To the Graduate Council:

I am submitting herewith a thesis written by Yingbo Guo entitled "Electrochemical Studies of Biofilm Detachment from the Gold Surface". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

James Q. Chambers

J. Q. Chambers, Major professor

We have read this thesis and
recommend its acceptance:

M J Sepaniak

John H Shibata

Accepted for the Council:

Lou Winkel

Vice Provost
and Dean of the Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a M. S. degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Yingbo Gu

Date Aug 15, 1991

ELECTROCHEMICAL STUDIES OF BIOFILM DETACHMENT FROM THE GOLD SURFACE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Yingbo Guo

December, 1991

DEDICATION

This thesis is dedicated to my parents

Mr. Wenyan Guo and Mrs. Baohua Hu

(郭文岩，胡宝华)

and my husband

Mr. Ziyang Zhang

(张子扬)

ACKNOWLEDGMENTS

I would like to take this opportunity to thank my professor, Dr. Chambers, for his guidance and patience during the process of this project and the preparation of this manuscript. I would also like to thank David Nivens for his valuable help over the past year. Additional thanks go to my friends, Gina Stanley, Beverly Swaile, and Tina Richey, for their kindness and help. Finally, I would like to express my special thanks to my husband, Ziyang, for his support and encouragement during my stay at UTK.

ABSTRACT

This research attempted to develop an effective method to detach a biofilm from a gold surface, so that repetitive measurements of the biomass can be made possible using a quartz crystal microbalance in an on-line configuration. This was an interdisciplinary effort involving both electrochemistry and microbiology.

The bacteria, *Caulobacter crescentus,* were used in the studies. They were allowed to grow in the growth media and to attach onto a gold surface. Both in situ and ex situ electrochemistry, including cyclic voltammetry (CV), controlled current and controlled potential electrolysis, were performed to detach the biofilm. According to an acridine orange direct counting technique using the fluorescence microscopy, continuous cyclic voltammetry during the growth period and ex situ electrolysis in 0.1 M NaOH solution after the growth period were found to effectively detach the biofilm. The surface population of bacteria generally decreased to 10 % of the control. As low as 0.5 % of the control population can be achieved. In situ CV, in situ electrolysis, and ex situ CV did not give consistent decrease of the surface population. Two mechanisms were proposed to account for these results.

It was concluded that, when combined with a quartz crystal microbalance in a suitable way, ex situ electrolysis in 0.1 M NaOH solution can detach the biofilm

effectively, so that continuous measurements of the surface biomass and bacterial regrowth were possible.

TABLE OF CONTENTS

CHAPTER PAGE

I. Introduction....1

A. Microbial growth....1

B. *Caulobacter crescentus*....5

C. Electrolysis: controlled potential and controlled current techniques....8

D. Cyclic voltammetry....11

E. Electrochemistry on gold electrode surface....12

F. Proposed research....17

II. Experimental....19

A. Electrochemical cell....20

B. Bacteria....23

C. Medium....23

D. Optical density measurements....24

E. Acridine orange direct counting (AODC)....25

F. Scanning electron microscopy....26

G. Sterilization....27

H. Procedure....27

III. Results and discussion....29

A. Background studies....29

B. The studies of bacteria detachment....33

(i). Continuous cyclic voltammetry studies....33

(ii). In situ cyclic voltammetry studies....38

(iii). In situ electrolysis studies....41

(iv). Ex situ CV studies....45

(v). Ex situ electrolysis studies....48

IV. Summary....53

List of references....54

Vita....58

LIST OF TABLES

TABLE PAGE

I. AODC results of bacteria surface population in the condition of continuous CV....35

II. AODC results of bacteria surface population in the condition of in situ CV....40

III. AODC results of bacteria surface population in the condition of in situ electrolysis....43

IV. AODC results of bacteria surface population in the condition of ex situ CV....46

V. AODC results of bacteria surface population in the condition of ex situ electrolysis....49

LIST OF FIGURES

FIGURE PAGE

1. Growth curves of (a) microbial cells and (b) biofilm....2

2. *Caulobacter crescentus* with stalks developed from the envelops....6

3. Current-potential curves at different times during (a) a bulk controlled potential electrolysis and (b) a bulk controlled current electrolysis....10

4. Cyclic voltammogram of an Au rotating disk electrode in 0.1 M NaOH....13

5. Cyclic voltammogram for glucose in air-saturated (A) 0.1 M NaOH, (B) 0.1 M phosphate buffer, pH 7.2, (C) 0.1 M acetate buffer, pH 4.8, and (D) 0.2 M nitric acid at rotating disk gold electrode....16

