Disinfection using a Novel Heterogeneous Catalyst

BY

Michael Kojo Boateng

Thesis submitted in partial fulfilment for the award of Doctor of Philosophy in Microbiology at the Faculty of Health and Life Sciences, De Montfort University, United Kingdom.

February 2010

Dedicated to my Family and Africa

ACKNOWLEDGEMENTS

This work was undertaken at the School of Pharmacy, Faculty of Health and Life Sciences, De Montfort University, Leicester, UK under the supervisions of Professor Katherine Huddersman (1st supervisor) and Dr Sussanah Elizabeth Walsh (2nd supervisor). It is a pleasure to thank those who made this thesis possible. Firstly, I would like to express my deepest appreciation and thanks to my supervisors for all their respective and collective support, guidance and motivation throughout my research. I am heartily thankful and grateful for their indispensable help during the writing up of the thesis.

I owe my deepest gratitude to Professor Richard Jenkins for the invaluable support gained from his expert technical knowledge and his critical review of my work.

I would like to extend my sincere appreciation to all the technical support I received from Dr John Hall for CLSM; Dr Toks Taiwo for ESR analysis; and Dr Mike Needham for both SEM and EDX analysis. Furthermore, I wish to thank all the technical staff at the Department of Microbiology and Chemistry for all their help.

I am very much grateful and thankful for the moral support and encouragement received from all my friends at the University and outside; particularly George, Anyolah, Gabi, Martha, Sylvia, Ethel, Vera and Penglei.

I am very much indebted to my family for all their support and prayers throughout my studies. Thank you Mama and my late Papa.

Finally, I would like to give the ultimate thanks to God Almighty for protecting and guiding me throughout my studies; and for helping me to overcome challenges, trials and tribulations during my studies. Thank you Jesus for your unconditional love, grace and mercy.

To God Be The Glory.

Abstract

Hydrogen peroxide (H_2O_2) is a strong oxidising agent, which is considered environmentally friendly because it can rapidly degrade to the innocuous products of water and oxygen. It is the aim of this work to investigate the potential of a novel catalyst in activating H_2O_2 so as to reduce the concentration of H_2O_2 used and the time taken for disinfection.

The antimicrobial properties of an iron based novel heterogeneous polyacrylonitrile catalyst (PCat) were examined against *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 6538 using a modified version of the European suspension test. Experiments were performed at room temperature and at 35°C (with or without bubbled air). H_2O_2 validation experiments were performed to establish the concentrations at which there is slow inactivation of the organisms. Further studies were then conducted at these concentrations to determine whether the addition of PCat increases the rate of microbial inactivation.

H_2O_2 at 0.2% w/v (against *Escherichia coli*), 0.5% w/v (against *Pseudomonas aeruginosa*) and 1% w/v (against *Staphylococcus aureus*) resulted in average log reductions of 4.76, 0.97, and 5.37 respectively after 60 minutes exposure at room temperature. Increased activity was seen against all of the organisms at 35°C (after 40 mins exposure time log reductions of >5.39, 4.09 and >5.59 were recorded). Bactericidal activity was significantly increased when PCat was combined with H_2O_2. At room temperature bactericidal activity was seen at 30 mins (*Escherichia coli*), 20 mins (*Pseudomonas aeruginosa*) and10 mins (*Staphylococcus aureus*); and at 35°C bactericidal activity occurred at 10 mins for all the tested organisms. Overall, greater increases in antimicrobial activity were seen when the tests were carried out at 35°C compared to room temperature. Bactericidal activity was observed 48hrs and 6months after first use of PCat at room remperature and 35°C. However, there was reduction in activity compared to the initial ‘fresh’ PCat tests at room temperature, but no significant ($p>0.05$) difference in activity at 35°C. Five percent and 7.5% w/v H_2O_2 with PCat showed sporicidal activity against *Bacillus subtilis* spores at 2hrs at room temperature, whereas without the catalyst sporicidal activity was absent.

In the presence of H_2O_2, leachate from PCat was found to contain very low amounts of iron which exhibited potent antimicrobial activity similar to that of PCat when it was left in the experimental solution. Traditional homogeneous iron salts of the same strength showed considerably less activity suggesting that there may be a structural conversion of iron in the leached solution resulting in the formation of new antimicrobial compounds.

Electron paramagnetic resonance studies using DMPO as radical scavenger indicated that PCat decomposes H_2O_2 to generate hydroxyl radicals ($^{\cdot}OH$ radicals). $^{\cdot}OH$ radicals were seen to be involved in the mode of antimicrobial action. These studies have demonstrated the potential biocidal use of the novel PCat when combined with low concentrations of H_2O_2 (below the current recommended levels). Reuse of the PCat may be possible and it appears to be more effective at 35°C than at room temperature.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS III
ABSTRACT IV
TABLE OF CONTENTS V
LIST OF FIGURES XI
LIST OF TABLES XVI
LIST OF ABBREVIATIONS XXI

Chapter 1

General Introduction

1.1. OVERVIEW 2
1.2. DEFINITION OF TERMS 5
1.3. BIOCIDES 8
1.3.1. Different Classes of Biocides 13
1.3.1.1. Alcohols 13
1.3.1. 2. Aldehydes 14
1.3.1.3. Biguanides 15
1.3.1.4. Chlorine compounds 16
1.3.1.5. Ethylene oxide 17
1.3.1.6. Iodine and Iodophors 17
1.3.1.7. Peroxygens 18
1.3.1.8. Quaternary Ammonium Compounds 19
1.3.1.9. Silver compounds 19
1.3.1.10. Copper compounds 20
1.4. BIOCIDAL PRODUCTS DIRECTIVE IN EUROPE 20
1.5. HYDROGEN PEROXIDE 21
1.5.1 Characteristics and synthesis of hydrogen peroxide 21
1.5.1.1. Commercial synthesis of hydrogen peroxide 22
1.5.1.2. *In vivo* synthesis of hydrogen peroxide 24
1.5.2. Antimicrobial activities of hydrogen peroxide 25
1.6. CATALYSTS 27
1.6.1 Homogeneous catalyst 28
1.6.2 Heterogeneous catalyst 28
1.6.3. Oxidation catalysts 29
1.7. FENTON'S REACTION 30

1.7.1. Applications of Fenton's reagent ... 32
1.7.2. Antimicrobial properties of hydrogen peroxide via Fenton's reaction ... 33
1.7.3. Lipid peroxidation ... 34

1.8. AIMS AND OBJECTIVES ... 37

Chapter 2

Antimicrobial Activities of Hydrogen Peroxide and Peracetic Acid their Activation by a Novel Heterogeneous Fentons-Like Modified PAN Catalyst

2.1. INTRODUCTION ... 39

2.2. TYPES OF BIOCIDE TESTING ... 40

2.3. MATERIALS AND METHODS ... 47

2.3.1. Materials ... 47
2.3.1.1. Organisms, media and maintenance. ... 47
2.3.1.2. Chemicals and reagents ... 47
2.3.1.3.. Equipment and its uses ... 48
2.3.2. Methods. ... 48
2.3.2.1 Preparation of bacterial suspension. ... 48
2.3.2.2 Preparation and maintenance of bacterial spores. ... 49
2.3.2.3. Stock solutions of test products. ... 50
2.3.2.4. Modified heterogeneous polyacrylonitrile (PAN) catalyst preparation. ... 50
2.3.2.5. Initial viable count method. ... 51
2.3.2.6. Neutralisation tests ... 53
2.3.2.7. Procedure for toxicity test ... 53
2.3.2.8. Modified European quantitative suspension test (Phase 1) ... 54
2.3.2.9. Antibacterial effects of H_2O_2 with or without modified PAN catalyst; or modified PAN mesh ... 56
2.3.2.10. Antibacterial effects of air, modified PAN mesh, and modified PAN catalyst respectively. ... 57
2.3.2.11. Antibacterial effects of homogeneous iron catalyst with or without H_2O_2 ... 57
2.3.2.12. Effects of pH and mildly alkaline (unbuffered) or phosphate buffered H_2O_2 solutions on bacterial survival; with or without modified PAN catalyst. ... 58
2.3.2.13. Antibacterial activity as a function of the reusability of different batches of modified PAN catalyst with H_2O_2 ... 59
2.3.2.14. Antibacterial effects of peracetic acid with or without modified PAN catalyst, homogeneous iron catalyst, non-modified PAN mesh or modified PAN mesh. ... 59
2.3.2.15 Sporicidal activity of H_2O_2 with or without modified PAN catalyst (Modified European Basic Sporicidal Activity Test - Phase 1, BSEN 14347:2005) ... 60
2.3.2.16. Statistical Analysis ... 60

2.4. RESULTS AND DISCUSSIONS ... 62

2.4.1. Effects of neutralisers on test products and on microorganisms. ... 62

2.4.2. Antibacterial effects of H_2O_2 with or without modified PAN catalyst; or modified PAN mesh 65
2.4.2.1. H_2O_2 validation tests 65
2.4.2.2. Antibacterial effects of H_2O_2 with heterogeneous modified PAN catalyst..... 73
2.4.2.3. Antibacterial effects of H_2O_2 with modified PAN mesh 78
2.4.3. Antibacterial effects of air, modified PAN mesh, and modified PAN catalyst respectively. 86
2.4.3.1. Antibacterial effects of water and air 86
2.4.3.2. Antibacterial effects of modified PAN mesh (MOPM) alone without H_2O_2.. 87
2.4.3.3. Antibacterial effects of modified PAN catalyst alone without H_2O_2 87
2.4.4. Antibacterial effects of homogeneous iron catalyst with or without H_2O_2 96
2.4.4.1. Antibacterial effects of homogeneous catalyst with H_2O_2 96
2.4.4.2. Antibacterial effects of homogeneous (ferric chloride) catalyst alone without H_2O_2 97
2.4.5. Effects of pH and mildly alkaline (unbuffered) or phosphate buffered H_2O_2 solutions on bacterial survival; with or without modified PAN catalyst 103
2.4.5.1. Effects of acidic distilled water on bacterial survival 103
2.4.5.2. Effects of acidic H_2O_2 on bacterial survival 105
2.4.5.3. Effects of mildly alkaline (unbuffered) 0.5% w/v H_2O_2 with or without modified PAN catalyst against *Ps. aeruginosa* 106
2.4.5.4. Effects of buffered 0.5% w/v H_2O_2 with or without modified PAN catalyst on *Ps. aeruginosa* 107
2.4.6. Antibacterial activity as a function of the reusability of different batches of modified PAN catalyst with H_2O_2 119
2.4.7. Antibacterial effects of peracetic acid with or without modified PAN catalyst, homogeneous iron catalyst, non-modified PAN mesh or modified PAN mesh. 128
2.4.8. Sporicidal activity of H_2O_2 with or without modified PAN catalyst 133

2.5. CONCLUSION 138

Chapter 3

Iron Leaching From the Novel Heterogeneous Modified PAN Catalyst and its Effect on Antibacterial Activity

3.1. INTRODUCTION 141

3.2. MATERIALS AND METHODS 143

3.2.1. Materials 143
3.2.1.1. Equipment and its uses. 143
3.2.2. Methods 144
3.2.2.1. Atomic Absorption Spectrophotometry (AAS) settings 144
3.2.2.2. The extent of iron leaching from modified PAN catalyst 144
3.2.2.3. The extent of iron leaching from reused modified PAN catalyst 145
3.2.2.4. Comparative differences of iron leaching between modified PAN catalyst batches 145

3.2.2.5. Digestion of modified PAN catalyst 145
3.2.2.6. Antibacterial effects of leachate from modified PAN catalyst in H_2O_2 solution. 146
3.2.2.7. Statistical Analysis 148

3.3. RESULTS AND DISCUSSIONS 149

3.3.1. The extent of iron leaching from modified PAN catalyst with or without microorganism........ 149
3.3.2. The extent of iron leaching from reused modified PAN catalyst........ 155
3.3.3. Differences in iron leaching between modified PAN catalyst batches. 161
3.3.4. Antibacterial effects of leachate from modified PAN catalyst in H_2O_2 solution.164

3.4. CONCLUSION 170

Chapter 4

Antimicrobial Action of Hydroxyl Radicals Produced in the Decomposition of Hydrogen Peroxide by the Novel Heterogeneous Modified PAN Catalyst

4.1. INTRODUCTION 172

4.2. MATERIALS AND METHODS 174

4.2.1. Materials 174
4.2.1.2. Equipment and its uses 174
4.2.2. Methods 174
4.2.2.1. Removal of trace elements from distilled water 174
4.2.2.2. Purification of spin trap DMPO 175
4.2.2.3. Determination and quantification by ESR of $^{\cdot}OH$ radicals 175
4.2.2.4. Total cell count as a means of qualitative assessment of antimicrobial mode of action 178
4.2.2.5. The effect of inoculum on the catalytic decomposition of H_2O_2 179
4.2.2.6. Antibacterial effects of H_2O_2/modified PAN catalyst system and H_2O_2/homogeneous iron catalyst system after continuous generation of $^{\cdot}OH$ radicals for 24 hrs 180
4.2.2.7. Statistical Analysis 182

4.3. RESULTS AND DISCUSSIONS 183

4.3.1. Determination and quantification of $^{\cdot}OH$ radicals by ESR 183
4.3.1.1. Identification of $^{\cdot}OH$ radicals by ESR spectra in the presence of DMPO 183
4.3.1.2. Quantification of $^{\cdot}OH$ radicals produced as a function of H_2O_2 concentration and nature of catalyst 189
4.3.1.3. Quantification of $^{\cdot}OH$ radicals in the leachate of modified PAN catalyst exposed to H_2O_2 194
4.3.2. Total cell count as a means of qualitative assessment of antimicrobial mode of action 200
4.3.3. The effect of inoculum on the catalytic decomposition of H_2O_2 202

4.3.4. Antibacterial effects of H_2O_2/modified PAN catalyst system and H_2O_2/homogeneous iron catalyst system after continuous generation of $^{\cdot}OH$ radicals for 24 hrs 212

4.4. CONCLUSION 216

Chapter 5

Characterisation of PAN Catalyst: Iron loading and bacterial attachment

5.1. INTRODUCTION 219

5.2. MATERIALS AND METHODS 221

5.2.1. Materials 221
5.2.1.1. Equipment and Application 221
5.2.2. Methods 221
5.2.2.1. Energy Dispersive X-ray (EDX) analysis for the distribution and quantification of iron on the modified PAN catalysts surfaces 221
5.2.2.2. Bacterial cell attachment to PAN catalyst mesh by SEM analysis 222
5.2.2.3. Qualitative assessment of live and dead microbial cells on PAN catalyst and in experimental solution using LIVE/DEAD kit on Confocal Laser Scanning Microscopy (CLSM) 223
5.2.2.4. Viable and total cell count of bacteria attached to PAN catalyst 224

5.3. RESULTS AND DISCUSSION 226

5.3.1. Energy Dispersive X-ray (EDX) analysis for the distribution and quantification of iron on the modified PAN catalysts surfaces. 226
5.3.2. The nature of bacterial cells attached to PAN catalyst mesh by SEM analysis ... 236
5.3.3. Confocal Laser Scanning Microscopy (CLSM) of microbial cells on PAN catalyst and in experimental solution using LIVE/DEAD kit 249
5.3.4. Viable and total cell count of bacteria attached to PAN catalyst 258

5.4. CONCLUSION 268

Chapter 6

MAIN CONCLUSIONS, RECOMMENDED APPLICATIONS AND FUTURE WORKS

6.1. MAIN CONCLUSIONS 270

6.2. RECOMMENDED APPLICATIONS 275

6.3. FUTURE WORK 277

REFERENCES 280

APPENDIX 1: (A) AND (B) SHOW EXPERIMENTAL SETUPS WHEN BUBBLE AIR WAS USED FOR ANTIMICROBIAL TESTING AT ROOM TEMPERATURE AND $35^{O}C$ RESPECTIVELY. 312

APPENDIX 2: PERKINELMER AANALYST 200/400 ATOMIC ABSORPTION SPECTROPHOTOMETRY USED FOR IRON DETERMINATION. 313

APPENDIX 3: COD REACTOR USED TO DIGEST SAMPLES FOR IRON DETERMINATION. 313

APPENDIX 4: A AND B INDICATE STANDARD CURVE FOR IRON MEASUREMENTS. 314

APPENDIX 5: BRUKER EMX 6/1 EPR SPECTROMETER USED FOR HYDROXYL RADICALS DETERMINATION. 315

APPENDIX 6: DR 3800 SC VIS SPECTROPHOTOMETER USED TO DETERMINE H_2O_2 CONCENTRATION. 315

APPENDIX 7: LEICA S430 FOR ELECTRON MICROSCOPY AND OXFORD INSTRUMENTS INCA-SIGHT FOR EDX ANALYSIS. 316

APPENDIX 8: EDWARDS SPUTTER COATER S150B FOR COATING SAMPLES PRIOR TO ELECTRON MICROSCOPY AND EDX ANALYSIS. 317

APPENDIX 9: TRUE CONFOCAL SCANNER LEICA TCS SP2 USED FOR CONFOCAL MICROSCOPY WORK. 318

LIST OF FIGURES

Fig 1.1: Descending order of resistance to antiseptics and disinfectants. 3

Fig. 1.2**:** Structure of the outer cell wall of (a) Gram-negative bacteria; (b) Gram-positive bacteria; and (c) mycobacteria.. 9

Figure 1.3: Synthesis of H_2O_2 via anthraquinone auto-oxidation 23

Figure 1.4: Reactions involved in the direct synthesis of H_2O_2 24

Fig 1.5: (A) and (B) representing homogeneous and heterogeneous phases respectively ... 27

Figure 1.6: The principal oxidative pathways occurring in living organisms and the main antioxidant systems transforming or inactivating free radicals or reactive oxygen species 35

Figure 2.1: Schematic diagram of the procedure for initial bacterial counts using the Miles & Misra approach. 52

Fig 2.2. A summary demonstration of the differences in the rate of antibacterial effects between 0.2% w/v H_2O_2 alone and in the modified PAN catalyst/H_2O_2 system. 85

Fig. 2.3. A summary of the differences in the rate of antibacterial activity of bubbled air and water, MOPM and water, and PCatCR and water against *Ps. aeruginosa*. 95

Fig. 2.4. A demonstration of the differences in the antibacterial activities between acidic H_2O_2 and modified PAN catalyst/H_2O_2 system. 117

Fig. 2.5. A demonstration of the differences in the antibacterial activities between 'Phosphate buffered H_2O_2 alone' and 'Phosphate buffered modified PAN catalyst/H_2O_2 system'against *Ps. aeruginosa*. 118

Fig. 2.6: A demonstration of the differences in the reusability of modified PAN catalyst.127

Figure 3.1: Amount of iron leaching from 1g modified PAN catalyst (PCatDC2) in distilled water in presence or absence of microorganisms at room temperature. 153

Figure 3.2: Amount of iron leaching from 1g modified PAN catalyst (PCatDC2) with 0.2% w/v H_2O_2 in presence or absence of *E. coli* at room temperature and 35^oC. 153

Figure 3.3: Amount of iron leaching from 1g modified PAN catalyst (PCatDC2) with 1% w/v H_2O_2 in presence or absence of *S. aureus* at room temperature and 35^oC. 154

Figure 3.4: Amount of iron leaching from reused 1g (48hrs between use) modified PAN catalyst (PCatDC2) with 0.2% w/v H_2O_2 in the presence or absence of *E. coli* at room temperature and 35^oC. 157
Figure 3.5: Amount of iron leaching from reused 1g modified PAN catalyst (PCatDC2) with 1% w/v H_2O_2 in presence or absence of *S. aureus* at room temperature and 35^oC. 159
Figure 3.6: Comparative leached iron concentrations from different batches of 1g modified PAN catalysts (PCatDC2, PCatDC3 and PCatDS) with 1% w/v H_2O_2 at room temperature. 163
Figure 4.1: Splitting profile of DMPO-$^{\cdot}$OH adducts. 184
Figure 4.2: Controls: (A) Spectra of purified distilled water with DMPO only at room temperature; (B) Spectra of 0.2% w/v H_2O_2 and DMPO alone as baseline; (C) Spectra of 0.2% w/v H_2O_2 and modified PAN catalyst alone without DMPO at room temperature.. 186
Figure 4.3: (A) Spectra showing formation of $^{\cdot}$OH radicals as a result of reaction mixture of 0.2% w/v H_2O_2, PCatDC3 and DMPO at room temperature; (B) Spectra showing formation of $^{\cdot}$OH radical as a result of reaction mixture of 0.1% w/v H_2O_2, PCatDC3 and DMPO at room temperature. 187
Figure 4.4: (A) Spectra showing formation of $^{\cdot}$OH radical as a result of reaction mixture of 0.2% w/v H_2O_2, modified PAN mesh (MOPM) and DMPO at room temperature; (B) Spectra showing formation of $^{\cdot}$OH radical as a result of reaction mixture of 0.2% w/v H_2O_2, non-modified PAN mesh (NMPM) and DMPO at room temperature. 188
Figure 4.5: Comparative hydroxyl radicals production from modified PAN catalysts using different concentrations of H_2O_2 at room temperature. 193
Figure 4.6: Hydroxyl radicals production in leachate of modified PAN catalyst exposed to H_2O_2 at room temperature. 199
Figure 4.7: Decomposition of H_2O_2 with or without *E. coli* or modified PAN catalyst (PCatDS) at room temperature. 207
Figure 4.8: Decomposition of H_2O_2 with or without *E. coli* or modified PAN catalyst (PCatDS) at 35^oC. 208
Figure 4.9: Decomposition of H_2O_2 with or without *E. coli* or modified PAN catalyst (PCatDC3) at room temperature. 209

Figure 4.10: Decomposition of H_2O_2 with or without *S. aureus* or modified PAN catalyst (PCatDS) at room temperature. 210
Figure 4.11: Decomposition of H_2O_2 with or without *S. aureus* or modified PAN catalyst (PCatDS) at 35°C. 211
Figure 4.12: Comparative decomposition of H_2O_2 with homogeneous iron catalysts and heterogeneous modified PAN catalysts after 24 hrs of preparation. 211
Fig. 5.1: Determination of iron on the modified PAN catalyst mesh (PCatDC3 = ferric chloride impregnated). 229
Fig. 5.2: Determination of iron on the modified PAN catalyst mesh (* PCatDC3 = ferric chloride impregnated) 230
Fig. 5.3: Determination of iron on the modified PAN catalyst mesh (PCatDC4 and *PCatDC4 = - ferric chloride impregnated). 231
Fig. 5.4: Determination of iron on the modified PAN catalyst mesh (PCatDS and * PCatDS = ferric sulfate impregnated). 232
Figure 5.5: Relative apparent concentration of iron within and between batches on modified PAN catalyst. 233
Fig. 5.6: SEM-EDX showing elemental distribution on surface of PAN catalyst mesh .. 235
Fig. 5.7:SEM of PAN catalyst surface control: (**A**) Circled area showing debris on the surface of PCatDS (ferric sulfate impregnated); (**B**) Surface of PCatDC3 (ferric chloride impregnated) indicating no debris or environmental microbial attachment 239
Fig. 5.8: SEM of PAN catalyst surface after immersion in aqueous suspension of *E. coli* for 1 hr Controls: (**A**) Black arrows indicating attachment on PCatDS (ferric sulfate impregnated) (Pink arrows showing areas of debris on the mesh); (**B**) Black arrows indicating *E. coli* attachment on PAN catalyst on PCatDC3 (ferric chloride impregnated). 240
Fig. 5.9: SEM of modified PAN mesh surface after immersion in aqueous suspension of *E. coli* for 1 hr Controls: (**A**) Black arrows indicating *E. coli* attachment on modified PAN mesh (MOPM = no iron impregnation); (**B**) Black arrows indicating *E. coli* attachment on modified PAN mesh after contact with an aqueous suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr and after neutralisation. 241

Fig. 5.10: SEM of PAN surfaces after immersion in aqueous suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr (**A**) Control: Black arrows indicating *E. coli* attachment on modified PAN mesh (no neutralisation); (**B**) Experiment: Black arrows indicating *E. coli* attachment on PAN catalyst mesh (PCatDS) (after neutralisation). 242
Fig. 5.11: SEM of PAN catalyst surface after immersion in aqueous suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr Experiment: (**A**) Black arrows indicating *E. coli* attachment on PCatDS) (no neutralisation). (Pink arrow showing areas of debris on the mesh); (**B**) Black arrows indicating *E. coli* attachment on PCatDC3) (after neutralisation). 243
Fig 5.12: SEM of PAN catalyst surface after immersion in aqueous suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr Experiment: (At low magnification) Black arrows indicating abundance of *E. coli* scattered on PCatDC3 (no neutralisation). 244
Fig. 5.13: SEM of PAN catalyst surface after immersion in aqueous suspension of *S. aureus* for 1 hr Control: (**A**) Black arrows indicating attachment of *S. aureus* on PCatDS; (**B**) Areas in circles indicating attachment of *S. aureus* on PCatDC3. 245
Fig. 5.14: SEM of modified PAN mesh surface after immersion in aqueous suspension of *S. aureus* containing 1% w/v H_2O_2 for 1 hr Control: (A) Areas in circles indicating attachment of *S. aureus* on modified PAN mesh (after neutralisation); (B) Areas in circle indicating attachment of *S. aureus* on modified PAN mesh (no neutralisation)............................... 246
Fig. 5.15: SEM of PAN catalyst surface after immersion in aqueous suspension of *S. aureus* containing 1% w/v H_2O_2 for 1 hr Experiment: (**A**) (At low magnification) Circled areas indicating attachment of *S. aureus* on PCatDS (after neutralisation); (**B**) (At low magnification) Circled areas indicating attachment of *S. aureus* on PCatDS) (no neutralisation)... 247
Fig. 5.16: SEM of PAN catalyst surface after immersion in aqueous suspension of *S. aureus* containing 1% w/v H_2O_2 for 1 hr Experiment: (**A**) (At low magnification) Circled areas indicating attachment of *S. aureus* on PCatDC3 (after neutralisation); (**B**) (At low magnification) Circled areas indicating attachment of *S. aureus* on PCatDC3) (no neutralisation)... 248

Fig. 5.17: Confocal Laser Scanning Microscopy using Live/Dead dyes of an aqueous suspension of *E. coli* Control: **A** and **B** indicate green fluorescence of live *E. coli* cells suspended in water (circled areas are clumped cells). 252

Fig. 5.18: Confocal Laser Scanning Microscopy using Live/Dead dyes of PAN catalyst surface Controls: **A,** without *E. coli* treatment; **B,** green fluorescence of separated live *E. coli* cells attached onto PAN catalyst fibres (cells suspended only in water); and **C,** green fluorescence a highly concentrated clumped live *E. coli* cells on PAN catalyst fibres (cells suspended only in water).. 253

Fig. 5.19: Confocal Laser Scanning Microscopy using Live/Dead dyes Positive Control: **A** and **B** indicate red fluorescence of dead *E. coli* cells treated with 70% alcohol for 1 hr... 254

Fig. 5.20: Confocal Laser Scanning Microscopy using Live/Dead dyes of experimental solution Experiment: **A** and **B** indicate different fields of view of red fluorescence of dead *E. coli* cells in experimental solution containing 0.2% w/v H_2O_2 together with PAN catalyst taken after neutralisation (circled areas are clumped cells). 255

Fig. 5.21: Confocal Laser Scanning Microscopy using Live/Dead dyes of experimental solution Experiment: **A** and **B** indicate different fields of view of red fluorescence of dead *E. coli* cells in experimental solution containing 0.2% w/v H_2O_2 together with PAN catalyst and without neutralisation. 256

Fig 5.22: Confocal Laser Scanning Microscopy using Live/Dead dyes of PAN catalyst surface treated with 0.2% w/v H_2O_2 for 1 hr Experiment: **A,** indicates green (live cells) and red (dead cells) fluorescence of *E. coli* cells attached to PAN catalyst fibre after neutralisation; **B,** a different field of view showing red (dead cells) fluorescence of *E. coli* cells on PAN catalyst fibre after neutralisation.. 257

Fig 5.23: Confocal Laser Scanning Microscopy using Live/Dead dyes of PAN catalyst surface treated with 0.2% w/v H_2O_2 for 1 hr Experiment: **A** and **B** indicate field of views of red (dead cells) fluorescence of *E. coli* cells on PAN catalyst fibre without neutralisation. 258

LIST OF TABLES

Table 1.1: Definition of terms 5
Table 1.2: Chemical structures and uses of biocides in antiseptics and disinfectants 10
Table 1.3: Summary of mechanisms of antibacterial action of antiseptics and disinfectants. 12
Table 1.4: Microbial susceptibility to alcohols 13
Table 1.5: Microbial susceptibility to aldehydes 15
Table 1.6: Microbial susceptibility to chlorhexidine 16
Table 1.7: Microbial susceptibility to chlorine compounds 17
Table 1.8: Microbial susceptibility to quaternary ammonium compounds 19
Table 1.9: Physical properties of hydrogen peroxide and water 22
Table 1.10: Half-life of free radicals 32
Table 2.1: Basic bactericidal activity 42
Table 2.2: Application test for the food, industrial, domestic, and institutional area: bactericidal activity. 43
Table 2.3: Amount of iron on different batches of modified PAN catalyst. 51
Table 2.4: Tests for initial microbial counts, neutralisation and toxicity controls. 64
Table 2.5: Antibacterial effects of 5% w/v and 3% w/v H_2O_2on *E. coli*, *Ps. aeruginosa* and *S. aureus* at roomtemperature. 69
Table 2.6: Antibacterial effects of 1% w/v H_2O_2 against *E. coli*, *Ps. aeruginosa* and *S. aureus* at room temperature 70
Table 2.7: Antibacterial effects of H_2O_2 with or without air (2.5L/min) on vegetative microorganisms at room temperature. 71
Table 2.8: Antibacterial effects of H_2O_2 with or without air (2.5L/min) on vegetative microorganisms at 35°C. 72
Table 2.9: Antibacterial effects of 1% w/v H_2O_2 with PAN catalyst against *E. coli*, *Ps. aeruginosa* and *S. aureus* at room temperature without bubbled air. 79
Table 2.10: Antibacterial effects of H_2O_2 with modified PAN catalyst (PCatCR; 1 ± 0.1g); with or without air (2.5L/min) on vegetative microorganisms at room temperature 80

Table 2.11: Antimicrobial effects of H_2O_2, PAN catalyst (PCatCR; 1 ± 0.1g) and with or without air (2.5 L/min) on vegetative microorganisms at 35°C. 81
Table 2.12: Effects of H_2O_2 with modified PAN catalyst (PCatDC1; 1 ± 0.1g) with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature. 82
Table 2.13: Antibacterial effects of H_2O_2 with modified PAN catalyst (PCatDC1; 1 ± 0.1g) with or without air (2.5 L/min) on vegetative microorganisms at 35°C. 83
Table 2.14: Antibacterial effects of H_2O_2, modified PAN mesh (MOPM) with bubbled air (2.5L/min) on vegetative microorganisms at room temperature and 35°C. 84
Table 2.15: Antibacterial effects of water and air (2.5 L/min) on vegetative microorganisms at room temperature and 35°C. 92
Table 2.16: Antibacterial effects of modified PAN mesh (MOPM) and air (2.5 L/min) on vegetative microorganisms at room temperature and 35°C. 93
Table 2.17: Antibacterial effects of modified PAN catalyst (PCatCR) with or without bubbled air (2.5 L/min) on vegetative microorganisms at room temperature and 35°C. 94
Table 2.18: Antibacterial effects of homogeneous ferric chloride catalyst (HmCl) with H_2O_2 with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature. 99
Table 2.19: Antibacterial effects of homogeneous ferric chloride catalyst (HmCl) and H_2O_2 with or without bubbled air (2.5L/min) on vegetative microorganisms at 35°C. 100
Table 2.20: The antibacterial effects of homogeneous ferric chloride catalyst (HmCl) with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature... 101
Table 2.21: The antibacterial effects of homogeneous ferric chloride catalyst (HmCl) with or without bubbled air (2.5L/min) on vegetative microorganisms at 35°C....................... 102
Table 2.22: Effects of acidic distilled water with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature. 110
Table 2.23: Effects of acidic distilled water with or without bubbled air (2.5L/min) on vegetative microorganisms at 35°C.. 111
Table 2.24: Effects of acidic H_2O_2 with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature. 112

Table 2.25: Effects of acidic H_2O_2 with or without bubbled air (2.5L/min) on vegetative microorganisms at 35°C. 113
Table 2.26: Effects of mildly alkaline (unbuffered) 0.5% w/v H_2O_2 with or without modified PAN catalyst (PCatDC1) against *Ps. aeruginosa* ATCC 15442 at room temperature or 35°C. 114
Table 2.27: Effects of phosphate buffered 0.5% w/v H_2O_2 with or without modified PAN catalyst (PCatDC1) against *Ps. aeruginosa* ATCC 15442 at room temperature or 35°C.. 115
Table 2.28: pH ranges for Table 2.26 (non-buffered system). 116
Table 2.29: pH ranges for Table 2.27 (buffered system). 116
Table 2.30: Antibacterial effects of reused modified PAN catalyst (PCatDC2) at three independent uses (48hrs between use) with 0.2% w/v H_2O_2 on *E. coli* at room temperature and 35°C. 122
Table 2.31: Antibacterial effects of reused modified PAN catalyst (PCatDS) at three independent uses (48hrs between use) with 0.2% w/v H_2O_2 on *E. coli* at room temperature and 35°C. 123
Table 2.32: Antibacterial effects of reused modified PAN catalyst (PCatDC3) at three independent uses (48hrs between use) with 1% w/v H_2O_2 on *S. aureus* at room temperature and 35°C. 124
Table 2.33: Antibacterial effects of reused modified PAN catalyst (PCatCR) at two independent uses (6months between use) with 0.2% w/v H_2O_2 on *E. coli* at room temperature and 35°C. 125
Table 2.34: Antibacterial effects of reused modified PAN catalyst (PCatCR) at two independent uses (6months between use) with 1% w/v H_2O_2 on *S. aureus* at room temperature and 35°C. 126
Table 2.35: Effects of peracetic acid (PAA) on *S. aureus* at room temperature. 130
Table 2.36: Effects of peracetic acid (PAA) with modified PAN catalyst (PCatDC3) on *S. aureus* at room temperature. 131
Table 2.37: Effects of 0.0002% w/v PAA with either modified PAN catalyst or homogeneous catalyst or non-modified PAN mesh or modified PAN mesh on *S. aureus* at room temperature. 132

Table 2.38: Effects of peracetic acid (PAA) and glutaraldehyde (GTA) on *B. subtilis* NCIB 8054 spores at room temperature. 136
Table 2.39: Effects of H_2O_2 with or without modified PAN catalyst on *B. subtilis* NCIB 8054 spores at room temperature. 137
Table 3.1: Amount of iron digested off different batches of modified PAN catalyst. 146
Table 3.2: The levels of iron leaching from reused modified PAN catalyst (PCatDC2) in 0.2% w/v H_2O_2 with or without *E. coli* at room temperature. 158
Table 3.3: The levels of iron leaching from reused modified PAN catalyst (PCatDC2) in 0.2% w/v H_2O_2 with or without *E. coli* at 35°C. 158
Table 3.4: The levels of iron leaching from reused modified PAN catalyst (PCatDC2) in 1% w/v H_2O_2 with or without *S. aureus* at room temperature. 159
Table 3.5: The levels of iron leaching from reused modified PAN catalyst (PCatDC2) in 1% w/v H_2O_2 with or without *S. aureus* at 35°C. 160
Table 3.6: Antibacterial effects of modified PAN catalyst (PCatDC2) leachate in H_2O_2 solution against *S. aureus* ATCC 6538 at room temperature. 168
Table 3.7: Antibacterial effects of modified PAN catalyst (PCatDS) leachate in H_2O_2 solution against *S. aureus* ATCC 6538 at room temperature. 169
Table 4.1: (A) Antimicrobial effects of H_2O_2 and modified PAN catalyst (PCatDC3) on vegetative microorganisms at room temperature; (B) Total cell count of experiments from (A). 201
Table 4.2: The antibacterial effects of modified PAN catalyst (PCatDS) with 0.2% w/v H_2O_2 (PCatDS) against *E. coli* as a result of continuous generation of OH radicals at 24 hrs. 215
Table 5.1: Control: (A) and (B) Determination of various elements on modified PAN mesh 234
Table 5.2: Enumeration of viable cells released from 0.5g PAN catalyst mesh (PCatDC3 = ferric chloride impregnated and PCatDS = ferric sulfate impregnated) treated with various concentrations of NaCl salts after 1 hr contact time in experiment using 0.2% w/v H_2O_2 against *E. coli*. 265

Table 5.3: Control: Enumeration of cells released from PAN catalyst mesh as a function of the NaCl salt concentration after 1hour contact time using only *E. coli* in the absence of H_2O_2. 266

Table 5.4: Total cell count of cells released from 0.5g PAN catalyst mesh (PCatDC3 = ferric chloride impregnated and PCatDS = ferric sulfate impregnated) treated with various concentrations of NaCl salts after 1hr contact time in experiment using 0.2% w/v H_2O_2 against *E. coli*. 267

LIST OF ABBREVIATIONS

Abbreviation	Term
µL	Microlitre
µm	Micrometre
µmole	Micromole
˙OH	Hydroxyl radical
AAS	Atomic Absorption Spectrophotometry
AOP	Advanced oxidation process
ATCC	American type culture collection
AU	Arbitrary units
BAC	Biological activated carbon
BPD	Biocidal products directive
BSE	Bovine spongiform encephalopathy
CD	Conjugated diene
CFU	Colony form units
CJD	Creutzfeldt-Jacob disease
Cl_2	Chlorine
CLSM	Confocal laser scanning microscopy
cm	Centimetre
COD	Chemical oxygen demand
Cu^{2+}	Copper ions
DMPO	5,5, dimethyl-1-pyrroline-N-oxide
DMU	De Montfort University
DNA	Deoxyribonucleic acid
EC	European commission
EDL	Electric double layer
EDX	Energy dispersive x-ray
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance
EU	European union
Fe	Iron
Fe^{2+} or Fe^{3+}	Iron ions
FMA	Formaldehyde
g/l	Gram per litre
GTA	Glutaraldehyde
H_2O_2	Hydrogen peroxide
H_2O_2/AL	Hydrogen peroxide alone
H_2SO_4	Sulfuric acid
HBV	Hepatitis B virus
PCat	PAN catalyst
HCl	Hydrochloric acid

HIV	Human immunodeficiency virus
HmCl	Homogeneous ferric chloride catalyst
HmFST	Homogeneous ferric sulfate catalyst
HNO_3	Nitric acid
HPV	Hydrogen peroxide vapour
hrs	Hours
IC	Initial count
kV	Kilovolts
L/min	Litre per minute
LPO	Lipid peroxidation
M	Molar
mg	Milligram
mg/L	Milligram per litre
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MOPM	Modified PAN mesh
MPa	MegaPascal
MRSA	Methicillin-resistant *Staphylococcus aureus*
mT	Millitesla
mV	Millivolts
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NCS	Neutraliser catalase solution
NGLC	neutraliser glycine
NL-PCatD	Non-leached PAN catalyst batch D- ferric chloride based
nm	Nanometre
NMPM	Non-modified PAN mesh
$NO^{\cdot}$	Nitric oxide
NST	Neutraliser sodium thiosulfate
NTC	Neutralisation test control
O_2	Oxygen
O_2^-	Superoxide
OPA	*Ortho*-phtalaldehyde
PAA	Peracetic acid
PAN	Polyacrylonitrile
PCatDC1	Ferric chloride PAN catalyst (Dralon-L type batch 1)
PCatDC2	Ferric chloride PAN catalyst (Dralon-L type batch 2)
PCatDC3	Ferric chloride PAN catalyst (Dralon-L type batch 3)
PCatCR	Ferric chloride PAN catalyst (Russian type)
PCatDS	Ferric sulfate PAN catalyst (Dralon-L type)
PMT	Photomultiplier
PPM	Parts per million

PUFAs	Polyunsaturated fatty acids
QAC	Quaternary ammonium compound
rev/min	Revolution per minute
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
S.D.	Standard Deviation
sdH_2O	Sterile distilled water
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
sq.	Square
TEM	Transmission electron microscopy
TNTC	Too numerous to count
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TTC	Toxicity test control
VBNC	Viable but non-culturable
v/v	Volume per volume
WD	Working distance
w/v	Weight per volume
w/w	Weight per weight
ZF	Zoom factor

CHAPTER 1

General Introduction

1.1. Overview

Microorganisms (e.g., bacteria, fungi, algae, viruses) are a diverse group of microscopic organisms that may live as single cells or as a cluster of cells. Microbes are ubiquitous and can be found on living things including humans, plants and animals. They can live in the air, on land, and in fresh or salt water environments. Some microorganisms can be pathogenic and can be harmful and cause diseases. Under non-sterile environments, the reduction of microbial populations generally requires the application of antimicrobial agents (Gardner and Peel, 1998).

Biocides are antimicrobial agents that contain disinfectant, antiseptic or preservative activity and many have been in use for years (Table 1.1 provides definition of terms). They were originally designed for applications in water and foodstuffs preservation, and later for antisepsis (Denyer and Maillard, 2002). However, since the start of the 20th century, there have been a significant number of biocides available for a broad spectrum of applications, ranging from simple cleaning to sterilisation. Physical or chemical antimicrobial agents are widely used as preservatives in pharmaceuticals, cosmetics and food products, whilst disinfectants and antiseptics are widely employed in hospitals and domestic environments (Hodges and Denyer, 1996). Furthermore, they are employed in certain specialised areas, such as cutting oils, fuels, paper, wood, paint, textiles and the construction industry (Moore and Payne, 2004). The antimicrobial activity of biocides is well documented, and different kinds of biocides show different levels of antimicrobial activity (Maillard, 2002). The susceptibility of microorganisms to biocides depends on the microbial type. Bacteria exhibit varying degrees of resistance to biocides. However, Gram-negative bacteria are generally more resistant than Gram-positive bacteria to biocides with bacterial spores and mycobacteria known to be the most resistant among all the bacteria types. On the other hand, prions are known to be most resistant to biocides among all the microbial groups (McDonnell and Russell, 1999; Maillard, 2002). The classification of microbial susceptibility to biocides is shown in Fig. 1.1.

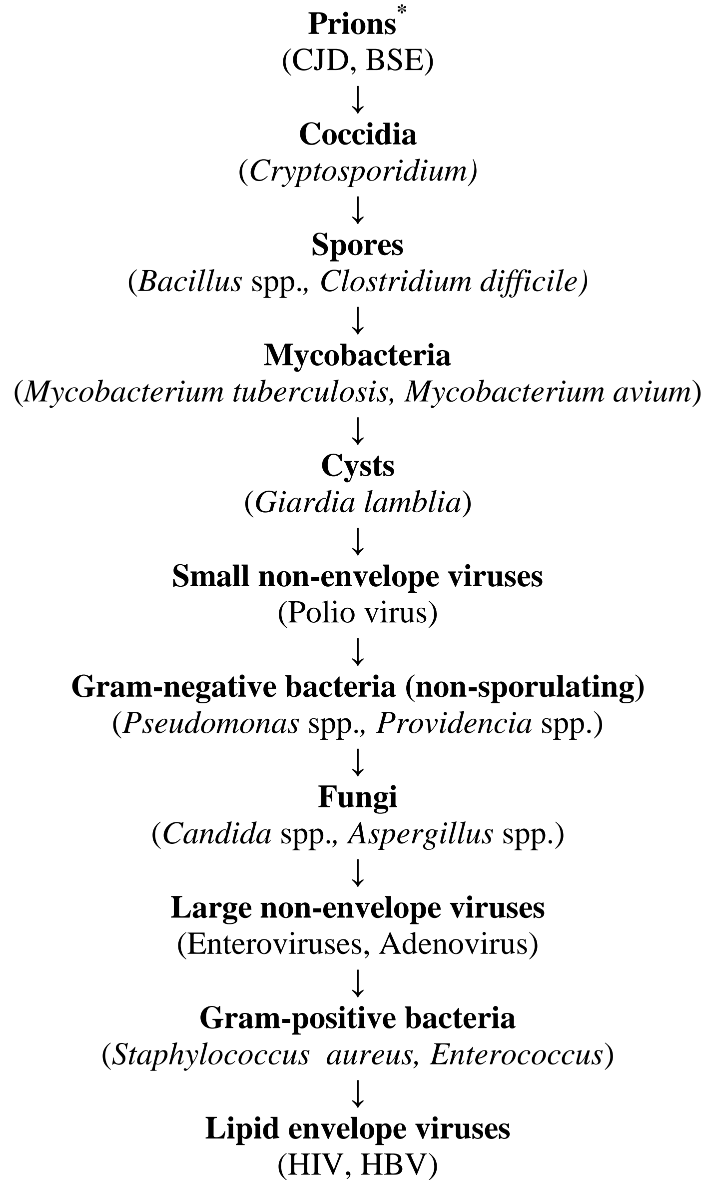


Fig 1.1: Descending order of resistance to antiseptics and disinfectants. The asterisk indicates that the conclusions are not yet universally agreed upon. (This figure has been reproduced from 'Antiseptics and Disinfectants: Activity, Action, and Resistance', by McDonnell, G. and Russell, A.D. 1999, Clinical Microbiology Review, volume 12, pages 147-179. Copyright (1999), with permission from American Society for Microbiology). CJD, Creutzfeldt-Jakob Disease; BSE, bovine spongiform encephalopathy; HIV, human immunodeficiency virus; hepatitis B virus. Examples are given in parentheses.

Hydrogen peroxide (H_2O_2) is a versatile and environmentally friendly oxidising agent that possesses many practical applications in various industries. It is used as a household antiseptic and as a disinfectant. H_2O_2 may be used alone, or in combination with compounds such as peracetic acid (Alasri *et al.,* 1992; Brinez, *et al.,* 2006) and formaldehyde (Alasri *et al.,* 1992). H_2O_2 can be decomposed into water and oxygen via enzymatic and non-enzymatic routes. Transition metal salts such as iron salts, ozone and UV-light can activate H_2O_2 to form hydroxyl radicals which are strong oxidants:

ozone and H_2O_2:

$O_3 + H_2O_2 \rightarrow {}^{\cdot}OH + O_2 + HO_2^{\cdot}$ **Equation 1.1**

iron salts and H_2O_2:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$ **Equation 1.2**

UV-light and H_2O_2:

$H_2O_2[+UV] \rightarrow 2{}^{\cdot}OH$ **Equation 1.3**

The oxidation processes involving activation of H_2O_2 by iron salts is known as Fenton's reaction (Neyens and Baeyens, 2003).

This thesis examines the antimicrobial activity of a novel heterogeneous catalyst to activate H_2O_2 against vegetative cells in planktonic form and bacterial spores.

1.2. Definition of Terms

Table 1.1: Definition of terms (Gardner and Peel, 1998; Block, 2001; Omidbakhsh, 2006)

Terms	Definitions
Activation	Initiation of a chemical or biological process (e.g. germination of bacterial spores).
Advance oxidation processes	Oxidation processes in which hydroxyl radical is the main oxidant involved.
Airbone infection	Infection caused by inhaling airbone dust particles or droplet nuclei carrying microbial contaminants.
Antibiotic	An organic chemical substance produced by microorganisms that has the capacity in dilute solutions to destroy or inhibit the growth of bacteria and other microorganisms.
Antimicrobial	Adjective describing an agent or action that kills or inhibits the growth of microorganisms.
Antisepsis	Prevention of infection by topical application of biocidal or biostatic agents to injured tissue.
Antiseptic	A chemical agent used for antisepsis.
Asepsis	Prevention of microbial contamination of living tissues or sterile materials by excluding, removing or killing microorganisms.
Autoclave	A vessel fitted with self-sealing door; not descriptive of modern pressure steam sterilisers but continued usage (as noun or verb) is convenient.
Bactericide	A chemical or physical agent that kills vegetative (non-sporing) bacteria.
Bacteriostat (*Bacteristat)*	An agent that prevents multiplication of bacteria.
Bioburden	The microbiologic load, that is, the number of contaminating organisms in the product before sterilisation.
Biocide	A physical or chemical that kills some or all types of microorganisms (often used in the inexact sense).
Biological safety cabinet	A completely or partly enclosed work station with laminar air flow through high-efficiency filters for personnel and production protection against infection and contamination.
Colony forming unit (CFU)	Is a measure of viable bacterial numbers
Contamination	Introduction of microorganisms to sterile articles, materials or tissues.

Table 1.1 cont….

Terms	**Definition**
Critical medical devices	Are those that come in contact with bloodstream or sterile areas of the body such as cardiac catheters, implants, or surgical instruments.
Culture medium	A nutrient solution or agar gel for isolating and identifying microorganisms.
Decontamination	Disinfection of used articles to make them safe to handle.
Disinfection	A process that is intended to kill or remove pathogenic microorganisms but which cannot usually kill bacterial spores.
Disinfectant	An agent that is used for disinfection.
Fungicide	An agent that kills fungi and their spores.
High-level disinfectant	An agent capable of killing bacterial spores when used in sufficient concentration under suitable conditions. It is therefore expected to kill all other microorganisms.
Inactivation	Death of microorganisms, destruction of enzyme activity or 'neutralisation' of the antimicrobial activity of a disinfectant.
Infection	Growth of microorganisms in the tissues of a host, with or without detectable signs or injury.
Infectious disease	The harmful results of infection by microorganisms.
Intermediate-level disinfectant	An agent that destroys all vegetative bacteria, including tubercle bacilli, lipid and some nonlipid viruses, and fungus spores, but not bacterial spores.
Low-level disinfectant	An agent that destroys all vegetative bacteria except tubercle bacilli, lipid viruses, some nonlipid viruses, and some fungus spores, but not bacterial spores.
Noncritical medical devices	Are those that touch intact (unbroken) skin but not the mucous membranes, such as blood pressure cuffs, stethoscopes, and bedpans.
Nosocomial	Hospital-acquired infections. Infections not present or incubating before admittance to the hospital but obtained during the patient's stay in the hospital.
Pathogen	Any disease-producing microorganism.
Planktonic	Describes growth of microbiologic organisms dispersed in solution, as in the case of free-swimming plankton.
Preservation	The process by which chemical or physical agents prevent biologic deterioration of substances.

Table 1.1 cont….

Terms	**Definition**
Sanitisers	An agent that reduces the number of bacterial contaminants to safe levels as judged by public health requirements.
Semicritical medical devices	Are those that only come into contact with mucous membranes of the body and do not contact the sterile part of the body. Example flexible endoscopes, aspirator tubes, bronchoscopes, laryngoscopes, and respiratory therapy equipment.
Spores (bacterial)	Thick-walled resting cells formed by certain Gram-positive bacteria (e.g. *Bacillus* and *Clostridium*), capable of survival in unfavourable natural environments and often highly resistant to heat and chemicals.
Spores (fungal)	Unicellular or multicellular reproductive cells, capable of survival in dry conditions with some resistance to chemicals but not highly resistant to heat.
Sporicide	An agent that kills bacterial spores.
Sterilant	An agent that kills all types of microorganisms.
Sterile	Term applied to organisms that are incapable of multiplication or articles that are free from living microorganisms.
Sterilisation	A process that is intended to kill or remove all types of microorganisms with an acceptably low probability of an organism surviving on any article.
Vegetative bacterium	A bacterium that is in the growth and reproductive phase.
Viable microorganism	A microorganism that is capable of multiplication in favourable conditions.
Viable but nonculturable	Viable cells that do not give rise to visible growth under nonselective conditions that normally support growth
Virucide	An agent that renders viruses non-infective.

1.3. Biocides

Antiseptics and disinfectants are widely used in hospitals and other healthcare settings for a variety of topical and hard-surface applications. In particular, they are a very important aspect of infection control practices and assist in the prevention of nosocomial infections (Larson, 1996). Growing concerns over the potential for microbial contamination and infection risks in the food and general consumer markets have also given rise to increased use of antiseptics and disinfectants by the general public. A wide variety of active chemical agents (or “biocides”) are found in these products, many of which have been utilised for hundreds of years for antisepsis, disinfection and preservation (Block, 1991). Despite this, less is known about the mode of action of these active agents than about antibiotics.

Generally, biocides have a broader spectrum of activity than antibiotics, and, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets (McDonnell and Russell, 1999). The widespread use of antiseptic and disinfectant products has prompted some speculation on the development of microbial resistance, in particular cross-resistance to antibiotics (McDonnell and Russell, 1999). It is deduced that accumulation of secreted materials outside the bacterial cell including capsule and slime that surrounds the bacteria might play a role in bacterial insusceptibility to biocides (Maillard, 2002). Different microbial entities respond differently to a biocidal challenge due to the different cellular and surface compositions among microbial cells (Russell, 2002). For example, one of the functions of the Gram-negative cell outer envelope is to act as an impermeable barrier that protects the microorganism from deleterious effects of antimicrobials such as biocides (Denyer and Maillard, 2002). Fig. 1.2 shows the structure of the outer cell wall of Gram-negative and Gram-positive bacteria as well as mycobacteria. A summary of the various types of biocides used in antiseptics and disinfectants and the mechanisms of antibacterial action are illustrated in Table 1.2 and Table 1.3 respectively**.** It is important to consider that many of these biocides may be used alone or in combination in a variety of products which vary considerably in activity against microorganisms. Furthermore, it must be noted that antimicrobial activity can be influenced by several

factors such as formulation effects, presence of organic load, synergistic effects, temperature, dilution, and test method (McDonnell and Russell, 1999).

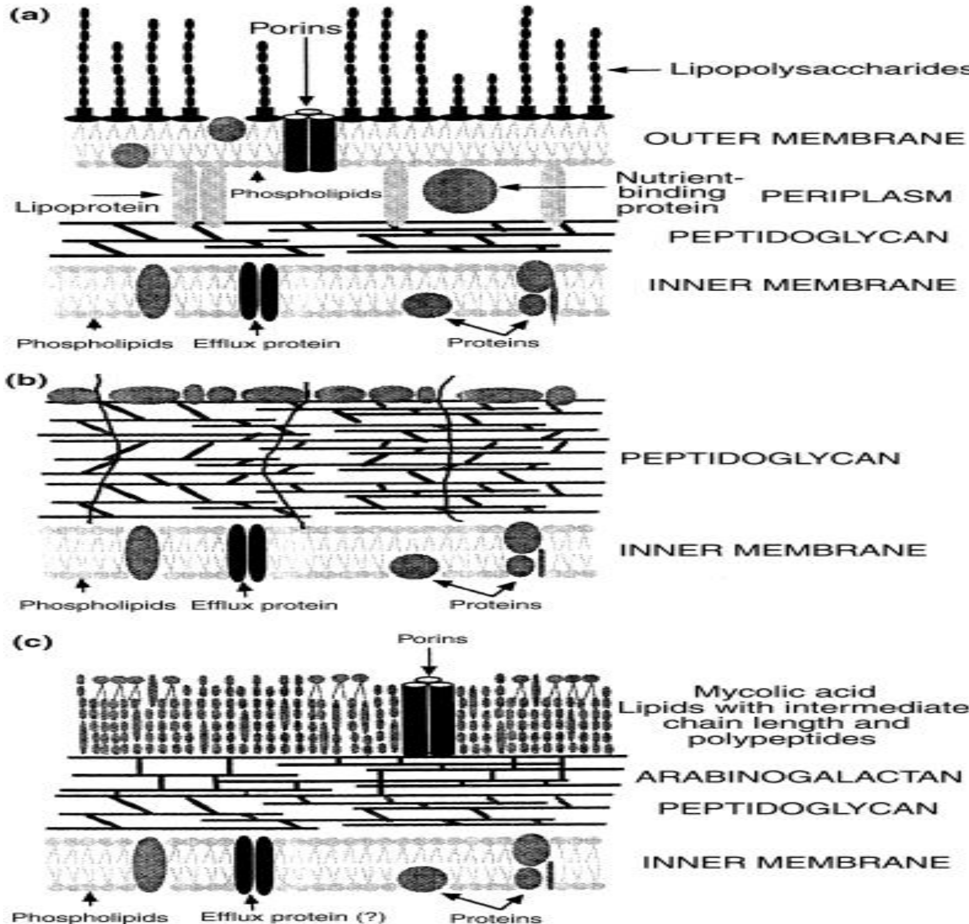


Fig. 1.2: Structure of the outer cell wall of (a) Gram-negative bacteria; (b) Gram-positive bacteria; and (c) mycobacteria. (This figure has been reproduced from 'Bacterial target sites for biocide action', by MAILLARD, J-Y. 2002, Journal of Applied Microbiology, volume 92, pages S16-S27. Copyright (2002), with permission from John Wiley and Sons – www.interscience.wiley.com.).

Table 1.2: Chemical structures and uses of biocides in antiseptics and disinfectants (This table has been reproduced from 'Antiseptics and Disinfectants: Activity, Action, and Resistance', by McDonnell, G. and Russell, A.D. 1999, Clinical Microbiology Review, volume 12, pages 147-179. Copyright (1999), with permission from American Society for Microbiology).

Agent	Biocide	Chemical structure	Application
Alcohols	Ethanol	CH_3 — CHOH	Antisepsis
	Isopropanol	CH_3, CH_3 > CHOH	Disinfection
			Preservation
Aldehydes	Glutaraldehyde	OH— $CCH_2CH_2CH_2C$ — HO	Disinfection
	Formaldehyde	H — C — HO	Sterilization
			Preservation
Anilides	General structure	C_6H_5.NH.COR	Antisepsis
	Triclocarban	Cl —(ring)— NHCONH —(ring, Cl)— Cl	
Biguanides	Chlorhexidine	Cl —(ring)— N(HCN)$_2$H(CH$_2$)$_6$N(HCN)$_2$H —(ring)— Cl (NH, NH)	Antisepsis
			Antiplaque agents
	Alexidine, polymeric biguanides		Preservation
			Disinfection
Bisphenols	Triclosan	Cl —(ring)— O —(ring)— Cl (Cl, OH)	Antisepsis
			Antiplaque agents
	Hexachlorophene	OH, Cl, Cl, Cl (ring) — CH_2 — (ring) OH, Cl, Cl, Cl	Deodorants
			Preservation
Diamidines	Propamidine		Antisepsis
			Preservation
	Dibromopropamidine	NH, NH_2 > C —(ring)— O—$(CH_2)_n$—O —(ring)— C < NH, NH_2	

Table 1.2 cont…

Agent	Biocide	Chemical structure	Application
Halogen-releasing agents	Chlorine compounds	◇OCl^-, $HOCl$, Cl_2	Disinfection Antisepsis
	Iodine compounds	◇I_2	Cleaning
Halophenols	Chloroxylenol (PCMX)	OH, CH_3, CH_3, Cl	Antisepsis Preservation
Heavy metal derivatives	Silver compounds	Ag	Preservation Antisepsis
	Mercury compounds	Hg	Disinfection
Peroxygens	Hydrogen peroxide	H_2O_2	Disinfection
	Ozone	O_3	Sterilization
	Peracetic acid	$CH_3.COOOH$	
Phenols and cresols	Phenol	OH	Disinfection Preservation
	Cresol	OH, CH_3	
Quaternary ammonium compounds	General structure	$[NR^1R^2R^3R^4]^+ X^-$	Disinfection Antisepsis Preservation
	Cetrimide, benzalkonium chloride	$[N(CH_3)_3C_nH_{2n+1}]^+ Br^-$; $[C_6H_5CH_2N(CH_3)_2C_nH_{2n+1}]^+ Cl^-$	Cleaning

Table 1.3: Summary of mechanisms of antibacterial action of antiseptics and disinfectants. (This table has been reproduced from 'Antiseptics and Disinfectants: Activity, Action, and Resistance', by McDonnell, G. and Russell, A.D. 1999, Clinical Microbiology Review, volume 12, pages 147-179. Copyright (1999), with permission from American Society for Microbiology).

Target	Antiseptic or Disinfectant	Mechanism of action
Cell envelope (cell wall, Outer membrane)	GTA,	Cross-linking of proteins
	EDTA, other permeabilisers	Gram –ve bacteria: removal of Mg^{2+}, releasing of LPS.
Cytoplasmic (inner) membrane	QACs	Generalised membrane damage involving phospholipid bilayers.
	CHXD	Low concentrations affect membrane integrity, high concentrations cause congealing of cytoplasm.
	Diamines	Induction of leakage amino acids
	PHMB, alexidine	Phase separation and domain formation of membrane lipids
	Phenols	Leakage; some cause uncoupling.
Cross-linking macromolecules	Formaldehyde	Cross-linking of proteins, RNA and DNA.
	GTA	Cross-ling of proteins in cell envelope and elsewhere in the cell.
DNA intercalation	Acridines	Intercalation of an acridine molecule between two layers of base pairs in DNA.
Interaction with thiol groups	Silver compounds	Membrane-bound enzymes (interaction with thiol groups).
Effects on DNA	Halogens	Inhibition of DNA synthesis
	H_2O_2, silver ions	DNA strand breakage.
Oxidising agents	Halogens	Oxidation of thiol groups to disulfides, sulfoxides, or disulfoxides.
	Peroxygens	H_2O_2: activity due to formation of free hydroxyl radicals, which oxidise thiol groups in enzymes and proteins; PAA: disruption of thiol groups in proteins and enzymes.

GTA, glutaraldehyde; EDTA, ethhylenediaminetetraacetic; QACs, quaternary ammonium compounds; CHXD, chlorhexidine, PHMB, polyhexamethylene biguanide; LPS, lipopolysaccharide; RNA, ribonucleic acid, DNA, deoxyribonucleic acid; PAA, peracetic acid; H_2O_2, hydrogen peroxide.

1.3.1. Different Classes of Biocides

1.3.1.1. Alcohols

Antimicrobial properties have been identified for a number of alcohols. However, ethyl ($CH_3.CH_2OH$), isopropyl ($CH_3.CHOH.\ CH_3$) and *n*-propyl ($CH_3.CH_2.CH_2OH$) alcohols are the most widely used. They are colourless liquids, which boil at approximately 80°C and they are flammable at concentrations that are recommended for disinfection (Gardner and Peel, 1998). Alcohols generally exhibit rapid antimicrobial activity against vegetative bacteria including acid-fast bacilli, viruses and fungi; but possess no sporicidal effects (McDonnell and Russell, 1999; Moore and Payne, 2004).

Alcohols are mostly used as hard-surface disinfectants and as skin antiseptics but are not recommended for sterilisation due to their poor sporicidal activity. However, it has been shown that the addition of 1% sodium or potassium hydroxide, or acids or 10% amy-m-cresol to 70% alcohol exhibited some sporicidal activity (Russell, 1990). 80% v/v ethyl alcohol is generally used to disinfect the surface of glass ampoules containing local anaesthetics, cleaned surfaces of hospital trolleys, dental bracket tables and cleaned laboratory benches. 70% v/v ethyl alcohol is unable to inactivate cell-free, dried HIV within 10 minutes and is therefore not suitable to inactivate HIV on surfaces (Hanson *et al.,* 1989). The mode of action of alcohols is not well understood, however, on the basis of increased activity in the presence of water, it is generally believed that they cause membrane damage and rapidly denature proteins resulting in subsequent interference with metabolism and cell lysis (Larson and Morton, 1991). Table 1.4 indicates microbial susceptibility to alcohols.

Table 1.4: Microbial susceptibility to alcohols

Gram-positive bacteria	highly susceptible
Gram-negative bacteria	highly susceptible
Acid-fast bacteria	susceptible (suspensions)
Bacterial spores	resistant
Lipophilic viruses	susceptible (suspensions)
Hydrophilic viruses	variable

1.3.1. 2. Aldehydes

There are two important aldehydes that are considered as disinfectants, namely glutaraldehyde ($CHO.CH_2$. $CH_2.CH_2.CHO$) and formaldehyde (H.CHO), however, others have also shown antimicrobial activity including *ortho*-phthalaldehyde which is now widely used (McDonnell and Russell, 1999; Cooke *et al.,* 2003; Yoshimasa *et al.,* 2004).

Glutaraldehyde: Glutaraldehyde (GTA) is a dialdehyde which is used as a disinfectant. It is used particularly for low-temperature disinfection of endoscopes, surgical instruments, and as a fixative in electron microscopy (McDonnell and Russell, 1999). GTA is known to possess broad-spectrum microbicidal activity against vegetative bacteria, bacterial and fungi spores, mycobacteria, mycelial and various types of viruses (Moore and Payne, 2004). The mode of action of GTA on microorganisms (Table 1.3) is considered to be based on its cross-linkage abilities with proteins, possibly by impeding normal enzymatic activity or due to the fixation of other important cellular structures resulting in inhibition of important survival functions (Maillard, 2002). GTA was formerly widely used but it has now been withdrawn from the United Kingdom market by its manufacturer because apart from many advances in the development of disinfectants with superior antimicrobial activity, GTA was known to cause toxic effects on skin and mucous membranes resulting in diseases such as severe dermatitis, conjunctivitis, sinusitis and asthma (Dartford and Gravesham NHS Trust 2006).

Formaldehyde: Formaldehyde (FMA) is a monoaldehyde and is used generally as a disinfectant and sterilant. FMA exhibits bactericidal, sporicidal and virucidal activities, however, its activity is much slower than GTA (McDonnell and Russell, 1999). Principally, it reacts with proteins, DNAs, RNAs and nucleic acids (McDonnell and Russell, 1999). As an alkylating agent, FMA is potentially mutagenic and carcinogenic (Ealy, 1991) and squamous cell carcinomas have been produced experimentally in rats (Yodaiken, 1981).

***Ortho*-phtalaldehyde:** *Ortho*-phtalaldehyde (OPA) is an aromatic dialdehyde and has been demonstrated as a potent bactericidal, sporicidal and virucidal compound (McDonnell and

Russell, 1999; Walsh *et al.,* 1999a, 1999b, 2001). There were growing concerns over the safety and efficacy of GTA and FMA and as a result, OPA was proposed as an alternative for their replacement (Alfa and Sitter, 1994; McDonnell and Pretzer, 2001; Cooke *et al.,* 2003; Yoshimasa *et al.,* 2004). It is now been accepted for use as a high level disinfectant at in-use concentration of 0.5% w/v (Walsh *et al.,* 2001). The mechanism of action by OPA is not fully understood, however, it has been suggested that its action is possibly similar to GTA (McDonnell and Russell, 1999; Walsh *et al.,* 1999b). Table 1.5 indicates microbial susceptibility to aldehydes.

Table 1.5: Microbial susceptibility to aldehydes

Gram-positive bacteria	highly susceptible
Gram-negative bacteria	highly susceptible
Acid-fast bacteria	moderately susceptible
Bacterial spores	susceptible (slow killing, species variation)
Lipophilic viruses	susceptible
Hydrophilic viruses	moderately susceptible
Fungi	fungistatic or fungicidal

1.3.1.3. Biguanides

Various biguanides have antimicrobial activity, including chlorhexidine, alexidine and the polymeric biguanides. Among the biguanides, chlorhexidine is the most important, and possesses broad antibacterial activity against both Gram-positive and Gram-negative bacteria (Moore and Payne, 2004). It is available as dihydrochloride, diacetate and gluconate. Chlorhexidine is probably the most widely preferred biocide in antiseptic products, particularly, in hand washing and oral products and also as a disinfectant and preservative (McDonnell and Russell, 1999). Chlorhexidine is active in the pH range 5.5-8. The optimum is known to be on the alkaline side because this gives rise to the availability of anionic groups on the surface of the bacteria to react with the cationic chlorhexidine and the free base precipitates above pH 8. On the other hand, soaps and other anionic detergents react with chlorhexidine and render it inactive (Gardner and Peel, 1998). Table 1.6 indicates microbial susceptibility to chlorhexdine.

Table 1.6: Microbial susceptibility to chlorhexidine

Gram-positive bacteria	highly susceptible
Gram-negative bacteria	moderately susceptible
Acid-fast bacteria	resistant
Bacterial spores	resistant
Lipophilic viruses	susceptible
Hydrophilic viruses	resistant

1.3.1.4. Chlorine compounds

The broad spectrum biocidal activity of chlorine (Cl_2) and the variety of organic and inorganic chlorine-releasing compounds is mediated by hypochlorous acid (HOCl), which is formed in aqueous solutions at pH 5-8 (Gardner and Peel, 1998). Chlorine compounds show high biocidal activity and due to their low toxicity to humans, they have found useful applications as sanitising agents in the food industry (Moore and Payne, 2004). Hypochlorites are well known oxidising agents that possess broad microbicidal activity. They attack multiple sites of the microbial cell wall including amino groups within proteins; they cause progressive oxidation of thiol groups to disulphides, sulphoxides and disulphoxides with inhibition of DNA synthesis leading to formation of chlorinated derivatives of nucleotide bases (McDonnell and Russell, 1999). Chlorine dioxide is effective against bacteria, fungi, protozoa and algae (Knapp and Bettsti, 2001). Furthermore, inorganic and organic chloramines and isocyanuric acids are also effective antimicrobial compounds (Moore and Payne, 2004). Table 1.7 indicates microbial susceptibility to chlorine compounds.

Table 1.7: Microbial susceptibility to chlorine compounds

Microorganism	Susceptibility
Gram-positive bacteria	highly susceptible
Gram-negative bacteria	highly susceptible
Acid-fast bacteria	moderately susceptible
Bacterial spores	susceptible (optimum pH 7.6)
Lipophilic viruses	susceptible
Hydrophilic viruses	susceptible (high concentration)
Amoebic cysts, algae	susceptible
Fungi	moderately susceptible
Prions	moderately susceptible (high concentrations)

1.3.1.5. Ethylene oxide

Ethylene oxide (C_2H_4O) (referred to as epoxyethane or dimethylene) is an alkylating agent and is one of the most important disinfectants among the oxides group. Chemical alkylating agents resemble ionising radiation in their potential for toxicity, mutagenicity and carcinogenicity. They share with ionising radiation a broad spectrum of biocidal action, with less than tenfold difference between the resistance of bacterial spores and the vegetative cells (Gardner and Peel, 1998). Ethylene oxide possesses a greater degree of antimicrobial efficacy against all types of microorganisms when it is used at suitable conditions of concentration, temperature and relative humidity for a sufficient time (Gardner and Peel, 1998; Block, 2001). Ethylene oxide gas has the capability for the sterilisation of medical instruments as well as other contaminated objects due to its superior penetration properties and its ability to inactive spore-forming bacteria, viruses and fungi at relatively low temperatures (Parker IV and Johnson, 1995; Jose-Moreira *et al.,* 2004). However, it is too slow for the decontamination of an instrument that must be used for successive patients in an operating session (Gardner and Peel, 1998).

1.3.1.6. Iodine and Iodophors

Aqueous solution of iodine with potassium iodide or alcohol has potent antimicrobial effects on vegetative bacteria, bacterial spores, moulds, yeasts and viruses (Gottardi, 1985). Duan *et al.* (1999) showed bactericidal, fungicidal and virucidal activity of iodine

incorporated in an enzyme-based disinfectant. Iodophors are organic complexes containing iodine trapped within micelles of a surface active agent (Gardner and Peel, 1998). The most widely used are povidone-iodine and poloxamer-iodine in both antiseptics and disinfectants. Iodophors are considered to be less effective against some types of fungi as well as spores (McDonnell and Russell, 1999). Microbial cell death by iodine is known to be due to the attacks on key proteins, nucleotides and fatty acids (Gottardi, 1991).

1.3.1.7. Peroxygens

The well-known peroxygens are hydrogen peroxide (H_2O_2), and peracetic acid (CH_3COOOH); (Block, 2001). H_2O_2 is a widely used biocide for disinfection, sterilisation and antisepsis (McDonnell and Russell, 1999). It possesses some activity against most types of microorganisms but enteric viruses and bacterial spores require a high concentration for lethal action (Gardner and Peel, 1998). In general greater activity is seen against Gram-positive than Gram-negative bacteria; however, the presence of catalase or other peroxidases in these organisms can increase tolerance in the presence of lower concentrations (McDonnell and Russell, 1999). H_2O_2 acts as an oxidant by producing hydroxyl free radicals ($^{\cdot}OH$) which attack essential cell components, including lipids, proteins, and DNA (McBain and Gilbert, 2001; Russell, 2003; Moore and Payne, 2004).

Peracetic acid (PAA) has a more potent biocidal action than H_2O_2. PAA shows a broad-spectrum of antimicrobial efficacy against bacteria, spores, viruses, and fungi at relatively low concentrations ($<0.3\%$); (Block, 2001). It is a strong oxidising agent and it is known to be corrosive under certain conditions (Moore and Payne, 2004). One of the great advantages of PAA is that it decomposes to innocuous safe by-products of water and oxygen (Moore and Payne, 2004), and also has a competitive advantage over H_2O_2 because it is free from decomposition by peroxidases and its activity is not impeded by the presence of organic matter (McDonnell and Russell, 1999). PAA is mainly used as low-temperature liquid high level disinfectant for medical instruments, flexible endoscopes, and haemodialysers; and also used for environmental surface disinfection (McDonnell and Russell, 1999). Little is known about the mechanism/s of action by PAA, however, it is

deduced that the mechanism of action of PAA on microorganisms is probably similar to that of H_2O_2 (Russell, 2003).

1.3.1.8. Quaternary Ammonium Compounds

The quaternary ammonium compounds (QACs) are cationic (positively charged) surface active disinfectants. Both characteristics contribute to their bactericidal activity by promoting uptake of the molecules by the microorganisms. The complex cations form salts with chloride or bromide; however, the associated anions play no part in the antimicrobial activity (Gardner and Peel, 1998). QACs have found useful applications in clinical settings such as preoperative disinfection of unbroken skin as well as disinfecting noncritical surfaces. QACs are also useful for hard-surface cleaning and deodorisation (Mcdonnell and Russell, 1999). It has been well-documented that QACs are membrane-active compounds that targets primarily the cytoplasmic membrane in bacteria and plasma membrane in yeasts (McDonnell and Russell, 1999). Table 1.8 indicates microbial susceptibility to quaternary ammonium compounds.

Table 1.8: Microbial susceptibility to quaternary ammonium compounds

Gram-positive bacteria	highly susceptible
Gram-negative bacteria	moderately susceptible
Acid-fast bacteria	resistant
Bacterial spores	resistant
Lipophilic viruses	susceptible
Hydrophilic viruses	resistant (except HBV)

HBV, hepatitis B virus

1.3.1.9. Silver compounds

Bactericidal activity of silver ions has been known since ancient times (Weber and Rutala, 1995). Silver and its salts (e.g. silver nitrate) have been used for medical applications and in controlling bacteria and other organisms in water (Liau *et al.,* 1997). The generation of silver ions can also be achieved through ion exchange using complexes of silver with other inorganic materials (e.g. silver-zeolites complexes); (Im *et al.,* 1996). Silver nano-particles

have demonstrated antimicrobial properties against both bacteria and viruses (Sondi and Salopek-Sondi, 2004; Elchiquerra *et al.,* 2005). Silver ions interact with a number of components of both bacterial, protozoal and fungal cells. It has been established that silver ions react with thiol (sulphydryl,-SH) groups in vital enzymes and inactivate them or react with DNA leading to dimerisation of pyrimidine via a photodynamic reaction and a probable inhibition of DNA synthesis (Liau *et al.,* 1997; Matsumura *et al.,* 2003).

1.3.1.10. Copper compounds

It is well established that low amounts of copper ions are utilised by microorganisms as important micronutrients and as vital cofactors for processing of metalloproteins and certain enzymes (Gadd, 1993; Nies, 1999). However, higher concentrations of copper can inhibit growth or death of microorganisms (Ibrahim *et al.,* 2008). The deleterious effect of copper against microorganisms is noted to occur by displacing essential ions resulting in the obstruction of functional groups of protein, inactivating enzymes, producing hydroperoxide free radicals, and altering membrane integrity (Nies, 1999).
Copper has been used to inactivate a range of microorganisms including *E. coli* (Noyce *et al.,* 2006), methicillin-resistant *Staphylococcus aureus* (Noyce *et al.,* 2006), influenza A virus (Noyce *et al.,* 2007), and *Clostridium difficile* (Wheeldon *et al.,* 2008). Recent work has demonstrated that copper-containing items offer the potential to significantly reduce microbial numbers in the clinical environment (Casey *et al.,* 2010). The combination of lactic acid and copper was used to control food-borne pathogens such as *E. coli* O157:H7 and *Salmonella* spp. (Ibrahim *et al.,* 2008).

1.4. Biocidal Products Directive in Europe

The complex requirements of the EU Biocidal Products Directive (BPD) (98/8EC) raises concerns on cost to manufacturers of registering even existing biocidal products; and directly or indirectly affecting the incentive to research and develop new products (Moore and Payne, 2004). The principle of BPD is that products can only be placed on the market if authorised in accordance with the laid down regulations; each product requires an authorisation (Claassens, 1998). The aims and objective of the BPD were to harmonise

biocidal products introduced to EU markets with a common set of data requirements both for the active substance and the biocidal formulation product itself; these rules hope to remove trade barriers within EU member states and to ensure proper protection for humans and the environment (Rasmussen and MacLellan, 2001). Annex 1 of the BPD requires stringent testing of all old and new active substances of biocidal products against new safety standards (European Commission, 1998). Although, it is envisaged that biocide manufacturers will recognise the advantages of BPD (improved protection of man and environment, harmonisation across EU etc) that is provided, it is noted that this may also undermine the efforts of the industry in delivering better hygiene with safe, effective, but affordable disinfectants (Claassens, 1998).

1.5. Hydrogen peroxide

1.5.1 Characteristics and synthesis of hydrogen peroxide

Hydrogen peroxide (H_2O_2) is an important chemical product with a wide variety of applications. It is a colourless liquid and is miscible in water and soluble in organic solvents (e.g. carboxylic esters) at concentrations above 65% (Jones, 1999). Table 1.9 compares some of the significant properties of hydrogen peroxide and water. H_2O_2 is thermodynamically unstable, particularly in dilute solutions and decomposes in the presence of enzymes such as catalase and peroxidases (McDonnell and Russell, 1999), the action of light (Fiorenza and Ward, 1997) and in the presence of transition metal ions (Halliwell and Gutteridge, 1984). Benzoic acid or other suitable substances are added as stabilisers to H_2O_2 (Moore and Payne, 2004). There are no toxic decomposition products, as a result, H_2O_2 is considered environmentally friendly because it can rapidly degraded into the innocuous products of water and oxygen (Miller, 1996) as indicated in the following reaction:

$2H_2O_2 = 2H_2O + O_2\uparrow$ **Equation 1.4**

Table 1.9: Physical properties of hydrogen peroxide and water (This table has been reproduced from 'Applications of hydrogen peroxide and derivatives' (page 14) by Jones, C.W. 1999, Cambridge, Royal Society of Chemistry. Copyright (1999) with permission from Royal Society of Chemistry – www.rsc.org).

Properties	Hydrogen peroxide	water
Melting point (^{o}C)	-0.43	0.0
Boiling point (^{o}C)	150.2	100
Heat of melting (J/g)	368	334
Heat of vaporization ($Jg^{-1}K^{-1}$)		
25 ^{o}C	1519	2443
b.p.	1387	2258
Specific heat ($Jg^{-1}K^{-1}$)		
liquid (25 ^{o}C)	2.629	4.182
gas (25 ^{o}C)	1.352	1.865
Relative density (g cm^{-3})		
0°C	1.4700	0.9998
20°C	1.4500	0.9980
25 ^{o}C	1.4425	0.9971
Viscosity (mPa s)		
0°C	1.819	1.792
20°C	1.249	1.002
Critical temperature (0°C)	457	374.2
Critical pressure (MPa)	20.99	21.44
Refractive index	1.4084	1.3330

1.5.1.1. Commercial synthesis of hydrogen peroxide

The demand for the use of H_2O_2 is continually increasing. Several methods and techniques are available for the synthesis of H_2O_2 (Samanta, 2008). H_2O_2 is synthesised commercially mainly by a process known as auto-oxidation of hydroanthraquinone (Fig. 1.3), that involves cyclic oxidation and catalytic hydrogenation reactions in a complex organic mixture (Hicks, 1975; Choudhary *et al.,* 2007). However, this process is only economically viable in commercial production. Recently, there have been a number of new greener approaches in H_2O_2 synthesis. One approach is an *in situ* generation of H_2O_2 that involves the selective reduction of oxygen by hydrazine over supported palladium catalyst prepared in an aqueous acidic medium in the presence of a bromine promoter at ambient

temperatures (Choudhary *et al.,* 2007). Voloshin, *et al.* (2007) showed direct synthesis of H_2O_2 through the oxidation of H_2 by O_2 to H_2O_2 (Figure1. 4). Due to the environmentally clean process of this approach, it has been suggested that it has the potential to replace the currently practiced hydroquinone auto-oxidation process (Choudhary and Jana, 2007; Voloshin, *et al.,* 2007; Samantha, 2008).

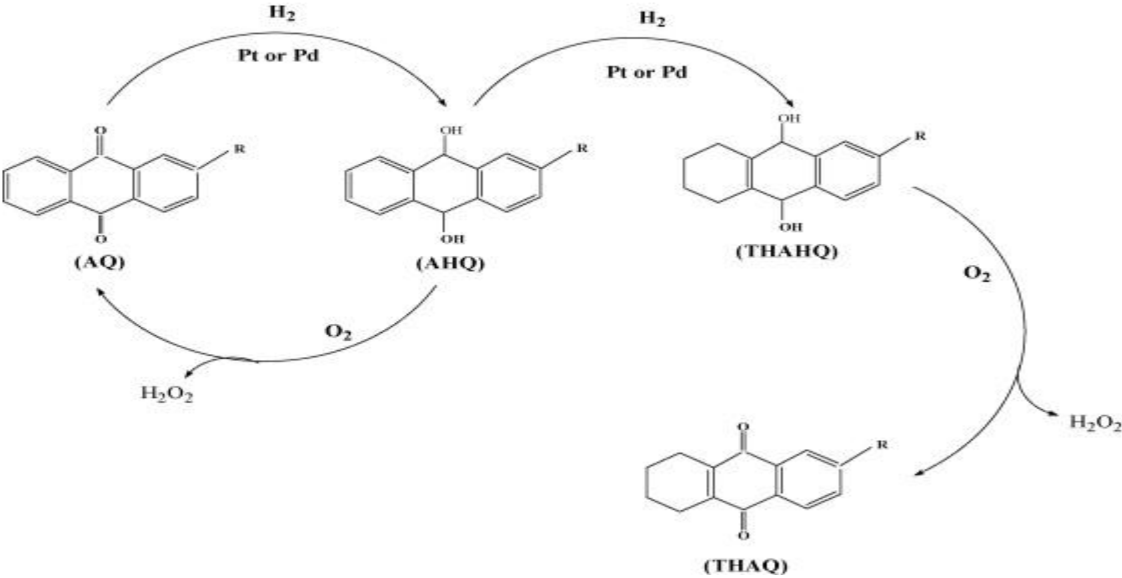


Figure 1.3: Synthesis of H_2O_2 via anthraquinone auto-oxidation
(This figure has been reproduced from 'Direct synthesis of hydrogen peroxide from hydrogen and oxygen: An overview of recent developments in the process', by Samanta, C. 2008, Applied Catalysis A: General. Vol. 350, pages 133-149. Copyright (2008), with permission from Elsevier).

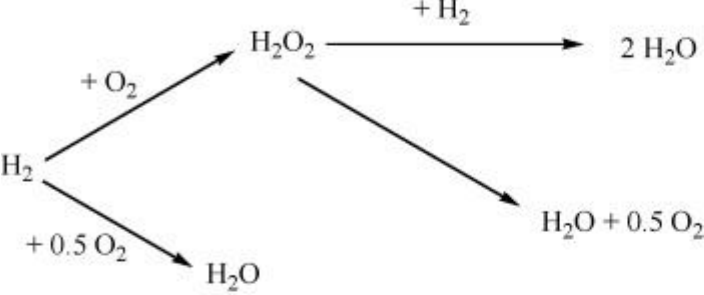


Figure 1.4: Reactions involved in the direct synthesis of H_2O_2
(This figure has been reproduced from 'Direct synthesis of hydrogen peroxide from hydrogen and oxygen: An overview of recent developments in the process', by Samanta, C. 2008, Applied Catalysis A: General. Vol. 350, pages 133-149. Copyright (2008), with permission from Elsevier).

1.5.1.2. *In vivo* synthesis of hydrogen peroxide

H_2O_2 is one of the most abundant reactive oxygen species (ROS) molecules available *in vivo*. It is continuously synthesised intracellularly as a by-product of the aerobic metabolism or extracellularly by stimulated host phagocytes (Sousa-Lopes *et al.,* 2004). H_2O_2 is formed in bacteria when molecular oxygen oxidises redox enzymes (Miller and Britigan, 1997; Messner and Imlay, 1999). For example, during cell respiration, the biomolecule NADPH is oxidised by dissolved oxygen to form the superoxide anion O_2^-:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^- \qquad \textbf{Equation 1.5}$$

The superoxide is converted to hydrogen peroxide in a slightly acidic medium by superoxide dismutase (SOD):

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \textbf{Equation 1.6}$$

Endogenously produced H_2O_2 can slowly oxidise proteins, forming methionine sulfoxide and carbonyl adducts (Moskovitz, *et al.,* 1995). Most lactic acid bacteria that grow aerobically are known to produce and excrete copious amount of H_2O_2 (Seki *et al.,* 2004). The genome of *Streptococcus pyogenes* showed that H_2O_2-producing oxidases for NADH, lactate, and α-glycerophosphate are present but those for pyruvate are absent (Gibson *et al.,* 2000). On the other hand, *Streptococcus pneumoniae* produces H_2O_2 via the action of

pyruvate oxidase catalysing a two-electron reduction of O_2 (Spellerberg *et al.,* 1996; Pericone *et al.,* 2003).

1.5.2. Antimicrobial activities of hydrogen peroxide

H_2O_2 has been known for its antimicrobial and/or antiseptic activities for very long time because of its efficacy, versatility and reasonable manipulation safety; and it is well known for its topical application in 3% concentrations (Labas *et al.,* 2008). Depending on the application, H_2O_2 is usually used at concentrations ranging from 1-20% (Mullen, 2002). Depending on the concentration and operating conditions, H_2O_2 have shown growth inhibition and/or inactivation against pathogenic microorganisms such as vegetative bacteria, fungi, viruses, mycobacteria and bacterial spores (Satter, 2002; Semchyshyn *et al.,* 2005; Labas *et al.,* 2008). In foods such as milk, H_2O_2 was used as disinfectant as early as 1904 (Labas *et al.,* 2008). In aquatic environments, tenacibaculosis, which is caused by *Tenacibaculum maritimum* (*T. maritimum*), is an economically important disease in a great variety of European cultured fish (Toranzo *et al.,* 2005; Avendana-Herrera *et al.,* 2006). Repetitive use of antimicrobial agents is indicating an emergence of resistant strains of *T. maritimum*t. 30 to 240 ppm of H_2O_2 were found to be efficacious, killing a high proportion of the fish pathogen *T. maritimum* in seawater after 30 min exposure time, hence, it has been recommended that 240 ppm of H_2O_2 can be use as a general disinfection preventative method for treating water culture and surface tanks before the introduction of fish (Avendana-Herrera *et al.,* 2006).

Medical devices are placed into different classifications according to the risk of infection involved in their use. In 1968, Spaulding proposed these classifications as critical, semicritical and noncritical medical devices, thus, Spaulding believed that medical devices should be cleaned and reprocessed according to the level of risk associated with their intended use (Omidbakhsh, 2006). Even though the application of potent antibiotic therapy has contributed to the reduction of the risk of infection of temporarily and long-term implants such as catheters, prostheses, heart valves, pacemakers, and defibrillators, however, infection of biomaterials still continues to be a major concern in clinical settings

(Pfeiffer *et al.,* 1994; Alt *et al.,* 1999). Local treatment with 3% H_2O_2 significantly reduced bacterial growth on typical clinically used biomaterials such as polyurethane and silicone tubing even for 1 month after treatment (Alt *et al.,* 1999).

Although, heat sterilisation of critical and semi-critical medical instruments continues to be the safest and preferred approach for instrument processing between patients. In exceptional cases, heat sensitive instruments may be cold sterilised or high level disinfected by immersion in liquid chemical high level disinfectants such as H_2O_2 (Acosta-Gio *et al.,* 2005).
Flexible endoscopes invariably become contaminated with microorganisms during clinical use (Foliente *et al.,* 2001). 7.5% H_2O_2 is used as a high level disinfectant (HLD) for 6 hours exposure time (Rutala and Weber, 1999; Acosta-Gio *et al.,* 2005). H_2O_2 fumigation processes (Omidbakhsh and Satter, 2006) such as vaporised hydrogen peroxide (VHP) was found to significantly reduce surface microbial contamination levels in hospital areas and therefore has been recommended for routine environmental disinfection practices within healthcare settings, particularly to be used during the occurrence of outbreaks (McDonnell *et al.,* 2007). 5% H_2O_2 dry fumigation system ran in three cycles showed good sporicidal activity when used in rooms, ambulances, and external and internal parts of ventilated equipment (Anderson *et al.,* 2006). A 7.66% solution of accelerated H_2O_2 showed sporicidal, bactericidal, virucidal, fungicidal, and mycobactericidal activities; and although the level of H_2O_2 dropped from as high as 7.66% to as low as 6.40% in 14 days, similar antimicrobial activity was observed during the reuse period (Satter *et al.,* 2002).

