Exploration of an Extracellular Polymeric Substance from Earthworm Gut Bacterium (*Bacillus licheniformis*) for Bioflocculation and Heavy Metal Removal Potential

Jayanta Kumar Biswas [1,2,*], Anurupa Banerjee [1], Binoy Sarkar [3], Dibyendu Sarkar [4,*], Santosh Kumar Sarkar [5], Mahendra Rai [6] and Meththika Vithanage [7]

1 Enviromicrobiology, Ecotoxicology and Ecotechnology Research Unit, Department of Ecological Studies, University of Kalyani, Kalyani, Nadia 741235, West Bengal, India; anu.banerjee90@gmail.com
2 International Centre for Ecological Engineering, University of Kalyani, Kalyani 741235, West Bengal, India
3 Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK; b.sarkar@lancaster.ac.uk
4 Department of Civil, Environmental and Ocean Engineering, Stevens Institute of Technology, 1 Castle Point on Hudson, Hoboken, NJ 07030, USA
5 Department of Marine Science, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700 019, West Bengal, India; sarkar.santosh@gmail.com
6 Department of Biotechnology, SGB Amravati University, Amravati 444602, Maharashtra, India; pmkrai@hotmail.com
7 Office of the Dean, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda 10250, Sri Lanka; meththikavithanage@gmail.com
* Correspondence: biswajoy2008@gmail.com (J.K.B.); dsarkar@stevens.edu (D.S.)

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Abstract: The present study shows the potential of an extracellular polymeric substance (EPS) produced by *Bacillus licheniformis* strain KX657843 isolated from earthworm (*Metaphire posthuma*) gut in the sorption of Cu(II) and Zn(II) and in flocculation. After harvesting bacterial cells from sucrose supplemented denitrifying culture medium, the EPS was extracted following ethanolic extraction method. The Fourier Transform Infrared Spectroscopy (FTIR) and ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) of EPS revealed its functional groups, electronegative constituents, unsaturated carbon, and carbonyl groups. The negatively charged functional groups of carbohydrates and protein moiety of the EPS endowed it with heavy metal binding capacity through electrostatic interactions. The highest flocculation activity (83%) of EPS was observed at 4 mg L^{-1} and pH 11. The metal sorption by EPS increased with increasing pH. At pH 8, the EPS was able to remove 86 and 81% Cu(II) and Zn(II), respectively, from a 25 mg L^{-1} metal solution. 94.8% of both the metals at 25 mg L^{-1} metal solutions were removed by EPS at EPS concentration of 100 mg L^{-1}. From Langmuir isotherm model, the maximum sorption capacities of EPS were calculated to be 58.82 mg g^{-1} for Cu(II) and 52.45 mg g^{-1} for Zn(II). The bacterial EPS showed encouraging flocculating and metal sorption properties. The potential to remove Cu(II) and Zn(II) implies that the EPS obtained from the earthworm gut bacteria can be used as an effective agent for environmental remediation of heavy metals and in bioflocculation.

Keywords: extracellular polymeric substances; earthworm; gut bacteria; flocculation; metal remediation; isotherm models

1. Introduction

Soil is the soul of infinite life forms ranging from miniscule microbes to macro-invertebrates. The latter groups are represented by earthworms, termites and ants, which are distinguished by their capacity to excavate the soil and produce a great impact on soil physical properties by creating organomineral structures, such as macro-voids, nests, mounds, galleries and caverns. These organisms have been described as "ecological engineers" of the soil [1]. Earthworms play a pivotal role in decomposition, nutrient cycling and maintenance of soil structure in the terrestrial ecosystem [2]. The gut of the earthworm constitutes an anoxic and organic substrate-rich microzone which provides a unique opportunity to the ingested heterotrophic soil bacteria for anaerobiosis [3]. The earthworm's alimentary tract provides a compelling environment to the gut associated microorganisms, and microorganisms experience a continuous change in the pH, salinity, nutrient levels and gases like oxygen, nitrogen, hydrogen etc. which can influence the production of extracellular polymeric substances (EPS) or biofilm by gut-inhabiting microorganisms [4].

EPS are a complex blend of high molecular weight microbial biopolymeric secretory by-products. These biopolymers mostly consist of proteins, polysaccharides, uronic acids, humic substances, lipids, and nucleic acids [5]. Synthesis of EPS and their composition are regulated by a variety of environmental conditions especially the nutrient level including the availability of carbon and nitrogen in the growth medium [6]. These EPS show unique physiological and physicochemical properties which make them suitable for several clinical, industrial and environmental applications [7,8]. Biopolymers have been recognized by many researchers as potential alternatives to conventional chemical polymers because of their ease of biodegradability, high efficiency, and non-toxic nature [9]. Microbial EPS offer an array of promises for their applications in foods, drug delivery, oil recovery, water purification, and metal removal in mining and industrial waste treatments, and also in downstream processes in fermentation and pharmaceutical industries [10].

In wastewater treatment and downstream processing industries, flocculating agents are used in the process of removing colloids, suspended solids and cell debris comprising main constituents of wastewater [11]. Synthetic polymers like acrylamide, acrylic acid and mixtures of their derivatives are oft-used flocculants for such treatments [12]. However, reports on some of these synthetic flocculants show their neurotoxic and carcinogenic effects, including disorders like neuropathies, ataxia, numbness of hands and feet, muscle weakness, and in some cases cerebellar alterations even at a sub-chronic concentration [13]. Hence, it is important to develop non-toxic, environmental-friendly, cost-effective and highly efficient flocculating agents for wastewater treatment. In this scenario, bioflocculants endowed with attributes of biodegradability, non-toxicity and eco-friendly nature are gaining importance [14]. EPS produced by microorganisms could be a promising alternative to the chemically synthesized flocculants [11].

