Cellulose-metallothionein biosorbent for removal of Pb(II) and Zn(II) from polluted water

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1. Introduction

Heavy metals have become a predominant contaminant of water because of constant growth in industrialization and urbanization (Chen et al., 2018). Once toxic heavy metals, such as lead (Pb) and zinc (Zn), enter the human body, they accumulate and cause various health problems. Lead has no known beneficial effects on human health, whereas Zn is essential (Zoroddu et al., 2019). However, Zn is toxic at high concentrations (Baran et al., 2018). Lead causes neurodevelopment disorders and Zn imparts an undesirable astringent taste to water, and both should be removed from drinking water (World Health Organization, 2017).

To date, toxic trace elements have been removed by conventional water treatment methods which include coagulation, flocculation, clarification, and filtration, and followed by disinfection (Gitis and Hankins, 2018; Singh et al., 2018). However, many of these techniques are suboptimal. Biosorption has emerged as an alternative method that is efficient, simple, and specific (Singh et al., 2018). The biosorption process involves interaction of a solid phase (biosorbent) with a liquid phase (water) containing the dissolved species to be adsorbed (metal ions). Biosorbents effectively remove heavy metals from water (Fakhre and Ibrahim, 2018). Cellulose is attractive as a biosorbent because it is stable, inert, and the most abundant polymer on Earth (Abouzeid et al., 2019). However, natural cellulose has not been used as a biosorbent because of its low metal-ion adsorption capacity (Hokkanen et al., 2016). To address this challenge, we have developed a functional cellulose modified with metallothionein (MT), which adsorbs metal ions, by using a carbohydrate-binding module (CBM) as a binder.

In this research, we constructed a biosorbent by fusion protein of MT from *Synechococcus elongatus* and CBM from *Clostridium thermocellum* (Figure 1). MTs are a group of well-conserved proteins that act as antioxidants. They are found in all living organisms and contain a sulfhydryl group that bind to heavy metals (Chaudhary et al., 2018; Mekawy et al., 2018). However, MT would not be easy to recover after dispersion. To address this, we investigated modifying MT by fusing it with CBM. Because CBM binds to cellulose by hydrophobic interaction (Chang et al., 2018), the cellulose-MT-CBM biosorbent would be stable and easily recycled (Yunus and Tsai, 2015). Although peptide-based biosorbents have been previously suggested for water treatment (Xu et al., 2002), our biosorbent is unique in that it can be prepared in a single-step without protein purification by using cellulose-binding ability of CBM. The MT can adsorb various toxic trace elements that may be present in polluted water. We have investigated the removal of toxic metal ions, which are contained in real mine wastewater, by using a novel biosorbent.

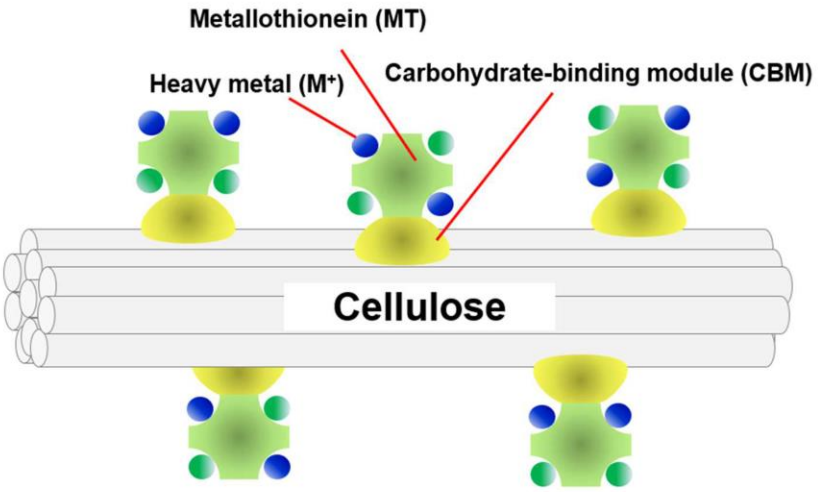


Figure 1. Illustration of cellulose-MT-CBM.