6. The schematic diagrams of the electrochemical cell and the flow system....21

7. CV of gold foil electrode in air-saturated 0.1 M NaOH solution....30

8. CV of a gold foil electrode in medium....31

9. Growth curve of *Caulobacter crescentus*....32

10. CV of a gold electrode at the first cycle
during continuous sweep.....34
11. AODC photographs
in the condition of continuous CV.....36
12. SEM photographs
in the condition of continuous CV.....37
13. CV of a gold electrode in the condition of in situ CV.....39
14. AODC photographs
in the condition of in situ CV.....42
15. AODC photographs
in the condition of in situ electrolysis.....44
16. AODC photographs
in the condition of ex situ CV in 0.1 M NaOH.....47
17. SEM photographs
in ex situ electrolysis in 0.1 M NaOH.....50
18. CV of a gold electrode in the condition of
ex situ electrolysis in 0.1 M NaOH.....51

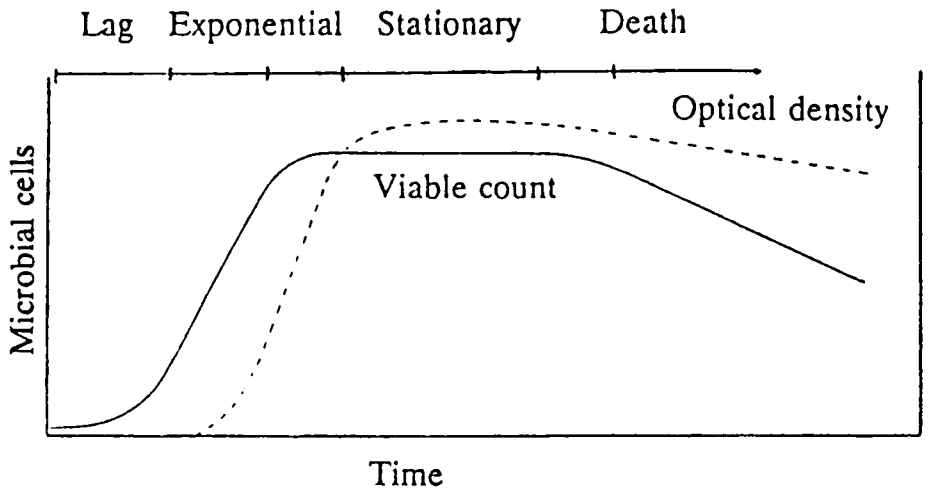
CHAPTER I

INTRODUCTION

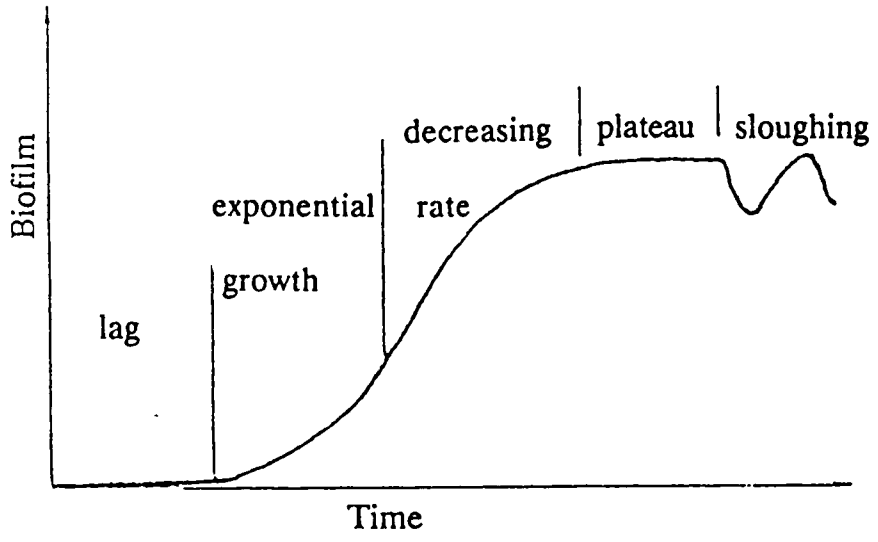
A. Microbial growth.

Although too small to be seen by the human eye, microorganisms play far more important roles in nature than their small sizes would suggest. They mineralize organic carbon, and convert nitrogen and sulfur containing compounds to CO_2, NH_3, NO_3^-, SO_4^{2-} and S^{2-}, so that these materials can be used cyclically for growth of higher plants and animals. On the other hand, some microorganisms are responsible for transmitting fatal diseases [1]. There are also many other things microorganisms can do. Although their existence was first accepted in 1677, microorganisms are recognized to a much better extent in this century, and the theory of microorganisms, i.e., microbiology, has achieved rapid progress, especially in the past two decades.

