Investigation of *Desulfovibrio vulgaris* Biocorrosion Mechanism and Its Mitigation
Using an Antimicrobial Enhanced by a D-amino Acid

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This thesis titled

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ABSTRACT

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Investigation of Desulfovibrio vulgaris Biocorrosion Mechanism and Its Mitigation

Using an Antimicrobial Enhanced by a D-amino Acid

Director of Thesis: Tingyue Gu

Biocorrosion, also known as microbiologically influenced corrosion (MIC), which is primarily caused by microbial respiration or metabolic products of microbes, has been a major factor in the initiation and acceleration of metal corrosion. Apart from the destruction of pipelines and economic losses, MIC is also suspected to cause the failure of human implants. Metabolites such as hydrogen sulfide are a threat to human health.

Sulfate reducing bacteria (SRB) often contribute to the MIC of pipelines and implants due to their wide distribution in industrial fields and in the human body. The biocatalytic cathodic sulfate reduction (BCSR) theory was proposed by Gu et al. (2009) to clarify the mechanism of MIC pitting corrosion caused by SRB. Extracellular electron transfer is the key step to explain how SRB MIC pitting happens. Riboflavin and flavin adenine dinucleotide (FAD), two common electron mediators used by many microbes, were found in lab tests to accelerate the electron transfer from metal surface to sessile cells, providing support to the BCSR theory. Starvation test using 304 and 316 stainless steel coupons suggested that SRB can utilize elemental iron as an electron donor when there is a local shortage of organic carbon underneath the SRB biofilm.

A mixture of D-amino acids and THPS has been proposed to mitigate. D-amino acids are biocide enhancers that disperse sessile cells in biofilms under an antimicrobial
stress. In order to achieve a better mitigation of implant MIC in the human body, a synergetic combination of a D-amino acid and an antibiotic was investigated. Decreased weight loss and MIC pit depth were obtained using a cocktail treatment of D-methionine and metronidazole.
DEDICATION

To all who encouraged and helped me during my MS study

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TABLE OF CONTENTS

Abstract ............................................................................................................................... 3
Dedication ........................................................................................................................ 5
Acknowledgments............................................................................................................... 6
List of Tables .................................................................................................................... 10
List of Figures .................................................................................................................. 12
Chapter 1 Introduction ................................................................................................... 19
Chapter 2 Literature review ........................................................................................... 22
  2.1 Stainless steel .................................................................................................... 22
  2.2 Biofilm formation and extracellular polymeric substances (EPS) .................... 23
  2.3 Sulfate reducing bacteria (SRB) ....................................................................... 25
  2.4 Mechanisms of MIC due to SRB .................................................................... 28
  2.4.1 Cathodic depolarization theory (CDT) ......................................................... 28
  2.4.2 Biocatalytic cathodic sulfate reduction (BCSR) theory .............................. 29
  2.5 Extracellular electron transfer to support BCSR .............................................. 31
  2.6 Mitigation of MIC ........................................................................................... 34
Chapter 3 MIC promoter investigation .......................................................................... 38
  3.1 Introduction ....................................................................................................... 38
  3.2 Extracellular electron transfer ........................................................................... 38
  3.3 Mechanisms of electron mediators by flavins .................................................. 39
  3.4 MIC and MFC ................................................................................................... 40
  3.5 Objectives ......................................................................................................... 41
  3.6 Materials and methods ...................................................................................... 42
  3.6.1 Bacterium and cultivation ............................................................................. 42
  3.6.2 Corrosion analysis ......................................................................................... 43
  3.6.3 Analysis of corroded coupons using scanning electron microscopy (SEM) and infinite focus microscopy (IFM) ................................................................. 44
  3.6.4 Measurement of sulfate concentration in culture medium ......................... 44
  3.7 Results ............................................................................................................... 45
  3.7.1 SRB cell count observation ........................................................................... 45
3.7.2 Corrosion analysis of corroded coupons .......................................................... 46
3.7.3 Sulfate concentration in test medium ............................................................ 49
3.8 Discussion ......................................................................................................... 50
  3.8.1 Electron transfer and microbial synergy in biofilms ..................................... 50
  3.8.2 Pathways for electron carrying mediators crossing cell wall ..................... 52
3.9 Conclusions ..................................................................................................... 54

Chapter 4 Starvation test to support BCSR theory .............................................. 68
  4.1 Introduction .................................................................................................... 68
  4.2 The influences of Cl\(^-\) and H\(^+\) on pitting corrosion ......................... 69
  4.3 Objectives .................................................................................................... 70
  4.4 Materials and methods ............................................................................... 70
    4.4.1 Starvation test ....................................................................................... 70
    4.4.2 Investigation of chloride ion effect on MIC pitting corrosion ............... 71
    4.4.3 Test of pH value effect on MIC pitting corrosion .................................. 71
  4.5 Results and discussion ............................................................................... 72
    4.5.1 Starvation test using 304 SS and 316 SS .............................................. 72
    4.5.2 Chloride ion influence on MIC test ..................................................... 74
    4.5.3 pH influence experiment ...................................................................... 75
  4.6 Conclusion .................................................................................................... 77

Chapter 5 Mitigation of MIC using a cocktail of an antibiotic and a D-amino acid ... 99
  5.1 Introduction .................................................................................................. 99
  5.2 Antibiotics against anaerobic microbes ..................................................... 99
  5.3 D-amino acids as antibiotic enhancers ...................................................... 100
  5.4 Objectives .................................................................................................. 101
  5.5 Materials and methods ............................................................................. 101
    5.5.1 Prevention test ..................................................................................... 101
    5.5.2 Removal test ....................................................................................... 102
  5.6 Results and discussion ............................................................................. 102
    5.6.1 Prevention of SRB biofilm establishment .......................................... 102
    5.6.2 Removal of established SRB biofilm ................................................ 104
  5.7 Conclusion .................................................................................................. 105
Chapter 6 summary ........................................................................................................ 126
6.1 Promoters for cross-cell wall electron transfer ................................................. 126
6.2 Starvation test to verify SRB MIC mechanism ................................................... 126
6.3 Mitigation using a cocktail of metronidazole and D-methionine ....................... 127

References .................................................................................................................... 128
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Composition of the 304 and 316 stainless steels (data from Sedriks, 1996)</td>
<td>23</td>
</tr>
<tr>
<td>2-2</td>
<td>CDT mechanism of MIC by SRB (von Wolzogen Kuehr and von der Vlugt, 1934)</td>
<td>28</td>
</tr>
<tr>
<td>3-1</td>
<td>Promoter test matrix</td>
<td>55</td>
</tr>
<tr>
<td>3-2</td>
<td>Sessile cell count after 7-day incubation</td>
<td>55</td>
</tr>
<tr>
<td>3-3</td>
<td>pH value after 7 days</td>
<td>56</td>
</tr>
<tr>
<td>3-4</td>
<td>General corrosion rate (mm/year) based on weight loss</td>
<td>56</td>
</tr>
<tr>
<td>3-5</td>
<td>Pitting corrosion rate (mm/year) based on maximum pit depth</td>
<td>57</td>
</tr>
<tr>
<td>3-6</td>
<td>Residual sulfate concentration after 7-day incubation</td>
<td>57</td>
</tr>
<tr>
<td>4-1</td>
<td>Starvation test matrix</td>
<td>78</td>
</tr>
<tr>
<td>4-2</td>
<td>Components of ATCC 1249 medium</td>
<td>79</td>
</tr>
<tr>
<td>4-3</td>
<td>ATCC 1249 medium minus carbon sources</td>
<td>79</td>
</tr>
<tr>
<td>4-4</td>
<td>Test matrix for chloride influence</td>
<td>79</td>
</tr>
<tr>
<td>4-5</td>
<td>Test matrix for pH influence</td>
<td>80</td>
</tr>
<tr>
<td>4-6</td>
<td>Acetate acid solution with pH 3-6</td>
<td>80</td>
</tr>
<tr>
<td>4-7</td>
<td>Phosphate buffer solution with pH 7-9</td>
<td>80</td>
</tr>
<tr>
<td>4-8</td>
<td>Sessile cell count after 7 days in 304 SS starvation test</td>
<td>81</td>
</tr>
<tr>
<td>4-9</td>
<td>Sessile cell count in 316 SS starvation test after 7 days</td>
<td>81</td>
</tr>
<tr>
<td>4-10</td>
<td>pH value after 7-day 304 SS starvation test</td>
<td>82</td>
</tr>
<tr>
<td>4-11</td>
<td>pH value after 7-day 316 SS starvation test</td>
<td>82</td>
</tr>
<tr>
<td>5-1</td>
<td>Mitigation test matrix</td>
<td>106</td>
</tr>
<tr>
<td>5-2</td>
<td>pH value in biofilm prevention test after 7-day treatment</td>
<td>106</td>
</tr>
</tbody>
</table>
Table 5-3. Sessile cell count in biofilm prevention test after 7-day treatment ............ 107

Table 5-4. Sessile cell count in removal test after 3-day culturing and then immersing in different concentrations of metronidazole and D-methionine for 3 hours...................... 121
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2-1.</td>
<td>The processes of biofilm development: (1) initiation, (2) attachment by EPS, (3) early 3-D structure formed, (4) maturation, (5) detachment (reprinted from Stoodley et al., 2002).</td>
<td>24</td>
</tr>
<tr>
<td>Figure 2-2.</td>
<td>Biofilm morphology of <em>D. vulgaris</em> on the surface of 304 SS coupons by using SEM.</td>
<td>27</td>
</tr>
<tr>
<td>Figure 2-3.</td>
<td>The scheme of <em>D. vulgaris Hildenborough</em> energy generation process coupling lactate oxidation with sulfate respiration. Sulfate processing steps include sulfate transportation, sulfate activation, and sulfate reduction (Pereira et al., 2007).</td>
<td>27</td>
</tr>
<tr>
<td>Figure 2-4.</td>
<td>Schematic illustration of SRB MIC mechanism showing the transport of extracellular electrons (Xu and Gu, 2014).</td>
<td>31</td>
</tr>
<tr>
<td>Figure 2-5.</td>
<td>Simplified EET pathways: (A) DET via contacted cytochrome or via pili, (B) MET via electron mediators (Schröder, 2007).</td>
<td>32</td>
</tr>
<tr>
<td>Figure 2-6.</td>
<td>Two types of PG with D-alanine terminus: (A) meso-diaminopimelic acid (DAP) type, (B) Lys-type (Royet and Dziarski, 2007).</td>
<td>37</td>
</tr>
<tr>
<td>Figure 3-1.</td>
<td>Chemical structure of riboflavin (Tan and Webster, 2012).</td>
<td>40</td>
</tr>
<tr>
<td>Figure 3-2.</td>
<td>Comparison of electron transfer mechanisms for biocathode in MFC and for BCSR theory in MIC. “Med(red)” represents the form of mediators carrying electrons; Med(ox) represents the form of mediators after releasing electrons. Cyt represents the c-type cytochrome embedded in cell membrane to assist electron transfer into cells.</td>
<td>41</td>
</tr>
<tr>
<td>Figure 3-3.</td>
<td>The working mechanism of mediated electron transfer in SRB biofilm formation. Green bacteria are capable of producing soluble mediators; blue ovals represent the SRB cells which are not capable of secreting electron mediators. Med$^{\text{red}}$ represents mediators carrying electrons; Med$^{\text{ox}}$ represents the mediators without electrons. Black membrane dots (•) displays membrane-bound proteins that are able of transfer electrons from the mediators into cell cytoplasm.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 3-4.</td>
<td>Possible working mechanism of electron transfer chain from outside the environment into cytoplasm; the green, blue, and purple shapes represent different types of cytochrome; cyt (red) denotes cytochrome carrying electrons; cyt (ox) denotes cytochrome without electrons.</td>
<td>53</td>
</tr>
<tr>
<td>Figure 3-5.</td>
<td>Planktonic cell count with and without an electron mediator. Initial cell concentration was $10^6$ cells/ml and the experiment was repeated three times.</td>
<td>58</td>
</tr>
</tbody>
</table>
Figure 3-6. Sani-Check SRB kit test tubes for SRB without mediator, SRB with 10 ppm riboflavin, SRB with 10 ppm FAD, respectively ............................................................. 58

Figure 3-7. Sessile SRB cells on 304 SS coupon surface after 7 days of incubation: (A) without any mediators, (B) with addition of 10 ppm FAD, (C) with addition of 10 ppm riboflavin ............................................................. 59

Figure 3-8. Surface morphology on 304 SS coupon in abiotic test: (A) with ATCC 1249 medium only, (B) with ATCC 1249 medium and 10 ppm FAD, (C) with ATCC 1249 medium and 10 ppm riboflavin after 7 days ............................................................. 60

Figure 3-9. Average pit depth for abiotic control with or without adding electron mediators (each column calculated from at least three duplicate coupons). Error bars represent standard deviation ............................................................. 61

Figure 3-10. Specific weight loss after 7-day of SRB incubation with or without an electron mediator (each column calculated from at least three duplicate coupons). Error bars represent standard deviation ............................................................. 61

Figure 3-11. Pit depth after 7-day exposure to SRB culture without an electron mediator. The maximum pit depth was 4.8 μm ............................................................. 62

Figure 3-12. Pit depth after 7-day exposure to SRB with addition of 10 ppm FAD. The maximum pit depth was 6.0 μm ............................................................. 63

Figure 3-13. Pit depth after 7-day exposure to SRB with addition of 10 ppm riboflavin. The maximum pit depth was 6.6 μm ............................................................. 64

Figure 3-14. Average pit depth after 7-day SRB incubation with and without an electron mediator (each column calculated from at least three duplicate coupons). Error bars represent standard deviation ............................................................. 65

Figure 3-15. Pit morphology on the surface of 304 SS coupon after 7-day exposure to: (A) SRB only without an electron mediator, (B) SRB with addition of 10 ppm FAD, (C) SRB with addition of 10 ppm riboflavin ............................................................. 66

Figure 3-16. Average surface pit diameter after 7-day SRB inclubation with and without an electron mediator. Error bars represent standard deviations ............................................................. 67

Figure 4-1. Images of SRB biofilms on different coupon surfaces ............................................................. 77

Figure 4-2. Anaerobic vials after 7-day starvation test using 304 SS coupons: (1) ATCC 1249 medium with 0% carbon source, (2) ATCC 1249 medium with 10% carbon source, (3) ATCC 1249 medium with 90% carbon source, (4) ATCC 1249 medium with 100% carbon source, and (5) full strength ATCC 1249 medium after 10 days of incubation .... 83
Figure 4-3. SEM images for 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in full strength ATCC 1249 medium: (A) Biofilm formation, (B) Surface morphology after removing the biofilm and corrosion products. ................................................................. 84

Figure 4-4. SEM images for 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in modified ATCC 1249 medium with 90% carbon source removed: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products. .................. 85

Figure 4-5. SEM images for 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in modified ATCC 1249 medium with 100% carbon source removed: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products. ................................. 86

Figure 4-6. SEM images for 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in full strength ATCC 1249 medium: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products. ................................................................. 87

Figure 4-7. SEM images for 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 90% carbon source removed: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products. ........................................... 88

Figure 4-8. SEM images for 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 100% carbon source removed: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products. ........................................... 89

Figure 4-9. Average pit depth of 304 SS coupons after the starvation test. .................. 90

Figure 4-10. Specific weight loss of 304 SS coupons after the starvation test. .......... 90

Figure 4-11. Average pit depth of 316 SS coupons after the starvation test. .......... 91

Figure 4-12. Specific weight loss of 316 SS coupons after the starvation test. .......... 91

Figure 4-13. Maximum pit depth of 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in full strength ATCC 1249 medium. The maximum pit depth was 4.6 μm. ....................... 92

Figure 4-14. Maximum pit depth of 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 90% carbon source removed. The maximum pit depth was 5.6 μm.. 92
Figure 4-15. Maximum pit depth of 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 100% carbon source removed. The maximum pit depth was 6.6 μm.