Generally, it has been established that the microbial growth inhibition by H_2O_2 is not a direct result of its oxidative properties in its molecular state, but rather the consequence of the activity of other strongly oxidant chemical species derived from it. Thus, H_2O_2 is known to be an excellent source of highly reactive species such as singlet oxygen, superoxide radical and hydroxyl radical which are very toxic to microorganisms (Labas *et al.,* 2006). It must be pointed out that the exact mechanism by which H_2O_2 produces toxic compounds to a number of microorganisms is not clearly understood. However, it is

generally known that due to its ability to produce the above reactive species with strong oxidative properties, it can therefore induce damage to nucleic acids, enzymes and membrane constituents (Lynch *et al.,* 1978; Labas *et al.,* 2006).

1.6. CATALYSTS

Catalysts speed up chemical reactions but can be recovered unchanged at the end of the reaction. They can also direct the reaction towards a specific product and allow chemistry to be carried out at lower temperatures and pressures with higher selectivity towards the desired product (Cole-Hamilton and Tooze, 2006). There are two main types of catalysts, either homogeneous or heterogeneous in nature. In effect, homogenous catalysts are in the same phase (Fig 1.5A), for example, a dissolved catalyst in a liquid mixture and hence all catalytic sites are available for reaction, whereas heterogeneous catalysts are present in different phases (Fig 1.5B) from reactants, for example, a solid catalyst in a liquid reaction mixture in which the reaction is taking place at the surface of the catalyst (Bowker, 1998; Cole-Hamilton and Tooze, 2006; Smith, 2009).

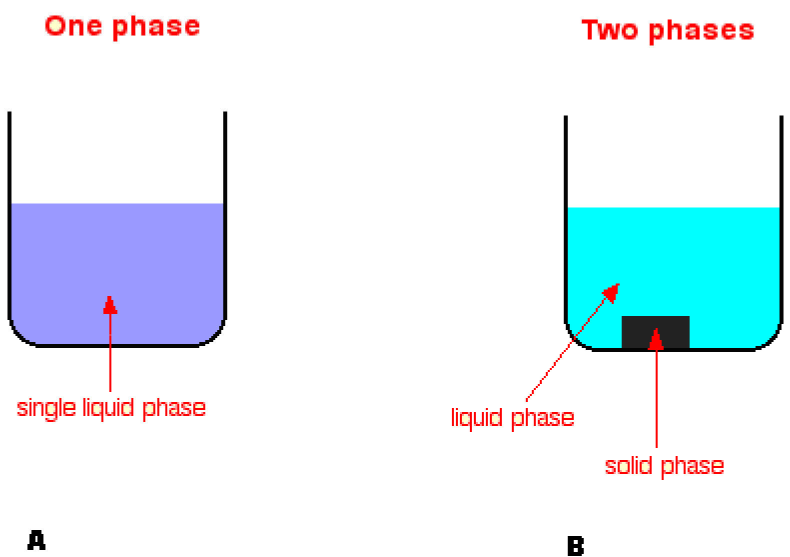


Fig 1.5: (A) and (B) representing homogeneous and heterogeneous phases respectively.

1.6.1 Homogeneous catalyst

As briefly described above, in homogeneous catalysis the catalyst is a molecule which facilitates the reaction. The reactant(s) coordinate to the catalyst or *vice versa* and are converted to product(s), which are then released from the catalyst. The catalyst and reactants are in the same phase (Bowker, 1998). Some examples of homogeneous catalysts include H^+(aq) which reacts as a catalyst in esterification, and chlorine free radicals in the break down of ozone. Thus, the formation of chlorine free radicals is due to the action of ultraviolet radiation on chlorofluorocarbons (CFCs). They react with ozone and give rise to the formation of oxygen molecules and regenerating chlorine free radicals (Bowker, 1998) as indicated in the following reaction:

$Cl + O_3 \rightarrow ClO + O_2$ **Equation 1.7**

$ClO + O \rightarrow Cl + O_2$ **Equation 1.8**

Ruthenium(III) acetylacetonate has been employed as a highly active homogeneous catalyst in the hydrolysis of sodium borohydride (Keceli and Ozkar, 2008). Homogeneous catalysts have many attractive properties, such as high selectivities. However, it is deduced that many homogeneous catalysts cannot be commercialised because of difficulties associated with separating the products from the catalyst (Cole-Hamilton, 2003). Heterogeneous catalysts applications appear to be gaining greater interest in catalysis compared to homogeneous catalysts (Ziolek, 2004).

1.6.2 Heterogeneous catalyst

The simplest model pertaining to heterogeneous catalysis involves the catalyst providing a surface on which the reactants or substrates temporarily become adsorbed (Bowker, 1998). Bonds in the substrate subsequently become weakened enough for new bonds to be created. Thus, the bonds between the products and the catalysts are weaker, so the products are released. Given as an example, in the Haber process for the manufacture of ammonia, finely divided iron acts as a heterogeneous catalyst. In effect, active sites on the metal allow partial weak bonding to the reactant gases, which are adsorbed onto the surface. As a result, the bond within the molecule of the reactant is weakened and the reactant molecules are

held in close proximity to each other. In this manner, the particularly strong triple bond in nitrogen is weakened and the hydrogen and nitrogen molecules are brought closer together than would be the case in the gas phase, so the rate of reaction increases (Kieboom *et al.,* 2000; Yiokari *et al.,* 2000; Armor, 2010).

Heterogeneous catalysis for the synthesis of ammonia:

$3H_2(g) + N_2(g) \leftrightarrow 2NH_3(g)$ – catalysed by Fe(s) **Equation 1.9**

For industrial applications, the development of heterogeneous catalysts is crucial for overcoming some of the limitations of homogeneous catalysts, such as the need to separate the product from the catalyst and the incompatibility with flow reactors (Berner *et al.,* 2006).

1.6.3. Oxidation catalysts

The development of catalysts and selective oxidation reactions is the main area of interest in catalysis (Centi and Perathoner, 1999). Most transition metals including Fe and Cu are used as oxidation catalysts in either homogeneous or heterogeneous state (Villabrille *et al.,* 2007). The need for efficient chemical processes with both low energy consumption and environmental impact has stimulated the quest for improved catalytic systems. One example is the development of oxidation reactions that use molecular oxygen and a metal catalyst at room temperature (Berner *et al.,* 2006). Supported noble metal catalysts have been traditionally considered a different class of materials from that of transition metals oxides, with different mechanisms and applications, but a few studies have been devoted to synergistic systems containing both components, especially when both have a role in the reaction mechanism (Centi and Perathoner, 1999). An example of this class of catalyst is that of heterogeneous Wacker type catalysts in which a transition metal oxide mediates the reoxidation by oxygen of the palladium ion reduced during the oxidation of the hydrocarbon (Centi and Perathoner, 1999). It has been observed that metal ions such as Zn^{2+} (zinc), Ni^{2+} (nickel), and Pd^{2+} (palladium) incorporated into the vanadyl hydrogen phosphate $VOHPO_4{\cdot}0{\cdot}5H_2O$ phase were catalytically active and the palladium incorporated compound particularly displays shape selective catalysis for different oxidation and

reduction reactions (Datta *et al.,* 2002). The most important oxygen sources for oxidation catalysis include dioxygen, (O_2), H_2O_2, alkyl hydroperoxides (ROOH), and hypochlorite anion (ClO^-); (Sherrington, 1988).

1.7. Fenton's reaction

A free radical is an atom or molecule that contains one or more unpaired electrons (Table 1.10) and can exist as anionic, cationic or neutral (Bergendi *et al.,* 1999). Transition metals are highly active as catalysts and have special oxygen transfer properties which improve the utility of H_2O_2 (Hicks, 1975; Henle and Linn, 1997). By far, the most common of these is iron which reacts with H_2O_2 to generate highly reactive hydroxyl radicals ($^{\cdot}OH$); (Henle and Linn, 1997). The reactivity of this system was first observed in 1894 by H.J.H Fenton hence the name 'Fenton's reaction or reagent', but its utility was not recognised until the 1930s once the mechanisms were identified (Wink *et al.,* 1994; Dunford, 2001).
The transition metal catalysts may be used in either the homogeneous phase or heterogeneous phase (Shah *et al.,* 2007). The reactive oxygen species (ROS) (e.g. superoxide, hydroxyl radical and hydrogen peroxide) are hypothesized to be responsible for electrophilic attack and/or degradation of organic contaminants (Smith *et al.,* 2002). The products of the Fenton's reaction are often cited as a model for biological processes such as *in vivo* oxidative damage during certain pathological conditions, carcinogenesis, drug-associated toxicity, postischemic reperfusion injury and arteriosclerosis (Wink *et al.,* 1994). The increased production of ROS and reduction of natural antioxidant species is known as 'oxidative stress' (Franco *et al.,* 2008; Gonsette, 2008). A typical Fenton's reagent is a combination of H_2O_2 and ferrous iron salts to produce hydroxyl radicals, ferric iron (Fe^{3+}), hydroperoxyl radicals ($HO_2^{\cdot}$) (Walling, 1975; Mortazavi *et al.,* 2005), and/or superoxide radicals ($O_2^{\cdot -}$); (Smith *et al.,* 2002). The ferrous iron (Fe^{2+}) initiates and catalyses the decomposition of H_2O_2, resulting in the generation of hydroxyl radicals.

The generation of the radicals involves a complex sequence in an aqueous solution according to the following classical Fenton's reactions:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + {}^{\bullet}OH$ **Equation 1.10**

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\bullet} + H^+$ **Equation 1.11**

$Fe^{2+} + {}^{\bullet}OH \rightarrow Fe^{3+} + HO^-$ **Equation 1.12**

${}^{\bullet}OH + H_2O_2 \rightarrow H_2O + HO_2^{\bullet}$ **Equation 1.13**

$HO_2^{\bullet} \rightarrow O_2^{\bullet -} + H^+$ **Equation 1.14**

$H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + HO^- + {}^{\bullet}OH$ **Equation 1.15**

The above reaction is noted to be a chain reaction in which ferrous iron is regenerated (Equation 1.11); (Dunford, 2002). Previous studies have shown that an acidic pH level near 3 is usually optimum for Fenton's oxidation processes (Zepp, 1992; Ndjou'ou and Cassidy, 2006; Papadopoulos *et al.,* 2007; Katsoyiannis *et al.,* 2008). The main features of the Fenton system are said to be based on reagent conditions, thus, $[Fe^{2+}]$, $[Fe^{3+}]$, $[H_2O_2]$ and the reaction characteristics (pH, temperature and the amount of organic and inorganic constituents); (Yoon *et al.,* 2001). Because these factors are essential in determining the overall reaction efficiency, it is therefore important to understand the mutual relationships between these parameters in terms of hydroxyl radical production and consumption (Neyens and Baeyens, 2003).

Table 1.10: Half-life of free radicals (This table has been reproduced from 'Chemistry, physiology and pathology of free radicals' by Bergendi, L., Benes, L., Durackova, Z., Ferencik, M. 1999, Life Science. Vol. 65, pages 1865-1874. Copyright (1999), with permission from Elsevier).

Intermediate	Formula	Half-life (sec)	Half-life
Hydroxyl radical	$^{\cdot}OH$	10^{-9}	
Alcoxyl radical	$RO^{\cdot}$	10^{-6}	
Singlet oxygen	$^{1}O_2$	10^{-5}	
Peroxynitrite anion	$ONOO^-$	0.05-1.0	
Peroxyl radical	$ROO^{\cdot}$	7	
Nitric oxide	$^{\cdot}NO$	1-10	
Semiquinone radical			days
Hydrogen peroxide	H_2O_2		enzyme decomposition
Superoxide anion radical	$O_2^{\cdot-}$		enzyme decomposition

1.7.1. Applications of Fenton's reagent

The advance oxidation processes (AOPs) that primarily generate highly reactive and non-selective $^{\cdot}OH$ are of great interest for the destruction of contaminants in wastewater (Laat *et al.,* 2004; Chen *et al.,* 2006). Fenton's reagent has the ability to effectively oxidise a diverse collection of contaminants (Kang and Hua, 2005). Its usage as an oxidising system relies heavily on the formation of hydroxyl radicals from H_2O_2 leading to a series of radical propagation and termination reactions (Kang and Hua, 2005). When sufficient amount of $^{\cdot}OH$ is produced, the Fenton's reagent is seen as a potential method for rapidly destroying contaminants primarily in aqueous conditions (Kang *et al.,* 2006) at near diffusion limited rates ($>10^9$ $M^{-1}S^{-1}$); (Ndjou'ou and Cassidy, 2006).

A considerable body of literature on Fenton's reagent has shown that its main application has been on environmental settings such as wastewater treatments (Garcia-Montano *et al.,* 2006; Yoon *et al.,* 2001). Chemical processes companies, such as oil refineries, petrochemical units, textile units, dye and dye intermediate manufacturing industries and many more dump their waste toxic compounds in the environment near to water courses causing water pollution (Ramirez *et al.,* 2007). Fenton's reagent has been applied in

treating some of these hazardous organic pollutants (Garcia-Montano *et al.,* 2006; Ramirez *et al.,* 2007).

Heterogeneous catalyst systems based on the Fenton or Fenton-like reactions are currently emerging as an alternative to the homogeneous version for many applications, including removal of natural organic matter from drinking water bodies (Murray and Parsons, 2004) and microbial decontamination in water treatment processes (Shah *et al.,* 2007; Dramou *et al.,* 2008). Apart from Fenton's reagent, there are several approaches within the AOPs that also generates $^{\cdot}OH$ for similar applications, these include O_3, O_3/ H_2O_2, UV, UV/ H_2O_2, and $O_3/UV/H_2O_2$ (Zhang *et al.,* 2006).

1.7.2. Antimicrobial properties of hydrogen peroxide via Fenton's reaction

The antimicrobial activity of H_2O_2 is widely known and well documented. H_2O_2 is bactericidal, fungicidal, virucidal and sporicidal (Gardner and Peel, 1998). However, a number of factors such as pH, temperature, concentration, the presence of transition metals as well as bacterial strain differences influence the activity of H_2O_2 (Brul and Coote, 1999). In biological systems, superoxide ($O_2^{\cdot-}$) is a moderately reactive compound capable of acting as an oxidant or reductant, however, H_2O_2 is a lot more reactive oxidant than $O_2^{\cdot-}$ and readily diffuses across cell membranes (Miller and Britigan, 1997). It is known that H_2O_2 causes significant cellular damage by attacking DNA (Henle and Linn, 1997). It does not directly oxidise DNA, but it reacts very rapidly with available transition metals (e.g. Cu(I) and/or Fe(II) salts) to form a hydroxyl radical species which attack phospholipids, organic acids (Hallwell and Gutteridge, 1984) and both sugar and base moieties, leading to sugar fragmentation, strand scission, and base adducts (Park and Imlay, 2003).

Oxidative DNA damage occurs in three steps:

reductant^{red} + Fe^{3+} → redunctant^{ox} + Fe^{2+} **Equation 1.16**

$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\cdot}OH + HO^-$ (Fenton's reaction) **Equation 1.17**

$^{\cdot}OH$ + DNA → H_2O + damage **Equation 1.18**

where reductant^{red} and redunctant^{ox} are reduced and oxidised reductant, respectively (Park and Imlay, 2002).

Imlay *et al.* (1988) and Park and Imlay (2003) demonstrated that killing of *Escherichia coli* (*E. coli)* by H_2O_2/ Fe^{2+} was attributed to DNA damage. A pool of iron (ca. 20 μM) observed in *E. coli* that is not integrated into proteins, is known to catalyse the Fenton reaction (Park and Imlay, 2003). It must be emphasised that some microorganisms possess enzymes such as superoxide dismutase and catalase that scavenge superoxide and decompose H_2O_2, thus preventing the formation of $^{\cdot}OH$ and/or intracellular transport of free iron (Srinivasan *et al.,* 2000). Hence anaerobic microorganisms in comparison to aerobic microorganisms that do not contain such enzymes are thus more susceptible to radical attacks (Fridovich, 1978; Srinivasan *et al.,* 2000). Fig 1.6 illustrates the major oxidant pathways and the most significant antioxidants including their synergistic effects.

1.7.3. Lipid peroxidation

Living organisms including plants have polyunsaturated fatty acids (PUFAs) in their membranes (Spiteller, 2001). The PUFAs promote fluidity of cellular membranes (Nakazawa and Masamoto, 1989) and belong to the most oxygen sensitive molecules in nature (Spiteller, 2001). A free radical prefers to steal electrons from the lipid membrane of a cell, initiating a free radical attack on the cell, a process referred to as 'lipid peroxidation' (LPO). LPO may occur via enzymatic process or nonenzymatic process (Spiteller, 2001). Biological membranes including lipoproteins are more susceptible to the damaging effects of LPO processes (Lubec *et al.,* 1996). It can be divided into three categories: initiation, propagation and termination. Reactive oxygen species target the carbon-carbon double bond of polyunsaturated fatty acids (Halliwell and Gutteridge, 1984).

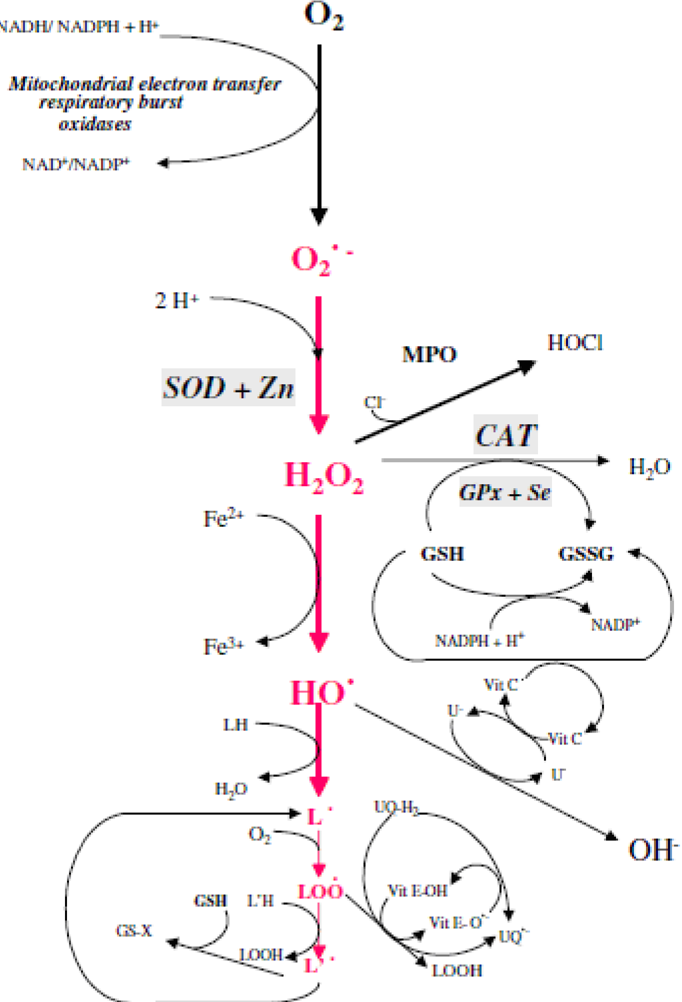


Figure 1.6: The principal oxidative pathways occurring in living organisms and the main antioxidant systems transforming or inactivating free radicals or reactive oxygen species: O_2, oxygen; $O_2^{\cdot}$, superoxide anion; H_2O_2. hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; LH, fatty acid, $L^{\cdot}$, fatty acid radical; $LOO^{\cdot}$, lipid peroxide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidise; MPO, myeloperoxidase; GSH, reduced glutathione; GSSG, oxidised glutathione; GS-X, glutathione linked to an oxidant; NADPH, nicotinamide adenine phosphate; U, uric acid; Vit C, vitamin C; Vit E, vitamin E; UQ, ubiquinone. (This figure has been reproduced from 'The oxidant/antioxidant equilibrium in horses by Kirschvink, N., Moffarts, B.D., Lekeux, P. 2008, The Veterinary Journal, Vol. 177, pages 178-191. Copyright (1999), with permission from Elsevier).

The double bond on the carbon weakens the carbon-hydrogen bond allowing for easy dissociation of the hydrogen by a free radical. A free radical will steal the single electron from the hydrogen associated with the carbon at the double bond. In turn this leaves the carbon with an unpaired electron and hence becomes a free radical. In an effort to stabilise the carbon-centred free radical, molecular rearrangement occurs (Halliwell and Gutteridge, 1984). The newly arranged molecule is known as a conjugated diene (CD). The CD then very easily reacts with oxygen to form a peroxyl radical. The peroxyl radical steals an electron from another lipid molecule in a process known as propagation. This process then continues in a chain reaction. Termination, however, occurs when typically two free radicals combine to form a stable species (Halliwell and Gutteridge, 1984).The reaction process of LPO is provided as follows:

$Lipid\text{-}H + {}^{\cdot}OH \rightarrow Lipid^{\cdot} + H_2O$ **Equation 1.19**

$Lipid^{\cdot} + O_2 \rightarrow Lipid\text{-}O_2^{\cdot}$ (after molecular rearrangement) **Equation 1.20**

$Lipid\text{-}O_2^{\cdot} + Lipid\text{-}H \rightarrow Lipid\text{-}O_2H + Lipid^{\cdot}$ **Equation 1.21**

LPO has been noted to play a major role in oxygen free radical toxicity in reperfused heart (Paradies *et al.,* 1999); and it is also implicated to play a role in the neurodegeneration of multiple sclerosis (Gonsette, 2008). Increased LPO has been detected in the presence of heavy-metals (redox active) in *Saccharomyces cerevisiae* induced with PUFAs linoleate and linolenate (Howlett and Avery, 1997). Fenton's reagent has been found to induce LPO and protein oxidation resulting in cell death in *Azotobacter vinelandii* (Onwurah, 1999).

1.8. Aims and Objectives

The prime aim of this study is to examine the antimicrobial effects of a novel fibrous heterogeneous Fenton-like oxidation catalyst developed at De Montfort University, Leicester (UK).

Main objectives for the research project are outlined as follows:

- To evaluate antimicrobial activities of hydrogen peroxide and peracetic acid and their activation by a novel heterogeneous Fentons-Like modified polyacrylonitrile (PAN) catalyst.
- To evaluate iron leaching from the novel heterogeneous modified PAN catalyst and its effect on antibacterial activity.
- To evaluate antimicrobial action of hydroxyl radicals produced in the decomposition of hydrogen peroxide by the novel heterogeneous modified PAN catalyst.
- To characterise the PAN catalyst, with respect to iron loading and bacterial attachment.

CHAPTER 2

Antimicrobial Activities of Hydrogen Peroxide and Peracetic Acid and their Activation by a Novel Heterogeneous Fentons-Like Modified PAN Catalyst.

2.1. Introduction

Hydrogen peroxide (H_2O_2) as reviewed in chapter 1 (Section 1.5), is an effective microbial disinfectant that destroys pathogens by oxidation. H_2O_2 is effective against both Gram-negative and Gram-positive bacteria, viruses, yeast and fungi (Alt *et al*., 1999); and bacterial spores (Russell, 1990; Gardner and Peel, 1998; Block, 2001). In general, H_2O_2 is known to exhibit more activity against Gram-negative than Gram-positive bacteria (Block, 2001). It has also been utilised to prevent biofilm formation in dental unit waterlines (Decoret *et al.,* 2005; Zanetti *et al*., 2003) as well as disinfecting contact lenses to remove pathogens such as the resistant cyst form of *Acanthamoeba* (Hughes and Kilvington, 2001). H_2O_2 is used as a high level disinfectant (Rutala, 1996). It is deduced that microbial destruction is much more rapid with liquid H_2O_2 than with the vapour type acting at the same temperature (Moore and Payne, 2004). However, H_2O_2 vapour (HPV) appears to be gaining popularity as an effective fumigator and sterilant. It has been applied for decontaminating surfaces against bacterial spores (Johnston *et. al.,* 2005), rooms, medical equipment and ambulances (Andersen *et al.,* 2006).

As discussed earlier, the reaction of H_2O_2 and iron (II)/(III) salts generates reactive oxidising agents, hydroxyl radicals ($^{\cdot}OH$) (Neyens and Baeyens, 2003), which are capable of destroying microbial cells through non-specific attack (McBain and Gilbert, 2001; Russell, 2003; Moore and Payne, 2004).

As mentioned in the preceding chapter, peracetic acid (PAA) is a strong oxidant and disinfectant. Its disinfectant activity is dependent on the release of active oxygen (Liberti and Notarnicola, 1999). PAA is available commercially in the form of a quaternary equilibrium mixture containing acetic acid (CH_3CO_2H), hydrogen peroxide (H_2O_2), peroxyacetic acid or peracetic acid (CH_3CO_3H), and water (H_2O); (Block, 2001; Kitis, 2004; Dell'Erba *et al.,* 2007) as portrayed by the following equation:

$$CH_3CO_2H + H_2O_2 \rightarrow CH_3CO_3H + H_2O \quad \textbf{Equation 2.1}$$

Because of its efficient broad spectrum antimicrobial activity demonstrated in various industries, PAA as a disinfectant of waste water effluents has been gradually gaining popularity (Stampi *et al.,* 2002). PAA has been used to control *Legionella* species on hospital water systems (Ditommaso *et al.,* 2005). Similarly, PAA has been employed as a

sterilant on dental equipment (Ceretta *et al.,* 2008). 0.2% PAA has also demonstrated significant virucidal activity when used in combination with 80% ethanol (Wutzler and Sauerbrei, 2000).

2.2. Types of Biocide testing

There are various established methods available for biocide testing. However, the testing of biocides has been a topic with long history of controversy. Thus, no single test appeared to be universally accepted which is a proof that no single test is completely satisfactory (Ross, 1966; Cremieux *et al.,* 2001). Biocides are used on surfaces, instruments, to treat water, linen, waste, etc., but also on living tissues such as hands and skin, mucous membrane and wounds. They are also widely used in human and veterinary medicine, in agriculture and food industry, as well as in domestic area. Hence, it is unlikely that one test can cover all these fields and domains (Reybrouck, 1998). From a historical perspective, disinfectants agents were continuously evaluated by early research workers including Bucholtz, Pasteur, Koch and Lister, who used viable counts or serial dilution tests performed in nutrient broths; later tests such as the Rideal-walker and Chick-Martin were established for quality control and to protect the public from inefficient disinfectant (Hugo, 1995). Rideal-Walker method is dependent on the estimation of phenol coefficient. Thus in the absence of organic matter, the bactericidal activity of both phenol and test disinfectant is determined against *Salmonella typhi* suspension (Rideal and Walker, 1913). This test is valid only for biocides resembling phenol and is misleading and inaccurate for others. The Chick-Martin test also determines the phenol coefficient of the test biocide, however, the biocides are tested in the presence of yeast suspension, and *S. typhi* as well as *S. aureus* are used for efficacy tests. Kelsey-Sykes method was later devised, in this method, tests were conducted in both clean and dirty conditions and the phenol coefficient was not determined. The Kelsey-Sykes procedure is noted to be cumbersome, slow and costly method (Mattila, 1987). The current quantitative tests that have been developed, compare the initial microbial load, which is subjected to the disinfectant, with the number of survivors. By subtracting the logarithm of the number of surviving organisms from the logarithm of initial inoculum size, these tests give the decimal log

reduction or biocidal effect (Reybrouck, 1998). These tests include quantitative suspension and carrier tests (Cremieux *et al.,* 2001). The former is the basic test for all contemporary testing schemes, and it examines whether microorganisms are killed by a disinfectant in relation to a range of concentrations and several times of exposure (Reybrouck, 1998). Tables 2.1 and 2.2 summarise the characteristics of the established European Committee for Standardisation (CEN) for phase 1 and 2 respectively. In the carrier test, an inert carrier is inoculated with microorganism and allowed to dry, after drying, the carrier is exposed to the use-dilution of the disinfectant for a given contact time, after which it is cultured in a nutrient broth; no growth shows activity of the test disinfectant, whereas growth shows failing (Reybrouck, 1998).

Table 2.1: Basic bactericidal activity. (Adapted from: Cremieux *et al.,* 2001).

Method and Reference	EN 1040 (1997) – Phase 1 Suspension Test	
	Dilution – neutralisation method (preferentially)	Membrane filtration method (if neutralisation fails or suitable neutraliser is not available)
Objective of the Test	To assess basic bactericidal activity of chemical disinfectants and antiseptics	
Test strains	*Pseudomonas aeruginosa* ATCC 15442 *Staphylococcus aureus* ATCC 6538	
Inoculum size	Initial viable cells subjected to test product/s: 1.5×10^7 cfu/mL	
Diluent for the test product and tested concentrations	Distilled water should be used and the product test solution is 1.25 times the required test concentration (maximum tested 80%). At least three dilutions (geometric range 2), including at least two-dilutions in the active range.	
Exposure time and temperature	1, 5, 15, 30, 45, or 60mins at 20°C	
Neutralising test products from the subcultures	Transfer of microorganism-test product mixture (1mL) into a suitable neutraliser validated simultaneously (dilution: 1/10)	Transfer of microorganism-test product mixture (0.1mL) into filtration equipment. Wash under conditions simultaneously with a possible use of neutraliser
Survivor counts	Survivor count in the neutralised by inclusion in agar medium	Membrane placed on agar medium
Incubation	Incubation at 20°C or 20°C at 48 hours (or adapted conditions for additional strains)	
Interpretation of results	Controls must fit with fixed values, and neutralisers for test products including filtration procedures should be validated. The $\log_{10}$ reduction should be calculated. The product passes the test if it demonstrates at least a 10^5 reduction in viability within 60mins (or less) with both test microorganisms. Further tests (phase 2, step 1 and step 20 are required to qualify the product for a specific use.	

ATCC, American Type Culture Collection; cfu, colony-forming units.

Table 2.2: Application test for the food, industrial, domestic, and institutional area: bactericidal activity. (Adapted from: Cremieux *et al.,* 2001).

Method and Reference	EN 1276 (1997) – Phase 2 Suspension Test	
	Dilution – neutralisation method (preferentially)	Membrane filtration method (if neutralisation fails or suitable neutraliser is not available)
Objective of the Test	To assess bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas	
Test strains	*Pseudomonas aeruginosa* ATCC 15442 *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 10536, *Enterococcus hirae* ATCC 10541. Additional strains: *Salmonella typhimurium, Lactobacillus brevis, Enterobacter cloacae* or any suitable strain according to the prescription of the standard	
Inoculum size	Initial viable cells subjected to test product/s: 1.5×10^7 cfu/mL	
Diluent for the test product and tested concentrations	Standard hard water should be used (distilled water for ready-to-use products) and the product test solution is 1.25 times the required test concentration (maximum tested 80%). At least three dilutions (geometric range 2), including at least two-dilutions in the active range.	
Interfering substances	Bovine albumin: clean (0.3 g/L) and dirty (3 g/L) conditions; skimmed milk (1% v/v); yeast extract; sucrose; buffers, sodium lauryl sulfate according to the use of the product	
Exposure time and temperature	5mins and additional times: 1, 15, 30, and 60mins at 20°C and additional temperatures: 4°C, 10°C, or 40°C	
Neutralising test products from the subcultures	Transfer of microorganism-test product mixture (1mL) into a suitable neutraliser validated simultaneously (dilution: 1/10)	Transfer of microorganism-test product mixture (0.1mL) into filtration equipment. Wash under conditions simultaneously with a possible use of neutraliser
Survivor counts	Survivor count in the neutralised by inclusion in agar medium	Membrane placed on agar medium
Incubation	Incubation at 20°C or 20°C at 48 hours (or adapted conditions for additional strains)	

Table 2.2 cont……..

Interpretation of results	Controls must fit with fixed values, and neutralisers for test products including filtration procedures should be validated. The $\log_{10}$ reduction should be calculated for each stain and each test. For general application: the product is considered bactericidal at a concentration that shows at least a 10^5 reduction in viability within 5mins at 20°C, in clean or dirty conditions with each of the four reference strains. For specific applications: the product is considered bactericidal at a concentration that shows at least a 10^5 reduction in viability within 5mins at 20°C, with each of the reference strains and if needed, with additional strains, in additional experimental conditions (i.e. contact time, temperature, interfering substances). Further tests (phase 2, step 2) may be needed to qualify the product for a specific application.

ATCC, American Type Culture Collection; cfu, colony-forming units.

The European Basic Sporicidal Activity Test - Phase 1, BSEN 14347:2005 is used to determine the basic sporicidal activity of a chemical disinfectant. In this method, a test suspension of bacterial spores is added to a sample of the test product. The mixture is maintained at 20°C ± 1°C for a specific contact time selected from one of the following 30min ± 10s, 60min ± 10s or 120min ± 60s. At the end of the contact time, an aliquot is taken and transferred into a tube containing a neutraliser to neutralised the test product. The numbers of surviving bacterial spores in each sample are determined (by counting the sporeforming bacteria) and the reduction is calculated. The product passes the test if it demonstrates at least a 4 log reduction in viable counts within 30min, 60min or 120min or less at 20°C.

There are a large number of works devoted to using modified polyacrylonitrile (PAN) fibres containing functional groups (Liu *et al.,* 1999 ; Gong, 2002) to ligate with metal ions such as Fe^{2+}/ Fe^{3+} which can be used as heterogeneous Fenton catalysts for the oxidative degradation of substances such as organic pollutants in wastewater (Ishtchenko *et al.,* 2003; Zhenbang *et al.,* 2010). It is noted that these fibrous PAN materials have advantages including large specific surface area, good adsorption performance, easy handling and convenient utility in various forms (Zhenbang *et al.,* 2010).

This chapter will examine the antimicrobial activity of various concentrations of H_2O_2 with or without a novel heterogeneous modified PAN catalyst (impregnated with ferric chloride or ferric sulfate) against *E. coli*, *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*Ps. aeruginosa*), and *Bacillus subtilis* (*B. subtilis*) spores.

The sequence of the study is outlined below:

Neutralisation and toxicity tests: This is to determine whether the neutraliser/s used can neutralise or inactivate the test products efficiently; and whether neutralisers are toxic or non-toxic to test organisms.

Hydrogen peroxide validation tests: To establish relatively low H_2O_2 concentrations for microbial inactivation that can be enhanced by the use of the catalyst.

Antimicrobial activity of hydrogen peroxide and heterogeneous catalyst: To examine the effects that different concentrations of H_2O_2 on microbial inactivation both with or without catalyst at room temperature and increased temperature.

Effects of air: To examine the effects of air on bacterial survival.

Effects of modified PAN mesh: To examine the antimicrobial activity of modified PAN mesh before iron impregnation.

Effects of PAN catalyst alone: To examine if modified PAN catalyst alone without H_2O_2 possesses any antimicrobial activity.

Effects of homogeneous catalyst: To compare the antimicrobial activity of H_2O_2 activated by a homogeneous Fentons-like catalyst to that of H_2O_2 activated by the heterogeneous modified PAN catalyst.

Effects of pH: To examine the influence of pH on the antimicrobial activity of H_2O_2. activated by the heterogeneous modified PAN catalyst.

Effects of alkaline/buffer on catalytic activity: To examine if catalytic activity will be reduced or enhanced in an alkaline or buffered environment.

Effects of reused modified PAN catalyst produced from different batches: To examine whether the modified PAN catalyst produced from different batches is reusable in its activation of H_2O_2, i.e. will the antimicrobial activity of the H_2O_2/catalyst system be reduced or remain the same after subsequent uses.

Effects of peracetic acid and PAN catalyst: To examine if the modified PAN catalyst would increase the antimicrobial activity of peracetic acid.

Effects of hydrogen peroxide and PAN catalyst on bacterial spores: To assess the sporicidal activity of H_2O_2/modified PAN catalyst system.

2.3. Materials and Methods

2.3.1. Materials

2.3.1.1. Organisms, media and maintenance

Tests organisms were *E. coli* ATCC 10536, *Ps. aeruginosa* ATCC 15442, *S. aureus* ATCC 6538, and *B. subtilis* NCIB 8054. All bacteria used were preserved and maintained on treated beads in a cryopreservative fluid purchased from Technical Service Consultants (Heywood, UK), and stored at -20°C. CM0003 nutrient agar (Oxoid, UK) solid culture medium was used for viable counts of all test microorganisms apart from *B. subtilis* NCIB 8054 (see Section 2.3.2.2). CM0001 nutrient broth (Oxoid, UK) liquid culture medium was used for all bacterial growth during the experiments except for that of *B. subtilis* NCIB 8054 (see Section 2.3.2.2). Bacteriological peptone, tryptone soya agar (TSA), tryptone soya broth (TSB), bacteriological sodium chloride, tryptone, bacteriological agar (No. 1), blood agar (No. 2) and yeast extract were all purchased from Sigma-Aldrich (Poole, UK). Defibrinated sterile sheep blood was purchased from Fisher Scientific (Loughborough, UK).

2.3.1.2. Chemicals and reagents

Stabilised 30% w/w Hydrogen peroxide (Sigma-Aldrich, Poole, UK) and 37.7% w/w peracetic acid (Sigma-Aldrich, Poole, UK) were stored at 4°C. Glutaraldehyde solution, Grade 1, 50% w/w (Sigma-Aldrich, Poole, UK) was stored at -20°C. Neutralisers used were catalase from bovine liver (Sigma-Aldrich, Poole, UK), glycine (Invitrogen Life Technologies, Paisley, UK), and sodium thiosulfate, anhydrous (Acros Organics, New Jersey). 1M hydrochloric acid (HCl), sodium hydroxide (NaOH), and sodium hydrogen carbonate ($NaHCO_3$) were purchased from Fisher Scientific (Loughborough, UK) and were used to control pH levels. Manganese (II) sulfate monohydrate was purchased from Sigma-Aldrich (Poole, UK). Buffer solution (50mM) was prepared from di-potassium hydrogen orthophosphate anhydrous (K_2HPO_4) (Fisher Chemicals, Loughborough, UK) and potassium dihydrogen orthophosphate (KH_2PO_4) (BDH Chemicals Ltd, East Grinstead, UK). Distilled water obtained using Fistreem Calypso distilled water system (Sanyo, Gallen

Kamp, PLC, Loughborough, UK). Polyacrylonitrile (PAN) mesh (Monarch Ltd, Kirby-In-Ashfield, UK), 99% hydroxylamine mono hydrochloride, 98% hydrazine di-hydrochloride, and transition metal salts (iron (III) chloride hexahydrate or iron (III) sulfate monohydrate) were all purchased from Sigma-Aldrich, (Poole, UK).

2.3.1.3. Equipment and its uses

Sorvall Legend^{TM} T/RT (Kendro laboratory Products, Inc, Newtown, CT, USA) was used to centrifuge all microorganisms. Hanna pH meter Instrument 8520 was used for all pH measurements. Pipette tips of volumes, 1-200µL, 200-1000µL; microcentrifuge tubes 1.5ml with cap; and 90mm single vent Petri dishes were all purchased from Fisher Scientific (Loughborough, UK). Biolab air pump (Biolab, Cheltenham, UK) was used as a source of air in the experiments. Pyrex gas distribution tubes (Barloworld Scientific Ltd, Stone, UK) were used for air dispersion. Electromechanical agitator VWR mini vortexer (Culus Listed Laboratory Equipment) was used for microtube vortexing. Clifton hotplate stirrer (Nickel Electro Ltd, Weston-Super-Mare, UK) and temperature controlled-water baths (Grant Instruments (Cambridge) Ltd, UK) were used to magnetically stir experiments and to perform some experiments at temperatures >30°C respectively. Orbital mechanical incubator S150 (Stuart Scientific, Stone, UK) was utilised for incubation. Glass beads (Fisher Scientific, Loughborough, UK) were used in the sporulation protocol. (See Section 2.3.2.2).

2.3.2. Methods

2.3.2.1 Preparation of bacterial suspensions

A single bead of stock culture was aseptically removed and inoculated into sterilised 10mL volume of nutrient broth and incubated at 37°C ± 1°C for 18-24 hours at a shaking speed of 100 rev/min. After incubation, the bacteria were harvested by centrifugation at 3200 *g* for 15 mins. Supernatants were discarded and the bacteria were resuspended and mixed by vortexing in sterile distilled water, ready to be used for various experimental procedures.

2.3.2.2 Preparation and maintenance of bacterial spores.

The method stipulated in European suspension requirements for basic sporicidal activity (BSEN 14347:2005) was followed for the preparation and maintenance of bacterial spores. One bead of stock culture of *Bacillus subtilis* (vegetative) in cryopreservative was inoculated into 50mL of TSB in a 100mL culture flask and incubated for 24 hours in an orbital mechanical shaker at 100 rev/min and at $37^{o}C \pm 1^{o}C$. 10ml of this culture was transferred into 1000mL Roux-bottles containing 150ml of sporulating agar (peptone USP 10g, yeast extract 2g, manganese sulfate 0.04g, bacteriological agar 15g and water to make up to 1000mL). The liquid culture was evenly distributed on the sporulating agar surface with sterile glass beads (diameter 3mm to 4mm). The Roux-bottles were well sealed with sterile foam plugs and incubated first at $37^{o}C \pm 1^{o}C$ for 2 days and for 21 days at $30^{o}C \pm 1^{o}C$. After 21 days of incubation, 20mL of sterile distilled water was pipetted into the Roux-bottles and the spores were washed off with the help of the glass beads and transferred by aspiration into a 100mL culture flask. The suspension was purified by filtration through two layers of sterile gauze (BSN Medical, Hamburg) into a 50mL centrifuge tube. The filtrates were centrifuged for 30mins at 3000 *g* at $10^{o}C$. The supernatants were discarded and pellets were suspended in 65% w/v 2-propanol (using not more than 50% of the use volume of the tube) for 3 hours at $20^{o}C$ to inactivate the remaining vegetative cells. After 3 hours, a volume of sterile distilled water equal to the volume of 65%w/v 2-propanol in the tube was added to the suspension and centrifuged for 30mins at 3000g at $10^{o}C$. The supernatants were discarded and pellets suspended in sterile distilled water. Centrifugation and washing in water was repeated 5 times. To confirm the purity of spores, the cultures were stained using the malachite green method and examined under light microscope. The number of remaining vegetative cells did not exceed 20% per field of view. Pour plate counts on TSA were performed to determine the number of viable spores present in the suspension. Contaminants of the suspension were assessed by pour plate counts on blood agar. The final spore cfu/mL was 6.96×10^{7}.

The bacterial spores suspensions were stored at $4^{o}C$ and used within 4 weeks.

2.3.2.3. Stock solutions of test products.

Thirty percent (w/w) hydrogen peroxide, 37.7% w/w peracetic acid, and 50% w/w glutaraldehyde were used as stock solutions for antimicrobial tests. 0.015% w/v catalase stock solutions were freshly prepared prior to each experiment. In brief, 0.015g of catalase powder (Catalase was obtained in the form of lyophilized powder and contained a minimum of 13,800 units/mg protein: One unit will decompose 1.0 µmole of H_2O_2 per min at pH 7.0) was evenly mixed in a 50mM buffer solution (100mL total volume) of di-Potassium hydrogen orthophosphate anhydrous (50mM) and Potassium dihydrogen orthophosphate (50mM) at pH 7. After mixing, the catalase solution was filtered into a sterilised 100mL bottle using a filter membrane of 0.20µm pore size (Sartorius Biotech, Goettengen, Germany) and a single use sterilised syringe (Sartorius Biotech, Goettengen, Germany). Five percent (w/v) glycine was prepared and sterilised through a filter membrane as described above. Five grams per litre (0.5% w/v) of sodium thiosulfate was prepared and sterilised by autoclaving.

2.3.2.4. Modified heterogeneous polyacrylonitrile (PAN) catalyst preparation

Two types of the catalyst were used for this project, one was impregnated with ferric chloride and the other by ferric sulfate. The former was prepared at De Montfort University under aseptic conditions; the latter was prepared commercially. The catalysts were prepared according to the method developed by Ishtchenko *et al.* (2003). The preparation of the catalyst requires two major steps, a modification step and impregnation step. Modification solutions were prepared from hydrazine hydrochloride and hydroxylamine hydrochloride. The original PAN mesh was submerged in boiling solution of hydrazine and hydroxylamine at pH 9.5 (NaOH was added to obtain the required pH of the modifying solution) for 2 hours. This procedure was followed by washing with sterilised distilled water. A further treatment with boiling 5% w/v NaOH for 30secs is ensued followed by further washing with sterile distilled water. The modified PAN mesh was then impregnated at room temperature for 19 hours with 0.1M solutions of the iron salts, followed by thorough washing with sterile distilled water. After washing, the modified mesh was dried

for 24 hours by wrapping it in sterilised tissue papers at room temperature, and finally stored in sterilised autoclavable sealed bags. Modified PAN catalyst was washed in a beaker with sterile distilled water before use. Table 2.3 shows amounts of iron per gram on the different modified PAN catalyst used. See Chapter 3, Section 3.2.2.1 and 3.2.2.5. for method used to determine iron on each batch of modified PAN catalyst.

Table 2.3: Amount of iron on different batches of modified PAN catalyst.

PAN catalyst type/batch	Amount of iron (mmol/g of PAN mesh)
PCatCR	0.120
PCatDC1	0.049
PCatDC2	0.054
PCatDC3	0.063
PCatDS	0.214

PCatCR = Laboratory produced PAN catalyst Russian made and impregnated with ferric chloride; PCatDC1, PCatDC2, PCatDC3 = Laboratory produced PAN catalysts based on Dralon L and impregnated with ferric chloride; PCatDS = Commercially produced PAN catalyst based on Dralon L and impregnated with ferric sulfate.

2.3.2.5. Initial viable count method

Bacteria: The Miles & Misra method (1938) as schematically shown in Figure 2.1 was used for the bacterial viability count as follows:

0.1mL of the bacterial suspension in distilled water was transferred into 0.9mL sterile distilled water in eppendorf tubes and serially diluted from 10^{-1} to a 10^{-6}. After vortexing each diluted/inoculated tube, 20µL of each dilution was plated out onto nutrient agar. Petri dishes were incubated for 18-24 hours at 37°C ± 1°C and the number of colonies recorded and colony forming units/mL (CFU/mL) calculated.

Spores: 1mL of the stock culture was serially diluted in universal bottles from 10^{-1} to 10^{-8}. One millilitre of each dilution sample (in duplicate) was mixed with 12 to 15mL melted TSA (pour plate) or blood agar. Petri dishes were incubated for a minimum of 4 and maximum of 7days at 37°C ± 1°C and growth was checked daily. Colonies were counted and averaged, and the number of CFU/mL of the spore forming bacteria determined.

Bacterial suspension in distilled water

0.1ml suspended culture

0.1ml (serial dilutions)

Serial dilutions from 10 fold to 1000, 000 fold in eppendorf tubes

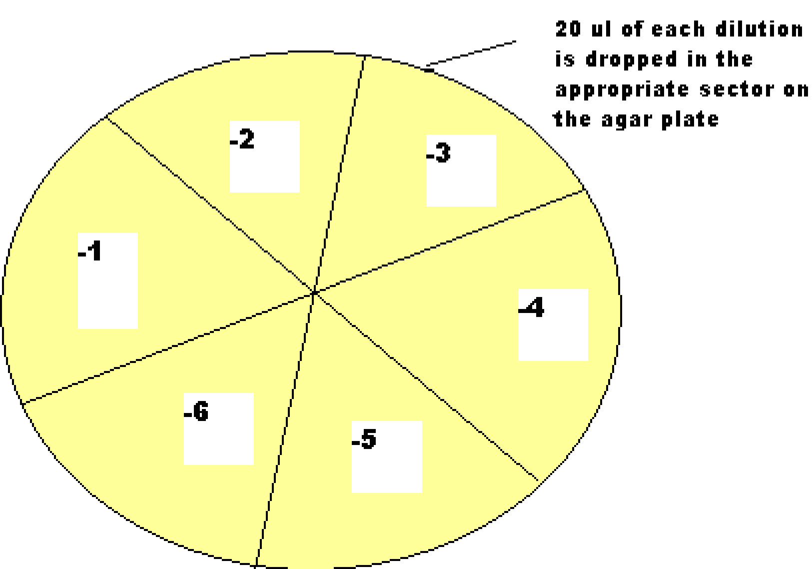


Figure 2.1: Schematic diagram of the procedure for initial bacterial counts using the Miles & Misra approach.

2.3.2.6. Neutralisation tests

Prior to undertaking the main experiments, neutralisation tests were performed to ensure that residual antimicrobial products were decomposed. Catalase from bovine liver was used for the neutralisation tests for H_2O_2.

0.1mL of bacterial or spore suspension in sterile distilled water was added to a solution of 2mL catalase (0.015% w/v) and 7mL sterilised phosphate buffer (pH 7.4) and mixed. 0.9mL of H_2O_2 stock solution was added to the mixture to make up a total volume of 10mL with a resultant concentration in H_2O_2 of 5%, 3%, 7.5%, 0.2%, 0.5% or 1% w/v. Five gram per litre sodium thiosulfate and 5% w/v glycine were used to neutralise peracetic acid (PAA) and glutaraldehyde (GTA) respectively. The neutralisation procedure was the same as described for catalase (9mL neutraliser + 0.1mL bacterial or spore suspension + 0.9mL PAA or GTA). Contact time for the reaction was 5 min ± 10s. After this time, the number of cfu/mL was enumerated using the procedure/s outlined in Section 2.3.2.5.

The effectiveness of the neutralisers for H_2O_2, PAA or GTA was determined by comparing the Log_{10}IC to Log_{10} NTC. Where IC is the initial number of bacteria or spores and NTC is the viable count of the bacteria or spores after exposure to the neutralisers as described.

2.3.2.7. Procedure for toxicity test

The toxicity tests were performed to examine if the neutraliser (catalase, sodium thiosulfate or glycine) possessed any toxicity to the test organisms. The procedure was similar to the neutralisation test as described above with exception that no H_2O_2 or PAA or GTA was added in the reaction mixture. 0.1mL of the bacterial or spore suspension was added to 9mL of the neutraliser with 0.9mL of distilled water making a total volume of 10mL, and left for a contact time of 5 min ± 10s. After this time, the number of cfu/mL was enumerated using the procedure(s) outlined in Section 2.3.2.5. The toxicity effect of the neutraliser was evaluated by comparing the Log_{10}IC to Log_{10} NTC and Log_{10} TTC. Where IC is the initial number of bacteria and TTC is the viable count of the toxicity test control.

2.3.2.8. Modified European quantitative suspension test (Phase 1)

The phase 1 test examines whether antimicrobial products, diluted in distilled water, possess a basic level of activity in the absence of any organic matter (Table 2.1) (Payne *et al.,* 1999; Cremieux *et al.,* 2001; European Standard EN 1040:2005).

The following studies based on this test were undertaken:

- Validation experiments to establish a range of low concentrations of H_2O_2 that can be used in the presence of the modified PAN catalyst to increase antibacterial activity (tested at both room temperature and 35°C; with or without bubbled air).
- Antibacterial effects of modified PAN catalyst together with H_2O_2 (tested at both room temperature and 35°C; with or without bubbled air).
- Antibacterial effects of modified PAN mesh together with H_2O_2 (tested at both room temperature and 35°C; with or without bubbled air).
- Effects of bubbled air (2.5L/min) on bacterial survival (tested at both room temperature and 35°C).
- Antibacterial effects of modified PAN mesh alone (tested at both room temperature and 35°C; with or without bubbled air).
- Antibacterial effects of modified PAN catalyst alone (tested at both room temperature and 35°C; with or without bubbled air).
- Antibacterial effects of homogeneous ferric chloride catalyst solution with or without H_2O_2 (tested at both room temperature and 35°C; with or without bubbled air).
- Effects of pH (acidic or alkaline or buffer) on bacterial survival (tested at both room temperature and 35°C; with or without bubbled air).
- Antibacterial activity of modified PAN catalyst together with H_2O_2 in mildly alkaline or phosphate buffered (pH 7.41) solution (tested at both room temperature and 35°C; with or without bubbled air).
- Antibacterial activity of re-used modified PAN catalyst together with H_2O_2 (tested at both room temperature and 35°C; with or without bubbled air).

- Antibacterial activity of peracetic acid with or without modified PAN catalyst, modified PAN mesh, non-modified PAN mesh or homogeneous ferric chloride solution (tested at room temperature).
- Sporicidal activity of H_2O_2 with or without modified PAN catalyst on *B. subtilis* spores.

The procedure used for the modified European quantitative suspension test was similar for all the above outlined studies. Unless specified, all tests were performed independently for three times. Unless specified, the total volume used in each experiment was 75mL in a 100mL conical flask. This comprised of 7.5mL bacterial suspension (See Table 2.4 for initial microbial number) and an appropriate concentration of test products (H_2O_2 or peracetic acid) in 75mL total volume. When the test was performed at room temperature, the mixture was stirred constantly using a magnetic stirrer to ensure a uniform distribution and to prevent sedimentation of the bacteria. Unless specified, contact times were 10, 20, 30, 40, 50 and 60mins.

When the test was performed at 35^oC, the reaction mixture was the same as described but was placed in a temperature-controlled water bath calibrated to the required temperature prior to use. Since the magnetic stirrer cannot be used in the water bath, a sterilised glass rod was used to stir the reaction mixture at regular intervals. The above procedure was performed with or without bubbled air. The volume of air used for all the experiments was 2.5 L/min supplied via the air pump equipment. Air was filtered and distributed into the mixture via a sterilised Pyrex gas distribution tube (See Appendix 1 for experimental setups).

One millilitre of aliquots were taken out of the experimental flask at appropriate time intervals and transferred into 9mL of neutraliser for 5mins ± 10s to inactivate biocidal activity. After neutralising the reaction, an aliquot of 0.1mL was serially diluted using the Miles & Misra procedure as described earlier (Section 2.3.2.5., Fig. 2.1). Plates were incubated at 37^oC ± 1^oC for 18-24 hours. Following incubation, the viable counts were assessed and log reductions were calculated as follows:

Log_{10} Reduction = Log_{10}IC – Log_{10}E where IC is the initial bacterial number before experiment, and E is the viable number after experiment.

2.3.2.9. Antibacterial effects of H_2O_2 with or without modified PAN catalyst; or modified PAN mesh

H_2O_2 validation tests: Using the procedure as described in Section 2.3.2.8, 5%, 3% and 1% w/v H_2O_2 were initially tested against *E. coli*, *Ps. aeruginosa* and *S. aureus* in the absence of air. 7.5mL of each bacterial suspension was added to a solution of 67.5ml making up a total experimental volume of 75ml containing the appropriate concentration of H_2O_2 (5% or 3% or 1% w/v). One millilitre of the suspension was neutralised in 9mL catalase solution (Section 2.3.2.6.) after each contact time starting from 10, 20, 30, 40, 50 and 60mins at room temperature. After neutralisation for 5mins, 0.1mL of the suspension was transferred into 0.9mL sterile distilled water in eppendorf tubes and serially diluted and enumerated using the Miles & Misra method described in Section 2.3.2.5. Using the same procedure described in this section, low concentrations of 0.2%, 0.5%, and 1%w/v H_2O_2 were further tested against *E. coli*, *Ps. aeruginosa* and *S. aureus* respectively and with or without bubbled air at room temperature or 35°C. The volume of air used for all the experiments was 2.5 L/min supplied via the air pump equipment; and was filtered and distributed into the mixture via a sterilised Pyrex gas distribution tube.

Effects of H_2O_2 with modified PAN catalyst or modified PAN mesh: In these tests, 1 ± 0.1g of either modified PAN catalyst (PCatDC1, PCatDC2, PCatDC3, PCatCR or PCatDS) or modified PAN mesh (modified PAN without iron salt impregnation) was added to either 0.2% or 0.5% or 1% w/v H_2O_2 (1g PAN mesh ≡ 75mL experimental solution) and tested against *E. coli*, *Ps. aeruginosa* and *S. aureus* respectively. One percent (w/v) H_2O_2 with the PAN catalyst was also tested against *E. coli* and *Ps. aeruginosa* respectively. The test procedure, contact times, neutralisation, and incubation were the same as described for 'H_2O_2 validation tests'.

2.3.2.10. Antibacterial effects of air, modified PAN mesh, and modified PAN catalyst respectively.

The antibacterial effects of water and air were examined by passing dispersed air (2.5L/min) into a 75mL total experimental volume containing 7.5mL of either *E. coli* or *Ps. aeruginosa* or *S. aureus* and 67.5mL sterilised distilled water at room temperature or 35^oC. After each contact time (10, 20, 30, 40, 50 and 60mins), a 0.1mL aliquot was serially diluted and plated out onto nutrient agar and incubated as described earlier (Section 2.3.2.8).

The antibacterial effects of modified PAN mesh or PAN catalyst alone were examing by exposing *E. coli*, *Ps. aeruginosa* or *S. aureus* to either 1 ± 0.1g of modified PAN mesh (room temperature & 35^oC; pH = 8.45-8.80) or PAN catalyst (room temperature = pH 3.47-3.54; 35^oC = pH 2.95-3.04). in sterile distilled water (7.5mL bacterial suspension + 67.5mL sterile distilled water) with or without bubbled air (2.5 L/min) at room temperature or 35^oC. After each contact time (10, 20, 30, 40, 50 and 60mins), a 1mL aliquot was neutralised in phosphate buffer (pH 7.41) solution for 5mins in order to remove any residual acidic solution; and serial dilutions and incubation were followed as described in Section 2.3.2.8.

2.3.2.11. Antibacterial effects of homogeneous iron catalyst with or without H_2O_2

Antibacterial effects of homogeneous iron catalyst with H_2O_2: In this procedure, the ferric chloride solution was used as the catalyst by weighing out an amount (0.033g) of the hydrated ferric salt containing the same amount of iron present on 1 ± 0.1g of the modified PAN catalyst and added to the experiment containing either 0.2% w/v or 0.5% w/v or 1% w/v H_2O_2 and tested against either *E. coli*, *Ps. aeruginosa* or *S. aureus* (with or without bubbled air) at room temperature or 35^oC (room temperature = pH 3.47-3.54; 35^oC = pH 2.95-3.04). Experimental procedure, neutralisation, contact times, dilutions and incubations procedures were followed as described in Section 2.3.2.8.

Antibacterial effects of homogeneous iron catalyst alone: The procedure was the same as described for using either modified PAN mesh or PAN catalyst alone in Section 2.3.2.10.

The only difference was that 0.033g of ferric chloride hexahydrate was added to the reaction mixture containing 7.5mL bacterial suspension, 67.5mL sterile distilled water (room temperature pH = 3.47-3.54; 35^oC pH = 2.95-3.04). After each contact time (10, 20, 30, 40, 50 and 60mins), a 1mL aliquot was neutralised in phosphate buffer (pH 7.41) solution for 5mins in order to remove any residual acidic solution; and serial dilutions and incubation were followed as described in Section 2.3.2.8. H_2O_2 was not used in this test. Note: Room temperature was between 22^oC to 25^oC.

2.3.2.12. Effects of pH and mildly alkaline (unbuffered) or phosphate buffered H_2O_2 solutions on bacterial survival; with or without modified PAN catalyst.

Effects of acidic distilled water on bacterial survival: Effects of pH activity on bacterial survival were examined by using 1M HCl to create acidic conditions (room temperature pH = 3.47-3.54; 35^oC pH = 2.95-3.04). Using a sterilised disposal Pasteur pipette, drops of 1M HCl were added to 67.5mL of sterile distilled water to obtain the required pH for either room temperature or 35^oC. 7.5mL of bacterial suspension (*E. coli*, *Ps. aeruginosa* or *S. aureus*) was then added to the acidic water making up a total volume of 75mL. Tests were performed with or without bubbled air. The pH was monitored during the duration of the experiment to make sure that it was maintained. Any drop of pH below the required level was controlled with 1M NaOH. After each contact time (10, 20, 30, 40, 50 and 60mins), 1mL aliquot was neutralised in phosphate buffer (pH 7.41) solution for 5mins in order to remove any residual acidic solution; and serial dilutions and incubation were followed as described in Section 2.3.2.8.

Effects of acidic H_2O_2 on bacterial survival: 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 solutions were prepared using 1M HCl to create acidic conditions (room temperature pH = 3.47-3.54; 35^oC pH = 2.95-3.04). These acid solutions were tested against *E. coli*, *Ps. aeruginosa* and *S. aureus* with or without bubbled air. pH was monitored and controlled as described above. Experimental procedure, neutralisation, contact times, dilutions and incubations procedures were followed as described in Section 2.3.2.8.

Effects of unbuffered mildly alkaline 0.5% w/v H_2O_2 with or without modified PAN catalyst against *Ps.aeruginosa*: Drops of 1M NaOH were used to create mildly alkaline

solutions of 0.5% w/v H_2O_2 (room temperature pH = 7.57-7.60; 35°C pH = 7.42-7.52), which were then tested against *Ps. aeruginosa*. Tests were performed in the presence or absence of 1 ± 0.1g of modified PAN catalyst and with or without bubbled air. Experimental procedure, neutralisation, contact times, dilutions and incubations procedures were followed as described in Section 2.3.2.8.

Effects of buffered 0.5% w/v H_2O_2 with or without modified PAN catalyst on *Ps. aeruginosa*: To create buffered conditions, all experimental constituents were prepared in phosphate buffer (pH 7.41), thus, bacterial cultures after centrifugation were re-suspended in sterilised phosphate buffer instead of sterilised distilled water. Similarly, 0.5% w/v H_2O_2 was also prepared in phosphate buffer. Tests were performed in the presence or absence of 1 ± 0.1g of modified PAN catalyst and with or without bubbled air. Experimental procedure, neutralisation, contact times, dilutions and incubations procedures were followed as described in Section 2.3.2.8.

2.3.2.13. Antibacterial activity as a function of the reusability of different batches of modified PAN catalyst with H_2O_2

The procedure for re-used catalyst experiments was the same as described previously in Section 2.3.2.9. The only difference was that in this procedure, modified PAN catalyst was reused 48 hours or 6 months after first use. Experimental procedure, neutralisation, contact times, dilutions and incubations procedures were followed as described in Section 2.3.2.8.

2.3.2.14. Antibacterial effects of peracetic acid with or without modified PAN catalyst, homogeneous iron catalyst, non-modified PAN mesh or modified PAN mesh.

In this procedure a range of peracetic acid (PAA) final in use concentrations of 0.18%, 0.1%, 0.05%, 0.025%, 0.006%, 0.003%, and 0.001% w/v in the presence or absence of 1 ± 0.1g modified PAN catalyst were tested against *S. aureus*. Further work was performed using 0.0002% w/v PAA with either modified PAN catalyst (PCatDC3 or PCatDS), 0.02g ferric chloride hexahydrate (the same amount of iron present on 1 ± 0.1g of PCatDC3),

non-modified PAN mesh (not chemically modified as described in Section 2.3.2.4.), or modified PAN mesh (chemically modified but not impregnated with iron salts). Experimental procedure, neutralisation, contact times, dilutions and incubations procedures were followed as described in Section 2.3.2.8. However, these tests were performed only at room temperature and without bubbled air.

2.3.2.15. Sporicidal activity of H_2O_2 with or without modified PAN catalyst (Modified European Basic Sporicidal Activity Test - Phase 1, BSEN 14347:2005).

This test examines whether products, diluted in distilled water, possess a basic level of sporicidal activity in the absence of any organic matter. The test procedure used was the same as described above (Section 2.3.2.8), but the total experimental volume was 37.5mL prepared in a 100mL conical flask. Final in-use concentration of either 5% or7.5% w/v H_2O_2 with or without 0.5g modified PAN catalyst were tested against *Bacillus subtilis* spores. The appropriate neutralisers as described in Section 2.3.2.6 were used. Reference test products of 0.05% and 0.1% w/v peracetic acid; and 1% and 3% w/v glutaraldehyde were used as test controls. Glutaraldehyde was activated to alkaline pH (pH 7.59-7.85) with $NaHCO_3$ (solid). The procedure for incubation and calculation of colony forming units of spore forming bacteria was the same as described in Section 2.3.2.8. All experiments were performed at room temperature and without bubbled air and the contact times used were 10, 30, 60 and 120mins.

2.3.2.16. Statistical Analysis

The cfu/mL counts (three replicates) after each contact time were calculated and converted into $\log_{10}$ cfu/mL prior to statistical evaluation. Microsoft Excel's Students' *t*-test was employed for all statistical analyses. A one tail test was employed to evaluate specifically whether there is increase or decrease (significant difference) in activity between two samples. For example whether catalyst/H_2O_2 system is more effective than using H_2O_2 alone or the statistical differences between the 'presence of bubbled air and absence of bubbled air'; or between 'antimicrobial activity at room temperature and at 35°C' or

‘differences in activity between catalyst impregnated with the same iron salts but from different batches’.

2.4. Results and Discussions

2.4.1. Effects of neutralisers on test products and on microorganisms.

Neutralising activity against test products; and neutraliser toxicity on test microorganisms were examined. The results are summarised in Table 2.4. The neutralisers, catalase solution (0.015% w/v), sodium thiosulfate solution (0.5% w/v), and glycine (5% w/v) showed efficient neutralising activity against 7.5% w/v and 5% w/v H_2O_2, 0.1% w/v PAA and 3% w/v GTA respectively. *E. coli*, *S. aureus* and *Ps. aeruginosa* survived the effects of 5% w/v H_2O_2 in the presence of 0.015% w/v catalase solution after 5mins contact time. There was no significant difference ($p>0.05$) between the log_{10} of the initial bacterial number and the log_{10} of the bacterial number enumerated in the neutralising control tests. Similarly, in the presence of the neutralisers catalase, sodium thiosulfate and glycine, the spores of *Bacillus subtilis* survived the effects of 7.5% w/v H_2O_2, 0.1% w/v PAA and 3% w/v GTA at 5mins contact time (Table 2.4). There was no significant difference ($p>0.05$) between the log_{10} of the initial bacterial spores number and the log_{10} of the bacterial spores number from the neutralising control tests. Furthermore, these neutralisers showed no toxicity to the test organisms as there was no significant difference ($p>0.05$) between the log_{10} of the initial number of bacteria or spores and the log_{10} of the number of bacteria or spores from the neutralisers in the absence of test products (H_2O_2, PAA and GTA).

The effectiveness of antimicrobial products such as disinfectants, sanitisers and antiseptics is measured by their ability to inactivate microbial agents at a particular contact time. Hence, the accurate determination and effectiveness of disinfectant products rely primarily on the efficient and effective neutralisation of the disinfectant as incomplete neutralisation of the test products would allow continuous killing of microbial agents beyond the required experimental contact times, leading to unreliable conclusions by exaggerating the activity of disinfectant (ASTM International, 2002). A review by Russell (1981) on the effective neutralisation of biocides provided three criteria for effective neutralisation:

1. The neutraliser should be capable of effectively inactivating or inhibiting the test product;
2. The neutraliser should not be toxic to the challenged microorganism and;
3. The combination of the neutraliser and test product should not form toxic compounds against the challenge organism.

The results of Table 2.4 showed that these neutralisers can efficiently and effectively inactive the test products at 5mins contact time. Equally, these neutralisers were not toxic to the test microorganisms (Table 2.4). The initial bacterial count, viable count of neutralisation and toxicity challenge tests all showed at least 8 log_{10} cfu/mL with negligible log reduction. Similarly, the initial spore count, viable count of neutralisation and toxicity challenge tests all showed at least 7 log_{10} cfu/mL with a negligible log reduction (Table 2.4).

Table 2.4: Tests for initial microbial counts, neutralisation and toxicity controls.

Control Tests	Mean log_{10}IC (± S.D.)	Mean log_{10}NTC (± S.D.)	Mean log_{10}TTC (± S.D.)
E. coli alone	8.21(0.08)		
E. coli + NCS. + 5% w/v H_2O_2		8.25(0.17)	
E. coli + NCS.			8.27(0.10)
Ps. aeruginosa alone	8.31(0.12)		
Ps. aeruginosa + NCS. + 5% w/v H_2O_2		8.26(0.08)	
Ps. aeruginosa + NCS.			8.46(0.09)
S. aureus alone	8.24(0.10)		
S. aureus + NCS. + 5% w/v H_2O_2		8.16(0.07)	
S. aureus + NCS.			8.18(0.02)
B. subtilis spores alone	7.84(0.01)		
B. subtilis spores + NCS. + 7.5% w/v H_2O_2		7.38(0.03)	
B. subtilis spores + NCS.			7.37(0.01)
B. subtilis spores + NST + 0.1% w/v PAA		7.31(0.10)	
B. subtilis spores + NST			7.37(0.05)
B. subtilis spores + NGLC + 3% w/v GTA		7.37(0.02)	
B. subtilis spores + NGLC			7.40(0.07)

S.D = standard deviation; log_{10}IC = log_{10} of initial count; log_{10}NTC = log_{10} of neutralisation test control; log_{10}TTC = log_{10} of toxicity test control; NCS = neutraliser catalase solution; NST = neutraliser sodium thiosulfate; NGLC = neutraliser glycine; PAA = Peracetic acid; GTA = glutaradehyde.

* Contact time = 5mins.

2.4.2. Antibacterial effects of H_2O_2 with or without modified PAN catalyst; or modified PAN mesh

2.4.2.1. H_2O_2 validation tests

The H_2O_2 validation tests were performed for two purposes:

1. To establish a concentration of H_2O_2 that would slowly inactivate the test organisms (longer contact times).
2. To use the catalyst with established H_2O_2 concentrations to speed up microbial inactivation rate (quicker inactivation contact times).

Various concentrations of H_2O_2 were initially considered and tested against *E. coli*, *Ps. aeruginosa* and *S. aureus*. The concentrations used were 5% w/v, 3% w/v, 1% w/v, 0.5% w/vand 0.2% w/v.

The effects of 5% w/v, 3% w/v and 1% w/v concentrations of H_2O_2 at room temperature were tested against *E. coli*, *Ps. aeruginosa*, and *S. aureus* for10 to 60mins contact times in the absence of air. Further tests using 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 were carried out against *E. coli*, *Ps. aeruginosa*, and *S. aureus* at both room temperature and 35^oC (with or without bubbled air). The antibacterial activity of 5% w/v and 3% w/v H_2O_2 are summarised in Table 2.5; and the activity of 1% w/v H_2O_2 against all three bacteria is presented in Table 2.6. The results for the antibacterial activity of 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 at room temperature and 35^oC are shown in Tables 2.7 and 2.8 respectively. Both 5% and 3% w/v completely inactivated *E. coli*, *Ps. aeruginosa*, and *S. aureus* within 10mins of contact (Table 2.5), as a result, these concentrations were excluded from subsequent experiments. However, 1% w/v H_2O_2 inactivated *E. coli* and *Ps. aeruginosa* at 20mins (Table 2.6); and showed 5.37 log reduction at 60mins against *S. aureus*. Clearly, 1% w/v H_2O_2 showed more antibacterial activity against *E. coli* and *Ps. aeruginosa* than *S. aureus*.

In the absence of air at room temperature, 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 showed 4.76, 0.97 and 5.37 log reductions against *E. coli, Ps. aeruginosa* and *S. aureus*

respectively at 60mins. At room temperature, the presence of air did not affect the reduction of *Ps. aeruginosa* and *S. aureus*, however, this was not the case observed for *E. coli* as it was inactivated (>5.58 log reduction) at 50mins ($p<0.01$) compared with when air was not added to the reaction.. Bacterial inactivation increased significantly at 35°C compared to activity at room temperature (Tables 2.7 & 2.8).

At 35°C, the presence or absence of bubbled air in combination with 0.2% w/v H_2O_2 inactivated *E. coli* at 40mins (Table 2.8). At the same time point and the same experimental conditions, 0.5% w/v H_2O_2 showed a 4.09 (without air) and a 3.06 (with air) log reductions against *Ps. aeruginosa* respectively; and 1% w/v H_2O_2 completely inactivated *S. aureus* at 40mins with or without air. The presence of air did not show any significant difference ($p>0.05$) in the experiment against *E. coli*, *Ps. aeruginosa* and *S. aureus* as compared to its absence at 35°C, although, at room temperature the presence of air caused an increase in activity against *E. coli* at 50 and 60mins. Overall, bacterial inactivation was dependent on H_2O_2 concentration, contact time and temperature.

From the results described above, it can be seen that high concentrations (5% w/v and 3% w/v) of H_2O_2 rapidly inactivated all the organisms, this was not true for lower H_2O_2 concentrations. 1% w/v had a relatively rapid bactericidal effect on *E. coli* and *Ps. aeruginosa* (both inactivated within 20mins) but had slow inactivating effects on *S. aureus* (within 60mins); (Tables 2.6, 2.7) at room temperature. 0.2% w/v, 0.5% w/v and 1% w/v were seen to inactivate *E. coli*, *Ps. aeruginosa* and *S. aureus* at a slow rate (Tables 2.7, 2.8). These concentrations were maintained for subsequent studies on the organisms so that any increases in activity caused by the catalyst could be easily observed.

The differences in the microbial responses to different concentrations of H_2O_2 may be explained due to catalase activity. The catalase enzyme is found widely in mammalian and non-mammalian aerobic cells containing a cytochrome system, and with a few exceptions, only strict anaerobes lack this enzyme (Deisseroth and Dounce, 1970). These enzymes use a two-electron transfer mechanism to decompose H_2O_2 into molecular oxygen and water

(Williams, 1927; Horst *et. al.,* 2006; Guwy *et al.,* 1999). Catalases scavenge H_2O_2 and protect cells from reactive oxygen species (Guwy *et al.,* 1999). Catalases are present in many aerobic bacteria such as *E. coli* (Guwy *et al.,* 1999), *Ps. aeruginosa* (Elkins *et al.,* 1999), and *S. aureus* (Martin and Chaven, 1987; Hart *et al.,* 2001). (See Chapter 4 for further information about decomposition of H_2O_2).

The *E. coli* strain employed in this study was catalase negative whilst the *Ps. aeruginosa* and *S. aureus* strains used in these tests were catalase-positive organisms, as a result, they are able to exhibit some degree of scavenging against low H_2O_2 concentrations but were unable to show such scavenging abilities and survive in the presence of higher amounts of H_2O_2 (5% w/v and 3% w/v). It was noted from the range of H_2O_2 concentrations used that *E.coli* was more susceptible to the effects of H_2O_2 as compared to *Ps. aeruginosa* and *S. aureus* possibly due to its lack of the catalase enzyme. Although, *Ps. aeruginosa* and *S. aureus* are both catalase-positive, their susceptibility to H_2O_2 varied considerably. *Ps. aeruginosa* was more susceptible to the effects of 1% w/v H_2O_2 than *S. aureus* and this may be due to differences in their respective catalase activity and in addition, possibly in differences in their respective cellular structures, as it is known that microbial cellular structures play significant role in biocide uptake (Maillard, 2002). *S. aureus* appeared to show stronger catalase activity than *Ps. aeruginosa*. Catalase enzymes are known to vary between bacterial species and strains; for example, ammonia-oxidising bacteria such as *Nitrosomonas europaea* was found to contain very high specific activity of catalase compared to other nitrifiers (Wood and Sørensen, 2006). Such variability may play a significant role in the degree and ability to which a particular microorganism is able to scavenge H_2O_2.

The data demonstrated (Table 2.7) that the presence of bubbled air in the reaction mixture (at room temperature) did not influence the activity of H_2O_2 on *Ps. aeruginosa* and *S. aureus*, but there was an increase in antibacterial activity ($p<0.01$) against *E. coli* in the presence of air than its absence from 30-60mins.

35°C experimental conditions were used to establish whether an increase in temperature would result in a corresponding increase in antibacterial activity. The temperature used

(35°C) was considered not to be lethal to the test organisms and also not high enough to destroy the activity of H_2O_2 as H_2O_2 is known to decompose at elevated temperatures (Lin *et al.,* 1991). It is well established that the efficacy by chemical disinfectants to inactivate microorganisms is reduced significantly at lower temperatures (Sobsey, 1989; Kim *et al.,* 2004) but increases at a relatively higher temperatures (Kim *et. al.,* 2004). An increased antibacterial activity against all of the organisms was observed at 35°C when 0.2%, 0.5% and 1% w/v H_2O_2 were used as compared to the same experimental conditions performed at room temperature (see Tables 2.7 and 2.8). There was no significant difference when air was used at 35°C as compared to when it was absent against all the three organisms. Overall, bacterial inactivation was dependent on H_2O_2 concentration, contact time and temperature. The H_2O_2 validation work presented here has shown that 0.2%, 0.5% and 1% w/v H_2O_2 are able to reduce viability of the test organisms at both room temperature and 35°C, but requires longer contact time particularly at room temperature to cause complete microbial inactivation. Further work was performed to establish if the addition of modified PAN catalyst can increase antimicrobial activity of 0.2%, 0.5% and 1% w/v H_2O_2 at a shorter contact time. This work is demonstrated in Section 2.4.2.2.

Table 2.5: Antibacterial effects of 5% w/v and 3% w/v H_2O_2 on *E. coli*, *Ps. aeruginosa* and *S. aureus* at room temperature.

Contact time/min	Mean Log_{10} reduction (± S.D.)					
	5% H_2O_2 *E. coli*	5% H_2O_2 *Ps. aeruginosa*	5% H_2O_2 *S. aureus*	3% H_2O_2 *E. coli*	3% H_2O_2 *Ps. aeruginosa*	3% H_2O_2 *S. aureus*
10	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)
20	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)
30	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)
40	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)
50	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)
60	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)	>5.56(0.00)	>5.52(0.00)	>5.41(0.00)

pH 5.53-5.95; S.D. = standard deviation; '1 Log_{10} reduction is a 10-fold reduction'

Table 2.6: Antibacterial effects of 1% w/v H_2O_2 against *E. coli*, *Ps. aeruginosa* and *S. aureus* at room temperature

Contact time/min	Mean Log_{10} reduction (± S.D.)		
	E. coli	*Ps. aeruginosa*	*S .aureus*
	1% H_2O_2	1% H_2O_2	1% H_2O_2
10	2.66(0.10)	3.70(0.16)	0.70(0.18)
20	>5.44(0.00)	>5.53(0.00)	1.43(0.75)
30	>5.44(0.00)	>5.53(0.00)	2.88(0.99)
40	>5.44(0.00)	>5.53(0.00)	3.89(0.66)
50	>5.44(0.00)	>5.53(0.00)	4.97(0.65)
60	>5.44(0.00)	>5.53(0.00)	5.37(0.40)

pH of 1% w/v H_2O_2 = 6.01; S.D. = standard deviation.

Table 2.7: Antibacterial effects of H_2O_2 with or without air (2.5L/min) on vegetative microorganisms at room temperature.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	0.2% H_2O_2 no bubbled air	0.2% H_2O_2 with bubbled air	0.5% H_2O_2 no bubbled air	0.5% H_2O_2 with bubbled air	1% H_2O_2 no bubbled air	1% H_2O_2 with bubbled air
10	1.01(0.36)	1.33(0.27)	0.52(0.12)	0.36(0.13)	0.70(0.18)	0.61(0.09)
20	2.20(0.42)	2.68(0.22)	0.81(0.24)	0.53(0.10)	1.43(0.75)	1.27(0.64)
30	2.67(0.52)	3.98(0.28)	0.88(0.25)	0.55(0.18)	2.88(0.99)	2.57(0.44)
40	3.52(0.57)	4.79(0.43)	0.86(0.32)	0.59(0.00)	3.89(0.66)	3.84(0.15)
50	4.21(0.19)	>5.58(0.00)	0.87(0.28)	0.72(0.07)	4.97(0.65)	5.07(0.21)
60	4.76(0.39)	>5.58(0.00)	0.97(0.22)	0.50(0.09)	5.37(0.40)	5.40(0.17)

pH 6.21 – 6.01; S.D. = standard deviation.

Table 2.8: Antibacterial effects of H_2O_2 with or without air (2.5L/min) on vegetative microorganisms at 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	0.2% H_2O_2 no bubbled air	0.2% H_2O_2 with bubbled air	0.5% H_2O_2 no bubbled air	0.5% H_2O_2 with bubbled air	1% H_2O_2 no bubbled air	1% H_2O_2 with bubbled air
10	2.63(0.30)	2.72(0.11)	2.48(0.28)	1.72(0.13)	1.81(0.18)	1.96(0.31)
20	4.30(0.37)	4.74(0.13)	3.23(0.14)	2.34(0.33)	4.45(0.18)	4.45(0.26)
30	5.29(0.17)	5.23(0.10)	3.91(0.18)	3.14(0.65)	5.13(0.15)	≥5.27(0.55)
40	>5.39(0.00)	>5.59(0.00)	4.09(0.13)	3.06(0.21)	>5.59(0.00)	>5.59(0.00)
50	>5.39(0.00)	>5.59(0.00)	4.22(0.20)	3.35(0.24)	>5.59(0.00)	>5.59(0.00)
60	>5.39(0.00)	>5.59(0.00)	4.40(0.13)	3.51(0.26)	>5.59(0.00)	>5.59(0.00)

pH 5.50-5.89; S.D. = standard deviation.

2.4.2.2. Antibacterial effects of H_2O_2 with heterogeneous modified PAN catalyst

The Fenton based reaction as discussed earlier (Chapter 1 Section 1.7) involves the reaction of iron salts (catalyst) with H_2O_2 to produce free hydroxyl radicals ($^{\cdot}OH$), very powerful oxidants.

The antimicrobial potency of the established concentrations of 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 in combination with 1 ± 0.1g of novel heterogeneous modified PAN catalyst were tested against *E. coli*, *Ps. aeruginosa* and *S. aureus* at room temperature and 35°C (with or without bubbled air) respectively. The reaction pathway in this study is presumed to be partly based on the Fenton's-like reaction because ferric (Fe^{3+}) chloride and sulfate salts were used to impregnate the modified PAN mesh to form the heterogeneous PAN catalyst.The initial PAN mesh was produced from Russian polyacrylonitrile fibres whilst the others were all produced in the UK using Dralon L as the source of polyacrylonitrile fibres. The types of modified PAN catalyst used will be referred in the text as follows: PCatCR (Russian type with ferric chloride impregnation); PCatDC1, PCatDC2, PCatDC3 (Dralon L types with ferric chloride impregnation); and PCatDS (Dralon L type with ferric sulfate impregnation); (see Table 2.3 for further details).

Table 2.9 shows results for antibacterial activity of 1% w/v H_2O_2 with modified PAN catalyst (PCatCR or PCatDS) against *E. coli, Ps. aeruginosa* and *S. aureus* at room temperature. The results for the antibacterial activity of 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 with PCatCR or PCatDC1 at room temperature and 35°C are shown in Tables 2.10, 2.11, 2.12, and 2.13. Fig. 2.2. shows a summary demonstration of the differences in the rate of antibacterial effects between H_2O_2 alone and modified PAN catalyst/H_2O_2 system.

The presence of modified PAN catalyst (PCatCR or PCatDS) with 1% w/v H_2O_2 inactivated all the bacteria within 10mins. There was significantly more antibacterial activity with either 'PCatCR or PCatDS and 1% w/v H_2O_2' (Table 2.9) than with '1% w/v H_2O_2 alone' (Table 2.6) against *E. coli* and *Ps. aeruginosa* ($p<0.01$) at 10mins. From 10-40mins, 'PCatCR or PCatDS with 1% w/v H_2O_2' showed significantly more antibacterial

activity ($p<0.05$) against *S. aureus* than when '1% w/v H_2O_2 alone' was used (compare Tables 2.9 & 2.6).

The addition of PAN catalyst (PCatCR) to 0.2% w/v and 0.5% w/v H_2O_2 at room temperature increased bacterial inactivation. There was total bacterial inactivation at 30mins against *E. coli* (in the presence of air), and 20mins against *Ps. aeruginosa* (with or without air) (Table 2.10), compared to much lower levels of inactivation seen when the catalyst was not present (Table 2.7). There was highly significant difference ($p<0.01$) between the presence of PCatCR and its absence against *E. coli* (from 10-40mins, with air; Fig 2.2) as well as *Ps. aeruginosa* (from 10-60mins, with or without air). The presence of bubbled air in these conditions did not show any significant difference against all three organisms.

Furthermore, the addition of PCatCR to either 0.2% w/v or 0.5% w/v or 1% w/v H_2O_2 showed an increase antibacterial activity at 35^oC as compared to room temperature (Tables 2.10 & 2.11). At 10mins, the addition of PCatCR to 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 completely inactivated *E. coli, Ps. aeruginosa* and *S .aureus* at 35^oC with or without air. There was highly significant increased ($p<0.01$) activity from 10-30mins in the presence of PCatCR as compared to its absence against *E. coli* at 35^oC (Tables 2.8 & 2.11). Similar significant increases ($p<0.01$) in activity were observed from 10-60mins when PCatCR was present against *Ps. aeruginosa* at 35^oC compared to when it was absent.
The presence of PCatDC1 in 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 showed similar increases in antibacterial activity as PCatCR when used under similar experimental conditions at both room temperature and 35^oC against *E. coli*, *Ps. aeruginosa* and *S. aureus* (see Tables 2.10 & 2.12; and 2.11 & 2.13). 0.2% w/v H_2O_2 with PCatDC1 completely inactivated *E. coli* at 30mins (room temperature; Fig 2.2) and after 20mins at 35^oC; and 0.5% w/v and 1% w/v H_2O_2 completely inactivated *Ps. aeruginosa* and *S. aureus* 20mins (room temperature) and after 10mins at 35^oC. There were slight variations in the activity between PCatCR and PCatDC1. Overall, there were no significant differences ($p>0.05$) between the antibacterial activity of PCatCR and PCatDC1 against *E. coli* and *Ps.*

aeruginosa at room temperature, and *Ps. aeruginosa* and *S. aureus* 35°C, however, PCatCR was significantly ($p<0.05$) more effective than PCatDC1 against *E. coli* after 10mins at 35°C and also against *S. aureus* after 10mins at room temperature.

0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 require the presence of the catalysts PCatCR, PCatDC1 and PcatDS to show increased antibacterial or bactericidal activity. Overall, bacterial inactivation was dependent on H_2O_2 concentration, presence or absence of modified PAN catalyst, contact time, and degree of temperature.

It has been demonstrated that the activities of the modified PAN catalysts significantly influenced the antibacterial activity of H_2O_2 to cause quicker bacterial inactivation against *E. coli*, *Ps. aeruginosa*, and *S. aureus* at room temperature and 35°C as compared to when the catalysts were absent from the reaction. From the data (Tables 2.9, 2.10, 2.12), it is clear that the modified PAN catalysts tend to provide synergistic antibacterial effects to 0.2%, 0.5%, and 1% w/v H_2O_2, although, other factors such as pH may also be a contributory factor in microbial inactivation (see Section 2.4.5. for further work on effects of pH on microbial survival).
A more rapid bactericidal activity was observed when the test organisms were subjected to H_2O_2 with PCatCR or PCatDC1 at 35°C than at room temperature. Classical Fenton's or Fenton's-like reaction are known to increase $^{\cdot}OH$ production at elevated temperatures (Changha and Jeyong, 2004; Tekbas *et al.,* 2008), which could result in an increase in antimicrobial activity. *Ps. aeruginosa* and *S. aureus* were inactivated within 10mins contact time when PCatCR or PCatDC1 with H_2O_2 was used, compared to slower activity seen in the absence of these catalysts (Table 2.8, 2.11, 2.13). It is likely that total inactivation for these three organisms would be even less than 10min as even at room temperature (with or without bubbled air), *S. aureus* was completely inactivated within 10mins of contact time. Similarly, PCatCR and PCatDC1 with 0.2%w/v H_2O_2 completely inactivated *E. coli* at 10 and 20mins respectively. Catalytic activity was found to be more pronounced at relatively higher H_2O_2 concentrations, as antibacterial potency was much higher against *E. coli* (using 1%w/v H_2O_2), *Ps. aeruginosa* (using 0.5%w/v, 1%w/v H_2O_2) and *S. aureus* (using 1%

H_2O_2). This may also be due to the possibility that in the presence of the catalyst, the amount of $^{\cdot}OH$ produced is dependent on H_2O_2 concentration.
Clearly, it was seen that at both room temperature and 35°C, PCatCR seem to be slightly more active than PCatDC1, the reason for this may be because PCatCR has twice as much iron content as PCatDC1 (see Table 2.3). These differences could also be due to differences in surface area of the mesh, extent of iron leaching, extent of washing off residual chemicals, for example, hydroxylamine, hydrazine, ammonia and salts.