Aquatic and soil systems are two of the most important habitats for microorganisms. Provided the necessary nutrients, such as carbon, nitrogen, sometimes minerals, etc., and a suitable environment (i.e., temperature, pH, and osmotic pressure), microorganisms will grow in size and weight, and divide into new cells. This results in increased cell numbers, and the typical growth curve is shown in Fig.1a. [2]. The curve exhibits several distinct phases: lag, exponential (or log),



(a)



(b)

Fig. 1. Growth curves of (a) microbial cells, and (b) biofilm.

stationary, and death phases. The curve starts with the lag phase, corresponding to the period where no obvious growth happens for the newly inoculated bacteria. In the exponential phase, the number of cells increases by a constant factor during each time unit. In a closed culture vessel, a population can not grow forever at an exponential rate. Thus, in the stationary phase the growth no longer increases after a certain time. This could happen either due to exhaustion of a required nutrient or to accumulation of an inhibitory product, e.g., organic acids or alcohols. The cells may remain alive and continue to metabolize, but often they die. In the latter case, growth enters the death phase.

The above discussion is based on batch cultures, which means a fixed volume of a culture medium. Although cells in the exponential phase are suitable for some microbiologic studies, they grow in an ever-changing environment. In later stages of the growth progress, drastic changes often take place. Continuous-flow cultures, in a device called the chemostat, i.e., a flow system of constant volume to which medium is added continuously and from which any overflow is continuously removed, yield cells in truly steady-state growth [2]. Therefore, the growth-limiting concentration of a required nutrient determines the population density, while the rate of flow of the medium determines the rate of growth. Chemostats are widely used in microbiological studies.

Microorganisms, primarily bacteria, adhere to surfaces ranging from the human tooth and intestine to the metal surface of pipes exposed to untreated water. The films formed this way are called biofilms. Extensive work has been done during

the last 10 years to characterize both the structure and the dynamics of biofilms [3,4]. According to Bryers and Characklis [5], biofilm development is considered to be the net result of the following physical, chemical and biological processes: (1) adhesion of dissolved nutrients at the wetted surface; (2) transport of microbial particles to the surface; (3) microorganism adhesion to the surface; (4) biofilm production due to metabolism; (5) biofilm detachment. The microorganism adhesion step is generally viewed as a two-step process: reversible adsorption followed by irreversible attachment. These five processes could occur simultaneously, although certain steps may predominant over others at specific times.

Many bacteria living in aqueous environments are found to be sessile, growing preferentially attached to a solid surface. The reason is that in dilute nutrient solutions such as sea water, the nutrients concentrated by adsorption on solid surfaces enhances bacterial activity [4,6]. Noticeably, many bacteria adhere to surfaces so tenaciously that they are dislodged neither by washing with water nor by staining procedures. Biofilms serve beneficial purposes such as removing organic and inorganic "contaminants" from natural streams and in waste water treatment processes, just like the effect of their counterparts in free water. Biofilms are undesirable in other cases, for example, impeding heat tranfer across surfaces, increasing fluid frictional resistances at the surfaces, and most drastically, increasing the rate of corrosion at the surface [7].

B. *Caulobacter crescentus.*

Caulobacter crescentus, being one of the *Caulobacter* family, is present mainly in aquatic communities. These bacteria, which are important to carbon cycling in our environment, will readily absorb and metabolize organic substances, no matter how meager the supply [8]. In addition, they have attracted great attention for a long time as a model system for cell development because of their developmental peculiarity in microbiological studies [9,10]. A scanning electron micrograph of characteristic cells is shown in Fig. 2. [8]. The cells are curve-shaped, usually with at least one appendage, named the stalk, derived from the cell envelope. With the stalks, the cells can attach themselves to a solid surface by means of adhesive material present at the stalk tip. These materials are believed to be acidic polysaccharides [11].

Caulobacter crescentus possesses a relatively rapid growth rate compared with other members of the *Caulobacter* family. It exhibits a generation time in dilute media of 2 to 5 hrs [8]. For example, the generation time in a medium of 25 mg/L glucose is about 3 hrs [12].

Caulobacter crescentus firmly attaches itself through the stalk to solid surfaces such as small particles suspended in the media, glass slides or metal foils submerged in the media. A possible role for the stalk has been proposed to be a mechanism to remove the cell body from the crowd of microorganisms on the surface that formed the biofilm . In this way, the cell could access both the nutrient absorbed on the surface and that flowing past the surface, since much of the stalk and the cell body would float in the water. In the meantime, the cell would avoid the close contact with



Fig. 2. *Caulobacter crescentus* with stalks developed from the envelops.

its competitors within the biofilm for the limiting nutrients on the surface [8].

For general cultivation of bacteria, several factors should be taken into account: ingredients, aeration, pH and temperature.

