Conference Abstract

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Marine bacterial biofilm in bioremediation of organic and inorganic pollutants

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Abstract

Increasing industrialization and unsustainable use of polycyclic aromatic hydrocarbons (PAHs) and toxic metals poses serious environmental pollution. Marine environment serves as the highly dynamic milieu and the inhabitant microbes possess *de-novo* potential in bioremediation. Use of biofilm-EPS (extracellular polymeric substances) forming microorganisms from marine microbiota have enormous metabolic potential for environmental clean-up. Many biofilm forming marine bacteria; *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Alcaligenes*, *Escherichia*, *Vibrio* and *Ochrobactrum* have been isolated and characterized from Bay of Bengal, Odisha, India. These isolates tolerate substantial level of PAHs (naphthalene, phenanthrene and pyrene) and toxic metals (Hg and Cd).

Key words: biofilms, bioremediation, marine bacteria, PAHs, toxic metals

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ABSTRACT

Increasing industrialization and unsustainable use of polycyclic aromatic hydrocarbons (PAHs) and toxic metals poses serious environmental pollution. Marine environment serves as the highly dynamic milieu and the inhabitant microbes possess de-novo potential in bioremediation. Use of biofilm-EPS (extracellular polymeric substances) forming microorganisms from marine microbiota have enormous metabolic potential for environmental clean-up. Many biofilm forming marine bacteria; *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Alcaligenes*, *Escherichia*, *Vibrio* and *Ochrobactrum* have been isolated and characterized from Bay of Bengal, Odisha, India. These isolates tolerate substantial level of PAHs (naphthalene, phenanthrene and pyrene) and toxic metals (Hg and Cd). Increase in the synthesis of biofilm-EPS by the marine bacterium *Pseudomonas mendocina* NR802 in presence of Ca^{2+} enhanced phenanthrene degradation by 15%. Biofilm of *Stenotrophomonas acidaminiphila* NCW-702 efficiently degraded 71.1±3.1% and 40.2±2.4% of phenanthrene and pyrene respectively, as compared to their planktonic forms (38.7±2.5% and 29.7±1% phenanthrene and pyrene respectively). Another biofilm-forming marine bacterium *Pseudomonas aeruginosa* N6P6 was isolated and found that phenanthrene and pyrene degradation was affected by biofilm growth and lasI expression. The respective phenanthrene degradation for 15, 24, 48, and 72 h old biofilm after 7 days was 21.5, 54.2, 85.6, and 85.7 %. However, the corresponding pyrene degradation was 15, 18.28, 47.56, and 46.48%, respectively. Similarly, highly mercury resistant biofilm forming marine bacterium *B. thuringiensis* PW-05 was found to volatilize 98.44% of Hg^{2+} in-vitro with respect to a terrestrial isolate which volatilized 60.06% of Hg. Furthermore, high expression of *merA* gene, responsible for Hg^{2+} volatilization, in marine *Bacillus* sp. confirmed the superiority of marine bacteria for bioremediation. A transgenic bacterium *Bacillus cereus* BW-03(pPW-05), constructed by transforming a plasmid harbouring *mer* operon, was able to remove >99% of mercury supplement in-vitro by simultaneous volatilization (>53%) and biosorption (~40%). Moreover, encapsulation of the transformant increased its mercury removal potential to almost 100%. In case of cadmium, functional groups of marine bacterial biofilm-EPS of *Pseudomonas aeruginosa* JP-11 was found to act as a capping agent for CdS nanoparticles synthesis and removed 88.66% of Cd from aqueous solutions. Thus, the potential of biofilm forming marine bacteria may be harnessed for various bioremediation applications.

INTRODUCTION

•Biofilms are multilayered, three dimensional sessile structures encapsulated in hydrated EPS on a substratum. It is a rich matrix of polysaccharides, proteins and nucleic acids.

•Biofilm development is a type of adaption cycle by microbial community, regulated by quorum sensing (QS). Biofilms are highly tolerant to the physical, chemical and biological stresses.

•Marine bacteria are potential candidates utilized for bioremediation of toxic metals (inorganic), hydrocarbon (organic) recalcitrant compounds through biofilm formation and production of EPS.

