The interaction between hydrogen peroxide and biofilms
by Xiaofeng Lu

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
© Copyright by Xiaofeng Lu (1996)

Abstract:
The interaction between biofilms consisting of catalase-positive bacteria Pseudomonas aeruginosa, Pseudomonas fluorescence and Klebsiella pneumoniae and hydrogen peroxide was investigated in a fiat plate reactor.

The experimental results using a hydrogen peroxide micro electrode showed that the penetrations of hydrogen peroxide into biofilms was greatly retarded. This was comparable to the results reported using other oxidizing antimicrobial agents.

An increase in dissolved oxygen concentration inside the biofilm measured by a dissolved oxygen microelectrode was found immediately after hydrogen peroxide treatment. Crude extracts from biofilms and planktonic cells using the same three experimental species all showed catalase activity, as determined by monitoring the breakdown of hydrogen peroxide. Thus the enzyme catalase is very likely to be active in the biofilm response to hydrogen peroxide treatment. A catalase inhibitor, 3-amino-1,2,4-triazole showed its inhibitory effect (IC50=50mM) when incubated with cell extracts. When this inhibitor was applied to either planktonic cells in the batch culture or to the biofilms in the reactor, inhibitory activity was observed only with planktonic cells (IC50=200mM). There was no significant inhibitory effect on response of the biofilms to hydrogen peroxide.

The conventional plate count method and total cell count method were employed to show the disinfectant effect of the hydrogen peroxide on the biofilm. The experiments showed that there was a significant difference between the total culturable cell count and total direct cell count (n=3, two sample test., $P<0.01$) after 2 hours treatment with 0.3% hydrogen peroxide.

The fluorescence probes CTC and DAPI were also used to show the respiratory activity in the biofilms. The results showed that there was a nonuniform distribution inside the biofilm after the hydrogen peroxide treatment. The greatest loss of respiratory activity . occurred near the interface between the biofilm and the bulk fluid.

THE INTERACTION BETWEEN HYDROGEN PEROXIDE AND BIOFILMS

by

Xiaofeng Lu

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Microbiology

MONTANA STATE UNIVERSITY-BOZEMA
Bozeman, Montana

December 1996

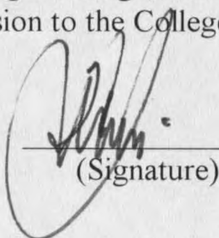
N378
L96

APPROVAL

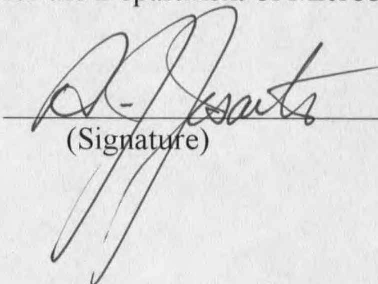
of a thesis submitted by

Xiaofeng Lu

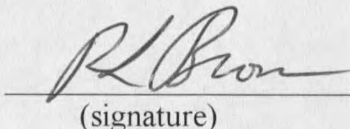
This thesis has been read by each member of the committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Zbigniew Lewandowski  (Signature) Dec. 17, 1996 Date

Approved for the Department of Microbiology

Al Jesaitis  (Signature) 12/17/96 Date

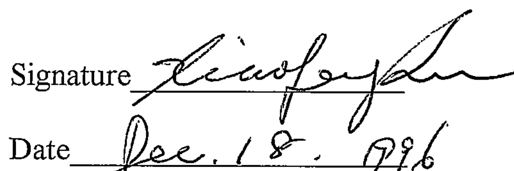
Approved for the College of Graduate Studies

Robert Brown  (signature) 12/21/96 Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for the master's degree at Montana State University-Bozeman, I agree that the Library shall make it available to borrowers under rules of the library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowed only for scholarly purposes, consistent with "fair use" as prescribed in the US Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.



Signature

Date

TABLE OF CONTENTS

Page

LIST OF TABLES vi

LIST OF FIGURES vii

ABSTRACT ix

INTRODUCTION 1

LITERATURE REVIEW 4

MATERIALS AND METHODS 21

Microorganisms and Culture Methods 21

Biofilm System Set-up 22

Construction of Hydrogen Peroxide Microelectrode 22

Hydrogen Peroxide Standardization 24

Calibration of Hydrogen Peroxide Microelectrode 25

Construction of Dissolved Oxygen Microelectrode 27

Calibration of Dissolved Oxygen Microelectrode 29

Measurement of Hydrogen Peroxide and Dissolved Oxygen concentration Profiles 31

Data Collection Set-up 31

Measurement in Biofilm System 33

Catalase Activity Assay and the Effect of Aminotriazole on Catalase 33

Inhibition of Catalase Activity in the Biofilms and the Batch Cultures 37

Respiratory Activity Assessment by CTC/DAPI Staining 38

Enumeration Methods....39

RESULTS....41

Growth of Biofilm....41

Characteristics of the Hydrogen Peroxide Microelectrode....41

Hydrogen Peroxide Concentration Profiles in the Biofilm Systems....42

Dissolved Oxygen Concentration Profiles in the Biofilm Systems....54

Catalase activity in the Cell Extracts from Planktonic Cells and Biofilms and the Effect of Aminotriazole on the Catalase....59

Inhibitory Effect of Aminotriazole on the Batch Cultures and the Biofilms....60

The Change of the Cell Counts with Hydrogen Peroxide Treatment....67

Effect of H_2O_2 on Respiratory Activity: Spatial distribution by CTC/DAPI Epiluorescence....67

DISCUSSION....74

Multispecies Biofilms....74

Hydrogen Peroxide Microelectrode....75

Dissolved Oxygen Microelectrode....77

Hydrogen Peroxide Penetration....77

The Antimicrobial Effect of Hydrogen Peroxide on the Biofilms....79

Respiratory Activity within Biofilm during Hydrogen Peroxide Treatment....81

CONCLUSIONS....83

REFERENCES....85

APPENDIX....92

LIST OF TABLES

Table Page

1. Antimicrobial activity of hydrogen peroxide toward bacteria, yeast and viruses 13
2. Sporicidal activity of hydrogen peroxide toward spore-forming bacteria and bacteria spores 14
3. Composition of modified Scheusner's mineral salts medium 40
4. Composition of phosphate buffer (pH 7.4) 40
5. Raw results from Case A. (Figure 13), hydrogen peroxide concentration profiles measurement in biofilms: with 0.3% hydrogen peroxide treatment 92
6. Raw results from Case B. (Figure 15), hydrogen peroxide concentration profiles measurement in biofilms: with 0.3% hydrogen peroxide treatment 93
7. Raw results of Figure 17. Dissolved oxygen (DO) concentration profiles: without hydrogen peroxide treatment 94
8. Raw results of Figure 18. Dissolved oxygen (DO) concentration profiles: with 0.3% hydrogen peroxide treatment 95
9. Raw results of Figure 24. Dissolved oxygen (DO) concentration profiles in biofilm: with 0.3% hydrogen peroxide and 1 M 3-amino-1,2,4-triazole 97

LIST OF FIGURES

Figure Page

1. Experimental set-up 23

2. Hydrogen peroxide microelectrode calibration curve 26

3. Dissolved oxygen microelectrode calibration curve 30

4. Apparatus for dissolved oxygen or hydrogen peroxide concentration profile measurement inside the biofilms 32

5. Hydrogen peroxide calibration curve at 280 nm wavelength 35

6. Total protein calibration curve using Protein Assay kit. 36

7. pH dependence of hydrogen peroxide microelectrode in hydrogen peroxide solution (0.3%) 43

8. The effect of phosphate buffer concentration on the sensitivity of a hydrogen peroxide microelectrode 44

9. Sensitivity and selectivity of hydrogen peroxide microelectrode at different applied potentials 45

10. The stirring effect on the signals of hydrogen peroxide microelectrode with different tips 46

11. Hydrogen peroxide concentration profile measurement control experiment 1. Profile measured in a hydrogen peroxide solution with no biofilm 47

12. Hydrogen peroxide concentration profile measurement control experiment 2. Profile measured in a hydrogen peroxide-free buffer with the biofilm 48

13. Hydrogen peroxide concentration profiles in biofilms: with 0.3% hydrogen peroxide treatment. Case A (600-micron biofilm) 50

14. The change in hydrogen peroxide concentration with time at one point deep (600-micron)within the biofilm (600 microns) exposed to a constant hydrogen peroxide concentration 51

15. Hydrogen peroxide concentration profiles in biofilms: with 0.3% hydrogen peroxide treatment. Case B (600-micron biofilm) 52

16. The change in hydrogen peroxide concentration with time at one point deep (700-micron)within the biofilm (700 microns) exposed to a constant hydrogen peroxide concentration 53

17. Dissolved oxygen concentration profiles: without hydrogen peroxide treatment..... 55

18. Dissolved oxygen concentation profiles: with 0.3% hydrogen peroxide treatment 56

19. The change in dissolved oxygen (DO) concentration with time at one point deep within the biofilm (400-micron) exposed to a constant hydrogen peroxide concentration 57

20. Dissolved oxygen concentation profile: with 0.3% hydrogen peroxide treatment after killing the biofilm 58

21. The effect of aminotriazole (AT) on the breakdown of hydrogen peroxide in the cell extracts from planktonic bacteria 61

22. The effect of aminotriazole (AT) on the breakdown of hydrogen peroxide in the cell extracts from biofilms 62

23. The decrease of specific catalase activity in the planktonic cells and in the biofilms in the presence of AT 63

24. Dissolved oxygen concentration profiles in the biofilm: with 0.3% hydrogen peroxide and 1 M 3-amino-1,2,4-triazole 64

25. The effect of aminotriazole (AT) on the degradation of hydrogen peroxide to oxygen. 65

26. Cell count results of biofilms treated with 0.3% hydrogen peroxide 68

27. Epifluorescence micrographs of frozen sections of the biofilms grown on plastic coverslip treated with 0.3% hydrogen peroxide 70

ABSTRACT

The interaction between biofilms consisting of catalase-positive bacteria *Pseudomonas aeruginosa*, *Pseudomonas fluorescence* and *Klebsiella pneumoniae* and hydrogen peroxide was investigated in a flat plate reactor.

The experimental results using a hydrogen peroxide microelectrode showed that the penetrations of hydrogen peroxide into biofilms was greatly retarded. This was comparable to the results reported using other oxidizing antimicrobial agents.

An increase in dissolved oxygen concentration inside the biofilm measured by a dissolved oxygen microelectrode was found immediately after hydrogen peroxide treatment. Crude extracts from biofilms and planktonic cells using the same three experimental species all showed catalase activity, as determined by monitoring the breakdown of hydrogen peroxide. Thus the enzyme catalase is very likely to be active in the biofilm response to hydrogen peroxide treatment. A catalase inhibitor, 3-amino-1,2,4-triazole showed its inhibitory effect (IC_{50}=50mM) when incubated with cell extracts. When this inhibitor was applied to either planktonic cells in the batch culture or to the biofilms in the reactor, inhibitory activity was observed only with planktonic cells (IC_{50}=200mM). There was no significant inhibitory effect on response of the biofilms to hydrogen peroxide.

The conventional plate count method and total cell count method were employed to show the disinfectant effect of the hydrogen peroxide on the biofilm. The experiments showed that there was a significant difference between the total culturable cell count and total direct cell count (n=3, two sample test., $P<0.01$) after 2 hours treatment with 0.3% hydrogen peroxide.

The fluorescence probes CTC and DAPI were also used to show the respiratory activity in the biofilms. The results showed that there was a nonuniform distribution inside the biofilm after the hydrogen peroxide treatment. The greatest loss of respiratory activity occurred near the interface between the biofilm and the bulk fluid.

INTRODUCTION

Biofilms are involved in many problems such as fouling of heat exchangers and cooling water towers, contamination in food processing, microbially influenced corrosion, and persistent infections associated with medical implants (Characklis, 1990). Frequently, antimicrobial agents are used to control biofilm accumulation and activity. Although antimicrobial agents, such as biocides and antibiotics, are highly effective in controlling planktonic microbial populations, they have been found to be less effective against biofilms or cell aggregates.

Hydrogen peroxide naturally exists in organisms as a results of cellular oxygen metabolism. It has been known for a long time that hydrogen peroxide is one of the metabolic intermediates. It is generated during the reduction of oxygen to water (Fridorich, 1978). Because of the toxic effect of hydrogen peroxide on enzyme and cellular function, organisms have evolved some enzymes that can destroy these toxic oxygen derivatives. Catalase-peroxidase-superoxide dismutase is the most common enzymatic system produced by respiring cells to successfully destroy hydrogen peroxide and other toxic oxygen derivatives (Brock et al., 1991). Within this system, catalase is the enzyme which directly breaks down the hydrogen peroxide into water and oxygen. During this process, an increase in dissolved oxygen concentration will be observed. To examine the protective response of biofilm organisms against hydrogen peroxide, we hypothesize that the increase in oxygen might be used as an indicator of breakdown of hydrogen peroxide in the biofilms. The ability of catalase to mediate the degradation of

hydrogen peroxide can be inhibited by specific catalase inhibitors, such as 3-amino-1,2,4-triazole (Paul et al., 1973; Gee et al., 1970) which can irreversibly inhibit the catalytic function of catalase (Heim et al., 1955). Thus the inhibition of oxygen evolution or hydrogen peroxide breakdown by aminotriazole would be suggestive of a catalase mediated protective response. Since biofilm are notorious in evasion of metabolic inhibitor, the effectiveness of this inhibition in biofilms needs to be measured.

Evidence already exists that hydrogen peroxide has reduced effectiveness as an oxidizing biocide when used against biofilms (Exner et al.,1987; Vincent et al., 1989; Wilson et al., 1990). The reason for this reduced efficiency is unclear. We hypothesize that in the process of penetration of hydrogen peroxide into the biofilm cluster, reaction-diffusion of hydrogen peroxide is expected to occur in biofilms. For catalase positive bacteria, as we used in our experimental system, catalase are likely to be active to degrade hydrogen peroxide, a toxic oxygen derivative to oxygen. This is beneficial to the effective growth of microorganism in biofilms. As a result of these reactions, the concentration of hydrogen peroxide decreases and so does its efficacy as an antimicrobial agent. Such a mechanism might be partially identical to that described by DeBeer et al for Chlorine (1994).

To test this hypothesis, we measured hydrogen peroxide concentration in aerobic biofilms consisting of *Pseudomonas aeruginosa, Pseudomonas fluorescence,* and *Klebsiella pneumoniae.* The reaction kinetics of hydrogen peroxide with biofilms and biocidal efficacy were identified. A combination of microelectrode measurements of dissolved oxygen and hydrogen peroxide gradient, microbial viability determined by CTC

and DAPI staining, and conventional direct viable count (C-DVC) were used to quantify biocidal effects of hydrogen peroxide on biofilms.

LITERATURE REVIEW

Hydrogen Peroxide: An Antimicrobial Agent

A Survey of Hydrogen Peroxide

Hydrogen peroxide (HP) was first reported by the French chemist Thenard in 1818, but it was the English physician Richardson who first recognized, in 1858, the ability of HP to get rid of foul odors and proposed its use as a disinfectant. Since disease often produced unpleasant odors, it was thought that chemicals that reduced these odors would serve as disinfectants. Richardson's proposal led to the early commercial use of hydrogen peroxide as a disinfectant under the trade name *Sanitas*.

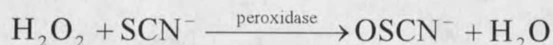
HP is considered to be very safe that it has been approved for use in foods in many countries (Schumb et al.,1955). Also it can be easily destroyed by enzymes, such as catalase and peroxidase to give the innocuous end products, oxygen and water (Brock et al., 1991). Early literature reveals that HP was satisfactory when it was used as a disinfectant for inanimate materials. For example, when HP was used in low concentrations, it was ideal for the preservation of milk and water (Heinemann,1913), and for the sterilization of cocoa milk beverage (Wilson et al.,1927). In 1950, an electrochemical process was developed to produce pure HP in high concentrations that were stable even at higher temperatures and thus had long-term storage and usage (Schumb et al, 1955). During last several decades, interest in HP has been increasing rapidly. Yoshpe-Purer and Eylan (1968) reported the use of low concentrations of HP for the sterilization of drinking water. Naguib and Hussein (1972) found that incubation of

0.1% (29.41 mM) HP with raw milk at 54^0C for 30 minutes could reduce the total bacterial count in raw milk by 99.999%, and the coliform, staphylococcal, salmonellae, and clostridial counts even by 100%. HP also showed its anti-virus effect. In 1973, Mentel & Schmidt reported the rapid virucidal activity of HP against rhinovirus. Work in the USSR indicated the practicability of HP for sterilization of spacecraft; this view was supported by the United States scientists Wardle & Renninger (1975). Pure HP is highly stable. Schumb et al, (1955) reported the discovery of factors that caused the decomposition of HP and led to the development of some effective stabilizers that could destroy contaminating material but was not active on HP itself. The thermal stability of a 3% (882 mM) HP solution was examined for retained biocidal activity. The time required for an unheated 3% (882 mM) HP solution to eliminate a 1×10^5 per milliliter inoculum was compared to that of a solution that had previously been subjected to 45^0C. This comparison revealed that there was no significant difference in the killing time between these two solutions for 7 bacteria strains and 1 fungus, the time it took to kill microorganism was 1 day and remained equally effective after 7 days (Turner, 1974). The studies mentioned above were for planktonic cells.

Mechanism of Action of Hydrogen Peroxide

HP naturally exists in tissues as a result of cellular oxygen metabolism. By various mechanisms, it protects us from infections by invading pathogenic microorganisms. HP is present in the saliva produced by membranes in the mouth, it is believed to act as a

powerful oxidant either alone or in combination with thiocyanate and peroxidase (Thomas & Aune, 1978):

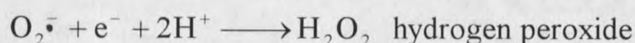


$$H_2O_2 + SCN^- \xrightarrow{\text{peroxidase}} OSCN^- + H_2O$$

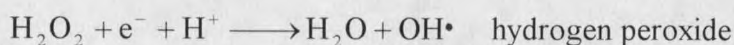
$$OSCN^- + RSH \longrightarrow H^+ + RSOSCN \quad \text{(sulfenyl thiocyanate)}$$

Although oxygen is required by respiring organisms, it is also toxic to them; cells are protected from excess oxygen by reducing it to water in a series of enzymatic steps. The following equations summarize the four-electron reduction of oxygen to water by stepwise addition of electrons. All of the intermediates formed are reactive and toxic to cells (Fridovich, 1975).

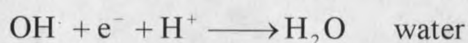
$$O_2^{\,0} + e^- \longrightarrow O_2^{\bar{\bullet}} \qquad \text{superoxide ion}$$



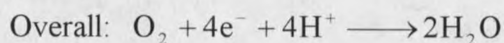
$$O_2^{\bar{\bullet}} + e^- + 2H^+ \longrightarrow H_2O_2 \quad \text{hydrogen peroxide}$$



$$H_2O_2 + e^- + H^+ \longrightarrow H_2O + OH\bullet \quad \text{hydrogen peroxide}$$

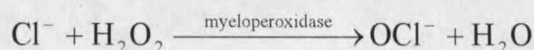


$$OH^{\cdot} + e^- + H^+ \longrightarrow H_2O \qquad \text{water}$$



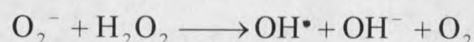
$$\text{Overall: } O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$$

It is seen that HP, the superoxide ion, and the hydroxyl radical are intermediates in the scheme for the reduction of oxygen to water. In 1968, Klebanoff found that in the presence of myeloperoxide enzyme, chloride in the bacteria maybe oxidized by HP to hypochlorite.