Recently the extensive usage of various heavy metal (e.g., Cu, Zn, Cd, Cr, Pb, Ni, Co) compounds in different industries has led to their increase in ecosystems [15]. Heavy metals are nonbiodegradable in nature, and as a result they persist in the environment for longer periods of time. Conventional treatment processes for the remediation of heavy metal ions include coagulation, chemical precipitation, electrodialysis, evaporative recovery, floatation, flocculation, ion exchange, nanofiltration, reverse osmosis and ultrafiltration [16]. Although effective, these methods are often highly expensive and result in the production of a huge amount of ineradicable toxic slurries which again pollutes the environment. Therefore, there is a need for an effective yet less expensive, environmentally safe method to minimize heavy metals from toxic to safe limits in the environment [17]. Recently, biological methods, including prokaryotic as well as eukaryotic microbial cells and their by-products like bacterial EPS, are used as emerging candidates for the biosorption and remediation of heavy metals at contaminated sites in an eco-friendly fashion [18,19]. Thus, utilizing bacterial EPS in environmental management is an emerging area of research. Previous studies report an L-asparagine monohydrate dependent EPS producing *Bacillus licheniformis* from the gut of the earthworm, *Metaphire posthuma*, and the modulating factors for microbial synthesis of EPS [4]. Since such bacterium harbors in the gut of the earthworm

which is highly acclaimed as a soil fertility enhancing and bioremediating organism and, in concert with other bacterial assemblage, it aids the earthworm gut acting as a bioreactor. The study on the role of the EPS synthesized by that bacterium in environmental management deserves special attention.

The particular gut isolate *Bacillus licheniformis* KX657843 has been tested for certain properties such as metal removal, phosphate solubilization, indole acetic acid (IAA) production, and its effect on seed germination has also been reported [20]. The present study focuses on studying the metal sorption and bioflocculating properties of the EPS produced by *Bacillus licheniformis* KX657843. Two main objectives of the present study are the following: (1) characterization of the EPS by Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS) while the Fourier transform infrared spectroscopy (FTIR) study of the EPS was done earlier [4], (2) examining the effects of some fundamental parameters on the sorption of heavy metal Cu(II) and Zn(II) and flocculation by the EPS obtained from *Bacillus licheniformis*.

2. Materials and Methods

2.1. Earthworm Sample

Earthworms (*Metaphire posthuma*) used in this study were of medium size (~10 cm in length and 5 mm in diameter), endogeic, and geophagous organism. Sterile bags were used for collecting the earthworm samples from the garden soil of University of Kalyani campus (Lat 22.9862° N, Long 88.4464° E). Further examination and isolation of bacteria were carried out from these earthworm samples.

2.2. Isolation and Characterization of the Microorganism

The isolation procedure of the bacteria from the gut of the earthworm *Metaphire posthuma* with a characteristic slimy, mucoid phenotypic nature (Supplementary information: Figure S1) and the detailed biochemical and molecular level identification have been previously reported [4,20]. *Bacillus licheniformis* strain (KX657843) able to produce EPS exclusively in the presence of L-asparagine monohydrate was isolated from the earthworm gut. The information regarding the biochemical characterization and the complete 16S rRNA gene sequencing analysis has been reported in previous works [4,20].

2.3. Production of EPS

EPS was extracted from the bacterial isolates employing an ethanolic extraction method [21]. Mucoid colonies obtained from 24 h cultures on plates of denitrifying agar medium supplemented with sucrose instead of citrate were scraped with a sterile glass slide and re-suspended in a sterile saline solution (0.85% NaCl). Sucrose was utilized since maximum amount of EPS was produced when sucrose was the carbon source. From the resulting suspension, cells were harvested by centrifugation at 9500× *g* for 30 min at 4 °C. The precipitation of the resultant supernatant was done by adding an equal volume of pre-chilled absolute ethanol and incubated the mixture at −20 °C for 1 h. Precipitated EPS was recovered by centrifugation at 9500× *g* for 20 min at 4 °C. Finally, the pellet obtained in the process was dialyzed against Milli Q water using cellulose membrane (14 KDa MWCO membrane) for 72 h employing two changes of Milli Q water per day in order to remove low molecular weight impurities including ethanol, and then the solution was dried using vacuum freeze drier [22].

2.4. Characterization of EPS

The purified EPS fraction was subjected to FTIR analysis using potassium bromide (KBr) disc method in order to study the functional groups. The EPS sample (2–5 mg) was mixed with KBr of IR spectroscopy grade in a smooth agate mortar. The mixture was then pressed under pressure of about 5–8 ton cm^{-2} for about 20–30 s [4,23]. The electrical charge on EPS was determined by electrophoresis method using dynamic light scattering in combination with an applied electric field [24]. The ^{1}H NMR and ^{13}C NMR analyses were conducted in D_2O solvent. To obtain ^{1}H NMR spectra, EPS sample (0.7 mL) was dissolved in D_2O, and a solution of tetramethylsilane (TMS) was used as the reference internal

standard. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 500 and 125.7 MHz, respectively. Mass spectra were collected with a JEOLJMS-AX 500 spectrophotometer.

2.5. Determination of Flocculation Activity

The flocculation activity was determined using an artificial colloidal suspension made of kaolin clay. In terms of surface charge behavior, the kaolin clay mimics wastewater sludge (zeta potential = −32 mV) [25] and provides better reproducible results than the sludge itself in terms of suspension stability. The flocculation activity was determined following a method described earlier [26] with minor modifications. Kaolin clay suspension (5 g L^{-1}, pH = 7) in 1% w/v aqueous $CaCl_2$ with EPS as the test bioflocculant was thoroughly vortexed for 1 min, and was kept at room temperature for 5 min. The EPS concentrations were varied in the range of 2–20 mg L^{-1}. The entire mixture was prepared to a final volume of 20 mL in test tubes. Finally, the optical density of the aqueous phase was measured with a spectrophotometer (UV-1800 UV-Vis Shimadzu, Kyoto, Japan) at 550 nm wavelength. A control treatment was set up in a similar way where extracted EPS was replaced with distilled water. Flocculation activity was determined by the following equation

$$\text{Flocculation activity}(\%) = \frac{B - A}{B} \times 100 \quad (1)$$

where, A is optical density of samples containing EPS, and B is the optical density of the control [9].

2.6. Effect of Operational Parameters on Flocculation Activity

The concentration/dosage of EPS that gave the best result in the above flocculation experiment was chosen for subsequent experiments, and the flocculation activities under different operational parameters were determined independently by the method mentioned above. All the experiments employed triplicate sets.

