Chemical effects of biofilm colonization on stainless steel
by Jyostna Pendyala

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physics
Montana State University

Abstract:
Microbially induced corrosion (MIC) of metals is a serious problem for industry. Currently the only way to combat MIC is by replacing the affected metal. In order to understand the mechanism of MIC, thus allowing control of biocorrosion, the effects of bacteria on long-and short-term surface chemistry of the metals at a micro scale (i.e., on the scale of atoms and electrons), must be better understood. Although there have been numerous investigations of this problem from a microbiological and engineering aspect, there has been little work investigating the problem from a surface chemistry point of view. The goal of this work is to determine the effects of biofilm colonization on the surface chemistry of stainless steel in the first 20-60 Å. The effects of biofilm (a mixed culture of Pseudomonas aeruginosa, Pseudomonas fluorescens and Klebsiella pneumoniae) colonization on 0.05pm- finish polished and unpolished 304 stainless steel was examined using Auger electron spectroscopy (AES), X-ray Photoelectron Spectroscopy (XPS) and Time of Flight Secondary Ion Mass Spectroscopy (TOF-SIMS).

Changes in the surface concentrations of the main alloying elements of an as-received unpolished 304 stainless steel were observed after biofilm colonization. In the oxide film close to the bulk stainless steel, there was an enrichment in the relative concentration of Cr with a corresponding decrease in the relative Fe concentration as compared to a control coupon exposed only to sterile media. There were no changes observed in the relative Ni concentration.

Electrochemically induced localized pits reveal a chemistry similar to unpolished stainless steel surfaces colonized by bacteria. The electrochemically treated surfaces also show the presence of blackened grains and visually unaffected areas whose chemistry has changed significantly.

The activity of the biofilm on the well characterized polished stainless steel surface causes penetration of organic material into the matrix of the surface oxide layer. This penetration is dominated by carbon and nitrogen on surfaces exposed to bacteria. There were no chemical changes observed due to the exposure of the surfaces to sterile media under identical flowing conditions.

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APPROVAL

of a thesis submitted by

Jyostna Pendyala

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Microbially induced corrosion (MIC) of metals is a serious problem for industry. Currently the only way to combat MIC is by replacing the affected metal. In order to understand the mechanism of MIC, thus allowing control of biocorrosion, the effects of bacteria on long- and short-term surface chemistry of the metals at a micro scale (i.e., on the scale of atoms and electrons), must be better understood. Although there have been numerous investigations of this problem from a microbiological and engineering aspect, there has been little work investigating the problem from a surface chemistry point of view. The goal of this work is to determine the effects of biofilm colonization on the surface chemistry of stainless steel in the first 20-60 Å. The effects of biofilm (a mixed culture of *Pseudomonas aeruginosa, Pseudomonas fluorescens and Klebsiella pneumoniae*) colonization on 0.05μm- finish polished and unpolished 304 stainless steel was examined using Auger electron spectroscopy (AES), X-ray Photoelectron Spectroscopy (XPS) and Time of Flight Secondary Ion Mass Spectroscopy (TOF-SIMS).

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CHAPTER 1

INTRODUCTION

Accumulation of microorganisms and their metabolic products on metal surfaces has been proposed to accelerate and enhance corrosion (Pope *et al.*, 1984; Walsh *et al.*, 1993). This phenomenon has been termed as microbially influenced corrosion (MIC), or biocorrosion, biodeterioration, biofouling and so on. Corrosion is the generic term involving natural, or electrochemical processes that effect the life and utility of materials. MIC on the other hand, can involve a plethora of organisms and mechanisms (Hamilton, 1990) and refers to the undesirable formation of biological deposits on material surfaces leading to a decrease in material performance and the useful life of the material (Characklis, 1988).

As early as 1910, Gaines suggested that iron bacteria and sulfur bacteria were partly responsible for the corrosion of buried ferrous metals. However it was not until two decades ago that MIC was recognized as a contributing factor in several types of routinely observed corrosion (Wolfram, 1991).

