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Short Communication

Assessment of 2,4-Di-tert-butylphenol induced modifications in extracellular polymeric substances of *Serratia marcescens*

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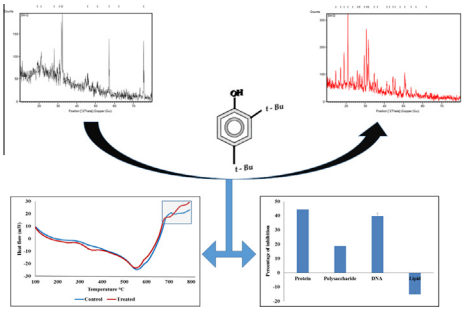
HIGHLIGHTS

- Effect of DTBP on EPS of *S. marcescens* was evaluated using biophysical techniques.
- Thermostability of *S. marcescens* EPS was unveiled for the first time.
- DTBP reduced the crystallinity index and crystallite size of *S. marcescens* EPS.
- DTBP increased the dislocation density and hydration in EPS.
- Modulating EPS will aid in disintegrating matured biofilm.

GRAPHICAL ABSTRACT



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ABSTRACT

Extracellular polymeric substances (EPS) play crucial roles in biofilm formation and biocorrosion resulting in heavy economic loss in an industrial setup. Hence, in an attempt to develop an agent to control the EPS across the hosts, the ability of 2,4-Di-tert-butylphenol (DTBP), a potent antioxidant, to modify the EPS of *Serratia marcescens* has been investigated in this study using biophysical methods. Protein, polysaccharides and eDNA components of EPS were inhibited significantly ($p < 0.05$) upon exposure to DTBP. DTBP treatment reduced the crystallite size and crystallinity index of EPS and increased the dislocation density of crystallites without inducing stress, besides increasing the hydration of EPS which reduced its thermal stability. On the whole, this study highlights the efficacy of DTBP to modulate secreted EPS of *S. marcescens* which in turn could facilitate the disruption of biofilms besides favouring the diffusion of antimicrobials into the cell aggregates resulting eradication of persistent biofilms.

1. Introduction

Biofilms are complex structures where microbial cells aggregate and embedded inside a hydrated matrix made of extracellular polymeric substances (EPS). Biofilm acts as a protective barrier and provides resistance to indwelling microbes against antibiotics, other bactericidal agents, degrading enzymes, desiccation, protozoan grazers and host immune responses. Biofilms are of immediate concern as they challenge mankind right from hospital acquired infections to economically important biocorrosions (Busalmen et al., 2002) besides fouling on all possible substrata in the environment (Dvorak et al., 2011). EPS provides mechanical stability, three dimensional architectures and physical integrity to biofilms thereby transforming the biofilm formers the most successful forms of life on earth (Flemming and Wingender, 2010). EPS has been reported to have polysaccharides, proteins, lipids and DNA each component of which is indispensable for the integrity of biofilm and survival of the indwelling organisms. EPS matrix

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acts as an external digestive system and also considered as a house of biofilms (Flemming and Wingender, 2010).

EPS plays crucial role in every stages of biofilm life cycle from attachment, aggregation to maturation and dispersion. Biofilm mode of life starts with the initial attachment of planktonic cells on solid substratum followed by EPS secretion which strengthen the microcolony formation followed by maturation and subsequent dispersion of cells which again initiate new biofilms on nearby surfaces. Dispersion is clinically important in disease dissemination and the dispersed EPS acts as a nutrient source for further colonisers. EPS was also found to be important in biocorrosion process as it facilitates redox reactions in the biofilm colonized microenvironments. Taken together, the importance of EPS in prevention of biofilm could be understood. Though, numerous antibiofilm agents with the potentials to inhibit the formation of biofilms were reported thus far, the availability of agents afflicting EPS is scarce which is indispensible for eradication of preformed biofilms or mitigating biocorrosions. In this work, the effect of an antioxidant compound 2,4-Di-tert-butylphenol (DTBP) possessing antibiofilm activity (Padmavathi et al., 2014) was evaluated against the secreted EPS of *Serratia marcescens*. *S. marcescens* is a Gram negative bacterium belonging to the family *Enterobacteriaceae* which typically forms biofilms of clinical and industrial concern (Bakkiyaraj et al., 2012; Ibrahim et al., 2009). *S. marcescens* produce cell bound and secreted EPS (Badireddy et al., 2008) wherein secreted EPS initiates the attachment of planktonic cells to solid substratum thereby facilitating biofilm mode of life cycle. Hence, the modulatory effect of DTBP on *S. marcescens* secreted EPS was investigated using biophysical techniques to delineate the structural changes as well.