The effect of pH on flocculation activity was assessed by adjusting the pH of the flocculant mixture at a pH range of 5–12 using 1N HCl and 1N NaOH. The effect of different concentrations of $CaCl_2$ on the flocculation activity was determined by varying the salt concentrations from 0 to 8%. Here, two different pH conditions were chosen: the neutral pH (7.0) and the pH (11.0) which gave the best flocculation activity in the pH effect experiment stated above. The effect of different cations on the flocculation activity was assessed by replacing $CaCl_2$ with Na^+, K^+, $Cu^{2+,}$ Zn^{2+} and Fe^{3+} at 1% w/v concentration. Thermal stability of the flocculant EPS was determined by incubating the EPS in water bath at a temperature range of 50–100 °C for 30 min at neutral as well as the pH which gave the best flocculation activity in the pH effect experiment.

2.7. Determination of Cu(II) and Zn(II) Adsorption Efficiency

Stock solutions (500 mM) of Cu^{2+} and Zn^{2+} were prepared by dissolving $CuSO_4{\cdot}5H_2O$ and $ZnSO_4{\cdot}7H_2O$, respectively, in Milli Q water, and passed through 0.45 µm pre-sterile syringe filters (Millipore filter paper). A stock solution of EPS (1000 mg L^{-1}) was also prepared using milli Q water. The batch adsorption system consisted of a total 30 mL of EPS and heavy metal solutions both at 25 mg L^{-1} concentrations. The pH of the solutions was adjusted using 1 N HCl and 1 N NaOH at a range of 5–8 keeping the concentrations of EPS and heavy metal ions constant. All experiments were conducted in triplicates, and a setup without EPS was treated as control. The suspensions were agitated on a shaker at 25 ± 2 °C at 150 rpm for 24 h. An aliquot of 6 mL from the batch suspensions was withdrawn, to which equal volume of ice-cold ethanol was added, and the suspension was centrifuged at 1000× *g* for 10 min [27]. The supernatant obtained was filtered through 0.45 µm syringe filter and further acid digested at 100 °C on hot plate with 1 mL concentrated HCl in order to remove any organic impurities which would otherwise interfere in the measurement of metal ions. Then the amount of residual metal ions remaining in the digested supernatants, both in the EPS treated and EPS free control, was determined using atomic absorption spectroscopy (AAS) (AAnalyst 200, PerkinElmer,

Waltham, MA, USA). A calibration was performed using individual standard reference solutions (Fluka Analytical, Buchs St. Gallen, Switzerland) for ensuring the quality control. The standard curve has been provided in (Supplementary information: Figure S2). The concentration range for both the metal ions taken was from 0–2 mg L^{-1}. The correlation coefficients (R^2) for Cu(II) and Zn(II) were 0.992 and 0.991 respectively.

2.8. Adsorption Isotherm

Adsorption isotherms of Cu(II) and Zn(II) on the extracted EPS were studied by varying the EPS concentrations from 0 to 100 mg L^{-1} (pH = 7.0) while keeping Cu(II) and Zn(II) concentrations constant at 25 mg L^{-1}. Other process parameters were similar as the above. The adsorption of Cu(II) and Zn(II) by EPS was tested using the linear Langmuir isotherm model represented by the equation given below:

$$\frac{C_e}{q} = \frac{C_e}{q_m} + \frac{1}{q_m\, b} \tag{2}$$

where, q represents the uptake of metal by EPS (mg g^{-1}) calculated by the formula given below (Equation (3)), C_e is the metal ion concentration at equilibrium (mg L^{-1}), q_m (mg g^{-1}) and b (L mg^{-1}) are the Langmuir constants, which determines the maximum metal adsorption capacity and the affinity between the biosorbent and the metal ion, respectively. The constants are determined from the linear plot of C_e/q versus C_e.

$$q = \frac{V\,(C_i - C_f)}{W} \tag{3}$$

where V is the volume of EPS solution (L), C_i and C_f are the initial and final metal ion concentrations (mg L^{-1}), respectively. W is the dry weight of EPS (g).

Furthermore, a dimensionless constant known as separation factor (RL) was calculated in order to test the thermodynamic favorability of adsorption using the following equation:

$$RL = \frac{1}{(1 + bC_i)} \tag{4}$$

where b is the Langmuir isotherm constant, and C_i is the initial metal ion concentration (mg L^{-1}). The RL parameter explains the isotherm as follows: RL > 1 means unfavorable; RL = 1 means linear; 0 < RL < 1 means favorable; and RL = 0 means irreversible adsorption [28,29].

The percentage removal potential of metal ions by EPS was calculated using the following equation [27]:

$$\text{Removal potential}(\%) = \frac{\text{Initial metal concentration} - \text{Final metal concentration}}{\text{Initial metal concentration}} \times 100 \tag{5}$$

The adsorption of Cu(II) and Zn(II) with EPS was also studied using the Freundlich isotherm model which assumes a heterogeneous adsorption surface and active sites with different energies. The isotherm is represented by the equation given below:

$$\text{Log } q = \log K_f + n^{-1} (\log C_e) \tag{6}$$

where q and C_e are same as described above. K_f is the Freundlich constant which indicates the adsorption capacity (L g^{-1}), and n represents the exponent known as adsorption intensity. The constants K_f (L g^{-1}) and n can be determined from the linear plot of log q versus log C_e [28,29].

2.9. Statistical Analyses

The means of replicates were compared by utilizing one–way analysis of variance (ANOVA). The differences of means of each parameter among different sets of conditions were tested by least

significant difference (LSD) test. Regression analysis was used to determine the degrees of correlation among related factors. Acceptance of the level of statistical significance was done at 5% level ($p < 0.05$).