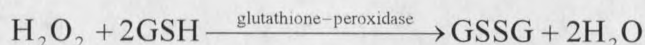
$$Cl^- + H_2O_2 \xrightarrow{\text{myeloperoxidase}} OCl^- + H_2O$$

while OCl^- (hypochlorite) is a well known oxidant and germicide. Another proposed mechanism by which HP participates in the destruction of bacteria involves the reactions of the superoxide ion with HP to produce the hydroxyl radical (Haber and Weiss, 1934 ; Fridovich, 1978).



$$O_2^- + H_2O_2 \longrightarrow OH^\bullet + OH^- + O_2$$

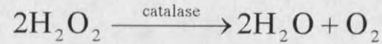
It was believed that the hydroxyl radical was the strongest oxidant ever known (Fridovich, 1975); and thus responsible for the HP-mediated killing of the bacteria. Transition metals, such as iron, are known to catalyze the formation of the hydroxyl radical in cells. Yoshpe-Purer & Eylan (1968) suggested in the case of water free of metal ions, the bacteria could provide the necessary metal ions themselves. Colobert (1962) once demonstrated that in the absence of metal ions in the culture medium, or if these ions are chelated with ethylene diaminetetracetic acid, there is no bactericidal action observed on *E. coli*. In 1962, Gould & Hutchius suggested that the antimicrobial action of HP is due to the oxidation of sulfhydryl groups and double bonds in proteins, lipids, and surface membranes. An enzymatic glutathione (GSH) detoxification system was proposed in 1980 by Voetman and his colleagues that could protect the phagocytes against toxic levels of HP. This system is as follows:



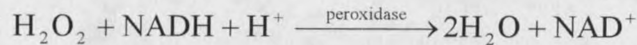
$$H_2O_2 + 2GSH \xrightarrow{\text{glutathione-peroxidase}} GSSG + 2H_2O$$

It should be noted that with such an array of toxic oxygen derivatives, e.g. H_2O_2, O_2^-, it is perhaps not surprising that organisms have developed enzyme systems that can

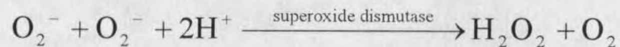
destroy these toxic oxygen products. The following reactions describe these enzymatic systems:



$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$



$$H_2O_2 + NADH + H^+ \xrightarrow{\text{peroxidase}} 2H_2O + NAD^+$$



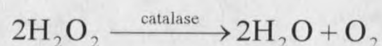
$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

Superoxide dismutase and catalase can work together to convert superoxide back to oxygen. It has been found that there is no enzymatic system to deal with hydroxyl radicals. This is very likely due to the short half life of hydroxyl radicals in water. However, by the mechanism of removal of HP from cells, organisms can be protected in part by preventing the formation of hydroxyl radicals in cells. (Brock et al., 1991).

Catalase and Aminotriazole

Catalase (H_2O: H_2O oxidoreductase; EC 1.11.1.6) was one of the first enzymes to be isolated in a high state of purity, and its crystallization from beef liver extracts was one of the early triumphs of biochemistry. Generally, all carefully characterized catalases are oligomers which consist of four 60,000-dalton subunits. Each subunit contains a single polypetide chain that associates with a single prosthetic groups, ferric protoporphyrin IX. The subunits apparently function independently of one another (Schonbaum & Chance, 1976). Catalase catalytically scavenges H_2O_2. It has been found that catalase is the most common enzyme produced by respiring cells which successfully destroys H_2O_2, it adequately protects cells from damage by metabolically produced HP (Brock et al., 1991).

The catalase reaction consists of the conversion of two molecules of H_2O_2 to one molecule of oxygen and two molecules of water.

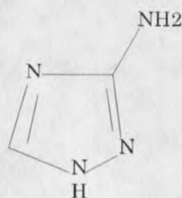


$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Catalase forms an enzyme-substrate complex called Compound I on reacting with the first molecule of H_2O_2. Reaction with the second molecule of H_2O_2 brings catalase back to its initial state (Schonbaum & Chance 1976).

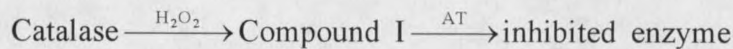
Bacteria can fall into two categories in terms of catalase activity, either catalase positive or catalase negative. Typical catalase positive bacteria are *Bacillus*, and *Micrococcus*. The bacteria used in this study were *Pseudomonas* and *Klesiella*, which include the organisms used in this study. The good examples of catalase negative bacteria are *Clostridium* and *Streptococcus.* Some microorganisms, which are unable to synthesize heme, can still produce a catalase. This catalase, which in contrast to heme-containing catalase, has been called pseudocatalase. Bacteria such as pediococci, lactobacilli, leuconostocs were found to contain pesudocatalase (Kono & Fridovich, 1983)

Many catalase inhibitors have been found in nature. These inhibitors are either specific or nonspecific to catalase. 3-amino-1H-1,2,4-triazole (AT) is a specific inhibitor of catalase. The structure of AT is as follows:



(AT: $C_2H_4N_4$. MW: 84.04)

In 1955, Heim et al. first found that AT could irreversibly inhibit the catalytic function of catalase in liver. The usual concentration of AT used for inhibitory purpose starts from a range of 20 mM to 200 mM (Kono, 1995; Gee et al 1970; Kono & Fridovich, 1983). The inhibition depended on the presence of peroxide or compounds which were susceptible to autooxidation, and thus proposed a reaction between Compound I (the enzyme-peroxide complex mentioned previously) and AT:



$$\text{Catalase} \xrightarrow{H_2O_2} \text{Compound I} \xrightarrow{AT} \text{inhibited enzyme}$$

Only the subunits of catalase with intact prosthetic groups are modified. and the inhibited product contained approximately one equivalent of AT per hematin. The derivative retains both the ferric, largely high-spin characteristics and the oligomeric structure of the native enzyme (Schonbaum & Chance 1976).

Hydrogen Peroxide as an Antimicrobial Agent

HP is active against a wide range of organisms: bacteria, yeast, fungi, viruses, and spores (Tables 1&2). Anaerobic microorganisms are even more sensitive to HP, probably because they are believed to lack catalase to break down the HP. Baldry (1983) found that 25 ppm (0.735 mM) or less of HP would prevent the growth of vegetative bacteria. From the Tables 1 and 2, it can be found that HP is slow in its action against yeast, some viruses, and especially bacterial spores. In general, HP has greater activity against Gram-negative than Gram-positive bacteria. The activity of HP is affected by changes in pH, with greater activity under the acid condition (Baldry, 1983). Destruction of spores is

greatly increased both with rise in temperature and increase in concentration. This makes HP an effective sporicide under these conditions. In the 40- 70 °C range, the time for 1% (294 mM) HP to kill half the spores decreased from one-half to one-third for each 10°C temperature rise (Curran et al., 1940). Leaper (1984) (Table 2) showed that an increase in temperature from 20-45°C reduced the time to kill spores 10-20 times, and an increased concentration from 17.7% (5.21 M) to 35.4% (10.41 M) caused a time reduction of 3 to 4 times.

In vivo antimicrobial activity of neutrophils and monocytes has been traced in part to a synergistic system consisting of HP, chloride ions and myeloperoxidase (a glycosylated hemoprotein in human leukocytes) (Klebanoff, 1968). Action of this system results in the immediate halting of DNA activity in *E.coli*. Also, destroy of DNA-membrane interaction, loss of DNA synthesis, and loss of cell viability was found throughout the critical early period of myeloperoxidase activity (Rosen et al., 1990). Klebanoff and Coombs (1992) found that this system also resulted in considerable viricidal potency as well. Their data suggested that when polymorphonuclear leukocyte (PMN) were stimulated, myeloperoxidase (MPO) released from degranulation would react with HP formed by the respiratory activity in order to oxidize chloride to a product (hypochlorous acid), while this product was toxic to HIV-1. These findings raised the possibility that this viricidal effect of stimulated PMN may influence the host defense against HIV-1. HP, in conjunction with human salivary peroxidase and thiocynate (HPT), has also been shown to be a natural oral antimicrobial agent. Application of a similar system,, lactoperoxidase/SCN^-/HP, has been considered for control of oral bacteria. This

system seemed to be well designed as an inhibitor of bacterial metabolism and growth in the oral environment. Lactoperoidase used HP to produce a more effective inhibitor and thus amplified the activity of HP (Thomas et al., 1994). In controlling the contamination of bacteria in milk, the lactoperoxidase/SCN/HP system has also been shown to be effective. Kamau et al. (1990) found that this system, using the inherent milk lactoperoxidase, effectively inhibited the growth of *L. monocytogenes* and *S.aureus*, especially at 10°C.

Industrial and Medical Applications of Hydrogen Peroxide

In recent years, HP has been widely applied as an antimicrobial agent in industrial areas, such as drinking water treatment. Studies (Tables 1 and 2) demonstrating its efficacy are very common throughout the scientific literature.

In the water treatment industry, the efficacy of HP alone has been studied. Strobel & Dieter (1990) summarized that the suitability of HP for the disinfection of swimming pool water, from a toxicological point of view, would primarily depend on whether the HP molecule was a stable mechanism or vehicle of transport into the body for the toxic O_2^- and $OH^{\cdot}$. Only on the basis of this knowledge, would the technical development of HP-based disinfection procedures for the water of public swimming pools be acceptable from a toxicological view point. HP efficacy in water treatment was also observed using an ozone-hydrogen peroxide system. The efficacy of ozone was compared to an ozone-HP system for inactivating *E.coli* in water system. It was found that there were some differences of the inactivation efficiency between these two systems.

Table 1. Antimicrobial Activity of Hydrogen peroxide Toward Bacteria, Yeast and Viruses

Organism	Concentration (ppm)	Lethality (minutes)	Temperature (°C)	Reference
Bacteria				
S. aureus	1000	60	-	Kunzman,1934
S .aureus	25.8×10^4	0.2	24	Toledo et al., 1973
E .coli	1000	60	-	Kunzman,1934
E .coli	500	10-30	37	Kunzman,1934
A .aerogenes	500	10-30	37	Nambudripad et al.,1949
Sarcina spp	500	150	37	Nambudripad et al.,1949
S. lactis	500	150	37	Nambudripad et al, 1949
S .liquefaceus	500	240	37	Nambudripad et al, 1949
Micrococcus spp	500	10	-	Wardle et al., 1975
S.epidermidis	500	10	-	Wardle et al., 1975
E.typhi	1000	60	-	Kunzman, 1934
Yeasts				
Torule spp.	500	180-210	37	Nambudripada & Iya 1951
Oidium spp.	500	180-210	37	Nambudripada & Iya 1951
Virus				
Rhinovirus 1A, 1B, 7	0.75×10^4	50-60	37	Mentel & Schmidt, 1973
Orthinosis virus	3.0×10^4	180	-	Nikolov & Papova, 1965
Rhinovirus 1A, 1B, 7	1.5×10^4	18-20	37	Mental & Schmidt, 1973
Rhinovirus 1A, 1B, 7	3.0×10^4	6-8	37	Mental & Schmidt, 1973
Poliovirus 1	1.5×10^4	75	20	Kline & Hull, 1960
Poliovirus 1	3.0×10^4	75	20	Kline & Hull, 1960

Table 2 Sporicidal Activity of Hydrogen Peroxide toward Spore-forming Bacteria and Bacteria Spores.

Organism	Concentration (ppm)	Lethality (minutes)	Temperature (^0C)	pH	Comment	Reference
B.subtilis	500	420-1080	37	-	b.c.	Nambudripad et al, 1949
B.megatheriu	500	420-1080	37	-	b.c.	Nambudropad etal, 1949
B.subtilis *	3.0×10^4	1440	37	4.3	spores	Baldy,1983
B.subtilis SA	25.8×10^4	7.3	24	3.8	s.s.	Toledo et al 1973
B.coagulans	25.8×10^4	1.8	24	3.8	s.s.	Toledo et al 1973
B.stearother-ophilus	25.8×10^4	1.5	24	3.8	s.s.	Toledo et al 1973
C.sporogenes	25.8×10^4	0,8	24	3.8	s.s.	Toledo et al 1973
B.subtilis var.globigii	25.8×10^4	2.0	24	3.8	s.s.	Toledo et al 1973
B.subtilis var.globigii	35×10^4	1.5	24	3.8	s.s.	Toledo et al 1973
B.subtilis SA	17.7×10^4	9.4	20	-	s.s.	Leaper,1984
B.subtilis SA	17.7×10^4	0.53	45	-	s.s.	Leaper,1984
B.subtilis SA	29.5×10^4	3.6	20	-	s.s.	Leaper,1984
B.subtilis SA	35.4×10^4	2.3	20	-	s.s.	Leaper,1984

b.c. Bacteria cultures; s.s., spore suspension. * carrier test

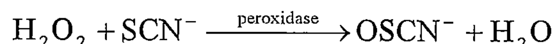
The combination of HP and ozone has been found to significantly improve the oxidation of taste and odor related compounds. It was concluded that the maintenance of an ozone residual was important for obtaining the best disinfection performance. Thus it is desirable to optimize the maintenance of an ozone residual prior to oxidation by an advanced oxidation process (Wolf et al.,1989; Finch, 1992). Scott, et al. (1992) and Ferguson, et al. (1990), also reported that ozone and peroxone (an advanced oxidation process for water treatment by combining ozone and HP) performed comparably in the disinfection process. Ozone residual appeared to be the most important determining factor in the bacteria inactivation. The lower applied ozone dosage is required to oxidize 2-methylisoborneal and geosmin (taste and odor compounds) as compared with ozone alone. HP, in combination with UV light , is also very effective for water treatment. HP concentrations of 2.5, 5.0 and 10.0 g/L (73.5, 147, 294 mM) were tested. In each case, synergistic inactivation was observed. At the highest concentration of HP (294 mM), a fraction ($1.3X10^{-3}$) of *E.coli* survived after 20 minutes. This fraction decreased to $3.1X10^{-6}$ with simultaneous UV irradiation. Dingman (1990) also found that the UV light /hydrogen peroxide system was effective in removing pseudomonas and fecal coliform bacteria and thus the swimming-pool water could be held within required chemical limits.

In hospitals, HP has been used both as an antimicrobial agent for equipment surface, and as application for infection. Domingue (1990) found that no viable *L. pneumophila* (a common pathogenic agent found in cooling towers and evaporative condensers) could be detected after a 24-hour exposure to 100 or 300 μg of HP per ml (29.4 or 88.2 M). Klapes (1990) demonstrated the feasibility of using vapor-phase

hydrogen peroxide (VPHP) as a surface decontaminate and sterilant. It was evaluated in a centrifuge application. VPHP cycles of 4, 8, 16, 32, minutes were examined for cidal activity against spores of *Bacillus*, *Subtilis* subsp, *Globigii* and *Bacillus stearothermophilus*. VPHP was shown to exhibit significant sporicidal capability. Flourney and Robinson (1990) tested the in vitro activity of 349 methicillin resistant *Staphylococcus aureus* (MRSA) isolates from veterans against eight antimicrobial agents. They found that HP exhibited very good activity against the test-isolate and may have some use as a topical agent for reduction of MRSA on skin and some mucous membrane. The combination of peracetic acid and hydrogen peroxide, tested by a checkerboard micromethod was found to be synergistic against bacillus spores. The minimal sporicidal concentration (MSC) of a biocide combination of peracetic acid and HP showed that synergy was maintained with increasing contact time and that the MSC could be reduced by two to eight times when compared with those of the individual biocides alone (Alasri et al.,1993). In particular, the disinfection efficacy, bactericidal, and detachment properties of peracetic acid, HP, and their combinations were studied against a polymicrobial biofilm grown in the continuous culture. The experiments showed that HP could give the best detachment properties. While the biocide combination of peracetic acid and HP, showed a complementary action between the two active substances, peracetic acid produced the bactericidal effect and HP allowed a successful detachment (Alasri et al., 1992). In 1992, Shimokawa and Nakayama observed the effect of combination of HP and an azole drug, clotrimazole (CTZ) to control *Candida albicans*. The results showed that the sensitivity

of *Candida albicans* cells to HP was found to increase markedly when they were grown in the presence of sub-growth-inhibitory concentration of CTZ.

HP in combination with peroxidase and thiocynate ion is continuing to be studied in the area of oral hygiene,or dental prophylaxis. Thomas and Thomas (1978) suggested the mechanism of the antimicrobial activity of the lactoperoidase-peroxide-thiocyanate system against *E.coli* in oral hygiene. In 1986, Fruitt and colleagues demonstrated that the reaction:



$$H_2O_2 + SCN^- \xrightarrow{\text{peroxidase}} OSCN^- + H_2O$$

is an important reaction in human and mouse oral environments. This reaction is in a state of dynamic equilibrium in vivo. The equilibrium concentrations of HP in whole saliva were calculated to range from 8 to 13 μM. This range is consistent with the reported estimate of 10 μM as the HP-tolerance limit for human cells. This calculation may partially explain why bacterial plaque metabolism may continue in human mouth in spite of continual generation of the antimicrobial agents, HOSCN and $OSCN^-$ by the salivary peroxidase system. Maruniak et al (1992) concluded that one of the mouth rinses, Perimed [R] (povidone, iodine, and hydrogen peroxide) was very effective in reducing plaque and gingivitis when used as a 2X daily mouth rinse.

In the food industry, especially those dealing with dairy products, HP is being considered as an effective antimicrobial agent in food. HP, in combination with thiocyanate ion, also can control *Listeria monocytogenes* in milk. Gaya et al (1991) observed the activity of lactoperoxidase-thiocyanate-hydrogen peroxide (LP) system on

four *Listeria monocytogenes* strains found in raw milk at refrigeration temperature. They found that the lactoperoxidase/SCN/HP system exhibited a bactericidal activity against *L.monocytogenes* at 4°C and 8°C. This system was shown to control development of *L. monocytogenes* in raw milk at a temperature of 4°C. .

Another important application of HP is in the contact lens industry. HP has been shown to be an effective disinfectant for soft contact lenses. Wilson et al (1991) and Lowe et al (1992) compared many disinfection solutions for soft contact lenses . They all found that HP was more effective against microbial films in lens cases and when used over longer disinfecting period, 3% HP gave adequate performance against fungi.

