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Research Paper

STUDIES ON CHARACTERIZATION OF BIOFILM ASSOCIATED EPS OF *Pseudomonas aeruginosa* AND ITS INTERACTION WITH THE ORGANIC AND INORGANIC POLLUTANTS

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Abstract

The present study was performed to explore the potential of biofilm associated EPS of a marine bacterial strain *Pseudomonas aeruginosa* PFL-P1 previously isolated from polycyclic aromatic hydrocarbon (PAH) contaminated site of Odisha coast for remediation of toxic organic and inorganic pollutants. Minimum inhibitory concentrations were determined against organic pollutants phenanthrene as well as pyrene and also against inorganic pollutant, lead (Pb). The biofilm associated EPS was extracted and characterized by colorimetric methods. Interaction of lead (Pb) with biofilm associated EPS was studied by fluorescence spectroscopy. The minimum inhibitory concentration of lead was observed. The strain was able to utilize phenanthrene and pyrene as carbon sources and was also resistant to lead, a toxic heavy metal. Characterization of EPS revealed a protein content of 36.1µg/mg and carbohydrate content of 44.5µg/mg in extracted EPS.

Key words: Bioremediation, EPS, Heavy metal and lead.

INTRODUCTION

The beneficial use of microbial biofilms in remediation of environmental pollutants plays an important role in bioremediation. Bioremediation is an environmental friendly and cost effective method that utilizes the microbes to decontaminate and degrade lots of pollutants into less harmful products. Biofilms are assemblage of microorganisms of microbial species attached to the biological or inert surfaces immobilized in a self-synthesized matrix comprising of water, proteins, carbohydrates and DNA, which gives protection from the contaminants. Biofilm forming bacterium isolated from marine

environment have been adapting them in adverse condition by secreting high amount of EPS and hence used in bioremediation of heavy metals and polycyclic aromatic hydrocarbons[1].The extra cellular polymeric substance (EPS) matrix which also provide a structure to the biofilm forming microbes and has been found to be metal binding and also has the potential to influence the heavy metal availability in the environment [2].The bacterial species *Pseudomonas* tends to resist lead (Pb) by precipitating the biofilm associated EPS, decontaminate the pollutants such as heavy metals and organic compounds. EPS also serve as a trap for metal and metalloids due to the presence of negatively charged functional groups which will help in the formation of the complexes with the heavy metals and organic contaminants and in their removal. The biofilm forming marine bacteria are adapted to survive and suited for bioremediation as they have the tolerance towards harsh environment. Therefore bacterial EPS can be used efficiently as a surface active agent for bioremediation [3]. The present study was performed to explore the potential of biofilm associated EPS of a marine bacterial strain *Pseudomonas aeruginosa* PFL-P1 previously isolated from polycyclic aromatic hydrocarbon (PAH) contaminated site of Odisha coast for remediation of toxic organic and inorganic pollutants.

MATERIALS AND METHODS

Determination of tolerance level of PFL-P1 In lead and PAH

For checking the ability of the strain *Pseudomonas aeruginosa* PFL-P1 to utilize different PAH and metal resistance, two nutrient media were utilised. The PAH solutions including phenanthrene and pyrene, were added separately in Bunshell Haas Broth (BHM) [Himedia] media whereas metal like lead was supplemented in Muller Hinton Agar (MHA) [Himedia]. The PAH stock solution was prepared as 50Mg/ml in acetone and added to autoclaved BHM broth varying its concentration from100 ppm to 5000 ppm. Similarly lead stock solution was prepared as10000 ppm by dissolving lead nitrate in distilled water and adding the filtered solution varying its concentration from 100ppm to 2000ppm in autoclaved MHA and pouring it in petriplates. The overnight culture of PFL-P1 was inoculated in both the cases and incubated at 37˚C for 24 hours. Observations were recorded in terms of MIC i.e., the minimum inhibition concentration of PAH's and metal that causes almost complete inhibition or give no visible growth over the solidified media plates.

Extraction of Biofilm associated EPS

Sterilized ceramic beads were used as substratum for the growth of biofilm forming bacterium *P.aeruginosa* PFL-P1. Luria bertani broth was used as a growth supplement in which over night cultures ofPFL-P1was inoculated and kept for incubation at 37˚C for 48 hours. After 48 hours, the media was discarded to remove the planktonic cells followed by vortexing to disintegrate the associated biofilm from the beads. The vortex sample was collected and centrifuged at 7500 rpm for 15mins in 4˚C. The supernatant was collected and stored at 4˚C for 24 hours. To this solution ice-cold ethanol was added followed by centrifugation at 7500 rpm for 15 mins at 4˚C.The supernatant was discarded. The pellet so obtained was washed with deionized water followed by brief centrifugation. The collected pellet was lyophilized, stored at 4˚C and used for further studies.

