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CHROMIUM REDUCTION USING BACTERIAL BIOFILMS

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Abstract— Biofilms are the aggregates of the bacterial colonies which are found to be the friends and foes to the mankind. Biofilms of bacteria can be employed in not only removal of organic waste but can also can be used in treatment of hexavalent chromium containing waste, an element which is found to be carcinogenic, mutagenic and irritative in nature. The organism forming biofilm is isolated from tannery effluent and is quantitatively and qualitatively analysed by using tube assay and microtiter plate assay. The isolate was identified by using 16srRNA technology with the help of barcode institute Aurangabad it is identified as *Bacillus vietnamiensis*. *Bacillus vietnamiensis* culture was able to reduce almost 100% of 500mg/l of hexavalent chromium at 6 pH and temprature of 35°C under normal aerobic conditions. Optimisation experiments proved temperature 35°C and pH 6 is more favourable for bacterial growth and as well as reduction process. The bacteria is found to be more potent as it is obtained from the indigenous source and found to be very efficient in reduction of hexavalent chromium..

Keywords— EPS-exopolysaccharide, PBS- phosphate buffered saline, QS- quorum sensing, TSB- Trypticase soya broth, AHL-acylhomoserine lactones, AI auto inducers.

I. INTRODUCTION

Bacterial biofilms are the architectural aggregations growing almost on every surface forming complex communities [1]. Biofilms are found since evolution began on the earth. In medical microbiology, biofilms are studied for chronic infections. Biofilms normally colonize on different household surfaces and due to low rate of disinfection practices and improper sanitization of different products leads to prevalence of illness by pathogens. The antibiotic era has

proved to be efficient in curing of different bacterial diseases as earlier people used to die of pneumonia and other different acute infections [2]. Biofilm is found to comprise of thoroughly structured matrix surrounded by their communities [3]. The cells of Biofilm produce their genes with different Pattern from their Plank tonic counterparts [4]. Pure cultures of biofilm producing bacteria and consortium in the nature presents their cell growth in the form of microcolonies enclosed in a specialized matrix. The specialized structure of biofilms can be observed by scanning confocal laser microscopy which helps to differentiate cells-cells signals and their cellular specialization [4]. The Cr(VI) reduction by bacteria is a wonderful mechanism which shows resistance of organism to Cr(VI). It penetrates the cell membrane of bacteria as it is a strong oxidizing agent. Bacteria Resistance of Cr (VI) is based on presence of plasmid and the sensitive cells cannot accumulate Cr (VI) as resistant cells [5].

II. BIOFILM STRUCTURE AND DIFFERENTIATION

A. Biofilm Formation

Biofilm formation is mainly classified as 3 main phases early, intermediate and matures [6]. In the earlier phase the free planktonic cells move through the surface using their flagella and go on aggregating to form the mass growth. The stages of growth of biofilms are depicted in (Figure 1 which is as follows). Phase 1: Primary adherence of the cells to the surface, Phase 2: Exopolysaccharide production of cells results in attachment of cells which is irreversible in nature, Phase 3: Results in primary development of biofilm structure, Phase 4: Maturation of biofilm structure and Phase 5: Moving and Scattering of the single cells from the biofilm layer. The figure 1 indicates the phases of growth production of *Bacillus* [7].

The interaction among the microbes and its substratum results in the production of cells of monolayer. Some bacteria

are not resistant to antibiotics and thus can be successfully used in treatment. It is necessary to analyze peri operative antibiotic prophylaxis. [2, 8]. The EPS produced during this stage is found around the bacterial microcolonies, it consists of polymers, polysaccharides and water molecules bound within the capsules [10]. The polymers are secreted by biofilms matrix which absorbs nutrients, metabolic products and intracellular products [11-13].