Figure 4-16. Maximum pit depth of 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in full strength ATCC 1249 medium. The maximum pit depth was 3.8 μm.

Figure 4-17. Maximum pit depth of 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 90% carbon source removed. The maximum pit depth was 4.3 μm.

Figure 4-18. Maximum pit depth of 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 100% carbon source removed. The maximum pit depth was 5.2 μm.

Figure 4-19. Specific weight loss of 304 SS coupons in abiotic test with chloride concentration variation.

Figure 4-20. Specific weight loss of 304 SS coupons in biotic test with chloride concentration variation.

Figure 4-21. Pit depth profile of 304 SS after 7 days in abiotic test using full strength ATCC 1249 medium without chloride removed. No well-defined pit was observed.

Figure 4-22. Pit depth profile of 304 SS in SRB biotic test after 7 days using full strength ATCC 1249 medium without chloride removed. The maximum pit depth was 3.9 μm.

Figure 4-23. Pit depth profile of 304 SS in SRB biotic test after 7 days using full strength ATCC 1249 medium with 90% chloride removed. The maximum pit depth was 4.1 μm.

Figure 4-24. Pit depth profile of 304 SS in SRB biotic test after 7 days using full strength ATCC 1249 medium with 99% chloride removed. The maximum pit depth was 3.8 μm.

Figure 4-25. pH value after 7 days of corrosion test in phosphate buffer solution and acetate buffer solution with different pH values.

Figure 4-26. Specific weight loss of 304 SS coupons after 7-day test in phosphate buffer solution and acetate buffer solution with different pH values.

Figure 5-1. Planktonic cell count after 7-day treatment with different antibiotic doses in the biofilm prevention test.
Figure 5-2. Images of sessile SRB cells on X65 coupons after 7-day biofilm prevention test: (A) no treatment, (B) with 500 ppm D-methionine treatment. ........................................ 108

Figure 5-3. Images of sessile SRB cells on X65 coupons after 7-day biofilm prevention test: (A) with 50 ppm metronidazole treatment, (B) treated with 50 ppm metronidazole + 50 ppm D-methionine, (C) treated with 50 ppm metronidazole + 100 ppm D-methionine. ........................................................................................................... 109

Figure 5-4. Images of sessile SRB cells on X65 coupons after 7-day biofilm prevention test: (A) treated with 100 ppm metronidazole, (B) treated with 100 ppm metronidazole + 50 ppm D-methionine, (C) treated with 100 ppm metronidazole + 100 ppm D-methionine. ........................................................................................................... 110

Figure 5-5. Images of sessile SRB cells on X65 coupons after 7-day biofilm prevention test: (A) treated with 200 ppm metronidazole, (B) treated with 200 ppm metronidazole + 50 ppm D-methionine, (C) treated with 200 ppm metronidazole + 100 ppm D-methionine. ........................................................................................................... 111

Figure 5-6. Specific weight loss in biofilm prevention test with treatment of 50 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.......................................................... 112

Figure 5-7. Specific weight loss in biofilm prevention test with treatment of 100 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.......................................................... 112

Figure 5-8. Specific weight loss in biofilm prevention test with treatment of 200 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.......................................................... 113

Figure 5-9. Average pit depth in biofilm prevention test with treatment of 50 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.......................................................... 113

Figure 5-10. Average pit depth in biofilm prevention test with treatment of 100 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.......................................................... 114

Figure 5-11. Average pit depth in biofilm prevention test with treatment of 200 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.......................................................... 114

Figure 5-12. Pit depth profile in prevention test after 7-day exposure to the test condition of no treatment. The maximum pit depth was 17.4 μm. ................................................... 115
Figure 5-13. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 500 ppm D-methionine. The maximum pit depth was 15.5 μm. .............................. 115

Figure 5-14. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 50 ppm metronidazole. The maximum pit depth was 12.2 μm. ..................... 116

Figure 5-15. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 50 ppm metronidazole + 50 ppm D-methionine. The maximum pit depth was 12.4 μm. .......................................................................................................................... 116

Figure 5-16. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 50 ppm metronidazole + 100 ppm D-methionine. The maximum pit depth was 11.6 μm. .......................................................................................................................... 117

Figure 5-17. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 100 ppm metronidazole. The maximum pit depth was 11.2 μm. ................. 117

Figure 5-18. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 100 ppm metronidazole + 50 ppm D-methionine. The maximum pit depth was 10.7 μm. .......................................................................................................................... 118

Figure 5-19. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 100 ppm metronidazole + 100 ppm D-methionine. The maximum pit depth was 8.4 μm. .......................................................................................................................... 118

Figure 5-20. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 200 ppm metronidazole. The maximum pit depth was 8.3 μm. ............... 119

Figure 5-21. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 200 ppm metronidazole + 50 ppm D-methionine. The maximum pit depth was 6.9 μm. .......................................................................................................................... 119

Figure 5-22. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 200 ppm metronidazole + 100 ppm D-methionine. The maximum pit depth was 6.5 μm. .......................................................................................................................... 120

Figure 5-23. Images of sessile cells on X65 coupons in biofilm removal test with no treatment. After 3 days of growth in the ATCC 1249 medium to establish mature SRB biofilms, coupons were removed from vials and immersed in Petri dishes with a PBS buffer: (A) without any treatment, (B) treated with 500 ppm D-methionine. ................ 122

Figure 5-24. Images of sessile cells on X65 coupons in biofilm removal test: (A) treated with 50 ppm metronidazole, (B) treated with 50 ppm metronidazole + 50 ppm D-methionine, (C) treated with 50 ppm metronidazole + 100 ppm D-methionine............. 123
Figure 5-25. Images of sessile cells on X65 coupons in biofilm removal test: (A) treated with 100 ppm metronidazole, (B) treated with 100 ppm metronidazole + 50 ppm D-methionine, (C) treated with 100 ppm metronidazole + 100 ppm D-methionine........... 124

Figure 5-26. Images of sessile cells on X65 coupons in biofilm removal test: (A) treated with 200 ppm metronidazole, (B) treated with 200 ppm metronidazole + 50 ppm D-methionine, (C) treated with 200 ppm metronidazole + 100 ppm D-methionine........... 125
CHAPTER 1 INTRODUCTION

Microbiologically influenced corrosion (MIC) has become an important research topic recently given the increasing failures of pipelines and biomedical implants. Several-billion dollars in losses are realized each year in the United States (Walsh et al., 1993; Jacobson 2007). However, the involvement of microbes in initiation, facilitation and acceleration of corrosion makes MIC difficult to investigate and predict. Increased MIC in oil pipelines is due to the application of water-flooding techniques that build up well pressure for enhanced petroleum recovery (Stetter et al., 1993). Due to incomplete water separation from oil, water ends in the oil transport pipelines with increasing fractions leading to more frequent water-wetting (of pipeline wall) operation condition. Water wetting induces the microbial proliferation in oil pipelines and thus promotes MIC. MIC pitting corrosion was mainly blamed for the failure of the Alaska oil pipelines in 2006.

Sulfate reducing bacteria (SRB) can commonly be found in oil reservoirs (Rosnes et al., 1991). These bacteria are often thought to be the main contributors to MIC in industrial pipelines (Javaherdashti, 1999). Boopathy et al. (2002) reported that SRB also exist in the human body, such as in periodontal pockets and the gut. SRB were discovered to be a key factor in the corrosion of biomedical implants and bowel diseases (Mylonaki et al., 2005). While theories explaining why SRB attack metal materials have been investigated since 1934 (von Wolzogen Kuehr and van der Vlugt, 1934), the exact mechanism of how SRB are involved in MIC pitting is still unclear.

A new mechanistic model of biocatalytic cathodic sulfate reduction (BCSR) was proposed by Gu et al. (2009) to elucidate the process of MIC pitting due to SRB from the
perspective of bioelectrochemistry and bioenergetics. The well-investigated theories from microbial fuel cells (MFCs) such as extracellular electron transfer (EET) support the assumptions of BCSR. In this work, two tests were performed to explain why and how MIC happens due to SRB. A starvation test was constructed to support BCSR theory from the perspective of bioenergetics. Secondly, electron transfer in BCSR was demonstrated by using electron mediators.

Presently, due to its popularity in oil pipelines, carbon steel has become the primary material in MIC research. Compared with carbon steel, stainless steel is more resistant to corrosion because of its specific components such as chromium and nickel that react with oxygen to form a protective passivation film (Kim and Young, 2013). However, stainless steel is still vulnerable to MIC attack. The passivation film of stainless steel can be disrupted by biofilm formation (Duan et al., 2006), and the anaerobic condition does not allow repair of the damaged oxide film.

Due to the potential economic losses and environmental issues caused by MIC, establishing green mitigation methods has become a common practice. Mitigation of MIC includes two types of methods: mechanical methods and chemical methods (Frey, 1998; Vide, 2002). Pigging is a common mechanical method used to scrub the biofilm covering surface of pipelines. However, not all pipes can be pigged. Chemical methods such as biocide treatment are commonly applied in industries. Tetrakis (hydroxymethyl) phosphonium sulfate (THPS) and glutaraldehyde are two green biocides, which are biodegradable (Frey, 1998). However, due to the protective nature of the biofilm, sessile cells are more difficult to remove than planktonic cells. Ten times or high biocide dosages
are needed to treat biofilm. Even 1000 times higher concentration of biocides can be necessary to achieve biofilm eradication (Mah and O’Toole, 2001). Safer and efficacious mitigation methods, which combine current biocides with new biocide enhancers, have been investigated by Xu et al. (2012a, 2012b, 2012c, 2013a). They found that the efficacy of a biocide can be highly enhanced when combined with certain D-amino acids.

Antibiotics have been employed in the clinical field to mitigate biofilms and inhibit MIC pitting of biomedical implants. Metronidazole, ciprofloxacin hydrochloride monohydrate, and nalidixic acid are three universal antibiotics used against anaerobic bacteria (i.e., SRB) (Cancet, 1979; Desai et al., 1998; Lewis et al., 2005; Prabhala et al., 1984). Since both planktonic cells and sessile cells in the human body can easily become resistant to antibiotic treatment, more effective methods are desired. Better efficacy of antibiotics against microbes could be achieved with the combination of antimicrobial enhancers. This is the perspective of antimicrobial treatment in this study. Safe and natural D-amino acids, such as D-methionine and D-tyrosine, have been investigated to signal the biofilm dispersal under an antimicrobial stress (Xu et al., 2013a; Xu et al., 2012a; Xu and Liu, 2011). In this work, a synergistic mixture of metronidazole enhanced by D-methionine was investigated as an MIC mitigation method.
CHAPTER 2 LITERATURE REVIEW

2.1 Stainless steel

Sedriks (1996) defined stainless steel as an iron alloy which has at least 10.5% chromium content. Olsson and Landolt (2003) discussed that the high content of chromium in stainless steel reacts with oxygen to form a dense and thin passive film consisting of a chrome-containing oxide. This stable film is resistant to aggressive environments. The 300 series, which has the compositional property of the classic 18/8 (18% Cr-8% Ni), is the most common type of stainless steel used in industry in the United States (Sedriks, 1996). The additional of nickel and chromium lead to a compact chromium oxide film, which further improves the corrosion resistance. The composition of two types 300 series stainless steel is shown in Table 2-1.

Primarily due to its compositional characteristics, stainless steel is widely used in oil pipelines (although not for main trunk lines due to high costs), cooling water systems, and clinical implants. However, some corrosive chemicals such as chlorides and organic acids can still induce pitting corrosion by removing the protective passivation film (Kaneko and Isaacs, 2000; Olsson and Landolt, 2003). Biofilms are capable of attaching to the surface of stainless steel. Various metabolites (i.e., H⁺, H₂S) and sessile cells can attack and disrupt the protective film, and, thus, cause MIC pitting.
Table 2-1. Composition of the 304 and 316 stainless steels (data from Sedriks, 1996)

<table>
<thead>
<tr>
<th>UNS Number</th>
<th>Name</th>
<th>Composition (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cr</td>
</tr>
<tr>
<td>S30400</td>
<td>304</td>
<td>18-20</td>
</tr>
<tr>
<td>S31600</td>
<td>316</td>
<td>16-18</td>
</tr>
</tbody>
</table>

2.2 Biofilm formation and extracellular polymeric substances (EPS)

Biofilms are considered to be complex communities of microbes attached to a biotic or abiotic surface by extracellular polymeric substances (EPS), which are secreted by the microbes (Wingender et al., 1999). Laspidou and Rittmann (2002) indicated that EPS impacted the aggregation of planktonic cells and the formation of biofilms. Stoodley et al. (2002) then showed that EPS including proteins, polysaccharides and some macromolecules produced by microorganisms determine the phenotypic characteristics and functional structures of the biofilms. The formation and development of biofilms are influenced by the changes in the microenvironments, such as pH, temperature, and nutrient conditions. Figure 2-1 shows the process of biofilm formation. The development of biofilms includes attachment, growth, and dispersal (O’Toole et al., 2000).
In biofilm formation, bacteria tend to live as a community initiating the formation of a biofilm (Costerton et al., 1987; Johnson, 2008). In the initiation of biofilm, Neu and Lawrence (1999) demonstrated that EPS assist bacteria monomers to adhere together and form three-dimensional or mushroom-shaped structures. Once a biofilm forms, detachment of the top layer of the biofilm occurs, releasing sessile cells that return to the planktonic system. The reduction of EPS, the starving of sessile cells, or extracellular environmental signals can mediate the dispersal of biofilms (Allison et al., 1998; O’Toole et al., 2000; Stoodley et al., 2002).
The characteristics of biofilm—especially mass transfer and protective mechanisms—are closely associated with external environmental factors and biofilm formation. Because the structure of the biofilm and EPS create a physical and chemical barrier between internal sessile cells and those of outside environments (O’Toole et al., 2000), this provides protection against some physical and chemical attacks such as water flooding, and antimicrobial agents (Neu and Lawrence, 1999). It has also been reported that the specific phenotype of biofilms can limit or decrease the transportation of biocides into biofilms (Videla, 2002; Donlan, 2000; Vance and Thrasher, 2005). Biofilms protect cells from the influence of antimicrobial agents and detrimental environments (Denkhaus et al., 2007).

