

BIOFILM FORMATION BY ACIDITHIOBACILLUS FERROOXIDANS ON PYRITE ORE

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ABSTRACT

Bioleaching is the assembly of metal cations from insoluble ores by microorganisms. Biofilms can enhance this process. Since *Acidithiobacillus* often appears in leaching heaps or reactors, this genus has aroused attention. In this study, biofilm formation and subsequent pyrite dissolution by the Gram-negative, non-spore forming rod, moderately thermophilic acidophile *Acidithiobacillus ferrooxidans* were investigated. This species is of particular in the industry because of its working pH which is 1.5-3 where most metal ions remain in solution resulting in easy separation. An indigenous laboratory scale bioreactor has been prepared where the oxidation of pyrite (FeS_2) carried by using the bacterial strain *Acidithiobacillus ferrooxidans*, were tested for enhancement of its biofilm formation on pyrite ore.

KEYWORDS: Bioleaching, Quorum sensing, growth kinetics.

1. INTRODUCTION

Biofilm is the structure formed by a community of microorganisms which adhere to the surface with the help of fimbriae, flagella, pili and microenvironment is formed by EPS (Extracellular Polymeric Substances) which include proteins, polysaccharides, lipids and DNA (Wood et al., 2011). Biofilm formation is a multistep process which includes formation of conditioning layer, bacterial adhesion, and bacterial growth and finally biofilm expansion (Figure 1). When talking of surfaces, biofilm can form on a variety of surfaces such as plastic, glass, metal, soil particles, sand, tissue and food. In nature biofilms can be formed by a single species or multiple species and can be a single layer or a three-dimensional network

(Kokare et al., 2009). The process of initial attachment of microorganisms to the surface, development of community and detachment follow specific mechanisms (Donlan, 2002). Cells in this biofilm structure communicate with each other via a process called Quorum sensing (QS), which is a regulatory mechanism which controls cell-to-cell communication and also attachment and detachment of the cells to and from the matrix respectively (Zhang and Dong, 2004).

1.1. Attachment

The attachment of the bacteria to the solid surfaces is dependent on various factors which include the adherence structures like pili, flagella or fimbriae present on the bacteria or the extracellular polymeric substances secreted by the cells. Other factors include the surface charge and the degree of the hydrophobicity of the substratum. Large number of bacteria attach to the hydrophobic plastics with little or no surface charge (Teflon, polyethylene, polystyrene), moderate number of bacteria attach to the hydrophilic metals with a positive or neutral charge and very few attach to the hydrophilic surface with a negative charge. The interactions involved in the attachment include both electrostatic and hydrophobic interactions. Two important events regulate the attachment of bacteria to the surface: i) that the bacteria encounter a surface and come close enough for attachment to occur which is dependent on a number of factors like culture concentration and bacterial motility; ii) that the bacterial outer surface adheres to the substratum (Fletcher and Loeb, 1979.). The hydrophobic interactions between the cell and the substratum allow the cell to overcome the repulsive forces active within a certain distance from the substratum.

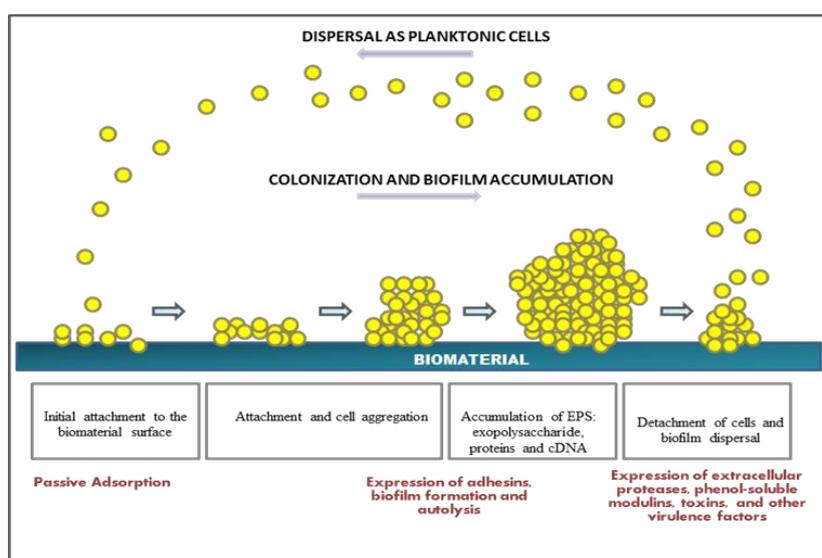


Figure 1: The Cycle of Biofilm (Arciola et al., 2012).

1.2 Role

Bacterial cells adherent to the matrix and existing as colonies in biofilm differ in physiology than their free-living counterparts. They transcribe genes which are switched off in planktonic forms. Biofilm formation thus involves the process of gene transcription and signal transduction.

