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Original Article

EXTRACELLULAR POLYMERIC SUBSTANCE (EPS) FROM *KOCURIA* SP. BRI 36: A KEY COMPONENT IN HEAVY METAL RESISTANCE

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ABSTRACT

Objective: Evaluation of Extracellular Polymeric Substance (EPS) induced heavy metal tolerance in *Kocuria* sp. BRI 36.

Methods: Initially, the effect of different concentrations of glucose (1-10 %) on EPS production by BRI 36 was examined. At optimum glucose concentrations, EPS levels were measured by varying heavy metal concentrations (10-50 ppm) of Pb^{2+}, Cd^{2+} and Cr^{3+}. Maximum tolerable concentration (MTC) and survival percentage of BRI 36 were determined under conditions that support EPS synthesis. Comparative analysis of extracted crude EPS was performed by Fourier Transform Infrared Spectroscopy (FTIR) to establish functional groups involved in the metal interaction.

Results: *Kocuria*sp. BRI 36 produced maximum EPS (1g/l) at 5% glucose. Increase in EPS production up to 89% (considering 1g/l as 100%) with an increase in concentrations of heavy metals up to 40 ppm. MTC levels of BRI 36 for heavy metals increased up to 700 ppm when it was cultivated in presence of 5% glucose indicating a major role of surface polymer in metal adsorption. The function of EPS as a protective cover was also evident from an increase in survival percentage of BRI 36 up to 39.4 at MTC. Comparative analysis of extracted crude EPS by FTIR revealed the involvement of O-H, C=O, and C-O-C groups in metal adsorption

Conclusion: Antarctic oceanic isolate *Kocuria* sp. BRI 36 has an ability to produced EPS under stress conditions of heavy metals. Simultaneously, its MTC values increased due to increase in EPS levels. These observations suggest the possibility to develop gentle, environmentally safe and cost-effective method for heavy metal removal.

Keywords: Exopolysaccharides, Fourier Transform Infrared Spectroscopy (FTIR), heavy metals, Maximum Tolerable Concentration (MTC)

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INTRODUCTION

Many bacteria produce biopolymers with varied chemical properties through the utilization of simple to complex substrates. They could either be intracellular or extracellular. The extracellular polymeric substances (EPS), also known as exopolysaccharides, have a high molecular weight and consist mainly of carbohydrates. However, the intracellular biopolymers are few and have very limited use [1]. EPS may be grouped into four major classes; polysaccharides, inorganic polyanhydrides (such as polyphosphates), polyesters, and polyamides [2].

Most marine bacteria can produce EPS, which occur in two forms viz. capsular polysaccharides in which the polymers are covalently bound to the cell surface; and slime polysaccharides that either remain attached (loosely bound) to the cell surface or are released into the environment [3, 4]. EPS produced by marine bacteria are thought to play a key role in the protection against the marine environment characterized by extreme physicochemical conditions such as low or high temperature, high pressure, low nutrient concentration, high salinity, and heavy metal presence [5]. Occurrence of heavy metals in soil and water are found to be important micronutrients, provided that the levels are in a limit [6, 7] or else they contaminate natural resources which in turn has an adverse effect on the ecosystem [8, 9]. BRI isolates from Antarctic oceanic region have an ability to grow under extreme environmental conditions [10]. Among them red-orange pigment producing *Kocuria*sp. BRI 36 was observed to tolerate and accumulate heavy metals [11]. The response of BRI 36 to different heavy metals at different concentrations in terms of amount and color of pigment produced might prove useful in the detection of heavy metal contamination [12]. Our recent studies showed the possible role of chromosomal genetic elements in heavy metal tolerance of BRI 36 [13].

The isolate under study was also found to produce EPS at high sugar concentration. Interestingly, heavy metal tolerance of BRI 36 in terms of maximum tolerable concentration (MTC) was found to increase considerably with an increase in EPS produced by the isolate. Role of EPS in metal resistance has been previously documented by various researchers [14-17]. These observations prompted us to undertake studies on EPS production, and its effect on heavy metal tolerance of *Kocuria* sp. BRI 36.

