ORIGINAL ARTICLE

Lead (Pb^{2+}) adsorption characteristics and sugar composition of capsular polysaccharides of cyanobacterium *Calothrix marchica*

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Abstract

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The ability of living cells of the cyanobacterium *Calothrix marchica* to adsorb Pb^{2+} was studied. *C. marchica* showed high efficiency to remove Pb^{2+}. The desorbing agent, EDTA could effectively desorb Pb^{2+} from cyanobacterial cells, suggesting that Pb^{2+} was mainly adsorbed on the cell surface, presumably binding with certain functional groups of capsular polysaccharides (CPS). Subsequently, production of CPS, their sugar contents and ability to absorb Pb^{2+} were studied in details. CPS production by *C. marchica* increased as growth proceeded. *C. marchica* showed the highest CPS content of 26.85 mg g^{-1} dry weight after 30-day of cultivation. The major components of CPS were carbohydrates and protein (37.6% and 34.7% of total CPS, respectively). The neutral sugars presented in CPS of *C. marchica* were xylose, arabinose, ribose, rhamnose,

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galactose, glucose, mannose and fructose. Acidic sugars including galacturonic and glucuronic acids were also found. The highest acidic sugar content was found after 30 days of cultivation. The extracted CPS of *C. marchica* could adsorb 64.15±5.09 mg Pb per g CPS. *C. marchica* cells with intact CPS adsorbed 3.42±0.07 mg Pb g^{-1} dry weight, which was not significantly different from cells without CPS. From these results, it can be concluded that *C. marchica* was able to remove Pb^{2+} rapidly and efficiently, and to produce high amount of CPS with acidic sugars 117.73±2.15 μg g^{-1} as the major component, suggesting that *C. marchica* could serve as one of the alternative bioadsorbers for Pb^{2+} in bioremoval systems.

Key words : Lead (Pb^{2+}), *Calothrix marchica*, cyanobacteria, capsular polysaccharides (CPS), Pb^{2+} adsorption

บทคัดย่อ

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ลักษณะการดูดซับตะกั่วและองค์ประกอบน้ำตาลของโพลิแซคคาไรด์ในไซยาโนแบคทีเรีย *Calothrix marchica*

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การศึกษาความสามารถของไซยาโนแบคทีเรีย *Calothrix marchica* โดยใช้เซลล์สดในการดูดซับตะกั่ว พบว่า *C. marchica* สามารถดูดซับตะกั่วได้อย่างมีประสิทธิภาพ และ EDTA สามารถล้างตะกั่วออกจากเซลล์ *C. marchica* ได้อย่างมีประสิทธิภาพหลังการดูดซับ ซึ่งแสดงว่าตะกั่วถูกดูดซับอยู่ที่ผิวเซลล์ของ *C. marchica* ด้วยการจับในตำแหน่งที่เฉพาะเจาะจงบนโพลิแซคคาไรด์ที่หุ้มเซลล์ (CPS) *C. marchica* ไว้ ดังนั้นจึงได้มีการศึกษาถึงรายละเอียดของการผลิต CPS องค์ประกอบน้ำตาลของ CPS และความสามารถของ CPS ในการดูดซับตะกั่ว ผลการศึกษาพบว่าการสร้าง CPS ของ *C. marchica* เพิ่มขึ้นเมื่อระยะเวลาในการเลี้ยงเพิ่มขึ้น โดยมีการสร้าง CPS ได้สูงสุด 26.85 มก./กรัมน้ำหนักแห้ง หลังจากการเลี้ยงได้ 30 วัน โดยใน CPS มีคาร์โบไฮเดรท 37.6% และโปรตีน 34.7% เป็นองค์ประกอบ ชนิดน้ำตาลที่พบใน CPS ของ *C. marchica* ได้แก่ น้ำตาลที่มีสมบัติเป็นกลางคือ ไซโลส อะราบิโนส ไรโบส แรมโนส กาแลคโตส กลูโคส แมนโนส และฟรุคโตส และน้ำตาลที่มีสมบัติเป็นกรดคือ กาแลคทูโรนิค และกลูคูโรนิค โดยน้ำตาลที่มีสมบัติเป็นกรดพบสูงที่สุดหลังจากการเพาะเลี้ยงได้ 30 วัน CPS ที่สกัดได้จากไซยาโนแบคทีเรียชนิดนี้สามารถดูดซับตะกั่วได้ 64.15±5.09 ผลการศึกษานี้สามารถสรุปได้ว่า *C. marchica* มีความสามารถในการดูดซับตะกั่วได้อย่างรวดเร็วและมีประสิทธิภาพสูง และสามารถสร้าง CPS ได้ในปริมาณมาก โดยองค์ประกอบหลักของ CPS เป็นน้ำตาลที่มีสมบัติเป็นกรดสูงถึง 117.73±2.15 ไมโครกรัม/กรัม ดังนั้น *C. marchica* จึงสามารถนำไปใช้เป็นทางเลือกหนึ่งในการกำจัดตะกั่วในระบบบำบัดแบบชีวภาพได้

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The contamination of heavy metal in the environment has greatly increased as a result of major anthropogenic emission and improper waste disposal (Fourest and Roux, 1992). Once the heavy metals are released into the environment, they are difficult to remove by physical or chemical means and most of them are toxic to organisms. The conventional methods to remove these toxic heavy metals are generally expensive and not satisfactorily effective at trace levels of heavy metal contamination (El-Enany and Issa, 2000). During the past decades, a promising new method using microbes as a biosorbent for heavy metal removal has emerged and received considerable attention (Volesky *et al*., 1993; Matheickal and Yu, 1996; Wang *et al*., 1998; Gupta *et al*., 2001).