•Biofilm forming marine bacteria are used for PAHs (phenanthrene, pyrene) and toxic metals (mercury, cadmium) remediation which are hazardous to the ecosystem and biomass due to their toxicity and carcinogenic effects.

MATERIALS AND METHODS

Collection of Sample

Sediment samples were collected from the Odisha coast of Bay of Bengal in sterile tubes and selective enrichment technique was used to isolate phenathrene and pyrene degrading isolates on Basal salt medium (BSM). The samples were serially diluted and plated onto seawater nutrient (SWN) agar medium supplemented with varying concentrations of Cd and Hg to isolate metal resistant marine bacteria. *P. mendocina* NR802, *S. acidaminiphila* NCW-702, *P. aeruginosa* N6P6, *B. thuringiensis* PW-05 and *P. aeruginosa* JP-11 were selected for further studies.

Confocal scanning laser microscopy (CSLM) and phenanthrene biodegradation by P. mendocina NR802

Staining with Syto9 and ConA-TRITC followed by CSLM studies was performed to monitor the biofilm architecture of *P. mendocina* NR802 grown on glass slides under various growth media (i.e. LB media, TRIS-G and TRIS- with 20 mM of Ca^{2+} and Mg^{2+}). Mid-log phase culture was inoculated into TRIS medium with phenanthrene (100 mg/l). After 7 days, 200 µl of this culture was transferred to screw capped glass tubes with glass beads, containing 2 ml of respective media (LB, TRIS, 20 mM Mg^{2+}-TRIS and 20 mM-Ca^{2+} TRIS media, supplemented with 1% glucose). After 48 h without shaking, free planktonic cells were removed from the tubes and biofilm grown on the glass beads was rinsed with sterile PBS. 5 ml of TRIS medium containing phenanthrene (100 mg/l) was added to each tube and incubated for 7 days. After incubation, the residual concentration of phenanthrene was extracted with an equal volume of n-hexane (Merck, India). Concentration of phenanthrene was determined in a UV/Vis spectrophotometer at 292 nm.

Biodegradation studies of phenanthrene and pyrene by S. acidaminiphila NCW-702

1 ml of the overnight culture was transferred to 100 ml TRIS medium, supplemented with 100 mg/l of phenanthrene and pyrene respectively. It was incubated in dark at 37°C and 160 rev/min with aeration for 7 days. This pre-enriched culture was further diluted to 1:10 in LB medium, and 2 ml of it was transferred to a screw-capped glass tubes with glass beads. After 48 h, free planktonic cells were carefully aspirated from the tubes and biofilms grown over the glass beads was rinsed with sterile PBS. 5 ml of TRIS medium with 100 mg/l phenanthrene and pyrene respectively were transferred to tubes with biofilm and incubated at 37°C under static condition. At regular time intervals, the residual concentration of phenanthrene/pyrene were extracted with an equal volume of n-hexane. Concentration of phenanthrene and pyrene was determined in an UV/Vis spectrophotometer in terms of absorbance at 292 and 335 nm, respectively.

Biofilm mediated PAH degradation by P. aeruginosa N6P6

Degradation of phenanthrene and pyrene by *P. aeruginosa* N6P6 biofilm (15h, 24h, 48h, 72h old) was performed over the period of 7 days following Mangwani et al.

Mercury volatilization potential of B. thuringiensis PW05 and B. cereus BW-03(pPW-05)

The isolate PW05 and transgenic *B. cereus* BW-03(pPW-05) was grown in SWN broth supplemented with 50 ppm of mercury as $HgCl_2$ for 48 h, and the cell suspension was harvested by centrifugation. The cell mass was suspended into 0.05 ml of 0.07 M phosphate buffer (pH 7.0) containing 0.5 mM EDTA, 0.2 mM magnesium acetate, 5 mM sodium thioglycolate and 50 ppm of $HgCl_2$ in a microtitre plate. The plate was covered with X-ray film and was incubated in dark for 60 min at 35 °C.

Removal of mercury by B. cereus BW-03(pPW-05) after encapsulation

The overnight grown culture of *B. cereus* BW-03(pPW-05) in SWN broth supplemented with 10 ppm $HgCl_2$ was mixed with 20 ml of sterilized 1% w/v sodium alginate solution, mixed thoroughly for 30 min to obtain a homogenous suspension and the suspension was extruded drop wise into the sterile hardening solution i.e. 4% w/v $CaCl_2$. The beads were washed with sterile milliQ water and inoculated into various concentrations of mercury supplementations (10, 20, 30 and 50 ppm) in SWN broth. After incubation of 24, 48 and 72 h, the supernatant was harvested after separating the beads and the mercury was analyzed using cold vapor atomic absorption spectrophotometer(AAS) (Perkin Elmer, USA).