Characterization of extracted EPS by colorimetric method

Estimation of protein content in extracted EPS by Bradford's Assay

The Comassie brilliant blue protein assay, commonly known as the Bradford assay is widely used because of its rapid and convenient protocol as well as its relative sensitivity. Determination of micro gram quantities of protein in the Brad ford comassie brilliant blue assay is accomplished by measurement of absorbance at 590nm. This most common assay enables rapid and simple protein quantification in cell lysates, cellular fractions, or recombinant protein samples, for the purpose of normalization of bio chemical measurements where Bovine Serum Albumin (BSA) can be used as standard. Brad ford reagent was prepared by dissolving1mg of comassie brilliant blue G-25 was in1mlof methanol 2ml of 85% of concentrated phosphoric acid(H_3PO_4). The final volume was made upto 20ml with mill Q water. This whole solution was filtered and stored in dark bottle at 4˚C.Stock solution of concentration 0.1mg/ml was prepared using BSA as standard. The extracted EPS was prepared as 1 mg/ml in milli Q water. The working standards were prepared as 2-10µg/ml in triplicates by diluting the standard BSA stock solution. Milli Q water was used as blank. 1 ml of Brad ford reagent was added to each sample. After 5 minutes of incubation, the absorbance was measured at 595nm in UV-visible spectrophotometer [Perkin Elmer Lambda 35]. The observed data for known concentration of standard was plotted against the measured absorbance. From the linear graphs obtained, the

value for the unknown i.e., EPS was forecasted.

Estimation of total carbohydrate content in extracted EPS by Phenol Sulphuric Acid method

Phenol Sulphuric acid method is the most reliable and easiest method [4] among the quantitative assays for carbohydrate estimation. This method is widely used to determine the total concentration of carbohydrate [5]. The results are expressed in the terms of a single carbohydrate which is used as standard, usually glucose. In this method, in hot acidic medium glucose is dehydrated to hydroxyl methyl furfural, this forms a yellow brown coloured product with phenol and has absorption maximum at 490nm [6]. The sulphuric acid causes all non reducing sugar to be converted to reducing sugars that this method determines the total sugar present in the sample. 20,40,60,80 and 100µl of working standard (with 0.1mg/ml conc.) Of glucose was taken in boiling tubes and the final volumes of each tube were made 1ml by adding distilled water.1ml of 5% Phenol and 5ml of 96% Sulphuric acid was added one by one in each tubes and shook well so that the Phenol and sulphuric acid get mixed thoroughly with working standard.After 10 minutes all the tubes were placed in water bath at 25-30°C for 15 minutes. Blank was set with 1ml of distilled water and O.D of each tube was taken at 490 nm with the help of spectrophotometer. Then the whole process following Phenol and Sulphuric acid method was repeated with 1mg/L of extracted EPS samples and the O.D.s of samples solutions was taken.

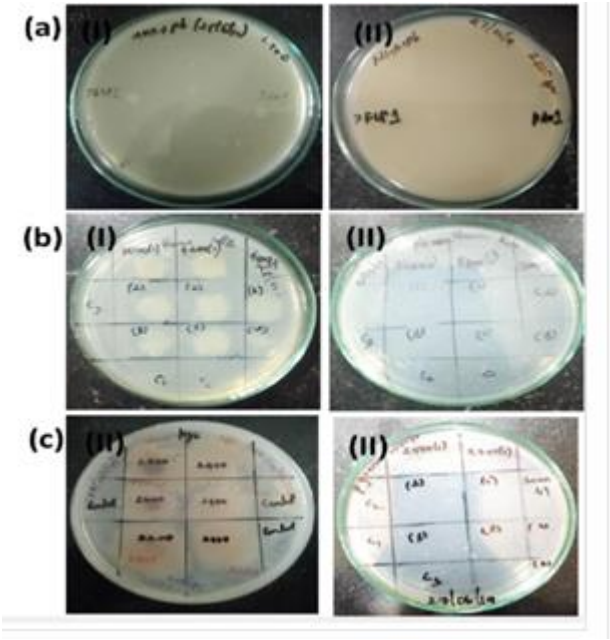
Fluorescence quenching titration using EEM fluorescence spectroscopy

The fluorescence spectra of the EPS solution were recorded with a fluorescence spectrophotometer (Hitachi Model F-7000 FL Spectrophotometer). Excitation (EX) emission (EM) spectra (EEM) were collected over an excitation of 250nm along with an emission range of 260–450nm. The band pass for excitation and emissions lits were adjusted to 5nm. The scanning speed was maintained at 240nm min^{-1}. 3 DEEM data were processed by software Origin9. Lyophilized EPS was reconstituted in Milli- Q water. 10ml of pristine EPS solution (30mgl^{-1}) was titrated with different concentrations (100,300,500,700and1000 µm) of Pb (II) at room temperature.