MIC has now been recognized as one of the key factors of material failure in heat exchangers, oil pipelines, submarine hulls and even in aircraft and nuclear power plants. MIC has also been implicated in the failure of medical implants leading to infections in the patient. Although biocide treatment has been shown to be effective in some cases, it

is only a temporary measure. The long term and sometimes only alternative is to replace the damaged material. This leads to grave economic consequences in industry. White *et al.* (1990) stated very aptly in their paper that "corrosion (is) the venereal disease of industry (it is painful, incapacitating, and expensive, but usually unmentionable)...". An even greater cause for concern is that even materials such as stainless steels, titanium etc., that were thought to be immune to corrosion attack have been found to succumb to MIC. Thus the industrial sector has an increased interest in understanding the mechanisms contributing to MIC and the interactions of the bacteria with surfaces at the micro- and nanoscales. A search for the "holy grail" of a material resistant to bacterial attachment and attack, has also begun.

Most of the biocorrosion occurs in the form of localized attack and pitting corrosion. The mechanisms through which microorganisms participate in pitting corrosion, however, remain obscure. Studies performed in this field have mainly been microbiological or electrochemical in origin. Although the studies focus on the chemical processes within the biofilm or at the surface, few have attempted to study the micro- and nanoscale chemistry of the liquid-solid interface. Much of the chemical evidence linking pitting corrosion to microbial processes is based on data obtained from surface deposits which have accumulated long after microorganisms exert their initial effects. Studies involving surfaces have mainly investigated bulk properties of the surface, which provides few if any clues to the surface properties involved or contributing to MIC.

Biofilm-forming bacteria are likely to induce subtle effects on the underlying metal surfaces during early stages of colonization and biofilm formation. Detection of near-surface chemical changes mediated by biofilms requires surface-sensitive analytical techniques. Auger Electron Spectroscopy (AES) , X-ray Photoelectron Spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectroscopy (TOF SIMS) obtain elemental information from the top 1-6 nm of the metal surface where biofilm microbial processes are likely to have their greatest influence. Few studies have combined surface spectroscopic analyses such as AES, XPS and TOF SIMS, with biological analyses. Hence there is little understanding of the metal-biofilm interface and the processes occurring there. This work aims to take the first steps in trying to address these issues.

The majority of the research in this topic until now has been concentrated on the microbiological and engineering aspects with little emphasis placed on the biofilm-surface interactions at the microscale. This research integrates the vast surface science resources of Image and Chemical Analysis Laboratory (ICAL) at MSU Physics, together with Microbiology and Engineering. Surface science has long pursued the understanding of solid-solid, solid-liquid and solid-gas interactions at the microscale. The combined interdisciplinary focus is being used to bring a broader perspective and approach to understanding and solving the issues of cell-solid and cell-liquid interactions. A review of the some of the microbiological and engineering work preceding this research will be presented in the following chapter.

Hypothesis and Objectives

The objectives of this thesis are based on the main hypothesis that biofilm colonization causes changes in the elemental chemistry and composition on the surface of stainless steel. Therefore the main objective of this work is to determine the effect of biofilm on the top 50 - 100 Å of the surface of stainless steel after the initial stages of colonization.

In order to achieve this objective, a list of specific tasks to be addressed are :

(i) Examine and determine surface chemistry of unpolished and polished coupons with the surface analytical techniques before any treatment (or exposure, as-received).

(ii) Expose unpolished and polished stainless steel coupons ("biocoupons") to biofilm and media under flowing conditions in a controlled environment for a fixed length of time. Simultaneously expose unpolished and polished stainless steel coupons ("control coupons") to sterile media alone under the same conditions as the biocoupons.

(iii) Reexamine the surface chemistry of these coupons with surface analytical techniques after the above treatments. Establish if there are quantifiable changes in the surface chemistry before and after treatments in terms of the bulk alloying elements of stainless steel, Fe, Cr and Ni, and in terms of changes in the passive layer.