2.3 Sulfate reducing bacteria (SRB)

SRB are anaerobic bacteria, which grow well in sulfate rich anaerobic environments, such as oil reservoirs and pipelines (due to seawater injection), and the human gut (Hao et al., 1996; Gibson et al., 1988; Boopathy et al., 2002). They are often considered to be the culprit in MIC of pipelines and biomedical implants. Figure 2-2 shows the SEM image of *Desulfovibrio vulgaris* growing on the surface of carbon steel. SRB can use sulfates as their terminal electron acceptors to release sulfides, and utilize other components such as organic carbon (Thauer et al., 2007). In this redox reaction, the energy produced maintains their metabolism. The scheme of sulfate reduction for energy production is described in Figure 2-3 (Pereira et al., 2007). Actually, the sulfate respiration in the cytoplasm is a complex process, which includes three steps: sulfate
activation, cytoplasmic APS reduction, and bisulfite reduction (Pereira, 2007; Hubert and Voordouw, 2007).

\[
\begin{align*}
\text{SO}_4^{2-} + \text{ATP} + 2\text{H}^+ & \rightarrow \text{APS} + \text{PPi} \text{ (Sulfate activation)} \\
\text{APS} + \text{H}^+ + 2\text{e}^- & \rightarrow \text{HSO}_3^- + \text{AMP} \text{ (APS reduction)} \\
\text{HSO}_3^- + 6\text{H}^+ + 6\text{e}^- & \rightarrow \text{HS}^- + 3\text{H}_2\text{O} \text{ (Bisulfite reduction)}
\end{align*}
\]

Reactions (1) and (2) show that in order to activate sulfate, up to 2 ATPs are consumed (Sperling et al., 1998). In Reaction (3), the net synthesis of HSO\(_3^-\) to HS\(^-\) costs almost 3 ATPs. The net energy generation combined Reactions (1), (2), and (3), is about 1 ATP, which means an energy gain for the whole process. The coupling of sulfate respiration and organic oxidation is utilized for energy production.

Metabolic products such as hydrogen sulfide produced by SRB can cause souring in reservoirs and induce serious corrosion (Bagarinao and Vetter, 1986). Hydrogen sulfide is toxic to the human body (Lewandowskiiy et al., 1997; Magot et al., 2000; Loubinoux et al., 2002). Rose et al. (2005) reported that hydrogen sulfide can even protect cancer cells from medical treatment, weakening the therapeutic function of drugs and, thus, leading to the proliferation and infiltration of cancer cells.
Figure 2-2. Biofilm morphology of *D. vulgaris* on the surface of 304 SS coupons by using SEM.

Figure 2-3. The scheme of *D. vulgaris* *Hildenborough* energy generation process coupling lactate oxidation with sulfate respiration. Sulfate processing steps include sulfate transportation, sulfate activation, and sulfate reduction (Pereira et al., 2007).
2.4 Mechanisms of MIC due to SRB

2.4.1 Cathodic depolarization theory (CDT)

Even though microbial activity to corrosion was reported by Garrett et al. in as early as 1891, the mechanism of MIC was still unknown until the Cathodic Depolarization Theory (CDT) was introduced by von Wolzogen Kuehr and von der Vlugt in 1934. CDT is recognized as the first theory to demonstrate the mechanism of MIC using electrochemical reactions (Table 2-2). Electrons originate from the iron dissolution. Water-derived protons are oxidized by accepting electrons to produce hydrogen (cathodic reaction). Hydrogen is used by the hydrogenase catalysis of SRB to release $H_2$ and electrons (cathodic depolarization) (Odom and Peck, 1984). SRB use these electrons for sulfate reduction and produce energy (sulfate respiration). FeS and Fe(OH)$_2$ are the corrosion products yielded in the whole corrosion process (Kakooei et al., 2012).

Table 2-2. CDT mechanism of MIC by SRB (von Wolzogen Kuehr and von der Vlugt, 1934).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anodic reaction (1)</td>
<td>$4Fe \rightarrow 4Fe^{2+} + 8e^-$</td>
</tr>
<tr>
<td>Water dissociation (2)</td>
<td>$8H_2O \rightarrow 8H^+ + 8OH^-$</td>
</tr>
<tr>
<td>Cathodic reaction (3)</td>
<td>$8H^+ + 8e^- \rightarrow 8H_{ads}$</td>
</tr>
<tr>
<td>Cathodic depolarization by hydrogenase (4)</td>
<td>$8H_{ads} \rightarrow 4H_2 \rightarrow 8H^+ + 8e^-$</td>
</tr>
<tr>
<td>Sulfate reduction (5)</td>
<td>$SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O$</td>
</tr>
<tr>
<td>Precipitation (6)</td>
<td>$Fe^{2+} + S^{2-} \rightarrow FeS$</td>
</tr>
<tr>
<td>Precipitation (7)</td>
<td>$3Fe^{2+} + 6OH^- \rightarrow 3Fe(OH)_2$</td>
</tr>
<tr>
<td>Overall reaction (8)</td>
<td>$4Fe + SO_4^{2-} + 4H_2O \rightarrow 3Fe(OH)_2 + FeS + 2OH$</td>
</tr>
</tbody>
</table>
MIC, caused by hydrogenase-positive *D. vulgaris*, provides evidence to support the CDT (Cypionka, 2000; Iverson, 1966; Videla and Herrera, 2005). However, this theory cannot explain the MIC caused by hydrogenase-negative SRB. Additionally, the role of biofilm in MIC is not demonstrated in CDT. A more comprehensive mechanism is required.

### 2.4.2 Biocatalytic cathodic sulfate reduction (BCSR) theory

Biocatalytic cathodic sulfate reduction (BCSR) theory was proposed by Xu and Gu (2011) to illustrate why and how MIC happens due to SRB from the perspective of bioelectrochemistry and bioenergetics. Bioenergetics explains why SRB corrode metal materials. Normally, SRB obtain energy from the oxidation of organic carbon (electron donor), such as lactate. When the organic carbon sources are rich enough, digestion of organic carbon releases energy to support the growth of SRB (Muyzer and Stams, 2008). In this process, oxidation of organic carbon happens in the cytoplasm where the electrons are used for respiration of sulfate (Thauer et al., 2007). The oxidation of lactate coupled with sulfate reduction is shown below as:

**Anodic reaction:** \[ \text{CH}_3\text{CHOHCOO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{CO}_2 + 4\text{H}^+ + 4\text{e}^- \]  

**Cathodic reaction:** \[ \text{SO}_4^{2-} + 9\text{H}^+ + 8\text{e}^- \rightarrow \text{HS}^- + 4\text{H}_2\text{O} \]

The $E^\circ$ value of \( \text{CO}_2 + \text{CH}_3\text{COO}^-/\text{CH}_3\text{CHOHCOO}^- \) couple is $-430\text{mV}$. The reduction potential of \( \text{SO}_4^{2-}/\text{HS}^- \) is $-217\text{mV}$, obtained under the standard conditions of 1M solutes (1 bar gases) at pH 7 and 25°C (Thauer et al., 2007). When Reaction (1) and Reaction (2) are combined together, the reaction cell potential is $+213\text{mV}$. Thus, this
redox reaction is thermodynamically favorable, i.e., energy is released (Thauer et al., 2007).

However, in some conditions, there may be inadequate carbon sources. A typical example is that the bottom layers of biofilms cannot gain enough organic carbon for their metabolism because of the mass transfer barrier of the biofilms. The sessile cells at the bottom are starving and need energy for survival. Since the reduction potential of Fe\(^{2+}/Fe^0\) is \(-447\text{mV}\), which is similar to the CO\(_2\) + CH\(_3\)COO\(^-)/CH\(_3\)CHOHCOO\(^-\) reduction potential (\(-430\text{mV}\)), the starvation of biofilms causes the bioenergetics of SRB to switch from oxidation of organic carbon to the oxidation of elemental iron, which induce MIC pitting. The reactions in BCSR theory are shown below as:

Anodic: \(4\text{Fe} \rightarrow 4\text{Fe}^{2+} + 8e^-\) (Iron dissolution) \(-E^\circ = +447\text{ mV}\) (3)

Cathodic: \(\text{SO}_4^{2-} + 9\text{H}^+ + 8e^- \rightarrow \text{HS}^- + 4\text{H}_2\text{O}\) (BCSR) \(E^\circ = -217\text{ mV}\) (4)

The \(E^\circ\) values are from Thauer et al. (2007). In the combined Reactions (3) and (4), the cell potential is +230mV, which was obtained by subtracting −217mV from +447mV. Gibbs free energy is obtained from the equation of \(\Delta G^\circ = -nF\Delta E^\circ\). From this equation, the coupling of Reactions (3) and (4) obtains a negative Gibbs free energy, which means these reactions are thermodynamically favorable and thus theoretically possible. However, this redox reaction cannot react forward without overcoming a high activation energy. SRB work as the biocatalyst in this redox reaction in the cytoplasm (Xu and Gu, 2011). A cross-cell wall transportation of electrons is required in this process. Figure 2-4 shows the electron transfer scheme of MIC caused by SRB.
In the development of BCSR theory, Xu and Gu (2011) indicated that only sessile cells can directly affect the iron dissolution without consideration of other factors, such as acid attacks. The biofilm of SRB is thought to be responsible for MIC pitting. Planktonic cells do not contribute to iron dissolution, since electrons cannot exist freely in bulk fluid. According to the BCSR theory, iron oxidation occurs extracellularly because of the insolubility of iron which means it cannot diffuse into the cytoplasm unlike soluble organic carbon. This means that extracellular electron transfer is needed for MIC pitting.

2.5 Extracellular electron transfer to support BCSR

The extracellular electron transfer chain is the key to understanding how MIC happens due to SRB. Research of microbial fuel cells (MFCs), which is related to bioelectrochemical energy conversion, involves the mechanism of electron transportation (Schröder, 2007). In the MFC field, the mechanisms of extracellular electron transfer are
mainly classified in two ways: direct electron transport (DET) and mediated electron transport (MET). These two ways for electron transfer are described in Figure 2-5.

Figure 2-5. Simplified EET pathways: (A) DET via contacted cytochrome or via pili, (B) MET via electron mediators (Schröder, 2007).

DET requires physical contact between the cells and the metal surfaces without other diffusional processes (Reguera et al., 2005). Membrane bound proteins associated with outer-membrane (OM) redox proteins such as c-type cytochromes or conductive
nanowires such as pili can be used for DET (Figure 2-5A), since living cells are not considered to be electrically conductive (Schröder, 2007). The microorganisms that are capable of electron transfer relying on DET are known as electrogenic microbes (Chang et al., 2006). Gorby et al. (2006) indicated that these pili probably are purposefully formed by electrogenic bacteria for external electron transfer and energy transport.

MET relies on electron mediators, which are soluble redox-active chemicals, to transport electrons (Figure 2-5B) (Zhou et al., 2012). Direct physical contact between cells and metal surfaces is not necessary. Mediators can be supplied in two ways: externally supplied mediators called exogenous mediators and self-secreted mediators known as endogenous mediators (Schröder, 2007; Wang et al., 2007). Riboflavin, quinone-containing humic acids, phenazines, and flavin adenine dinucleotide (FAD) are well-known endogenous mediators, and ferrocyanide is one of the common exogenous electron mediators (Chen et al., 2013; Durliat et al., 1988; Schröder, 2007).

The research by Hernandez and Newman (2001) show that endogenous humic acids, which have quinone structures, can be reduced to a hydroquinone capable of chelating with insoluble ions to work as electron donors for anaerobic respiration. Through this process, humic acids control the extracellular electrons transferred from microbes to other microbes or electron acceptors. Flavins like riboflavin and FAD are widely used chemical mediators. Riboflavin is also known as water-soluble Vitamin B₂, which is a precursor for the synthesis of FAD and flavin mononucleotide (FMN) (Fischer and Bacher, 2008). Furthermore, FAD and FMN are cofactors of enzymes. They are capable of accepting electrons and catalyzing redox reactions (Tan and Webster, 2012).
The structure of the isoalloxazine ring in flavins mainly determines the capability of electron chelating (Albert, 1950, 1953). The chelation of riboflavin with ferric iron assists to shuttle exogenous electrons and accelerates the oxidation of ferric iron.

Apart from self-secreted mediators, microbes can benefit from electron mediators secreted by other microbes. This often happens in a biofilm consortium, where sessile cells synergistically live together in a biofilm community (O’Toole et al., 2000). *Shewanella oneidensis* have the capability of secreting mediators for electron transportation in both aerobic and anaerobic environments (Canstein et al., 2008; Marsili et al., 2008). *Shewanella* have been proven to synergistically coexist with SRB under anaerobic conditions (McLeod et al., 2004). Therefore, electron transfer shuttles secreted by *Shewanella* can accelerate the electron transfer of SRB, and then increase the respiration of sulfate. MIC pitting due to SRB will be promoted through this process. These mediators are labeled as “MIC promoters” or “MIC accelerators” because they accelerate MIC (Gu and Xu, 2010). The mediator promotion of MIC pitting caused by SRB could support the BCSR theory by demonstrating that the electron transfer chain from extracellular iron oxidation to cytoplasm sulfate reduction is a key bottleneck in the MIC mechanism.

### 2.6 Mitigation of MIC

Several strategies used for MIC mitigation have been developed. Pigging, biocides, and antibiotics are often used to mitigate biofilms (Lewis et al., 2005; Videla, 2002). In industry, pigging is a device used to remove the corrosion deposits in oil pipelines. However, this method is limited to straight pipelines. Tetrakis (hydroxymethyl)
phosphonium sulfate (THPS), glutaraldehyde and other biocides are widely employed to eliminate microorganisms in the oil and gas industry (Little et al., 2007). Because of the diffusion barrier of biofilms, it is difficult for biocides to deal with sessile cells. Ten times or higher concentrations of biocides are required for sessile cells (Mah and O’Toole, 2001b). Given that large amounts of biocides are costly and environmental problems from biocide discharging, it is obvious that better methods for MIC mitigation are desired.

In clinical fields, antibiotics are utilized to inhibit microbial growth in the human body. Due to the presence of various microbes in the human body, MIC may directly contribute to the corrosion of implants. Furthermore, toxic by-products such as hydrogen sulfide generated by microbes can lead to clinical problems, such as impairment of colonic mucosa (Gibson et al., 1991; Roediger et al., 1997; Lewis et al., 2005). Different antibiotics have been studied to inhibit MIC in vitro. Lewis et al. (2005) showed that SRB can be sensitive to metronidazole, which is a nitroimidazole antibiotic. The activity of the SRB was reduced by treatment with metronidazole. However, because of the protective structure of the biofilms and their resistance to antimicrobials, microbes in the human body are difficult to eliminate (Costerton et al., 1999). Better protocols are needed to improve the mitigation of implant MIC by using antibiotics.

In dealing with the problems of MIC mitigation, D-amino acids can be applied to strengthen the effects of biocides and antibiotics (Xu et al., 2012a, 2013b). D-amino acids naturally exist in plants, animals and even in human beings in small amounts (Konno et al., 2009). They affect the properties of peptidoglycan (PG) (Cava et al., 2011). The PG
has negative effects on the osmotic pressure of cells, and sustains the morphology and components of the cells (Turner et al., 2013). The structure of PG is exhibited in Figure 2-6. D-amino acids can work as signal molecules to replace the D-alanine (D-ala) terminus in the cell wall; thus, regulating biofilm dispersal (Lam et al., 2009). D-tyrosine (D-tyr), D-methionine (D-met), D-tryptophan (D-trp), and D-leucine (D-leu) have been verified to have the signal ability increase biofilm dissembling at a low concentration of 10 nM in aerobic cultures of *Bacillus subtilis* (Kolodkin-Gal et al., 2010). Planktonic cells are more susceptible to antimicrobial treatments. Xu et al. (2012a, 2013a) investigated the synergistic treatment of THPS with D-methionine and D-tyrosine to mitigate SRB biofilms. Their research indicated that the combination of 50ppm THPS and 100ppm D-methionine could increase the mitigation as effectively as mitigation by applying 500 ppm THPS alone (Xu et al., 2013).

