Applied Microbiology and Biotechnology (2019) 103:5411–5420
https://doi.org/10.1007/s00253-019-09852-6

ENVIRONMENTAL BIOTECHNOLOGY

Fabrication of biobeads expressing heavy metal-binding protein for removal of heavy metal from wastewater

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Received: 21 February 2019 / Revised: 9 April 2019 / Accepted: 11 April 2019 / Published online: 8 May 2019

Abstract

Heavy metals, being toxic in nature, are one of the most persistent problems in wastewater. Unabated discharge of large amount of heavy metals into water bodies are known to cause several environmental and health impacts. Biological remediation processes like microbial remediation and phytoremediation are proved to be very effective in the reduction of heavy metal pollutants in wastewater. To circumvent the issues involved several peptides and proteins are being explored. Metal-binding capacity, accumulation, and tolerance of heavy metals in bacteria can be upsurge by overexpressing the genes which code for metal-binding proteins. In the present study, an attempt has been made to bioremediate heavy metal toxicity by overexpressing metal-binding proteins. Two expression cassettes harboring top4 metal-binding protein (T4MBP) and human metallothionein 3 (HMP3) were designed under the control of constitutive CaMV 35S promoter and transformed into *E.coli* TBI cells. *E.coli* over expressing HMP3 and T4MBP were immobilized in biobeads which were explored for the detoxification of water contaminated with copper and cadmium. Effects on the concentration of heavy metal before and after treatment with beads were estimated with the help of ICP-OES. Noteworthy results were obtained in the case of copper with 87.2% decrease in its concentration after treatment with biobeads. Significant decrement of 32.8% and 27.3% was found in case of zinc and cadmium, respectively. Mechanisms of binding of proteins with heavy metals were further validated by molecular modeling and metal-binding analysis. HMP3 protein was found to be more efficient in metal accumulation as compared with T4MBP. The fabricated biobeads in this study definitely offer an easy and user-handy approach towards the treatment of toxic wastewater.

Keywords Biobeads · Metallothionein · Heavy metal toxicity · Expression cassette · HMP · T4MBP

Introduction

Rivers, which are the major source of water in India, are getting contaminated by heavy metals. Heavy metal is a collective term, which can be used for metals and metalloids having an atomic density five times greater than water, i.e., 4 g/cm^3 (Hawkes 1997). Aquatic bodies have been used as dumping areas for discharging agricultural, industrial, and military operation effluents having toxic heavy metals which deteriorate the flora and fauna of aquatic ecosystem leading to an alarming situation (Pazirandeh et al. 1995). Active research targeting heavy metals and their removal from wastewaters and sediments have been focused on the development of novel materials which possess increased affinity, capacity, and selectivity for target metals (Gadd and White 1993). For fulfilling the set target, the vital role of microorganisms to sequester, precipitate, or alter the oxidation state of various heavy metals has been extensively carried out (Macaskie 1990; Gadd and White 1993; Shen and Wang 1993). Metallothioneins also known as phytochelatins in plants is the family of proteins and peptides having most of the selective heavy metal-binding molecules (Butt and Ecker 1987; Kagi and Schaffer 1988). Metallothioneins are low molecular weight, cysteine-

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-019-09852-6) contains supplementary material, which is available to authorized users.

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rich proteins that have been found in a broad range of eukaryotic species (Hammer 1986; Kagi and Schaffer 1988) and in a few prokaryotes (Olafson et al. 1979; Higham et al. 1986). There are varieties of heavy metals which includes Co^{2+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, and Cu^{2+} coordinately binds with high affinity to the cysteine residue of metallothionein (Cismowski et al. 1991). In order to increase the metal-binding affinity and biosorptive potentiality of bacterial cells for heavy metals, the amplified expression of metallothioneins (Pazirandeh et al. 1995; Berka et al. 1988; Ghanbarinia et al. 2015; Choi et al. 2018) or metal-binding protein motif (Pazirandeh et al. 1998) proves to be a promising technology for development of bacterial-based biosorption of heavy metals (Pena-Montenegro and Dussan 2013). The uses of free bacteria towards remediation of heavy metal toxicity possess several disadvantages which include low mechanical strength and difficulty in the separation of the biomass from the effluent (Shakya et al. 2015). This problem can be tackled by immobilizing the bacterial cell via whole cell immobilization technique which offers several advantages (Abdelmajeed et al. 2013). In the present study, an attempt has been made to bioremediate heavy metal by overexpressing metal-binding protein in the presence of CaMV 35S promoter. To the best of our knowledge, this is the first report on the use of CaMV 35S promoter towards the expression of genes in bacteria. Recombinant bacteria were encapsulated in the form of calcium-alginate biobeads and further tested for removal of three potential heavy metals like copper, cadmium, and zinc from toxic water.

Material and methods

Materials Restriction enzymes and pre-stained DNA marker were obtained from Thermo Fisher Scientific and used according to the manufacturer's instructions. All other commonly used chemicals such as $CuCl_2$, $ZnCl_2$, and $CdCl_2$ were purchased from SRL Ltd.