As discussed above, the presence of PCatCR or PCatDC1 enhanced the antimicrobial activity of H_2O_2 greatly at either room temperature or at 35°C. The mechanism/s of increased bactericidal action of H_2O_2 in the presence of modified PAN catalyst is not fully understood. However, $^{\cdot}OH$ production and involvement cannot be ruled out considering the accumulated evidence from the literature based on the by-products formed in the Fenton or Fenton's-like reactions (Luo *et al.,* 1994; Park and Imlay, 2003), and as observed, catalytic activity is increased at an elevated temperature. It is therefore likely that an increase in $^{\cdot}OH$ production is dependent on H_2O_2 concentration and temperature (See Chapter 4 for further details on $^{\cdot}OH$ production). H_2O_2 is not as reactive as the $^{\cdot}OH$ product it can form. Like H_2O_2, $^{\cdot}OH$ passes easily through membranes and cannot be kept out of cells. As discussed previously, reactive oxygen species (ROS) such as $^{\cdot}OH$ are by-products of normal cellular metabolism, however, it must be stressed that aerobic cells have evolved several defence mechanisms. Antioxidant enzymes, such as catalase (as used in this study) and SOD, constitute the primary defences of cells (Luo *et al.,* 1994).

The effective antibacterial activity shown by the modified PAN catalyst/ H_2O_2 system may be due to excessive oxidative stress. Oxidative stress is a well-known phenomenon that results whenever the concentrations of reactive oxygen species (ROS) exceed the antioxidant capacity of the cells (Santoro and Thiele, 1997). The cellular damage caused by oxidative stress is due to the chain reactions that are established between the ROS and the biomolecules of the cells, such as DNA, proteins and lipids, which result in damage to cellular physiology and morphology (Belo *et al.,* 2005). It is possible that $^{\cdot}OH$ as well as

other ROS are produced consistently in the modified PAN catalyst/ H_2O_2 system, especially at 35°C, in high concentrations giving rise to non-specific rapid oxidative attack to the cellular membranes of the organisms. The mode of action of antimicrobial activity pertaining to $^{\cdot}OH$ will be considered in Chapter 4.

The literature shows a number of different $^{\cdot}OH$ production systems (Advanced oxidation process) such as O_3, O_3/ H_2O_2, UV, UV/ H_2O_2, O_3/UV/H_2O_2 (Zhang *et al.,* 2006); electro-Fenton (Sires *et al.,* 2007) and photo-Fenton (Fe^{2+}/UV/H_2O_2) (Tamimi *et al.,* 2008) systems developed for various applications. Numerous sources in the literature so far indicate that most of the applications of these systems including the classical Fenton systems are geared towards environmental treatments, particularly for wastewater treatments (Gogate and Pandit, 2004) with limited applications on their use as disinfectant, although the use of H_2O_2 alone is well documented as a disinfectant product for many settings including hospitals for high level disinfection (Foliente *et al.,* 2001; Satter *et al.,* 2002; Omidbakhsh, 2006). For example, the electrochemically generated Fenton's reagent (electro-Fenton - Fe^{2+}/H_2O_2 generated at the cathode) has been applied to remove chemical oxygen demand (COD) from landfill leachate (Zhang *et al.,* 2006); the UV/H_2O_2 and photo-Fenton systems have been applied in the degradation of gallic acid (3,4,5-trihydroxybenzoic) in wastewater (Benitez *et al.,* 2005). Drinking water disinfection has employed the use of O_3 (Guzel-Seydim *et al.,* 2004); and UV and sunlight (Khaengraeng and Reed, 2005). UV/ H_2O_2 has been used to reduce bacterial population on and within fresh produce without affecting the shelf-life stability (Hadjok *et al.,* 2008); equally, UV/ H_2O_2 advanced oxidation process (AOP) and AOP-biological activated carbon (BAC) have been used respectively to remove disinfection by-products such as trihalomethanes and haloacetic acids in drinking water (Toor and Mohseni, 2007). There are many advantages compared to disadvantages of the $^{\cdot}OH$ generation systems, however, it must be said that these systems generally are capital intensive and usually require complex chemistry for specific application; hence, a new approach for $^{\cdot}OH$ generating system that is simple to develop, less capital intensive with possible re-usage would be useful.

Although currently the number of applications for $^{\cdot}OH$ systems focused on microbial inactivation is limited, it appears that the potential for heterogeneous catalysts on microbial inactivation is gradually becoming a focal point of investigation by other workers. For example, recent work of a heterogeneous copper based catalyst and H_2O_2 was successfully used to decontaminate flood water via the formation of $^{\cdot}OH$ (Shah *et al.,* 2007). Fifty milligram of copper immobilised on ion-exchange resin with 22 mM H_2O_2 was used to decontaminate *E. coli* contaminated water. The initial bacterial load of 2.4 x 10^8 cells/mL was reduced by more than 99% after 10min and 100% at 40min; and the control test using polymer or H_2O_2 alone were not as effective as the catalyst/H_2O_2 system (Shah *et al.,* 2007). This work correlates with the present study which has demonstrated that the bacterial viability is rapidly decreased as a function of time in the catalyst/H_2O_2 system and also more effective than the controls, H_2O_2 alone (Tables 2.6, 2.7, 2.8), modified PAN mesh/H_2O_2 system (Table 2.14), modified PAN mesh and water alone (Table 2.16), and modified PAN catalyst and water alone (Table 2.17).

2.4.2.3. Antibacterial effects of H_2O_2 with modified PAN mesh

The control experiments involving modified PAN mesh (MOPM) with H_2O_2 were performed to establish whether the chemicals (hydrazines, hydroxylamines and sodium hydroxides) used to treat the PAN mesh prior to catalyst impregnation influence or increase the antibacterial effects of H_2O_2 against the test organisms.

0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 with MOPM at room temperature and 35°C were tested against *E. coli, Ps. aeruginosa* and *S. aureus* respectively. The results shown in Table 2.14 indicate that MOPM did not possess any catalytic activity, thus, it did not increase or influence the antibacterial activity of H_2O_2. This is confirmed by comparing these results with the antibacterial activity of 0.2% w/v, 0.5% w/v and 1%w/v H_2O_2 in the absence of MOPM (compare Tables 2.7, 2.8; & 2.14). This suggests that the antibacterial activities exhibited by 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 in the presence of MOPM were mainly derived from H_2O_2 activity, and therefore the presence of MOPM did not play any significant ($p>0.05$) role in antibacterial activity in the overall reaction.

Table 2.9: Antibacterial effects of 1% w/v H_2O_2 with PAN catalyst against *E. coli*, *Ps. aeruginosa* and *S. aureus* at room temperature without bubbled air.

Contact time/min	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	1% H_2O_2 with PCatCR	1% H_2O_2 with PCatDS	1% H_2O_2 with PCatCR	1% H_2O_2 with PCatDS	1% H_2O_2 with PCatCR	1% H_2O_2 with PCatDS
10	>5.44(0.00)	>5.42(0.00)	>5.53(0.00)	>5.52(0.00)	>5.60(0.00)	>5.63(0.00)
20	>5.44(0.00)	>5.42(0.00)	>5.53(0.00)	>5.52(0.00)	>5.60(0.00)	>5.63(0.00)
30	>5.44(0.00)	>5.42(0.00)	>5.53(0.00)	>5.52(0.00)	>5.60(0.00)	>5.63(0.00)
40	>5.44(0.00)	>5.42(0.00)	>5.53(0.00)	>5.52(0.00)	>5.60(0.00)	>5.63(0.00)
50	>5.44(0.00)	>5.42(0.00)	>5.53(0.00)	>5.52(0.00)	>5.60(0.00)	>5.63(0.00)
60	>5.44(0.00)	>5.42(0.00)	>5.53(0.00)	>5.52(0.00)	>5.60(0.00)	>5.63(0.00)

pH of 1% w/v H_2O_2 + PCatCR = 3.47-3.54 and 1% w/v H_2O_2 + PCatDS = 4.13-4.10; S.D. = standard deviation; PCatCR = ferric chloride form of PAN catalyst (Russian type); PCatDS = ferric sulfate form of PAN catalyst (Dralon-L type).

Table 2.10: Antibacterial effects of H_2O_2 with modified PAN catalyst (PCatCR; 1 ± 0.1g); with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature

Contact time (min)	Mean Log_{10} reduction (± S.D.)				
	E. coli	*Ps. aeruginosa*		*S. aureus*	
	0.2% w/v H_2O_2 and bubbled air	0.5% w/v H_2O_2 no bubbled air	0.5% w/v H_2O_2 and bubbled air	1% w/v H_2O_2 no bubbled air	1% w/v H_2O_2 and bubbled air
10	2.80(0.82)	4.72(0.39)	5.17(0.56)	>5.64(0.00)	>5.64(0.00)
20	4.95(0.39)	>5.74(0.00)	>5.74(0.00)	>5.64(0.00)	>5.64(0.00)
30	>5.65(0.00)	>5.74(0.00)	>5.74(0.00)	>5.64(0.00)	>5.64(0.00)
40	>5.65(0.00)	>5.74(0.00)	>5.74(0.00)	>5.64(0.00)	>5.64(0.00)
50	>5.65(0.00)	>5.74(0.00)	>5.74(0.00)	>5.64(0.00)	>5.64(0.00)
60	>5.65(0.00)	>5.74(0.00)	>5.74(0.00)	>5.64(0.00)	>5.64(0.00)

pH 3.47-3.54; S.D. = standard deviation; PCatCR = ferric chloride form of PAN catalyst (Russian type).

Note: 0.2% w/v H_2O_2 with PCatCR without air against *E .coli* was not performed.

Table 2.11: Antimicrobial effects of H_2O_2, PAN catalyst (PCatCR; 1 ± 0.1g) and with or without bubbled air (2.5 L/min) on vegetative microorganisms at 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	0.2% w/v H_2O_2 no bubbled air	0.2% w/v H_2O_2 and bubbled air	0.5% w/v H_2O_2 no bubbled air	0.5% w/v H_2O_2 and bubbled air	1% w/v H_2O_2 no bubbled air	1% w/v H_2O_2 and bubbled air
10	>5.53(0.00)	>5.53(0.00)	>5.62(0.00)	>5.62(0.00)	>5.64(0.00)	>5.64(0.00)
20	>5.53(0.00)	>5.53(0.00)	>5.62(0.00)	>5.62(0.00)	>5.64(0.00)	>5.64(0.00)
30	>5.53(0.00)	>5.53(0.00)	>5.62(0.00)	>5.62(0.00)	>5.64(0.00)	>5.64(0.00)
40	>5.53(0.00)	>5.53(0.00)	>5.62(0.00)	>5.62(0.00)	>5.64(0.00)	>5.64(0.00)
50	>5.53(0.00)	>5.53(0.00)	>5.62(0.00)	>5.62(0.00)	>5.64(0.00)	>5.64(0.00)
60	>5.53(0.00)	>5.53(0.00)	>5.62(0.00)	>5.62(0.00)	>5.64(0.00)	>5.64(0.00)

pH 2.95-3.04; S.D. = standard deviation; PCatCR = ferric chloride form of PAN catalyst (Russian type).

Table 2.12: Effects of H_2O_2 with modified PAN catalyst (PCatDC1; 1 ± 0.1g) with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	0.2% w/v H_2O_2 no bubbled air	0.2% w/v H_2O_2 and bubbled air	0.5% w/v H_2O_2 no bubbled air	0.5% w/v H_2O_2 and bubbled air	1% w/v H_2O_2 no bubbled air	1% w/v H_2O_2 no bubbled air
10	2.28(0.13)	3.14(1.01)	4.78(0.46)	≥5.39(0.00)	3.58(0.58)	3.13(0.20)
20	4.79(0.06)	≥5.30(0.28)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
30	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
40	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
50	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
60	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)

pH 3.41-3.54; S.D. = standard deviation; PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1).

Table 2.13: Antibacterial effects of H_2O_2 with modified PAN catalyst (PCatDC1; 1 ± 0.1g) with or without bubbled air (2.5 L/min) on vegetative microorganisms at 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	0.2% w/v H_2O_2 no bubbled air	0.2% w/v H_2O_2 and bubbled air	0.5% w/v H_2O_2 no bubbled air	0.5% w/v H_2O_2 and bubbled air	1% w/v H_2O_2 no bubbled air	1% w/v H_2O_2 no bubbled air
10	4.07(0.40)	≥4.85(0.69)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
20	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
30	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
40	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
50	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)
60	>5.46(0.00)	>5.46(0.00)	>5.59(0.00)	>5.59(0.00)	>5.36(0.00)	>5.36(0.00)

pH 3.38-3.45; S.D. = standard deviation; PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1).

Table 2.14: Antibacterial effects of H_2O_2, modified PAN mesh (MOPM) with bubbled air (2.5L/min) on vegetative microorganisms at room temperature and 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	RT 0.2% w/v H_2O_2 with MOPM	35°C 0.2% w/v H_2O_2 with MOPM	RT 0.5% w/v H_2O_2 with MOPM	35°C 0.5% w/v H_2O_2 with MOPM	RT 1% w/v H_2O_2 with MOPM	35°C 1% w/v H_2O_2 with MOPM
10	0.42(0.37)	1.73(0.03)	0.66(0.17)	1.68(0.06)	0.35(0.09)	1.64(0.11)
20	1.70(0.39)	3.55(0.11)	0.80(0.13)	2.60(0.07)	1.07(0.60)	3.61(0.09)
30	3.10(0.17)	5.52(0.64)	0.94(0.18)	3.27(0.06)	2.21(0.61)	4.82(0.14)
40	3.62(0.00)	>5.55(0.00)	0.89(0.31)	3.44(0.06)	2.93(0.85)	≥5.50(0.17)
50	4.60(0.35)	>5.55(0.00)	0.99(0.15)	3.55(0.06)	4.39(0.82)	>5.60(0.00)
60	>5.50(0.00)	>5.55(0.00)	1.05(0.07)	3.72(0.11)	5.17(0.51)	>5.60(0.00)

pH 7.40-8.00; S.D. = standard deviation; RT = room temperature; MOPM = modified PAN mesh (not impregnated with iron salts).

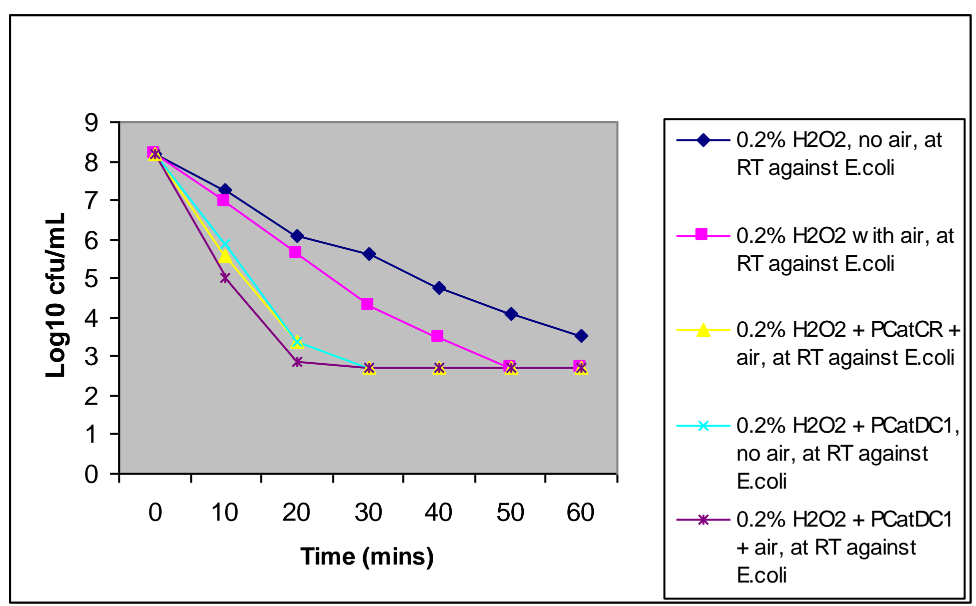


Fig 2.2: A summary demonstration of the differences in the rate of antibacterial effects between 0.2% w/v H_2O_2 alone and in the modified PAN catalyst/H_2O_2 system. RT = room temperature; PCatCR = ferric chloride form of PAN catalyst (Russian type); PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1).

Note: Log10 in graph is Log_{10}

The above summary graph clearly demonstrates that the rate of antimicrobial activity increases faster in the modified PAN catalyst/H_2O_2 system compared to when H_2O_2 is used alone against *E. coli*.

2.4.3. Antibacterial effects of air, modified PAN mesh, and modified PAN catalyst respectively.

The effects of bubbled air (2.5L/min) in distilled water; modified PAN mesh (MOPM); and modified PAN catalyst (PCatCR) against *E. coli*, *Ps. aeruginosa* and *S. aureus* were examined to establish if they possess any antibacterial activity in the absence of H_2O_2 at room temperature and 35°C. The antibacterial activities of water with bubbled air, MOPM and PCatCR are presented in Tables 2.15, 2.16 and 2.17 respectively. Fig. 2.3. shows a summary of the differences in the rate of antibacterial activity of bubbled air and water, MOPM and water, and PCatCR and water.

2.4.3.1. Antibacterial effects of water and air

Bubbled air (2.5L/min) and distilled water (without MOPM and H_2O_2) at both room temperature and 35°C (Table 2.15) showed no toxic effect against *E. coli*, *Ps. aeruginosa* and *S. aureus*.

It is well documented that an increase in air or oxygen pressure in microbial cell cultures can cause oxidative stress and consequently affect cell physiology and morphology, thus, despite the fact that oxygen is an essential nutrient for aerobic organisms, it can also be a toxic agent that can damage cells by the action of reactive oxygen species (ROS) (Belo *et al.,* 2005). ROS are by-products of normal cellular metabolism but generation is stimulated by many different stress conditions, including oxygen pressure increase (Moradas-Ferreira *et al.,* 1996). Considering the potential toxicity of air as explained, an attempt was made to assess whether using air alone without H_2O_2 at room temperature or at 35°C would exert any undue effect on bacterial viability. The results showed (Table 2.15) that bubbled air (2.5 L/min) alone at room temperature had no antibacterial effect on the test organisms within the range of contact times used. Similarly, the effect of air alone at raised temperature (35°C) showed no significant ($p>0.05$) effect on the test organisms within the contact times used (Table 2.15). However, the increase in antibacterial activity shown by both '0.2% w/v H_2O_2' (Table 2.7) and 'Catalyst with H_2O_2' (Table 2.10 & 2.12) in the presence of bubbled air, could be due to the oxygen of the air participating in the chemical

reaction under these experimental conditions (oxygenated Fenton's reaction) and or it enhanced mixing bringing the microbial organisms/hydrogen peroxide closer to the catalyst.

2.4.3.2. Antibacterial effects of modified PAN mesh (MOPM) alone without H_2O_2

Tests employing the modified PAN mesh (MOPM) with air at room temperature or 35°C were performed to establish if MOPM would display any antibacterial activity against the microorganisms. Table 2.14 shows that MOPM possessed no influential or catalytic activity on the antimicrobial effects of H_2O_2 (see Table 2.7), it was thought prudent to double check this by comparing the activity of the modified PAN mesh to the effects of 'water and air'; and 'modified PAN catalyst alone' as these comparisons should then help to establish whether these three different control experimental conditions can exhibit any antibacterial activity on their own or not. The outcome of the tests indicated that the MOPM at both room temperature or at 35°C with bubbled air had no antibacterial activity on the test organisms (Table 2.16). There was no significant difference ($p>0.05$) between the effects of 'MOPM alone' and 'water and air alone'.

2.4.3.3. Antibacterial effects of modified PAN catalyst alone without H_2O_2

The modified PAN catalyst (PCatCR) and bacterial interaction tests were performed to examine whether PCatCR alone (without H_2O_2) could exhibit any antibacterial activity against the test organisms.

PCatCR and water with bubbled air (without H_2O_2) at room temperature exhibited some antibacterial activity against the three organisms tested (Table 2.17). At 60mins there were log reductions of 1.14, 2.96 and 1.09 against *E. coli*, *Ps. aeruginosa* and *S. aureus* respectively. There was no significant difference ($p>0.05$) of activity when PCatCR was tested at 35°C against *E. coli* in comparison to the activity at room temperature. However, from 10-60mins, there was a highly significant increase in antibacterial activity ($p<0.01$) with or without bubbled air by PCatCR against *Ps. aeruginosa* at 35°C compared to room temperature (Table 2.17). At 60mins, there were 5.09 (without bubbled air) and 5.76 (with

bubbled air) log reductions against *Ps. aerugionsa* at 35°C. PCatCR with or without bubbled air exhibited some antibacterial activity against *S. aureus*. At 60mins, *S. aureus* was reduced by 1.09 (without bubbled air at room temperature) and 3.76 (with bubbled air at 35°C) log reductions. The increased activity at 35°C was significant from 10-60mins compared to room temperature. There was significant increase in activity ($p<0.01$) when PCatCR was used against all the test organisms compared to the activity observed when either 'bubbled air and water alone' was used or MOPM alone was used. *Ps. aeruginosa* was more susceptible to the effects of PCatCR particularly at 35°C than *E. coli* and *S. aureus*. (see Tables 2.15, 2.16, 2.17).

The modified PAN catalyst alone without the presence of H_2O_2 appears to exhibit some antibacterial activity against *E. coli, Ps. aeruginosa* and *S. aureus* especially at elevated temperatures. *Ps. aeruginosa* demonstrating more susceptibility to the catalyst effects than the other test organisms. It is of interest to note that the antibacterial effect of the catalyst was more pronounced on the catalase-positive organisms (*Ps. aeruginosa* = Gram-negative; and *S. aureus* = Gram-positive) than the catalase-negative organism (*E. coli* = Gram-negative). It is possible that there may be two different mechanisms of action for 'modified PAN catalyst and microbial interaction' and 'modified PAN catalyst-hydrogen peroxide and microbial interaction'. This is an interesting assumption because at least in the latter case, it is well documented that hydroxyl radicals are produced and are implicated in cellular destruction; on the other hand, nothing is known on the former as this appears to be an unexpected outcome with no known related documentation in the literature as regards to disinfection.

This outcome however, raises a few experimental questions such as the following:

- What is the nature of the modified PAN catalytic active sites and are they playing any role?
- What role does increased temperature (35°C) play in the reaction? Although increased temperature increases rate of reaction, could it be causing any other reaction/s with and on the catalyst?

- Are chemicals leaching out at higher concentrations and catalysed into reactive species by the catalyst?
- What role does air play in the mixture, thus, are water (H_2O) molecules especially oxygen (O_2) playing any role with PAN catalyst?
- What role does pH play in the antibacterial activity seen?

It is known from earlier works that some metal salts of variable valence (iron, copper, manganese etc) can cause oxidation by reacting with molecular oxygen (Oxygenated Fenton's reaction) (Yasina *et al.,* 1968). On the other hand, silver oxide (Ag_2O) can be activated by ultraviolet light and can react with water molecules to produce $^{\cdot}OH$ (He *et al.,* 2008). It has been shown that the presence of dissolved oxygen (aerated conditions) with Ag-zeolite resulted in the production of ROS causing bactericidal activity (Inoue *et al.,* 2002).

The understanding of some or all of the above raised questions may help elucidate this phenomenon pertaining to antibacterial activity of the modified PAN catalyst alone in water.

It should be noted that the pH in the catalyst containing experiments was much lower [pH 3.47-3.54 (room temperature); 2.95-3.04 (35°C)] than the pH seen in the controls discussed previously, but identical to the pH recorded in the hydrogen peroxide and catalyst experiments (where much more rapid antibacterial activity was recorded). It was therefore possible that some or all of the activity seen in Table 2.17 was due to the effect of pH on the organisms, so further experiments were carried out to investigate this. The reaction mixture of modified PAN mesh alone as mentioned produced an alkaline environment (pH 8.80), and that there were no significant antibacterial effects under this condition against all the tests organisms (Table 2.16). (see Section 2.4.6. on the effect of pH on bacterial survival).

The other possible scenario to consider is whether these organisms possess some form of selective affinity (adsorption properties) in binding to the modified PAN catalyst active sites perhaps so tightly which may result in essential products such as K^{+} and Na^{+} unable to enter or leave cells, leading to eventual microbial death? Would one particular

microorganism binds more tightly to the PAN catalyst than others and as a result be more susceptible to inactivation than others? Under these circumstances, it could be suggested that *Ps. aeruginosa* may have more affinity or bind more tightly to the PAN catalyst resulting in its higher inactivation rate, followed by *S. aureus*, and *E. coli*.

Zeta potential is a measure of the magnitude of the repulsion or attraction between particles. Thus, colloidal particles dispersed in a solution are electrically charged due to their ionic characteristics and dipolar attributes. Particles, for example, in natural waters exhibit a surface charge and hence an electric double layer (EDL) is formed; EDL therefore, determines adsorption characteristics, ion-exchange properties and aggregation processes. Such phenomena can be seen to control the behaviour and distribution of organic and inorganic trace constituents in natural waters (Harrison and Mora, 1996).

Cell surface properties are recognized as the key factors that influence bacterial adhesion to surfaces. Among the critical surface properties are surface hydrophobicity, extracellular polymers, and surface electrostatic charge (Sanin et *al.,* 2003). Hydrophobic interactions define the strong attraction between hydrophobic molecules and surfaces in water. In biological systems hydrophobic interactions are the strongest non-covalent interactions and are considered a determining factor in microbial adhesion to surfaces (Sanin *et al.,* 2003). The net surface electrostatic charge, typically measured by the zeta potential on the cell surface, determines the electrostatic interaction between bacterial cells and surfaces (Boonaert and Rouxhet, 2000). Considering these factors, it may be possible to hypothesise that opposite charges between Gram-negative bacteria (carries a negative charge) and protonated PAN catalyst (positively charged) attract each other, leading to a strong electrostatic bond resulting in cellular damage and eventual cell death. However, Gram-positive organism (*S. aureus*) may possibly also get attracted to possible lone pair electrons within the PAN catalyst, such as exist on nitrogen which would be an area of negative charge. For example, in a study, two surfactant lipid preparations (SLPs), 8N8 (water-in-oil emulsion) and W60C (a liposome) were tested against Gram-positive bacteria and non-enteric-Gram-negative bacteria. Both compounds have bactericidal activity against Gram-positive bacteria and non-enteric-Gram-negative bacteria. Zeta potential measurements suggested that resistance displayed by Gram-negative bacilli to 8N8 might be caused by

ionic repulsion due to identical charges on the surface of the emulsion particles and bacteria. In contrast, the positive charge on the surface of W60C facilitates its attraction to the negatively-charged bacteria in deionised water. However, addition of 50 μmol ethylene diamine tetra acetic acid in 100 mmol Tris buffer to either SLPs (8N8 or W60C) yielded bactericidal activity against Gram-negative bacilli (Hamouda and Baker, 2000). Electron microscopy examination showed that 8N8 caused the bacteria to lyse by disrupting bacterial cell wall; while W60C fuses and internalises within the cell, causing damage without immediate cell lysis (Hamouda and Baker, 2000). Although an attempt is being made in this discussion to explain the antibacterial phenomenon of the modified PAN catalyst, the question here again is, if antibacterial effect was indeed based on electrostatic bonding then why is one Gram-negative organism (*Ps. aeruginosa*) more susceptible than the other (*E. coli*)?

Antimicrobial activity of metal catalysts particularly silver (Ag) is well known. For example, catalyst based on silver nano-particles deposited on activated carbon (Ag/C) developed electrochemically, have been used in controlling microorganisms in water (Kumar *et al.,* 2004). A study showed that significant reductions of *Legionella pneumophila* were effected by Ag/Zn zeolite coatings within 2 hours of contact (Rusin *et al.,* 2003). Titanium dioxide (TiO_2) has been used to inactivate *E. coli* (Sunada *et al.,* 2003) and TiO_2 photocatalytic activities have been used for disinfection (Ireland *et al.,* 1993; Cho *et al.,* 2004; Cho *et al.,* 2005). It is possible that there may be new products formed on the PAN mesh that uniquely possess antimicrobial properties and are able to act without H_2O_2. As mentioned earlier, several unknown parameters may be responsible in this phenomenon, and at this moment it is uncertain what mode of action is taken place. It is even possible that inactivation may be due to physical removal of bacteria from solution by sorption, as sorption could increase with contact time. However, if such scenario is the case, why is *Ps. aeruginosa* more susceptible to sorption than *E. coli* and *S. aureus*?

Table 2.15: Antibacterial effects of water and air (2.5 L/min) on vegetative microorganisms at room temperature and 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	RT sdH_2O with bubbled air	35°C sdH_2O with bubbled air	RT sdH_2O with bubbled air	35°C sdH_2O with bubbled air	RT sdH_2O with bubbled air	35°C sdH_2O with bubbled air
10	0.18(0.06)	0.02(0.18)	0.40(0.09)	0.04(0.05)	0.61(0.10)	0.65(0.13)
20	0.17(0.24)	-0.08(0.17)	0.19(0.00)	0.07(0.10)	0.75(0.05)	0.58(0.14)
30	0.27(0.34)	-0.16(0.21)	0.48(0.05)	0.16(0.13)	0.66(0.06)	0.60(0.21)
40	0.33(0.20)	-0.03(0.27)	0.21(0.21)	0.27(0.07)	0.56(0.21)	0.41(0.17)
50	0.05(0.00)	-0.02(0.06)	0.29(0.17)	0.33(0.15)	0.40(0.02)	0.53(0.12)
60	0.02(0.09)	-0.02(0.06)	0.23(0.07)	0.43(0.10)	0.46(0.08)	0.60(0.16)

S.D. = standard deviation; RT = room temperature; sdH_2O = sterile distilled water; **NOTE**: negative (-) log reduction means there were more cfu/mL in the experiment than control.

Table 2.16: Antibacterial effects of modified PAN mesh (MOPM) and air (2.5 L/min) on vegetative microorganisms at room temperature and 35°C.

Contact time (min)	Mean Log reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	RT sdH_2O with MOPM and bubbled air	35°C sdH_2O with MOPM and bubbled air	RT sdH_2O with MOPM and bubbled air	35°C sdH_2O with MOPM and bubbled air	RT sdH_2O with MOPM and bubbled air	35°C sdH_2O with MOPM and bubbled air
10	0.22(0.05)	0.07(0.01)	-0.02(0.07)	0.08(0.06)	0.50(0.08)	0.61(0.03)
20	0.07(0.19)	0.03(0.06)	0.06(0.09)	0.15(0.32)	0.62(0.17)	0.58(0.16)
30	-0.09(0.06)	0.03(0.08)	0.08(0.06)	0.22(0.21)	0.69(0.11)	0.56(0.10)
40	-0.01(0.17)	0.03(0.05)	0.06(0.09)	-0.003(0.05)	0.60(0.14)	0.51(0.12)
50	-0.34(0.26)	-0.13(0.16)	0.02(0.05)	0.05(0.35)	0.59(0.09)	0.58(0.05)
60	0.31(0.12)	-0.13(0.11)	0.08(0.06)	0.23(0.07)	0.57(0.09)	0.70(0.03)

pH 8.45-8.80; S.D. = standard deviation; RT = room temperature; sdH_2O = sterile distilled water; MOPM = modified PAN mesh**. NOTE**: negative (-) log reduction means there were more cfu/mL in the experiment than control.

Table 2.17: Antibacterial effects of modified PAN catalyst (PCatCR) with or without bubbled air (2.5 L/min) on vegetative microorganisms at room temperature and 35°C.

Contact time (min)	Mean Log reduction (± S.D.)						
	E. coli		*Ps. aeruginosa*			*S. aureus*	
	RT sdH_2O with PCatCR and bubbled air	35°C sdH_2O with PCatCR and bubbled air	RT sdH_2O with PCatCR and bubbled air	35°C sdH_2O with PCatCR, no bubbled air	35°C sdH_2O with PCatCR, with bubbled air	RT sdH_2O with PCatCR, no bubbled air	35°C sdH_2O with PCatCR and bubbled air
10	0.71(0.35)	0.78(0.15)	1.17(0.01)	4.60(0.48)	4.71(0.15)	0.89(0.10)	1.35(0.12)
20	0.98(0.20)	1.22(0.21)	1.82(0.10)	4.83(0.25)	5.04(0.30)	0.82(0.19)	1.68(0.29)
30	0.95(0.22)	1.32(0.08)	2.38(0.09)	4.96(0.38)	5.18(0.17)	0.91(0.13)	2.33(0.28)
40	1.19(0.03)	1.58(0.24)	2.65(0.13)	5.07(0.45)	5.50(0.10)	0.97(0.04)	2.92(0.62)
50	1.04(0.11)	1.38(0.13)	2.67(0.10)	4.91(0.22)	5.66(0.17)	1.13(0.11)	3.35(0.61)
60	1.14(0.22)	1.58(0.13)	2.96(0.07)	5.09(0.21)	5.76(0.17)	1.09(0.07)	3.76(0.46)

pH 3.47-3.54 (RT), 2.95-3.04 (35°C); S.D. = standard deviation; RT = room temperature; sdH_2O = sterile distilled water; PCatCR = ferric chloride form of PAN catalyst (Russian type).

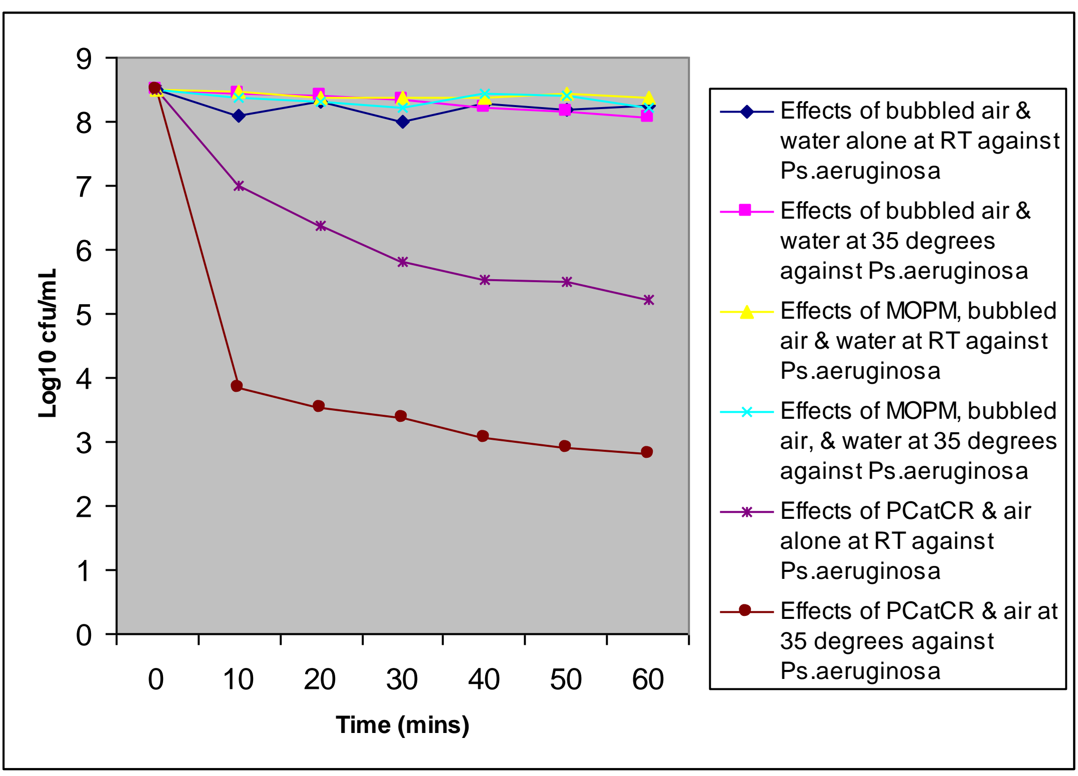


Fig. 2.3: A summary of the differences in the rate of antibacterial activity of bubbled air and water, MOPM and water, and PCatCR and water against *Ps. aeruginosa*.

RT = room temperature; MOPM = modified PAN mesh; PCatCR = ferric chloride form of PAN catalyst (Russian type).

Note: Log10 in graph is Log_{10}

The above summary graph clearly shows that 'bubbled air and water' and 'MOPM and water' do not exert any undue antimicrobial activity, in contrast, PCatCR exhibited significant ($p<0.01$) antimicrobial activity.

2.4.4. Antibacterial effects of homogeneous iron catalyst with or without H_2O_2

2.4.4.1. Antibacterial effects of homogeneous catalyst with H_2O_2

An attempt was made to assess the antibacterial activity of the homogeneous solution (HmCl) by adding 0.033g of $FeCl_3.6H_2O$ to the flask. This amount of iron is equal to the amount impregnated on 1g of the heterogeneous modified PAN catalyst (PCatCR). This allows a comparison of hetero and homogeneous activity and also may identify some advantages and disadvantages of the two systems (heterogeneous and homogeneous). The pH conditions of the homogeneous tests were the same pH as used for the heterogeneous conditions (room temperature pH 3.47-3.54; 35°C pH 2.95-3.04).

The activity of HmCl with H_2O_2, with or without bubbled air significantly inactivated *E. coli*, *Ps. aeruginosa* and *S. aureus* at both room temperature and 35°C as compared to the antibacterial effect of H_2O_2 in the absence of HmCl (Tables 2.7, 2.18, 2.8 2.19). 0.2% w/v H_2O_2 with HmCl (0.033g) inactivated *E. coli* by 4.87 (without bubbled air) and >5.50 (with bubbled air) log reductions at 30mins and 40mins respectively at room temperature. The same experimental conditions completely inactivated *E. coli* at 20mins at 35°C. The reaction of '0.2% w/v H_2O_2 with HmCl' showed significantly increased ($p<0.01$) activity compared to '0.2% w/v H_2O_2 alone' against *E. coli* from 10-60mins without air, and 10-40mins with air at room temperature. And there was significant increased activity ($p<0.05$) of '0.2% w/v H_2O_2 with HmCl' over '0.2% w/v H_2O_2 alone' from 10-30mins at 35°C (with or without air). Comparatively, there was no significant difference ($p>0.05$) between the antibacterial activities of '0.2% w/v H_2O_2 with HmCl' and '0.2% w/v H_2O_2 with PCatCR' at room temperature, however, the latter showed an increased activity ($p<0.05$) within 10mins at 35°C (see Tables 2.18, 2.19; and 2.10, 2.11).

0.5% w/v H_2O_2 with HmCl (0.033g) inactivated *Ps. aeruginosa* by ≥5.67 (without bubbled air) and ≥5.77 (with bubbled air) log reductions after 10mins at room temperature. The same experimental conditions also completely inactivated *Ps. aeruginosa* at 10mins at 35°C. These results were very similar as those seen with PCatCR (Compare Tables 2.18, 2.19; and 2.10, 2.11). 1% w/v H_2O_2 with HmCl (0.033g) in the presence or absence of bubbled air completely inactivated *S. aureus* at 10mins at both room temperature and 35°C.

These results mirrored those seen with PCatCR, which was equally effective (compare Tables 2.18, 2.19; and 2.10, 2.11). Overall, bubbled air did not have any significant effect ($p>0.05$) on all the experimental conditions tested; and bacterial inactivation was dependent on H_2O_2 concentration, homogeneous catalyst, contact time and increased temperature. The results for the homogeneous catalyst were very similar to those seen with the heterogeneous catalyst; proving equally effective against the test organisms.

It could be said that both homogeneous catalyst and heterogeneous modified PAN catalyst (PCatCR) enhanced the antimicrobial activities of H_2O_2 but PCatCR was slightly more effective as an antimicrobial at both room temperature and 35°C particularly against *E. coli*.

2.4.4.2. Antibacterial effects of homogeneous (ferric chloride) catalyst alone without H_2O_2

The antibacterial activities of homogeneous catalyst in the absence of H_2O_2 were tested under the same pH conditions (room temperature pH 3.47-3.54; 35°C pH 2.95-3.04) as the heterogeneous modified PAN catalyst (PCatCR) reactions to evaluate similarities in bacterial viability, and to try to provide some understanding into whether the antibacterial effects observed under PCatCR (without H_2O_2) conditions were directly due to the mesh (PCatCR) or compounds leaching from it or due to any other means.

HmCl solution without H_2O_2 at pH 3.47-3.54 (room temperature) and 2.95-3.04 (35°C) with or without air reduced the viability of the test organisms. *Ps. aeruginosa* was more susceptible to this than *E. coli* and *S. aureus* at 35°C but not at room temperature (Tables 2.20 & 2.21). At room temperature and 60mins contact time, the log reductions were 1.02 (without bubbled air) and 1.21 (with bubbled air) against *E. coli*; 0.31 (without bubbled air) and 0.24 (with bubbled air) against *Ps. aeruginosa*; and 0.93 (without bubbled air) and 0.94 (with bubbled air) against *S. aureus*. At 35°C and 60mins contact time, the log reductions were 1.59 (without bubbled air) and 1.49 (with bubbled air) against *E. coli*; 2.56 (without bubbled air) and 2.81 (with bubbled air) against *Ps. aeruginosa*; and 1.34 (without bubbled air) and 1.75 (with bubbled air) against *S. aureus*.

At both room temperature and 35°C, the presence of bubbled air did not showed any significant activity ($p>0.05$) against the test organisms compared to when it was absent from 10-60mins (Table 2.20). Overall, there was significant ($p<0.01$) increase in activity at 35°C from 10-60mins than at room temperature against *Ps. aeruginosa* and *S. aureus*. There was no significant difference ($p>0.05$) between the effects of HmCl alone and PCatCR alone against *E. coli* at both room temperature and 35°C (Tables 2.17, 2.20 & 2.21). However, a highly significant increase ($p<0.01$) activity was observed in the reaction using PCatCR alone compared to HmCl alone against *Ps. aeruginosa* at both room temperature and 35°C (from 10-60mins). Furthermore, no significant difference ($p>0.05$) was observed between PCatCR alone and HmCl alone at room temperature (10-60mins) against *S. aureus*, however, there was significant increase ($p<0.05$) in activity by PCatCR from 50-60mins when the test was conducted at 35°C compared to the HmCl results.
In the absence of H_2O_2, the modified PAN catalyst (PCatCR) showed increased antibacterial activity compared to the homogeneous iron (III) catalyst (HmCl); (see Tables 2.17; and 2.20, 2.21). *Ps. aeruginosa* was most susceptible to the effects of HmCl particularly at 35°C followed by *S. aureus*, with *E. coli* showing the least susceptibility. Both PCatCR and homogeneous catalyst alone have exhibited some antibacterial activities particularly against *Ps. aeruginosa* and *S. aureus* and it is possible at this stage to assume that these activities may in part due to the acidic environment that both catalysts produced. Also a possible oxidation caused by molecular oxygen/air and iron salts as mentioned earlier could not be ruled out in the reaction. An examination of bacterial survival in water at an acidic pH was performed to provide further insight and is discussed in section 2.4.5.1.

Table 2.18: Antibacterial effects of homogeneous ferric chloride catalyst (HmCl) with H_2O_2 with or without bubbled air (2.5 L/min) on vegetative microorganisms at room temperature.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	0.2% w/v H_2O_2 with HmCl, no bubbled air	0.2% w/v H_2O_2 with HmCl, with bubbled air	0.5% w/v H_2O_2 with HmCl, no bubbled air	0.5% w/v H_2O_2 with HmCl, with bubbled air	1% w/v H_2O_2 with HmCl, no bubbled air	1% w/v H_2O_2 with HmCl, with bubbled air
10	3.44(0.12)	3.88(0.33)	≥5.67(0.17)	≥5.77(0.00)	>5.39(0.00)	>5.39(0.00)
20	4.14(0.21)	4.37(0.51)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
30	4.87(0.35)	≥5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
40	≥5.40(0.17)	>5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
50	>5.50(0.00)	>5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
60	>5.50(0.00)	>5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)

pH 3.47-3.54; S.D. = standard deviation; HmCl = homogeneous ferric chloride catalyst.

Table 2.19: Antibacterial effects of homogeneous ferric chloride catalyst (HmCl) and H_2O_2 with or without bubbled air (2.5 L/min) on vegetative microorganisms at 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	0.2% w/v H_2O_2 with HmCl, no bubbled air	0.2% w/v H_2O_2 with HmCl, with bubbled air	0.5% w/v H_2O_2 with HmCl, no bubbled air	0.5% w/v H_2O_2 with HmCl, with bubbled air	1% w/v H_2O_2 with HmCl, no bubbled air	1% w/v H_2O_2 with HmCl, with bubbled air
10	4.14(0.11)	4.34(0.07)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
20	>5.50(0.00)	>5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
30	>5.50(0.00)	>5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
40	>5.50(0.00)	>5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
50	>5.50(0.00)	>5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)
60	>5.50(0.00)	>5.50(0.00)	>5.77(0.00)	>5.77(0.00)	>5.39(0.00)	>5.39(0.00)

pH 2.95-3.04; S.D. = standard deviation; HmCl = homogeneous ferric chloride catalyst.

Table 2.20: The antibacterial effects of homogeneous ferric chloride catalyst (HmCl) with or without bubbled air (2.5 L/min) on vegetative microorganisms at room temperature**.**

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	sdH_2O with HmCl, no bubbled air	sdH_2O with HmCl and bubbled air	sdH_2O with HmCl, no bubbled air	sdH_2O with HmCl and bubbled air	sdH_2O with HmCl, no bubbled air	sdH_2O with HmCl and bubbled air
10	0.69(0.21)	1.07(0.53)	0.16(0.02)	0.19(0.07)	0.59(0.12)	0.84(0.17)
20	0.90(0.24)	0.99(0.25)	0.18(0.15)	0.29(0.09)	0.73(0.09)	0.75(0.10)
30	0.82(0.12)	1.18(0.76)	0.22(0.09)	0.25(0.11)	0.69(0.05)	0.87(0.24)
40	1.12(0.05)	1.20(0.49)	0.11(0.01)	0.25(0.23)	0.74(0.14)	0.84(0.21)
50	1.04(0.30)	1.04(0.48)	0.19(0.06)	0.23(0.15)	0.80(0.24)	0.99(0.23)
60	1.02(0.14)	1.21(0.41)	0.31(0.10)	0.24(0.02)	0.93(0.16)	0.94(0.21)

pH 3.47-3.54; S.D. = standard deviation; HmCl = homogeneous ferric chloride catalyst; sdH_2O = sterile distilled water.

Table 2.21: The antibacterial effects of homogeneous ferric chloride catalyst (HmCl) with or without bubbled air (2.5 L/min) on vegetative microorganisms at 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	sdH_2O with HmCl, no bubbled air	sdH_2O with HmCl and bubbled air	sdH_2O with HmCl, no bubbled air	sdH_2O with HmCl and bubbled air	sdH_2O with HmCl, no bubbled air	sdH_2O with HmCl and bubbled air
10	0.89(0.23)	0.79(0.06)	0.29(0.03)	0.39(0.07)	0.89(0.21)	1.16(0.31)
20	0.63(0.21)	1.19(0.22)	0.73(0.48)	0.99(0.39)	0.78(0.18)	1.63(0.48)
30	0.93(0.43)	1.26(0.18)	1.45(0.02)	1.66(0.61)	0.86(0.07)	1.66(0.41)
40	1.21(0.68)	1.60(0.28)	2.14(0.45)	2.34(0.70)	0.88(0.27)	1.84(0.27)
50	1.26(0.56)	1.71(0.46)	2.53(0.09)	2.61(0.91)	0.95(0.13)	1.75(0.41)
60	1.59(0.38)	1.49(0.43)	2.56(0.12)	2.81(0.68)	1.34(0.20)	1.75(0.26)

pH 2.95-3.04; S.D. = standard deviation; HmCl = homogeneous ferric chloride catalyst; sdH_2O = sterile distilled water.

2.4.5. Effects of pH and mildly alkaline (unbuffered) or phosphate buffered H_2O_2 solutions on bacterial survival; with or without modified PAN catalyst

Antibacterial effects of acidic distilled water of pH 3.47-3.54 (room temperature) and 2.95-3.04 (35°C); and acidic H_2O_2 of pH 3.47-3.54 (room temperature) and 2.95-3.04 (35°C) were examined and compared with the effects of modified PAN catalyst (PCatCR) and homogeneous iron (III) catalyst (HmCl) under similar conditions to evaluate the contributory effects of pH in microbial inactivation. Further tests were performed to assess the effectiveness of modified PAN catalytic (PCatDC1) activity in a mildly alkaline (pH ~8)or phosphate buffered (pH 7.41) H_2O_2 solution at room temperature and 35°C. Tables 2.22 and 2.23 summarise the antibacterial effects of acidic distilled water at room temperature and 35°C respectively. Tables 2.24 and 2.25 summarise antibacterial effects of acidic H_2O_2 at room temperature and 35°C respectively. Fig. 2.4. shows a summary demonstration of the differences in the antibacterial activities between acidic H_2O_2 and modified PAN catalyst/H_2O_2 system. Tables 2.26 and 2.27 summarise the catalytic activity of PCatDC1 in mildly alkaline (controlled with 1M NaOH) and phosphate buffered 0.5% w/v H_2O_2 solutions respectively against *Ps. aeruginosa*. Tables 2.28 and 2.29 show the effects of PAN catalyst on pH in mildly alkaline (controlled with 1M NaOH) and phosphate buffered 0.5% w/v H_2O_2 solutions respectively. Fig. 2.5. shows a summary demonstration of the differences in the antibacterial activities between 'Phosphate buffered H_2O_2 alone' and 'Phosphate buffered modified PAN catalyst/H_2O_2 system'.

2.4.5.1. Effects of acidic distilled water on bacterial survival

Acidic distilled water of pH 3.47-3.54 (room temperature) and 2.95-3.04 (35°C) with or without air reduced viability of the tested organisms (Tables 2.22 & 2.23). The log reductions indicated by the effects of acidic distilled water at room temperature at 60mins were 0.59 (without bubbled air) and 0.37 (with bubbled air) against *E. coli*; 1.41 (without bubbled air) and 1.51 (with bubbled air) against *Ps. aeruginosa*; and 0.63 (without bubbled air) and 1.44 (with bubbled) against *S. aureus*. The log reductions caused by acidic distilled

water at 35°C after 60mins were 1.29 (without bubbled air) and 1.40 (with bubbled air) against *E. coli*; 3.09 (without bubbled air) and ≥4.04 (with bubbled air) against *Ps. aeruginosa*; and 1.62 (without bubbled air) and 3.59 (with bubbled) against *S. aureus*. There was no significant difference ($p>0.05$) between the reaction involving 'Acidic water alone' and 'PCatCR alone' and 'HmCl alone' at the same pH against *E. coli* at both room and 35°C. From 10-60mins contact time, 'PCatCR alone' showed significantly increased activity ($p<0.05$) compared to 'Acidic water' when experiments were performed in the presence of air against *Ps. aeruginosa* at both room temperature and 35°C. In contrast, when both conditions (PCatCR alone' and 'Acidic water') were carried out in the absence of air, no significant difference was observed at 35°C between them.
In the absence of air, no significant differences in activity were observed between 'Acidic water alone' and 'HmCl alone' at room temperature. No significant difference was observed between the activities of 'Acidic water alone' and 'HmCl alone' against *Ps. aeruginosa* at 35°C with or without air. There was no significant difference between 'Acidic water alone' and 'PCatCR alone' when activity was tested against *S. aureus* at both room temperature and 35°C. *Ps. aeruginosa* was most susceptible to the effects of acidic distilled water followed by *S. aureus*, and *E. coli* respectively. It could be deduced that in the absence of H_2O_2, the homogeneous or heterogeneous iron catalyst alone was not entirely responsible for the antibacterial effects exhibited as shown previously, but the reduced viability was also dependent on the low pH of the experimental condition (see Tables 2.17, 2.20, 2.21, 2.22 and 2.23). Acidity or alkalinity is a factor that profoundly affects all living microbial cells (Tan *et al.,* 1998). Thus, all microorganisms require specific pH condition at which they can grow or survive. The pH of the natural environment is usually between 5 and 9 and most microorganisms require these pH ranges to grow (Middelbeek and Drijver-de Haas, 1992). *E. coli*, *Ps. aeruginosa* and *S. aureus* are known to survive and grow well within these pH ranges (pH 5-9) as they are neither acidophiles (pH <2) or alkalophiles (pH >9) (Middelbeek and Drijver-de Haas, 1992). Overall, the acidic conditions used: pH 3.47-3.54 at room temperature; and 2.95-3.04 at 35°C resulted in some reduction of the viability of the test organisms (particularly at 35°C). The statistical differences in activities between 'Acidic water alone', 'HmCl alone', and

'PCatCR alone' suggest that, to some extent the presence of air contributed to the antimicrobial activities of both homogeneous or heterogeneous catalyst but not the acidic water. However, 'PCatCR alone' demonstrated greater antimicrobial activity than both 'Acidic water alone' and 'HmCl alone' even though all the tests were performed at the same pH. This may suggest that the increased activity observed from 'PCatCR alone' resulted not only from low pH but also may be due to other factors as mentioned previously.

2.4.5.2. Effects of acidic H_2O_2 on bacterial survival

As shown above (Section 2.4.5.1), acidic water showed variable effects on the microorganisms and some microorganisms were more susceptible to acidic conditions than others. This suggests that acidic environment may have contributed to microbial inactivation when the catalyst was present. The antibacterial activity of acidic H_2O_2 prepared at the same pH (room temperature = pH 3.47-3.54; 35^oC = 2.95-3.04) as 'PAN catalyst with H_2O_2' was assessed and compared with the activity exhibited by 'PAN catalyst with H_2O_2'. This was done to further demonstrate the effects of pH in the reaction, and to investigate whether the presence of the PAN catalyst in the reaction resulted in different antimicrobial activity levels to an acidic solution.

Acidic H_2O_2 of pH 3.47-3.54 (room temperature) and 2.95-3.04 (35^oC) with or without bubbled air reduced the viability of *E. coli*, *Ps. aeruginosa* and *S. aureus* (Tables 2.24 & 2.25). Acidic 0.2% w/v H_2O_2 inactivated *E. coli* by ≥5.11(without bubbled air) and ≥5.46(with bubbled air) log reductions after 40mins at room temperature. The same experimental conditions completely inactivated *E. coli* after 20mins at 35^oC. Acidic 0.5% w/v H_2O_2 inactivated *Ps. aeruginosa* by 3.88 (without bubbled air) and ≥4.81 (with bubbled air) log reductions after 40mins at room temperature. The same experimental condition completely inactivated *Ps. aeruginosa* after 10mins at 35^oC. Acidic 1% w/v H_2O_2 inactivated *S. aureus* by 3.23 (without bubbled air) and 3.45 (with bubbled air) log reductions after 60mins at room temperature. The same experimental condition at 35^oC completely inactivated *S. aureus* at 20mins (without bubbled air) and 30mins (with bubbled air). A significant increase ($p<0.05$) in activity was seen when '0.2% w/v H_2O_2 with

PCatCR'was used as opposed to 'Acidic 0.2% w/v H_2O_2' (from 10-30mins) at room temperature in the presence of air against *E. coli* (Fig. 2.4). '0.5% w/v H_2O_2 with PCatCR' showed significantly more activity (<0.05) from 10-40mins than 'Acidic 0.5% w/v H_2O_2'against *Ps. aeruginosa* at room temperature (Fig. 2.4). The activity of '1% w/v H_2O_2 with PCatCR' from 10-60mins was significantly greater ($p<0.05$) than that of 'Acidic 1% w/v H_2O_2'against *S. aureus* at room temperature with or without air (Fig. 2.4). Similarly, the activity of '1% w/v H_2O_2 with PCatCR' was significantly greater ($p<0.05$) than 'Acidic 1% w/v H_2O_2 at 10mins (without air) and 10-20mins (with air) at 35^oC against *S. aureus*. Acidic H_2O_2 showed significantly ($p<0.05$) greater bacterial inactivation than acidic H_2O; 'PCatCR and water alone'; and 'HmCl and water alone' (compare Tables 2.24, 2.25; and 2.7, 2.8; 2.17; 2.20, 2.21), but the activity of 'PCatCR with H_2O_2, was greater than all the aforementioned conditions.

Reduced bacterial viability was dependent on pH, increased temperature, contact time, and the presence of air. *Ps. aeruginosa* was more susceptible to these conditions followed by *E. coli* and *S. aureus* respectively. Overall, it can be seen that both the heterogeneous or homogeneous catalysts require H_2O_2 to exhibit an increased antimicrobial activity and that the overall efficacy of the catalyst is not caused by a decrease in pH alone.

2.4.5.3. Effects of mildly alkaline (unbuffered) 0.5% w/v H_2O_2 with or without modified PAN catalyst against *Ps. aeruginosa*

The effects of mildly alkaline H_2O_2 with or without modified PAN catalyst (PCatDC1) were examined to establish whether the alkaline environment would affect catalytic activity.

Mildly alkaline 0.5% w/v H_2O_2 (alkalinity controlled with 1M NaOH) with or without bubbled air at room temperature had a low antibacterial effect against *Ps. aeruginosa*, which increased slightly as temperature was raised to 35^oC (Table 2.26). The log reductions indicated at 60mins were 0.80 (without bubbled air) and 0.87 (with bubbled air) at room temperature; and 2.87 (without bubbled air) and 1.85 (with bubbled air) at 35^oC. Addition of modified PAN catalyst (PCatDC1) with or without bubbled air at room temperature or 35^oC had a greater antibacterial effect on *Ps. aeruginosa.* Bacterial inactivation was

increased by raised temperature. At room temperature, *Ps. aeruginosa* was completely inactivated at 30mins (without bubbled air) and 20mins (with bubbled air). On the other hand, there was a log reduction of ≥5.07 (without bubbled air) and complete inactivation at 10mins (with bubbled air) against *Ps. aeruginosa* at 35°C. The addition of bubbled air when 'Alkaline 0.5% w/v H_2O_2 with PCatDC1' was used resulted in significantly increase activity ($p<0.05$) from 10-20mins at room temperature and 10mins at 35°C than when air was absent. Overall, there was statistically significant ($p<0.01$) increase in antibacterial activity at both room temperature and at 35°C in the presence of PCatDC1 compared to when it was absent. The addition of PCatDC1 to the mildly alkaline hydrogen peroxide appeared to reduce the initial pH to an acidic pH (Table 2.28).

It was noted that the pH of the solution remained very similar (alkaline) from start to finish when H_2O_2 alone was used, but when catalyst was included the pH dropped down over the hour to 3.6-3.9 (Table 2.28). This pH shift may have been responsible or contributory for some of the antimicrobial activity observed. This observation raised a question of whether the PAN catalyst is only active at acidic environment. Hence further studies looking at activity in the presence of a buffer were conducted (see section 2.4.5.4).

2.4.5.4. Effects of buffered 0.5% w/v H_2O_2 with or without modified PAN catalyst on *Ps. aeruginosa*

Traditional homogeneous Fenton (Fe^{2+}) or Fenton-like (Fe^{3+}) reactions are thought to be effective at acidic pH, but ineffective at pH above 5 due to precipitation of the iron salts (Salles *et al.,* 2007). Conversely, the photoferrioxalate system, which involves oxalate ions and UV-visible light, allows Fe^{3+} to dissolve at slightly acidic and near-neutral pHs and hence maintains the catalytic activity of iron (Cho *et al.,* 2004). The literature has not shown, whether heterogeneous catalysts based on iron complexes/oxides via the Fenton or Fenton-like pathways are active and assist in antimicrobial activity with H_2O_2 in a buffered solution medium. However, Li *et al.* (2008) demonstrated the treatment of wastewater with a heterogeneous electro-Fenton system at neutral pH.

The present work examined the antimicrobial effects of buffered (pH 7.41) 0.5% w/v H_2O_2 with or without modified PAN catalyst (PCatDC1) against *Ps. aeruginosa* at both room temperature and 35^oC.

Phosphate buffered 0.5% w/v H_2O_2 (pH 7.41) with or without bubbled air at room temperature had mild antibacterial effects on *Ps. aeruginosa*, which increased as temperature was raised to 35^oC (Table 2.27). The log reductions at 60mins were 1.85 (without bubbled air) and 1.90 (with bubbled air) at room temperature; and 3.89 (without bubbled air) and 3.37 (with bubbled air) at 35^oC. Addition of modified PAN catalyst (PcatDC1) with or without bubbled air at room temperature or 35^oC resulted in an increase in antibacterial effect against *Ps. aeruginosa* (Fig. 2.5). The log reductions indicated at 60mins were 2.25 (without bubbled air) and 2.43 (with bubbled air) at room temperature; however, at 35^oC there was a log reduction of 5.01 (without bubbled air) at 40mins, and a complete inactivation at 20mins (with bubbled air). In the absence of PCatDC1, the presence of bubbled air showed no significant ($p>0.05$) effect when compared to 'Phosphate buffered 0.5% w/v H_2O_2' at room temperature and 35^oC. There was significant increase in activity in the presence of PCatDC1 compared to when it was absent from 10-60mins at both room temperature and 35^oC. Furthermore, the presence of PCatDC1 in 'Phosphate buffered 0.5% w/v H_2O_2', demonstrated a highly significant ($p<0.01$) increase activity at 35^oC compared to that observed at room temperature from 10-60mins (Fig. 2.5). The presence of bubbled air resulted in a slight increase in antibacterial activity at 35^oC when PCatDC1 was added to the reaction, however, was not statistically significant ($p>0.05$). The presence of PCatDC1 under these conditions did not appear to alter the initial buffered alkaline pH to acidic pH (Table 2.29). However, antibacterial activity was much higher using PCatDC1 in mildly alkaline 0.5% w/v H_2O_2 (alkalinity controlled with 1M NaOH) than using PCatDC1 in phosphate buffered 0.5% w/v H_2O_2 (see Tables 2.26 & 2.27). This may indicate that the pH shift (from alkaline to acidic) contributes, but is not completely responsible for the increased in antimicrobial activity seen with the presence of the PAN catalyst.

The work pertaining to 'buffered H_2O_2 with PCatDC1' has shown that the antimicrobial activity of H_2O_2 is clearly enhanced by the presence of the modified PAN catalyst. This

work has therefore demonstrated that the modified PAN catalyst can be used at neutral pH, but it is a lot more active at lower pH and at higher temperatures (35°C).

Table 2.22: Effects of acidic distilled water with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	Acidic H_2O, no bubbled air	Acidic H_2O, with bubbled air	Acidic H_2O, no bubbled air	Acidic H_2O, with bubbled air	Acidic H_2O, no bubbled air	Acidic H_2O, with bubbled air
10	0.04(0.14)	0.29(0.23)	0.71(0.29)	0.67(0.23)	0.53(0.10)	1.00(0.68)
20	0.18(3.40)	0.28(0.08)	0.61(0.15)	1.19(0.52)	0.60(0.07)	1.20(0.67)
30	0.21(0.10)	0.28(0.20)	1.00(0.58)	1.19(0.58)	0.59(0.26)	1.27(0.61)
40	0.56(0.44)	0.58(0.23)	0.95(0.49)	1.10(0.31)	0.73(0.09)	1.21(0.91)
50	0.47(0.25)	0.63(0.41)	1.24(0.60)	1.37(0.39)	0.61(0.20)	1.29(0.80)
60	0.59(0.12)	0.37(0.09)	1.41(0.98)	1.51(0.86)	0.63(0.29)	1.44(0.88)

pH 3.47-3.54; S.D. = standard deviation.

Table 2.23: Effects of acidic distilled water with or without bubbled air (2.5L/min)on vegetative microorganisms at 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	Acidic H_2O, no bubbled air	Acidic H_2O with bubbled air	Acidic H_2O, no bubbled air	Acidic H_2O with bubbled air	Acidic H_2O, no bubbled air	Acidic H_2O with bubbled air
10	0.38(0.14)	0.97(0.18)	2.35(1.64)	2.80(1.30)	0.57(0.15)	1.63(0.37)
20	0.60(0.40)	1.09(0.08)	2.79(1.88)	2.98(1.35)	0.69(0.15)	2.88(0.75)
30	0.94(0.16)	1.20(0.17)	2.67(1.64)	3.20(0.88)	0.96(0.60)	3.28(0.73)
40	0.99(0.20)	1.21(0.22)	3.21(1.81)	3.52(1.22)	1.15(0.64)	3.36(0.65)
50	1.05(0.47)	1.17(0.15)	2.85(1.68)	3.83(1.28)	1.46(0.74)	3.45(0.66)
60	1.29(0.20)	1.40(0.12)	3.09(1.34)	≥4.04(1.52)	1.62(0.93)	3.59(1.00)

pH 2.95-3.04; S.D. = standard deviation.

Table 2.24: Effects of acidic H_2O_2 with or without bubbled air (2.5L/min) on vegetative microorganisms at room temperature**.**

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	Acidic 0.2% w/v H_2O_2, no bubbled air	Acidic 0.2% w/v H_2O_2 with bubbled air	Acidic 0.5% w/v H_2O_2, no bubbled air	Acidic 0.5% w/v H_2O_2 with bubbled air	Acidic 1% w/v H_2O_2, no bubbled air	Acidic 1% w/v H_2O_2 with bubbled air
10	2.43(0.47)	2.16(0.61)	2.30(0.78)	2.53(0.82)	0.69(0.26)	1.00(0.24)
20	3.40(0.71)	3.35(0.41)	2.76(0.88)	3.41(0.85)	0.72(0.26)	1.86(0.82)
30	4.21(0.91)	4.46(0.50)	3.71(1.09)	4.25(0.79)	1.30(0.12)	2.33(0.94)
40	≥5.11(0.60)	≥5.46(0.00)	3.88(0.66)	≥4.81(0.66)	2.03(0.30)	2.83(0.99)
50	≥5.30(0.28)	>5.46(0.00)	4.52(0.92)	≥5.34(0.17)	2.90(0.23)	3.23(0.99)
60	>5.46(0.00)	>5.46(0.00)	4.83(0.64)	≥5.34(0.35)	3.23(0.47)	3.45(0.97)

pH 3.47-3.54; S.D. = standard deviation.

Table 2.25: Effects of acidic H_2O_2 with or without bubbled air (2.5L/min) on vegetative microorganisms at 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)					
	E. coli		*Ps. aeruginosa*		*S. aureus*	
	Acidic 0.2% w/v H_2O_2, no bubbled air	Acidic 0.2% w/v H_2O_2 with bubbled air	Acidic 0.5% w/v H_2O_2, no bubbled air	Acidic 0.5% w/v H_2O_2 with bubbled air	Acidic 1% w/v H_2O_2, no bubbled air	Acidic 1% w/v H_2O_2, with bubbled air
10	4.09(0.48)	≥4.57(0.96)	>5.54(0.00)	>5.54(0.00)	3.08(0.10)	3.12(0.24)
20	>5.46(0.00)	>5.46(0.00)	>5.54(0.00)	>5.54(0.00)	≥5.47(0.00)	4.58(0.53)
30	>5.46(0.00)	>5.46(0.00)	>5.54(0.00)	>5.54(0.00)	>5.47(0.00)	>5.47(0.00)
40	>5.46(0.00)	>5.46(0.00)	>5.54(0.00)	>5.54(0.00)	>5.47(0.00)	>5.47(0.00)
50	>5.46(0.00)	>5.46(0.00)	>5.54(0.00)	>5.54(0.00)	>5.47(0.00)	>5.47(0.00)
60	>5.46(0.00)	>5.46(0.00)	>5.54(0.00)	>5.54(0.00)	>5.47(0.00)	>5.47(0.00)

pH 2.95-3.04; S.D. = standard deviation.

Table 2.26: Effects of mildly alkaline (unbuffered) 0.5% w/v H_2O_2 with or without modified PAN catalyst (PCatDC1) against *Ps. aeruginosa* ATCC 15442 at room temperature or 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)							
	Alkaline 0.5% w/v H_2O_2, no bubbled air at RT	Alkaline 0.5% w/v H_2O_2 with bubbled air at RT	Alkaline 0.5% w/v H_2O_2, no bubbled air at 35°C	Alkaline 0.5% w/v H_2O_2, with bubbled air at 35°C	Alkaline 0.5% w/v H_2O_2 with PCatDC1, no bubbled air at RT	Alkaline 0.5% w/v H_2O_2 with PCatDC1 and bubbled air at RT	Alkaline 0.5% w/v H_2O_2 with PCatDC1, no bubbled air at 35°C	Alkaline 0.5% w/v H_2O_2 with PCatDC1 and bubbled air at 35°C
10	0.80(0.25)	0.40(0.11)	1.37(0.14)	1.43(0.09)	1.98(0.17)	2.59(0.31)	3.34(0.78)	>5.56(0.00)
20	0.81(0.18)	0.51(0.24)	2.36(0.17)	1.57(0.09)	5.10(0.15)	>5.56(0.00)	≥5.07(0.50)	>5.56(0.00)
30	0.85(0.24)	0.67(0.11)	2.56(0.13)	1.73(0.22)	>5.56(0.00)	>5.56(0.00)	>5.56(0.00)	>5.56(0.00)
40	0.80(0.15)	0.60(0.11)	2.63(0.06)	1.87(0.45)	>5.56(0.00)	>5.56(0.00)	>5.56(0.00)	>5.56(0.00)
50	1.00(0.24)	0.73(0.13)	2.69(0.11)	1.87(0.36)	>5.56(0.00)	>5.56(0.00)	>5.56(0.00)	>5.56(0.00)
60	0.80(0.25)	0.87(0.21)	2.87(0.09)	1.85(0.23)	.>5.56(0.00)	>5.56(0.00)	>5.56(0.00)	>5.56(0.00)

RT = room temperature; S.D. = standard deviation; PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1).

No buffers used, NaOH used to adjust initial pH to ~ 8

Table 2.27: Effects of phosphate buffered 0.5% w/v H_2O_2 with or without modified PAN catalyst (PCatDC1) against *Ps. aeruginosa* ATCC 15442 at room temperature or 35°C.

Contact time (min)	Mean Log_{10} reduction (± S.D.)							
	0.5% w/v H_2O_2 in buffer, no bubbled air at RT	0.5% w/v H_2O_2 in buffer with bubbled air at RT	0.5% w/v H_2O_2 in buffer, no bubbled air at 35°C	0.5% w/v H_2O_2 in buffer with bubbled air at 35°C	0.5% w/v H_2O_2 in buffer with PCatDC1, no bubbled air at RT	0.5% w/v H_2O_2 in buffer with PCatDC1 and bubbled air at RT	0.5% w/v H_2O_2 in buffer with PCatDC1, no bubbled air at 35°C	0.5% w/v H_2O_2 in buffer with PCatDC1 and bubbled air at 35°C
10	1.08(0.25)	1.37(0.04)	3.04(0.07)	2.89(0.06)	1.38(0.45)	1.44(0.25)	4.04(0.24)	3.78(0.52)
20	1.49(0.37)	1.46(0.07)	3.57(0.18)	3.13(0.11)	1.79(0.15)	1.81(0.38)	4.78(0.71)	≥5.40(0.28)
30	1.69(0.11)	1.71(0.32)	3.65(0.12)	3.36(0.10)	1.97(0.11)	2.14(0.45)	4.89(0.61)	>5.56(0.00)
40	1.79(0.07)	1.61(0.20)	3.79(0.42)	3.41(0.07)	2.08(0.14)	2.29(0.58)	5.01(0.49)	>5.56(0.00)
50	1.92(0.18)	1.75(0.10)	3.90(0.31)	3.39(0.15)	2.19(0.17)	2.33(0.54)	5.10(0.45)	>5.56(0.00)
60	1.85(0.24)	1.90(0.22)	3.89(0.35)	3.37(0.11)	.2.25(0.17)	2.43(0.59)	5.24(0.28)	>5.56(0.00)

RT = room temperature; S.D. = standard deviation; PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1). pH of phosphate buffer = 7.41

Table 2.28: pH ranges for Table 2.26 (non-buffered system).

time /min	pH of 0.5%w/v H_2O_2 at mildly alkaline conditions							
	No bubbled air at RT	With bubbled air at RT	No bubbled air at 35°C	With bubbled air at 35°C	With PCatDC1, no bubbled air at RT	With PCatDC1and bubbled air at RT	With PCatDC1, no bubbled air at 35°C	With PCatDC1 and bubbled air at 35°C
0	7.52-7.84	7.42-7.48	7.43-7.71	7.57-7.62	7.24-7.69	7.42-8.02	7.28-7.56	7.44-7.59
60	7.52-8.69	7.02-7.44	7.48-7.62	7.43-7.90	3.59-3.80	3.59-3.80	3.60-3.89	3.60-3.76

RT = room temperature; PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1). No buffers used, NaOH used to adjust initial pH to ~ 8

Table 2.29: pH ranges for Table 2.27 (buffered system).

time /min	pH of 0.5%w/ H_2O_2 at phosphate buffer conditions							
	No bubbled air at RT	With bubbled air at RT	No bubbled air at 35°C	With bubbled air at 35°C	With PCatDC1, no bubbled air at RT	With PCatDC1and bubbled air at RT	With PCatDC1, no bubbled air at 35°C	With PCatDC1 and bubbled air at 35°C
0	7.34-7.38	7.36-7.37	7.32-7.34	7.33-7.35	7.11-7.14	7.08-7.09	7.00-7.02	7.03-7.11
60	7.34-7.38	7.35-7.38	7.31-7.34	7.32-7.35	7.05-7.07	7.02-7.12	6.99-7.02	7.01-7.05

RT = room temperature; PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1). pH of phosphate buffer = 7.41

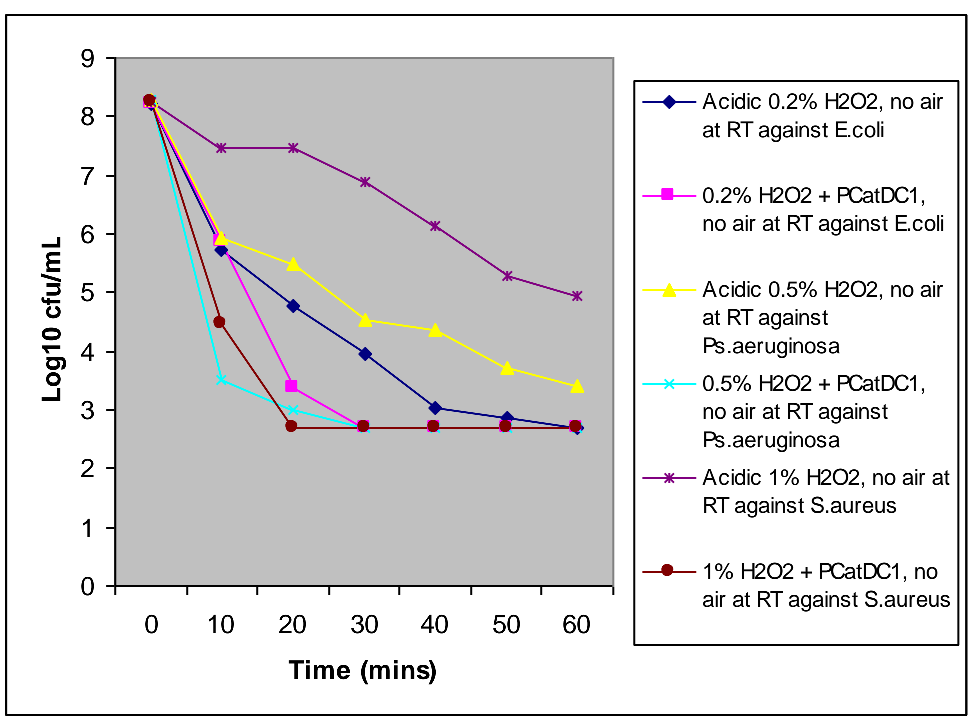


Fig. 2.4: A demonstration of the differences in the antibacterial activities between acidic H_2O_2 and modified PAN catalyst/H_2O_2 system.

RT = room temperature; PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1). pH of acidic 0.2-1% w/v H_2O_2 is 3.47-3.54; pH of 0.2-1% w/v H_2O_2 with PCatDC1 is 3.41-3.54.

Note: Log10 in graph is Log_{10}

The above graph shows that the antimicrobial activity of PAN catalyst/H_2O_2 system is not only due to the low pH of the solution, but that the catalyst can actually increase the antimicrobial activity of H_2O_2.

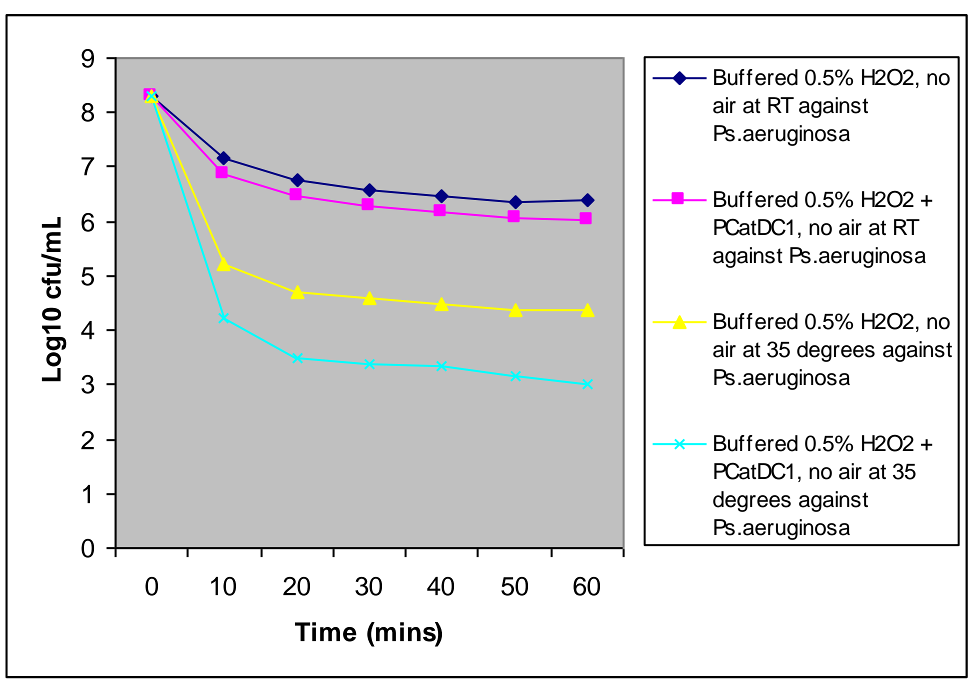


Fig. 2.5: A demonstration of the differences in the antibacterial activities between 'Phosphate buffered H_2O_2 alone' and 'Phosphate buffered modified PAN catalyst/H_2O_2 system'against *Ps. aeruginosa*.

RT = room temperature; PCatDC1 = ferric chloride form PAN catalyst (Dralon-L type batch 1).

Note: Log10 in graph is Log_{10}

The above graph shows that the antimicrobial activity of 'Phosphate buffered modified PAN catalyst/H_2O_2 is greater than that of 'Phosphate buffered H_2O_2 alone' (especially at 35°C; $p<0.01$). These differences in activity suggest that the modified PAN catalyst can be used at neutral pH. However, it was evident that the phosphate buffer is reducing activity. This could be due to phosphate ligating to active Fe site forming iron phosphate which would be insoluble and probably bind quite strongly to Fe stopping H_2O_2 coming on. It has been shown in the literature that phosphate buffer can prevent $^{\cdot}OH$ formation in the Fenton's systems (Dyke *et al.,* 1996).

2.4.6. Antibacterial activity as a function of the reusability of different batches of modified PAN catalyst with H_2O_2

The reusability of different types of heterogeneous catalysts for a variety of applications has been described (Hsu *et al.,* 2007; Shaterian and Yarahmadi, 2008; Tekbas *et al.,* 2008). However, a number of literature searches did not provide any evidence of reusability of heterogeneous iron catalyst for antimicrobial purposes. Applications for many reusable heterogeneous catalysis have focused on environmental treatments as opposed to antimicrobial studies.

A range of modified PAN catalysts (PCatDC2, PCatDS; PCatDC3, PcatCR) with H_2O_2 were assessed to establish whether they are reusable, whether antibacterial activity was dependent on the particular modification method used (chloride or sulfate), and whether there was any reproducibility between different batches (chloride only). Tables 2.30 and 2.31 show the reusability (48hrs between use) of PCatDC2 and PCatDS respectively with 0.2% w/v H_2O_2 against *E. coli* at both room temperature and 35°C. Table 2.32 summarises the reusability (48hrs between use) of PCatDC3 with 1% w/v H_2O_2 against *S. aureus* at both room temperature and 35°C. Table 2.33 summarises the reusability (6 months between use) of PCatCR with 0.2% w/v H_2O_2 against *E. coli* at both room temperature and 35°C; and Table 2.34 summarises the reusability (6 months between use) of PCatCR with 1% w/v H_2O_2 against *S. aureus* at both room temperature and 35°C. Fig. 2.6: shows a summary demonstration of the differences in the reusability of modified PAN catalyst.

The antibacterial effects of PCatDC2 with 0.2% w/v H_2O_2 showed complete inactivation of *E. coli* at 30mins (first use); 40mins (second use) and 50mins (third use) at room temperature (Fig. 2.6). However, at 35°C, complete inactivation of *E. coli* occurred at 10mins (first use) and 20mins (second and third uses respectively). In the absence of air, the three uses of PCatDC2 at both room temperature and 35°C showed significantly greater activity compared to when 0.2% w/v H_2O_2 alone was used. Thus, there was significant increase activity from 10-60mins at first and second uses ($p<0.05$), and 30-60mins at third use ($p<0.05$) at room temperature as compared to when 0.2% w/v H_2O_2 was used alone (Fig. 2.6). However, significantly increase activity occurred from 10-20 for all three uses

($p<0.05$) when tests were conducted at 35^oC as compared to the same condition when 0.2% w/v H_2O_2 was alone.

PCatDS with 0.2% w/v H_2O_2 showed complete inactivation of *E. coli* at 40mins during all three reuses at room temperature. At 35^oC, complete inactivation of *E. coli* occurred after 20mins during all the three uses.

PCatDC3 with 1% w/v H_2O_2 resulted in complete inactivation of *S. aureus* at 20mins (first use) and 30mins (both second and third uses) at room temperature. At 35^oC, *S. aureus* was completely inactivated at 10mins (first use) and at 20mins (both second and third uses). From 10-40mins, there was significant increased activity with all three uses of PCatDC3 compared to when 1% w/v H_2O_2 alone was used at room temperature. Equally, there was significantly increased activity from 10-30mins ($p<0.05$) when PCatDC3 was used three times at 35^oC compared to when 1% w/v H_2O_2 was used alone.

The antibacterial effects of PCatCR with 0.2% w/v H_2O_2 showed complete inactivation of *E. coli* at 30mins (first use) and 20mins (second use) at room temperature. However, at 35^oC, complete inactivation of *E. coli* occurred at 10mins for both first and second uses (see Table 2.33). Furthermore, PCatCR with 1% w/v H_2O_2 at room temperature completely inactivated *S. aureus* at 10mins (first use) and 30mins (second use). At 35^oC, complete inactivation of *S. aureus* occurred at 10mins (first use) and 20mins (second use; see Table 2.34). There was significant increase ($p<0.05$) in activity after the two uses of PCatCR either with 0.2% w/v H_2O_2 or with 1%w/v H_2O_2 compared to when H_2O_2 was used alone.

Antibacterial effects of modified PAN catalysts appeared to be lower on reuse and some of the effects seen may be due to pH or other factors. Interestingly results varied depending on which type of catalyst was used. Although, there was a generally significant increase ($p<0.05$) in activity from 10-20mins (room temperature) and at 10mins (35^oC) when catalyst was first used compared to the second and third uses, there was not much difference in bacterial inactivation between 2nd and 3rd uses of the catalysts. These differences were observed mainly for most of the ferric chloride impregnated catalysts (PCatDC2, PCatDC3 and PCatCR). It is worth to note that no significant difference ($p>0.05$) was observed between all the three uses of PCatDS (ferric sulfate impregnated catalyst) at either room temperature or at 35^oC as this observation was not always true for all the ferric chloride catalysts (PCatCR, PCatDC2, PCatDC3) used. This may have been

due to there being approximately four times more iron on the PCatDS than PCatDC2, and PCatDC3 (Table 2.3); and approximately two times more than on PCatCR (Table 2.3). In support of the above observation, it was noted that PCatCR which has approximately twice as much iron as PCatDC2 and PCatDC3, showed no significant difference when it was used twice with 0.2% w/v H_2O_2 at either room temperature or 35^oC. However, when the H_2O_2 concentration was increased to 1% w/v, there was a significant difference from 10-20mins at room temperature between first and second use but no difference between first and second use at 35^oC. The above deductions may suggest that the reusability of the heterogeneous PAN catalyst depends on several factors which include H_2O_2 concentration, type of catalyst, i.e. ferric chloride or ferric sulfate impregnation, amount of iron on the support mesh, and temperature.

Comparing the reused modified PAN catalysts to the control tests (Tables 2.7 & 2.8), it is evident that the PAN catalysts are reusable as they showed significantly increased activity compared to the control tests (H_2O_2 alone). This clearly demonstrates a competitive advantage of the heterogeneous modified PAN catalyst over the homogeneous iron catalyst, (as demonstrated in Section 2.4.4.), because, although the homogeneous iron catalyst works as efficiently as the PAN catalyst, it is not reusable.

Table 2.30: Antibacterial effects of reused modified PAN catalyst (PCatDC2) at three independent uses (48hrs between use) with 0.2% w/v H_2O_2 on *E. coli* at room temperature and 35°C.

Contact time/min	Mean Log_{10} reduction (± S.D.)					
	0.2% w/v H_2O_2 with PCatDC2 at room Temp. (1st Use)	0.2% w/v H_2O_2 with PCatDC2 at room Temp. (2nd Use)	0.2% w/v H_2O_2 with PCatDC2 at room Temp. (3rd Use)	0.2% w/v H_2O_2 with PCatDC2 at 35°C (1st Use)	0.2% w/v H_2O_2 with PCatDC2 at 35°C (2nd Use)	0.2% w/v H_2O_2 with PCatDC2 at 35°C (3rd Use)
10	1.62(0.09)	2.14(0.03)	1.41(0.06)	>5.50(0.00)	3.23(0.06)	3.34(0.05)
20	4.74(0.25)	3.32(0.07)	2.73(0.02)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)
30	>5.50(0.00)	4.67(0.13)	3.66(0.10)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)
40	>5.50(0.00)	>5.50(0.00)	4.61(0.36)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)
50	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)
60	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)

pH range 3.77-3.50 (room temperature); 3.63-3.48 (35°C); S.D. = standard deviation; Temp. = temperature**.**

PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2).

Table 2.31: Antibacterial effects of reused modified PAN catalyst (PCatDS) at three independent uses (48hrs between use) with 0.2% w/v H_2O_2 on *E. coli* at room temperature and 35°C.

Contact time/min	Mean Log_{10} reduction (± S.D.)					
	0.2% w/v H_2O_2 with PCatDS at room Temp. (1st Use)	0.2% w/v H_2O_2 with PCatDS at room Temp. (2nd Use)	0.2% w/v H_2O_2 with PCatDS at room Temp. (3rd Use)	0.2% w/v H_2O_2 with PCatDS at 35°C (1st Use)	0.2% w/v H_2O_2 with PCatDS at 35°C (2nd Use)	0.2% w/v H_2O_2 with PCatDS at 35°C (3rd Use)
10	1.84(0.42)	1.97(0.30)	2.25(0.22)	3.01(0.006)	3.30(0.29)	3.60(0.18)
20	3.38(0.48)	3.56(0.43)	3.64(0.07)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)
30	≥5.22(0.48)	4.98(0.54)	4.76(0.12)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)
40	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)
50	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)
60	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)

pH range pH range 4.65-4.37 (room temperature); 4.50-4.11 (35°C); S.D. = standard deviation; Temp. = temperature

PCatDS = ferric sulfate form PAN catalyst (Dralon-L type).

Table 2.32: Antibacterial effects of reused modified PAN catalyst (PCatDC3) at three independent uses (48hrs between use) with 1% w/v H_2O_2 on *S. aureus* at room temperature and 35°C.

Contact time/min	Mean Log_{10} reduction (± S.D.)					
	1% w/v H_2O_2 with PCatDC3 at room Temp. (1st Use)	1% w/v H_2O_2 with PCatDC3 at room Temp. (2nd Use)	1% w/v H_2O_2 with PCatDC3 at room Temp. (3rd Use)	1% w/v H_2O_2 with PCatDC3 at 35°C (1st Use)	1% w/v H_2O_2 with PCatDC3 at 35°C (2nd Use)	1% w/v H_2O_2 with PCatDC3 at 35°C (3rd Use)
10	1.66(0.18)	1.26(0.11)	1.01(0.02)	>5.41(0.00)	3.71(0.08)	3.71(0.03)
20	>5.41 (0.00)	4.29(0.23)	3.77(0.03)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)
30	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)
40	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)
50	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)
60	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)	>5.41(0.00)

pH range 3.84-3.46 (room temperature); 3.50-3.37 (35°C); S.D. = standard deviation; Temp. = temperature; PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 3).