General roles of biofilms

1. Bacterial cells growing in biofilms also have the advantage over less sensitivity to antibacterial agents as the extracellular matrix prevent the penetration of such agents into the cell. Also decreased metabolic activity of the biofilm cells contribute to their decreased sensitivity towards antimicrobial agents (Landini et al., 2010).

2. Protection from environment: The EPS of biofilm has structural role. It also prevents the microbial cells from antimicrobial agents by preventing their penetration. It prevents the entry of surrounding substances into the cells or within the community. These characteristics depend on factors like the agent and the EPS matrix, more effect with the antibiotics that are hydrophilic and positively charged.

3. Nutrient availability: Biofilm formed by multiple species live with a cooperative environment i.e. mutually syntrophic relationship. The close proximity allows inter species substrate exchange and removal of metabolic products.

4. Acquisition of new genetic trait: This is particularly important for the evolution point of view. If a free planktonic cell has to become a biofilm cell it must switch on or increase the transcription of genes the product of which confers a characteristic feature of the biofilm cell and switch off or decrease the expression of certain genes. For example, *Pseudomonas aeruginosa* in sessile state increases the transcription of *algC* gene for alginate synthesis almost four times. This simultaneously leads to the decrease in flagellum synthesis which as it destabilizes the biofilm (Kokare et al., 2009).

2. MATERIALS AND METHODS

2.1 Microbial strain and growth media

Acidithiobacillus ferrooxidans, obtained from the ATCC. *A. ferrooxidans* was grown in modified 9K basal salts medium which consisted of: excessive concentrations of phosphate, magnesium and ammonium. The pH of the medium was adjusted to 1.7 with concentrated 1N

H₂SO₄. All chemicals used in the growth medium were of laboratory grade, and purchased from Merck, Germany.

2.2 Preparation of reactor vessel

2.2.1 Bioreactor for continuous ferrous oxidation

A plastic jar was taken were wiped with absolute ethanol further it was kept under UV light for sterilization and fitted agitator with motor which was at around 7 volts. The bioreactor used to evaluate the biological oxidation of Fe²⁺ iron in this study. One litre of 9K⁻ basal salt media was added and the media was inoculated with the pure culture of *A. ferrooxidans*. Further, in the media slides were added of 1x1cm dimensions containing pyrite ores on it.

2.2.2 Preparation of glass slides

The glassslides (25x50mm) were washed with absolute ethanol. Slides were placed on aluminum foil, covered, and dried in an oven. A 1 cm x 1 cm area was marked at the center of the slides with a hydrophobic marker for the deposition of bacterial suspension. The glass slides were finally sterilized by autoclaving at 121⁰C for 20 min. Pyrite ores were applied on the slides with the help of adhesives and kept for drying.

2.3 Direct viable count

One ml of the inoculated 9K⁻ media were taken and viable cells were counted. From this one ml small amount of media was taken on hemocytometer. In a conical microfuge tube, add 10 microliters of 0.4% trypan blue solution was added and gently it was swirled (finger vortex) the cell suspension and 10 microliter of it was removed using sterile technique. 10 microliters of cell suspension were combined with 10 microliters of trypan blue in the microfuge tube. 10 microlitres of this mixture was then taken on hemocytometer and analysed for viable cell count.

2.4 Determination of adsorption parameter

The slides were taken on equal intervals and adsorption rate was studied by analyzing it microscopically. The initial rate of bacterial adsorption was determined with the experimental results using the equation: Adsorption rate= $K_{ads} B_3 (SB-B_2)$

Where, K_{ads} is the adsorption rate constant for bacteria on the support, B_3 , the rate of bacterial adsorption on the solid support is proportional to the concentration of bacteria, SB, maximum adsorption capacity of the support, B_2 , concentration of adsorbed bacteria.

2.5 Growth kinetics study: Growth kinetics were studied and analyzed. The graph was plotted against cell number versus time interval.

3. RESULTS

3.1 Designing of the bioreactor: The reactor had the capacity of 2l media. Figure 2 represents the image of the indigenously prepared bioreactor.



Figure 2: Bioreactor used for pyrite oxidation in the laboratory.

3.2 Initial cell number in the mother culture

1ml of the undiluted culture was taken and spread over one haemocytometer square by a micropipette through the gap between the slide and the coverslip. The cells were observed under a microscope at 100x magnification and the cells count was performed.

3.3 Counting of number of adsorbed cells on pyrite cell surface

The number of cells present in the liquid culture was calculated at an interval of 12 hours from which indirectly the number of cells adsorbed on the pyrite surface was estimated. Table 1 shows the observed number of adsorbed cells with different time intervals.

