index) were determined.

2.4. Statistical analysis

Statistical analysis was performed using SPSS software for Windows with analysis of variance (ANOVA) with Duncan's Multiple-Range Test (DMRT) at the 0.05 probability level.

3. Results and Discussion

In vitro binding of Pb by γ-PGA revealed that the Pb adsorption occurred at pH 5.0 (Fig. 1.). γ-PGA was stable at pH 7; however, the adsorption activity was more than 90% at pH 5-6. While γ-PGA was most stable at 30°C, the Pb adsorption activity was more than 90% at 40-50°C (Fig. 2.). The maximum removal efficiency of Pb was 87.9% (Table 1.). Because γ-PGA is an anionic polymer of glutamic acid, it has been used as a chelating material as it is capable of binding metal ions via its carboxylic and amide groups [10]. Furthermore, the metal ions may also interact with amide groups of γ-PGA [6,10]. In previous studies, γ-PGA was effective in binding several metal ions [6,7,8]. In these studies the heavy metal ions were bound with γ-PGA using a similar chelation mechanism which was followed by conformational change of the γ-PGA into an enveloped aggregate precipitate [11].

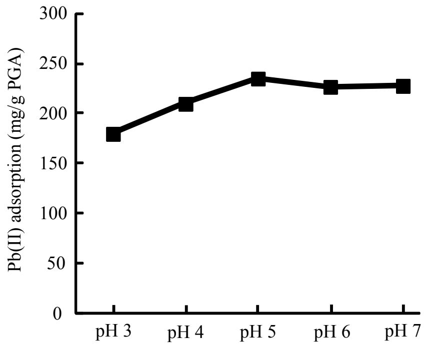
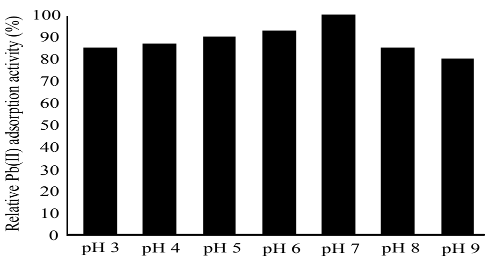
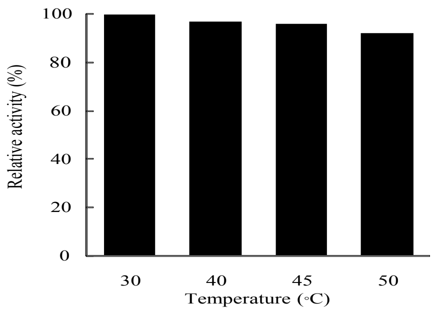


Fig. 1. Effect of pH on Pb adsorption by γ-PGA



(a)



(b)

Fig. 2. Effect of pH and temperature on stability of γ-PGA; (a) pH and (b) temperature

Table 1. Biosorption of lead by γ-PGA

Initial lead conc. (mg/L)	Residual lead conc. (mg/L)	% Removal of lead
70.06	8.46	87.92
93.82	39.80	57.57
243.59	157.37	35.39
411.86	330.77	19.69
703.20	589.74	16.13

Table 2. Effect (mean ± standard error) of Pb and γ-PGA on growth parameters of *Brassica chinensis* L.

