INVESTIGATION OF SULFATE-REDUCING BACTERIA GROWTH BEHAVIOR FOR THE MITIGATION OF MICROBIOLOGICALLY INFLUENCED CORROSION (MIC)

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Chapter 1 Introduction

When pure metals or their alloys are exposed to water, corrosion occurs immediately. Corrosion is an electrochemical process consisting of two partial reactions, an anodic reaction by which metal becomes corroded and a cathodic reaction where some species are reduced. In some cases, the presence of microorganisms affects the corrosion reactions by forming a biofilm on the metal surface although no new electrochemical mechanisms are present in the corrosion process (Beech and Gaylarde, 1999). The dissolution of metals both directly and indirectly related to the activities of microorganisms is known as microbiologically influenced corrosion (MIC) or biocorrosion. MIC is not a new corrosion mechanism; it involves the activities of microorganisms in corrosion processes. All materials can be attacked by microorganisms, including metals, minerals, organic materials and plastics. Therefore, MIC has become a multidisciplinary subject that integrates the fields of materials science, chemistry, microbiology and biochemistry (Thierry and Sand, 2002).

At the end of the 19th century, the first reported suggestion that microorganisms might influence the metal corrosion process was made by Garret in 1891. He found that the corrosion of lead-sheathed cable was affected by the action of bacteria metabolites. Later reports provided evidence that iron and sulfur bacteria can be linked to the corrosion of the interior and exterior of water pipes (Gaines, 1910). Von Wolzogen Kuhr and van der Vlugt in 1934 published the first paper that attempted to interpret MIC mechanisms in electrochemical terms. During the decades of the 1960s and 1970s, the

cathodic depolarization theory (CDT) was the prevalent explanation for the corrosion of ferrous metal caused by sulfate-reducing bacteria (SRB). At the same time, more mechanistic studies were published either objecting to or validating the anaerobic corrosion of iron by the cathodic depolarization theory (Booth and Tiller, 1962; Iverson, 1966).

In the ensuing decades, there has been considerable attention given to understanding the nature and mechanisms of MIC, but it was not until the late 1970s that a good understanding of MIC processes was obtained. In practical applications, MIC is probably not the result of one single organism acting by one mechanism; rather, it is a result of a consortium of different microorganisms acting via different mechanisms. Tiller (1985) mentioned that MIC problems were often subtle, hidden behind traditional corrosion, and often overlooked, suggesting that a sophisticated methodology and equipment for the detection and analysis was needed.

Videla (1996) stated that the participation of microbes could provoke or increase the corrosion of iron without changing the electrochemical mechanisms of corrosion. The microorganisms are capable of causing corrosion directly by converting element metal into metal ions. They can also secrete extracellular products that are corrosive in the absence of microbes. Under field conditions, corrosive microorganisms grow along with other microorganisms in a synergistic consortium. This mixed microbial consortia and the countless organic and inorganic chemical species in a micro-habitat make MIC a complicated process to study.

Mild steel and stainless steel are the most frequently used engineering materials in the oil and gas industry. The metals are known to suffer from localized corrosion by the presence of microorganisms. Considerable scientific attention has been devoted to investigating the microbial corrosion process of steel and iron in the presence of SRB (Starosvesky et al., 1999). SRB are non-fermentative anaerobes that obtain their energy for growth from the oxidation of organic substances using inorganic sulfur oxy-acids or nitrate as terminal electron acceptors whereby sulfate is reduced to sulfide (Feio et al., 2000). Biogenic sulfide may result in the corrosion of mild steel in an anaerobic environment (Lee and Characklis, 1993). However, the mechanism of how SRB influence the anaerobic corrosion of ferrous metal continues to be controversial (Rainha and Fonseca, 1997).

In oilfield operations, the active participation of microorganisms has been blamed for the deteriorating effects including the corrosion of equipment and installations, plugging of petroleum formation, and souring of the reservoir and fluids. Although there have been different estimations for the cost of MIC, some figures from individual companies or sectors of the industry indicate MIC is costly. Detailed studies carried out in the United States indicate that MIC costs various industries between $16 and $18 billion (NBS, 1978). In the oil and gas industry 34% of the corrosion damage experienced by one oil company was believed to be related to microorganisms (Jack et al., 1992). In the 1950s, it was reported that MIC-related costs of repair and replacement of piping materials used in different types of service in the United States was approximately 0.5 to 2 billion dollars per year (Beech and Gaylarde, 1999). In the United Kingdom, it was

suggested that 50% of corrosion failures in pipelines were microbiologically influenced (Booth, 1964); while, it was also proposed that around 20% of all corrosion damages to metallic materials was associated with MIC (Flemming, 1996). Furthermore, the losses due to damage of equipment by MIC are combined with those resulting from biofouling although they do not cause the same damage. In 1971, it was reported that biofouling problems in cooling water systems caused $300 million in damages (Purkiss, 1971).

A good example of MIC was from in the Chevron Oil Production Company in the United States. Pinhole leaks were detected in several segments of a new oil and water gathering system only 18 months after the new system began operation. Internal examination of the leaking piping indicated that serious damages due to microorganisms had occurred beneath the deposits of fracture sand and/or iron sulfide (Strickland et al., 1996).

Another historical case of microbial corrosion was the water injection system of a Brazilian offshore plant (Videla et al., 1989). The performance of carbon steel and steel N-80 related to biofouling and biocorrosion were investigated in detail with injected seawater under different marine conditions. Both types of steel tested in this system showed poor resistance to the seawater and were significantly damaged. The problem was thought to be associated with microorganisms since chemical considerations of the injection seawater alone were not capable of causing the severe corrosion and type of the attack on metal surfaces.

Luo et al. (1994) reported the results of experiments performed by the BP Corporation. The tests confirmed that the corrosion rate of steel specimens could be

accelerated in the presence of microorganisms when they tested the corrosion of steel in two different systems. The corrosion rate of mild steel increased with exposure time in the system inoculated with bacteria, while a relatively low and constant corrosion rate was obtained in the sterile system.

The reality of all these studies suggested that MIC was a relevant area of research both in the laboratory studies and industrial investigations. A timely topic is the study of the growth, morphology of microorganisms and their interactions with ferrous metals, leading gradually to the MIC mitigation methods that are friendly to the environment.

Chapter 2 Literature Review

2.1 General corrosion

Corrosion, the destruction or deterioration of a material caused by interactions with its environment, has been recognized as a major problem in the world. It is estimated that in the United States, the direct costs of corrosion are approximately 4.9% of the gross national product, which is greater than the combined cost of all the fires, floods, hurricanes, and earthquakes in this nation. Indirect costs of corrosion are much harder to determine, but they may be at least not less than those direct costs (Bradford, 1992).

Metal corrosion can be attributed to air, water, soil, and/or microbial consortia. Metal corrosion is a natural process, essentially an electrochemical process that causes metal to react with its environment, to become oxidized and released from the metal surface at an anodic site, while the electrons produced from metal oxidation reduce the chemical species that contact the metal surface at a cathodic site (Horn and Jones, 2002). Regardless of various corrosion forms, including uniform corrosion, pitting or cracking corrosion, all metals are corroded by this same basic mechanism (Fontana and Greene, 1967).

Apart from the direct and indirect costs, corrosion also leads to the depletion of natural resources. It is estimated that 40% of all steel produced is used to replace the steel lost due to corrosion. Furthermore, many metals, especially those in alloying, are difficult to recycle with present technologies. Finally, energy resources are also expended in

producing replacement metals, which are deteriorated or lost from corrosion (Bradford, 1992).

2.2 Microbiologically influenced corrosion (MIC)

In a variety of environmental situations microorganisms produce many kinds of corrosive metabolic by-products, making microorganisms a constant threat to the stability and performance of such metals as cast iron, mild steel and stainless steel, copper, aluminum and their alloys. Beech and Gaylarde (1999) classified that the types of organisms related with corrosion failures of materials could be classified into groups: sulfate-reducing bacteria (SRB), iron-oxidizing/reducing bacteria, manganese oxidizing bacteria, sulfur-oxidizing bacteria, and bacteria that secret organic acids and extracellular polymeric substances (EPS) or slime. These species are capable of coexisting within the biofilms that occur on the metal surface where they form the microbial communities. The activities and characteristics of these organisms will be provided below in detail.

2.2.1 Metal-reducing bacteria (MRB)

As early as 1981, it was shown that corrosion reactions of metals can be affected by a variety of types of bacteria, such as *Pseudomonas* and *Shewanella*, which can carry out manganese and/or iron oxide reduction (Obuekwe et al., 1981; Myers and Nealson, 1988). Corrosion of iron and its alloys was shown to accelerate in the presence of these microorganisms by dissolving the corrosion resistant oxide films or the protective films

were replaced with less stable reduced metal films on the metal surfaces. If the protective passive films on stainless steel surfaces is lost or damaged, corrosion is promoted and higher corrosion rate ensues. However, it was not until recently that these bacteria were considered seriously in corrosion processes although they participated in industrial corrosion in a wide range of natural and man-made systems (Beech and Gaylarde, 1999).

2.2.2 Metal-depositing bacteria (MDB)

Bacteria of the genera *Siderocapsa, Gallionella, Leptothrix, Sphaerotilus, Crenothrix and Clonothrix,* have been shown to occur frequently in the biotransformation of oxides of iron and manganese (Gounot, 1994). The oxidation of Fe^{2+} to Fe^{3+} is a very common phenomenon occurring on a surface with the participation of bacteria in these genera, such as *Gallionell* and *Leptothrix.* These bacteria were observed in the form of tubercles (discrete macroscopic deposits) and associated with pitting attack on the steel surfaces. Microscopic examination indicated that the sheathed filamentous bacteria among these bacteria were very important in the corrosion of steels (Keevil et al., 1989; Lutey, 1992; Tatnall, 1981). While the cells are not very distinctive from each other in appearance, the long sheathed filaments are readily detected and identified from other life forms under the microscope.

Aside from iron oxidizers, manganous ions can also be oxidized to manganic ions by the bacteria leading to further corrosion of metal (Little et al., 1997). The deposits on the metal surface due to MDB can decrease or damage the stability of protective passive

oxide films covered on the surfaces of corrosion resistant steels and alloys, thus aggravating the corrosion. Furthermore, the formation of a biofilm containing the sheath-forming, manganese-depositing bacterium accumulating on the metal surfaces was responsible for the corrosion of stainless steels in aquatic systems (Dickinson et al., 1997).

2.2.3 Slime-producing bacteria

Slime-producing microorganisms have also been reported to be associated with localized attacks of steels. These organisms produce large quantities of extracellular polymeric substances (EPS) during their growth within biofilms covered on the steel surfaces including *Clostridium spp.*, *Flavobacterium spp.*, *Bacillus spp.*, *Desulfovibrio spp.*, *Desulfotomaculum spp.* and *Pseudomonas spp.* (Pope et al., 1984). The sticky polymers they produce referred to as "slime" affect both the attachment of the cells to the surface and the permeation of substances through the deposit on the surface.

Microscopic amounts of EPS ($10ng/cm^2$) can induce or provoke the initiation of microbial corrosion of stainless steels in natural seawater, but the mechanisms of the EPS in the MIC of stainless steels are still not very clear. McEvoy and Colbourne (1988) showed that tested copper tubes were corroded seriously with the well-developed biofilm. A correlation of the pitting sites on the copper surface and copious amounts of biofilms was observed with the Scanning Electron Microbiology (SEM) method (Keevil et al., 1989).

2.2.4 Acid-producing bacteria (APB)

Certain bacteria can produce large amounts of inorganic or organic acids as by-products during their metabolism, leading to serious corrosion damage to equipment. Organic acid-producing bacteria have been blamed for the corrosion of carbon steel in some cases (Soracco et al., 1988). Little et al. (1988) also reported that the corrosion of cathodic protected stainless steel was promoted by certain acetic-producing bacteria. Fungi that produce organic acids were also known to accelerate the biocorrosion of steel and aluminum. The mechanism of how acids affected the corrosion of mild steel was well understood in the metallurgical literature (Shreir, 1963.), while the effects of the acids produced by the bacteria and their concentrations on the corrosion process of mild steel remain obscure under MIC conditions.

2.2.5 Sulfate-reducing bacteria (SRB)

SRB are the most troublesome groups of organisms among all microorganisms involved in MIC of steels and other metals in oil, gas and shipping industries (Hamilton, 1994). Considerable efforts have been focused on the influence of SRB on mild steels and alloys. Costerton and Boivin (1991) assessed the MIC damage due to SRB at upwards of some hundreds of million US dollars in the United States in production, transport, and storage of oil, not including the costs for the lost oil and clean-up of environmental pollution. The serious problems also included the reduction of the quality of petroleum products and the increase of refinery costs through H_2S production (Herbert,

1986; Cochrane et al., 1988; Frezer and Bolling, 1991). In addition, SRB growth in seawater injection systems led not only to the corrosion of the equipment, but also contamination of the oil and gas with H_2S and viable SRB inside.

SRB are defined as obligate anaerobes which obtain energy for growth from the oxidation of organic substances, using sulfate as the external electron acceptor and reducing sulfate to sulfide (Postgate, 1984; Widdel, 1988). Corrosion related to SRB activities is primarily realized as a localized attack, usually in the form of pitting, occurring on the metal surface. SRB include all unicellular bacteria that can reduce sulfate to sulfide (Nielsen, 1987; Mohanty et al., 2000). SRB are often thought to be strictly anaerobic, however, some genera can still grow well at low dissolved oxygen concentrations (Hardy and Hamilton, 1981; Abdollahi et al., 1990); Dilling and Cypionka (1990) reported that some SRB were even capable of respiring oxygen with hydrogen and acting as an electron donor. SRB can grow in conditions within pH range from 5.0 to 10.0 and temperature from 5°C to 50°C with the best temperature being between 25°C and 40°C (Javaherdashi, 1999). Extensive reviews on the ecology and physiology of SRB had been provided by Postgate (1984) and Widdel (1988).

In many industrial environments, SRB have been shown to have an affinity for adhering to available surfaces and developing patchy biofilms (Costerton and Geesey, 1979; Dewar, 1986; Rosnes et al.; 1990). It is these sessile (biofilm) SRB that are responsible for localized corrosion of mild steel in industrial and aquatic environments (Costerton and Boivin, 1991). The factors that could affect SRB behavior and resultant

corrosion of mild steel include nutrient availability, temperature, sulfide inhibition and adhesion of cells to the metal surfaces (Costerton, and Boivin, 1991).

2.3 Characteristics of MIC

2.3.1 General characteristics of MIC

In principle, corrosion is an interfacial process and the electrochemical mechanisms remain valid for MIC. However, the presence of microorganisms growing at interfaces can influence not only the anodic and cathodic reactions, but also such interfacial properties as pH value, salts, redox potential and conductivity. Sequeira (1988) summarized the contributions of microorganisms involved in the corrosion processes: (1) a direct influence on the anodic and cathodic reactions as well as the corrosion rate; (2) alteration of film resistance on the metal surface by metabolic production of aggressive species; (3) creation of a corrosive environment. These organisms could adhere to the available surfaces, enclose themselves in sticky extracellular polymeric substances (EPS) and form biofilms. Generally, the main components of biofilms are water, EPS, cells, entrapped particles and precipitates, adsorbed ions and polar and apolar organic molecules (Schaule et al., 1999). Biofilms are characterized by their heterogeneity with the thickness ranging from a very thin layer (less than 100nm) to centimeters. Videla (2001) presented four steps in the procedure of biofilm formation on the metal surface: transport of organic material to metal surface, transport of planktonic cells to the surface to become the sessile cells, attachment of the cells to a surface and the growth of cells

within biofilm. The mechanisms interpreted the whole corrosion process with microbial participation, but the modification of the interface structure due to biofilm accumulation should be regarded as the main cause of MIC (Videla, 1986; 2001).

2.3.2 Role of SRB biofilm

Mild steel and carbon steel are the most widely used materials used in industrial structures and they are known to be attacked by patchy biofilms accumulated on the surfaces due to SRB. In general, on the carbon steel surfaces, biofilm accumulation of SRB forms on an unstable and continuously growing layer of inorganic products. SRB cell adhesion is initiated on amorphous corrosion products, and later many areas of the carbon steel surface are found to be covered by bacteria colonies, also by some kinds of corrosion products (Beech and Gaylarde, 1999). Figure 1 shows the biofilm formed by SRB on the metal surface observed under SEM.

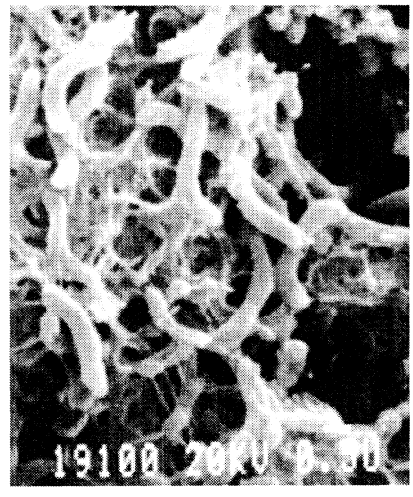


Figure 1. SEM image of a biofilm formed by SRB on the surface of mild steel (Beech and Gaylarde, 1999).