3. Results

3.1. Characteristics of EPS

The FTIR spectroscopy determined the functional groups present on the surface of the EPS [4]. A strong signal at 3378 cm^{-1} confirmed the existence of an alcoholic and/or carboxylic OH stretching, and a signal at 2940 cm^{-1} was attributed to the CH stretching. The presence of C≡C was proved by the signal at 2149 cm^{-1}; the conjugated C=O or the H– bonded carboxylic C=O stretching was assigned to the signal at 1637 cm^{-1}; and the 777 cm^{-1} band confirmed the presence of C–X (alkyl halides) stretching vibrations. A zeta potential value of −27.4 mV was obtained for the extracted EPS sample [4]. Both ^{1}H NMR and ^{13}C NMR spectra derived proton signal positions (shielding and deshielding environment) confirmed that the EPS possessed electronegative substituents or groups, unsaturated carbon, CH_2-chain and carbonyl groups. For ^{1}H NMR spectrum of the EPS (Figure 1a), the signals obtained from δ-1.9 ppm and δ-2.3 ppm resembled CH_2 protons. Signals from δ-2.0 ppm confirmed the presence of -CH proton attached to vinylic carbon. Signals from δ-3.5-3.8 ppm were assigned to the presence of electronegative group deshielding, and signals δ-3.9-4.8 ppm confirmed electronegative oxygen coming from the ester functional group. Figure 1b represents the ^{13}C NMR spectrum of the EPS. Signals δ-27.58-32.20 ppm resembled to-CH_2 protonic group. The presence of electronegative group was indicated from signals δ-54.78-63.30 ppm. Signals δ-75.07-80.20 ppm confirmed the presence of unsaturated carbon atoms. The signal of δ-104.13 ppm indicated the presence of unsaturation. The presence of carbonyl group was determined by signal δ-175.49 ppm. Characteristic ^{1}H NMR and ^{13}C NMR signal positions of the EPS are shown in Table 1. The MS-ESI (Mass Spectrometry-Electrospray Ionization) depicts the mass spectra of EPS (Figure 2), and this study further confirmed the molecular weight of the EPS sample while the mass 392 *m*/*z* positive ion mode confirmed the identity of the EPS sample.

Table 1. Characteristic ^{1}H NMR and ^{13}C NMR signal positions with corresponding functional groups of the extracellular polymeric substance (EPS) produced by *Bacillus licheniformis* strain KX657843 isolated from earthworm (*Metaphire posthuma*) gut.

Signal Positions	Functional Groups Present in EPS
	^{1}H NMR signal positions
δ-1.9 ppm	-CH_2 protons
δ-2.0 ppm	-CH proton attached to vinylic carbon
δ-2.3 ppm	-CH_2 protons
δ-3.5-3.8 ppm	presence of electronegative group deshielding
δ-3.9-4.8 ppm	peak due to electronegative oxygen comes from ester functional group
	^{13}C NMR signal positions
δ-27.58-32.20 ppm	-CH_2 protonic groups
δ-54.78-63.30 ppm	presence of electronegative group
δ-75.07-80.20 ppm	presence of unsaturated carbon atoms
δ-104.13 ppm	presence of unsaturation
δ-175.49 ppm	presence of carbonyl group

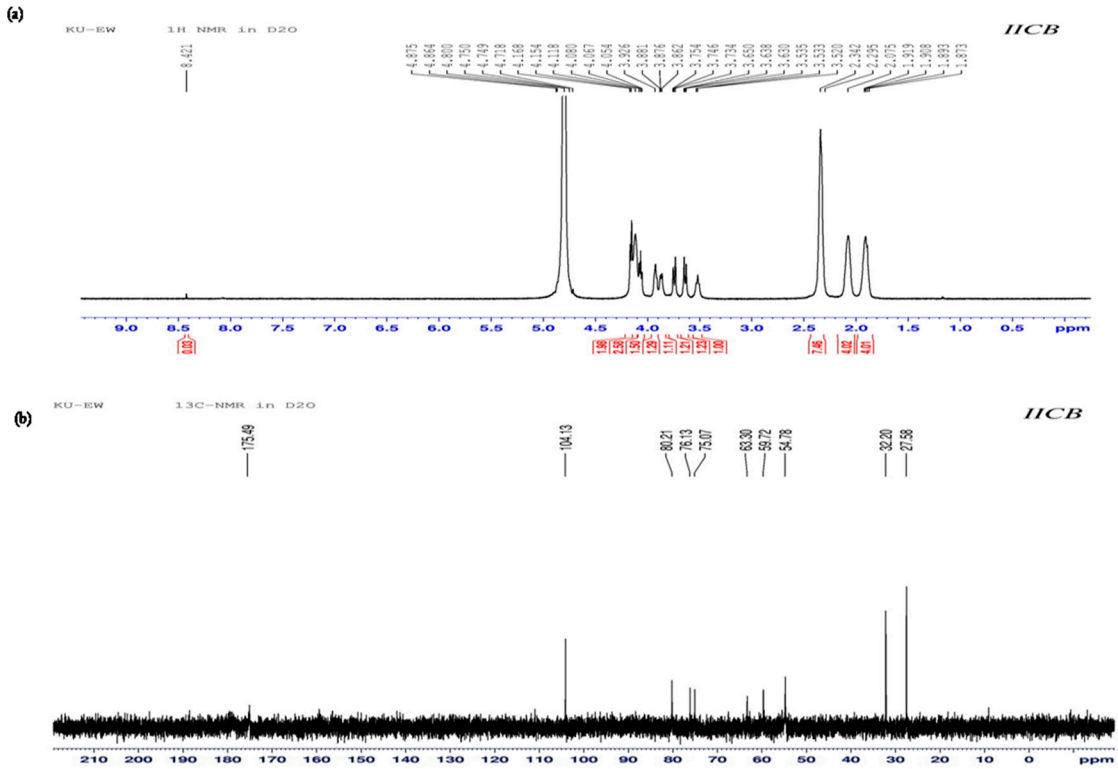

Figure 1. (**a**) ^{1}H Nuclear Magnetic Resonance (NMR) and (**b**) ^{13}C NMR signal positions of extracellular polymeric substance (EPS).

