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Extracellular Polymeric Substance Produced by *Lysinibacillus* sp. SS1 in Response to Petroleum Crude Oil Stress Facilitates Removal of Heavy Metals from Aqueous Solutions

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The use of microbial extracellular polymeric substances (EPS) as bioflocculants opposed to synthetic and organic flocculants in the treatment of heavy metal polluted wastewater is currently gaining importance. The present study focuses on the characterization and application of EPS produced by *Lysinibacillus* sp. SS1 in heavy metal removal. *Lysinibacillus* sp. SS1 (10%v/v), isolated from used engine oil contaminated soil, produced EPS and circular white spongy masses on the utilization of 3% petroleum crude oil (PCO) in the Bushnell Haas medium in 10 d. Analysis of extracted EPS and masses revealed that it contained carbohydrates, lipids, and proteins in proportions of 1.3: 94.6: 3.6 and 2.3: 91.5: 6.2, respectively. Characterization by LCMS and FT-IR analysis confirmed that they were predominantly lipids with proteins and polysaccharide groups. Masses could remove 48% of lead and 40% of mercury from aqueous solutions by adsorption on their surface as confirmed by SEM-EDAX. Extracted EPS (100 mg/L) could remove 94.9% of lead from aqueous solution (150 mg/L) in 72 h by precipitation of lead as nanoparticles of sizes ranging from 30–40 nm. This study shows the potential of *Lysinibacillus* sp. SS1 and its EPS in response to PCO in heavy metal removal and recovery.

Keywords: extracellular polymeric substance, lead, *Lysinibacillus* sp. SS1, mercury, nanoparticles, petroleum crude oil

INTRODUCTION

The increasing urbanization and industrialization in the present-day scenario have led to extensive effluent dumping into water bodies. Wastes from industries such as dyeing, chemical, petroleum, and polymer generate huge quantities of wastewater (Agunbiadea *et al*. 2018). These effluents are heavily loaded with toxic heavy metals that are extremely harmful and are often ingested by animals and humans due to their non-biodegradable and extremely stable nature (Zhu *et al. 2019*). High dosages of these heavy metals can lead to extreme health conditions and can also be fatal in certain cases (Beni and Esmaili 2020). Water treatment in modern times has to focus on heavy metal removal, apart from the removal of suspended solids and microbial contamination.

Flocculation is most commonly used in industries for the removal of heavy soluble particles by forming continuous masses called flocs. Floc formation is induced by the aggregation of suspended matter and colloids by a stimulant called a flocculating agent (Sajayan *et al.* 2017). There are many kinds of flocculating agents – inorganic (aluminum sulfate and polyaluminum chloride), organic (polyethyleneimine and polyacrylamide), and natural

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flocculants (chitosan, guar gum, *etc.*) (Renault *et al*. 2009). Although organic and inorganic flocculants have been used for the treatment of wastewater, they have also been found to be harmful to the ecosystem, which has given rise to a need for the use of bioflocculants (Subudhi *et al.* 2016; Hassimi *et al.* 2020).

EPS are molecules secreted by microorganisms under physiological stress encountered in the natural environment (Decho and Guteirrez 2017). Bioflocculants are mainly EPS composed of exopolysaccharides, glycoproteins, glycolipids, proteins, and lipids produced by microorganisms in response to stress (More *et al.* 2014). The usage of bioflocculants could be extremely useful due to its biodegradability, non-toxicity, and absence of secondary pollutant production (Bukhari *et al.* 2018). The EPS can be produced at low cost and are favorable to the environment and living organisms around them (Ates 2015). There have been numerous reports on the utilization of microbial EPS as bioflocculants for the treatment of various industrial wastewater samples (Agunbiadea *et al.* 2018; Ayangbenro *et al.* 2019; Tawila *et al.* 2019; Fan *et al*. 2019).

Many bioflocculants have been worked upon in recent years; however, most of them are cation dependent (Joshi *et al.* 2017). These bioflocculants do not work unless an additional salt such as $CaCl_2$ or cation such as Zn^{2+} or Ca^{2+} is added (Makapela *et al.* 2016). The addition of salts and ions increases the cost and also acts as a pollutant burdening the users and the environment (Okaiyeto *et al.* 2013). Hence, progress is being made towards the usage of cation independent bioflocculants.

