

Role of Bacterial Bioflocculant on Antibiofilm Activity and Metal Removal Efficiency

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Abstract

In the present study, bioflocculant derived from bacteria are widely explored in bioremediation such as removing heavy metal and inhibiting biofilm formation. The bacteria isolated from sewage identified as *Proteus mirabilis* using 16S rRNA gene made up of D-Mannose, D-Fructose, D-Sucrose, and D-Galactose could be identified by Gas liquid Chromatography Mass Spectrometry (GCMS). Fourier transform infrared (FTIR) analysis of the bioflocculant indicated the presence of -COOH, -OH, and -NH groups which showed the characteristic of polysaccharide and protein. The removing percentage of heavy metals achieved by the precipitated bioflocculant were 82.63 ± 1.20 , 72.076 ± 0.42 , 57.36 ± 1.05 , and $44.7 \pm 1.053\%$, respectively for Zn^{2+} , Cd^{2+} , Cu^{2+} , and Hg^{2+} at optimum conditions. Bioflocculant were exhibited excellent biofilm inhibition against *P. aeruginosa* where maximum reduction (73%) were achieved at the minimum amount of 80µg/ml dose. Similarly, maximum reduction (80%) were achieved at the minimum amount of 70µg/ml dose for *S. aureus*. It was concluded that the bioflocculant produced have the potential in metal removal and biofilm inhibition.

Keywords: Heavy metals, Bioflocculant, Flocculation, Antibiofilm, Polysaccharides.

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INTRODUCTION

Microorganisms produced their metabolites are called as bioflocculant during their growth. Non-toxic nature of the bioflocculant are alternative to synthetic flocculant involving in bioremediation and biomedical application^{1,2}. Several bacterial species are capable of producing bioflocculant like *Alcaligenes latus*, *Bacillus firmus*³, *Aspergillus* sp.⁴, *Streptomyces* and *Cellulomonas*⁵. The bioflocculants made up of microbial metabolic products such as glycoprotein and complex carbohydrates⁶. It is mainly affected by environmental factors such pH, Temperature, dosage of inoculum, carbon and nitrogen sources^{7,8}. These key factors affecting bioflocculant production varies for different microbial species.

Generally, bacteria produces a variety of polysaccharides which are classified based on location such as, intracellular, extracellular, and capsular polysaccharides. They are produced via different biosynthesis pathways⁹. In addition, polysaccharides can be used as bioflocculant, heavy metal removing agents¹⁰, bioadsorbents, encapsulating materials. They are high molecular weight biopolymers such as polysaccharide, glycoprotein, glycolipids, and protein flocculants^{11,12}. They described that the functional groups identification of the bioflocculant are significant factor for the flocculating activity¹³.

For decades, attention have focused to remove hazardous pollutants to the environment. But, currently practicing chemical and physical methods for removing the metal from the contaminated environments are not environmentally friendly, costly, non-specific^{14,15}. In addition, many of these processes are not successful¹⁶. Therefore, bioflocculant is the best alternative to remove contaminants. Various uses of bioflocculant in bioremediation processes are involved to remove the toxic materials, and heavy metals, synthesis of nanoparticles and biomass recovery^{2,17-19}. In addition, bioflocculant could be acted as a way of preventing biofilm formation.

Considering the significance of bioflocculant in metal sequestration and antibiofilm activity, the present study was focused at searching the strain producing the bioflocculant and simultaneously acting as an antibiofilm agent under optimum conditions. Also, amount of metal uptake and biofilm inhibition percentage of the

bioflocculant was estimated. Finally, it emphasized to search over the structural and functional components responsible for flocculation and antibiofilm formation.

MATERIALS AND METHODS

Identification of Bacterial strains

Totally, 20 bacterial strains were previously isolated from the wastewater. Potential strain showing highest flocculating activity were screened for further research. Test bacteria were recognized as *Proteus mirabilis* through partial sequencing of 16S rRNA genes. Nucleotide sequences were finally deposited in GenBank (MG996004).

Production and partial purification of bioflocculant

Bioflocculant producing media was prepared based on the methods of Zhang et al.²⁰. Components of the media such as KH_2PO_4 (2.0 g), Glucose (15.0 g), $(\text{NH}_4)_2\text{SO}_4$ (0.2 g), Yeast extract (0.5 g), K_2HPO_4 (5.0 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), NaCl (0.1 g), were dissolved in 1L of distilled water (pH=7) under optimum conditions. 0.5 ml culture of *P. mirabilis* isolated from wastewater, inoculated into the medium and kept it for 72 hrs incubation at 30°C, 160 rpm/min. The broth was centrifuged (8000'g, 20 min) to pelletize the cells. The upper portion of the bioflocculant were added with thrice the volume of ethanol. This washing step was repeated several times to obtain the precipitate. Thereafter, they were assayed for flocculation activity.