2. Materials and methods

2.1. Plasmid construction and protein expression

The gene for MT from *S. elongatus* PCC7942 (Shi et al., 1992) was synthesized by Eurofins Genomics (Tokyo, Japan). Genomic DNA from *C. thermocellum* NBRC 103400 was obtained from NBRC (Kisarazu, Japan). The polymerase chain reaction (PCR) was performed using PrimeSTAR® HS DNA polymerase (Takara Bio Inc., Otsu, Japan) with the oligonucleotide primers (Eurofins Genomics) listed in Table S1. The gene for MT was amplified from the vector containing the synthesized MT gene using primers MT-F and MT-R. The gene for CBM 3 (Hong et al., 2007) was cloned from genomic DNA of *C. thermocellum* using the primers CBM-F and CBM-R. The genes of MT and CBM were fused by overlap PCR using the primers OL-F, OL-R, IF-F, and IF-R. The amplified gene was inserted into pET-15b vector (Merck Biosciences GmbH, Schwalbach Ts., Germany), which was cut by *Nco* I and *Xho* I, using an In-Fusion HD Cloning Kit to obtain the pET-MT-CBM vector (Figure S1). The nucleotide sequence of the MT-CBM fusion gene (648 bp) was verified by DNA sequencing (Eurofins Genomics).

To express the target protein, *Escherichia coli* BL21(DE3) strain (Nippon Gene Co. Ltd, Toyama, Japan) was transformed with the pET-MT-CBM vector. The transformant was grown at 37°C in Luria-Bertani (LB) medium containing 100 μg/mL ampicillin. When the optical density of the culture medium measured at a wavelength of 600 nm (OD_{600}) reached 0.45, the expression of the protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration of 1 mM) to the medium and culturing at 15°C for 24 h. The bacterial cells expressing the proteins were harvested by centrifugation at 8,000 ×*g* for 10 min and lysed by ultrasonication at 30 kHz for 5 min (Vibra-Cell™ VCX 130, Sonics & Materials, Inc., Newtown, USA). After centrifugation of the cell lysate at 8,000 ×*g* for 10 min, the supernatant was recovered and used directly in biosorption studies.

2.2. Preparation of the cellulose-MT-CBM biosorbent

Biosorption of the protein onto cellulose is illustrated in Figure 2. To immobilize the MT-CBM fusion protein on cellulose and obtain the cellulose-MT-CBM biosorbent, Whatman filter paper No. 1 was cut into rectangular pieces (1 g) and then suspended in 5 mL of the crude protein mixture (protein concentration of 250 µg/mL) obtained in Section 2.1 for 2 h at 25°C. The filter paper and cellulose-MT-CBM biosorbent were analyzed by using attenuated total reflectance Fourier transform infrared (ATR-FTIR) (JASCO 360 FTIR Spectrometer, JASCO, Japan). The point of zero charge (pzc) for the cellulose-MT-CBM was determined by the pH drift method (Bakatula et al., 2018).