The present study deals with the extraction and characterization of spongy white mass and EPS released by *Lysinibacillus* sp. SS1 in response to PCO. The ability of the bioflocculant and spongy mass to remove heavy metals such as lead and mercury from water samples was studied. This report is one among very few reports on purification, characterization, and application of bioflocculants produced in response to PCO.

MATERIALS AND METHODS

Bacterium, Substrates, and Chemicals

The bacterium *Lysinibacillus* sp. SS1 used in this study was isolated from soil procured from a motor garage in Moodabidri, Dakshina Kannada, India by screening for degradation of PCO. PCO was procured from a petroleum refinery industry situated in Mangalore, India. All the other substrates and chemicals used were of analytical grade unless otherwise mentioned.

Production of EPS Using *Lysinibacillus* sp. SS1

Lysinibacillus sp. SS1 (16 h culture, 10%v/v, $1X10^8$ CFU/ml) was inoculated into sterile BH broth (g/L: $MgSO_4$ 0.2, $CaCl_2$ 0.02, KH_2PO_4 1.0, K_2HPO_4 1.0, NH_4NO_3 1.0, $FeCl_2$ 0.05) supplemented with 3%v/v PCO as sole carbon source in 250 ml Erlenmeyer flasks. The flasks were agitated at 80 rpm for 10 days at 27 °C as performed previously.

Extraction and Purification of EPS

The bacterial cells were separated by centrifuging the culture broth at 10,000 rpm for 20 min (Ayangbenro *et al.* 2019). The spongy white masses, which floated in the supernatant, were separated by filtration. They were then washed with hexane and distilled water twice. The supernatant was collected and mixed with cold ethanol in 1:4 ratios by rapid stirring. After keeping this mixture at 4 °C overnight, it was then centrifuged at 8000 rpm for 15 min at 15 ˚C. The supernatant was discarded and the precipitate was collected. This was dissolved in distilled water and mixed with an equal volume of a chloroform-butanol mixture (ratio 2:1). It was then added to the extraction funnel and allowed to stand for 12 h (Goveas and Sajankila 2020). The solvent fraction was then evaporated by heating at 60 °C and then air-dried completely to get dried flakes of partially purified EPS.

Characterization of Masses and Purified EPS

The presence of proteins, lipids, and carbohydrates in the masses and purified EPS was checked by qualitative tests using Molisch's reagent, ninhydrin, and Sudan Black, respectively (Abdalsadiq *et al.* 2018). The quantitative analysis of proteins, lipids, and carbohydrates in the masses and purified bioflocculant was performed by Lowry's method (Lowry *et al.* 1951), phenol/sulfuric acid method (Dubois *et al.* 1956), and silphia/phosphovanilin method (Knight *et al.* 1972), respectively.

The masses and dried purified EPS (100 mg each) were analyzed by Fourier transform infrared (FT-IR) spectroscopy (Perkin Elmer System 2000, England) to determine their functional groups. The samples were mixed with potassium bromide (200 mg) to form a pellet and analyzed over a frequency range of 370–4000 cm^{-1}. The samples were then solubilized in acetonitrile and analyzed using liquid chromatography – mass spectrophotometer (LCMS) (1260 Infinity LC fitted with 6410 Triple Quadrupole MS, Agilent Technologies, USA), as per François *et al.* (2012) with full scan for negative mode from 100–1000 (m/Z).

Removal of Lead and Mercury by Masses

Aqueous solutions of lead and mercury (25 mg/L) were incubated with the masses (0.5%w/v) for 72 h at 100 rpm in triplicates. Distilled water with masses plus solutions

of lead and mercury devoid of masses were maintained as the controls. The residual heavy metal concentration in the solution after 72 h of incubation was estimated by AA240FS atomic-absorption spectrophotometer (Varian Company, USA). The percentage removal of heavy metals by the masses was calculated as per the following equation:

$$Removal\ (\%) = \frac{Co - Cr}{Co} * 100 \quad (1)$$

where C_o and C_r are the initial and final residual concentration of heavy metals in the solution.

The masses were separated from aqueous solutions of heavy metals and washed gently with sterile distilled water twice. They were then analyzed for structural differences and the presence of heavy metals on their surface by scanning electron microscopy and energy-dispersive X-ray spectroscopy (SEM/EDAX). The masses were coated with gold and microscopic images were taken using a field emission SEM unit (JEOLJSM-7800F).