Characteristics of the bioflocculant

Carbohydrate content of the bioflocculant using glucose as the standard solution were measured using Phenol-sulfuric acid method²¹. Lowry method was assessed in measuring the protein content of the bioflocculant²². The monomeric unit of the bioflocculant was obtained by hydrolysis of the bioflocculant with HCl at 121°C for 2 hrs. They were further subjected to the analysis of FT-IR. The scanning electron microscopy (SEM: SUPRA 55 SAPPHIRE; Germany) image of the bioflocculant was obtained.

Gas Chromatograph-Mass Spectrometer

The extracted samples were subjected to GC/MS analysis (TQ8030, Shimadzu, Japan). For identifying sugars components, carrier gas used was helium the with a flow rate of 1.0 mL min⁻¹, maintaining the temperature of 300°C with an

electron ionization mode (70 eV). Compounds were identified by the NIST1 library database using chromatograms.

Heavy metal removal rate

The bioflocculant (0.8mg/100ml) was applied for the bioflocculation test against heavy metal solutions containing Cd²⁺, Zn²⁺, Cu²⁺, and Hg²⁺ at the concentrations of 0-50 mg L⁻¹, respectively. The amount of heavy metals were identified by inductively coupled plasma atomic emission spectroscopy (ICP-AES; iCAP 7000, Thermo Fischer

Scientific, Bremen, Germany). All experiments were repeated thrice. The percentage of heavy metal removal was measured based on the following equation:

$$\% R = (C_i - C_f) / m$$

Where, C_i – amount of heavy metal of test solution initially; C_f – amount of heavy metal of test solution finally; m (g) is the weight of bioflocculant.

Antibiofilm assay

Crystal violet assay was performed which was examined to quantify the biofilm inhibition

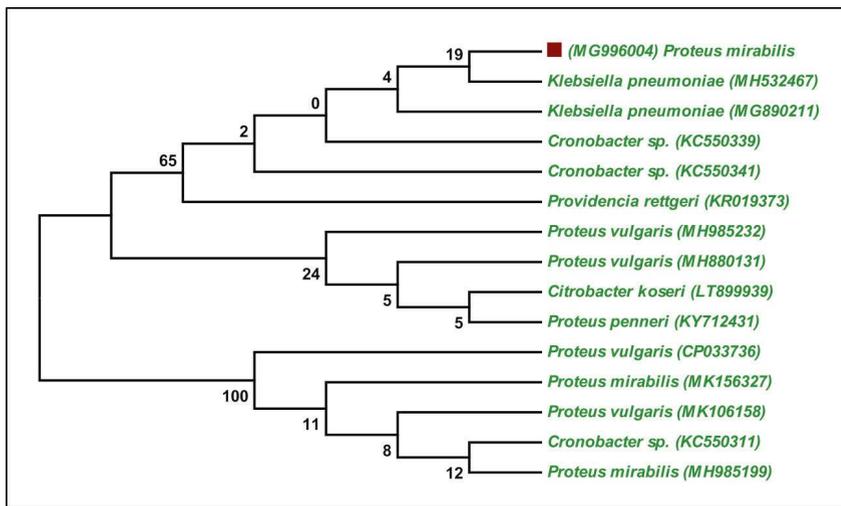


Fig. 1. Phylogenetic tree based on 16S rRNA sequences

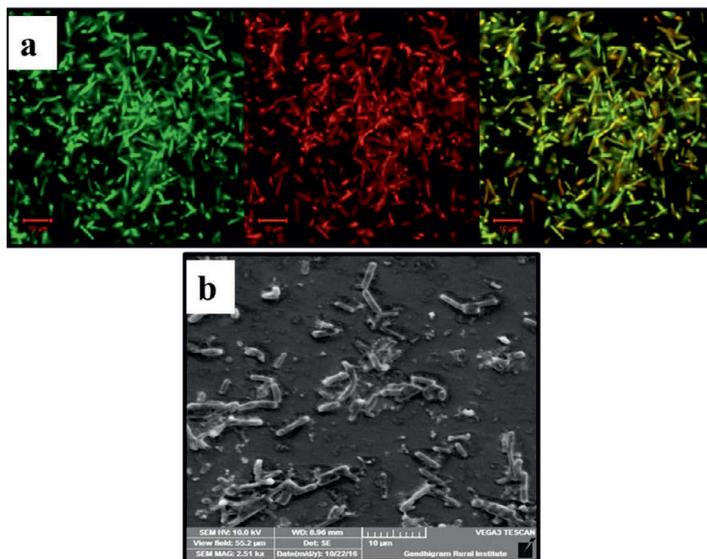


Fig. 2. a) Image of Gram negative bacteria *Proteus mirabilis* observed under the confocal microscope; b) SEM image of Gram negative bacteria *Proteus mirabilis*.

