Indian J. Microbiol. (March 2008) 48:49–64

REVIEW

Microbial extracellular polymeric substances: central elements in heavy metal bioremediation

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Received: 27 September 2007 / Final revision: 26 November 2007 / Accepted: 8 January 2008

Abstract Extracellular polymeric substances (EPS) of microbial origin are a complex mixture of biopolymers comprising polysaccharides, proteins, nucleic acids, uronic acids, humic substances, lipids, etc. Bacterial secretions, shedding of cell surface materials, cell lysates and adsorption of organic constituents from the environment result in EPS formation in a wide variety of free-living bacteria as well as microbial aggregates like biofilms, bioflocs and biogranules. Irrespective of origin, EPS may be loosely attached to the cell surface or bacteria may be embedded in EPS. Compositional variation exists amongst EPS extracted from pure bacterial cultures and heterogeneous microbial communities which are regulated by the organic and inorganic constituents of the microenvironment. Functionally, EPS aid in cell-to-cell aggregation, adhesion to substratum, formation of flocs, protection from dessication and resistance to harmful exogenous materials. In addition, exopolymers serve as biosorbing agents by accumulating nutrients from the surrounding environment and also play a crucial role in biosorption of heavy metals. Being polyanionic in nature, EPS forms complexes with metal cations resulting in metal immobilization within the exopolymeric matrix. These complexes generally result from electrostatic interactions between the metal ligands and negatively charged components of biopolymers. Moreover, enzymatic activities in EPS also assist detoxification of heavy metals by transformation and subsequent precipitation in the polymeric mass. Although the core mechanism for metal binding and / or transformation using microbial exopolymer remains identical, the existence and complexity of EPS from pure bacterial cultures, biofilms, biogranules and activated sludge systems differ significantly, which in turn affects the EPS - metal interactions. This paper presents the features of EPS from various sources with a view to establish their role as central elements in bioremediation of heavy metals.

Keywords Microbial exopolymer · biofilm, bioflocs, activated sludge, biogranule, heavy metals, biosorption, bioreduction, bioremediation, biodegradation

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Introduction

Extracellular polymeric substances (EPS) are biosynthetic polymers produced by both prokaryotic and eukaryotic microorganisms growing in natural as well as artificial environments either as single species, in binary association or in heterogenous communities. Irrespective of their origin, EPS are localized at or outside the bacterial cell surface and comprised of a variety of high molecular weight organic macromolecules such as polysaccharides, proteins, nucleic acids, phospholipids along with other nonpolymeric constituents of low molecular weight [1]. Although, these biogenic polymers mediate contact and exchange processes with biotic and abiotic environments, they may not be essential for growth and viability in free-living bacterial cultures. But

during growth under natural environment, bacterial EPS play important role in cell adhesion, formation of microbial aggregates such as biofilms, flocs, sludges and biogranules [2–4] and protect cells from hostile environments. They are also involved in the degradation of particulate substances, sorption of dissolved materials including heavy metals, [5] leaching of minerals from sulphidic ores as well as biocorrosion [6].

Over the last few decades studies on the use of microorganisms for environmental restoration have primarily focused attention towards exploiting microbial potential for remediation of heavy metal contamination in both terrestrial and aquatic systems. Bioremediation of toxic metals and radionuclides from polluted sediments and waste stream employ living and / or non-living microbial biomass or isolated biopolymers as agents for biosorption [7, 8]. These relatively simple and inexpensive technologies try to exploit the cation-binding ability of microbial biomass / biopolymer to form a stable, non-toxic complex. The electrostatic interactions between the metal ligands and negatively charged biopolymeric substances outside the cells lead to formation of stable complexes. Though availability of cheap biomass and immobilization techniques have made bioremediation process advantageous, the metal-binding capacity depends on the nature of biomass preparation and availability of specific biopolymers. Moreover, microbial EPS differs both in specificity and metal-sorption capacity with only few functional groups potentially involved in cation-binding. In spite of all these constraints, the ease of application of purified EPS and that in biofilm, activated sludges or biogranules in bioremediation have demonstrated efficiency in several studies which have led to a large number of reports favoring this approach [9–16].

The aim of this review is to provide an overview of the structural and functional aspects of extracellular polymers with special emphasis on its isolation, composition, production and degradation. It also focuses attention towards the cation binding ability of EPS from isolated bacteria, biofilms, activated sludges and biogranules with an endeavor to establish the role EPS as the sole component in heavy metal bioremediation.

Microbial extracellular polymeric substances

Ever since the introduction of the term, extracellular polymeric substances (EPS) have been defined variously in the literature. As early as 1982, it was coined as substances of biological origin that participate in the formation of microbial aggregates [17]. While, in relation to biofilm structure and function, it was described as "organic polymers of microbial origin, which in biofilm systems are frequently responsible for binding cells and other particulate materials together (cohesion) and to the substratum (adhesion)" [18]. In recent times, scientists believe that EPS are biopolymers resulting from different microbial processes like active secretion, shedding of cell surface materials, cell lysis and adsorption from the environment [1]. They are located at or outside the cell surface independent of their origin and are distinguished as bound EPS and soluble EPS based on their physical state [19]. In simple experimental terms,.soluble EPS can be extracted by centrifugation alone, while the bound one requires additional treatment for their extraction [1]. Metabolically, the soluble EPS are actively secreted by bacteria and are biodegradable, while the bound one remains attached to active and inert biomass or are molecules resulting from cell lysis [20].