The medium used should contain at least the following ingredients [8]: (1) phosphate as the pH buffer, as well as one important mineral nutrient; (2) a carbon source, almost always glucose, at a concentration of 0.2 % (1 mM); (3) trace amounts of metal ions, such as Mg^{2+}, Ca^{2+}, NH_4^+, etc. *Caulobacter crescentus* is sensitive to phosphate concentration; growth occurs at the maximal rate with 1 mM phosphate. Ammonia is the ideal nitrogen source; another choice can be an amino acid. Metal ions are sometimes supplied in the so-called mineral solutions, like Hunter's and Wolfe's solutions. Vitamin is beneficial, too. One thing interesting is that *Caulobacter crescentus* prefers nutrient-poor habitats to nutrient-rich ones. All nutrients seem to have low upper limits for uninhibited growth [8]. The reason for this is still not clear.

Caulobacter crescentus is generally cultivated aerobically and the growth rate decreases by limiting aeration. Oxygen-limiting cell division is frequently anomalous, giving rise to siblings of unequal size [13]. It was concluded that growth was dependent on oxygen respiration.

The growth of *Caulobacter crescentus* was found to be optimal at 25-30 ^{0}C [8]. The pH of the suitable medium is near neutrality, i.e., pH 6.8-7.2. This is consistent with the fact that *Caulobacter* family is usually found in nature within the pH range of about 5-9.

C. Electrolysis: controlled potential and controlled current techniques.

Bulk electrolysis generally utilizes electrodes with relatively large areas, and involves large currents and time scales on the order of minutes and hours. Hydrodynamic flow of electrolyte solution over the electrode surface is often involved, which gives the advantages of a quickly attained steady-state, a higher mass transfer rate, and minimized double-layer charging current [14].

Consider the redox reaction for an O/R couple:

$$O + n\,e \rightleftharpoons R \qquad (1)$$

If the kinetics of electron transfer is rapid, the concentration of the redox species in equilibrium at the electrode-solution interface is determined by the Nernst equation:

$$E = E^{0'} + (RT/nF) \ln (C_o / C_R) \qquad (2)$$

where $E^{0'}$ is the formal potential of O/R couple, R is the gas constant, T is the temperature, n is the number of electrons transferred, and C_o and C_R are the concentrations of O and R at the electrode surface, respectively.

In controlled potential techniques, the potential of the working electrode is maintained constant with respect to a reference electrode. It is usually the most desirable method for bulk electrolysis, since the potential of the working electrode basically controls the degree of an electrolytic process in most cases. A counter electrode is often supplied to provide a fairly uniform current distribution across the surface of the working electrode.

For a redox reaction with both O and R soluble and only the oxidant present

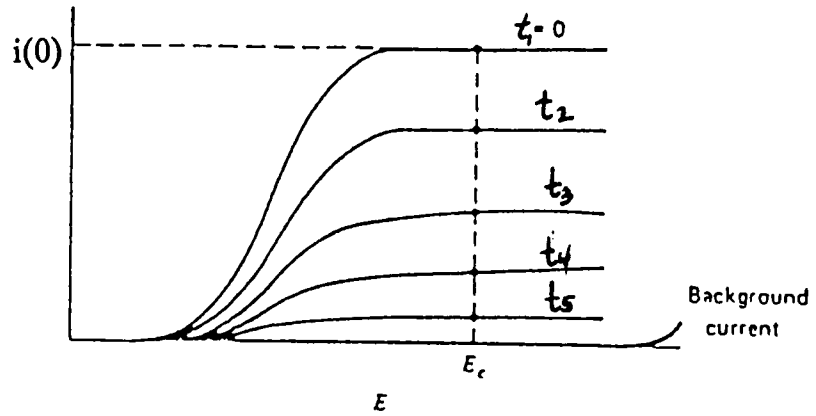
initially, the i-E behavior is governed by

$$E = E_{1/2} + (RT/nF) \ln \{(i_l - i)/i\} \quad (3)$$

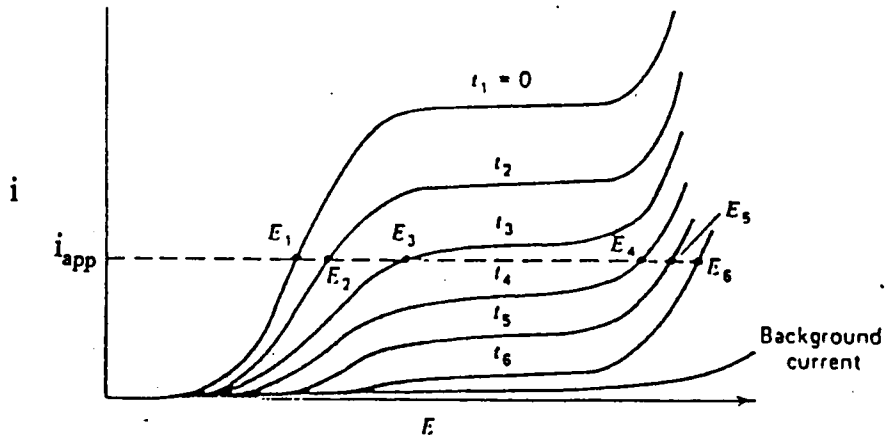
where i_l is the limiting current, and other symbols as defined above. This equation assumes steady-state conditions of uniform hydrodynamic flow to the electrode surface.