Quorum sensing mechanism [14] is used to study the various phases of development of biofilm. It helps to study the mechanism of production of biofilm, release of the cells and detection of chemical signaling molecules it also regulates the gene expression within the cells based on the density gradient [15, 16] well defined groups of signaling found in bacteria are oligopeptides, acylhomoserine lactones and auto inducers [16]. Oligopeptides are the molecules used by gram positive bacteria for communication [17, 18] while gram negative ones use AHLs but AI – 2 is found to be universal signaling molecules used to communicate among the inter and intraspecies [16].

Boles and Horswill have studied QS in *Staphylococcus aureus* Agr, which helps in sensing the systems controls in switching between the free cells, biofilm layer [19].

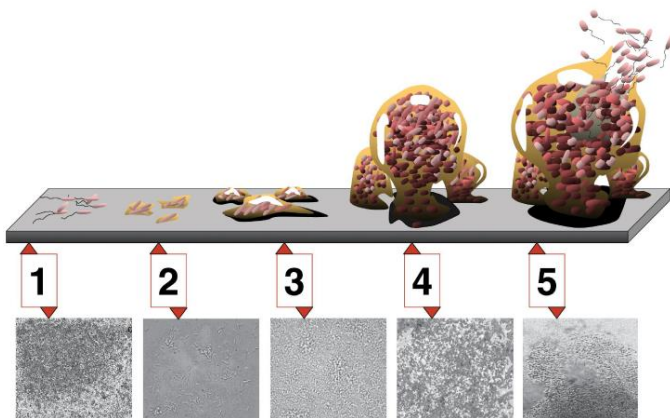


Fig 1. Different phases of biofilm development [7].

B. Role of surfaces in the origin of cells

Prokaryotic cells have mainly existed from a membrane consisting of protein and nucleic acid with other Cytoplasmic contents in a highly structured membrane. The bacterial cells which randomly float or swim have also evolved in the studies. Biofilms operate mainly on well structured and harmonized community, which consists of prokaryotic with membranes of specific functions [20]. Generally the crystals exhibit high levels of orders, clay show extreme structure aeries of alternating bands of various charges and composition. Cairns-Smith [4] has compared the arrangement of enzyme molecules which is having the structure similar to clay molecules. The Physical proximity from one cell to another cell gives a selective pressure for creating complex interaction among the earlier communities.

III. MATERIALS AND METHODS

A. Bacterial Strains and Growth Condition

The bacteria were isolated from chromium contaminated sites. Here the tannery effluent was taken as the sample for isolating the indigenous bacteria. The sample was incubated into the nutrient agar plates and further, the colonies obtained on the plates were incubated on NA plates containing 1000 mg of $K_2Cr_2O_7$. The organism which was able to grow and form a confluent colony was further tested for biofilm production. The bacterium was streaked on NB plates and incubated for 24 hours at 37° C from a preserved culture. The organisms were grown in different temperatures of 20°C, 30°C, 35°C, and 40°C to analyze their optimum growth. In other plates, colonies were used to inoculate 5ml of nutrient broth (NB) in phosphate-buffered saline (PBS) which was incubated at 37° C for 24 hours. The grown preculture for 18 hours was further used for centrifugation (100ml NB in PBS). The bacteria which were taken from centrifugation was further harvested and washed with sterile Millipore-Q water twice [2] further the biofilm assay was carried out.

IV. GROWTH AND ANALYSIS OF BIOFILM LAYERS

Production of bacterial biofilms can be examined & studied by variety of systems. The primary stages of biofilm formation of different bacteria help to know the pattern of aggregation. The static assays are helpful in observing the preliminary stages of biofilm formation which can be detected within 60 minutes duration [26]. To study the cell death in biofilm which are treated with antimicrobial agents' colony based biofilm is very essential.

A. Colony Biofilm Assay

Biofilm colonies are cultured on a semipermeable membrane on agar plates [27] Fig 2 indicates the growth of biofilm on the agar plate. These type of biofilms can be used for mass growth by providing the essential nutrients on a fresh agar plates. Biofilms with different carbon source can be cultivated on the agar plate. These biofilm colonies can be used in studying the antibiotic resistant properties of the bacterial cells. It provides an example to study the effect antibiotics on the cells [26].