Production and extraction

Production of EPS by bacteria in culture or in aggregates depends on a number of factors, such as microbial species, phases of growth, nutritional status and the environmental conditions. In *Rhodopseudomonas acidophila*, the phases of growth, substrate type and C/N ratio have influenced the anaerobic production of water-soluble EPS which was inversely proportional to bacterial dry weight. The amount of EPS produced decreased in order of the following carbon sources: benzoate >acetate >butyrate >propionate >succinate >malonate. A high NaCl concentration however resulted in more EPS production contrary to $CaCl_2$ [21]. Similarly, in anaerobically growing *Schewanella* spp. the terminal electron acceptors were found to influence the quantity and composition of capsular exopolymers. Nitrate- and fumarate-grown cells produced low EPS compared to trimethylamine N-oxide (TMAO)-grown cells. During growth on TMAO, the exopolymer appear as a continuous, convoluted layer covering the entire cell surface, which influence a variety of cell interactions, including aggregation, attachment to surfaces and binding of aqueous metal species [22].

Enhanced production of EPS was induced by the so-called stressful culture conditions. This was true in biofilms of some enterobacteriaceae members where availability of carbon substrates both inside and outside the cell and balance between carbon and other limiting nutrients influenced the synthesis of EPS inside a biofilm. Presence of excess carbon and limitations of nitrogen, potassium or phosphate also promoted synthesis of EPS [2]. However, for a number of bacterial species increased EPS synthesis was found to be genetically controlled and differentially expressed genes in biofilm state regulate its composition and development.

Toxic substances including heavy metals stimulated the production of EPS in *Rhodopseudomonas acidophila* but reduced the cell dry weight [23]. Likewise, exposure of *Pseudomonas putida* unsaturated biofilm to chromium resulted in elevated production of extracellular carbohydrate, protein and DNA [24].

In aerobic biogranules, polymer synthesis is influenced by the type of reactors used, composition of substrate and their rate of loading, hydraulic retention time, hydrodynamic shear force, settling time and periodical feast-famine regime in sequence batch reactors (SBR), culture temperature, etc. [25, 26]. It has been postulated that environmental modifications either alter the composition of the microbial consortium by an increase or decrease in EPS producer organisms or the existing microbial community may re-regulate the metabolic pathway of EPS production [27]. However, it is yet to demonstrate whether the genes for EPS production are expressed before or after bacterial granulation.

In a standard protocol, extraction of EPS is carried out following appropriate physical or chemical methods or combinations thereof. The different methods of extraction and analysis of EPS in general [19] and that of biofilm [28] in particular have been reviewed. As convention, physical methods like low- and high-speed centrifugation, blending, ultrasonication or heat treatment have often been employed in pure cultures as well as in activated sludge systems. High-speed centrifugation was most effective for bacteria in culture but not for bound EPS from microbial aggregates. However, heat treatment has been used effectively in many studies but it causes significant lysis and disruption of cells leading to the contamination of EPS. Chemical extraction methods involve the use of ethylene diamine tetraacetic acid (EDTA), cation exchange resins, NaOH and NaCl. Removal of the divalent cations, mainly Ca(II) and Mg(II) by cation exchange resin and EDTA lead to effective release of EPS in biofilm systems, activated sludge and pure bacterial cultures but use of EDTA also causes destabilization of cell wall and release of intracellular macromolecules in the EPS. Critical evaluation of protocols have emphasized that extraction by cation exchange resin seems to be the method of choice for the separation of EPS from the microbial cells [29].

In extracting EPS from aerobic/sulfate reducing and nitrifying/denitrifying biofilm samples, centrifugation with formaldehyde gave the greatest carbohydrate yield, while steam extraction yielded highest protein with insignificant cell lysis [30]. Extraction of EPS with dicyclohexyl-18-crown-6 ether which selectively binds alkaline and alkaline earth metals but not heavy metals was comparable with that of cation exchange resin extraction [31]. Removal of polysaccharide and proteins from aerobic biofilms by either EDTA or NaOH was enhanced by the alternating current especially within the first 0.5 h of current application [32]. To overcome the leakage of intracellular materials during extraction of EPS from *Pseudomonas fluorescens* and *Alcaligenes denitrificans* biofilms, Dowex resin and sonication were used following pretreatment with glutaraldehyde. The later act as a protectant against cell lysis and pre-treatment was effective when combined with sonication [33]. Irrespective of the methods used, the extracted EPS are further purified and analyzed to determine its macromolecular composition, physico-chemical properties and biological functions [34–36].

Compositional analysis

In general microbial EPS from diverse sources are chemically complex and represent a mixture of macromolecules, such as proteins, polysaccharides, lipids and nucleic acids. Compositional analysis and extraction methods of EPS from pure bacterial cultures, biofilms, activated sludges and biogranules are presented in Table 1. The exopolysaccharides represent the major component of the macromolecules accounting some 40–95% of the microbial EPS [37]. Production of exopolysaccharides by diverse microbial groups growing under *in vitro* and *in vivo* conditions including biofilms have been reviewed extensively [2, 38–41].

Being composed of a number of monosaccharides and non-carbohydrate substituents, EPS are distinguished as homo- and heteropolysaccharides. While the homopolysaccharides are neutral, majority of the heteropolysaccharides are polyanionic due to the presence of either uronic acids (glucuronic acid, galacturonic acid and mannuronic acid) or ketal-linked pyruvate. Inorganic residues, such as phosphate or rarely sulphate, may also confer polyanionic status [42]. In very few cases EPS may even be polycationic, as exemplified by the adhesive polymer obtained from *Staphylococcus epidermidis* strains associated with biofilms [43]. The free, capsular and biofilm EPS of *Pseudomonas* NCIMB 2021 were found to be carbohydrate-protein copolymers [44]. The carbohydrate moieties were acidic and *O*- and *N*-acetylated with relatively less uronic acid detected in the capsular EPS compared to either biofilm or free exopolymers. *O*- and *N*-acetylation were maximum in the biofilm exopolymer. In contrast to exopolysaccharides, reports pertaining to the EPS proteins are scarce. Exopolymer from *Pseudomonas putida* biofilm were heterogenous with proteins representing the largest fraction (75%) followed by polysaccharide and DNA [45]. The EPS produced by pure cultures of bacteria isolated from the activated sludge were mainly composed of proteins

Table 1 Extraction and biochemical composition of EPS from different microbial resources