RESULTS

Determination of tolerance level of PFL-P1 in lead and PAH

After incubating the inoculated culture of PFL-P1 over media supplemented with varying concentration of lead (Pb), the minimum inhibitory concentration of lead was observed as 2000ppm. The strain could utilize upto 4500 ppm and 2400 ppm of phenanthrene and pyrene respectively.



Metal and PAHS concentrations used for deducing the minimum inhibitory concentration (MIC) for *Pseudomonas aeruginosa* pfl-P1. Growth observed in (a) Lead with 1700 ppm and 2000 ppm prepared in MHA; (b) Phenanthrene varying from 4000 ppm to 6000 ppm in BHM; (c) Pyrene varying from 2200 ppm to 3000 ppm in BHM.

Characterization of different EPS components by colorimetric methods.

Biofilm associated EPS was extracted by ethanol precipitation method. The standard curve was plotted between known concentrations of BSA on X-axis with their respective measured absorbance at 595nm on Y-axis (figure 1). Bradford's assay was utilized to deduce concentration of protein in the extracted EPS which resulted in presence of 36.1µg/mL of protein in 1mg/mL of EPS sample.

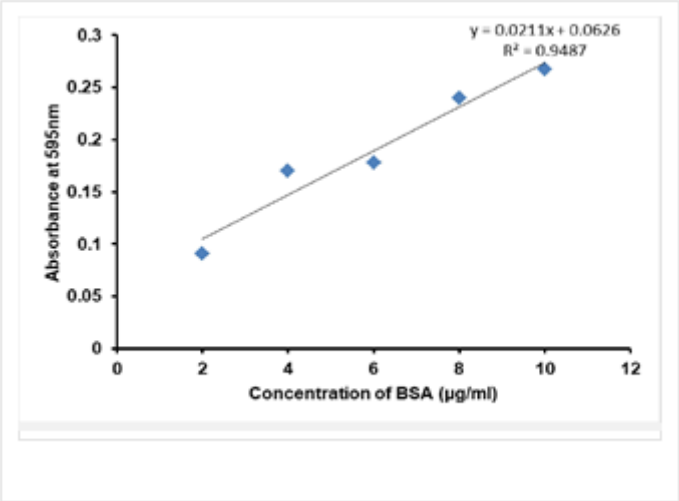
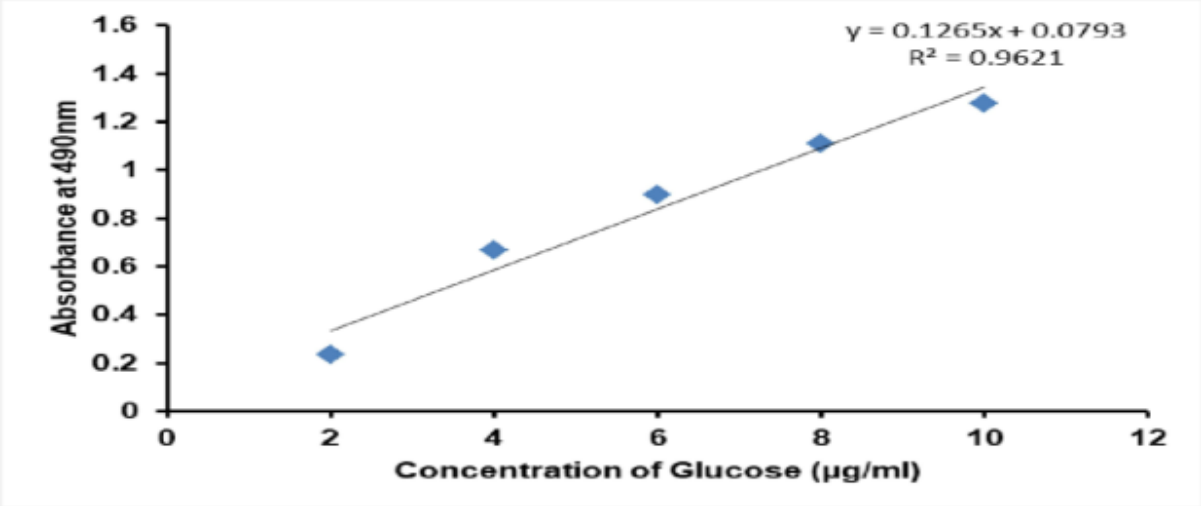