Developing an expression cassette

pUCPMAGUS (Dey and Maiti 1999) vector was obtained from Dr. Nrisingha Dey, Institute of Life Science Bhubaneswar, India. The vector was further modified to harbor *GFP* reporter gene at *Xho*I and *Sac*I site by replacing the *GUS* reporter gene from the respective sites. The modified vector was further used for cloning of metallothionein. A gene coding for metal-binding protein designed by an iGEM-2014 team, Hannover named as top4 metal-binding protein (T4MBP) (iGEM part no: BBa_K148002; supplementary file 1) and *human metallothionein 3* (HMP3) gene (Accession ID: KJ897206.1) (Yang et al. 2011) were synthesized in the form of double-stranded g-blocks by Integrated DNA Technology (IDT). Sequence of T4MBP was obtained from http://2014.igem.org/Team:Hannover/Team.

Efficacy testing of CaMV 35S in bacterial system using CLSM

E. coli (strain TB-1) was transformed with pUCPMACaMV35SGFP vector, having CaMV 35S promoter upstream of the *GFP* reporter gene. A single colony was grown overnight in 10 ml Luria Broth (LB) having appropriate antibiotic (ampicillin) at 180 RPM /37 °C. The overnight grown culture was centrifuged at 3000 RPM for 10 min at room temperature. The obtained pellet was washed with PBS buffer, twice, and re-suspended in 250 μl of phosphate buffer saline (PBS). A tiny drop of about 10 μl was smeared on the slide and covered with coverslip. The prepared slides were kept for 15 to 20 min for stabilizing the cells. The prepared slides were analyzed for expression of the GFP protein using confocal laser scanning microscopy as described in Sahoo et al. (2009).

Molecular modeling of T4MBP and human metallothionein

The protein structure models for the top4 metal-binding protein and human metallothionein-3 were generated based on nonlinear context-specific alignment potential and probabilistic consistency algorithm integrated in threading-based approach of RaptorX tool (Kallberg et al. 2012). The generated models were ranked based on the alignment score of the target and template sequences, the highest ranked best model for these sequences were selected. These modeled structures were analyzed for the identification of putative metal-binding sites using the superposition method. The modeled structures were aligned with the available crystal structures to identify the crucial residues that are involved in the formation of putative metal-binding site. The coordinates of the metal ions were transferred and superimposed to the existing modeled structure.

Encapsulation of the expression cassette in biobeads

Transformed *Ecoli*-TB1 cells with *T4MBP* and *HMP* genes, respectively, having CaMV 35S promoter were encapsulated in 1% and 1.5% *w*/*v* sodium alginate solutions in 100 mM $CaCl_2$ solution. A volume of 100 ml overnight grown culture was centrifuged at 3500 RPM for 15 min at room temperature. Pellet down cells were washed twice with PBS buffer, and dissolved in 10 ml of sodium alginate solution. Spherical beads were formed by tumbling sodium alginate solution in calcium chloride solution in