A characteristic i-E plot is shown in Fig. 3a [14]. It can be seen that the limiting current plateau decreases with time and eventually attains the background level. Because the current can be maintained at the limiting current value during the procedure, controlled potential electrolysis is a very efficient way to carry out a bulk electrolysis.

In controlled current electrolysis, the current passing through the cell is held constant. As long as the applied current i_{app} is smaller than the limiting current corresponding to the prevailing concentration of the oxidant, the electrode potential is governed by the redox reaction of O/R couple, and the reaction proceeds with 100% current efficiency (see Fig. 3b.). If the applied current is greater than the limiting current, the electrode potential is forced to shift to a more negative value until a second process occurs to help carry a portion of the current. The current efficiency then drops below 100%. The simplicity of the theory and the instrumentation is an important advantage of controlled current techniques, although it is more difficult to maintain 100 % current efficiency during controlled current electrolysis than during controlled potential electrolysis.



(a)



(b)

Fig. 3. Current-potential curves at different times during (a) a bulk controlled potential electrolysis, and (b) a bulk controlled current electrolysis.

D. Cyclic voltammetry.

Cyclic voltammetry is perhaps the most effective and versatile electroanalytical technique for the study of redox systems. The method enables a wide potential range to be rapidly scanned in search of redox couples. Once located, a couple can then be characterized from the potentials of peaks on the cyclic voltammogram and from changes caused by variation of the scan rate [15].

In cyclic voltammetry, the current, both cathodic and anodic, is followed during the complete excursions of the applied triangular potential sweep. For the reaction $O + n\,e \rightleftharpoons R$, if the potential scan begins at a value well positive of $E^{0'}$ for the reduction, only nonfaradaic currents flow for awhile. When the potential moves near $E^{0'}$, the reduction of O begins and the current starts to flow. As the potential becomes more negative, the surface concentration of O drops, hence the flux to the surface and the current increase. As the potential passes $E^{0'}$, the surface concentration drops to almost zero, mass transfer of O to the surface reaches a maximum rate, and then declines as the depletion effect sets in. Therefore a peak-shaped current-potential curve can be obtained. If the scan direction is reversed following the process, R becomes reoxidized and an anodic current flows, resulting in a similar shape for the i-E response as for the forward step.

Assuming semi-infinite, linear diffusion, a quiet solution, and rapid electron transfer compared with mass transfer, the peak current can be expressed as

$$i_p = 0.4463\ nFAC_o^* (nF/RT)^{1/2} v^{1/2} D_o^{1/2} \qquad (4)$$

where C_o^* is the bulk concentration of species O, v is the scan rate, D_o is the

diffusion coefficient, and others as defined above.

The theory of cyclic voltammetry has been extended to quasi-reversible and totally irreversible systems, multicomponent systems and multistep charge transfers, electrode reaction with coupled chemical reactions [14]. When coupled with other techniques, such as rotating disk electrode studies, it is an extremely powerful tool in electrochemistry.

E. Electrochemistry on gold electrode surface.

A great deal is known about the surface chemistry of gold electrodes [16,17,18]. The important properties of gold electrodes that have led to an increased interest in their use for anodic oxidations in recent years include the following: (1) inertness at anodic potential; (2) wide working range; (3) ability to chemisorb intermediate species and thereby catalyze electrode processes; (4) facile oxide stripping process. Gold electrodes can be used in electrocatalysis as applied to fuel cell processes and electroorganic oxidation reaction in general. They have also found important applications as the working electrodes employed in pulsed electrochemical detectors for liquid chromatography [19,20].

The standard potential of Au^{+}/Au and Au^{3+}/Au couples are 1.68 V and 1.29 V vs. NHE at 25 ^{0}C, respectively. A typical cyclic voltammogram in 0.1 M sodium hydroxide solution is shown in Fig. 4. [19]. The peak at ca. 0.3 V (A) is due to the formation of surface oxide (positive scan). The intermediate in the oxide formation, i.e., AuOH, has been implicated as a reactive species in the mechanism of oxygen