2.4 Mechanism of MIC due to SRB

Over the past decades, an extensive progress has been made to understanding the interaction between SRB activities within biofilm and the corrosion process of ferrous metal. Hamilton (1985) raised the issue of the cathodic reaction with the participation of SRB during corrosion process in neutral anaerobic environment. The overall reaction of MIC due to SRB can be expressed as:

$$4Fe + SO_4^{2-} + 4H_2O \Leftrightarrow 3Fe(OH)_2 + FeS + 2OH^- \quad (2.1)$$

Among many mechanisms proposed to explain how the metal dissolution proceeds in the presence of SRB, the cathodic polarization theory is the most prevalent explanation whereby protons may act as an electron acceptor at the cathode in the absence of oxygen (Von Wolzogen Kuhr and van der Vlugt, 1934). The typical reactions of this theory are provided below:

Anodic reaction: $4Fe \Leftrightarrow 4Fe^{2+} + 8e^- \quad (2.2)$

Electrolytic dissociation of water: $8H_2O \Leftrightarrow 8H^+ + 8OH^- \quad (2.3)$

Cathodic reaction: $8H^+ + 8e^- \Leftrightarrow 8H \quad (2.4)$

Cathodic depolarization by SRB: $SO_4^{2-} + 8H \Leftrightarrow S^{2-} + 4H_2O \quad (2.5)$

Corrosion product: $Fe^{2+} + S^{2-} \Leftrightarrow FeS \quad (2.6)$

Corrosion product: $3Fe^{2+} + 6OH^- \Leftrightarrow 3Fe(OH)_2 \quad (2.7)$

Overall reaction: $4Fe + SO_4^{2-} + 4H_2O \Leftrightarrow 3Fe(OH)_2 + FeS + 2OH^-$

On the other hand, Costello (1974) proposed that hydrogen sulfide, H_2S, instead of hydrogen ion could act as cathodic reactant, i.e.

$$2H_2S + 2e^- \Leftrightarrow 2HS^- + H_2 \qquad (2.8)$$

The process of corrosion of ferrous metal due to SRB by the cathodic depolarization theory is shown in Figures 2 and 3. The removal of adsorbed hydrogen atoms is utilized to reduce sulfate to sulfide by the bacteria hydrogenase. The activity of hydrogenase of SRB combines the adsorbed hydrogen atoms to produce H_2 gas first and then regenerates protons, which effectively facilitates the hydrogen evolution and increase the corrosion rate accordingly (Von Wolzogen Kuhr and van der Vlugt, 1934; Thierry and Sand, 2002). Without the bacteria, the process would stop at Equation (2.4), because the surface would be covered by a layer of adsorbed hydrogen atoms without the depolarization of the cathode (i.e. the removal of these hydrogen atoms). The produced sulfide would react with available proton to form hydrogen sulfide, H_2S, which is known as a very corrosive species related to the corrosion of ferrous materials (Borenstein, 1994).

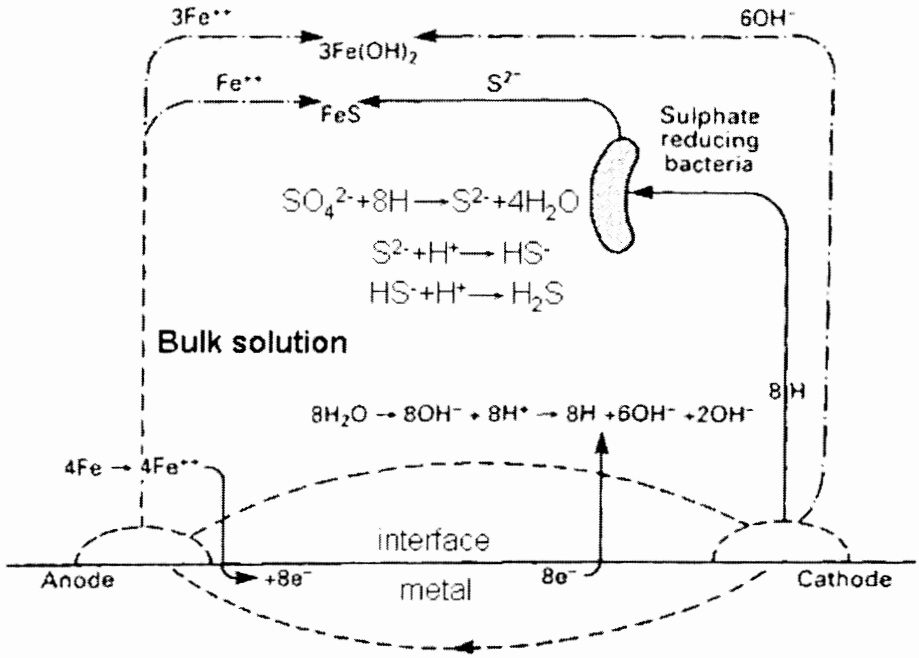


Figure 2. Influence of SRB on corrosion of ferrous metals after Nelson (1961).

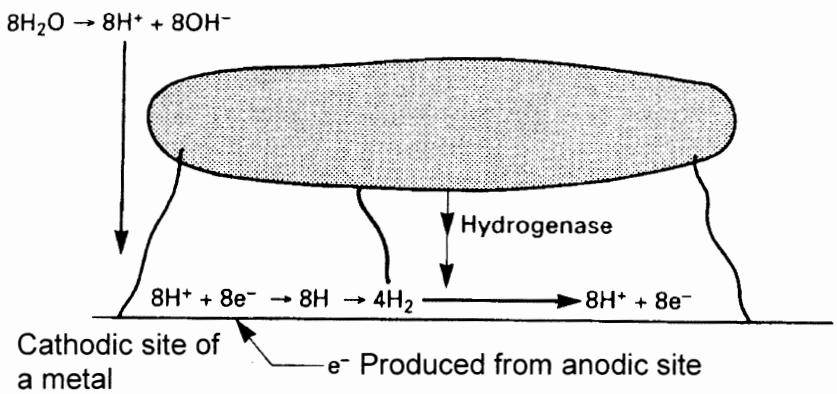


Figure 3. Cathodic depolarization of surface due to utilization of hydrogen by hydrogenase of SRB after Flemming and Geesey (1991).

2.5 Factors related to the corrosion of mild steel due to SRB

2.5.1 Ferrous ion and iron sulfides

The concentration of ferrous ions in the medium has been considered the most significant parameter in the corrosion of mild steel mediated by SRB. The differences of corrosion of mild steel due to the variation of ferrous ion concentration lie in the different physical forms of iron sulfide. It is found that all the iron sulfides (mackinawite, greigite, pyrrhotite, marcasite, and pyrite) can be formed in the presence of ferrous ion and *Desulfovibrio desulfuricans* (Rickard, 1969; Morse et al., 1987). It is difficult to distinguish the biogenic iron sulfides from those produced by purely inorganic processes under the same conditions.

Mara and Williams (1972) noted that in a medium containing a low iron concentration there was an adherent iron sulfide film on the mild steel surface. They also found that after this protective film was broken, the rate of corrosion was not only higher, but also independent of the growth of SRB. The film breakdown was attributed to the transformation of mackinawite to non-protective greigite. In a similar study, King et al. (1973) found that an increase of ferrous ion concentration in the medium resulted in the breakdown of the protective mackinawite film. Once the film was ruptured, the corrosion increased at a rate proportional to the concentration of ferrous ions. In a medium containing sufficient ferrous ions, no protective film was observed and a high corrosion rate was obtained (Booth et al., 1965; 1968).

Lee and Characklis (1993) investigated the effects of suspended iron sulfide on the corrosion of mild steel in an anaerobic biofilm reactor where the ferrous ion concentration increased from 0 to 60mg/l. When the increase of ferrous ion concentration reached 60mg/l, iron sulfide particles were able to penetrate through the protective iron sulfide film, resulting in the rupture of the protective film. Intergranular attacks were also found on the metal surface under SEM examination. King and Miller (1971) proposed that the produced iron sulfide (FeS) acted as the absorber of molecular hydrogen and FeS would be regenerated and maintained as cathode by the reaction of the hydrogen evolution system. In this situation where the area covered by biofilm acts as anode while the area covered by iron sulfide becomes cathode, corrosion is continuous and the corrosion rate of metal remains high.

2.5.2 Sulfate nutrient limitation

The general energy limitation for the growth of bacteria is the carbon source. However, in many oil and gas systems with mixed populations of organisms, the limiting nutrient ion could be the sulfate instead of the carbon sources for the growth of SRB. In some cases, the sulfate concentration in a system has a direct influence on the growth and activity of SRB and the amount of sulfide produced (Sanders, 1988). It was found that the initiation of biocorrosion due to SRB only occurred in the presence of sulfate species when Fonseca et al. (1998) tested the corrosion of mild steel under different media both

with and without sulfate ions. Mohanty et al. (2000) also found that a high sulfate concentration in the medium could inhibit the sulfate reduction rate of SRB.

2.5.3 Cell attachment inhibition

An important step in biofilm formation according to Videla (2001) is the attachment of planktonic cells, which becomes the sessile organisms that are adsorbed to the metal surface, leading to the continuous biofilm accumulation and an increase of biofilm thickness. These patchy biofilms formed on the metal surface are blamed for the localized corrosion of steels. If the migration of free SRB cells on the metal surface at different sites is inhibited, the formation of patchy biofilms on the metal surface due to bacteria accumulation would not occur. Therefore, immobilization of living SRB cells would be a possible way to inhibit microbial corrosion. Immobilization of microorganism is a technique that restricts the cell mobility by aggregating the cells or by confining then into, or attaching them to, a solid support (Chung et al., 1998).

The porous cell immobilization materials should have characteristics such as high affinity and degree of cell attachment to them, being nontoxic, cheap, resistance to microbial degradation and exhibiting high stability during long-term culture. These properties justify the selection of Celite beads as the support material for SRB immobilization in this study. As an inexpensive natural product, Celite is derived from the fossilized shells of diatoms. The main chemical constituent is silica, SiO_2, which accounts for 90% of Celite by weight, with small amounts of other inorganic oxides such

as Al_2O_3, Fe_2O_3, and CaO present (Hull et al.; 1953). Porous Celite beads are used commonly as supports for the immobilization of whole cells (Chun and Agathos, 1991) in the fermentation industries and they are commercially available in different forms with various particle sizes, shapes and porosities.

2.6 Investigation techniques in MIC

There are various techniques for MIC investigation. Currently, experimental methods used to assess and monitor the damage or attack of biocorrosion can be divided into two categories: microbiological and electrochemical methods.

2.6.1 Microbiological methods

The quantitative investigation of microorganisms in MIC is restricted to the planktonic population of SRB in liquid samples in many investigations. The planktonic SRB cell numbers can be obtained under an optical microscope using a hemocytometer (Neubauer chamber, Hausser Scientific) with serial dilutions (Penn, 1991). However, Ruseska et al. (1982) suggested that the traditional assessment of planktonic bacteria activities in liquid state could be different from the bacteria activities in bioflims. Hamilton (1985) reported that assessment of the activity of the microorganisms within the biofilm should be the most important in biofilm quantification.

It was reported that the quantification of the sessile bacteria population had more advantages (Geesey et al., 1978; Gilbert and Herbert, 1987). The biofilm could represent

more properly the microbial population attacking the metal in terms of cells number or biomass. In the evaluation of biocidal activity, the sessile microorganisms represent the susceptibility more accurately than the equivalent microorganisms dispersed in the bulk solution. This is because the protective action of EPS increases the resistance of the sessile bacteria to biocides by hindering the penetration of biocides to deeply embedded cells in the biofilm or by altering the surface properties of the bacteria.

The methods for the biofilm quantification include viable counts, biomass assessment and activity measurements (Videla, 2001). Viable counts can provide the estimation of the number of viable bacteria embedded in a biofilm without special equipment. Plate counting to obtain colony forming units (CFU) is the most widely used method to monitor biofilms. Biofilms can be removed from a metal surface with mild sonication. Numbers of viable suspended bacteria can be determined by counting the number of CFU of bacteria colonies after the bacteria were plated on suitable solid media. For SRB quantification, many agar media had been attempted by earlier researchers to grow these organisms using the plating method. But those usually resulted in poor and slow growth, typically 7 days at room temperature (Iverson, 1966).

Radiorespirometric techniques have been mainly used to investigate the activity of SRB in the oil and gas industry. This method is also able to obtain quantification of the amount of biogenic sulfide in undisturbed biofilm conditions similar to those occurring in the corrosive environment (Hamilton and Maxwell, 1986; Sanders and Hamilton, 1986).

2.6.2 Electrochemical methods

The participation of microorganisms in the corrosion process of steels changes the properties of the interface between the metal and the bulk solution. These modifications of metal surface conditions result in many effects including changes from the general corrosion to the localized corrosion, or to corrosion inhibition. However, the electrochemical techniques are still useful in explaining the phenomenon of biofouling and biocorrosion induced by microorganisms. All electrochemical measurements are indirect methods based on Faraday's law, and can give instantaneous results. Furthermore, they are thought to be more powerful and efficient techniques in the investigation of corrosion mechanisms.

2.6.2.1 Open-circuit potential measurement

When a sample is immersed in a corrosive medium and the sample is not connected to any instrumentation, there is an assumption of a potential termed the corrosion potential (relative to a reference electrode), E_{corr}. E_{corr} can be defined as the potential where the oxidation rate is exactly equal in magnitude to the reduction rate when measured with an electrometer.

Tuovinen and Cragnolino (1986) used open circuit potential measurements to study corrosion caused by microorganisms. This technique can be used as an indicator of the development of active corrosion since it only yields the qualitative information about the corrosion process, and the potential is measured on the basis of a standard reference

electrode with a long time stable performance. In open circuit potential measurements, it is considered that the rises in potential of the steel may result from the low redox-potential, which is produced by the bacteria in their initial development. The drop of potential to a more noble state may be the indication of the formation of protective iron sulfide film (Hadley, 1943; Wanklyn and Spruit, 1952), as shown in Figure 4.

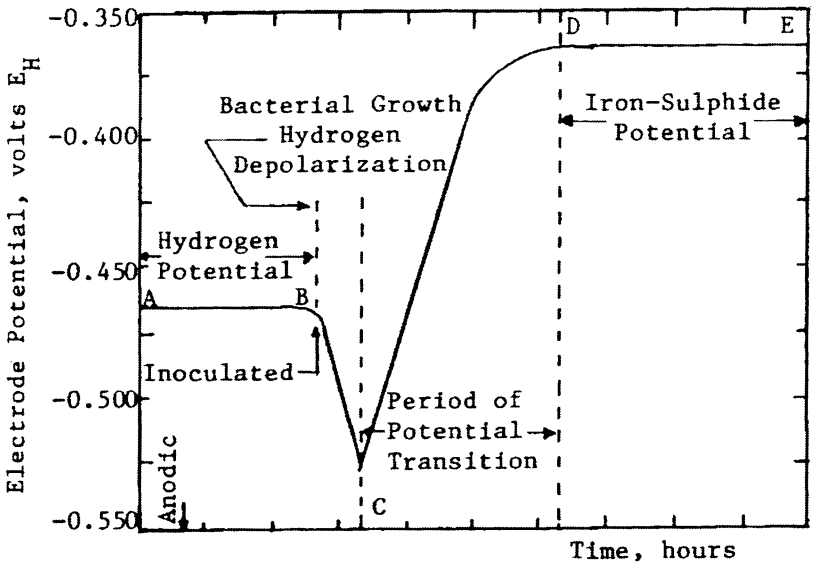


Figure 4. Potential-time curve of mild steel in the presence of SRB (Hadley, 1943).

2.6.2.2 Potentiodynamic polarization measurement

The potentiodynamic scan include polarizing the electrode probes with a linear voltage ramp within a wide potential range (i.e., -2V to +2V relative to a reference electrode) and monitoring the current response. The polarization plots from this measurement have various shapes corresponding to different forms of corrosion behavior of the materials (Wagner and Traud, 1938). Using the method of potentiodynamic scan,

the corrosion rate can be also obtained based on the mixed potential theory, which regards the anodic and cathodic polarizations as two complete independent processes (Wagner and Traud, 1938). The Tafel plot is used to determine corrosion current density, I_{corr}, at which the corrosion rate is calculated. To obtain the value of I_{corr}, the linear portion of the Tafel plot can be extrapolated to corrosion potential E_{corr}, whereby the correspondent value of corrosion current density from the intersection of E_{corr} is I_{corr}, as shown in Figure 5 (Obuekwe et al., 1981).

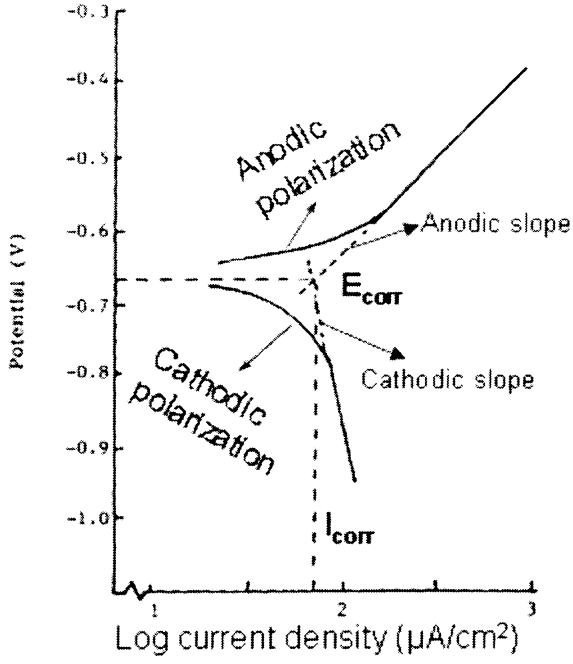


Figure 5. Tafel plot from data obtained during polarization of mild steel after Hill et al. (1987).

Another advantage of potentiodynamic scan is determining the pitting potential E_p, which is important in localized corrosion caused by SRB, by investigating the anodic

polarization curves. Pitting potential E_p is the potential value where the current density begins to increase substantially in the passive range. Figure 6 shows the behavior of stainless steel through anodic polarization curves in seawater contaminated with SRB and the pitting potential was determined (Erauzkin, 1988).