Cyanobacteria represent one of the potential choices for biological treatment of wastewater because they increase O_2 content of water via photosynthesis and remove heavy metals via adsorption/absorption processes (El-Enany and Issa, 2000). The sorption of heavy metals on cyanobacterial cell is dependent on cell surface characteristics (Goudey, 1987). Crist *et al.* (1994) demonstrated that many functional groups (such as carboxyl, hydroxyl, sulfate and other charged-groups) making up the cyanobacterial cell wall played important roles in metal removal. These functional groups are parts of carbohydrates, proteins and lipids of cyanobacterial mucilage that covers their cell surface. The major part of mucilage is exocellular polysaccharides (EPS) found both in culture medium and on cell surface (capsular polysaccharides or CPS). They act as the binding sites for metals and the capacity of cyanobacteria in removing metal ions depends on their affinity and specificity to metal ions (Xue and Sigg, 1990).

Lead (Pb) is one of the most important metals often found in effluents discharged from industries involved in batteries, painting, publishing and etc. Exposure to Pb can produce anemia, diseases of the liver and kidneys, brain damage and ultimately death (Jain *et al*., 1989). Various techniques have been developed to remove Pb from the environment. These include the applications of chemical and biological materials such as cyanobacteria (Wang *et al*., 1998). Many kinds of cyanobacteria were use to remove heavy metals in Thailand. For example, *Tolypothrix tenuis, Calothrix parietina* show high ability to remove Hg (Inthorn *et al*., 2001), *Scytonema schmidlei, Anabaena cylindrica, A. torulosa* have high Cd (1 mg L^{-1}) removal efficiency at 96-98% (Inthorn *et al*., 2002), *Gloeocapsa sp., Nostoc paludosum, N. piscinale, N. punctiforme, N. commune, Oscillatoria agardhii, Phormidium molle* and *Tolypothrix* could remove 90-96% of Pb (1 mg L^{-1}) (Dungkokkruad, 2002).

Inthorn *et al.* (2002) reported that the cyanobacterium *C. marchica* was able to remove Pb from wastewater. The cells of this cyanobacterium are covered with a mucilage sheath, thus, we speculated that it would have high capacity for removing Pb from solution and serve as one of the potential alternative biomaterials for removal Pb from contaminated water.

To improve our understanding of metal removal mechanisms, knowledge on their cell surface characteristics and interactions with metal ions is crucial. In this study, Pb^{2+} removal by a cyanobacterium *C. marchica* was investigated. The objectives of this study were; (1) to investigate the roles of CPS, the major components of the mucilage of *C. marchica*, in adsorbing Pb^{2+}, (2) to study the relationship between the amount of *C. marchica*'s CPS and its growth, and (3) to characterize the composition of CPS produced by *C. marchica*.

Materials and methods

1. Preparation of *C. marchica* biomass

Calothrix marchica TISTR 8109 was obtained from Microbiological Resources Center (MIRCEN), the Thailand Institute of Scientific and Technological Research (TISTR). Stock culture was grown at pH 7 in Medium-18 (Inthorn *et al*., 2001) under the continuous illumination of 400 μE m^{-2} s^{-1} at 25°C. Medium-18 consisted of (per liter); 1.50 g $NaNO_3$, 380 mg $MgSO_4.7H_2O$, 110 mg $CaCl_2.2H_2O$, 70 mg NaCl, 120 mg K_2HPO_4, 10 mg $Fe_2(SO_4)_3.7H_2O$, 9 mg $NaMoO_4.2H_2O$, 3 mg H_3BO_3, 2 mg $MnSO_4.4H_2O$, 0.3 mg $ZnSO_4.7H_2O$, 0.08 mg $CuSO_4.5H_2O$, 0.04 mg $CoCl_2.6H_2O$. The pH was adjusted to 7 and the medium was autoclaved before use. Upon harvesting, the cyanobacterium was washed three times with Milli-Q water and separated by centrifugation at 1000 μg for 5 minutes at 4°C. These cells were used in the experiments thereafter. All the experiments were conducted in triplicate.