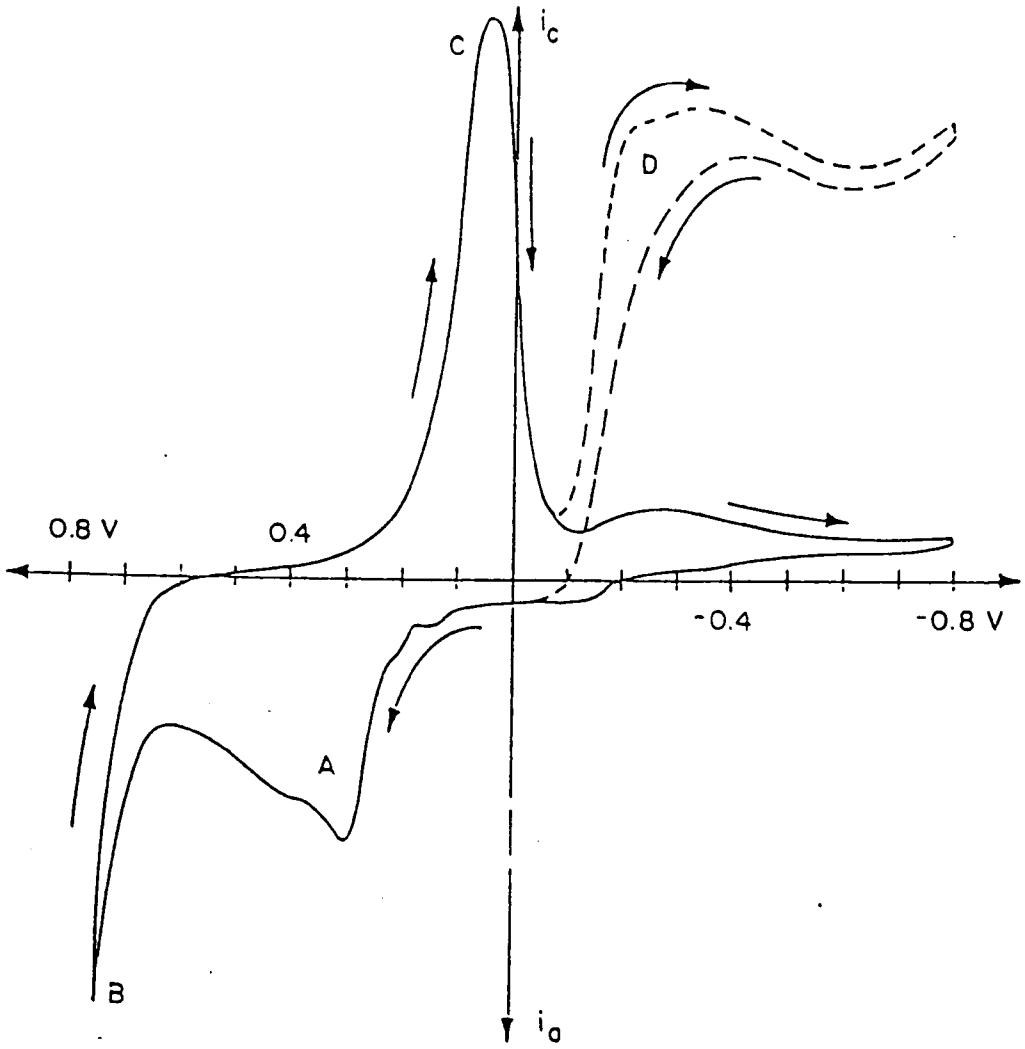


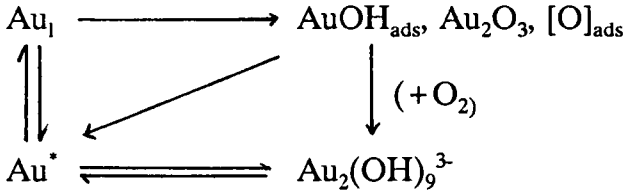
Fig. 4. Cyclic voltammogram of an Au rotating disk electrode in 0.1 M NaOH.

Conditions: 200 rev/min, 100 mV/s scan rate.

transfer from H_2O to various organic substrates. The final stable oxide, hydrous gold oxide, can be cathodically dissolved by a negative potential scan. The peak at ca. 0.1 V (C) corresponds to this process. The response beyond ca. 0.6 V (B) is due to the evolution of oxygen. The plateau current (D) is due to the reduction of oxygen in an air-saturated solution. Similar behavior is found in 1 M H_2SO_4, where the oxide is formed at a potential approximately 60 mV x 13 = 780 mV more positive than in base [21].

Spectroscopic studies of single-crystal Au electrodes have clearly demonstrated the existence of adsorbed hydroxyl radicals (AuOH) in alkaline solutions at potential values considerably below the onset of anodic formation of the "normal" phase oxide on Au surface [22]. The anodic formation of gold oxide on Au electrodes in acidic solutions also has been shown to proceed via an AuOH intermediate [23,24,25]. Voltammetric evidence for the formation of AuOH is usually concluded to be a shoulder on the anodic wave for gold oxide formation during the positive scan [26]. The adsorbed hydroxyl radicals might be present initially at unusual surface micro structures, such as Au adatoms, grain boundaries, or steps in a surface plane.

A detailed reaction scheme at the gold surface was proposed by Burke et al. [27] as follows,



where Au^* is an adatom present at equilibrium on Au surface. The subscript ads

defines those species as adsorbed at the surface.