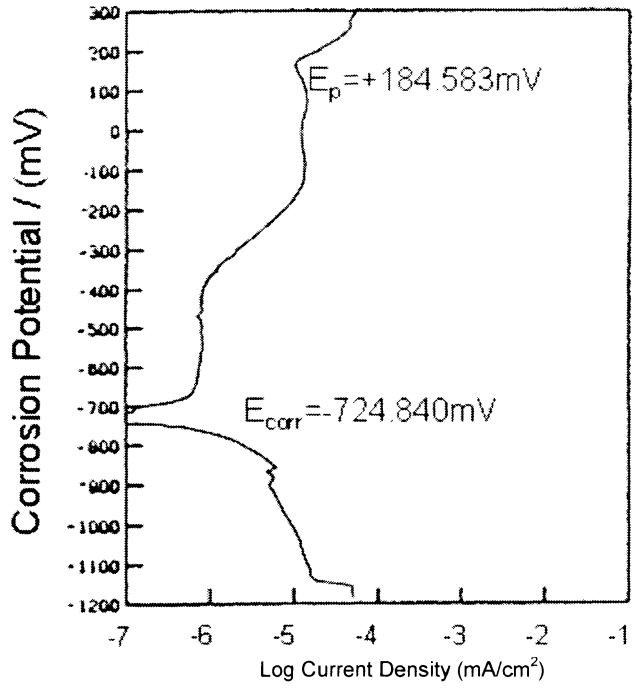


Figure 6. Anodic polarization curves of stainless steel in artificial seawater contaminated with SRB after Erauzkin (1988).

2.6.2.3 Linear polarization resistance (LPR) measurement

The measurement of linear polarization resistance (LPR) is a technique that uses the slope at the origin of the straight line obtained by polarizing the electrode probe in a

small range of potential (not more than ±10mV), and the value corrosion potential E_{corr} should be included in this potential range (Stern and Geary, 1957). Compared to non-electrochemical techniques, this measurement has many advantages because it can be implemented with simple, easy operating instruments to obtain the polarization resistance, R_p, and the corrosion rate, CR, relatively quickly (2-10 minutes). It can also avoid damaging the electrode probe and changing dramatically the properties of the corroding system by excessive polarization (Sequeira, 1988). However, it must also be noticed that the experimental value of polarization resistance, R_p, can be influenced by the Ohmic resistance from surface layers, or electrolyte layer between the test and reference electrode. An underestimation of corrosion rate could be obtained due to the uncompensated Ohmic resistance (Mansfeld, 1976). Another disadvantage of the LPR technique is that the Tafel slope constants need to be assumed *a priori*. This may be problematic when the mechanism is unknown. In order to avoid assuming Tafel constants, potentiodynamic sweep technique can be incorporated to determine the Tafel slope.

2.6.3 Detection devices

The direct optical microscopic examination of the samples continues to be the simplest, quickest and most widely used method to determine the presence and quantification of bacteria. However, it is not possible to distinguish different kinds of bacteria by this method alone. Recently, phase-contrast, fluorescence and confocal laser

microscopes were successfully employed in the identification of microorganisms (Caldwell et al., 1992; Walker and Keevil, 1994). If necessary, stain solutions such as methylene blue, crystal violet, safranin or fluorescent dyes (i.e. acridine orange) could be used to help the cells' identifications (Chamberlain et al., 1988; Schaule et al., 1993). The SEM technique is the most widely employed to study the morphology of microorganisms, the distribution of the colonies on the metal surface, as well as the presence of EPS (Beech and Gaylarde, 1999). Atomic Force Microscope (AFM) is more useful in studying biocorrosion because it can provide more information about the progression of pitting in coupons. The length, width and depth of the pits can be determined by AFM. In this way, corroded volume of the coupon can be calculated (Xu et al.; 1999).

The chemistry of corrosion products (crystalline or amorphous), thought to be related to microbial activities, on the metal surface can be revealed by chemical spectroscopy. X-ray diffraction (XRD), energy dispersive X-ray analysis (EDAX), Electron Dispersion Spectroscopy (EDS) have been commonly used to obtain chemical information on corrosion products (Marquis, 1988). These techniques provide information on the chemical composition of the corrosion products and microbial deposits, which helps us to gain insight into the possible electrochemical reactions involved in MIC corrosion process.

2.7 Mitigation of MIC

Mitigation of MIC is widely considered to be difficult and expensive. The ideal protection against MIC would be a clean system without any bacteria (Edyvean and

Videla, 1991). The application of organic biocides and biostats is the most commonly adopted treatment in order to achieve microbiological control of microbial activity in the oil and gas industry. The organic biocides include glutaraldehyde, quaternary ammonium compounds, formaldehyde, acrolein, amine and diamine. The selection of biocides must carefully follow prescribed criteria and those chosen must be specifically relevant to the bacteria, design and operation of the system in order to kill the planktonic and sessile bacteria (biofilms) or retard bacteria growth. The concentration of biocides should be minimized, and the contact time should not be too long for achieving the effective control of microbial activities (Sanders, 1988). While the application of biocides is successful in some situations, there are increasing environmental concerns raised by their uses. Microorganisms are also capable of developing resistance to biocides after prolonged uses. Coating and cathodic protection methods are also used to combat microbial corrosion (Videla, 1996). However, such measurements are usually not feasible for applications in such cases as the oil and gas industry.

Another method to mitigate microbial activities of SRB is the addition of nitrate and/or nitrite and introduction of nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) such as *Thiobacillus denitrificans* in the injection water that had been demonstrated to inhibit biogenic H_2S production (Jenneman et al., 1986, 1997; McInerney et al., 1992, 1996). Montgomery et al. (1990) proposed that a sulfide-resistant strain of *Thiobacillus denitrificans*, strain F could be effective in controlling sulfide production by SRB when both organisms were grown in the co-culture. Several explanations have been proposed for the reduction of H_2S production after nitrate injection in oil fields. The control

mechanism could be using the sulfide resistant nitrate-reducing bacteria to outcompete SRB for electron donors and using nitrate as an electron acceptor (Dalsgaard and Bak, 1994; Sandbeck and Hizman, 1995). Furthermore, the metabolic activities of nitrate-reducing bacteria in the presence of sufficient nitrate could remove H_2S present in the system by the reaction (Gevertz et al., 2000): sulfide + nitrate → sulphur + nitrite. This treatment could be effective in controlling SRB activities; however, biofouling cannot be controlled. Furthermore, NR-SOB was also reported to cause MIC in recent investigations (Voordouw et al., 2002). Therefore, methods with higher efficiency and fewer environmental damages and possibly reduced costs need to be developed.

Chapter 3 Research Objectives and Test Matrices

3.1 Research objectives

Based on the published literature, it is necessary to mitigate MIC due to SRB from a different perspective using biochemical engineering approaches. Several parameters that influence SRB growth were investigated in a pure culture of a strain of SRB. The principle questions that were addressed in this work included:

1) How the key sulfate nutrient species affect SRB growth and corrosion of steel?
2) How the interactions between the biofilm and iron sulfide films influence the corrosion process?
3) Can microcarriers for cell immobilization be used to help inhibit SRB biofilm formation on the metal surface and thus reduce the corrosion rate?
4) Are there synergistic effects of glutaraldehyde and EDTA (a common chelator) combination on the growth of SRB and the corrosion rate of steel?
5) What are the necessary components in solid media for SRB growth? And, what the appropriate experimental conditions are to achieve a rapid growth of SRB on solid media. This leads to the development of a more efficient solid medium for quantification and analysis of sessile SRB cells.

3.2 Test matrices

3.2.1 Test conditions

In the current study, *Desulfovibrio desulfuricans* ATCC strain 7757 was used. Experiments were carried out both in specially sealed vials and in glass cells with a special rotating cylinder. The anaerobic condition was implemented by purging with filtered nitrogen. The medium for liquid culture was based on the ATCC 1249 medium (Modified Barr's Medium) for the growth of *Desulfovibrio desulfuricans* ATCC strain 7757 (Atlas and Parks, 1997). Modified Barr's Medium is a very commonly applied medium to cultivate SRB (Atlas and Parks, 1997). The composition of the medium is:

Component I: $MgSO_4$, 2.0g

Sodium Citrate, 5.0g

$CaSO_4$, 1.0g

NH_4Cl, 1.0g

Distilled water, 400ml

Component II: K_2HPO_4, 0.5g

Distilled water, 200ml

Component III: Sodium lactate, 3.5g

Yeast Extract, 1.0g

Distilled water, 400ml

(The pH of each component above is adjusted to 7.5 before autoclaving.)

Component IV: Filter-sterilize 5% ferrous ammonium sulfate $Fe(NH_4)_2(SO4)_2$, which is not autoclavable. 0.1ml of this solution is added to 5.0ml of medium prior to inoculation.

It must be noticed that in this medium, ferrous ammonium sulfate requires filter sterilization because it is heat sensitive and thus cannot be autoclaved, making the procedure of medium sterilization inconvenient. For this reason, Ferrous sulfate plus ammonium sulfate were used to replace ferrous ammonium sulfate $Fe(NH_4)(SO_4)_2$. The replacement would produce the same ionic strength in the solution. A joint effort with Jhobalia (2004) led to the finding that the use of ferrous sulfate plus ammonium sulfate, in terms of cell growth and corrosion rate, was similar between the original medium and the replacement. Hence, in all experiments carried out in this work, ferrous sulfate and ammonium sulfate were used in equivalent molar amounts of ferrous, ammonium and sulfate ions in $Fe(NH_4)(SO_4)_2$. Different ferrous ion concentrations were obtained by changing the amount of $FeSO_4$ added into the medium. $NaSO_4$ was added to the medium to achieve the concentrations of sulfate ion as required.

Modified component IV: $FeSO_4$, 2.1g

$(NH_4)_2SO_4$, 1.0g

Distilled water, 30ml

(10ml of this solution is added to 1000ml of medium prior to inoculation.)

UNC C1018 mild steel coupons were tested in the experiments. The compositions of the coupon were provided in Table 1. Corrosion rates were obtained using the conventional weight loss and the linear polarization resistance (LPR) methods.

Table 1. Composition of UNS C1018 mild steel coupons used in experiments

Element	Wt %	Element	Wt %	Element	Wt %
Al	0.066	Ca	0.0004	Mn	0.84
Ni	0.03	Sb	0.009	Ti	0.002
As	0.01	Co	0.007	Mo	0.028
P	0.016	Si	0.036	V	0.002
B	0.0009	Cr	0.052	Nb	0.012
Pb	0.036	Sn	0.005	Zr	0.006
C	0.2	Cu	0.02	Fe	balance
S	0.009	Ta	0.005		

The morphology of the coupon surface was analyzed using the Scanning Electron Microscope (SEM) and the Electron Dispersion Spectroscopy (EDS) methods. Planktonic SRB cell numbers were counted under an optical microscope using a hemocytometer with serial dilutions (Penn, 1991).

To investigate the SRB growth on a solid surface, different media were tested. The medium compositions in one liter distilled water are:

(1) Medium 1: 50g Wort Agar, 1g Yeast Extract.

(2) Medium 2: 50g Wort Agar, 1g Yeast Extract, 5g Salts (Sodium chloride).

(3) Medium 3: 50g Wort Agar, 1g Yeast Extract, 5g Salts (Sodium chloride), 2g Magnesium sulfate ($MgSO_4$), 5g Sodium citrate, 3.5g Sodium lactate.

3.2.2 Test Matrices

The experiments were carried out in an order following the experimental plan, as shown in Tables 2 and 3.

Table 2. Test matrix for SRB growth in liquid media

Test strain	*Desulfovibrio desulfuricans* (ATCC 7757)
Test medium	Modified ATCC 1249 medium
Coupon Material	UNS C1018 mild steel
Temperature (°C)	37
pH	7.0±0.1
Total initial Fe^{2+} concentration (ppm)	0, 25, 50, 100, 143
Total sulfate concentration (g/l)	1.93, 2.5, 3.5, 4.5, 6.5,
Celite beads concentration (weight/volume)	5g/100ml
Glutaraldehyde concentration (ppm)	0, 50, 100, 250, 500, 1000, 2000
Glutaraldehyde introduction	At inoculation time, 1 day later
EDTA concentrations (ppm)	200, 500, 1000, 2000
Combination of Glutaraldehyde and EDTA concentrations (ppm)	Glutaraldehyde: 250 EDTA: 200, 500, 1000, 2000
Experimental setup	Anaerobic vials, glass cells

Table 3. Test matrix for SRB growth on solid media

Test strain	*Desulfovibrio desulfuricans* (ATCC 7757)
Test media	Medium 1, medium 2, medium 3
Temperature (°C)	37, 25
pH	7.0±0.1, 4.8±0.1, 4.9±0.1, 5.8±0.1
Atmosphere	Pure N_2, 30%H_2+70%N_2, air
SRB inoculum source	Planktonic, biofilm

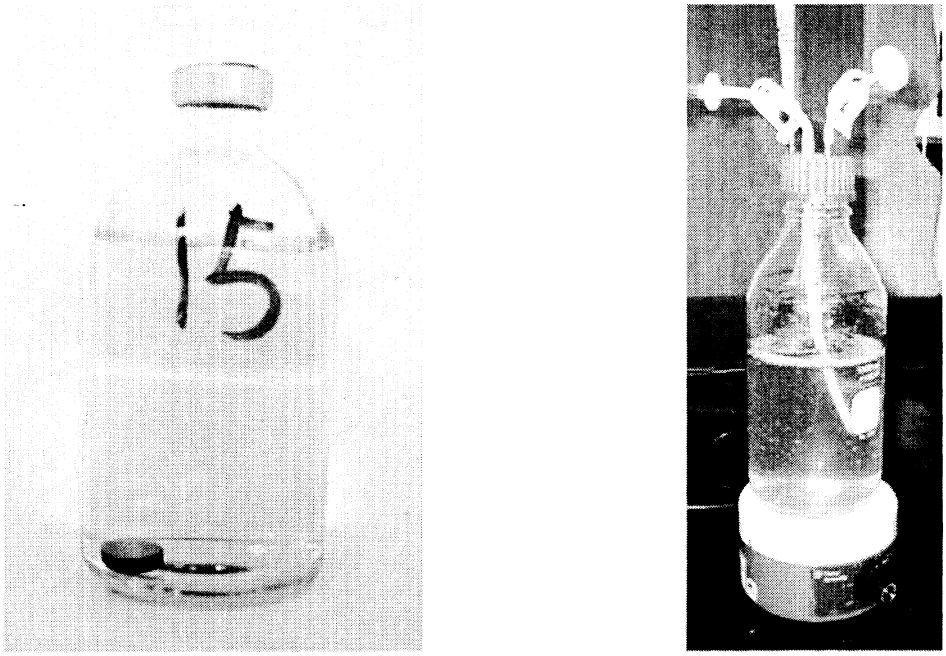
Chapter 4 Experimental Setup

The experiments were performed in sealed anaerobic vials and special glass cells, each with a rotating cylinder. The anaerobic condition was maintained by purging filtered nitrogen in all the experiments. Special care was taken at all times to avoid any microbial contamination.

4.1 Anaerobic vial experiments

Before the experiments, all the devices including flasks, medium bottles, vials, caps needles, syringes and other items involved in the experiments were first cleaned and sterilized. The medium (modified ATCC 1249 medium) for growing SRB cells was prepared according to the formulation described in Chapter 3 using deoxygenated distilled water. After mixing each component in the solution, the flask was covered with a sponge and aluminum foil. They were then autoclaved at 121°C for 15 minutes followed by an exhaust cycle of 20 minutes. After sterilization, all the medium components were transferred to a laminar flow hood, which had been pre-sterilized by UV light. When the components were cooled, they were transferred to a sterilized deoxygenation bottle. Filtered nitrogen was then bubbled through the medium for approximately 30 minutes to remove dissolved oxygen in the liquid. During this procedure, the medium was continuously stirred. This procedure took place while the medium was still warm enough to remove as much oxygen as possible. The medium was then transferred to the anaerobic

chamber (glove box) with a clean nitrogen environment where inoculation took place. After distributing the medium in the vials to the desired volumes (50ml in each vial without a coupon; 100ml in each vial with a coupon), each vial was inoculated with SRB broth from a one-week old culture. The inoculum to medium volume ratio was 1%. Polished, cleaned and de-greased metal coupons (sterilized with ethanol) were placed in the vials which were then sealed and placed in an oven of 37°C. Figure 7 shows a vial and a deoxygenation bottle used in this work. A hemocytometer counting chamber was employed to observe and count cells from the planktonic sample under an optical microscope at 400X magnification.



(a) (b)

Figure 7. Devices for experiments in anaerobic vials.
(a) Anaerobic vial, and (b) deoxygenation bottle.

The metal coupons used in the anaerobic vials were common 1018 mild carbon steel. They were disk shaped like a small coin with a thickness of 3.0mm and a diameter of 11.5mm. The coupons were polished with 400 grit Si-C sand paper, rinsed with ethanol, and then coated with Teflon leaving only the top disk surface exposed. To get rid of all moisture and volatile substances from the coated coupons, after the Teflon dried overnight, the coated coupons were heated in an oven at 200°F first, and then the temperature was increased by 50°F every 30 minutes. When the temperature reached 350 °F, the coupons were cooked for 2 more hours and then allowed to cool in the oven. The exposed surface of Teflon coupons were then polished again with 400 and 600 grit papers, respectively. The coupons were rinsed with ethanol and subjected to ultrasonic bursts for 15 seconds to remove all forms of dirt and grease on the coupon surfaces. The coupons were then weighed on a balance scale to obtain the initial weight. After this, they were immediately transferred to a desiccator and the desiccator was vacuumed to stay ready for the experiments.

At the end of the experiments typically lasting for one week, the coupons were taken out and cleaned with ethanol and bursts of ultrasonication. To study the biofilm on the coupon surface, sterilized deoxygenated distilled water was used in placing of alcohol to wash the coupon surface. To remove the FeS film from the coupon surface, Clarke's Solution (Haynes and Baboian, 1983) was used. The coupons were then reweighed to obtain the loss in weight.