percentage²³.

$$\text{Biofilm inhibition} = \frac{(\text{OD of untreated control}) - (\text{OD of treated sample})}{\text{OD of untreated control}} \times 100$$

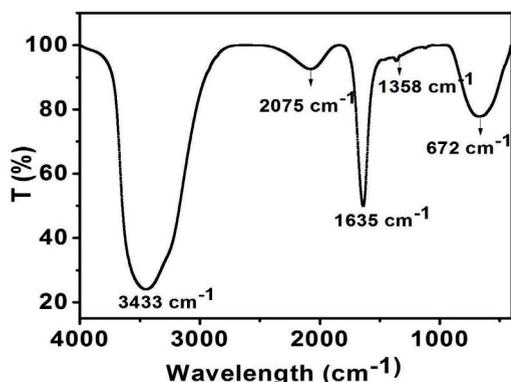


Fig. 3. FTIR spectra of bioflocculant showing presence of hydroxyl, amino, and carboxylic group.

Biofilm of *Pseudomonas aeruginosa* and *Staphylococcus aureus* grown separately on glass slides in well plates were added with the bioflocculant. They kept the incubation for 24 hrs at 37°C and washed with phosphate buffered saline (0.1 M; pH 7.4) to discard non-adherent cells. In the same way, control were also subjected to the same procedure without the bioflocculant. The glass slides were observed under confocal microscopy (CLSM, LSM 510, Carl Zeiss, and Germany) after the Acridine orange staining. All the experiments were conducted thrice.

Production of bioflocculant

Totally, 20 bacterial strains were isolated from the sewage water. In which, strain showing highest flocculating activity were selected for bioflocculant production. The strain were identified as *P. mirabilis* (MG996004) through 16S rRNA sequence²¹. Phylogenetic tree based on 16S rRNA sequences were constructed (Fig. 1). Morphological structure of *P. mirabilis* were identified (Fig. 2). Totally, 2 g of bioflocculant was obtained from 1L fermentation broth

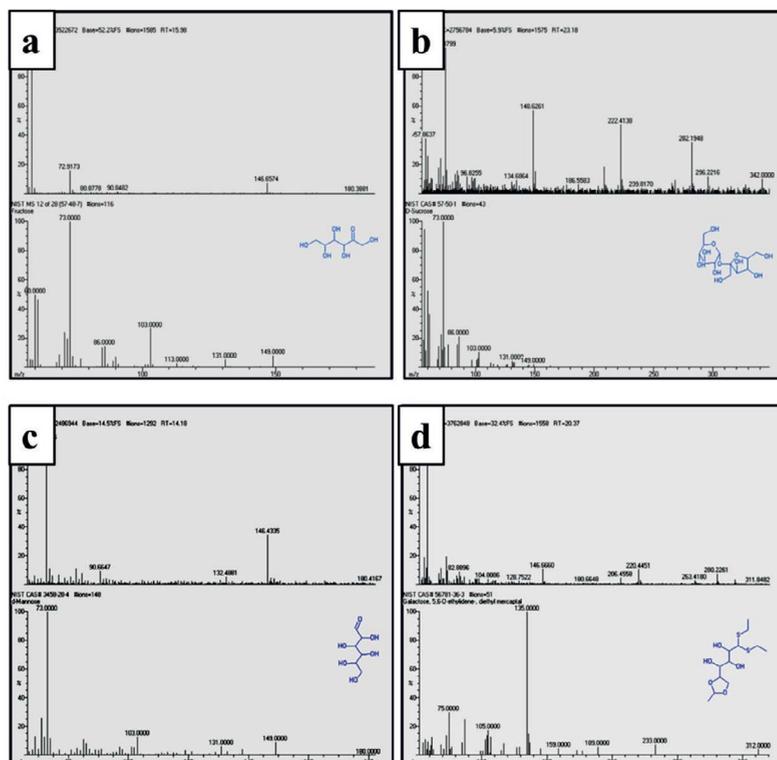


Fig. 4. GC/MS chromatogram of monomeric sugar units identified in the bioflocculant a) D-Fructose, b) D-Sucrose, c) D-Mannose, d) D-Galactose.

under optimal conditions²⁰. The production of biofloculant was mainly controlled by the factors, such as the components of the culture medium and the cultivation conditions²⁴. The key factors, such as the concentration of carbon sources, temperature, shaking speed, nitrogen sources, pH, and inoculum size have to be focused on the flocculating activity of the biofloculant by *Proteus mirabilis*²⁵.