MATERIALS AND METHODS

Chemicals and reagents

All the media components were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). The stock solutions of cadmium, lead and chromium at the concentrations of 1000 ppm each were purchased from Sigma-Aldrich. All chemicals and reagents used were of analytical grade.

Analysis of EPS

*Kocuria*sp. BRI 36 was cultivated on Mineral Salt Medium (MSM) at 30±2 °C for 48 h with 10% inoculum. The isolate was then grown in MSM containing 5 % glucose at 30±2 °C for 48 h. The occurrence of slimy colonies at the end of incubation period indicated EPS production by BRI 36. The isolate was then grown in MSM containing 5 % glucose at 30±2 °C for 48 h with shaking at 120 rpm. The contents were centrifuged (7826 xg for 20 min) and the culture cell-free broth was used to estimate EPS as carbohydrates following phenol-sulphuric acid method [18].

EPS production at different sugar concentrations

The production of Extracellular Polymeric Substance (EPS) was studied by growing the isolate at different concentrations of glucose (1–10%) and EPS as carbohydrates were estimated as mentioned above.

Effect of heavy metals

*Kocuria*sp. BRI 36 was cultivated in MSM amended with 5% glucose. The effect of Pb^{2+}, Cd^{2+} and Cr^{3+} at different concentrations (10 ppm–50 ppm) was examined individually on EPS production, The EPS estimation was carried out as mentioned above.

Maximum tolerable concentration (MTC)

MTC of Kocuria sp. BRI 36 was determined by growing the isolate in MSM supplemented with 5% glucose. Cd2+, Pb2+and Cr3+were added individually at concentrations ranging from 200-700 ppm. The samples were incubated for 48 h at 30±2 °C with shaking at 120 rpm.

Extraction of EPS

*Kocuria*sp. BRI 36 was grown in Mineral Salt Medium (MSM) with 5% glucose at 30±2 °C for 48 h with shaking at 120 rpm and biomass was separated by centrifugation at 7826xg for 15 min. The cell-free broth was then treated with 20% trichloroacetic acid (TCA) to precipitate protein fraction. Precipitated proteins were separated by centrifugation at 7826 xg for 15 min. The clear cell-free broth was used for EPS precipitation by adding two volumes of chilled ethanol. EPS obtained by filtration was calculated gravimetrically on the dry weight basis [19]. It was used as crude EPS for further experiments.

Fourier transform infrared spectroscopy (FTIR)

One milligram of crude dry EPS was directly used for FTIR (Brucker, tensor 37) analysis. The conditions used were 16 scans at a resolution of 4 cm^{-1} measured between 400 cm^{-1} and 4000 cm^{-1}.

RESULTS AND DISCUSSION

*Kocuria*sp. BRI 36 was found to produce EPS when grown in presence of 5% glucose. Increase in glucose concentration up to 10 % resulted in increase in total carbohydrate in cell-free broth with a maximum yield of 1 mg/ml at 5% glucose concentration (table 1).

Table 1: Effect of different glucose concentrations on EPS production

Glucose concentration (%)	EPS production[a] (mg/ml)	% Survival
1	0.164±0.0005	16.44
2	0.250±0.0005	25.06
3	0.449±0.0005	44.90
4	0.50±0.0005	50.39
5	1.00±0.05	100.00
6	0.394±0.001	39.42
7	0.362±0.0005	36.29
8	0.339±0.0005	33.94
9	0.258±0.0005	25.84
10	0.049±0.001	4.96

[a]mean±SD, n = 3.

In order to examine the effect of heavy metals on EPS production, the isolate was cultivated in presence of increasing concentrations of heavy metals individually along with 5% glucose. We observed an increase in carbohydrate content (0.2 to 89 % considering 1g/l as 100% yield) in cell-free broth up to 30 ppm metal concentration for lead and chromium while, in presence of 40 ppm cadmium, maximum EPS yield was detected (table 2). Metal-induced EPS synthesis has previously reported in various microorganisms [22].