Removal of Lead by Purified EPS of *Lysinibacillus sp.* SS1

An aqueous solution of lead (25 mg/L) was incubated with the purified EPS (200 mg/L) for 24 h at 100 rpm in triplicates. The residual concentration of lead in the solution was determined by AAS and the percentage removal of lead by bioflocculant was calculated, as per Equation 1.

Study of Effect of Factors on Lead Removal by Purified EPS by One Factor at a Time (OFAT) Approach

The OFAT approach involves the concept of studying the effect of one factor by varying its value, whereas all the other factors are fixed at a constant value. The next factor is then varied by fixing the previously varied factor at a value wherein the maximum output was attained (Goveas *et al*. 2020). In this study, lead concentration (25–150 mg/L), EPS concentration (100–500 mg/L), and time (12–72 h) were varied by the OFAT method. All the experiments were performed in triplicates. The effect of these factors on the lead removal was estimated by measurement of residual lead in the solution and calculation of percentage removal of lead.

SEM-EDAX Analysis

The precipitate obtained on incubation of aqueous solution of lead with purified bioflocculant was checked for its structure and composition by SEM-EDAX analysis. The sample was prepared, as mentioned earlier.

RESULTS AND DISCUSSION

Extraction and Purification of EPS from the Culture Broth

When *Lysinibacillus* sp. SS1 was grown in BH medium in presence of PCO as the sole carbon source, many solid white spongy masses were observed to be formed 7 d after incubation, which then increased in size (Figure 1a). These masses were carefully separated and washed with hexane and distilled water and kept for further use at 4 ºC (Figure 1b).

The yield of EPS obtained on purification from the culture broth was found to be 6.75 g/L. *Halobacillus* sp. Mvuyo isolated from Algoa Bay sediment samples produced a bioflocculant yield of 3.08 g/L 3 d post-incubation (Cosa *et al.* 2013). *Pseudomonas boreopolis* G22 produced a maximum bioflocculant yield of 2.08 g/L after 15 d of incubation (Guo *et al.* 2018).

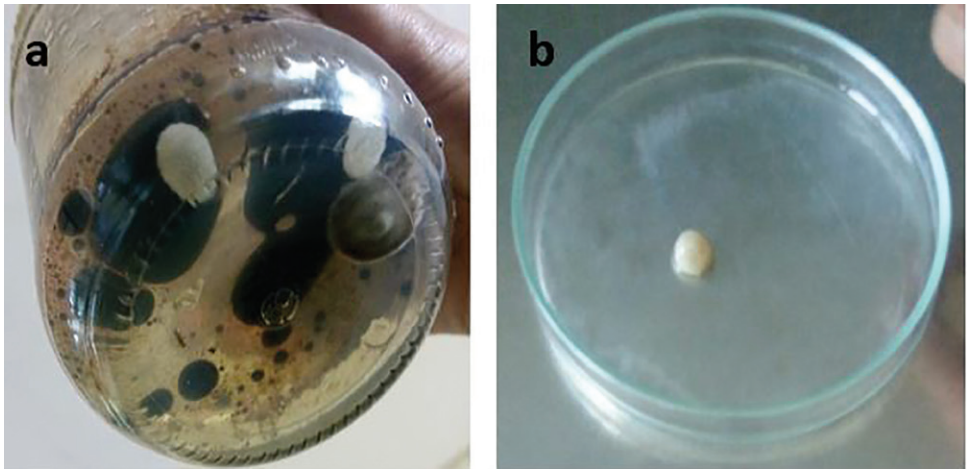

Figure 1. White spongy circular masses formed in the culture medium of exposure of *Lysinibacillus* sp. SS1 to PCO (a and b).

Characterization of EPS and Masses

Qualitative and quantitative characterization. The qualitative analysis of both the extracted EPS and separated masses revealed the presence of carbohydrates, proteins, as well as lipids. EPS and masses were both found to contain carbohydrates, proteins, and lipids in the ratio 1.8: 3.6: 94.6 and 2.3: 6.2: 91.5.