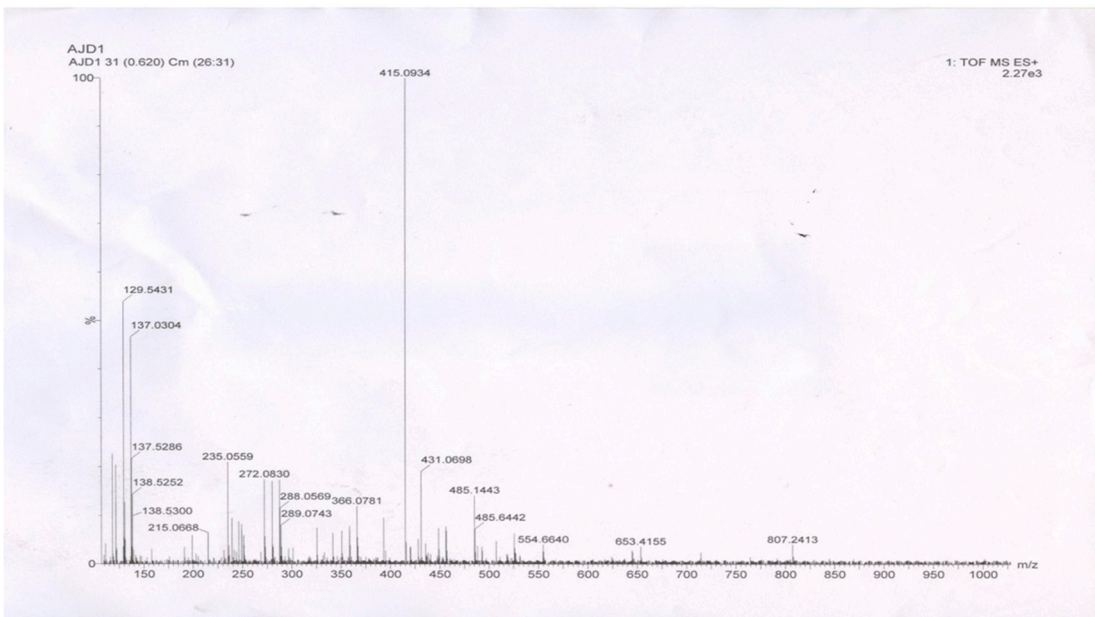

Figure 2. Spectrum of EPS sample.

3.2. Flocculation Activity of EPS

The flocculating efficiency obtained with respect to the varying dosage of EPS is shown in Figure 3a. The results showed that maximum flocculation activity (74%) was obtained at 4 mg L^{-1} concentration of EPS. This concentration was chosen for the rest of the assays. A further increase in EPS concentration resulted in a decrease in the flocculation activity, and there was a high rate of decline of flocculation activity at EPS concentrations of 18 and 20 mg L^{-1}.

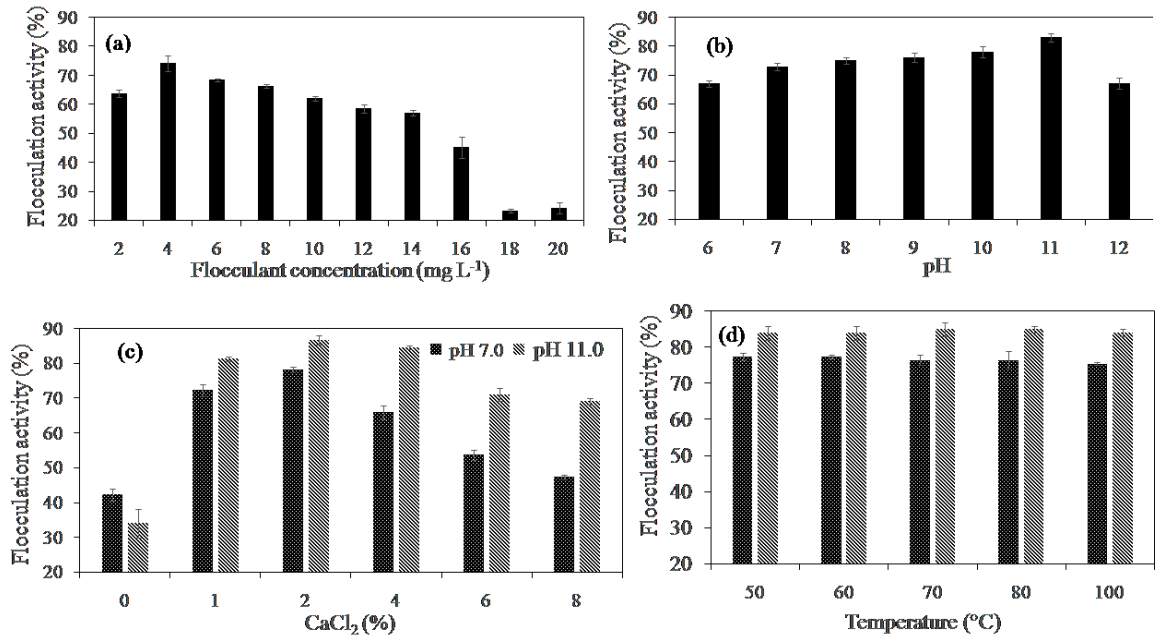

Figure 3. Effect of different controlling factors on flocculation activity performed by *Bacillus licheniformis* strain KX65783 isolated from earthworm (*Metaphire posthuma*) gut: (**a**) varied EPS (flocculant) dosage at a fixed pH = 7 and $CaCl_2$ concentration (2%); (**b**) varied pH at a constant EPS concentration (4 mgL^{-1}) and $CaCl_2$ concentration (2%); (**c**) varied $CaCl_2$ concentration at a fixed EPS concentration (4 mgL^{-1}) and pH 7 and 11; (**d**) varied temperature at a fixed EPS concentration (4 mgL^{-1}), pH 7 and 11, and $CaCl_2$ concentration (2%). The error bars in the graphs represent the standard deviations.

The effect of pH on the flocculation activity demonstrated that the EPS failed to show flocculation activity at pH ≤ 5. Flocculation activity increased gradually from pH 6 to 11 with no significant variation between pH 7–10. Maximum flocculation activity of 83% was observed at pH 11 (Figure 3b). The study on the effect of varying dosage of $CaCl_2$ on the flocculation activity showed that 2% (0.2 M) dosage of $CaCl_2$ possessed the maximum flocculation activity of 72% and 81% at pH 7 and 11, respectively (Figure 3c).

