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Acinetobacter haemolyticus MG606 produces a novel, phosphate binding exobiopolymer

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ABSTRACT

The present study evaluated an extracellular, novel biopolymer produced by *Acinetobacter haemolyticus* MG606 for its physicochemical properties and phosphate binding mechanism. The exobiopolymer (EBP) was characterized to be majorly polysaccharide in nature consisting of 48.9 kDa heteropolysaccharide composed of galactose, glucose, xylose, lyxose, allose, ribose, arabinose, mannose and fructose. Maximum phosphate binding efficiency of 25 mg phosphate/g of EBP was described by Langmuir isotherm and further, the physicochemical and spectroscopic studies revealed that phosphate appeared to bind predominantly with the polysaccharide fraction, and to a relatively lesser extent to protein fraction of EBP. The electrostatic interactions with amino groups and ligand exchange with hydroxyl groups of EBP were found to be primary basis for phosphate binding mechanism. The results of this study implicate the feasibility of the EBP for commercial bioremediation processes.

1. Introduction

Extracellular biopolymers (EBP) produced by microorganisms have been identified as a novel, safe, biodegradable platform for remediation of environmental pollutants. Although, their application in bioremediation is still in infancy, EBP from diverse microbial genera have been investigated for potential applications in removal of ionic contaminations, notably heavy metals, as well as organic contaminants like pesticides and oil spills. Several groups have reported affinity of EBP for cationic contaminants such as arsenic, lead, mercury, chromium and other heavy metal ions (More, Yadav, Yan, Tyagi, & Surampalli, 2014). Interestingly, a vast amount of literature exist demonstrating cation-binding capacity of EBP; however, anion-binding by EBP has largely remained unexplored due to non-consideration of seriousness of these anions unlike other toxic metal cations. The role of anions, particularly phosphate, as an environmental concern has only been realized recently (Rockstrom et al., 2009).

An increased incidence of eutrophication in water bodies has been linked to phosphorus accumulation. Phosphate remediation in wastewater can be achieved by physicochemical methods such as adsorbents, sand filters, membrane filters, precipitation and electrocoagulation. However, these methods suffer from disadvantages of long-term high processing costs and limited reusability of the removed phosphorus. These issues have paved the way for development of biological methods of phosphate removal such as enhanced biological phosphorus removal (EBPR), agricultural waste and assimilation by bacteria, algae and plants (El-Bestawy, Hussein, Baghdadi, & El-Saka, 2005; Nguyen, Ngo, Guo, & Nguyen, 2012). EBPR is currently used for the bioremediation of phosphate due to its low operating cost, reduced sludge production, easier management and reuseability of byproducts and effluent. EBPR is achieved through polyphosphate accumulating organisms (PAOs) present in sludge, which accumulates phosphate intracellularly in the form of polyphosphates (Oehmen et al., 2007). Although the role of EBP present in activated sludge and its participation in EBPR through phosphate sorption has been realized in the recent years, however, studies were either restricted to quantitative analysis of total phosphorus bound to EBP (Li, Ren, Wang, & Kang, 2010; Oosthuizen & Cloete, 2001) or mostly focussed on phosphorus speciation in EBP extracted from activated sludge (Zhang et al., 2013a,b). In the related reports, phosphate binding was studied in EBP produced by a community of PAOs and the role of specific microorganism producing the phosphate-binding EBP was not considered; also, the composition of phosphate-binding components and the mechanism of phosphate-EBP interaction were not completely elucidated.

We have earlier reported the phosphate-binding ability of EBP produced by an environmental isolate of *Acinetobacter haemolyticus* (Genbank accession number KP701480) as well as an EBP-overproducing mutant (Kaur, Ganguli, & Ghosh, 2013a; Kaur,

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Sharma, Ganguli, & Ghosh, 2013b). The current investigation was aimed at identification and characterization of EBP components responsible for phosphate binding in EBP produced by the mutant strain *A. haemolyticus* MG606. Further, the nature and mechanism of phosphate interaction with the polysaccharide fraction of EBP was investigated by physical and chemical methods.

2. Materials and methods

2.1. Bacterial strain, culture conditions and EBP extraction

A. haemolyticus MG606 was grown in EBP production medium and EBP was extracted as described previously (Kaur et al., 2013b). Briefly, the bacterium was grown at 30 °C in EBP production medium composed of ammonium sulphate (1 g/L), calcium chloride dihydrate (0.7 g/L), dextrose (1 g/L), dipotassium hydrogen phosphate (1 g/L), magnesium sulphate heptahydrate (0.3 g/L), peptone (5 g/L), potassium dihydrogen orthophosphate (1 g/L), sodium chloride (0.1 g/L) and agar (3 g/L). The final pH of medium was adjusted to 7.0 ± 0.2. After 48 h of growth, the cultures were centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was mixed with double volume of chilled ethanol to precipitate crude EBP. The crude EBP was reprecipitated with 10% cetyl pyridinium chloride and sodium chloride and then centrifuged at 12,000 × *g* for 10 min. The pellet was washed twice with deionized water, dialyzed for 48 h with deionised water and then lyophilized to obtain purified EBP.

2.2. Physicochemical characterization of EBP

The elemental composition (C, H, N and S) and biochemical composition was determined as described previously (Kaur et al., 2013a,b).