In view of the enhancement of D-amino acids to the efficacy of biocides, it may be postulated that D-amino acids will cooperate with antibiotics to improve their effectiveness and reduce the dosage. The dispersal of sessile cells into planktonic cells by D-amino acids may make antibiotics more effective. This research focused on investigating a D-amino acid to function as an antimicrobial enhancer combined with an antibiotic against SRB biofilms in the human body.
Figure 2-6. Two types of PG with D-alanine terminus: (A) meso-diaminopimelic acid (DAP) type, (B) Lys-type (Royet and Dziarski, 2007).
CHAPTER 3 MIC PROMOTER INVESTIGATION

3.1 Introduction

As described in the Literature Review, biofilms are responsible for MIC development. The electron transfer process in biofilms is the key to understand the mechanism of MIC. The BCSR theory described in Chapter 2 illustrates the mechanism of MIC caused by SRB, when electrons from iron dissolution are transferred into cytoplasm of SRB for sulfate respiration (Gu and Xu, 2010; Gu, 2012; Thauer et al., 2007). The electron transfer chain explains how SRB biofilms attack steel materials.

3.2 Extracellular electron transfer

Many microorganisms, which have the ability to use electrons crossing the cell wall, are widely utilized in MFC research to convert chemical energy to electrical power (Chang et al., 2006; Gu, 2012; Zhou et al., 2013; Lovley, 2008; Logan and Regan, 2006). EET, which demonstrates the electron transport between electrodes and microbial cells is well developed in the MFC study (Schröder, 2007; Torres et al., 2010).

As mentioned in Chapter 2, DET and MET are the two main ways of EET (Du et al., 2007). Electrogenic microbes mainly use DET for electron transport. However, most of the microbes are not electrochemically active. MET is an effective pathway for the microbes transferring electrons (Mclean et al., 2010). In synergistic biofilms, mediators actively secreted from some microbes can be utilized locally by themselves or for other microbes in the same biofilm community to transfer electrons (Zhou et al., 2012).
3.3 Mechanisms of electron mediators by flavins

Mechanisms of mediators that can shuttle electrons have been investigated. Different structures of mediators will determine the distinct pathway for electron transfer. One of the typical mediators is flavins. In recently updated research, flavins like riboflavin and FAD have been found to follow the proton-coupled electron transfer (PCET) mechanism for extracellular electron transfer rather than simple chelation or binding with metal elements due to the complicated chemical scheme of their isoalloxazine ring (Tan and Webster, 2012). The active sites of the chemical structure of riboflavin are N3 and N5 (Figure 3-1). The charge distribution and electron accepting ability of riboflavin are highly affected by these active nitrogen atoms (Tan and Webster, 2012). In an aqueous solution, flavoquinone which is the fully oxidized state of riboflavin (Fl) is reduced to the flavosemiquinone radical (Fl•−) by accepting one electron from an electron donor. Then, this unstable Fl•− is immediately reduced and protonated to flavohydroquinone (FlH₂), which is the stable reduced form of riboflavin, carrying two electrons and two protons. The proton addition reaction occurs in this reduction reaction. The intermediate reaction products such as reduced Fl²− and protonated FlH• have also been detected (Tan and Webster, 2012). The proton addition reactions have been proven to occur spontaneously at room temperature and atmospheric pressure. In this process, two electrons are accepted by flavins from various electron donors; varying degrees of reduced flavins are capable of chelation or binding with metal ions. Afterwards, the reduced flavins can shuttle electrons into cells for respiration and metabolism.
3.4 MIC and MFC

Because of the significant contribution of the EET process, electron mediators are widely utilized in MFCs. Electrons released from carbon oxidation in biofilms covering anodes are transferred into the solid anode surface of MFC. This electron transfer direction is opposite to that in MIC, whereby electrons flow from iron oxidation into the SRB biofilms for sulfate reduction. However, biofilm electron transfer is reversible.

A typical example is the biocathode, which exploits microbes to form biofilms for catalyzing a reduction reaction, i.e., electron utilization by an electron acceptor (oxidant). The biocathode has been constructed to improve the practicality and sustainability of MFC by eliminating the need for expensive platinum to catalyze the reduction reaction (Clauwaert et al., 2007a, 2007b). The working mechanism of electron transfer within the biocathode is shown in Figure 3-2. In the biocathodic region, the electrons are transferred from the anode to the solid cathode via an external circuit, and then from the cathode to the biofilm (Castelle et al., 2008; He and Angenent, 2006). Either DET or electron mediators can be applied to transport electrons from the cathode surface to the biofilm. Therefore, in this case, the electron transfer direction in the biocathode is exactly the
same as the direction in the MIC, which is electron transfer from outside the cells to the cytoplasm. The well-established knowledge obtained from MFC can be utilized to build the connection between MFC and MIC, and then to support the BCSR theory in MIC.

Figure 3-2. Comparison of electron transfer mechanisms for biocathode in MFC and for BCSR theory in MIC. “Med(red)” represents the form of mediators carrying electrons; Med(ox) represents the form of mediators after releasing electrons. Cyt represents the $c$-type cytochrome embedded in cell membrane to assist electron transfer into cells.

3.5 Objective

The aim of this work was to demonstrate the promotion effects of riboflavin and FAD on EET process in MIC by using SRB based on the BCSR theory. The sessile cells
and planktonic cells were enumerated to identify the influence of riboflavin and FAD to cell growth. The changes of coupon weight, pit depth and pit diameter during addition of riboflavin and FAD were measured in order to gain insight into their roles in promotion of electron transport and MIC pitting. In light of these results and the data, the conclusion, which riboflavin and FAD promote the electron transport between steel materials and cell cytoplasm, could be offered. Meanwhile, these observations also provided insights into the role of electron transfer to MIC pitting.

3.6 Materials and methods

3.6.1 Bacterium and cultivation

The test matrix for promoter test is shown in Table 3-1. A laboratory SRB strain *D. vulgaris* (ATCC 7757) was cultured in ATCC 1249 medium with added 100 ppm L-cysteine (Fisher Scientific, Pittsburgh, PA, USA) as oxygen scavenger and 200 ppm Fe$^{2+}$. The culture medium included 4.1 g MgSO$_4$·7H$_2$O, 1.0 g CaSO$_4$, 1.0 g NH$_4$Cl, 0.5 g K$_2$HPO$_4$, 5.0 g sodium citrate, 1.0 g yeast extract, and 4.5 ml sodium lactate in 1000 ml of distilled water. The medium was autoclaved at 121 °C for 20 minutes. After sterilization, the medium was cooled to room temperature and deoxygenated by sparging nitrogen gas for at least 45 minutes. The final pH of the SRB culture medium was 7.1 ± 0.1.

For the experiment, 10 ppm riboflavin or 10 ppm FAD purchased from Sigma-Aldrich (St. Louis, MO, USA) served as the electron mediator for MIC test in each 120 ml vial (Catalog No. 22374, Wheaton Industries Inc., Millville, NJ, USA), respectively. The 304 SS coupons were prepared through consecutively polishing with 180, 400, 600
grit abrasive papers and cleaned with 75% isopropanol (C\text{3}H\text{8}O) (v/v). Before transfer into the anaerobic chamber, coupons were sterilized with UV light for at least 15 minutes. Each test vial contained three coupons, 1 ml SRB seed, 100 ml culture medium, 100 ppm L-cysteine, 200 ppm Fe\textsuperscript{2+}, and either 10 ppm riboflavin or FAD. A \textit{D. vulgaris} seed culture was used to inoculate each vial to an initial cell concentration of approximately \(10^6\) cells/ml. Incubation lasted 7 days at 37 \(^\circ\)C. All manipulations were completed in an anaerobic glove-chamber environment filled with nitrogen. One ml of the culture medium was taken out by syringe from the test vials every 12 hours for planktonic cell counting. Motile SRB cells were observed using hemocytometer under a light microscope at 400X magnification.

3.6.2 \textit{Corrosion analysis}

After the 7-day incubation, coupons were removed from the vials for corrosion analysis. Deoxygenated phosphate buffered solution (PBS), which included 8.0 g NaCl, 0.2 g KCl, 1.4 g Na\textsubscript{2}HPO\textsubscript{4}\textbullet2H\textsubscript{2}O, and 0.2 g KH\textsubscript{2}PO\textsubscript{4} in 1000 ml distilled water adjusted to pH 7.4, was prepared to rinse the coupons. A sterilized brush-like dipstick from the SRB test kit (Sani-Check\textsuperscript{®} Product #100, Biosan Laboratories, Warren, MI, USA) was utilized to scrape off biofilms from the coupon surface. In order to distribute cells evenly, each dipstick and coupon was sonicated for 15 seconds. Then the dipstick was inserted into the SRB test kit, which included a solid SRB culture medium. The SRB sessile cell count was obtained by comparing the length of time for a FeS black color to appear on the dipstick as calibrated from the vendor (Biosan Laboratories, Inc., 2013). This cell count method provides the Most Probable Number (MPN) of cell counts. Weight loss of
corrosion coupons was obtained after clearing surface biofilms and corrosion products with Clark’s Solution, which is the ASTM G1-03 solution included 0.5 g Sb$_2$O$_3$, and 1.3 g SnCl$_2$ in 25 ml concentrated HCl for preparing corrosion specimen (ASTM G1-3, 2003). The cleaned coupons were then washed in pure isopropanol and dried before weighing.

3.6.3 *Analysis of corroded coupons using scanning electron microscopy (SEM) and infinite focus microscopy (IFM)*

After incubation for 7 days, biofilms on the surface of test coupons were observed using Scanning Electron Microscopy (SEM, Model JSM-6390, JEOL, Tokyo, Japan). Biofilm-covered coupons were first washed with the distilled water to remove planktonic cells, fixed with 4% (v/v) glutaraldehyde at room temperature for at least two hours, then dehydrated at increasing concentrations of isopropanol (25%, 50%, 75%, 100% by volume) for 10 minutes each step, dried with a critical point dryer, and coated with Au. SEM was performed to scan the entire surface of the coupons at magnification of 100-4000X. The size of pits was scrutinized by SEM after removing the biofilms and corrosion products on the coupon surface. Pit depth was measured by Infinite Focus Microscope (IFM, Model ALC13, ALICONA, Austria).

3.6.4 *Measurement of sulfate concentration in culture medium*

The method for determining the concentration of sulfate in the test medium was adapted from the turbidimetric assay used by Xu et al. (2012). The mechanism of this measurement relates to the turbidity caused by precipitated barium sulfate measured at 420 nm wavelength (Kolmert et al., 2000).
3.7 Results

3.7.1 SRB cell count observation

The growth curves of planktonic cells with and without promoters are shown in Figure 3-6. Each data point was the average of three cell counts. The similar growth curves implied that both 10 ppm FAD and 10 ppm riboflavin had no effect on planktonic cell growth after 7-day incubation in anaerobic vials.

The sessile cell counts in Table 3-2 from the Biosan SRB test kit were measured more than three times. Figure 3-6 shows the FeS color responses from the SRB test kit. Results of the sessile cell counts indicated that they were not influenced by either 10 ppm riboflavin or 10 ppm FAD. The SRB sessile cell counts were all $10^2$ cells/cm$^2$. Compared to the typical sessile cell count, which was $10^6$ cells/cm$^2$ on C1018 coupons under the same growth condition, these cell counts on 304 SS are much smaller (Xu et al., 2013). The lower sessile cell counts in the coupon surface led to less severe corrosion of SS by *D. vulgaris*. The established biofilms on the coupon surfaces were observed under SEM shown in Figure 3-7. The similar biofilm morphologies in these SEM images displayed that riboflavin and FAD made no difference in sessile cell coverage. Considering the average coupon surface area of 1.1 cm$^2$ and the sessile cells observed by SEM shown in Figure 3-7, the total sessile cells on the coupon surface should be much more than $10^2$ cells/cm$^2$, which might indicate the inconsistency of Table 3-2 and Figure 3-7. However, the SEM images are just the visible images for a few locations with living cells and dead cells. They could not differentiate the living cells and dead cells, and they should be not used to count cells because they did not reflect the overall coverage. Sessile cell counts
obtained by the SRB test kit in Table 3-2 demonstrated the living SRB sessile cells not the dead cells on coupon surfaces. The SEM images in Figure 3-7 and the sessile cell counts in Table 3-2 both suggested that 10 ppm riboflavin and FAD have no influence on the SRB sessile cell growth.

3.7.2 Corrosion analysis of corroded coupons

The various abiotic controls, which were ATCC 1249 medium only, medium with 10 ppm FAD and medium with 10 ppm riboflavin, were constructed to discern the influence of the riboflavin addition and the FAD addition to abiotic corrosion. The similar surface morphologies of abiotic controls observed by SEM are shown in Figure 3-8. Additionally, the pit depth in this abiotic test was measurement by IFM shown in Figure 3-9. The maximum pit depth of medium only was 2.1 ± 0.2 μm, which was similar to the pit depth when adding 10 ppm FAD, and 10 ppm riboflavin, corresponding to the value of 2.1 ± 0.2 μm and 2.0 ± 0.1 μm, respectively (Figure 3-9). Therefore, it can be concluded that the mediators--riboflavin and FAD alone had no influence on the surface morphology and pitting corrosion of 304 SS coupons without SRB.

In the biotic test with SRB, the specific weight loss, pit depth, pit surface diameter, and pH value data were collected after 7-day incubation. The pH value in each test condition is shown in Table 3-3. It was found that the pH values in SRB cultures with and without an electron mediator were all 6.8.

The influence of promoters on pitting corrosion is demonstrated by the specific weight loss data in Figure 3-10. Results in Figure 3-10 showed that the average weight loss (g/cm²) for coupons immersed in the culture medium with 10 ppm FAD and 10 ppm
riboflavin was 0.0003 ± 0.0001, and 0.0004 ± 0.0001 g/cm², respectively, which was about two times greater than the weight loss in the medium without mediators (0.0002 ± 0.0001 g/cm²). The p-values of these two tests were 0.03 and 0.008, which were much smaller than the normal acceptable threshold value of 0.05. The differences in the specific weight loss in the test conditions were statistically significant. For these three experimental conditions, the only difference in culture media was 10 ppm riboflavin or 10 ppm FAD. Therefore, increases in weight loss corresponded only to the addition of the promoters. General corrosion rates were assessed based on weight loss (Table 3-4). It was averaged over the entire surface. In contrast, with addition of riboflavin and FAD, the corrosion rates were 0.028 ± 0.006 mm/year and 0.022 ± 0.005 mm/year, respectively, almost 2 times larger than 0.012 ± 0.006 mm/year of biotic control without promoters, indicating the significant ability of these promoters accelerate MIC pitting.

The acceleration of pitting corrosion of 304 SS coupons by the mediators was also reflected by the pit depth data. Pit depth was measured by IFM after removing biofilms and corrosion products. IFM was used to scan the entire coupon surface first at a smaller magnification. When a well-defined pit or a pit cluster was found, IFM was set to zoom to obtain a clear image. Compared to the maximum pit depth of 4.8 µm in biotic control without mediators (Figure 3-11), the maximum pit depth due to SRB with the addition of 10 ppm FAD and 10 ppm riboflavin were 6.0 µm and 6.6 µm after the 7-day test, respectively (Figures 3-12 and 3-13). There was more than a 25% increase in both cases.

The average pit depth data was obtained from at least three duplex coupons in each test condition. The addition of 10 ppm FAD and 10 ppm riboflavin increased the
average pit depth to 5.6 ± 0.2 µm, and 6.0 ± 0.4 µm respectively, which was almost 30% larger than the average pit depth of 4.6 ± 0.4 µm without mediators (Figure 3-14). This increase in pit depth directly indicated that the electron mediators could accelerate MIC pitting caused by SRB.