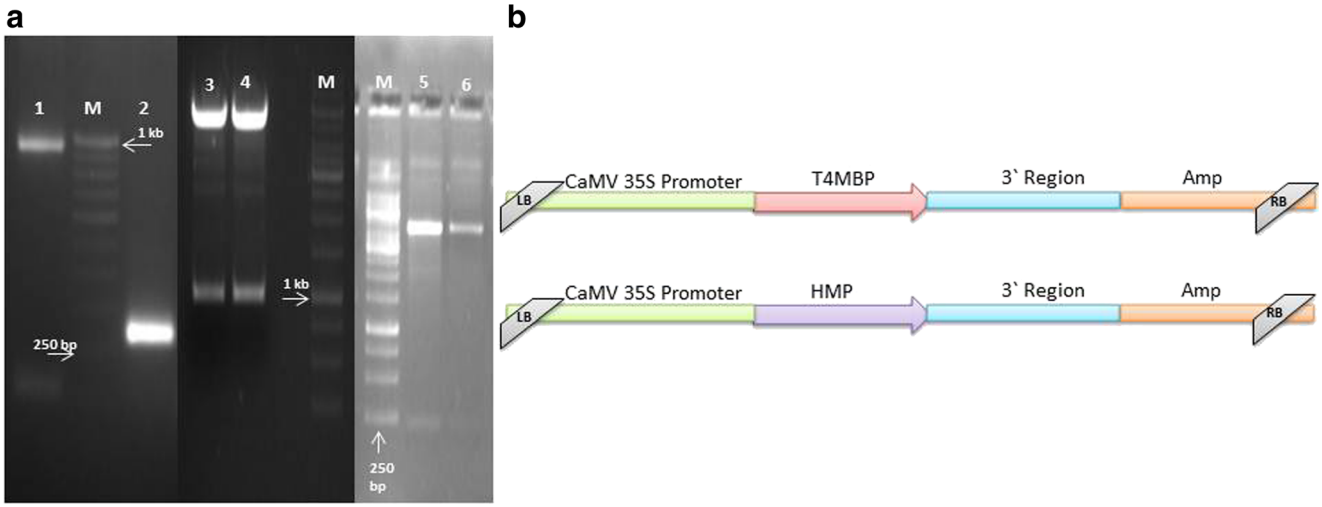

Fig. 1 Cloning and schematic representation of construct formation. **a** Lanes 1 and 2 represent PCR amplification of *T4MBP* and *HMP* gene, and lanes 3 and 4 and lanes 5 and 6 represent restriction digestion of T4MBP and HMP, respectively. b The restriction enzyme site used to assemble these promoter constructs are given as X (*XhoI)*, Sc (*SacI*), E (*EcoRI*), and H (*HindIII*). The right and left border were denoted as RB and LB, position of the rbcS polyadenylation (3-Region) and Antibiotic resistant site ampicillin as Amp

drop wise manner using a Pasteur pipette. Hardenings of the developed biobeads were done by keeping in a calcium chloride solution for 4 h.

Evaluating the formed beads against toxic heavy metals using ICP-OES

Respective biobeads having *T4MBP* and *HMP* genes were tested for purification of potent heavy metals like copper, cadmium, and zinc. Twenty well-round beads were incubated for 16 h at a constant temperature of 25 °C with a mild agitation of 80 RPM. Beads were filtered out from the water sample and the filtrate water was digested with HNO_3 to prevent any further activity. Three water samples were treated with the bio beads among which one is the artificial water prepared in the lab having 1 mM $CuCl_2$, $CdCl_2$, and $ZnCl_2$ (This sample was taken in triplicate to be assured with the accuracy of the system), desired amount was added and the water was filtered with Whatman filter paper 1 to remove any sediments. The other two samples were taken from sewage treatment plant designated as sewage treatment plant (STP) inlet and outlet.

Water samples were subjected to Agilent 710, induced couple plasma optical emission spectrometry (ICP-OES) for estimation of the heavy metals before and after treatment with biobeads. The concentration of heavy metals was determined in milligram per liter against the linearity curve plotted against the standard.

Adsorption rate, capacity, and statistical analysis

Samples as mentioned in Table 3 were tested in triplicates to ensure the reproducibility of experiments. Mean values were used for calculation of adsorption rate (Q) and adsorbent capacity (q) of formulated biobead were calculated as $Q = [(C_i\text{-}C_f)/C_i]X100$ and $q = [(C_i\text{-}C_f)XV]X0.05M$ where Q is the adsorption rate (%), q is the adsorption capacity (mg/g), Ci is the initial concentration of heavy metal ions (mg/l), Cf is the final concentration of heavy metal ions (mg/L), V is the volume of wastewater (L), and M is the immobilized biological adsorbent dosing weight (g/l) (Li and Zhou 2018). Groups were compared by one-way analysis of variance (ANOVA). A P value of < 0.05 was considered significant.

Results

Developing an expression cassette

After confirming the efficacy of CaMV 35S promoter, *T4MBP* and *HMP3* genes were cloned in PUCPMA GFP vector as described (Ranjan et al. 2012). Appropriate PCR

Table 1 List of primer used for amplification of T4MP and HMP3 gene

Gene	Forward primer	Reverse primer
T4MBP	5′GCCAAGCTTCTCGAGATGGTGGTTAT CAACGGCGTCAA3′	5′TAGAATTCGAGCTCTTATTA CTTACAGCTGCAAGATG3′
HMP3	5′CGGAAGCTTCTCGAGATGGATCCGGA AACCTGCCCGTG3′	5′TAGAATTCGAGCTCCTGGCAGCAGCT GCATTTTTCCGC3′