(iv) Determine if surface chemistry changes can be accelerated electrochemically and whether any useful parallels to the changes occurring due to biofilm colonization can be drawn.

(v) Determine if there are differences in surface chemistry between colonized and uncolonized areas by the biofilm on the biocoupon.

CHAPTER 2

LITERATURE REVIEW

Biofilms and Stainless Steel

This review focuses on work relating to stainless steel, biofilms, MIC and surface analyses. Before reviewing current literature, a brief introduction to stainless steels, bacteria, biofilms, their physiology and interactions with surfaces from both microbiological and engineering standpoints will be given.

Bacteria

The majority of bacteria are unicellular and possess rigid cell walls. They do not possess a true nucleus. The DNA exists as a single circular molecule that is not surrounded by a nuclear membrane. Reproduction occurs through cell division and replication of the DNA and respiration through the plasma membrane (see Fig. 1 below; Characklis and Marshall, 1990). They may be spherical (cocci), rod shaped (bacilli) or spiral shaped (spirilla), and vary in size from ~ 0.2 to 2 μm in width and 1 to 10 μm in length. Due to their small size, bacteria have large surface/volume ratios. Hence the bacteria are easily affected by any unfavorable change in their environment. However, the simplicity of their structure and the rapidity with which they can utilize nutrients enables

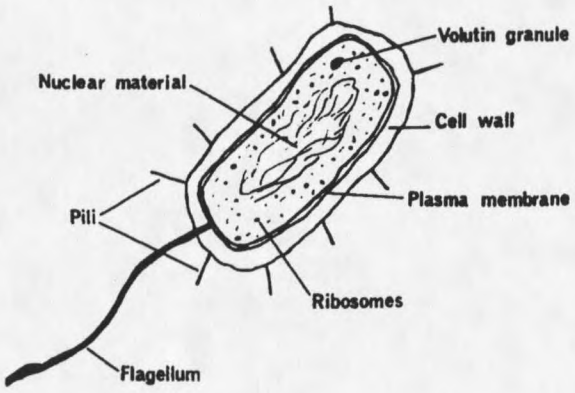

Figure 1: Bacterial cell

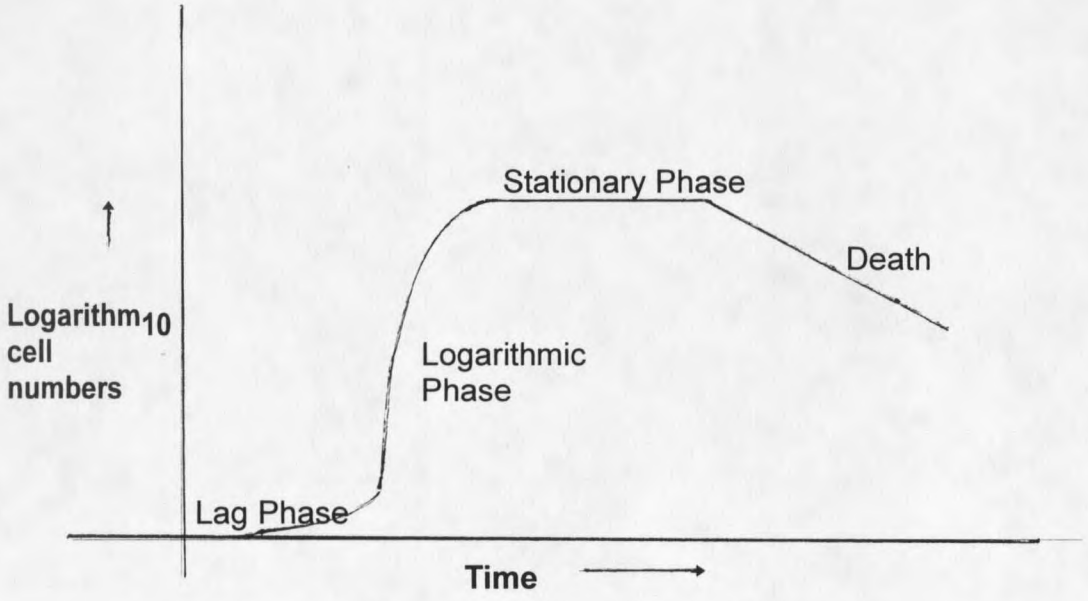

Figure 2: Microbial Growth Curve

them to multiply (and perhaps to mutate) exceedingly fast (Miller,1970).