As noted above, the corrosion rate based on the results of weight loss was measured to investigate the development of general corrosion. However, in MIC research, general corrosion rate calculated by weight loss cannot properly reflect pitting corrosion caused by microorganisms, because it is not uniform corrosion as in the case of hydrochloric acid or sulfuric acid corrosion. Biofilms form unevenly and localize in specific areas to cause pitting corrosion. Therefore, pit depth and distribution are more relevant in MIC pitting than the general corrosion rate. Considering the worst-case scenario, assuming constant pit growth, Table 3-5 represents extrapolated yearly pit growth rates. They were 0.289 ± 0.012 mm/year and 0.307 ± 0.019 mm/year for FAD and riboflavin, respectively, while the value was only 0.237 ± 0.018 mm/year without mediators. In comparison with the general corrosion rate obtained from weight loss, the pitting corrosion rate from pit depth was approximately ten times higher, implying a more severe pinhole leak threat.

The surface morphology of coupons and size of pits were also supposed to be affected by the addition of mediators in the presence of SRB. In contrast to the clear and shining surface of coupons in the abiotic test (Figure 3-8), there was obvious pit aggravation on the coupon surface with SRB (Figure 3-16). Meanwhile, in comparing the SEM image in the biotic control (Figure 3-15A), when 10 ppm FAD and 10 ppm
riboflavin were added, crater-like pits were seen after the removal of biofilms (Figures 3-15B, 15C). The maximum pit diameter of test coupons after exposure to SRB without mediators was 4.2 µm, which was much smaller than the pit diameter of 6.2 µm exposed to 10 ppm FAD, and 10.4 µm exposed to 10 ppm riboflavin. Figure 3-16 presents the average pit surface diameter in these three conditions. With addition of 10 ppm riboflavin, the average pit diameter was 8.9 ± 1.3 µm, which was about 3 times larger than the value of 3.8 ± 0.8 µm in SRB culture without mediators. Such results revealed that the promotion of riboflavin and FAD not only influence pit depth, but also pit surface diameter. Those corrosion analysis data verified that MIC pitting corrosion due to SRB can be accelerated by adding electron mediators.

3.7.3 Sulfate concentration in test medium

The residual sulfate concentration directly corresponds to the sulfate consumption through sulfate reduction, and sulfide production. In addition, sulfate reduction is directly related to electron uptake. Therefore, the concentration of residual sulfate could be an indirect mean of correlating extracellular electron transfer and MIC pitting. Table 3-6 illustrated that by adding FAD and riboflavin, the residual sulfate concentrations were reduced from 1.7 mM without mediators to 1.2 mM and 0.8 mM with FAD and riboflavin, respectively. There was about 29% and 53% increase of sulfate consumption with 10 ppm FAD and 10 ppm riboflavin. Combined with the results of specific weight loss in Figure 3-10 demonstrated about 82% and 133% weight loss increase with adding 10 ppm FAD and 10 ppm riboflavin. The correlated trend of sulfate consumption and weight loss
indicated that sulfate would be the terminal electron acceptor rather than other oxidants. The presence of electron mediators promoted sulfate consumption.

3.8 Discussion

The study described above demonstrated that electron mediators promoted MIC pitting caused by *D. vulgaris*. The increases of corrosion parameters including weight loss, pit depth and pit diameter were observed due to the exogenous addition of riboflavin and FAD. Several findings such as synergy among different bacteria, and the theory about the facilitation of electron transfer by mediators can verify more completely and specifically the electron transfer pathway and the influence of mediators to the MIC pitting corrosion.

3.8.1 Electron transfer and microbial synergy in biofilms

The results described in previous tests can be accepted as being valid, since the electron transfer is an essential factor in MIC due to SRB. For DET, a direct connection between cell wall and metal surface is required if there is no pilus structure (Chang et al., 2006; Du et al., 2007; Schröder, 2007). This means that only one monolayer of electrogenic cells will involve in the electron transfer for MIC pitting. Pilus networking among various cells to metal surface enables multiple layers of sessile cells to participate in electron transport, thus, allowing several layers of sessile cells to attack. This could aggravate MIC pitting. In MET, since electron mediators can dissolve into the medium and distribute widely in the biofilm formation, more layers of sessile cells might utilize these mediators for shuttling electrons, thus promoting MIC pitting (Gu, 2012; Zhou et al., 2012).
Biofilm consortia that include various species of bacteria instead of a pure strain microbe are more common. Multispecies of microbes in biofilms live synergistically and benefit each other. Soluble mediators, secreted by non-electrogenic bacteria, could be embedded in this biofilm matrix. They can be used by other electrogenic species such as the corrosive SRB species to promote electron transport for energy production (Zhou et al., 2012). In addition to chemical mediators, some nutrients such as fatty acids are secreted by one special species (like APB) to be used by other microbes like SRB. The scheme of electron transfer by mediators in biofilm consortium is shown in Figure 3-4. The synergetic activities of consortia in biofilms enable various bacteria to maximize their metabolic benefits to each other and maintain the integrity and stability of the whole community (Wolfaardt et al., 1994).

With the insight of the synergetic function of multispecies bacteria in the biofilm formation, the corrosive activities of SRB are not only dependent on their own growth condition, but also growth corresponding to the advantageous metabolic activities of other bacterial species in the biofilm matrix. The MIC pitting corrosion caused by biofilm consortium could be more severe than pure SRB biofilm due to the synergetic benefits of microbes in a biofilm community.
3.8.2 Pathways for electron carrying mediators crossing cell wall

Cross-cell wall electron transfer is a critical step for the redox reactions in cell cytoplasm using extracellular electrons. However, the pathway for mediators taking electrons into cells is still unclear. One reasonable assumption is that mediators pass electron to membrane-bound redox active proteins, for example $c$-type cytochromes. $C$-type cytochrome is a common structure embedded in the cell membrane. Esteve-Núñez et al. (2008) demonstrated that extracytoplasmic cytochromes are almost exclusively located in the periplasm. Outer membranes have the capability to store electrons, and carry out extracytoplasmic electron transfer. Current research shows that electrons from
the oxidation of organic carbons passing out of cells are mainly dependent on the \( c \)-type cytochromes in the phospholipid bilayer (Cournet et al., 2010). The pathway in this research indicates that the electrons in cell cytoplasm are transferred via a menaquinone pool to the inner membrane anchored \( c \)-type cytochrome, then to a periplasmic \( c \)-type cytochrome, and finally to the outer membranes (OM) anchored \( c \)-type cytochromes (Gralnick et al., 2006; Myers and Myers, 2004, 2002, 1997).

Figure 3-4. Possible working mechanism of electron transfer chain from outside the environment into cytoplasm; the green, blue, and purple shapes represent different types of cytochrome; cyt (red) denotes cytochrome carrying electrons; cyt (ox) denotes cytochrome without electrons.

The reversed electron transport pathway from extracellular sites transfer into the cytoplasm of cells has still not been investigated completely. Based on the electron storage activity of cytochrome and the developed intracellular electron transfer method, the hypothesis that the extracellular electron transport method into cells is similar to
intracellular electron transfer out of cells should be investigated (Figure 3-4). The assumption indicates that the reduced mediators carrying electrons first interact with the OM-anchored c-type cytochromes to reduce the OM cytochromes. Through this process, electrons in reduced mediators will be released into the OM cytochromes, which may transfer these electrons to the periplasmic c-type cytochromes. These reduced cytochromes have the ability to contact with the inner membrane anchored c-type cytochromes and traffic electrons to them. When electrons arrive at the inner membranes, various oxidants in cell plasmas, including sulfate, and nitrate, may accumulate to obtain electrons for respiration and energetics. The electron transport into cells through cytochrome requires energy consumption, this may be provided by the cell metabolism. The oxidant reduction by accepting the extracellular electrons produce energy more than the consumption for extracellular electron transfer. The net gain in energy is used for other metabolic activities of cells (Thauer et al., 2007). Further investigations into the exact pathways for mediators carrying electrons into cells are desired.

3.9 Conclusions

1) Riboflavin and FAD did not increase the planktonic and sessile SRB cell counts under test conditions.

2) Without SRB, riboflavin and FAD did not cause significant pitting corrosion of 304 SS.

3) Significant increases of pit depth, pit diameter, and weight loss were observed when a promoter was added to SRB cultures with 304 SS coupons.
4) Riboflavin and FAD worked as electron transfer shuttles to accelerate sulfate reduction and, thus, MIC pitting. This is consistent with the assumption of BCSR.

Table 3-1. Promoter test matrix

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRB strain</td>
<td><em>D. vulgaris</em> (ATCC 7757)</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Culture medium</td>
<td>ATCC 1249 medium</td>
</tr>
<tr>
<td>Initial pH</td>
<td>7.0 ± 0.1 in the medium</td>
</tr>
<tr>
<td>Promoter solution</td>
<td>Riboflavin (10 ppm), FAD (10 ppm)</td>
</tr>
<tr>
<td>Incubation time</td>
<td>7 days</td>
</tr>
<tr>
<td>Material</td>
<td>304 SS</td>
</tr>
</tbody>
</table>

Table 3-2. Sessile cell count after 7-day incubation

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Sessile cell count (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRB culture without mediator</td>
<td>≥10²</td>
</tr>
<tr>
<td>SRB culture with 10 ppm FAD</td>
<td>≥10²</td>
</tr>
<tr>
<td>SRB culture with 10 ppm riboflavin</td>
<td>≥10²</td>
</tr>
</tbody>
</table>
Table 3-3. pH value after 7 days

<table>
<thead>
<tr>
<th>Mediator</th>
<th>pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRB culture without mediator</td>
<td>6.8 ± 0.04</td>
</tr>
<tr>
<td>Abiotic medium without mediator</td>
<td>6.9 ± 0.07</td>
</tr>
<tr>
<td>SRB culture with 10 ppm FAD</td>
<td>6.8 ± 0.05</td>
</tr>
<tr>
<td>SRB culture with 10 ppm riboflavin</td>
<td>6.8 ± 0.03</td>
</tr>
</tbody>
</table>

Table 3-4. General corrosion rate (mm/year) based on weight loss

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Corrosion rate based on weight loss (mm/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.012 ± 0.006</td>
</tr>
<tr>
<td>10 ppm FAD</td>
<td>0.022 ± 0.005</td>
</tr>
<tr>
<td>10 ppm riboflavin</td>
<td>0.028 ± 0.006</td>
</tr>
</tbody>
</table>
Table 3-5. Pitting corrosion rate (mm/year) based on maximum pit depth

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Corrosion rate based on pit depth (mm/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.237 ± 0.019</td>
</tr>
<tr>
<td>10 ppm FAD</td>
<td>0.289 ± 0.012</td>
</tr>
<tr>
<td>10 ppm riboflavin</td>
<td>0.307 ± 0.019</td>
</tr>
</tbody>
</table>

Table 3-6. Residual sulfate concentration after 7-day incubation

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Residual ([\text{SO}_4^{2-}]) (mM)</th>
<th>Increase in sulfate consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.7</td>
<td>0%</td>
</tr>
<tr>
<td>10 ppm FAD</td>
<td>1.2</td>
<td>29%</td>
</tr>
<tr>
<td>10 ppm riboflavin</td>
<td>0.8</td>
<td>53%</td>
</tr>
</tbody>
</table>
Figure 3-5. Planktonic cell count with and without an electron mediator. Initial cell concentration was $10^6$ cells/ml and the experiment was repeated three times.

Figure 3-6. Sani-Check SRB kit test tubes for SRB without mediator, SRB with 10 ppm riboflavin, SRB with 10 ppm FAD, respectively.
Figure 3-7. Sessile SRB cells on 304 SS coupon surface after 7 days of incubation: (A) without any mediators, (B) with addition of 10 ppm FAD, (C) with addition of 10 ppm riboflavin.
Figure 3-8. Surface morphology on 304 SS coupon in abiotic test: (A) with ATCC 1249 medium only, (B) with ATCC 1249 medium and 10 ppm FAD, (C) with ATCC 1249 medium and 10 ppm riboflavin after 7 days.
Figure 3-9. Average pit depth for abiotic control with or without adding electron mediators (each column calculated from at least three duplicate coupons). Error bars represent standard deviation.

Figure 3-10. Specific weight loss after 7-day of SRB incubation with or without an electron mediator (each column calculated from at least three duplicate coupons). Error bars represent standard deviation.
Figure 3-11. Pit depth after 7-day exposure to SRB culture without an electron mediator. The maximum pit depth was 4.8 μm.
Figure 3-12. Pit depth after 7-day exposure to SRB with addition of 10 ppm FAD. The maximum pit depth was 6.0 μm.
Figure 3-13. Pit depth after 7-day exposure to SRB with addition of 10 ppm riboflavin. The maximum pit depth was 6.6 μm.
Figure 3-14. Average pit depth after 7-day SRB incubation with and without an electron mediator (each column calculated from at least three duplicate coupons). Error bars represent standard deviation.
Figure 3-15. Pit morphology on the surface of 304 SS coupon after 7-day exposure to: (A) SRB only without an electron mediator, (B) SRB with addition of 10 ppm FAD, (C) SRB with addition of 10 ppm riboflavin.
Figure 3-16. Average surface pit diameter after 7-day SRB incubation with and without an electron mediator. (Error bars represent standard deviations).
CHAPTER 4  STARVATION TEST TO SUPPORT BCSR THEORY

4.1 Introduction

The BCSR theory proposed by Gu et al. (2009) was discussed in Chapter 2. It indicates that the redox reaction coupling of iron oxidation and sulfate reduction occurs due to the biocatalysis of SRB biofilm. The promoter tests in Chapter 3 corroborate the theory of electron transfer. In this Chapter, a starvation experiment that was constructed and explained will further support BCSR theory from the perspective of bioenergetics.

In nutrient rich conditions, the energy for growth of SRB is obtained from the digestion of organic carbon in SRB cytoplasm. In this process, electrons flow from the carbon oxidation to the sulfate reduction in the cytoplasm (Pereira et al., 2007). However due to dense biofilm formation causing a diffusion barrier, starving SRB may cause the bioenergetics of SRB to switch from oxidation of organic carbon to oxidation of elemental iron. Gu et al. (2009b) used the BCSR theory to explain the mechanism of MIC pitting caused by SRB biofilm. In MIC, the electron donor is Fe$^0$ rather than carbon sources. Thermodynamically, $E^o$ value of $\text{Fe}^{2+}/\text{Fe}^0$ is $-447\text{mV}$, which is more negative than $-430 \text{ mV}$ of CO$_2$ + acetate/lactate. This means that the Fe$^0$ is more energetic than lactate under the conditions defined for $E^o$. Iron oxidation coupled with sulfate reduction provides energy for SRB metabolism.

Additionally, as demonstrated in Chapter 2 and Chapter 3, iron oxidation occurs outside of the cells. The electron transfer from iron dissolution to sulfate reduction is extracellular. Either electron mediators or pili, or direct cell wall contact with a steel surface is required for electron transfer. According to the research of Sherar et al. (2011),
the structure of pili were observed in the carbon source starvation condition. This finding supports the BCSR theory that electrons are transferred from iron oxidation when there is a lack of organic carbon. The starvation makes the biofilm more aggressive toward steels.

4.2 The influences of Cl\textsuperscript{-} and H\textsuperscript{+} on pitting corrosion

In order to clearly demonstrate the mechanism of MIC by SRB, the effects of other corrosive factors (i.e., chloride ions, H\textsuperscript{+}) should also be carefully considered. One critical factor is chloride. Sea water contains chloride ion.