Table 1: Number of cells adsorbed on pyrite with time.

Time (Hour)	No. of cells in the media	No. of cells on pyrite
12	4×10^5	1×10^5
24	3.04×10^5	1.96×10^5
36	2.5×10^5	2.5×10^5
48	1.8×10^5	3.2×10^5
60	0.04×10^5	4.96×10^5
72	0.03×10^5	4.97×10^5
84	0.04×10^5	4.96×10^5
96	0.04×10^5	4.96×10^5

*No. of cells on pyrite = (Initial – at a particular time interval) number of cells in the media.

3.4 Preparation of slides: A blank slide was utilised as positive control. Slides were observed under light microscope (Applied Biosystems) and pictures were taken to check for the presence of biofilm as shown in figure 3. Due to blockage of light transmission path biofilm was only visualised in the cases of 12 h and 24 h growth.

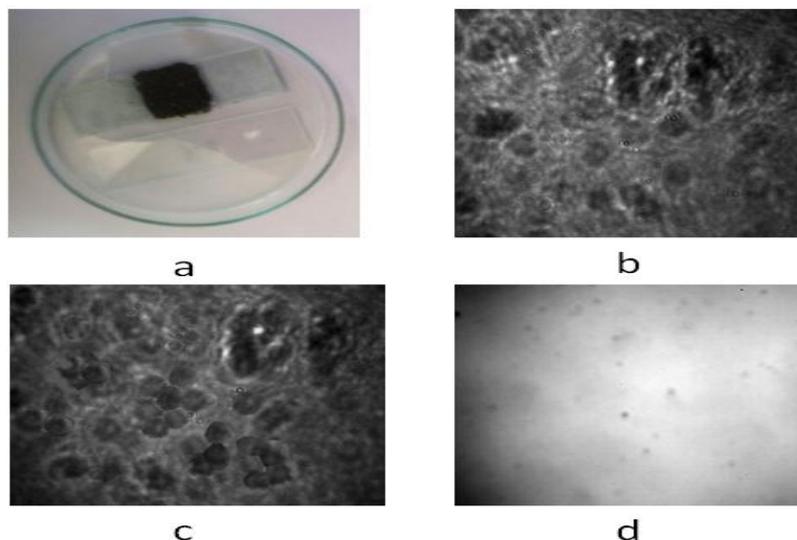


Figure. 3. (a) The control slide and the biofilm deposited slide. The brown square portion indicates biofilm deposited on pyrite. (b) Biofilm formed after 12 h (c) Biofilm formed after 24 h (d) The control slide which is clear indicating no adsorption of bacterial cells on the empty glass surface.

3.5 Growth kinetics of bacterial adsorption: As depicted in figure 4, the growth curve of the bacteria it is clearly evident that after initial attachment the bacteria goes through a logarithmic phase in which the attachment process of the cells increases exponentially after which the curve becomes steady indicating the stationary phase.

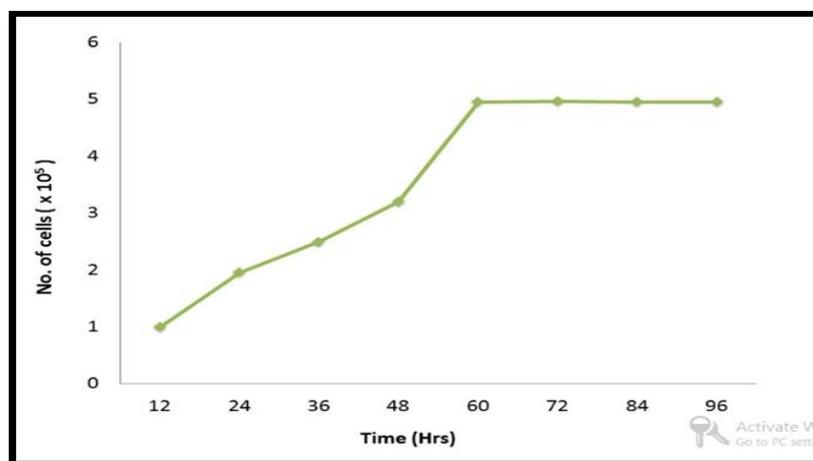


Figure 4: Growth curve of *Acidithiobacillus ferrooxidans* on pyrite.

4. CONCLUSIONS

The slow detachment rate of *Acidithiobacillus ferrooxidans* and high initial adhesion rate to pyrite demonstrate that this organism is a good biofilm former under the standard conditions. Robust biofilms can be observed on pyrite surfaces with different strategies of changing the initial pH of the medium, supplementing an extra energy source, adding ferric ions or phosphate starvation, exchanging exhausted medium with fresh medium, bioleaching of pyrite continues.

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