FT-IR spectroscopy. The FTIR spectrum of both the masses and purified EPS showed significant similarities with few differences. Both the products showed significant peaks at around 3400–3450 cm^{-1}, confirming the presence of hydroxyl (O-H) and amine groups (Figures 2a and 2b) (Deng *et al.* 2003). The presence of sharp peaks at 1458 cm^{-1} and 1637 cm^{-1} (masses) plus 1436 cm^{-1} and 1678 cm^{-1} (EPS) are characteristic of antisymmetric extension of C=O, confirming the presence of carboxyl groups (Sun *et al.* 2015). The weak bands observed at 921 cm^{-1} and 979 cm^{-1} in the case of masses and EPS, respectively, are the characteristics of the β-glycosidic linkage of monomers of sugars (Guo *et al.* 2013). The presence of a weak vibration band at 1757 cm^{-1}, peaks between 800–900 cm^{-1}, and at 1070 cm^{-1} in the FT-IR spectrum of EPS are characteristics of esters (C=O stretching) and aromatic rings (Figure 2a). The presence of a medium peak at 711.37 cm^{-1} and 1109 cm^{-1} in the spectrum of masses are also characteristics of ester linkages and aromatic rings (Figure 2b) (Ferhat *et al.* 2011). Hence, the findings of FTIR analysis confirmed that both the masses and EPS comprised of carbohydrates, lipids, and proteins. Studies have shown that hydroxyl, carboxylic, and amine groups are the functional groups that play a major role in flocculation of heavy metals (Zhang *et al.* 2013; Fan *et al.* 2019).

LCMS analysis. LCMS analysis of the masses was done and several peaks were detected. The peaks of lower values correspond to the removal of methyl groups, indicating its presence in the sample (Figure 3a). The peaks 258.87, 284.89, 409.12, and 410.13 denote the loss of nine methylene groups (Patowary *et al.* 2016). The

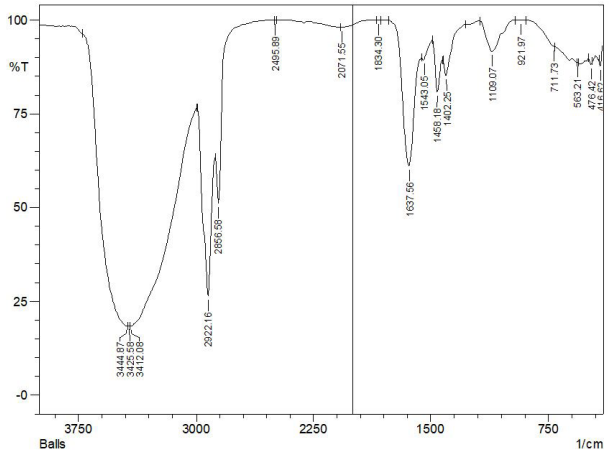

Figure 2a. FT-IR spectrum of masses produced on growth of *Lysinibacillus* sp. SS1 in PCO supplemented medium.

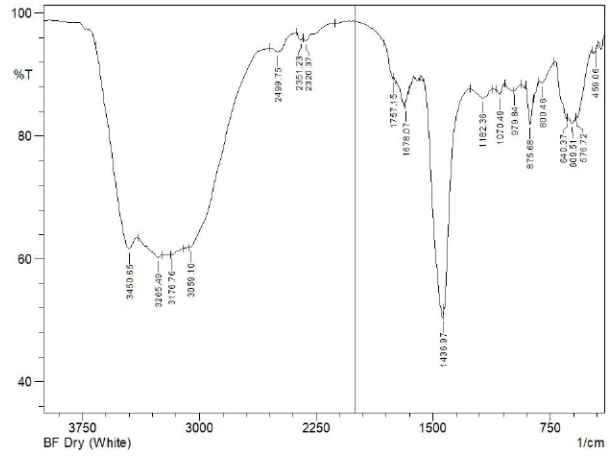

Figure 2b. FT-IR spectrum of purified EPS produced by *Lysinibacillus* sp. SS1 in response to PCO.