Table 2.33: Antibacterial effects of reused modified PAN catalyst (PCatCR) at two independent uses (6months between use) with 0.2% w/v H_2O_2 on *E. coli* at room temperature and 35°C.

Contact time/min	Mean Log_{10} reduction (± S.D.)			
	0.2% w/v H_2O_2 with PCatCR at room Temp. (1st Use)	0.2% w/v H_2O_2 with PCatCR at room Temp. (2nd Use)	0.2% w/v H_2O_2 with PCatCR at 35°C (1st Use)	0.2% w/v H_2O_2 with PCatCR at 35°C (2nd Use)
10	2.80(0.82)	2.27(0.03)	>5.53(0.00)	>5.65(0.00)
20	4.95(0.39)	3.26(0.46)	>5.53(0.00)	>5.65(0.00)
30	>5.65(0.00)	>5.65(0.00)	>5.53(0.00)	>5.65(0.00)
40	>5.65(0.00)	>5.65(0.00)	>5.53(0.00)	>5.65(0.00)
50	>5.65(0.00)	>5.65(0.00)	>5.53(0.00)	>5.65(0.00)
60	>5.65(0.00)	>5.65(0.00)	>5.53(0.00)	>5.65(0.00)

S.D. = standard deviation; Temp. = temperature;

PCatCR = ferric chloride form PAN catalyst (Russian type)

Table 2.34: Antibacterial effects of reused modified PAN catalyst (PCatCR) at two independent uses (6months between use) with 1% w/v H_2O_2 on *S. aureus* at room temperature and 35°C.

Contact time/min	Mean Log_{10} reduction (± S.D.)			
	1% w/v H_2O_2 with PCatCR at room Temp. (1st Use)	1% w/v H_2O_2 with PCatCR at room Temp. (2nd Use)	1% w/v H_2O_2 with PCatCR at 35°C (1st Use)	1% w/v H_2O_2 with PCatCR at 35°C (2nd Use)
10	>5.64(0.00)	2.94(0.52)	>5.64(0.00)	≥5.11(0.51)
20	>5.64(0.00)	4.28(0.09)	>5.64(0.00)	>5.68(0.00)
30	>5.64(0.00)	>5.68(0.00)	>5.64(0.00)	>5.68(0.00)
40	>5.64(0.00)	>5.680.00)	>5.64(0.00)	>5.68(0.00)
50	>5.64(0.00)	>5.68(0.00)	>5.64(0.00)	>5.68(0.00)
60	>5.64(0.00)	>5.68(0.00)	>5.64(0.00)	>5.68(0.00)

S.D. = standard deviation; Temp. = temperature;

PCatCR = ferric chloride form PAN catalyst (Russian type)

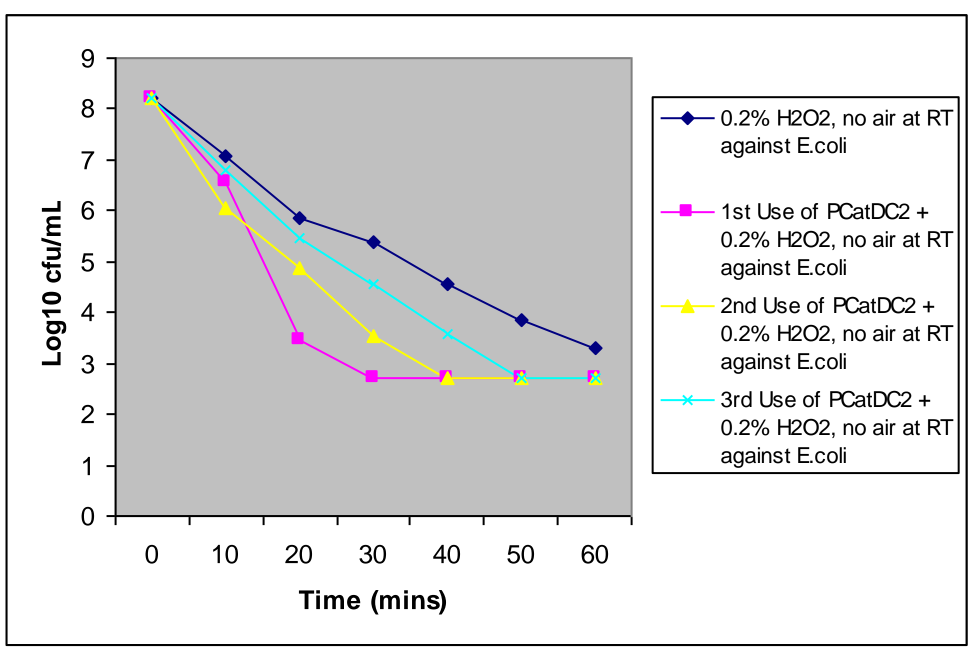


Fig. 2.6: A demonstration of the differences in the reusability of modified PAN catalyst. RT = room temperature; PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2).

Note: Log10 in graph is Log_{10}

The above summary graph shows that the modified PAN catalyst can be used at least two times and activity at 3rd use is still significantly greater than when H_2O_2 alone is used.

2.4.7. Antibacterial effects of peracetic acid with or without modified PAN catalyst, homogeneous iron catalyst, non-modified PAN mesh or modified PAN mesh.

As described earlier, peracetic acid (PAA) is a powerful oxidant and disinfectant, with a reduction potential of 1.06V (Zhao *et al.,* 2008).

Although H_2O_2 contributes to the disinfectant abilities of PAA, it is known that PAA is a more potent antimicrobial agent than H_2O_2, being rapidly active at low concentration against a wide spectrum of microorganisms (Baldry, 1983; Block, 2001; Kitis, 2004).

Due to the presence of H_2O_2 in PAA, an attempt was made to assess whether the modified PAN catalyst (PCatDC3 or PCatDS) or homogeneous iron chloride catalyst (HmCl) could provide synergism to a low concentration of PAA against *S. aureus*. Further work was also performed to establish the effects of PAA with non-modified PAN mesh (NMPM) or modified PAN mesh (MOPM) against *S. aureus*.

Table 2.35 summarises the antibacterial effect of 0.18-0.001% w/v PAA at room temperature; and Table 2.36 shows the effects of adding PCatDC3 under the same conditions. Table 2.37 shows the antibacterial effects of 0.0002% w/v PAA with or without PCatDC3 or PCatDS or HmCl or NMPM or MOPM at room temperature.

0.18-0.001% w/v PAA with or without PCatDC3 completely inactivated *S. aureus* at 10mins (Tables 2.35 and 2.36) and 0.0002% w/v PAA completely inactivated *S. aureus* at 30mins (Table 2.37). Greenspan and MacKellar (1951) showed that 0.001% w/v PAA is bactericidal (5mins inactivation against *S. aureus*), 0.003% fungicidal and 0.3% being sporicidal. Interestingly, the addition of either PCatDC3 or PCatDS to 0.0002% w/v PAA showed a highly significant ($p<0.01$) reduction of antibacterial activity from 20-60mins. At 60mins, there were log reductions of 1.07 and 2.60 for the activities of PAA/PCatDC3 and PAA/PCatDS respectively as compared to complete bacterial inactivation at 30mins when PCatDC3 and PCatDS were absent in the reaction (Table 2.37). The reduced levels of activity seen when the PAN catalyst was used in combination with 0.0002% w/v PAA were investigated further to establish whether the inhibition was due to the combined effects of

chemicals on the PAN fibre. The addition of either HmCl or NMPM or MOPM to 0.0002% w/v PAA did not reduce the antibacterial activity of PAA. *S. aureus* was completely inactivated at 30mins, 20mins and 30mins using HmCl, NMPM, and MOPM respectively (Table 2.37). There were no significant differences ($p>0.05$) between the antibacterial activities of '0.0002% w/v PAA'; '0.0002% w/v PAA with HmCl'; '0.0002% w/v PAA with NMPM'; and '0.0002% w/v PAA with MOPM'. However, the addition of PCatDC3 or PCatDS to 0.0002% w/v PAA resulted in highly significant ($p<0.01$) reductions in antibacterial activity from 20-60mins as compared to the activities of '0.0002% w/v PAA'; '0.0002% w/v PAA with HmCl'; '0.0002% w/v PAA with NMPM'; and '0.0002% w/v PAA with MOPM'. These observations suggest that the significantly reduced antibacterial activity seen when PCatDC3 or PCatDS was present in 0.0002% w/v PAA may be due to the combined chemicals (including chloride or sulfate iron salts) on the mesh. There was no inhibition of activity arising from the use of non-modified PAN mesh or from the modified PAN mesh (but not impregnated with iron salts), suggesting that inhibition was not due to the leakage of chemicals used in the production of the PAN fibre or any leakage of the modification chemicals, hydrazine or hydroxylamine or indeed of ammonia from the modified PAN mesh. It also suggested that there was little reaction between the PAA and the functional groups on either the non-modified PAN mesh (cyano groups) or the modified PAN mesh (hydrazine, amide, oxime, and carboxylate groups). As there was no inhibition of activity with homogeneous ferric chloride catalyst, the suggestion is that at pH 3.31, PAA was protonated and did not complex the iron cation. Another possibility is that a PAA-Iron complex was formed but that it was active. Interestingly, these inhibitory effects demonstrated by PCatDC3 or PCatDS were not seen when they were used with PAA between the concentrations ranging from 0.18-0.001% w/v. It is likely that these concentrations were high enough to overcome any antagonistic reaction resulted from PCatDC3 or PCatDS. Clearly, the present work has shown that the heterogeneous modified PAN catalyst did not increase the antibacterial activity of PAA, despite H_2O_2 being known to be present in PAA, but rather reduced the activity of low concentrations of PAA.

Table 2.35: Effects of peracetic acid (PAA) on *S. aureus* at room temperature.

Contact time/min	Mean Log_{10} reduction (± S.D.)						
	0.18% w/v PAA	0.1% w/v PAA	0.05% w/v PAA	0.025% w/v PAA	0.006% w/v PAA	0.003% w/v PAA	0.001% w/v PAA
10	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
20	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
30	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
40	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
50	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
60	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)

pH range 3.40-4.36 (room temperature); S.D. = standard deviation; PAA = peracetic acid.

Table 2.36: Effects of peracetic acid (PAA) with modified PAN catalyst (PCatDC3) on *S. aureus* at room temperature.

Contact time/min	Mean Log_{10} reduction (± S.D.)						
	0.18% w/v PAA and PCatDC3	0.1% w/v PAA and PCatDC3	0.05% w/v PAA and PCatDC3	0.025% w/v PAA and PCatDC3	0.006% w/v PAA and PCatDC3	0.003% w/v PAA and PCatDC3	0.001% w/v PAA and PCatDC3
10	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
20	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
30	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
40	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
50	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
60	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)

pH range 3.33-4.12 (room temperature); S.D. = standard deviation; PAA = peracetic acid; PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 3).

Table 2.37: Effects of 0.0002% w/v PAA with either modified PAN catalyst or homogeneous catalyst or non-modified PAN mesh or modified PAN mesh on *S. aureus* at room temperature.

Contact time/min	Mean Log_{10} reduction (± S.D.)					
	0.0002% PAA pH 4.57	0.0002% PAA with PCatDC3 pH 3.92	0.0002% PAA with PCatDS pH 4.54	0.0002% PAA with HmCl pH 3.31	0.0002% PAA with NMPM pH 5.36	0.0002% PAA with MOPM pH 4.94
10	1.14(0.21)	0.85(0.14)	1.34(0.43)	2.37(0.69)	1.47(0.85)	1.01(0.24)
20	5.02(0.45)	0.88(0.16)	1.29(0.27)	4.64(0.66)	>5.53(0.00)	2.89(1.05)
30	>5.53(0.00)	1.08(0.15)	1.70(0.40)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
40	>5.53(0.00)	1.01(0.07)	2.15(0.39)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
50	>5.53(0.00)	1.07(0.15)	2.18(0.41)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)
60	>5.53(0.00)	1.07(0.04)	2.60(0.67)	>5.53(0.00)	>5.53(0.00)	>5.53(0.00)

S.D. = standard deviation; PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 2); PCatDS = ferric sulfate form PAN catalyst (Dralon-L type); HmCl = homogeneous ferric chloride catalyst; NMPM = non-modified PAN mesh; MOPM = modified PAN mesh.

2.4.8. Sporicidal activity of H_2O_2 with or without modified PAN catalyst

The most clinically important spore formers are derived from members of the genera *Bacillus* and *Clostridium* (Russell, 1990). Bacterial spores are more resistant to chemical and physical agents such as heat, UV radiation and oxidising agents (e.g. H_2O_2) than their vegetative form and other vegetative bacteria (Russell, 1990; Setlow and Setlow, 1994; Popham *et al.,* 1995; Sagripanti *et al.,* 2007). Spore structure (Figure 2.2) and chemical composition are known to play major roles in spore resistance (Setlow, 2006; Kim *et al.,* 2006).

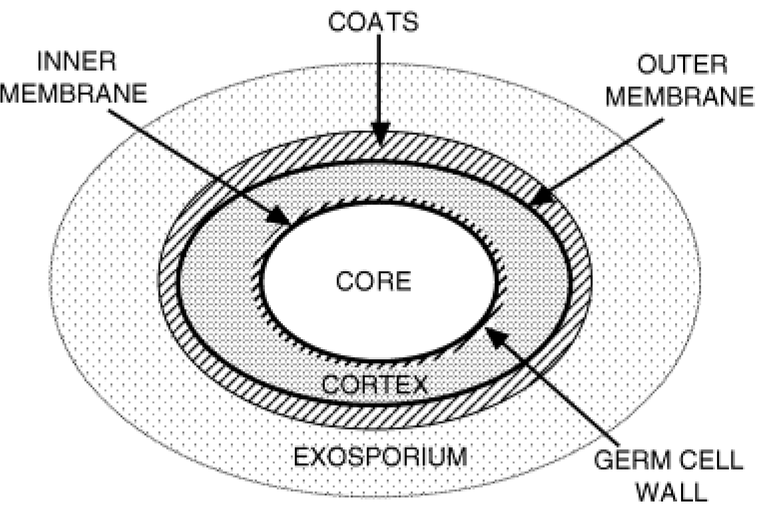


Figure 2.2: Spore structure (Adapted from: Setlow, 2006).
(This figure has been reproduced from 'Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals', by Setlow, P. 2006, Journal of Applied Microbiology, volume 101, pages 514-525. Copyright (2006), with permission from John Wiley and Sons – www.interscience.wiley.com.).

The number of chemical sporicides available to disinfect *Bacillus* spores are few and they are known to possess a high degree of toxicity; their activity is limited at low temperatures and they are impeded by organic matter (Kida *et al.,* 2004). Given as an example, formaldehyde (FMA) and glutaraldehyde (GTA) are well known chemical sporicides that possess toxicological properties and require adequate precautions for use (Kida *et al.,* 2004;

Rideout *et al.,* 2005). Six percent (w/v) H_2O_2 is used as sterilant for 6 hours (Rutala *et al.,* 1993). It is noted that inactivation of spores by H_2O_2 is greatly enhanced by increased temperature and increased concentration (Block, 2001). The sporicidal activity of $^{\cdot}OH$ has been studied. Thus, a modified Fenton reagent formulation involving cupric chloride, ascorbic acid, and sodium chloride to produce $^{\cdot}OH$ have been shown to exhibit effective sporicidal activity under aerobic conditions (Cross *et al.,* 2003).
The sporicidal effects of H_2O_2 with or without modified PAN catalyst (PCatDC3 or PCatDS) against *B. subtilis* NCIB 8054 spores was examined. Table 2.38 shows the control tests with peracetic acid (PAA) and glutaraldehyde (GTA) against *B. subtilis* NCIB 8054 spores. Table 2.39 summarises the sporicidal activities of 5% w/v and 7.5% w/v H_2O_2 with or without PCatDC3 or PCatDS.

PAA and GTA showed sporicidal and moderate activity respectively. Sporicidal effects occurred within 30mins using 0.05% w/v PAA and within 10mins using 0.1% w/v PAA. At 120mins, log reductions of 3.11 and 3.81 were observed using 1% w/v and 3% w/v GTA respectively. 0.1% w/v PAA showed significantly increased activity ($p<0.01$) at 10mins compared to 0.05% w/v PAA. There was no significant difference between the activity of 1% w/v and 3% w/v GTA respectively.

At 120mins, 5% w/v H_2O_2 showed a 3.45 log reduction against *B. subtilis* NCIB 8054 spores. However, in the presence of PCatDS, a log reduction of 4.91 occurred at 120mins.When PCatDC3 was used, sporicidal activity (>6.84 log reduction) was observed at 120mins. There was no significant difference from 10-30mins between the reaction involving '5% w/v H_2O_2 with PCatDS' and '5% w/v H_2O_2 alone', however, there was significant increase in activity ($p<0.05$) from 60-120mins by '5% w/v H_2O_2 with PCatDS' compared to '5% w/v H_2O_2 alone'. Similarly, significantly increase activity ($p<0.01$) was observed at 120mins when '5% w/v H_2O_2 with PCatDC3' was used compared to '5% w/v H_2O_2 alone'.

At 120mins, 7.5% w/v H_2O_2 gave a 4.22 log reduction against *B. subtilis* NCIB 8054 spores. However, the addition of PCatDS gave a log reduction of 2.50 after 60 minutes, and sporicidal activity at 120mins (>6.84 log reduction). When PCatDC3 was added, a log reduction of ≥6.28 occurred after only 60mins. There was no significant difference at 10mins between the reaction involving '7.5% w/v H_2O_2 with PCatDS' and '7.5% w/v H_2O_2 alone', however, there was significant increased activity ($p<0.05$) from 30-120mins with '7.5% w/v H_2O_2 with PCatDS' compared to '7.5% w/v H_2O_2 alone'. Similarly, there was significant increase in activity ($p<0.01$) from 10-120mins when '7.5% w/v H_2O_2 with PCatDC3' was used compared to '7.5% w/v H_2O_2 alone'.

The data demonstrated that the presence of the modified PAN catalysts (PCatDS or PCatDC3) contributed significantly to the increased sporicidal activity of H_2O_2. However, as shown in Table 2.39, the ferric chloride impregnated catalyst (PCatDC3) appears to provide a greater synergistic/catalytic effect to H_2O_2 than the ferric sulfate impregnated catalyst (PCatDS). The catalyst/ H_2O_2 system was more effective than 1-3% w/v GTA, but less effective than 0.5-0.1% w/v PAA.

According to the literature, 6% w/v or 7.5% w/v H_2O_2 is used as sterilant for 6 hours (Rutala *et al.,* 1993; Rutala and Weber, 1999; Acosta-Gio *et al.,* 2005), the study presented here has shown that 5% w/v or 7.5% w/v H_2O_2 with PAN catalyst is sporicidal at 2 hours; potentially the use of the catalyst could present an opportunity to shorten the 6 hours sterilising contact time of H_2O_2 to 2 hours.

Table 2.38: Effects of peracetic acid (PAA) and glutaraldehyde (GTA) on *B. subtilis* NCIB 8054 spores at room temperature.

Contact time (min)	Mean Log_{10} reduction (± S.D.)			
	0.05% w/v PAA pH 3.66	0.1% w/v PAA pH 3.52	1% w/v GTA pH 7.59-7.85	3% w/v GTA pH 7.59-7.85
10	2.46(0.26)	>6.84(0.00)	1.13(0.08)	1.93(0.03)
30	>6.84(0.00)	>6.84(0.00)	2.33(0.24)	2.64(0.12)
60	>6.84(0.00)	>6.84(0.00)	2.76(0.13)	2.93(0.13)
120	>6.84(0.00)	>6.84(0.00)	3.11(0.30)	3.81(0.48)

S.D. = standard deviation; PAA = peracetic acid; GTA = glutaraldehyde.

Table 2.39: Effects of H_2O_2 with or without modified PAN catalyst on *B. subtilis* NCIB 8054 spores at room temperature.

Contact time/min	Mean Log_{10} reduction (± S.D.)					
	5% w/v H_2O_2 pH 5.67	5% w/v H_2O_2 with PCatDS pH 4.19	5% w/v H_2O_2 with PCatDC3 pH 3.43	7.5% w/v H_2O_2 pH 5.53	7.5% w/v H_2O_2 with PCatDS pH 4.13	7.5% w/v H_2O_2 with PCatDC3 pH 3.360
10	1.02(0.03)	1.00(0.60)	0.23(0.08)	0.04(0.11)	0.28(0.17)	1.48(0.06)
30	1.26(0.12)	1.33(0.56)	0.49(0.04)	0.19(0.08)	0.95(0.14)	2.41(0.08)
60	1.90(0.09)	2.91(0.37)	1.62(0.31)	0.95(0.01)	2.50(0.16)	≥6.28(0.54)
120	3.45(0.27)	4.91(0.90)	>6.84(0.00)	4.22(0.71)	>6.84(0.00)	>6.84(0.00)

S.D. = standard deviation; PCatDS = ferric sulfate form PAN catalyst (Dralon-L type); PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 3)

2.5. Conclusion

This study has demonstrated that the antimicrobial activity of H_2O_2 is significantly increased in the presence of novel PAN catalyst (impregnated with iron salts), this is possibly due to the generation of hydroxyl radicals as the mode of killing (see Chapter 4 for mode of action). Increases in antimicrobial activity were higher at 35°C than at room temperature, both in the presence or absence of air (2.5 L/min). Homogeneous ferric chloride catalyst with H_2O_2 also demonstrated high antimicrobial activity at both room temperature and at 35°C, with no significant difference compared to activity of the PAN catalyst with H_2O_2. However, the heterogeneous modified PAN catalyst is reusable, whilst the homogeneous catalyst is not, which potentially represents a competitive advantage over the homogeneous catalyst. The reusability of the PAN catalyst was found to be dependent on factors such as H_2O_2 concentration, type of catalyst i.e. ferric chloride or ferric sulfate impregnation, amount of iron on the support mesh, and temperature.

The PAN catalysts were reusable at least three times, however, catalytic activity appears to reduce on subsequent uses at room temperature but remain more active on reuse at 35°C. PAN catalysts appear to show activity around neutral pH, with more activity at 35°C than at room temperature. The presence of the phosphate buffer may have compromised the reaction by binding to the active iron site preventing access by hydrogen peroxide. It is possible that if the PAN catalyst had been washed and adjusted with NaOH until no change in pH when put in water, thus to remove the protonation of the catalyst, and although there might have still be a reduced activity but it could have provided an insight whether it was due to phosphate binding. However, this activity was less than when the PAN catalyst is at acidic pH (normal pH of the PAN catalyst in solution). Although acidic water alone containing the same pH as PAN catalyst in solution (control) showed a reduction in viability of these microorganisms, it was not as great as the activity seen with the PAN catalyst alone in water. PAN catalyst alone may possess some antimicrobial activity, but the mechanism of action at this stage is not clear. The antimicrobial activity of the PAN catalyst alone in water was low (except with *Ps. aeruginosa* and elevated temperatures), but it did show more antimicrobial activity than the homogeneous catalyst used alone in water.

PAN catalysts were more active in acidic environments than in alkaline or buffered environments. Overall, it can be seen that both the heterogeneous and homogeneous catalysts require H_2O_2 to exhibit an increased antimicrobial activity and that the overall efficacy of the catalyst is not caused by a decrease in pH alone.

The modified PAN mesh (MOPM) did not show any antimicrobial activity whether used alone or with H_2O_2 against *E. coli*, *Ps. aeruginosa*, and *S. aureus*.

PAN catalysts significantly reduced the antimicrobial activity of low concentrations of peracetic acid (PAA), however, modified PAN mesh (MOPM), non-modified PAN mesh (NMPM), and homogeneous iron catalyst did not inhibit the activity of PAA.

PAN catalysts with H_2O_2 at room temperature showed sporicidal activity against *Bacillus subtilis*; however, the ferric chloride impregnated catalyst showed increased spore reduction compared to the ferric sulfate impregnated catalyst.

In general the PAN catalyst/H_2O_2 systems successfully passed the tests pertaining to the basic bactericidal and sporicidal activities as stipulated and required by the European Standard Phase 1 Suspension Tests as described in Section 2.2.

CHAPTER 3

Iron Leaching From the Novel Heterogeneous Modified PAN Catalyst and its Effect on Antibacterial Activity.

3.1. Introduction

Metals are natural constituents of the environment. Trace metals (alternatively called trace elements) are found in extremely low amounts, these include iron, selenium, copper, zinc etc. Iron is widely available in the environment, in water, soil, plants (Salinas and March, 1984; Tautkus *et al.,* 2004), and rocks (Pournaghi-Azar and Fatemi, 2000). Iron is essential for bacteria and human host, participating in a variety of basic metabolic pathways (Andrews *et al.,* 2003). It is needed for many cellular processes such as catalysis, electron transfer, oxygen metabolism, regulation and signal transduction (Yang *et al.,* 2006). Besides the many benefits of iron to most living organisms, excessive amounts can be harmful as it can generate reactive oxygen species (ROS) to destroy cellular components (Touati, 2000).

Many methods and instruments have been developed in the determination of trace metals; these may require the digestion of samples (biological or non-biological) to determine total amount of trace metals. Atomic absorption spectroscopy (AAS) is an analytical instrument which is widely employed in the field of trace metal analysis (Beaty and Kerber, 1993; Ogiyama *et al.,* 2006). AAS involves atomising the specimen, often by spraying a solution of the sample into a flame, and then studying the absorption of radiation from an electric lamp producing the spectrum of the element to be determined (Mendham *et al.,* 2000). Sample digestion is a critical step in most analytical methods for routine determination of trace elements in biological samples such as foods (Demirel *et al.,* 2008) and microbial cells (Jackson *et al.,* 2006; Zaied *et al.,* 2008). These digestion procedures include dry washing methods (samples are subjected to crucible furnace at increased temperatures), wet digestion in open vessels (require the use of concentrated acids), and closed-vessel acid decomposition in microwave oven systems (Demirel *et al.,* 2008). AAS has been used to identified the presence of lead (Pb), iron (Fe), cadmium (Cd), nickel (Ni), chromium (Cr), cobalt (Co), copper (Cu), zinc (Zn) and manganese (Mn) in wild edible mushrooms (Mendil *et al.,* 2004) and it has also been used to determine iron in microbial cells (Jackson *et al.,* 2006; Zaied *et al.,* 2008) as well as trace elements in wastewater bodies (Segura *et al.,* 2004).

The present study employed AAS to determine both the amount of iron leached from the heterogeneous modified PAN catalyst in the presence and absence of microorganism. The antimicrobial activity of the leachate from the heterogeneous modified PAN catalyst was also examined.

3.2. Materials and Methods

3.2.1. Materials

Thirty seven percent (w/v) hydrochloric acid (HCl), 98% w/v sulphuric acid (H_2SO_4) were purchased from Sigma-Aldrich (UK). Heterogeneous modified PAN catalyst was prepared as previously described in Chapter 2, Section 2.3.2.4. For materials on bacteria preparation, growth, cultures, and testing, see Chapter 2, Section 2.3.1.1.

3.2.1.1. Equipment and its uses.

PerkinElmer AAnalyst 200/400 Atomic Absorption Spectrophotometry (AAS) (PerkinElmer Life and Analytical Sciences, USA) was used for iron determination (see Appendix 2). A multi-position heated reactor (HACH – model: HH/45600.00) (Camlab Limited, Cambridge, England) (see Appendix 3) was used to digest iron from PAN catalyst. Sorvall Legend™ T/RT (Kendro laboratory Products, Inc, USA) was used to centrifuge all microorganisms. The fume cupboard used was a Labcaire T400 Schools Model, Bio-decontamination Solutions (Labcaire Systems Ltd, North Somerset, UK). Minisart, 0.2µm pore size single use sterile filters (Sartorius Biotech, Goettengen, Germany) were used in conjunction with 10mL volume syringes (Becton Dickinson, Madrid, Spain) for filtration procedures.

3.2.2. Methods

3.2.2.1. Atomic Absorption Spectrophotometry (AAS) settings

The following settings on AAS were used for iron (Fe) determination for all the tests:

Acetylene flow (L/min) = 2.50

Oxidant flow (L/min) = 10.00

Wavelength (nm) = 248.33

Slit (mm) = 1.8/1.35

3.2.2.2. The extent of iron leaching from modified PAN catalyst

The amount of iron leached from the modified PAN catalyst was determined both in the presence and absence of microorganisms. When microorganisms were used, these were prepared according to the experimental procedure as described in Chapter 2 Sections 2.3.2.1 and 2.3.2.9. However, no neutralisation procedure was carried out. Control tests had either 1 ± 0.1g PAN catalyst with 75mL of sterile distilled water or 67.5mL distilled water with 7.5mL bacteria suspension (*E. coli* or *S. aureus*). Experimental samples were the same as the controls but included the appropriate amount of H_2O_2. Contact times were 10, 20, 30, 40, 50, and 60mins. At the appropriate contact time, 5mL aliquot of the solution phase is taken with a 10mL volume syringe and filtered through 0.2µm pore size single use filter into a universal bottle. The iron content of the filtered solution was then measured using AAS instrument as follows: The AAS instrument was first blanked with the appropriate solution (in tests where H_2O_2 was used, H_2O_2 solution was used as blank). Sterilised distilled water was used as blank in control experiments where no H_2O_2 was involved. The following iron standards were used 0.5, 1.0, 2.0. 4.0 and 10 parts per million (ppm) equivalent to mg/L to calibrate the instrument. A standard curve was produced by the instrument (see Appendix 4). Samples were then measured and iron content in sample was provided in ppm by the instrument. Each test was performed independently in triplicate at room temperature or 35°C.

NOTE: Iron content of sterile distilled water was measured, averaged and subtracted from the iron content determined in each experiment to give the true value of leached iron from the modified PAN catalyst.

3.2.2.3. The extent of iron leaching from reused modified PAN catalyst.

Modified PAN catalyst used in Section 3.2.2.2. was reused 48 hours after first use to compare the differences in the amount of iron leached into solution between first use and reuse. The experimental procedure was the same as described in Section 3.2.2.2.

3.2.2.4. Comparative differences of iron leaching between modified PAN catalyst batches

These tests were performed to compare the rate of iron leaching from different batches of the modified PAN catalysts, and were performed in the absence of microorganisms. The experimental procedure was the same as described in Section 3.2.2.2.

3.2.2.5. Digestion of modified PAN catalyst

1g x 3 modified PAN catalysts were digested both to determine the amount of iron on the catalyst and to compare the relative amount leached from the catalyst in the previous experiments.

0.1g mesh of the modified PAN catalyst was inserted into each of 10 digestion vials/tubes (10 tubes with 0.1g PAN catalyst each) containing 10mL volume of 37% w/v HCl (for ferric chloride impregnated catalyst) or 98% w/v H_2SO_4 (for ferric sulfate impregnated catalyst). These tubes were then placed into the Hach reactor at 150^oC and digested for 2 hours. After cooling, digested PAN catalyst mesh was removed from the tubes. The digested solutions were combined together in a 100mL Duran bottle and filtered through 0.2μm pore size filter. A 10^{-2} dilution of the digested combined solution made, and AAS measurement was performed. Table 3.1. shows the amount of iron in ppm/g and mmol/g of the modified PAN catalyst mesh.

Table 3.1: Amount of iron digested off different batches of modified PAN catalyst.

PAN catalyst type/batch	Amount of iron (ppm/g of mesh)
PCatDC2	30
PCatDC3	35
PCatDS	120

PCatDC2, PCatDC3 = Dralon L ferric chloride impregnated PAN catalysts; PCatDS = Dralon L ferric sulfate impregnated PAN catalyst; ppm/g = parts per million per gram

3.2.2.6. Antibacterial effects of leachate from modified PAN catalyst in H_2O_2 solution.

1 ± 0.1g of the modified PAN catalyst was added to 67.5mL of H_2O_2 (concentration of 1.11% w/v) and magnetically stirred for 1hour at room temperature. The modified PAN catalyst was then removed and 7.5mL bacteria suspension (*S. aureus*) in sterile distilled water was added making up to a total volume of 75mL of a 1% w/v H_2O_2 solution. Experimental procedure, neutralisation, contact times, dilutions and incubations procedures were followed as described in Chapter 2, Sections 2.3.2.8 and 2.3.2.9. The modified PAN catalyst was reused 48 hours after first use. The procedure was the same as described above. In the experiment where 1 ± 0.1g of modified PAN catalyst was left in the reaction for the duration of test, the procedure was the same as described in Chapter 2, Sections 2.3.2.8. and 2.3.2.9.

Controls:

The antimicrobial activity of the amount of iron determined from leachate of either PCatDC2 (ferric chloride impregnated PAN catalyst) or PCatDS (ferric sulfate impregnated PAN catalyst) was carried out and compared to the antimicrobial activity of leachate described previously. The control tests were performed as follows: Either 3.42mL of a 10ppm Fe stock solution (≡ 0.456ppm of iron from leachate of PCatDC2) or 3.32mL of a 10ppm Fe stock solution (≡ 0.443ppm of iron from leachate of PCatDS) was added to an

experimental total volume of 75mL containing 1% w/v H_2O_2 and *S. aureus* (7.5mL volume). Experimental procedure, neutralisation, contact times, dilutions and incubations procedures were followed as described in Chapter 2, Sections 2.3.2.8 and 2.3.2.9.

The following calculations show how the amount of iron from iron salts ($FeCl_3.6\ H_2O$ or $Fe_2(SO_4)_3$) was obtained and used for the control experiments:

Molecular weight of $FeCl_3.6\ H_2O \equiv 270.2$ (g/mol)

Molecular weight of $Fe_2(SO_4)_3 \equiv 399.88$ (g/mol)

Atomic mass of Fe (iron) ≡ 56 (g/mol)

0.456 mg/L = [iron] from leachate of PCatDC2 ($FeCl_3.6\ H_2O$) found in 75mL experimental volume of 1% w/v H_2O_2 solution after 1 hour contact time

0.443 mg/L = [iron] from leachate of PCatDS ($Fe_2(SO_4)_3$) found in 75mL experimental volume of 1% w/v H_2O_2 solution after 1 hour contact time

0.456ppm or 0.443ppm [Fe] needed in 75mL experimental solution was prepared from a 10ppm [Fe] stock solution in 100 mL volume, (prepared from $FeCl_3.6\ H_2O$ and $Fe_2(SO_4)_3$) using the following procedure:

Preparation of 10 ppm (in 100mL) [Fe] from $FeCl_3.6\ H_2O$

> ppm x required volume x gravimetric factor = milligrams to be weighed
>
> 10mg/L x 0.100L x $FeCl_3.6\ H_2O$/Fe = milligrams to be weighed
>
> 10mg/L x 0.100L x 270.2/56 = 4.8mg of $FeCl_3.6\ H_2O$
>
> 4.8mg of $FeCl_3.6\ H_2O$ was dissolved in 100mL sterilise distilled water to give a 10ppm of Fe stock solution. The same procedure was used to prepare a 10ppm stock solution for $Fe_2(SO_4)_3$.

The volume of Fe stock solution (10ppm) needed to make up a concentration of either 0.456ppm or 0.443ppm [Fe] in 75mL experimental total volume was achieved by the following calculations:

> ppm_B x $volume_B$ = ppm_A x $volume_A$ where A and B refer to before dilution and after dilution respectively.

Example, $volume_B$ = (0.456ppm x 75mL) ÷ 10ppm = 3.42mL

So the addition of 3.42mL of 10ppm stock solution ($FeCl_3.6\ H_2O$) to 71.58mL of experimental constituents making up total volume of 75mL produced 0.456ppm iron

concentration (ferric chloride); and the addition of 3.32mL of 10ppm ($Fe_2(SO_4)_3$) to 71.68mL of experimental constituents making up total volume of 75mL produced 0.443ppm iron concentration (ferric sulfate) .

3.2.2.7. Statistical Analysis

The Student *t*-test was employed for all statistical analysis (using Microsoft Excel). To compare whether one type of modified PAN catalyst leaches more iron than another type of catalyst, one tail test was used. Similarly, one tail test was used to evaluate statistical significance between ‘the presence of microorganism and absence of microorganism’ or ‘condition at room temperature and at 35°C’. Statistical analysis for antibacterial effects of leachate solution was the same as described in Chapter 2, Section 2.3.2.16.

3.3. Results and Discussions

3.3.1. The extent of iron leaching from modified PAN catalyst with or without microorganism.

The extent of iron leaching from modified PAN catalyst (PCatDC2) was examined in the presence or absence of microorganisms. Figure 3.1. shows the extent of iron leaching from PCatDC2 in distilled water in the presence or absence of either *E. coli* or *S. aureus* at room temperature. Figure 3.2. indicates the extent of iron leaching from PCatDC2 in 0.2% w/v H_2O_2 in the presence or absence of *E. coli* at both room temperature and 35^oC. Figure 3.3. shows the extent of iron leaching from PCatDC2 in 1% w/v H_2O_2 in the presence or absence of *S. aureus* at both room temperature and 35^oC.

At room temperature, PCatDC2 with or without *E. coli* or *S. aureus* leached Fe into distilled water. The leaching of iron increases as a function of time. More iron had leached from the PAN catalyst at 60mins than at 10mins. Statistical observations demonstrated an interesting anomaly between iron leached from PCatDC2 alone and that leached when either *E. coli* or *S. aureus* was present. Significantly more iron was found to be leached ($p<0.05$) at 20, 30, 50 and 60mins when PCatDC2 was left alone in distilled water than when *E. coli* was present, however, no significant difference ($p>0.05$) was observed between the two (present or absent of *E. coli*) conditions at 10 and 40mins. Similarly, more leaching was found with PCatDC2 alone in water at 30, 50 and 60mins than when *S. aureus* was present, but no significant difference was detected between the two conditions at 10, 20 and 40mins. Generally, the results show that less iron was detected in the leachate solution in the presence of *E. coli* or *S. aureus* than without their presence, suggesting that perhaps some iron was adsorbed by these microorganisms (surface sorption) or that they inhibited release of iron (Figure 3.1). It also possible to suggest that the insignificant differences observed between PCatDC2 alone and the presence of organisms in-between contact times could also be due to an uneven distribution of the iron on the PAN mesh, whereby a certain section of the catalyst may leach less or more than another section depending on amount of iron present. Therefore even if the organisms were present, the section of the catalyst with

relatively higher amount of iron could still leach more iron in solution equivalent to when the organisms were absent (see Chapter 5 for iron distribution on the PAN mesh).

The catalyst PCatDC2 leached Fe in 0.2% w/v H_2O_2 with or without *E. coli* at room temperature or 35°C. The leaching of iron increases with time and temperature. The amount of iron in the leachate solution was less in the presence of *E. coli* (Figure 3.2) than when it was absent, although, this difference was not statistically significant either at room temperature or at 35°C. However, leaching of iron from PCatDC2 was significantly higher ($p<0.05$) from 20-60mins at 35°C than at room temperature.

The catalyst PCatDC2 leaches Fe in 1% w/v H_2O_2 with or without *S. aureus* at room temperature or 35°C. The leaching of iron increases as a function of time, H_2O_2 concentration, and temperature. Iron in leachate solution was reduced by the presence of *S. aureus* (Figure 3.3), but this reduction was found to be statistically insignificant ($p>0.05$) at both room temperature or at 35°C.

In all the experiments displayed in Figs. 3.1-3.3, between 0.3%-1.3% w/w of the total iron available leached into solution at room temperature and 1%-2.5% w/w of iron leached into solution at 35°C. (see Table 3.1. for total amount of iron on modified PAN catalyst mesh).

Example of the calculation on percentage of iron leached from 1g of modified PAN catalyst is as follows:

$$\%\text{ of Fe leached} = \frac{\text{amount of iron in leachate (mg)}}{\text{amount of Fe/g on mesh (mg)}} \times 100$$

e.g. 0.1mg/L of iron leached in 75mL volume of solution

(0.1mg x 75mL) ÷ 1000mL = 0.0075mg of Fe leached per gram of mesh

30mg/L of Fe digested from 1g of mesh in 100mL solution

(30mg x 100mL) ÷ 1000mL = 3mg of Fe on 1g of mesh

therefore % of Fe leached from 1g of mesh = (0.0075mg ÷ 3mg) x 100 = 0.25%

Generally, iron leaching from the PAN catalyst is dependent on contact time; temperature; presence or absence of H_2O_2 and its specific concentration; and the presence or absence of microorganism, thus, iron leached more at longer contact times than shorter contact times at both room temperature and 35^oC. Iron also leaches more at 35^oC than at room temperature. This was expected since it is well known that chemical reactions increase with increase temperature.

In another study, the activity of 100mg iron-based mesoporous silica catalyst with H_2O_2 (0.1Mol^{-1}) for the oxidation of phenol, showed that after 4hrs reaction time, iron leached between the ranges of <0.1ppm to 0.8ppm at room temperature; increasing the reaction temperature to 40^oC showed an increase in iron concentration to 8.2ppm; and iron leached into solution accounted for homogeneous contribution in the reaction (Xiang *et al.,* 2009). This aforementioned work in correlation to the present study showed that heterogeneous iron-based catalysts do leach out during their activity, and it is possible that there is a homogeneous iron catalyst contribution in the antimicrobial activity shown in Chapter 2 as a result of iron leached from the heterogeneous phase of the PAN.

The leaching of iron from PCatDC2 was found to be affected by concentration of H_2O_2. Thus, there was a significantly ($p<0.05$) greater amount of iron leached from 20-60mins in 1% w/v H_2O_2 than in 0.2% w/v H_2O_2. This may be possible because 1% w/v H_2O_2 may be able to break up the ligands holding the iron on the PAN mesh faster than 0.2% w/v H_2O_2 resulting in more iron leaching into solution. It was interesting to note that no significant difference was observed in the amount of iron leached into solution between 'catalyst/ H_2O_2 system' and 'catalyst/organism/ H_2O_2 system', which was not true between 'catalyst/water system' and 'catalyst/organism/water system'. The reason for these differences would require careful interpretation. Although, in all cases, iron leached more in the absence of the organisms than in their presence (Figs. 3.1-3.3).

The literature shows that different types of heterogeneous catalyst leach into solution, resulting in relatively reduced activity when the catalyst is reused (Iyer and Thakur, 2000; Kang *et al.,* 2010). For example, a heterogeneous copper catalyst was used to selectively synthesised diphenylaniline or triphenylamine, it was found that catalytic activity decreased

after reused, as a result of a considerable amount of catalyst leaching out in the reaction mixture (Patil *et al.,* 2010). It can be said that the relatively reduced antimicrobial activity observed when PAN catalysts were reused as shown in Chapter 2, Section 2.4.6. may, in part, be due to leaching of iron, however, other factors may also be important.

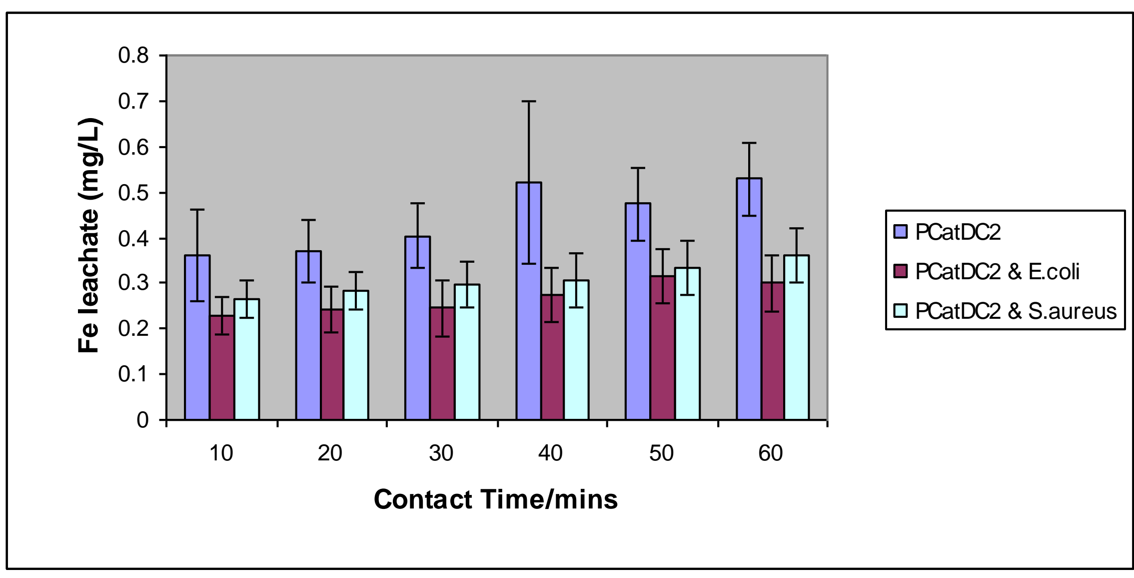


Figure 3.1: Amount of iron leaching from 1g modified PAN catalyst (PCatDC2) in distilled water in presence or absence of microorganisms at room temperature.

PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2).

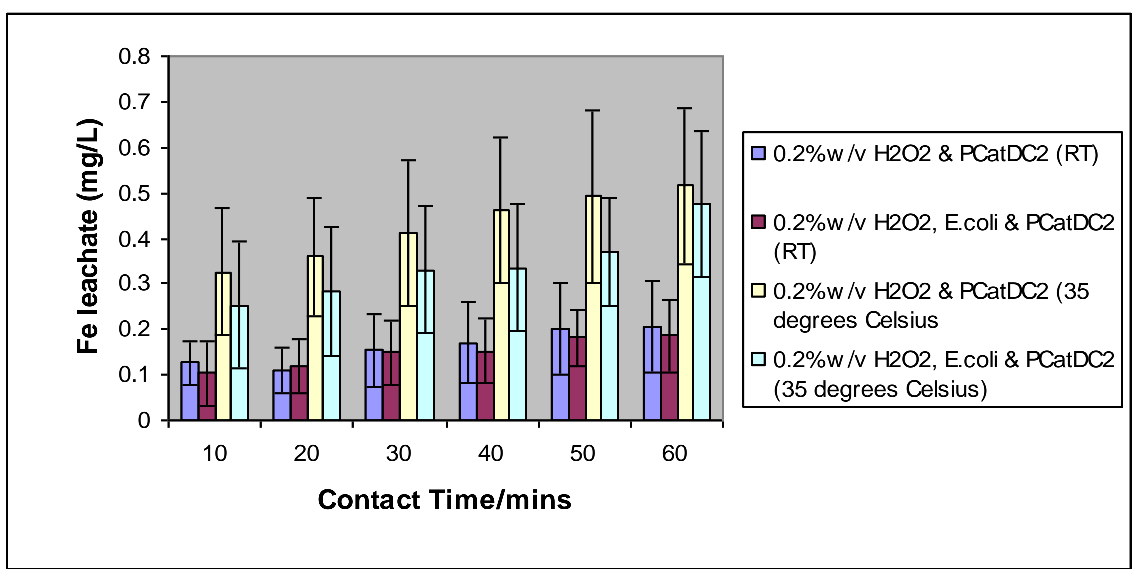


Figure 3.2: Amount of iron leaching from 1g modified PAN catalyst (PCatDC2) with 0.2% w/v H_2O_2 in presence or absence of *E. coli* at room temperature and 35°C.

PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2); RT = room temperature.

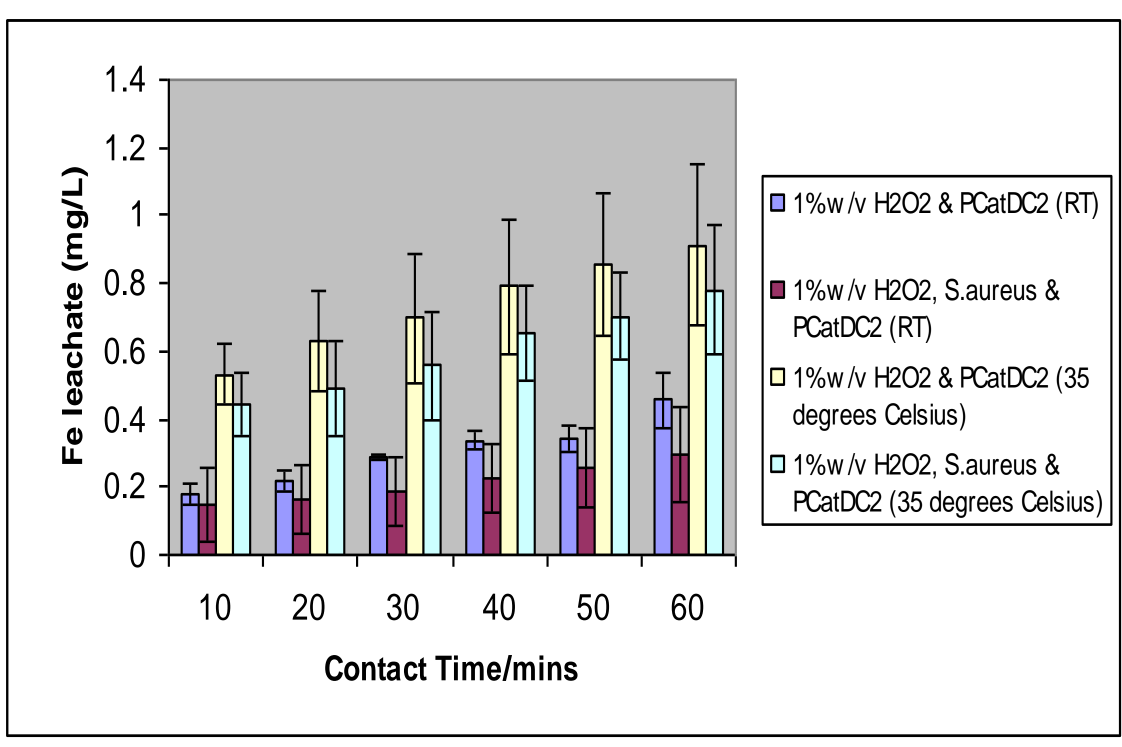


Figure 3.3: Amount of iron leaching from 1g modified PAN catalyst (PCatDC2) with 1% w/v H_2O_2 in presence or absence of *S. aureus* at room temperature and 35°C.

PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2); RT = room temperature

3.3.2. The extent of iron leaching from reused modified PAN catalyst.

The extent of iron leaching from reused modified PAN catalyst (PCatDC2) was examined in the presence or absence of microorganism. Figure 3.4. shows the extent of iron leaching from reused PCatDC2 (48hrs between use) in 0.2% w/v H_2O_2 in the presence or absence of *E. coli* at both room temperature and 35^oC. Tables 3.2 and 3.3 show the levels of iron leaching from reused PCatDC2 in 0.2% w/v H_2O_2 with or without *E. coli* at room temperature and 35^oC. Figure 3.5. indicates the extent of iron leaching from reused PCatDC2 (48hrs between use) in 1% w/v H_2O_2 in the presence or absence of *S. aureus* at both room temperature and 35^oC. Tables 3.4 and 3.5 show the levels of iron leaching from reused PCatDC2 in 1% w/v H_2O_2 with or without *S. aureus* at room temperature and 35^oC respectively.

Reused PCatDC2 leaches in 0.2% w/v H_2O_2 with or without *E. coli* at room temperature and 35^oC. There was no significant difference between the amount of iron leached when PCatDC2 was tested alone and when *E. coli* was present at both room temperature and 35^oC. Iron leaches less at room temperature with or without *E. coli* than at 35^oC. There was a slight increase in the amount of iron leached at 35^oC when PAN catalyst was reused compared to first use (Fig. 3.4; Table 3.3), but this increase was not statistically significant ($p>0.05$). Generally, there were no statistical differences observed between the amount of iron leached at first use and second use whether in the presence or absence of *E. coli*, (Table 3.3).

Reused PCatDC2 leached in 1% w/v H_2O_2 with or without *S. aureus* at room temperature and 35^oC (Fig 3.5). Iron leached less at room temperature than at 35^oC. At either room temperature or 35^oC, no significant differences were observed in the amount of iron leached at all time points whether there was *S. aureus* present in the reaction or not (Fig 3.5, Tables 3.4, 3.5). It was observed that iron leached slightly more from reused PCatDC2 at room temperature, than when first used, but this increase was statistically insignificant. Similarly, an increased in the amount of iron observed at 35^oC when PCatDC2 was first used was statistically insignificant compared to iron leached at reuse.

There was between 0.1%-0.4% of the iron present leached at room temperature and 0.4-1.5% of iron present leached at 35°C (using 0.2% w/v H_2O_2 from 10mins to 60mins contact time); and between 0.8%-1.4% of iron leached at room temperature and 1.25% -1.6% of iron leached at 35°C using 1%w/v H_2O_2 from 10mins to 60mins contact time (see Table 3.1. for total amount of iron on modified PAN catalyst mesh).
Generally, the PAN catalyst appeared to leach less iron on reuse than when first used. However, the increase in iron concentration on reuse in the presence of 1% w/v H_2O_2 compared to first use at room temperature may be attributed to the wet condition of the PAN catalyst prior to testing (as it was not completely dry at 48 hours after first use when it was retested). The PAN catalyst was not washed after first use and it is possible that there could be a breaking down of the ligands holding the iron to the modified PAN, resulting in a higher iron release on reimmersion of the catalyst into solution during reuse. Thus, H_2O_2 may have caused weaknesses in the ligands holding the iron during first use of the PAN catalyst, therefore, further use of the PAN catalyst in the presence of H_2O_2 may have led to a quicker release of iron into solution.

The reduction in iron concentration of the leachate on reuse could be correlated to the slightly lower antibacterial activity of the modified PAN catalyst on reuse as shown in Chapter 2, Section 2.4.6. Although, the amount of iron leached into solution may be considered as very low, it may be possible that reduced amount of iron on the modified PAN catalyst as a result of leaching into solution during first use leads to slightly reduce catalytic activity on second use. On the other hand, it is also possible that reduced antimicrobial activity on reuse of catalyst may not entirely be due to loss of iron from the PAN catalyst but also be due to dead microbial cells logged onto the active sites of the catalyst causing reduction in activity on reuse (see Chapter 5 for further work on live and dead microbial cells presence on the PAN catalyst). As suggested, these dead and live microbial cells on the PAN catalyst could be responsible for the reduced amount of iron leached from the PAN catalyst in the presence of microbial load (Tables 3.2, 3.3, 3.4, 3.5). It is possible that H_2O_2 may not have gained complete access to catalytic active sites to

react with the catalysts due to the presence of microbial cells (dead or alive) blocking these sites.

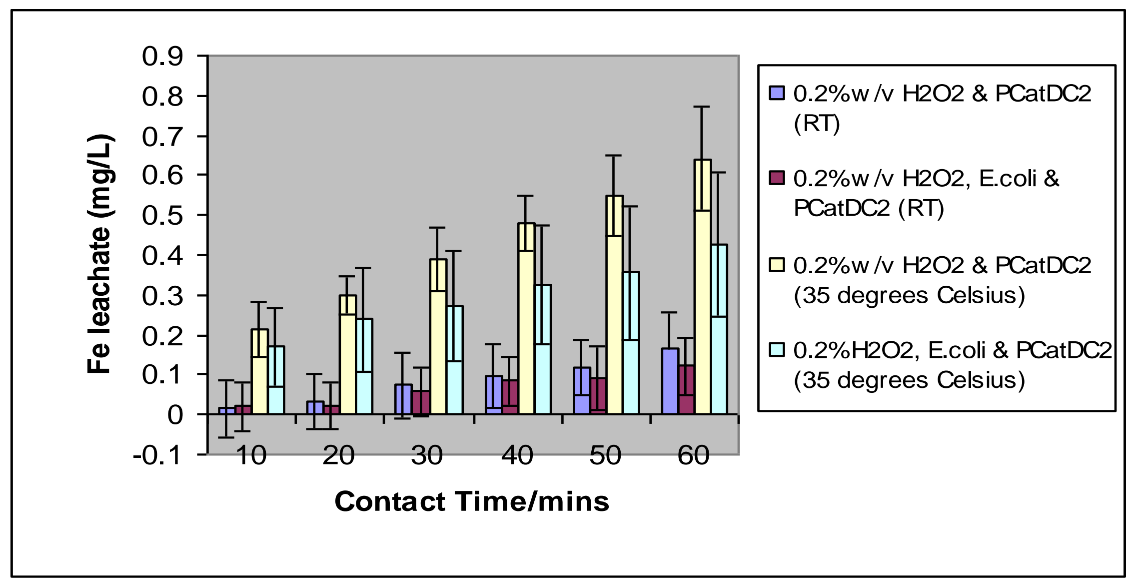


Figure 3.4: Amount of iron leaching from reused 1g (48hrs between use) modified PAN catalyst (PCatDC2) with 0.2% w/v H_2O_2 in the presence or absence of *E. coli* at room temperature and 35°C.

PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2); RT = room temperature.

Table 3.2: The levels of iron leaching from reused modified PAN catalyst (PCatDC2) in 0.2% w/v H_2O_2 with or without *E. coli* at room temperature.

Contact time/min	Mean Iron mg/L(± S.D)			
	1st use PCatDC2 Alone	2nd use PCatDC2 alone	1st use PCatDC2 & *E. coli*	2nd use PCatDC2 & *E. coli*
10	0.126(0.05)	0.015(0.07)	0.103(0.07)	0.020(0.06)
20	0.111(0.05)	0.033(0.07)	0.118(0.06)	0.023(0.06)
30	0.155(0.08)	0.073(0.08)	0.149(0.07)	0.057(0.06)
40	0.170(0.09)	0.096(0.08)	0.153(0.07)	0.084(0.06)
50	0.201(0.10)	0.118(0.07)	0.181(0.06)	0.093(0.08)
60	0.205(0.10)	0.164(0.09)	0.187(0.08)	0.121(0.07)

S.D. = standard deviation; PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2)

Table 3.3: The levels of iron leaching from reused modified PAN catalyst (PCatDC2) in 0.2% w/v H_2O_2 with or without *E. coli* at 35^oC.

Contact time/min	Mean Iron mg/L(± S.D)			
	1st use PCatDC2 Alone	2nd use PCatDC2 alone	1st use PCatDC2 & *E. coli*	2nd use PCatDC2 & *E. coli*
10	0.326(0.14)	0.214(0.07)	0.253(0.14)	0.169(0.10)
20	0.359(0.13)	0.299(0.05)	0.284(0.14)	0.239(0.13)
30	0.411(0.16)	0.391(0.08)	0.331(0.14)	0.272(0.14)
40	0.460(0.16)	0.479(0.07)	0.336(0.14)	0.325(0.15)
50	0.493(0.19)	0.548(0.10)	0.371(0.12)	0.355(0.17)
60	0.515(0.17)	0.640(0.13)	0.377(0.16)	0.427(0.18)

S.D. = standard deviation; PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2)

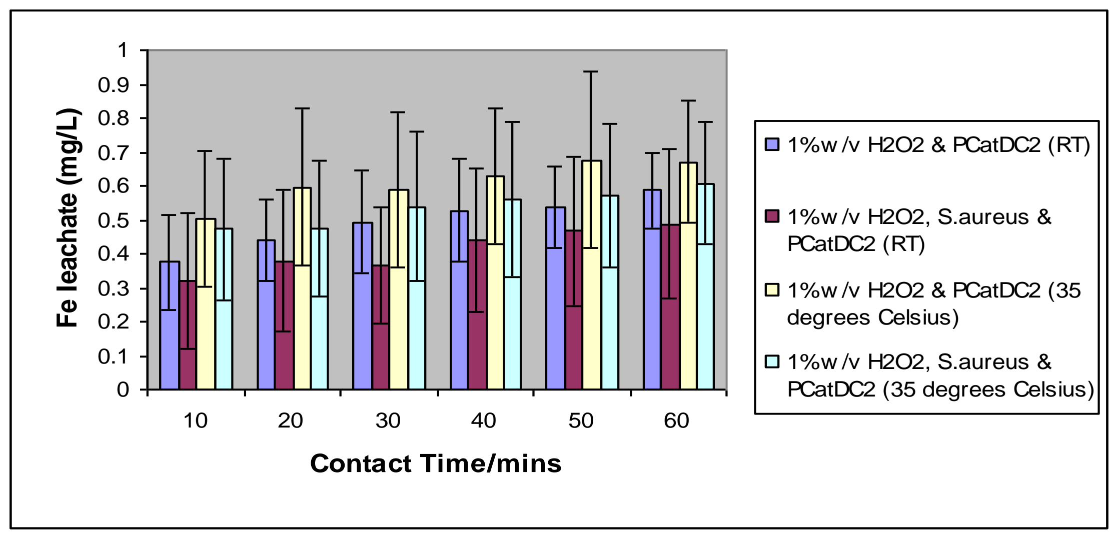


Figure 3.5: Amount of iron leaching from reused 1g modified PAN catalyst (PCatDC2) with 1% w/v H_2O_2 in presence or absence of *S. aureus* at room temperature and 35^oC.
PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2); RT = room temperature

Table 3.4: The levels of iron leaching from reused modified PAN catalyst (PCatDC2) in 1% w/v H_2O_2 with or without *S. aureus* at room temperature.

Contact time/min	Mean Iron mg/L(± S.D)			
	1st use PCatDC2 Alone	2nd use PCatDC2 alone	1st use PCatDC2 & *S. aureus*	2nd use PCatDC2 & *S. aureusi*
10	0.180(0.03)	0.377(0.14)	0.149(0.11)	0.320(0.20)
20	0.216(0.03)	0.439(0.12)	0.163(0.10)	0.379(0.21)
30	0.289(0.01)	0.494(0.15)	0.188(0.10)	0.487(0.17)
40	0.338(0.03)	0.528(0.15)	0.255(0.10)	0.441(0.21)
50	0.345(0.04)	0.538(0.12)	0.257(0.12)	0.468(0.22)
60	0.456(0.08)	0.587(0.11)	0.292(0.14)	0.486(0.22)

S.D. = standard deviation; PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2)

Table 3.5: The levels of iron leaching from reused modified PAN catalyst (PCatDC2) in 1% w/v H_2O_2 with or without *S. aureus* at 35^oC.

Contact time/min	Mean Iron mg/L(± S.D)			
	1st use PCatDC2 Alone	2nd use PCatDC2 alone	1st use PCatDC2 & *S. aureus*	2nd use PCatDC2 & *S. aureus*
10	0.532(0.09)	0.505(0.20)	0.443(0.09)	0.472(0.21)
20	0.629(0.15)	0.597(0.23)	0.491(0.14)	0.476(0.20)
30	0.697(0.19)	0.590(0.23)	0.559(0.16)	0.540(0.22)
40	0.791(0.20)	0.629(0.20)	0.651(0.14)	0.561(0.23)
50	0.858(0.21)	0.677(0.26)	0.703(0.13)	0.571(0.21)
60	0.913(0.24)	0.669(0.18)	0.780(0.19)	0.608(0.18)

S.D. = standard deviation; PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2)

3.3.3. Differences in iron leaching between modified PAN catalyst batches.

The differences of the amount of iron leaching between batches of modified PAN catalyst (PCatDC2, PCatDC3 and PCatDS) was examined and compared. (see Table 2.3. for differences in modified PAN catalysts). Figure 3.6 shows the differences in the amount of iron leaching from PCatDC2 or PCatDC3 or PCatDS in 1% w/v H_2O_2 at room temperature. The ferric chloride impregnated PAN catalysts (PCatDC2 & PCatDC3) leached a higher percentage of their total (0.5%-2.5%) iron content than the ferric sulfate impregnated PAN catalyst (0.2%-0.3%) (PCatDS) , but the total amount of iron released by PCatDC2 and PCatDS was similar (Figure 3.6). The amount of iron leached over the course of the experiment varied greatly between the ferric chloride impregnated PAN catalysts. PCatDC3 leached significantly ($p<0.01$), at 10-60mins contact time, more iron than PCatDC2. Similar significant increases ($p<0.01$) of iron leached were seen with PCatDC3 compared to PCatDS at the same time points. PCatDC3 leached the highest percentage of total iron present (2%-2.5%), followed by PCatDC2 (0.5%-1.3%), with PCatDS showing the least % leaching (0.2%-0.3%). See Table 3.1. for total amount of iron on modified PAN catalyst mesh.

These differences of iron leaching rate between batches may support the explanation provided earlier on, that there is uneven distribution of iron on the modified PAN mesh. Perhaps the modification and impregnation processes of the PAN mesh may have contributed to variations in iron leachate levels between batches. It is possible that the iron may not have been held well by the ligands in some batches and therefore these batches may release iron into solution in the presence of H_2O_2 much more than others whose ligands are able to hold iron much firmly. It is also possible that poor iron impregnation may have ensued, giving rise to an uneven distribution, as already mentioned (see Table 2.3 for amount of iron on modified PAN catalysts). For example, there may be more iron accumulation on one batch, but less iron accumulation on another batch. Also, there could be more iron accumulation on some sections on a particularly batch, but less iron on other

sections of the same batch. Such variations could lead to differences in amount of iron leaching between and within batches of the PAN catalysts.

As pointed out above, the ferric chloride based catalyst PCatDC3 leached significantly more than the ferric sulfate based catalyst. Interestingly, antimicrobial activity shown in Chapter 2, Section 2.4.6. did not indicate much of a difference in the activity between the different batches of the modified PAN catalysts, even though the ferric sulfate PAN catalyst (PCatDS) had more iron impregnated than the ferric chloride catalysts (PCatDC2, PCatDC3). This may suggest that irrespective of the amount of iron leached from the respective PAN catalysts at 60mins contact time, antimicrobial activity of the different batches of PAN catalysts may not be affected. This is possibly because, the amount of iron that is left on the PAN mesh after leaching may be still sufficient to induce bacterial death. However, the iron leached into solution may not be irrelevant but rather iron leached into solution may have a homogeneous contribution in antimicrobial activity. It is also possible that leachate contained other compounds as well as iron, that could be contributing to the antimicrobial activity and this needs to be investigated further. The antimicrobial activity of leachate with H_2O_2 is examined in Section 3.3.4.

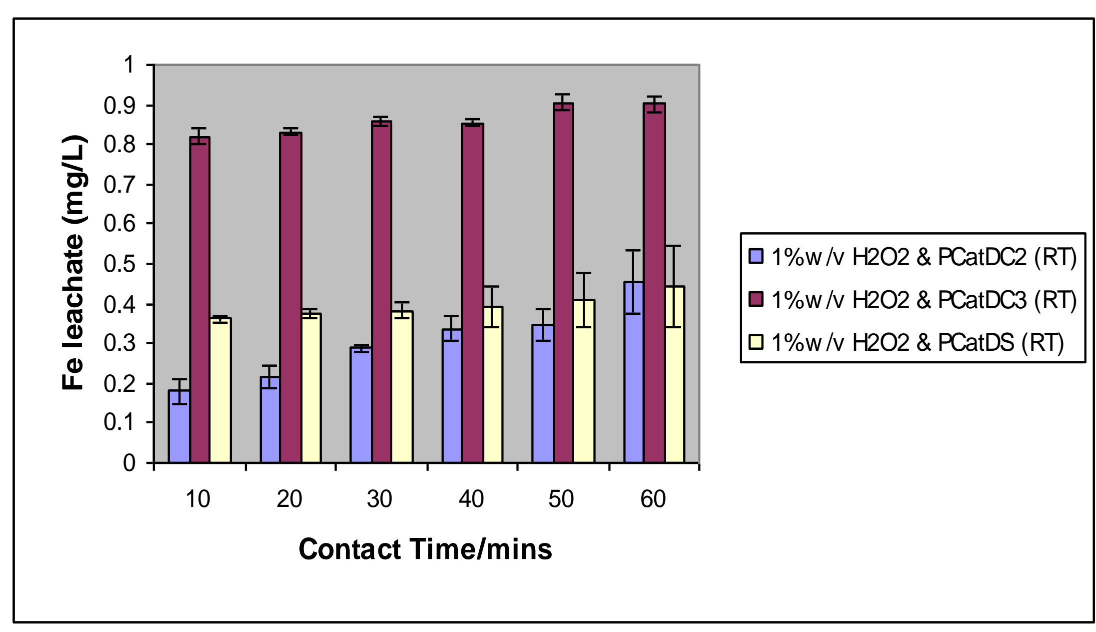


Figure 3.6: Comparative leached iron concentrations from different batches of 1g modified PAN catalysts (PCatDC2, PCatDC3 and PCatDS) with 1% w/v H_2O_2 at room temperature.

PCatDC2 & PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batches 2 & 3); PCatDS = ferric sulfate form of PAN catalyst (Dralon-L type); RT = room temperature.

3.3.4. Antibacterial effects of leachate from modified PAN catalyst in H_2O_2 solution.

The antibacterial effects of leachate from ferric chloride (PCatDC2) or ferric sulfate (PCatDS) impregnated modified PAN catalysts in 1% w/v H_2O_2 was tested and compared with the antibacterial activity of the known amount of iron in the leachate against *S. aureus* at room temperature. Tables 3.6. and 3.7. summarise the results of the antibacterial activities of PCatDC2 and PCatDS leachate respectively.

The PAN catalyst (PCatDC2 or PCatDS) and leachate from PAN catalyst (PCatDC2 or PCatDS) in 1% w/v H_2O_2 showed similar antibacterial activity against *S. aureus* at room temperature with no significant difference in activity ($p>0.05$) at all time points of the experiment (Table 3.6). However, PCatDS demonstrated a significant increase ($p<0.01$) in activity within 10mins, but no significant difference after 20mins time point as compared to the leachate of PCatDS (Table 3.7).

An equivalent amount of iron to that found in the leachate solution (ferric chloride 0.456ppm [Fe] or ferric sulfate 0.443ppm [Fe]) was tested in the homogeneous state and did not produce the same effect as the leachate solution. The antibacterial effect of the homogeneous iron solution was less active than that observed in 1% w/v H_2O_2 leachate solution. The leachate solution from PCatDC2 exhibited significant increased activity from 10-30mins compared to the homogeneous iron solution (0.456ppm) (Table 3.6); equally, the leachate of PCatDS showed significantly increased activity from 10-40mins compared to the activity of a solution containing 0.443ppm homogeneous iron (Table 3.7). One percent (w/v) H_2O_2 alone was less active than both leachate and homogeneous iron solutions respectively (Tables 3.6 & 3.7). Leachate from reused modified PAN catalyst (PCatDS) showed increased antimicrobial activity compared to the control 1% w/v H_2O_2 as well as homogeneous iron catalyst, but slightly less activity than when it was first used. The leachate from PCatDS when first used showed significantly increased ($p<0.05$) antimicrobial from 10-20mins compared to leachate of reused PCatDS (Table 3.7).

The faster antibacterial activities of leachate from the PAN catalysts, compared to the activity of known amounts of iron in solution, appeared to suggest that there could be a possible chemical change when iron is impregnated to the modified PAN mesh. Hence, the modification of the PAN mesh with hydroxylamines, hydrazines, NaOH and subsequent impregnation of iron salts may possibly lead to the formation of new compounds that possessed the potent antibacterial activity shown (Tables 3.6. & 3.7). Perhaps these compounds are more active in the presence of H_2O_2 than its absence, although the present work did not undertake the antimicrobial activity of leachate in water (without H_2O_2). Such work is warranted for further studies.

It has been established that the main functional groups formed after the reaction of PAN with hydroxylamines and hydrazines are oximes, amides, imidines, hydrazides and carboxylate groups. These groups possess lone pair electrons and as a result, they all have the ability to ligate iron (Chi, 2008). It is possible to consider that the antibacterial activity of leached PAN catalyst may be due to a complex of iron and a nitrogen containing ligand. A number of new antimicrobial compounds have been developed from hydrazines and hydroxylamines. For example, a synthesised amphipathic derivative of 1-isonicotinyl-2-palmitoyl hydrazine used in association with *m*-fluorophenylalanine has bactericidal activity against *Mycobacterium avium* (Rastogi and Goh, 1990). The reaction of hydrazine with other compounds such as new 3,4-dihydropyrimidine derivatives has resulted in antimicrobial activity against a number of microbes including *Mycobacterium tuberculosis* (Modha *et al.,* 2001). Also, various hydrazines have been utilised in combination with 1*H*-pyrazole-3-carboxylic acids to form new pyrazolo[3,4-*d*]pyridazin, which showed antimicrobial activity against Gram-negative and Gram-positive bacteria, and fungi (Akbas and Berber, 2005). The preparation of pyrazolylhydrazonomalononitriles that were reacted with hydrazine monohydrate yielded 10 novel heterocyclic disazo dyes and these compounds exhibited antimicrobial activity (Karci *et al.,* 2009). Prepared compound 1-(1-benzofuran-2-yl)-2-mesitylethanone was reacted with hydroxylamine to yield 1-(1-benzofuran-2-yl)-2-mesitylethanoneoxime and 1-(1-benzofuran-2-yl)-2-mesitylethanonesemicarbazone, from which other derivatives obtained exhibited antimicrobial activity against *S. aureus* ATCC 6538, *E. coli* ATCC 25922, and *Candida*

albicans ATCC 10231 (Kirilmis *et al.,* 2008). In another work, nafimidone oxime was synthesised by the reaction of nafimidone and hydroxylamine hydrochloride, most of the derivatives showed anticonvulsant and antimicrobial activities (Karakurt *et al.,* 2001).

As shown from the literature above, hydrazines and hydroxylamines compounds can react with other compounds to form novel antimicrobial agents, hence, it would be appropriate to suggest that the antimicrobial activity of the leachate from the PAN catalysts may be as a result of novel antimicrobial compounds formed from the compounds involved in the PAN catalyst preparation. These new antimicrobial compounds may involve iron because the control antimicrobial tests involving modified PAN mesh (modified PAN mesh without iron impregnation) (Tables 2.14 and 2.16) with or without H_2O_2 showed little antimicrobial activity compared to when PAN catalyst was used (Tables 2.10, 2.11, 2.12, 2.13, 2.17). The increased antibacterial activity of the leachate was synergised or influenced by the presence of H_2O_2. This is because when one compares the antibacterial activity of PAN catalyst in the absence of H_2O_2 and the activity of the leachate with H_2O_2, it can be seen that although the former showed some antibacterial activity, the activity was not as high as the latter case (compare Tables 2.17., 3.6. and 3.7). It is therefore appropriate at this stage to assume that the antimicrobial activity of the PAN catalyst, with or without H_2O_2 may possess more than one mode of action. Although, $^{\cdot}OH$ is implicated as one component of the mode of action due to the reaction of iron salts and H_2O_2, the activity of the leachate from the modified PAN catalysts suggest there may be other additional modes of action in the experimental reaction. It is envisaged that a full analysis of the components of the leachate including speciation of any iron complexes within the leachate could help to identify these new antimicrobial products.

The comparably reduced antibacterial activity of leachate of reused PCatDS (Table 3.7) may be due to less leached compounds from the PAN catalyst, perhaps as a result of more compounds leached out during first use. This is supported by the iron leaching work, which demonstrated that generally iron leaches less on reuse of the PAN catalyst (see Tables 3.2, 3.3 & 3.5). The antibacterial activity tests using reused PAN catalysts also support this idea (Chapter 2, Section 2.4.6), as they showed less antibacterial activity after first use. However, the leachate of reused PCatDS showed much higher antimicrobial activity than

when H_2O_2 was used alone and much more than homogeneous iron catalyst with an equivalent level of iron (Table 3.7). This further demonstrates the antimicrobial potency of the leachate from the PAN catalysts.

Table 3.6: Antibacterial effects of modified PAN catalyst (PCatDC2) leachate in H_2O_2 solution against *S. aureus* ATCC 6538 at room temperature.

Contact time/min	Mean Log_{10} reduction (± S.D.)			
	1% w/v H_2O_2 with PCatDC2 pH 3.60	Leachate from PCatDC2 in 1% w/v H_2O_2 pH 3.40	1% w/v H_2O_2 with 0.456ppm HmCl pH 4.03	1% w/v H_2O_2 pH 5.98
10	1.95(1.00)	2.31(0.45)	0.82(0.22)	0.43(0.23)
20	>5.30(0.00)	>5.30(0.00)	2.37(0.34)	1.21(0.68)
30	>5.30(0.00)	>5.30(0.00)	4.22(0.57)	2.25(0.44)
40	>5.30(0.00)	>5.30(0.00)	4.70(0.1)	3.92(0.43)
50	>5.30(0.00)	>5.30(0.00)	>5.36(0.00)	4.57(0.51)
60	>5.30(0.00)	>5.30(0.00)	>5.36(0.00)	5.10(0.35)

S.D. = standard deviation; PCatDC2 = ferric chloride form PAN catalyst (Dralon-L type batch 2);
HmCl = homogeneous ferric chloride catalyst

Table 3.7: Antibacterial effects of modified PAN catalyst (PCatDS) leachate in H_2O_2 solution against *S. aureus* ATCC 6538 at room temperature.

Contact time (min)	Mean Log_{10} reduction (± S.D.)				
	1% w/v H_2O_2 PCatDS pH 4.48	Leachate from PCatDS in 1% w/v H_2O_2 pH 4.23	1% w/v H_2O_2 with 0.443ppm HmFST pH 4.24	Leachate from reused PCatDS in 1% w/v H_2O_2 pH 3.89	1% w/v H_2O_2 pH 6.02
10	>5.41(0.00)	1.50(0.07)	0.83(0.08)	0.77(0.26)	0.56(0.03)
20	>5.41(0.00)	>5.36(0.00)	1.85(0.04)	3.19(0.37)	0.89(0.15)
30	>5.41(0.00)	>5.36(0.00)	3.31(0.10)	≥5.26(0.17)	2.67(0.09)
40	>5.41(0.00)	>5.36(0.00)	4.44(0.57)	>5.36(0.00)	3.82(0.80)
50	>5.41(0.00)	>5.36(0.00)	≥5.16(0.35)	>5.36(0.00)	4.61(0.64)
60	>5.41(0.00)	>5.36(0.00)	>5.36(0.00)	>5.36(0.00)	4.91(0.79)

S.D. = standard deviation; PCatDS = ferric sulfate form of PAN catalyst (Dralon-L type); HmFST = homogeneous ferric sulfate catalyst.

3.4. Conclusion

The present study has demonstrated that iron leaches from the modified PAN catalysts to different extents. It was found that iron leaches less at room temperature than at 35^oC, and also iron leaches more at higher H_2O_2 concentrations than at lower concentrations. The presence of microbial load in the reaction was found to reduce the concentration of iron in solution. The extent that iron leaches is dependent on contact time, the temperature, the absence or presence of H_2O_2 or microbial load.
It was found that less iron leaches from the modified PAN catalyst on subsequent reuse. Iron leaching was seen to vary between batches of the catalyst, however, ferric chloride impregnated PAN catalyst PCatDC3 (produced at De Montfort University) appeared to leach more than the ferric sulfate impregnated PAN catalyst (that was commercially produced).

It was found that the leachate from the modified PAN catalysts exhibited the same antibacterial activity as normal modified PAN catalysts at room temperature. In comparison, the homogeneous phase Fentons catalyst using the same amount of iron as found in the leachate, showed much less antibacterial activity. These outcomes, suggest that there is a possible chemical change when iron is impregnated onto the modified PAN mesh or that leached iron reacts with other compounds present in the leachate (possibly hydrazine, hydroxylamine, ammonia) resulting in the formation of a novel antimicrobial product in the leachate. Finally, it has been demonstrated that the leachate from the reused PAN catalyst was less active than the leachate from the PAN catalyst when it was first used. However, it was still more active than H_2O_2 alone or homogeneous catalysts with the same amount of iron.

CHAPTER 4

Antimicrobial Action of Hydroxyl Radicals Produced in the Decomposition of Hydrogen Peroxide by the Novel Heterogeneous Modified PAN Catalyst.

4.1. Introduction

Free radicals such as nitric oxide ($NO^{\cdot}$), superoxide ($O_2^{\cdot-}$), and hydroxyl radical ($^{\cdot}OH$) are molecular fragments that possess one or more unpaired electrons in atomic or molecular orbits resulting in their considerable reactivity (Kirschvink *et al.*, 2008). As extensively discussed from preceding chapters, $^{\cdot}OH$ radicals are generated whenever hydrogen peroxide (H_2O_2) comes into contact with copper ions (Cu^{+}) or iron ions (Fe^{2+} or Fe^{3+}) (Halliwell, 1995; Bergendi *et al.,* 1999; Urbanski and Beresewicz, 2000). The $^{\cdot}OH$ radical is known to be the most reactive oxygen radical among all the oxygen-centred radical species in chemistry and biology (Cheng *et al.,* 2003). It has tremendous potential for causing biological damage and is assumed to be involved in a number of pathological processes (Halliwell, 1995; Vidrio *et al.,* 2008; Okuno *et al.,* 2001). Due to the importance of the $^{\cdot}OH$ radical, its identification and quantification have been intensively studied. Electron spin resonance (ESR)-spin trapping method has been extensively employed because of its sensitivity and selectivity in the detection of the $^{\cdot}OH$ radical (Cheng *et al.,* 2003; Shi *et al.,* 2005; Zhou *et al.,* 2006; Taiwo *et al.,* 2006; Danilczuk *et al.,* 2007; Kim and Metcalfe, 2007). The technique is able to detect and identify molecules consisting of unpaired electrons (paramagnetic species) such as free radicals, transition metal ions and defects in materials (Robert *et al.,* 2002; Kim and Metcalfe, 2007). Most free radicals, particularly in aqueous conditions, are extremely reactive with a very short lifetime making it very difficult for their direct detection. For example the half-life of $^{\cdot}OH$ radical and $O_2^{\cdot-}$ is of the order of a few nanoseconds and tenths of milliseconds respectively (Table 1.10) (Bergendi *et al.,* 1999; Bacic and Mojovic, 2005). Therefore direct ESR measurements of free radicals in aqueous conditions are practically impossible to achieve. Spin-trap agents such as nitrone and nitroso compounds have been employed to overcome this limitation by rapidly reacting with free radicals in aqueous solutions to produce a relatively long-lived spin adduct nitroxide radical ($R_2N\text{-}O^{\cdot}$) that can then be easily detected by ESR at room temperature (Kim and Metcalfe, 2007).

The present study examined the generation of $^{\cdot}OH$ radicals by the reaction of modified PAN catalyst with H_2O_2 as well as its antimicrobial action. The spin-trap compound 5,5-dimethy-1-pyrroline-N-oxide (DMPO) was employed to assess the extent of the production of $^{\cdot}OH$ radicals. DMPO was chosen because of its high stability and water solubility, the robustness of its spin adducts as well as its simple spectrum yielding benefits in terms of signal versus noise ratio leading to more reliable results (Cheng *et al.,* 2003; Bacic and Mojovic, 2005; Kim and Metcalfe, 2007). This chapter also investigates the rate of H_2O_2 decomposition by the heterogeneous modified PAN catalyst as a function of time.

4.2. Materials and Methods

4.2.1. Materials

Chelex® 100, DMPO (stored as frozen), and activated charcoal were purchased from Sigma-Aldrich (UK). Bacterial culture media, H_2O_2 and catalase from bovine liver were as described in Chapter 2, Sections 2.3.1.1. and 2.3.1.2. Dr Lange round cuvette (LCW 906) and LCW 058A reagent (stored between 2-8°C) purchased from Hach Lange Ltd (Manchester, UK) were used to determine H_2O_2 concentration.

4.2.1.2. Equipment and its uses

Bruker EMX 6/1 EPR spectrometer (see Appendix 5) was used to record ESR spectra of $^{\cdot}OH$ radicals. DR 3800 SC Vis Spectrophotometer (Hach Lange Ltd, Manchester, UK) (see Appendix 6) was used to determine H_2O_2 concentration. Thoma counting chamber (1/400mm^2, depth 0.02mm); (Hawksley, Sussex, UK) was used to assess total cell count.

4.2.2. Methods

4.2.2.1. Removal of trace elements from distilled water

Chelex 100 was used as an ion-exchange resin to remove trace elements from distilled water (Corsini *et al.,* 1987) and was used for all preparations in this $^{\cdot}OH$ radical study. About 5-10g of the Chelex was placed in a 50mL column held firmly by a clamp and distilled water was poured through the column eluting over the chelex with the result that trace elements such as iron were adsorbed onto the chelex. Maximum care was taken to prevent any untreated/unchelated water from contaminating the treated water. The water purity was confirmed by conductivity tests on the pre-treated (average conductivity = 44mV) and post-treated (average conductivity was less than 0) water.

4.2.2.2. Purification of spin trap DMPO

$^{\cdot}OH$ radicals were analysed as spin adducts of DMPO. Prior to use, DMPO was purified with activated charcoal. DMPO was dissolved in purified water (described above) and activated charcoal was added and mixed well by shaking. The mixed solution was filtered through a Whatman syringe filter pore size 0.45μm to obtain a clear purified DMPO solution and stored frozen as 250 μL aliquots until needed. The concentration of the purified DMPO solution was determined by UV-VIS spectrophotometer at $\lambda = 255$ using a molar extinction coefficient for DMPO of $\varepsilon_{234} = 7700\ M^{-1}cm^{-1}$ in water (Qui *et. al.,* 1994; Taiwo *et. al.,* 2006). Concentration of DMPO stock solution after purification was 60.4 mM.

4.2.2.3. Determination and quantification by ESR of $^{\cdot}OH$ radicals

$^{\cdot}OH$ radicals spectra were recorded on Bruker EMX 6/1 EPR spectrometer with 100 kHz modulation at room temperature. ESR spectrometer settings were as follows: modulation amplitude, 2.0 G; modulation frequency, 100 kHz; sweep width, 100 G; time constant, 10.24 ms; field set, 3492 G; sweep time, 83.89 s; microwave frequency, 9.79 GHz; microwave power 10 mW. The concentration of DMPO prepared from the stock solution for all the tests was 20 mM. These conditions provided a stable ESR signal for all the tests. An arbitrary weight of 0.02g of the modified PAN catalysts as mesh (PCatDC3 = ferric chloride impregnated or PCatDS = ferric sulfate impregnated), modified PAN mesh (chemically modified without iron impregnation), and non-modified mesh (raw PAN mesh without any modification) were used. Concentrations of H_2O_2 used were 0.2% w/v and 0.1% w/v. Control and experimental total volume was 500 μL. Stock solutions of DMPO or H_2O_2 were kept on ice during the experiments. Experimental conditions were as follows:

Controls:

(1). **Hydrogen peroxide alone:** A solution of 100μL volume was taken from 1% w/v H_2O_2 stock solution and added to 170μL volume of DMPO solution (stock solution 60.4mM) and 230μL of purified distilled water making up a total volume of 500μL in a 1.5mL microcentrifuge tube with cap. This mixture representing 0.2% w/v H_2O_2 and 20mM

DMPO and was incubated at room temperature for 10mins. The reaction mixture was then rapidly transferred into an ESR quartz flat cell for ESR detection.

(2). **Hydrogen peroxide with either modified PAN mesh (MOPM) or non-modified PAN mesh (NMPM):** The solution containing 0.2% w/v H_2O_2 with 20mM DMPO was first prepared as above in (1), followed by the addition of either 0.02g of MOPM or NMPM respectively and incubated for 10mins at room temperature. The mesh was removed and the solution of the reaction mixture was then rapidly transferred into an ESR quartz flat cell for ESR detection.

Experiments:

(1). **Hydrogen peroxide with modified PAN catalysts (PCatDC3 or PCatDS):** Solutions containing either 0.2% w/v or 0.1% w/v H_2O_2 with 20mM DMPO respectively were first prepared as above in control (1) and either 0.02g of PCatDC3 or PCatDS was added and mixed well by shaking and incubated for 10mins. The reaction mixture was shaken at regular intervals. After 10mins reaction time, ESR detection was performed and $^{\cdot}OH$ radical spectra were recorded.

Note: Total experimental volume and constituents were the same as described above for control in (1) but for 0.1% w/v H_2O_2, 50μL was added from 1% w/v H_2O_2 stock solution to give the final experimental concentration of 0.1% w/v H_2O_2.

(2). **Modified PAN catalyst in contact with hydrogen peroxide for 1hr:** An experiment was performed to establish whether the heterogeneous modified PAN catalyst was able to generate $^{\cdot}OH$ radicals after being in contact with H_2O_2 for more than 10mins. The experiment was performed as follows: 0.02g PCatDC3 was left in contact with H_2O_2 in the absence of DMPO (solution contained 100μL from 1% w/v H_2O_2 stock solution and 230μL purified water) for 1 hour at room temperature. After 1 hour, 170μL of DMPO stock solution (resultant concentration 20mM) was added making a final H_2O_2 concentration of 0.2% w/v, and the mixture was incubated at room temperature for 10mins, followed by ESR detection and $^{\cdot}OH$ radical spectra were recorded.