Biofilms and Hydrogen Peroxide

Studies of microbial colonization of surfaces have shown that most microorganisms on surfaces exist in biofilms. Biofilms are microcolonies of bacterial cells embedded in a polymer matrix and attached to a surface by the way of adhesive polysaccharides they excrete. Biofilms have significant implications in human medicine and commerce. It has been shown that hydrogen peroxide as an antimicrobial agent has reduced effectiveness when used against biofilms. Wilson et al (1990) found that the contact lens storage cases of individuals, who used a HP antimicrobial system showed significantly lower incidence of biofilm contamination when compared to case of individuals who used other chemical disinfectants. However, biofilms in these storage cases were not always disinfected by the addition of fresh solution of HP,i.e. , HP demonstrated decreasing effectiveness. Also, when HP was used in water transmission

systems in hospitals (endoscopes, nebulizers, tap water systems, dental units etc.), it showed effective antimicrobial efficacy, but a distinct reduction of influence on biofilms (Exner et al., 1987). Vincent et al (1989) in a study of invading a biofilm in a hemodialysis system, also found that the efficiency of disinfectant HP was lower in the biofilm than in static studies with bacterial suspensions. The reason for this kind of reduced efficiency is unclear and is the subject of this thesis. Richards and Gagnon (1993) thought that it was due to a shielding matrix of polymerized carbohydrates adherent to the implant surface which protected the enclosed bacteria from immune defenses and antibiotics. This complex, of surface, bacteria, and matrix, was termed a biofilm. Biofilms can be removed by mechanical cleaning and traditional disinfection procedures such as chlorination (Characklis, 1990), while disinfection is usually directed against the metabolism processes of the organism. Hence, disinfection is not equivalent to biofilm removal. Christensen (1990) showed that the biofilm removal could be accomplished with HP at levels well below those required for total disinfection and point suggested a mechanism whereby the extracellular biopolymer matrix rather than intracellular components were being degraded

The different modes of hydrogen peroxide biocidal activity make a common mechanism unlikely. However, there are some common stages in any biocide process involving biofilms. These are 1) diffusion of HP into biofilm clusters or into laminar biofilms followed by 2) consumption or reaction, either by the glycocalyx matrix or the bacteria themselves. If the HP or an active species created from HP diffuses into the biofilm, it will react with the biofilm or enzymes (such as catalase). Measuring the HP

profiles in and above the biofilm will help explain and quantify the HP consumption process. Time-related depth profiles of organism viability will help relate antimicrobial efficiency to HP penetration. If HP is consumed near the surface of the biofilm and does not penetrate into the biofilm, then the growth of biofilm near the substratum will not be inhibited. Thus, the goal of this initial research will involve determination of oxidant and HP concentration profile and their relationship to organism viability.

MATERIALS AND METHODS

Microorganisms and Culture Method

The microorganisms used in the experiments were *Pseudomonas aeruginosa*, *Pseudomonas fluorescence* and *Klebsiella pneumoniae*. They were taken from the culture collection at the Center for Biofilm Engineering at Montana State University and kept in a -70°C freezer as frozen stock cultures. The steps for making frozen stock culture are as follows: 1) Streak bacteria onto a plate made with R2A medium (Difco Laboratories). 2) Take one single colony from an uncontaminated plate and streak to make a confluent lawn. Incubate at room temperature for 24 hours. 3) Make up a 20% glycerol and 2% peptone solution. 4) Two ml glycerol-peptone solution is mixed with a sample of the confluent colony using an inoculating loop to resuspend the bacteria. 5) Put 1 ml of the resuspended bacteria into a sterile cryo vial and seal.

In our study, 1 ml portions of stock cultures of *Pseudomonas aeruginosa* (7.7×10^{9} CFU/ml), *Pseudomonas fluorescence* (4.8×10^{10} CFU/ml), *Klebsiella pneumoniae* (7.2×10^{10} CFU/ml) were used to inoculate the reactor. A modified Scheusner's mineral salts nutrient solution was used to grow biofilms. The media composition is given in Table 3.

Biofilm System Set-up

Aerobic biofilms were grown on a flat plate reactor. The experimental set-up is shown in figure 1 . One 20-liter carboy was used to supply autoclaved concentrated (24×) nutrient. A 33 gallon plastic vessel was used to store the distilled dilution water. The final nutrient solution (dilution water and concentrated nutrient) were pumped into a mixing chamber (Master Flex). Air also went into this mixing chamber through a 0.2 μm bacterial air vents (Gelman Science) from an air valve. A closed recycle route including two pumps (6-600 rpm, Cole-Parmer Instr. Co.) and a flat plate reactor made of polycarbonate (total volume 410 mL) was employed. Waste materials were pumped out from the mixing chamber into a sink. Several removable plastic coverslips were placed in the reactor to accumulate samples of biofilms that were used for CTC/DAPI staining and direct plate count analysis. The reactor was operated at a dilution rate of 3.2 h^{-1}. This dilution rate greatly exceeded the growth rate of planktonic cells in the reactor ($< 0.15\ h^{-1}$) and thus ensured that the activity of biofilm microorganisms dominated that of suspended cells in the system.

Construction of Hydrogen Peroxide Microelectrode

A glass covered platinum wire was used as a H_2O_2 probe. To prepare this microelectrode, a 100 μm diameter (pure TC grade) platinum wire (California Wire Co.) was dipped into a saturated KCN solution while applying power of +0.25v AC with

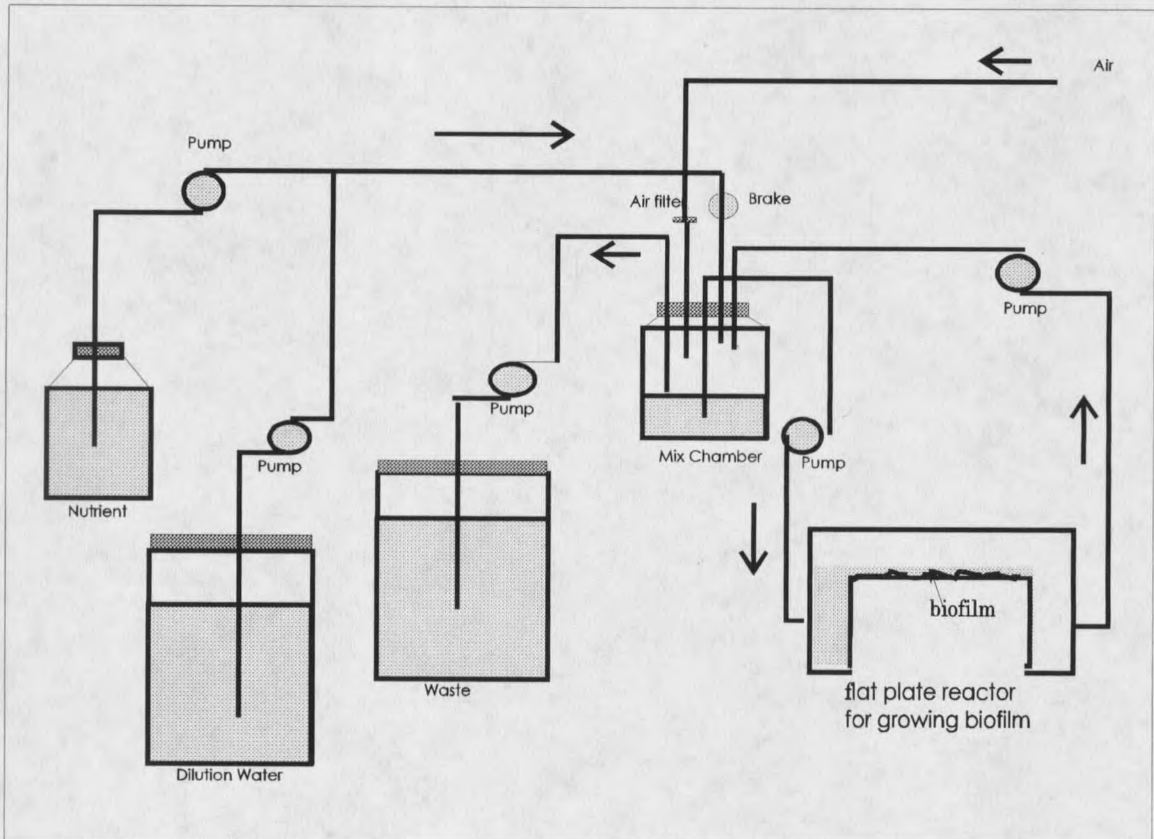


Figure 1 . Experimental set-up

respect to a graphite counter electrode. A tip 10 μm-20 μm in diameter was produced and checked with a light microscope for the correct size. This wire was then inserted into a 1 mm diameter glass capillary of Schott 8533 glass (Schott Glaswerke), which had been pulled by hand using a propane torch to get a tapered pipet shape. An electrode puller (Micro Electrode Puller, Stoelting Co.) was used to seal the wire in the glass capillary by melting the glass. This was done by adjusting the capillary so that the tip of the wire was 1.5 cm above the heat loop, attaching a weight to the suspended end of the glass capillary and setting the heat at 80% of full power. The glass capillary elongated and dropped by gravity, finally sealing the platinum wire. The tip of the electrode was ground flat to expose the platinum wire using a rotary grinder (Model EG-4, Narishige Co.) under observation by a video monitor. The exposed tip of the glass capillary was recessed about 2 μm by quickly dipping into KCN solution with an applied potential half of the previous one. After it was carefully washed in a sonication bath with deionized water and then acetone at least three times, the electrode was then dipped into a cellulose acetate solution (1 gram cellulose acetate (No. C-3782, Sigma Chemical Co.) to 20 ml acetone (HPLC Grade, A949-1, Fisher Scientific)). The membrane-covered electrode was allowed to air-dry overnight. Thus the hydrogen peroxide microelectrode was ready to use.

Hydrogen Peroxide Standardization

The hydrogen peroxide working solution used in this study was at a concentration of 0.3% (w/w), prepared by using 30% H_2O_2 (HX06035-2, EM Science). Before the 30% hydrogen peroxide was used, it was standardized. The standardization procedures were as

follows: First, add 3 ml concentrated H_2SO_4 to 100 ml distilled water in a 250 ml flask and swirl to mix using a magnetic stirbar. Second, pipet 100 μl 30% hydrogen peroxide into the above solution. Determine the density of 30% hydrogen peroxide solution by pipetting 100 μl volume in a beaker and weigh. Third, titrate 100 μl 30 % hydrogen peroxide with 0.1 N $KMnO_4$ (Lot No. 934987-18 , Fisher Scientific) until faint pink tinge appears. Finally, based on the readings of titration, get the actual concentration of hydrogen peroxide solution.

Calibration of Hydrogen Peroxide Microelectrode

When a potential of +0.8 volts is applied between the platinum cathode and the SCE reference electrode, hydrogen peroxide is oxidized to oxygen. This creates a current which is proportional to the hydrogen peroxide concentration in the solution surrounding the tip of the probe. The calibration procedures were as following: First, a picoammeter/DC voltage source (Hewlet Packard 4140B) was used to apply a potential of +0.8 volts between the platinum cathode and the SCE reference electrode. Second, prepare nominal ~~10~~ 100 mM hydrogen peroxide by diluting 1 ml of 30% hydrogen peroxide (HX06035-2, EM Science) to 100 ml distilled water. Third, add this solution 100 μl at a time to 200 ml distilled water with stirring and record current readings. Fourth, measure the actual concentration of hydrogen peroxide solution and get a series of relationship between current signals and hydrogen peroxide concentration. Finally, plot a calibration curve based on experimental data. An example of a calibration curve is shown in Figure 2.

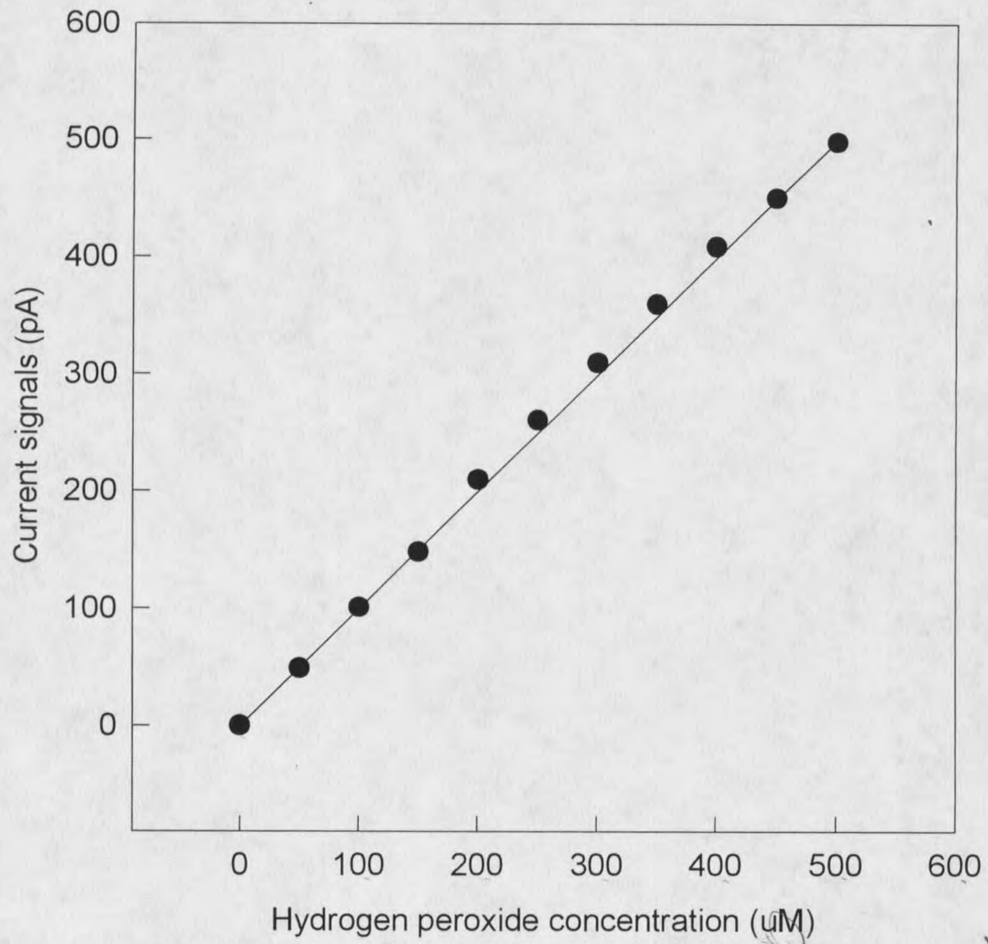


Figure 2. Hydrogen peroxide microelectrode calibration curve (tip diameter: 20 μm).

Construction of Dissolved Oxygen Microelectrode

The dissolved oxygen microelectrode was constructed as described by Revsbech (1983). It had five components: 1) a platinum cathode (working electrode); 2) a Ag/AgCl reference electrode; 3) a silver guard cathode; 4) an outer glass casing; and 5) an electrolyte solution.

The working microelectrode was made from a 100 microns (pure TC grade) platinum wire (California Wire Co.). One end was electrochemically etched (+0.25 AC) in a saturated KCN solution to a diameter of 5-10 microns and then rinsed in distilled water. The platinum wire was inserted into a glass capillary of Schott 8533 glass (Schott Glaswerke), which had been pulled by hand using a propane torch to get a tapered shape. Soda-lime glass was used to make a shaft. A glass tubing (15 cm in length) was pulled once over a propane torch, and the capillary was broken off. The tapered end of the shaft was then inserted into the 8533 capillary containing the platinum wire, and the two parts were fused in a flame. An electrode puller (Micro Electrode Puller, Stoelting Co.) was used to seal the wire in the glass capillary by melting the glass. This was done by adjusting the capillary so that the tip of the wire was 1.5 cm above the heating coil. Heat was gradually increased until the glass around the wire and the electrode dropped down. Under a microscope the glass was recessed 5-10 μm by moving a platinum heating loop close to the electrode tip. The exposed platinum was then electroplated with gold by inserting the tip into a $HAuCl_4$ solution and applying a potential of 2.0 volts for 2-3 seconds.

A 0.5 mm diameter, 99.99% pure silver wire was used for the Ag/AgCl reference electrode. The tip of a 3 cm long wire was polished with fine grained sandpaper followed

by cleaning in nitric acid and rinsing in distilled water. One centimeter of the wire was then submerged in a 0.1M HCl solution, and a current density of 0.4 mA/cm^2 was applied for 2 hours until the wire was uniformly covered with AgCl. It was then rinsed with distilled water. The outer casing with a tip of 16-20 μm was made from a 5$^3/_4$ inch Pasteur Pipet (Fisher Scientific). Heat was applied to the narrow end by a propane torch, and the glass was pulled by hand. The second pull was done by gravity, and a thin capillary was obtained by slowly moving a platinum heating loop towards the glass. The desired tip diameter was obtained by pushing the tip against a solid glass rod under a microscope. The tip opening was then shrunk to 2-3 μm by moving a platinum heating loop close to the tip. It was then covered with an uncured silicone (ACE Hardware Corp.), by capillary suction to a depth of 10-20 μm.

The next step was to insert a working microelectrode into the outer casing until the distance between the tip of the electrode and the tip of the outer casing was about 10 μm. Epoxy (ACE Hardware Corp.) was used to spot seal the working electrode to the outer casing and allowed to dry overnight. When the epoxy was dry, the reference and the guard cathodes were fixed to the outer casing with the epoxy. The distance between the tip of the platinum cathod and the tip of the guard cathode was about 100 μm.

The following step was to inject the electrolyte containing K_2CO_3(0.3M), $KHCO_3$(0.2M), and KCl(1.0M) into the outer casing. This process needed a vacuum pump (Model 1400, Sargent-Welch Scientific Co.) to make sure the tip of the platinum wire was immersed in the electrolyte.

Calibration of Dissolved Oxygen Microelectrode

When a potential of -0.8 volts is applied between the platinum cathode and the Ag/AgCl reference electrode, oxygen is reduced on the gold-tip of the cathode. This creates a current which is proportional to the oxygen concentration in the solution surrounding the tip of the probe. Because the calibration curve is linear, we only need two points, the currents associated with zero oxygen concentration and the currents related to saturated oxygen concentration. A picoammeter/DC voltage source (Hewlet Packard 4140B) was used to apply a potential of -0.8 volts between the platinum cathode and the Ag/AgCl reference electrode. This -0.8 volts potential was also applied between the guard cathode and the reference electrode. Some of the nutrient solution used in the experiments was transferred to a 300 ml beaker. The tip of the dissolved oxygen microelectrode was then submerged in the solution. Air or medical oxygen gas from compressed gas tanks was supplied to obtain a saturated oxygen concentration. When the current stabilized, the current reading was associated with the saturated oxygen concentration. Next, nitrogen gas was supplied to the solution to remove all the dissolved oxygen. When the current stabilized, the current reading was associated with zero oxygen concentration. Figure 3 shows an example of a calibration curve.

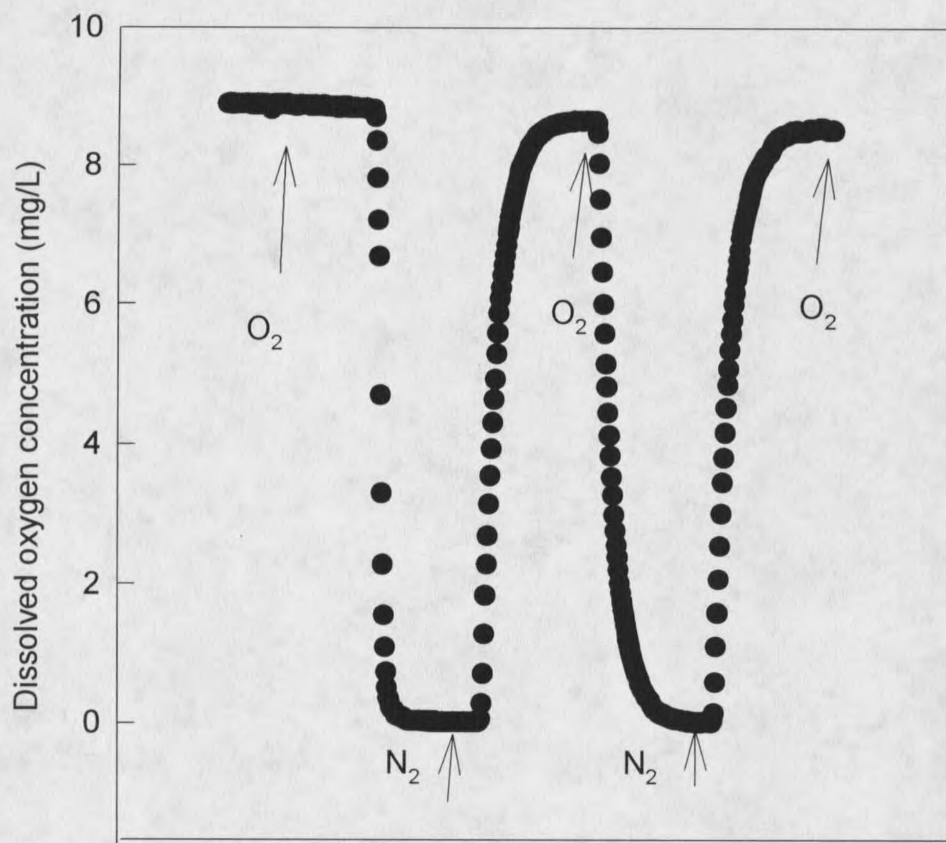


Figure 3. Dissolved oxygen microelectrode calibration curve (tip diameter: 8 μm).