Table 2: Effect of different concentration of heavy metals on EPS production

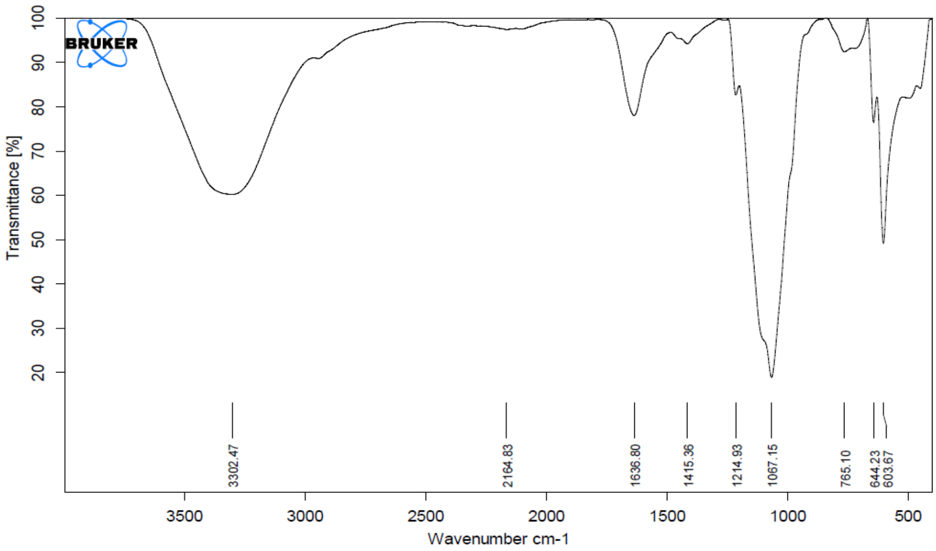
Metal concentration (ppm)	EPS production by*Kocuria sp.* BRI 36 (mg/ml)		
	Chromium	Lead	Cadmium
10	1.071±0.0005	1.27±0.005	1.20±0.005
20	1.151±0.001	1.78±0.005	1.23±0.005
30	1.300±0.02	1.89±0.005	1.27±0.005
40	1.071±0.0005	1.23±0.005	1.78±0.005
50	1.063±0.0005	1.20±0.005	1.29±0.005

mean±SD, n = 3.

*Kocuria*sp. BRI 36 possesses the ability to tolerate high concentrations of heavy metals in the range of 300 to 600 ppm isolate demonstrated MTC of 500 ppm for each of Cd^{2+}, Pb^{2+} and Cr^{3+} with survival percentage in the range of 16.1 to 29 % [11]. In the present investigation, we examined the effect of an increase in the concentration of glucose on MTC of *Kocuria* sp. BRI 36. We observed an increase in MTC values and improved percent survival of BRI 36 (table 3). Maximum MTC of 700 ppm was noted for Pb^{2+} followed by Cd^{2+}(600 ppm) and Cr^{3+}(600 ppm). Interestingly, percent survival increased from 36.12 to 39.4% (table 3) at MTC. These observations along with an increase in EPS levels (as mentioned above) suggest a significant role of EPS in metal tolerance. Earlier many researchers discussed enhanced EPS levels in microorganisms when cultivated under stress [23-26]. Synthesis of EPS is one of the mechanisms adopted by microorganisms to protect themselves from permeation of toxic metal ions. EPS, acting as a protective shield, sequesters metal ions, thereby inhibiting their penetration. The interaction might immobilize metal ions. Thus, as suggested by Gupta and Diwan EPS production can be considered as constitutive nonspecific mechanism displayed by microorganisms in the response of heavy metals [22].

Table 3: Increase in MTC and survival rate of *Kocuria* sp. BRI 36 at 5% glucose

Isolate	Metals	At 5% glucose	
		MTC (ppm)	% Survival
BRI 36	Pb^{2+}	700	36.12
	Cr^{3+}	600	35.3
	Cd^{2+}	600	39.4