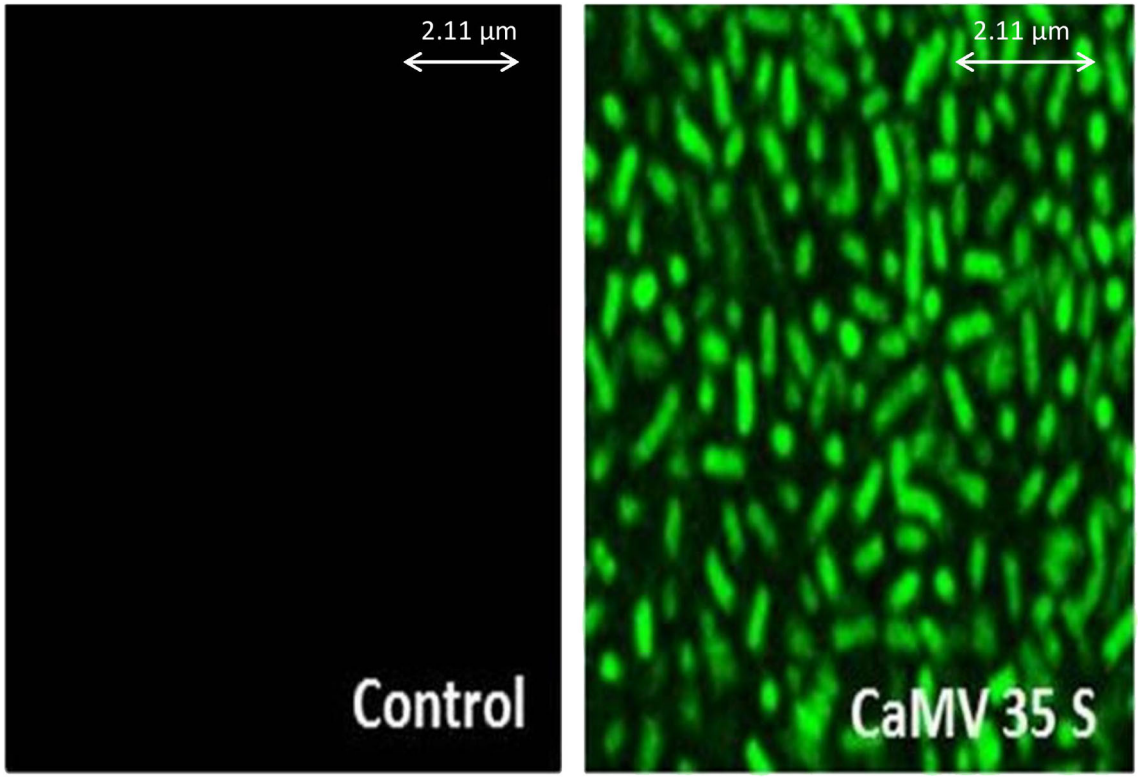

Fig. 2 Bacterial TB1 cells expressing GFP gene in the presence of CaMV 35S promoter observed in CLSM

amplification was estimated by a sharp band of the respective size of both genes comparable with DNA ladder. Size of the insert was further verified by restriction digestion with *Xho1* and *Sac1* enzymes (Fig. 1). For final confirmation of the sequence, cloned genes were sequenced using both forward and reversed primer (Table 1) and the obtained results were aligned using NCBI-BLAST.

Efficacy testing of CaMV 35S in bacterial system using confocal laser scanning microscopy (CLSM)

CaMV 35S is a strong promoter from plant pararetrovirus and widely used for ectopic gene expression in plants. In the preliminary study done by Sahoo et al. (2009), it was found that this bacteria also offers strong expression in wild strain of *E.coli*, i.e., TB1, hereby the effectiveness of this promoter was reconfirmed against *GFP* reporter gene by using CLSM technique. In the present study, it was found that CaMV 35S is well expressed in the cell of TB1 strain with high intensity (Fig. 2). These results indicate that this promoter is not only of high importance for the plant system but also suitable for enhanced bacterial expression, which implies that this promoter hold high importance and can be exploited in bio-industrial world.

Molecular modeling of T4MBP and human metallothionein

The structural alignment of these models on the crystal structure exhibited the RMSD of <1A. Analysis of the modeled structures revealed that only the cysteine residues (shown in Table 2) were involved in the formation of the metal-binding site for the top4 metal-binding site and the human metallothionein3 receptor. The top4 metal-binding sequence has shown the presence of three metal-binding sites with the arrangement of three metal ions in each site whereas the human metallothionein sequence has also shown the similar ability of occupying two metal-binding sites in combination with three and four metal ions at the metal-binding site (shown in Fig. 3). Further, the 3D structure reveals the presence of 2-turn alpha helices near the metal-binding site that might be essential for

Table 2 The table represents the list of active site residues that involved in metal binding

Receptor name	Cysteine residues list
Top4 metal-binding protein	177, 181, 186, 188, 204, 206, 210, 212, 215, 244, 248, 253, 255, 259, 261, 265, 267, 273, 275, 280, 282, 286, 292, 294, 298, 200
Human metallotheonein protein	6, 8, 14, 16, 20, 22, 25, 27, 30, 34, 35, 37, 38, 42, 45, 49, 51, 64, 66, 67

the placement of metal ions in the metal-binding site. Metallothioneins has the capacity to chelate seven zinc or cadmium atoms and 12 copper atoms per molecule due to the presence of multiple thiolate bonds of cysteine (Kagi and Schaffer 1988). Modeling of HMP3 and T4MBP metal-binding protein had also shown the maximum presence of cysteine residue, which may establish the fundamental role of cysteine residue in the metal-binding capacity of metallothionein.

Encapsulation of the expression cassette in biobeads

Different sodium alginate concentrations with a different molarity of calcium chloride were tested for formation of beads Out of which 1% sodium alginate with 100 mM calcium chloride solution exhibited best results in terms of shape, i.e., perfect round beads formation at this concentration and molarity (Fig. 4).