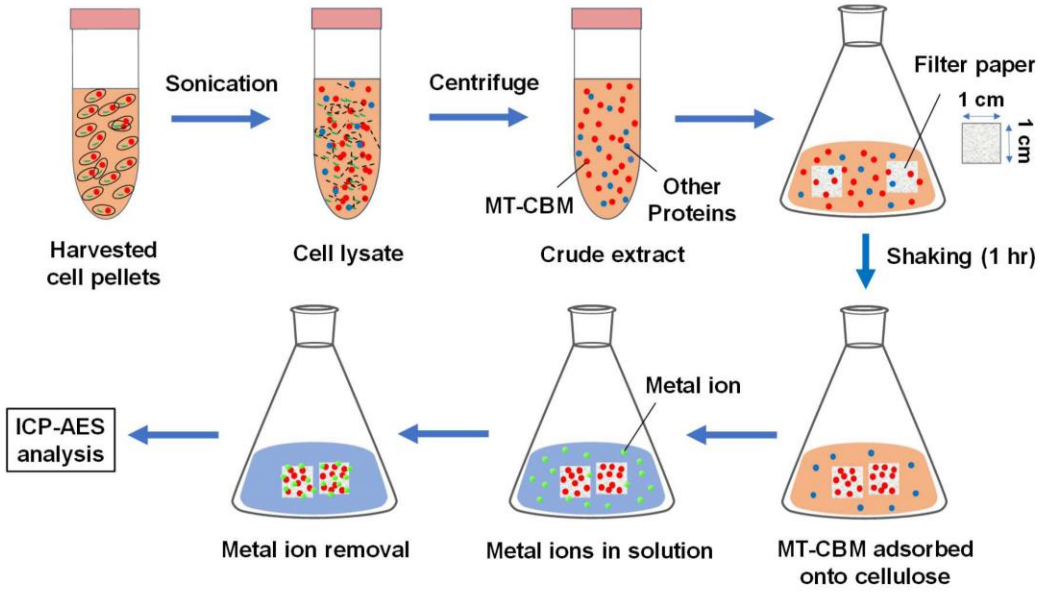


Figure 2. The process of immobilization of the MT-CBM protein on cellulose.

2.3. Metal ion adsorption experiments

A metal ion stock solution was prepared using $PbCl_2$ and $ZnCl_2$ (Wako Pure Chemical Industries Ltd, Tokyo, Japan), which were dissolved in distilled water. Solutions with different final concentrations of the metal ions were obtained by dilution of the stock solution. Metal adsorption was investigated in batch experiments. The effect of pH (2.0–9.0) on the removal of Pb(II) and Zn(II) was investigated by mixing 1 g of the prepared biosorbent with 100 mL of a 20 mg/L metal solution in a 250 mL Erlenmeyer flask. Then, the flasks were placed on a shaker at 100 rpm for 1 h. The adsorbent was recovered by centrifugation at 8,000 $\times g$ for 10 min and the supernatant was filtered through a 0.45-µm filter. The metal ion concentration in the supernatant was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (ICPE-9820, Shimadzu Corporation, Kyoto, Japan). The effect of the contact time (0–60 min) was studied by varying this while keeping all other parameters fixed.

Biosorption was analyzed using the Langmuir isotherm model:

$$Q_{eq} = \frac{Q_{max} \cdot K \cdot C_{eq}}{1 + K \cdot C_{eq}}, \qquad (1)$$

where Q_{eq} is the quantity adsorbed (mg/g), C_{eq} is the equilibrium concentration of the adsorbate (mg/L), Q_{max} is the Langmuir constant (mg/g), and K is the equilibrium constant.

2.4. Semi-continuous adsorption system and recyclability of the cellulose-MT-CBM biosorbent

The reusability of the adsorbent in a semi-continuous system was tested seven times with the experimental setup illustrated in Figure 3. To determine the recoverability and reusability

of the biosorbent, a column (syringe of mean diameter, D_{50} = 2.5 cm and height, h = 7 cm) was prepared by placing 2 g of cellulose in a 30-mL syringe. Then, 10 mL of crude extract containing overexpressed MT-CBM protein (total protein concentration: 250 µg/mL) was added to be adsorbed onto the cellulose, and the column was kept at 25°C in an incubator for 1 h. After washing three times with deionized water, a Pb(II) or Zn(II) aqueous solution was allowed to percolate through the column. The filtrate was collected for metal analysis. A valve was used to regulate the flow rate of the filtrate from the column. To desorb the adsorbed metal ions, 10 mL of 20 mM EDTA buffer (pH 8) was added to the column. To regenerate the column, 10 mL of fresh MT-CBM protein was added. A column containing only cellulose was used for control experiments. Treatment of actual mine wastewater collected from Chingola, Copperbelt, Zambia was investigated using the column.