2. Pb^{2+} removal by whole cell and extracted CPS from *C. marchica*

Pb^{2+} removal by whole cell cyanobacterium: To study whether Pb^{2+} removal by *C. marchica* is related to its CPS, cyanobacterial cells at the stationary phase were harvested and Pb^{2+} removal

by the cyanobacterial cells with and without CPS was compared. Cells without CPS were prepared by extracting the CPS from cyanobacterial cells by both cold and hot water (Sangar and Dugan, 1972; Hayashi *et al*., 1995). Cold water extraction was carried out by suspending freeze-dried cyanobacterial cells in a beaker containing water at room temperature for 1 night. Cells were then separated by centrifugation at 1400 μg for 15 min at 4°C. After cold extraction, these cells were used in hot water extraction at 80°C for 1 h. The cyanobacterial cells were separated and freeze-dried. One hundred mg of freeze-dried cyanobacterial cells without CPS were added into 100 ml of 10 mg L^{-1} Pb^{2+} solution at pH 4. As a control, freeze-dried cyanobacterial cells (with intact CPS) were also used to absorb Pb^{2+} in the same manner. The suspension was then shaken at 120 rpm on a shaker at 25°C for 90 min, and then filtered with 0.2 μm sartolon polyamide membrane. The residual Pb^{2+} concentrations were determined by Atomic Absorption Spectrophotometer (GBC Avanta, Australia). The Pb^{2+} removal by cyanobacterial cells was calculated from the difference between the initial Pb^{2+} concentration and the residual Pb^{2+} concentration at the completion of the sorption experiment.

Pb^{2+} removal by extracted CPS: To confirm the roles of *C. marchica*'s CPS in Pb^{2+} removal, 20 mg of extracted CPS were added into 100 ml of 10 mg L^{-1} of Pb^{2+} solution. This was shaken at 120 rpm on a shaker at 25°C for 90 min and filtered with 0.2 μm sartolon polyamide membrane. The residual Pb^{2+} concentrations were determined as described above.

3. Desorption of Pb^{2+} from the loaded cell

Since adsorption of Pb is rapid and reaches equilibrium within a few minutes (Ruangsomboon *et al*., manuscript submitted), it is assumed that Pb adsorption mainly occurs on cyanobacterial cell surfaces. To prove this assumption, desorption of Pb from the loaded biomass was conducted. Cyanobacterial cells of 0.1 g wet weight were first loaded with Pb by adding to 50 mL of 10 mg L^{-1} Pb solution, and shaken at 120 rpm on a shaker at 25°C for two periods of time, 60 min and 24 h. Adsorption at 60 min represents adsorption onto cell surface, while 24 h includes Pb uptake into the cells (Volesky *et al*., 1993).

The cells were then separated by centrifugation at 1400 μg for 15 min. The residual Pb^{2+} concentrations were then measured and compared to the initial Pb^{2+} concentration. The loss of Pb^{2+} was assumed to be as Pb^{2+} on the cyanobacterial cells. To check if the retention of Pb on the cyanobacterial cell walls was held by chemical adsorption process or simply by physical trapping, the Pb loaded biomass was desorbed by desorbing agent, with Milli-Q water as a control. Loaded biomass was washed once with 50 mL of deionized water and added to 10 mL of desorbing solution (10 mM EDTA) in 250 mL Erlenmeyer flasks. The flasks were incubated at 25°C while shaking at 120 rpm for 15 min. Cells were separated, and the same procedures for desorption were repeated twice. The desorbed Pb was then estimated.

4. EPS and CPS production at different growth stages

C. marchica cells were harvested and the mucilage or polysaccharides secreted from cyanobacterial cells and covered or attached on cyanobacterium cell surface (CPS) were determined at various growth phases as follows: lag phase (6^{th} day), early log phase (8^{th} day), mid log phase (18^{th} day), stationary phase (24^{th} day) and death phase (30^{th} day). The growth phase was determined based on chlorophyll-a content and dry weight. Polysaccharides secreted from cyanobacterial cells and dissolved in media (EPS) were determined at each growth phase. After harvest, cyanobacterial cells at each growth phase were freeze-dried and kept in a desiccator until use in CPS extraction.

5. Extraction and determination of *C.marchica*'s polysaccharides

Capsular polysaccharides (CPS) were extracted by both cold and hot water (Sangar and Dugan, 1972; Hayashi *et al*., 1995). Cold water extraction was carried out by suspending 500 g of freeze-dried cyanobacterial cells in 5-L beaker

containing 2-L water at room temperature for 1 night. Cells were then separated by centrifugation at 1400 µg for 15 min at 4°C. After cold extraction, these were used in hot water extraction at 80°C for 1 h. The CPS in the supernatant of both cold and hot water extraction was subsequently precipitated by hexadecyl trimethylammonium bromide at 4°C for 24 h, and centrifuged at 4,000 rpm at 4°C for 15 min. The CPS was collected and supernatant was decanted (Domenico *et al.*,1989).

The obtained pellets of CPS were dehydrated by a stepwise washing (in each step the volume of solution used was 100 mL); 1) in saturated sodium acetate in 95% ethanol, 2) in 95% ethanol, 3) in absolute ethanol, and 4) in diethyl ether. In each step, the CPS was immersed in washing solution and stirred by magnetic stirrer bar for 15 min. It was then separated by centrifugation at 1400 µg for 15 min. In the last step, CPS was separated from diethyl ether by centrifugation, and dried in a hood until the residual diethyl ether was completely evaporated (Hayashi *et al*., 1995). The dried CPS was kept in desiccator for use in the subsequent experiments. Exopolysaccharides (EPS) dissolved in the culture medium were determined according to phenol sulfuric acid method (Bois *et al*., 1956).