The influence of other ions on flocculation activity (Table 2) showed that K^+ ions and Na^+ ions showed lower flocculation activity than the other ions examined, while the best results were observed with Ca^{2+} ions.

Table 2. Flocculation activity (%) of the extracted EPS in the presence of different ions. The numerical values indicate the means of replicates (n = 3) ± standard deviations.

Ions	Flocculation Activity (%)
$CaCl_2$	72 ± 1
$FeCl_3$	51.7 ± 1.3
$ZnCl_2$	38.5 ± 1.5
$CuSO_4$	46.5 ± 1.5
NaCl	12.5 ± 3.0
KCl	16.5 ± 1.5

The effect of temperature on flocculation activity showed that the flocculation activity was retained even after heating the EPS for 30 min at 100 °C. The flocculation activity was almost constant for all the temperatures both at pH 7 and 11 (Figure 3d).

3.3. Adsorption Efficiency of Cu(II) and Zn(II) at Varying pH

The metal adsorption efficiency showed a similar trend for both Cu(II) and Zn(II) ions at a concentration of 25 mg L^{-1}. There was an increase in removal potential of metal ions with increasing pH for both the metal ions. At pH 5, the EPS was able to remove 54 and 42% of Cu(II) and Zn(II), respectively. At pH 8, the EPS could remove 86 and 81% of Cu(II) and Zn(II), respectively (Figure 4a). The percentage of sorption of Cu(II) increased from 54% at pH 5 to 86% at pH 8. Similarly, for Zn(II) the sorption increased from 42% at pH 5 to 81% at pH 8.

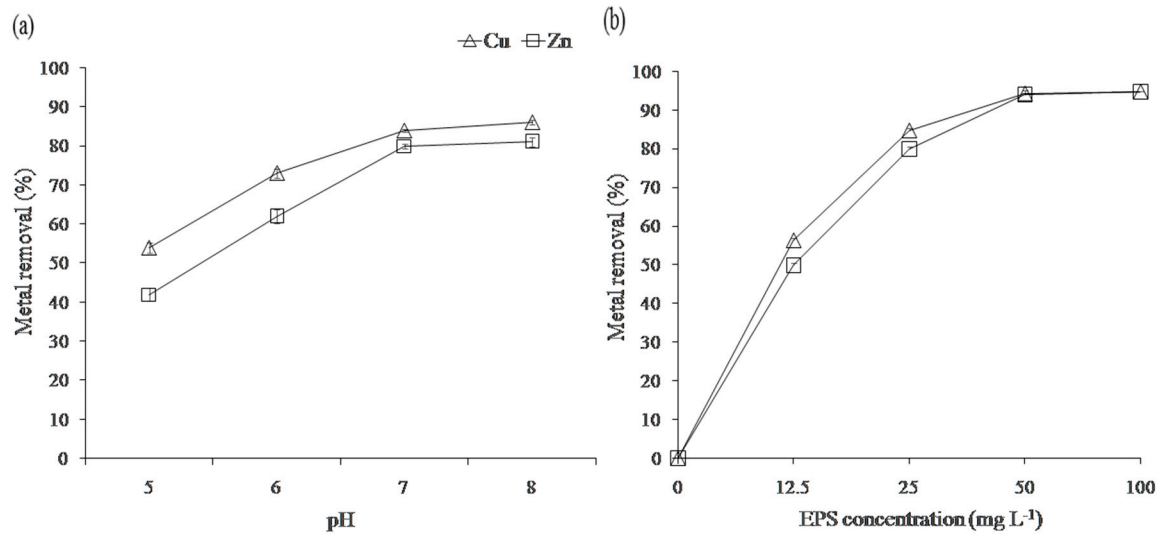

Figure 4. Effect of increasing (**a**) pH at a constant concentration of 25 mg L^{-1} for both metals and EPS and (**b**) EPS concentration on sorption of metals by EPS at a constant pH = 7.0 and a fixed concentration at 25 mg L^{-1} for both metals. The error bars in the graphs represent respective standard deviation.

3.4. Adsorption Isotherms

The effect of EPS dosage on Cu(II) and Zn(II) sorption is shown in Figure 4b. The adsorption of Cu(II) and Zn(II) increased as EPS concentration increased from 12.5 to 50 mg L^{-1}. At 100 mg L^{-1} EPS concentration, the adsorbent got saturated with no further increase in adsorption of the metal ions. The metal removal potential for Cu(II) increased from 56.4 to 94.4% when the EPS concentration increased from 12.5 to 50 mg L^{-1}. Similarly, for Zn(II), the removal potential increased from 50 to 94% when the EPS concentration increased from 12.5 to 50 mg L^{-1}. The further increase in EPS concentration failed to increase the metal sorption. At 100 mg L^{-1} EPS concentration, the metal removal potential was 94.8% for both the metal ions.

The data for adsorption of metal ions by EPS at different concentrations were tested with the Langmuir and Freundlich isotherm models. The correlation coefficients (R^2) of fitting the adsorption data to the Langmuir model were 0.999 for both Cu(II) and Zn(II), which showed that the adsorption of metal ions adequately fitted to the Langmuir model. The maximum adsorption capacities (q_m) obtained from the Langmuir plots were: 58.82 mg g^{-1} for Cu(II) and 52.45 mg g^{-1} for Zn(II) (Table 3). The values for the constant b were: 0.27 L mg^{-1} for Cu(II) and 0.29 L mg^{-1} for Zn(II). The correlation coefficients (R^2) of fitting the adsorption data to the Freundlich model were: 0.95 and 0.97 for Cu(II) and Zn(II), respectively. The values for the constants K_f and n^{-1} obtained from the Freundlich model fitting were: 12.59 and 2.94 for Cu(II), and 12.61 and 2.0 for Zn(II), respectively (Table 3). The R^2 values indicated that the Langmuir isotherm model gave a better fitting to the adsorption data than the Freundlich model. The values of R_L for adsorption of Cu(II) and Zn(II) by EPS were 0.13 and 0.12, respectively.

Table 3. Langmuir and Freundlich isotherm constants for the sorption of Cu(II) and Zn(II) on EPS.