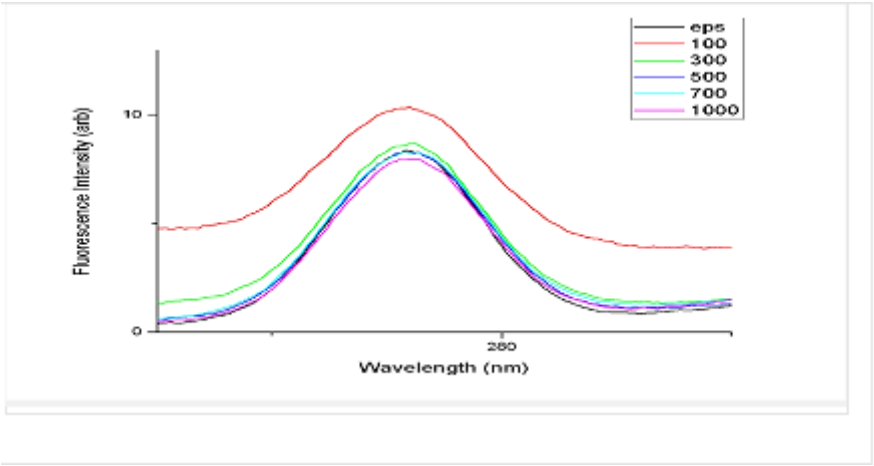
Fig. 1: Concentration of protein in EPS.

The Phenol- Sulphuric acid method was used to determine the total carbohydrate content in the extracted EPS. From the standard curve of known concentration of glucose absorbance at 490nm (figure 2), the total carbohydrate was quantified as 40.45µg/mL in 1mg/mL of extracted EPS sample.



Fluorescence quenching titration using EEM fluorescence spectroscopy

Fluorescence spectroscopy was performed to evaluate the quenching property of heavy metals. Different concentration of Pb was treated with 30mg/L of EPS. However, no definite pattern of EPS quenching was observed (Figure 3).



DISCUSSION

In the present study fluorescence quenching was performed to analysis the quenching property of heavy metals. However as a result no definite pattern of EPS quenching was observed. Some studies had reported that only one fluorescence peak was identified in quenching of *P. pseudoalcaligenes* NP103 EPS. The fluorescence peak was located at an excitation of 220nm and emission of 310nm.Two excitation wavelengths 220 – 230 and 270 – 280 nm are reported to be indicators of protein like fluorophores [7].The fluorescence intensity of plots of EPS of *P. pseudoalcaligenes* NP103 decreased with increased concentration of Pb. Similar results of decreased fluorescence intensities of protein like fluorophore of sludge EPS on interaction with pb was reported [8]. The present study shows that the minimum inhibitory concentration of lead was observed as 2000ppm. The strain could utilize up to 4500 ppm and 2400 ppm of phenanthrene and pyrene. The pure bacterial have the settling ability than the sludge EPS which makes it more suitable agents to be used in heavy metals sequestration and they had reported the potential of EPS extracted from *klebsiella* sp. and observed that EPS derived from this bacterium can only remove lead (Pb) present in lower concentration [9]. Thus the bacteria have been designated for assessing pollution through their tolerance and biosorption of heavy metals. The EPS matrix also provides a structure to a biofilm forming microbes and has found to be metal binding potential to influence the heavy metal availability in the environment [10]. The bacterial species *Pseudomonas* tends to resist lead (Pb) by precipitating the biofilm associated EPS also serves as a trap for metal and metalloids due to the presence of negatively charged functional groups which will help the formation of the complexes with heavy metals and organic contaminants and in their removal. Therefore it had been reported that bacterial EPS can be used efficiently as a

surface active agent for bioremediation [11]. Thus the bacteria have designated for assessing pollution through their tolerance and biosorption of heavy metals. Polycyclic aromatic hydrocarbons are unique in nature and are of greater importance due to their persistence, toxicity and carcinogenic in nature, however many marine bacteria have been reported to have the potential for the bioremediation [12,13].

CONCLUSION

Marine bacteria have diverse metabolic potential and are resistant to various harsh environments. *P. aeruginosa* strain was able to utilize phenanthrene and pyrene as carbon sources and was also resistant to lead, a toxic heavy metal. Cells inside biofilm are sheltered with in an EPS matrix which protects them from toxic environments. The biofilm EPS is composed of proteins, polysaccharides, nucleic acids, lipids and humic acid substances. Characterization of EPS revealed a protein content of 36.1µg/mg and carbohydrate content of 44.5µg/mg in extracted EPS. The ecological implications of EPS form land PAH sequestration capabilities will be investigated further.

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