Microbial growth occurs exponentially and consists of four phases (shown in Fig. 2 above): (i)*Lag Phase*: the addition of bacteria to the medium does not result in a doubling of the population, (ii) *Logarithmic Phase*: the cells begin to divide steadily, (iii) *Stationary Phase*: Growth plateaus, and (iv) *Death* (Characklis and Marshall, 1990).

Bacteria can withstand a wide range of temperatures from -10 °C to 90 °C (although most grow between 20-50 °C) and pH from 0 to 11. They can be aerobic, i.e., use oxygen to respire or anaerobic, i.e., they use other electron acceptors. Different groups of bacteria are known to use exclusively one or both mechanisms to respire. Bacteria can exist in two forms, the planktonic form and the sessile form. Planktonic bacteria are freely swimming or floating bacteria while sessile bacteria are attached to a substratum. The two strongest traits of bacteria are their ability to adapt to non-ideal conditions and exist in a state of suspension. In this way the bacteria remain viable (live, but in a frozen state) for long periods in extremely unfavorable conditions (Tatnall, 1991).

Biofilms and their structure

Biofilms are a collection of microorganisms, predominantly bacteria, attached to a surface in an aqueous environment and embedded within a three-dimensional gelatinous organic matrix of extracellular polymers secreted by the bacteria (Bryers, 1993; Characklis and Marshall, 1990). As seen in Fig. 3, the bacterial microcolony is the basic structural and functional unit of the microbial biofilm (Costerton, 1995). These microcolonies can be