Source	EPS composition	mg g^{-1} EPS	Extraction technique	Reference
Bacteria				
Hyphomonas MHS-3	Neutral hexose Acetyl groups Uronic acid / Pyruvate / Sulphate	710 75 <5	Capsular EPS removed by blending cells with EDTA and NaCl, centrifuged (16000 x g, 20 min), EPS precipitated with 4-vol 2-propanol, dialyzed and freeze-dried.	108
Methylobacterium organophilum NCIB 11278 KC-1	Total sugar Reducing sugar Protein Pyruvic acid Uronic acid Acetic acid	804 779 61 51 124 6	EPS in culture supernatant precipitated with 2-volume ethanol; precipitate dissolved in distilled water, dialyzed and re-precipitated before freeze-drying.	109
Alteromonas macleodii subsp. *fijiensis*	Neutral sugars Uronic acids Amino sugars Proteins Sulphate esters	420 380 16 40 50	EPS precipitated from culture supernatant by ethanol, precipitate washed with water ethanol mixture and dried.	110
Thiobacillus ferrooxidans R1	Total sugar Lipids Free fatty acid Nitrogen Phosphorus	522 369 55 5 7	Cells (grown in $FeSO_4$) homogenized (Ultra Turrax) with Tris-Cl (pH 7), Zwittergent 3-12 and EGTA at 4ºC, centrifuged (12000xg), supernatant dialyzed in water (pH 2) and freeze-dried.	6
Pseudomonas sp. NCIMB 2021	Free EPS Neutral sugar Protein Capsular EPS Neutral sugar Protein	 13.35 9.31 13.32 14.14	Culture centrifuged (10,000 X g, 30 min), supernatant dialysed and lyophilized (-60° C). Cell pellet extracted with EDTA (10 mM), centrifuged (10,000 x g, 10 min) and lyophilized.	44
Rhodovulum sp. PS88	Polysaccharides Protein RNA	16.0 44.2 48.5	Cell pellet shaken in phosphate buffer and centrifuged (5,000 x g, 15 min).	51
Pseudomonas putida	Free EPS Carbohydrate Protein Capsular EPS Carbohydrate Protein	 214 345 154 483	Free EPS precipitated by 2-vol ice-cold acetone from culture supernatant, dialyzed and lyophilized. Cells blended (5min), centrifuged (17,000 g, 20min), EPS precipitated as above.	57
Pseudoalteromonas CAM 025	Neutral sugars Proteins Uronic acid Sulphates	740 20 220 50	Culture supernatant pressure filtered (cellulose nitrate filter), EPS precipitated by cold ethanol (-2ºC).	25
Acinetobacter sp. 12S	Carbohydrate Pyruvic acid Uronic acid Fatty acid Mineral component	445 36 67 94 243	Culture dialyzed and centrifuged (12,000 x g, 10 min), supernatant concentrated, EPS precipitated by 1.5 isopropanol, washed and dried.	111
Enterobacter cloacae	Polysaccharides Proteins Nucleic acids Uronic acids	22.0 130.0 28.0 56.0	Sonication (40W, 2 min) of bacterial suspension in ultra pure water, centrifugation (20 000 x g, 20 min) and supernatant re-centrifuged (10,000 x g, 1 min).	46
Serratia marcecens	Polysaccharides Proteins Nucleic acids Uronic acids	40.0 206.0 21.0 7.0		

Table 1 (*Continued*)

Source	EPS composition	mg g^{-1} EPS	Extraction technique	Reference
Rhodopseudomonas acidophila	Carbohydrate Protein Nucleic acid	6.5 58.4 5.4	Cell pellet extracted in 2% EDTA and centrifuged (12,000 rpm, 30 min).	36
Sludge				
Aerobic activated sludge	Carbohydrate Protein Humic substances Uronic acid DNA	40.5 54.6 50.4 4.2 0.35	Sludge treated with formaldehyde and NaOH (1N, 4ºC, 3h), centrifuged (20,000 x g, 4ºC, 20 min), filtered through 0.2µm membrane, dialyzed and lyophilized.	34
Activated sludge	Polysaccharide Protein Humic acid Uronic acid Nucleic acid	140 343 61 15 46	Sonication (40W, 2 min) of concentrated sludge in ultra pure water, centrifugation (20,000 x g, 20 min, 4ºC), solution of EPS stored at -18ºC.	46
Aerobic granular sludge	Protein Carbohydrate Total organic carbon	210 26 203	Sludge homogenized (phosphate buffer, 10 min, 980 rpm), adjusted to pH 11 (1M NaOH), heated at 80ºC for 30 min and centrifuged.	100
Biofilm				
Unsaturated biofilm of *Pseudomonas aeruginosa*	Proteins Polysaccharide DNA	178 525 529	Biofilm broken by vigorous vortexing in 0.9% NaCl + EDTA, centrifuged (12,000 x g, 30 min, 4ºC).	54
Cyanobacterial mat	Polysaccharide Protein Uronic acid Organic carbon Total nitrogen	305 127 118 88 18	Fresh samples extracted with 10% NaCl (15 min, 40°C), centrifuged (8,220×*g*, 15 min, 4°C), supernatant precipitated with ethanol and dialyzed.	112

and low quantities of polysaccharides and uronic acids in comparison with EPS extracted from activated sludges [46]. Differences in protein composition of capsular, biofilm and free EPS of *Pseudomonas* NCIMB 2021 were confirmed by polyacrylamide gel electrophoresis (SDS-PAGE) and Fourier transform infrared (FTIR) spectroscopic analysis [44]. The protein profiles of EPS extracted from glucose and acetate-fed activated sludge showed distinctive differences between the specific proteins from the two carbon sources [47]. Activated sludge floc was reported to immobilize and accumulate large amounts of enzymes in EPS matrix [48]. Likewise,extracellular enzymes like hydrolases, lyases, glycosidases, esterases, etc. have readily been observed in the EPS matrix of biofilms [49, 50].