Synthesis of cadmium sulfide (CdS) NPs and Cd removal from aqueous solutions

Cadmium nitrate and sodium sulfide were used as precursors for the synthesis of CdS NPs. D-glucose ($C_6H_{12}O_6$) or EPS was used as a capping agent for the reaction mixture. Cd adsorption experiments were performed by adsorbent (pristine EPS, functionalized EPS and NPs incorporated EPS) in aqueous solution of desired concentration (25, 50, 75 and 100 ppm) of Cd salt at optimum pH at required temperature (298 K, 308 K and 318 K) for 24 h and 48 h.

RESULTS

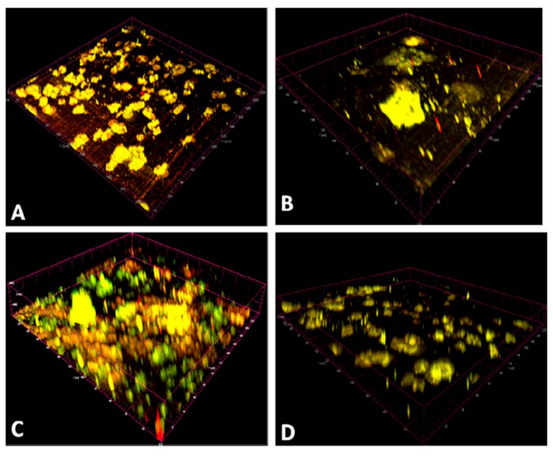


Fig. 1. CSLM images showing *P. mendocina* NR802 biofilm (48 h old) grown in different medium. **(A)** LB **(B)** Tris-G **(C)** Tris-G + 20 mM Ca2+ **(D)** Tris-G + 20 mM Mg2+. Biofilms were stained with Syto9 and ConA-TRITC. Syto9 specifically stains the live cells whereas ConA-TRITC binds bacterial EPS. Green and red colors indicate the presence of exopolysaccharides and live cells in *P. mendocina* NR802 biofilm, respectively.

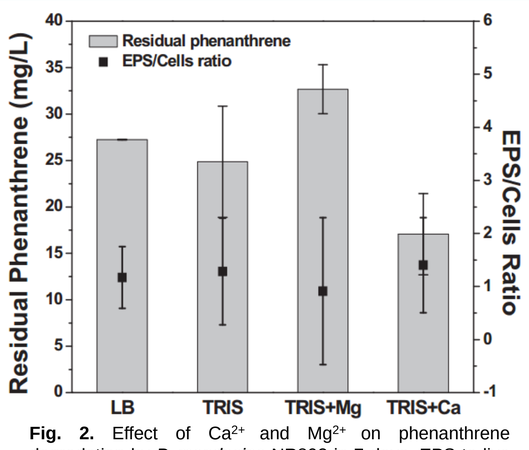


Fig. 2. Effect of Ca^{2+} and Mg^{2+} on phenanthrene degradation by *P. mendocina* NR802 in 7 days. EPS to live cells ratios are shown to indicate the correlation between phenanthrene degradation and EPS/cells ratio in biofilm grown in respective media.

Mangwani N, Shukla SK, Rao TS, & Das S (2014). *Colloids Surf B* 114, 301-309.

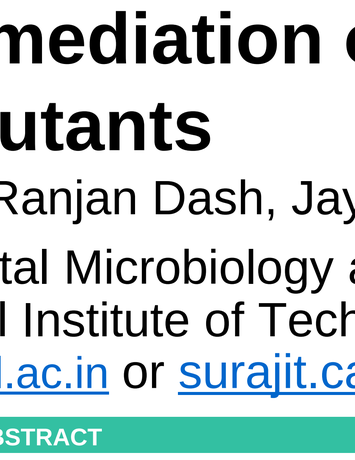


Fig. 3. 3D surface plot of *S. acidaminiphila* NCW-702 biofilm growth over the period of 7 days constructed using IMAGE J.