Different types of biofilms system indicate the adherence of bacterial cells on the surface even in the liquid medium. The above mentioned assay is one among the best procedures, to study the adherence and aggregation of bacterial cells. These biofilms are developed for meeting nutritional needs and removal of wastes for analyzing the assays. The organism can be either grown in plants or broth [27]. An advantage of biofilm growing on plate is that they are derived from clonal growth of original population of bacteria which are deposited on the substratum. in liquid media the bacteria which are motile may move from aggregates of cells [23]. The bacteria in the biofilms have very less ability to detach and move or drift from the biofilm layer. Thus, biofilm colonies are useful in visualizing the effects of antimicrobial agents though there

is change in cell ratio; it is attributed to cell death rather than detachment.

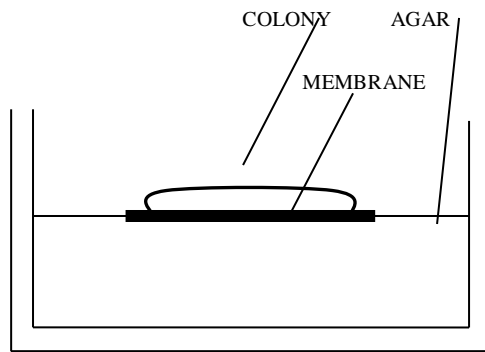


Fig 2: Side view of a colony biofilm assay

B. Tube Assay

Qualitative assessed biofilm development was done by the tube assay method [30]. Trypticase soya broth (TSB) with an amendment of sucrose (5%) in which bacteria were grown and kept for incubation for 24 hours at 37°C at both rotary and static conditions. The broth from the tubes was decanted, dried, and was stained with 0.1% of crystal violet. By observation of the film on the wall and base of the tube, the biofilm formation was confirmed. Here liquid interface will not indicate the biofilm formation [30].

C. Microtiter plate assay

Quantitative estimation of biofilm formation was done by microtiter plate assay. The dilution of grown cultures was done by 100 times. In the 96 well of Elisa plate 200µl of cultures were added. In static and stained condition with 0.1% (w/v) solution of crystal violet, the cultures were put in a triplicate and were further incubated for 24 hours at different time intervals of 20 °C, 30°C, 35°C and 40°C to observe the growth of biofilms. Using 96% of ethanol excess stain was washed off and plates dried and using the micro ELISA auto reader Optical density (OD) wavelength was measured for 570nm [30].

D. Analyzing the growth of biofilm

The optimization process is carried out to enhance the growth of the organism. The selected parameters used here are temperature and pH. The experiment is to analyze the effect of temperature on the growth of the organisms and to determine the optimum temperature in which the organisms can be grown. 5% of isolated culture is inoculated into the 200ml of TSB medium and kept for incubation at 20°C, 30°C, 35°C, 40°C temperature, and OD is measured at 570 nm at a time interval of 24 hours. To determine the optimum pH favorable for the growth of the organism the culture is inoculated into the 200ml of TSB medium with varying pH of 5pH, 6pH, 7pH, 8pH, 9pH, and later the OD of biofilms is measured at 570 nm at a time interval of 24 hours.

The classification was done according to absorbance as weakly adherent (weak biofilm producer), $0.10 \geq A > 0.06$; moderately adherent (moderate biofilm producer), $0.20 \geq A > 0.16$; and strongly adherent (strong biofilm producer), $A > 0.24$.