The EPS matrix of *Rhodovulum* sp. PS88, a marine photosynthetic bacterium contained a relatively high amount of RNA as a major constituent in addition to protein and sugars [51]. Continuous cultivation of the strain PS88 under aerobic condition in the dark led to a high production of exocellular RNA which attained a concentration of 460 mg / L of the medium [52]. Production of extracellular DNA (eDNA) as a component of EPS has been proved to be essential for saturated biofilm stability during the early stages of biofilm growth [53].while, the unsaturated biofilm of *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Rhodococcus erythropolis* and *Vivrio paradoxus* contained eDNA along with polysaccharides and protein when grown as either single- or dual-species [54, 55]. Epifluorescence and electron microscopy revealed a stable filamentous network of eDNA closely bound to the cell surface of environmental bacterial isolates indicating a new function of extracellular DNA, possibly as a structural component.

Interactions of EPS with heavy metals

Removal of heavy metals from the environment using microbial biosorbents has been studied extensively as a major challenge in bioremediation. Extracellular polymeric substances produced by many microorganisms are of particular relevance to the bioremediation process because of their involvement in flocculation and binding of metal ions from solutions [56, 57]. The use of isolated biopolymers in biosorption phenomenon seems to be more economical, effective and safe alternative to chemical methods such as precipitation, coagulation, ion exchange, electrochemical

and membrane processes. As a non-living sorbent, EPS is preferred for its easy availability in the treatment process and avoidance of pathogenecity issues of the organism concerned [58].

Exopolysaccharides and other biopolymers exhibit excellent metal-binding properties with varying degrees of specificity and affinity. The binding of cations to bacterial biopolymers generally occurs through electrostatic interaction with negatively charged functional groups such as uronic acids, phosphoryl groups associated with membrane components or carboxylic groups of amino acids. In addition, there may also be cationic binding by positively charged polymers or coordination with hydroxyl groups. Extracellular polymers surrounding the cells were able to chelate some metals and bind them to the cell surface [59].

Exopolymers of aquatic microorganisms act as polyanions under natural conditions by formation of salt bridges with carboxyl groups of acidic polymers (polysaccharides containing uronic acids) or by forming weak electrostatic bonds with hydroxyl groups on polymers containing neutral carbohydrates. Although bacterial EPS have been shown to exhibit selectivity in complexing metal ions, in most cases, the type of macromolecules playing a key role in metal binding has not been determined. While, a large number of metals have been reported to cross-link polysaccharide [60], protein part of EPS also plays a major role in complexation of metal ions [61]. Proteins rich in acidic amino acids, including aspartic and glutamic acid, also contribute to the anionic properties of EPS. Nucleic acids are polyanionic due to the phosphate residues in the nucleotide moiety. As negatively charged components of EPS, uronic acids, acidic amino acids and phosphate-containing nucleotides are expected to be involved in electrostatic interactions with multivalent cations [62].

EPS and heavy metal immobilization

The structural and compositional variations amongst EPS from diverse microbial cells and aggregates have attracted special attention for their possible utilization in heavy metal binding and bioremediation. The central role of these biopolymers from planktonic cells, biofilms, activated sludge and biogranules in metal biosorption and / or transformation are discussed under the following sections with brief consideration of their potential applications.

Metal sorption by planktonic cell EPS

Extracellular polymers of bacterial origin are plausible carriers for metals in soil or aquifer systems. A wide variety of bacteria in culture have been shown to produce various biopolymers. Metal binding by these growing cultures, intact biomass or isolated biopolymers have been evaluated (Table 2). Batch adsorption isotherms were employed to screen polymers of 13 bacterial strains, including five subsurface isolates for their ability to mobilize lead and cadmium adsorbed to an aquifer sand. Metal adsorption by over 90% was achieved at an extracellular polymer concentration of 10.6 mg l^{-1}. The sorption isotherm indicated a low affinity for the sand sorbent and suggested that the polymer would be mobile in the porous sand medium [63]. Under conditions of unbalanced growth, *Rhizobium etli* M4 [64] produced large quantities of EPS differentiated as a capsular form (EPScap) and a more freely dispersed soluble EPS. Cells and capsular EPS rapidly bound Mn(II) ions and preferentially to Pb(II) and Cu(II), but showed higher affinity for Pb(II) [65]. *Paenibacillus polymyxa* (P13), a strain isolated from fermented sausages, produced EPS (mannose polymer) under hyperosmotic stress which showed strong ability to bind Cu(II) [66] and the maximum binding capacity was double than those reported for other members of the Bacillaceae [56, 67]. Likewise, *Paenibacillus jamilae* CECT 5266 grown in aqueous extracts of two-phase olive mill waste (TPOMW) produced EPS capable of absorbing heavy metals in the following order from a multi-metal sorption system: Pb>Cd>Cu>Zn>Ni>Co. Lead was preferentially complexed by the polymer (228 mg g^{-1}) [68] .

Cyanobacterial cells possessing a thick capsular polysaccharide investment outside the cell contain large number of binding sites for trapping metal ions and are quite promising in heavy metal bioremediation. The released polymeric substances (RPS) in cyanobacterial members are complex heteropolymers composed of several monosaccharides (glucose, galactose, mannose, ribose, arabinose, xylose, fucose and rhamnose) and abundant uronic acid subunits like glucuronic acid and galacturonic acid which owing to their carboxyl groups, efficiently bind metals. Additional methyl and / or amino sugars are sporadically been found. Among eleven unicellular and filamentous RPS-producing cyanobacteria *Cyanothece* CE 4 and *Cyanospira capsulate* showed quick and effective Cu(II) adsorption [40].