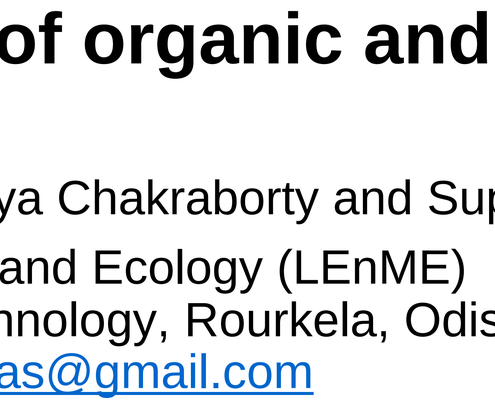


Fig. 4. *S. acidaminiphila* NCW-702 biofilm (after 48 h) after SYTO9 and ConA-TRITC staining. Green and red colours indicate the presence of cells (I) and exopolysaccharides (II) in *S. acidaminiphila* NCW-702 biofilm. (III) Superimposed image of (I) and (II). (IV) Superimposed image of (I) and (II) with vertical cross sections showing biofilm thickness.

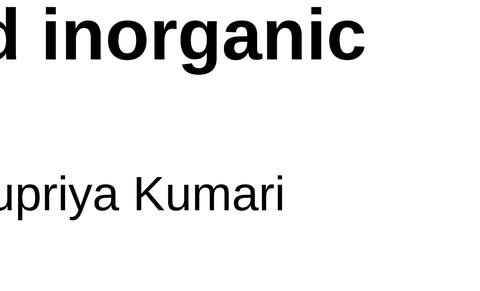
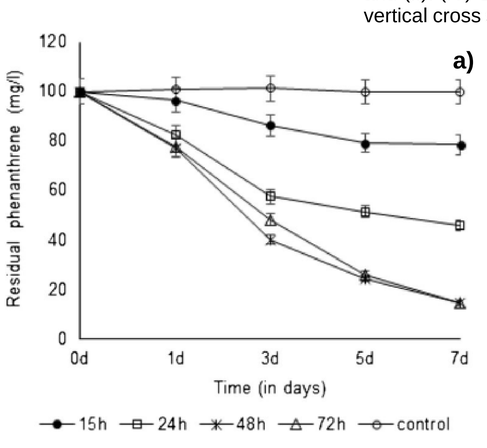


Fig. 5. Phenanthrene and pyrene degradation with *S. acidaminiphila* NCW-702 biofilm and planktonic cultures over the period of 15 days.

Mangwani N, Shukla SK, Kumari S, Rao TS, Das S (2014). *J. Appl. Microbiol.* 18,12602.



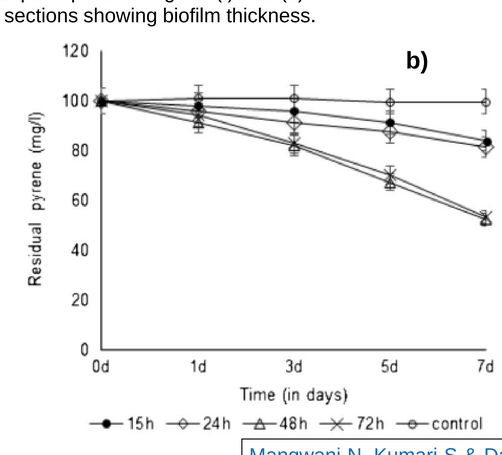


Fig. 6. Effect of biofilm growth phase on phenanthrene **(a)** and pyrene **(b)** degradation over the period of 7 days.. Maximum degradation was observed when 48 h biofilm was used for degradation. Over the period of 7 days, degradation of both phenanthrene and pyrene was significantly affected by biofilm growth phase (two-way ANOVA $P < 0.05$).

Mangwani N, Kumari S & Das S (2015). *Appl. Microbiol. Biotechnol.* DOI 10.1007/s00253-015-6868-7.