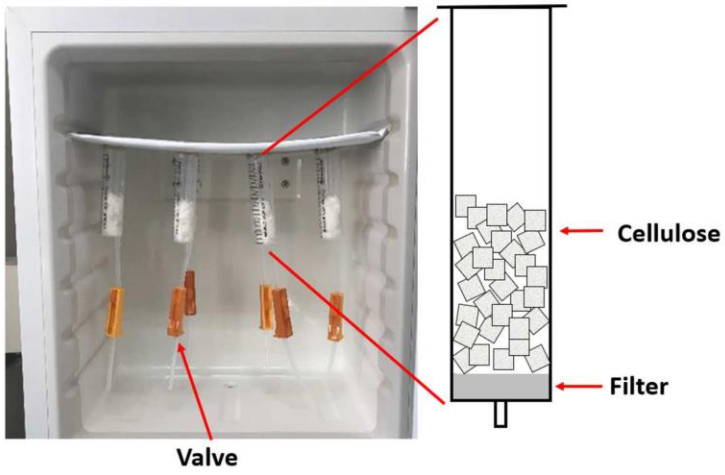


Figure 3. Setup for the cellulose-MT-CBM biosorbent regeneration experiments.

2.5. X-ray photoelectron spectroscopy analysis

X-ray photoelectron spectroscopy (XPS) measurements were performed by using a JPS-9200 spectrometer (JEOL Ltd, Tokyo, Japan) to explore the electronic states of Pb and Zn on

the biosorbent using monochromatic Mg Kα radiation. To compensate for surface charge effects, the binding energies were calibrated using C 1s at 284.80 eV.

3. Results and Discussion

3.1. Verification of plasmid construction, protein expression, and protein binding ability to cellulose

Figure 4 shows the results of SDS-PAGE analysis for the protein expressed in *E. coli* BL21(DE3) and biosorption of the protein to cellulose. Figure 4(a) shows the supernatant of the cell lysate of *E. coli* expressing MT-CBM. We found that the MT-CBM was successfully expressed in a soluble form with a molecular weight of 23.1 kDa. To verify the binding ability of the MT-CBM on cellulose, filter papers were added to the cell lysate. After 1 h, the band for MT-CBM disappeared, indicating that the MT-CBM in the cell lysate was adsorbed on cellulose (Figure 4(b)). This result showed that MT-CBM could be purified using cellulose in a single step. This result is consistent with previous studies where CBM was observed to bind to cellulosic material (Hong et al., 2007). We then used the cellulose-based biosorbent (cellulose-MT-CBM) for further studies. The FTIR spectra are obtained for pure cellulose and cellulose-MT-CBM (Figure S2). The results showed that the pure cellulose had no distinguishable functional groups compared with the cellulose-MT-CBM, where broad and strong bands at 3251 cm^{-1} (amine (-NH)), 1625 cm^{-1} (amide group (C=O), and 1302 cm^{-1} (C-O stretching) confirmed the presence of bound protein on the cellulose.

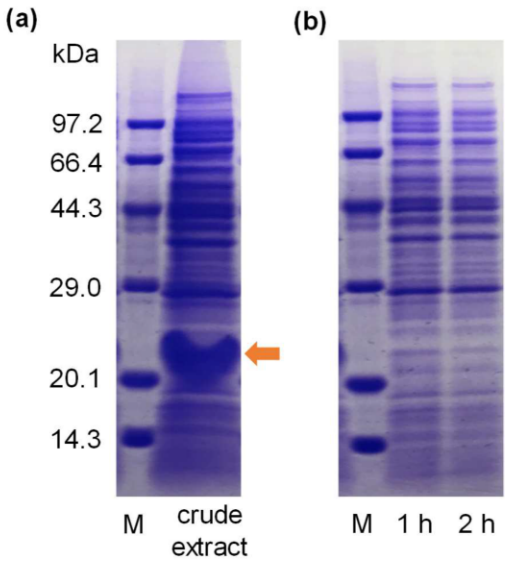


Figure 4. SDS-PAGE of (a) crude extract after expression of MT-CBM and (b) after addition of filter paper to crude extract (1 h and 2 h). M: protein marker.

3.2. Metal ion adsorption experiments

3.2.1. The effect of contact time

Contact time is an important factor affecting the efficiency of biosorption because it provides valuable information on how fast the removal process occurs. Time course of metal ion adsorption is shown in Figure S3. Equilibrium of the biosorption of Pb(II) and Zn(II) on the cellulose-MT-CBM reached equilibrium within 10 min. This rapid metal sorption is highly desirable for biosorbents for practical applications.