6. Composition of *C. marchica*'s mucilage and CPS

Protein and Carbohydrate Composition of *C. marchica*'s mucilage: The mucilage extracted from the death phase of *C. marchica*, was used. Ten milligrams of mucilage were dissolved in 10 mL of Milli-Q water. Carbohydrate fraction of mucilage was determined by the phenol sulfuric acid method (Bois *et al*., 1956; Nayak and Pattabiraman, 1981) and the protein fraction was determined by the Lowry method (Wilkins *et al*., 1996). Glucose and bovine serum albumin were used as carbohydrate and protein standard, respectively.

Sugar composition of *C. marchica*'s CPS: Ten mg of whole CPS were firstly hydrolyzed with 10 mL of 2 N H_2SO_4 for 8 h at 100°C, and then neutralized by barium carbonate to pH 5.5. The barium carbonate was subsequently removed by filtering with 1.2 µm membrane paper filter, and the supernatant was evaporated. The residue was dissolved in 1,000 µl of Milli-Q water and filtered with 0.2 µm PTFE membrane. Twenty-five µl of this solution were injected into an ion chromatograph (Dionex 4000I, USA) with pulsed amperometric detector equipped with an Au electrode and a Dionex CarboPac PA1 columns (2x250 mm) (Colombini *et al*., 2002). Linear calibration curves of sugar standards were obtained from the concentration ranges of 10-150 mg L^{-1}. All sugar standards are of analytical grade (Fluka, Italy). Since peaks of arabinose/ribose, and galactose/glucose were partially overlapped, they were reported as the single peak component.

Results and discussion

1. Pb^{2+} removal by whole cell and the extracted CPS

Pb^{2+} removal by whole cell of C. marchica: Pb^{2+} removal as a function of time by *C. marchica* is shown in Figure 1., which reveals that it effectively removed Pb^{2+} within a short time after exposure to Pb^{2+} solution. The Pb^{2+} adsorbed to cells of *C. marchica* reached 42.2% within 10 min. After that Pb^{2+} uptake continued slowly until reaching its equilibrium point at around 60 min. After 120 min of exposure, the residual Pb^{2+} in the solution with and without *C. marchica* cells was 392.28 (39.3% of initially added) and 998.10 (100% of initially added) µg L^{-1}, respectively. This indicates that *C.marchica* cells, which are covered with gelatinous sheath have high capability to adsorb Pb^{2+}. Our previous study showed that Pb^{2+} adsorption characteristics by this cycanobacterium followed the monolayer model of Langmuir with the maximum Pb^{2+} uptake (qmax) of 74.04 mg g^{-1} dry weight (Raungsomboon *et al*., revision). This qmax was much lower than the cyanobacterium *Gloeocapsa gelatinosa*, that had a qmax of 232.56 mg Pb^{2+} g^{-1} dry wt. (Raungsomboon *et al.,* revision). However, it was much higher than those of other types of cyanobacteria reported earlier (e.g. 13.6 mg g^{-1} for Pb^{2+} removal by Phormidium, Wang *et*

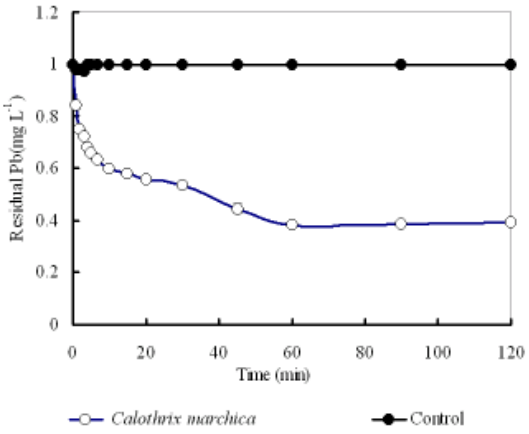

Figure 1. Time-course of Pb^{2+} removal by cells of the cyanobacterium *C. marchica* (initial Pb^{2+} concentration of 1 mg L^{-1} at pH 4 and temperature of 25°C).

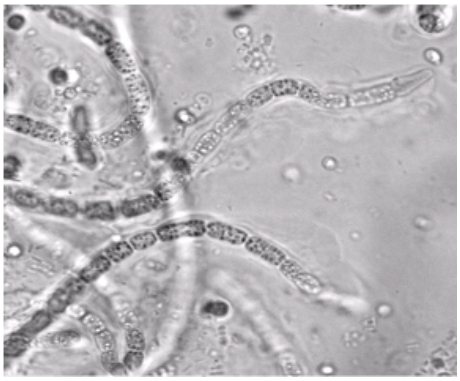

Figure 2. Cell and colony characteristics of *C. marchica* used in the present study (15-day cultured, 100X)

al., 1998). This suggests that *C. marchica* has the capacity to be used as an effective absorbent for metal ion removal.