Evaluating the formed beads against toxic heavy metals using ICP-OES

Artificial wastewater, STP inlet water, and STP outlet water were treated with developed biobeads having respective expressed protein genes and subjected to ICP-OES for effect on copper, cadmium, and zinc before and after treatment. Effect on copper: A sharp decrease in copper concentration was observed when samples were treated with the developed biobeads. 87.2% (mean value) decrease was observed in the artificial-treated samples while 50.3 and 48.56% decrease was obtained in STP inlet and outlet respectively with *HMP* gene. 57.1%, 34.32%, and 31.8% decrement was found in artificial wastewater, STP outlet, and inlet respectively when treated with T4MBP (Fig. 5). Effect on zinc: Though less than copper, but significant decrement was observed in case of zinc. 32.8%, 18.6%, and 17.8% decrease was obtained in artificial wastewater, STP outlet, and inlet respectively when treated

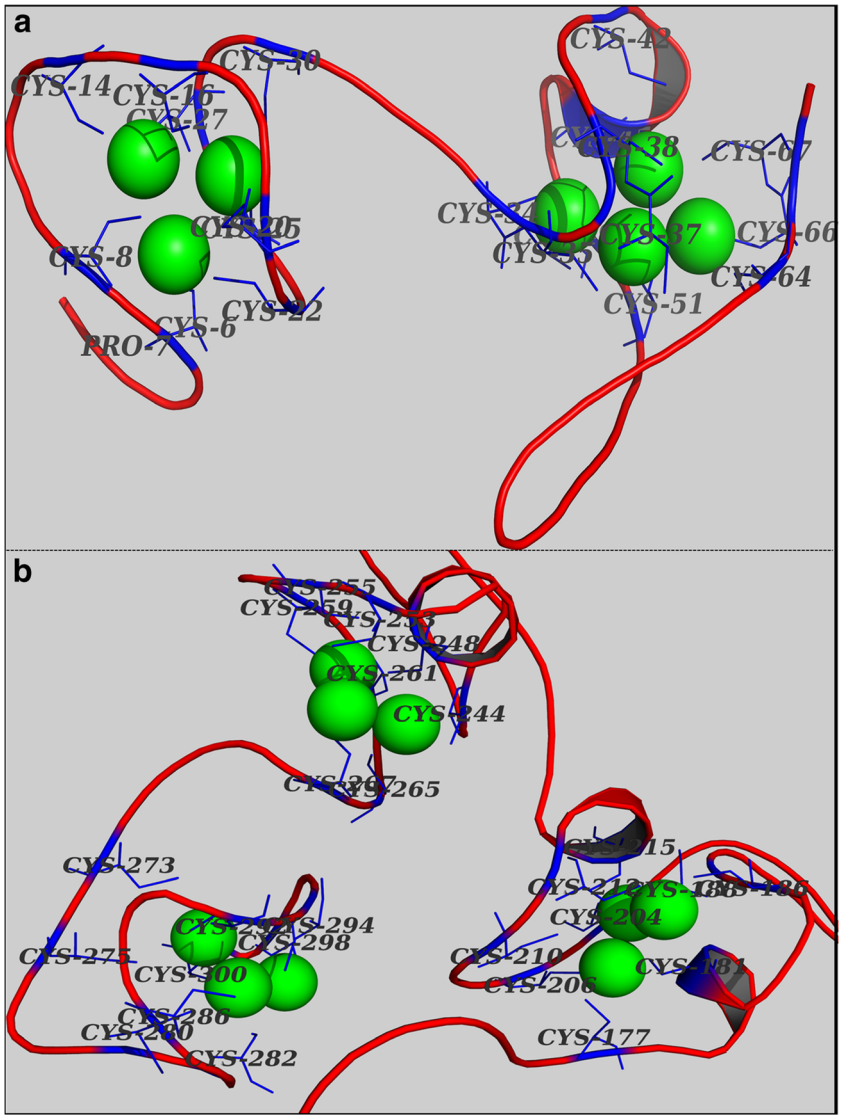

Fig. 3 The cartoon representation of protein models. The 3D structure of T4MBP (**a**) and human metallothionein 3 (**b**) proteins were shown. Each segment, i.e., protein, metal-binding site, and ions were shown in red-blue-green colors. The cartoon indicates protein, line indicates metal-binding site, and sphere indicates ions

with HMP biobeads, while 21.7%, 15.2%, and 12.5% were observed in artificial wastewater, STP outlet, and inlet respectively in the case of T4MBP (Fig. 5). Effect on cadmium: Least decrement was found in the samples having cadmium with 27.25%, 9%, 18% decrement was observed in artificial wastewater, STP outlet, and inlet respectively with HMP biobeads, while 3.5, 7, and 13% decrease was obtained in samples treated with T4MBP-biobeads (Fig. 5).