3.2.2. Effect of pH on metal ion adsorption

The effect of the initial pH of the solution on metal ion adsorption on cellulose-MT-CBM and untreated cellulose (control) is shown in Figure 5(a). The influence of pH on the

biosorption of Pb(II) and Zn(II) indicated that the biosorption increased with pH. From pH 2.0 to 7.0, the percentage of Pb(II) removed increased from 0.8 % to 94 %, and that of Zn(II) increased from 52 % to 60 %. The slightly lower biosorption yield observed for Zn(II) could be attributed to the possible inert nature of one of the MT binding sites to Zn as reported previously (Harrison et al., 2002).

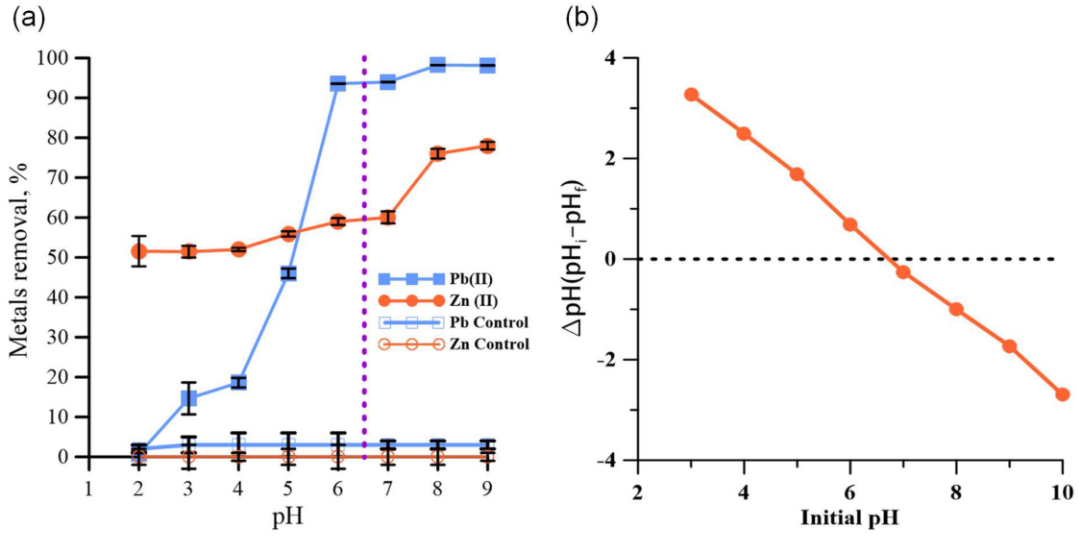


Figure 5. (a) Effect of pH on the adsorption of Pb(II) and Zn(II) on cellulose-MT-CBM (biomass dosage: 10 g/L, initial metal ion concentration: 20 mg/L, temperature: room temperature; the purple dotted line indicates the pzc of the biosorbent) and (b) pzc of the cellulose-MT-CBM biosorbent.

At lower pH values, the binding sites are protonated and the metal ions are in solution because they cannot access the binding sites on the cellulose-MT-CBM. At higher pH values, the binding sites are deprotonated, negatively charged, and more favorable for adsorption. The control had negligible biosorption capacity at all pH values because of a lack of binding ability. The percentages of Pb(II) and Zn(II) removed increased significantly at pH 6.5. This

higher adsorption could be related to the pzc of the cellulose-MT-CBM (Fig. 5b). At pH > pzc, the biosorption of Pb(II) and Zn(II) to the biosorbent is favorable because of the presence of negatively charged functional groups of the protein (Bakatula et al., 2018). Therefore, negatively charged functional groups at pH 6.5 would be able to attract and sequester positively charged metal ions.

