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Influence of metal ions on biofilm formation by *Arthrobacter* sp. SUK 1205 and evaluation of their Cr(VI) removal efficacy

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ABSTRACT

This study explores biofilm formation by the chromate resistant and reducing *Arthrobacter* sp. SUK 1205 isolated from Sukinda chromite mining regions of Odisha, India. Formation of biofilm by the isolate was pronounced in complex peptone-yeast extract-glucose medium and was positively influenced by Cr(VI), Mg(II) and Ni(II). The optimum pH and temperature for biofilm formation was 7.0 and 37 °C, respectively. Architectural analysis following Scanning Electron Microscopy and Confocal Laser Scanning Microscopy revealed increase in total biomass, thickness and production of extracellular polymeric substances in the biofilm. Biofilms developed on glass beads under the influence of metal ions were used for Cr(VI) removal in synthetic Minimal salts (MS) medium and complete removal of 0.5 mM Cr(VI) was achieved within 4 days with chromium induced biofilms. Experiments were also conducted to reuse and recycle the biofilms to assess their Cr(VI) removal efficacy and also to evaluate their utility in removal of Cr(VI) from metal contaminated waste water.

1. Introduction

Almost all microorganisms during their growth in natural conditions form biofilms and display a variety of structural and functional properties. Formation of biofilm normally occurs under the influence of various mechanical, environmental, biochemical and genetic factors and is considered to be more productive and metabolically active than planktonic cells. Extracellular polymeric substances (EPS) form the matrix of the biofilm in which the bacterial cells remains embedded. Chemically EPS are composed mostly of polysaccharides, proteins and nucleic acids (Flemming and Wingender, 2010) and render resistance to antibiotics, heavy metals as well as biocides (Shemesh et al., 2010; Hoiby et al., 2011). Moreover, they play critical role in metal adsorption and immobilization (Aguilera et al., 2008). Resistance towards metals in biofilms can be attributed to the fact that the biofilm matrix prevents the entry of reactive and charged heavy metals or metalloids ions as well as hydrophobic or large molecules by rendering diffusional barriers to these molecules. They are mostly immobilized by biofilms, often reduced to comparatively less toxic forms and/or sorbed by the EPS matrix of the biofilm. Also, there occurs a selection within biofilm which increases the population of mutant microbial strains better adapted to heavy metal contaminated sites (Stewart and Franklin, 2008). Biofilm EPS mediated metal removal can be considered as an important strategy for mitigation of heavy metal contaminated sites and can perform a significant role in bioremediation of contaminated water bodies (Flemming and Wingender, 2010).

Heavy metal contaminations of the environment are very common due to anthropogenic activities including mining and disposal of industrial wastes. Microorganisms indigenous to such metal contaminated industrial wastewater and mining sites have evolved various adaptive strategies to negate heavy metal stresses which consist of processes like sequestration of heavy metals, reduction of a toxic metal to a less toxic form and efflux of metals from the cell (Outten et al., 2000; Teitzel and Parsek, 2003). These metal tolerant and resistant isolates are reported to produce biofilms with significant amount of EPS to shield the cell mass from the toxic effects of heavy metals.

Earlier studies on interaction between biofilm and heavy metals were mainly aimed at the sorption and removal of heavy metals from liquid (Huang et al., 2000), wastewater treatment systems (White and Gadd, 1998) and also bioaccumulation of metals from fresh water systems (Morin et al., 2008). Biofilms formed by *Pseudochrobactrum saccharolyticum* LY10 alone (Long et al., 2015), consortium of *Bacillus subtilis* and *B. cereus* (Sundar et al., 2011) and sulphate reducing bacteria (Smith and Gadd, 2000) have been reported to remove Cr(VI). Apart from chromium, biofilm formation by *E. coli* K 12, *Pseudomonas* sp. (Perrin et al., 2009) and *Pseudomonas mendocina* NR802 (Mangwani et al., 2014) were positively influenced by nickel, magnesium and calcium.

Arthrobacter spp., the common Gram-positive soil bacteria are reported to survive in areas contaminated with heavy metals such as

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chromium. They could reduce Cr(VI) to its trivalent form (Dey and Paul, 2013, 2012a,b; Camargo et al., 2004) and lead to the precipitation of Cr(III). Further, they help in removing Cr(VI) from water by adsorption (Silva et al., 2012). The biofilm EPS contains several functional groups such as carboxyl, phosphoryl, hydroxyl, amino and sulfhydryl which have the ability to absorb wide range of organic and inorganic particles, however, their sorption capacity varies with the chemical nature of the functional groups.

Several researchers (Quintelas et al., 2009; Cordoba et al., 2008; Camargo et al., 2004) have reported biofilm formation by different *Arthrobacter* spp. and removal of Cr(VI) utilizing bioreactor systems. However, detailed studies on the influence of heavy metals on biofilm growth and structure with special emphasis on EPS content and hexavalent chromate reduction have not been explored so far.

In this study, we report for the first time the effect of Ni(II), Mg(II) and Cr(VI) on the growth of biofilm of a Cr(VI) tolerant and reducing bacterium *Arthrobacter* sp. SUK 1205. Experiments have been carried out to assess the effect of these metals on the development of biofilm by *Arthrobacter* sp. SUK 1205 and it was found that Ni(II), Mg(II) and Cr (VI) have a promotive effect on biofilm formation. Confocal scanning laser microscopy (CSLM) imaging and scanning electron microscopic (SEM) images were employed to visualize the changes induced by them. Experiments were also executed to evaluate the Cr(VI) removal efficiency of these biofilms from contaminated waste water systems.