BRUKER
Transmittance [%]
Wavenumber cm-1
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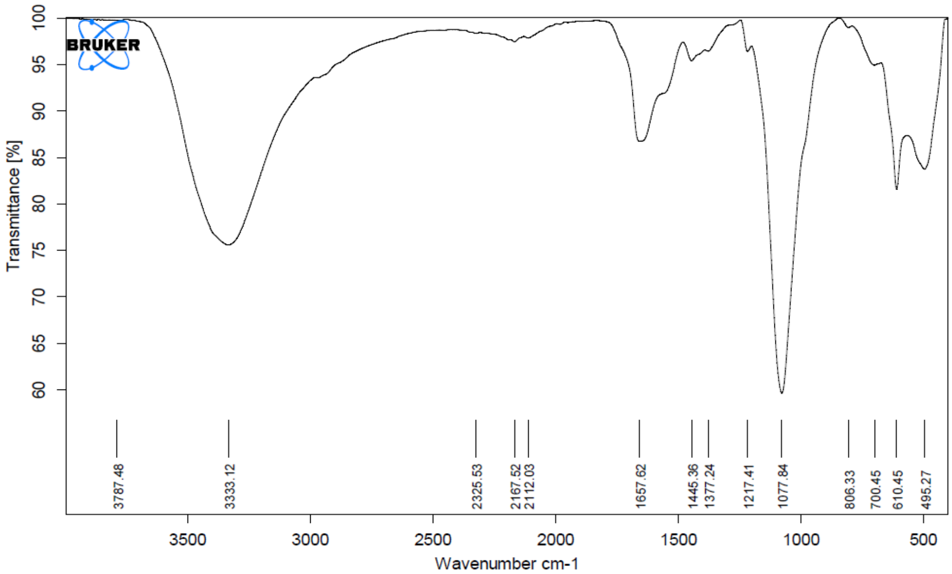
2164.83
1636.80
1415.36
1214.93
1067.15
765.10
644.23
603.67

a)


BRUKER
Transmittance [%]
Wavenumber cm-1
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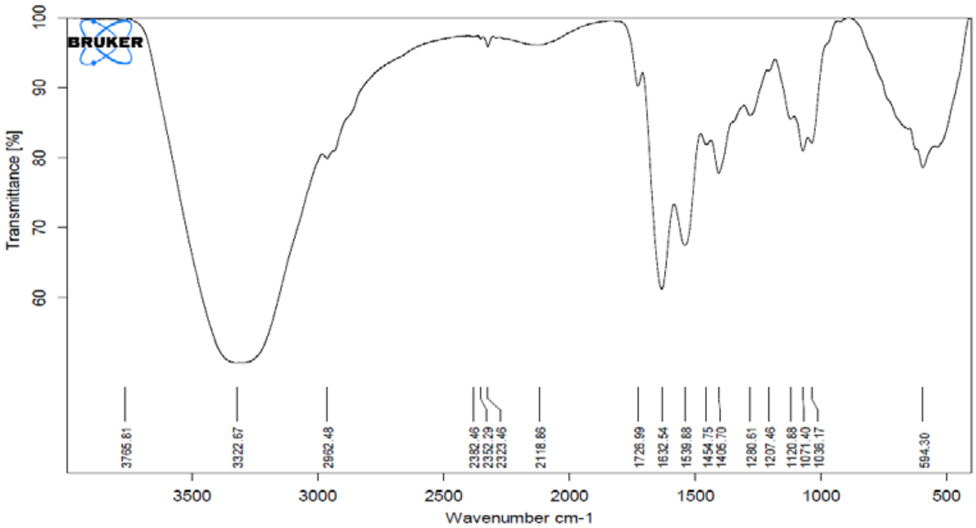
3333.12
2325.53
2167.52
2112.03
1657.62
1445.36
1377.24
1217.41
1077.84
806.33
700.45
610.45
495.27

b)