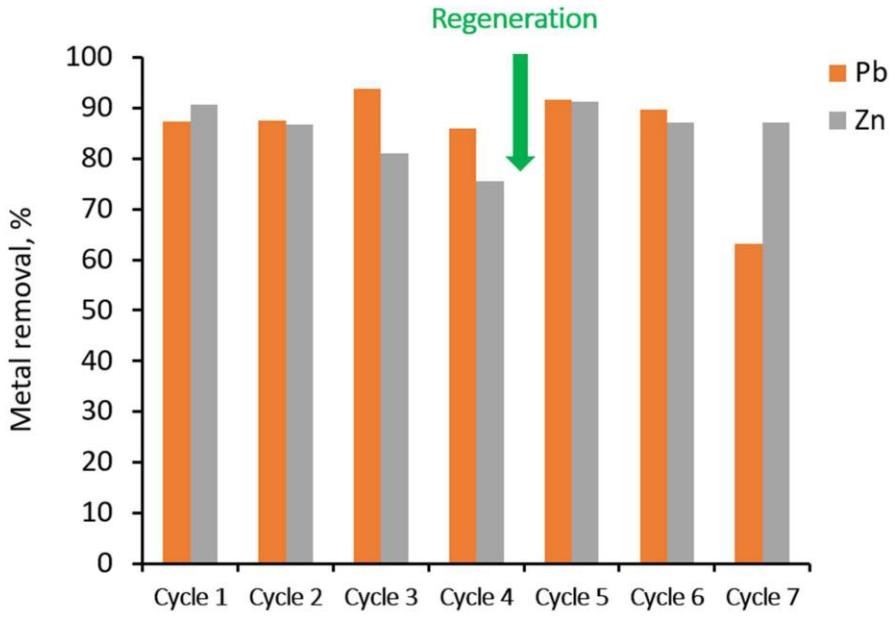
3.2.3. Biosorption capacity

The adsorption isotherm was studied to understand the equilibrium distribution of Pb(II) and Zn(II) between the aqueous phase and surface of the biosorbent. The Langmuir isotherm model was suitable for the experimental data as shown by a good fit with model data (Figure S4). This indicates that the biosorption of Pb(II) and Zn(II) onto the cellulose-MT-CBM takes place via a monolayer mechanism. The maximum biosorption capacities for Pb(II) and Zn(II) were higher than those obtained with other cellulose-based biosorbents. For Pb (II), Dhir and Kumar, (2010) found a Q_{max} value of 41.84 mg/g for wheat straw compared with 39.02 mg/g for the cellulose-MT-CBM in the present study. For Zn (II), Tian et al., (2017) found a Q_{max} value of 5.38 mg/g on a cellulose nanofibril aerogel compared with 29.28 mg/g on the cellulose-MT-CBM in the present study. These results indicate that the cellulose-MT-CBM biosorbent can be used for the removal of heavy metals from water.

3.3. Recyclability of the cellulose-MT-CBM biosorbent

Recyclability of the cellulose-MT-CBM biosorbent was examined because waste has a huge negative impact on the natural environment. The results for the biosorbent recyclability experiments are shown in Figure 6. The cellulose-MT-CBM was regenerated seven times and the bound metal ion was desorbed by 20 mM EDTA. In cycle 4, the adsorption ability slightly decreased. The adsorption ability could be restored by addition of MT-CBM to the

184 cellulose, which resulted in an increase in the metal biosorption. These results show that the
185 biosorbent could be used for multiple metal sorption/desorption cycles without any
186 significant loss in its efficiency.