Measurement of Hydrogen Peroxide and Dissolved Oxygen Concentration Profiles

Data Collection Set-up

Figure 4 shows the data collection set-up for measuring chemical profiles. We used the amperometric method to measure the hydrogen peroxide and dissolved oxygen concentrations in the liquid and/or biofilms. Each electrochemical cell consisted of a stable voltage source and an ammeter (Picoammeter/DC voltage source (Hewlet Packard 4140B)); the electrodes; and some nonactive species (phosphate buffer) at an applied potential in the solution. For hydrogen peroxide measurements, the electrodes used were hydrogen peroxide microelectrode and the saturated calomel (SCE) reference electrode. The applied potential was +0.8 volts Vs SCE. For the dissolved oxygen measurement, we used a combined dissolved oxygen microelectrode. The applied potential was -0.8V Vs Ag/AgCl reference electrode.

A micromanipulator (Model M3301L, World Precision Instruments.) was used to move the microelectrodes. It was equipped with a stepper motor (Model 18503, Oriel) and manipulated by a computer controller (Model 20010, Oriel). The measured signal was directed to a computer containing a data acquisition system (Model 810WW, Digital PC).

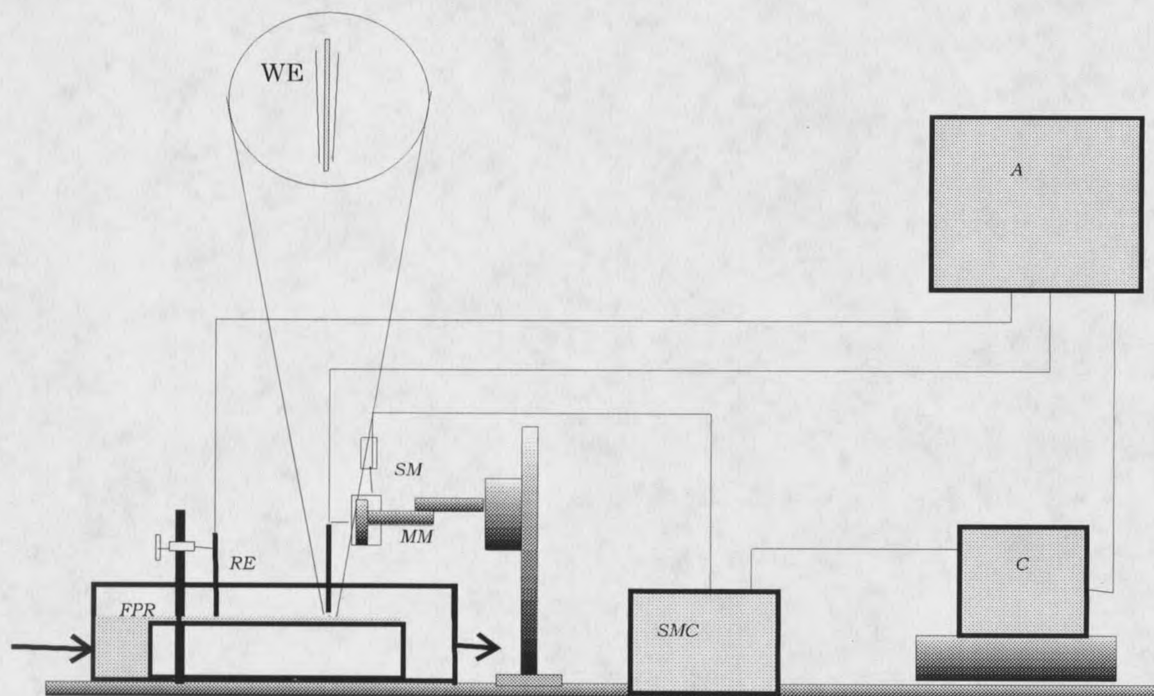


Figure 4. Apparatus for dissolved oxygen or hydrogen peroxide concentration profile measurement inside the biofilms. A: Pico-ammeter / DC-voltage source; C: Computer for data acquisition system; FPR: Flat plate reactor; MM: Micromanipulator; RE: SCE reference electrode; SM: Stepping motor; SMC: Stepping motor controller; WE: Working electrode.

Measurement in Biofilm System

H_2O_2 and DO concentration profiles were measured in the described biofilm system. The tips of the H_2O_2 or DO probes were located to reach the biofilm clusters by observing an inverted microscope. Approximately 1 micron accuracy of microelectrode movement in the Z-direction can be achieved by using a stepper motor (Model 18503, Oriel.) mounted on a micromanipulator (Model M0003L, World Precision Instruments) The movement was automated by connecting the stepper motor controller (Model 20010, Oriel) to the computer with a data acquisition software developed at the Center for Biofilm Engineering at Montana State University. When the tip reached the bottom of the biofilm cluster, the tip was pulled back 1500-2000μm. By setting up the steps, delay times, and collecting times, the DO and H_2O_2 concentration profiles in the biofilms were collected either without hydrogen peroxide or at different times while treated with hydrogen peroxide in the system.. These profiles then were saved to disk and processed by software of Microsoft Excel .

Catalase Activity Assay and the Effect of
Aminotriazole on Catalase

Planktonic cells and biofilm samples were collected in 250 ml centrifuge bottles respectively. These bottles were centrifuged in a RC5C centrifuge (Sorvall Instruments) for 20 minutes at 10000 rpm at 4^0C. The pellet on the bottom of the bottle was then resuspended using 10 ml 0.1 M phosphate buffer in a plastic tube. The cell suspension was sonicated using a probe sonicator for 1 minute on ice. After sonication, the sample

was centrifuged again at 10,000 rpm for 20 minutes. The supernatant was collected for catalase activity assay and total protein assay. Catalase activity was assayed according to Beers and Sizer (1952). The principle of this assay is that the decrease in ultraviolet absorption by hydrogen peroxide as a function of time can be used to follow the catalase-peroxide reaction. At any wave-length in a range from 200 nm to 400 nm, it is possible to use optical density increases linearly with peroxide concentration in accordance with the Beer-Lambert law. The reaction products, oxygen and water do not absorb light in this spectral region nor does catalase at the concentration of 10^{-9} M level; hence the ultraviolet absorption is a direct measure of peroxide concentration in the catalase peroxide system. To do this assay, first we use the standardized hydrogen peroxide solution (mentioned above) to plot a hydrogen peroxide standard curve by hydrogen peroxide concentration vs. absorbance. Figure 5 is a example of the standard curve. One ml of samples (supernatants) and one ml buffered hydrogen peroxide were then pipetted in each quartz one cm cuvettes. The reading of optical density was taken every 10 seconds at 280 nm wavelength and hydrogen peroxide concentration was interpolated as a function of time from the standard curve. Protein in the sample was determined by the method of Lowry et al. (1951) using the Lowry protein assay kit (Sigma Chemicals). A standard curve was prepared using the Protein Standard (catalog No. 690-10, Sigma Chemicals). The wave length of absorbance used in this assay was 600 nm. The protein concentration of the sample was interpolated from the standard curve (Figure 6) by plotting protein concentration vs. absorbance. The specific catalase activity was expressed as μmol of H_2O_2consumed/minute/mg protein. This assay was also conducted on both biofilms and

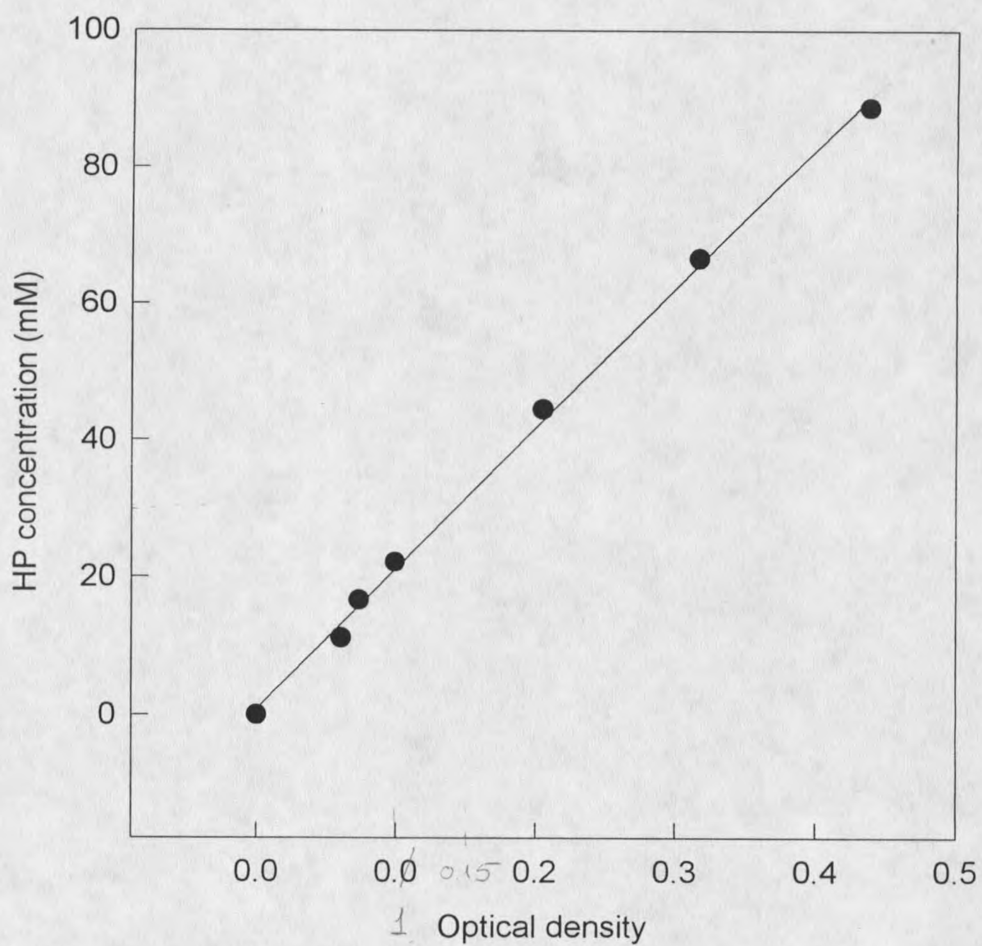


Figure 5. Hydrogen peroxide calibration curve at 280 nm wavelength. ($Y=204.6X+0.86$; $R^2=0.9980$).

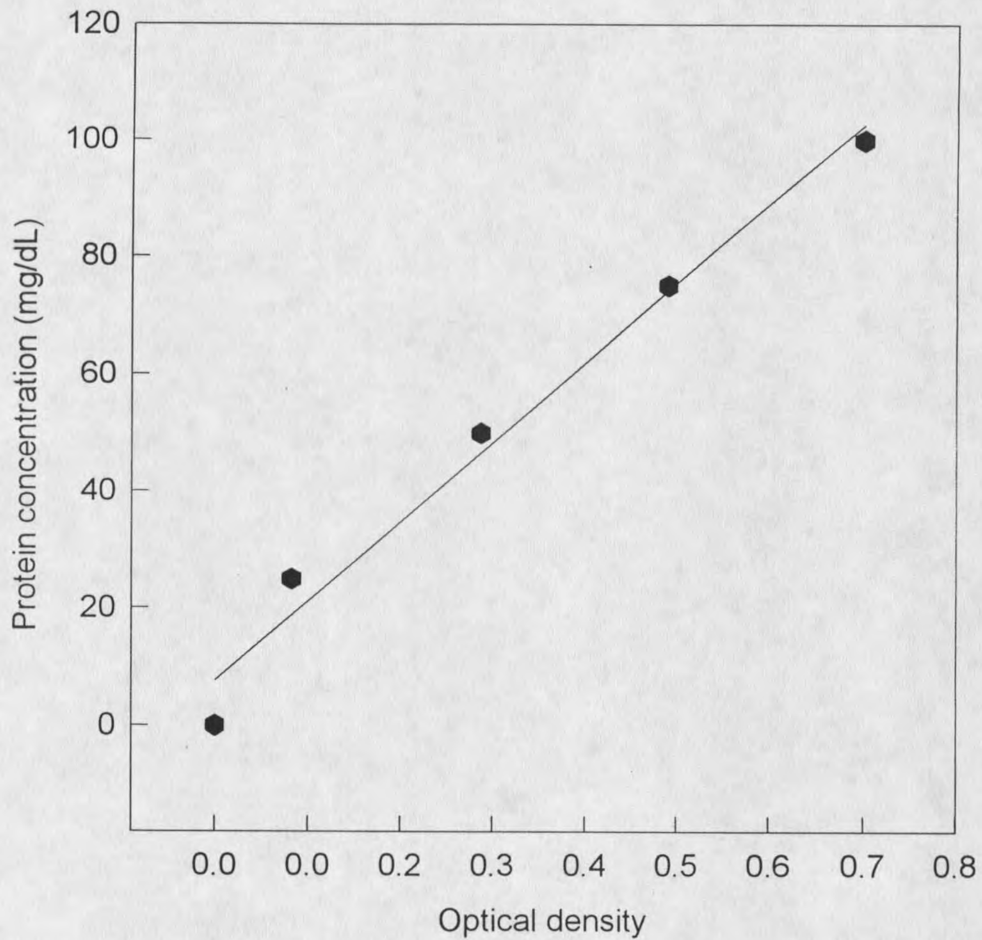


Figure 6. Total protein calibration curve using Protein Assay Kit (Sigma). Wavelength is 600 nm. ($Y=135.7X+7.6899$; $R^2= 0.9814$)

planktonic organisms exposed to varying amounts of catalase inhibitor, 3-amino-1,2,4-triazole. We pretreated the supernatant samples with this inhibitor for 1 hour, and mixed it with hydrogen peroxide to monitor the change of the concentration of hydrogen peroxide again to see if there was any inhibitory effect of aminotriazole on the catalase activity.

Inhibition of Catalase Activity in the Biofilms and the Batch Cultures

To check the catalase activity in the biofilm and in the suspended bacterial system, we used the specific inhibitor, 3-amino-1,2,4-triazole (AT) (Aldrich Chemical Co.), and pretreated either the biofilms or batch (suspension) cultures consisting of the same three species (*Pseudomonas aeruginosa* (7.7×10^9 CFU/ml), *Pseudomonas fluorescens* (4.8×10^{10} CFU/ml), and *Klebsiella pneumoniae* (7.2×10^{10} CFU/ml)). Batch cultures were prepared using the autoclaved modified Scheusner's mineral salts nutrient solution (Table 3), and incubated on a platform shaker (Thermolyne) at 150 rpm at room temperature for 48 hours. Different concentrations of AT solution were prepared right before the experiments. For the biofilm testing, we pumped a specific concentration of the inhibitor to the reactor and incubated for 1 hour and pumped 0.3% hydrogen peroxide to the system. According to Kono (1995), AT solution was stable during a 120 minutes incubation time. So in our experimental condition, AT should be stable during this 1-hour incubation. For the batch cultures, we mixed the cultures with the inhibitor to reach a specific concentration of the inhibitor for 1 hour, followed by mixing with hydrogen peroxide working solution to reach a concentration of 0.3%. The dissolved oxygen

concentration profiles were collected again to see the inhibitory effects on either the biofilms or batch cultures.

Respiratory Activity Assessment by CTC/DAPI Staining

Plastic unbreakable coverslips covered with biofilm were collected by withdrawing them at different times during 0.3% hydrogen peroxide treatment. The slides were then placed in a staining container with the biofilm side up. Respiratory activity within biofilms was determined with 5-cynao-2,3-ditolyl tetrazolium chloride (CTC) (Polysciences, Inc.) by the following procedures (Rodriguez et al. 1992, 1993). The biofilm slides were immersed in 0.04% CTC solution for 1 hour at 25°C. The samples were then fixed with 5% formalin and immediately stained with 1μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigama Chemical Co.) for 5 minutes. The biofilms were embedded and removed from the substratum by a cryoembedding technique (F.P.Yu et al., 1994) with Tissue-Tek OCT compound (Miles Inc.). The samples were then wrapped in aluminum foil and stored at -70°C before cryotomy. Frozen sections were cut with a cryostat (Reichert-Jung Cryocut 1800, Leica) operated at -19°C. The 5-μm thick sections were collected on glass slides for observation under an epifluorescence microscopy. The sections were examined with an Olympus BH-2 microscope with epifluorescence illumination (100-W mercury lamp). An Olympus B filter cube unit with an excitation filter (BP490), a dichroic mirror (DM500), and a barrier filter (AFC+O515) were employed to simultaneously visualize the CTC-formazan and DAPI fluorescence within the sectioned biofilms by the different color of each stain. The nonrespiring bacteria showed green-color when stained with DAPI, while

the respiring cells were green but contained intracellular crystals of red CTC-formazan. Filter block G fitted with an O590 barrier was used to visualize the red CTC-formazan crystals by excluding DAPI fluorescence; while, a U excitation filter cubic unit with an excitation filter (UG-1), a dichroic mirror (DM 400), and a barrier filter (L420) was employed for visualizing the DAPI fluorescence alone.

Enumeration Methods

Biofilm bacteria in hydrogen peroxide-treated and untreated samples were assayed by scraping the biofilms off the slide followed by homogenizing the cell suspension in an ice bath for 3 minutes with a homogenizer (Tekmar). One set of the samples was processed by a conventional direct serial dilution viable count (C-DVC) method using R2A agar (Difco Laboratories). After a 48-hour incubation at 30^{0}C, the viable cells and/or colonies on R2A agar plates were enumerated using a colony counter (American Optical Co.). The area density of biofilm bacteria on the substratum is expressed as colony forming unit (CFU) per square centimeter. Another set of samples was prepared in a series of dilutions followed by DAPI (0.1 mg/100ml) staining for 5 minutes. The DAPI-stained samples were collected on a 0.2-µm black polycarbonate membrane (Nuclepore), and the membrane was transferred to a glass slide. The enumeration of the sample on the slide was done under a microscope. The data collected this way were used as "total cell counts".

Table 3. Composition of Modified Scheusner's Mineral Salts Medium

Nutrients	Concentrations
K_2HPO_4	0.7 (g/L)
KH_2PO_4	0.3 (g/L)
$(NH_4)_2SO_4$	0.1 (g/L)
$MgSO_4.7H_2O$	0.01 (g/L)
Glucose	40 (mg/L)
Yeast Extracts	15 (μg/L)

Table 4. Composition of Phosphate Buffer (pH 7.4)

Chemicals	Concentrations
KH_2PO_4	0.236 (g/L)
Na_2HPO_4	0.405 (g/L)

RESULTS

Growth of Biofilm

After inoculation of the bacteria species into the flat plate reactor, all the experimental conditions, including the position of the reactor and the flow rate of the nutrients, were kept constant. In about 24 hours, the biofilms started growing into white irregular dots and patches. Then they became biofilm clusters which gradually grew thicker and bigger. Finally they covered almost all the bottom surface of the reactor. This growth process needed about 4-5 days. These biofilms were used in the reported experiments.