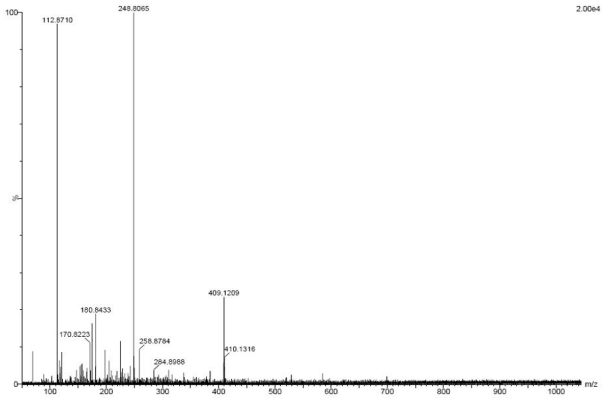

Figure 3a. ESI spectrum of the masses produced on growth of *Lysinibacillus* sp. SS1 in PCO supplemented medium.

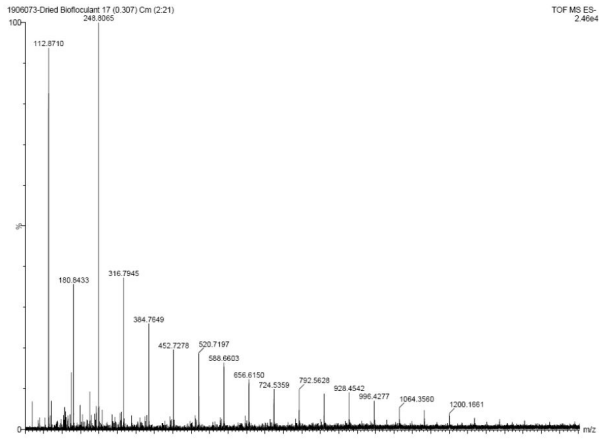

Figure 3b. ESI spectrum of the purified EPS produced by *Lysinibacillus* sp. SS1 in response to PCO.

peak corresponding to 284.80, which is found in 100% composition, denotes the loss of oleic acid (Abdalsadiq *et al.* 2018).

LCMS analysis of the purified EPS revealed the presence of lipopeptides and protein in the sample. This is denoted by the peak obtained at 1200.61 (Figure 3b). Furthermore, 1064.35 corresponds to $[C_{64}H_{117}O_{11} + Na]^+$. The peak 996.42 was produced by the loss of one group of methylene (CH_2=14) whereas peaks 724.53, 588.6603, 452.72, 316.79, 364.76, and 248.80 correspond to the loss of nine methylene groups (CH_2=14) (Abdalsadiq *et al.* 2018).

Removal of lead and mercury by masses. The masses could remove 48% of lead (13–25 mg/L) and 40% of mercury (15–25 mg/L) in 24 h when used at a concentration of 0.5 g/L. In order to confirm if the heavy metals were adsorbed on the surface of masses, SEM-EDAX analysis of the masses exposed to heavy metals and maintained as control was performed. The plain mass was found to have a clear ribbon-like appearance (Figure 4a), whereas the masses incubated in heavy metal solutions depict shrinkage of the surface with visualization of fine precipitate (Figures 4b and 4c). EDAX analysis confirmed the presence of lead and mercury on the surface of agglomerates incubated in aqueous solutions of lead and mercury, respectively (data not shown); in the case of plain mass, heavy metals were found to be absent. Exopolysaccharide produced by *Paenibacillus peoriae* removed 80% of 100 mg/L lead by adsorption, which was confirmed by SEM analysis (Fella-Temzi *et al.* 2018)

Removal of lead by purified EPS of Lysinibacillus sp. SS1. The purified EPS (200 mg/L) could remove –16% of lead from an aqueous solution containing 25 mg/L of lead in 24 h. An EPS producing bacteria UPI-7 could successfully remove 61% of 50 mg/L lead by biosorption in 72 h (Priyatharshini *et al*. 2019).

Effect of Parameters on Lead Removal by Purified EPS

Effect of lead concentration. Aqueous solutions of different lead concentrations (25–150 mg/L) were incubated with 200 mg/L of EPS for 24 h at 100 rpm. It was observed that the percentage removal of lead increased with an increase in lead concentration (Figure 5a) and reached a maximum of 63% for 150 mg/L of lead.