Characteristics of biofloculant

The biofloculant produced from *Proteus mirabilis* (MG996004) included 68% (wt/wt) carbohydrate, and 15% (wt/wt) protein. These components were confirmed by FTIR spectroscopy by identifying the functional group of the biofloculant (Fig. 3). The broad peak at 3433 cm⁻¹, which was confirmed it as hydroxyl and amino group²⁶. Sharp peak observed at 1635 cm⁻¹ was

attributed to the characteristic vibration of amide group. Weak peak at 1,358 cm⁻¹ was correlated to the carboxylate group of the biofloculant²⁷. The adsorption band at 672 cm⁻¹ could be related to the binding between sugar monomers²⁸. Finally, spectrum revealed the peaks for polysaccharides, protein and identified the presence of the functional groups: hydroxyl (-OH), amide (-CO-NH) and carboxyl (-COO-)²⁵. These functional groups act as a binding site for divalent cations, mainly involved in flocculating processes.

GC/MS analysis of biofloculant was found the monomers, viz., D-Mannose, D-fructose, D-Sucrose, D-Galactose (retention times of 14.18, 15.98, 23.18, and 20.37 min, respectively) and compared with NIST Library (Fig. 4). The results indicated that the biofloculant made up of four sugar monomers^{29, 30}. HR-SEM showed the outer

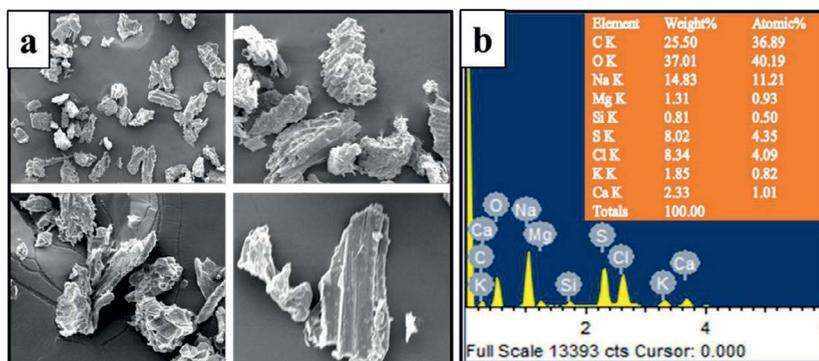


Fig. 5. (a) Irregular shape of the biofloculant; (b) SEM-EDAX spectra depicting the presence of C, K, O, Na, Ca, Mg, and Cl in the biofloculant.

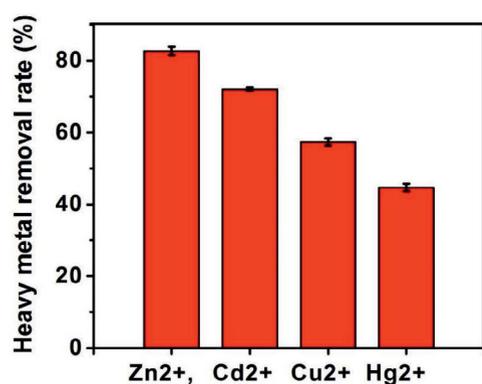


Fig. 6. Removal percentage of Cu²⁺, Zn²⁺, Hg²⁺, Cd²⁺ using biofloculant. The given data represents the mean value of three independent experiments. Error bars indicate mean ± SD.

structure of the biofloculant. Representative HR-SEM image showed well-dispersed, irregular shaped biofloculant (Figs. 5(a)) with negatively charged zeta potential possessing best flocculating property²². The configuration of the biofloculant were major factor involved in flocculating activity²¹. During flocculation, they bonded tightly and intertwined each other due to intermolecular interaction in between them^{22,31}. The elemental analysis for the biofloculant were shown the presence of C, K, Na, Mg, Cl, and O at particular proportions²⁹ (Figs 5(b)).