The catalytic oxidation of glucose at a gold electrode was studied by Johnson et al. [22,28,29]. Typical cyclic voltammograms are given in Fig. 5. [29]. The mechanism of glucose oxidation in alkaline solution is about the same as proposed before. It was suggested by Johnson that in low capacity buffers of acidic and neutral pH values, AuOH could be generated electrochemically within the diffusion layer by cathodic stripping of surface oxide and reduction of dissolved oxygen. Glucose is oxidized to the corresponding carboxylic acid first, followed by the cleavage of the C_1-C_2 bond, and the conversion of the C_2 and C_6 to the corresponding carboxylates.

Besides glucose, other organic compounds, such as alcohols, alkanolamines, amino acids, and sulfur compounds, can be oxidized at gold electrode in 0.1 M NaOH solution with similar mechanisms. Amino acids, the fundamental blocks of proteins, are the main composition of the bacteria cell envelop.

Upon applying a positive potential excursion which causes the formation of surface oxide followed by a negative potential excursion to strip the oxide formed, a "clean" Au surface can be restored, and hence the catalytic reactivity of Au surface can be regained. There are some organic compounds that are not electroactive at the oxide-free Au surface but are adsorbed on the surface. These compounds can also be oxidatively desorbed simultaneously with the oxide formation process to give useful anodic signals [19].

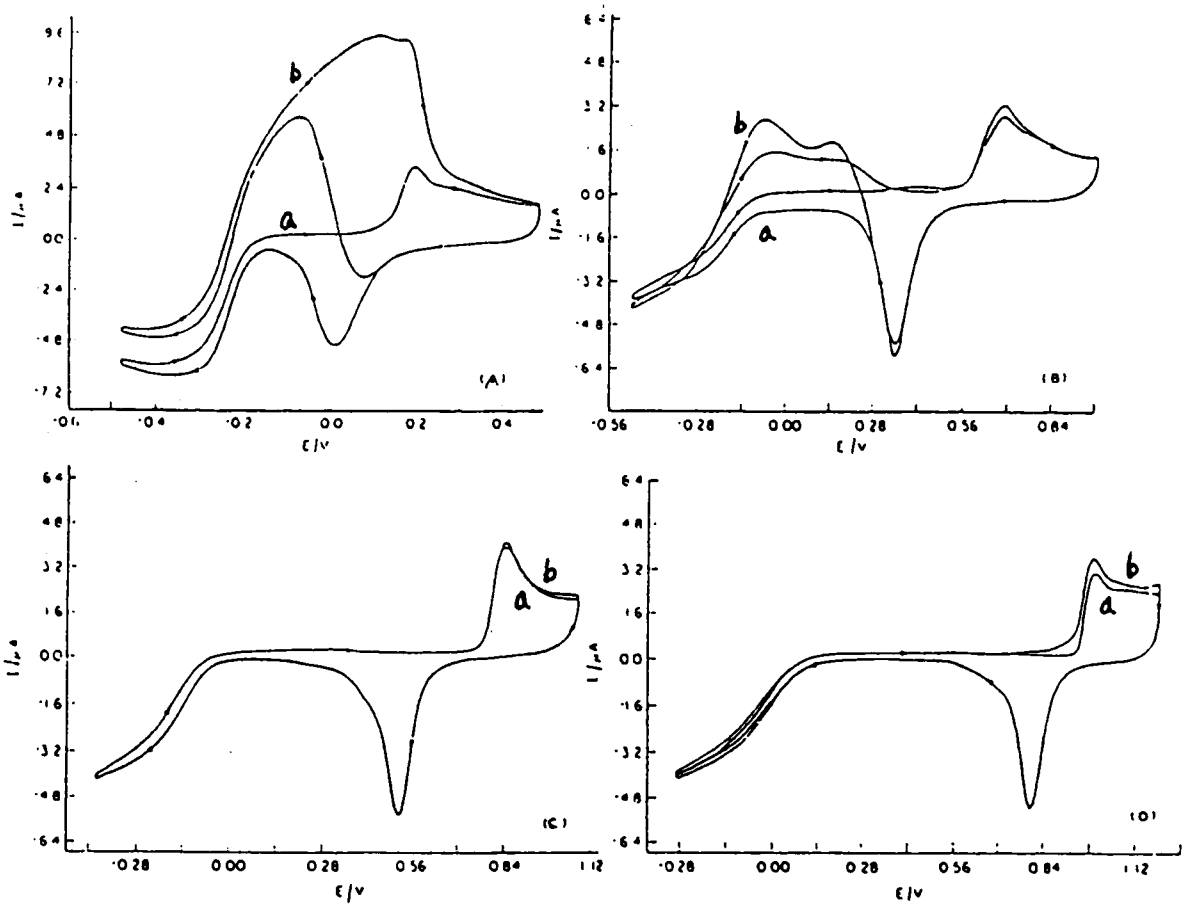


Fig. 5. Cyclic voltammogram for glucose in air-saturated (A) 0.1 M NaOH, (B) 0.1 M phosphate buffer, pH 7.2, (C) 0.1 M acetate buffer, pH 4.8, and (D) 0.2 M nitric acid at rotating disk gold electrode. ω = 94.2 rad/s, v = 0.1 V/s, A_d = 0.008 cm^2. (a) residual response; (b) [glucose]/mM: (A) 0.25; (B), (C), (D) 10.0. See the work of Johnson et al. in ref. [29].