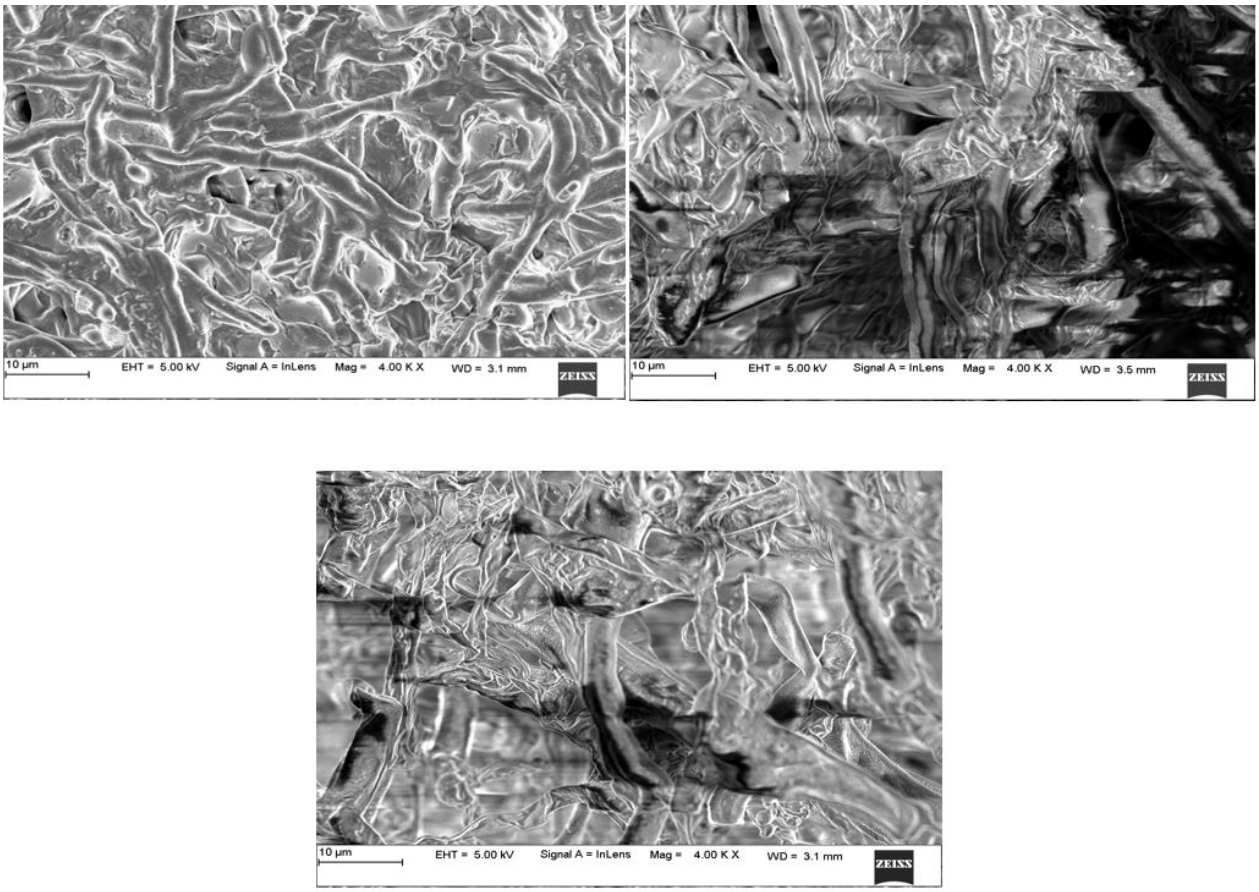

Figure 4. SEM analysis of plain mass (a): mass incubated in aqueous solution of lead (b), and mass incubated in aqueous solution of mercury (c).