The following tests were performed to examine whether the leachate from the modified PAN catalyst was capable of generating $^{\cdot}OH$ radicals:

(3). **Leachate of modified PAN catalyst left in contact with hydrogen peroxide for 1hr:** 0.02g of PCatDC3 was left in 100µL of H_2O_2 (from 1% w/v H_2O_2 stock solution) and 230µL purified distilled water for 1 hour. The PCatDC3 was then taken out, followed by an addition of 170µL of DMPO stock resulting in a final concentration of 20mM and final H_2O_2 concentration of 0.2% w/v H_2O_2. The solution was left for 10mins, followed by ESR detection and $^{\cdot}OH$ radical spectra were recorded

(4). **Leachate from modified PAN catalyst left in contact with water only:** 0.02g of either PCatDC3 or PCatDS was left in 500µL of purified distilled water for 10mins. An aliquot of 230µL of this solution was added to a solution mixture of 0.2% w/v H_2O_2 (100µL from 1% w/v H_2O_2 stock solution) and 20mM of DMPO (170µL volume of DMPO 60.4mM stock solution) making up a total volume of 500µL and incubated at room temperature for 10mins, followed by ESR detection and $^{\cdot}OH$ radical spectra were recorded.

(5).**Leachate from modified PAN catalyst left in contact with hydrogen peroxide for 24hr**: 0.02g of either PCatDC3 or PCatDS was left in 100µL of H_2O_2 (from 1% w/v H_2O_2 stock solution) and 230µL purified distilled water for 24 hours. The catalysts were then taken out from the reaction and 170µL volume of DMPO solution from 60.4mM stock solution added. The reaction was then incubated at room temperature for 10mins, followed by ESR detection and $^{\cdot}OH$ radicals spectra were recorded.

Processing of ESR spectra: Bruker WIN-EPR version: 921201 software was used to process all ESR spectra. The true spectra of ˙OH radicals were obtained by subtracting control (baseline) test (spectra produced from H_2O_2 and DMPO) from spectra of experiments (spectra produced from reaction of modified PAN catalyst, H_2O_2 and DMPO). DMPO-˙OH intensity (EPR intensity) values (for quantifying ˙OH radicals) recorded in arbitrary units were obtained using the 'view' option which provides information about the X-axis, Y-axis, and intensity coordination. Microsoft excel was used to plot graphs and to calculate averages and standard deviations to determine the differences in EPR intensities between experiments. Each experiment was performed at least three times.

4.2.2.4. Total cell count as a means of qualitative assessment of antimicrobial mode of action

Total cell number (live or dead) cells were evaluated after each contact time in the presence of PCatDC3 with either 0.5% w/v or 1% w/v H_2O_2 against *Ps. aeruginosa* or *S. aureus* at room temperature. The Procedure for bactericidal activity was as described in Chapter 2 Section 2.3.2.9. The Procedure for total cell count after neutralisation for a particular contact time; or initial bacterial number was as follows: Using a sterile disposable Pasteur pipette, a drop of neutralised experimental solution after contact time was placed into the centre of the round platform on the counting chamber and covered with a coverslip. Care was taken to make sure the drop filled the platform and the coverslip without overflowing into the trench on the slide. The coverslip was pressed gently with the thumb until Newton's rings were seen to have uniformly distributed over the area of contact. Phase contrast microscopy was used to count the cells by initially using X10 objective to focus on the grid engraved on the platform, and then finally switched to X40 phase objective to count the cells contained in five sets of 16 squares. The average number of cells/small square were calculated and used to determine the number of cells/mL of the original bacterial suspension (initial bacterial suspension before experiment) or in experiment respectively.

The following were the procedures used to calculate average number of cells/small square and number of cells/mL respectively:

Calculation for total cell count:

Each small square of the counting chamber = 1/400 sq. mm

Depth = 0.02mm

Therefore 0.0025 x 0.02 = 0.00005mm^3 (volume of each square)

0.00005 x 16 = 0.0008mm^3 (16 squares)

0.0008 x 5 = 0.004mm^3 (five sets of 16 squares)

So the total cell count is calculated either by dividing the average of the number of cells counted in each of the five sets of 16 squares by 0.0008mm^3 or divide the number of cells counted from five sets of 16 squares by 0.004mm^3.

The above calculation will only give cells per mm-cubed so to calculate cells per mL, there are 1000 cubic millimeters in one cubic centimeter (same as a millilitre) so cells per mm-cubed is multiplied by 1000 to obtain cells/mL taken into account the dilution factors used.

4.2.2.5. The effect of inoculum on the catalytic decomposition of H_2O_2

The following were the experimental parameters and conditions (total experimental volume was 75mL as described in Chapter 2, Section 2.3.2.8. and 2.3.2.9).

(1). 0.2% w/v H_2O_2 with or without 1g PCatDS or *E. coli* at room temperature or 35^oC at different contact times.

(2). 1% w/v H_2O_2 with or without 1g PCatDS or *S. aureus* at room temperature or 35^oC at different contact times.

(3). 0.2% w/v H_2O_2 with or without 1g PCatDC3 or *E. coli* at room temperature alone at different contact times.

(4a). 13mL of 200ppm stock solution of homogeneous ferric chloride ($FeCl_3.6\ H_2O$) was added to a mixture of 0.45mL of 33.3% w/v H_2O_2 stock solution and 61.55mL sterile distilled water making up total experimental volume of 75mL containing 0.2% w/v H_2O_2 and 35ppm $FeCl_3.6\ H_2O$ and left at room temperature for 24 hrs followed by analysis of H_2O_2 concentration.

(4b) 45mL of 200ppm stock solution of homogeneous ferric sulfate (($Fe_2(SO_4)_3$) was added to a mixture of 0.45mL of 33.3% w/v H_2O_2 stock solution and 29.55mL sterile distilled water making up total experimental volume of 75mL containing 0.2% w/v H_2O_2 and

120ppm ($Fe_2(SO_4)_3$ and left at room temperature for 24 hrs followed by analysis of H_2O_2 concentration.

Analysis for H_2O_2: The rate of decomposition of H_2O_2 was determined as a function of H_2O_2 concentration (0.2% w/v or 1% w/v H_2O_2), catalyst (PCatDS or PCatDC3, iron (III) chloride or iron (III) sulfate solution), temperature (room temperature or 35^oC), duration of contact (10mins to 60mins), and type of microorganism (*E. coli* or *S. aureus*).
The concentrations of H_2O_2 were assessed by adding 0.2mL of experimental solution to 5mL of reagent LCW 058A in a Dr Lange round cuvette (LCW 906). The cuvette was closed and inverted a few times and left for 2mins at room temperature. Test number 058 for H_2O_2 determination was selected on the DR 3800 SC VIS spectrophotometer and standard sample (provided by Hach Lange) was used to set the instrument to zero. This was followed by inserting the sample cuvette in the instrument and reading the results. The method used here is based on the principle that H_2O_2 and peracetic acid (PAA) react with molybdates to form yellow peroxomolybdates (Hach Lange, Manchester, UK). The reagent LCW 058 contained molybdates which reacts with H_2O_2 solution resulting in the formation of yellow peroxomolybdates, from which H_2O_2 concentration can be directly measured as described above.
H_2O_2 concentration range of the instrument is: 1-10g/L (0.1%-1% w/v).

4.2.2.6. Antibacterial effects of H_2O_2/modified PAN catalyst system and H_2O_2/homogeneous iron catalyst system after continuous generation of $^{\cdot}OH$ radicals for 24 hrs

Procedure for bactericidal activity was as described in Chapter 2 Sections 2.3.2.8 and 2.3.2.9.
Experimental parameters and conditions were as follows:
(1). **Antimicrobial effects of hydrogen peroxide at 0hrs (control):** 0.45mL of 33.3% w/v H_2O_2 stock solution was added to 67.05mL of sterile distilled water and 7.5mL *E. coli* suspension was added making up a total experimental volume of 75mL of 0.2% w/v H_2O_2 and bactericidal activity examined at time 0 hrs.

(2). **Antimicrobial effects of hydrogen peroxide at 24hrs (control):** 0.45mL of 33.3% w/v H_2O_2 stock solution was added to 67.05mL of sterile distilled water (making up 67.5mL volume) and left at room temperature for 24 hrs. After 24hrs, 7.5mL *E. coli* suspension was added making up a total experimental volume of 75mL of 0.2% w/v H_2O_2 and bactericidal activity examined.

(3). **Antimicrobial effects of hydrogen peroxide with modified PAN catalyst (PCatDS) at 0hrs (experiment):** 1g of PCatDS was added to a mixture containing 0.45mL of 33.3% w/v H_2O_2 stock solution, 67.05mL of sterile distilled water and 7.5mL *E. coli* suspension making up total experimental volume of 75mL of 0.2% w/v H_2O_2 and bactericidal activity examined at time 0 hrs.

(4). **Antimicrobial effects of hydrogen peroxide with modified PAN catalyst (PCatDS) added after 24hrs of H_2O_2 preparation (experiment):** 0.45mL of 33.3% w/v H_2O_2 stock solution was added to 67.05mL of sterile distilled water (volume of initial mixture 67.5mL) and left at room temperature for 24 hrs (as described in control 2). After 24 hrs, 1g of PCatDS was added followed by 7.5mL *E. coli* suspension making up a total experimental volume of 75mL of 0.2% w/v H_2O_2 and bactericidal activity examined.

(5). **Antimicrobial effects of hydrogen peroxide with modified PAN catalyst (PCatDS) after 24hrs of preparation (experiment):** 1g PCatDS was added to a mixture of 0.45mL of 33.3% w/v H_2O_2 stock solution and 67.05mL of sterile distilled water (volume of initial mixture 67.5mL) and left at room temperature for 24 hrs. After 24 hrs, 7.5mL *E. coli* suspension was added making up a total experimental volume of 75mL of 0.2% w/v H_2O_2 and bactericidal activity examined.

(6). **Antimicrobial effects of hydrogen peroxide with homogeneous ferric chloride at 0hrs:** 13mL of 200ppm stock solution of homogeneous ferric chloride ($FeCl_3.6\ H_2O$) was added to 0.45mL of 33.3% w/v H_2O_2 stock solution and 54.05mL sterile distilled water was then added, (volume of initial mixture 67.5mL) and 7.5mL *E. coli* suspension making up a total experimental volume of 75mL of 0.2% w/v H_2O_2 and 35ppm $FeCl_3.6\ H_2O$ (final concentrations), and bactericidal activity examined at time 0 hrs.

7). **Antimicrobial effects of hydrogen peroxide with homogeneous ferric chloride after 24hrs:** 13mL of 200ppm stock solution of homogeneous ferric chloride ($FeCl_3.6\ H_2O$) was

added to 0.45mL of 33.3% w/v H_2O_2 stock solution and 54.05mL sterile distilled water was then added, (volume of initial mixture 67.5mL) and left at room temperature for 24 hrs. After 24hrs, 7.5mL *E. coli* suspension was added making up a total experimental volume of 75mL of 0.2% w/v H_2O_2 and 35ppm $FeCl_3.6\ H_2O$ (final concentrations) and bactericidal activity examined.

Note: The concentrations of homogeneous iron salts used were the same as concentrations found when 1g of mesh was digested (see Table 3.1).

4.2.2.7. Statistical Analysis

The Student *t*-test was employed for all statistical analysis (using Microsoft Excel). To compare whether one type of modified PAN catalyst is more effective (e.g. either producing more $^{\cdot}OH$ radicals or decomposing H_2O_2 faster) than another type of catalyst, one tail test was used. Similarly, one tail test was used to evaluate statistical significance between 'the presence of microorganism and absence of microorganism' or 'condition at room temperature and at 35^oC'. Statistical analysis for antibacterial effects was the same as described in Chapter 2, Section 2.3.2.16.

4.3. Results and Discussions

4.3.1. Determination and quantification of $^{\cdot}OH$ radicals by ESR

4.3.1.1. Identification of $^{\cdot}OH$ radicals by ESR spectra in the presence of DMPO

The ESR spectra of DMPO in the presence of H_2O_2 or H_2O_2/modified PAN catalyst systems were obtained to identify the production of $^{\cdot}OH$ radicals. Figure 4.2A shows the spectra of 20mM DMPO in purified distilled water; Figure 4.2B shows the spectra of 20mM DMPO in 0.2% w/v H_2O_2; Figure 4.2C is 0.2% w/v H_2O_2 together with modified PAN catalyst (PCatDC3). Figures 4.3A and 4.3B show the reaction of 0.2% w/v H_2O_2, PCatDC3 and 20mM DMPO; and 0.1% w/v H_2O_2, PCatDC3 and 20mM DMPO respectively. Figures 4.4A and 4.4B show the spectra of the reaction of 0.2% w/v H_2O_2, modified PAN mesh (MOPM) and 20mM DMPO; and 0.2% w/v H_2O_2, non-modified PAN mesh (NMPM) and 20mM DMPO respectively. The spectra produced in Figures 4.2A, B, and C showed the absence of $^{\cdot}OH$ radicals. However, Figures 4.3A and B exhibited the spectra of $^{\cdot}OH$ radicals, and showed relative differences in $^{\cdot}OH$ radical production based on EPR signal intensity, thus, the signal decreased in intensity as the concentration of H_2O_2 was lowered. $^{\cdot}OH$ radicals were also detected in the presence of MOPM or NMPM with 0.2% w/v H_2O_2 respectively (Fig. 4.4A and B).

The data presented here strongly suggest that modified PAN catalysts (PCatDC3 = ferric chloride impregnated) are able to generate $^{\cdot}OH$ radicals in the presence of H_2O_2 (Fenton-like reaction, equation 2.1-2.4). This was shown by direct spin trapping of $^{\cdot}OH$ radicals by DMPO with characteristic ESR signals. Specific spin traps react with radicals at a double bond, resulting in a spin trap radical adduct, whose ESR signal is characteristic for identifying the original radical (Rohn and Kroh, 2005). The spin trap agent DMPO reacts with $^{\cdot}OH$ radicals to form DMPO-$^{\cdot}OH$ adduct (Han, 2004; Rohn and Kroh, 2005; Linxiang *et al.,* 2007):

DMPO + $^{\cdot}OH \rightarrow$ DMPO-$^{\cdot}OH$ **Equation 4.1**

The control tests involving the reactions of 'water and DMPO'; 'H_2O_2 and DMPO'; and 'H_2O_2 and PAN catalyst' (Figures 4.2A, 4.2B and 4.2C) displayed spectra not specific to the DMPO-$^{\cdot}OH$ adduct. However, the reaction involving 'H_2O_2, DMPO and PAN catalyst'

or ‘H_2O_2, DMPO and modified PAN mesh (MOPM) or non-modified PAN mesh (NMPM)’ produced typical DMPO-˙OH radical adduct spectra (Figures 4.3A and 4.3B; and 4.4A and 4.4B). These spectra are composed of a quartet with peak height ratio of 1:2:2:1 (Han, 2004) resulting from the splitting profile of DMPO-˙OH adduct (Figure 4.1).

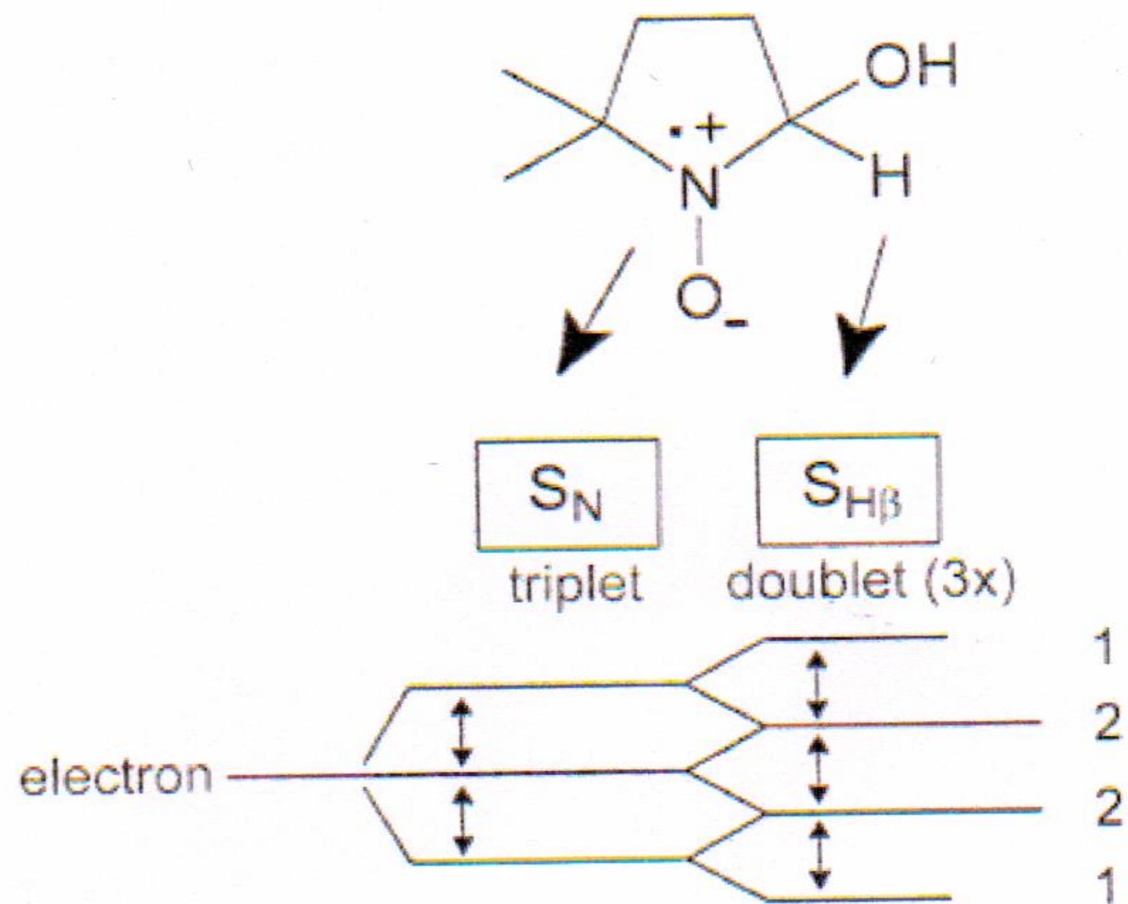


Figure 4.1: Splitting profile of DMPO-˙OH adducts. Triplet splitting of the signal caused by the nitrogen; further doublet splitting due to the interactions with the hydrogen atom at the β-site. As a result there is a four-line signal with an intensity pattern of 1:2:2:1. (This figure has been reproduced from ‘Electron spin resonance – A spectroscopic method for determining the antioxidant activity’, by Rohn, S. and Kroh, L.W. 2005, Molecular Nutrition & Food Nutrition, volume 49, pages 898-907. Copyright (2005), with permission from Wiley-VCH Verlag GmbH & Co.KGaA).

The presence of ˙OH radicals in the modified PAN mesh as well as the non-modified PAN mesh was not expected, as both materials were not impregnated with iron, it was therefore

surprising to observed that these materials contained $^{\cdot}OH$ radicals (Figures 4.4A and 4.4B). However, the intensities were decreased about fourfold (MOPM) and twentyfold (NMPM) from when the catalyst (PCatDC3) was present. The explanations for these observations are given at Section 4.3.1.2.

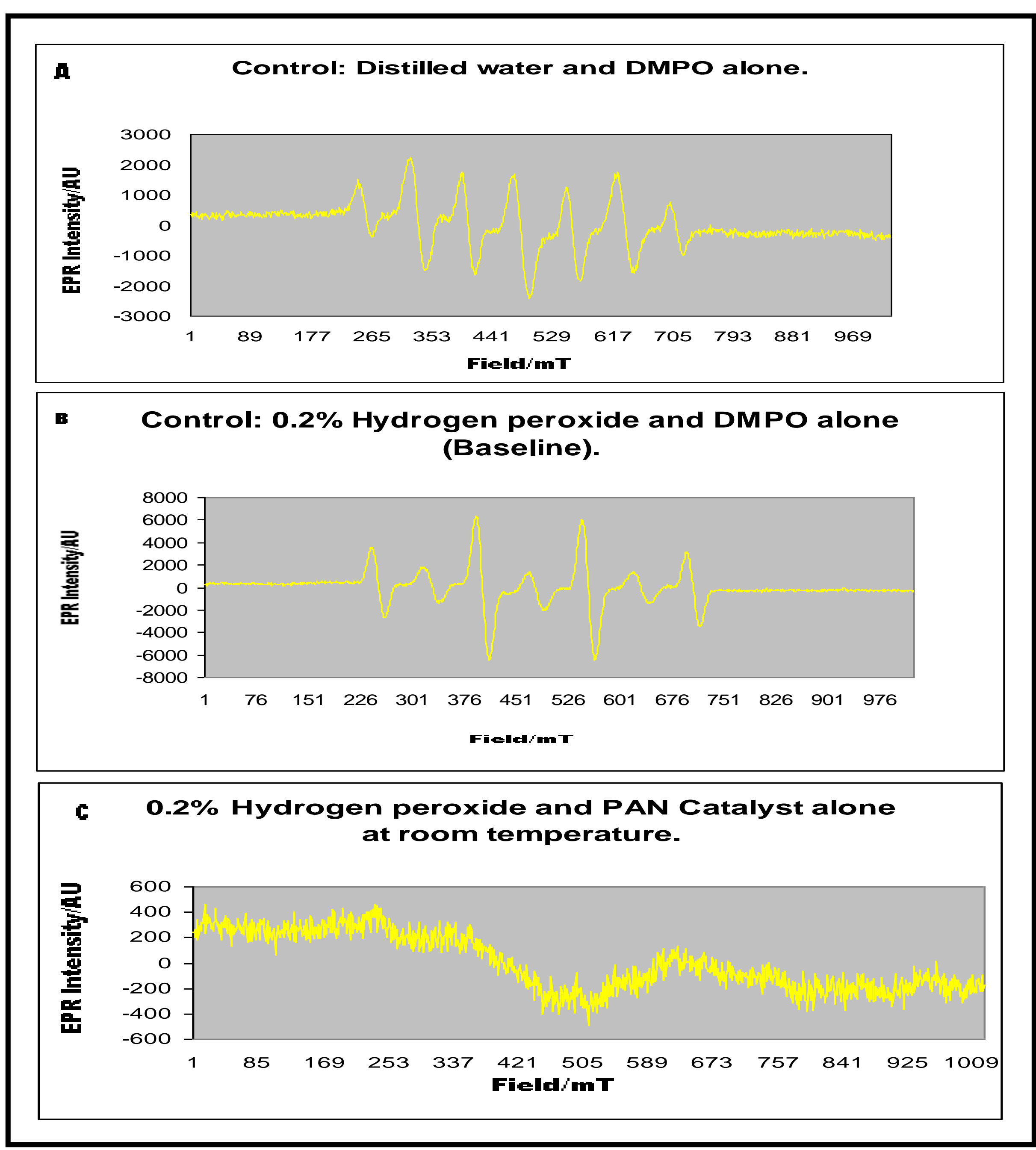


Figure 4.2: Controls: (A) Spectra of purified distilled water with DMPO only at room temperature; (B) Spectra of 0.2% w/v H_2O_2 and DMPO alone as baseline; (C) Spectra of 0.2% w/v H_2O_2 and modified PAN catalyst alone without DMPO at room temperature.
EPR = electron paramagnetic resonance; AU = arbitrary unit; mT = millitesla

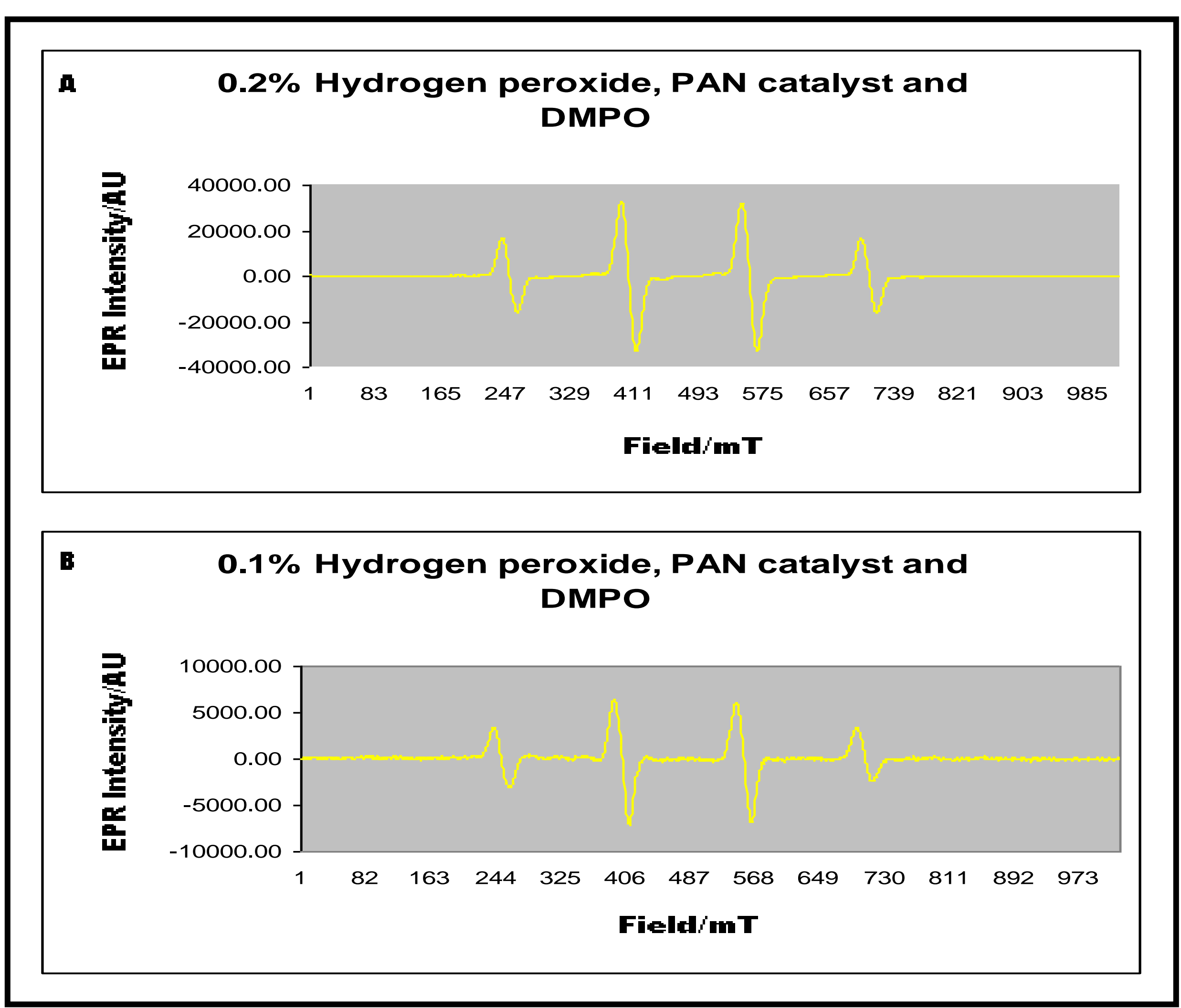


Figure 4.3: (A) Spectra showing formation of ˙OH radicals as a result of reaction mixture of 0.2% w/v H_2O_2, PCatDC3 and DMPO at room temperature; (B) Spectra showing formation of ˙OH radical as a result of reaction mixture of 0.1% w/v H_2O_2, PCatDC3 and DMPO at room temperature.

EPR = electron paramagnetic resonance; AU = arbitrary unit; mT = millitesla

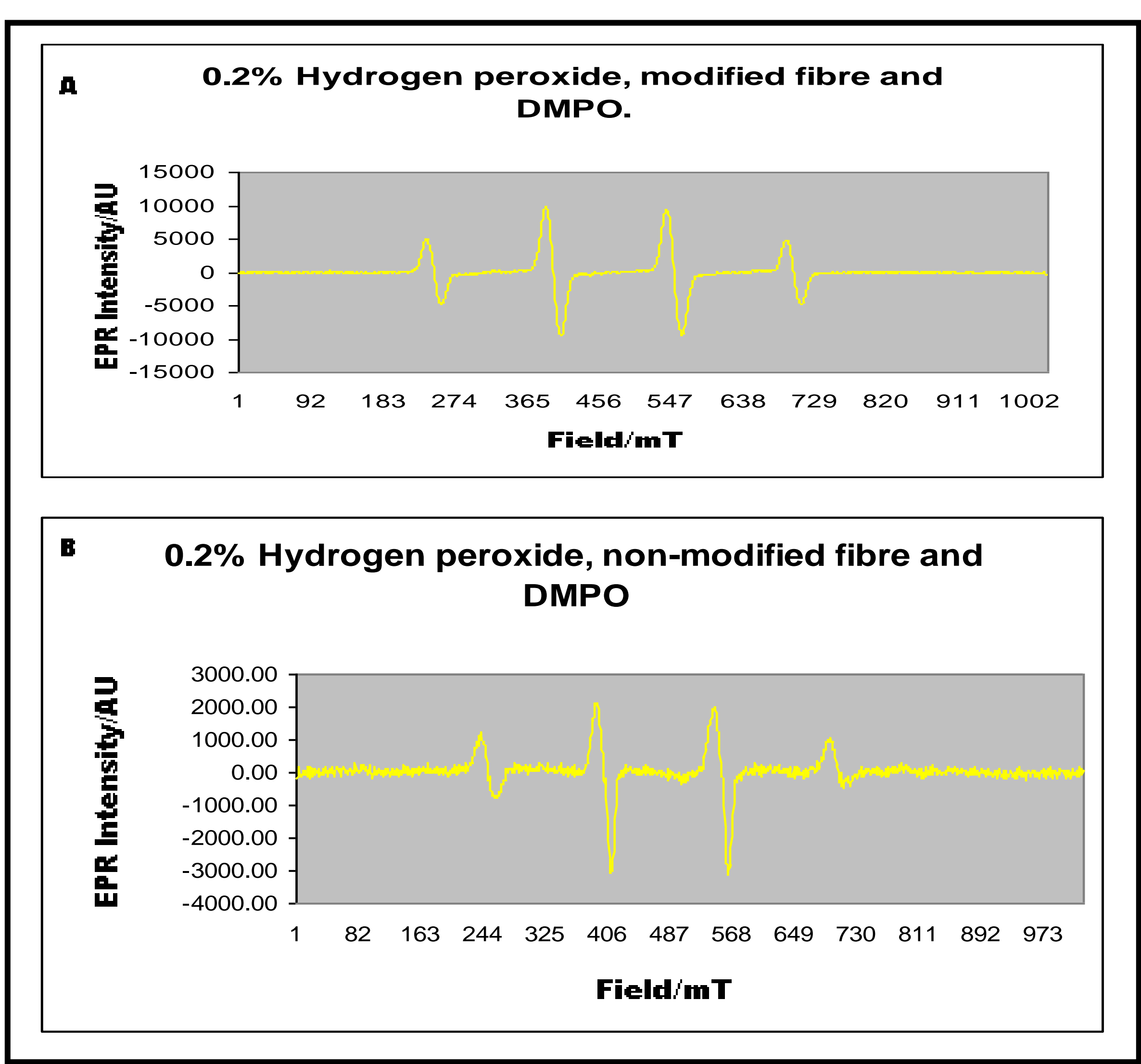


Figure 4.4: (A) Spectra showing formation of ˙OH radical as a result of reaction mixture of 0.2% w/v H_2O_2, modified PAN mesh (MOPM) and DMPO at room temperature; (B) Spectra showing formation of ˙OH radical as a result of reaction mixture of 0.2% w/v H_2O_2, non-modified PAN mesh (NMPM) and DMPO at room temperature.

EPR = electron paramagnetic resonance; AU = arbitrary unit; mT = millitesla

4.3.1.2. Quantification of $^{\cdot}OH$ radicals produced as a function of H_2O_2 concentration and nature of catalyst

The extent of $^{\cdot}OH$ radicals production was assessed in the reactions involving H_2O_2 in the presence of either ferric chloride impregnated modified PAN catalyst (PCatDC3) or ferric sulfate impregnated modified PAN catalyst (PCatDS) or modified PAN mesh (MOPM) or non-modified PAN mesh (NMPM). Figure 4.5 demonstrates differences in $^{\cdot}OH$ radical production. Ferric chloride impregnated PAN catalyst produced more $^{\cdot}OH$ radicals than the ferric sulfate impregnated PAN catalyst (Fig. 4.5A and 4.5B respectively) when 0.2% w/v H_2O_2 was used, but this difference was not statistically significant ($p>0.05$), even though the amount of iron on PCatDS was approximately four times more than on PCatDC3. In general, 0.2% w/v H_2O_2 with modified PAN catalysts (PCatDC3 or PCatDS) produced more $^{\cdot}OH$ radicals than when using 0.1% w/v H_2O_2, thus, increased $^{\cdot}OH$ radical production is a function of concentration of H_2O_2. However, when 0.1% w/v H_2O_2 was used it was clear that there was little difference between the chloride and sulfate impregnated catalysts and their ability to produce $^{\cdot}OH$ radicals (Figures 4.5C and 4.5D). As reviewed in Chapter 1, it is well known that the Fenton reaction is more active in acidic pH than in an alkaline pH (Lindsey and Tarr, 2000), and the formation of $^{\cdot}OH$ radical in the Fentons or Fenton-like reactions is known to be dependent on several factors including pH, iron and H_2O_2 concentrations (Zang *et al.,* 1995; Lindsey and Tarr, 2000). The formation of $^{\cdot}OH$ radical has been found to increased linearly with H_2O_2 concentration (Lindsey and Tarr, 2000; Kim and Metcalfe, 2007). Firstly, it is possible that the significant antimicrobial activity observed in Chapter 2, Section 2.4.2.2. as compared to Section 2.4.5.3. may be due to increased $^{\cdot}OH$ radical production as the former had acidic environment and the latter was of alkaline environment resulting in possibly less $^{\cdot}OH$ radical formation (therefore correlating to the lower antimicrobial activity observed). Secondly, the increase in antimicrobial activity observed when higher concentrations of H_2O_2 with PAN catalyst were used as compared to lower concentrations could also be said to be due to increased $^{\cdot}OH$ radical production (see Chapter 2, Section 2.4.2.2.). This assertion is supported by the differences of $^{\cdot}OH$ radical production observed between '0.2% w/v H_2O_2/PAN catalysts' and '0.1% w/v H_2O_2/PAN catalysts' systems (Figures A, B & C, D). In these conditions, the former

produced significantly ($p<0.01$) more $^{\cdot}OH$ radicals than the latter. And although the amount of catalyst used in both experimental conditions was the same, it may suggests that the increase or decrease of $^{\cdot}OH$ radical production in these systems is dependent on H_2O_2 concentration.
Interestingly, the reaction of either MOPM or NMPM in the presence of 0.2% w/v H_2O_2 produced $^{\cdot}OH$ radicals but these amounts were significantly less ($p<0.01$) than when PCatDC3 or PCatDS was used at the same H_2O_2 concentration (Figures 4.5E and 4.5F).

As mentioned earlier, the production of $^{\cdot}OH$ radicals derived from MOPM and NMPM was not expected as these materials were not treated with iron salts; however, unlike in aqueous conditions, it is known that unpaired electrons in transition metals and crystal structures are relatively stable and therefore can be measured easily with a direct ESR method (Kim and Metcalfe, 2007). Thus, it is possible for free radicals to be trapped in solid materials such as polymers including PAN (homopolymer and copolymer PAN) during their manufacturing processes via free radical polymerisation (Braunecker and Matyjaszewski, 2007; Yu *et al.,* 2007). Increased density of chemical crosslinks and physical entanglements leads to restricted mobility of molecules, even polymer chain segments. The consequence, of such phenomenon observed during free-radical crosslinking polymerisation is radical trapping (Pavlinec and Moszner, 2003). It has been established that the lifetime of free radicals trapped in polymers could be in minutes, months or even more (Pavlinec and Moszner, 2003). From the above explanation, it is therefore not really surprising to observe $^{\cdot}OH$ radicals from the MOPM and NMPM when it was exposed to H_2O_2, as this phenomenon may be due to trapped radicals resulting from the production of PAN fibre. It is possible to assume that the presence of H_2O_2 did not play any significant role for the detection of the $^{\cdot}OH$ radicals in MOPM and NMPM since firstly, MOPM and NMPM were not impregnated with iron salts (H_2O_2 react with iron salts to generate $^{\cdot}OH$ radicals), and secondly, these radicals may have already been trapped and present in MOPM and NMPM before exposure to H_2O_2.

However, it is also possible that these previously trapped radicals in the PAN polymer matrix could induce dissociation of H_2O_2 to form $^{\cdot}OH$ radicals:

$H_2O_2 + X = XOH + {}^{\cdot}OH$ **Equation 4.2**

where X is trapped radical.

The $^{\cdot}OH$ radical is trapped by DMPO in solution to give DMPO-OH as observed. It could be useful to subject MOPM and NMPM to a solution of DMPO in the absence of H_2O_2 to establish whether $^{\cdot}OH$ radical can still be identified from the PAN polymer in the absence of H_2O_2. Such work could demonstrate whether $^{\cdot}OH$ radicals were already trapped in the PAN polymer and as such can be detected in the absence of H_2O_2 or otherwise require the presence H_2O_2 before they could be generated from trapped radicals. At this stage, it is not known whether some of $^{\cdot}OH$ radicals observed, were previously trapped in the PAN matrix due to the manufacturing processes of PAN, and that H_2O_2 contributed in generating more of the $^{\cdot}OH$ radicals when reacted with the unknown free trapped radicals; or it was H_2O_2 which was completely responsible for the generation of all the $^{\cdot}OH$ radicals observed from MOPM and NMPM. On the other hand, it is also possible that H_2O_2 reacts with functional groups on the catalyst to produce a variety of compounds some of which may be free radical initiators. These could be investigated in future studies.

The amounts of $^{\cdot}OH$ radicals produced by MOPM and NMPM were fairly similar (Figure 4.5E and 4.5F), however, these amounts were significantly ($p<0.01$) less than when the PAN catalysts were used. This is likely, because the PAN catalysts contained iron salts, whereas MOPM and NMPM did not, and in the presence of H_2O_2, the PAN catalysts would have the ability to generate more and even continuous $^{\cdot}OH$ radicals compared to MOPM and NMPM. In addition, it is possible that $^{\cdot}OH$ radicals produced from MOPM and NMPM were less as a result to the permeability of the matrix to the solution.

As shown in Chapter 2, Sections 2.4.2.3. and 2.4.3.2, the $^{\cdot}OH$ radicals produced by MOPM and NMPM do not exhibit any antimicrobial activity in either the presence or absence of H_2O_2. One possible explanation could be that the trapped $^{\cdot}OH$ radicals in both MOPM and NMPM diffuse and decayed rapidly (half-life = few nanoseconds) when they were put into

solution, and hence, were unable to exert any significant antimicrobial effect, unlike the conditions where the modified PAN catalysts were present with H_2O_2. Bamford *et al.* (1955) showed that free radicals become trapped by the polymers with a resulting decrease in their accessibility for reaction; and as mentioned earlier, the increased of density of chemical crosslinks and physical entanglements give rise to restricted mobility of molecules, and this phenomenon is seen to be occurring with trapped radicals in polymers (Pavlinec and Moszner, 2003). From the above given perspectives, another explanation may be that the MOPM and NMPM did not show any significant antimicrobial activity as a result of decrease or no accessibility of $^{\cdot}OH$ radicals to cells, and possibly also due to the nature to which they are trapped or held in the PAN. And as mentioned earlier, these radicals may be generated within the matrix of the mesh and not in the solution phase resulting in limited contact with microbial presence in the solution. It is therefore plausible to suggest that in order for a free radical to cause an effect it must be free, mobile and accessible to react with the substrate, but not trapped in solid materials, as this could prevent its significant activity. Hence, it can be said that the PAN catalyst generates un-trapped free radicals and therefore are accessible for reaction, whereas the MOPM and NMPM contained trapped radicals and not accessible for reaction, hence, the former showed higher antimicrobial activity compared to the latter.

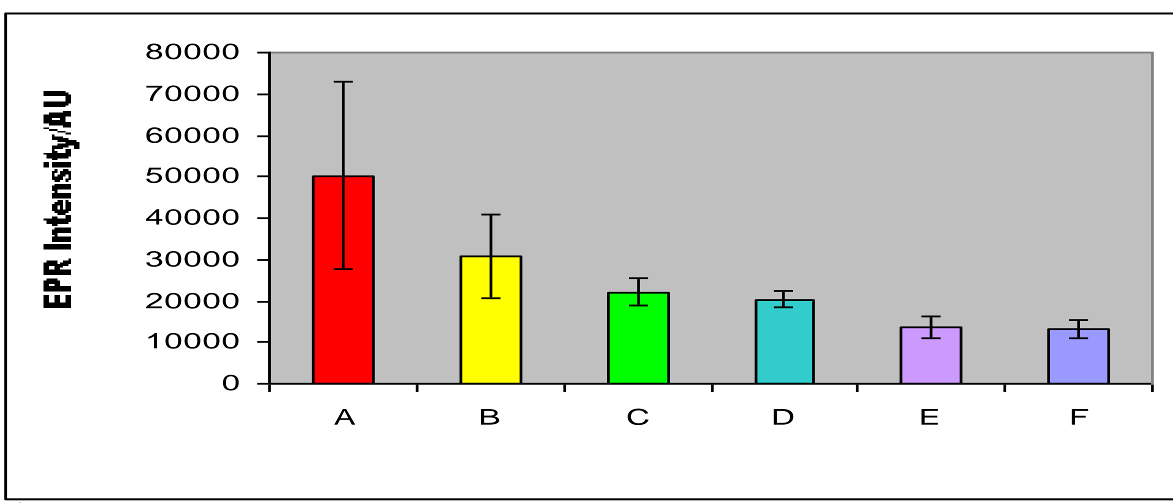


Figure 4.5: Comparative hydroxyl radicals production from modified PAN catalysts using different concentrations of H_2O_2 at room temperature.

EPR = electron paramagnetic resonance; AU = arbitrary unit

Key	**Experimental Parameters**
A	0.2% w/v H_2O_2 + DMPO + ferric chloride impregnated PAN catalyst (PCatDC3)
B	0.2% w/v H_2O_2 + DMPO + ferric sulfate impregnated PAN catalyst (PCatDS)
C	0.1% w/v H_2O_2 + DMPO + PCatDC3
D	0.1% w/v H_2O_2 + DMPO + PCatDS
E	0.2% w/v H_2O_2 + DMPO + modified PAN mesh (MOPM)
F	0.2% w/v H_2O_2 + DMPO + non-modified PAN mesh (NMPM)

Note: DMPO and H_2O_2 were mixed first before catalysts or MOPM or NMPM added for 10mins incubation.

4.3.1.3. Quantification of $^{\cdot}OH$ radicals in the leachate of modified PAN catalyst exposed to H_2O_2

The extent of $^{\cdot}OH$ radical production in the leachate of modified PAN catalysts (PCatDC3 or PCatDS) exposed to 0.2% w/v H_2O_2 was assessed. Figure 4.6 summarises differences in $^{\cdot}OH$ radical production from leachate of PCatDC3 or PCatDS exposed to 0.2% w/v H_2O_2. The amount of $^{\cdot}OH$ radical levels produced in the leachate of PCatDC3 exposed to 0.2% w/v H_2O_2 for 1hr ((PCatDC3 removed from H_2O_2 solution after 1hr before DMPO added) was less than when PCatDC3 was left in the H_2O_2 solution (PCatDC3 remained in H_2O_2 solution for 1hr and DMPO added) under the same contact time (Fig. 4.6G and 4.6H), however this difference was not statistically significant ($p>0.05$). The amount of $^{\cdot}OH$ radicals determined in the leachate of PCatDC3 or PCatDS in purified water (Catalysts left in water for 10mins, removed and water solution tested), indicated that PCatDS leachate produced significantly ($p<0.05$) more $^{\cdot}OH$ radicals than the leachate from PCatDC3 (Fig. 4.6I and 4.6J). This is likely, since PCatDS contained approximately, four times more iron than PCatDC3, although, the data presented in Figure 4.5 has shown that PCatDC3 produced more $^{\cdot}OH$ radicals than PCatDS when both catalysts were in solution (not leachate). Interestingly, leachate from PCatDS exposed to 0.2% w/v H_2O_2 for 24hrs (PCatDS left in H_2O_2 solution and removed after 24hrs before DMPO added) produced $^{\cdot}OH$ radicals, but the amount was less than when PCatDS was left in H_2O_2 solution (PCatDS remained in H_2O_2 solution after 24hrs and DMPO added) under similar experimental conditions, however the difference was not statistically significant (>0.05) (Fig. 4.6K and 4.6L). Furthermore, radical levels were very similar to those seen after 10 minutes exposure to the catalyst (Figure 4.6I and 4.6J). It was noticed that catalyst left in the presence of DMPO (Fig 4.5) produced relatively more $^{\cdot}OH$ radicals than catalyst left in solution without DMPO (Fig 4.6) irrespective of contact times. Presumably, $^{\cdot}OH$ radical is accumulative in the presence of DMPO and this perhaps explains the differences in intensities between the two graphs. However, it should be noted that the levels of $^{\cdot}OH$ radicals produced were lower than or similar to those seen with modified PAN mesh and non-modified PAN in Fig 4.5. It has been found that the amount of trapped radicals found in polymers can be significant, and when these polymers such as PAN are heat treated at temperatures around

500-600°C, they can demonstrate the large concentrations of free radicals with maximum ESR signals (Zhao and Jang, 1996). It is therefore not necessarily surprising to observed more $^{\cdot}$OH radicals in MOPM and NMPM than in the leachate. It should be noted that firstly, MOPM and NMPM were left in H_2O_2 solution in the presence of DMPO, whereas PAN catalyst was removed from solution before DMPO added to the solution, it is therefore plausible to assume that DMPO might be able to access the majority of the trapped or presumably generated $^{\cdot}$OH radicals in the former, whereas DMPO would trap limited amounts of radicals present in the leachate because the PAN was not in solution and some $^{\cdot}$OH radicals may have decayed long before DMPO was added (half-life of OH radicals = few nanoseconds).

It has been demonstrated that the solution of leachates from the PAN catalyst (either leached in water or H_2O_2) were capable of generating $^{\cdot}$OH radicals in the presence of H_2O_2. However, the extent of $^{\cdot}$OH radical production was dependent on time of contact of catalyst with the solution phase. Thus, decreased $^{\cdot}$OH radical production was related to an increase in the contact time and vice versa. This could be related to the likely decrease in H_2O_2 with time leading to a lower $^{\cdot}$OH radical formation, or it could be linked to a loss of activity of active species or both. Prior to the addition of DMPO, and at the same contact times, it has been demonstrated that the PAN catalysts which were left in the solution when $^{\cdot}$OH radicals production was measured produced relatively more $^{\cdot}$OH radicals than the leachate from the PAN catalyst (Figures 4.6G, 4.6H, 4.6K, & 4.6L). This is probably due to the continued presence of the iron on the modified PAN catalyst and its potential for continual leaching. This is also possible that, as shown in Chapter 3, Section 3.3.1. the amount and percentage of iron leached into solution is relatively small, compared to the total amount of iron on the PAN mesh.

Dramou *et al.* 2008 produced a copper based heterogeneous catalyst by immobilising copper on a commercial ion-exchange resin, Amberlite IRC 148 to create a polymer-metal complex. This catalytic complex was used for decontamination processes through the generation of $^{\cdot}$OH radicals in the presence of H_2O_2. It was identified that the system

generated $^{\cdot}OH$ radicals through a series of reactions in which H_2O_2 reacts with a ligand-bound transition metal (copper). Unlike the classical Fenton's reaction, it was concluded that $^{\cdot}OH$ radicals do not diffuse freely into water but remain bound to the catalyst to form a polymer-metal-radical complex; and this complex reacts with *E. coli* resulting in its inactivation. This observation by Dramou and workers may be similar to that seen in the present study as more $^{\cdot}OH$ radicals were generated when modified PAN catalyst is in solution than from the leachate. However, as shown in Chapter 3, Section 3.3.4., the degree of antimicrobial activity of either the leachate from the PAN catalyst or PAN catalyst remaining in solution were similar, hence, this further suggests that the active species providing antimicrobial activity is leached from the catalyst into solution. There is also a possible structural change when iron is impregnated to the modified mesh as can be seen from the differences of antimicrobial activities between PAN catalyst leachate and the solutions of iron salts (homogeneous Fentons catalyst equivalent to iron found in leachate) shown in Chapter 3, Section 3.3.4, where the former had higher activity than the latter, suggesting that the leachate may contain other species (possibly complexed with iron) which were also contributing to antimicrobial activity rather than just $^{\cdot}OH$ radicals alone causing microbial kill. Hence, a homogeneous contribution in the overall reaction cannot be ruled out. Thus, when the modified PAN catalyst is in solution, it can be said that both the catalyst and its leachate provide antimicrobial activity. It is possible that $^{\cdot}OH$ radical-microbial interactions occur in two phases, thus, one occurring possibly on the surface of the PAN catalyst; and the other occurring in solution with leached iron-hydroxylamine-hydrazine complex from the PAN mesh. This assertion is supported by Cho *et al.* (2005) whose TiO_2 photocatalytic disinfection against MS-2 phage and *E. coli* demonstrated that *E. coli* was inactivated by both free (solution phase) and surface-bound $^{\cdot}OH$ radicals but MS-2 phage was inactivated mainly by free $^{\cdot}OH$ radicals in the solution phase, and therefore concluded that microbial inactivation by reactive oxygen species (ROS) are very different depending on the type of organism tested. As already discussed from the literature, $^{\cdot}OH$ radicals attack microbial cells by oxidising biomolecule substrates such as proteins, DNA and lipids (Halliwell and Gutteridge, 1984; Miller and Britigan, 1997; Park and Imlay, 2003), and this form of radical attack is not ruled out from the current work.

However, due to a possible chemical changes occurring in relation to the significant antimicrobial activity exhibited by the leachate as shown in Chapter 3, it is possible to postulate that the mode of antimicrobial activity may involve multiple mechanisms which warrant further work.

It appears that possible continuous generation of $^{\cdot}OH$ radicals even after 24 hrs is due to a very slow decomposition rate of H_2O_2 by the novel heterogeneous PAN catalyst, or even by the 'novel leached PAN compounds' (iron-hydroxylamine-hydrazine complex system). It would be interesting to examine the mechanism/s involved in the continuous generation of $^{\cdot}OH$ radicals by the PAN catalyst or its leachate. The ability of leached PAN catalyst to generate $^{\cdot}OH$ radicals even after 24hrs of contact with H_2O_2 should be treated with keen research interest as it may find applications in continuous flow processes. However, as the levels of $^{\cdot}OH$ radicals produced were lower or similar to those seen with MOPM and NMPM in Fig 4.5, these results are not conclusive. On the other hand, it must be considered that MOPM did not show any increase in antimicrobial activity of H_2O_2 as demonstrated in Chapter 2, and as discussed previously, this suggests that the trapped or generated $^{\cdot}OH$ radicals identified from both MOPM and NMPM are not capable to cause any antimicrobial effects, possibly due to the fact that they may be generated in the polymer matrix and may not be accessible to the solution phase. This observation may require further investigation to ascertain some or all of the following:

- How are $^{\cdot}OH$ radicals generated from trapped radicals localised in MOPM and NMPM (PAN)? Were the $^{\cdot}OH$ radicals already trapped in the polymer matrix as a result of the manufacturing of PAN or were they generated by the reaction between H_2O_2 and other trapped radicals in the PAN?
- What role does H_2O_2 play in generating $^{\cdot}OH$ radicals from MOPM and NMPM in the absence of iron salts?
- Are the $^{\cdot}OH$ radicals generated from MOPM and NMPM accessible for reaction in solution or only in the polymer matrix or both?

• Are the $^{\cdot}OH$ radicals generated only at the trapping centres within the polymer matrix and if so, are they released into the solution phase? Or are the trapped radicals released when the MOPM and NMPM come in contact with solution?
• Does DMPO access $^{\cdot}OH$ radicals only within the polymer matrix or in solution or both?
• What effect/s do other trapped radicals exert on one another particularly on $^{\cdot}OH$ radicals, i.e. are there competition in the reaction leading to antagonising effect/s?

It is envisaged that an investigation of all or some of the above raised issues may help elucidate why $^{\cdot}OH$ radicals generated from trapped radicals from MOPM and NMPM showed no significant antimicrobial activity.

From the given perspectives, it is unlikely that MOPM and NMPM could continuously generate $^{\cdot}OH$ radicals similar to that seen with the PAN catalyst and its leachate. Furthermore, the PAN catalyst and its leachate caused significant antimicrobial activity in the presence of H_2O_2, whereas the MOPM showed no activity (the antimicrobial activity of NMPM was not tested but it is likely to show the same results as MOPM), this phenomenon shows variability in the nature of the $^{\cdot}OH$ radicals identified in the test materials/solutions.

As discussed earlier, although leachate showed similar level of antimicrobial activity to the PAN catalyst, which suggests that other compounds other than $^{\cdot}OH$ radicals may be involved in antimicrobial activity. However, it must be noted that $^{\cdot}OH$ radicals may still play a significant antimicrobial activity in the reaction, as this was clearly demonstrated with solutions of iron salts (homogeneous Fentons catalyst equivalent to iron found in leachate) in Chapter 3, which showed higher antimicrobial activity compared to the control of H_2O_2 alone. At this stage the unknown mechanism/s are the subject for future work and as mentioned earlier, it is possible that radical production is not entirely responsible for the catalyst's antimicrobial activity.

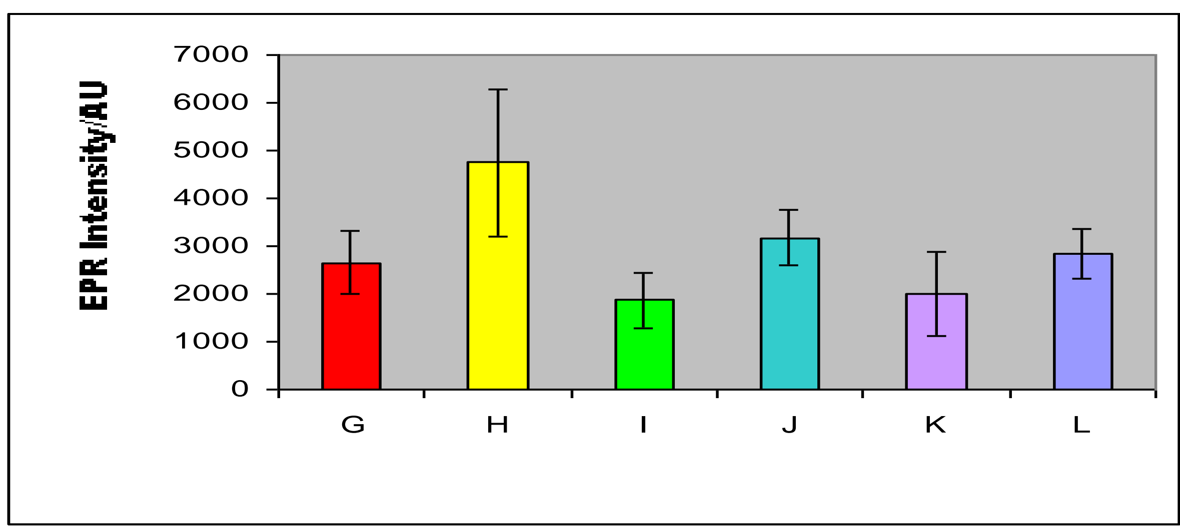


Figure 4.6: Hydroxyl radicals production in leachate of modified PAN catalyst exposed to H_2O_2 at room temperature.

EPR = electron paramagnetic resonance; AU = arbitrary unit

Key Experimental Parameters

G 0.2% w/v H_2O_2 + DMPO + PCatDC3 leachate (PCatDC3 left in H_2O_2 for 1hr and removed before DMPO added)

H 0.2% w/v H_2O_2 + DMPO + PCatDC3 (PCatDC3 left in H_2O_2 for 1hr before DMPO added)

I 0.2% w/v H_2O_2 + DMPO + PCatDC3 leachate (PCatDC3 left in H_2O for 10mins and removed before DMPO and H_2O_2 added)

J 0.2% w/v H_2O_2 + DMPO + PCatDS leachate (PCatDS left in H_2O for 10mins and removed before DMPO and H_2O_2 added)

K 0.2% w/v H_2O_2 + DMPO + PCatDS leachate (PCatDS left in H_2O_2 for 24 hrs and removed before DMPO and H_2O_2 added)

L 0.2% w/v H_2O_2 + DMPO + PCatDS (PCatDS left in H_2O_2 for 24 hrs, and DMPO added)

4.3.2. Total cell count as a means of qualitative assessment of antimicrobial mode of action

A qualitative examination was performed to investigate the mode of action in the modified PAN catalyst/ H_2O_2 system by comparing microbial death (log reduction) to total cell number (live and dead cells together) from the experimental solution after each contact time. Table 4.1. summarises the results of the experiments. As already shown in Chapter 2, it can also be seen from Table 4.1A that both *Ps. aeruginosa* and *S. aureus* were totally inactivated at 20mins contact time; however, total cell number (i.e. live and dead cells together) from the same experimental solution was found to be high and relatively the same as initial viable bacterial number (live cells only) (Table 4.1B). At the longest experimental contact time (60mins), there were 2.88×10^7 (representing 0.70 log reduction) and 2.38×10^7 (representing 0.71 log reduction) cells/mL total cell count for *Ps. aeruginosa* and *S. aureus* respectively as compared to the initial viable count of 10^8 cfu/mL. This suggests that the mode of action by the modified PAN catalyst/ H_2O_2 system against both Gram negative (*Ps. aeruginosa*) and Gram positive (*S. aureus*) cells is not through lysis or rupture of the microbial cells but possibly through intact cell death as total cell number was relatively similar to initial viable count.

Table 4.1: (**A**) Antimicrobial effects of H_2O_2 and modified PAN catalyst (PCatDC3) on vegetative microorganisms at room temperature; (**B**) Total cell count of experiments from (**A**).

Contact time (min)	(**A**) Mean Log_{10} reduction (± S.D.) of experiment		(**B**) Mean Log_{10} reduction (± S.D.) of total cell count of experimental solution from (**A**)	
	Ps. aeruginosa 0.5% w/v H_2O_2 with PCatDC3	*S. aureus* 1% w/v H_2O_2 with PCatDC3	*Ps. aeruginosa* 0.5% w/v H_2O_2 with PCatDC3	*S. aureus* 1% w/v H_2O_2 with PCatDC3
10	3.62(0.36)	2.04(1.00)	0.21(1.63)	-0.01(0.14)
20	>5.46(0.00)	>5.39(0.00)	0.13(0.50)	0.35(0.33)
30	>5.46(0.00)	>5.39(0.00)	-0.20(0.08)	0.27(0.91)
40	>5.46(0.00)	>5.39(0.00)	0.36(0.45)	0.71(0.82)
50	>5.46(0.00)	>5.39(0.00)	0.01(0.00)	0.99(0.90)
60	>5.46(0.00)	>5.39(0.00)	0.70(0.46)	0.71(0.22)
		Mean Log_{10}IC		Mean Log_{10}ITCC
	Ps. aeruginosa	8.16	*Ps. aeruginosa*	9.02
	S. aureus	8.09	*S. aureus*	9.37

S.D. = standard deviation; PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 3); Log_{10}IC = Log_{10} of initial count; Log_{10}ITCC = Log_{10} of initial total cell count.

4.3.3. The effect of inoculum on the catalytic decomposition of H_2O_2

The focus of this work was to evaluate the decomposition of H_2O_2 by the modified PAN catalyst in the presence or absence of *E. coli* (catalase-negative) or *S. aureus* (catalase-positive). Several methods are available for the decomposition of H_2O_2, with decomposition achieved via enzymatic or non-enzymatic routes. The enzyme catalase, as employed in this project as a neutraliser, decomposes H_2O_2 into water and oxygen (Williams, 1927; Beers and Sizer, 1952; Fidaleo and Lavecchia, 2003); and some transition metal catalysts such as iron and copper decompose H_2O_2 to generate $^{\cdot}OH$ radicals. The rate of H_2O_2 decomposition depends on the type of system and the experimental condition used. In the present work, ferric chloride impregnated PAN catalyst (PCatDC3) and ferric sulfate impregnated PAN catalyst (PCatDS) showed slow but different H_2O_2 decomposition rates. In the absence of both PCatDS and *E. coli*, 2.4g/L (0.24% w/v) H_2O_2 at room temperature and 35°C showed 4% (representing 0.10g/L H_2O_2 reduction) and 5% (representing 0.12g/L H_2O_2 reduction) decomposition of H_2O_2 respectively at 4 hrs (240mins) (Figures 4.7 and 4.8); increasing slightly at 24hrs to 8% decomposition (representing 0.19g/L H_2O_2 reduction) (Figure 4.7). The addition of *E. coli* made little difference to the rate of decomposition of 2.4g/L H_2O_2 and resulted in a 4.2% (representing 0.1g/L H_2O_2 reduction) and 6.2% (representing 0.15g/L H_2O_2 reduction) decrease in H_2O_2 concentration at room temperature and 35°C respectively at 4hrs. At 4 hrs, the presence of PCatDS with H_2O_2 resulted in a significant reduction in H_2O_2 concentration of 8% (representing 0.19g/L H_2O_2 reduction) and 13% (representing 0.31g/L H_2O_2 reduction) at room temperature ($p<0.01$ after 6hrs) and 35°C ($p<0.01$ after 30mins) respectively (Figures 4.7 and 4.8); which increased to a 21% (representing 0.50g/L H_2O_2 reduction) reduction at 24 hrs at room temperature (Figure 4.7). The presence of PCatDS, *E. coli* and H_2O_2 at room temperature and 35°C at 4 hrs resulted in a 9% (representing 0.22g/L H_2O_2 reduction) and 15% (representing 0.35g/L H_2O_2 reduction) reduction in H_2O_2 concentration respectively; however, 25% (representing 0.60g/L H_2O_2 reduction) decomposition was observed at 24hrs at room temperature. It was found that ferric chloride impregnated PAN catalyst (PCatDC3) decomposes H_2O_2 slightly faster than the ferric sulfate impregnated PAN

catalyst (PCatDS) particularly in the absence of *E. coli*. Thus, at 24hrs, there were 34% (representing 0.82g/L H_2O_2 reduction) and 25.4% (representing 0.61g/L H_2O_2 reduction) reductions in H_2O_2 by 'PCatDC3 alone' and 'PCatDC3 with *E. coli* respectively, as compared to 20% (representing 0.48g/L H_2O_2 reduction) and 25% (representing 0.60g/L H_2O_2 reduction) produced by PCatDS under the same experimental conditions (compare Figures 4.7 and 4.9).

The presence of catalase-positive *S. aureus* in 10g/L (1% w/v) H_2O_2 with or without PCatDS, resulted in an increase in H_2O_2 decomposition at both room temperature and 35°C. In the absence of any microorganisms, the rate of 10g/L H_2O_2 decomposition with or without PCatDS was similar to that of 2.4g/L H_2O_2. However, in contrast to tests with *E. coli*, *S. aureus* decomposed H_2O_2 by 34% (representing 3.40g/L H_2O_2 reduction) and 25% (representing 2.50g/L H_2O_2 reduction) at room temperature and 35°C respectively at 60mins (Figures 4.10 and 4.11). At room temperature, decomposition stopped after 60mins remaining stable even after 24hrs; decomposition, however, continued at 35°C but in a slower manner after 60mins, resulting in 30% (representing 3.00g/L H_2O_2 reduction) reduction at 4hours (Figure 4.11). Interestingly, the effect of PCatDS on the rate of decomposition of H_2O_2 was significantly less ($p<0.01$) throughout the contact time than when *S. aureus* alone was present with H_2O_2. Thus, PCatDS with 10g/L H_2O_2 at 60mins resulted in 11% (representing 1.10g/L H_2O_2 reduction) and 10% (representing 1.00g/L H_2O_2 reduction) reduction of H_2O_2 concentrations at room temperature and 35°C respectively; and further decomposition at 24 hrs at room temperature was 17% (Figures 4.10 and 4.11).

The mixture involving *S. aureus*, PCatDS and H_2O_2 at room temperature and 35°C showed higher H_2O_2 decomposition as compared to when PCatDS only was used. However surprisingly, the rate of decomposition of H_2O_2 was significantly ($p<0.01$) lower at all contact times, than when *S. aureus* alone reacted with H_2O_2 (Figures 4.10 and 4.11). Four possible explanations could be attributed to this outcome. Since catalase is released by *S. aureus* and the PAN catalyst both have the ability to decompose H_2O_2, there may be competition between catalase and the catalyst to react with H_2O_2 when they are both

present in solution; and this could result in the prevention of the either catalase or the PAN catalyst gaining full access in order to react properly with H_2O_2; secondly, and as already shown, the presence of the catalyst with H_2O_2 generates $^{\cdot}OH$ radicals which would attack and reduce or totally inactivate *S. aureus*, giving the catalyst a competitive advantage over *S .aureus* in the decomposition of H_2O_2 when both are present in solution; and thirdly, there could be the possibility that catalyst leachate complexes interact with catalase preventing its mode of action. The fourth point is that acidic pH is known to denature catalase activity (Samejima and Yang, 1963), and as already shown in previous chapters, the PAN catalyst in solution produced an acidic environment, hence, such environment may have reduced the activity of catalase allowing the catalyst to have much more competitive advantage in the decomposition of H_2O_2, hence, if the second and third explanations given above are true and since it has also been found that the catalyst decomposes H_2O_2 relatively slowly, it should not therefore be too surprising to observe reduced H_2O_2 decomposition by PAN catalyst/*S. aureus*/ H_2O_2 system compared to the reaction containing only *S. aureus* and H_2O_2.

The catalase enzyme is known to exhibit a high degree of structural complexity. The enzyme molecules are oligomers composed of four tetrahedrally-arranged 60,000 Da subunits. Each subunit consists of a single polypeptide chain and is associated with ferric protoporphyrin IX as prothetic group (Fidaleo and Lavecchia, 2003).

The general enzyme-catalysed reaction is shown as:

$2H_2O_2 \rightarrow 2H_2O + O_2$ **Equation 4.3**

It is believed that the manner in which the reaction mechanism proceeds involves the cycling of catalase between its ferric ground state (ferricatalase) and a two-electron-oxidised state (compound I) (Fidaleo and Lavecchia, 2003):

Ferricatalase + $H_2O_2 \rightarrow$ compound I + H_2O **Equation 4.4**

Compound I + $H_2O_2 \rightarrow$ Ferricatalase + $H_2O + O_2$ **Equation 4.5**

As mentioned earlier, factors affecting H_2O_2 decomposition by catalase include pH, temperature and H_2O_2 concentration (Williams, 1927; Fidaleo and Lavecchia, 2003; Horst *et al.,* 2006). Previous work (Chapter 2) showed that the antimicrobial activity of 1% w/v (10g/L) H_2O_2 completely inactivated *S. aureus* after 40mins at 35°C and had around a 5 log

reduction after 60mins at room temperature; whereas the ferric sulfate impregnated PAN catalyst with 1% w/v H_2O_2 inactivated *S. aureus* at 10mins at room temperature. Hence, one crucial question that could be asked is, does catalase activity still continue in the presence of H_2O_2 even when catalase-positive organisms are known to be killed? H_2O_2 decomposition tests have clearly shown that catalase was still active with or without PAN catalyst even after more than 4 hrs from the initial reaction (Figures 4.10 and 4.11), so even when the vegetative cell has been killed and that its metabolic processes have stopped, catalase seems to be continuing to be active. Further tests to assess the rate of H_2O_2 decomposition in the presence of dead catalase-positive organisms in comparison to live cells would be interesting. It is important to take into consideration catalase activity when selecting a particular concentration of H_2O_2 for an antimicrobial test particularly when the aim is to reuse the solution, as this enables one to know whether the initial H_2O_2 concentration has been reduced or completely decomposed prior to reuse. As shown in this project, due to the relative catalase activities of *Ps. aeruginosa* (0.5% w/v H_2O_2) and *S. aureus* (1% w/v H_2O_2) the concentration of H_2O_2 used against these organisms was increased as compared to catalase-negative *E. coli* (0.2% w/v H_2O_2).

Thirty five ppm [Fe] or [$FeCl_3.6\,H_2O$] homogeneous ferric chloride or 120ppm [Fe] or [$Fe_2(SO_4)_3$] homogeneous ferric sulfate, which was equivalent to the concentration of iron digested from 1g of the respective PAN catalysts (see Table 3.1) showed rapid decomposition of 2.4g/L H_2O_2 below measurable range (H_2O_2 concentration range of the instrument = 1g/L – 10g/L) (Figure 4.12) at 24hrs as compared to PAN catalyst which still showed relatively high H_2O_2 concentrations, with percentage decompositions of 21% for PCatDS and 34% for PCatDC3 respectively. The rapid decomposition of H_2O_2 by homogeneous iron catalyst, rapidly generates $^{\cdot}OH$ radicals, however, this mechanism is also seen as one of the disadvantages of the homogeneous Fenton's system, as the continuous generation of more $^{\cdot}OH$ radicals may require routine addition of H_2O_2 (Neyens and Baeyens, 2003). Hence, this provides an advantage in the application of the heterogeneous modified PAN catalyst over the homogeneous Fentons catalyst because as shown, the

heterogeneous modified PAN catalyst can continuously generate ˙OH radicals even after 24 hrs and possibly beyond this time, as a result of the slow decomposition of H_2O_2.
From Chapter 2, Section 2.4.6. it has been shown that the PAN catalysts can be reused two times. The H_2O_2 solution itself could be reused due to its slow decomposition by the catalyst even after 24hrs.

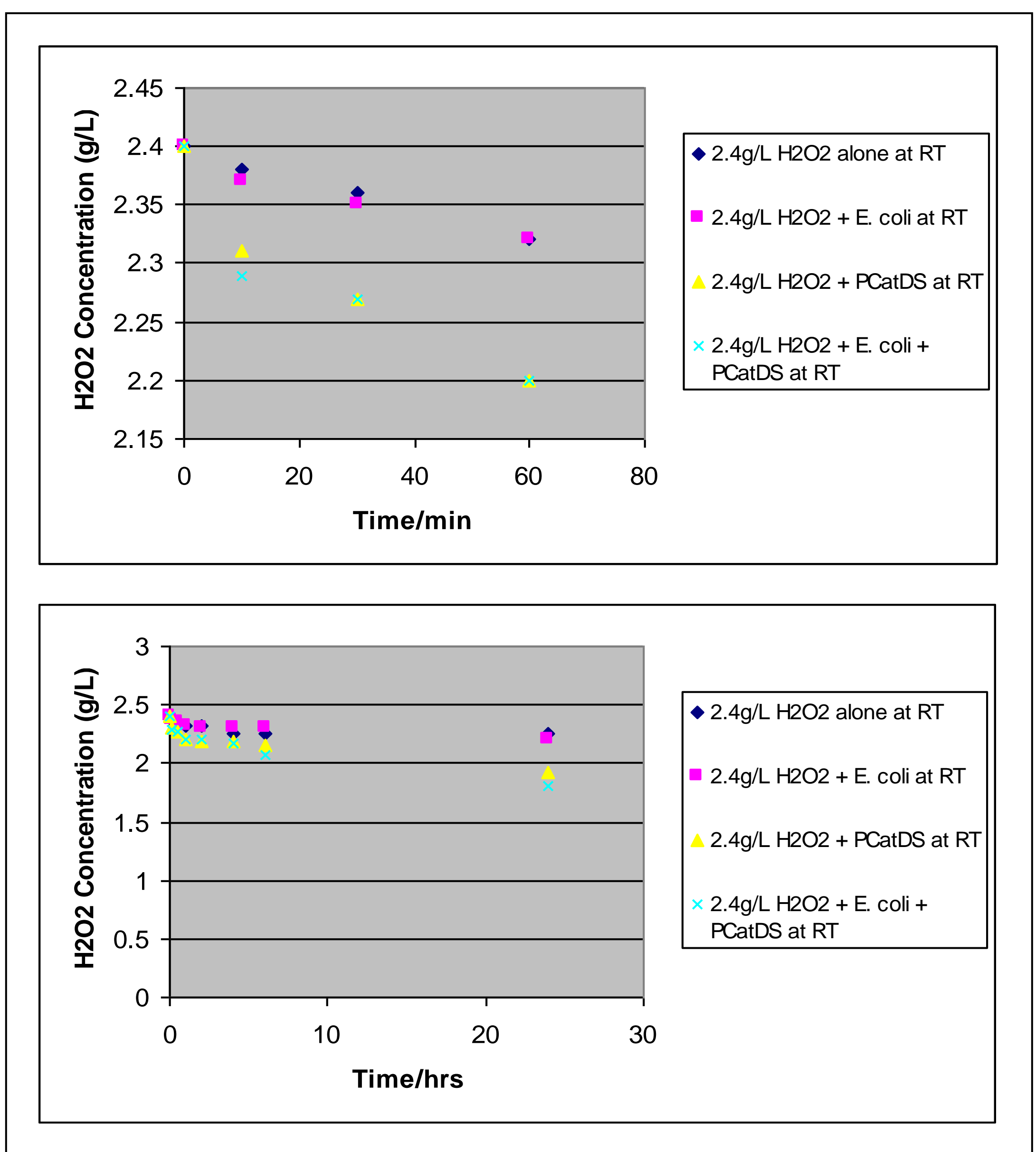


Figure 4.7: Decomposition of H_2O_2 with or without *E. coli* or modified PAN catalyst (PCatDS) at room temperature.

RT = room temperature; PCatDS = ferric sulfate form PAN catalyst (Dralon-L type)

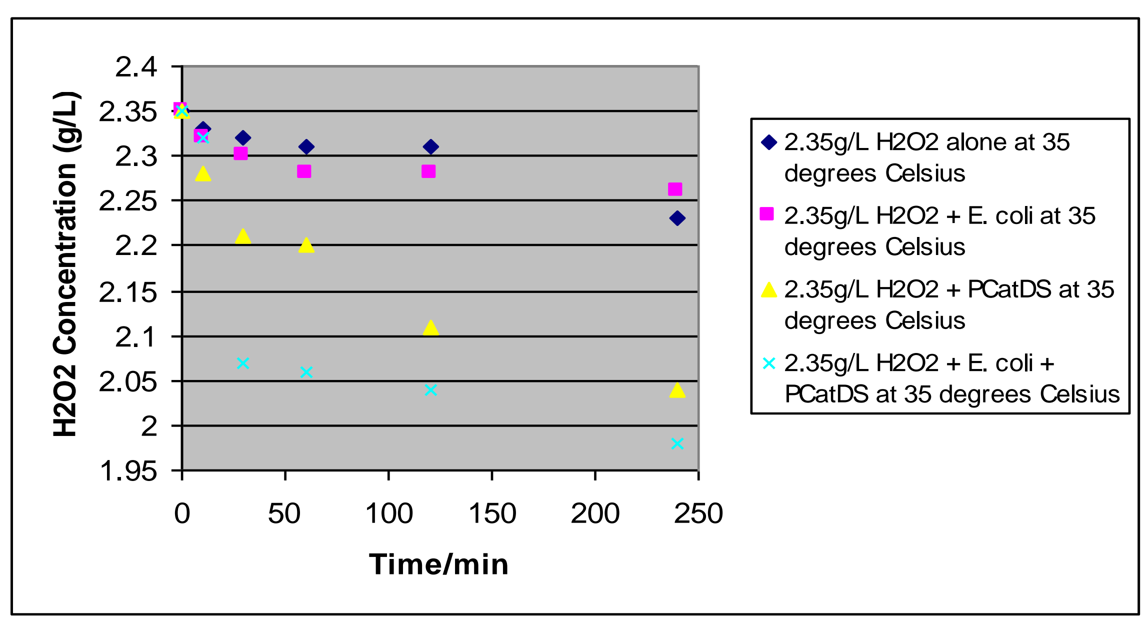


Figure 4.8: Decomposition of H_2O_2 with or without *E. coli* or modified PAN catalyst (PCatDS) at 35°C.

PCatDS = ferric sulfate form PAN catalyst (Dralon-L type)

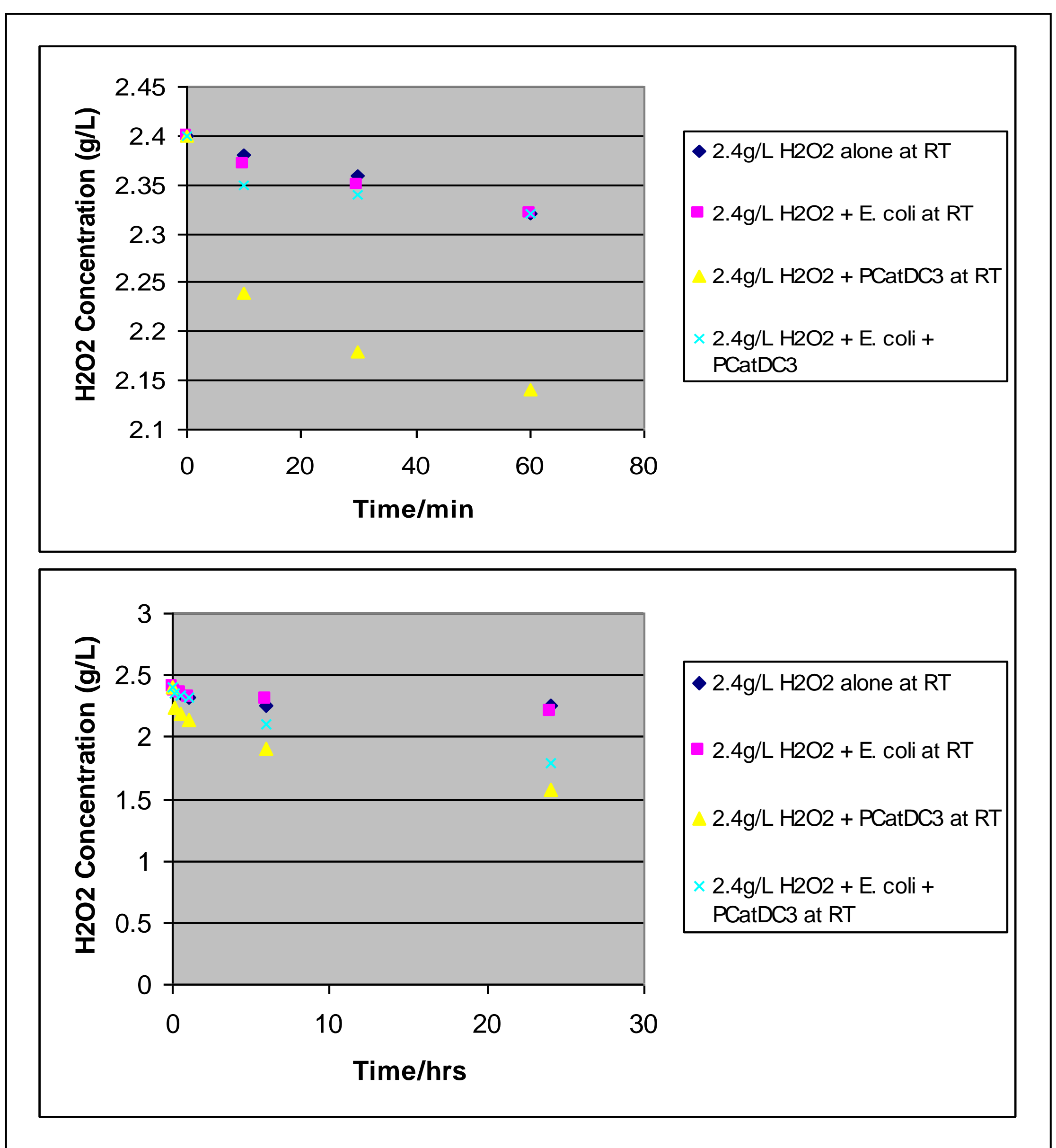


Figure 4.9: Decomposition of H_2O_2 with or without *E. coli* or modified PAN catalyst (PCatDC3) at room temperature.

RT = room temperature; PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 3)

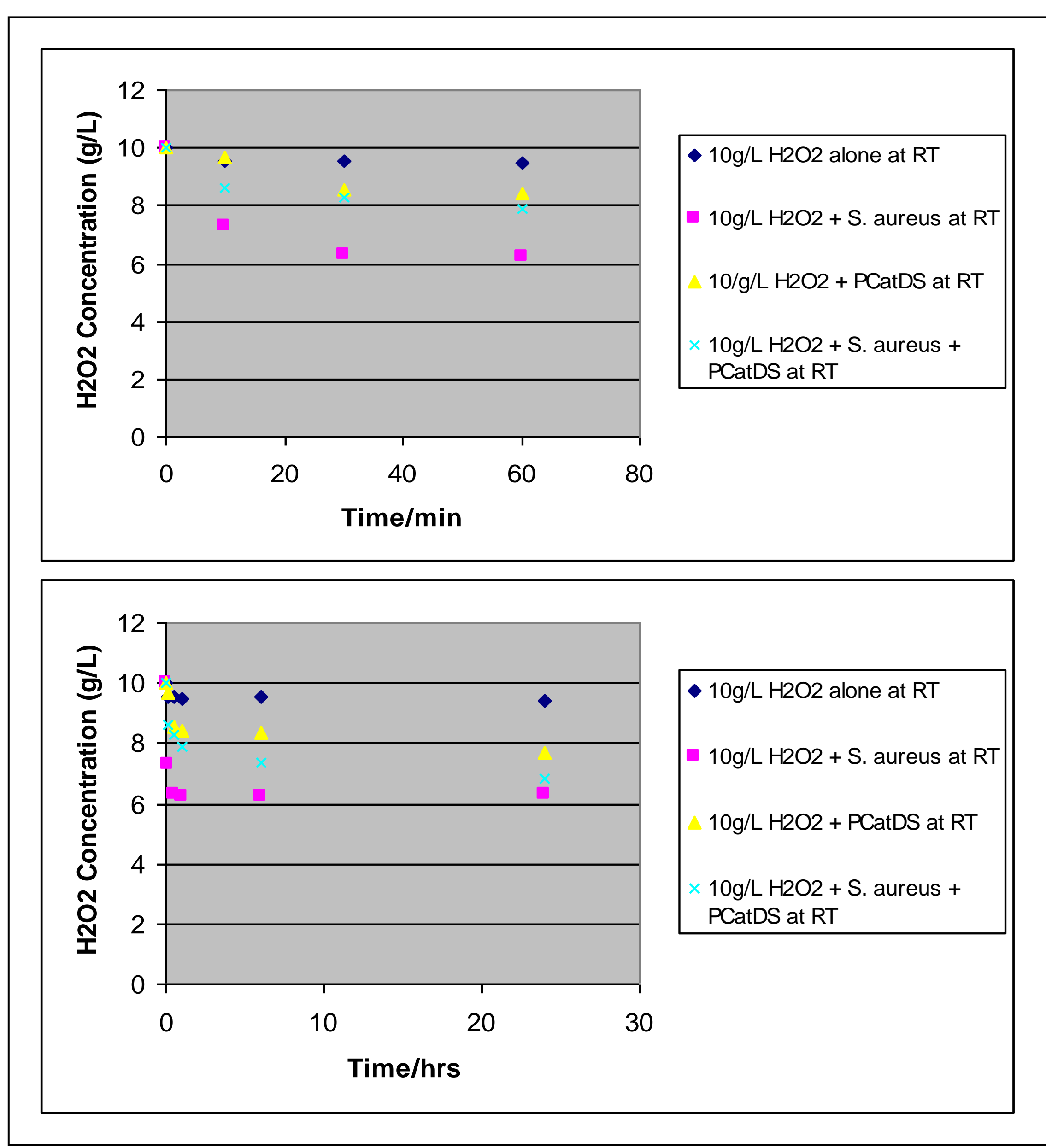


Figure 4.10: Decomposition of H_2O_2 with or without *S. aureus* or modified PAN catalyst (PCatDS) at room temperature.

RT = room temperature; PCatDS = ferric sulfate form PAN catalyst (Dralon-L type)

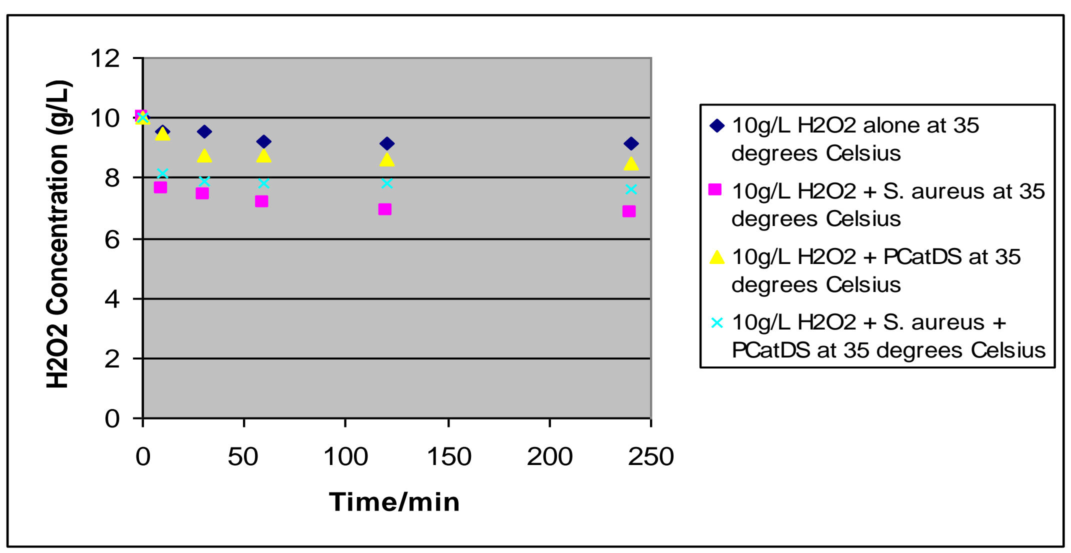


Figure 4.11: Decomposition of H_2O_2 with or without *S. aureus* or modified PAN catalyst (PCatDS) at 35°C.

PCatDS = ferric sulfate form PAN catalyst (Dralon-L type)

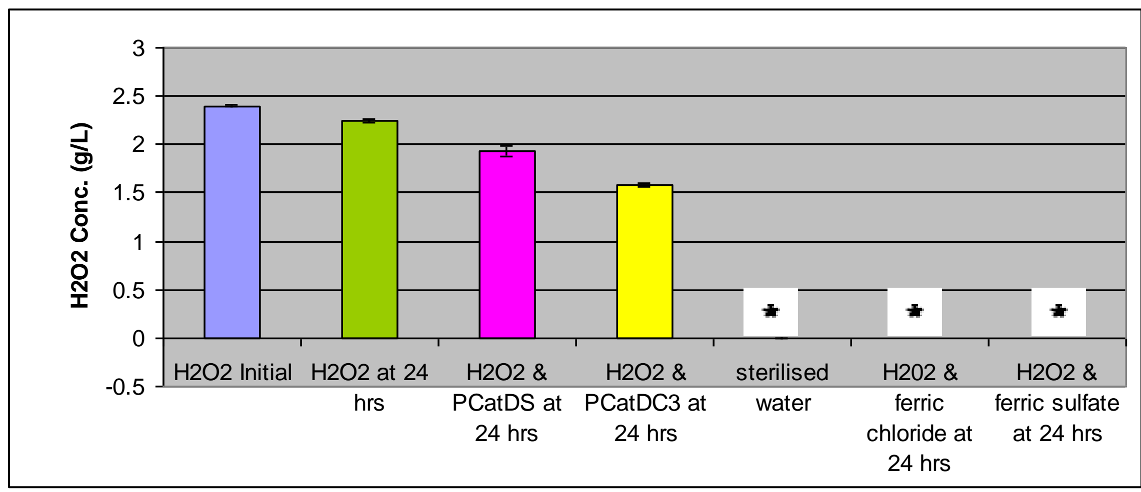


Figure 4.12: Comparative decomposition of H_2O_2 with homogeneous iron catalysts and heterogeneous modified PAN catalysts after 24 hrs of preparation.