Treatment		Germination	Shoot length	Root length	Fresh weight	Dry weight	Vigour index
Pb (μM)	γ-PGA (mg/L)	(%)*	(cm)*	(cm)*	(g)*	(g)*	
0	50	100.00±0.00 a	1.43 ±0.43 ab	5.27 ±1.84 a	0.0176 ±0.004 abc	0.0009 ±0.0003 b	670.00
	100	96.67±5.77 a	1.38 ±0.43 ab	5.02 ±2.25 ab	0.0172 ±0.005 bc	0.0013 ±0.0004 a	618.69
	500	96.67±5.77 a	1.53 ±0.55 ab	4.80 ±1.61 ab	0.0185 ±0.005 abc	0.0011 ±0.0005 ab	611.92
	1,000	100.00±0.00 a	1.87 ±0.69 a	4.66 ±1.78 abc	0.0252 ±0.007 a	0.0014 ±0.0003 a	653.00
50	0	100.00±0.00 a	1.41 ±0.43 b	3.12 ±1.07 defg	0.0175 ±0.005 ac	0.0012 ±0.0004 ab	453.00
	50	100.00±0.00 a	1.43 ±0.40 ab	3.93 ±1.41 bcde	0.0168 ±0.004 bc	0.0012 ±0.0003 ab	536.00
	100	100.00±0.00 a	1.65 ±0.51 ab	4.62 ±1.37 abc	0.0198 ±0.007 abc	0.0012 ±0.0003 ab	627.00
	500	100.00±0.00 a	1.64 ±0.48 ab	4.03 ±1.58 abcde	0.0191 ±0.004 abc	0.0013 ±0.0003 ab	567.00
	1,000	100.00±0.00 a	1.67 ±0.58 ab	4.30 ±1.44 abcd	0.0205 ±0.004 abc	0.0014 ±0.0004 a	597.00
100	0	96.67±5.77 a	1.59 ±0.50 ab	2.29 ±0.77 fgh	0.0173 ±0.005 bc	0.0013 ±0.0005 ab	375.08
	50	100.00±0.00 a	1.42 ±0.46 ab	2.82 ±1.08 efg	0.0166 ±0.005 bc	0.0012 ±0.0005 b	424.00
	100	100.00±0.00 a	1.51 ±0.53 b	3.87 ±1.30 bcde	0.0177 ±0.006 abc	0.0011 ±0.0003 ab	538.00
	500	100.00±0.00 a	1.24 ±0.45 b	3.74 ±0.89 bcde	0.0144 ±0.006 c	0.0012 ±0.0004 ab	498.00
	1,000	100.00±0.00 a	1.60 ±0.38 ab	3.223 ±1.19 defg	0.0236 ±0.029 ab	0.0012 ±0.0004 ab	482.30
250	0	100.00±0.00 a	1.62 ±0.52 ab	1.400 ±0.66 h	0.0196 ±0.006 abc	0.0015 ±0.0006 a	302.00
	50	100.00±0.00 a	1.68 ±0.47 ab	2.117 ±0.86 gh	0.0190 ±0.005 abc	0.0014 ±0.0005 a	380.00
	100	100.00±0.00 a	1.60 ±0.53 ab	2.990 ±0.88 efg	0.0181 ±0.005 abc	0.0015 ±0.0009 a	459.00
	500	100.00±0.00 a	1.29 ±0.40 b	3.320 ±1.17 defg	0.0171 ±0.004 bc	0.0013 ±0.0004 ab	461.00
	1,000	100.00±0.00 a	1.47 ±0.57 ab	3.050 ±1.24 defg	0.0195 ±0.006 abc	0.0014 ±0.0005 a	452.00
Control		100±0.00 a	1.50 ±0.47 ab	3.49 ±2.11 cdef	0.0185 ±0.006 abc	0.0011 ±0.0003 ab	499.00

* Different letters indicate statistically significant differences at the 0.05 probability level

The effects of Pb and γ-PGA on the percentage of germination of *B. chinensis* L. seedlings produced no significant differences among all treatments (Table 2.). However, Pb significantly inhibited root length growth. The inhibition of seedling growth is a common effect of exposure to heavy metals which has also been found in *Allum cepa* [12] and Indian mustard (*Brassica juncea* L.) [13]. Lead reduces root growth and cause mitotic irregularities [12], [13].

The addition of Pb alone clearly decreased the vigour index, while in contrast, the addition of γ-PGA with no added Pb maintained the vigour of seedlings and the addition of 1,000 mg/L increased the vigour index for all levels of added Pb. The addition of γ-PGA resulted in a noticeable increase in the root length with more lateral roots compared to the treatments without γ-PGA. However, the fresh and dry weights were not significantly different. Other studies reported that plants transport free metal ions by diffusion along the concentration gradient with results of these studies suggesting that γ-PGA became bound to Pb and formed Pb-PGA complexes which had difficulty in penetrating the cell walls of the plant roots causing a reduction in the Pb uptake [15], [16]. Furthermore, these studies considered that the concentration of soluble Pb became very low because of the formation of the Pb-PGA complexes affecting lead influx or the transport rate by plantlet roots.