4.2 Glass cell experiments

The schematic of an electrochemical glass cell is shown in Figure 8. The potentials were measured against a Saturated Calomel Reference Electrode (Ag/AgCl), which was connected to the cell via a Luggin capillary and a porous wooden plug. A concentric platinum ring was used as a counter electrode. The reference electrolyte was 1M KCl solution at pH 7.

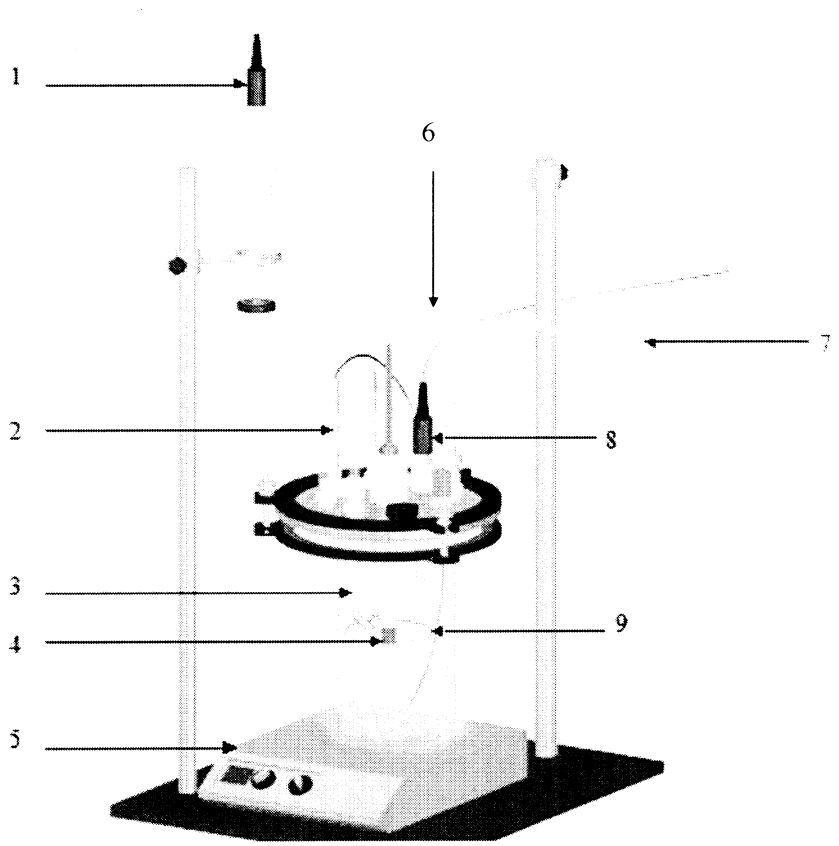


Figure 8. Schematic of an electrochemical glass cell: 1. Reference electrode; 2. Temperature probe; 3. Luggin capillary; 4. Working electrode; 5. Hot plate; 6. Gas output; 7. Bubbler for gas; 8. pH electrode; 9. Counter electrode. (Figure was drawn by Danniel Mossier at Ohio University, 2004.)

The glass cell and all the accessories including the fittings were sterilized in autoclave before the start of experiment. The pH probe was cleaned and sterilized using hydrochloric acid and 70% ethanol. The preparation of medium was the same as described earlier. After the medium was cooled down, it was transferred to the glass cell aseptically. The entire setup of glass cell was made in a laminar flow hood to avoid any contamination. Then the glass cell was placed on the hot plate and fastened. The temperature of the liquid in the glass cell was maintained constant at 37°C on a hot plate.

Cylindrical coupons of 1018 mild carbon steel with diameter 1.20cm and exposed surface area 5.40cm^2, were used in these experiments. The coupons were polished and cleaned following the same procedure for disk coupons. The deoxygenation of the medium was achieved by purging filtered nitrogen through a gas bubbler inserted into the medium. After purging nitrogen gas through medium for 45 minutes, the shaft with the working electrode mounted (the cylindrical mild steel coupon) was introduced into the glass cell.

Electrochemical measurements of the linear polarization resistance (LPR) method and the potentiodynamic polarization method were performed using a Gamry PC4 (http://www.gamry.com) monitoring system controlled by the computer. The polarization resistance, R_p, is defined according Equation (2.9), as the tangent of the polarization curve at the corrosion potential under steady-state polarization conditions using low amplitudes of perturbation (Sequeira, 1998).

$$R_p = \left(\frac{dE}{dI}\right)_{a_i, E=E_{corr}} \tag{2.9}$$

In the charge-transfer reaction, I_{corr} is associated with R_p and can be determined through the well-known Stern-Geary equation (Stern and Geary, 1957):

$$I_{corr} = \frac{\beta_a \beta_c}{2.303(\beta_a + \beta_c)} \frac{1}{R_p} \frac{1}{A} \qquad (2.10)$$

where β_a = anodic Tafel slope (V/decade);
β_c = cathodic Tafel slope (V/decade);
I_{corr} = corrosion current density (A/m^2);
R_p = polarization resistance (Ohm);
A = exposed surface area (m^2).

To match with the corrosion rate from the weight loss method, in this work the value of β_a was adjusted to 0.16V/decade. The value of β_c was 0.12V/decade, the same as that found from experimental results (Geogre, 2003.). A is the exposed coupon surface area to the solution, which was 5.4cm^2 for cylindrical coupons tested in this work.

Once corrosion current density is obtained, the corrosion rate can be calculated according to the following equation (Sun, 2003):

$$CR = \frac{I_{corr} M_w}{\rho n F} = 1.16 I_{corr} \qquad (2.11)$$

where CR = corrosion rate (mm/yr);
M_w = molecular weight of iron;
I_{corr} = corrosion current density (A/m^2);
n = number of electrons transferred during the reaction;
F = Faraday's constant;
ρ = density of iron (kg/m^3).

4.3 Plating SRB on solid media

Isolated, pure colonies can be obtained by the streak-plate technique on solid medium surfaces. The devices used in the experiments were cleaned and sterilized first. The solid media for SRB growing were prepared according to the formulations described in Section 3 using distilled water. The pH was adjusted to the certain value before sterilization. The sterilization of the media in an autoclave was similar to that for experiments in anaerobic vials. After sterilization, all the media solutions were transferred immediately to a laminar flow hood, which had been pre-sterilized by UV light. When the medium was still as warm as 50°C, the liquid agar solution was poured carefully onto the bottom of Petri dishes. The liquid agar hardened 1 hour later, and then a loopful of stock solution containing SRB was spread over a small area at one edge of the plate in order to make an effective use of the agar surface. The loop rested gently on the surface of the agar and then was moved across the surface without digging into the agar. The two steps were repeated 2 or 3 more times to make sure that all the agar surface was streaked with SRB. This procedure of streaking (Prescott and Harley, 2002) was carried out in the laminar flow hood in order to avoid any contamination.

The inoculated plates were incubated in an inverted position (with the agar surface facing down) to prevent the water condensation falling onto the agar surface and contaminating or interfering with discrete colony formations. The plates were then placed in anaerobic jars where the atmosphere was replaced with nitrogen or the gas mixture of nitrogen and hydrogen. The jars containing the plates were inoculated in an oven at 37°C or at the room temperature (25°C).

For plating of SRB in a biofilm from the coupon surface, the coupon was first cleaned with distilled water to remove the substances from the coupon surface after it was taken out at the end of the experiment. Then the coupon was immersed in a 20ml vial, which holds 10ml distilled water. The biofilm was removed by sonication bursts for 60 seconds. This solution was then used as the sessile SRB sources. If necessary, a dilution of this solution was employed before plating to count the SRB colonies.

Chapter 5 Results and Discussions

The experiments were carried out according to the test matrices, discussed in Chapter 3. All experiments were performed following the procedure provided in Chapter 4. Most cultures lasted for 7 days for experiments in vials and 4-5 days for experiments in glass cells. Inoculations were performed by adding a small volume of a one-week old SRB culture. The inoculum volume was 1% of the liquid volume in the culture. The initial cell count right after inoculation was around 2×10^6 cells/ml. The samples were taken out for cell count at regular time intervals (every 24 hours) with sterile needles and syringes. The experiments were implemented and the explanations of results were given in the following order:

(1) Blank experiments without SRB in the medium.

(2) Experiments with various initial ferrous ion concentrations in the medium.

(3) Experiments with different initial sulfate ion concentrations in the medium.

(4) Experiments with Celite beads as microcarriers.

(5) Experiments for SRB growing on solid media to develop a new solid medium.

(6) Experiments with glutaraldehyde, biocide enhancer EDTA or the combination of glutaraldehyde and EDTA.

5.1 Blank results (without SRB)

The blank experiments without introducing SRB into the sterile medium were carried out in both anaerobic vials and glass cells. In this work, corrosion rate in mm/yr

was calculated based on the coupon weight loss that was translated into the corresponding coupon volume loss and then the coupon thickness loss. Because pits were distributed unevenly and sometimes took up only around 2~3% of the total coupon surface area, the highest local corrosion rate around a pit could be up to 50 times the average. Figure 9 shows the results of weight loss corrosion rate of coupons in vials, Figure 10 is the corrosion rate obtained with the LPR method, both of which indicate that the medium solution itself was not corrosive. Even in a medium with a high ferrous ion (143ppm or 100ppm) concentration, the corrosion rate of mild steel was very low (0.02mm/yr with Fe^{2+}143ppm) in the absence of SRB. However, the participation of SRB in the medium shifted the open circuit potential of the metal to a more negative value, which accelerated the propagation rate of corrosion of mild steel (Figures 11 and 12).

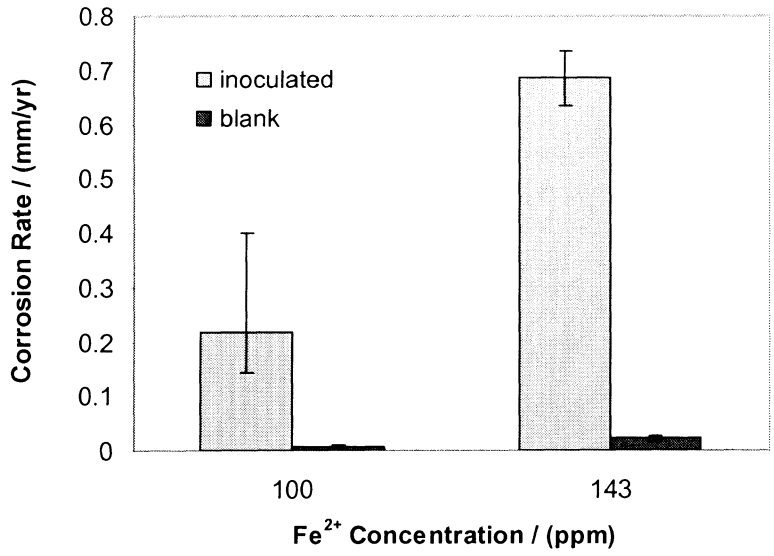


Figure 9. Comparison of weight loss corrosion rates in vials with and without SRB in the medium with high initial Fe^{2+} concentrations. Error bars represent the differences between the maximum and minimum corrosion rates. The culture medium volume in each vial was 100ml.

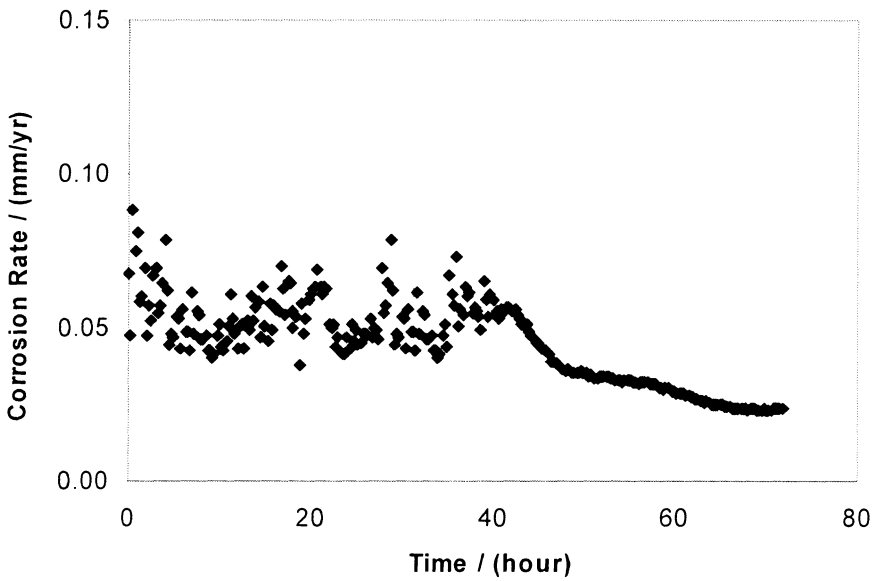


Figure 10. LPR corrosion rate in a glass cell without SRB containing an initial 25ppm Fe^{2+} concentration in the medium.

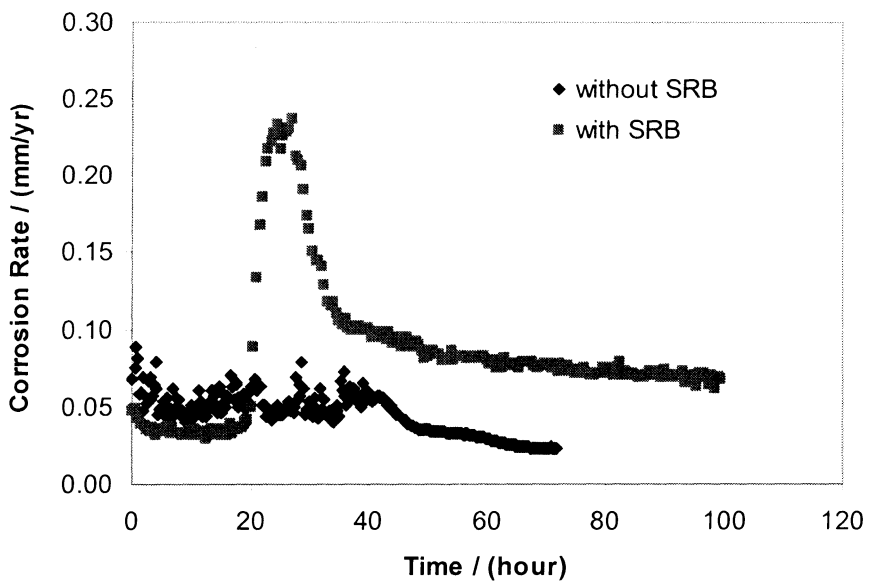


Figure 11. Comparison of LPR corrosion rates in glass cells with and without SRB in the medium containing 25ppm initial Fe^{2+} concentration.

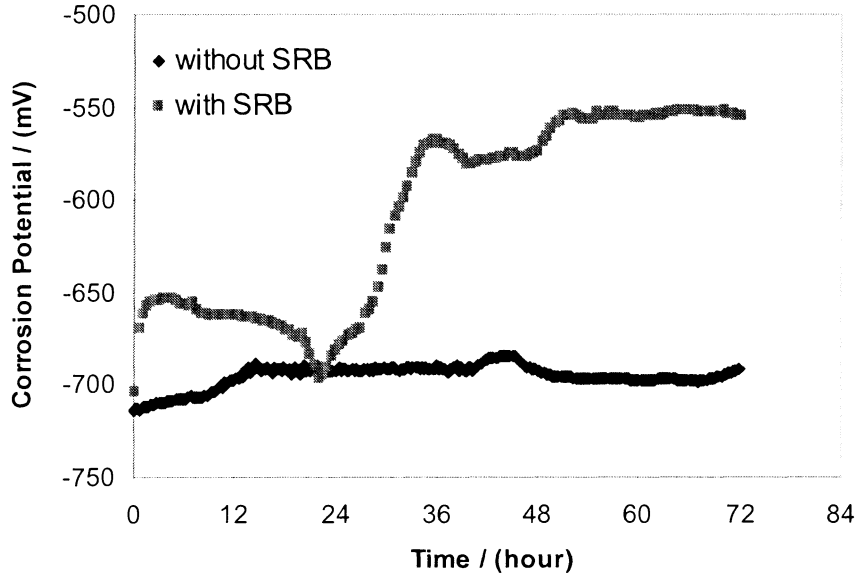


Figure 12. Comparison of corrosion potential with time in glass cells in the presence and also absence of SRB.

5.2 Effect of initial ferrous ion concentration

The experiments with a wide range of initial ferrous ion concentrations from 0ppm to 143ppm were conducted in anaerobic vials. Different initial ferrous ion concentrations (0ppm, 25ppm, 100ppm) were also studied in glass cells. SRB planktonic cell populations were counted with a hemocytometer and corrosion rates of mild steel were calculated from the experiments with various initial ferrous ion concentrations in the medium. Figure 13 shows the morphology of *Desulfovibrio desulfuricans* (ATCC 7757 strain), visualized at a high magnification. SRB cells are usually curved rod shaped with flagella. The diameter of them is less than 1μm.