The anaerobic sulphate reducing bacteria (SRB) are of considerable interest in biotechnology. Marine isolates of SRB released EPS in the liquid media during growth which formed complex with Ni(II), Cr(VI) and Mo(IV). High molybdenum content was detected in the EPS than other metals and it was assumed to be complexed with the uronic acids present in the EPS [69]. Kinetics of Cu(II) and Zn(II) biosorption by *Desulfovibrio desulfuricans* was studied and scanning electron micrographs indicated that metal biosorption may be related to the production of

Table 2 Metal-binding potential of EPS produced by bacteria in culture

EPS producer	Metal biosorbed	Amount, mg g^{-1} EPS	Remarks	Reference
Marine sulphate reducing bacteria	Mo(VI) Ni(II) Cr(III)	2.14 0.43 0.2	Metal-binding by growing cultures, analyzed by energy dispersive x-ray analysis (EDAX).	69
Methylobacterium organophilum	Pb(II) Cu(II)	184.2 200.3	Biosorption equilibrium attained after 30 min at neutral pH. Uronic, pyruvic and acetic acid helped in metal binding.	109
Alteromonas macleodii subsp. *fijiensis*	Pb(II) Cd(II) Zn(II)	316 125 75	Sorption studied in single and bi-component mixtures. Langmuir model indicated same binding site for Zn and Cd, preferential binding of Pb in bi-metallic state.	110
Pseudomonas aeruginosa Cu^r	Cu(II)	320	EPS of Cu-resistant strain (Cu^r) showed better metal-binding ability than the sensitive one (Cu^s).	113
Rhizobium etli M4	Mn(II)	67	Sorption equilibrium attained after 3h at pH 5.2-5.8. Isotherm obeyed both Langmuir and Freundlich models and depends on anion in the order: sulphate > nitrate > chloride.	65
Enterobacter cloaceae AK-I-MB-71a	Cr(VI)	8.3	Optimum EPS production and metal binding in 100 mg/L Cr(VI), X-ray fluorescence spectrometry confirmed metal sorption.	114
Chryseomonas luteola TEM05	Cd(II) Co(II)	64.1 55.2	Sorption by immobilizing EPS in calcium alginate beads. Langmuir isotherm model fitted the experimental data.	13
Paenibacillus polymyxa P13	Cu(II)	1602	EPS production associated with hyperosmotic stress leading to metal sorption. Adsorption isotherm obeyed Langmuir model. Optimum sorption at pH 6 and 25ºC.	66
Paenibacillus jamilae CECT 5266	Pb(II) Cd(II) Cu(II) Zn(II) Ni(II) Co(II)	228 55 40 37 15 10	EPS produced using two-phase olive oil mill waste as substrate, showed preferential binding to lead in multi-metal sorption system.	68

EPS [70]. Polarographic analysis of the culture filtrate of *Desulfococcus multivorans* indicated substantial production of an extracellular copper-binding compound. However, the partially purified compound was not the sole molecule for metal acquisition by the bacterium [71].

In *Rhodopseudomonas acidophila*, EPS having a higher protein content than carbohydrate was produced when the organism was grown in presence of toxic Cu(II), Cd(II) and Cr(VI). The protein in the EPS was suggested to be the major metal-binding component which increased simultaneously with the removal of metal ions keeping the carbohydrate content unaltered [23]. Using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopic techniques, it was recently found that nucleic acid in EPS produced by *Bacillus subtilis* ATCC 7003 and *P. aeruginosa* PAO1 form monodentate complexes with Fe centers on goethite (α-FeOOH) [72]. Correspondence between spectral data and quantum chemical calculations demonstrate that phosphodiester groups of nucleic acids mediate the binding of EPS to mineral surfaces.

Biofilm EPS and metal binding

Biofilm originates when a mass of microbial cells immobilized in the heterogeneous mass of EPS gets attached to a solid surface. They commonly occur in natural environments as coatings around the soil particles, in water-sediment interface, on surfaces of bioreactors, industrial production processes, sewers, etc. [2, 73]. In nature monospecies biofilms are rare and majority comprised of a mixture of microbial species which may also include higher trophic groups of protozoans and metazoans [74]. Besides water (nearly 97%) and microbial cells, biofilm matrix is complexed with secreted polymers, nutrients and metabolites, cell lysis products, particulate material and detritus from the surrounding surfaces. Biofilm EPS containing all major classes of macromolecules are anionic in nature due to the presence of uronic acids or ketal-linked pyruvates and ionisable functional groups which interact with other molecules, minerals and heavy metals [75, 76]. Several experimental findings have established that biofilm EPS plays

a substantial role in the sorption of inorganic substances and assist in the biomineralization of metal ions (as sulphides, phosphates, carbonates, oxides and hydroxides), thus concentrating and depositing fine grained minerals within the matrix (Table 3). Several stereoscopic as well as microscopic techniques are employed for describing the elemental composition and localization of the metal precipitate trapped in the biofilm matrix [10].

Metal binding to biofilm EPS is influenced by surrounding pH, metal concentration, presence of organic matter and biomass. In addition, carbohydrate : protein (C/P) ratio also plays a significant role in monitoring the state of biofilm process to remove heavy metals in the wastewater. In batch experiments, binding of Cu(II), Pb(II) and Ni(II) occurred with lowering of C/P ratio of EPS when exposed to Cu and Pb ions [77]. Using a rotating-disk biofilm reactor it was demonstrated that biofilms were more resistant to Cu, Pb and Zn than the stationary-phase or logarithmically growing planktonic cells [78]. The EPS encasing a biofilm protect cells from heavy metal stress by binding the metal ions or by retarding their diffusion within the biofilm. Metals (Fe, Au, La, Cu) bound to biofilm of *Pseudomonas aeruginosa* PAO1 significantly exceeded those bound by planktonically grown cells [77, 79].