According to the results obtained, maximum decrement was observed for copper, which is nearby 90%, which leads to an inference that biobeads encapsulating *HMP* gene possess a potent remedy for Cu toxicity in wastewater. For all three heavy metals, HMP biobeads had worked well while a significant decrease was found in the case of artificial wastewater. Although T4MBP Bi-beads was working in a noteworthy manner, but when comparing both these genes, HMP had bagged more points. On comparing artificial water samples with STP water samples, large decrement was observed in the case of artificial water samples, which may be due to more availability of free ion adsorption rate and capacity of developed biobeads were calculated (Table 3) which also endorse that biobeads expressing HMP has high adsorbent capacity for heavy metals.

Discussion

In the present era, bioremediation is highly explored for purification of wastes contaminated with toxic metals (Feng and Guo 2012). The eventual goal is to use microorganism in biosorption of metal polluted water, stream, and soil (Valls et al. 2000). Expression of heterologous genetically modified and native peptides in microorganisms appears to be an attractive solution in the field of research in environmental engineering (Srivastava and Majumder 2008; Naureen and Rehman 2016). Scientist had identified few microorganisms possessing the heavy metal removal capability, among which the prevalent degraders in biofilters are bacteria and fungi (Strandberg et al. 1981 and Dixit et al. 2015; Srivastava and Majumder 2008).

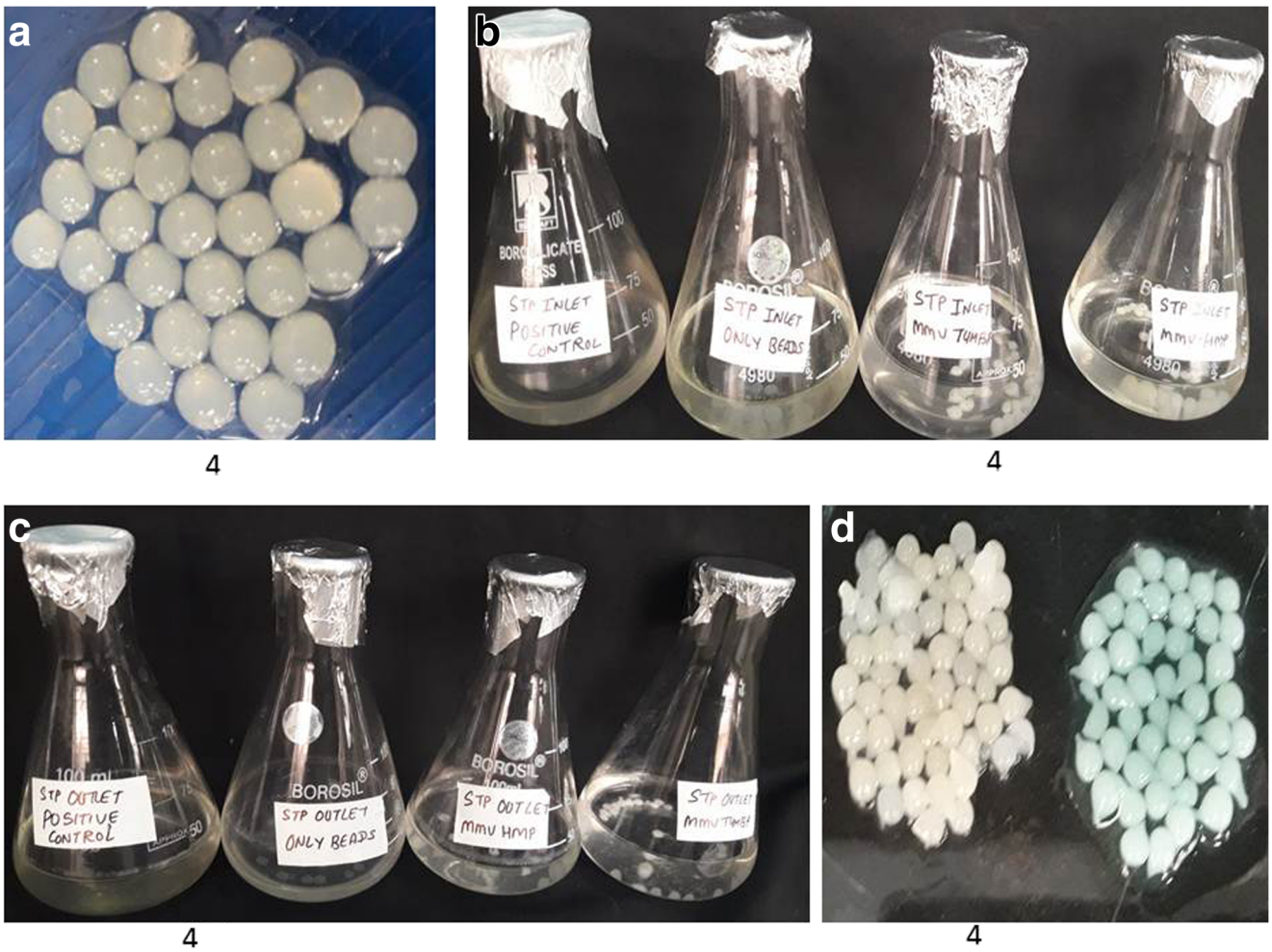

Fig. 4 Formation of biobeads. **a** Encapsulation of TB-1 bacterial cells having T4MBP and HMP3 metallothionein. **b**, **c** Treatment of sewage treatment plant inlet and outlet water with developed biobeads. **d** Biobeads before and after treating toxic water