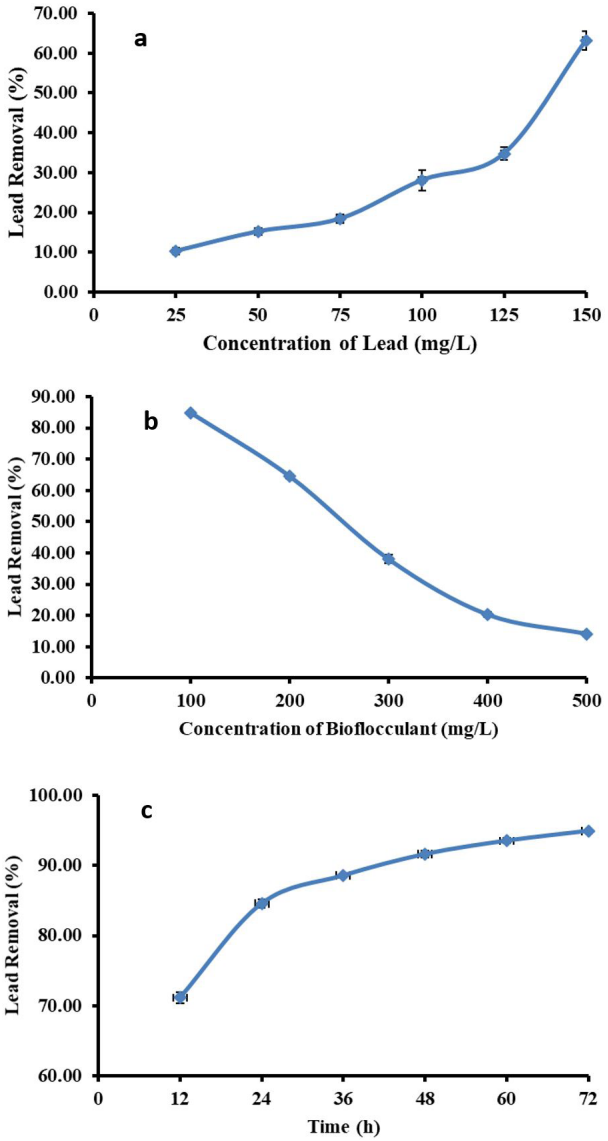

Figure 5. Effect of lead concentration (a), EPS concentration (b), and time (c) on the lead removal by purified EPS of *Lysinibacillus* sp. SS1.

Effect of EPS concentration. When varying concentrations (100–500 mg/L) of purified EPS were incubated with aqueous solutions of 150 mg/L of lead it was surprising to note that on an increase in the concentration of EPS, the percentage removal of lead was found to decrease (Figure 5b). About 84.4% of the total lead was removed when 100 mg/L EPS was added in 24 h. EPS produced by a Gram-negative bacteria consortium (100 mg/L) removed 75% of lead added in 24 h (Gawali *et al.* 2014).

Effect of time. An aqueous solution of 150 mg/L of lead was incubated with 100 mg/L of EPS for time periods of 12–72 h. A sharp increase in percentage removal of lead was observed up to 24 h, beyond which a steady increase was seen up to 72 h (Figure 5c). Hence, from OFAT studies, maximum percentage removal of 94.9% in 150 mg/L of lead by 100 mg/L EPS was obtained in 72 h. EPS produced by *Cloacibacterium normanens* removed 80% of nickel (50 mg/L) in 2 h (Nouha *et al.* 2016). EPS produced by *Pseudomonas* sp. W6 could remove 65% of lead from polluted water in 12 h (Kalita and Joshi 2017).

SEM-EDAX Analysis

When the EPS (100 mg/L) was added to an aqueous solution of lead (150 mg/L) after agitation for a few hours, a fine white precipitate appeared and subsequently formed masses attached to the bottom of the glass after 72 h (Figure 6). This white precipitate was carefully removed and analyzed by SEM-EDAX to determine its structure and composition. The SEM-EDAX analysis of the white precipitate showed the presence of intensely

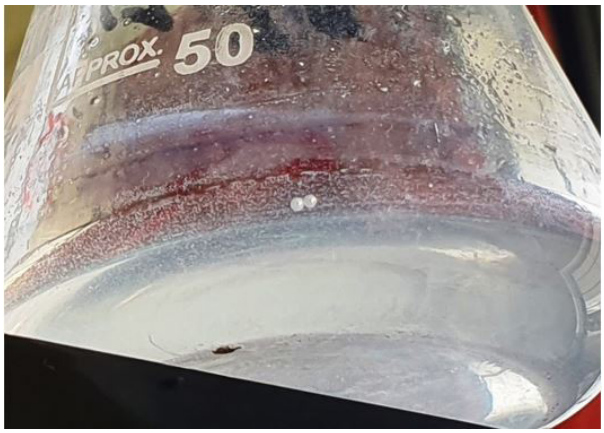

Figure 6. Formation of white precipitate and aggregate on agitation of an aqueous solution of lead with extracted EPS of *Lysinibacillus* sp. SS1.

aggregated lead nanoparticles of sizes ranging from 30–40 nm (Figures 7 and 8). Hence, it is established that the EPS results in the formation of nanoparticles on incubation with lead solution, which then form masses and are precipitated out of the solution. This can aid in the removal of lead from industrial effluents rich in lead and also produce nanoparticles that can be effectively used in various applications. Silver nanoparticles of 10–60-nm size were formed on the addition of polysaccharide-based bioflocculant produced from *Streptomyces* sp. to aqueous silver nitrate solution (Manivasagan *et al.* 2015). EPS extracted from *Shewanella oneidensis*, *Aeromonas hydrophila*, and *Pseudomonas putida* could reduce silver ions to nanoparticles (Li *et al.* 2016).