Characteristics of the Hydrogen Peroxide Microelectrode

Some preliminary experiments were done to test the specific characteristics of the hydrogen peroxide microelectrode. Figure 2 shows an example of a calibration curve of the microelectrode. This curve indicates that there is a linear relationship between the current signal and the concentration of hydrogen peroxide. As shown in Figure 7, this kind of microelctrode is also very sensitive to changes in pH. This figure shows that the current signal is quite stable around pH 7. This information tells us that in order to get good stable relationship between the current signal and the hydrogen peroxide concentration, we should use a neutral range of pH. Another experiment was conducted to test the response of the hydrogen peroxide microelectrode in phosphate buffers of different concentrations (Figure 8). The results demonstrate that this electrode is stable over the

length of a typical experiment (2-3 hours) and independent of buffer concentration except in extremely low concentration solutions.

The sensitivity and selectivity of this hydrogen peroxide microelectrode were examined by using the applied potential range from -1.0 to + 2.0 volt (Figure 9). The same trend was found in the background signal and in the hydrogen peroxide in the solution. The selectivity of the microelectrode is defined as the ratio of the signals measured in the presence and absence of hydrogen peroxide and in the presence of an interfering ion. From the experiment, the maximum selectivity was observed at the applied potential of +0.8 v.

It has been shown that an amperometric measurement may have a strong stirring effect because of the change of the rate of mass transfer due to the different stirring conditions. A cellulose acetate film was desposited on the tip of the electrode to minimize the stirring effect. The experimental data (Figure 10) show that none of the electrodes with tips less than 25 micron displayed significant stirring sensitivity.

Hydrogen Peroxide Concentration in the Biofilm Systems

Two control experiments (shown in Figures 11 and 12) were performed prior to measuring hydrogen peroxide concentration profiles in the biofilms. First (Figure 11), we measured the hydrogen peroxide concentration in the biofilm without hydrogen peroxide solution flowing through. A flat response was observed, which indicated that no hydrogen

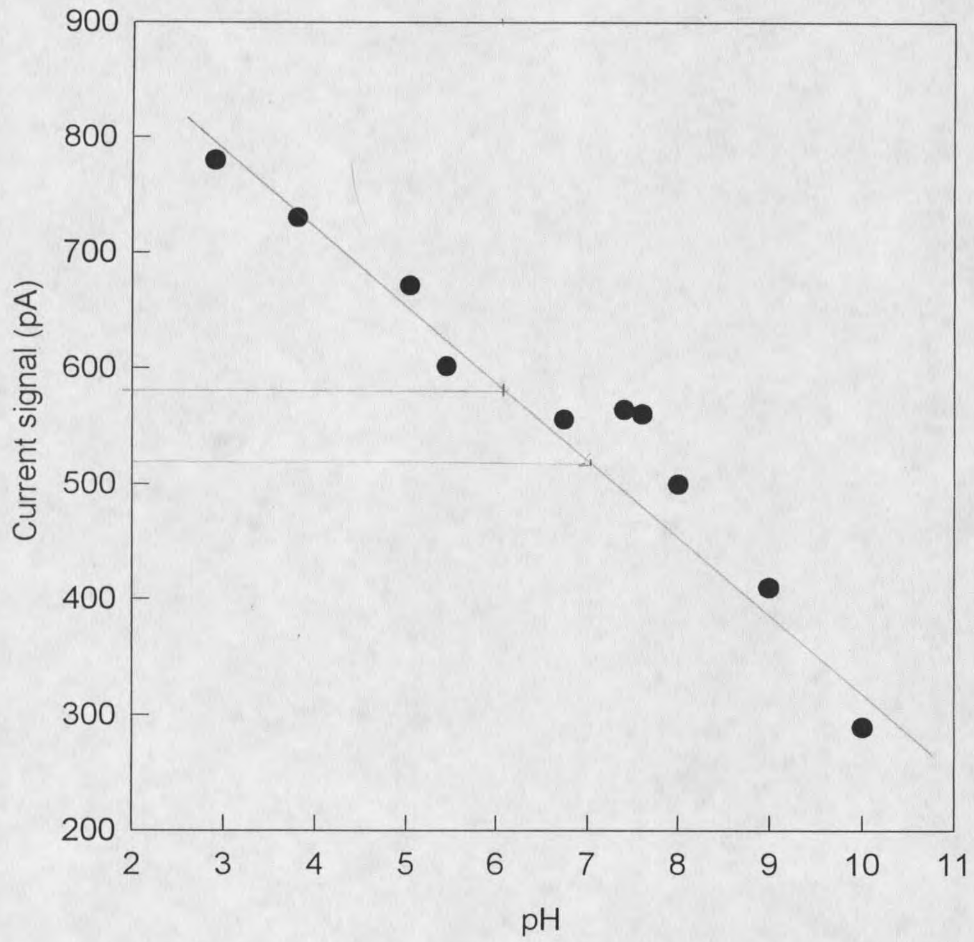


Figure 7. pH dependence of hydrogen peroxide microelectrode (tip diameter: 25 μm) in hydrogen peroxide solution (0.3%)

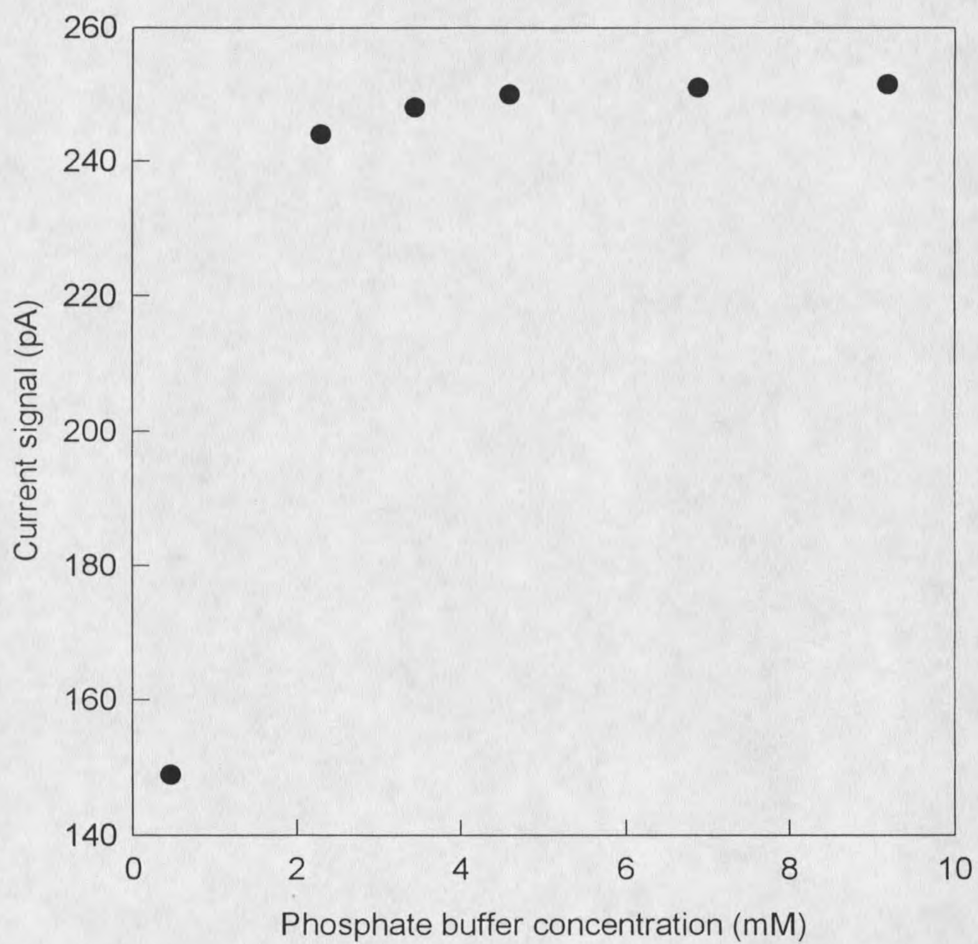


Figure 8. The effect of phosphate buffer concentration on the sensitivity of a hydrogen peroxide microelectrode. The pH of the phosphate buffer was 7.4. The concentration of hydrogen peroxide was 0.3%.

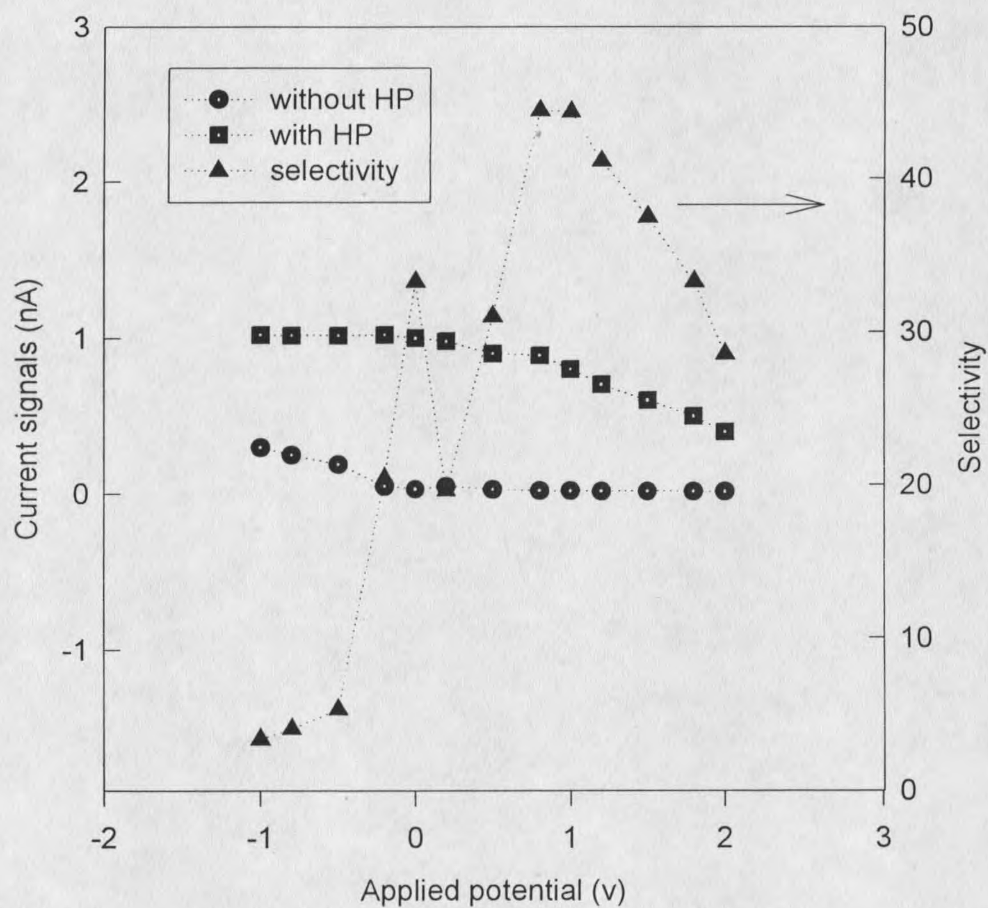


Figure 9. Sensitivity and selectivity of hydrogen peroxide (HP) microelectrode at different applied potentials. Selectivity is the ratio of signals from the solution with and without HP.

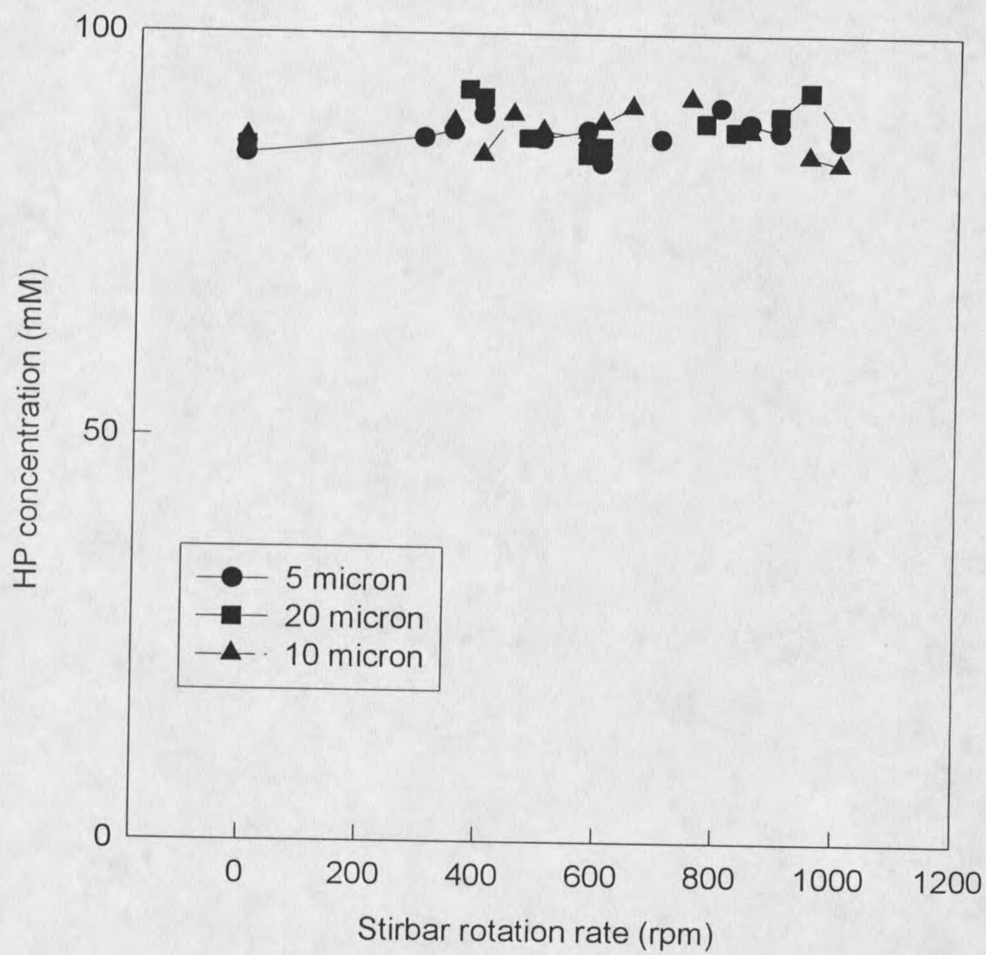


Figure 10. The stirring effect on the signals of hydrogen peroxide microelectrodes with different tips.

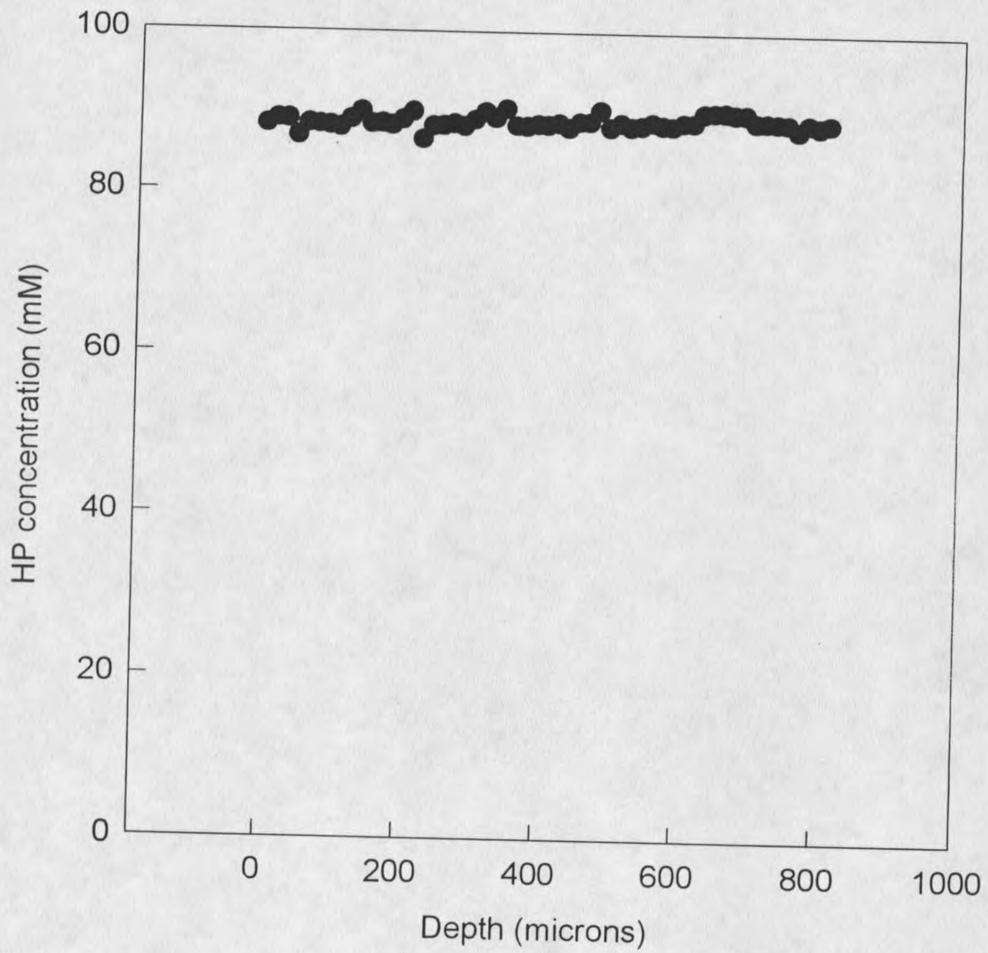


Figure 11. Hydrogen peroxide concentration profile measurement control experiment 1 with hydrogen peroxide and no biofilm.