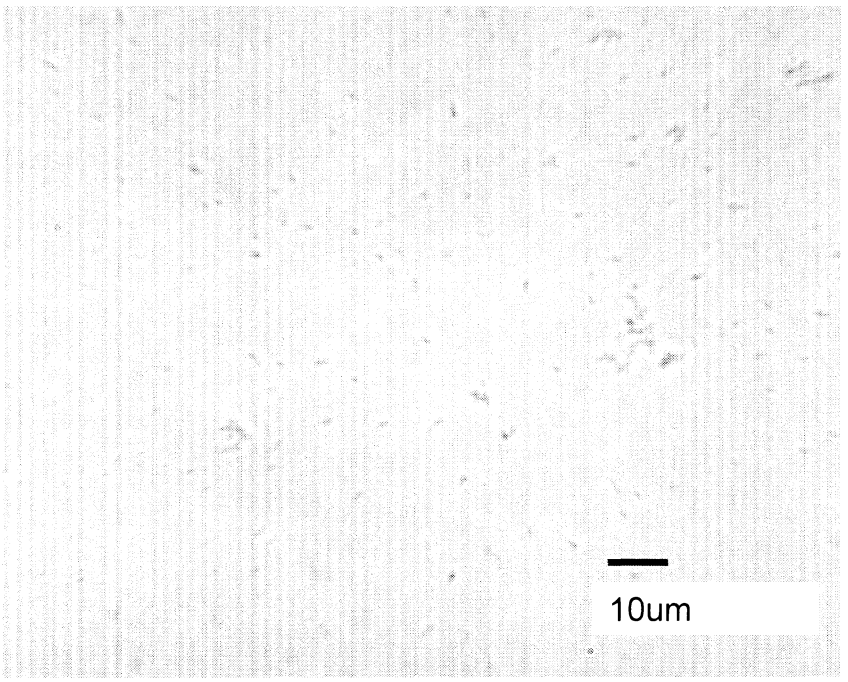


Figure 13. *Desulfovibrio desulfuricans* (ATCC 7757 strain) under epifluorescent microscope at 1000X magnification.

Figure 14 indicates that the ferrous ion (Fe^{2+}) concentration in the medium also influenced the cell growth pattern. When the concentration was higher than 50ppm, the cells propagated more quickly than with lower initial Fe^{2+} concentration. In an iron-rich medium (50ppm), cell numbers declined more quickly with time compared with that in a medium containing low ferrous ion concentration.

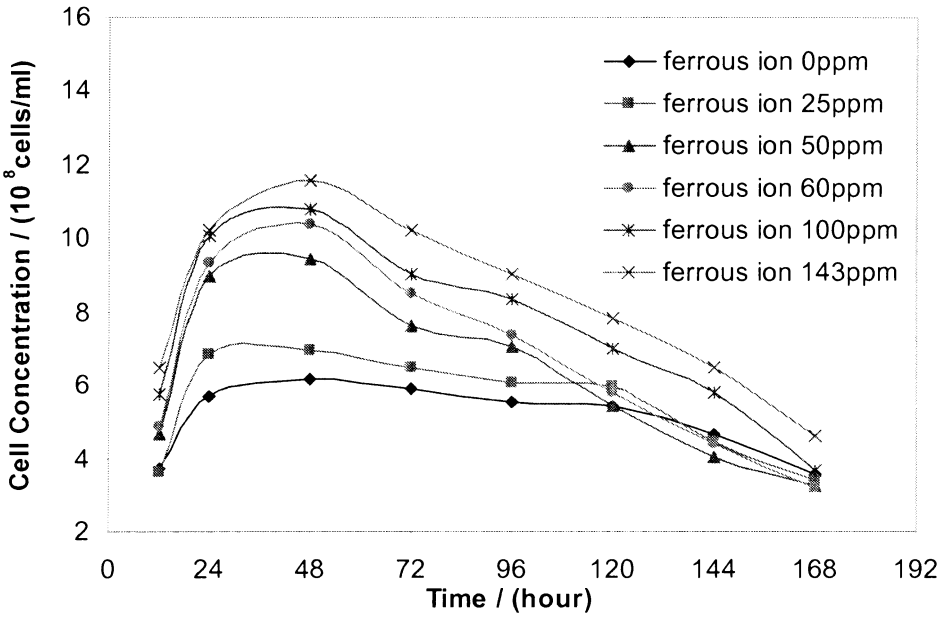


Figure 14. SRB growth rates with time at various initial ferrous ion concentrations in the medium.

The corrosion rates (Figures 15 to 17) from experiments both in vials and glass cells demonstrate that the ferrous ion concentration in the medium had a significant role in the biocorrosion process and in the determination of corrosion rate of mild steel. Figure 18 indicates the differences of corrosion potential between various ferrous ion concentrations in the medium. Figure 19 is a typical microscopic picture of the clean polished coupon surface before the experiments. After the experiments, there were some pits on the coupon surface, as shown in Figures 20 to 22.

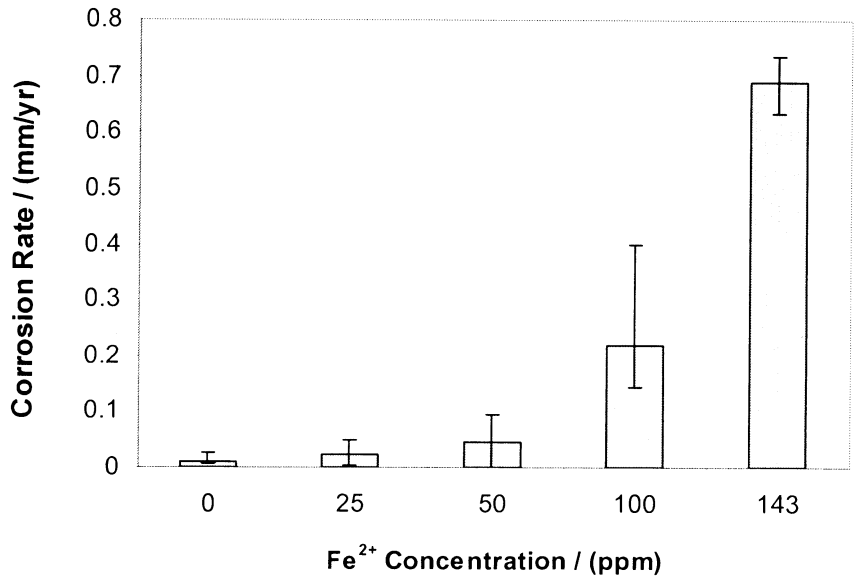


Figure 15. Weight loss corrosion rates containing different initial ferrous ion concentrations in the medium at 37°C for 1 week after inoculation. Error bars represent the differences between the maximum and minimum corrosion rates.

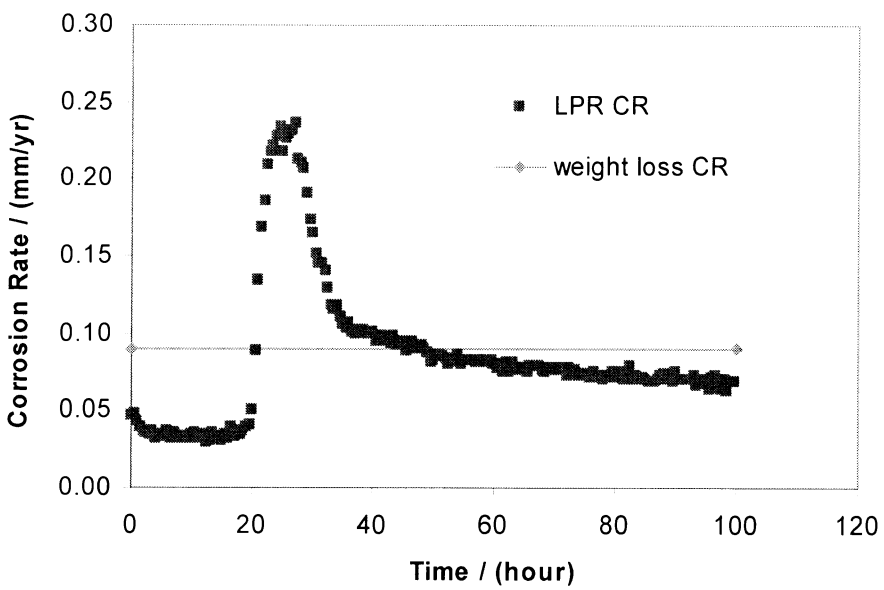


Figure 16. LPR corrosion rate (CR) in the medium containing 25ppm initial Fe^{2+} concentration.

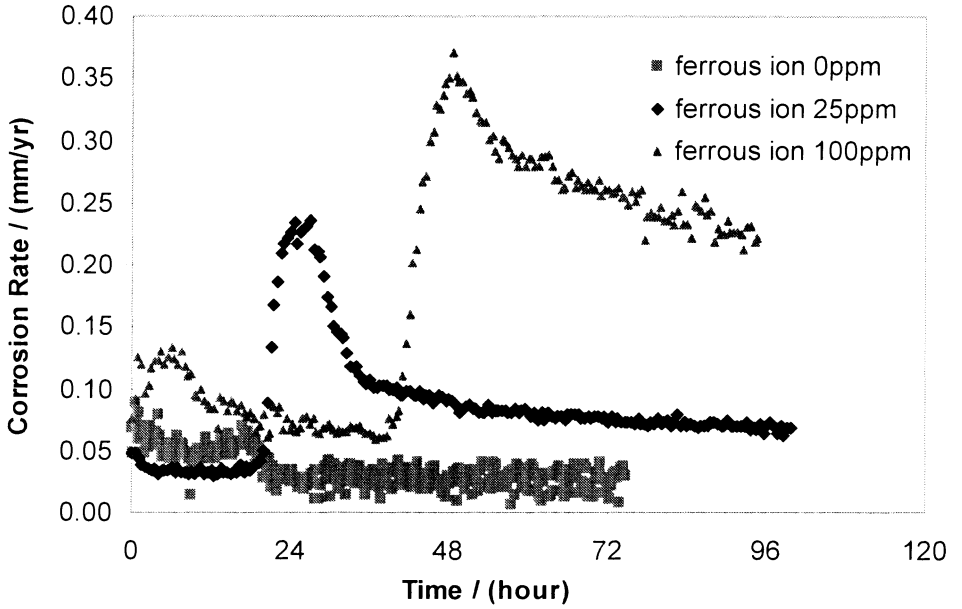


Figure 17. LPR corrosion rates at different initial ferrous ion concentrations.

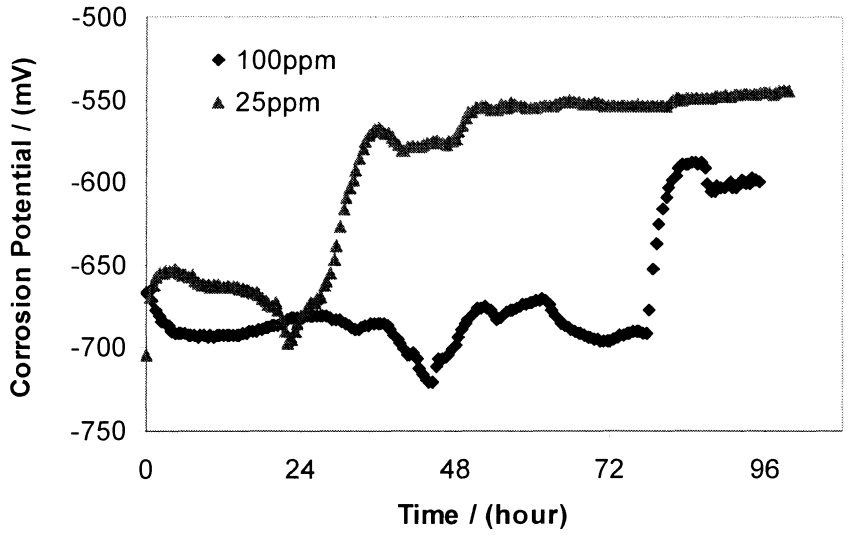


Figure 18. Corrosion potential at different initial Fe^{2+} concentrations in the medium.

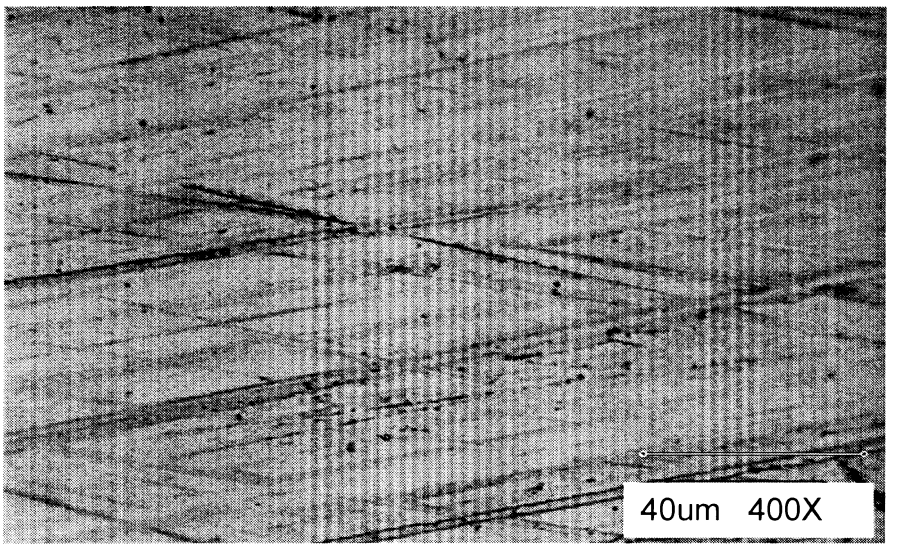


Figure 19. Cleaned disk coupon surface after polishing under a microscope at 400X magnification.

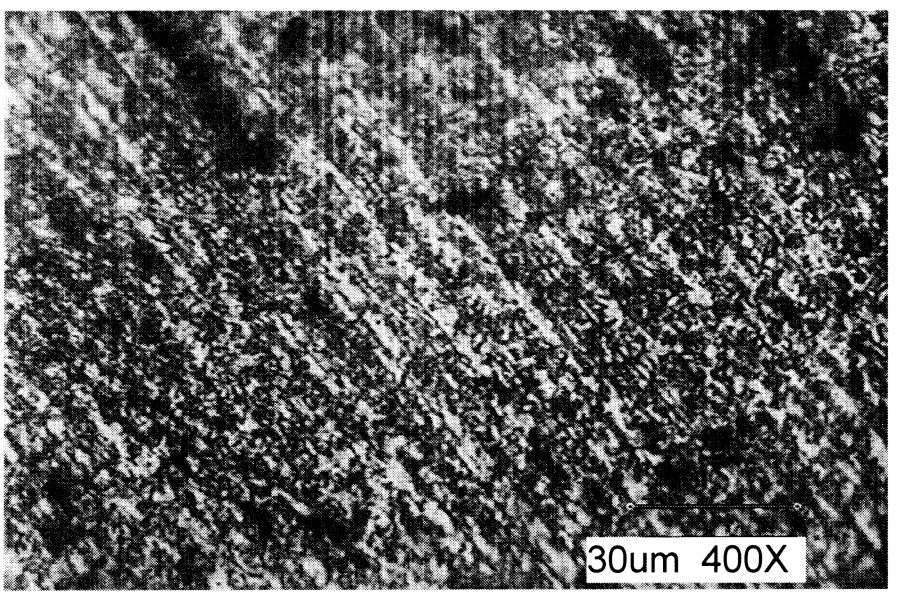


Figure 20. Disk coupon surface after cleaning at the end of the experiment in a medium containing 0ppm Fe^{2+} concentration under a microscope at 400X magnification

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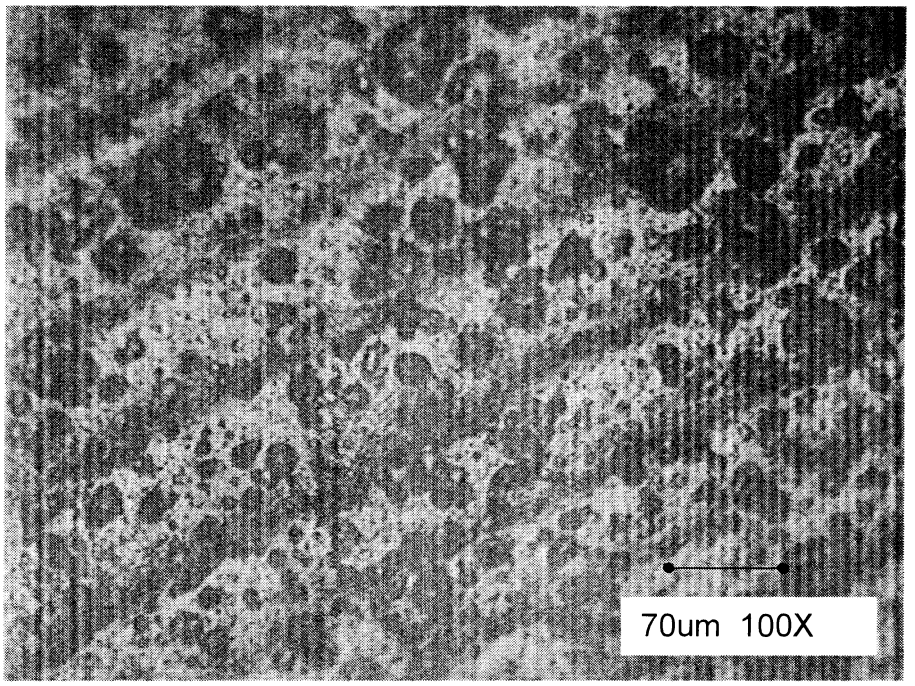

Figure 21. Microscopic picture of a cylindrical coupon surface after cleaning at the end of the experiment in a medium containing 25ppm initial Fe^{2+} concentration.