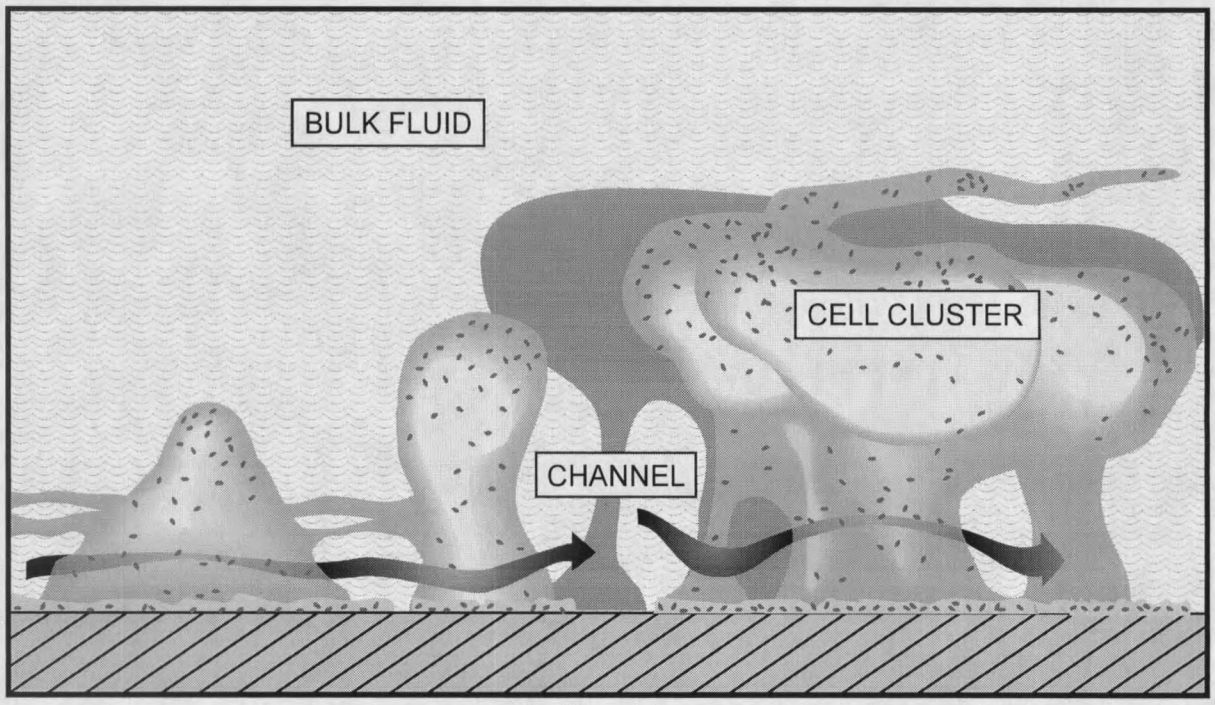

Figure 3: Schematic of biofilm (Center for Biofilm Engineering, MSU)

composed of single-species or multiple-species. The microcolonies are interspersed with water channels that are more permeable and contain less dense matrix material (Costerton and Lappin-Scott, 1995). These water channels form a network throughout the biofilm and provide access from the bulk fluid to the colonized surface (Costerton *et al.*, 1994). Researchers argue that the persistence of these water channels deep in the biofilm together with the development of mushroom shaped microcolonies indicates some form of cell-cell communication (Costerton, 1995). The further discovery that convective flow is possible through the water channels and that they allow the passage of large particles has revolutionized the concept of bacterial growth in biofilms. All these findings suggest that the water channels may actually represent a primitive circulatory system (Costerton *et al.* 1995). Thus the biofilm structure possesses a complex architecture and assumes a high degree of cooperation between microorganisms. Bacteria and other microorganisms have often been dismissed as primitive. However they are the most successful lifeform on earth having survived eons in time and many extreme environments. They pose a challenge to scientific understanding.

Advantages of Biofilm Growth Mode

The biofilm mode of growth allows sessile bacteria to trap and retain scarce organic compounds. It also helps in the breakdown of complex nutrients requiring time or the cooperation of other bacterial species. Biofilm formation changes the

microenvironment at the colonized surface and renders its inhabitants less vulnerable to hostile physical, chemical, and biological factors (Costerton and Lappin-Scott, 1995).

Biofilm Accumulation

Biofilms accumulate as a result of the microbes' ability to adsorb to a substratum, replicate and produce extracellular polymers. The initial surface colonization and the subsequent biofilm formation is a complex series of chemical, physical and biological processes (Fig. 4; Bryers, 1994):

1. Macromolecules present in the bulk fluid adsorb to the surface resulting in a "pre-conditioning" of the substratum.
2. Transport of planktonic cells from the bulk fluid to the substratum.
3. Reversible adsorption of cells to the substratum for a finite time.
4. The desorption of cells from the substratum either due to fluid shear forces or other physical, chemical and biological factors.
5. Irreversible adsorption occurs when some cells remain adsorbed permanently to the substratum.
6. The attached cells grow at the expense of the substrate and nutrients in the bulk fluid.
7. Cell replication and extracellular polymer production occur along with the formation of microcolonies and biofilm growth.

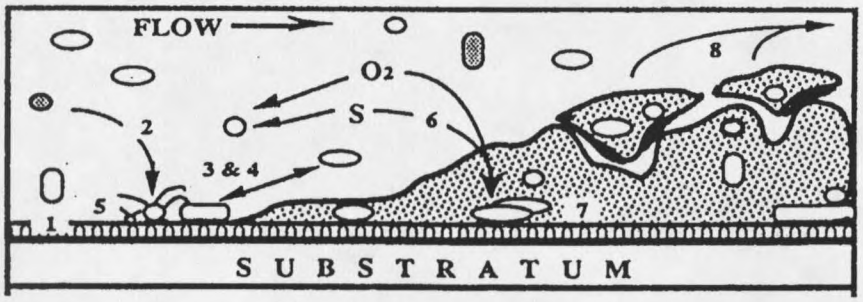

Figure 4: Biofilm accumulation (Bryers and Characklis, 1992)