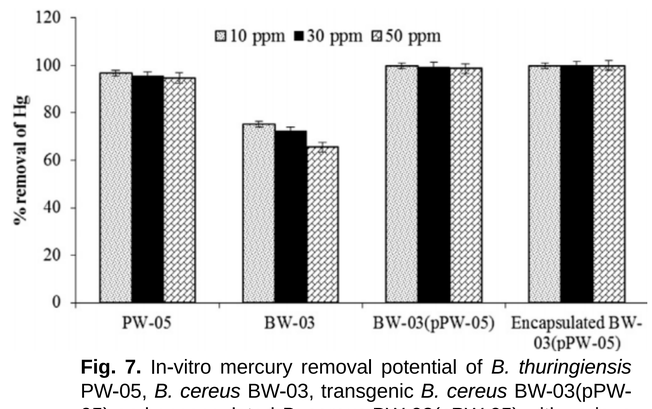


Fig. 7. In-vitro mercury removal potential of *B. thuringiensis* PW-05, *B. cereus* BW-03, transgenic *B. cereus* BW-03(pPW-05) and encapsulated *B. cereus* BW-03(pPW-05) with various concentrations of $HgCl_2$.

Table. 1. Percentage removal of cadmium from aqueous solution.

Adsorbents	% Removal of Cd (24 h)	% Removal of Cd (48 h)
Pristine EPS	57.41 ± 0.48	61.88 ± 0.45
Functionalized EPS	77.07 ± 0.99	80.81 ± 0.89
CdS NPs incorporated functionalized EPS	86.46 ± 0.71	88.66 ± 0.61

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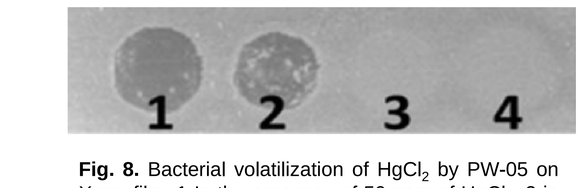

Fig. 8. Bacterial volatilization of $HgCl_2$ by PW-05 on X-ray film. 1-In the presence of 50 ppm of $HgCl_2$, 2-in the presence of 10 ppm of $HgCl_2$, 3-PW-05 grown without Hg, 4-sensitive strain of *E. coli* (DH5α).

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Dash HR, & Das S (2015). *Int. Biodeter. Biodegr.* 103, 179-185.

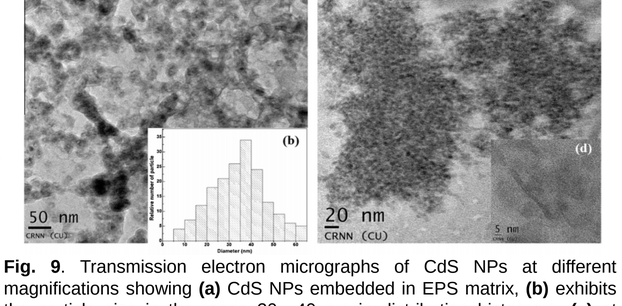

Fig. 9. Transmission electron micrographs of CdS NPs at different magnifications showing **(a)** CdS NPs embedded in EPS matrix, **(b)** exhibits the particle size in the range 20– 40 nm in distribution histogram, **(c)** at higher magnification show large number of dispersed NPs and (d) lattice parameter of CdS NPs with high level of crystallinity at 5 nm resolution.

SUMMARY

- *P. mendocina* NR802 in presence of Ca^{2+} enhanced phenanthrene degradation by 15%. Within 7 days, phenanthrene degradation was found to be 82.9 ± 4.37% in presence of calcium.
- Biofilm of *S. acidaminiphila* NCW-702 efficiently degraded 71.1±3.1% and 40.2±2.4% of phenanthrene and pyrene respectively, as compared to their planktonic forms (38.7±2.5% and 29.7±1% phenanthrene and pyrene respectively).
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- The transgenic *Bacillus cereus* BW-03(pPW-05), removed >99% of mercury supplement in-vitro by simultaneous volatilization (>53%) and biosorption (~40%). Encapsulation of the transformant increased its mercury removal potential to almost 100%.
- CdS NPS were embedded in EPS matrix of *P. aeruginosa* JP-11 and formed homogenous uniform sized NPs in the range of 20–40 nm. CdS NPs incorporated functionalized EPS could remove 88.66% of Cd from aqueous solutions after 48 h.
- Therefore, marine bacteria synthesizing biofilm-EPS can be effectively utilized in bioremediation of organic and inorganic pollutants from toxic contaminated sites.

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