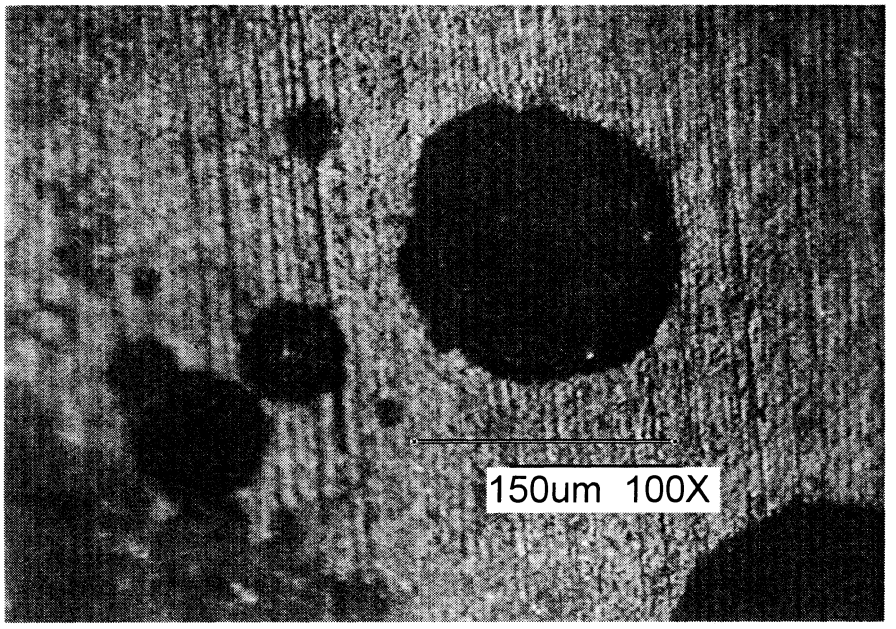

Figure 22. Cylindrical coupon surface after cleaning under a microscope after it was inoculated for 2 weeks in a medium containing 100ppm initial Fe^{2+} concentration.

The differences in the corrosion rate of mild steel due to the variation of ferrous ion concentration lie in the different physical forms of iron sulfides. It has been pointed out that in a solution containing a low iron concentration there was an adherent iron sulfide film on the mild steel surface, and the biofilm accumulation was followed on the adherent film (Mara and Williams, 1972). When the ferrous ion concentration in the medium increases to greater than 50ppm, the solution reaches super-saturation very quickly and the precipitation of iron sulfide ($Fe^{2+} + HS^- \Rightarrow FeS + H^+$) takes place. The super-saturation and precipitation process inhibit the protective film formation, or even break the film. Once loose iron sulfide particles penetrate through the protective iron sulfide film and contact with the mild steel surface, the corrosion rate increases greatly (King and Wakerley, 1973). Iron sulfide is semi-conductive and cathodic to the mild steel, therefore, with the biofilm on the mild steel surface the hydrogen sulfide is continuously produced, which keeps the iron sulfide cathodically active. In this situation where the area covered by biofilm acts as anode while the area covered by iron sulfide becomes cathode, metal corrosion is continuous and corrosion rate remains high.

In Figure 23, many pits can be seen on the coupon surface before surface cleaning under SEM observation. Figure 24 also shows the size of one pit on the coupon surface before surface cleaning. After the coupon was cleaned with distilled water and a series of ethanol washes to remove all the substances from the coupon surface, a big pit with a diameter of 50μm could still be seen on the coupon surface (Figure 25). The EDS scan shows that iron and sulfur were the most dominant elements on the metal surface (Figure 26), confirming the speculation that iron sulfides films covered the metal surface.

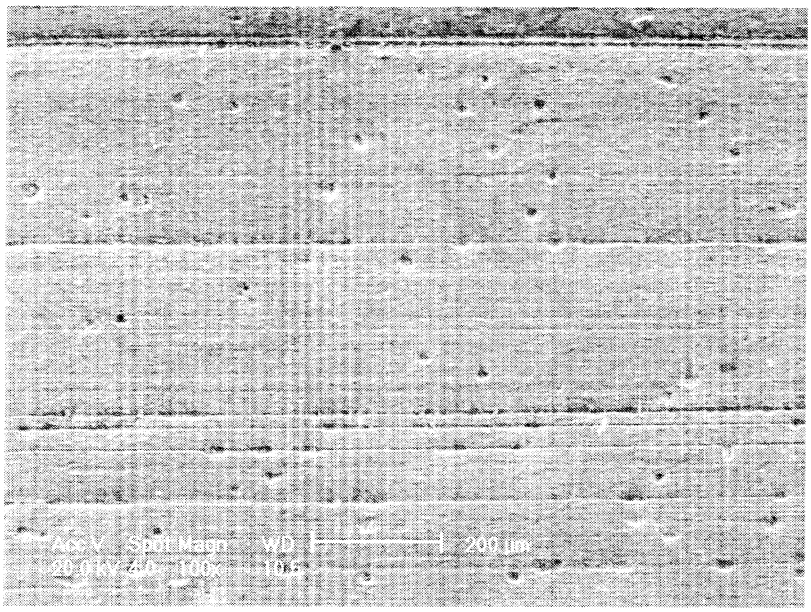

Figure 23. SEM picture of the coupon surface before coupon surface cleaning in a culture containing 100ppm initial Fe^{2+} concentration showing pits on the metal surface at 100X magnification.

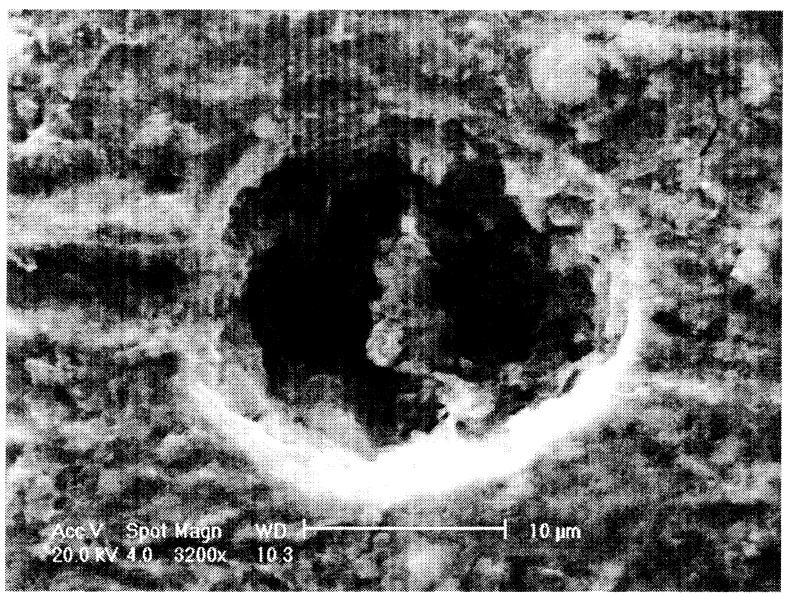

Figure 24. SEM picture of the pit on the coupon surface before coupon surface cleaning. The sample was treated with DI water wash and was kept in a desiccator before SEM examination. The coupon was from a 5-day old culture in the glass cell.

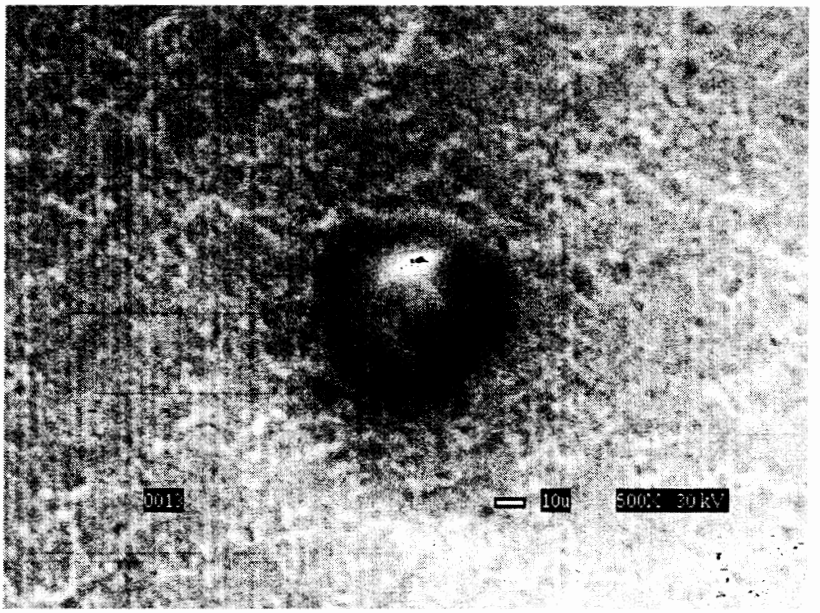

Figure 25. SEM picture showing the pit in the figure above on the coupon surface after surface cleaning.

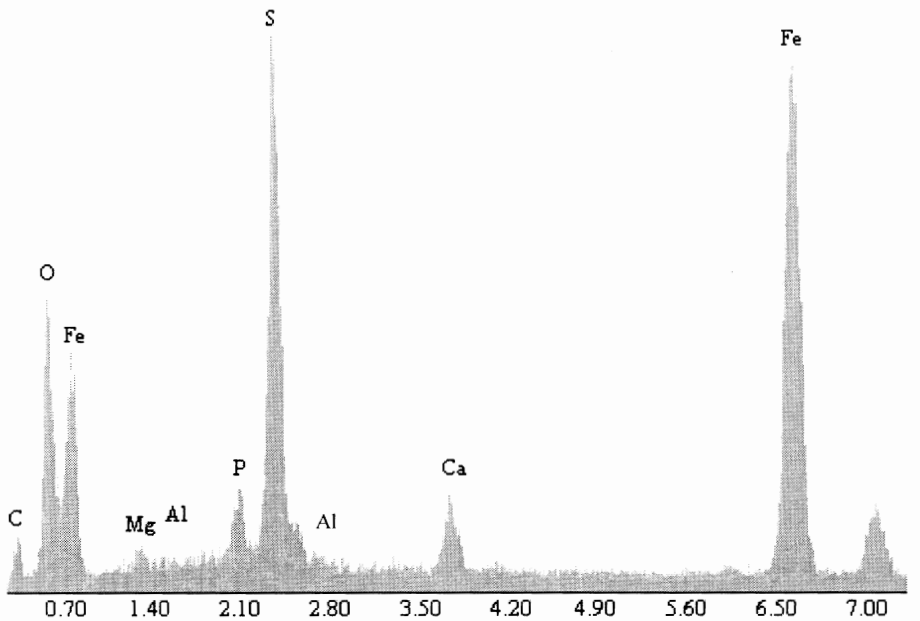

Figure 26. EDS scan of coupon surface showing the presence of sulfur and iron.

5.3 Effects of sulfate concentration

The effects of different initial sulfate ion (SO_4^{2-}) concentrations on SRB growth rate and corrosion of mild steel were performed in anaerobic vials. Figure 27 shows SRB growth rate curves at different initial sulfate concentrations in the medium. It indicates that sulfate reduction was decreased as the initial sulfate concentration increases within the range of 1.93g/l to 6.5g/l. This is thought to be due to the increasing toxicity of sulfates towards SRB metabolism or sulfate reduction (Mohanty, 2000). Figure 28 shows a lower corrosion rate was observed when the SRB growth was hindered.

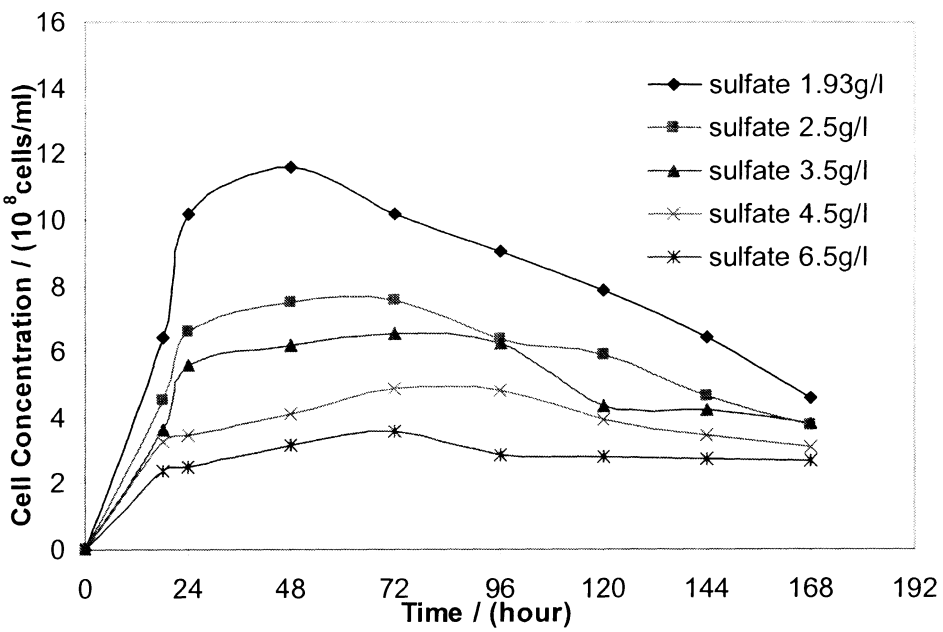

Figure 27. SRB growth rates with time at different initial sulfate concentrations in the medium from experiments in vials.

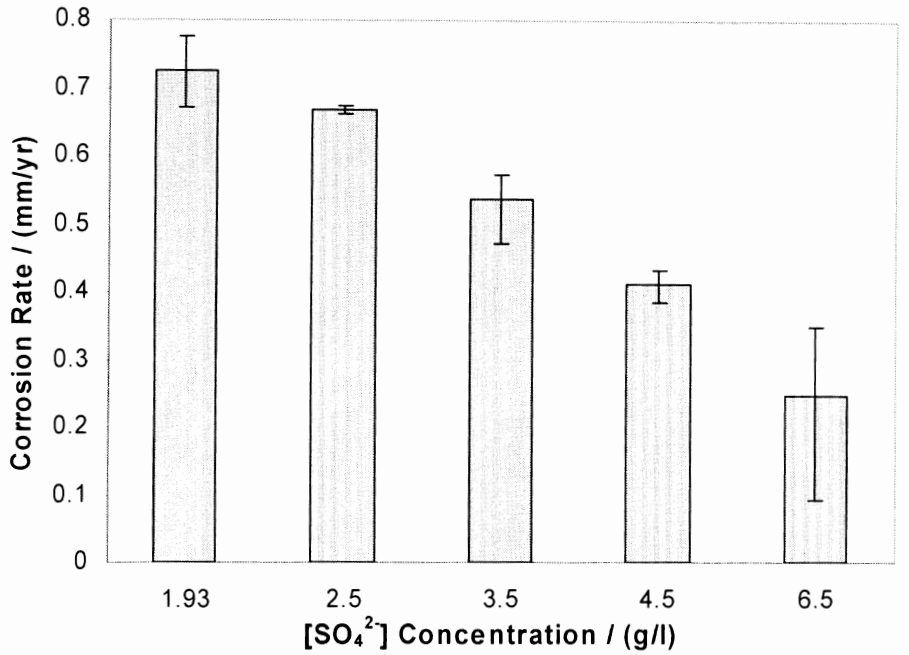

Figure 28. Weight loss corrosion rates with different initial sulfate concentrations in the medium at 37°C 1 week after inoculation from experiments in vials. Error bars represent the differences between the maximum and minimum corrosion rates.

5.4 Effect of Celite beads as microcarriers in the glass cell

SRB cell attachment plays an important role in the biofilm formation on the metal surface, as well as the corrosion process of mild steel. SRB corrosion inhibition using Celite beads as microcarriers to slow biofilm formation was tested in glass cells. Figure 29 shows that the planktonic SRB cell population (counted with a hemocytometer) could be lowered with Celite beads introduced into the medium. There was only a slight decrease in corrosion rate of mild steel, as shown in Figure 30. Further experiments are needed to improve the effect of using Celite beads as the cell immobilization support.

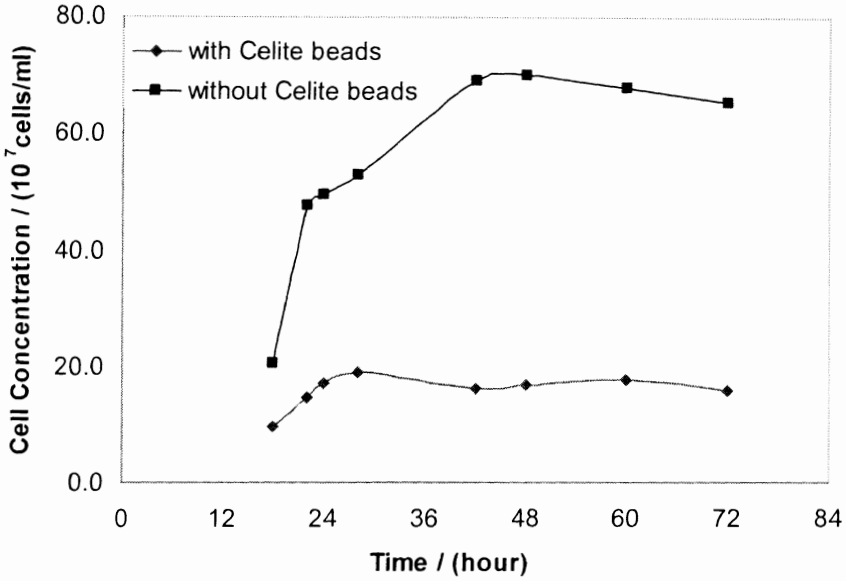

Figure 29. SRB planktonic cell growth rates with and without Celite beads in a medium containing 25ppm initial Fe^{2+} concentration in a glass cell.