Heavy metals removing efficiency

In the study, the metal ions such as Zn²⁺, Cd²⁺, Cu²⁺, and Hg²⁺ within the arrange of 0–50 mg L⁻¹ in aqueous solutions could be effectively

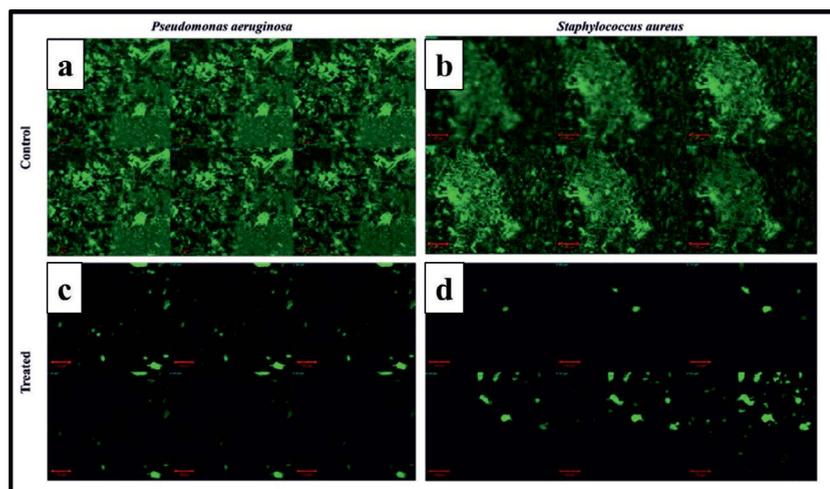


Fig. 7. (a) CLSM images of *P. aeruginosa* biofilm formation; (b) CLSM images of *S. aureus* biofilm formation; (c) CLSM images of *P. aeruginosa* biofilm formation treated with bioflocculant (80 µg/ml); (d) CLSM images of *S. aureus* biofilm formation treated with bioflocculant (70 µg/ml); Each experiment was replicated thrice.

flocculated by the bioflocculant. The heavy metal removing percentage achieved by the precipitated bioflocculant was 82.63 ± 1.20 , 72.076 ± 0.42 , 57.36 ± 1.05 , and $44.7 \pm 1.053\%$, respectively for Zn^{2+} , Cd^{2+} , Cu^{2+} , and Hg^{2+} at optimum conditions. When compare the treated sample with untreated sample, concentration of metals in treated sample was completely reduced that showed adsorption and bioflocculating ability of bioflocculant. In addition, concentration of bioflocculant reported was very less on comparing other reported literature^{32,33}. It was interpreted that the electro-negative properties of the biological material and the metals regulated the metal-removal capacity. In addition, the interactions between metal cations and negatively charged functional group of bioflocculant such as carboxylic group, and the phosphate group found in bioflocculant (Fig. 6) were highly enhanced under constant optimum conditions³³.

Antibiofilm assay

Crystal violet staining (Biofilm assay) assay are used to quantify the measurement of biofilm. Bioflocculant were showed excellent biofilm inhibition against *P. aeruginosa* where maximum reduction (73%) were achieved at the amount of 80µg/ml dose. Similarly, maximum reduction (80%) were achieved at the amount of 70µg/ml dose for *S. aureus*³⁴. Control (without bioflocculant) did not show any inhibiting percentage exhibited

dense bacterial population (Fig. 7). High inhibition percentage and minimum dosage requirement were showed that, bioflocculant was acting as a best antibiofilm agent. They were further visualized by confocal microscope^{35,36}. Previously reports suggested that, bioflocculant reduced cell-cell interaction by inhibiting the attachment of bacterial cells³⁷ and also polysaccharides found in the bioflocculant might be responsible for biofilm inhibition³⁸.

CONCLUSION

Out of 20 bacterial strains, bacterial strain producing highest flocculating activity were chosen for further research. The main constituents of the bioflocculant were proteins, polysaccharides as the functional groups in the bioflocculant which was being confirmed by FTIR. They were mainly responsible for its flocculating activity and antibiofilm activity. Structural characteristics of the bioflocculant were confirmed by SEM analysis. Further, monomer unit of the bioflocculant were demonstrated by GCMS. The flocculating efficiency suggested that the bioflocculant was considered as an effective material for the treatment of heavy metal removing process. High inhibition percentage and minimum dosage requirement were showed that, bioflocculant was acting as a best antibiofilm agent.

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DATA AVAILABILITY

All data generated during the study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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