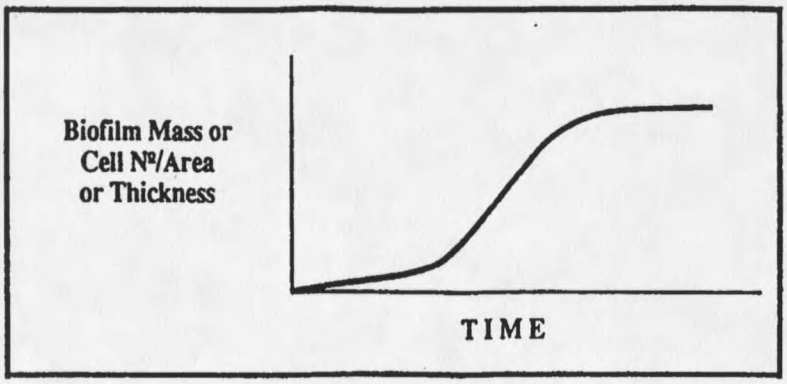

Figure 5: Biofilm accumulation curve (Bryers and Characklis, 1992)

8. Detachment of biofilm material occurs either continuously due to erosion brought about by shear forces, or randomly, where portions of the biofilm detach (sloughing) (Bryers and Characklis, 1992; Characklis and Marshall, 1990).

This progression occurs as a sigmoid curve (shown in Figure 5). The curve is divided into: (i) initial events,(ii) exponential or logarithmic accumulation and (iii) plateau or steady state (Characklis and Marshall, 1990).

Stainless Steel

Stainless Steels are iron based alloys containing at least 10.5% chromium up to 30% chromium. Chromium combines with oxygen to form a transparent chromium oxide film on the surface that makes the steel resistant to corrosion. This film is also known as the passive film (American Iron and Steel Institute (AISI), 1967). In addition to their corrosion resistance, the numerous grades of steel offer many advantageous physical and mechanical properties. Today stainless steel is ubiquitous with uses varying from nuclear fuel storage tanks made of steel to automobiles and aircrafts with steel parts.

Within the stainless steel family, there are martensitic, ferritic, austenitic and precipitation-hardenable steels. Austenitic steels account for two-thirds of the total stainless steel production. Austenitic steels have a face-centered cubic lattice crystal structure and may contain from 6-22% nickel and 16-28% chromium(AISI, 1967). These steels are extremely ductile and strong and hence are extensively used in the piping and

tubing industry. Martensitic steels principally contain 11.5-18% chromium while ferritic steels contain 14 -27% chromium besides iron.

The austenitic steels comprise the AISI 300 series. The most common among these are types 304, 304L, 316 and 316L. These alloys contain mainly iron, chromium, nickel and some manganese, molybdenum and silicon with less than 1% carbon, sulfur and phosphorus depending on the grade. The addition of minor alloys is thought to increase the passivating ability and decrease the activity of weak points in the film (Hermas *et al.*, 1995). Silicon increases oxidation resistance at high temperatures and is thought to improve resistance to pitting corrosion. Molybdenum increases resistance to localized corrosion by reducing the oxidation of iron. However, studies performed by Scott *et al.* (1991) have shown that alloys containing between 6-9% molybdenum have been susceptible to corrosion. Carbon is added as it is a good austenite (phase) stabilizer (Bruemmer,1992). However if an unstabilized austenitic stainless steel is slowly cooled or reheated in the temperature range 500-800 °C (known as sensitization), diffusion of carbon to the grain boundaries occurs (Laws and Goodhew, 1991). The precipitation of chromium-rich carbides occurs along the grain boundaries, leaving a region depleted of chromium. Impoverishment of chromium is hypothesized to be the reason for poor corrosion resistance (Joshi and Stein, 1972). Hence only a low amount of carbon is added to the alloys. Sulfur is added mainly to improve the machinability of the alloys (ASM, 1977). Corrosion of stainless steel, in general, is fueled by the breakdown of the passive film.