Here, we had approached to overexpress metallothionein in genetically engineered *E.coli* cells, which were further immobilized to provide a biosorbent system for removal of heavy metal toxicity. These peptides have high cysteine content, hereby efficiently provides the pockets for heavy metals binding. Studies had been reported on the construction of genetically engineered microorganism for accumulation of heavy metals. A group of scientists had reported co-expression of metal-binding protein and mercury transport system in *E.coli* and reported that maximum bioaccumulation of metals occurs when protein is highly expressed outside the cytosol (Chen and Wilson 1997). Similar studies were conducted by targeting the mouse metallothionein I (MT) protein to the cell surface of the heavy metal-tolerant Ralstonia eutropha ch34 strain. The engineered bacteria were found to have an enhanced ability for arresting Cd^{2+} ions from the external media (Valls et al. 2000). These investigators had used a different range of heavy metal concentration from 5 to 30 mM and showed that due to periplasmic expression of metallothionein, non-viable cells can also be efficiently used; in our study, we had tested the engineered viable cells against 0.5 mM concentration of heavy metals and found significant results. Through multiple thiolate bonds of cysteines, metallothionein has the capacity to chelate seven atoms of Zn or Cd, or 12 atoms of Cu per molecule (Kagi and Kojima 1987). Similar results were found in our study, modeling of HMP3 and T4MBP had

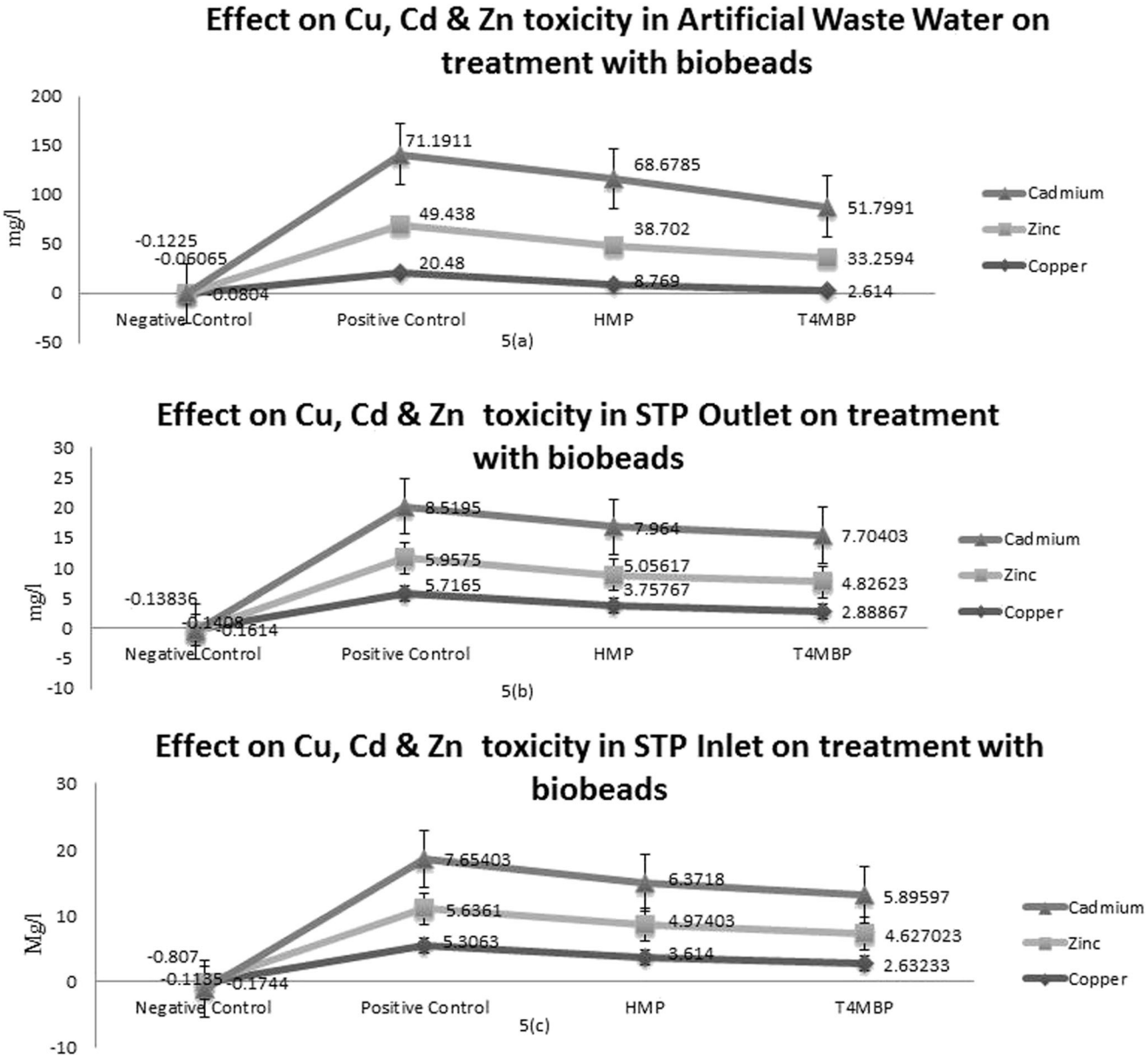

Fig. 5 Graphical representation of effects on Cu, Cd, and Zn toxicity on treatment with biobeads