4. Conclusions

γ-PGA is an effective Pb biosorbent. Furthermore, it is capable of alleviating Pb phytotoxicity in *Brassica chinensis* L. seedlings. The effect of γ-PGA on *Brassica chinensis* L. grown in Pb contaminated soil will be investigated further.

References

[1] Sharma P, Dubey RS. Lead toxicity in plants. *Braz J Plant Physiol* 2005;17:35-52.

[2] Sanethaog C. Distribution of zinc lead and cadmium in vegetable growing areas, suburb of Saraburi province. Master's thesis: Kasetsart University; 2008.

[3] Codex Alimentarius Commission. Lead: Maximum Levels. Vol.1. COdwx Stan; 2001, p. 230.

[4] Shih IL, Van YT. The production of poly-(γ-glutamic acid) from microorganisms and its various applications. *Bioresour Technol* 2001;79:207-225.

[5] Inbaraj BS, Chiu CP, Ho GH, Yang J, Chen BH. Removal of cationic dyes from aqueous solution using an anionic poly-gamma-glutamic acid-based adsorbent. *J Hazard Mater* 2006;137:226-234.

[6] McLean IL, Beauchemin D, Clapham L, Beveridge TJ. Metal-binding characteristics of the gamma-glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. *Appl Environ Microbiol* 1990;56:3671-3677.

[7] Inbaraj BS. Wang JS, Lu JF, Siao FY, Chen BH. Adsorption of toxic mercury (II) by an extracellular biopolymer poly (γ-glutamic acid). *Biores Technol* 2009;100:200-207.

[8] Mark SS, Crusberg TC, Dacunha CM, Iorio D. A heavy metal biotrap for wastewater remediation using poly-gamma-glutamic acid. *Biotechnol Prog* 2006;22:523-531.

[9] Goto A, Kunika M. Biosynthesis and hydrolysis of poly(γ-glutamic acid) from *Bacillus subtilis* IFO3335. *Biosci Biotechnol Biochem* 1992;56:1031-1035.

[10] Siao FY, Lu JF, Wang JS, Inbaraj BS, Chen BH. *In vitro* binding of heavy metals by and edible biopolymer poly (γ-glutamic acid). *J Agric Food Chem* 2009;57:777-784.

[11] He JY, Zhu C, Ren YF, Yan YP, Jiang DA. Genotypic variation in grain cadmium concentration of lowland rice. *J Plant Nutr Soil Sci* 2006;169:711-716.

[12] Lui D, Jiang W, Wang W, Zhao F, Lu C. Effects of lead on root growth, cell division and nucleolus of *Allum cepa*. *Environ Pollut* 1994;86:1-4.

[13] Lui D, Jiang W, Wang W, Zhao F, Lu C. Uptake and accumulation of lead by roots, hypocotyls and shoots of Indian mustard [*Brassica juncea* (L.)]. *Bioresour Technol* 2000;71:273-277.

[14] Munzuroglu O, Geckil H. Effects of metals on seed germination, root elongation, and coleoptile and hypocotyl growth in *Triticum aestivum* and *Cucumis sativus*. *Arch Environ Contam Toxicol* 2002;43:203-213.

[15] Cseh E, Fodor F, Varga A, Za'ray G. Effect of lead treatment on the distribution of essential elements in cucumber. *J Plant Nutrition* 2000;23:1095-1105.

[16] Aidid SB, Okamoto H. Effects of lead, cadmium and zinc on the electric membrane potential at the xylem/symplast interface and cell elongation of *Impatiens balsamina*. *Environ Exp Bot* 1992;32:439-448.