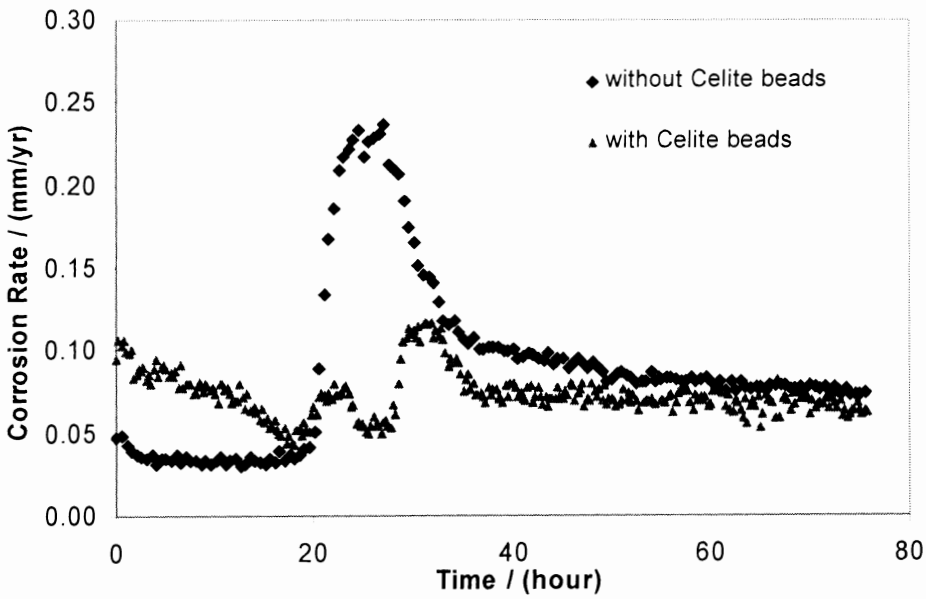

Figure 30. LPR corrosion rates with and without Celite beads in a medium containing 25ppm initial Fe^{2+} concentration in a glass cell.

5.5 SRB growth on solid media

Experimental results (Figures 31 to 33) indicate that Wort Agar plus Yeast Extract were sufficient to obtain a rapid and excellent growth of SRB. The medium compositions are given in Chapter 3. In media 1 and 2, the initial pH was important for SRB growth on the solid media. In medium 3, the introduction of other chemicals increased the solution pH to 5.8±0.1 and SRB colonies were found grown on this solid medium surface since SRB can grow within a wide pH range from 5.5 to 10.0. As for the experimental results in Figures 34 to 37, the pH value of the media was adjusted to 7.0±0.1. Figure 34 shows that SRB would grow rapidly on the surface of medium 1 at room temperature under pure N_2 atmosphere without any other added hydrogen donors such as sodium lactate or hydrogen gas. Figure 35 indicates that there was almost no difference in SRB growth on the plates under pure N_2 atmosphere or 30% H_2+70% N_2 atmosphere. Thus, H_2 is not necessary for the newly developed medium. Figure 36 also shows SRB would grow with similar results when exposed to sterile air atmosphere at room temperature, indicating that this SRB strain could have a strong oxygen tolerance. There results indicate that *Desulfovibrio desulfuricans* (ATCC 7757 strain) may be a facultative anaerobe instead of a strictly obligate anaerobe. At 37°C, excellent SRB growth could be obtained overnight. At room temperature (25°C), it took one extra day to achieve similar results on the agar surface. Figure 37 shows that the bacteria from biofilm could also grow very well on the newly developed medium, which facilitates the analysis and quantification of SRB cells in biofilms. It can also be used to select and preserve SRB cells.

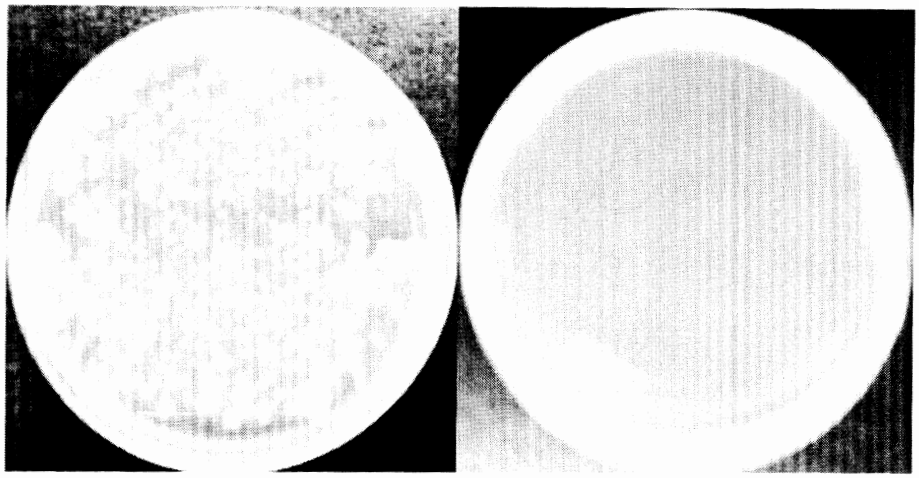

Figure 31. Comparison of the plates streaked with *Desulfovibrio desulfuricans* (ATCC 7757 strain) after 1 day at different pH on agar surface of medium 1 under pure N_2 atmosphere at 37°C. The initial pH of left plate was adjusted to 7.0±0.1 and the right 4.9±0.1.

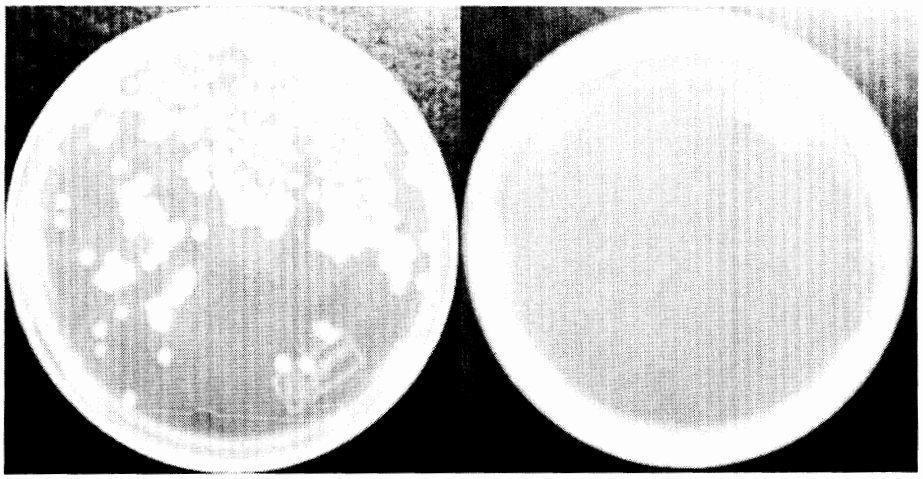

Figure 32. Comparison of the plates streaked with *Desulfovibrio desulfuricans* (ATCC 7757 strain) after 1 day at different pH on agar surface of medium 2 under pure N_2 atmosphere at 37°C. The initial pH of left plate was adjusted to 7.0±0.1 and the right 4.8±0.1.

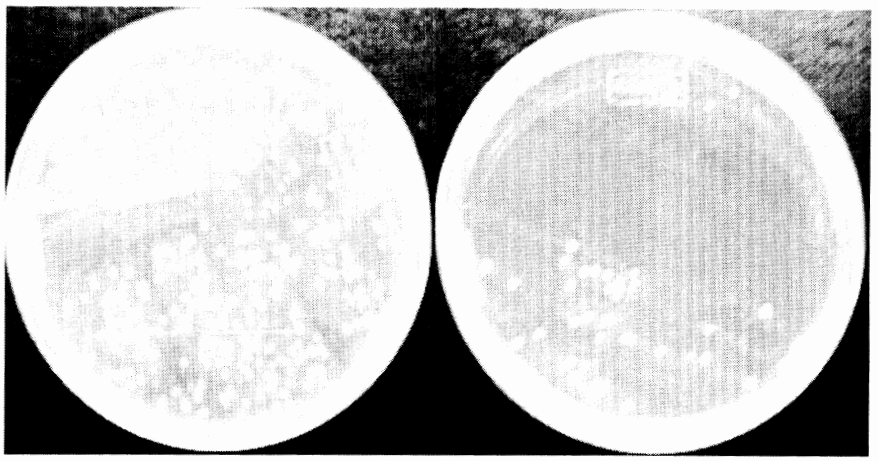

Figure 33. Comparison of the plates streaked with *Desulfovibrio desulfuricans* (ATCC 7757 strain) after 1 day at different pH on agar surface of medium 3 under pure N_2 atmosphere at 37°C. The initial pH of left plate was adjusted to 7.0±0.1 and the right 5.8±0.1.

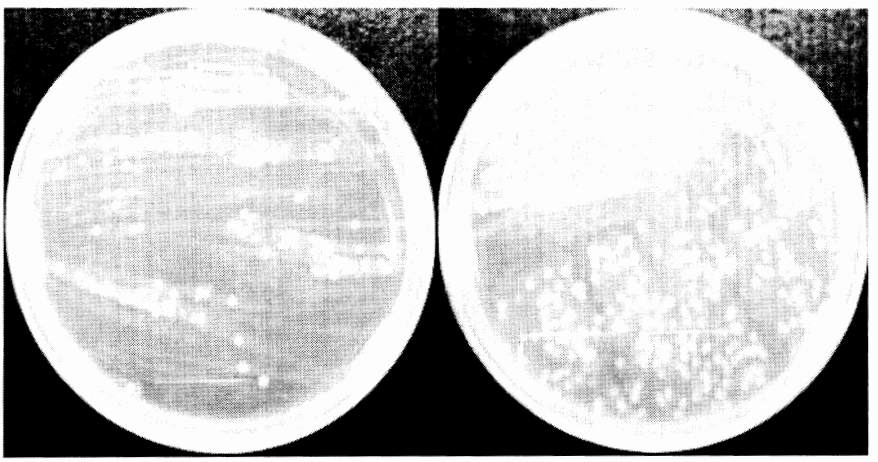

Figure 34. Comparison of SRB growth at different temperatures under pure N_2 atmosphere on the agar surface of medium 1. The left plate was at room temperature (25°C) after 2 days, and the right plate was at 37°C after 1day.

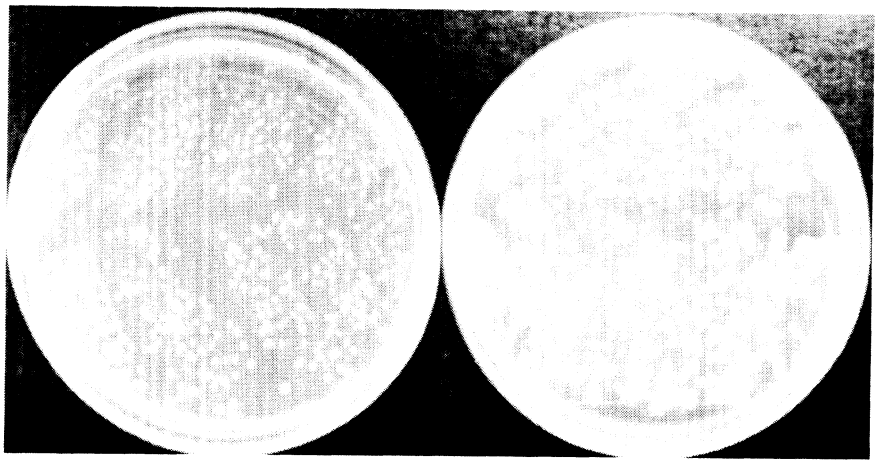

Figure 35. Comparison of SRB growth under different atmospheres on medium 1 at 37°C after 1 day. The left plate was under 30% H_2 +70% N_2 atmosphere, and the right plate was under pure N_2 atmosphere.

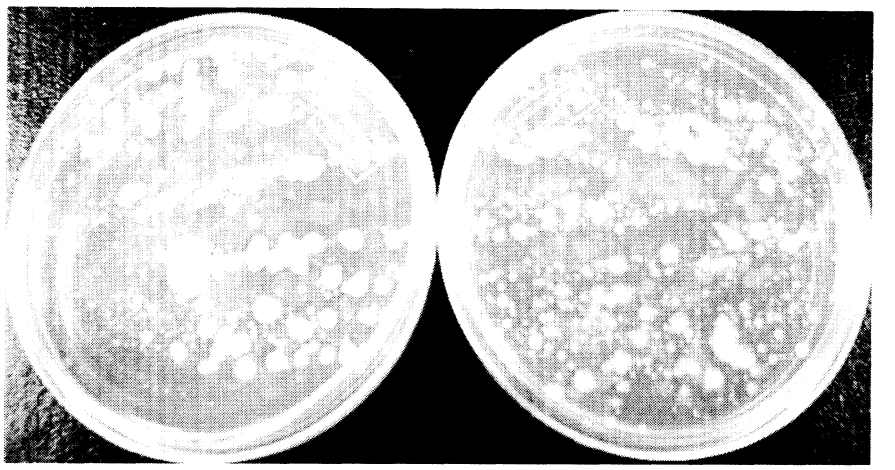

Figure 36. Comparison of SRB growth at different temperatures on medium 1 under sterile air atmosphere. The left plate was at 37°C after 1 day, and the right plate was at room temperature (25°C) after 2 days.

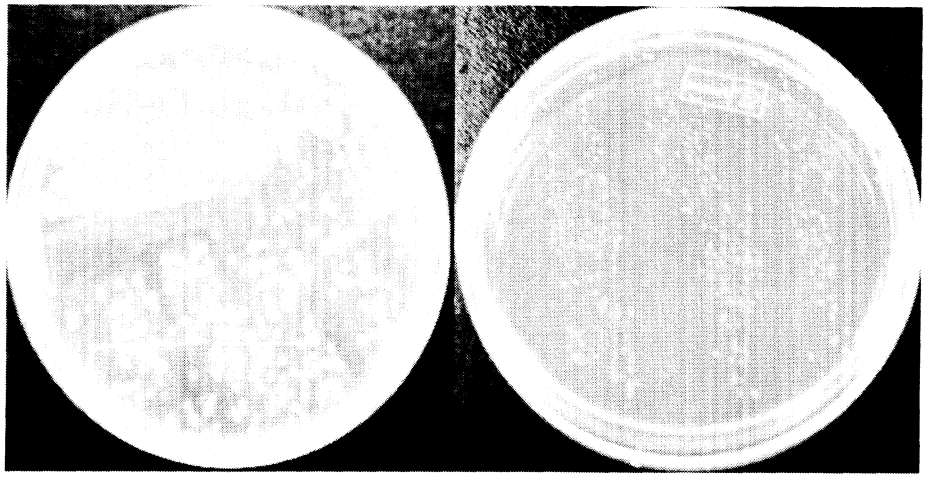

Figure 37. Comparison of SRB growth on medium 1 under N_2 atmosphere at 37°C from different inoculum sources after 1 day. The left plate was inoculated with a planktonic SRB solution, and the right plate was inoculated with a cell suspension obtained from a biofilm.

To confirm that the colonies on the plates are *Desulfovibrio desulfuricans*, a big round colony on the agar surface was used to inoculate the same liquid medium used for the experiments in vials at 37°C. It took relatively longer time to grow in the liquid medium possibly due to the extra time needed for cells to adapt to the new liquid medium environment. In Figure 38 it can be seen that the solution became totally black after about 1 week. This indicates sulfate reduction to sulfide. Microscopic examinations showed that the viable cells had the same shape and motility as those cells from liquid cultures inoculated using normal planktonic SRB cells in liquid in the experiments in vials.

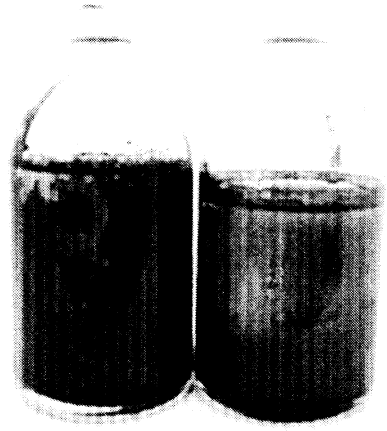

Figure 38. Liquid medium with SRB inoculated with a big round colony on the agar surface. The colony was incubated at 37°C after 1 day on the solid medium 1. The liquid solution became totally black after about 7 days. The vial on the right was a control using an inoculum from a liquid SRB stock solution.

5.6 Effects of glutaraldehyde and EDTA on cell growth and corrosion rate

The application of biocides is currently the most popular treatment of MIC in aqueous systems. Glutaraldehyde, as a biocide, was tested to determine its effectiveness in controlling the planktonic bacteria in this work. As used herein, biocide is referred to glutaraldehyde in this work. Experiments were carried out to test glutaraldehyde with a chelator to control both the planktonic and sessile SRB involved in MIC. The chelator used in this work was the disodium salt dihydrate of Ethylenediamine Tetraacetic Acid (EDTA). EDTA has been proven to have synergistic effects with antibiotics in the treatment of aerobic cell growth (Raad et al.; 2001). The effects of different glutaraldehyde concentrations (0 to 2000ppm), and the time of glutaraldehyde introduction (at inoculation time, after 1 day), were studied. The influences of EDTA alone and its combination with glutaraldehyde on the planktonic bacteria killing or

growth retardation were also investigated. The study was aimed at establishing the efficiency of the glutaraldehyde enhancement by EDTA and a dosing strategy.

5.6.1 Adding glutaraldehyde at inoculation time

Figure 39 indicates that when glutaraldehyde concentration was lower than 1000ppm, cell growth was retarded for several days. Usually, it takes 1 day for the solution to become completely black that is the indication of strong SRB propagation in the absence of glutaraldehyde. At a glutaraldehyde concentration of 50ppm, it took 3 days for solution to become black. At a glutaraldehyde concentration of 250ppm, it took about 7 days to become black. At a high glutaraldehyde concentration of 2000ppm, cell growth was suppressed effectively. However, a very high glutaraldehyde concentration to control bacteria growth is not applicable in the industries.