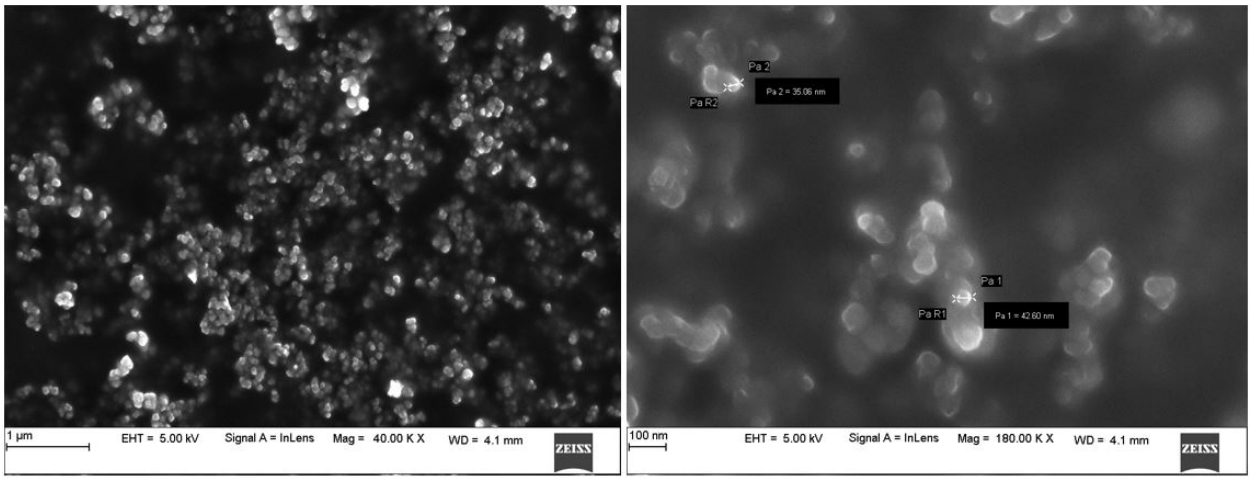

Figure 7. SEM analysis of white precipitate showing the formation of aggregates of lead nanoparticles of sizes ranging from 30–40 nm.

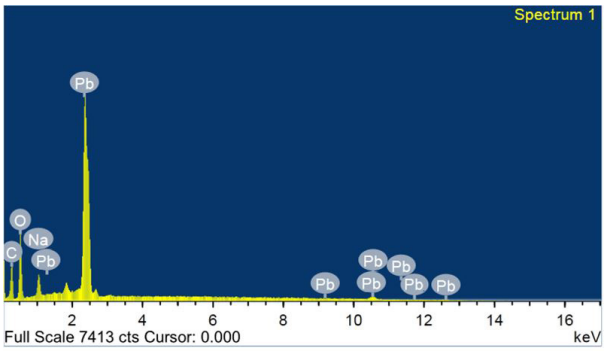

Figure 8. EDAX analysis of white precipitate confirming the presence of lead in the aggregates of nanoparticles.

CONCLUSIONS

Lysinibacillus sp. SS1 isolated from motor garage soil could effectively utilize PCO as a sole source of carbon and produce solid masses and EPS in response to it. Quantitative analysis *via* characterization by FT-IR and LC-MS revealed that these products were composed mainly of lipids with trace amounts of carbohydrates and proteins. Both the masses and purified EPS could effectively remove heavy metals such as lead and mercury from aqueous solutions, indicating their potential to be used in the treatment of heavy metal contaminated wastewater samples. Further, these products could also be exploited for the removal of other harmful pollutants (*e.g.* dyes, pesticides/insecticides) and for the removal of suspended solids and bacteria.

STATEMENT ON CONFLICT OF INTEREST

The author has no conflict of interest to declare.

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