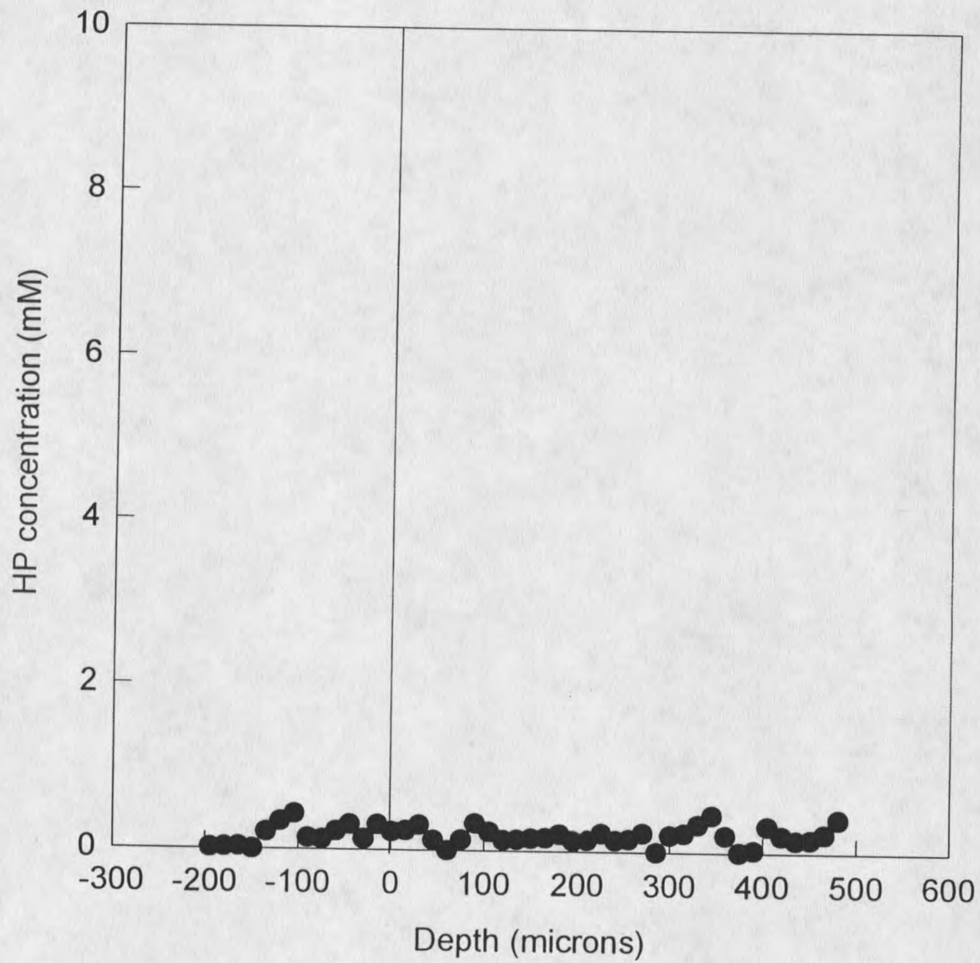


Figure 12. Hydrogen peroxide concentration profile measurement control experiment 2. Profile measured in a hydrogen peroxide-free buffer within the biofilm. Negative numbers on the x-axis represent the bulk fluid, and zero corresponds to the interface of bulk fluid and biofilm.

peroxide was produced spontaneously from the biofilm. In the second control experiment (Figure 12), we took the hydrogen peroxide profiles in the flat plate reactor with hydrogen peroxide flowing through but no biofilm in the reactor. Also a flat curve was observed, indicating that neither the hydrogen peroxide solution itself nor hydrodynamic characteristics perturbed hydrogen peroxide measurement by the microelectrode.

The experiments were performed using the hydrogen peroxide microelectrode to measure transient hydrogen peroxide concentration in the biofilm. A 0.3% hydrogen peroxide (88.8mM) solution was used. Several locations in the biofilm were examined for hydrogen peroxide penetration. In Case A, the biofilm was around 600 microns, Case B 700 microns (Figures 13 and 15). The hydrogen peroxide profiles were measured at 5 minutes, 30 minutes, 60 minutes and 120 minutes into the experiment. Figures 14 and 16 showed the change in hydrogen peroxide concentration with time at one point deep within the biofilm exposed to a constant hydrogen peroxide concentration (Case A: at 600-micron; Case B: at 700-micron). From the experimental data, we did find that the hydrogen peroxide penetrated the biofilm. However, it seemed that the penetration of hydrogen peroxide into the biofilm was a very slow and complicated process or it was rapidly consumed by a component of the biofilm. Even after 2 hours treatment, hydrogen peroxide had barely penetrated into the biofilms. Raw data for the hydrogen peroxide concentration profiles are tabulated in the Appendix (Tables 5, 6) .

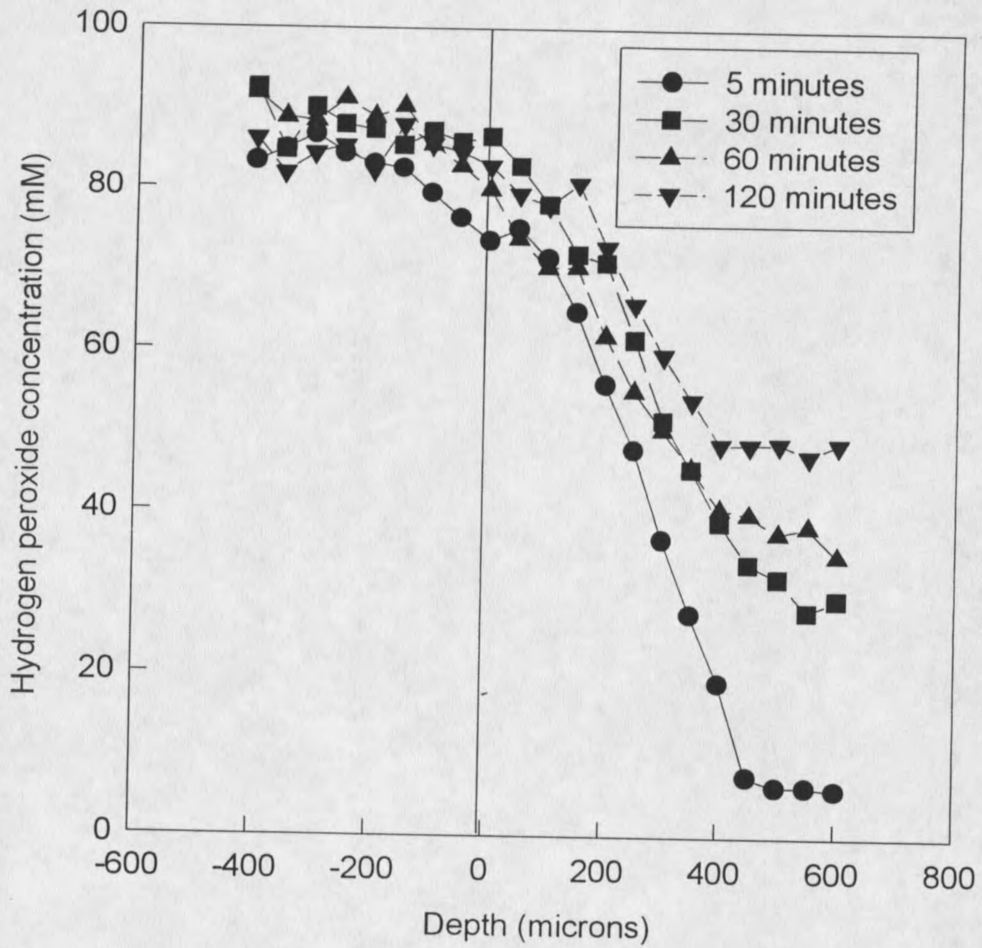


Figure 13. Hydrogen peroxide concentration profiles in biofilms: with 0.3 % hydrogen peroxide treatment. Case A (600 μm biofilm). Zero represents the interface of bulk fluid and biofilm, and negative number on the x-axis corresponds to the bulk fluid.

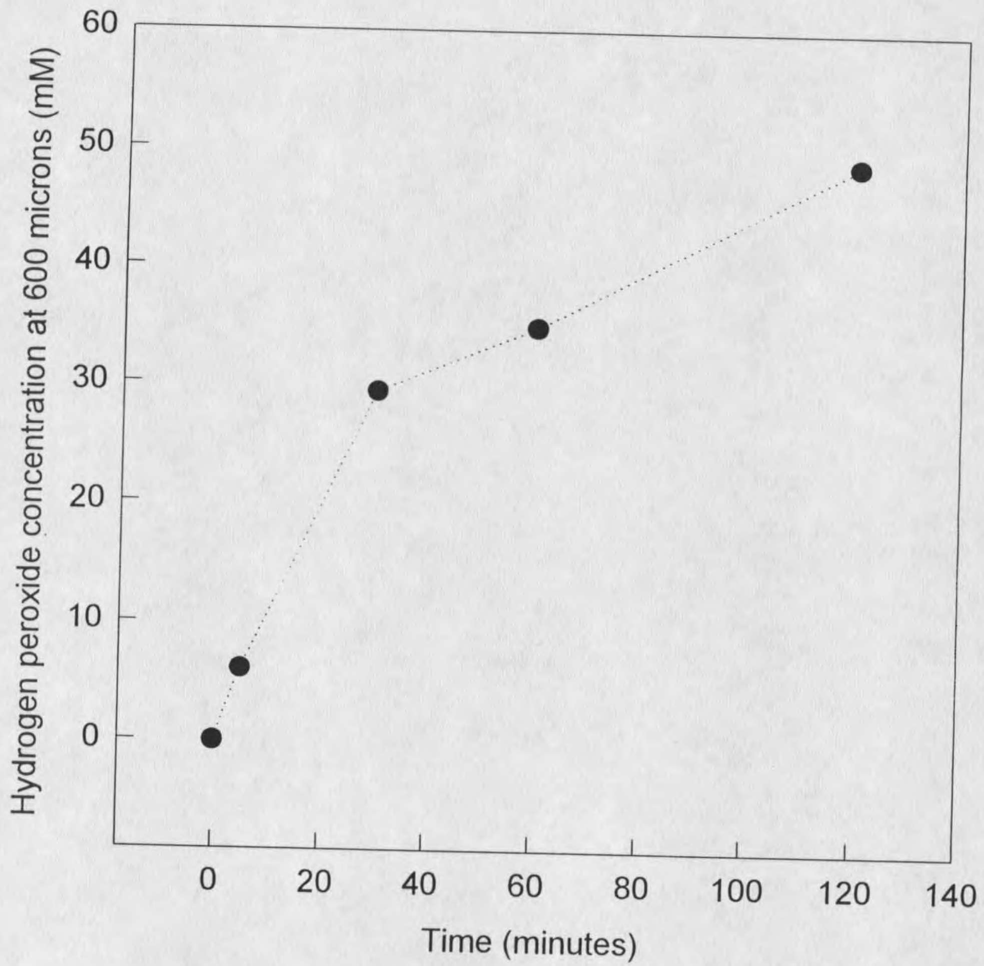


Figure 14. The change in hydrogen peroxide concentration with time at one point deep (600 μm) within the 600-micron biofilm exposed to a constant hydrogen peroxide concentration.

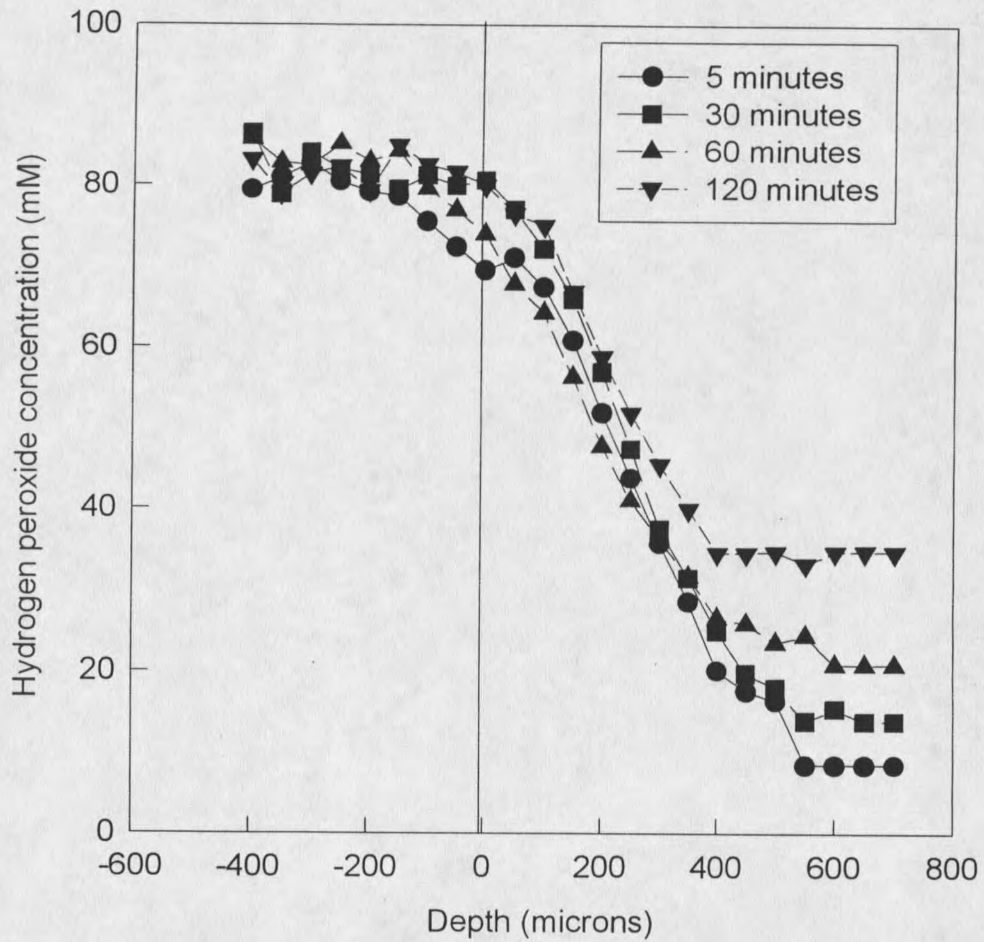


Figure 15. Hydrogen peroxide concentration profiles in biofilms: with 0.3% hydrogen peroxide treatment. Case B (700 μm biofilm). Negative numbers on the x-axis represent the bulk fluid, and zero corresponds to the interface of bulk fluid and biofilm.

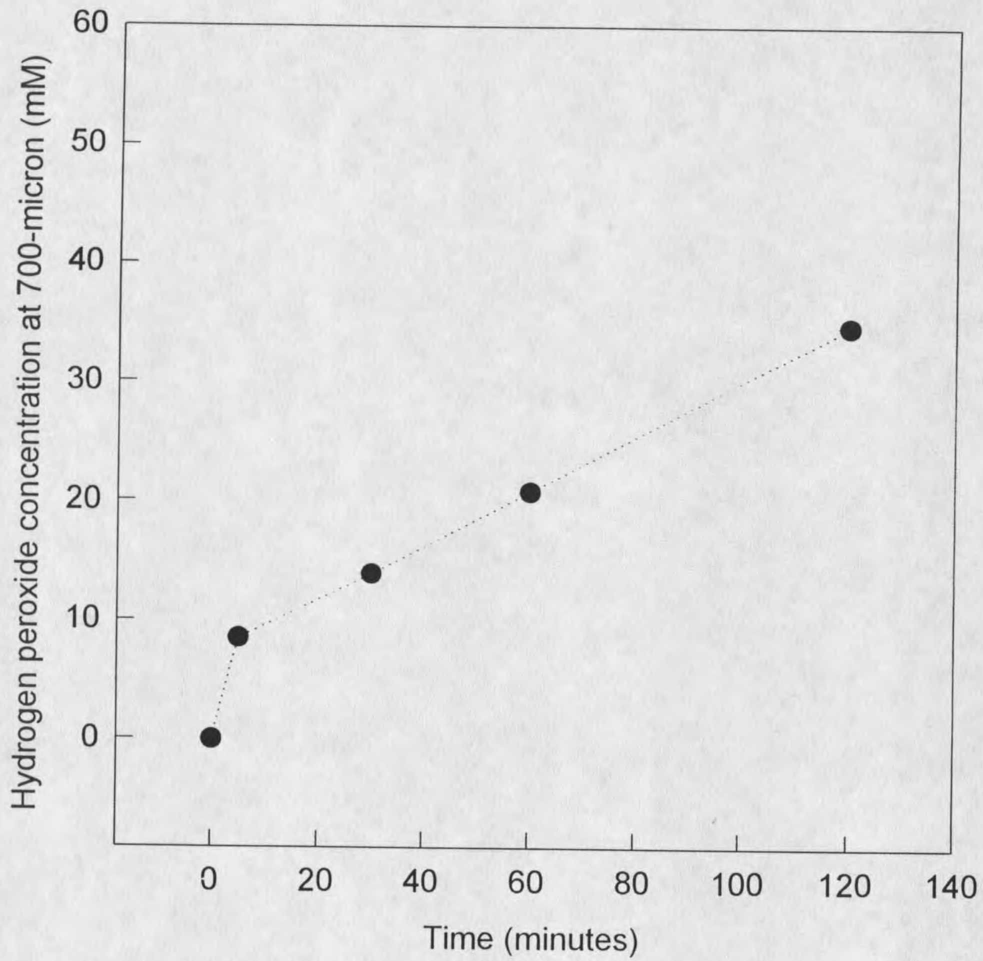


Figure 16. The change in hydrogen peroxide concentration with time at one point deep within the biofilm (700-micron) exposed to a constant hydrogen peroxide concentration.

Dissolved Oxygen Concentration Profiles in the Biofilm Systems

Before and after taking the experimental dissolved oxygen concentration profiles, the dissolved oxygen probe was calibrated in air or medical oxygen saturated water and nitrogen saturated water. Figure 3 shows the dissolved oxygen calibration curve. A very interesting phenomenon was found when we introduced hydrogen peroxide into the biofilm system. Figure 17 shows the dissolved oxygen concentration profiles in the biofilms without hydrogen peroxide. This is a typical S-shaped profile depicting oxygen consumption by the biofilm. However, when we pumped hydrogen peroxide into the system and measured the dissolved oxygen (DO) concentration profiles, the results, which are shown in Figure 18, were obtained. These curves indicate that hydrogen peroxide was degraded by a process which produced oxygen. At the end of the experiment, we used commercial bleach (20%; the Clorox Company) to kill the biofilm but not remove the biofilm for about 4 to 5 hours. Then we took the DO profiles again; these results are shown in Figure 19, which is a flat curve. It indicated that the oxygen increase was due to the reaction of hydrogen peroxide with the biofilms. Since the three species that we used are catalase positive bacteria, we hypothesized that this increase of oxygen was due to the activity of catalase.

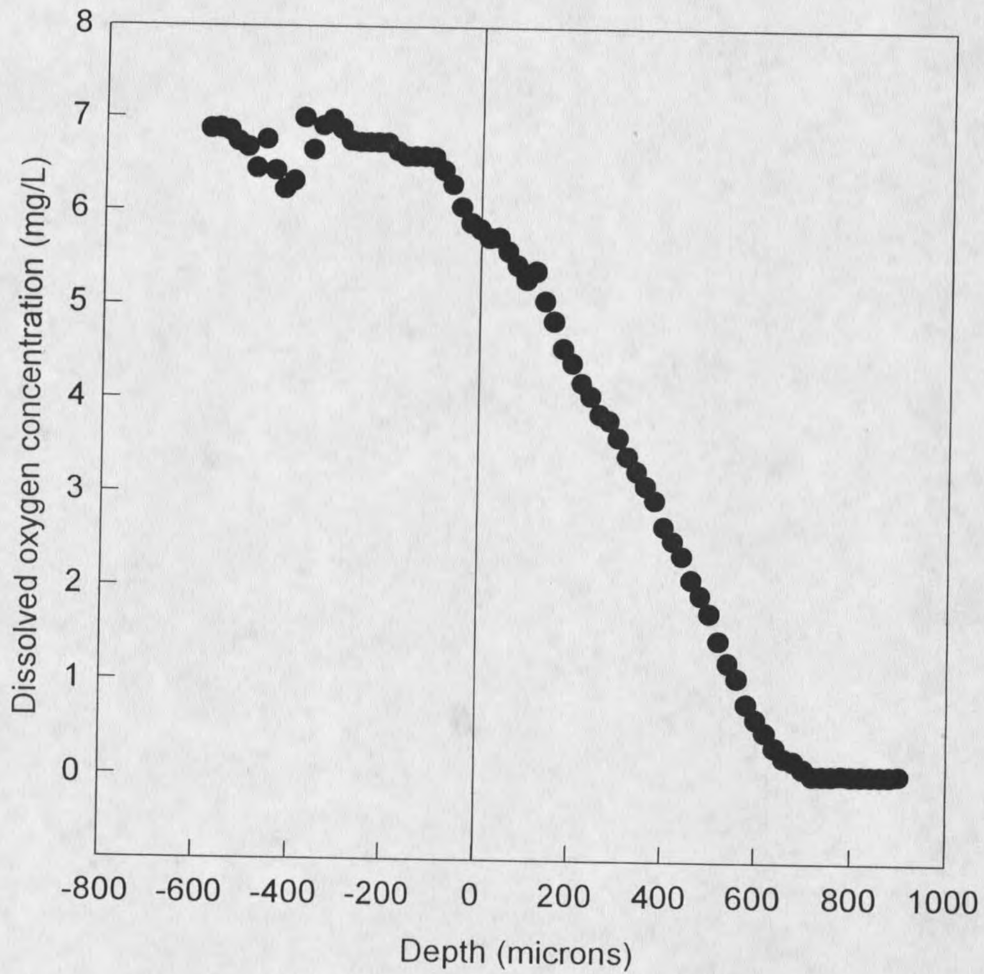


Figure 17. Dissolved oxygen concentration profiles: without hydrogen peroxide treatment. Negative numbers on the x-axis represent the bulk fluid, and zero corresponds the interface of bulk fluid and biofilm.