Table 3 Descriptive analysis of readings obtained through ICP-OES on treating toxic water with the developed biobeads (# mean values of each reading are provided in the given table)

Heavy Metal	S. No	Biobeads	Artificial water			STP outlet			STP inlet		
			Reading (mg/l)	Adsorption rate (Q)	Adsorption capacity (q)	Reading (mg/l)	Adsorption rate (Q)	Adsorption capacity (q)	Reading (mg/l)	Adsorption rate (Q)	Adsorption capacity (q)
Copper (Cu)	1	Negative control	− .0804 ± 03744	-NA-	-NA-	− 0.1614 ± .01010	-NA-	-NA-	0.1744 ± .00163	-NA-	-NA-
	2	Positive control	20.480 ± .07768	-NA-	-NA-	5.7165 ± .11267	-NA-	-NA-	5.3063 ± .06970	-NA-	-NA-
	3	35S-T4MBP	8.769 ± 11721	57.1	1220.83	3.75767 ± .07093	34.32	204.1	3.614 ± .06493	31.8	177.08
	4	35S-HMP	2.614 ± .05227	87.2	1861.45	2.88867 ± .20576	49.56	294.79	2.63233 ± .18282	50.3	278.75
Zinc (Zn)	1	Negative control	− .0935 ± .03285	-NA-	-NA-	− 0.1408 ± .01154	-NA-	-NA-	− 0.131 ± .01750	-NA-	-NA-
	2	Positive control	49.4380 ± 1.22111	-NA-	-NA-	5.9575 ± .03325	-NA-	-NA-	5.6361 ± .18237	-NA-	-NA-
	3	35S-MT4MBP	38.7020 ± .77975	21.7	1117.70	5.05617 ± .06241	15.2	93.75	4.97403 ± .11451	12.5	69.37
	4	35S-HMP	33.2594 ± 1.63998	32.8	1685.41	4.82623 ± .05809	18.6	117.70	4.627023 ± .25809	17.8	105.20
Cadmium (Cd)	1	Negative control	− .1225 ± .01989	-NA-	-NA-	− 0.1484 ± .01004	-NA-	-NA-	− 807.09 ± .00855	-NA-	-NA-
	2	Positive control	71.1911 ± 1.57056	-NA-	-NA-	8.5195 ± .01004	-NA-	-NA-	7.65403 ± .18679	-NA-	-NA-
	3	35S-MT4MBP	68.6785 ± 2.07785	3.5	262.5	7.964 ± .06266	7	57.29	6.3718 ± .22466	13	133.33
	4	35S-HMP	51.7991 ± 1.30519	27.25	2020.83	7.70403 ± .04233	9	84.37	5.89597 ± .28768	18	183.33

shown the maximum presence of cysteine residue, which might establish the fundamental role of cysteine residue in the metal-binding capacity of metallothionein.

We had also compared two metal-binding proteins and found that although both are working significantly well in their function, yet HMP3 possess, the better potentiality for biosorbent up to 87.2%, 32.8%, and 27.25% for accumulation of Cu, Zn, and Cd, respectively. The efficiency of the developed bacterial system decreases relatively in case of STP water, which may be due to the presence of heavy metals in more complex form in comparison of heavy metal present in artificial wastewater. The developed expression system in *E.coli* provides several advantages for the study of foreign gene such as metallotheonein, their interaction, localization, and stability in the microorganism. Here, CaMV 35S promoter was used, which is a strong, constitutive viral promoter, thus provides potentiality for better expression of ectopic gene. Our developed biobeads containing the immobilized genetically engineered E.coli TB1 cells may provide a fast and competent approach for the development of heavy metal removal system. Our developed biobeads, strongly expressing T4MBP and HMP, could be fast, easy, and user handy for purification of water possessing heavy metal toxicity.

Acknowledgments We are grateful to the Director, DEI, Dayalbagh for providing infrastructure and Institute of Life Science, Bhubaneswar for Confocal facility. We sincerely acknowledge Ms. Mrinalini Prasad, Ms. Gauri Sharma, and Ms. Simran Singh for finding various literatures and actively supporting in other research activities.

Funding This study was funded by the Department of Biotechnology, Government of India, New Delhi, for DBT-iGEM grant-2017.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human or animal participants performed by any of the authors.

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