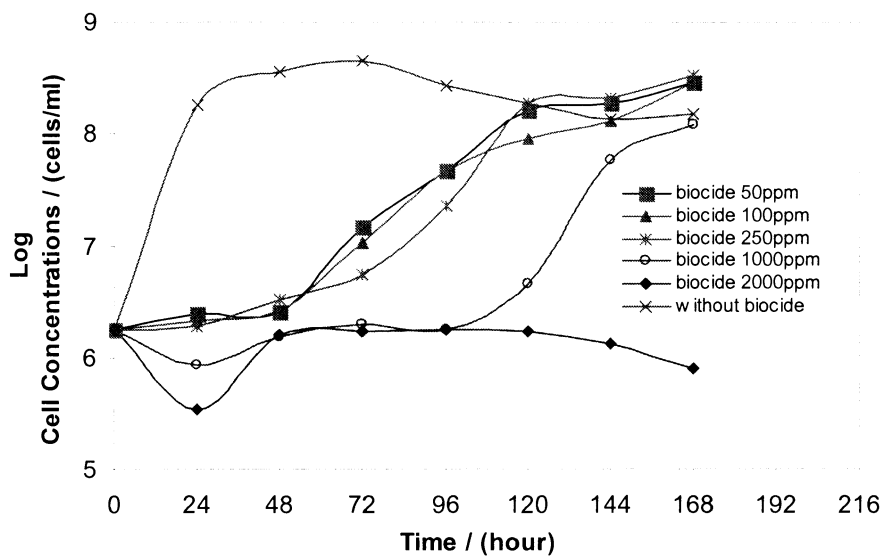

Figure 39. Planktonic cell growth in a medium with glutaraldehyde added at inoculation time.

The corrosion rates of coupons were also studied with different glutaraldehyde concentrations in the culture. Figure 40 indicated that a lower corrosion rate was obtained with 2000ppm glutaraldehyde in the medium. Figure 40 also demonstrates that the addition of glutaraldehyde could change some properties of the interface between the metal surface and the liquid solution, resulting in different corrosion rates of coupons. With different initial ferrous ion concentrations in the medium, it was found that when Fe^{2+} concentration was greater than 50ppm, the corrosion rates decreased due to the addition of glutaraldehyde; while in the medium with Fe^{2+} concentration lower than 50ppm, the addition of glutaraldehyde led to a high corrosion rate. The surface examinations under a microscope showed there were no pits on the surfaces of coupons from the culture with glutaraldehyde added into the medium containing low ferrous ion concentrations. More experiments needed to be performed to confirming these interesting results.

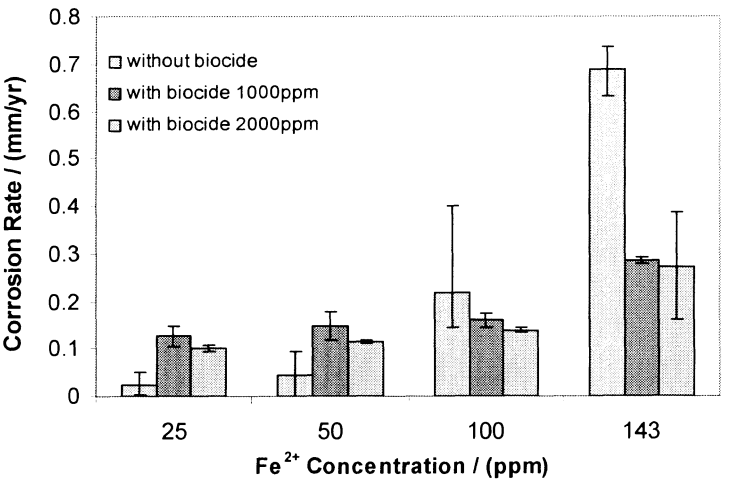

Figure 40. Comparison of weight loss corrosion rates in vials with and without glutaraldehyde. Error bars represent the differences between the maximum and minimum corrosion rates.

The experiments carried out in the glass cell confirmed the effectiveness of glutaraldehyde. Figure 41 is the corrosion potential differences with and without glutaraldehyde added to the medium. Figures 42 and 43 were the corrosion rates of cylindrical coupons obtained with the LPR method based on the corroded area on the coupon surface. In Figure 43, the reduction of corrosion rate appears to be small due the use of glutaraldehyde. It is probably because of the already low corrosion rate. More experiments are currently underway to investigate the biocide effects on corrosion rate including experiments using weight loss, coupon surface pit analysis and quantification of sessile SRB cells in the biofilm on a coupon surface.

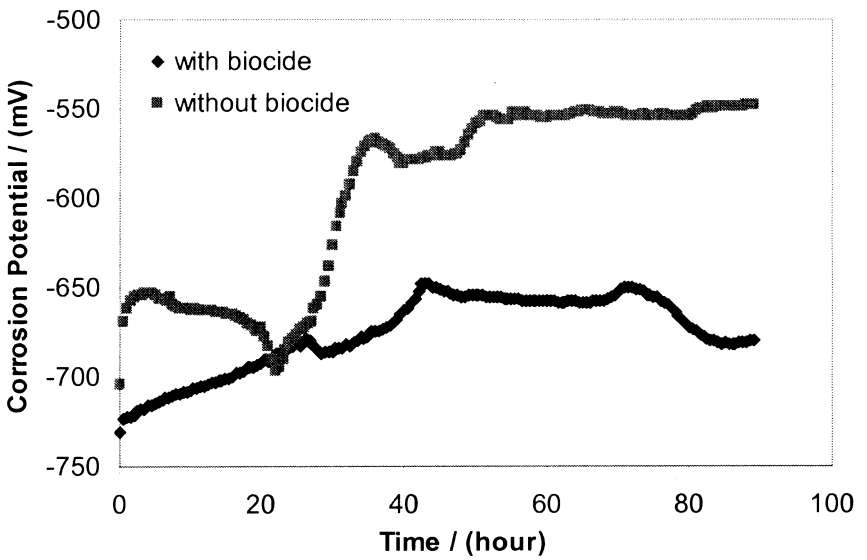

Figure 41. Corrosion potential with and without 500ppm glutaraldehyde in a medium containing 25ppm initial Fe^{2+} concentration. The experiment was carried out in a glass cell.

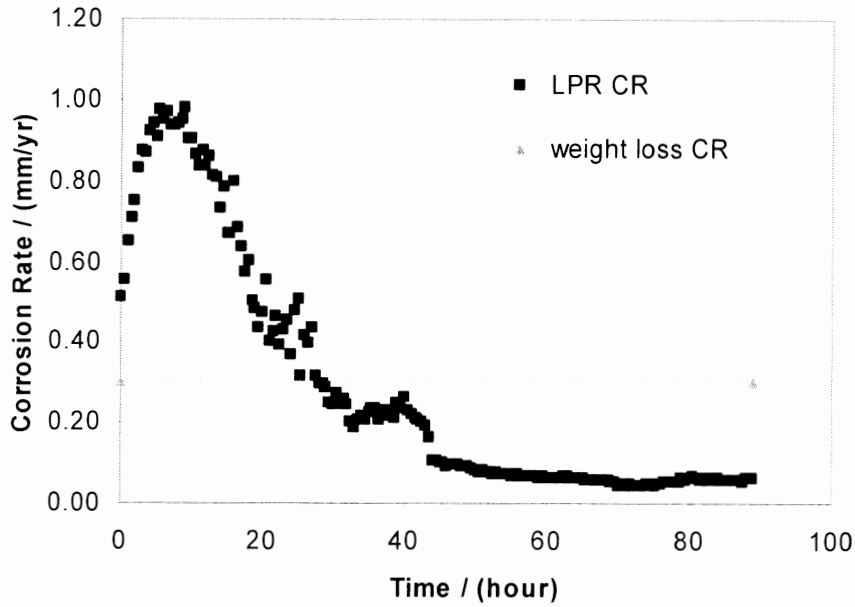

Figure 42. Corrosion rate with 500ppm glutaraldehyde in a medium containing 25ppm initial Fe^{2+} concentration. The experiment was carried out in a glass cell.

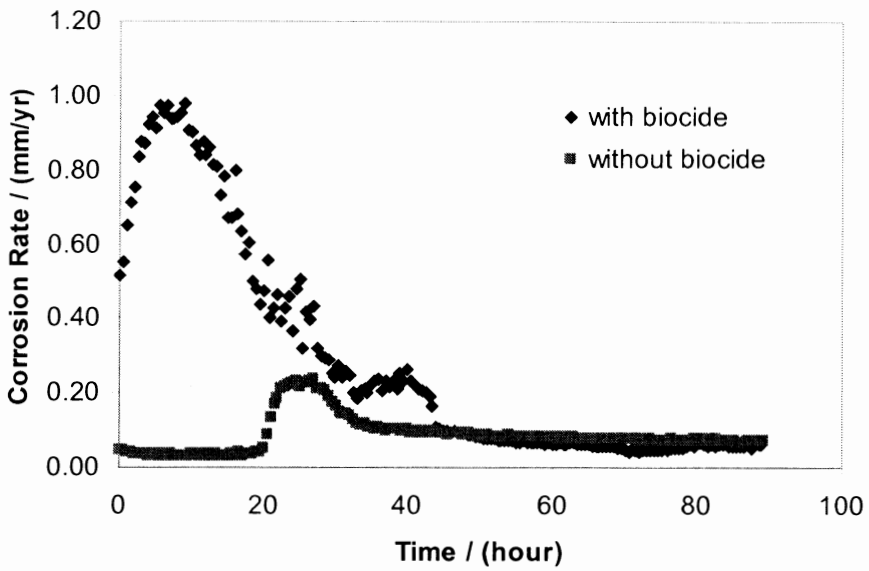

Figure 43. LPR corrosion rates in glass cells with and without 500ppm glutaraldehyde in a medium containing 25ppm initial Fe^{2+} concentration.

5.6.2 Adding glutaraldehyde after 1 day of growth

After 1day, the culture was already found to have a good growth with an SRB cell count of 1.8×10^{8} cells/ml, therefore, a high glutaraldehyde concentration was needed to achieve the inhibitory effect of SRB cell growth. Compared to those results where glutaraldehyde was added at inoculation time, the concentration of glutaraldehyde as high as 2000ppm was still not enough to control SRB cell growth effectively (Figure 44) since the culture already had a large cell population. This means that the initial SRB cell counts has a great impact on biocide effectiveness. Jhobalia (2004) reported a very small lethal dosage of 50ppm glutaraldehyde added to SRB cultures with a small initial SRB level of 100 cells/ml cell count.

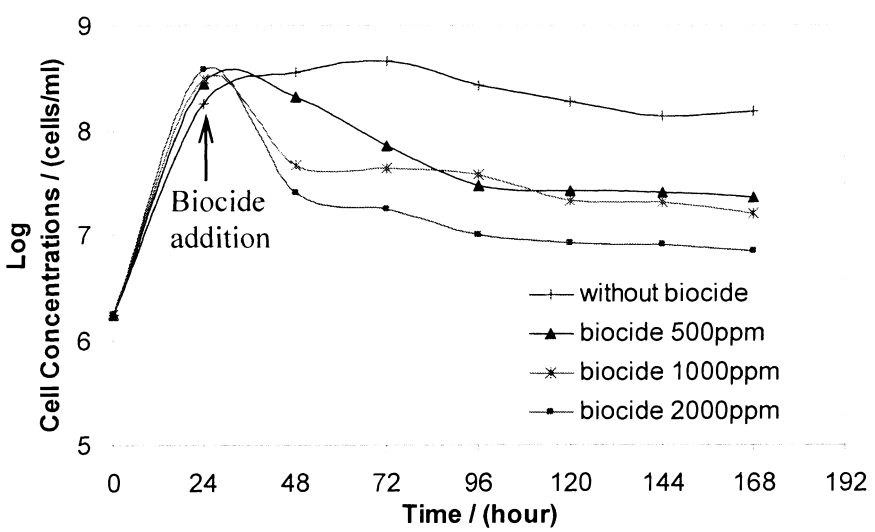

Figure 44. Planktonic cell growth in a medium with glutaraldehyde added after 1 day.

5.6.3 Adding glutaraldehyde and EDTA

Figure 45 indicates that EDTA alone was not able to inhibit the SRB cell growth effectively. However, when it was combined with glutaraldehyde, it was effective to suppress the SRB cell growth compared with the introduction of only glutaraldehyde or EDTA alone into the medium. Figure 46 shows that the combination of glutaraldehyde and EDTA was more effective in controlling planktonic bacteria than the use of glutaraldehyde alone. In Figure 46, it can be seen that the cell population was about 10^3 times lower than that without glutaraldehyde and EDTA.

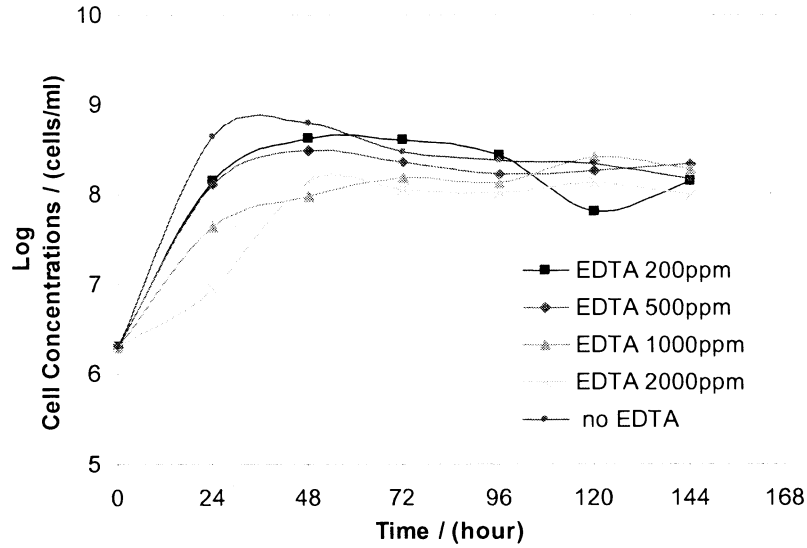

Figure 45. Planktonic cell growth rates at different concentrations of EDTA.

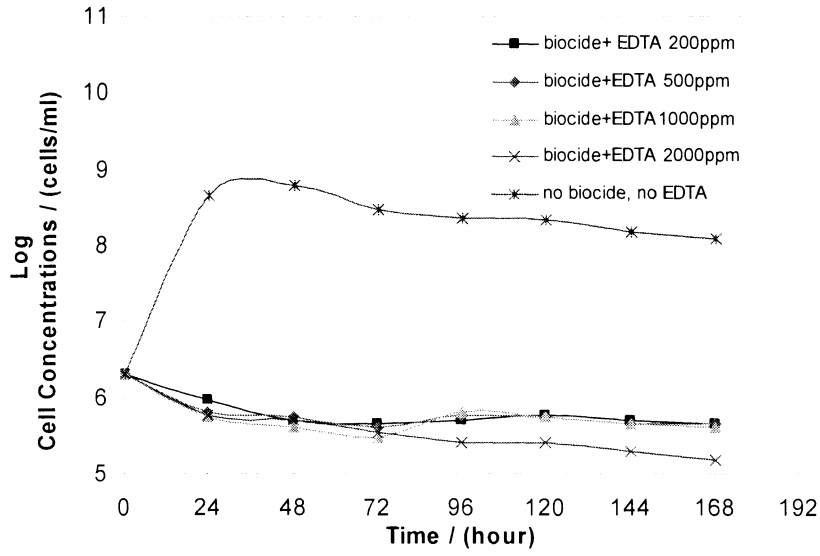

Figure 46. Planktonic cell growth rates at different concentrations of EDTA and 250ppm glutaraldehyde.

Chapter 6 Conclusions

SRB growth behavior and biocorrosion due to SRB on mild steel surfaces were investigated in anaerobic vials and electrochemical glass cells using a pure culture of an SRB species *Desulfovibrio desulfuricans*. The conclusions obtained in this study were below:

(1) SRB growth rate increased with the increase of the initial ferrous ion concentration in the medium.

(2) The protective iron sulfide film formation could be affected by the ferrous ion concentration in the medium. Low corrosion rate was achieved in a culture medium containing low ferrous ion concentration; high corrosion rate was obtained in an iron-rich medium (higher than 50ppm).

(3) The increase of SO_4^{2-} concentration within the range of 1.93g/l to 6.5g/l decreased the planktonic SRB growth and the corrosion rate of mild steel.

(4) Celite beads as microcarriers had a very limited effect on reduction of corrosion rate even though they reduced the planktonic cell count by 3-fold.

(5) A new solid medium was successfully developed for fast plating of SRB without using hydrogen gas. The value of pH was very important for SRB growth at different medium compositions.

(6) SRB growth rate was retarded at various levels of glutaraldehyde in the medium. High SRB cell concentrations at the point of glutaraldehyde introduction in the medium limited the effectiveness of glutaraldehyde. The effectiveness of glutaraldehyde on the SRB growth control was considerably improved by the addition of EDTA. The synergistic effects of the combined use of glutaraldehyde and EDTA at lower concentrations on planktonic SRB growth control, biofilm treatment, as well as on the corrosion rate should be further investigated.

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Investigation of Sulfate-Reducing Bacteria Growth Behavior for the Mitigation of Microbiologically Influenced Corrosion (MIC) (94 pp.)

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SRB in the oil and gas industry have created serious problems. Since the 1980s considerable efforts have been devoted to understanding the corrosion process due to SRB, while the interactions between the biological systems and the environment still remain obscure. A thorough study of the parameters that affect SRB growth with respect to mitigating SRB related MIC is mandatory because the growth behavior of SRB is believed to be an important factor in the MIC process due to SRB.

This study focused on the factors influencing the growth of SRB and the corrosion of steel. The ATCC 7757 strain of *Desulfovibrio desulfuricans* was used as a representative of SRB in this work. Corrosion due to SRB only occurs in the presence of sulfate ions; however, experimental results in this work showed that high concentrations of sulfate ions inhibited sulfate reduction by SRB.

The ferrous ion concentration plays a significant role in the corrosion process and corrosion rate of steel. Experimental results indicated high concentrations of ferrous ion aggravated the corrosion of steel.

The adhesion of SRB to metal surfaces affects the corrosion process substantially. Celite beads exhibited some inhibition of cell migration to the biofilm by reducing the planktonic SRB cell count considerably. However, it reduced the corrosion rate only to a small extent.