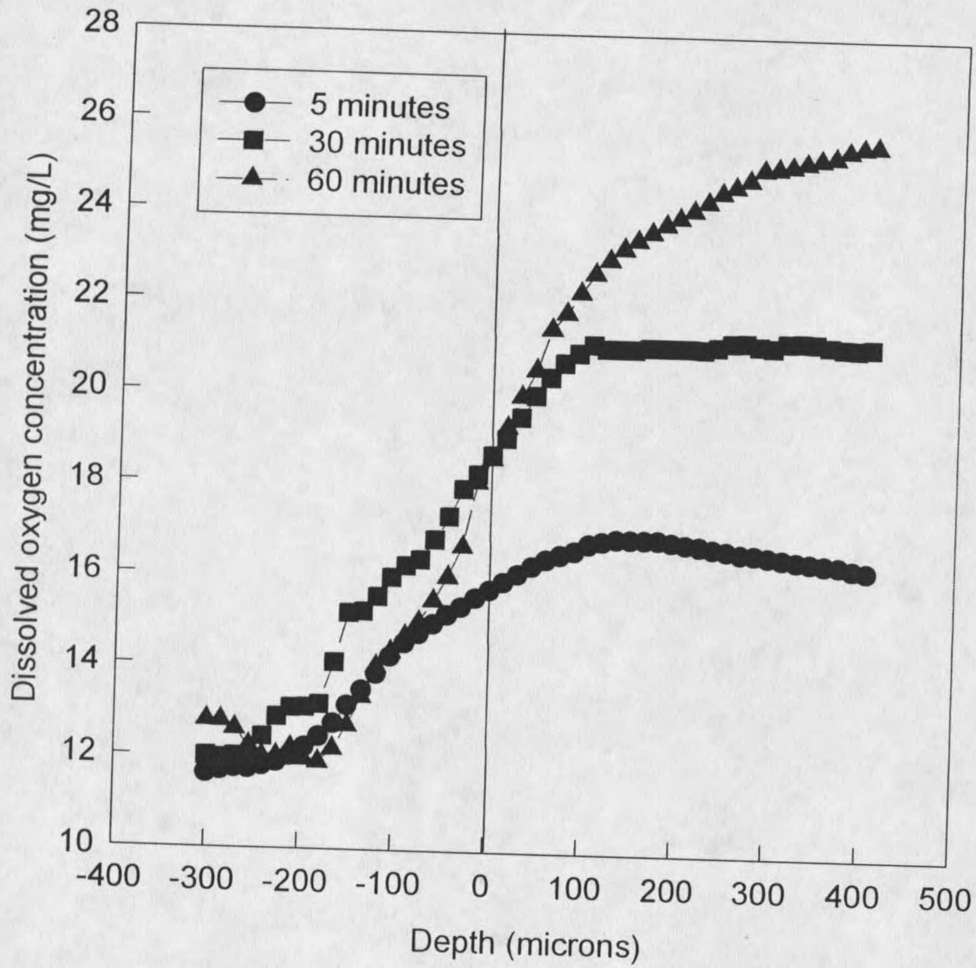


Figure 18. Dissolved oxygen concentration profiles: with 0.3% hydrogen peroxide treatment. Negative number on the x-axis correspond the bulk fluid, and zero represents the interface of bulk fluid and biofilm.

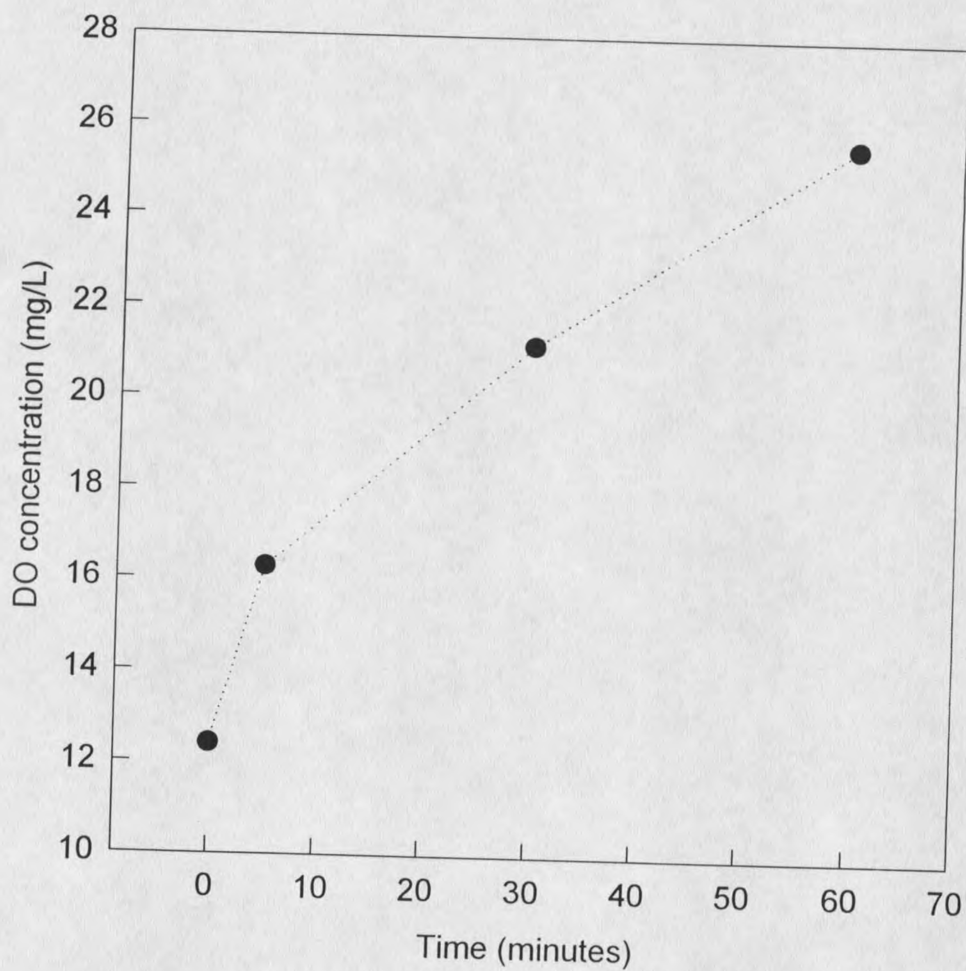


Figure 19. The change in dissolved oxygen (DO) concentration with time at one point deep within the biofilm (400-micron) exposed to a constant hydrogen peroxide concentration.

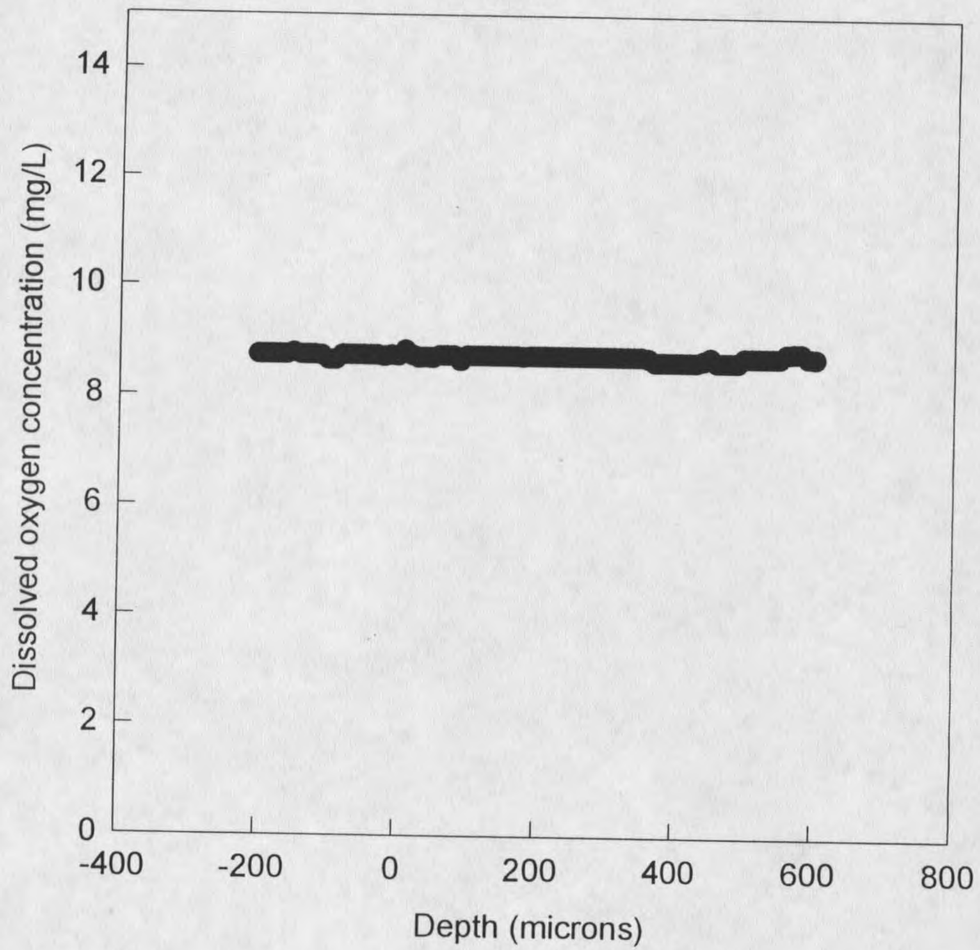


Figure 20. Dissolved oxygen concentration profiles: with hydrogen peroxide treatment after killing the biofilm. Negative numbers on the x-axis represent the bulk fluid, and zero corresponds to the interface of bulk fluid and biofilm.

Catalase Activity in the Cell Extracts from Planktonic Cells and Biofilms and the Effect of Aminotriazole On the Catalase

To test this hypothesis, we did a cell extract assay to see whether or not the catalase was active either in planktonic cells or in biofilms. The experimental data demonstrated that the cell extracts either from planktonic cells and biofilms showed similar catalase activity (Figure 20, 21) using a standard assay according to Beers and Sizer (1952). This result demonstrated that catalase existed in the planktonic cells and the biofilm of our experimental system and probably was involved in the breakdown of hydrogen peroxide to produce oxygen. When the chemical 3-amino-1,2,4-triazole (AT) was introduced into the cell extracts, the ability of breakdown of hydrogen peroxide in the cell extracts decreased with the increase of the concentration of AT (Figure 20, 21). This result showed that AT could be used as an inhibitor to inhibit the catalatic function of catalase equally in the planktonic cells or the biofilms. These two results suggest that catalase was active when hydrogen peroxide was introduced to our experimental system. By using the Lowry protein assay, a total protein concentration in the cell extracts was obtained. Under our experimental condition, this number in the planktonic cell system was 7.047 mg/ml, while in the biofilm system it was 5.608 mg/ml. A specific activity of catalase was defined as that amount of catalase catalyzing the degradation of 1 μmol of H_2O_2 per minute per mg protein. From our experimental data, the specific activity of catalase in the absence of catalase inhibitor was 69.42 units / mg protein in the planktonic cells, while the specific activity of catalase in the biofilm was 69.83 units / mg protein. This was close to the range of catalase activity (70-108 units) in bacteria soluble extracts

(Kono and Fridovich, 1983). Figure 22 shows the decrease of specific catalase activity in the planktonic cells and in the biofilms in the presence of AT. A dose dependent inhibitory effect was found from these curves. The IC_{50} was around 50 mM.

Inhibitory Effect of Aminotriazole on the Batch Cultures and the Biofilms

The inhibitor for catalase, 3-amino-1,2,4-triazole (AT), was then applied to our experimental system. We pretreated the biofilms with this inhibitor for 1 hour, and pumped hydrogen peroxide into the system and measured DO concentration profiles again. Different inhibitor concentrations, from 50 mM to 1 M were used. Figure 24 shows that even the concentration of AT introduced was as high as 1 M, the DO concentration profiles still showed their increase in the biofilm with the increase of times. Figure 25-A showed that inside the biofilm at one point, the DO concentration did not have significant change in the presence of an increasing amount of AT. Another experiment was done using a three species (*Pseudomonas aeruginosa*, *Pseudomonas fluorescence* and *Klebsiella pneumoniae*) batch culture. Also, two groups of data (with or without AT pretreatment), compared to each other, showed that, unlike with biofilms, catalase inhibitor 3-amino-1,2,4-triazole did have a distinct dose-dependent effect on the catalase activity of suspended bacteria (Figure 25-B).

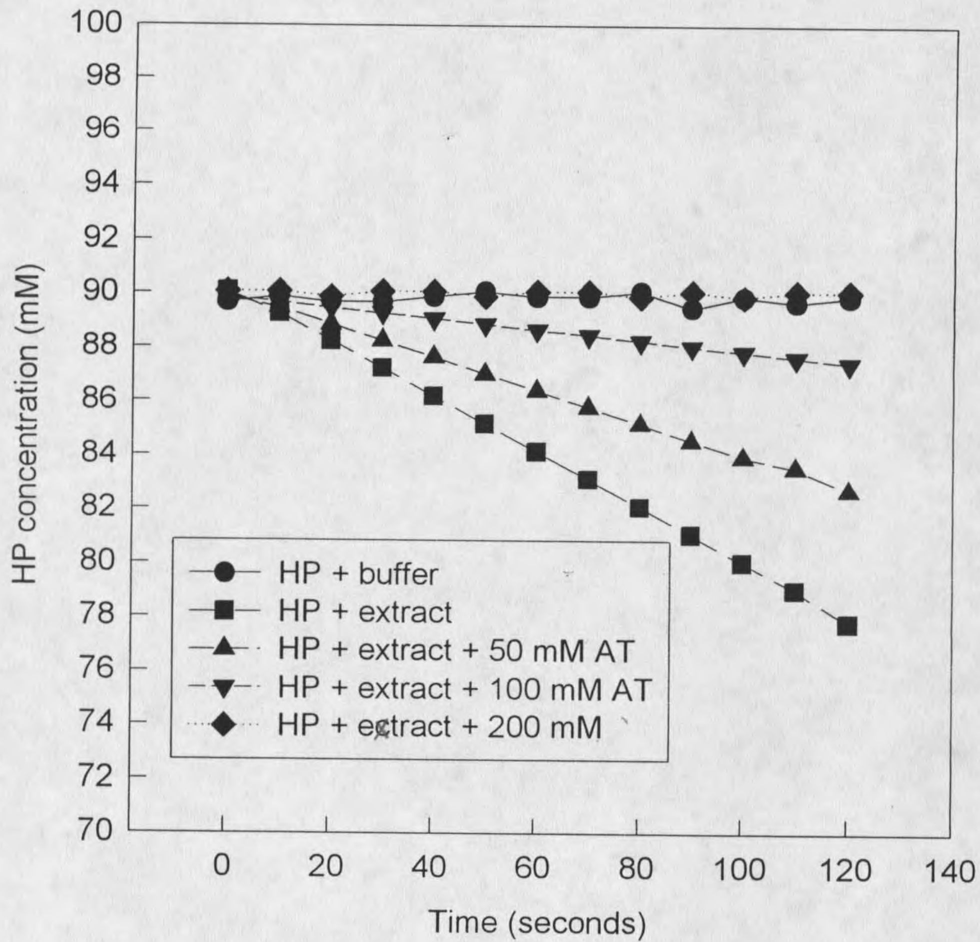


Figure 21. The effect of aminotriazole (AT) on the breakdown of hydrogen peroxide in the cell extract of planktonic bacteria. AT: aminotriazole; HP: hydrogen peroxide. The initial concentration of hydrogen peroxide was 0.3 % (0.088 M)..

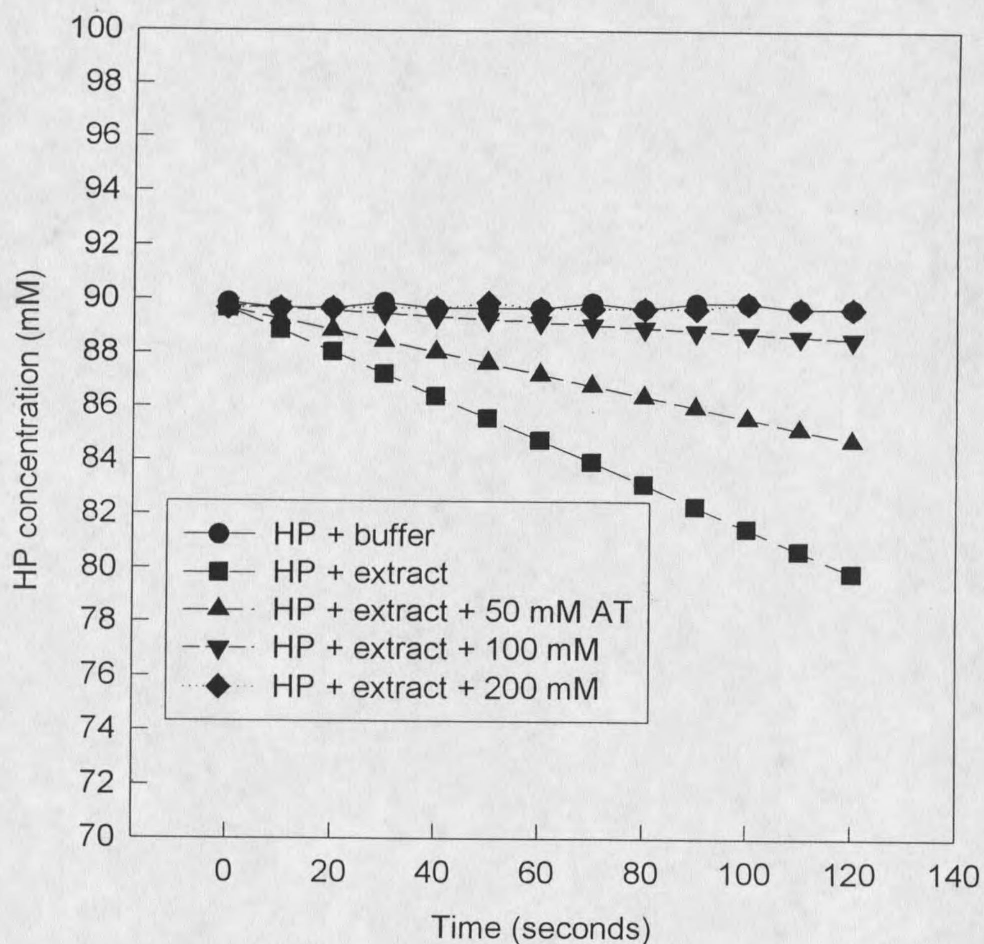


Figure 22. The effect of aminotriazole (AT) on the breakdown of hydrogen peroxide in the cell extract from biofilms. AT: aminotriazole; HP: hydrogen peroxide. The initial concentration of hydrogen peroxide was 0.088 M.