The filaments of *C. marchica* are covered with thin colorless mucilage sheaths (Figure 2). The filaments usually attach to each other, and form a large colony whose size increases when culture time is prolonged. This makes it very interesting for the real application in wastewater treatment pond because it is relatively easy to remove *C. marchica* cells from the treatment system as the colony is large. To elucidate the mechanisms of Pb^{2+} removal in this cyanobacterium, the roles of *C. marchica*'s mucilage sheath (CPS) in Pb^{2+} removal were examined. Pb^{2+} removal by cells with and without CPS was compared. Results showed that the cyanobacterial cells with intact CPS adsorbed 3.42±0.07 mg Pb g^{-1} dry weight or 36.3% of initial amount of Pb^{2+} added in the solution. However, this Pb^{2+} adsorption capacity was not significantly different from cell without CPS, which adsorbed 3.17±0.12 mg Pb g^{-1} dry weight or 33.6% of the initial amount of Pb^{2+} added (data not shown).

It seems that after extraction of its CPS, *C. marchica* could still remove Pb^{2+} effectively. Normally, filaments of *C. marchica* form sheath-like layers and this characteristic may affect its

metal removal ability. The cells or colony inside the sheath may not be exposed to Pb^{2+}. However, the high temperature during CPS extraction (80°C) could break down colony of *C. marchica*, thus the surface area, and also the Pb^{2+} binding surface area was increased. Furthermore, during the CPS extraction, high temperature would kill the cells, the consequence is destruction of the cell membranes, thereby exposing intracellular components as well as surface binding sites to Pb^{2+} (Al-Ashes and Duvnjak, 1995).

Pb^{2+} removal by extracted CPS: Additionally, a separate experiment was carried out such that the extracted CPS was directly added to Pb^{2+} solution and left for 90 min, after which the Pb^{2+} removal ability of CPS was determined. It was found that 64.15±5.09 mg Pb was absorbed per gram of this extracted CPS. Therefore, it was confirmed that CPS of *C. marchica* alone could remove Pb^{2+} from the solution without the function (metabolism) of cyanobacterial cells.

2. Desorption of Pb^{2+} from the loaded cells

Since adsorption of Pb^{2+} by *C. marchica* is rapid and reach equilibrium within a few minutes, it is likely that Pb^{2+} adsorption mainly occurs on cyanobacterial cell surface. To confirm this, ethylenediamine tetraacetic acid (EDTA) was used to desorb Pb^{2+} from the loaded cells. EDTA was used successfully in many studies as metal ion desorber (Xie *et al*., 1996; Zhou *et al*., 1998; Ahuja *et al*., 1999) and thus it was expected that EDTA would desorb Pb^{2+} in our case as well.

The loaded Pb on the biomass was expressed as absolute mass of Pb on the total cell mass. The results show that the Pb loaded on *C. marchica* cells after exposure to Pb solution for 24 h were significantly higher than at 60 min (Table 1). After *C. marchica* cells was exposed to Pb^{2+} solution, Pb^{2+} was desorbed by EDTA. Table 1 compares the amounts of Pb^{2+} desorped by using Milli-Q water (control) and EDTA (desorbing agent). Milli-Q water could desorb a lower amount (less than 10.4%) of Pb than could EDTA, which was able to desorb approximately 30-40% of the initially added Pb^{2+} from cyanobacterial cells. These results indicate that Pb^{2+} formed a complex, presumably with the functional groups on cyanobacterial cells and thus could not be washed out by water as suggested in other studies (Ahuja *et al*., 1999, Schiewer and Volesky, 2000).

The results in Table 1 also show that EDTA could not desorb all of the Pb^{2+} from the loaded cells during the time course of the experiment. The amounts that could not be desorbed were probably transported into the cells, strongly bound to cell surface, or desorbed by the binding sites located in the inner part of the sheath. Thus, the time duration applied for desorption was probably not sufficiently long to desorb all of these Pb^{2+} from different parts. This assumption is supported by the results in Table 1 that in the 3rd desorption the amount of Pb^{2+} desorbed was still high when compared to the 1st and 2nd desorptions. It is likely that extending the desorption time would result in more Pb^{2+} desorbed from the loaded cells. In

Table 1. Desorption of Pb from the loaded *C. marchica* biomass by EDTA (mean ± S.E. of three replicate).

Exposure time of loaded biomass	Pb on loaded biomass (μg)	Desorbing agent	Pb desorbed (μg)			Total Pb desorbed (μg)
			1st desorption	2nd desorption	3rd desorption	
60 min	187±1.4 [a]	EDTA	40.6±0.6	20.0±0.2	13.9±0.4	74.5±0.5 [a]
	189±1.0 [a]	Milli-Q	4.8±0.0	3.1±0.1	2.5±0.1	10.4±0.0 [b]
24 hr	255±12.0 [b]	EDTA	32.7±1.3	25.7±2.0	19.3±1.1	77.6±4.4 [a]
	226±1.3 [b]	Milli-Q	6.7±0.1	9.4±0.0	7.5±0.0	23.5±0 [c]

The same superscript letter in each column denotes no significant difference with 95 % confidence limit (p?0.05).

addition, it is also possible that EDTA was not a suitable desorbing agent for Pb^{2+} in *C. marchica*. Such possibility was reported by Ahuja *et al.* (1999), who found that Na_2CO_3 and H_2SO_4 were more effective in desorbing Co from the cyanobacterium *Oscillatoria anguistissima* than EDTA.