PCatDS = ferric sulfate form PAN catalyst (Dralon-L type); PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 3)

* = below detection limit (H_2O_2 measuring range 1-10 g/L)

4.3.4. Antibacterial effects of H_2O_2/modified PAN catalyst system and H_2O_2/homogeneous iron catalyst system after continuous generation of $^{\bullet}OH$ radicals for 24 hrs

The work presented in Section 4.3.1.3. has shown that the presence of the modified PAN catalyst with H_2O_2 may be able to generate $^{\bullet}OH$ radicals even after 24 hrs of contact, as a result of slow decomposition of H_2O_2. However, it has not been shown whether the [$^{\bullet}OH$] radicals generated at 24 hrs (Fig 4.6L) is sufficient to exhibit any antimicrobial activity in comparison to the results shown when the PAN catalyst or homogeneous iron catalyst with H_2O_2 were tested at time 0hrs. Hence, the purpose of this section of work was to examine and compare the antimicrobial efficacies between modified PAN catalyst and homogeneous Fentons catalyst left in 0.2% w/v H_2O_2 for 24 hrs at room temperature. The microorganism chosen for this work was *E. coli*. Table 4.2 summarises the experimental parameters and the results of the tests.

In the absence of ferric sulfate impregnated PAN catalyst (PCatDS), it was found that the antimicrobial activity of 0.2% w/v H_2O_2 prepared and tested immediately (at time 0 hrs) showed similar antimicrobial activity to 0.2% w/v H_2O_2 prepared and tested after 24 hrs, with log reductions after 60mins of >5.50 and ≥5.17 respectively (Table 4.2A and 4.2B). Equally, the antimicrobial activity of PCatDS in 0.2% w/v H_2O_2 which was prepared and tested immediately (at time 0 hrs), was similar to the antimicrobial activity when PCatDS was added to a 0.2% w/v H_2O_2 prepared 24 hrs earlier (no significant difference), with both experimental conditions showing total microbial inactivation at 30mins (Table 4.2C and 4.2D). These outcomes clearly support the previous work which stipulates that H_2O_2 hardly decomposes when left at room temperature for 24 hrs.

When 0.2% w/v H_2O_2 with PCatDS was prepared and left at room temperature for 24 hrs before examination for antimicrobial activity, results showed no significant difference in antimicrobial activity over the respective controls comprising just H_2O_2 (Table 4.2), but less antimicrobial activity than when tested soon after preparation. There was highly significant increase ($p<0.01$) from 10-40mins in the reaction tested soon after preparation compared to the one tested after 24hrs (Table 4.2C and 4.2E). This was probably due to the

reduction in H_2O_2 because the catalyst was left in the H_2O_2 solution for 24hrs, and as already discussed previously it is possible that a longer contact period of the PAN catalyst with H_2O_2 lead to a reduction of $^{\cdot}OH$ radical production, resulting in less antimicrobial activity compared to when catalyst had shorter contact with H_2O_2 solution.

Conversely, the homogeneous catalyst prepared in 0.2% w/v H_2O_2 and left for 24 hrs before testing results showed very little antimicrobial activity. Thus, the log reduction at 60mins was 1.71 compared to total microbial inactivation at the same contact time by the PAN catalyst/ H_2O_2 solution tested after 24hrs (Table 4.2G). The latter reaction demonstrated a highly significant increase ($p<0.01$) in antimicrobial activity throughout the experimental contact times compared to the reaction of the former. The homogeneous catalyst showed a good level of activity when tested immediately after the H_2O_2 was added (Table 4.2F). At 10 and 20mins the activity seen was greater than that seen in Table 4.2C with the catalyst and H_2O_2. The homogeneous catalyst decomposes H_2O_2 rapidly, and unlike the PAN catalysts, H_2O_2 was completely decomposed at 24 hrs (Figure 4.12). The low log reduction which resulted from the activity of the homogeneous catalyst/ H_2O_2 mixtures tested at 24hrs, may not have been a consequence of $^{\cdot}OH$ radical attack, but was possibly due to the low acidic environment. This assertion can be supported with the results shown when 'acidic water' (pH 3.47-3.54) was tested against *E. coli* in Chapter 2, Table 2.22, and although the homogeneous catalyst/ H_2O_2 mixtures at 24hrs showed a slightly higher activity than the 'acidic water', this may be because the former is expected to be more acidic than the latter, hence activities in the two conditions would vary as a result of the different pHs.

This work has further demonstrated that there is continuous generation of $^{\cdot}OH$ radicals in the presence of the PAN catalyst and H_2O_2 (Figure 4.6), and these radicals may be able to exhibit effective antimicrobial activity even after 24hrs; which further confirms that $^{\cdot}OH$ radicals may play an antimicrobial role in this novel system. It can therefore be suggested that the modified PAN catalyst could act in a flow reactor and give continuous antimicrobial protection (sustained protection with time in a tank).

The reusability tests of the PAN catalyst indicated in Chapter 2, Section 2.4.6. showed that antimicrobial activity reduces on subsequent reuse of the PAN catalyst, which fits in well with the data presented here, that the generation of $^{\cdot}OH$ radicals reduces after longer contact of the PAN catalyst with H_2O_2, resulting in reduced antimicrobial activity due to a number of reasons, which include some deactivation of the catalyst resulting from loss of iron through leaching, a reduction in H_2O_2 concentration, and inactive catalytic active sites.

Table 4.2: The antibacterial effects of modified PAN catalyst (PCatDS) with 0.2% w/v H_2O_2 (PCatDS) against *E. coli* as a result of continuous generation of OH radicals at 24 hrs.

Experimental conditions	Mean Log_{10} reduction (± S.D.)						
	A	B	C	D	E	F	G
Time H_2O_2 added	0 hrs	0 hrs	0 hrs	0 hrs	0 hrs	0 hrs	0 hrs
Time PCatDS added	—	—	0 hrs	24 hrs	0 hrs	—	—
Time $FeCl_3$ salt added	—	—	—	—	—	0 hrs	0 hrs
Time of testing	0 hrs	24 hrs	0 hrs	24 hrs	24 hrs	0 hrs	24 hrs
Contact time (min)	pH 5.70	pH 5.65	pH 4.64	pH 4.76	pH 4.46	pH 3.47	pH 3.14
10	1.01(0.19)	1.06(0.08)	1.84(0.42)	2.16(0.33)	1.23(0.10)	3.44(0.12)	0.50(0.11)
20	2.08(0.12)	2.16(0.13)	3.38(0.48)	3.63(0.34)	1.86(0.18)	4.14(0.21)	0.64(0.17)
30	3.28(0.08)	3.05(0.78)	≥5.22(0.48)	≥5.50 (0.00)	3.06(0.12)	4.87(0.17)	0.79(0.23)
40	4.00(0.02)	3.40(0.13)	>5.50(0.00)	>5.50(0.00)	4.65(0.13)	≥5.40(0.17)	1.13(0.06)
50	5.17(0.35)	4.35(0.58)	>5.50(0.00)	>5.50(0.00)	≥5.34(0.28)	>5.50(0.00)	1.31(0.05)
60	>5.50(0.00)	≥5.17(0.35)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	>5.50(0.00)	1.71(0.28)

S.D. = standard deviation; PCatDS = ferric sulfate form PAN catalyst (Dralon-L type)

4.4. Conclusion

The present study has established that the PAN catalyst in the presence of H_2O_2 generates $^{\cdot}OH$ radicals. The increase or decrease of $^{\cdot}OH$ radical generation was found to be dependent on concentration of H_2O_2 and time. Thus, higher H_2O_2 concentrations produced relatively more $^{\cdot}OH$ radicals than lower concentrations; equally, more $^{\cdot}OH$ radicals are generated when PAN catalyst react with H_2O_2 at shorter contact times than at longer contact times. Leachates from PAN catalyst in either H_2O_2 or in water may generate low levels of $^{\cdot}OH$ radicals, but this needs to be confirmed with further work. PAN catalyst or leachate from the PAN catalyst in the presence of H_2O_2 may be generating $^{\cdot}OH$ radicals even after 24 hrs, but as the levels of radicals seen with the modified fibre controls were higher, it is not certain that these are entirely responsible for the leachates antimicrobial activity. It is possible that another mode of action is also adding to the catalyst's activity. The modified PAN mesh and the non-modified PAN mesh were both found to produce a similar amount of $^{\cdot}OH$ radicals. This unexpected outcome was assumed to be as a result of trapped radicals in the polymer/fibres due to the free radical polymerisation processes used in manufacturing such polymers. The radicals produced by the modified PAN mesh and the non-modified PAN mesh in the presence of H_2O_2 can be seen as insignificant to the study in so far as these two materials did not affect antimicrobial activity in the presence of H_2O_2. It is also possible that H_2O_2 reacts with functional groups on the catalyst to produce a variety of compounds some of which may be free radical initiators.

Overall, it has been shown that H_2O_2 kept at room temperature for 24 hrs does not decompose significantly. The PAN catalyst decomposes H_2O_2, however, the rate of decomposition was relatively slow taking more than 48 hrs to decompose H_2O_2 by 50%. Although still quite slow, decomposition occurs at a higher rate at 35^oC than at room temperature. The presence of *E. coli* slightly increased the ability of the PAN catalyst to decompose H_2O_2. This was not true of PAN catalyst in the presence of *S. aureus* where the decomposition of H_2O_2 with *S. aureus* alone showed increased decomposition of H_2O_2 compared to when the PAN catalyst was also present. The ferric chloride impregnated PAN

catalyst decomposes H_2O_2 faster than the ferric sulfate impregnated PAN catalyst, so this may be why the antimicrobial activity of the former is slightly higher than the latter (Chapter 2). Figure 4.5. showed that there is rapid generation of $^{\cdot}OH$ radicals as a result of faster H_2O_2 decomposition for the chloride catalyst. The slow decomposition of H_2O_2 by the PAN catalyst may be directly responsible for the continuous generation of $^{\cdot}OH$ radicals; and this assertion is supported by the different antimicrobial activities of PAN catalyst/ H_2O_2 and homogeneous catalyst/ H_2O_2, which shows that the former possesses far superior antimicrobial activity than the latter after 24 hrs contact of catalyst with the H_2O_2. H_2O_2 was completely decomposed at 24 hrs by the homogeneous catalyst resulting in complete cessation of $^{\cdot}OH$ radical generation. Thus the PAN catalyst/ H_2O_2 system is suitable for continuous disinfection.

CHAPTER 5

Characterisation of PAN Catalyst: Iron loading and bacterial attachment.

5.1. Introduction

Transmission electron microscope (TEM) and scanning electron microscope (SEM) provide an exceptional combination of information about chemical composition and structure, which is often sufficient to completely characterise a solid-state material on the nanometre scale (Wirth, 2008). SEM can be used in conjunction with energy dispersive X-ray (EDX) which provides chemical information as a result of the excitation characteristics of the sample. Emitted X-rays from the sample are collected by a detector and are used for qualitative and standard based quantitative chemical analysis (Wirth, 2008).

It has been established that the bulk of molecular interactions in the environment take place at the surface or interface of an environmental particle in contact with water; and this environmental particle could be an inorganic mineral, organic matter, a microbial, or a composite (Sawunyama *et al.,* 2001). Interaction between bacteria and solid surfaces has been a subject of importance in many scientific disciplines, thus, the adhesion of bacteria to surfaces has been examined with a number of artificial and natural materials (Mills *et al.,* 1994). Bacterial attachment to solid surfaces are often related to electrostatic, van der Waals, acid-base interactions and/or hydrophobic forces (Xu and Logan, 2006). SEM has widely been used to investigate bacteria adhesion to surfaces such as stainless steel (Xu *et al.,* 2007), plants (Carmichael *et al.,* 1999), and polymers (Kodjikian *et al.,* 2003). Although, scanning electron micrographs can show bacteria aggregation and adhesion on surfaces, it does not provide evidence of live cells on the attached material in real time. To overcome this limitation of SEM, a more direct approach using confocal laser scanning microscopy (CLSM) could be used to differentiate live and dead bacteria cells by staining. LIVE/DEAD *Bac*Light Bacterial Viability kit (henceforth referred to as the LIVE/DEAD kit) from Molecular Probes provides two colour fluorescence assay of bacterial viability that has been proven useful for a diverse array of bacterial genera and has been utilised to identify many Gram-negative and Gram-positive bacterial species (Maukonen *et al.,* 2000).

The stains of the LIVE/DEAD kit contain a mixture of nucleic acid stains that differ both in their spectral characteristics and their ability to penetrate healthy bacterial cell membranes

(Molecular Probes, Invitrogen Detection Technologies, 2004). Bacteria with intact cell membranes fluoresce green, while bacteria with damaged membranes fluoresce red, and the background remains virtually nonfluorescent (Maukonen *et al.,* 2000; Auty *et al.,* 2001; Ross, 2001; Leuko *et al.,* 2004).

The present study investigated the following:

1. The amount of iron and its distribution on the PAN mesh surface using EDX.
2. Bacterial attachment onto the modified PAN catalyst mesh as a function of time using SEM.
3. Qualitative assessment to differentiate live and dead bacteria cells on the modified PAN catalyst mesh and in experimental solution using LIVE/DEAD kit by CLSM.
4. Quantification and enumeration of 'viable count' and 'total cell count' of bacteria adhered to the modified PAN catalyst mesh.

5.2. Materials and Methods

5.2.1. Materials

Heterogeneous PAN catalyst (Table 2.3) and modified PAN mesh (chemically modified without iron impregnation), grade 1, 50% glutaraldehyde (GTA) and absolute alcohol (Sigma-Aldrich, UK), silica gel (Fisher Scientific, UK), aluminium pin stubs and spectra tabs (Agar Scientific, Essex, UK), catalase, hydrogen peroxide and phosphate buffer similar as described previously in Chapter 2, bacteriological grade peptone and sodium chloride (NaCl) (Oxoid Ltd, UK), and L7012 LIVE/DEAD *Bac*Light Viability kit for microscopy and quantitative assays (Molecular Probes, Inc. Oregon).

5.2.1.1. Equipment and Application

Leica S430 for electron microscopy (see Appendix 7); Oxford Instruments INCA-Sight (see Appendix 7) for EDX analysis; Edwards Sputter Coater S150B (see Appendix 8) for coating samples prior to electron microscopy and EDX analysis; and True Confocal Scanner Leica TCS SP2 (Leica Microsystems, Heidelberg, Germany) (see Appendix 9) used for confocal microscopy work.

5.2.2. Methods

5.2.2.1. Energy Dispersive X-ray (EDX) analysis for the distribution and quantification of iron on the modified PAN catalysts surfaces

Elemental composition of the PAN catalyst were analysed without the presence of bacteria. PAN catalyst was glued firmly by spectra tabs onto aluminium pin stubs and samples sputter coated with gold for 1min. Elements on the PAN mesh were then analysed using Oxford INCA detector, working distance (WD) of 19mm, and accelerating voltage (EHT) of 20 kV.

Standard used were as follows:

C = $CaCO_3$, O = SiO_2, Al = Al2O_3, Cl = KCl, Fe = Fe, Au = Au

5.2.2.2. Bacterial cell attachment to PAN catalyst mesh by SEM analysis

Bacterial attachment on the PAN catalyst was analysed after bacteria (*E. coli* or *S. aureus*) were treated with H_2O_2 and 1g PAN catalyst for 1 hr (the same bactericidal procedure as described in Chapter 2, Section 2.3.2.9. was followed). The experiments were performed in two sets containing the same experimental constituents (75mL total volume); in one set, the 1g PAN catalyst used in the experiment was neutralised after the stated contact time in 10mL catalase solution (0.015% w/v), whereas the other set was left un-neutralised. These two sets of experiments were undertaken to examine the differences in bacterial attachment on both neutralised and un-neutralised PAN catalysts, thus whether residue H_2O_2 (un-neutralised) on the catalyst will affect bacterial attachment as compared to the neutralised test.

Control: 1g PAN catalyst was left in a bacterial suspension (7.5mL of bacteria of either *E. coli* or *S. aureus* and 67.5mL sterile distilled water making up 75mL total volume) without H_2O_2 for 1 hr.

Neutralised, un-neutralised PAN catalyst or PAN catalyst from the control was fixed in 2% GTA before SEM analysis. The procedure of fixation was as follows:

1. 1g of PAN catalyst exposed to *E. coli* or *S. aureus* with or without H_2O_2 after 1 hr contact time were fixed for 1hr at room temperature in 10mL volume of 2% GTA prepared in 50mM phosphate buffer (pH 7.4-7.6).

2. The samples were dehydrated in a series of aqueous alcohol of the following concentrations by weight of alcohol:

50%	10mins
70%	10mins
80%	10mins
90%	10mins
100%	10mins
100%	10mins
100%	10mins

3. Critical dehydration of the samples was achieved by exposure to silica gel (water absorbent) in a desiccator for 24 hrs.

The samples were then gold sputtered and analysed by SEM using a secondary electron detector (SE1), EHT between 9.82-10kV and WD between 4-8mm.

5.2.2.3. Qualitative assessment of live and dead microbial cells on PAN catalyst and in experimental solution using LIVE/DEAD kit on Confocal Laser Scanning Microscopy (CLSM)

Experiments: *E. coli* cells treated with 1g ferric sulphate impregnated PAN catalyst (PCatDS) with or without H_2O_2 were stained with LIVE/DEAD kit and analysed by CLSM. Bactericidal tests as described previously in Chapter 2, Section 2.3.2.9., were performed for 1 hr contact time for PAN catalysts treated with *E. coli* in the presence of 0.2% w/v H_2O_2. The experiments were performed in two sets containing the same experimental constituents (75mL total volume); in one set, the experimental solution and 1g PAN catalyst used in the experiment were neutralised in catalase solution as described above for the catalyst, and as described in Chapter 2, Section 2.3.2.9. for the solution. For the other set the experimental solution and catalyst were left un-neutralised.

0.02g of either the neutralised or un-neutralised PAN catalyst was added to a mixture of two nucleic acid stains of the LIVE/DEAD kits, comprising SYTO 9 and propidium iodide in the ratio of 1:1 (100µl: 100µl; according to manufacturer's instructions) in microcentrifuge tubes, and incubated at room temperature in the dark for 15mins. Strands of the stained PAN catalysts were then loosened with forceps into smaller fine fibrils onto a microscopic glass slide and the loosened fibres covered with a coverslip glued onto it, followed by CLSM observation.

Six microlitre of LIVE/DEAD kit mixture was added to either 1mL of the neutralised or un-neutralised experimental solution, mixed thoroughly, and incubated at room temperature in the dark for 15mins. Five microlitre of the stained bacteria were trapped between a microscope slide and 18mm square coverslip, followed by CLSM observation.

CLSM analysis were performed using the following settings:

1. Objective lense: HCX PL APO 40x/0.85 CORR CS, 0.11
2. Working distance: 0.24mm
3. Green channel, detection at 488nm; red channel, detection at 543nm.
4. Red-green images (12bits), 512 by 512 pixels.
5. 16 line averaging was used to remove photomultiplier (PMT) noise.

NOTE: The same settings were maintained for all other examinations. Images were initially viewed at 1x zoom for bacterial aggregates, with digital increase in the zoom factor for subsequent observation of observed individual cells.

Controls: To confirm the suitability of the LIVE/DEAD kit the following control tests were performed:

1. 0.1mL of *E. coli* suspension in water was subjected to 0.9mL of 70% alcohol for 1 hr. 6μL of LIVE/DEAD kit mixture were added, mixed thoroughly, and incubated at room temperature in the dark for 15mins. Five microlitre of the stained bacteria were trapped between a microscope slide and 18mm square coverslip, followed by CLSM examination as described.
2. 0.1mL of *E. coli* suspension were added to 0.9mL water for 1 hr, followed by staining and CLSM protocol followed as described.
3. Six microlitre of the stain mixture was added to 0.02g PAN catalyst treated with only a suspension of *E.coli* but no H_2O_2 (1mL bacteria volume) or 0.02g PAN catalyst (1mL water volume) for 1 hr, followed by CLSM examination as described.

5.2.2.4. Viable and total cell count of bacteria attached to PAN catalyst

VIABLE COUNT*:* Experimental preparation was the same as described for bactericidal activity above and in Chapter 2, Section 2.3.2.9. After 1 hr contact time, the 1g PAN catalyst was either neutralised or left un-neutralised. 0.5g of either the neutralised or un-neutralised catalyst was added to 20mL volume containing 0.1% w/v sterilised peptone, and 0.5% w/v, 1% w/v, 2% w/v, 3% w/v and 4% w/v NaCl prepared in 0.1% w/v peptone in a universal bottle. These mixtures were vigorously shaken in a GallenKamp flask shaker for 10mins at an arbitrary unit speed of 8. After shaking, 100μL of the solution was spread

onto a dried nutrient agar in a Petri dish and after solution was well soaked into the nutrient agar, the Petri dishes were inverted and incubated and cfu/mL of bacteria colonies was calculated as previously described in Chapter 2, Section 2.3.2.8. PAN catalyst treated with only *E. coli* suspension in water was used as control and was treated as above.
TOTAL CELL COUNT*:* Total cell count as previously described in Chapter 4, Section 4.2.2.5. was used to assess the experimental solutions above.

5.3. Results and Discussion

5.3.1. Energy Dispersive X-ray (EDX) analysis for the distribution and quantification of iron on the modified PAN catalysts surfaces.

EDX was used to examine the distribution and amount of iron present on both different batches of the modified PAN catalyst mesh and on different types of the modified PAN catalyst mesh. Figures 5.1 and 5.2 demonstrate the presence of Carbon (C), Nitrogen (N), Oxygen (O), Aluminium (Al), Chlorine (Cl), Titanium (Ti), Iron (Fe), and Gold (Au) on different locations on the surfaces of ferric chloride impregnated catalyst prepared in the same batch (PCatDC3 and *PCatDC3). Figure 5.3 shows the presence of Carbon (C), Nitrogen (N), Oxygen (O), Chlorine (Cl), Titanium (Ti), Iron (Fe), and Gold (Au) at different locations on the surfaces of ferric chloride impregnated catalysts from the same batch (PCatDC4 and *PCatDC4Figure 5.4 identifies the presence of Carbon (C), Oxygen (O), Chlorine (Cl), Aluminium (Al), Sulphur (S), Calcium (Ca), Iron (Fe), Rubidium (Rb) and Gold (Au) at different locations on the surfaces of ferric sulfate impregnated catalyst from the same batch (PCatDS and *PCatDS). Table 5.1 shows the presence of Carbon (C), Nitrogen (N), Oxygen (O), Chlorine (Cl), Aluminium (Al), Titanium (Ti), Copper (Cu) and Gold (Au) at different locations on the surfaces of modified PAN mesh (not impregnated with iron (III) salts) prepared from the same batch (MOPM and *MOPM) used as controls. Apart from Ti, Au, Al and Rb, all the remaining elements identified were directly derived from the PAN fibre or as a result of modification of the PAN and impregnation with iron salts. Ti is known to alloy with Al (Shapovalova and Onishchenko, 1979), and as Al stubs were used to hold samples for EDX analysis, it was not surprising to observe the appearance of these elements. Similarly, Rb is known to alloy Au (Grosch and Range, 1996) and as Au was used to coat the samples, so it was likely for such elements to be seen in the spectra.

The data revealed that iron distribution on the surface of the PAN fibre varies within batches and between batches for both ferric chloride and ferric sulfate impregnated catalysts. Thus, different sections from the same batch of ferric chloride impregnated catalysts (PCatDC3, *PCatDC3); and PCatDC4, *PCatDC4) showed different amounts of iron on the PAN fibre (Figures 5.1A, 5.1B, 5.2A, 5.2B, 5.3B, 5.3D). This was also true when different sections from the same batch of ferric sulfate impregnated catalyst (PCatDS, *PCatDS) were examined (Figures 5.4B, 5.4D). The same lack of agreement was observed when different batches were analysed. Comparatively, the iron concentration on all the ferric sulfate PAN catalysts was greater than on the ferric chloride PAN catalysts (Figure 5.5). The variation of peak heights indicated in the elemental spectra, is a clear visual indication that there is an uneven distribution of the iron on the PAN fibre either within batches or between batches (compare Figures 5.1C and 5.2C; 5.3A and 5.3C; 5.4A and 5.4C; compare Figures 5.1C, 5.2C and 5.3A, 5.3C).

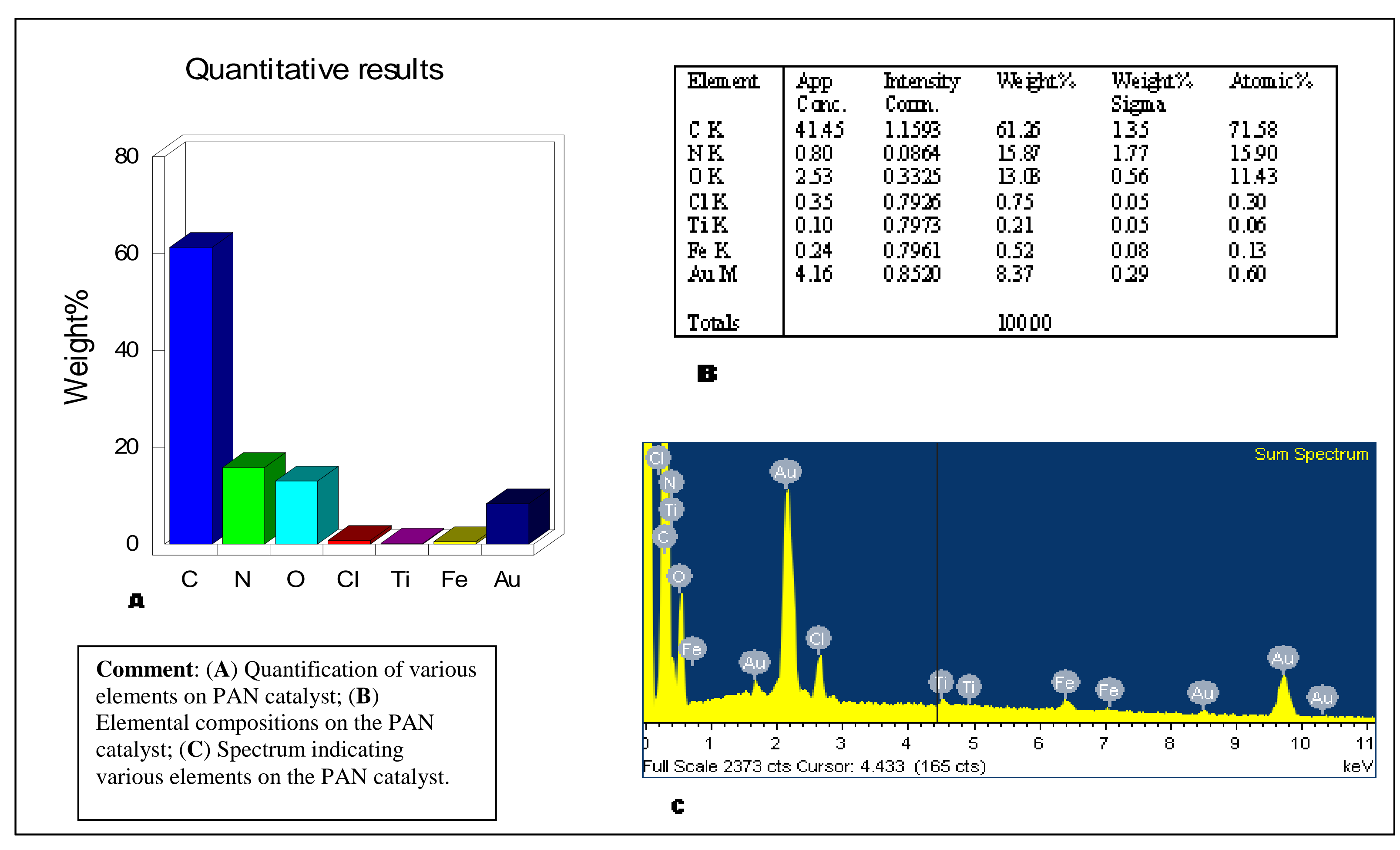
The above differences in the amount of iron on the PAN fibre, as well as its corresponding uneven distribution either within batches or between batches appears to support the differences in the extent of iron leaching described in Chapter 3, Section 3.3.1. As shown, the amounts of iron leached from PAN catalyst within batches or between batches were different. Equally, the amounts of iron digested from 1g of PAN catalyst mesh from different batches was different (Table 3.1). It was suggested that such variations could be due to uneven distribution of the iron on the PAN fibre. It could also possibly be due to high or low affinity of the iron to some of the ligands chelating the iron to the fibre. EDX analysis presented here seems to support the former suggestion. However, EDX is noted to scan only near surface components on materials (Walzak *et al.,* 1998), hence would only scan the surface of the PAN material and therefore is only able to quantify the amount of iron on the surface of the PAN suggesting that iron residing inside the mesh may not be fully quantified. It must be clarified that the penetration depth of the beam on the EDX was not known in this work, however, it is understood that the penetration depth of electrons within a material is associated with the thickness of the material that is traversed by an electron beam of certain known energy (Maqbool *et al.,* 2009). This suggests that the depth of EDX beam penetration depends on the material(s) used. Lee *et al.* (2006) suggested that

electron penetration depth is correlated with the selection of accelerating beam voltage (ABV), thus, the understanding of the electron penetration depth of EDX in certain materials helps in selecting an appropriate ABV.

From the iron leaching work in Chapter 3, it was seen that the ferric sulfate impregnated PAN catalyst has more iron than the ferric chloride PAN catalyst and the former leaches less than the latter. Therefore, it is a reasonable assumption that the differences in the extent of iron leaching from the PAN catalyst may not just be due to the uneven distribution of the iron, but also to the conditions under which they were produced. For example, the ferric chloride PAN catalyst was produced in the laboratory, whereas the ferric sulfate PAN catalyst was produced commercially, and these two different conditions could affect the final product and nature of the catalyst.

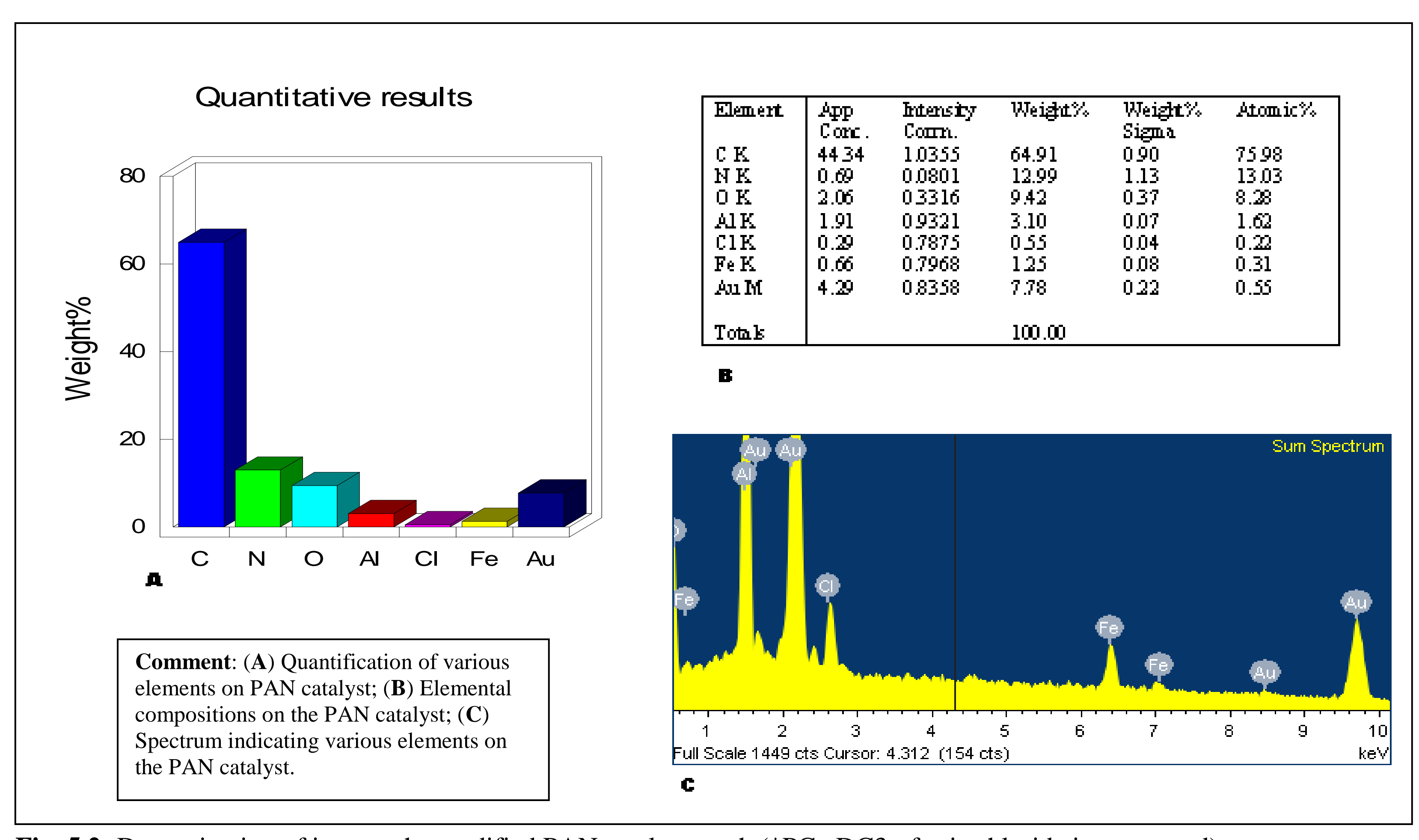
Further, it could be suggested that these variations of iron on the PAN may be contributing factors responsible for the variations observed in the antimicrobial activities between different batches of the PAN catalyst and the observed differences in the reusability tests (Chapter 2).

EDX analysis of modified PAN fibre control showed no indication of the presence of iron on the fibre (Tables 5.1A and 5.1B). Figure 5.6 showed EDX mapping on the PAN mesh surface indicating the spread of elements, and it confirmed that iron only binds to the polyacrylonitrile fibre but not the polypropylene strands in the mesh, as expected.



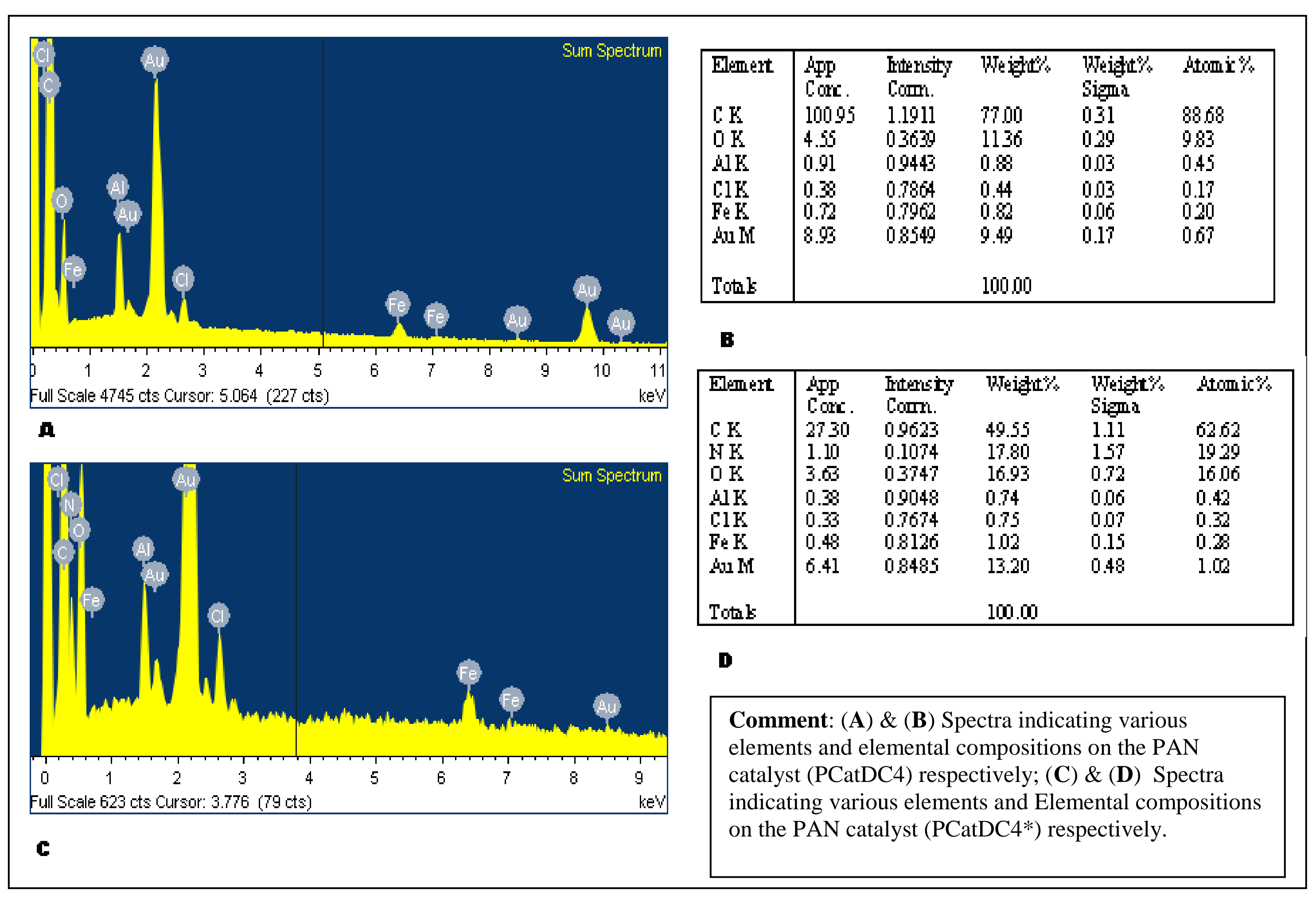
Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
C K	41.45	1.1593	61.26	1.35	71.58
N K	0.80	0.0864	15.87	1.77	15.90
O K	2.53	0.3325	13.03	0.56	11.43
Cl K	0.35	0.7926	0.75	0.05	0.30
Ti K	0.10	0.7973	0.21	0.05	0.06
Fe K	0.24	0.7961	0.52	0.08	0.13
Au M	4.16	0.8520	8.37	0.29	0.60
Totals			100.00		

Fig. 5.1: Determination of iron on the modified PAN catalyst mesh (PCatDC3 = ferric chloride impregnated).



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
C K	44.34	1.0355	64.91	0.90	75.98
N K	0.69	0.0801	12.99	1.13	13.03
O K	2.06	0.3316	9.42	0.37	8.28
Al K	1.91	0.9321	3.10	0.07	1.62
Cl K	0.29	0.7875	0.55	0.04	0.22
Fe K	0.66	0.7968	1.25	0.08	0.31
Au M	4.29	0.8358	7.78	0.22	0.55
Totals			100.00		

Fig. 5.2: Determination of iron on the modified PAN catalyst mesh (*PCatDC3= ferric chloride impregnated)



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
C K	100.95	1.1911	77.00	0.31	88.68
O K	4.55	0.3639	11.36	0.29	9.83
Al K	0.91	0.9443	0.88	0.03	0.45
Cl K	0.38	0.7864	0.44	0.03	0.17
Fe K	0.72	0.7962	0.82	0.06	0.20
Au M	8.93	0.8549	9.49	0.17	0.67
Totals			100.00		

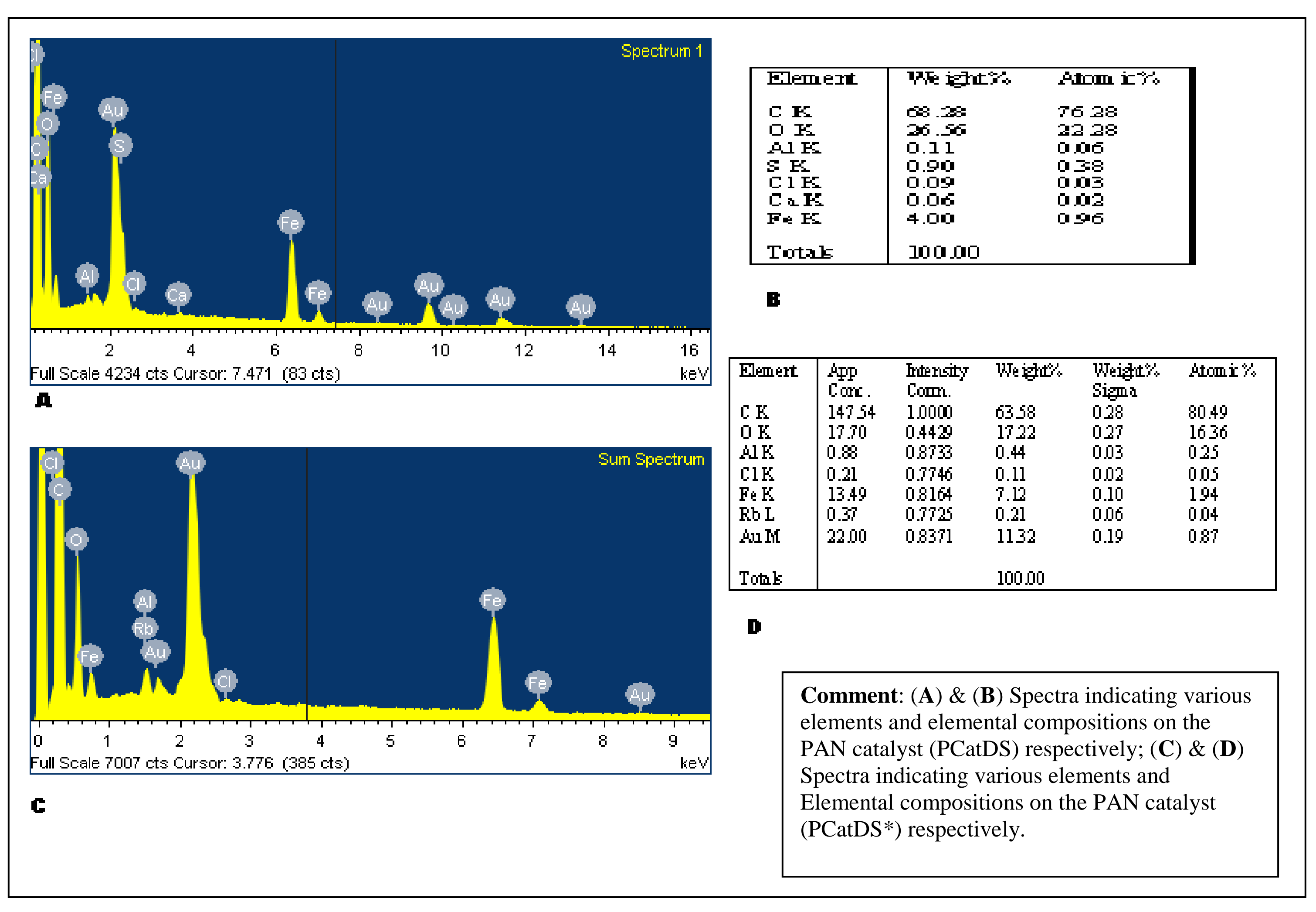
B

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
C K	27.30	0.9623	49.55	1.11	62.62
N K	1.10	0.1074	17.80	1.57	19.29
O K	3.63	0.3747	16.93	0.72	16.06
Al K	0.38	0.9048	0.74	0.06	0.42
Cl K	0.33	0.7674	0.75	0.07	0.32
Fe K	0.48	0.8126	1.02	0.15	0.28
Au M	6.41	0.8485	13.20	0.48	1.02
Totals			100.00		

D

Comment: (**A**) & (**B**) Spectra indicating various elements and elemental compositions on the PAN catalyst (PCatDC4) respectively; (**C**) & (**D**) Spectra indicating various elements and Elemental compositions on the PAN catalyst (PCatDC4*) respectively.

Fig. 5.3: Determination of iron on the modified PAN catalyst mesh (PCatDC4 and *PCatDC4 = ferric chloride impregnated).



Element	Weight%	Atomic%
C K	68.28	76.28
O K	26.56	22.28
Al K	0.11	0.06
S K	0.90	0.38
Cl K	0.09	0.03
Ca K	0.06	0.02
Fe K	4.00	0.96
Totals	100.00	

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
C K	147.54	1.0000	63.58	0.28	80.49
O K	17.70	0.4429	17.22	0.27	16.36
Al K	0.88	0.8733	0.44	0.03	0.25
Cl K	0.21	0.7746	0.11	0.02	0.05
Fe K	13.49	0.8164	7.12	0.10	1.94
Rb L	0.37	0.7725	0.21	0.06	0.04
Au M	22.00	0.8371	11.32	0.19	0.87
Totals			100.00		

Comment: (**A**) & (**B**) Spectra indicating various elements and elemental compositions on the PAN catalyst (PCatDS) respectively; (**C**) & (**D**) Spectra indicating various elements and Elemental compositions on the PAN catalyst (PCatDS*) respectively.

Fig. 5.4: Determination of iron on the modified PAN catalyst mesh (PCatDS and *PCatDS= ferric sulfate impregnated).

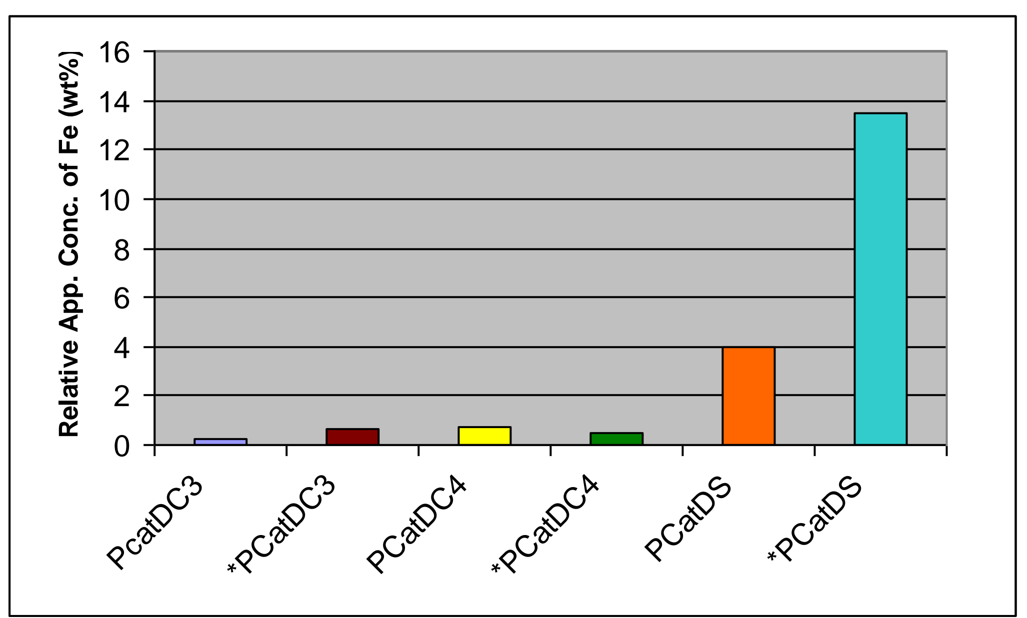


Fig. 5.5: Concentration of iron within and between batches on modified PAN catalyst.

App. Conc. = apparent concentration; Fe = iron, wt% = weight percentage; PCatDC3 and *PCatDC3 are catalyst fibres from different sections of the same batch of ferric chloride PAN catalyst; PCatDC4 and *PCatDC4 are catalyst fibres from different sections of the same batch of ferric chloride PAN catalyst; PCatDS and *PCatDS are catalyst fibres from different sections of the same batch of ferric sulfate PAN catalyst.

Table 5.1: Control: (A) and (B) Determination of various elements on modified PAN mesh

Element	App. Conc.	Intensity	Weight%	Weight% Sigma	Atomic%
C	28.50	1.30	60.48	1.28	68.97
N	0.70	0.09	22.33	1.54	21.84
O	1.09	0.30	10.07	0.57	8.61
Cl	0.07	0.80	0.25	0.04	0.10
Au	2.10	0.85	6.87	0.31	0.48
Totals			100.00		

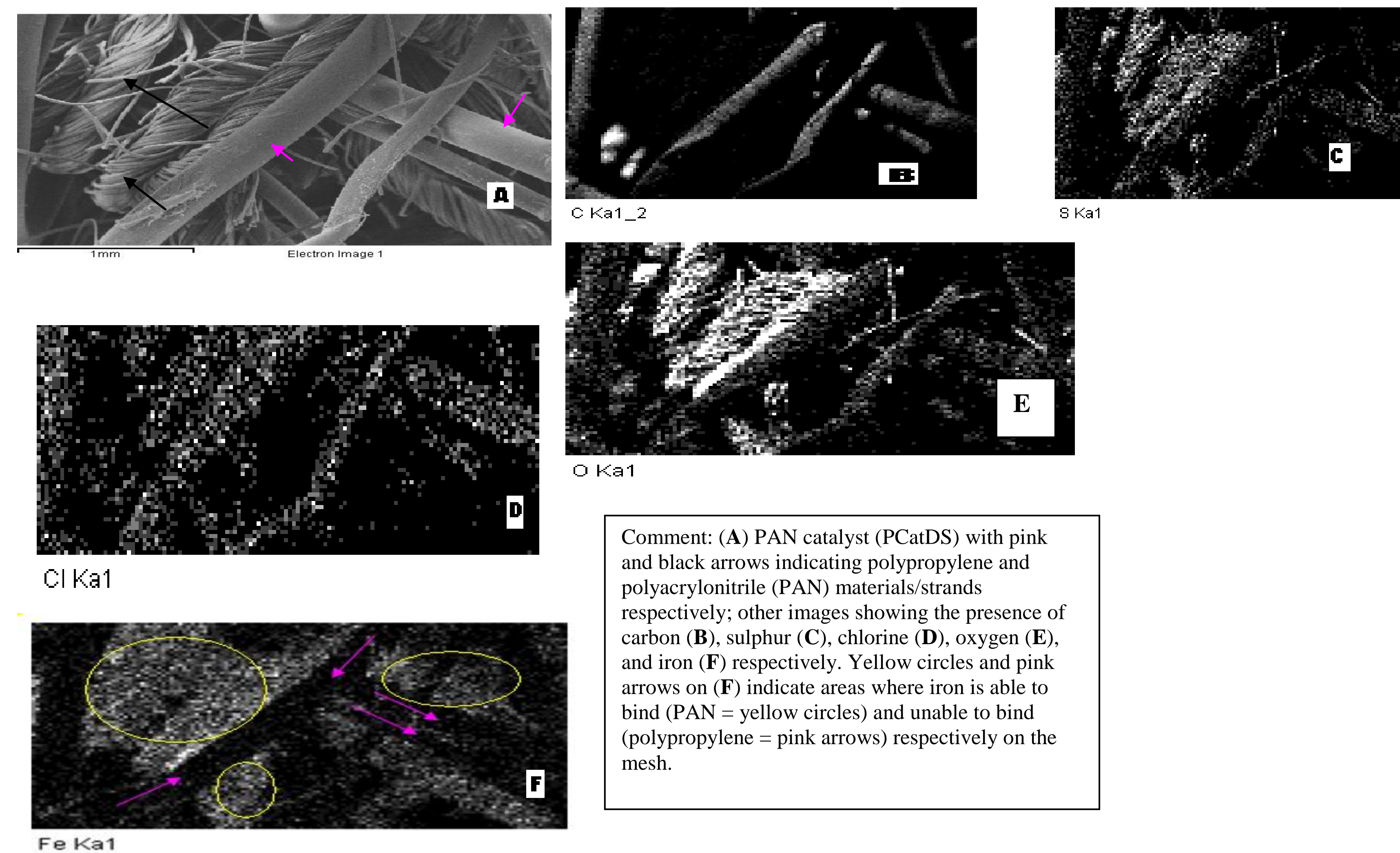
A

Element	App. Conc.	Intensity	Weight%	Weight% Sigma	Atomic%
C	27.60	0.97	62.13	0.71	72.74
N	5.79	0.08	15.36	0.93	15.42
O	13.86	0.32	9.36	0.26	8.22
Al	24.00	0.94	5.60	0.07	2.92
Cl	0.81	0.78	0.23	0.02	0.09
Ti	0.45	0.80	0.12	0.02	0.04
Cu	1.34	0.76	0.39	0.05	0.09
Au	25.50	0.82	6.81	0.14	0.49
Totals			100.00		

B

Comments: Elemental assessment on the modified PAN mesh surface showing the absence of iron.App.

Conc. = Apparent concentration.



Comment: (**A**) PAN catalyst (PCatDS) with pink and black arrows indicating polypropylene and polyacrylonitrile (PAN) materials/strands respectively; other images showing the presence of carbon (**B**), sulphur (**C**), chlorine (**D**), oxygen (**E**), and iron (**F**) respectively. Yellow circles and pink arrows on (**F**) indicate areas where iron is able to bind (PAN = yellow circles) and unable to bind (polypropylene = pink arrows) respectively on the mesh.

Fig. 5.6: SEM-EDX showing elemental distribution on surface of PAN catalyst mesh

5.3.2. The nature of bacterial cells attached to PAN catalyst mesh by SEM analysis.

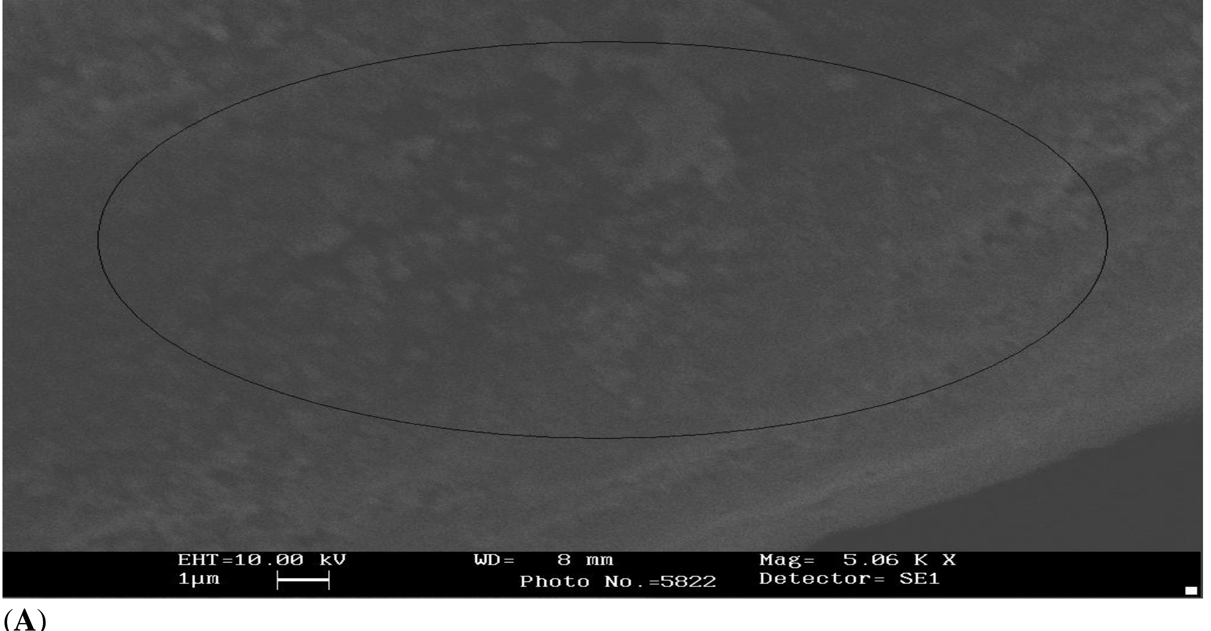
E. coli and *S. aureus* attachment onto the PAN catalyst mesh was observed by SEM to establish whether bacterial adhesion was present (whether the cells are intact or lysed) on the PAN catalyst mesh after antimicrobial treatment. If bacterial adhesion was not present on the PAN catalyst mesh this could possibly be due to cell lysis as a result of the antimicrobial activity of PAN catalyst/ H_2O_2 system. The control samples with no microbial and/or H_2O_2 treatment in Figures 5.7A and 5.7B show that there is no microbial attachment on the surfaces of either the ferric sulfate (PCatDS) or ferric chloride (PCatDC3) impregnated PAN catalyst mesh. Control samples treated with a suspension of *E. coli* in water showed *E. coli* attachment on PCatDS, PCatDC3 (Figures 5.8A and 5.8B), and modified PAN mesh (MOPM) (Figure 5.9A). Control samples treated with a suspension of *E. coli* containing 0.2% w/v H_2O_2 after 1 hr contact showed *E. coli* attachment on both neutralised MOPM and un-neutralised MOPM (Figures 5.9B and 5.10A). PAN catalyst samples treated with a suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr contact time, showed the attachment of *E. coli* on both neutralised or un-neutralised PAN catalysts (Figures 5.10B, 5.11A and 5.11B, 5.12). The treatment with *S. aureus* in the presence or absence of 1% w/v H_2O_2 showed similar trend of attachment on MOPM, PCatDS, and PCatDC3 to *E. coli* (Figures 5.13A and 5.13B; 5.14A and 5.14B; 5.15A and 5.15B; 5.16A and 5.16B).

The results portrayed above indicated that microorganisms were not attached to the commercially produced ferric sulfate PAN catalyst (PCatDS) and the laboratory produced ferric chloride PAN catalyst (PCatDC3) respectively, prior to experimental use; however, the commercially produced catalyst (PCatDS) appeared to have a large amount of debris on it surfaces (Figure 5.7A) as compared to PCatDC3, which showed a relatively clean and smooth surface. These differences in surface character between PCatDS and PCatDC3 may be due to the conditions under which the two catalysts were produced and stored. PCatDC3 was prepared under strict aseptic and sterilised conditions including a robust washing process which may have reduced any adherence of debris and environmental microbes to

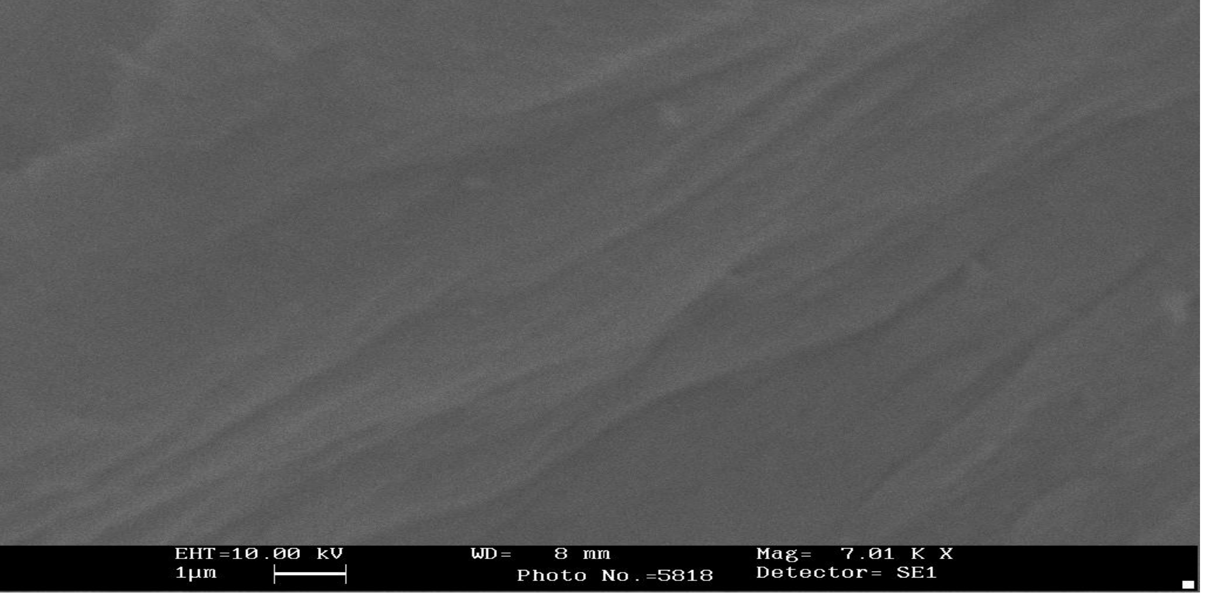
the catalyst and was stored in an environment free from debris. This might not have been the case for the preparation and storage of PCatDS as it was received prior to use from an unsterile environment. Although, microorganisms were not found on the surfaces of PCatDS.

It is known that the production of PCatDS was run at a high speed on rollers and is therefore likely to have been abraded, which may appear as debris on the SEM micrograph. These differences in surface characteristics as portrayed above were equally evident in the control when PCatDS and PCatDC3 were treated with either *E. coli* or *S. aureus* (Figures 5.8A, 5.8B, 5.12A and 5.12B). Although, debris seems to be prevalent on the surfaces of PCatDS, it did not affect the identification of *E. coli* and *S. aureus* on the PAN mesh. It is possible that this debris may have contributed to the reduced antimicrobial activity of the commercially produced catalyst in comparison to those produced at the laboratory even though, as shown from the EDX analysis, the commercially produced catalyst contained more iron than the laboratory produced ones. Thus, some of the debris may have prevented H_2O_2 gaining access to the catalytically active sites preventing the generation of $^{\cdot}OH$ radicals leading to relatively reduced antimicrobial activity. Other control tests further suggest that *E. coli* and *S. aureus* were able to bind to the modified PAN mesh (MOPM) either in the absence (Figures 5.9A) or presence of H_2O_2 (Figures 5.9B, 5.10A; and Figures 5.14A, 5.14B) and this further suggests that microbial binding does not necessarily depend on the PAN mesh being impregnated with iron salts. Thus, the microbes are capable of binding to the PAN mesh whether impregnated or not. Experimental work involving PAN catalyst (either PCatDS or PCatDC3) and H_2O_2 showed the attachment of either *E. coli* or *S. aureus* cells on the PAN catalyst at 1hr contact time and that the further step of neutralisation did not affect attachment (compare Figures 5.10B and 5.11A; 5.11B and 5.12; 5.15A and 5.15B; 5.16A and 5.16B).

Comparing control and experimental samples, SEM micrographs indicated that microbial cells maintained the same cellular appearance before and after their exposure to the toxic effects of $^{\cdot}OH$ radicals. Hence, this may support total cell count experiments shown in Chapter 4, Section 4.3.3., that microbial cells do not lyse during $^{\cdot}OH$ radical attack, but maintain their intact cellular structure. However, the SEM identification of intact cell structures attached to the PAN catalyst after exposure to $^{\cdot}OH$ radical attack did not show whether these cells were alive or dead, hence, further work was required to assess the viability of these cells.



(**A**)



(**B**)

Fig. 5.7: SEM of PAN catalyst surface control: (**A**) Circled area showing debris on the surface of PCatDS (ferric sulfate impregnated); (**B**) Surface of PCatDC3 (ferric chloride impregnated) indicating no debris or environmental microbial attachment.

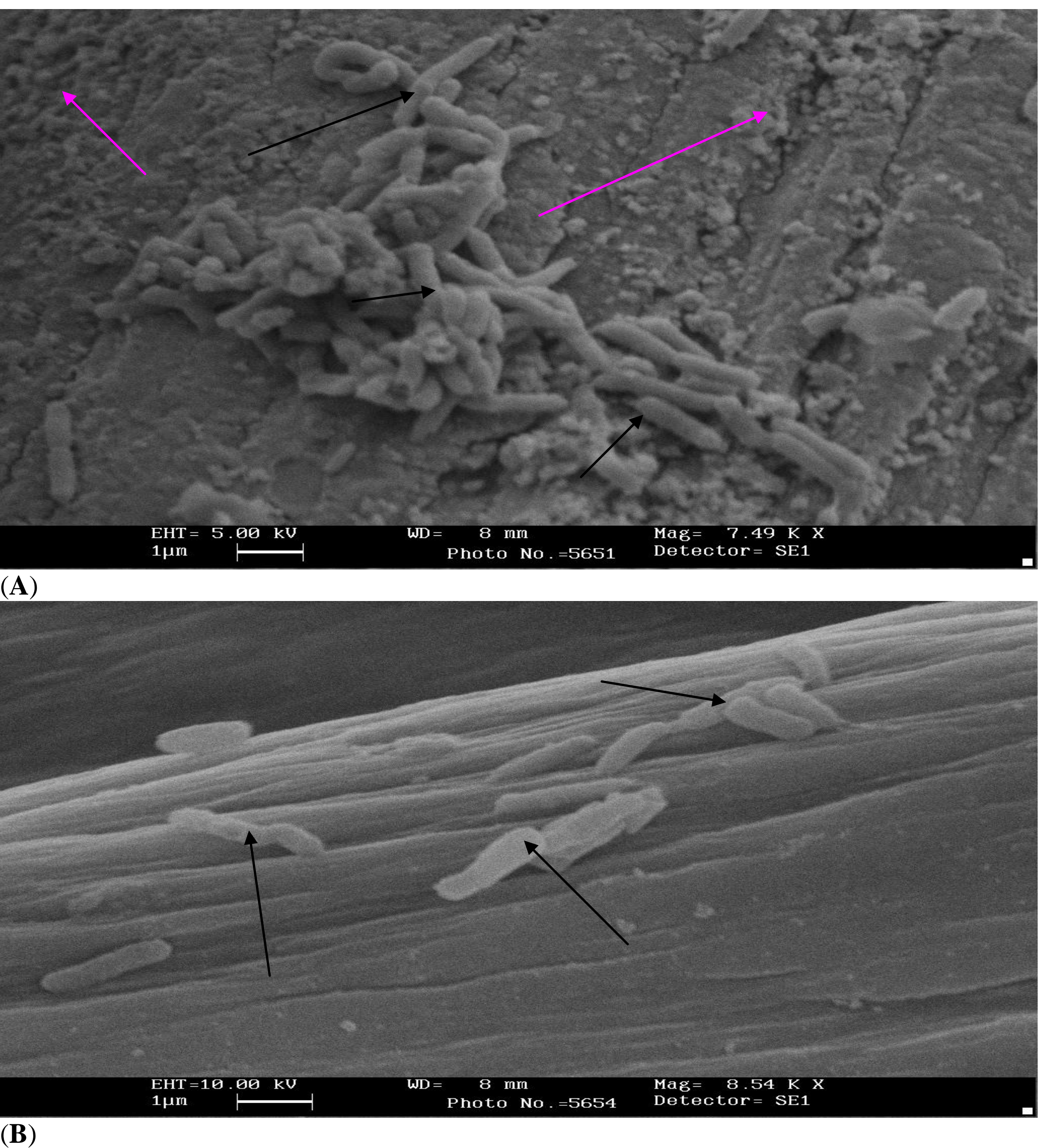


Fig. 5.8: SEM of PAN catalyst surface after immersion in aqueous suspension of *E. coli* for 1 hr Controls: (**A**) Black arrows indicating attachment on PCatDS (ferric sulfate impregnated) (Pink arrows showing areas of debris on the mesh); (**B**) Black arrows indicating *E. coli* attachment on PAN catalyst on PCatDC3 (ferric chloride impregnated).

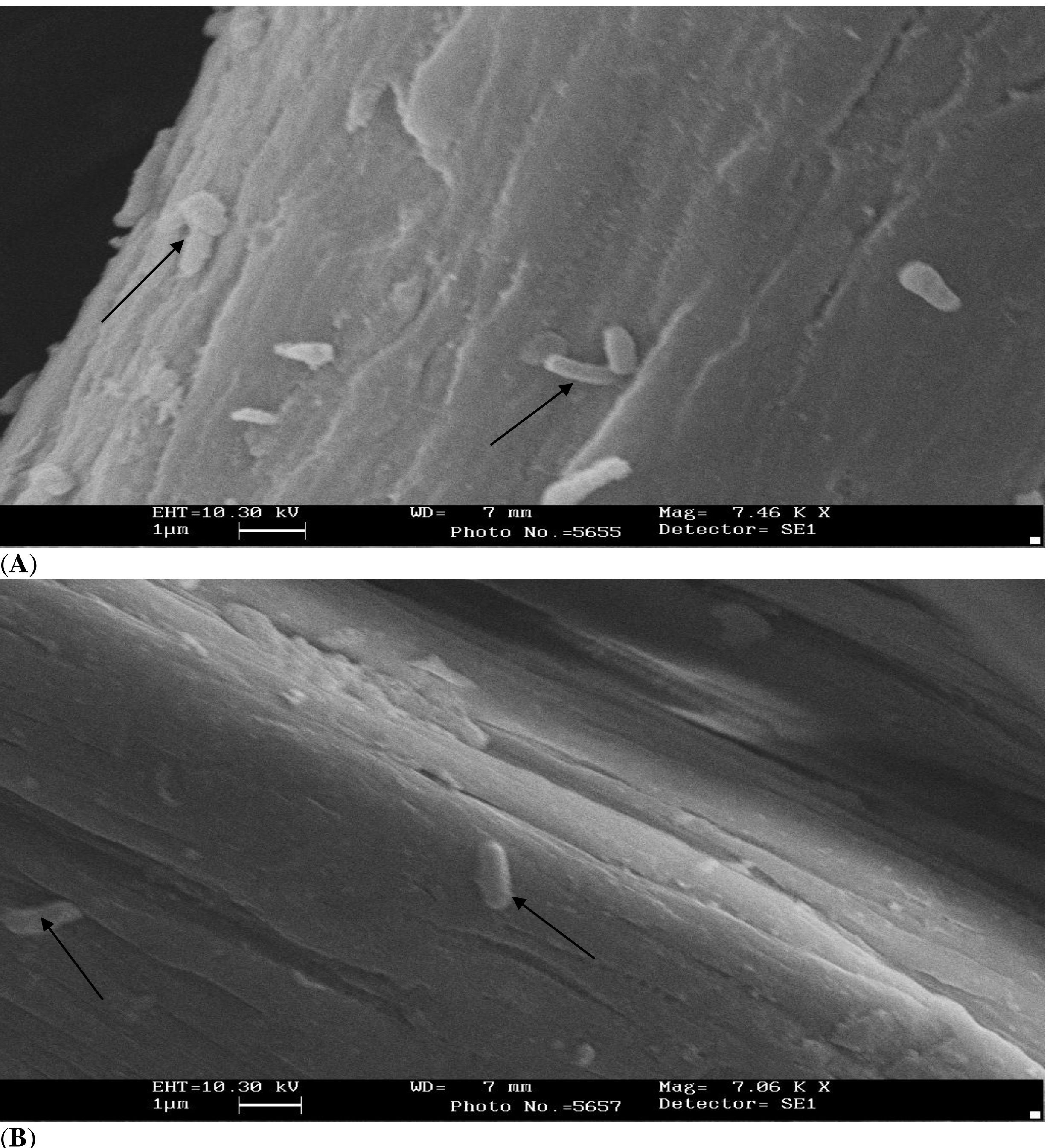
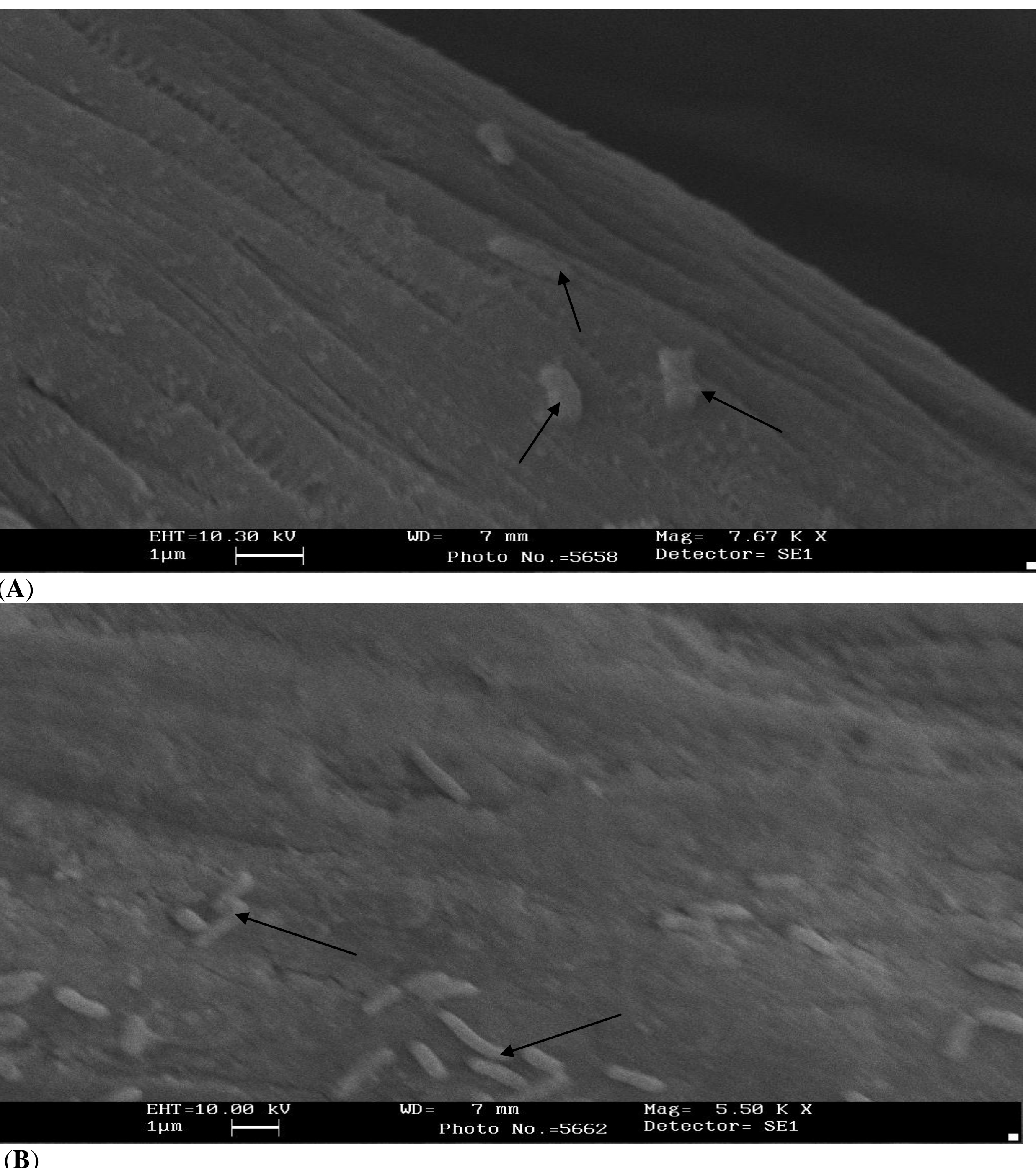


Fig. 5.9: SEM of modified PAN mesh surface after immersion in aqueous suspension of *E. coli* for 1 hr Controls: (**A**) Black arrows indicating *E. coli* attachment on modified PAN mesh (MOPM = no iron impregnation); (**B**) Black arrows indicating *E. coli* attachment on modified PAN mesh after contact with an aqueous suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr and after neutralisation.



(**A**)

(**B**)

Fig. 5.10: SEM of PAN surfaces after immersion in aqueous suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr (**A**) Control: Black arrows indicating *E. coli* attachment on modified PAN mesh (no neutralisation); (**B**) Experiment: Black arrows indicating *E. coli* attachment on PAN catalyst mesh (PCatDS) (after neutralisation).

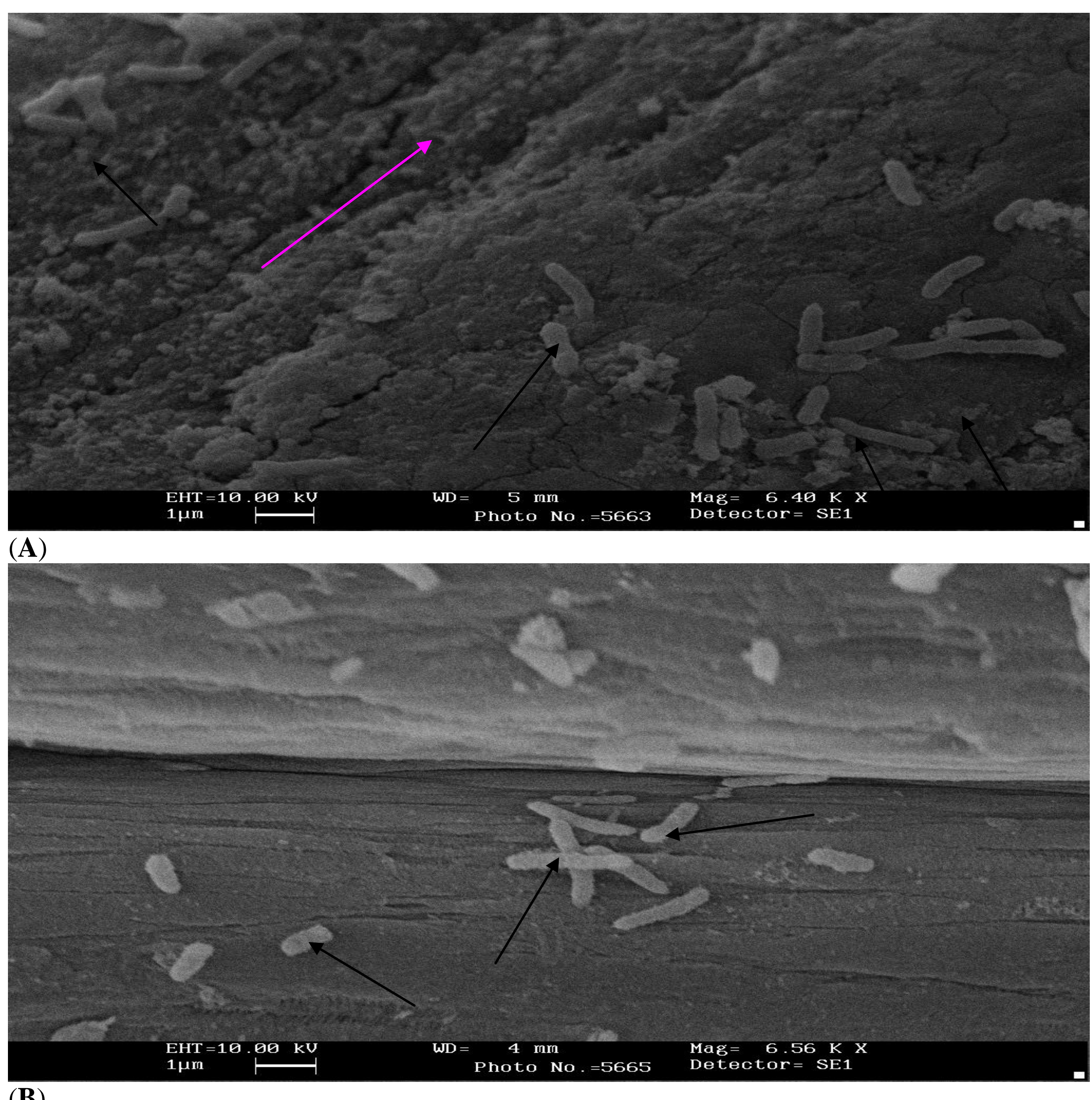


Fig. 5.11: SEM of PAN catalyst surface after immersion in aqueous suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr Experiment: (**A**) Black arrows indicating *E. coli* attachment on PCatDS) (no neutralisation). (Pink arrow showing areas of debris on the mesh); (**B**) Black arrows indicating *E. coli* attachment on PCatDC3 (after neutralisation).

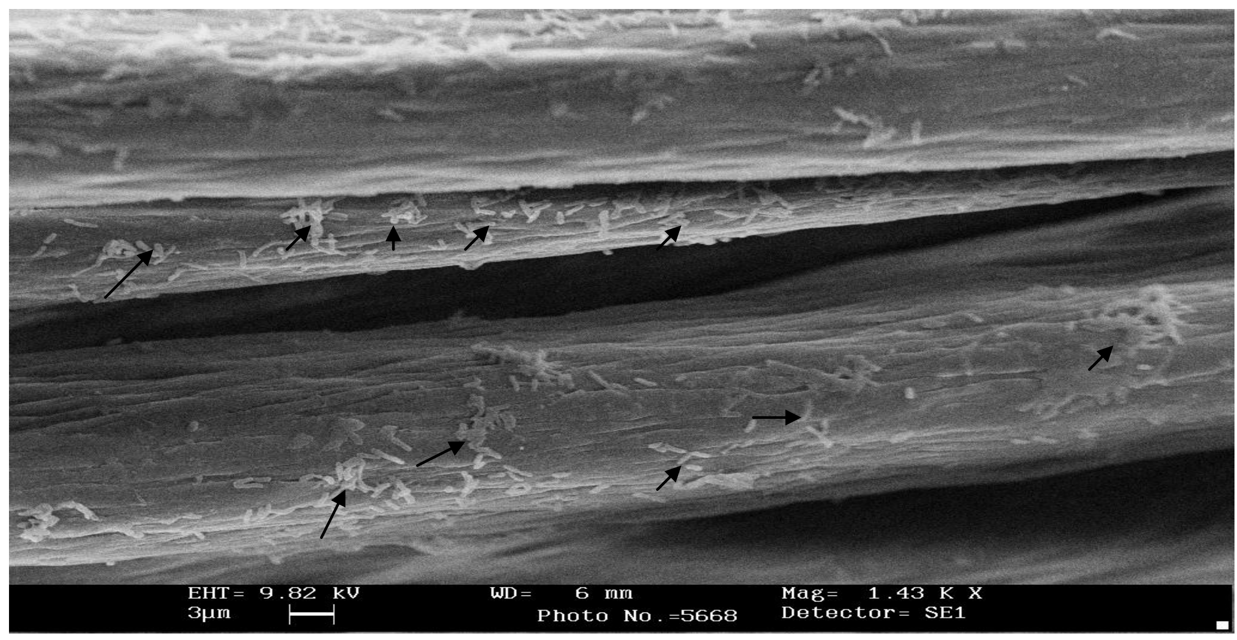


Fig 5.12: SEM of PAN catalyst surface after immersion in aqueous suspension of *E. coli* containing 0.2% w/v H_2O_2 for 1 hr Experiment: (At low magnification) Black arrows indicating abundance of *E. coli* scattered on PCatDC3 (no neutralisation).

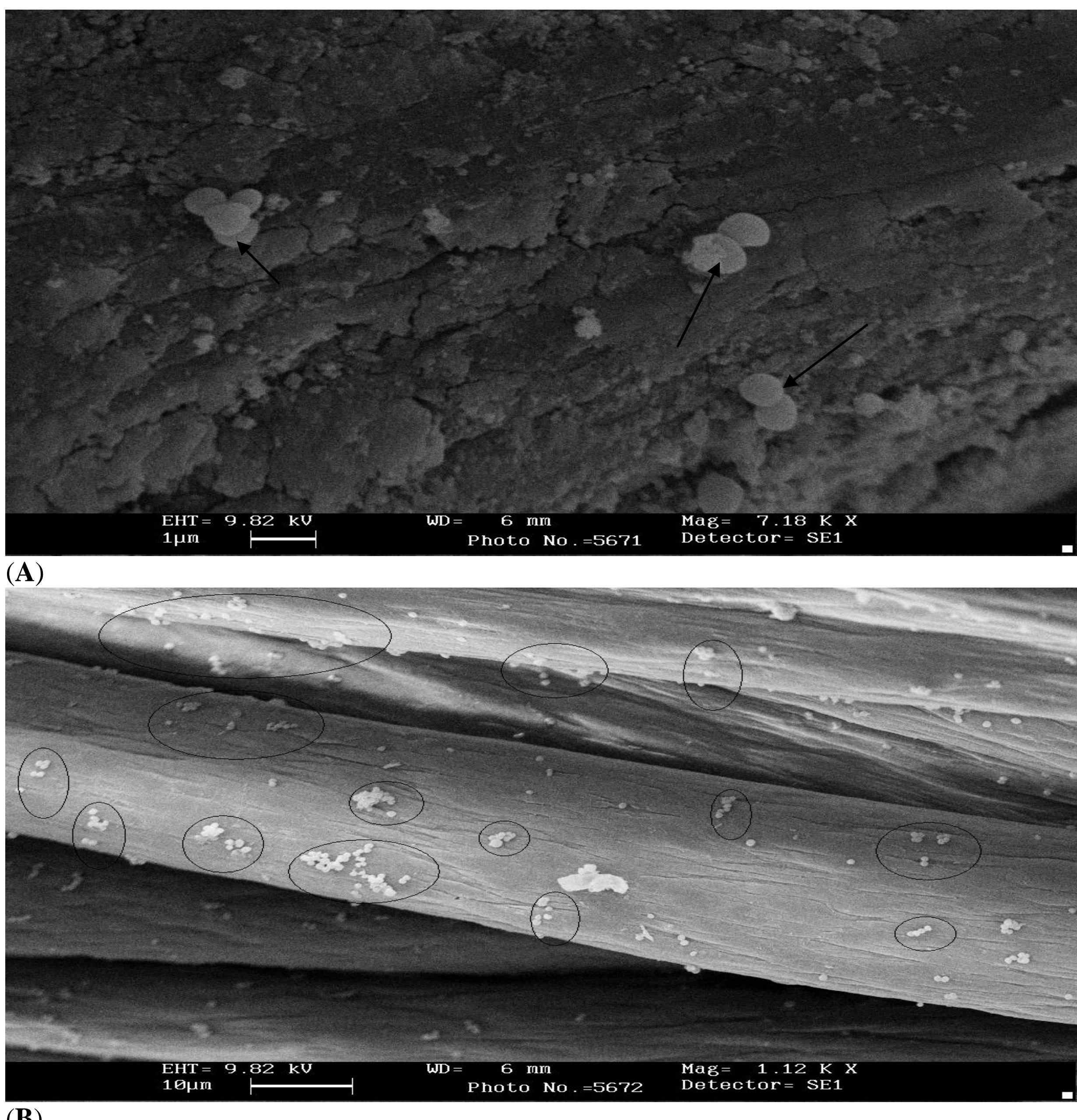


Fig. 5.13: SEM of PAN catalyst surface after immersion in aqueous suspension of *S. aureus* for 1 hr Control: (**A**) Black arrows indicating attachment of *S. aureus* on PCatDS; (**B**) Areas in circles indicating attachment of *S. aureus* on PCatDC3.