The mechanism of pitting corrosion of stainless steel induced by the chloride ion has been well investigated. It mainly includes diffusion and capillary adsorption (Cheng et al., 1999; Kaneko and Isaacs, 2000; Tsutsumi et al., 2007). Cl\textsuperscript{-} damages the oxide passivation film (Galvele, 1976; Newman, 1987). In anaerobic condition, with presence of microbes, such as SRB, chloride ion can attack stainless steel with migrating to the local corrosion and increasing the local Cl\textsuperscript{-} concentration (Antony et al., 2007).

The hydrogen ion (H\textsuperscript{+}) is another critical factor that affects passivation film formation and pitting corrosion of steel. The passivation film on stainless steel is stable in both neutral and alkaline conditions (Lambert and Muir, 1965). In acidic environments, H\textsuperscript{+} has high diffusion and absorbing ability in the metal surface (Ningshen et al., 2006). It actively affects the cathodic reactions and also the anodic oxidation. The mechanic structure of the protective film will be disrupted by the involvement of hydrogen ions in pitting corrosion.
4.3 Objectives

In order to clearly support the BCSR theory of MIC pitting, starvation tests of 304 SS and 316 SS were constructed. Biofilm formation and surface morphology with various carbon source reductions were observed to connect the growth condition of cells to the MIC pitting corrosion. Weight loss and pit depth data were calculated to link the bioenergetics of SRB to biocorrosion. To study the effects of chloride ion and hydrogen ion on pitting corrosion, biotic tests in different concentrations of Cl\(^-\) and abiotic experiments in both acidic and alkaline conditions were established to eliminate the influence of these factors.

4.4 Materials and methods

4.4.1 Starvation test

The test matrix of starvation is described in Table 4-1. Three carbon source reductions of ATCC 1249 medium were used. Test materials were 304 SS and 316 SS disc. After 3 days of incubation in full strength ATCC 1249 medium for mature biofilm establishment, coupons were transferred from test vials to new sterilized vials with 100 ml fresh ATCC 1249 medium with carbon source reductions of 0%, 90%, and 100%, respectively. All these manipulations were finished in an anaerobic chamber as described in Chapter 3. After 7 days, coupons were removed from vials for corrosion analysis and biofilm observation following the similar procedures in processing the test coupons in Chapter 3.

The method used to prepare the test medium was mixing full strength medium with different carbon concentration. Table 4-2 shows the typical components of ATCC
Table 4-3 represents the ATCC 1249 medium minus any carbon sources, which means that 5.0 g of sodium citrate, 1.0 g of yeast extract, and 4.5 ml of sodium lactate were eliminated from the standard ATCC 1249 medium. The 100 ml test medium with 90% carbon source removed condition was prepared by mixing 90 ml zero carbon medium (Table 4-3) with 10 ml full strength ATCC 1249 medium.

4.4.2 Investigation of chloride ion effect on MIC pitting corrosion

Table 4-4 shows the test matrix of chloride influence experiment to demonstrate the effect of chloride ion on MIC pitting of stainless steel coupons, prepared in the same way as for the promoter test in the Chapter 3. Three Cl⁻ concentrations of 100% (i.e., same as that in the standard ATCC 1249 medium) 10%, and 1% were assembled. Since the main contribution of Cl⁻ concentration in ATCC 1249 medium is 1 g of NH₄Cl, the 10% chloride concentration medium was prepared by mixing 10 ml full strength ATCC 1249 medium with 90 ml ATCC 1249 medium without any addition of NH₄Cl. For the 1% chloride concentration medium, 1 ml full ATCC 1249 medium was used with 99 ml ATCC medium without any NH₄Cl. All these processes were manipulated in an anaerobic chamber. After 7 days, coupons were removed from test vials to obtain corrosion data, and biofilm images.

4.4.3 Test of pH value effect on MIC pitting corrosion

Acetate buffer solutions of pH 4, 5, 6 were used to investigate the influence of H⁺ on pitting corrosion without the biofilm. Test matrix is shown in Table 4-5. The steel material utilized in this test was 304 SS. The different pH buffer solutions were prepared following the instruction of J. Lambert and T.A. Muir (1973) shown in Table 4-6.
The second step of the pH influence test was to employ the phosphate buffer solution of pH 7, 8, 9 to clear the effects of alkaline conditions on the pitting corrosion (Table 4-7). Table 4-7 represents the components of the phosphate buffer solution (Lambert and Muir 1973). The corrosion analysis data including weight loss, pH value and pit depth were obtained after 7 days.

4.5 Results and discussion

4.5.1 Starvation test using 304 SS and 316 SS

After 7 days of incubation in the test media with different carbon source reductions, the sessile cell counts were measured, as listed in Table 4-8 (304 SS) and Table 4-9 (316 SS). The sessile cell counts in both the 304 and starvation test decreased from $10^3$ cells/cm$^2$ (full strength medium) to $10^2$ cells/cm$^2$ with 90% carbon source removed, and then to 10 cells/cm$^2$ with 100% carbon source removed. This sessile cell count decrease indicated that the organic carbon affected the sessile cell growth. The SEM images of biofilm formation in the 304 SS starvation test (Figures 4-3A, 4-4A, and 4-5A), and biofilm formation in the 316 SS starvation test (Figures 4-6A, 4-7 A, and 4-8A) also showed the biofilm density decrease with carbon source reduction. SEM images provided the visible confirmation of the sessile cell counts in Tables 4-8 and 4-9. The color of the culture medium shown in Figure 4-2 gradually became lighter because of less FeS precipitation. This provided the evidence that planktonic cells were influenced by the starvation condition. Additionally, compared with the decrease of sessile counts in 304 SS and 316 SS starvation tests, the pH value (Tables 4-10, and 4-11) shows obvious reduction under starvation conditions. Especially, in 0% concentration of organic carbon
condition, the pH value was around acidic 5. This implies that the lack of carbon source may impact the metabolism of *D. vulgaris*.

Underneath the biofilm, the surface morphology of 304 SS and 316 SS coupons also reflected starvation conditions. In the 304 SS starvation test, the maximum pit diameter of 11.6 µm and 7.9 µm were yielded in the 0% carbon concentration starvation, and 10% carbon source starvation, respectively. It was more than 30% larger than the pit diameter of 5.9 µm in full strength medium condition (Figures 4-3B, 4-4B, and 4-5B). Even though the pit size in the starvation test using 316 SS (Figures 4-6B, 4-7B, and 4-8B) was much smaller than that of same test condition using 304 SS, this was because of the more corrosion resistant property of 316 SS. It still shows the same trend of increase in 0%, 90%, and 100% carbon source reductions. The average pit depth data of 304 SS starvation in Figure 4-9 shows an increase from 4.0 ± 0.7 µm in 100% carbon source medium to 5.3 ± 0.2 µm in 10% carbon concentration medium, and 6.1 ± 0.5 µm in 0% carbon concentration medium. The maximum pit depth profiles of 304 SS starvation obtained by IFM are shown in Figures 4-13, 4-14, 4-15. They match the SEM biofilm observation and average pit depth analysis. Average pit depth data of 316 SS in Figure 4-11 and maximum pit depth profiles in Figures 4-16, 4-17, 4-18 also helped to prove that with carbon source reduction, the biofilm became more aggressive and induced deeper pit depth despite the factor that less carbon source led to lower biofilm density on the coupon surface.

Specific weight loss is another factor that reflects the severity of MIC pitting corrosion. Figure 4-10 shows the weight loss of 304 SS starvation increased from
0.00019 g/cm$^2$ (full strength medium) to 0.00025 g/cm$^2$ (90% organic carbon removed), and to 0.00044 g/cm$^2$ (100% carbon removed), respectively. Figure 4-12 also shows the similar changes of weight loss using 316 SS coupons in carbon source reduction experiments. Both pit depth and weight loss consistently suggested that lack of carbon source caused *D. vulgaris* to switch from organic carbon to elemental iron, causing more severe MIC pitting.

4.5.2 *Chloride ion influence on MIC test*

As described before, chloride ions can dissolve the protective passive film and cause pitting corrosion. The chloride source in the starvation test originated from 1g/L NH$_4$Cl in the medium. The experiment was constructed to demonstrate the influence of different chloride concentrations on MIC pitting corrosion. Figure 4-19 shows the weight loss of abiotic control in ATCC 1249 medium with 99%, 90%, 0% chloride removed. The weight loss in the abiotic test without SRB growing shows no decrease along with chloride concentration changes. The pit depth profile of the abiotic test in full strength medium without chloride removed presented in Figure 4-21, indicates that no well-defined pit was obtained by IFM. Both weight loss and pit depth in abiotic test with chloride concentration decrease proved that at least with 1 g/L NH$_4$Cl, chloride didn’t play a significant role in corrosion. Comparing the weight loss of different chloride concentration changes in abiotic control (<0.0001 g/cm$^2$) to the weight loss of 0.0002 g/cm$^2$ in biotic test, it implies that the main contribution of pitting corrosion did not originate from chloride.
In the biotic test, weight loss of 0.00015 g/cm² (99% chloride removed) and 0.00015g/cm² (90% chloride removed) were smaller than the weight loss of 0.0002 g/cm² in full strength medium without any chloride removed (Figure 4-20). The difference was very small and insignificant. This demonstrated that the chloride concentration may not influence the MIC pitting corrosion without considering other factors in this test. From Figures 4-22 to 4-24, comparing the maximum pit depth in full strength medium test with 90% chloride removed medium and 99% chloride removed medium (3.9 µm vs 4.0 µm and 3.8 µm, respectively), there was no significant difference. Both the weight loss data and pit depth profile suggested that the effect of chloride on MIC pitting was also not significant in this work.

4.5.3 pH influence experiment

Due to the pH decrease in the starvation test (Tables 4-8 and 4-9), the influence of pH on corrosion should be included. The test material here was 304 SS. Acetate buffer solutions of 100 ml with pH value of 4, 5, 6 were added in each 125 ml test vial with three 304 SS coupons. A slight pH value drop in each pH buffer condition is shown in Figure 4-25 after 7 days of incubation. Figure 4-26 reflects that the specific weight loss was 0.0003 g/cm², 0.0002 g/cm², 0.0002 g/cm² under the acidic condition of pH 4, 5, 6, respectively. The weight loss in alkaline condition of pH 7, 8, 9 was 0.0001 g/cm², 0.0002 g/cm², and 0.0002 g/cm², respectively. After 7 days of incubation in full strength ATCC 1249 medium, the weight loss of 304 SS coupons was 0.0002 g/cm² with a pH value of 7.0 in the medium. With carbon source reduction (90%, 100%), weight loss increased to 0.0003 g/cm² with pH value of 6, and 0.0004 g/cm² with pH value of 5.
Compared to the weight loss in starvation, and pH influence test, even though H\textsuperscript{+} contributed to the corrosion, there still was approximately 0.0002 g/cm\textsuperscript{2} weight loss change in 0% carbon starvation test obtained from the addition of SRB. The weight loss change of 0.0001 g/cm\textsuperscript{2} in 10% carbon starvation test came from the corrosive activities of SRB. Overall, even considering the influence of pH on the corrosion, the weight loss data in pH influence test still supported the bioenergetics of SRB in starvation test as the critical factor for more severe MIC pitting.

Both the chloride influence test and the pH influence test demonstrated that without adding SRB, Cl\textsuperscript{-} and H\textsuperscript{+} had not influenced the corrosion. However, compared with the corrosion analysis data with SRB involvement, the significant-changes illustrated that SRB biofilm’s switching of electron donor was responsible for the MIC pitting. Thus, it can be concluded that BCSR could explain the underlining mechanism of MIC pitting by SRB.

In fact, elemental iron metabolized as a fuel material (electron donor) by microbes has been studied elsewhere (Chastain and Kral, 2010; Xu and Gu, 2011; Dake Xu et al., 2013). Xu and Gu (2014) constructed a \textit{D. vulgaris} starvation test using carbon steel. Their results verified that the increased weight loss and pit depth of carbon steel observed were due to the aggressive SRB biofilm induced by carbon source reduction. SRB cells form dense and mature biofilm on the carbon steel surface shown in Figure 4-1. Biofilms on the 304 SS had the isolated island structures (Figure 4-1). The structures and morphology of biofilms on these two different materials were different. This provides the visual evidence that the sessile cell counts on the stainless steel surface ($10^3$ cells/cm\textsuperscript{2})
were much lower than on the carbon steel surface ($10^6$ cells/cm$^2$). Weight loss and pit depth obtained from the stainless steel starvation test were also much smaller than that from carbon steel starvation test reported by Xu and Gu (2014). The 304 SS and 316 SS starvation test results combined with the starvation test using carbon steel support the BCSR theory that SRB sessile cells switched from carbon source oxidation to iron oxidation causes MIC pitting.

Figure 4-1. Images of SRB biofilms on different coupon surfaces.

4.6 Conclusion

1) Weight loss, pit diameter and pit depth increased after the 7-day incubation of pre-established SRB biofilm in a reduced carbon source medium.
2) The starvation condition allowed the SRB biofilm to become more corrosive toward 304 SS and 316 SS despite a reduced sessile cell density.

3) The SRB switching from organic carbon oxidation to elemental iron oxidation occurred when there was a lack of carbon sources.

4) The results of this work supported the BCSR theory that elemental iron replaced organic carbon as an electron donor, leading to MIC pitting due to SRB.

5) Chloride and pH did not influence MIC in the test conditions. The change in the bioenergetics of SRB was responsible for MIC pitting.