The ultrastructure and elemental composition of EBP was determined using a scanning electron microscopy (JSM-6510LV, Jeol) equipped with EDS (INCAx-act, Oxford Instruments). The surface area and porosity of EBP was determined by Brunauer, Emmett and Teller (BET) method using Micromeritics ASAP 2020 analyzer and nitrogen gas as sorbent. The surface area was calculated using BET equation while micropore volume was calculated using Barrett, Joyner and Halenda (BJH) analysis.

Molecular weight of EBP was determined by gel permeation chromatography (GPC) on Ultrahydragel 500 and Ultrahydragel 120 column in series using Waters Alliance HPLC-GPC (Waters 2695 separation module coupled with Waters 2414 refractive index detector). The mobile phase used was 0.2 M sodium nitrate in water.

The monosaccharide components of EBP were determined by gas chromatography coupled with a mass spectrometer (GCMS) (Pierre et al., 2012; Senila, Gog, Senila, Roman, & Silaghi-Dumitrescu, 2011). Briefly, EBP was hydrolyzed with 2 M trifluoroacetic acid at 121 °C for 1 h. The hydrolyzed sample was lyophilized and dried extract was derivatized with *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA). The mixture was heated for 30 min at 80 °C, centrifuged and sodium sulphite was added to remove moisture present in the sample and again centrifuged at 12,000 × *g* for 10 min. The supernatant was collected, vacuum dried and injected into GCMS (GCMS QP 2010, Shimadzu) equipped with DB-5 column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies).

A preweighed sample of EBP was placed in sample pan of thermogravimetric analysis (TGA) apparatus (Mettler Toledo) and heated at 15 °C/min under a constant flow of nitrogen gas. The sample was heated until no further change in weight was observed and degradation temperature was determined from TGA curve.

The dynamic viscosity of aqueous solution of EBP was determined by Brookfield viscometer (DV-11+/Pro, Brookfield). All measurements were performed at ambient temperature (25 °C).

2.3. Phosphate sorption by EBP

Standard phosphate solution was prepared by dissolving 100 mg of potassium dihydrogen phosphate in 1 L of deionised water. The working concentrations ranging from 2 mg/L to 10 mg/L were prepared by suitably diluting the stock solution with deionised water.

To determine sorption of phosphate on EBP, 2.5 mL phosphate solution (2 to 10 mg/L) was mixed with an equal volume of EBP solution (100 to 1000 mg/L), vortexed for 10 min and kept undisturbed for indicated time. The mixture was then filtered through 0.22 μm membrane filter and phosphate concentration was determined by molybdenum blue stannous chloride method using potassium dihydrogen orthophosphate as standard (0–10 mg/L) (APHA, 1998). The colour produced by reaction of ammonium molybdate with stannous chloride in presence of phosphate was measured by recording absorbance at 690 nm. The standard plot between phosphate concentration (1–5 mg/L) and absorbance was linear ($y = 0.027x + 0.005$; $R^2 = 0.991$).

The sorption data for optimized contact time and EBP concentration was fitted to isotherm equations using MATLAB (Foo & Hameed, 2010). The goodness of fit was determined based on coefficient of determination (R^2) and rigorous error functions (Foo & Hameed, 2010; Ho, 2004). The best fitting isotherm model was selected based on corrected Akaike information criterion (AICc) (Akpa & Unuabonah, 2011).

The reversibility of phosphate binding was determined by desorption studies. Phosphate-bound EBP was prepared by sorption of phosphate (1 mg/L) on EBP (100 mg/L) for 4 h. Phosphate-bound EBP was recovered by filtration, mixed with 0.1 N NaOH (1 mg EBP/10 mL) and kept undisturbed for 4 h. The desorbed phosphate was determined by measuring phosphate concentration as described above.

2.4. Mechanism of phosphate binding

2.4.1. Effect of enzyme and chemical treatment, competing anions and pH

The effect of chemical/enzyme treatment, competing anions and pH on phosphate sorption by EBP was determined at 1 mg/L phosphate and 100 mg/L EBP.

An aqueous solution of EBP (1 g/L) was incubated for 16–18 h with a cocktail of 1 mg/L proteolytic (protease [4000 U/mg], trypsin [1000–1500 U/mg] and proteinase K [30 U/mg] in equal weight proportions), 1 mg/L amylolytic (amylase [2000 U/mg], cellulose [0.3 U/mg] and beta-galactosidase [500 U/mg] in equal weight proportions) enzymes and their combination (1 mg/L proteolytic and 1 mg/L amylolytic cocktail) at 37 °C and lyophilized. For chemical modifications, 50 mg powdered EBP was mixed with 50 mL of 4% v/v glutadaldehyde or 1:100 concentrated hydrochloride acid:anhydrous methanol mixture (v/v) or 1:2 formaldehyde:formic acid (v/v) and allowed to react for 24 h. The reaction mixture was then extensively dialyzed against deionized water to remove unreacted reactants and then lyophilized (Jianlong, 2002; Micheletti et al., 2008). A low concentration of glutaraldehyde (4%, v/v) was selected to minimize interchain crosslinking, and subsequent precipitation, observed at higher glutaraldehyde concentrations. Phosphate binding was determined with treated EBP as described above.

To determine the effect of competing ions, EBP and phosphate (1 mg/L) were incubated until equilibrium was reached (240 min). Following equilibrium, potassium salts of sulphate, chloride or nitrate were added to EBP solution at 10, 50 and 100 mg/L