Autotrophic biofilms accumulate substantial quantities of metals from contaminated water. Biofilms in running water sorbed three to five times more Cd than in still water. Cd(II) sorption was 75% greater in high-biomass biofilm and light enhanced metal sorption by 40%. Metal toxicity to photosynthesis was evident in thin biofilms but photosynthesis by

Table 3 Role of biofilm and its EPS in heavy metal bioremediation

Biofilm type	Biofilm support / analysis	Metal sorption / reduction	Reference
Citrobacter sp.	Biofilms grown in carbon-free continuous culture in an air-lift fermentor using raschig rings as support, scanning electron microscopic (SEM) and confocal laser scanning microscopic (CLSM) analysis confirmed uptake.	Uptake of 0.18 mM UO_2 via uranyl phosphate which subsequently removed Ni(II) by intercalative ion-exchange to uranyl phosphate crystals.	115
Pseudomonas aeruginosa CMG156	Biofilms grown on polyvinyl chloride (PVC) cylinders, scanning electron microscope with energy-dispersive X-ray microanalysis confirmed Cu sorption.	Sorption of 85% of initial 6.39 mg Cu(II) l^{-1}	116
Burkholderia cepacia	Biofilms formed on hematite and alumina surfaces, x-ray microprobe analysis. EXAFS (extended x-ray absorption fine structure) data and TEM showed metal binding.	90% of the total Pb uptake occurred at pH < 4.5, nanoscale crystals of pyromorphite ($Pb_5(PO_4)3(OH)$) formed on the biofilm.	117
Desulfovibrio desulfuricans	Biofilm grown in anaerobic condition using two flat-plate flow reactors filled with either hematite or quartz or both	Pb(II) immobilization more on quartz than hematite; Pb(II) deposits partially reoxidized in hematite biofilms	118
Escherichia coli PHL628	Biofilms grown under batch and continuous system in microwell dishes, two-photon laser scanning microscopy (2P-LSM) used for detection of metal	Zn(II) distributed evenly in thin biofilms but in thick ones zinc was located only at the surface with depth <20μm.	119
Photosynthetic biofilm	Sand blasted glass plates used as support, flame atomic absorption spectroscopic (FAAS) analysis adopted for metal sorption.	Adsorption of 3.2 mg Zn (II) g^{-1} dry biofilm in 12 h dark and 12 h light treatment	16
Mixed biofilm	Biofilm developed on rotating drum reactors, microbial communities identified using ARISA (automated ribosomal intergenic spacer analysis).	Cu(II) uptake 25-31% of initial 100, 200 & 500ppb, copper contamination inhibited EPS production in biofilm	120
Pseudomonas aeruginosa PAO1	Single layered biofilm on zinc selenide (ZnSe) prism surface; metal-binding observed using attenuated total reflectance infrared spectroscopy (ATR-IR).	Cr(III) binding more than Ni(II) and Co(II), Cr binding causes contraction of EPS and move cells closer to ZnSe surface.	121
Arthrobacter viscosus	Mini-columns partially filled with granular activated carbon (GAC) and/or zeolite served as support in a continuous flow system, interactions between metal ions and functional groups confirmed by FTIR.	Maximum uptake values ranged from 0.57 - 3.58 mg Cr g^{-1} Support.	122
Escherichia coli PHL628	Biofilms grown in batch and continuous system in microwell dishes, detection of Cu by scanning electrochemical microscope (SECM).	Cu(II) was located in the upper region of the biofilm with a penetration depth <150 μm.	123

thicker biofilms occurred even at 100 μg·Cd l^{-1} [80]. A long-period x-ray standing wave technique was applied to probe the allocation of Pb(II) in *Burkholderia cepacia* biofilms growing on hematite (α-Fe_2O_3) or corundum (α-Al_2O_3) surfaces and determine quantitatively the partitioning of Pb(II) at the biofilm-metal oxide interface [81]. The reactive sites on the metal oxide surfaces were activated by the formation of a monolayer biofilm and thus form a dominant sink for Pb(II) ions at submicromolar concentrations.

Immobilization of precipitated metal sulphide inside a biofilm of sulphate reducing bacteria is considered an effective method to reduce the mobility of metal ion in wastewater [82]. In a stirred tank bioreactor growth of SRB biofilm with Cd(II) and sulphate led to accumulation of CdS and simultaneous increase in protein and polysaccharide content in biofilm, indicating sulphide precipitation and entrapment by biofilm EPS. Similarly binding of Cu took place through precipitation of CuS at the biofilm surface or in the liquid phase, followed by entrapment of precipitated CuS by the polymer [83].

Metal remediation by activated sludge EPS

Activated sludge process, the common biological process of wastewater treatment, involves degradation of organic pollutants under aerobic conditions by a community of microorganisms contained in flocs. Floculation represents a major portion of the activated sludge and are composed of heterotrophic bacteria belonging to genera *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Alcaligenes*, *Citrobacter*, *Zoogloea* etc. Production of EPS by these sludge bacteria plays an important adhesive role in floc formation and are partly secreted by the microorganisms and partly derived from molecules present in the wastewater or from cell lysates [84]. EPS in activated sludge system are differentiated into soluble and bound forms [20], of which the soluble one forms a protective barrier that prevents any harmful effects of heavy metals on floculating microorganisms.

A combination of flocculation and settling was the mechanism by which metal removal was achieved in activated sludge. Using adsorption isotherms and Freudlich model it has been demonstrated [85] that the affinity of sludge EPS for metals was in the order Zn>Cu>Cr>Cd>Co>Ni>CrO_4. The metal (Cd, Pb and Ni) complexation potential of EPS extracted from activated sludges and those produced by the pure cultures of bacteria isolated from the same sludges was compared [46]. Biosorption order for the metals was Pb> Ni >Cd irrespective of the origin of the EPS. However, EPS from sludges exhibited greater metal complexation than that of pure cultures of bacteria. Moreover, the carboxylic and phosphoric groups were reported to play a major role in the complexation of metals similar to those reported earlier [86–88]. Further, the metal sorption capacity of the activated sludge was highly dependent on metal species and the C/N ratio. The increase in C/N ratio resulted in an increase in Cd(II) but decrease in Cu(II) sorption capacity. A different behavior was observed for Zn(II) and Ni(II) and it was not possible to indicate the relationship between maximum adsorptive capacity of these metals and C/N ratio [89].