3. EPS and CPS production at different growth stages

The production of EPS and CPS during *C. marchica* growth is shown in Table 2. Production of EPS in culture media and EPS covered on cell (CPS) by *C. marchica* increased when growth proceeded, from about 14 mg g^{-1} biomass at the beginning to more than 25 mg g^{-1} biomass after 30 days of cultivation. The amounts of EPS and CPS produced at the lag phase (6 days) and early log phase (8 days) was not significantly different ($p \leq 0.05$). However, after early log phase EPS and CPS production rapidly increased. Among the various growth phases, the yield of extractable CPS at death phase (30 days) was highest (26.85 mg g^{-1} biomass, Table 2). Generally, synthesis of polysaccharides occurs inside the cells, and subsequently the completed polysaccharide chains are transported through the cytoplasmic membrane to the cell surface. Such polysaccharides are known as exocellular polysaccharides (EPS) (Standford, 1979). Thus, the longer cultured time usually results in the higher exocellular polysaccharides production, which supported the result of this study.

The dependence of CPS production on growth stage was also reported in red algae *Porphyridium* sp. (Arad *et al.*, 1988). In another strain of cyanobacteria, *Gloeothece magna*, Mohamed (2001) reported that the CPS production of 15.5 mg g^{-1} biomass was also found in the dead phase. EPS could play the role in metal adsorption in the similar manner as CPS, especially for living system. This is due to during cyanobacterial growth, EPS was released to the environment and thus could adsorb to metal in solution. The consequence may be the aggregation or precipitation of metal. In this study *C. marchica* released the highest amount of EPS at the death phase.

From reviewing the literature, *C. marchica* has higher EPS than other cyanobacteria (Table 3), except *Anabaena* strain WSAF sp., *Chlorogloeopsis* sp. strain 6912 and *Phormidium*. Thus it seems that *C. marchica* may have higher metal adsorption capacity than other cyanobacteria which contain the lower amount of EPS. However, metal removal ability also depends on the amount of acidic sugar in the EPS. In the present study, the quantity of EPS in culture medium was not sufficient for further analysis of sugar composition or Pb^{2+} removal ability. We, therefore, focused only on the roles of CPS.

4. Compositions of *C. marchica*'s mucilage and CPS

Protein and carbohydrate composition of

Table 2. The amount of *C. marchica*'s CPS, EPS and biomass cultured under control conditions.

Growth condition	CPS (mg g^{-1} dry biomass)	EPS (mg L^{-1} culture medium)	Total dry biomass (g L^{-1})
Lag phase (6 Days)	14.24	6.3±0.3 [a]	0.1±0.0 [a]
Early log phase (8 Days)	14.55	7.8±0.5 [a]	0.1±0.0 [a]
Mid log phase (18 Days)	24.07	18.2±0.2 [b]	0.3±0.0 [b]
Stationary phase (24 Days)	26.03	22.9±0.6 [c]	0.4±0.0 [c]
Death phase (30 Days)	26.85	26.3±0.4 [d]	0.4±0.0 [c]

There was only one value for CPS under each condition because all of the biomass was combined to give the sufficient amount for analysis (except for dry biomass determination that the values are average ± SE of three replicate). The same superscript letter in each column denotes no significant difference with 95% confidence limit ($p<0.05$).

Table 3. Comparison of dissolved exopolysaccharide (EPS) produced by different strains of cyanobacteria.

Cyanobacteria	EPS (μg mL^{-1})	Reference
Spirulina	9	(Nicolaus *et al*., 1999)
Oscillatoria	1.5	(Nicolaus *et al*., 1999)
Phormidium	29	(Nicolaus *et al*., 1999)
Anabaena torulosa	3.7	(Nicolaus *et al*., 1999)
Anabaena sphaerica	1	(Nicolaus *et al*., 1999)
Anabaena strain WSAF sp.	55.2	(Nicolaus *et al*., 1999)
Anabaena variabilis	3.5	(Nicolaus *et al*., 1999)
Nostoc linckia	0.015	(Nicolaus *et al*., 1999)
Scytonema hofmanni	4	(Nicolaus *et al*., 1999)
Tolypothrix tenuis	7.4	(Nicolaus *et al*., 1999)
Fischerella muscicola	*1.5*	*(Nicolaus et al., 1999)*
Chlorogloeopsis sp. strain 6912	30	(Nicolaus *et al*., 1999)
Gloeocapsa gelatinosa	33.01	(Raungsomboon *et al*., revision)
Calothrix marchica	26.01	This study

C. marchica's mucilage: The metal removal ability of cyanobacteria depends on both the amount of mucilage and the mucilage compositions, both of which should be considered when biomaterials are selected for use as biosorbent for heavy metals. The bacterial mucilage is composed of two major components: polysaccharides (CPS) and proteins. The relative amounts of these two components vary to a large degree on the type of organisms (Decho and Herndl, 1995).