Metal Ions	Langmuir Isotherm			Freundlich Isotherm		
	b (L mg^{-1})	q_m (mg g^{-1})	R^2	n^{-1}	K_f	R^2
Cu(II)	0.27	58.82	0.9993	2.94	12.59	0.952
Zn(II)	0.29	52.45	0.9993	2.0	12.61	0.971

4. Discussion

The majority of the influences of earthworms on nutrient cycling and biogeochemical processes of the ecosystem is due to the joint activities of earthworms and the microorganisms selectively activated within their functional domains [2,30]. The gut of the earthworm is dominated by high amounts of mucus, plant derived saccharides, organic acids, several denitrification-derived gases such asnitrous oxide (N_2O), molecular hydrogen (H_2) and molecular nitrogen (N_2). The earthworm gut also experiences increased total organic carbon, nitrogen, and moisture contents which provide an optimal environment for the activation of dormant microbes and support the germination of ingested endospores [3,31].

The earthworm gut environment can influence EPS producing microorganisms [4]. The isolation of an EPS producing bacterial strain *Bacillus licheniformis* with accession number KX65784 from the earthworm gut (*Metaphire posthuma*) has been reported earlier [4,20]. The isolated strain was unique in producing EPS only in the presence of L-asparagine monohydrate and maximum EPS yield was observed when sucrose was the carbon substrate [4]. The phenotypic and genotypic characterization, optimal conditions for growth and EPS production of the strain isolated from the earthworm gut were also reported earlier [4]. The current study focuses on the application aspect of the EPS produced by the above bacterial strain.

The FTIR and NMR analyses act as powerful tools for studying and speculating several functional groups [32], carbohydrate compositions and structure of complex substances in biomolecules such as EPS. The ^{1}H NMR and ^{13}C NMR spectra (Figure 1a,b) confirmed that the isolated EPS possessed electronegative substituents or groups, unsaturated carbon, CH_2-chain and carbonyl groups. The signals from δ-1.9 ppm and δ-2.3 ppm resembling -CH_2 protons, and signals from δ-3.5-3.8 ppm indicating the presence of electronegative group deshielding were in accordance to earlier report [26]. Previous authors [26] similarly reported a strong signal at δ-1.87 ppm indicating the presence of alkyl -CH_2 protons, while the δ-3.5-3.8 ppm signals indicated the presence of electronegative group. The FTIR analysis showed that majority of the functional groups in the isolated EPS belonged to alkanes, alkynes, carboxylic and alcoholic groups. The results of FTIR and NMR analyses obtained by previous studies [33–35] were in conformity with our results. The EPS composition consisted of carbohydrates with some amounts of proteins and sugar acids, and also traces of nucleic acids. Mass spectra (Figure 2) revealed the mass of the extracted EPS. The anionic nature of the EPS extracted from the gut of the earthworm was confirmed from the zeta potential value [4].

The biological functioning of EPS and EPS producing bacteria are determined by the occurrence of the charged (positive or negative) functional groups. The presence of a significant quantity of negatively charged functional groups, such as carboxyls and hydroxyls, in the EPS evident from the NMR and IR studies indicated a high carbohydrate to protein ratio in the biomolecules. This feature of the EPS might offer a vast promise in the removal and remediation of heavy metals from polluted environments by this EPS [23,28,36].

Microbial EPS are an emerging group of bioflocculants that can potentially replace synthetic flocculants in various applications. A large number of extracellular biopolymeric flocculants have been isolated from diverse groups of microorganisms, but their chemical compositions and activities differ greatly [11,37]. An important parameter in flocculation is the dosage of the applied flocculant. An insufficient dose would affect the bridging mechanism and formation of flocs, while an overdose would inhibit sedimentation of suspended particles and cause high viscosity in the flocculating

medium [38]. In the present study, the bioflocculant showed a flocculation efficiency of 74% at 4 mg L^{-1} concentration of EPS (Figure 3a). Different microbial strains have different flocculation efficiency at different concentrations. The ability of *Bacillus licheniformis* strains to produce bioflocculant has been reported [39,40]. Studies showed that bioflocculant from the strain *Bacillus licheniformis* produced 700 U mL^{-1} flocculation activities after cultivation at 37 °C for 48 h [40]. Another study on *Bacillus* sp. showed that this strain produced a maximum of 83.45% flocculation activity at 12 mg L^{-1} flocculant concentration [11]. Another essential parameter for flocculation is the system pH. In the present study, the flocculation efficiency showed pronounced activity in a basic medium, with the maximum activity at pH 11 (Figure 3b). Bioflocculants exhibit varying degree of electrical states at varying pH. Alteration of pH alters the charge status on bioflocculant which in turn alters the surface characteristics of suspended materials. This results in a change of the flocculation ability of the flocculants [41,42]. The present study found conformity with other reports where alkaline pH favored bioflocculation of suspended particles [37,38,43]. The present study failed to flocculate the suspended kaolin particles at an acidic pH below 6. The increase in flocculation activity at higher pH than at lower pH could be due to the fact that at higher pH the OH^- ions might disrupt the formed complexes between kaolin and the bioflocculant, while lower pH might cause the adsorption of H^+ ions by both the bioflocculant and kaolin particles leading to reduced flocculation activity [44]. These results were supported by other reports where bioflocculant produced by *Bacillus megaterium* strain caused flocculation in the pH range of 7 to 12 with maximum flocculation at pH 9 and acidic pH inhibited flocculation [45].

Another important parameter for flocculation is the presence of cations. Cations neutralize and stabilize negative charges of functional groups of both kaolin particles and the bioflocculant [38]. In the present study, the effect of varying concentrations of $CaCl_2$ on the flocculation activity showed that maximum activity was observed at 2% salt concentration (Figure 3c). Here too, the flocculation activity was better at pH 11 than low pH values. The flocculation activities against various cations are shown in Table 2 indicating that flocculation efficiency was more in the case of divalent cations. The maximum value was for Ca^{2+} ions. Divalent cations neutralize negative charges on both the kaolin particles and bioflocculant, which minimizes the repulsive forces, thereby enhancing adsorption of bioflocculant onto the surface of the kaolin clay leading to agglomeration of flocs and sedimentation of the suspended particles [45]. This result was supported by other reports where divalent cations enhanced flocculation while monovalent ions failed to cause effective flocculation [37,38]. In spite of being a trivalent cation, Fe^{3+} failed to increase the bioflocculation activity in the present study. This could be due to the fact that there was an antagonistic effect between Fe^{3+} and the bioflocculant. Fe^{3+} could have caused an alteration in the charge of kaolin surfaces and at the same time covered the reactive adsorption sites. Consequently, a competition between the positively charged particles and the adsorption sites might have occurred causing an antagonistic effect of Fe^{3+} ions and subsequent decrease in flocculation activity [46]. The result was in accordance with other reports [47] where Fe^{3+} showed least or no flocculation activity.