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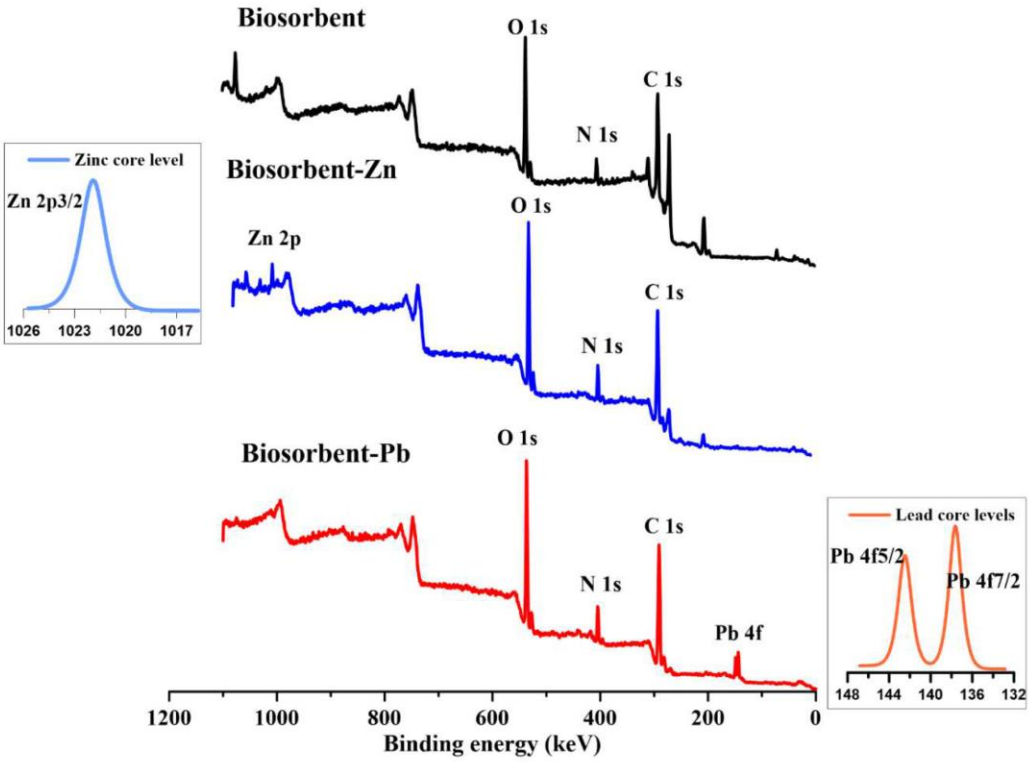
188 **Figure 6**. Pb(II) and Zn(II) removal efficiencies of the cellulose-MT-CBM biosorbent for
189 different cycles (initial metal ion concentration: 20 mg/L, pH: 6.5, temperature: room
190 temperature).

191 **3.4. XPS analysis**

192 XPS measurements of cellulose-MT-CBM after metal adsorption were performed to
193 confirm the electronic state(s) of Pb(II) and Zn(II) on the biosorbent. XPS wide scan spectra
194 of cellulose-MT-CBM before and after Pb(II) and Zn(II) adsorption depicted core levels of C
195 1s, O 1s, N 1s, Pb 4d, and Zn 2p (Figure 7). Two new peaks at 141 and 136 eV appeared after
196 Pb (II) adsorption, which were attributed to the Pb 4f orbital (Qiao et al., 2019). One new
197 peak at 1022 eV was attributed to the Zn 2p orbital (Zhou et al., 2016). These results indicate

198 that Pb(II) and Zn(II) are adsorbed on the biosorbent. The source of N 1s is the MT-CBM
199 protein bound on the cellulose.



200

201 **Figure 7.** Typical XPS wide scan spectra of the cellulose-MT-CBM before and after Pb(II)
202 and Zn(II) adsorption.

203 Metal adsorption by cellulose-MT-CBM could be attributed to the formation of N:
204 Pb^{2+} and N: Zn^{2+} complexes, in which a lone pair of electrons from the N atom in -NH_2 group
205 is donated to a shared bond between the nitrogen atom and Pb^{2+} or Zn^{2+}. Additionally, the
206 oxygen atom could be responsible for heavy metal removal via ionic interactions with Pb^{2+}
207 and Zn^{2+}. The O 1s peak at 532.29 eV was modeled after curve fitting and attributed to the
208 oxygen-rich functional groups such as -COOH group, which interact with heavy metals to
209 form O: Pb^{2+} or O: Zn^{2+}. The XPS results confirm the FTIR data that identified organic
210 functional groups such as -NH_2 and -COOH groups on the biosorbent (Figure S2).

Furthermore, the removal of heavy metals by the cellulose-MT-CBM occurs via heavy metal complexation with the thiol group of cysteine-rich MTs (Diep et al., 2018) and ion exchange with the O atom in the biosorbent as previously reported (Jana et al., 2016).