The carbohydrate fraction of *C. marchica*'s mucilage (death phase) was slightly higher than the protein fraction. The mucilage contained 375.9±8.36 mg g^{-1} (37.6 % by dry wt.) and 346.7 ±11.04 mg g^{-1} (34.7% by wt.) of carbohydrates and proteins, respectively. The ratio of carbohydrate to protein in CPS of *C. marchica* was similar to that of Nostoc, which was 16.2% and 14.2%, respectively (Hu *et al*., 2003). However, in some algae protein constituted only a small fraction of the mucilage. For example, the carbohydrate and protein fractions were respectively 55-83% and 5.2-10.5% for *Botryococcus braunii*'s (Allard and Casadevall, 1990), and 68% and 3% for *Chlamydomonas angustae*'s (Allard and Tazi, 1993).

Sugar composition of C. marchica's CPS: CPS of cyanobacterial mucilage often shows diverse physical and chemical properties, depending on its specific functional groups. These include high adsorptive capability, aggregation and dissolution. In this study, heavy metal removal potential of CPS was inferred from its neutral sugar and acidic sugar monosaccharide contents. Acidic sugars contain carboxylic group(-COOH) that can be protonated when the pH of solution reaches its pKa value (2.9-3.4). The consequence is the deprotonated COO- that prompts the binding to cations. Such acidic sugars are the important components of CPS that affect cyanobacterial metal removal, and thus, indicate the potential of CPS to bind with heavy metals including Pb^{2+}.

The sugar composition may slightly vary both qualitatively and quantitatively, especially with the age of the culture. The sugar composition of *C. marchica*'s CPS is shown in Table 4. The CPS is composed of both neutral and acidic sugars. The neutral sugars are rhamnose, arabinose, ribose, galactose, glucose, mannose, fructose and xylose. The acidic sugars were galacturonic and glucuronic acids. *C. marchica*'s CPS contained different sugars at different growth phase. Acidic sugars are absent in the early log phase. The amount of all sugar varied randomly in each growth phase. The

Table 4. Sugar composition (mg g^{-1} CPS) of *C. marchica*'s CPS. (n=3, in all cases S.E. is lower than 0.001).

Growth condition	Sugar								Total sugar
	Rhamnose	Arabinose+ Ribose	Galactose+ Glucose	Mannose	Fructose	Xylose	Galacturonic	Glucuronic	
lag phase	2.66 [a]	1.53 [a]	1.78 [a]	1.27 [a]	1.74 [a]	3.19 [a]	0.79 [a]	1.34 [a]	14.30 [a]
early log phase	1.75 [b]	1.44 [b]	1.27 [b]	0.06 [b]	2.43 [b]	1.75 [b]	0.00 [b]	0.00 [b]	8.70 [b]
mid log phase	1.75 [b]	1.46 [b]	0.94 [c]	0.14 [c]	1.19 [c]	0.42 [c]	1.49 [c]	1.64 [c]	9.03 [c]
stationary phase	2.39 [c]	1.98 [c]	1.10 [d]	0.38 [d]	1.79 [a]	1.22 [d]	1.69 [d]	1.89 [d]	12.44 [d]
death phase	1.09 [d]	1.66 [d]	1.01 [d]	0.02 [e]	1.90 [d]	1.35 [e]	1.60 [e]	2.78 [e]	11.42 [e]

The same superscript letter in each column denotes no significant difference with 95% confidence limit (p?0.05).

Table 5 Total acidic sugar (μg g^{-1} dry biomass) produced by *C. marchica* at various growth phases (n=3, mean ± SE.).

Growth condition	Acidic sugar content in mg g^{-1} CPS	Acidic sugar content in μg g^{-1} dry biomass
Lag phase	2.13±0.03 [a]	30.37±0.46 [a]
Early log phase	0.00±0.00 [b]	0.00±0.00 [b]
Mid log phase	3.13±0.04 [c]	75.33±0.86 [c]
Stationary phase	3.57±0.08 [d]	93.04±1.13 [d]
Death phase	4.38±0.05 [e]	117.73±2.15 [e]

The same superscript letter in each column denotes no significant difference with 95% confidence limit (p±0.05).

amount of total monosaccharides significantly depended on growth phase ($p<0.05$, Table 4). Before reaching the death phase the dominant monosaccharide was neutral sugar and at death phase this was the acidic sugars. The highest amount of individual monosaccharide found in lag phase, early log phase, mid log phase, stationary phase, and death phase was xylose, fructose, rhamnose, rhamnose, and glucuronic acid, respectively. The highest total sugar content of CPS was found in lag phase as 14.30 mg g^{-1} CPS, whereas the highest acidic sugar was found in death phase (Table 5). For other cyanobacteria it was reported that the neutral sugar occurred widely and glucose was very often the most important monosaccharide found. The acidic sugar was absent or found in the lesser amount (Nicolaus *et al*., 1999).