The experiment on the effect of temperature on the flocculation ability of EPS showed that the EPS was thermally stable (Figure 3d). Flocculation activity was retained even when the EPS was exposed to a temperature of 100 °C for 30 min. The result was akin to other reports where thermal stability of EPS and retention of flocculation activity were shown [38,40,48]. The flocculation activity was higher at pH 11 than at pH 7 even at a varying temperature. Reports suggested that flocculants rich in carbohydrate backbone showed greater thermal stability than those of protein and nucleic acid backbones [38]. The FTIR and NMR analyses of the EPS in our studies showed the presence of carbohydrate in the EPS backbone, thus confirming the thermal stability of the EPS.

Utilizing EPS produced by bacteria in metal adsorption is one of the most profoundly driven approaches in metal remediation [18]. One of the important parameters for metal adsorption by bacterial EPS is the initial pH of the solution. The pH not only affects the speciation of metal ions in the solution, but also influences the surface charges and dissociation of binding sites of the biosorbent [15].

In the present study, the adsorption was low for both the metal ions at pH 5, while the adsorption increased with an increase of pH values from 5 to 8 (Figure 4a).

A low pH is responsible for raising the concentration of hydrogen ions in the solution, which increases the positive charges of functional groups present on the biosorbent surfaces. As a result, an electrostatic repulsion can occur between the positively charged functional groups and the positively charged metal ions. Additionally, a competition between metal ions and hydrogen ions for binding sites on EPS surfaces can also arise, which would result in fewer binding sites available on the surface of EPS to bind the metal ions. At high pH on the other hand, the biosorbent surface becomes negatively charged due to deprotonation of metal binding sites, and consequently the metal ions get electrostatically attracted and adsorbed on the surface [28,36]. Carboxylic groups play an important role in the pH-responsive protonation-deprotonation reactions as discussed above and participate in the complexation of metal ions resulting in the best adsorption performance at pH 7 [49]. The EPS isolated in the present study showed the presence of carboxyl groups [4], which might be the reason behind the better adsorption of metal ions at pH 7 by the isolated EPS. There are reports showing a constant adsorption of Cu(II) from pH 1–9, while for Zn(II) there was an initial increase in adsorption at initial increase in pH from 1–3 after which up to pH 9 there was a constant adsorption without any change [36]. Similarly, reports showed that there was a constant increase in the amount of Cu(II) adsorbed by EPS from 0.09 mmol g^{-1} EPS at pH 3.0 to 0.37 mmol g^{-1} EPS at pH 7.0 [28].

Another important parameter for metal sorption by bacterial EPS is the initial concentration of the EPS used. The capacity of sorption of metal ions by EPS in this study reached a constant value when the concentration of EPS was 50 mg L^{-1} and it remained constant even at 100 mg L^{-1}. This can be due to the fact that, an increase of the biosorbent dosage resulted in an increase in the number of sites available for adsorption at lower EPS concentrations. A higher EPS concentration, on the other hand, led to a saturation of binding sites on the biosorbent surface. Higher concentration of EPS could have resulted in interference between the binding sites themselves. Another reason which led to the formation of the plateau region as seen in Figure 4b could be due to the fact that there was an insufficient number of metal ions in the solution with respect to the number of available binding sites [28,29,50]. A previous study [28] showed that an increase in EPS concentration from 26 to 135 mg L^{-1} resulted in the increase in adsorption of Cu(II), while further increase in EPS concentration failed to increase the adsorption of Cu(II). Similarly, reports showed an increase in adsorption of metal ions with initial increase in EPS concentration while further increase failed to increase the adsorption of metal ions [29,36].

Optimization of adsorption systems and determination of the efficiency of adsorption is accomplished by equilibrium adsorption isotherms. The Langmuir isotherm postulates the monolayer adsorption on homogenous surfaces, and Freundlich isotherm assumes the multilayer formation on heterogenous surfaces [28]. The model parameter values obtained (Table 3) indicated that the adsorption of metal ions onto the EPS synthesized by *Bacillus licheniformis* isolated from the earthworm gut fitted to the Langmuir model better than the Freundlich model. These results indicated a monolayer adsorption mechanism taking place between EPS and the metal ions, in accordance to the Langmuir model [15,28,36].

5. Conclusions

The EPS derived from *Bacillus licheniformis* KX657843 isolated from the earthworm *Metaphire posthuma* showed potential flocculating and metal sorption properties. The zeta potential, FTIR and NMR analyses confirmed the polyanionic nature of the EPS. The majority of components of the extracted EPS were carbohydrates, proteins, sugars and nucleic acids. The EPS also possessed electronegative groups, unsaturated carbon, CH_2-chain and carbonyl groups. The ability to remove heavy metal Cu(II) and Zn(II) from aqueous solutions implied that the EPS could be used as an effective adsorbent for heavy metal sorption in aqueous solutions. The study also confirmed that the adsorption of metal ions by the EPS was in accordance with the Langmuir isotherm model. Thus, the present study

demonstrated that microbial EPS might find applications in addressing multifarious environmental problems including heavy metal remediation, water/wastewater treatment and industrial waste treatment. The emerging potentials of earthworm gut microflora as an environmental management tool warrant future research investigations.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/1/349/s1, Figure S1: The isolation of the bacteria with a characteristic slimy, mucoid phenotypic nature from the gut of the earthworm *Metaphire posthuma*, Figure S2. Atomic absorption spectroscopy (AAS) standard curve for metals Cu(II) and Zn(II).

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