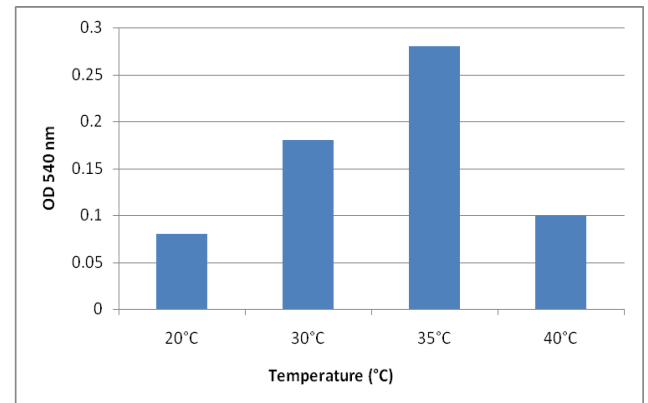


Figure 3: Temperature optimization of biofilm production

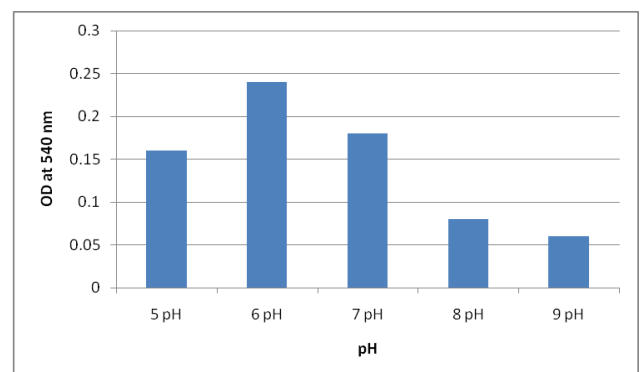


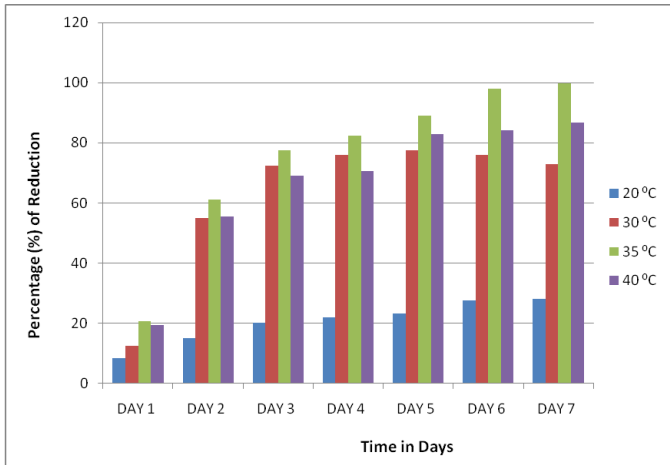
Figure 4: pH optimization of biofilm production

The results optimization of pH and temperature for the formation of biofilms are denoted in figure 3 and figure 4 respectively. Figure 3 indicates that biofilm production is maximum at 35°C and a minimum at 20°C. At 30°C, it is moderate. This indicates that the biofilm formed at 35°C is thicker and is found to be stronger biofilm than compared to other temperatures [31].

Figure 4 indicates the biofilm production at different pH. It is found from the figure 4, the organism grows and form biofilms at different pH of 5,6,7,8 & 9 but the biofilm production is found to be maximum at pH 6 or almost neutral pH & is moderate at pH 5 & 7 and weak biofilms are formed at pH 8 & 9 [31].

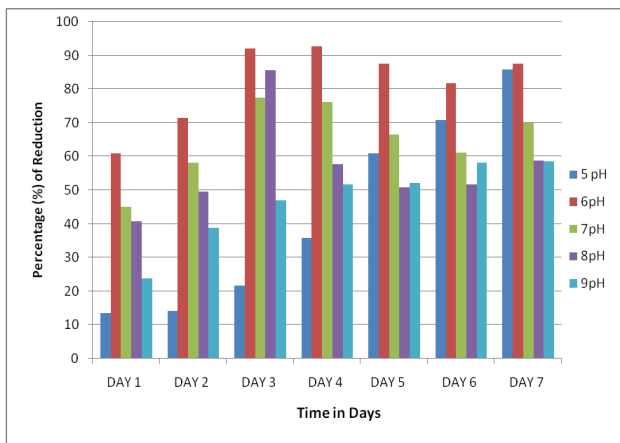
E. Analysis of reduction of hexavalent chromium