Among various growth stages and on the dry weight basis of CPS, the highest acidic sugar production (4.38±0.05 mg g^{-1} CPS or 38.3 % of total sugar) (Table 5) was found in the death phase. In contrast, no acidic sugar was found in early log phase. In this study we mainly focused on the total amount of acidic sugars (galacturonic and glucuronic acids), because they played an important role in metal binding. Thus, for selecting the optimal culture condition based on the criterion that *C. marchica* cells produced the highest amount of acidic sugars per unit of biomass, the amounts of acidic sugar in CPS were converted to dry weight basis of cyanobacterial biomass. The highest acidic sugar per dry biomass was 117.73±2.15 μg g^{-1}, which was found in death phase. Therefore, harvesting this cycanobacterium at death phase would be the reasonable strategy for obtaining the biomass with high acidic sugar content and Pb^{2+} removal capability.

5. The possible role of cyanobacterial mucilage on Pb^{2+} removal

Since algae have high potential to adsorb heavy metals, many researchers have tried to explain metal adsorption mechanisms by algae. For example, Eccles (1999) reported that the functional groups responsible for metal biosorption by biological organisms included carboxyl group from uronic acids, phosphate from polysaccharides, amino from cystidine and, hydroxyl from tyrosine-phenolic. Xia and Rayson (2002) reported that carboxylates were the dominant chemical moieties involved in the binding of heavy metal in algae.

The composition of CPS can affect its ability to remove heavy metals (Decho and Herndl, 1995). In this study, the two functional groups that possibly exist in the mucilage sheath of *C.marchica* were carboxylic (acidic sugar of carbohydrate) and amine groups (protein fraction). However, only carboxylic group (pKa 2.9-3.4) (Reddad *et al*., 2002) would play the role in Pb^{2+} adsorption in this study because the solution pH used in this study was set at pH 4. Amine (pKa 8) is not dissociated under pH 4. Generally, pH of the solution and the pKa of the ionizable groups determine the charge of CPS. When the pH of solution is higher than this pKa value, the carboxyl groups on the cyanobacterial cells protonate and are present in the negatively-charged form (COO-). In this form they could bind to the positive charged Pb^{2+} ions. Thus, when cyanobacterial cells are added to Pb^{2+} solution with pH above the pKa value of carboxyl group, these cyanobacterial cells could act as adsorbing media for Pb^{2+}. Since acidic sugars make up the components of *C. marchica*'s CPS (Table 4 and 5) and our working pH was 4, we therefore assume that the main mechanism for Pb^{2+} ions removal from the solution is through binding with the COO- or other similar functional groups of these acidic sugars. The significantly higher amounts of total acidic sugar content at stationary phase than at lag phase and mid log phase may be one of the reasons to explain that Pb^{2+} adsorption by *C. marchica* at stationary phase (18.28±0.99 mg Pb g^{-1} dry wt.) was higher than at lag phase (2.53±0.03 mg Pb g^{-1} dry wt.) and mid log phase (11.64±0.95 mg Pb g^{-1} dry wt., Table 6).

Conclusions

Various evidence from this study showed that *C. marchica* was able to effectively remove Pb^{2+} from the solution. The adsorbed Pb^{2+} onto *C. marchica*'s mucilage sheath could be removed by EDTA but not by water, which indicates that functional groups on *C. marchica* cell wall adsorb Pb^{2+} through cation exchange reaction. The ability to remove Pb^{2+} was related to the presence of mucilage sheath (capsular polysaccharides) covering on its cell surface. Analyzing this mucilage sheath revealed that it is made up of both carbohydrates and proteins. The carbohydrate fraction was composed of neutral and acidic sugars, which neutral sugars were the major composition. *C. marchica* provided the highest acidic sugar per

Table 6. The amount of Pb^{2+} adsorbed (mg Pb g^{-1} dry wt.) by *C. marchica* at different growth stages.

Growth phase	Pb^{2+} adsorbed (mg Pb g^{-1} dry wt.)	Total acidic sugar (µg g^{-1} dry biomass)
Lag phase	2.53±0.03 [a]	30.37±0.46 [a]
Mid log phase	11.64±0.95 [b]	75.33±0.86 [b]
Stationary phase	18.28±0.99 [c]	93.04±1.13 [c]

(initial Pb^{2+} = 2 mg L^{-1}, pH 4, temperature = 25°C)
The amount of acidic sugar (n = 3, mean ± SE.) was also given. The same superscript letter in each column denotes no significant difference with 95% confidence limit ($p \leq 0.05$).

dry biomass at death phase. The results suggest that the main mechanism of Pb^{2+} removal under working pH 4 was through binding with certain functional groups of *C. marchica*'s CPS (mainly COO- of acidic sugars). The ability to produce high amount of CPS containing acidic sugars indicates that *C. marchica* has high potential to be developed as bioadsorbent for Pb^{2+} removal in contaminated waters.

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