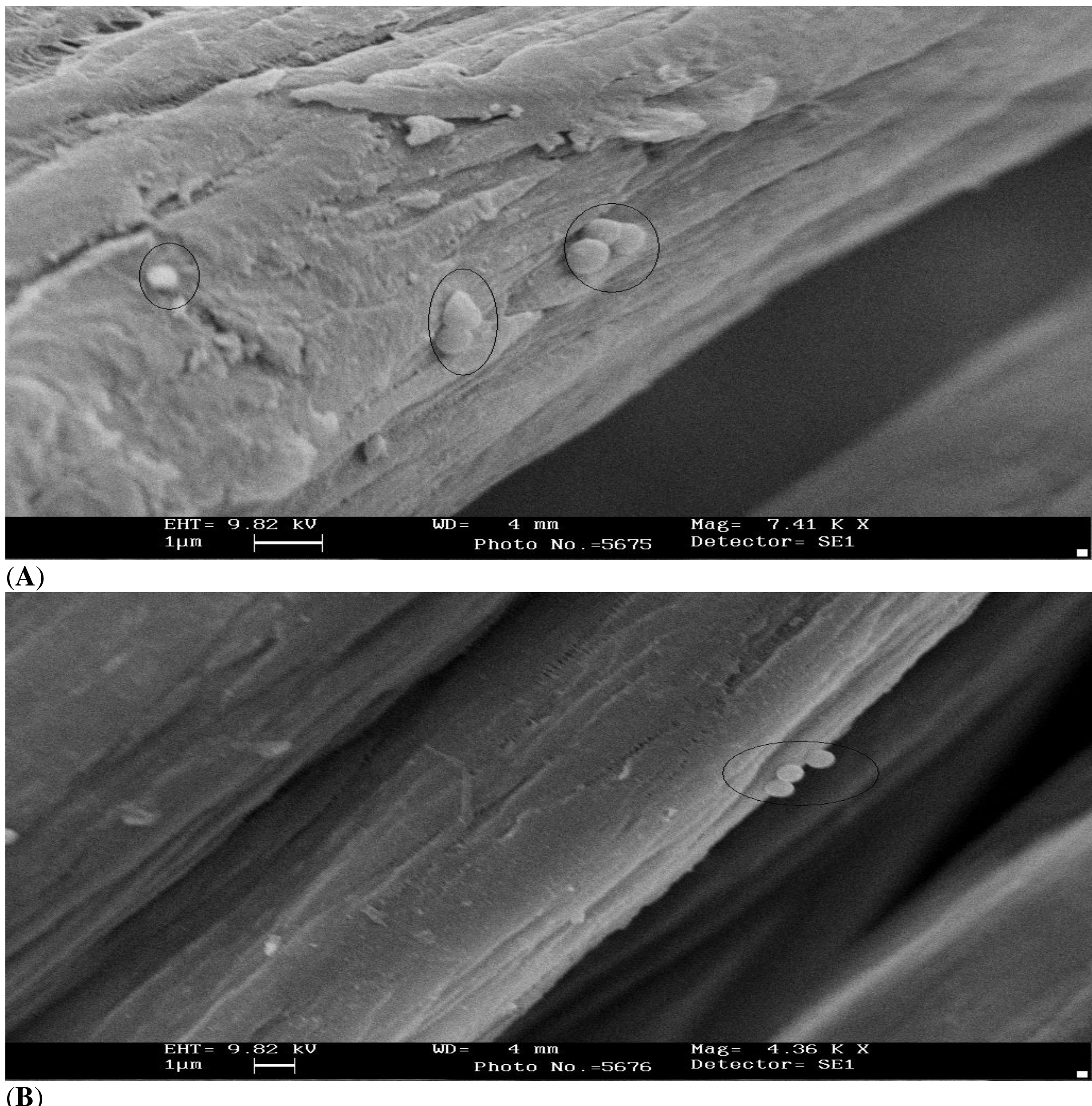
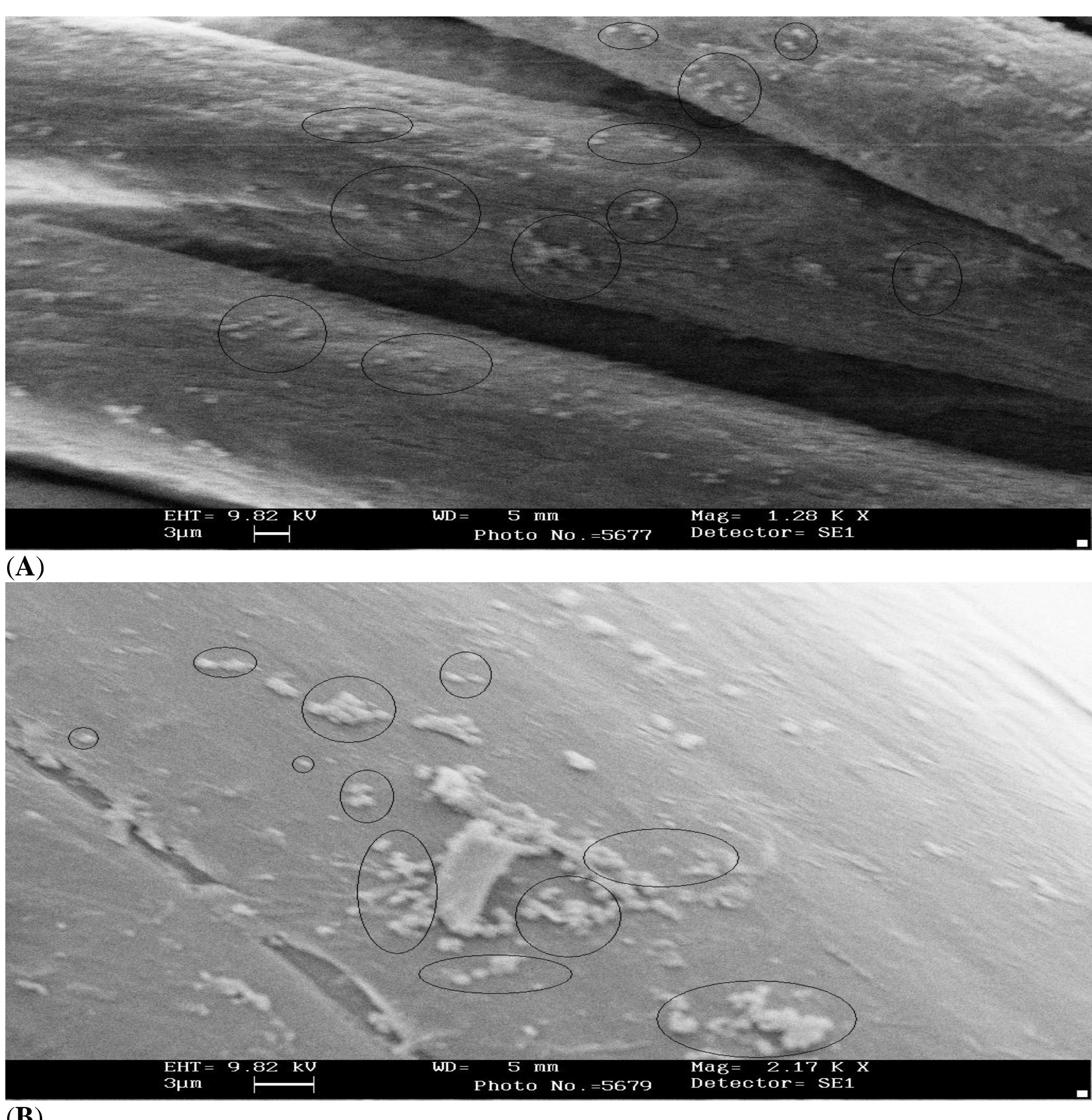


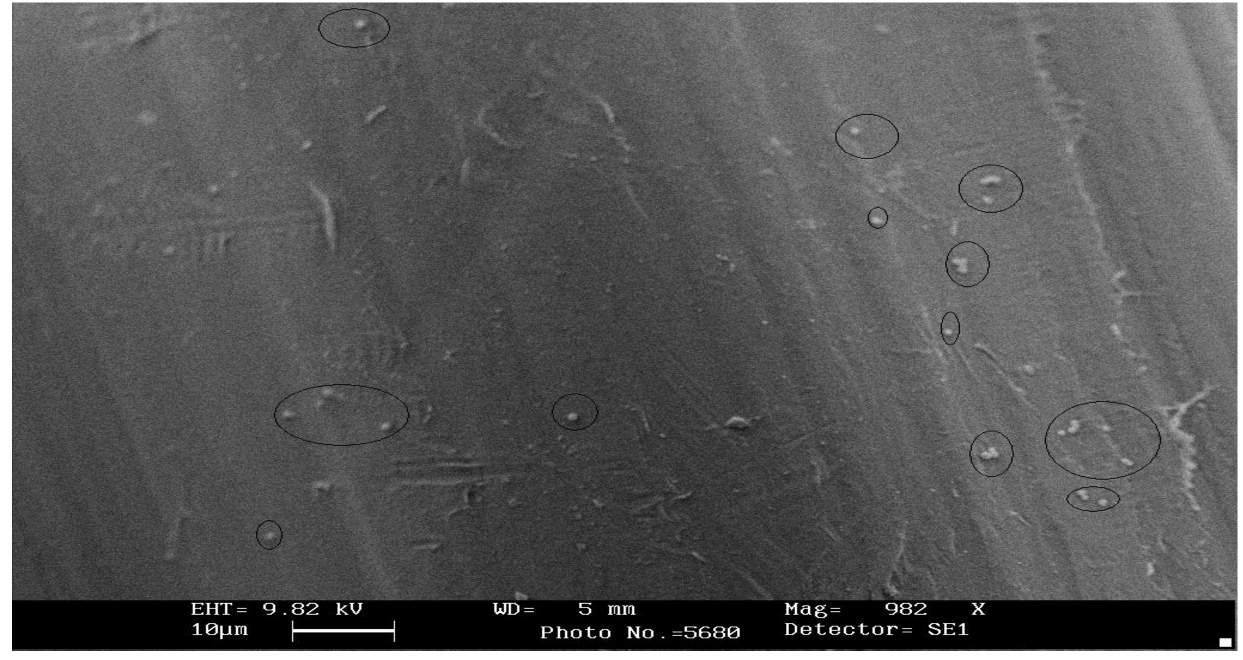
Fig. 5.14: SEM of modified PAN mesh surface after immersion in aqueous suspension of *S. aureus* containing 1% w/v H_2O_2 for 1 hr Control: (**A**) Areas in circles indicating attachment of *S. aureus* on modified PAN mesh (after neutralisation); (**B**) Areas in circle indicating attachment of *S. aureus* on modified PAN mesh (no neutralisation).



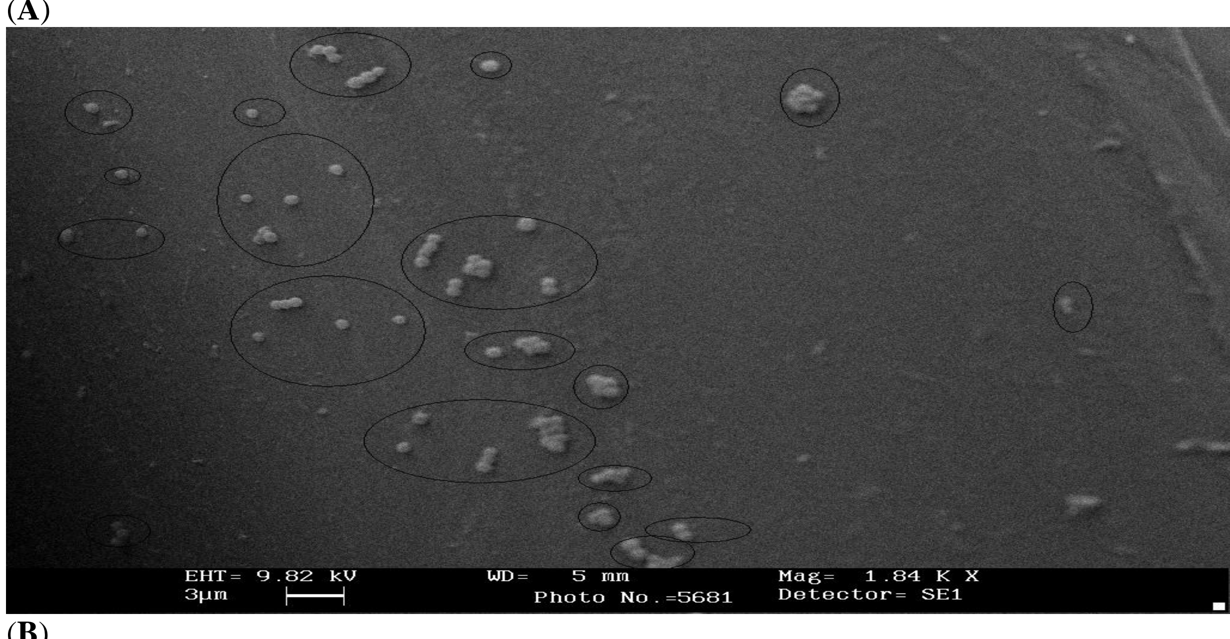
(**A**)

(**B**)

Fig. 5.15: SEM of PAN catalyst surface after immersion in aqueous suspension of *S. aureus* containing 1% w/v H_2O_2 for 1 hr Experiment: (**A**) (At low magnification) Circled areas indicating attachment of *S. aureus* on PCatDS (after neutralisation); (**B**) (At low magnification) Circled areas indicating attachment of *S. aureus* on PCatDS) (no neutralisation).



(**A**)



(**B**)

Fig. 5.16: SEM of PAN catalyst surface after immersion in aqueous suspension of *S. aureus* containing 1% w/v H_2O_2 for 1 hr Experiment: (**A**) (At low magnification) Circled areas indicating attachment of *S. aureus* on PCatDC3 (after neutralisation); (**B**) (At low magnification) Circled areas indicating attachment of *S. aureus* on PCatDC3) (no neutralisation).

5.3.3. Confocal Laser Scanning Microscopy (CLSM) of microbial cells on PAN catalyst and in experimental solution using LIVE/DEAD kit

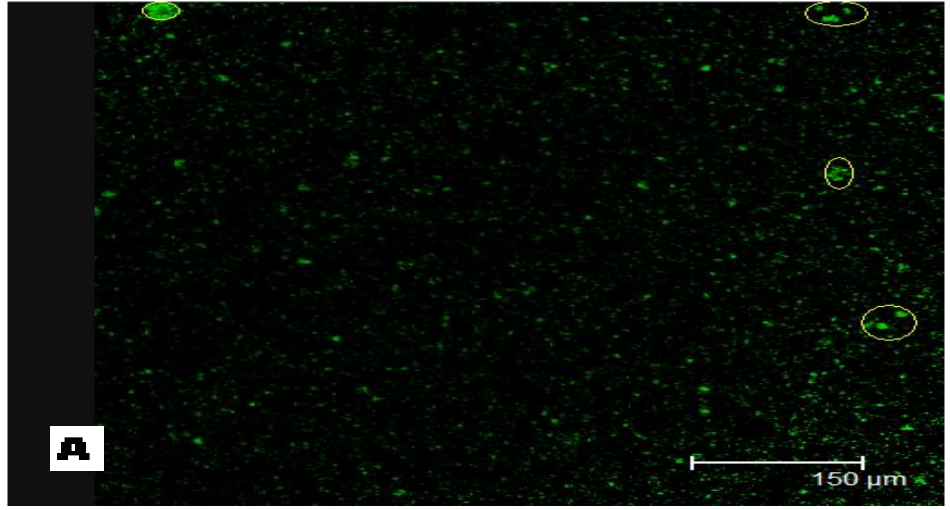
The microbial cells on both PAN catalyst and in experimental solution were qualitatively assessed using LIVE/DEAD kit to establish as to whether the cells were alive or dead after 1 hr contact time in the catalyst/hydrogen peroxide system. LIVE/DEAD kit contained a mixture of SYTO 9 and propidium iodide stains; the former binds to live bacterial cells with intact membranes to give a green fluorescence, whereas the latter binds or penetrates damaged bacterial membranes to fluoresce red (Molecular Probes, Invitrogen Detection Technologies, Paisley, UK). The LIVE/DEAD kit has been utilised in a number of studies to identify live and dead cells after antimicrobial activity in real time. For example, it has been employed to distinguish live and dead cells of *Lactobacillus paracasei*, *Lactobacillus salivarius*, *Bifidobacterium* sp. (Gardiner *et al.,* 2000; Auty *et al.,* 2001); *Pseudomonas fragi*, *Listeria monocytogenes* (Maukonen *et al.,* 2000); extremophilic archaea (Leuko *et al.,* 2004); and *Pseudomonas aeruginosa* and *S. aureus* (Newman *et al.,* 2006).

In the present study both live and dead *E. coli* cells were identified on the PAN catalyst (PCatDS) and predominantly dead cells were identified in the experimental solutions. Control tests involving *E. coli cells* suspended in water revealed predominantly green fluorescence (live cells) (Figures 5.17A and 5.17B) equally, only live *E. coli* cells were observed on PCatDS when *E. coli* cells were subjected to PCatDS in water alone (Figures 5.18B and 5.18C). Stained PCatDS without the presence of *E. coli* showed no microbial attachment to the PAN fibre (Figure 5.18A). This suggests that the surface debris observed using SEM did not contain environmental microbes (Fig 5.7A).
Further control tests involving *E. coli* cells subjected to 70% alcohol showed positive identification of red fluorescence (dead cells) (Figures 5.19A and 5.19B).
Neutralised or un-neutralised experimental solutions after 1hour contact time with PCatDS in 0.2% w/v H_2O_2 with *E. coli*, showed predominately red fluorescence indicating dead *E. coli* cells (Figures 5.20A and 5.20B; and 5.21A and 5.21B). However, the central sections of clumped cells in the neutralised experimental solution gave a yellow fluorescence. The

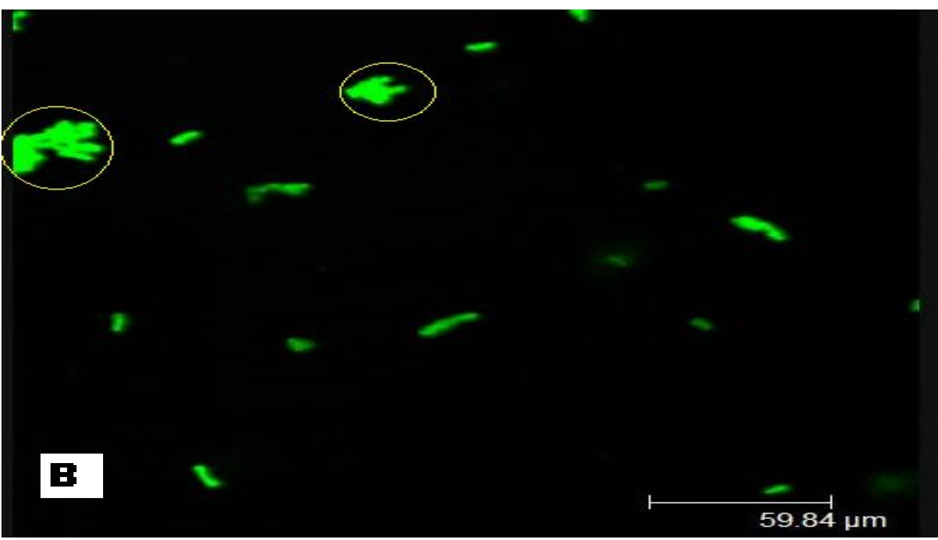
reason for this was possibly due to the inability of propidium iodide stain to penetrate into the damaged cell membrane completely and that the concentration of SYTO 9 in these areas was high. Another reason could also be that the cells may be in the process of dying which resulted in the yellow fluorescence when propidium iodide was present. To elucidate whether the above assertions are true, further work could be performed by staining the bacteria with individual dyes separately instead of mixing them together as was done in the present work. Such work could demonstrate a characterised effect of propidium iodide in the absence of SYTO 9 on both clumped and single cells. Furthermore, the mixture could be prepared with a decreased volume of SYTO 9 and an increased volume of propidium iodide and then compared with the results of the present work. As mentioned earlier, both SYTO 9 and propidium iodide are known to differ in their spectra characteristics which correlate to their differing ability to penetrate healthy bacterial cells. When used alone, SYTO 9 generally labels all bacteria in population including those with intact cell membranes and those with damaged membranes. Thus, SYTO 9 is a cell-permeant nucleic acid stain that exhibits a large fluorescence enhancement upon binding to nucleic acid such as ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in both live and dead eukaryotic cells as well as Gram-positive and Gram-negative bacteria (Molecular Probes, Invitrogen Detection Technologies, 2004). However, the presence of propidium iodide reduces the activity of SYTO 9 by giving damaged cells a red fluorescence (Molecular Probes, Invitrogen Detection Technologies, 2004). Propidium iodide specifically binds to DNA in cells only with permeabilised membranes. Because of this property, it is commonly use in evaluating cell cycle, aneuploidy and apoptosis. For example, propidium iodide is employed to differentiate necrotic cells or cells in late stages of apoptosis with permeabilised membranes from viable cells or early stages of apoptosis where membranes are intact and cannot bind to propidium iodide (Molecular Probes, Invitrogen Detection Technologies, 2004). The above explanations could suggest that a complete penetration of propidium iodide to enable it bind to all the DNA in damaged cells was compromised as a result of clumped cells. It is therefore appropriate at this stage to assume that the yellow fluorescence in clumped cells may in fact be dead *E. coli* and not live cells, as it has been shown that *E. coli* cells under these experimental conditions were killed in 40mins (Chapter

2, Section 2.4.6.). It is known that some bacteria can be in a viable but non-culturable (VBNC) state, in this state, bacteria are still viable and exhibit metabolic activity and respiration, but cannot be shown as colony forming units using the conventional plate counts and remain hidden (Sardessai, 2005; Smith *et al.,* 2002). Hence, if live cells were identified under these experimental conditions, then it is possible that they are VBNC cells which would explain the observed results. Another possibility is that clumping protects some of the cells from the biocides action.

Interestingly, a mixture of live and dead cells was identified on PCatDS after neutralisation (Figures 5.22A and 5.22B); however, only dead cells were observed on PCatDS when it was not neutralised (Figures 5.23A and 5.23B), presumably because the H_2O_2 had not been destroyed and was still active with the catalyst in killing cells. The identification of live cells on the PAN catalyst in the presence of H_2O_2 after 1 hour contact time was unexpected because cells in solution were completely killed at 40mins contact time. Cells were not expected to survive on the surface of the PAN catalyst since there are likely to be more $^{\bullet}OH$ radicals generated at the PAN catalyst surface than in solution, and it can be assumed that microbial cells closer to the surface of the PAN catalyst would be inactivated faster than those in solution. However, this assumption appears not to have been the case, rather it has now been shown that microbial cells in experimental solution are completely killed, whilst some cells on the PAN catalyst manage to escape inactivation. The data suggests that there may be relative protection for microbial cells on the PAN catalyst compared to in solution. As mentioned earlier, if the cells are clumped then H_2O_2 may not be able to inactivate them. The mechanism of microbial cell adhesion/interaction with the PAN catalyst which leads to protection from inactivation warrants further work.



ZF = 1



ZF = 5.01

Fig. 5.17: Confocal Laser Scanning Microscopy using Live/Dead dyes of an aqueous suspension of *E. coli* Control: **A** and **B** indicate green fluorescence of live *E. coli* cells suspended in water (circled areas are clumped cells).

Images were taken at X40 magnification with additional digital magnification (zoom factor, ZF as shown above).

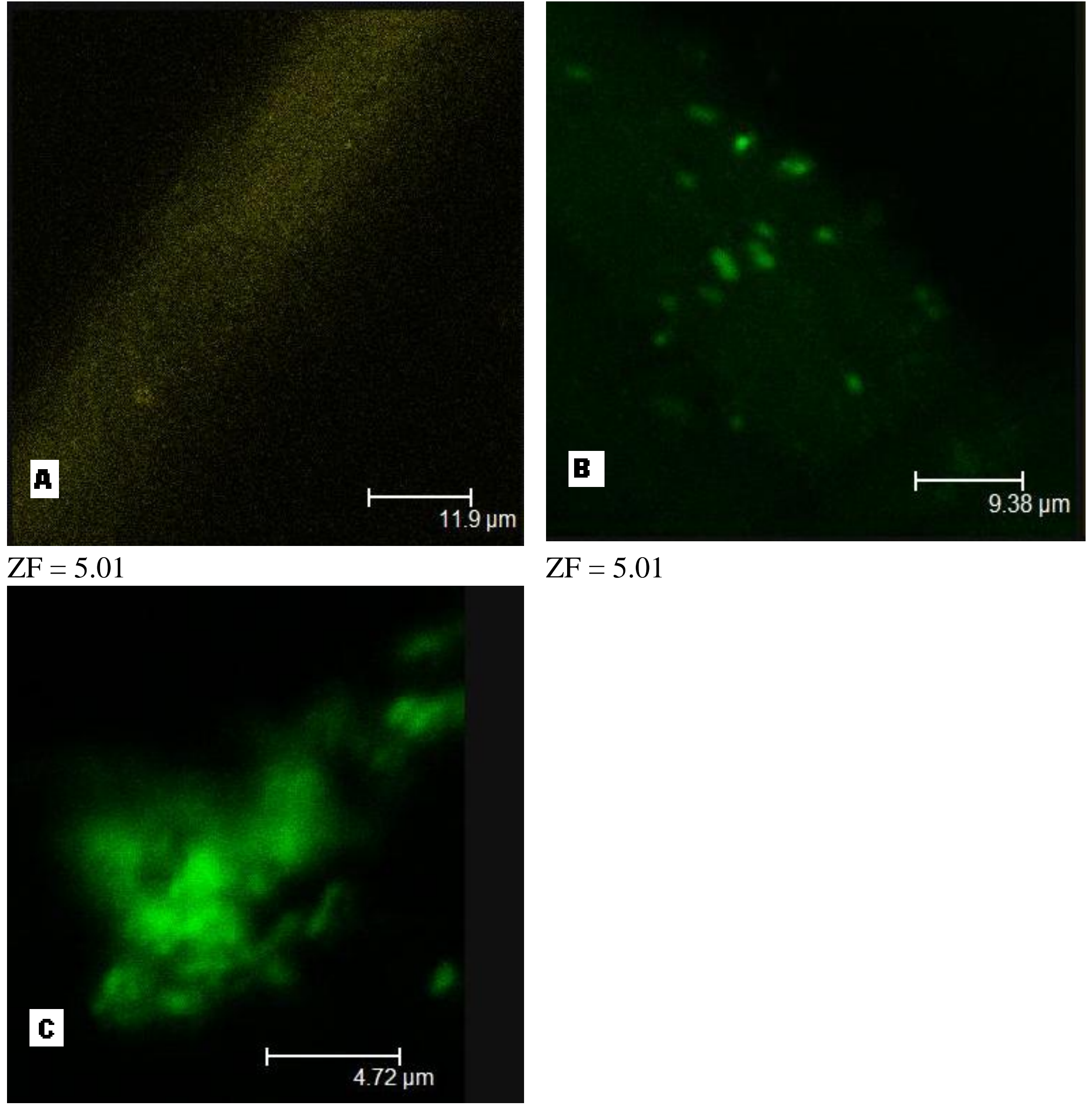


Fig. 5.18: Confocal Laser Scanning Microscopy using Live/Dead dyes of PAN catalyst surface Controls: **A,** without *E. coli* treatment; **B,** green fluorescence of separated live *E. coli* cells attached onto PAN catalyst fibres (cells suspended only in water); and **C,** green fluorescence a highly concentrated clumped live *E. coli* cells on PAN catalyst fibres (cells suspended only in water).

Images were taken at X40 magnification with additional digital magnification (zoom factor, ZF = 5.01 for all three micrographs).

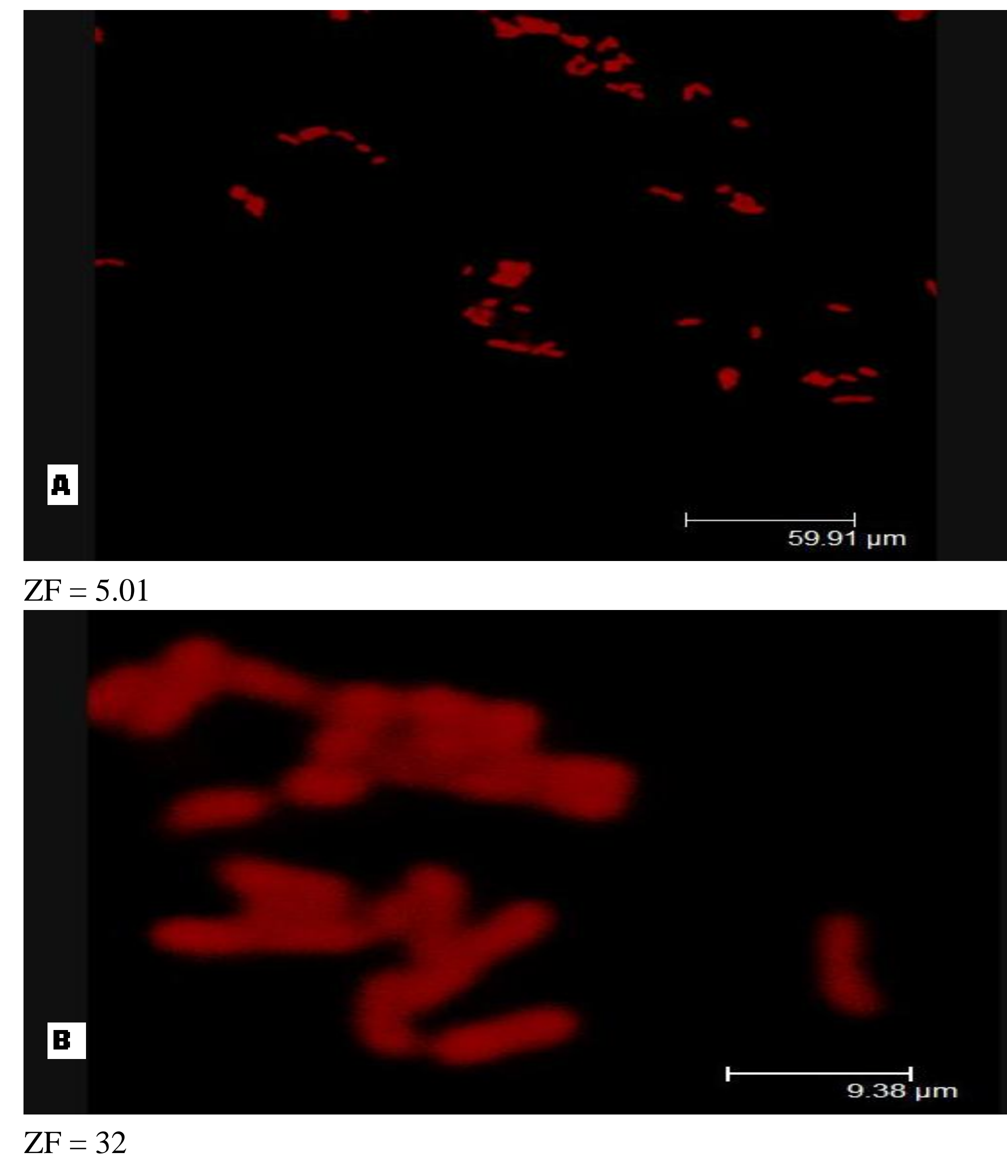
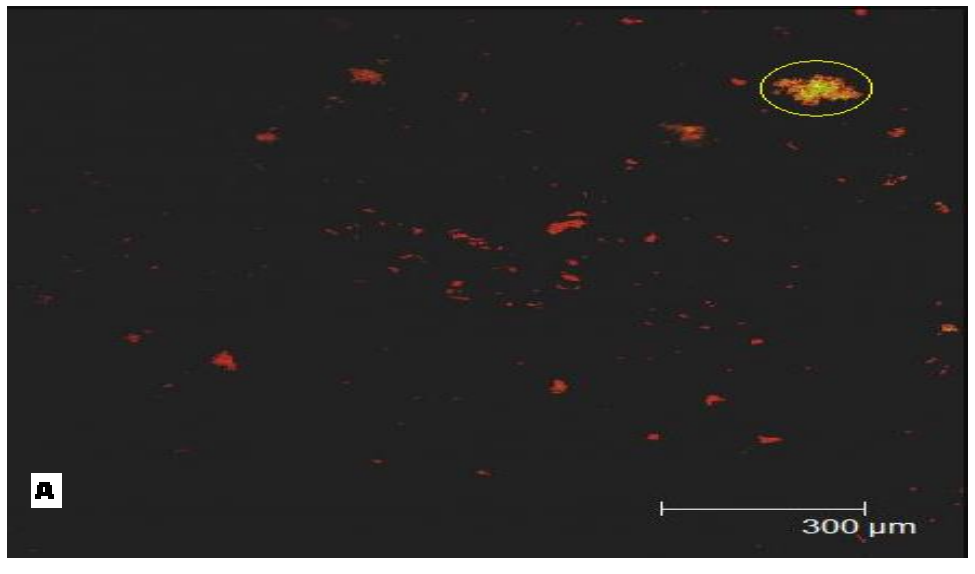
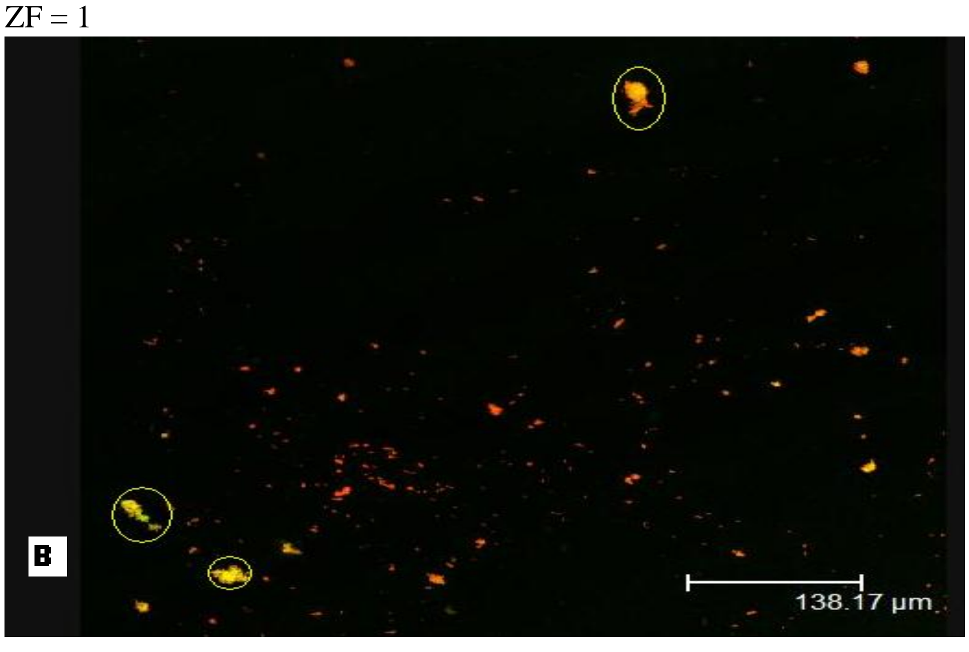


Fig. 5.19: Confocal Laser Scanning Microscopy using Live/Dead dyes Positive Control: **A** and **B** indicate red fluorescence of dead *E. coli* cells treated with 70% alcohol for 1 hr.

Images were taken at X40 magnification with additional digital magnification (zoom factor, ZF as shown above).



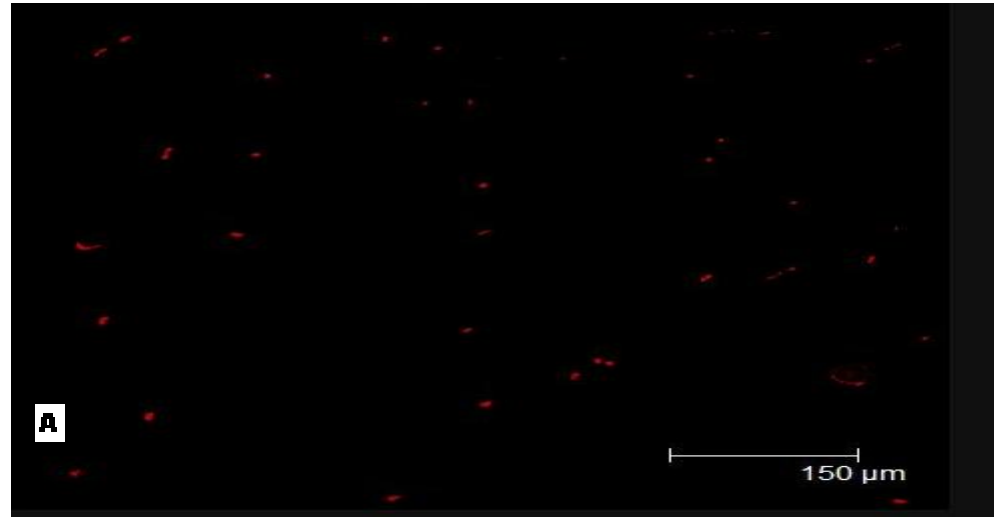
ZF = 1



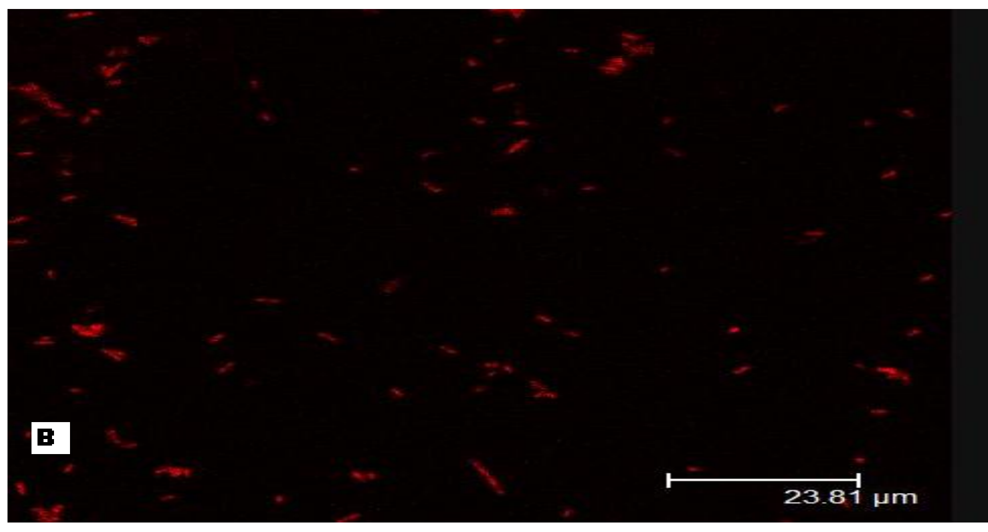
ZF = 1

Fig. 5.20: Confocal Laser Scanning Microscopy using Live/Dead dyes of experimental solution Experiment: **A** and **B** indicate different fields of view of red fluorescence of dead *E. coli* cells in experimental solution containing 0.2% w/v H_2O_2 together with PAN catalyst taken after neutralisation (circled areas are clumped cells).

Images were taken at X40 magnification with additional digital magnification (zoom factor, ZF as shown above).



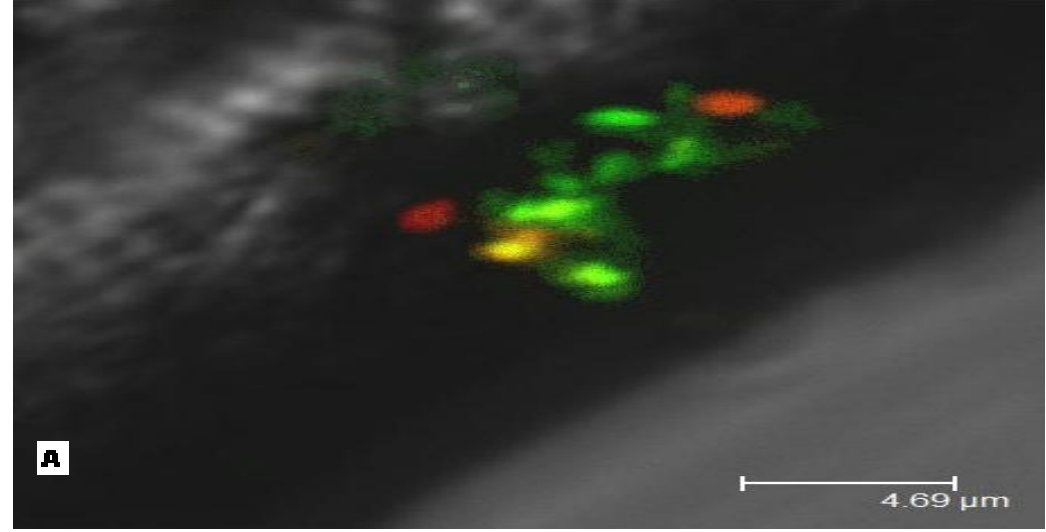
ZF = 1



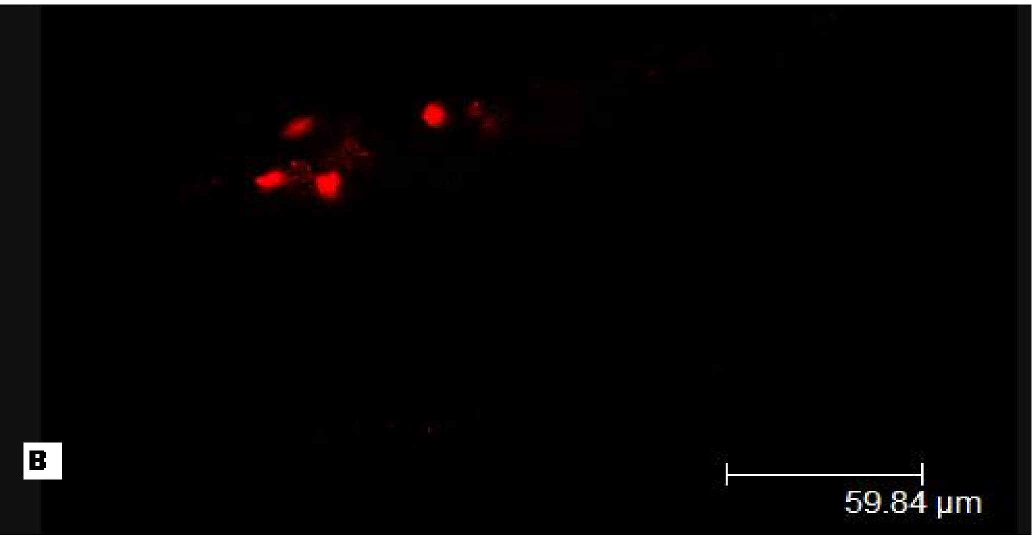
ZF = 1

Fig. 5.21: Confocal Laser Scanning Microscopy using Live/Dead dyes of experimental solution Experiment: **A** and **B** indicate different fields of view of red fluorescence of dead *E. coli* cells in experimental solution containing 0.2% w/v H_2O_2 together with PAN catalyst and without neutralisation.

Images were taken at X40 magnification with additional digital magnification (zoom factor, ZF as shown above).



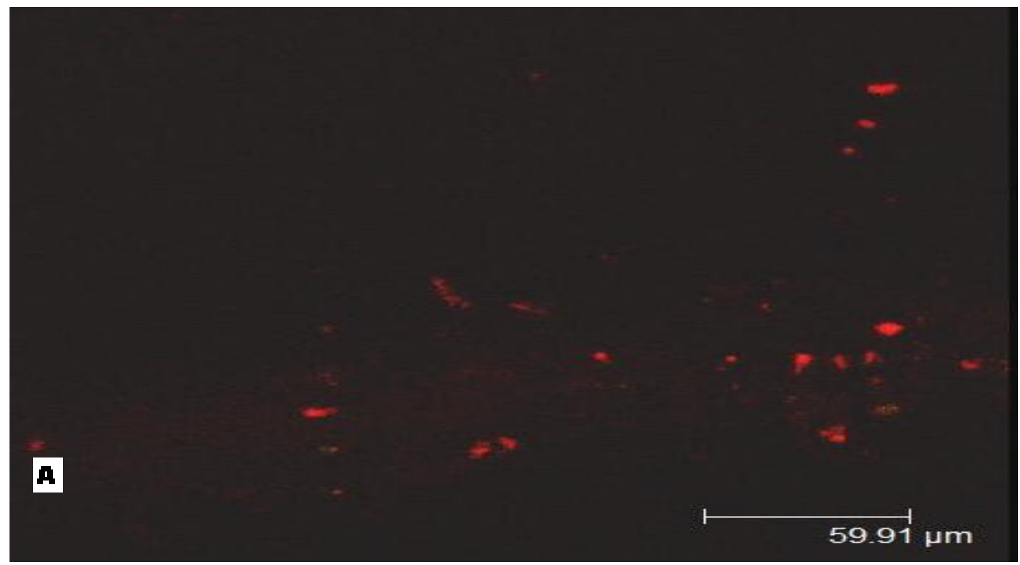
ZF = 5.01



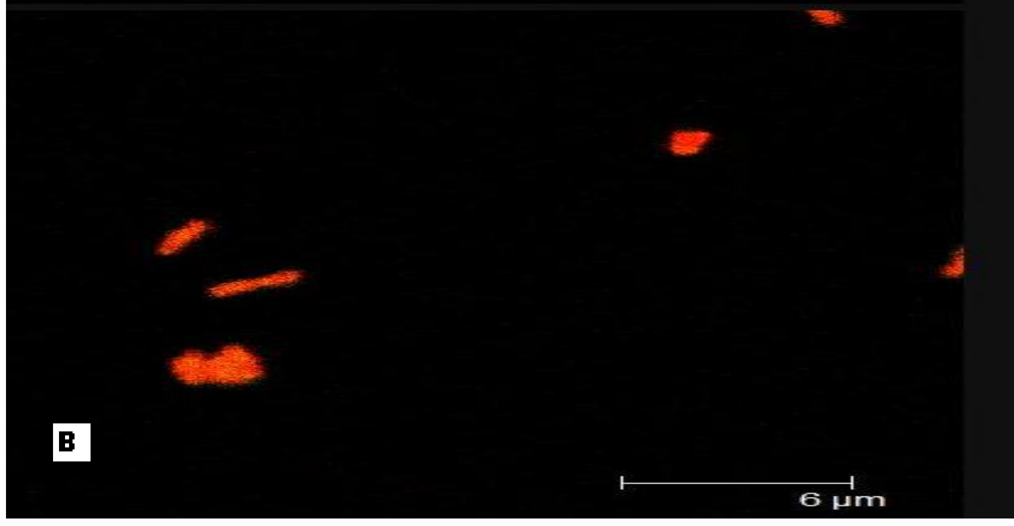
ZF = 5.01

Fig 5.22: Confocal Laser Scanning Microscopy using Live/Dead dyes of PAN catalyst surface treated with 0.2% w/v H_2O_2 for 1 hr Experiment: **A,** indicates green (live cells) and red (dead cells) fluorescence of *E. coli* cells attached to PAN catalyst fibre after neutralisation; **B,** a different field of view showing red (dead cells) fluorescence of *E. coli* cells on PAN catalyst fibre after neutralisation.

Images were taken at X40 magnification with additional digital magnification (zoom factor, ZF as shown above).



ZF = 5.01



ZF = 5.01

Fig 5.23: Confocal Laser Scanning Microscopy using Live/Dead dyes of PAN catalyst surface treated with 0.2% w/v H_2O_2 for 1 hr Experiment: **A** and **B** indicate field of views of red (dead cells) fluorescence of *E. coli* cells on PAN catalyst fibre without neutralisation.

Images were taken at X40 magnification with additional digital magnification (zoom factor, ZF as shown above).

5.3.4. Viable and total cell count of bacteria attached to PAN catalyst

PAN catalyst mesh treated with 0.2% w/v H_2O_2 and *E. coli* for 1 hr was washed in NaCl solutions and the solution was then assessed for viability and total count of *E. coli* cells. After 1 hr contact time, both neutralised and un-neutralised PAN catalyst mesh (0.5g of PCatDC3 impregnated with ferric chloride) yielded 10^2 cfu/mL (Table 5.2A). In comparison 10^7 cfu/mL viable *E. coli* cells was obtained from the control test when PCatDC3 treated with *E. coli* suspension (no H_2O_2) was washed with NaCl solution (Table 5.3). 0.5g PAN catalyst mesh (PCatDS ferric sulfate impregnated) used under the same experimental condition as PCatDC3 resulted in 10^3 cfu/mL of viable *E. coli* cells on neutralised PCatDS and 10^2 cfu/mL on un-neutralised PCatDS (Table 5.2B. Total cell numbers obtained in the NaCl solutions used to wash PCatDC3 or PCatDS was 10^7 cells/mL (Table 5.4A and 5.4B). In general, some viable *E. coli* cells appear to have remained attached to the modified PAN catalyst mesh after treatment with H_2O_2 or may have attached themselves later from the solution. Also present on the PAN catalyst mesh are relatively high number of dead *E. coli* cells (Table 5.4A and 5.4B).

As shown in the previous section, live and dead cells were identified as present on the PAN catalyst after contact with microorganisms in H_2O_2 solution; however, these were only assessed by a qualitative approach. The present work devised a quantitative approach to assess viable cells present on the PAN catalyst after 1 hour contact time with a view to understanding some of the mechanism/s of microbial attachment to the PAN catalyst which allowed them to evade inactivation.

Due to the chemical characteristics of the PAN catalyst such as its initial protonated nature (the impregnation step is acidic), as well as, the presence of lone pair electrons on the nitrogen atoms (only when the pH rises to neutral) which form part of the ligating system, it was assumed that microbial cells may interact with the PAN catalyst by ionic/electrostatic bonding. Hence, various concentrations of NaCl were used to release cells bound to the PAN catalyst into solution for viability assessment. It was found that *E. coli* cells were able to avoid inactivation by attaching themselves to the PAN catalyst (either ferric chloride impregnated PAN catalyst (PCatDC3) or ferric sulfate impregnate PAN catalyst (PCatDS))

even after the longest contact time with the H_2O_2 solution of 1hr. About 10^2 cfu/mL *E. coli* cells (live cells) were found to have bound to the PAN catalyst whether neutralised or un-neutralised (Tables 5.2A and 5.2B). Control tests involving PAN catalyst treated with only *E. coli* cells in the absence of H_2O_2 enumerated 10^7 cfu/mL of *E. coli* (Table 5.3). Although 10^2 cfu/mL may be seen as a relatively small viable number on the catalyst, it must be noted that only half (0.5g) the amount of the catalyst in Chapter 2 (1g) was used in the present study, which suggests that viable bacterial number would have proportionally increased if 1g of the PAN catalyst was used and assessed. Hence 10^2 cfu/mL could be seen as an important bacteria number on 0.5g PAN catalyst after the experimental contact time of 1hr.

In the qualitative live and dead cells assessments using the confocal microscope, it was observed that only dead red cells were present on the PAN catalyst when it was not neutralised, however, the quantitative assessment presented here has shown otherwise. Thus, viable microbial cells were still present on the un-neutralised PAN catalyst just as they were present on the neutralised catalysts after the longest experimental contact time. This discovery could suggest that the stain mixture of the LIVE/DEAD kit perhaps was unable to penetrate deep enough into the PAN catalyst fibre as a result was unable to stain completely all live and dead cells present on the PAN catalyst fibres. Or that the confocal microscope can only pick up those cells on the surface of the fibres and not those held deeper within the fibres.

As shown in Chapter 4, Sections 4.3.1.2., the PAN catalyst/ H_2O_2 system generates $^{\cdot}OH$ radicals which are probably involved in antimicrobial activity, and the data presented here has shown that viable cells were present on the PAN catalyst after the expected inactivation time. However, it is interesting to note that un-neutralised PAN catalysts, that presumably could possess residual H_2O_2, were still unable to inactivate attached *E. coli* cells. This phenomenon is clear evidence that the PAN catalyst offers protection to *E. coli* cells compared to cells in solution. In particular if cells are clumped together, it is also possible that the cells in the interior are protected from $^{\cdot}OH$ radical attack.

Establishing the mechanisms by which microbes bind to surfaces is challenging due to the biochemical and biophysical complexity of the microbial cell, the number of substrates of

variable properties, as well as different environmental factors that may influence the overall process (Abu-Lail and Camesano, 2006). The surface of a bacterial cell is made up of a complex mixture of proteins, phospholipids, polysaccharides, lipopolysaccharides (LPS), and other biopolymers. Thus, LPS and proteins located in the outer membrane of bacteria, as well as extracellular polymeric substances on bacterial surfaces have been found to influence bacterial adhesion (Xu and Logan, 2006). Atomic force microscopy (AFM) is seen as a powerful tool for probing microbial-surface interactions and for characterising both adhesion forces and cell surface properties on a variety of microbial cell types (Li and Logan, 2004; Ubbink and Schar-Zammaretti, 2005; Abu-Lail and Camesano, 2006). AFM studies has revealed that biopolymers play an important role in *E. coli* adhesion to surfaces (Abu-Lail and Camesano, 2006). Similarly, AFM analysis showed that the adhesion of *Ps. aeruginosa* to silicon surfaces was controlled by extracellular polymers, in addition, steric and electrostatic forces each contributed to the interfacial interactions between *Ps. aeruginosa* and the silicon surface (Atabek and Camesano, 2007). The effect of the hydrophobicity and electrostatic charge on the surface of bacterial surfaces in relation to their adsorption to inorganic porous supports with SiO_2 or Al_2O_3 as the main components was investigated. Gram-negative and Gram-positive bacteria were characterised by water contact angle and zeta-potential measurements. The influence of microbial charge on adsorption was examined by varying the ionic strength of the suspending liquid. It was found that the amount of *E. coli* cells adsorbed to the support materials increased with increasing electrolyte concentration. Furthermore, the effect of cell surface hydrophobicity on the extent of adsorption was demonstrated at high ionic strength (0.15M NaCl) where charge effects were reduced. It was concluded that the support used in the study promoted the adsorption by hydrophilic bacteria (Krekeler *et al.,* 1991).

Considering the evaluation made from the literature as briefly summarised above, it could be suggested that the possible adhesion mechanism between *E. coli* and the PAN catalyst was due to electrostatic interaction. The PAN catalyst is protonated after impregnation processes with iron salts and as a result may exhibit a predominantly positively charged surface which would interact with negatively charged surfaces such as *E. coli*. It is even possible for positively charged biomolecules to interact with the lone pair electrons (only at

pH greater than ~4.5 when the carboxylic acid group is ionised to the carboxylate group, the amine group is deprotonated at higher pH around neutral) residing on oxygen and nitrogen atoms present on the PAN catalyst as shown by EDX analysis.

Oxygen and nitrogen atoms are present in ligands such as oximes, amides, imidines, azides and carboxylate groups that have been shown to be present on the catalyst by FT-IR-ATR studies (Chi, 2008) and these ligate with the iron on the PAN. At pH of above 4.5 and below 8.5 the carboxylic groups are present as negatively charged carboxylate groups and the amine groups are protonated and are positively charged. That is the catalyst has both cationic and anionic sites and is known to act as an ion-exchange resin (Chi, 2008). It is therefore possible that the catalyst could be attractive to both Gram-negative and Gram-positive bacteria. It is equally possible that not all sites on the ligands bind with the iron, as a result, these could then ligate with opposite charges that may come in contact with it. Hence, it is possible to assume that some Gram-positive organisms such as *S. aureus* could bind to the PAN catalyst (as shown in SEM micrograph) in a similar manner to that of *E. coli* and resist total inactivation. It must be stressed that the microbial inactivation by PAN catalyst when used alone (in the absence of H_2O_2) as demonstrated in Chapter 2, Section 2.4.3.3., particularly the deleterious effects exhibited against *Ps. aeruginosa* could partly be due to electrostatic bonding. Although, it is possible that not all the bacteria attracted to the catalyst was killed by this action. Further work on assessing viability of all the three organisms (*E. coli*, *S. aureus* and *Ps. aeruginosa*) on the PAN catalyst used with or without H_2O_2 would be useful.

The antimicrobial activity of the leached compound/s from the PAN catalyst as shown in Chapter 3, Section 3.3.4. may have a competitive advantage over the antimicrobial activity of the PAN catalyst itself. This is because microbes could be killed by the leached compound/s at a similar rate to when the PAN catalyst is present. In the absence of the PAN catalyst, microbes have nothing to attach themselves to so can not escape inactivation via this mechanism. In summary, it must be pointed out that the bacterial inactivation data shown in Chapter 2 involving PAN catalyst with or without H_2O_2 may not be fully accurate

as some of the bacteria in the experiment may have evaded inactivation by adhering to the PAN catalyst. The activity of the leachate from the PAN catalyst could be seen as more reliable and accurate data as microbes could not escape inactivation via this mechanism.

The following issues arise and need to be examined in relation to these microbe-PAN catalyst interactions:

1. Does the microbe bind to the catalytic active sites resulting in the inability of H_2O_2 to access these sites and generate $^{\cdot}OH$ radicals?
2. If H_2O_2 is able to access available catalytically active sites and generate $^{\cdot}OH$ radicals, how and why do microbes bound to the PAN still escape inactivation?
3. Could this microbial escape of inactivation be regarded as a microbial innate resistance due to its ability to associate with the catalyst to avoid $^{\cdot}OH$ radicals attack?
4. Could this binding affinity of the microbe makes the PAN catalyst susceptible to biofilm formation?

An investigation of some or all of the above raised issues may help understand in detail the mode by which *E. coli* escapes inactivation when it binds to the PAN catalyst; how H_2O_2 generates $^{\cdot}OH$ radicals on the PAN catalyst (on active sites) and/or in solution in the presence or absence of microbes; and whether the PAN catalyst promotes biofilm formation or not. Such knowledge could possibly promote other new formulations that would avoid or significantly reduce microbial adhesion and make the PAN catalyst more efficacious in its antimicrobial activity.

As shown in Chapter 3, Section 3.3.1, solutions containing microbial loads had lower iron concentrations than those without, from the present study it is possible that microbial adhesion to the PAN catalyst prevents iron/compounds leaching from the catalyst into solution.

As shown from the LIVE/DEAD kit analysis, the total cell count presented here (Tables 5.4A and 5.4B) also shows that there were large numbers of dead cells as compared with the viable count (Table 5.2A and 5.2B) on the PAN catalyst fibre. Furthermore, the large number for the total cell count presented here and Chapter 4 in relation to the SEM data

(Section 5.3.2) as well as LIVE/DEAD kit results gave good indication that inactivated cells still maintain their intact cell membrane and do not lyse.

Table 5.2: Enumeration of viable cells released from 0.5g PAN catalyst mesh (PCatDC3 = ferric chloride impregnated and PCatDS = ferric sulfate impregnated) treated with various concentrations of NaCl salts after 1 hr contact time in experiment using 0.2% w/v H_2O_2 against *E. coli*.

Washing solution	Neutralised PCatDC3 after 1 hr contact with 0.2% w/v H_2O_2 and *E. coli* CFU/mL	Un-neutralised PCatDC3 after 1 hr contact with 0.2% w/v H_2O_2 and *E. coli* CFU/mL
0.1% peptone	0	0
0.5% NaCl in 0.1% peptone	0	0
1% NaCl in 0.1% peptone	3.2×10^2	5.6×10^2
2% NaCl in 0.1% peptone	9×10^1	0
3% NaCl in 0.1% peptone	1.3×10^1	0
4% NaCl in 0.1% peptone	0	0
A	*E. coli* suspension in water	Initial CFU/mL (No H_2O_2 or catalyst involved) 1.8×10^8

Washing solution	Neutralised PCatDS after 1 hr contact with 0.2%w/v H_2O_2 and *E. coli* CFU/mL	Un-neutralised PCatDS after 1 hr contact with 0.2%w/v H_2O_2 and *E. coli* CFU/mL
0.1% peptone	1.23×10^2	7.3×10^1
0.5% NaCl in 0.1% peptone	2.03×10^2	7.6×10^2
1% NaCl in 0.1% peptone	2.3×10^1	6×10^1
2% NaCl in 0.1% peptone	1.9×10^2	0
3% NaCl in 0.1% peptone	1.84×10^3	1.2×10^2
4% NaCl in 0.1% peptone	1.6×10^2	0
B	*E. coli* suspension in water	Initial CFU/mL (No H_2O_2 or catalyst involved) 1.8×10^8

PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 3); PCatDS = ferric sulfate form of PAN catalyst (Dralon-L type); CFU/mL = colony forming unit/mL.

Table 5.3: Control: Enumeration of cells released from PAN catalyst mesh as a function of the NaCl salt concentration after 1hour contact time using only *E. coli* in the absence of H_2O_2.

Test solutions of washed PAN catalyst mesh after 1 hr contact with *E. coli* suspension in water	CFU/mL	
0.1% peptone		
0.5% NaCl in 0.1% peptone		
1% NaCl in 0.1% peptone		
2% NaCl in 0.1% peptone	2.17×10^7	
3% NaCl in 0.1% peptone	4.50×10^7	
		Initial CFU/mL (No H_2O_2 or catalyst involved)
	E. coli suspension in water	1.8×10^8

CFU/mL = colony forming unit/mL

Table 5.4: Total cell count of cells released from 0.5g PAN catalyst mesh (PCatDC3 = ferric chloride impregnated and PCatDS = ferric sulfate impregnated) treated with various concentrations of NaCl salts after 1hr contact time in experiment using 0.2% w/v H_2O_2 against *E. coli*.

Washing solutions used for PCatDC3	Mean total cell count	
	Neutralised PCatDC3 after 1 hr contact with 0.2% w/v H_2O_2 and *E. coli* Total cell/mL	Un-neutralised PCatDC3 after 1 hr contact with 0.2% w/v H_2O_2 and *E. coli* Total cell/mL
0.1% peptone	3.5×10^7	1.08×10^8
0.5% NaCl in 0.1% peptone	1.4×10^7	1.1×10^8
1% NaCl in 0.1% peptone	2.06×10^7	4.1×10^7
2% NaCl in 0.1% peptone	2.06×10^7	1.9×10^7
3% NaCl in 0.1% peptone	5.3×10^7	3.6×10^7
4% NaCl in 0.1% peptone	2.6×10^7	2.2×10^7
A		Total cell/mL
	E. coli suspension in water	1.3×10^9

Washing solutions used for PCatDS	Mean total cell count	
	Neutralised PCatDS after 1 hr contact with 0.2% w/v H_2O_2 and *E. coli* Total cell/mL	Un-neutralised PCatDS after 1 hr contact with 0.2% w/v H_2O_2 and *E. coli* Total cell/mL
0.1% peptone	5.1×10^6	2.9×10^7
0.5% NaCl in 0.1% peptone	5.9×10^7	5.2×10^7
1% NaCl in 0.1% peptone	1.5×10^7	1.4×10^7
2% NaCl in 0.1% peptone	1.1×10^7	5.6×10^7
3% NaCl in 0.1% peptone	1.5×10^8	2.7×10^7
4% NaCl in 0.1% peptone	9.6×10^7	7.1×10^7
B		TC/mL
	E. coli suspension in water	1.3×10^9

TC/ml = total cell/mL; PCatDC3 = ferric chloride form PAN catalyst (Dralon-L type batch 3); PCatDS = ferric sulfate form of PAN catalyst (Dralon-L type).

5.4. Conclusion

EDX elemental analysis performed in the present work revealed that iron is not evenly distributed on the surface of the PAN fibre. Iron distribution was found to vary slightly within and between batches (Figure 5.5). However, on average, the commercially produced PAN catalysts were found to contain about 17 times more iron than the laboratory produced ones (Figure 5.5). SEM examination revealed both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) microorganisms attached to the PAN catalyst with their structure visually intact after antimicrobial testing. The surfaces of the commercially produced PAN catalyst showed a larger amount of debris than that of the laboratory produced ones, it was suggested that this debris may have been contributing to the slightly reduced antimicrobial activity of the former even though the former contained more iron. LIVE/DEAD kit examination by CLSM has confirmed that whilst microbial cells in experimental solution were completely killed, some cells were able to avoid inactivation by attaching to the PAN catalyst. Thus, the PAN catalyst appeared to offer protection to some of the microbes. Viability assessment with NaCl washing of the PAN catalyst suggested that the microbes possibly attached themselves to the PAN catalyst by ionic interaction. Even though NaCl can break electrostatic/ionic bonding, it is likely that not all the cells bound to the PAN catalyst was released by this method. Possibly, due to the nature of the catalyst mesh, some cells could reside deep inside the mesh matrix, hence, a different and robust method could be devised for future work, and fibres of the PAN could be used instead of the mesh. Although, total cell count, as compared to the viable count indicated large numbers of dead cells on the PAN catalyst, the actual mode of microbial evasion of inactivation is not clearly understood at this stage.

CHAPTER 6

MAIN CONCLUSIONS, RECOMMENDED APPLICATIONS AND FUTURE WORK

6.1. Main Conclusions

This project resulted in the following novel findings:

This study has shown that the antimicrobial activity of H_2O_2 is significantly enhanced in the presence of a novel heterogeneous PAN catalyst (PCat) at both room temperature and at 35°C. This is supported by similar work from the literature where a heterogeneous copper catalyst/H_2O_2 system demonstrated higher antimicrobial activity compared to H_2O_2 alone (Shah *et al.,* 2007). 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 in the presence of PCat inactivated *E. coli*, *Ps. aeruginosa* and *S. aureus* at 30, 40, and 10mins respectively at room temperature, but all the bacteria were inactivated at 35°C at 10mins. The absence of PCat in 0.2% w/v, 0.5% w/v and 1% w/v H_2O_2 showed significantly less antimicrobial activity. One percent (w/v) H_2O_2 with PCat was found to be bactericidal with all the tests organisms at 10mins at room temperature. This outcome suggests that the use of the catalyst/ H_2O_2 system has an advantage in terms of both time and cost saving over using just H_2O_2 alone**.** Bacteria inactivation was found to be dependent on exposure/contact time, H_2O_2 concentration, and temperature. Homogeneous catalysts (simple iron salts) with H_2O_2 were equally effective as the heterogeneous PAN catalyst/ H_2O_2 system. It was shown that PCat alone (without H_2O_2) significantly reduced viability of *Ps.aeruginosa* (5.76 log reduction at 60mins) at 35°C but not at room temperature. Homogeneous catalyst (simple iron salts) under the same experimental conditions also showed antimicrobial activity but were not as effective as PCat. However, the acidic environment (pH 2.95-3.04) which is produced by PCat or homogeneous catalyst were found to play a role in the reduced microbial viability. In general the PAN catalyst/H_2O_2 systems successfully passed the tests pertaining to the basic bactericidal and sporicidal activities as stipulated and required by the European Standard Phase 1 Suspension Tests as described in Section 2.2.

This study has shown that PCat is active in buffered solutions (maintained at pH 7.41) and is capable of inactivating bacteria at neutral pH at both room temperature and 35°C, but activity is much greater at 35°C. PCat in an alkaline solution (unbuffered) adjusted with 1M NaOH showed a swift shift of pH from alkaline (pH 7.42-8.02) to acidic pH (3.59-3.80)

resulting in increased antimicrobial activity in comparison to that shown in the buffered solution. This again suggests that increase in the antimicrobial activity of PCat is favoured by an acidic environment.

This study has shown that impregnated iron on the PAN catalyst leaches into solution. This is supported by the literature indicating iron leachate from heterogeneous catalyst (Xiang *et al.,* 2009). The concentration of leached iron varies within and between batches. However, ferric chloride impregnated PAN catalysts appeared to leach relatively more (0.680ppm of iron at 60mins) than the ferric sulfate impregnated PAN catalyst (0.443ppm of iron at 60mins). Iron leaches less in the presence of microbial load than in their absence, but the difference was not statistically significant. Iron was found to leach less during subsequent uses of the PAN catalyst and this is a possible reason for reduced antimicrobial activity of the PAN catalyst on subsequent uses. The rate of iron leaching was dependent on contact time, temperature and the presence or absence of microbial load.

PCat was reusable at least 2 times, reusable both 48 hrs and 6 months after first use. However, activity decreases during subsequent uses particularly at room temperature, whilst activity at $35^{o}C$ was mostly maintained even at 3rd use (>5.50 log reduction at 20mins). This suggests that there could be an ageing effect after 6 months. The literature has shown that different types of heterogeneous catalyst leach into solution, resulting in relatively reduced activity when the catalyst is reused (Iyer and Thakur, 2000; Kang *et al.,* 2010).

This study has shown that peracetic acid (PAA) which is known to contain H_2O_2, did not exhibit any increased antimicrobial effect when PCat was added as compared to catalyst/H_2O_2 system. Rather, the presence of PCat antagonised the antimicrobial activity of 0.0002% PAA, resulting in significantly less activity. This was not seen with either homogeneous iron salts, or the modified PAN mesh (not impregnated with iron salts) or the initial non-modified PAN mesh and is thought to arise by complexation of the peracetic acid with iron (active catalytic site) on the PAN catalyst thus making it unavailable for biocidal activity against the microorganisms.

The presence of PCat with either 5% w/v or 7.5% w/v H_2O_2 significantly increased sporicidal activity against *Bacillus subtilis* at 2 hrs at room temperature with ferric chloride impregnated PAN catalyst compared to when 5% w/v or 7.5% w/v H_2O_2 was used alone. However, the ferric sulfate impregnated PAN catalyst seemed slightly less active and exhibited sporicidal activity only with 7.5%w/v H_2O_2 at 2 hrs.

This study has discovered that the leached solution of the PAN catalyst contained iron and possibly some other unknown compounds. It is possible that the other unknown compounds are ammonia (decomposition product of hydrazine and hydroxylamine) and residual hydrazine or hydroxylamine which may have remained trapped within the fibres of the catalyst during the modification process due to incomplete washing. It was shown that leached PAN catalyst solution was equally as potent in its antimicrobial activity as the PAN catalyst. A homogeneous equivalent, containing the same number of moles of iron as iron (III) chloride or iron (III) sulphate, to the leached iron from the PAN catalyst displayed less antimicrobial activity. This difference in activity, suggests that there is a possible chemical conversion when iron is impregnated to the modified PAN fibre and that it is this chemically converted iron that leaches out. It was further demonstrated that a leachate from a reused PAN catalyst also showed relatively higher antimicrobial activity than when H_2O_2 was used alone, however, the activity of this leached solution was less than that of the leached solution from first use of the catalyst. It is possible that the concentration of the active species in the leachate from the re-used catalyst would also decrease on catalyst reuse.

Fe and Cu have been employed as oxidation catalysts in either homogeneous or heterogeneous state to generate $^{\cdot}OH$ radicals (Villabrille *et al.,* 2007). Other systems such as Advanced Oxidation Processes (AOPs), are also being used to generate $^{\cdot}OH$ radicals for many applications (Laat *et al.,* 2004; Chen *et al.,* 2006). The presence of the PAN catalyst or leachate from the PAN catalyst with H_2O_2 generates $^{\cdot}OH$ radicals. However, the PAN catalyst in a solution of H_2O_2 generates significantly more $^{\cdot}OH$ radicals than the leachate from the PAN catalyst in H_2O_2 solution (results for the leachate were not conclusive and

another mechanism of action may be involved). New complexes may be formed when iron is impregnated onto the modified PAN fibre that are able to activate the decomposition of H_2O_2 into hydroxyl radicals. These could be hydroxamic, hydrazone, amidine, amide and carboxylic complexes of iron. It is possible that fragments of these complexes leach out and activate H_2O_2 in the solution phase. Certain complexes of iron are well known for their ability to decompose H_2O_2 and are also known to have a longer lifetime than the simple iron salts (Tachiev *et al.,* 1999; Salem *et al.,* 2000; Tolvanen *et al.,* 2009).

Increased amount of $^{\cdot}OH$ radicals is dependent on increased concentration of H_2O_2. Ferric chloride impregnated PAN catalyst generates relatively more $^{\cdot}OH$ radicals than the ferric sulfate impregnated PAN catalyst and this was thought to be the reason why the former showed higher antimicrobial activity than the latter. The extent of the generation of $^{\cdot}OH$ radicals was also seen to be time dependent. Thus, the presence of the PAN catalyst in H_2O_2 generates more $^{\cdot}OH$ radicals at shorter contact time than at longer contact times. This may be due to the drop in hydrogen peroxide concentration as the contact time increases. $^{\cdot}$ The novel generation of $^{\cdot}OH$ radicals was supported by the high level of antimicrobial activity exhibited by a PAN catalyst with H_2O_2 solution prepared 24hrs before antimicrobial testing; and very little antimicrobial activity shown by a homogeneous catalyst with H_2O_2 solution prepared 24hrs before antimicrobial testing. The former case displayed a reasonable concentration of H_2O_2 after 24hrs prior to antimicrobial testing, whereas the latter resulted in complete decomposition of H_2O_2 prior to antimicrobial testing at 24hrs. Thus, the PAN catalyst decomposes H_2O_2 slowly, whereas the homogeneous catalyst decomposes H_2O_2 rapidly therefore no H_2O_2 is left to generate $^{\cdot}OH$ radicals at 24hrs. The PAN catalyst showed higher decomposition rate of H_2O_2 at 35^oC than at room temperature, which suggests that a larger amount of $^{\cdot}OH$ radicals may being produced, and equally, suggests why antimicrobial activity was higher at 35^oC than at room temperature. It was found that the modified PAN mesh (not impregnated with iron salts) and non-modified PAN mesh (not impregnated with iron salts) unexpectedly produced $^{\cdot}OH$ radicals in the presence of H_2O_2. This observation was attributed to trapped radicals in the polymer matrix resulted from the manufacturing of the PAN via free radical polymerisation (Braunecker and Matyjaszewski, 2007; Yu *et al.,* 2007). However, the levels of $^{\cdot}OH$

radicals observed did not produce any significant antimicrobial activity compared to the activity showed by the PAN catalyst and its leachate. As reviewed from the literature in Chapter 1, it has been widely established that ·OH radicals have highly potent antimicrobial properties, and the reason for the inability of ·OH radicals generated from modified PAN mesh and non-modified PAN mesh showing no significant antimicrobial activity is at this stage unknown. It was assumed that possibly these radicals were generated and trapped by DMPO in the matrix of the mesh, but not in the solution phase and that these radicals may not be accessible to reaction due to their location, and the nature in which they are held in the matrix. It was assumed that H_2O_2 possibly reacts with functional groups on the catalyst to produce a variety of compounds some of which may be free radical initiators. This observed phenomenon would require further and careful interpretation.

EDX elemental analysis revealed that iron was not evenly distributed within and between batches of the PAN catalyst. Ferric sulfate impregnated PAN catalyst (commercially produced) had a relatively higher amount of iron than the ferric chloride impregnated catalyst (Produced at De Montfort University). EDX analysis showed that the former had about 17 times more iron on the PAN surface than the latter. SEM examination showed rough surfaces on the ferric sulfate impregnated PAN catalyst whereas the surface of the ferric chloride impregnated PAN catalyst was relatively smooth. This rough surface appearance on the catalyst was assumed to be a collection of debris. The presence of the rough surface or debris may be responsible for the lower antimicrobial activity induced by the commercially produced PAN catalyst than the De Montfort University produced ones. Thus, some of the debris may have prevented H_2O_2 gaining access to the catalytically active sites preventing the generation of ·OH radicals leading to relatively reduced antimicrobial activity. Another possibility for the reduced activity is that the sulphate form of the catalyst is not as active as the chloride form, however to prove this a laboratory prepared sulphate catalyst would have to be prepared and its antibacterial activity compared with that of the laboratory prepared chloride catalyst. SEM analysis further showed that both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) were attached to the PAN catalyst and that their cell structure was visibly intact after antimicrobial activity. This was

supported by high cell number observed when total cell (live and dead cells) count of experimental solution after the longest contact time (60mins) was performed, which suggests that microbial cells do not lyse during $^{\cdot}OH$ radical attack.

LIVE/DEAD kit microbial staining method in conjunction with confocal laser scanning microscopy (CLSM) revealed that *E. coli* was completely inactivated (dead) in the solution containing PAN catalyst and H_2O_2 at the longest contact time (60mins). However, both live and dead cells were observed to be attached to the neutralised PAN catalyst fibre after the longest contact time (60mins); this was not observed on the PAN catalyst which was not neutralised.

Live cells and dead cells were assessed by viable cell count and total cell counts respectively from the PAN catalyst by NaCl washing. It was suggested that the PAN catalyst seems to offer relative protection to microbes enabling them to resist inactivation compared to when the microbes were in solution. The microbial attachment to the PAN catalyst and thus, their ability to avoid inactivation is thought to be due partly or fully to electrostatic attraction between bacteria and the PAN catalyst. Consequently, not all the bacteria initially dosed were inactivated in the PAN catalyst/ H_2O_2 system, as some of these bacteria avoid inactivation by adhering to the PAN catalyst. The antimicrobial activity exhibited by the leachate from the PAN catalyst/ H_2O_2 solution could be seen as more reliable in terms of bacterial inactivation as both bacteria and antimicrobial compounds were all in solution with no catalyst mesh surface to attach to and avoid inactivation.

6.2. Recommended Applications

The outcomes of this project suggest that the PAN catalyst and its leachate, and depending on the concentration of H_2O_2 could be applied as low level, or intermediate level or high level disinfectant. One percent (w/v) H_2O_2 with either PAN catalyst or its leachate could be recommended for either low or intermediate level disinfection with 10mins contact time; whereas 5% w/v or 7.5% w/v H_2O_2 could be used for high level disinfection with 2hrs contact time saving at most 4hrs in relation to the recommended 6 hrs for high level

disinfection when 7.5% w/v H_2O_2 is used (Rutala and Weber, 1999; Acosta-Gio *et al.,* 2005).

The system developed here could be recommended for water treatments such as wastewater decontamination; swimming pool disinfection; and drinking water disinfection. However, the complexes released by leaching need to be identified before its use as a disinfectant in swimming pools and drinking water, as it is not known whether these unknown leached compounds pose any health hazard or not. There are several chemicals and processes that are available for microbial decontamination of water. For example, chlorine has been widely used to disinfect wastewater (Stampi *et al.,* 2002; Shah *et al.,* 2007), drinking water (King *et al.,* 1988) and swimming pools because of its fast reaction and lasting disinfection potential (Glauner *et al.,* 2005). However, chlorine is known to produce disinfection by-products such as trihalomethanes known to be potentially mutagenic and/or cancerous (Stampi *et al.,* 2002; Glauner *et al.,* 2005). Conversely, new emerging technologies including ozonation and UV light systems have been used for water disinfection. However, these processes are noted to be technologically intensive (Shah *et al.,* 2007). Homogeneous Fenton systems are considered unsuitable for such treatments as it requires secondary processes for removal of catalysts from water (Shah *et al.,* 2007); and as shown in this project, the homogeneous catalyst decomposes H_2O_2 very rapidly and as a result, there is a potential cost problem associated with H_2O_2 consumption in this system unlike the heterogeneous catalyst. Furthermore, the homogeneous catalyst causes very undesirable staining and discolouration to material surfaces, as seen in this project, whereas the heterogeneous system developed here showed no staining or discolouration on surfaces. There is also a sludge problem with homogeneous Fentons reagent as the iron salts precipitate out at the pH of natural waters.

The PAN catalyst/ H_2O_2 system can be used for wastewater treatment by exhibiting two functions, thus, by inactivating pathogenic microorganisms and by removing organic pollutants via oxidation (Chi, 2008).

The safety of foods such as raw meat, poultry, fish and fresh produced from microbial contamination has been a major concern. The PAN catalyst/ H_2O_2 system can be applied in the food industry as a cost-effective disinfection system against pathogenic foodborne

organisms such as *Campylobacter* (Yoda and Uchimura, 2006) and *Salmonella* sp (Malorny *et al.,* 2004).

The novel heterogeneous catalyst used here is inexpensive to develop, and very efficient in its activity especially in the presence of low concentrations of H_2O_2. It may serve as a potential alternative for the above mentioned treatments, particularly in developing countries where water quality and sanitation is less than adequate (Kehoe *et al.,* 2004).

6.3. Future Work

As shown, the antimicrobial activity of the present system employed a modified version of phase 1 of the European quantitative suspension test methods (BSEN 1040:2005 and BSEN 14347:2005), which tested the basic bactericidal and sporicidal activity of the developed system in the absence of organic matter/interfering substances and hard water. According to the requirements of the methods as portrayed in Chapter 2, the results of the present work passed these tests. Therefore, for the system developed here to be used for any application including those recommended above, future work should be performed using the phase 2, step 1 as well as phase 3 of the European quantitative suspension test methods. Phase 2, step 1 (BSEN 1276:1997) stipulates that suspension tests are performed under laboratory conditions which are representative of practical use (i.e. test prepared in hard water and in the presence of interfering substances); and phase 3 is field tests under practical conditions (in-use trials) (Payne *et al.,* 1999).

In the absence of H_2O_2, the PAN catalyst working with air alone showed some selective antimicrobial activity on the test bacteria, hence, future work should explore the mechanism/s for these effects. Work may be performed with deoxygenated water in the absence of air to elucidate the contribution of air/molecular oxygen in the reaction.

The antimicrobial activity of the system in buffered solution was tested against only *Ps. aeruginosa*, future work should be performed on all the remaining tests organisms including *Bacillus subtilis* spores and many other microganisms such as mycobacteria and viruses to establish different responses by the organisms in a buffered environment.

The PAN catalyst is reusable, and it decomposes H_2O_2 very slowly in the presence or absence of microbial load making it suitable for a continuous flow reactor. The present work performed all experiments in static batch mode whereas future work should focus on dynamic flow reactors which are a more convenient engineering set-up for industrial application. The amount of hydrogen peroxide used as the auxillary oxidant together with the PAN catalyst needs to be optimised for each microorganism so that the minimum amount is used to give satisfactory antibacterial activity in a suitable time frame. The amount of hydrogen peroxide to be used needs to be minimised, as there are cost implications associated with its use.

In the presence of H_2O_2, the PAN catalyst inactivates all *E. coli* present in solution, but at the same time offers some protection to some *E. coli* which may be electrostatically attached to the catalyst mesh; future work should firstly, assess if the remaining tests organisms including *Bacillus subtilis* spores were able to avoid inactivation through this means. Secondly, future work should seek to investigate the mechanism/s by which this microbial-PAN catalyst interaction leads to the avoidance of microbial inactivation by $^{\cdot}OH$ radicals attack, and a study on the pathway of $^{\cdot}OH$ radical generation on the PAN catalyst would be useful in this context. Thirdly, due to this microbial-PAN catalyst interaction, future work should design an experiment to assess if the PAN catalyst is susceptible to biofilm formation on it. Other work performed by a member of our research group has suggested that if H_2O_2 is present with the catalyst then this prevents the formation of a biofilm (unpublished work). These assessments may help to direct chemical modifications to the PAN catalyst in preventing microbial attachment during its antimicrobial applications.

Further work should focus on establishing how $^{\cdot}OH$ radicals are generated from the modified PAN mesh and the non-modified, and why these radicals showed no antimicrobial activity.

As shown, the leachate from the PAN catalyst in the presence of H_2O_2 showed potent antimicrobial activity against *S. aureus*. Firstly, future work therefore should be performed under similar conditions against the remaining tests organisms including *Bacillus subtilis* spores; secondly, leached PAN catalyst solution in the absence of H_2O_2 (with or without air) should be tested against all the tests organisms to compare antimicrobial activity with that of the PAN catalyst in the absence of H_2O_2; and thirdly and very importantly, there is a possible structural conversion when the iron is impregnated onto the modified PAN catalyst, hence, it is believed that iron may being leached into solution as a novel complex forming a new novel antimicrobial. Future work such as speciation by inductively coupled plasma mass spectroscopy (ICP-MS) or liquid chromatography-mass spectrometry (LC-MS/MS) should be performed to establish the identity of these new antimicrobial compounds. Preliminary antimicrobial tests could be performed to establish whether ammonia, hydrazine or hydroxylamine are responsible for the antimicrobial activity of the leachate from the PAN catalyst. Furthermore iron (III) complexes of ammonia, hydrazine, hydroxylamine and carboxylate and mixtures need to be synthesised and tested for their antibacterial activity with or without hydrogen peroxide against a range of microorganisms. In addition the activities of these iron (III) complexes needs to be compared with those of the iron(III) simple salts used as the homogeneous catalysts in this work, namely iron(III) sulphate and iron(III) chloride.

As reviewed from the literature in Chapter 1, photo-Fenton system involving UV, iron salts and H_2O_2 are now widely used in advanced oxidation processes; future work could seek to explore whether employing UV systems in conjunction with the PAN catalyst/H_2O_2 system gives comparable antimicrobial efficiency with those shown in this project.

H_2O_2 can be produced by some microorganisms (see Chapter 1), and future work could explore the potential use of the PAN catalyst in the presence of H_2O_2-generating microorganisms with the view towards bioremediation. However, considerations should be made on the pathogenic activities of these microorganisms before these experiments are pursued.

REFERENCES

ABU-LAIL, N.I. AND CAMESANO, T.A. (2006) The solvent polarity on the molecular surface properties and adhesion of *Escherichia coli. Colloids and Surfaces B: Biointerfaces.* Vol. 51: 62-70.

ACOSTA-GIO, A.E., RUEDA-PATINO, J.L., AND SANCHEZ-PEREZ, L. (2005) Sporicidal activity in liquid chemical products to sterilise or high-level disinfect medical and dental instruments. *American Journal of Infection Control.* Vol. 33: 307-9.

AKBAS, E. AND BERBER, I. (2005) Antibacterial and antifungal activities of new pyrazolo[3,4-*d*]pyridazin derivatives. *European Journal of Medicinal Chemistry.* Vol. 40: 401-405.

ALASRI, A., ROGUES, C., MICHEL, G., CABASSUD, C., APTEL, P. (1992) Bactericidal properties of peracetic acid and hydrogen peroxide, alone and in combination, and chlorine and formaldehyde against bacterial water strains. *Canadian Journal of Microbiology.* Vol. 38(7): 635-642.

ALFA, M.J. AND SITTER, D.L. (1994) In-hospital evaluation of ortho-phthalaldehyde as a high level disinfectant for flexible endoscopes. *Journal of Hospital Infection.* Vol. 26: 15-26.

ALT, E., LEIPOLD, F., MILATOVIC, D., LEHMANN, G., HEINZ, S., AND SCHOMIG, A. (1999) Hydrogen peroxide for prevention of bacterial growth on polymer biomaterials. *The Annals of Thoracic Surgery.* Vol. 68: 2123-8.

ANDERSON, B.M., RASCH, M., HOCHLIN, K., JENSEN, F.-H., WISMAR, P. AND FREDRIKSEN, J.-E. (2006) Decontamination of rooms, medical equipment and ambulances using an aerosol of hydrogen peroxide disinfectant. *Journal of Hospital Infection.* Vol. 62: 149-155.

ANDREWS, S.C., ROBINSON, A.K., AND RODRIQUEZ-QUINONES, F. (2003) Bacterial iron homeostasis. *FEMS Microbiology Review.* Vol. 27: 215-237.

ARMOR, J.N. (2010) A history of industrial catalysis. *Catalysis Today* (2010), doi:10.1016/j.cattod.2009.11.019.

ASHRAF, S.S., RAUF, M.A., ALHADRAMI, S. (2006) Degradation of methyl using Fenton's reagent and the effect of various salts. *Dyes and Pigments.* Vol. 69: 74-78.

ASTM INTERNATIONAL (2002) Standard test methods for evaluation of inactivators of antimicrobial agents. *Designation: E 1054-02.* ASTM International, 100 Barr Harbor Drive, P.O. Box C700, West Conshohocken, PA 19428-2959. USA.

ATABEK, A. AND CAMESANO, T.A. (2007) Atomic force microscopy study of the effect of lipopolysaccharides and extracellular polymers on adhesion of *Pseudomonas aeruginosa. Journal of Bacteriology.* Vol. 189(23): 8503-8509.

AUTY, M.A.E., GARDINER, G.E., McBREARTY, S.J., O'SULLIVAN, E.O., MULVIHILL, D.M., COLLINS, J.K., FITZGERALD, G.F., STANTON, C., AND ROSS, R.P. (2001) Direct *in situ* viability assessment of bacteria in probiotic diary products using viability staining in conjunction with confocal scanning laser microscopy. *Applied and Environmental Microbiology.* Vol. 67(1): 420-425.

AVENDANO-HERRERA, R., MAGARINOS, B., IRGANG, R., AND TORANZO, A.E. (2006) Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*). *Aquaculture.* Vol. 257: 104-110.

BACIC, G. AND MOJOVIC, M. (2005) EPR spin trapping of oxygen radicals in plants: a methodology overview. *Annals New York Academy of Sciences.* Vol. 1048: 230-243.

BALDRY, M.G.C (1983) The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. *Journal of Applied Bacteriology.* Vol. 54: 417-423.

BAMFORD, C.H., JENKINS, A.D., INGRAM, D.J.E., AND SYMONS, M.C.R. (1955) Detection of free radicals in polyacrylonitrile by paramagnetic resonance. *Nature.* Vol. 175: 894-895.

BEATY, R.D. AND KERBER, J.D. (1993) Concepts, Instrumentation and Techniques in Atomic Absorption Spectrophotometry. 2nd ed. Norwalk, CT, The Perkin-Elmer Corporation. Pp. 1-1-1-7.

BEERS Jr, R.F. AND SIZER, I. (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *The Journal of Biological Chemistry.* Vol. 195(1): 133-40.

BELO, I., PINHEIRO, R., AND MOTA, M. (2005) Morphological and physiological changes in *Saccharomyces cerevisiae* by oxidative stress from hyperbaric air. *Journal of Biotechnology.* Vol. 115: 397-404.

BENITEZ, F.J., REAL, F.J., ACERO, J.L, LEAL, A.I., AND GARCIA, C. (2005) Gallic acid degradation in aqueous solutions by UV/H_2O_2 treatment, Fenton's reagent and the photo-Fenton system. *Journal of Hazardous Materials.* Vol. B126: 31-39.

BERGENDI, L., BENES, L., DURACKOVA, Z., FERENCIK, M. (1999) Chemistry, physiology and pathology of free radicals. *Life Science.* Vol. 65(18/19): 1865-1874.

BERNER, S., BIELA, S., LEDUNG, G., GOGOLL, A., BACKVALL, J.-E., PUGLIA, C., AND OSCARSSON, S. (2006) Activity boost of a biomimetic oxidation catalyst by immobilisation onto a gold surface. *Journal of Catalysis.* Vol. 244: 86-91.

BLOCK, S.S (1991) Historical review, In: BLOCK, S.S. (ed) Disinfection, sterilisation, and preservation, 4th ed. Philadelphia, Lea & Febiger. Pp. 3-17.

BLOCK, S.S. (2001) Definition of Terms. In: BLOCK, S.S. (ed), Disinfection, sterilisation and preservation, 5th, ed. Philadelphia, Lippincott Williams & Wilkins. Pp. 19-28.

BLOCK, S.S. (2001) Peroxygen Compounds, In: BLOCK, S.S. (ed), Disinfection, sterilisation and preservation, 5th, ed. Philadelphia, Lippincott Williams & Wilkins. Pp. 185-201.

BOONAERT, C.J.P. AND ROUXHET, P.G (2000) Surface of lactic acid bacteria: relationships between chemical composition and physicochemical properties. *Applied and Environmental Microbiology.* Vol. 66: 2548-2554.

BOWKER, M. (1998) The basis and application of heterogeneous catalysis. New York, Oxford University Press. Pp. 1-87.

BRAUNECKER, W.A. AND MATYJASZEWSKI, K. (2007) Controlled/living radical polymerisation: features, developments, and perspectives. *Progress in Polymer Science.* Vol. 32: 93-146.

BRINEZ, W.J., ROIG-SAGUES, A., HERNANDEZ HERRRO, M., LOPEZ-PEDEMONTE, T., GUAMIS, B. (2006) Bactericidal efficacy of peracetic acid in combination with hydrogen peroxide against pathogenic and non-pathogenic strains of *Staphylococcus* spp. *Listeria* spp. and *Escherichia coli*. *Food Control*. Vol. 17(7): 516-521.