BRUKER
Transmittance [%]
Wavenumber cm-1
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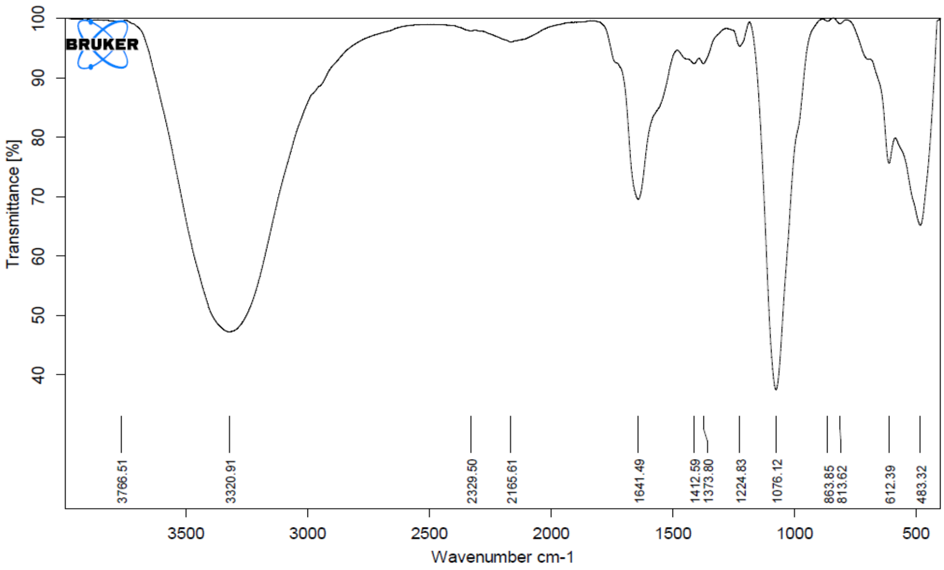
2962.48
2382.46
2352.29
2323.46
2118.86
1726.99
1632.54
1539.88
1454.75
1405.70
1280.61
1207.46
1120.88
1071.40
1038.17
594.30

c)



d)

Fig. 1: FTIR spectra of a) control, b) cadmium treated EPS, c) lead treated EPS and d) chromium treated EPS

Fourier Transform Infrared Spectroscopy (FTIR) analysis of crude EPS produced by *Kocuria* sp. BRI 36 revealed distinct stretching frequencies of chemical groups (fig. 1a). The dominant peaks were observed at 3302.47, 2164.83, 1636.80, 1415.36, 1214.14, 1067.15, 765.10, 644.23 and 603.67 $^{cm-1}$respectively. This is in accordance to the peak assignment of 3400–3200 $^{cm-1}$(O-H or H bond), 3000–2800 $^{cm-1}$(C-H bond), 1850–1600 $^{cm-1}$ (C=O bond), 1400–1370 $^{cm-1}$(C-O bond), 1240 $^{cm-1}$(C=O bond) and 900–1150 $^{cm-1}$(C-O-C bond) [27]. However, analysis of EPS extracted from BRI 36 cultivated in metal-containing media demonstrated shifts in wave numbers (fig. 1b, 1c, 1d) as compared to control (fig. 1a). Noticeable alterations were observed in $Pb^{2+}$and Cd^{2+}loaded EPS samples indicating metal adsorption to carboxyl groups present on EPS. Moreover, shifts observed at 1076 to 1120^{cm-1} are due to the characteristic of uronic acid, O-acetyl ester linkage bond [28]. Our observations are in line with the explanation specified by Zhang *et al.* and Guibaud*et al.* that, metal adsorption involves 'O' of the polymeric structure interacts with metal in adsorption process to reduce the electron cloud density of functional groups containing oxygen and causing shifts in vibration frequency and intensity [29, 30]. Involvement of O-H, C=O, and C-O-C groups in heavy metal adsorption by EPS was also reported by Perez *et al.* [31] during their studies on *Paenibacillusjamilae.*

CONCLUSION

Maximum Tolerable Concentration (MTC) of *Kocuria* sp. BRI 36 for Pb^{2+}, $Cd^{2+}$and Cr^{3+}increased and percent survival at MTC increased considerably when the isolate was grown in presence of glucose. This may be attributed to increased levels of an extracellular polymeric substance (EPS) observed in presence of metals. The assumption was further established by Fourier Transform Infrared Spectroscopy (FTIR) analysis of samples. The comparative studies confirmed the involvement of O-H, C=O, and C-O-C groups present on exopolysaccharides in metal adsorption. Therefore, use of surface polymer produced by *Kocuria* sp. BRI 36 might prove an effective biocontrol method by reducing the availability of heavy metals, thus reducing adverse effect caused by their uptake and/or accumulation.

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AUTHORS CONTRIBUTIONS

All the experimental part of the work was completed by Anuradha Mulik. Rama Bhadekar contributed through planning of the work, result analysis, a compilation of the work and manuscript writing.

CONFLICTS OF INTERESTS

No conflict of interest was reported by the authors

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