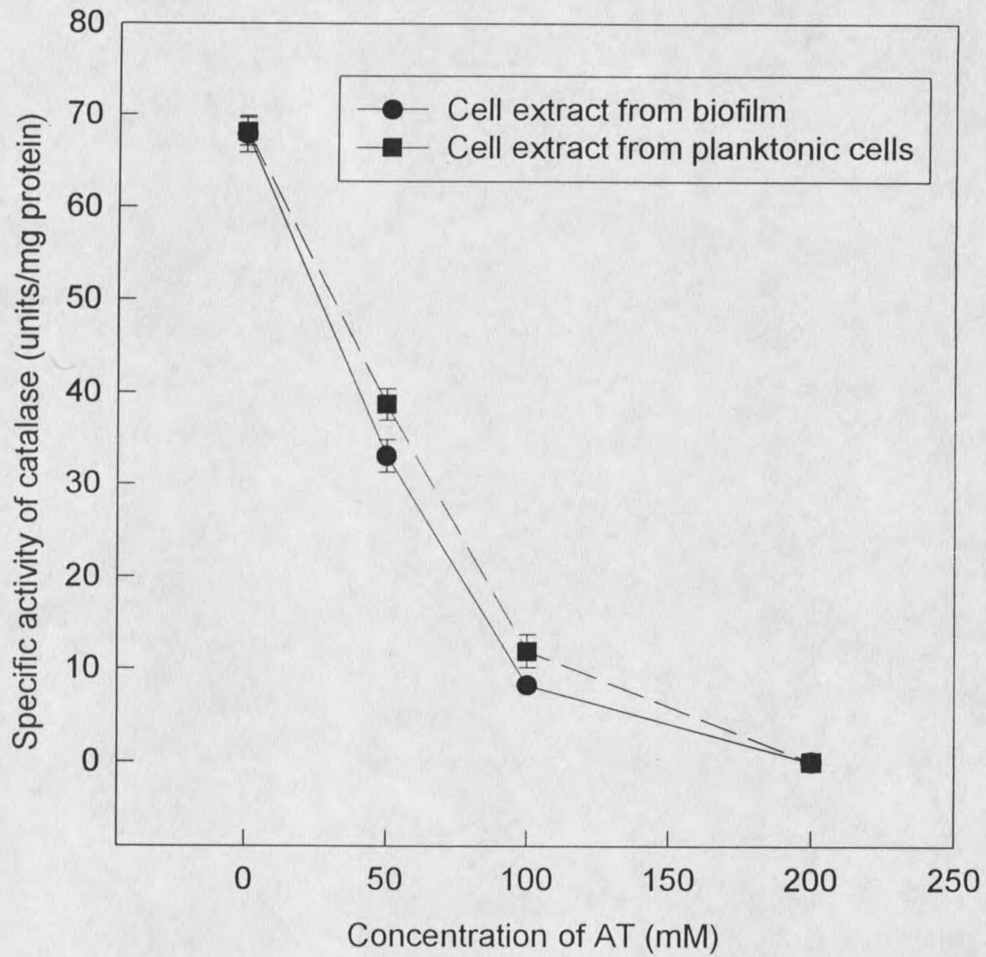


Figure 23. The decrease of specific catalase activity in the planktonic cells and in the biofilms in the presence of aminotriazole (AT). (n=3, Bars indicate standard errors.)

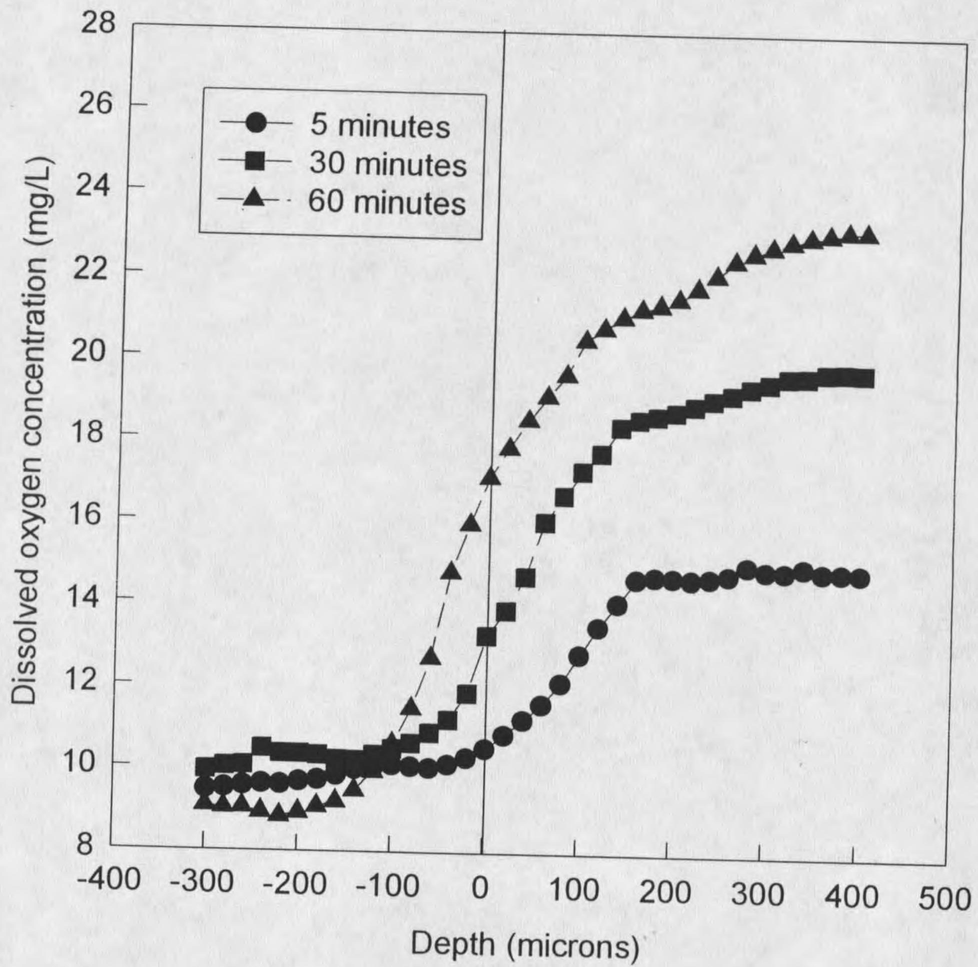


Figure 24. Dissolved oxygen concentration profiles in biofilm: with 3% hydrogen peroxide and 1 M 3-amino-1,2,4-triazole. Negative numbers on the x-axis represent the bulk fluid, and zero corresponds to the interface of bulk fluid and biofilm.

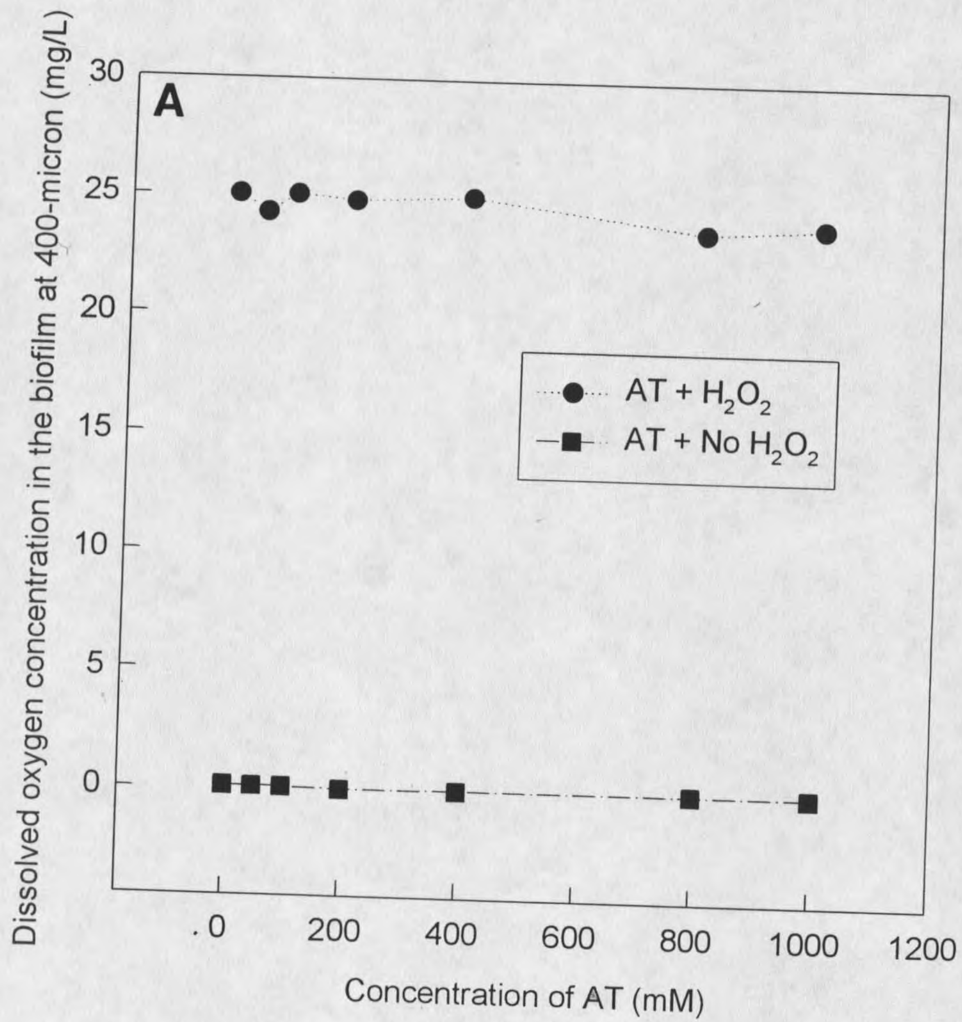


Figure 25. The effect of aminotriazole (AT) on the degradation of hydrogen peroxide to oxygen. (A) In the biofilm: the curves represent the concentration of dissolved oxygen at the bottom of 400 μm thickness biofilm using different concentrations of AT with or without hydrogen peroxide treatment. Incubation time was 60 minutes.

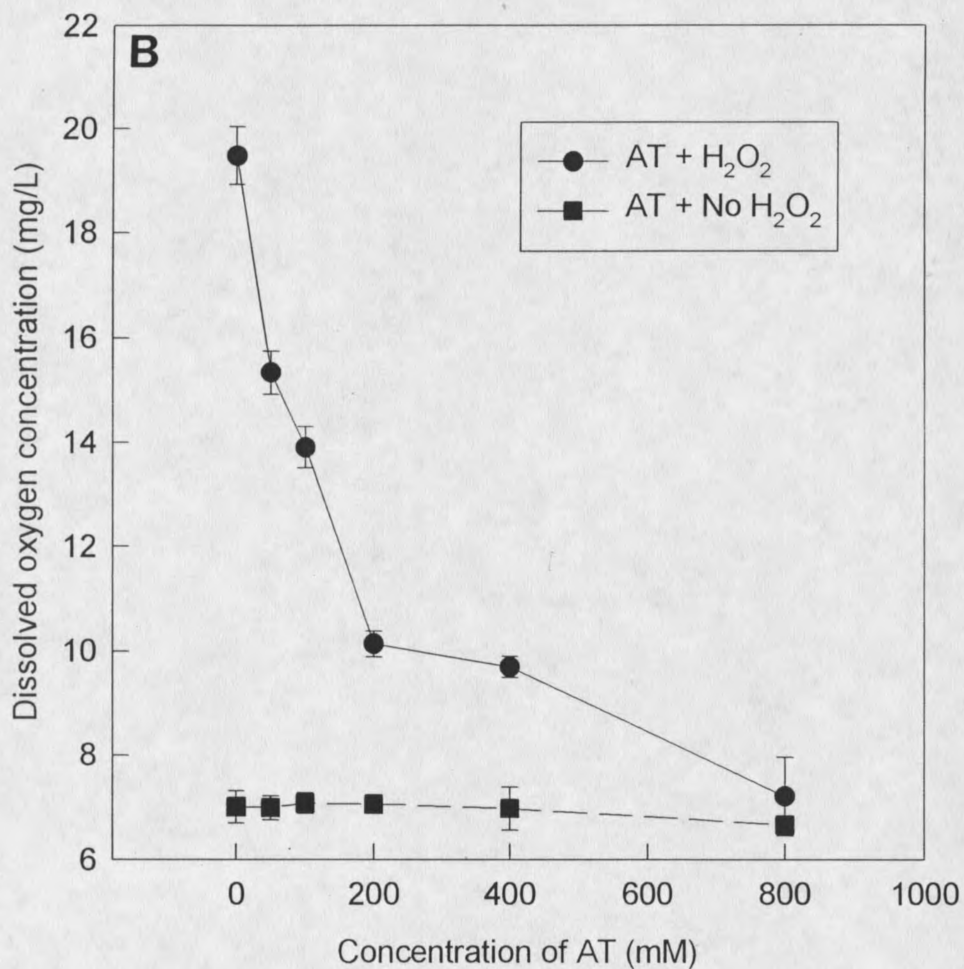


Figure 25 (cont'd). The effect of aminotriazole (AT) on the degradation of hydrogen peroxide to oxygen. (B) In the batch culture. the curves represent the concentration of dissolved oxygen in the batch cultures using different concentrations of AT with or without hydrogen peroxide treatment. (n=3. Bars indicate standard errors.)

The Change of the Cell Counts With Hydrogen Peroxide Treatment

The decreases in total cell direct counts and the conventional plate counts of attached cells during exposure to 0.3% hydrogen peroxide are shown in Figure 26. The initial total cell density on the slide before treatment was 8.85±0.07 log cells/cm^2. The mean concentrations of cultureable cells were: 8.41±0.03 log CFU/cm^2 for *K. pneumoniae*; 8.07±0.11 log CFU/cm^2 for *P. aeruginousa*; and 7.96±0.08 log CFU/cm^2 for *P.fluorecence*. Differences in the concentration of unexposed cell populations at time zero between total direct count and total culturable cells were not statistically significant ($P > 0.5$). After 2 hour treatment, only 0.02 log decrease in total cells direct count was observed, while there was a reduction in the mean count of culturable cells of 1.16 log for *K.pneumoniae*, 1.04 log for *P. aeruginousa*, and 1.09 log reduction for *P.fluorecence*. The difference between total cells and total culturable cells after a 2 hour disinfection was statistically significant ($P < 0.01$), thus showing the disinfection effect.

Effect of H_2O_2 on Respiratory Activity: Spatial distribution by CTC/DAPI Epiluorescence

In our experiments, CTC and DAPI were used to visually convey the effect of hydrogen peroxide on bacterial respiratory activity within biofilms. Respiring cells within biofilms reduced CTC to red CTC-formazan intracellular crystals. Both respiring and non-respiring cells all can stained green with DAPI. For respiring cells, a green background cover with red crystals can be observed. While non-respiring cells always stain green.

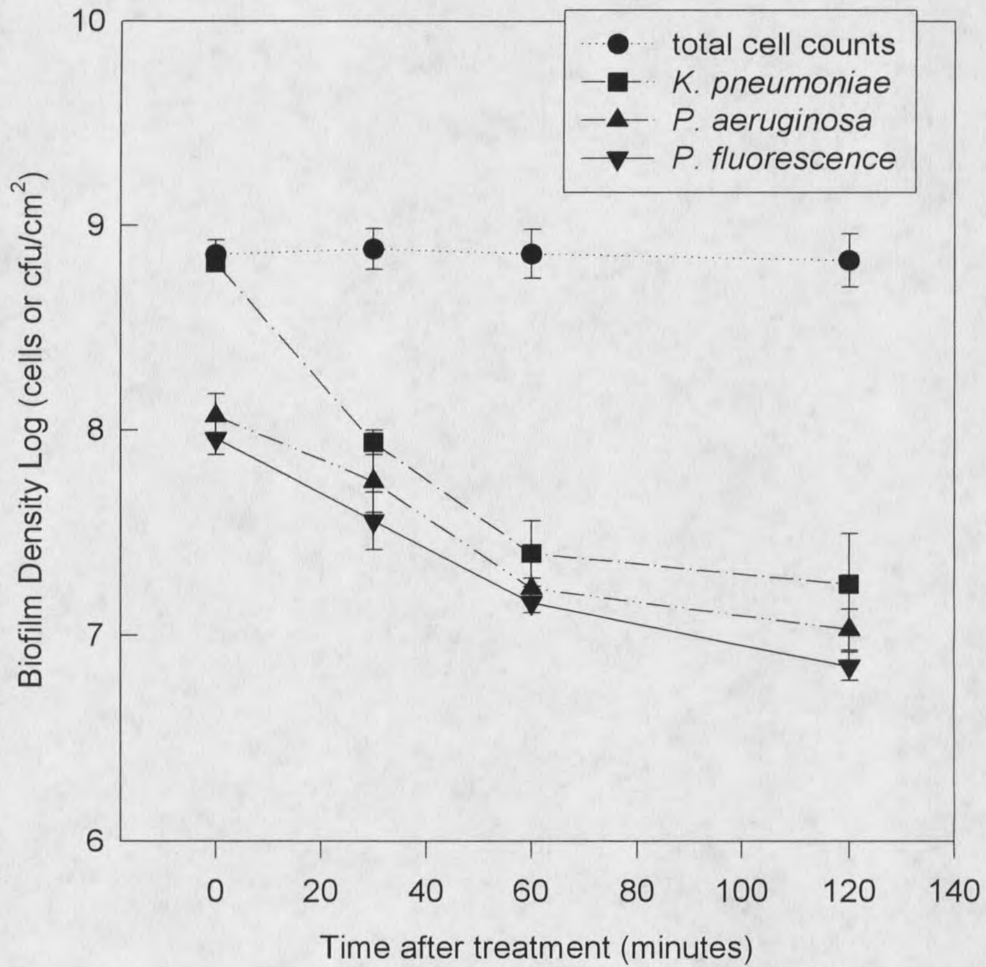


Figure 26. Cell count results of biofilms treated with 0.3% hydrogen peroxide.(n=3. Bars indicate standard errors)

By the cryosectioning technique, the spatial distribution of respiring and non-respiring cells within biofilms could be observed by epiflurescent microscopy.

Figure 27 illustrates representative patterns of respiratory activity within biofilms in response to treatment with 0.3% hydrogen peroxide. Respiring cells dominated most of the biofilms before treatment (Figure 27-A). Bacteria within the untreated biofilm appeared uniformly red at this stage because the CTC staining was more intense than the DAPI staining. As treatment proceeded, respiratory activity gradually decreased and the combination of stains yielded a yellowish color. Figure 27-B shows that nonrespiring (green) cells started appearing at the biofilm-bulk fluid interface after about 30 minutes of treatment. After about 60-minute treatment, nonrespiring cells constituted a greater fraction of the biofilm, and some of the biofilm biomass detached (Figure 27-C). At the end of experiment (2-hour treatment, Figure 27-D), a large portion of the biofilm showed non-respiratory activity (green stain). Note that the loss of respiratory activity in the biofilm was not spatially uniform. Most of the respiratory activity loss occurred near the biofilm-bulk fluid interface. A small portion of nonrespiring cells was also found near the substratum-biofilm interface.