An initial concentration of 500 mg/l of hexavalent chromium were prepared by dissolving 1.417 gm of $K_2Cr_2O_7$ in distilled water. The reduction process was carried out by using the Diphenyl Carbazide assay method.



Temperature optimization

The reduction of the initial concentration of 500mg/lit hexavalent chromium was found in different temperatures from 20°C-40°C. The reduction started after 24 hours and consequently increased till day 7, at temperature 20°C the reduction was maximum of 28% on day 7, at temperature 30°C the reduction was maximum of 77.4% on day 5, at temperature 35°C the reduction was maximum of 99.8% on day 7, and at the temperature of 40°C the reduction was maximum of 87% on day 7. At temperature 35°C the organism shows a maximum amount of reduction this proves to be a favorable condition for the reduction process.



pH optimization

The reduction of the initial concentration of 500 mg/lit of Hexavalent Chromium was found at different pH from 5 pH-9 pH. The reduction started after 24 hours and consequently increased till day 7, 5 pH was maximum on day 7 of 86%, 6 pH was maximum on day 4 of 93%, 7 pH was maximum on day 4 of 76.1%, 8 pH was maximum on day 3 of 86%, and 9 pH was maximum on day 7 of 60%. At pH 6 the organism showed the maximum amount of reduction which indicates pH 6 is a favorable condition for the reduction process.

V. IDENTIFICATION OF THE ORGANISM

The isolate which was able to form strong biofilm and maximum reduction was further identified by using 16srRNA technology. The organism is identified as *Bacillus* Species and has 99% homology with other *Bacillus* strains.

VI. CONCLUSION

The application of biofilms mainly includes the removal of toxic elements from environment. The specific secondary metabolites and the gain of robustness were produced by the bacteria in the biofilm. Biofilms provide huge microbial mass which helps in the treatment of the toxic wastes. Biofilms can be developed in both aerobic and anaerobic zones. The intrinsic properties of the biofilms vary with the environmental conditions. Biofilm treatment varies with influent characteristics and nutrient uptake. Biofilm study is still under the infancy. It acts as a friend in bioremediation and considered as a foe in the biomedical field. But very less information is obtained till now. The stability and growth of biofilm has yet to be analyzed. The enzymes produced extracellular decomposed carbohydrates, proteins and organic phosphates which in turn help in reduction of environmental contaminants found in the ecosystem. The interaction among the different organisms also responds in activating the extracellular enzymes helpful in degradation and balancing the ecosystem.

The major disadvantage of biofilms include high corrosiveness, and lack of machine stability. Biodispersants are designed to ensure that microorganisms are dispersed into the process water. They are effective in performing a function using a multiple mechanisms and are treated as a substitute to a biocide. Biofilms also are accountable for a huge variety of nonsocial infections. It includes the surfaces of medical devices, abrasion dressings, and other devices.

Colony biofilms have been used in determining antibiotic-resistance and to study the viability of the cells. The tube assay method is used to determine the qualitative assessment of biofilm. The microtiter plate enables researchers to quickly analyze attachment of many bacterial strains or growth circumstances in the experiment before the performance of the labor-intensive microscopic qualification. *Bacillus* species gram positive bacteria isolate from tannery effluent used in the reduction process is found to be very efficient in reducing 93% of 500 mg/ Lt of hexavalent chromium at pH 6 and temperature at 35°C. This organism is found to be very efficient in maximum reduction of hexavalent chromium without any amendments at natural conditions. So this organism can be used in future studies in treatment of chromium containing waste water.

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