Table 4-1. Starvation test matrix

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRB strain</td>
<td><em>D. vulgaris</em> (ATCC 7757)</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Culture medium</td>
<td>ATCC 1249 medium, modified ATCC1249 medium</td>
</tr>
<tr>
<td>Initial pH</td>
<td>7.0 ± 0.1 in the medium</td>
</tr>
<tr>
<td>Carbon source concentration</td>
<td>0%, 10%, 100% of that in the ATCC 1249 medium</td>
</tr>
<tr>
<td>Incubation time</td>
<td>3 days in full-strength medium to obtain mature biofilms, 7 days in subsequent carbon starvation test</td>
</tr>
<tr>
<td>Coupon</td>
<td>304 SS and 316 SS</td>
</tr>
</tbody>
</table>
Table 4-2. Components of ATCC 1249 medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component I</td>
<td>MgSO₄</td>
<td>2.0 g</td>
</tr>
<tr>
<td></td>
<td>Na₃C₆H₅O₇ (Trisodium Citrate)</td>
<td>5.0 g</td>
</tr>
<tr>
<td></td>
<td>CaSO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td></td>
<td>NH₄Cl</td>
<td>1.0 g</td>
</tr>
<tr>
<td></td>
<td>Distilled Water</td>
<td>400 ml</td>
</tr>
<tr>
<td>Component II</td>
<td>K₂HPO₄</td>
<td>0.5 g</td>
</tr>
<tr>
<td></td>
<td>Distilled Water</td>
<td>200 ml</td>
</tr>
<tr>
<td>Component III</td>
<td>NaC₃H₅O₇ (Sodium Lactate)</td>
<td>3.5 g</td>
</tr>
<tr>
<td></td>
<td>Yeast Extract</td>
<td>1.0 g</td>
</tr>
<tr>
<td></td>
<td>Distilled Water</td>
<td>400 ml</td>
</tr>
<tr>
<td>Component IV</td>
<td>Filter-sterilized 5% (wt) (NH₄)₂Fe(SO₄)₂ (ferrous ammonium sulfate). Add 2 ml of this solution to 100 ml of medium before inoculation.</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-3. ATCC 1249 medium minus carbon sources

<table>
<thead>
<tr>
<th>Component</th>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component I</td>
<td>MgSO₄</td>
<td>2.0 g</td>
</tr>
<tr>
<td></td>
<td>CaSO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td></td>
<td>NH₄Cl</td>
<td>1.0 g</td>
</tr>
<tr>
<td></td>
<td>Distilled Water</td>
<td>400 ml</td>
</tr>
<tr>
<td>Component II</td>
<td>K₂HPO₄</td>
<td>0.5 g</td>
</tr>
<tr>
<td></td>
<td>Distilled Water</td>
<td>200 ml</td>
</tr>
<tr>
<td>Component IV</td>
<td>Filter-sterilized 5% (wt) (NH₄)₂Fe(SO₄)₂ in distilled water. Add 2 ml of this solution to 100 ml of medium before inoculation.</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-4. Test matrix for chloride influence

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRB strain</td>
<td><em>D. vulgaris</em> (ATCC 7757)</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Culture medium</td>
<td>ATCC 1249 medium, modified ATCC 1249 medium</td>
</tr>
<tr>
<td>Initial pH</td>
<td>7.0 ± 0.1 in the medium</td>
</tr>
<tr>
<td>Chloride concentration</td>
<td>1%, 10%, 100% of that in the ATCC 1249 medium</td>
</tr>
<tr>
<td>Incubation time</td>
<td>7 days in subsequent chloride test</td>
</tr>
<tr>
<td>Coupon</td>
<td>304 SS</td>
</tr>
</tbody>
</table>
Table 4-5. Test matrix for pH influence

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions (Abiotic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37˚C</td>
</tr>
<tr>
<td>Test solutions</td>
<td>Acetate buffer solution, phosphate buffer solution</td>
</tr>
<tr>
<td>Initial pH</td>
<td>Acetate buffer solution (pH 4, 5, 6), phosphate buffer solution (pH 7, 8, 9)</td>
</tr>
<tr>
<td>Incubation time</td>
<td>7 days</td>
</tr>
<tr>
<td>Coupon</td>
<td>304 SS</td>
</tr>
</tbody>
</table>

Table 4-6. Acetate acid solution with pH 3-6*

<table>
<thead>
<tr>
<th>pH</th>
<th>vol. of 0.1M acetic acid (ml)</th>
<th>vol. of 0.1M sodium acetate (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>982</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>847</td>
<td>153</td>
</tr>
<tr>
<td>5</td>
<td>357</td>
<td>643</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>948</td>
</tr>
</tbody>
</table>

*Consisted of the following solutions: (1) 0.1M acetic acid 2) 0.1M sodium acetate (tri-hydrate) (13.6g/L) per the instruction of Lambert and Muir (1973).

Table 4-7. Phosphate buffer solution with pH 7-9**

<table>
<thead>
<tr>
<th>pH</th>
<th>vol. of phosphate (ml)</th>
<th>vol. of 0.1M HCl (ml)</th>
<th>vol. of 0.1M NaOH (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>756</td>
<td>244</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>955</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>955</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

**Consisted of the following solutions: (1) 0.1M disodium hydrogen phosphate (14.2g/L) (2) 0.1M HCl (3) 0.1 M NaOH per the instruction of Lambert and Muir (1973).
Table 4-8. Sessile cell count after 7 days in 304 SS starvation test

<table>
<thead>
<tr>
<th>Test condition</th>
<th>Sessile Cell Count (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full medium (ATCC 1249 Medium)</td>
<td>$\geq 10^3$</td>
</tr>
<tr>
<td>Full medium with 90% carbon source removed</td>
<td>$\geq 10^2$</td>
</tr>
<tr>
<td>Full medium with 100% carbon source removed</td>
<td>$\geq 10$</td>
</tr>
</tbody>
</table>

Table 4-9. Sessile cell count in 316 SS starvation test after 7 days

<table>
<thead>
<tr>
<th>Test condition</th>
<th>Sessile Cell Count (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full medium (ATCC 1249 Medium)</td>
<td>$\geq 10^3$</td>
</tr>
<tr>
<td>Full medium with 90% carbon source removed</td>
<td>$\geq 10^2$</td>
</tr>
<tr>
<td>Full medium with 100% carbon source removed</td>
<td>$&lt; 10$</td>
</tr>
</tbody>
</table>
Table 4-10. pH value after 7-day 304 SS starvation test

<table>
<thead>
<tr>
<th>Test condition</th>
<th>pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 1249 medium with 100% carbon</td>
<td>5.6 ± 0.02</td>
</tr>
<tr>
<td>source removed</td>
<td></td>
</tr>
<tr>
<td>ATCC 1249 medium with 90% carbon</td>
<td>6 ± 0.01</td>
</tr>
<tr>
<td>source removed</td>
<td></td>
</tr>
<tr>
<td>Full strength ATCC 1249 medium</td>
<td>6.7 ± 0.02</td>
</tr>
</tbody>
</table>

Table 4-11. pH value after 7-day 316 SS starvation test

<table>
<thead>
<tr>
<th>Test condition</th>
<th>pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 1249 medium with 100% carbon</td>
<td>5.1 ± 0.01</td>
</tr>
<tr>
<td>source removed</td>
<td></td>
</tr>
<tr>
<td>ATCC 1249 medium with 90% carbon</td>
<td>5.7 ± 0.01</td>
</tr>
<tr>
<td>source removed</td>
<td></td>
</tr>
<tr>
<td>Full strength ATCC 1249 medium</td>
<td>6.6 ± 0.03</td>
</tr>
</tbody>
</table>
Figure 4-2. Anaerobic vials after 7-day starvation test using 304 SS coupons: (1) ATCC 1249 medium with 0% carbon source, (2) ATCC 1249 medium with 10% carbon source, (3) ATCC 1249 medium with 90% carbon source, (4) ATCC 1249 medium with 100% carbon source, and (5) full strength ATCC 1249 medium after 10 days of incubation.
Figure 4-3. SEM images for 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in full strength ATCC 1249 medium: (A) Biofilm formation, (B) Surface morphology after removing the biofilm and corrosion products.
Figure 4-4. SEM images for 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in modified ATCC 1249 medium with 90% carbon source removed: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products.
Figure 4-5. SEM images for 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in modified ATCC 1249 medium with 100% carbon source removed: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products.
Figure 4-6. SEM images for 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in full strength ATCC 1249 medium: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products.
Figure 4-7. SEM images for 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 90% carbon source removed: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products.
Figure 4-8. SEM images for 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 100% carbon source removed: (A) Biofilm formation, (B) Surface morphology after removing biofilm and corrosion products.
Figure 4-9. Average pit depth of 304 SS coupons after the starvation test.

Figure 4-10. Specific weight loss of 304 SS coupons after the starvation test.
Figure 4-11. Average pit depth of 316 SS coupons after the starvation test.

Figure 4-12. Specific weight loss of 316 SS coupons after the starvation test.
Figure 4-13. Maximum pit depth of 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in full strength ATCC 1249 medium. The maximum pit depth was 4.6 μm.

Figure 4-14. Maximum pit depth of 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 90% carbon source removed. The maximum pit depth was 5.6 μm.
Figure 4-15. Maximum pit depth of 304 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 100% carbon source removed. The maximum pit depth was 6.6 μm.

Figure 4-16. Maximum pit depth of 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in full strength ATCC 1249 medium. The maximum pit depth was 3.8 μm.
Figure 4-17. Maximum pit depth of 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 90% carbon source removed. The maximum pit depth was 4.3 μm.

Figure 4-18. Maximum pit depth of 316 SS coupons in starvation test incubated in full-strength ATCC 1249 medium for 3 days and then followed by 7-day incubation in ATCC 1249 medium with 100% carbon source removed. The maximum pit depth was 5.2 μm.
Figure 4-19. Specific weight loss of 304 SS coupons in abiotic test with chloride concentration variation.

Figure 4-20. Specific weight loss of 304 SS coupons in biotic test with chloride concentration variation.
Figure 4-21. Pit depth profile of 304 SS after 7 days in abiotic test using full strength ATCC 1249 medium without chloride removed. No well-defined pit was observed.

Figure 4-22. Pit depth profile of 304 SS in SRB biotic test after 7 days using full strength ATCC 1249 medium without chloride removed. The maximum pit depth was 3.9 µm.
Figure 4-23. Pit depth profile of 304 SS in SRB biotic test after 7 days using full strength ATCC 1249 medium with 90% chloride removed. The maximum pit depth was 4.1 µm.

Figure 4-24. Pit depth profile of 304 SS in SRB biotic test after 7 days using full strength ATCC 1249 medium with 99% chloride removed. The maximum pit depth was 3.8 µm.
Figure 4-25. pH value after 7 days of corrosion test in phosphate buffer solution and acetate buffer solution with different pH values.

Figure 4-26. Specific weight loss of 304 SS coupons after 7-day test in phosphate buffer solution and acetate buffer solution with different pH values.
CHAPTER 5  MITIGATION OF MIC USING A COCKTAIL OF AN ANTIBIOTIC AND A D-AMINO ACID

5.1 Introduction

As mentioned in Chapter 2, THPS and glutaraldehyde are two common green biocides, which can be degraded and used safely. Due to increased environmental restrictions, a more effective biocide treatment with a biocide enhancer is desired. A synergistic cocktail of 1 ppm (w/w) D-tyrosine and 50 ppm THPS (active concentration) had equal efficacy as 100 ppm THPS in preventing biofilm establishment and removal of well-developed *D. vulgaris* biofilm in lab tests (Xu et al., 2012). In large industrial applications, one ppm of D-tyrosine costs much less than high concentrations of THPS. D-methionine combined with THPS was tested to assist biofilm dispersal and to mitigate MIC pitting. Xu et al. (2013) elucidated that 50 ppm THPS enhanced by 100 ppm D-methionine was more efficacious in MIC mitigation than 500 ppm THPS alone.

Instead of the biocide application used in industrial fields, antibiotics are commonly used to inhibit microbial growth in clinical treatment. Considering the distribution of SRB in the human body, methods of SRB mitigation in clinical field should be introduced.

5.2 Antibiotics against anaerobic microbes

In the human body, SRB can use organic carbon sources (i.e. lactate) as electron donors to produce hydrogen sulfide by sulfate respiration (Lewis et al., 2005). SRB are suspected as the cause of biocorrosion of implant materials (Eliades and Athanasion, 2002; Jayaraman et al., 1999). Different antibiotics have been studied to inhibit SRB
growth in vitro. SRB have been found to be sensitive to metronidazole. The colonic ulceration caused by anaerobic microbes (i.e., SRB) has been prevented with pretreatment of metronidazole (Ohkusa et al., 1987). Metronidazole is favorable in pharmacokinetic and pharmacodynamic properties (Löfmark et al., 2010).

The mechanism of metronidazole against anaerobic microbes is mainly related to inhibition of DNA synthesis and structural breakage (Diniz et al., 2000; Land and Johnson, 1999; Löfmark et al., 2010). Metronidazole passes through anaerobic cell walls through passive diffusion into the cytoplasm. It is oxidized by accepting one electron to convert to a new form of short-lived nitroso free radical in the cytoplasm (Löfmark et al., 2010). This converted form of metronidazole, which is cytotoxic, interacts with the DNA molecule and disrupt DNA synthesis causing structural degradation and cell death (Diniz et al., 2000; Land and Johnson, 1999). Metronidazole is less active in aerobic microbes, since aerobic bacteria lack the necessary electron transfer proteins in their cytoplasm to active metronidazole.

5.3 D-amino acids as antibiotic enhancers

Metronidazole is metabolized in the liver, and eliminated from the urine (Löfmark et al., 2010). A high concentration of metronidazole may cause pressure for metabolism, and damages both the liver and kidney. A better working method of metronidazole to treat anaerobic microbial biofilm is required to increase the efficacy and lower the dosage. At first, in vitro lab tests are needed.

As described before, D-amino acids have been shown to signal biofilm dispersal and to prevent biofilm formation (Kolodkin-Gal et al., 2010; Konno et al., 2009). With
mixture of D-amino acids and biocides, MIC mitigation has been enhanced. D-amino acids may enhance the mitigation of MIC using antibiotics.

5.4 Objectives

A mitigation test was constructed to demonstrate the enhancing effect of D-methionine to antibiotics treatment of an SRB biofilm. Planktonic and sessile cell counts were enumerated to reflect the influence of the cocktail treatment. Weight loss and pit depth data were obtained to prove the enhancement of mitigation by using the binary combination of D-methionine and metronidazole. Optimal dosage of metronidazole and D-methionine was determined.

5.5 Materials and methods

In the mitigation test, carbon steel X65 coupons were used as test materials instead of the 304 SS coupons because the fluffy biofilm formation on the surface of 304 SS coupons were too easy to treat in short-term lab tests. The starting sessile cell counts are not sufficiently high to reflect large logarithmic reductions after an antibiotic treatment. In order to obtain worst-case scenario, the relatively dense and mature biofilm on carbon steel surface was used as the target for antibiotics treatment. The surface area of this X65 coupon was around 4.0 cm$^2$. The test matrix is described in Table 5-1.

5.5.1 Prevention test

One X65 coupon with 1 ml *D. vulgaris* seed culture were introduced in 100 ml ATCC 1249 medium in each 120 ml anaerobic vial. The culture media in the vials were treated with different concentrations of metronidazole and D-methionine. Incubation lasted for 7 days. The planktonic cell count in each vial was enumerated every 12 hours
using a hemocytometer under a light microscope at 400X magnification. After 7 days, coupons were taken out from anaerobic vials for sessile enumeration and biofilm observation by SEM. Weight loss data and pit depth data were obtained after removing biofilms and corrosion products from coupon surfaces.

5.5.2 Removal test

One ml *D. vulgaris* seed culture was used to inoculate in 100 ml ATCC 1249 medium without any treatment chemicals in an anaerobic vial for mature biofilm to establish. After 3 days, X65 coupons with mature biofilms were taken out from the vials and immersed in Petri dishes containing a phosphate buffer solution (PBS) with different treatment doses of metronidazole and D-methionine for 3 hours. There were 8.0 g NaCl, 0.2 g KCl, 1.4 g Na₂HPO₄·2H₂O, and 0.2 g KH₂PO₄ in each 1000 ml distilled water in PBS. The pH value of the buffer was adjusted to 7.4. All the manipulations were done in an anaerobic glove-box. Coupons after treatment were transferred outside of the chamber for sessile cell counting and biofilm observation under SEM.

5.6 Results and discussion

5.6.1 Prevention of SRB biofilm establishment

The growth condition of planktonic cells treated with different concentrations of metronidazole is needed to find the optimal dosage of mitigation. Figure 5-1 elucidates that 200 ppm metronidazole was more effective in inhibiting the growth of planktonic SRB cells. Only a slight decrease of planktonic cell count was observed with 50 ppm and 100 ppm metronidazole. Table 5-3 shows the sessile cell counts with different antibiotics treatments in the biofilm prevention test. Sessile cells treated with 50 ppm and 100 ppm
metronidazole revealed no significant reduction by adding D-methionine. When treated with a 200 ppm concentration of metronidazole, the sessile cell count reflected a 4-log reduction. The addition of 100 ppm D-methionine into 200 ppm metronidazole made one more log reduction to 10 cells/cm². SEM images of biofilm formation represented in Figures 5-2 to 5-5 were also reproducible with the sessile cell counts (Table 5-3). Sessile cells were barely visible in the test condition of 200 ppm metronidazole + 100 ppm D-methionine (Figure 5-5). pH values in Table 5-2 were above 6, which indicated unlikely acidic pH influence to the mitigation test.