2. Methods

2.1. Extraction of EPS

S. marcescens was cultured in Luria–Bertani (LB) Broth (HiMedia, Mumbai, India) with (treated) and without (control) 250 μg mL^{-1} of 2,4-Di-tert-butylphenol (DTBP) at 28 °C for 24 h at 160 rpm. The concentration of 250 μg mL^{-1} was decided based on our earlier report (Padmavathi et al., 2014). 24 h grown control and treated *S. marcescens* were centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatants were filtered through 0.2 μm mixed cellulose ester filters (Advantec, Ehime, Japan). Three volumes of ice cold isopropanol was added to the filtered supernatants and incubated at −25 °C for overnight (Singh et al., 2011). The secreted EPS was centrifuged and collected as a pellet which was again resuspended in 1X phosphate buffered saline (PBS-137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4). EPS samples were dialyzed against 3000 Da molecular weight cut off membranes (HiMedia, Mumbai, India) to remove salt and other impurities. The dialysis was performed for 3 days with MilliQ ultrapure water (Merck Millipore, Darmstadt, Germany) interchanged thrice a day. Dialyzed EPS was lyophilized (Alpha 2–4 CD plus, Christ, Osterode am Harz, Germany) and stored at −25 °C until further use.

2.2. Emulsification index

Emulsifying activity of control and treated EPS were assessed according to Padmavathi and Pandian (2014). Two mL of EPS (1 mg mL^{-1}) dissolved in MilliQ water was taken in a screw capped test tube containing equal volume of olive oil. The tubes were vortexed vigorously for 2 min and kept undisturbed for 24 h at room temperature. The emulsification index was calculated by the following formula:

$$\text{Emulsification index } (E_{24}) = (\text{Height of the emulsified layer} / \text{Height of the total liquid column}) \times 100$$

2.3. Quantification of EPS components

Control and treated EPS were dissolved in MilliQ water and subjected to protein, carbohydrate, lipid and extracellular DNA (eDNA) estimation. Proteins were estimated with Bradford protein estimation kit (Genie, Bangalore, India) using bovine serum albumin (BSA) as standard (Singh et al., 2011). Carbohydrates were measured by phenol–sulfuric acid method (Padmavathi et al., 2014) using glucose as standard and lipids were estimated by vanillin ortho phosphoric acid method. eDNA was quantified using Nanospectrophotometer (Bio-Spec Nano, Shimadzu, Kyoto, Japan) using MilliQ water as blank. The inhibition percentage of EPS components upon treatment with DTBP was calculated using the following formula:

$$\text{Percentage of inhibition} = [(\text{control OD} - \text{treated OD})/\text{control OD}] \times 100$$

2.4. UV–Vis and Fourier Transform Infrared Spectroscopy

Control and treated EPS samples were taken for spectroscopic studies and further biophysical characterization. One mg mL^{-1} EPS was dissolved in MilliQ water and scanned using UV–Vis Spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) from 200 to 800 nm (Singh et al., 2011). For FTIR analysis lyophilized EPS was ground with potassium bromide (KBr) and scanned from 400 to 4000 cm^{-1} (Padmavathi and Pandian 2014) in a FT-IR spectrometer (Nicolet iS5, Thermo Scientific, Marietta, GA, USA) with a spectral resolution of 4 cm^{-1} and KBr pellet was used as background reference.

2.5. X-ray diffraction (XRD) analysis

The structural properties of control and treated EPS were studied by X-ray diffraction (XRD) spectrophotometer (X' pert Pro, PANalytical, Almelo, The Netherlands) using Cu-Kα (λ = 1.54060 Å) radiation source generated at 40 kV and 30 mA over a 2θ scan range of 10–80° with scan step time of 10 s at 25 °C. Specimen and irradiated length were set as 10 mm with 240 mm goniometer radius and the distance between focus and divergence slit was 91 mm. The d-spacings and full width at half maxima [FWHM] were recorded at their respective 2θ angles. Crystallite size (D), strain (ε) and dislocation intensity (δ) were calculated (Usha et al., 2013) for the samples using the following formulae:

$$\text{Crystallite size } D = \frac{k\lambda}{\beta \cos\theta}$$

where k is 0.9, λ is 1.54 Å, β is the FWHM in radians.

$$\text{Strain } \varepsilon = \frac{\beta \cos\theta}{4}$$

$$\text{Dislocation intensity } \delta = \frac{1}{D^2}$$

Crystallinity index (CrI) was calculated based on the height of the major peak (I_m) and the amorphous (I_{am}) using the following formula (Sathitsuksanoh et al., 2011).

$$\text{CrI } (\%) = [(I_m - I_{am})/I_{am}] \times 100$$