3.5. Treatment of actual mine wastewater by the cellulose-MT-CBM biosorbent

To demonstrate industrial application of the cellulose-MT-CBM, the biosorbent was used to treat contaminated surface water from an industrial source. The Mushishima stream in Chingola, Zambia, which flows into the Kafue River, is affected by effluent from the active Nchanga Mine (Sracek et al., 2012). It is essential to evaluate the performance of the biosorbent in a multi-metal system that reflects real effluent as opposed to simulated metal ion solutions. Table 1 shows the water quality data obtained before and after treatment with the cellulose-MT-CBM using the setup illustrated in Figure 3. Treated water from the site showed complete removal of toxic elements with below the detection limit of ICP-AES. These results show that the cellulose-MT-CBM is effective and be applied in the mining industry as a clean-up technique.

Table 1. Concentrations of metal ions in mine wastewater before and after treatment with the cellulose-MT-CBM biosorbent.

Toxic trace elements of concern	Before (mg/L)	After (mg/L)	WHO acceptable Limit (mg/L)
Pb	0.19	< 0.001	0.01
Cu	14.56	< 0.001	2.00
Ni	7.74	< 0.001	0.07
Cd	0.34	< 0.001	0.10

4. Conclusion

In this study we developed a novel biosorbent composed of cellulose and a fusion protein. The fusion protein was constructed from metallothionein (MT) and a carbohydrate-binding module (CBM), where CBM binds to cellulose and MT captures heavy metal ions in solution. The biosorbent had maximum biosorption capacities of 39.02 mg/g for Pb(II) and 29.28 mg/g for Zn(II) ions. The resulted cellulose-MT-CBM biosorbent showed regeneration and reusability after repeated using seven times in a semi-continuous system. The biosorbent was applied to purify multiparameter contaminated real mining wastewater and showed complete removal of Pb(II), Cu(II), Ni(II), and Cd(II) to below detection limit. Because of these capabilities, this biosorbent has great potential for efficient removal of toxic trace elements from polluted water.

Declarations of interest: none

Appendix A. Supplementary material

Acknowledgments

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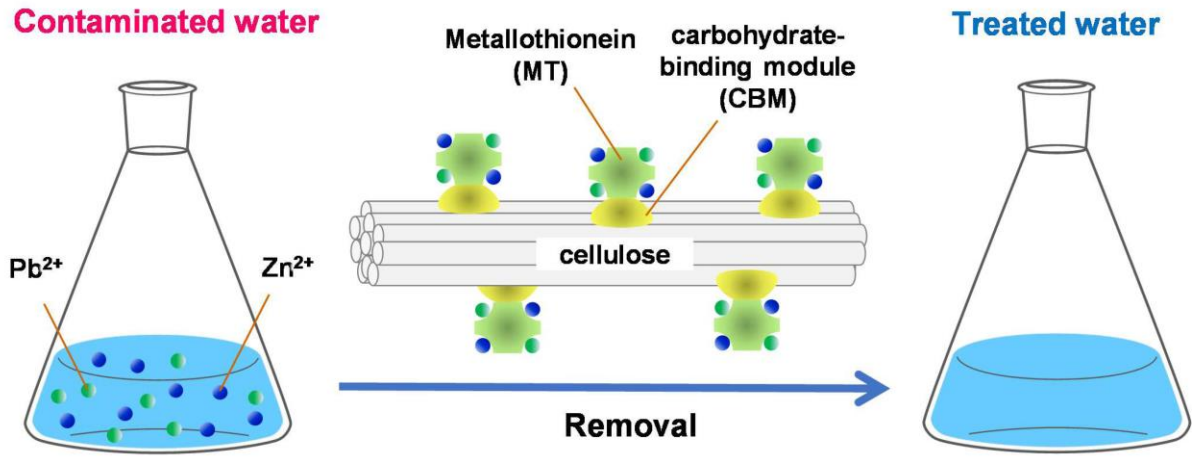
Contaminated water
Metallothionein (MT)
carbohydrate-binding module (CBM)
Treated water
Pb2+
Zn2+
cellulose
Removal