2. Materials and methods

Arthrobacter sp. SUK 1205, (MTCC Accession No. 8731 and NCBI Genbank Accession No. JQ312666), a chromium resistant and reducing bacterium previously isolated from chromite mine overburden of Sukinda valley, Odisha, India (Dey and Paul, 2013) was used in this study. The strain was regularly subcultured on peptone-yeast extract-glucose (PYEG) agar medium (Wang and Xiao, 1995) supplemented with 2 mM Cr(VI) (as K_2CrO_4). The medium contained (g/L): peptone, 10.0; yeast extract, 5.0; glucose, 3.0 and agar agar, 20.0 (pH 7.0). Overnight grown cultures were stored at 4 °C for future use.

2.1. Biofilm formation assay

2.1.1. Preparation of inoculum

The isolate was grown in PYEG medium for 24 h at 35 °C under continuous shaking (120 rpm). Cells were harvested aseptically by centrifugation at 10,000 rpm for 10 min at 4 °C and washed 2–3 times with ice cold 10 mM Tris buffer at pH 7.0. The pellet was suspended in the same buffer at 5% the original culture volume and was used as inoculum for all the experiments. The initial inoculum density was kept at 10^7 cells/mL and the isolate was screened for its biofilm forming ability by tube assay (Christensen et al., 1982), an indirect method for quantification of biofilm.

2.1.2. Tube assay

In tube assay method (Christensen et al., 1982), the PYEG broth (2 mL) was inoculated with 20 µL of freshly prepared inoculum (10^7 cells/mL), incubated at 37 °C under static condition and monitored for a period of 12 days. Tubes in triplicates were taken out at 24 h interval, decanted gently and washed with sterile normal saline. The tubes were then dried and stained with 0.1% (w/v) crystal violet. Subsequently, the tubes were washed with distilled water to remove the excess stain followed by destaining with 33% acetic acid. Optical density of the dye was determined at 590 nm.

2.1.3. Influence of environmental factors on biofilm formation

Biofilm formation by *Arthrobacter* sp. SUK 1205 was studied under various environmental conditions like variation of carbon source, temperature, pH, different metals and concentration of Cr(VI) for a period of 8 days. Peptone yeast extract broth was supplemented with 0.3% of fructose, sucrose, xylose, arabinose, maltose and raffinose as sole carbon source. PYEG broth was adjusted to different pH range (6.0–8.0) to study its effect on the formation of biofilm and the final pH was recorded. Effect of temperature on biofilm formation was studied at 32 and 37 °C. Effect of metals on biofilm formation was determined by supplementing PYEG broth with 1 mM Cd(II), Ni(II), Zn(II), Fe(III), Mn (II), Mg(II) and Cu(II). The influence of Cr(VI) concentration on biofilm formation was evaluated in the range of 1–10 mM. In each experiment uninoculated tube was used as blank.

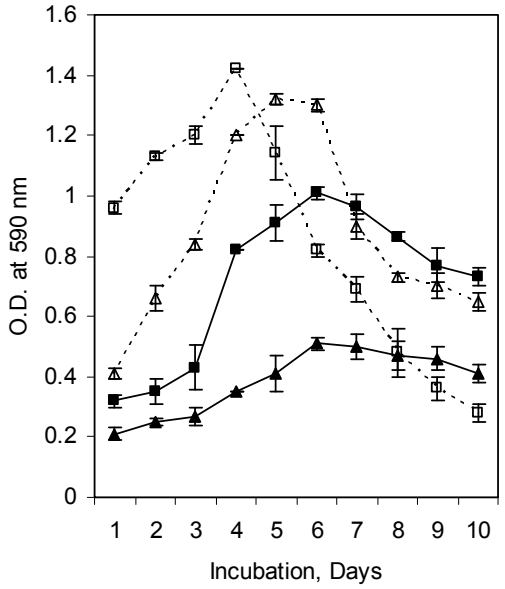

Fig. 1. Comparative analysis of biofilm and planktonic growth of *Arthrobacter* sp. SUK 1205 in PYEG and MS media supplemented with 0.1% glucose (Biofilm growth: ■- PYEG, -▲- MS, Planktonic growth: □- PYEG, - Δ - MS).

Table 1
Chemical analysis of extracellular polymeric substance (EPS) matrix produced by *Arthrobacter* sp. SUK 1205.

Method of extraction	Carbohydrate (µg/g dry wt.)	Protein (µg/g dry wt.)	DNA (µg/g dry wt)
Physical method of extraction			
Warm water treatment	2628.6 ± 1.2	227.6 ± 2.1	2.1 ± 1.3
Chemical method of extraction			
2% EDTA	567.3 ± 1.5	56.6 ± 0.8	–
1 mM NaOH	832.8 ± 1.8	78.6 ± 0.3	–
NaOH – formaldehyde	1284.8 ± 2.3	234.0 ± 1.2	1.2 ± 0.9

Carbohydrate, protein and DNA was estimated following methods of Dubois et al. (1956), Lowry et al. (1951) and diphenylamine method respectively. Results represent mean ± standard deviation of triplicate sets.

Table 2
Compositional analysis of the biofilm EPS of *Arthrobacter* sp. SUK 1205 following GC-MS analysis.

Peaks found	Identified sugar	Moles/g dry wt.	Relative Mol %
1	Rhamnose	0.1373	24.22
2	Ribose	0.0368	6.49
3	Mannose	0.1676	29.56
4	Galactose	0.0977	17.23
5	Glucose	0.1276	22.50

Biofilm EPS was extracted from 8 days old biofilm grown in PYEG medium following warm water treatment (70 °C) and vortexed for 10 min.