BRUL, S. AND COOTE, P. (1999) REVIEW: Preservative agents in foods mode of action and microbial resistance mechanisms. *International Journal of food Microbiology.* Vol. 50: 1-17.

CARMICHAEL, I., HARPER, I.S., COVENTRY, M.J., TAYLOR, P.W.J., WAN, J., AND HICKEY, M.W. (1999) Bacterial colonisation and biofilm development on minimally processed vegetables. *Journal of Applied Microbiology* Vol. 85(Symp. Suppl.): 45S-51S.

CASEY, A.L., ADAMS, D., KARPANEN, T.J., LAMBERT, P.A., COOKSON, B.D., NIGHTINGALE, P., MIRUSZENKO, L., SHILLAM, R., CHRISTIAN, P., AND ELLIOT, T.S.J. (2010) Role of copper in reducing hospital environment contamination. *Journal of Hospital Infection.* Vol. 74: 72-77.

CENTI, G. AND PERATHONER, S. (1999) Oxidation catalyst: new trends. *Current Opinion in Solid State & Materials Science.* Vol. 4: 74-79.

CERETTA, R., PAULA, M.M.S., ANGIOLETTO, E., MEIER, M.M., MITELLSTADT, F.G., PICH, C.T., JUNIOR, S.A., AND ANGIOLETTO, E. (2008) Evaluation of the effectiveness of peracetic acid in the sterilisation of dental equipment. *Indian Journal of Medical Microbiology.* Vol. 26(2): 117-122.

CHANGHA, L. AND JEYONG, Y. (2004) Temperature dependence of hydroxyl radical formation in the hv/Fe^{3+}/H_2O_2 and Fe^{3+}/H_2O_2 systems. *Chemosphere.* Vol. 56(10): 923-934.

CHEN, W.R., SHARPLESS, C.M., LINDEN, K.G., AND SUFFET, I.H. (2006) Treatment of volatile organic chemicals on the EPA contaminant candidate list using ozonation and the O_3/H_2O_2 advanced oxidation process. *Environmental Science & Technology.* Vol. 40: 2734-2739.

CHENG, S.-A., FUNG, W.-K., CHAN, K.-Y., AND SHEN, P.K. (2003) Optimising electron spin resonance detection of hydroxyl radical in water. *Chemosphere.* Vol. 52: 1797-1805.

CHI, T.G. (2008) Synthesis, chemical characterisation and scale up of a heterogeneous catalyst for the oxidation of organic pollutants in wastewater. PhD Thesis – De Montfort University, Leicester, UK. Pp. 337.

CHICK, H. AND MARTIN, C. (1908) The Chick-Martin test for disinfectants. *The Journal of Hygiene.* Vol. 8: 654-697.

CHO, M., CHUNG, H., CHOI, W., YOON, J. (2004) Linear correlation between inactivation and OH radical concentration in TiO_2 photocatalytic disinfection. *Water Research.* Vol. 38: 1069-1077.

CHO, M., CHUNG, H., CHOI, W., YOON, J. (2005) Different Inactivation Behaviours of MS-2 Phage and *Escherichia coli* in TiO_2 Photocatalytic disinfection. *Applied and Environmental Microbiology.* Vol. 71(1): 270-275.

CHO, M., LEE, Y., CHUNG, H., AND YOON, J. (2004) Inactivation of *Escherichia coli* by photochemical reaction of ferrioxalate at slightly acidic and near-neutral pHs. *Applied and Environmental Microbiology.* Vol. 70(2): 1129-1134.

CHOUDHARY, V.R. AND JANA, P. (2007) Synergistic effect of two halogen promoters present in acidic reaction medium or catalyst on the H_2O_2 formation (in H_2-to- H_2O_2 oxidation) and destruction over Pd/C (or Al_2O_3) catalyst. *Journal of Catalysis.* Vol. 246: 434-439.

CHOUDHARY, V.R., JANA, P., SAMANTA, C. (2007) Generation of hydrogen peroxide via the selective reduction of oxygen by hydrazine sulfate over Br-promoted Pd/ Al_2O_3 catalyst in an aqueous medium at ambient conditions. *Applied Catalysis A: General.* Vol. 323: 202-209.

CLAASSENS, M.L.M. (1998) The EU Biocidal Products Directive and the Consequences for Disinfectants. *International Biodeterioration and Biodegradation.* Vol. 41: 303-307.

COLE-HAMILTON, D.J. (2003) Homogeneous catalysis – New approaches to catalyst separation, recovery, and recycling. *Science.* Vol. 299(5613): 1702-1706.

COLE-HAMILTON, D.J. AND TOOZE, R.P. eds. (2006) Catalyst separation, recovery and recycling. Netherlands, Springer. Pp. 1-8.

COOKE, R.P.D., GODDARD, S.V., WHYMANT-MORRIS, A., SHERWOOD, J., AND CHATTERLY, R. (2003) An evaluation of CIDEX OPA (0.55% *ortho*-phthalaldehyde) as an alternative to 2% glutaraldehyde for high-level disinfection of endoscopes. *Journal of Hospital Infection.* Vol. 54(3): 226-231.

CORSINI, A., WADE, G., WAN, C.-C., AND PRASAD, S. (1987) Speciation of soluble manganese in lake water with Chelex-100 and polyacrylate resin, XAD-7. *Canadian Journal of Chemistry.* Vol. 65: 915-918.

CREMIEUX, A., FRENEY, J., AND DAVIN-REGLI, A. (2001) Methods of testing disinfectants. In: BLOCK, S.S. ed. Disinfection, sterilisation, and preservation, 5th ed. Philadelphia, Lippincott Williams & Wilkins. Pp. 1305-1325.

CROSS, J.B., CURRIER, R.P., TORRACO, D.J., VANDERBERG, L.A., WAGNER, G.L., AND GLADEN, P.D. (2003) Killing of *Bacillus* spores by aqueous dissolved oxygen, ascorbic acid, and copper ions. *Applied and Environmental Microbiology.* Vol. 69(4): 2245-2252.

DANILCZUK, M. AND BOSNJAKOVIC, A. (2007) Direct ESR and spin trapping methods for the detection and identification of radical fragments in Nafion membranes and model compounds exposed to oxygen radicals. *Journal of Power Sources.* Vol. 172: 78-82.

DARTFORD AND GRAVESHAM NHS TRUST (2006) Decontamination Policy: Decontamination of medical devices/equipment and reusable surgical instruments. Ref: TW010, VERS: 2.1. pp. 1-37.

DATTA, A., AGARWAL, M., AND DASGUPTA, S. (2002) Novel vanadium phosphate phases as catalysts for selective oxidation. *Proceedings of the Indian Academy of Sciences.* Vol. 114(4): 379-390.

DECORET, D., AMOUSSOU, Y., LAGNEAU, C., LISSAC, M., MORRIER, JJ., AND BARSOTTI, O. (2005) A simulated-use evaluation of hydrogen peroxide disinfectant for preventing biofilm formation in dental unit waterlines. *European Cells and Materials.* Vol. 10(Suppl.4): 24-25.

DEISSEROTH, A. AND DOUNCE, A.L. (1970) Catalase: Physical and chemical properties, mechanism of catalysis, and physiological role. *Physiological Review.* Vol. 50: 319-375.

DELL'ERBA, A., FALSANISI, D., LIBERTI, L., NOTARNICOLA, M., AND SANTORO, D. (2007) Disinfection by-products formation during wastewater disinfection with peracetic acid. *Desalination.* Vol. 215: 177-186.

DEMIREL, S., TUZEN, M., SARACOGLU, S., AND SOYLAK, M. (2008) Evaluation of various digestion procedures for trace element contents of some food materials. *Journal of Hazardous Materials.* Vol. 152(3): 1020-1026.

DENYER, S.P. AND MAILLARD, J-Y. (2002) Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. *Journal of Applied Microbiology Symposium Supplement.* Vol. 92: 35S-45S.

DITOMMASO, S., BIASIN, C., GIACOMUZZI, M., ZOTTI, C.M., CAVANNA, A., AND MOIRAGHI, A.R. (2005) Peracetic acid in the disinfection of a hospital water system contaminated with *Legionella* species. *Infection Control and Hospital Epidemiology.* Vol. 26(5): 490-493.

DRAMOU, B.J., SHAH, V., PINTO, J.M. (2008) A kinetic model for microbial decontamination of water based on a modified Fenton reaction. *Energy and Environmental Science.* Vol. 1: 395-402.

DUAN, Y., DINEHART, K., HICKEY, J., PANICUCCI, R., KESSLER, J., AND GOTTARDI, W. (1999) Properties of an enzyme based low level iodine disinfectant. *Journal of Hospital Infection.* Vol. 43: 219-229.

DUNFORD, H.B. (2002) Oxidations of iron(II)/(III) by hydrogen peroxide: from aquo to enzyme. *Coordination Chemistry Reviews.* Vol. 233-234: 311-318.

DYKE, B.R.V., CLOPTON, D.A., AND SALTMAN, P. (1996) Buffered-induced anomalies in the Fenton chemistry of iron and copper. *Analytica Chimica Acta.* Vol. 242: 57-61.

EALY, J.B. (1991) An alternative to formaldehyde: Avoiding the carcinogenic risks. *Science Teacher*. Vol. 58(4): 26-27.

ELCHIGUERRA, J.L., BURT, J.L., MORONES, J.R., CAMACHO-BRAGADO, A., GAO, X., LARA, H.H., AND YACAMAN, M.J. (2005) Interaction of silver nanoparticles with HIV-1. *Journal of Nanobiotechnology.* Vol. 3: 6.

ELKINS, J.G., HASSATT, D.J., STEWART, P.S., SCHWEIZER, H.P., AND McDERMOTT, T.R. (1999) Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Applied and Environmental Microbiology.* Vol. 65(10): 4594-4600.

EUROPEAN COMMISSION (1998) Directive 98/8EC of the European Parliament and the Council of 16 February 1998 concerning the placing of biocidal products on the market.

Official Journal of the European Communities no. L123 of 24 April 1998. Office for official publications of the European Communities, L-2985 Luxembourg.

FIDALEO, M. AND LAVECCHIA, R. (2003) Kinetic study of hydrogen peroxide decomposition by catalase in a flow-mix microcalorimetric system. *Thermochimica Acta.* Vol. 402: 19-26.

FIORENZA, S. AND WARD, C.H. (1997) Microbial adaptation to hydrogen peroxide and biodegradation of aromatic hydrocarbons. *Journal of Industrial Microbiology and Biotechnology.* Vol. 18: 140-151.

FOLIENTE, R.L., KOVACS, B.J., APRECIO, R.M., BAINS, H.J., KETTERING, J.D., AND CHEN, Y.K. (2001) Efficacy of high-level disinfectants for reprocessing GI endoscopes in simulated-use testing. *Gastrointestinal Endoscopy.* Vol. 53: 456-62.

FRANCO, R., SCHONEVELD, O., GEORGAKILAS, A.G., PANAYIOTIDIS, M.I. (2008) Oxidative stress, DNA methylation and carcinogenesis. *Cancer Letters.* Vol. 266: 6-11.

FRIDIVOCH, I. (1978) The biology of oxygen radicals. *Science.* Vol. 201: 875-880.

GADD, G.M. (1993) Interactions of fungi with toxic metals. *New Phytologist.* Vol. 124: 25-60.

GARCIA-MONTANO, J., TORRADES, F., GARCIA-HORTAL, J.A., DOMENECH, X., PERAL, J. (2006) Combining photo-Fenton process with aerobic sequencing batch reactor for commercial hetero-bireactive dye removal. *Applied Catalysis B: Environmental.* Vol. 67: 86-92.

GARDINER, G.E., O'SULLIVAN, E., KELLY, J., AUTY, M.A.E., FITZGERALD, G.F., COLLINS, J.K., ROSS, R.P., AND STANTON, C. (2000) Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. *Applied and Environmental Microbiology.* Vol. 66(6): 2605-2612.

GARDNER, J.F. AND PEEL, M.M. (1998) Sterilization, disinfection, and infection. 3rd. Florida, Harcourt Brace & Company. Pp. 1-5; 153-168.

GIBSON, C.M., MALLETT, T.C., CLAIBORNE, A., AND CAPARON, M.G. (2000) Contribution of NADH oxidase to aerobic metabolism of *Streptococcus pyogenes. Journal of Bacteriology.* Vol. 182: 448-455.

GLAUNER, T., WALDMANN, P., FRIMMEL, F.H., AND ZWIENER, C. (2005) Swimming pool water – fractionation and genotoxicological characterisation of organic constituents. *Water Research.* Vol. 39: 4494-4502.

GOGATE, P.R. AND PANDIT, A.B. (2004) A review of imperative technologies for wastewater treatment I: oxidation technologies at ambient conditions. *Advances in Environmental Research.* Vol. 8: 501-551.

GONG, B.L. (2002) Synthesis of polyacrylaminoimidazole chelating fibre and properties of concentration and separation of trace Au, Hg, and Pd from samples. *Talanta.* Vol. 57: 89-95.

GONSETTE, R.E. (2008) Neurodegeneration in multiple sclerosis: The role of oxidative stress and excitotoxicity. *Journal of the Neurological Sciences.* Vol. 274: 48-53.

GOTTARDI, W. (1985) The influence of the chemical behaviour of iodine on the germicidal action of disinfection solutions containing iodine. *Journal of Hospital Infection.* Vol. 6(Suppl. A): 1-11.

GREENSPAN, F.P. AND MACKELLAR, D.G. (1951) The application of peracetic acid germicidal washes to mould control of tomatoes. *Food Technology.* Vol. 5: 95-97.

GROSCH, G.H. AND RANGE, K.-J. (1996) Chemical trends in gold alkali alloys: A density functional theory study on stability and charge transfer Part 1: Gold alkali alloys of the formula MAu. *Journal of Alloys and Compounds.* Vol. 233(1-2): 30-38.

GUWY, A.J., MARTIN, S.R., HAWKES, F.R., AND HAWKES, D.L. (1999) Catalase activity measurement in suspended aerobic biomass and soil samples. *Enzyme and Microbial Technology.* Vol. 25: 669-676.

GUZEL-SEYDIM, Z.B., GREENE, A.K., AND SEYDIM, A.C. (2004) Use of ozone in the food industry. *Lebensm.-Wiss. u.-Technol.* Vol. 37: 453-460.

HACH LANGE LTD, Hydrogen peroxide LCW 058 Edition 90/06, Hach Lange Ltd Pacific Way, Salford, Manchester, M50 1DL, UK.

HADJOK, C., MITTAL, G.S, AND WARRINER, K. (2008) Inactivation of human pathogens and spoilage bacteria on the surface and internalised within fresh produce by using a combination of ultraviolet light and hydrogen peroxide. *Journal of Applied Microbiology.* Vol. 104: 1014-1024.

HALLIWELL, B. (1995) Oxygen radicals, nitric oxide and human inflammatory joint disease. *Annals of the Rheumatic Diseases.* Vol. 54: 505-510.

HALLIWELL, B. AND GUTTERIDGE, J.M.C. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal.* Vol. 219: 1-14.

HAMOUDA, T. AND BAKER, Jr. J.R. (2000) Antimicrobial mechanism of action of surfactant lipid preparations in enteric Gram-negative bacilli. *Journal of Applied Microbiology.* Vol. 89: 397-403.

HAN, S.-K. (2004) Enhancing effect of organic substances on hydroxyl radical generation during ozonation of water: Stopped-flow ESR technique. *Bulletin of the Korean Chemical Society.* Vol. 25(12): 1907-1910.

HANSON, P.J.V., GOR, D., JEFFRIES, D.J., AND COLLINS, J.V. (1989) Chemical inactivation of HIV on surfaces. *British Medical Journal.* Vol. 298: 862-864.

HARRISON, R.M AND De MORA, S.J (1996) Introduction chemistry for environmental sciences, 2nd ed. Cambridge, Press Syndicate. Pp. 133-138.

HART, M.E., CRUM, R.M., ST. JOHN-TIDWELL, M.A., AND MALAKOWSKY, C.A. (2001) Isolation of stable hemolysin and catalase variants of Staphylococcus aureus S6C includes one with an exoprotein-deficient phenotype. *Current Microbiology.* Vol. 43(2): 134-139.

HE, J., MORIYA, Y., OKETA, T., SASABE, S., NISHIDA, H., CHEN, L., ZHU, S., ZHANG, D., OMORI, H., AND CAO, G. (2008) Inactivation Mechanism of Microorganisms by the Synergy of Silver and Light Irradiation, and the Application in Household Electrical Appliances. *Chinese Journal of Biotechnology.* Vol. 24(6): 1091-1097.

HENLE, E.S. AND LINN, S. (1997) Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. *The Journal of Biological Chemistry.* Vol. 272(31): 19095-19098.

HICKS, J. (1975) Comprehensive chemistry. 2nd ed. London, The MacMillan Press. Pp. 331-343; 544-561.

HODGES, N.A. AND DENYER, S.P. (1996) Preservative testing. In: SWARBRICK, J. AND BOYLEN, J.C. (eds) Encyclopaedia of pharmaceutical technology. New York, Marcel Dekker. Pp. 21-37.

HORST, F., RUEDA, E.H., AND FERREIRA, M.L. (2006) Activity of magnetite-immobilised catalase in hydrogen peroxide decomposition. *Enzyme and Microbial Technology.* Vol. 38(7): 1005-1012.

HOWLETT, N.G. AND AVERY, S.V. (1997) Induction of lipid peroxidation during heavy metal stress in *Saccharomyces cerevisiae* and influence of plasma membrane fatty acid unsaturation. *Applied and Environmental Microbiology.* Vol. 63(8): 2971-2976.

HSU, H., SELVIN, R., AND HER, T. (2007) Highly active and reusable heterogeneous catalysts for acylation of anisole. *Journal of Thermal Analysis and Calorimetry.* Vol. 89(2): 379-383(5).

HUGHES, R. AND KILVINGTON, S. (2001) Comparison of hydrogen peroxide contact lens disinfection systems and solutions against *Acanthamoeba polyphaga. Antimicrobial Agents and Chemotherapy.* Vol. 47(7): 2038-2043.

HUGO, W.B. (1995) A brief history of heat, chemical and radiation preservation and disinfection. *International Biodeterioration & Biodegradation.* Vol. 36(3-4): 197-217.

IBRAHIM, S.A., YANG, H., AND SEO, C.W. (2008) Antimicrobial activity of lactic acid and copper on growth of *Salmonella* and *Escherichia coli* O157:H7 in laboratory medium and carrot juice. *Food Chemistry.* Vol. 109: 137-143.

IM, K., TAKASAKI, Y., ENDO, A., KURIYAMA, M. (1996) antibacterial activity of A-Type zeolite supporting silver ions in distilled water. *Journal of Antibacterial and Antifungal Agents.* Vol. 24: 269-274.

IMLAY, J.A., CHIN, S.M., LINN, S (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science.* Vol. 240: 640-642.

INOUE, Y., HOSHINO, M., TAKAHASHI, H., NOGUCHI, T., MURATA, T., KANZAKI, Y., HAMASHIMA, H., SASATSU, M. (2002) Bactericidal activity of Ag-

zeolite mediated by reactive oxygen species under aerated conditions. *Journal of Inorganic Biochemistry.* Vol. 92: 37-42.

IRELAND, J.C., KLOSTERMANN, P., RICE, E.W., AND CLARK, R.M. (1993) Inactivation of *Escherichia coli* by Titanium Dioxide Photocatalytic Oxidation. *Applied and Environmental Microbiology.* Vol. 59(5): 1668-1670.

ISHTCHENKO, V.V., HUDDERSMAN, K.D., AND VITKOVSKAYA, R.F. (2003) Part 1. Production of a modified PAN fibrous catalyst and its optimisation towards the decomposition of hydrogen peroxide. *Applied Catalysis A: General.* Vol. 242:123-137.

IYER, S., AND THAKUR, V.V. (2000) The novel use of Ni, Co, Cu, and Mn heterogeneous catalysts for the Heck reaction. *Journal of Molecular Catalysis A: Chemical.* Vol. 157: 275-278.

JACKSON, C.J., CARR, P.D., KIM, H.-K., LIU, J.-W., HERALD, P., MITI, N., SCHENK, G., SMITH, C.A., AND DAVID, L. (2006) Anomalous scattering analysis of Agrobacterium radiobacter phosphotriesterase: the prominent role of iron in the heterobinuclear active site. *Biochemical Journal.* Vol. 397: 501-508.

JOHNSTON, M.D., LAWSON, S., AND OTTER, J.A. (2005) Evaluation of hydrogen peroxide vapour as a method for the decontamination of surfaces contaminated with *Clostridium botulinum* spores. *Journal of Microbiological Methods.* Vol. 60: 403-411.

JONES, C.W. (1999) Applications of hydrogen peroxide and derivatives. Cambridge, The Royal Society of Chemistry. Pp. 1-32, 37-72.

JOSE-MOREIRA, A., MANSANO, R.D., PINTO, T.D.J.A., RAUS, R., ZAMBON, L.D.S., SILVA, M.V.D., AND VERDONCK, P.B. (2004) Sterilisation by oxygen plasma. *Applied Surface Science.* Vol. 235: 151-155.

KANG, C., HUANG, J., HE, W., AND ZHANG, F. (2010) Periodic mesoporous silica-immobilised palladium (II) complex as an effective and reusable catalyst for water-medium carbon-carbon coupling reactions. *Journal of Organometallic Chemistry.* Vol. 695: 120-127.

KANG, N. AND HUA, I. (2005) Enhanced chemical oxidation of aromatic hydrocarbons in soil systems. *Chemosphere.* Vol. 61: 909-922.

KANG, N., HUA, I., SURESH, P., RAO, C. (2006) Enhanced Fenton's destruction of non-aqueous phase perchloroethylene in soil systems. *Chemosphere.* Vol. 63(10): 1685-1698.
KARAKURT, A., DALKARA, S., OZALP, M., OZBEY, S., KENDI, E., AND STABLES, J.P. (2001) Synthesis of some 1-(2-naphthyl)-2-(imidazole-1-yl)ethanone oxime and oxime ether derivatives and their anticonvulsant and antimicrobial activities. *European Journal of Medicinal Chemistry.* Vol. 36: 421-433.
KARCI, F., SENER, N., YAMAC, M., SENER, I., AND DEMIRCALI, A. (2009) The systhesis, antimicrobial activity and absorption characteristics of some novel heterocyclic disazo dyes. *Dyes and Pigments.* Vol. 80: 47-52.
KECELI, E. AND OZKAR, S. (2008) Ruthenium(III) acetylacetonate: A homogeneous catalyst in hydrolysis of sodium borohydride. *Journal of Molecular Catalysis A: Chemical.* Vol. 286: 87-91.
KEHOE, S.C., BARER, M.R., DEVLIN, L.O., AND McGUIGAN, K.G. (2004) Batch process solar disinfection is an efficient means of disinfecting water contaminated with *Shigella dysenteriae* type I. *Letters in Applied Microbiology.* Vol. 38: 410-414.
KHAENGRAENG, R. AND REED, R.H. (2005) Oxygen and photoinactivation of *Escherichia coli* in UVA and sunlight. *Journal of Applied Microbiology.* Vol. 99: 39-50.
KIDA, N., MOCHIZUKI, Y., AND TAGUCHI, F. (2004) An effective iodide formulation for killing Bacillus and *Geobacillus* spores over a wide temperature range. *Journal of Applied Microbiology.* Vol. 97: 402-409.
KIEBOOM, A.P.G., MOULIN, J.A., van LEEUWEN, P.W.N.M., AND van SANTEN, R.A., (2000) History of catalysis. *Studies in Surface Science and Catalysis.* Vol. 123: 3-28.
KIM, H., HAHN, M., GRABOWSKI, P., McPHERSON, D.C., OTTE, M.M., WANG, R., FERGUSON, C.C., EICHENBERGER, P., AND DRIKS, A. (2006) The *Bacillus subtilis* spore coat protein interaction network. *Molecular Microbiology.* Vol. 59(2): 487-502.
KIM, J., CHO, M., OH, B., CHOI, S. AND YOON, J. (2004) Control of bacterial growth in water using synthesised inorganic disinfectant. *Chemosphere.* Vol. 55: 775-780
KIM, J.K. AND METCALFE, I.S. (2007) Investigation of the generation of hydroxyl radicals and their oxidative role in the presence of heterogeneous copper catalysts. *Chemosphere.* Vol. 69: 689-696.

KING, C.H., SHOTTS, JR. E.B., WOOLEY, R.E., AND PORTER, K.G. (1988) Survival of coliforms and bacterial pathogens within protozoa during chlorination. *Applied and Environmental Microbiology.* Vol. 54(12): 3023-3033.

KIRILMIS, C., AHMEDZADE, M., SERVI, S., KOCA, M., KIZIRGIL, A., AND KAZAZ, C. (2008) Synthesis and antimicrobial activity of some novel derivatives of benzofuran: Part 2. The synthesis and antimicrobial activity of some novel 1-(1-benzofuran-2-yl)-2-mesitylethanone derivatives. *European Journal of Medicinal Chemistry.* Vol. 43: 300-308.

KIRSCHVINK, N., MOFFARTS, B.D., LEKEUX, P. (2008) The oxidant/antioxidant equilibrium in horses. *The Veterinary Journal.* Vol. 177: 178-191.

KITIS, M. (2004) Disinfection of wastewater with peracetic acid: a review. *Environmental International.* Vol. 30: 47-55.

KNAPP, J.E. AND BETTISTI, D.L. (2001) Chlorine dioxide. In: BLOCK, S.S. (ed), Disinfection, sterilisation and preservation, 5th, ed. Philadelphia, Lippincott Williams & Wilkins. Pp. 215-227.

KODJIKIAN, L., BURILLON, C., ROQUES, C., PELLON, G., FRENEY, J., AND RENAUD, F.N.R. (2003) Bacterial adherence of *Staphylococcus epidermidis* to intraocular lenses: A bioluminescence and scanning electron microscopy study. *Investigative Ophthalmology and Visual Science.* Vol. 44: 4388-4394.

KREKELER, C., ZIEHR, H., AND KLEIN, J. (1991) Influence of physicochemical bacterial surface properties on adsorption to inorganic porous supports. *Applied Microbiology and Biotechnology.* Vol. 35: 484-490.

KUMAR, V.S., NAGARAJA, B.M., SHASHIKALA, V., PADMASRI, A.H., MADHAVENDRA, S.S., RAJU, B.D., AND RAO, K.S.R. (2004) Highly efficient Ag/C catalyst prepared by electro-chemical deposition method in controlling microorganisms in water. *Journal of Molecular Catalysis A: Chemical.* Vol. 223: 313-319.

LAAT, J.D., LE, G.T., LEGUBE, B. (2004) A comparative study of the effects of chloride, sulfate and nitrate ions on the rates of decomposition of H_2O_2 and organic compounds by Fe(II)/ H_2O_2 and Fe(III)/ H_2O_2. *Chemosphere.* Vol. 55: 715-723.

LABAS, M.D., ZALAZAR, C.S., BRANDI, R.J., AND CASSANO, A.E. (2008) Reaction kinetics of bacteria disinfection employing hydrogen peroxide. *Biochemical Engineering Journal.* Vol. 38: 78-87.

LARSON, E.L. (1996) Antiseptics, In: OLMSTAD, R.N. (ed) APIC infection control & applied epidemiology: principles & practices. St Louis, Mosby-Year Book, Inc. pp. 19-1-19-7, G1-G17.

LARSON, E.L. AND MORTON, H.E. (1991) Alcohols. In: BLOCK, S.S. (ed), Disinfection, Sterilisation and Preservation, 4th, ed. Philadelphia, Lipincott Williams & Wilkins. Pp. 191-203.

LEE, S., YOUNAN, H., SIPING, Z., AND ZHIQIANG, M. (2006) Studies on electron penetration versus beam acceleration voltage in Energy-Dispersive X-ray microanalysis. *Proceedings of the IEEC International Conference. Kuala Lumpur, October 2006.*

LEUKO, S., LEGAT, A., FENDRIHAN, S., AND STAN-LOTTER, H. (2004) Evaluation of the LIVE/DEAD *Bac*Light kit for detection of extremophilic archaea and visualisation of microorganisms in environmental hypersaline samples. *Applied and Environmental Microbiology.* Vol. 70(11): 6884-6886.

LI, J., AI, Z., AND ZHANG, L. (2008) Design of a neutral electro-Fenton system with Fe@Fe_2O_3/ACF composite cathode for wastewater treatment. *Journal of Hazardous Materials.* Doi: 10.1016/j.jhazmat.2008.07.109.

LI, X. AND LOGAN, B.E. (2004) Analysis of bacterial adhesion using a gradient force analysis method and colloid probe atomic force microscopy. *Langmuir.* Vol. 20(20): 8817-8822.

LIAU, S.Y., READ, D.C., PUGH, W.J., FURR, J.R., RUSSELL, A.D. (1997) Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterialaction of silver ions. *Letters in Applied Microbiology.* Vol. 25(4): 279-283.

LIBERTI, L. AND NOTARNICOLA, M. (1999) Advanced treatment and disinfection for municipal wastewater reuse in agriculture. *Water Science and Technology.* Vol. (40): 235-45.

LIN, C.C., SMITH, F.R., ICHIKAWA, N., BABA, T., AND ITOW, M. (1991) Decomposition of hydrogen peroxide in aqueous solutions at elevated temperatures. *International Journal of Chemical Kinetics.* Vol. 23(11): 971-987.

LINDSEY, M.E., AND TARR, M.A. (2000) Quantitation of hydroxyl radical during Fenton oxidation following a single addition of iron and peroxide. *Chemosphere.* Vol. 41(3): 409-417.

LINXIANG, L., ABE, Y., KANAGAWA, K., SHOJI, T., MASHINO, T., MOCHIZUKI, M., TANAKA, M., AND MIYATA, N. (2007) Iron-chelating agents never suppress Fenton reaction but participate in quenching spin-trapped radicals. *Analytica Chimica Acta.* Vol. 599: 315-319.

LIU, R.X., TANG, H.X., AND LI, Y. (1999) Application of the modified polyacrylonitrile fibre with amino-carboxyl-tetrazine groups for the preconcentration of trace heavy metal ions. *Journal of Applied Polymer Science.* Vol. 11: 7-14.

LUBEC, B., HAYN, M., DENK., W., BAUER, G. (1996) Brain lipid peroxidation and hydroxyl radical attack following the intravenous infusion of hydrogen peroxide in an infant. *Free Radical Biology and Medicine.* Vol. 21(2): 219-223.

LUO, Y., HAN, Z., CHIN, S.M., AND LINN, S. (1994) Three chemically distinct types of oxidants formed by iron-mediated Fenton reaction in the presence of DNA. *Proceedings of the National Academy of Sciences.* Vol. 91: 12438-12442.

LYNCH, R. AND FRIDOVICH, I. (1978) Effects of superoxide on the erythrocyte membrane. *Journal of Biological Chemistry.* Vol. 253: 1838-1845.

MAILLARD, J-Y. (2002) Bacterial target sites for biocide action. *Journal of Applied Microbiology Symposium Supplement.* Vol. 92: 16S-27S.

MALORNY, B., PACCASSONI, P.F., BUNGE, C., MARTIN, A., AND HELMUTH, R. (2004) Diagnostic Real-time PCR for Detection of *Salmonella* in Food. *Applied and Environmental Microbiology.* Vol. 70(12): 7046-7052.

MAQBOOL, A., KORDESCH, M.E., AND AHMAD, I. (2009) Electron penetration depth in amorphous AIN exploiting the luminescence of AIN: Tm/AIN: Ho bilayers. *Current Applied Physics.* Vol. 9(2): 417-421.

MARTIN, S.E. AND CHAVEN, S. (1987) Synthesis of catalase in *Staphylococcus aureus* MF-31. *Applied and Environmental Microbiology.* Vol. 53(6): 1207-1209.

MATSUMURA, Y., YOSHIKATA, K., KUNISAKI, S., TSUCHIDO, T. (2003) Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Applied and Environmental Microbiology.* Vol. 69(7): 4278-4281.

MATTILA, T. (1987) A modified Kelsey-Sykes method for testing disinfectants with 2,3,5-triphenyltetrazolium chloride reduction as an indicators of bacterial growth. *Journal of Applied Bacteriology.* Vol. 62: 551-554.

MAUKONEN, J., MATTILA-SANDHOLM, T., AND WIRTANEN, G. (2000) Metabolic indicators for assessing bacterial viability in hygiene sampling using cells in suspension and swabbed biofilm. *Lebensm.-Wiss.U.-Technol.* Vol. 33: 225-233.

McBAIN, A.J. AND GILBERT, P. (2001) Biocide tolerance and the harbingers of doom. *International Biodeterioration & Biodegradation.* Vol. 47: 55-61.

McDONNELL, C., BONFIELD, P., AND HERNANDEZ, V.D. (2007) The safe and effective fumigation of hospital areas with a new fumigation method based on vaporised hydrogen peroxide. *American Journal of Infection Control.* Vol. 35(5): E33-E34.

McDONNELL, G. AND PRETZER, D. (2001) New and developing Chemical Antimicrobials. In: BLOCK, S.S. (ed), Disinfection, sterilisation and preservation, 5th, ed. Philadelphia, Lippincott Williams & Wilkins. Pp. 437-441.

McDONNELL, G. AND RUSSELL, A.D. (1999) Antiseptics and Disinfectants: Activity, Action, and Resistance, *Clinical Microbiology Review,* Vol. 12 (1); 147-179.

MENDHAM, J., DENNEY, R.C., BARNES, J.D., AND THOMAS, M.J.K. (2000) Vogel's textbook of quantitative chemical analysis, 6th ed. Harlow, Pearson Education Limited. Pp. 428-434, 584-596.

MENDIL, D., an ULUÖZLÜ, O.D., an HASDEMIR., AND CA LAR, A. (2004) Determination of trace elements on some wild edible mushroom samples from Kastamonu, Turkey. *Food Chemistry.* Vol. 88(2): 281-285.

MESSNER, K.R. AND IMLAY, J.A. (1999) The identification of primary sites of superoxide and hydrogen peroxide formation in aerobic respiratory chain and sulfite

reductase complex of *Escherichia coli*. *The Journal of Biological Chemistry.* Vol. 274(15): 10119-10128.
MIDDELBEEK, E.J. AND DRIJVER-DE HAAS, J.S. (1992) Environmental factors influencing growth. In: CARTLEDGE, T.G. ed. In vitro cultivation of micro-organisms. Butterworth-Heinemann Ltd, Oxford. Pp. 146-175.
MILES, A.A. AND MISRA, S.S. (1938) Estimation of the bactericidal power of the blood. *The Journal of Hygiene.* Vol. 38: 732-749.
MILLER, R.A. AND BRITIGAN, B.E. (1997) Role of oxidants in microbial pathophysiology. *Clinical Microbiology Reviews.* Vol. 10(1): 1-18.
MILLS, A.L., HERMAN, J.S., HORNBERGER, G.M., AND DeJESUS, T.H. (1994) Effect of solution ionic strength and iron coatings on mineral grains on the sorption of bacterial cells to quartz sand. *Applied and Environmental Microbiology.* Vol. 60(9): 3300-3306.
MODHA, J., DATTA, N., AND PAREKH, H. (2001) Synthesis and biological evaluation of some new 3,4dihydropyrimidin-4-ones. *IL FARMACO.* Vol. 56: 641-646.
MOLECULAR PROBES, INVITROGEN DETECTION TECHNOLOGIES (2004) LIVE/DEAD® *Bac*Light Bacterial Viability Kits, Invitrogen Ltd, 3 Fountain Drive, Paisley, PA4 9RF, UK.
MOORE, S.L. AND PAYNE, D.N. (2004) Types of antimicrobial agents. In: FRAISE, A.P., LAMBERT, P.A., AND MAILARD, J-Y. (eds), Principles and practice of disinfection, preservation and sterilisation. Oxford, Blackwell Publishing Inc. pp. 10-77.
MORADAS-FERREIRA, P., COSTA, V., PIPER, P., AND MAGER, W. (1996) The molecular defences against reactive oxygen species in yeast. *Molecular Microbiology.* Vol. 19: 651-658.
MORTAZAVI, S.B., SABZALI, A., REZAEE, A. (2005) Sequence-Fenton reaction for decreaing phenol formation during benzene chemical conversion in aqueous solutions. *Iranian Journal of Environmental Health Science & Engineering.* Vol. 2(2): 62-71.
MOSKOVITZ, J., RAHMAN, M.A., STRASSMAN, J., YANCEY, S.O., KUSHNER, S.R., BROT, N., WEISSBACH, H. (1995) *Escherichia coli* peptide methionine sulfoxide

reductase gene: regulation of expression and role in protecting against oxidative damage. *Journal of Bacteriology.* Vol. 177: 502-507.
MULLEN, K. (2002) Disinfectants and Public Health Biocides. In: KNIGHT, D.J. AND COOKE, M. (eds) The Biocides Business: Regulation, safety, and Applications. Germany, Wiley-VCH. Pp. 252-266.
MURRAY, C.A. AND PARSONS, S.A. (2004) Removal of NOM from drinking water: Fenton's and photo-Fenton's processes. *Chemosphere.* Vol. 54: 1017-1023.
NDJOU'OU, A-C. AND CASSIDY, D. (2006) Surfactant production accompanying the modified Fenton oxidation of hydrocarbons in soil. *Chemosphere.* Vol. 65: 1610-1615.
NEWMAN, G.R., WALKER, M., HOBOT, J.A., AND BOWLER, P.G. (2006) Visualisation of bacterial sequestration and bactericidal activity within hydrating hydrofiber® wound dressings. *Biomaterials.* Vol. 27: 1129-1139.
NEYENS, E. AND BAEYENS, J. (2003) A review of classic Fenton's peroxidation as an advanced oxidation technique. *Journal of Hazardous Materials.* Vol. B98: 33-50.
NICHOLAS, JW. AND SØRENSEN, J. (2006) Catalase and superoxide dismutase activity in ammonia-oxidising bacteria. *FEMS Microbiology Ecology.* Vol. 38(1): 53-58.
NIES, D.H. (1999) Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology.* Vol. 51: 730-750.
NOYCE, J.O., MICHELS, H., AND KEEVIL, C.W. (2006) Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant *Staphylococcus aureus* in the healthcare environment. *Journal of Hospital Infection.* Vol. 63: 289-297.
NOYCE, J.O., MICHELS, H., AND KEEVIL, C.W. (2006) Use of copper cast alloys to control *Escherichia coli* O157 cross-contamination during food processing. *Applied and Environmental Microbiology.* Vol. 72: 4239-4244.
NOYCE, J.O., MICHELS, H., AND KEEVIL, C.W. (2007) Inactivation of influenza A virus on copper versus stainless steel surfaces. *Applied and Environmental Microbiology.* Vol. 73: 2748-2750.
OGIYAMA, S., SAKAMOTO, K., SUZUKI, H., USHIO, S., ANZAI, T., AND INUBUSHI, K. (2006) Measurement of concentrations of trace metals in arable soils with

animal manure application using instrumental neutron activation analysis and the concentrated acid digestion method. *Soil Science and Plant Nutrition.* Vol. 52: 114-121.
OKUNO, T., KAWAI, H., HASEGAWA, T., UENO, H., AND NAKAMURO, K. (2001) Enhancement of hydroxyl radical formation from superoxide anion radical in the presence of organic selenium compounds. *Journal of Health Science.* Vol. 47(3): 240-247.
OMIDBAKHSH, N. (2006) A new peroxide-based flexible endoscope-compatible high-level disinfectant. *American Journal of Infection Control.* Vol. 34: 571-7.
OMIDBAKHSH, N. AND SATTER, S.A. (2006) Broad-spectrum microbicidal activity, toxicologic assessment, and materials compatibility of a new generation of accelerated hydrogen peroxide-based environmental surface disinfectant. *American Journal of Infection Control.* Vol. 34: 251-257.
ONWURAH, I.N.E. (1999) Lipid peroxidation and protein oxidation in *Azotobacter vinelandii* exposed to mercury, silver, crude oil and Fenton reagent. *Toxicology Mechanisms and Methods.* Vol. 18(4): 167-176.
PAPADOPOULOS, A.E., FATTA, D., LOIZIDOU, M. (2007) Development and optimisation of dark Fenton oxidation for the treatment of textile wastewaters with high organic load. *Journal of Hazardous Materials.* Vol. 146: 558-563.
PARADIES, G., PETROSILLO, G., PISTOLESE, M., VENOSA, N.D., SERENA, D., RUGGIERO, F.M. (1999) Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. *Free Radical Biology and Medicine.* Vol. 27(1/2): 42-50.
PARK, S. AND IMLAY, J.A (2003) High levels of intracellular cysteine promote oxidative DNA damage by driving the Fenton reaction. *Journal of Bacteriology.* Vol. 185(6): 1942-1950.
PARKER IV, H.H., AND JOHNSON, R.B. (1995) Effectiveness of ethylene oxide for sterilisation of dental handpieces. *Journal of Dentistry.* Vol. 23(2): 113-115.
PATIL, N.M., GUPTE, S.P., AND CHAUDHARI, R.V. (2010) Heterogenised copper catalysts for the amination of arylhalide: synthesis, characterisation, and catalytic applications. *Applied Catalysis A: General.* Vol. 372: 73-81.

PAVLINEC, J. AND MOSZNER, N. (2003) Dark reactions of free radicals trapped in densely crosslinked polymer networks after photopolymerisation. *Journal of Applied Polymer Science.* Vol. 89: 579-588.

PAYNE, D.N., BABB, J.R., AND BRADLEY, C.R. (1999) An evaluation of the suitability of the European suspension test to reflect *in vitro* activity of antiseptics against clinically significant organisms. *Letters in Applied Microbiology.* Vol. 28: 7-12.

PERICONE, C.D., PARK, S., IMLAY, J.A., WEISER, J.N. (2003) Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the Fenton reaction. *Journal of Bacteriology.* Vol. 185(23): 6815-6825.

PFEIFFER, D., JUNG, W., FEHSKE, W., KORTE, T., MANZ, M., AND MOOSDORF, R. (1994) Complications of pacemaker-defibrillator devices: diagnosis and management. *American Heart Journal.* Vol. 127: 1073-80.

POPHAM, D.L., SENGUPTA, S., AND SETLOW, P. (1995) Heat, Hydrogen Peroxide, and UV resistance of *Bacillus subtilis* Spores with Increased Core Water Content and with or without Major DNA-Binding Proteins. *Applied and Environmental Microbiology.* Vol. 61(10): 3633-3638.

POURNAGHI-AZAR, M.H. AND FATEMI, B.M. (2000) Simultaneous determination of ferric, ferrous and total iron by extraction differential pulse polarography: application to the speciation of iron in rocks. *Microchemical Journal.* Vol. 65(3): 199-207.

QUI, Q-S., LIANG, A.G., ZHENG, H.J., AND CHEN, P. (1994) Ca^{2+} calmodulin-stimulated superoxide generation by purified plasma membrane from wheat roots. *Plant Science.* Vol. 101(2): 99-104.

RAMIREZ, J.H., COSTA, C.A., MADEIRA, L.M., MATA, G., VICENTE, M.A., ROJAS-CERVANTES, M.L., LOPEZ-PEINADO, A.J., MARTIN-ARANDA, R.M. (2007) Fenton-like oxidation of orange II solutions using heterogeneous catalysts based on saponite clay. *Applied Catalysis B: Environmental.* Vol. 71: 44-56.

RASMUSSEN, K. AND MACLELLAN, M.A. (2001) The control of active substances used in biocides in the European Union by means of a review. *Environmental Science and Policy.* Vol. 4: 137-146.

RASTOGI, N. AND GOH, K.S. (1990) Action of 1-Isonicotinyl-2-Palmitoyl Hydrazine against the *Mycobacterium avium* Complex and Enhancement of Its Activity by *m*-Fluorophenylalanine. *Antimicrobial Agents and Chemotherapy.* Vol. 34(11): 2061-2064.

REYBROUCK, G. (1998) The testing of disinfectants. *International Biodeterioration & Biodegradation.* Vol. 41: 269-272.

RIDEAL, S. AND WALKER, J.T.A. (1913) Approved techniques of the Rideal-Walker test. *The American Journal of Public Health.* Vol. 3(6): 575-581.

RIDEOUT, K., TESCHKE, K., DIMICH-WARD, H., AND KENNEDY, S.M. (2005) Considering risks to healthcare workers from glutaraldehyde alternatives in high-level disinfection. *Journal of Hospital Infection.* Vol. 59: 4-11.

ROBERT, R., BARBATI, S., RICQ, S., AND AMBROSIO, M. (2002) Intermediates in wet oxidation of cellulose: identification of hydroxyl radical and characterisation of hydrogen peroxide. *Water Research.* Vol. 36(19): 4821-4829.

ROHN, S. AND KROH, L.W. (2005) Electron spin resonance – A spectroscopic method for determining the antioxidant activity. *Molecular Nutrition & Food Research.* Vol. 49: 898-907.

ROSS, P.W. (1966) A new disinfectant. *Journal of Clinical Pathology.* Vol. 19: 318-320.

RUSIN, P., BRIGHT, K., AND GERBA, C. (2003) Rapid reduction of *Legionella pneumophila* on stainless steel with zeolite coatings containing silver and zinc ions. *Letters in Applied Microbiology.* Vol. 36: 69-72.

RUSSELL, A.D. (1981) 'Neutralisation procedures in the evaluation of bactericidal activity', In: COLLINS, C.H., ALLWOOD, M.C., BLOOMFIELD, S.F., AND FOX, A. (eds) Disinfectants: Their use and evaluation of effectiveness, London, Academic Press, Inc. pp. 45-59.

RUSSELL, A.D. (1990) Bacterial spores and chemical sporicidal agents. *Clinical Microbiology Reviews.* Vol. 3(2): 99-119.

RUSSELL, A.D. (2002) Introduction of biocides into clinical practice and the impact on antibiotic-resistance bacteria. *Journal of Applied Microbiology Symposium Supplement.* Vol. 92: 121S-135S.

RUSSELL, A.D. (2003) Similarities and differences in the responses of microorganisms to biocides. *Journal of Antimicrobial Chemotherapy.* Vol. 52: 750-763.

RUTALA, W.A (1996) APIC guidelines for selection and use of disinfectants. *American Journal of Infection Control.* Vol. 24: 313-342.

RUTALA, W.A. AND WEBER, D.J. (1999) Infection control: the role of disinfection and sterilisation. *Journal of Hospital Infection.* Vol. 43 (Supplement): S43-S55.

RUTALA, W.A., GERGEN, M.F., WEBER, D.J. (1993) Sporicidal activity of chemical sterilants used in hospitals. *Infection Control and Hospital Epidemiology.* Vol. 14: 713-718.

SAGRIPANTI, J-L., CARRERA, M., INSALACO, J., ZIEMSKI, M., ROGERS, J., AND ZANDOMENI, R. (2007) Virulent spores of *Bacillus anthracis* and other *Bacillus* species deposited on solid surfaces have similar sensitivity to chemical decontaminants. *Journal of Applied Microbiology.* Vol. 102: 11-21.

SALEM, I.A., EL-MAAZAWI, M., ZAKI, A.B. (2000) Kinetics and mechanisms of decomposition reaction of hydrogen peroxide in the presence of metal complex. *International Journal of Chemical Kinetics.* Vol. 32(11): 643-666.

SALINAS, F. AND MARCH, J.G. (1984) Iron determination in the environment by solvent extraction and atomic absorption spectrophometry. *International Journal of Environmental Chemistry.* Vol. 18(3): 209-214.

SALLES, M.O., PAIXAO, T.R.L.C., AND BERTOTTI, M. (2007) Hydrogen peroxide monitoring in photo-Fenton reactions by using a metal hexacyanoferrate modified electrode. *International Journal of Electrochemical Science.* Vol. 2: 248-256.

SAMANTA, C. (2008) Direct synthesis of hydrogen peroxide from hydrogen and oxygen: An overview of recent developments in the process. *Applied Catalysis A: General.* Vol. 350(2): 133-149.

SAMEJIMA, T., AND YANG, J.T. (1963) Reconstitution of acid-denatures catalase. *The Journal of Biological Chemistry.* Vol. 238(10): 3256-3261.

SANIN, S.L., SANIN, F.D., AND BRYERS, J.D. (2003) Effect of starvation on the adhesive properties of xenobiotic degrading bacteria. *Process Biochemistry.* Vol. 38: 909-914.

SANTORO, N. AND THIELE, D.J. (1997) Oxidative stress responses in the yeast *Saccharomyces cerevisiae*. In: HOHMABNNE, S. AND MAGER, W.H (eds), Yeast stress responses. Heidelberg, Springer-Verlag. Pp. 172-211.

SARDESSAI, Y.N. (2005) Viable but non-culturable bacteria: their impact on public health. *Current Science.* Vol. 89(10): 1650.

SATTAR, S.A., ADEGBUNRIN, O., AND RAMIREZ, J. (2002) Combined application of simulated reuse and quantitative carrier tests to assess high-level disinfection: Experiments with an accelerated hydrogen peroxide-based formulation. *American Journal of Infection Control.* Vol. 30: 449-57.

SAWUNYAMA, P., JACKSON, M., AND BAILEY, G.W. (2001) Interactions of methyl orange and cycldextrin/sodium-montmorillonite systems probed by UV-Visible Spectroscopy. *Journal of Colloid and Interface Science.* Vol. 237: 153-157.

SEGURA, M., CAMARA, C., MADRID, Y., REBOLLO, C., AZCARATE, J., KRAMER, G.N., GAWLIK, G.M., LAMBERTY, A., AND QUEVAUVILLER, P. (2004) Certified reference materials (CRMs) for quality control of trace-elements determinations in wastewaters. *Trends in Analytical Chemistry.* Vol. 23(3): 194-202.

SEKI, M., IIDA, K-I., SAITO, M., NAKAYAMA, H., YOSHIDA, S-I. (2004) Hydrogen peroxide production in *Streptococcus pyogenes*: Involvement of lactate oxidase and coupling with aerobic utilisation of lactate. *Journal of Bacteriology.* Vol. 186(7): 2046-2051.

SEMCHYSHYN, H., BAGNYUKOVA, T., STOREY, K., AND LUSHCHAK, V. (2005) Hydrogen peroxide increases the activities of *soxRS* regulon enzymes and the levels of oxidised proteins and lipids in *Escherichia coli. Cell Biology International.* Vol. 29: 898-902.

SETLOW, B. AND SETLOW, P. (1994) Heat Inactivation of *Bacillus subtilis* Spores Lacking Small, Acid-Soluble Spore Proteins Is Accompanied by Generation of Abasic sites in Spores DNA. *Journal of Bacteriology.* Vol. 176(7): 2111-2113.

SETLOW, P. (2006) Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology.* Vol. 101: 514-525.

SHAH, S., DZIKOVSKI, B., SHAH, V. (2007) Development of a new approach for microbial decontamination of water using modified Fenton's reaction. *Environmental Pollution.* Vol. 148: 674-678.

SHAPOVALOVA, O.M. AND ONISHCHENKO, T.N. (1979) Distribution of iron and aluminium in titanium alloys. *Metal Science and Heat Treatment.* Vol. 21(3): 230-232.

SHATERIAN, H.R. AND YARRAHMADI, H. (2008) Sodium hydrogen sulfate as effective and reusable heterogeneous catalyst for the one-pot preparation of amidoalkylnaphthols. *ARKIVOC.* Vol. II: 105-114.

SHERRINGTON, D.C. (1988) Polymer-supported metal complex oxidation catalysts. *Pure and Applied Chemistry.* Vol. 60(3): 401-414.

SHI, H., TIMMINS, G., MONSKE, M., BURDICK, A., KALYANARAMAN, B., LIU, Y., CLEMENT, J.-L., BURCHIEL, S., AND LIU, K.J. (2005) Evaluation of spin trapping agents and trapping conditions for detection of cell-generated reactive oxygen species. *Archives of Biochemistry and Biophysics.* Vol. 437: 59-68.

SIRES, I., GARRIDO, J.A., RODRIGUEZ, R.M., BRILLAS, E., OTURAN, N., AND OTURAN, M.A. (2007) Catalytic behaviour of the Fe^{3+}/ Fe^{2+} system in the electro-Fenton degradation of the antimicrobial chlorophene. *Applied Catalysis B: Environmental.* Vol. 72: 382-394.

SMITH, B.A., WATTS, R.J., ATKINSON, D.A. (2002) Destruction of DNAPLs by modified Fenton's reagent. In: Platform Abstracts of the Third International Conference on Remediation of Chlorinated and Recalcitrant Compounds. Monterey. California. May, 20-23, 2002.

SMITH, R. (2009) Surface characterisation of heterogeneous catalysts by XPS: Part I. *Platinum Metals Review.* Vol. 53(1): 55-56.

SMITH, R.J., NEWTON, A.T., HARWOOD, C.R., AND BARRER, M.R. (2002) Active but nonculturable cells of *Salmonella enterica* serovar Typhimurium do not infect or colonise mice. *Microbiology.* Vol. 148: 2717-2726.

SOBSEY, M.D. (1989) Inactivation of health-related microorganisms in water by disinfection processes. *Water Science and Technology.* Vol. 21: 179-195.

SONDI, I. AND SALOPEK-SONDI, B. (2004) Silver nano-particles as antimicrobial agent: A case study on *E.coli* as a model for Gram-negative bacteria. *Journal of Colloid and Interface Science.* Vol. 275: 177-182.

SOUSA-LOPES, A., ANTUNES, F., CYRNE, L., MARINHO, H.S. (2004) Decreased cellular permeability to H_2O_2 protects *Saccharomyces cerevisiae* cells in stationary phase against oxidant stress. *FEBS Letters.* Vol. 578: 152-156.

SPAULDING, E.H. (1968) Chemical disinfection of medical and surgical materials. In: BLOCK, S.S. ed. Disinfection, sterilisation and preservation. Philadelphia, Lea & Fabiger. Pp. 517-531.

SPELLERBERG, B., CUNDELL, D.R., SANDROS, J., PEARCE, B.J., IDANPAAN-HEIKKILA, I., ROSENOW, C., AND MASURE, H.R. (1996) Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae. Molecular Microbiology.* Vol. 19: 803-813.

SPITELLER, G. (2003) The relationship between changes in the cell wall, lipid peroxidation, proliferation, senescence and cell death. *Physiologia Plantarum.* Vol. 119: 5-18.

SRINIVASAN, C., LIBA, A., IMLAY, J.A., VALENTINE, J.S., GRALLA, E.D. (2000) Yeast lacking superoxide dismutase(s) show elevated levels of ‘free iron’ as measured by whole cell electron paramagnetic resonance. *The Journal of Biological Chemistry.* Vol. 275(38): 29187-29192.

STAMPI, S., LUCA, G.D., ONORATO, M., AMBROGIANI, E., AND ZANETTI, F. (2002) Peracetic acid as an alternative wastewater disinfectant to chlorine dioxide. *Journal of Applied Microbiology.* Vol. 93: 725-731.

SUNADA, K., WATANABE, T., AND HASHIMOTO, K. (2003) Studies on photokilling of bacteria on TiO_2 thin film. *Journal of Photochemistry and Photobiology.* Vol. 156: 227-233.

TACHIEV, G., ROTH, J.A., AND BOWERS, A.R. (1999) Kinetics of hydrogen peroxide decomposition with complexed and ‘free’ iron catalysts. *International Journal of Chemical Kinetics.* Vol. 32(1): 24-35.

TAIWO, F.A., POWERS, H.J., NAKANO, E., GRIFFITHS, H.R., AND NUGENT, D.F. (2006) Free radical reactions in atherosclerosis: An EPR spectrometry study. *Spectroscopy.* Vol. 20: 67-80.

TAMIMI, M., QOURZAL, S., BARKA, N., ASSABBANE, A., AND AIT-ICHOU, Y. (2008) Methomyl degradation in aqueous solutions by Fenton's reagent and the photo-Fenton system. *Separation and Purification Technology.* Vol. 61: 103-108.

TAN, Y., WANG, Z-X., AND MARSHALL, K.C. (1998) Modelling pH Effects on Microbial Growth: A Statistical Thermodynamic Approach. *Biotechnology and Bioengineering.* Vol. 59(6): 724-731.

TAUTKUS, S., STEPONENIENE, L., AND KAZLAUSKAS, R. (2004) Determination of iron in natural and mineral waters by flame atomic absorption spectrometry. *Journal of the Serbian Chemical Society.* Vol. 69(5): 393-402.`

TEKBAS, M., YATMAZ, H.C., AND BEKTAS, N. (2008) Heterogeneous photo-Fenton oxidation of reactive azo dye solutions using iron exchanged zeolite as a catalyst. *Microporous and Mesoporous.* Vol. 115(3): 594602.

THE EUROPEAN STANDARD (BSEN 1040:2005) Chemical Disinfectants and Antiseptics – Quantitative Suspension test for the evaluation of Basic Bactericidal activity of Chemical Disinfectants and Antiseptics – Test Method and Requirements (Phase 1). Brussels, European Committee for Standardisation.

THE EUROPEAN STANDARD (BSEN 1276:1997) Chemical Disinfectants and Antiseptics – Quantitative Suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas – Test method and requirements (Phase 2, step 1). Brussels, European Committee for Standardisation.

THE EUROPEAN STANDARD (BSEN 14347:2005) Chemical Disinfectants and Antiseptics – Basic Sporicidal activity – Test Method and Requirements (Phase 1/ Step 1). Brussels, European Committee for Standardisation.

TOLVANEN, P., MAKI-ARVELA, P., SOROKIN, A.B., SALMI, T., AND MURZIN, D.Y. (2009) Kinetics of starch oxidation using hydrogen peroxide as an environmentally

friendly oxidant and an iron complex as a catalyst. *Chemical Engineering Journal.* (2009), doi:10.1016/j.cej.2009.02.001.

TOOR, R. AND MOHSENI, M. (2007) UV-H_2O_2 based AOP and its integration with biological activated carbon treatment for DBP reduction in drinking water. *Chemosphere.* Vol. 66: 2087-2095.

TORANZO, A.E., MAGARINOS, B., AND ROMALDE, J.L. (2005) A review of the main bacterial fish diseases in mariculture system. *Aquaculture.* Vol. 246: 37-61.

TOUATI, D. (2000) Iron and oxidative stress in bacteria. *Archives of Biochemistry and Biophysics.* Vol. 373: 1-6.

UBBINK, J. AND SCHAR-ZAMMARETTI, P. (2005) Probing bacterial interactions: integrated approaches combining atomic force microscopy, electron microscopy and biophysical techniques. *Micron.* Vol. 36(4): 293-320.

URBANSKI, N.K. AND BERESEWICZ, A. (2000) Generation of $^{\cdot}OH$ initiated by interaction of Fe^{2+} and Cu^{+} with dioxygen; comparison with the Fenton chemistry. *Acta Biochimica Polonica.* Vol. 47(4): 951-962.

VIDRIO, E., JUNG, H., AND ANASTASIO, C. (2008) Generation of hydroxyl radicals from dissolved transition metals in surrogate lung fluid solutions. *Atmospheric Environment.* Vol. 42: 4369-4379.

VILLABRILLE, P., ROMANELLI, G., GASSA, L., VAZQUEZ, P., AND CACERES, C. (2007) Synthesis and characterisation of Fe- and Cu-doped molybdovanadophosphoric acids and their application in catalytic oxidation. *Applied Catalysis A: General.* Vol. 324: 69-76.

VOLOSHIN, Y., HALDER, R., LAWAL, A. (2007) Kinetics of hydrogen peroxide synthesis by direct combination of H_2 and O_2 in a microreactor. *Catalysis Today.* Vol. 125: 40-47.

WALLING, C. (1975) Fenton's reagent revisited. *Accounts of Chemical Research.* Vol. 8: 125-131.

WALSH, S.E., MAILLARD, J-Y., RUSSELL, A.D. (1999a) *Ortho*-phthalaldehyde: a possible alternative to glutaraldehyde for high level disinfection. *Journal of Applied Microbiology.* Vol. 86: 1099-1046.

WALSH, S.E., MAILLARD, J-Y., RUSSELL, A.D., HANN, A.C. (2001) Possible mechanisms for the relative efficacies of *ortho*-phthalaldehyde and glutaraldehyde against glutaraldehyde-resistant *Mycobacterium chelonae. Journal of Applied Microbiology.* Vol. 91: 80-92.

WALSH, S.E., MAILLARD, J-Y., SIMONS, C., RUSSELL, A.D. (1999b) Studies on the mechanisms of the antibacterial action of *ortho*-phthalaldehyde. *Journal of Applied Microbiology.* Vol. 87: 702-710.

WALZAK, M.J., DAVIDSON, R., AND BIESINGER, M. (1998) The use of XPS, FTIR, SEM/EDX, Contact angle, and AFM in the characterisation of coatings. *Journal of Materials Engineering and Performance.* Vol. 7(3): 317-323.

WEBER, D.J. AND RUTALA, W.A. (1995) Use of metals as microbicides for the prevention of nosocomial infections. In: RUTALA, W.A. ed. Chemical germicides in health care. Morin Heights, Polyscience Publications. Pp. 271-286.

WHEELDON, L.J., WORTHINGTON, T., LAMBERT, P.A., HILTON, A.C., LOWDEN, C.J., AND ELLIOT, T.S. (2008) Antimicrobial efficacy of copper surfaces against spores and vegetative cells of *Clostridium difficile*: the germination theory. *Journal of Antimicrobial Chemotherapy.* Vol. 62: 522-525.

WILLIAMS, J. (1927) The decomposition of hydrogen peroxide by liver catalase. *The Journal of General Physiology.* Vol. 11(4): 309-337.

WINK, D.A., NIMS, R.W., SAAVEDRA, J.E., UTERMAHLEN JR. W.E., FORD, P.C. (1994) The Fenton oxidation mechanism: reactivities of biologically relevant substrates with two oxidising intermediates differ from those predicted for the hydroxyl radical. *Proceedings of the National Academy of Sciences.* Vol. 91: 6604-6608.

WIRTH, R. (2008) Focused ion beam (FIB) combined with SEM and TEM: Advanced analytical tools for studies of chemical composition, microstructure and crystal structure in geomaterials on a nanometre scale. *Chemical Geology.* Doi: 10.1016/j.chemgeo.2008.05.019.

WOOD, N.J. AND SØRENSEN, J. (2001) Catalase and superoxide dismutase activity in ammonia-oxidising bacteria. *FEMS Microbiology Ecology.* Vol. 38: 53-58.

WUTZLER, P. AND SAUERBREI, A. (2000) Virucidal efficacy of a combination of 0.2% peracetic acid and 80% (v/v) ethanol (PAA-ethanol) as a potential hand disinfectant. *Journal of Hospital Infection.* Vol. 46: 304-308.

XIANG, L., ROYER, S., ZHANG, H., TATIBOUET, J-M., BARRAULT, J., AND VALANGE, S. (2009) Properties of iron-based mesoporous silica for the CWPO of phenol: A comparison between impregnation and co-condensation routes. *Journal of Hazardous Materials.* Vol. 172: 1175-1184.

XU, C., ZHANG, Y., CHENG, G., AND ZHU, W. (2007) Localised corrosion behaviour of 316L stainless steel in the presence of sulfate-reducing and iron-oxidising bacteria. *Materials Science and Engineering A.* Vol. 443: 235-241.

XU, L.-C. AND LOGAN, B.E. (2006) Adhesion forces between functionalised latex microspheres and protein-coated surfaces evaluated using colloid probe atomic force microscopy. *Colloids and Surfaces B: Biointerfaces.* Vol. 48: 84-94.

YANG, J., SANGWAN, I., LINDEMANN, A., HAUSER, F., HENNECKE, H., FISCHER, H.-M., AND O'BRIAN, M.R. (2006) *Bradyrhizobium japonicum* senses iron through the status of haem to regulate iron homeostasis and metabolism. *Molecular Microbiology.* Vol. 60: 427-437.

YASINA, L.L., MILLER, V.B., AND SHLYAPNIKOV, Y.A. (1968) Effect of an iron salt on oxidation of polypropylene. *Academy of Sciences of the USSR.* Vol. 11: 2635-2636.

YIOKARI, C.G., PITSELIS, G.E., POLYDOROS, D.G., KATSAOUNIS, A.D., AND VAYENAS, C.G. (2000) High-Pressure Electrochemical Promotion of Ammonia Synthesis over an Industiral Iron Catalyst. *The Journal of Physical Chemistry A.* Vol. 104(46): 10600-10602.

YODA, K. AND UCHIMURA, M. (2006) An Outbreak of *Campylobacter jejuni* Food Poisoning Caused by Secondary Contamination in Cooking Practice at a High School. *Japanese Journal of Infectious Diseases.* Vol. 59: 408-409.

YODAIKEN, R.E. (1981) The uncertain consequences of formaldehyde toxicity. *Journal of the American Medical Association.* Vol. 246: 1677-1678.

YODAIKEN, R.E. (1981) The uncertain consequences of formaldehyde toxicity. *Journal of the American Medical Association.* Vol. 246: 1677-1678.

YOON, J., LEE, Y., KIM, S. (2001) Investigation of the reaction pathway of OH radicals produced by Fenton oxidation in the conditions of wastewater treatment. *Water Science and Technology.* Vol. 44(5): 15-21.

YOSHIMASA, T., MASAHIRO, A., KOSUKE, H., SEITA., T., YOSHITAKA, H., TADAKATSU, M., AKIRA, S., AND FUMITAKA, T. (2004) Effectiveness of *ortho*-phthalaldehyde (OPA) disinfection procedure for bronchoscope. *Journal of the Japan Society for Bronchology.* Vol. 26(8): 746-750.

YU, T.-L., HUANG, B.-H., HUNG, W.-C., LIN, C.-C., WANG, T.-C., AND HO, R.-M. (2007) Preparation and characterisation of poly(ε-caprolactone)-*b*-polyacrylonitrile (PCL-*b*-PAN) and poly($_L$-lactide)-*b*-polyacrylonitrile (PLLA-*b*-PAN) copolymers and aluminium and lithium alkoxides containing double-headed initiators. *Polymer.* Vol. 48: 4401-4411.

ZAIED, K.A., ABDEI-MAGEED, H.N., FAYZALLA, E.A., SHARIEF, A.E., AND ZEHRY, A.A. (2008) Effect of yeast and bacterial recombinants on the uptake of heavy metals from wastewater. *Australian Journal of Basic and Applied Sciences.* Vol. 2(3): 685-700.

ZANETTI, F., LUCA, G.D., TARLAZZI, P., AND STAMPI, S. (2003) Decontamination of dental unit water systems with hydrogen peroxide. *Letters in Applied Microbiology.* Vol. 37: 201-206.

ZEPP, R.G. (1992) Hydroxyl radical formation in aqueous reactions (pH 3-8) of iron(II) with hydrogen peroxide: The photo-Fenton reaction. *Environmental Science & Technology.* Vol. 26: 313-319.

ZHANG, H., ZHANG, D., AND ZHOU, J. (2006) Removal of COD from landfill leachate by electro-Fenton method. *Journal of Hazardous Materials.* Vol. B135: 106-111.

ZHANG, L., LI, J., CHEN, Z., TANG, Y., YU, Y. (2006) Preparation of Fenton reagent with hydrogen peroxide generated by solar light-illuminated nano-Cu_2O/MWNTs composites. *Applied Catalysis A: General.* Vol. 299: 292-297.

ZHANG, L-Y., STONE, K., AND PRYOR, W.A. (1995) Detection of free radicals in aqueous extracts of cigarette tar by electron spin resonance. *Free Radical Biology & Medicine.* Vol. 19(2): 161-167.

ZHAO, L.R. AND JANG, B.Z. (1996) Switching behaviours in semiconducting carbon fibres. *Journal of Materials Science Letters.* Vol. 15: 99-101.

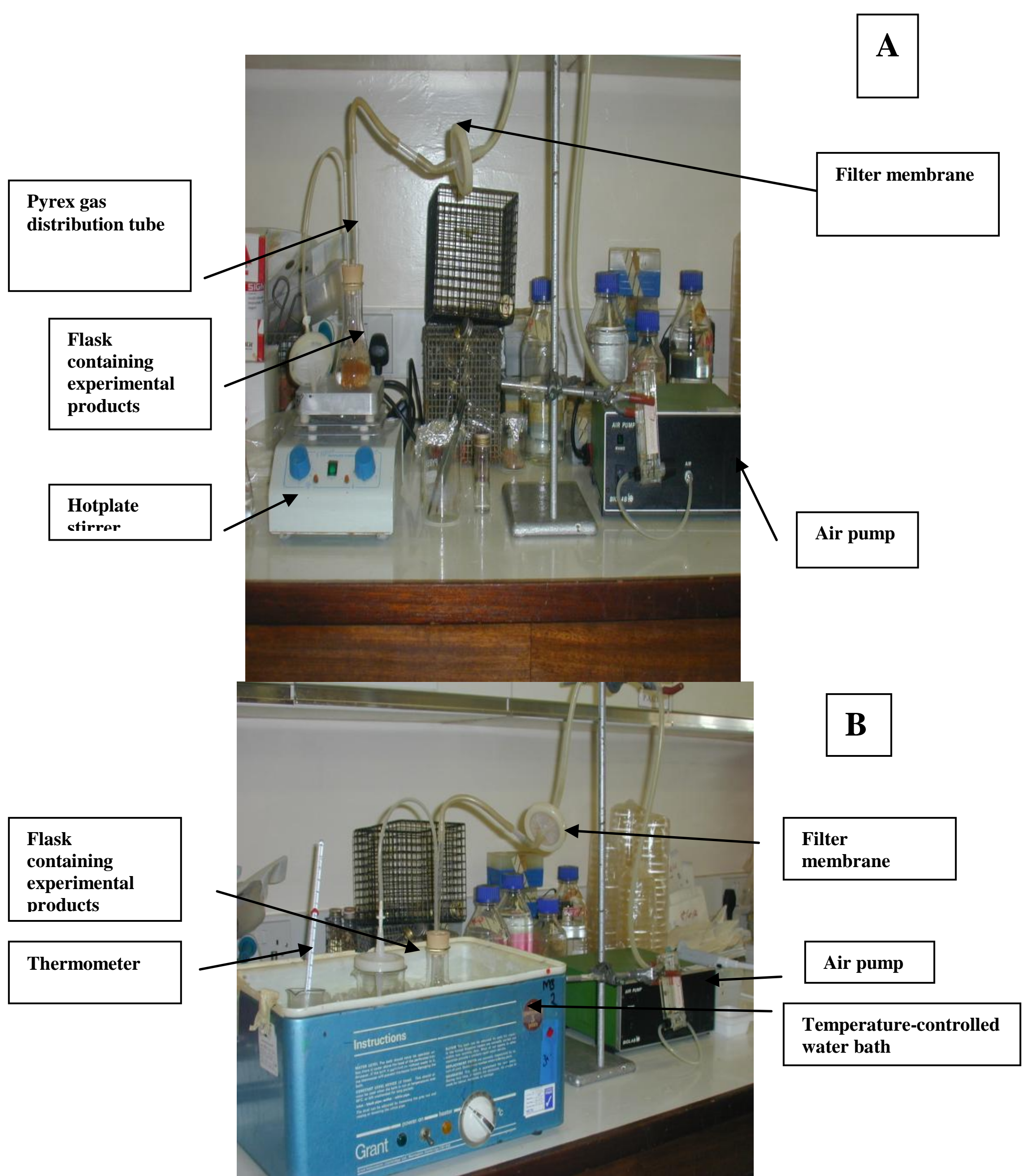
ZHAO, X., CHENG, K., HAO, J., AND LIU, D. (2008) Preparation of peracetic acid from hydrogen peroxide, part II: Kinetics for spontaneous decomposition of peracetic acid in the liquid phase. *Journal of Molecular Catalysis A: Chemical.* Vol. 284: 58-68.

ZHENBANG, H., YONGCHUN, D., AND SIMING, D. (2010) Comparative study on the mechanical and thermal properties of two different modified PAN fibres and their Fe complexes. *Materials and Design.* Vol. 31: 2784-2789.

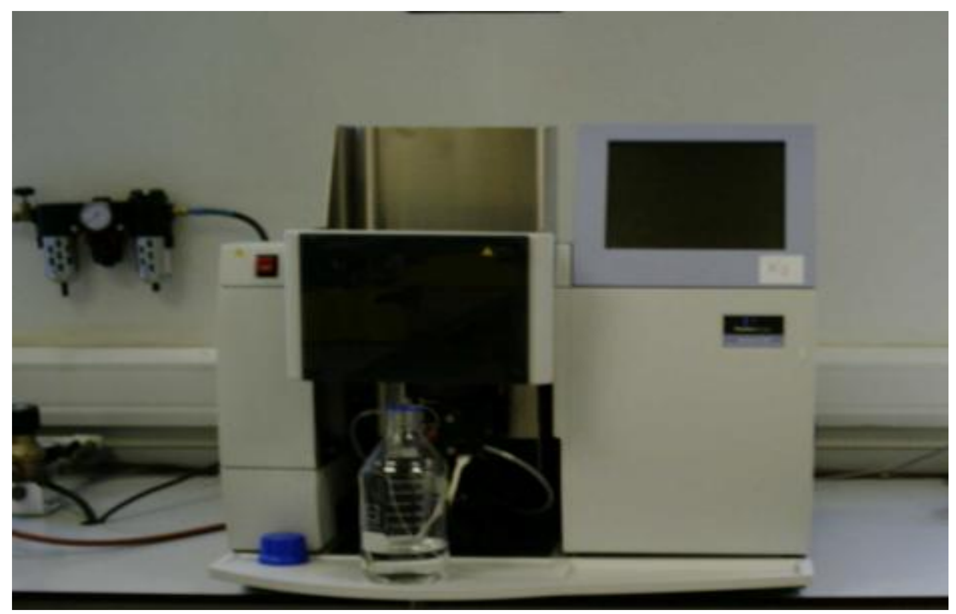
ZHOU, K., YIN, J.-J., AND YU, L.L. (2006) ESR determination of the reactions between selected phenolic acids and free radicals or transition metals. *Food Chemistry.* Vol. 95: 446-457.

ZIOLEK, M. (2004) Catalytic liquid-phase oxidation in heterogeneous system as green chemistry goal – advantages and disadvantages of MCM-41 used catalyst. *Catalysis Today.* Vol. 90(1-2): 145-150.

APPENDICES



Appendix 1: (A) and (B) show experimental setups when bubble air was used for antimicrobial testing at room temperature and 35°C respectively.

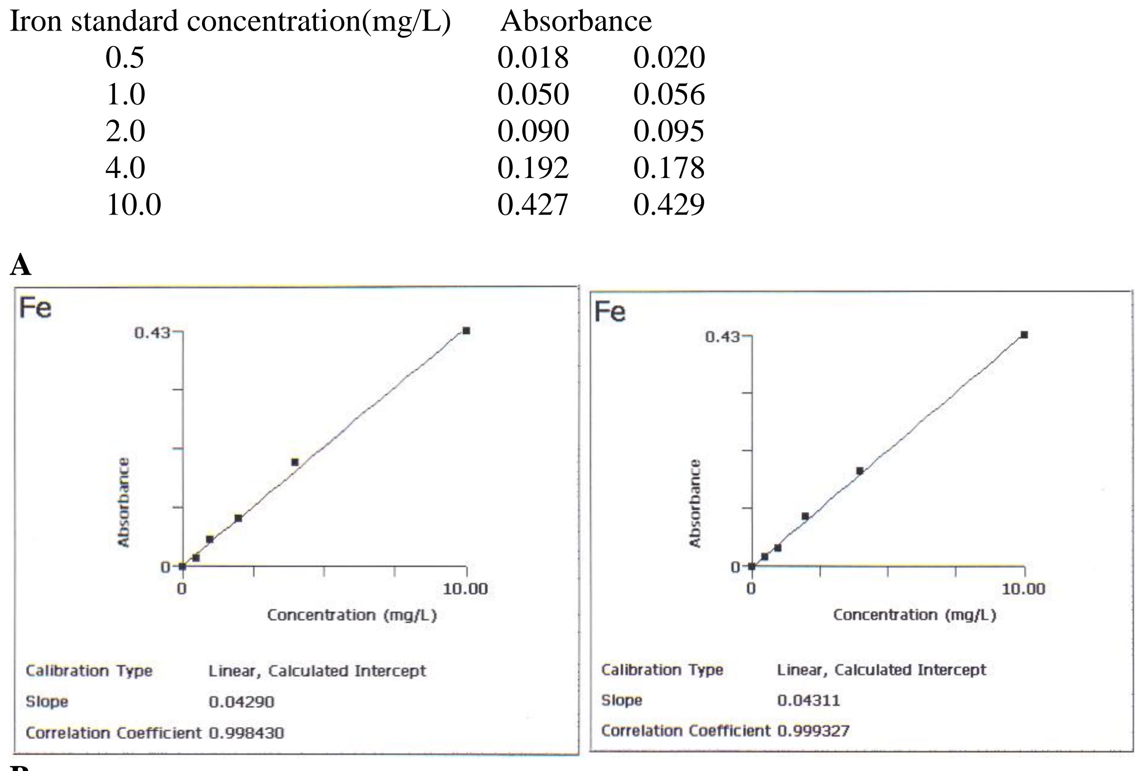


Appendix 2: PerkinElmer AAnalyst 200/400 Atomic Absorption Spectrophotometry used for iron determination.

Appendix 3: Cod reactor used to digest samples for iron determination.

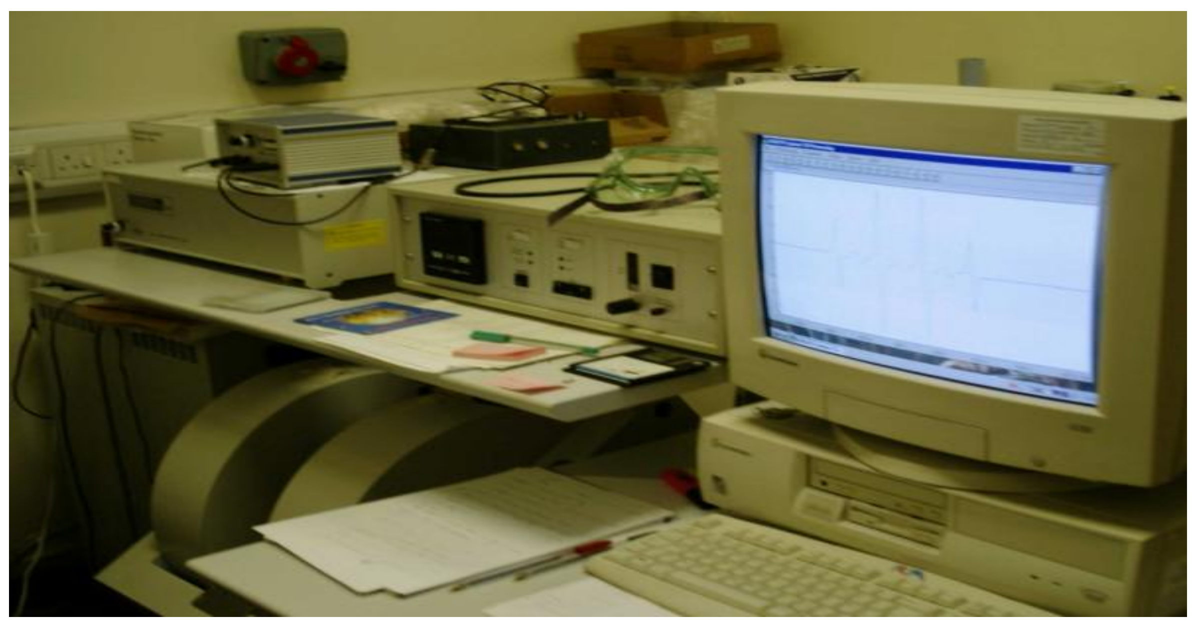
Iron standard concentration(mg/L)	Absorbance	
0.5	0.018	0.020
1.0	0.050	0.056
2.0	0.090	0.095
4.0	0.192	0.178
10.0	0.427	0.429

A

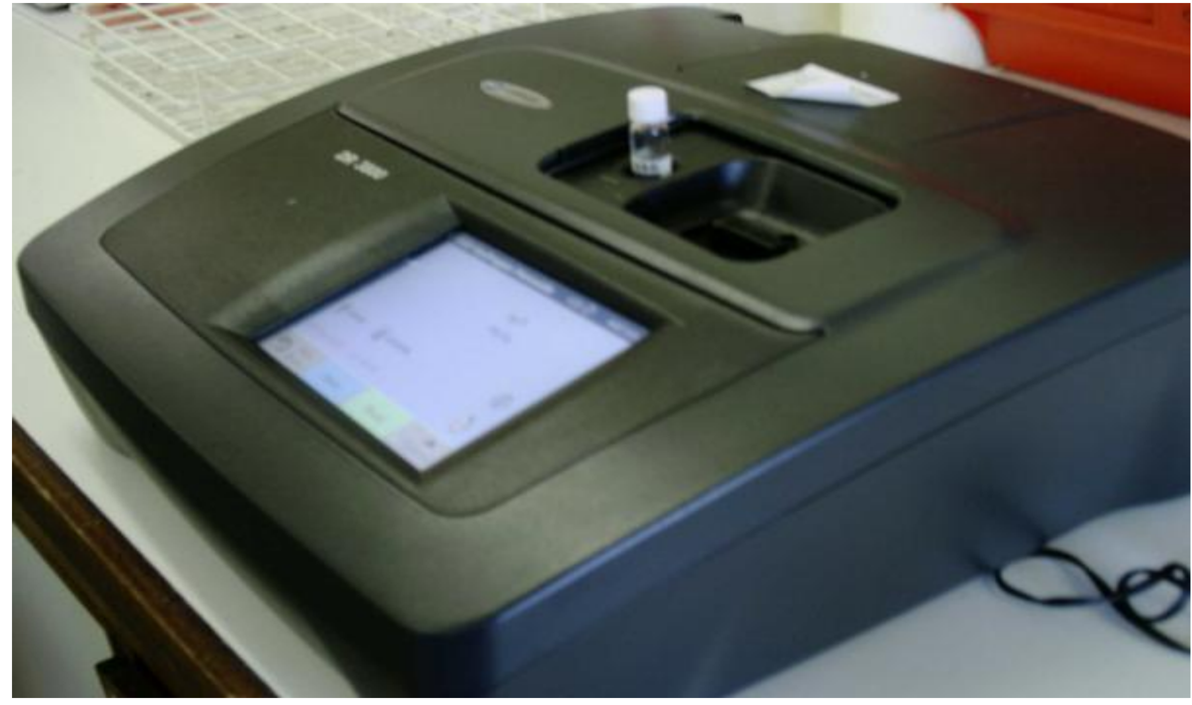


B

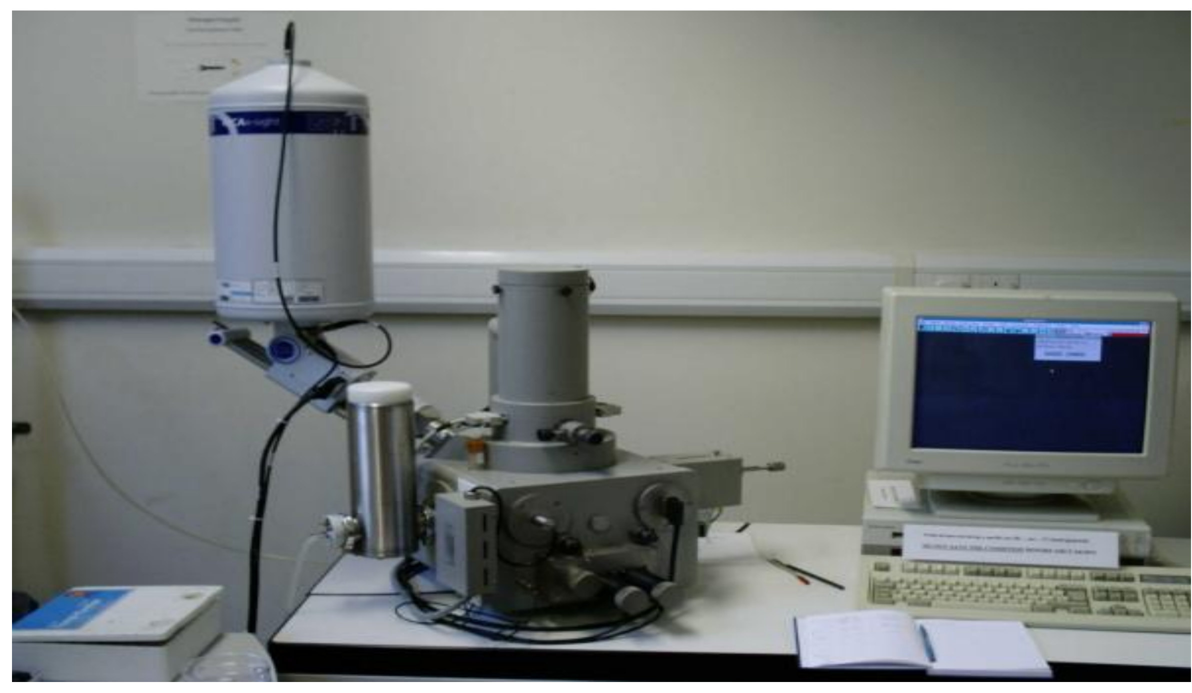
Appendix 4: A and B indicate standard curve for iron measurements.



Appendix 5: Bruker EMX 6/1 EPR spectrometer used for hydroxyl radicals determination.

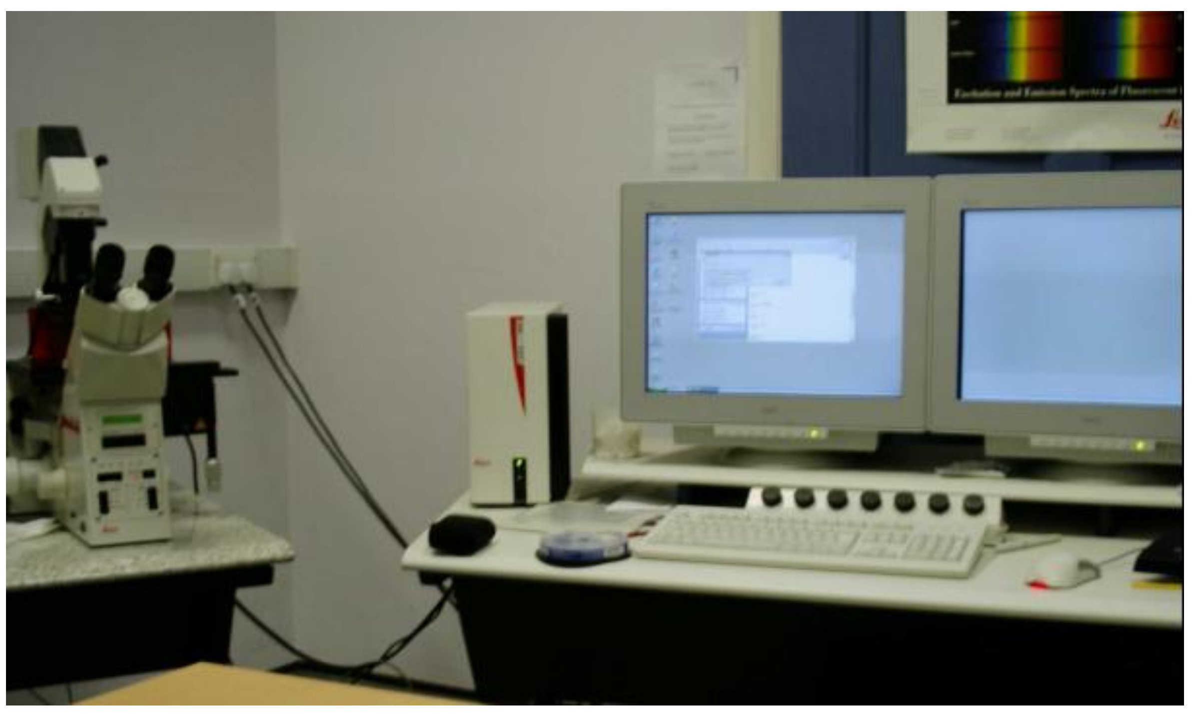


Appendix 6: DR 3800 SC Vis Spectrophotometer used to determine H_2O_2 concentration.



Appendix 7: Leica S430 for electron microscopy and Oxford Instruments INCA-Sight for EDX analysis.

Appendix 8: Edwards Sputter Coater S150B for coating samples prior to electron microscopy and EDX analysis.



Appendix 9: True Confocal Scanner Leica TCS SP2 used for confocal microscopy work.