Specific weight loss in the prevention test is shown in Figures 5-6, 5-7 and 5-8. In Figure 5-6, the weight loss of carbon steel X65 treated with 50 ppm metronidazole + 100 ppm D-methionine was 0.0036 g/cm², indicating an almost 32% reduction from 0.0053 g/cm² without treatment. Compared with the 15% reduction of weight loss treated with a high concentration of 500 ppm D-methionine, suggests that the cocktail of 50 ppm metronidazole and 100 ppm D-methionine had a better effect on MIC mitigation than 200 ppm metronidazole alone. The D-amino acid alone was not effective. Figure 5-7 and Figure 5-8 also displays the similar trend of weight loss changes for treatments with 100 ppm metronidazole and 200 ppm metronidazole, respectively. In Figure 5-8, the weight loss decreased from 0.0053 g/cm² without antibiotics treatment to 0.0032 g/cm² with 200 ppm metronidazole. By adding 100 ppm D-methionine, the weight loss decreased furthermore to 0.0025 g/cm². This additional 22% reduction was caused by the addition of D-methionine.
The pit depth reduction trends shown in Figures 5-9, 5-10, and 5-11 were consistent with the changes in weight loss. In Figure 5-11, there was 54% pit depth decrease from the average pit depth of 16.2 µm without treatment to that of 7.5 µm treated with 200 ppm metronidazole. A further decrease of pit depth to 6.0 µm was produced by adding 100 ppm D-methionine. Both the weight loss data and pit depth data proved that antibiotics treatment could be more effective by adding the D-methionine enhancer. The IFM image of maximum pit depth in each test condition is exhibited in Figures 5-12 to 5-22. The maximum pit depth profile was consistent with the average pit depth data.

5.6.2 Removal of established SRB biofilm

Table 5-4 shows the sessile cell counts in the removal test. It indicates a 2-log reduction of sessile cell count from $10^4$ cells/cm$^2$ without treatment to $10^2$ cells/cm$^2$ with 100 ppm metronidazole + 50 ppm D-methionine, or 100 ppm metronidazole + 100 ppm D-methionine treatment. A further cell count decrease to 10 cells/cm$^2$ was obtained by the 200 ppm metronidazole + 50 ppm D-methionine, or 200 ppm metronidazole + 100 ppm D-methionine treatment. Similar results were reflected in the biofilm formation shown in Figures 5-23 to 5-26. Biofilm became fluffier when treated with 200 ppm metronidazole. Sessile cells were barely visible in the test condition of 200 ppm metronidazole + 100 ppm D-methionine. Both sessile cell counts and biofilm observation supported the mitigation effect of metronidazole enhanced by D-methionine in the 3-hour biofilm removal test and the 7-day biofilm prevention test.
The mechanism of how D-methionine enhances antibiotic treatment might be following the research of Cava et al. (2011), Lam et al. (2009), Xu et al. (2012, 2013). D-methionine works as a trigger to disperse the SRB biofilm rather than being biocidal toward SRB cells. The dispersed sessile cells would be easier to be treated once they become planktonic cells. The exact mechanism of D-methionine working as an enhancer to antibiotic treatment should be further investigated.

5.7 Conclusion

1) D-methionine enhanced the efficacy of metronidazole prevention of SRB biofilm establishment and removal of SRB biofilm.

2) D-methionine alone with a high concentration of 500 ppm was inadequate.

3) The decrease of weight loss and pit depth with treatment using a cocktail of D-methionine and metronidazole proved that D-methionine can be used as an enhancer for MIC mitigation using antibiotics.

4) The optimal dosage of metronidazole and D-methionine for MIC mitigation was 200 ppm metronidazole + 100 ppm D-methionine. In both the prevention test and the removal test, sessile cell count was decreased to 10 cells/cm$^2$ from the initial sessile cell count of $10^6$ cells/cm$^2$ and $10^4$ cells/cm$^2$, respectively.
Table 5-1. Mitigation test matrix

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRB strain</td>
<td><em>D. vulgaris</em> (ATCC 7757)</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Culture medium</td>
<td>ATCC 1249 medium</td>
</tr>
<tr>
<td>Initial pH</td>
<td>7.0 ± 0.1 in the medium, 5.69 ± 0.1 in the treatment solution</td>
</tr>
<tr>
<td>Antibiotics solution</td>
<td>50, 100, 200 (ppm)</td>
</tr>
<tr>
<td>Test time</td>
<td>Prevention test (7 days), removal test (3 days)</td>
</tr>
<tr>
<td>Material</td>
<td>Carbon steel X65</td>
</tr>
</tbody>
</table>

Table 5-2. pH value in biofilm prevention test after 7-day treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abiotic control</td>
<td>6.8 ± 0.03</td>
</tr>
<tr>
<td>No treatment</td>
<td>6.6 ± 0.04</td>
</tr>
<tr>
<td>500 ppm D-met</td>
<td>6.6 ± 0.01</td>
</tr>
<tr>
<td>50 ppm Metronidazole</td>
<td>6.2 ± 0.05</td>
</tr>
<tr>
<td>50 ppm D-met + 50 ppm Metronidazole</td>
<td>6.6 ± 0.02</td>
</tr>
<tr>
<td>100 ppm D-met + 50 ppm Metronidazole</td>
<td>6.3 ± 0.03</td>
</tr>
<tr>
<td>100 ppm Metronidazole</td>
<td>6.0 ± 0.03</td>
</tr>
<tr>
<td>50 ppm D-met + 100 ppm Metronidazole</td>
<td>6.1 ± 0.02</td>
</tr>
<tr>
<td>100 ppm D-met + 100 ppm Metronidazole</td>
<td>6.0 ± 0.04</td>
</tr>
<tr>
<td>200 ppm Metronidazole</td>
<td>6.1 ± 0.02</td>
</tr>
<tr>
<td>50 ppm D-met + 200 ppm Metronidazole</td>
<td>6.1 ± 0.02</td>
</tr>
<tr>
<td>100 ppm D-met + 200 ppm Metronidazole</td>
<td>6.1 ± 0.02</td>
</tr>
<tr>
<td>Treatment</td>
<td>Sessile cell count (cells/cm²)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>No treatment</td>
<td>≥10⁶</td>
</tr>
<tr>
<td>500 ppm D-met</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>50 ppm metronidazole</td>
<td>≥10⁶</td>
</tr>
<tr>
<td>50 ppm metronidazole + 50 ppm D-met</td>
<td>≥10⁶</td>
</tr>
<tr>
<td>50 ppm metronidazole + 100 ppm D-met</td>
<td>≥10⁶</td>
</tr>
<tr>
<td>100 ppm metronidazole</td>
<td>≥10⁶</td>
</tr>
<tr>
<td>100 ppm metronidazole + 50 ppm D-met</td>
<td>≥10⁶</td>
</tr>
<tr>
<td>100 ppm metronidazole + 100 ppm D-met</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>200 ppm metronidazole</td>
<td>≥10²</td>
</tr>
<tr>
<td>200 ppm metronidazole + 50 ppm D-met</td>
<td>≥10</td>
</tr>
<tr>
<td>200 ppm metronidazole + 100 ppm D-met</td>
<td>≥10</td>
</tr>
</tbody>
</table>
Figure 5-1. Planktonic cell count after 7-day treatment with different antibiotic doses in the biofilm prevention test.

Figure 5-2. Images of sessile SRB cells on X65 coupons after 7-day biofilm prevention test: (A) no treatment, (B) with 500 ppm D-methionine treatment.
Figure 5-3. Images of sessile SRB cells on X65 coupons after 7-day biofilm prevention test: (A) with 50 ppm metronidazole treatment, (B) treated with 50 ppm metronidazole + 50 ppm D-methionine, (C) treated with 50 ppm metronidazole + 100 ppm D-methionine.
Figure 5-4. Images of sessile SRB cells on X65 coupons after 7-day biofilm prevention test: (A) treated with 100 ppm metronidazole, (B) treated with 100 ppm metronidazole + 50 ppm D-methionine, (C) treated with 100 ppm metronidazole + 100 ppm D-methionine.
Figure 5-5. Images of sessile SRB cells on X65 coupons after 7-day biofilm prevention test: (A) treated with 200 ppm metronidazole, (B) treated with 200 ppm metronidazole + 50 ppm D-methionine, (C) treated with 200 ppm metronidazole + 100 ppm D-methionine.
Figure 5-6. Specific weight loss in biofilm prevention test with treatment of 50 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.

Figure 5-7. Specific weight loss in biofilm prevention test with treatment of 100 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.
Figure 5-8. Specific weight loss in biofilm prevention test with treatment of 200 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.

Figure 5-9. Average pit depth in biofilm prevention test with treatment of 50 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.
Figure 5-10. Average pit depth in biofilm prevention test with treatment of 100 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.

Figure 5-11. Average pit depth in biofilm prevention test with treatment of 200 ppm metronidazole plus different concentrations of D-methionine after 7 days. Error bars represent standard deviations.
Figure 5-12. Pit depth profile in prevention test after 7-day exposure to the test condition of no treatment. The maximum pit depth was 17.4 µm.

Figure 5-13. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 500 ppm D-met. The maximum pit depth was 15.5 µm.
Figure 5-14. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 50 ppm metronidazole. The maximum pit depth was 12.2 μm.

Figure 5-15. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 50 ppm metronidazole +50 ppm D-methionine. The maximum pit depth was 12.4 μm.
Figure 5-16. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 50 ppm metronidazole + 100 ppm D-methionine. The maximum pit depth was 11.6 µm.

Figure 5-17. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 100 ppm metronidazole. The maximum pit depth was 11.2 µm.
Figure 5-18. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 100 ppm metronidazole + 50 ppm D-methionine. The maximum pit depth was 10.7 μm.

Figure 5-19. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 100 ppm metronidazole + 100 ppm D-methionine. The maximum pit depth was 8.4 μm.
Figure 5-20. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 200 ppm metronidazole. The maximum pit depth was 8.3 µm.

Figure 5-21. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 200 ppm metronidazole + 50 ppm D-methionine. The maximum pit depth was 6.9 µm.
Figure 5-22. Pit depth profile in prevention test after 7-day exposure to SRB with addition of 200 ppm metronidazole + 100 ppm D-methionine. The maximum pit depth was 6.5 μm.
Table 5-4. Sessile cell count in removal test after 3-day culturing and then immersing in different concentrations of metronidazole and D-methionine for 3 hours

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sessile cell count (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>≥10³</td>
</tr>
<tr>
<td>500 ppm D-met</td>
<td>≥10³</td>
</tr>
<tr>
<td>50ppm metronidazole</td>
<td>≥10⁴</td>
</tr>
<tr>
<td>50ppm D-met + 50ppm metronidazole</td>
<td>≥10⁴</td>
</tr>
<tr>
<td>100ppm D-met + 50ppm metronidazole</td>
<td>≥10³</td>
</tr>
<tr>
<td>100ppm metronidazole</td>
<td>≥10⁴</td>
</tr>
<tr>
<td>50ppm D-met + 100ppm metronidazole</td>
<td>≥10²</td>
</tr>
<tr>
<td>100ppm D-met + 100ppm metronidazole</td>
<td>≥10²</td>
</tr>
<tr>
<td>200ppm metronidazole</td>
<td>≥10³</td>
</tr>
<tr>
<td>50ppm D-met + 200ppm metronidazole</td>
<td>≥10</td>
</tr>
<tr>
<td>100ppm D-met + 200ppm metronidazole</td>
<td>≥10</td>
</tr>
</tbody>
</table>
Figure 5-23. Images of sessile cells on X65 coupons in biofilm removal test with no treatment. After 3 days of growth in the ATCC 1249 medium to establish mature SRB biofilms, coupons were removed from vials and immersed in Petri dishes with a PBS buffer: (A) without any treatment, (B) treated with 500 ppm D-methionine.
Figure 5-24. Images of sessile cells on X65 coupons in biofilm removal test: (A) treated with 50 ppm metronidazole, (B) treated with 50 ppm metronidazole + 50 ppm D-methionine, (C) treated with 50 ppm metronidazole + 100 ppm D-methionine.
Figure 5-25. Images of sessile cells on X65 coupons in biofilm removal test: (A) treated with 100 ppm metronidazole, (B) treated with 100 ppm metronidazole + 50 ppm D-methionine, (C) treated with 100 ppm metronidazole + 100 ppm D-methionine.
Figure 5-26. Images of sessile cells on X65 coupons in biofilm removal test: (A) treated with 200 ppm metronidazole, (B) treated with 200 ppm metronidazole + 50 ppm D-methionine, (C) treated with 200 ppm metronidazole + 100 ppm D-methionine.
CHAPTER 6  SUMMARY

6.1  Promoters for cross-cell wall electron transfer

Experimental data in the promoter investigation using *D. vulgaris* identified the role of electron transfer in SRB MIC and supported the BCSR theory of Gu et al. (2009). More than a 40% increase of weight loss of 304 SS was observed with the addition of promoters. Pit depth also increased from 4.8 µm to 6.6 µm and 6.0 µm from adding riboflavin and FAD, respectively. Promoters improved the transfer of electron released from extracellular iron oxidation into SRB cytoplasm for sulfate respiration and energy generation. While the energy harvest benefited sessile cell growth, it caused more severe corrosion. Thus, by adding promoters, acceleration of extracellular electron transfer demonstrated that SRB accept electrons from elemental iron for sulfate reduction. This proves that electron transfer is a key bottleneck in MIC due to SRB. The detection of unusually high concentrations of electron mediators should be a factor in forensic cases for severe MIC attacks. Addition of an electron mediator can be a tool in accelerating MIC lab tests to shorten time duration.

6.2  Starvation test to verify SRB MIC mechanism

The increased MIC pitting by *D. vulgaris* under organic carbon starvation supported the BCSR theory. Under starvation, less sessile cells were observed, but larger weight loss and pit depth were obtained due to the need for sessile cells to use elemental iron as electron donor when the usual electron donating organic carbon is in short supply.
6.3 Mitigation using a cocktail of metronidazole and D-methionine

The synergistic mixture of metronidazole and D-methionine enables better mitigation of SRB biofilms. The sessile cell counts in both the prevention test and the removal test treated with 500 ppm D-methionine alone showed only a 1-log reduction. This indicates that D-methionine alone is not adequate. With the treatment of 200 ppm metronidazole, there was a 4-log reduction of sessile cell count in the prevention test, and a 1-log reduction in the biofilm removal test. When D-methionine was added as an enhancer, one additional log reduction and 2 additional log reduction in the prevention test and the removal test were obtained, respectively. Corrosion analysis data including weight loss and pit depth also improved outcome when D-methionine was added to the antibiotic treatment solution. Therefore, D-methionine cooperated with metronidazole as an antimicrobial enhancer to improve the efficacy of antibiotic treatment against SRB biofilm. The optimal dosage of this cocktail was 200 ppm metronidazole + 100 ppm D-methionine under the test condition for the \textit{D. vulgaris} biofilm. Xu et al. (2012) found that only 1 ppm D-tyrosine could enhance the efficacy of THPS against the \textit{D. vulgaris} biofilm. It is expected that the low concentration of D-tyrosine might also enhance the antibiotic treatment as well. More work is recommended to test additional D-amino acids (including mixtures) in combination with different antimicrobials.
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Accepted.
(MFCs) and microbial electrolysis cells (MECs) for wastewater treatment,