The effect of extraction protocols on EPS from activated sludge on metal binding ability has been evaluated [4]. Qualitative analysis of exopolymer extracted by different chemical and physical methods demonstrated protein and carbohydrate as the predominant components. The extracted polymer showed a greater affinity for Pb(II) than for Cd(II). Although metal binding to sludge EPS extracted by physical methods were more or less identical (except by heating) the complexation properties were greatly modified by chemical methods [90]. Comparative biosorptive properties of soluble and bound EPS from sludges towards metallic cations revealed that both types of polymer had an affinity for metals in the following order: Cu > Pb > Ni > Cd. However, the soluble one showed stronger biosorption properties for Cu(II), Pb(II) and Ni(II) than bound EPS. Such a variation was attributed to the different metal adsorption sites, which appear to exist on exopolymers [91]. Additional reports documenting the metal binding ability of the activated sludge and their EPS from wastewater have been presented in Table 4.

Biogranules and metal removal

A process of self-immobilization of microorganisms, where cell-to-cell aggregation results in the formation of somewhat dense and compact aggregates attaining a spherical form is termed biogranulation. Formation of these granular structures is mediated by auto-aggregation (genetically identical cells) and co-aggregation (different species) abilities of interacting bacteria and does not require any carrier for its formation as in biofilm. The aerobic granules are commonly formed in sequencing batch reactors (SBR), while anaerobic granules are produced mostly in upflow anaerobic sludge blanket (UASB) reactors and have attracted much interest because of their, superior metal binding capabilities [92].

Formation of the three-dimensional structure of the granules is mediated through a sequence of events [93] and are primarily composed of mixed species microbial consortia held together in a matrix of their own EPS along with other embedded particles [94–99]. As in other microbial aggregates, such as biofilms and bioflocs, the EPS produced in biogranules are also composed of variable amounts of

Table 4 Biosorption of heavy metals by activated sludge and their EPS

Sludge system	Metal removal	Remarks	Reference
Dried activated sludge used in batch system as a function of initial pH in single- and bi-metallic condition.	Cr(VI) and Ni(II)	Mono-component equilibrium data fitted to both Langmuir and Freundlich models, while the multi-component sorption fitted to the later one.	124
EPS extracted from activated sludge of wastewater treatment plants used at pH 7.0.	Cd(II), Cu(II), Pb(II)	Polarographic method studied the complexation parameters of EPS with metals, binding sites and complexation constant found linked to proteins, polysaccharides and humic acid.	87
Activated sludge used in batch and continuous-flow stirred reactors, biosorption in single and binary component system.	Pb(II), 0.793 mM min^{-1} Cu(II), 0.242 mM min^{-1}	Equilibrium data fitted to Freundlich adsorption isotherm, forward rate constants were deduced from a mathematical model based on continuous system (liquid) and batch system (solid phase)	125
Pretreated activated sludge (PAS) in free- and immobilized (Ca-alginate) form used at pH 5.0 or less.	Cu(II), Zn(II), Cr(III)	Adsorption rate higher in free PAS than in immobilized ones due to polymeric matrix reaction, sorption fitted to Langmuir isotherm, carboxyl groups involved in metal binding.	126
Dried activated sludge used in batch systems, particle size <0.063 mm, pH 4.0, 20ºC.	Cu(II), 294 mg g^{-1}	Equilibrium data fitted to Langmuir and Freundlich isotherms and pseudo-second order kinetic model.	14
Dried sewage sludge (SS) compared with water treatment plant sludge, landfill leachate sludge and anaerobically digested sewage sludge.	Cd(II), 0.38mM g^{-1} SS	Equilibrium fitted to Langmuir isotherm, binding occurs via an ion exchange as well as electrostatic interaction between carboxylate groups and Cd(II).	15
Dried activated sludge used in batch systems.	Cd(II), Pb(II)	Reaction fitted to pseudo-second-order rate expression, equilibrium data fitted to both Langmuir and Freundlich models, FT-IR analysis showed binding with amide I group.	127
Powdered waste sludge (PWS) from paint industry used in batch kinetics, particle size 64 μm, pre-treatment with 1% H_2O_2,.	Cu(II), 116 mg g^{-1}	Pseudo-first and -second order kinetic models used to correlate data, Langmuir isotherm found fit.	128
EPS produced by anaerobic sludge of sulfate-reducing bacteria used.	Cd(II), 2720 mg g^{-1} EPS	Fourier transform infrared (FTIR) spectra demonstrated hydroxyl and carboxyl groups of EPS bind metal ions, sorption data fitted to Freundlich isotherm.	129
EPS extracted from different activated sludges used at initial pH 4–8.	Cu(II) > Pb(II) >> Cd(II)	Differential pulse polarography (DPP) and Ruzic's model used to investigate biosorption at the different pH, presence of hydroxylated and solid forms of Pb and Cu detected.	130
Activated sludge from a continuous-flow anaerobic-anoxic-oxic system in a biological nutrient removal process used in batch system.	Cd(II), Ni(II), and Zn(II)	Biosorption showed active and passive uptake stages, kinetic data simulated by pseudo-second-order rate equations and fitted to Freundlich isotherm.	131

proteins, polysaccharides, nucleic acids, humic-like substances, lipids and glycoproteins, but the content of EPS in biogranules have been found to be much higher than that of bioflocs and biofilms [3]. Moreover, extracellular polymers likely play an important role in maintaining the structural and functional integrity of the granule. The spatial distribution of cells and EPS in the granules has been probed using a number of different stains [fluorescein-isothiocyanate (FITC), concanavalin A (Con A) lectin conjugates, calcofluor white and SYTO [63] either singly or in combination [100–102].

The biogranules, by virtue of their physical characteristics and biochemical composition, particularly of EPS matrix, have been identified as potential biosorbent for heavy metals (Table 5). The biosorption of Zn(II) by acetate-fed aerobic granules was related to both the initial Zn(II) and

granule concentrations [103]. The concentration gradient of Zn(II) was the main driving force for biosorption of the metal by the granules. The feasibility of aerobic granules as a novel type of biosorbent for the removal of individual Cd(II), Cu(II) and Zn(II) from aqueous solution has been assessed and it implies that aerobic granules are effective biosorbing agents for the removal of Cd(II), Cu(II) and Zn(II) from industrial wastewater [104].

EPS and reduction nprecipitation of heavy metals

One of the modern approaches for bioremediation of heavy metals is based on the ability of microorganisms to effectively catalyze changes in the oxidation states of metals that in turn influence their solubility. Certain heavy metals like, Cr, Mo, Se, U, Au can be immobilized following enzymatic reduction of these elements into an insoluble lower redox state. Under oxidizing conditions, they occur as highly soluble and mobile ions, however, under reducing conditions they usually form insoluble phases.

The ability of the extracellular polymeric substances to bind and possibly reduce heavy metals and radionuclides may represent a novel feature in some anaerobic organisms. EPS of *Shewanella oneidensis* MR-1 bind and reduce metals in association with specific lipoproteins. Resting cell suspensions when incubated anaerobically with U(VI) become surrounded by thin (nm) filamentous structures that were heavily mineralized with nanocrystalline uraninite (UO_2) following reduction of U(VI). The EPS was able to bind and reduce metals in association with specific lipoproteins that include c-type cytochromes. However, the exact ultrastructure, composition, and mechanisms of cellular and extracellular components involved in metal reduction and precipitation are not completely understood [105]. Application of X-ray absorption near edge structure (XANES) spectroscopy showed reduction and immobilization of U(VI) in biofilms of sulfate reducing bacterium, *Desulfovibrio desulfuricans* G20 grown in flat-plate continuous-flow reactors. Uranium was immobilized in the biofilms as a result of two processes: (1) enzymatically and (2) chemically, by reacting with microbially generated H_2S and precipitated as uraninite [11].

Pseudomonas putida cultured as unsaturated biofilm on membranes overlaying iron-deficient solid media either containing potassium dichromate or dichromate coated hematite demonstrated enzymatic reduction of Cr(VI) and extracellular DNA binding of Cr(III) [24]. A mixed culture of SRB biofilm has also been investigated for their ability to reduce chromate to insoluble and less toxic trivalent form [106]. Mass balance studies showed that majority of chromate was transferred to insoluble form which accumulated at the base of the biofilm cells as sediment while bacterial biofilm did not retain significant amount of the metal. In another technical-scale bioremediation system, a

Table 5 Biosorption of heavy metals by biogranules

Biogranules	Metal removal	Remarks	Reference
Acetate-fed aerobic granules having particle size 1.0 mm diameter used.	Zn(II), 270 mg g^{-1}	Biosorption kinetics was related to the ratio of initial metal concentration and granule concentration.	103
Aerobic granules used in batch system.	Cd(II), 172.7 mg g^{-1} Cu(II), 59.6 mg g^{-1} Zn(II), 164.5 mg g^{-1}	Preparation of a general model based on thermodynamics of biosorption including Langmuir, Freundlich and Sips or Hill equations.	104
Aerobic granular sludge in two-series batch experiments.	Cu(II), 246.1 mg g^{-1} Zn(II), 180 mg g^{-1}	Two-kinetics model developed to fit the sorption data, aerobic granules highly efficient for the removal of dissolved Cu(II) and Zn(II) from wastewater.	12
Aerobic granules in batch systems used under different initial metal ion and granule concentration.	Ce(IV), 357 mg g^{-1}	Biosorption conformed first-order kinetics model.	132
Aerobic granular sludge used in batch system at initial pH 2 - 7.	Ni(II)	A thermodynamic equilibrium isotherm fit the experimental data, electrostatic attraction and ion-exchange occurred between granules and metal ions.	133
Anaerobic granules prepared from sludge of wastewater treatment plant used in batch system.	Pb(II), 1.23 mM g^{-1} Cd(II), 0.53 mM g^{-1} Cu(II), 0.87 mM g^{-1} Ni(II), 0.44 mM g^{-1}	Equilibrium data fitted in Langmiur isotherm model, non-viable biomass treated with Ca showed better efficiency.	134

packed-bed bioreactor containing Hg(II)-reducing bacterial biofilms was used for treatment of contaminated wastewater [107]. In this process, the mercury load of wastewater decreased by up to 99% during its flow through the bioremediation system. Residual Hg was removed from the reactor effluent by physical adsorption and biological reduction.

Conclusion

In conclusion, extracellular polymeric substances are abundant and seem to be ubiquitous in microbial cultures as well as aggregates of diverse types formed under different ecological conditions. They are best defined as bound and soluble EPS by the methods used in their extraction or separation rather than by their physical states. Production of EPS is greatly enhanced under so-called stressful cultural conditions imposed by nutrient limitations and toxic substances including heavy metals. Studies on interactions between metals and EPS produced by bacteria have established the phenomenon of biosorption as one of the vital processes in bioremediation of toxic heavy metal contaminated waste water systems, the components of EPS as a whole playing the central role. However, the structural complexity of the EPS contributed by its major components (polysaccharides, proteins and nucleic acids) has made it difficult to elucidating the functional role and relative contribution of each EPS component in biosorption of metal(s). It is urged to develop a simple model system which will provide an insight into the basic mechanism(s) of EPS-metal binding, highlight the specific role of each EPS component and justify the interaction(s) amongst the components thereof. Such an understanding will aid in engineering the extracellular polymeric substances with enhanced characteristics of metal sorption for effective bioremediation of heavy metals of environmental concern.

Acknowledgements This research was partly supported through Deutscher Akademischer Austausch Dienst (DAAD) Fellowship to A. Pal.

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