F. Proposed research.

D. E. Nivens et al. have proposed the use of the quartz crystal microbalance (QCM) to monitor the attachment of *Caulobacter crescentus* onto a gold surface as an on-line, nondestructive biomass measurement [30]. Based on the piezoelectric effect of the crystal, QCM measurement correlates the mass deposition on the surface to the frequency change of the crystal with the use of Sauerbrey equation. When a living biofilm is present on the crystal surface, a good sensitivity, i.e., 2×10^4 organisms per cm^2 is realized. The method has been found to be accurate, assuming that the mass of the biofilm is not more than two percent of the mass of the crystal and that the biofilm behaves as a rigid layer. At a certain point when the biofilm gets too thick, however, these two conditions no longer fit and the Sauerbrey equation fails to give good quantitative results. Then it is necessary to detach the biofilm so that repetitive measurements can be performed in an on-line configuration.

A principal goal of the research project described in this thesis was to discover an in situ electrochemical protocol to remove a biofilm attached to the gold QCM surface. Electrooxidation was proposed for the oxidative cleaning of living microbes on the surface for several reasons. (1) Bacteria grow indiscriminately on the quartz and the gold surface [30]. (2) A gold surface can be made catalytic for the oxidation of a variety of organic substrates including carbohydrates and sugars, and may lead to multielectron oxidations and partial oxidation to CO_2 [19]. Since bacteria attach to the surface via structural organelles such as polysaccharides, it is likely that these units can be oxidized under the same conditions. (3) The solution conditions used in

oxidation studies on Au surface can match those in which bacteria grow and form the biofilm. This is an interdisciplinary effort involving both electrochemistry and microbiology.

The detailed experimental, results and discussion are presented in the following chapters.

CHAPTER II

EXPERIMENTAL

The experiments described below were designed to grow biofilms on gold electrodes that could be subjected to various electrochemical protocols.Generally two modes of operations were followed: (1) continuous electrolysis during biofilm growth, and (2) electrolysis after biofilm growth, both in situ in the growth medium and ex situ in less complex electrolyte solutions. Biofilm growth generally took 2-3 days. Biofilm population on control and electrochemically treated electrodes were assayed by fluorescence microscopy and scanning electron microscopy techniques.

Initially, the gold working electrode was fabricated as a patterned gold thin film on microscope slides, which were compatible with the direct counting microscopy technique. Gold was sputtered using an electron beam sputtering instrument onto the chromium thin film, which served as the underlayer to make gold attach better than directly onto the glass slide. The thickness of gold was 1000 Angstroms and that of chromium was 40 Angstroms. The patterned electrodes resembled the QCM electrical contacts, that is, the gold attached to the microscope slide in the same way as it did to the quartz crystal and in about the same pattern. Unfortunately, the gold thin films just barely attached on the glass slide, even though the underlayer was

used, and lifted off while immersed in the solution. Subsequently, gold foil electrodes were used for the bacteria attachment studies. They proved to be the right choice for the study, because they were easy to handle and could be used repetitively.

A. Electrochemical cell.

A tall one-liter flask was used as the electrochemical cell for the following experiments. It was sealed with a rubber stopper, and covered with aluminum foil to prevent contamination. The rubber stopper contained different ports for the medium inlet drip tube, outlet line and the ventilation tube, so that a flow system could be established throughout the experiment. A Masterflex pump (1-100 RPM) was used to send the fresh medium into and get the waste out of the flask. A schematic diagram of the experiment set-up is shown in Fig. 6. In addition, the rubber stopper contained ports for the counter electrode, the lead of the working electrode, and a bridge tube. The bridge tube was used to connect the reference electrode to the bulk medium. It was filled with saturated potassium chloride, and had a vicor frit (from EG & G Princeton Applied Research) at its tip to make a separate compartment, electrochemically and microbiologically. One working electrode (Au foil, 1.6cm^2), spot welded to a tungsten wire, was led out of the flask through the stopper, so that continuous voltammetric measurements could be possible during the experiment. The working volume of the flask was 500 mL, and the flow rate was 0.33 h^{-1}. The rate was chosen to allow wash out of most of the bulk phase bacteria, and formation of the biofilm as a monolayer.

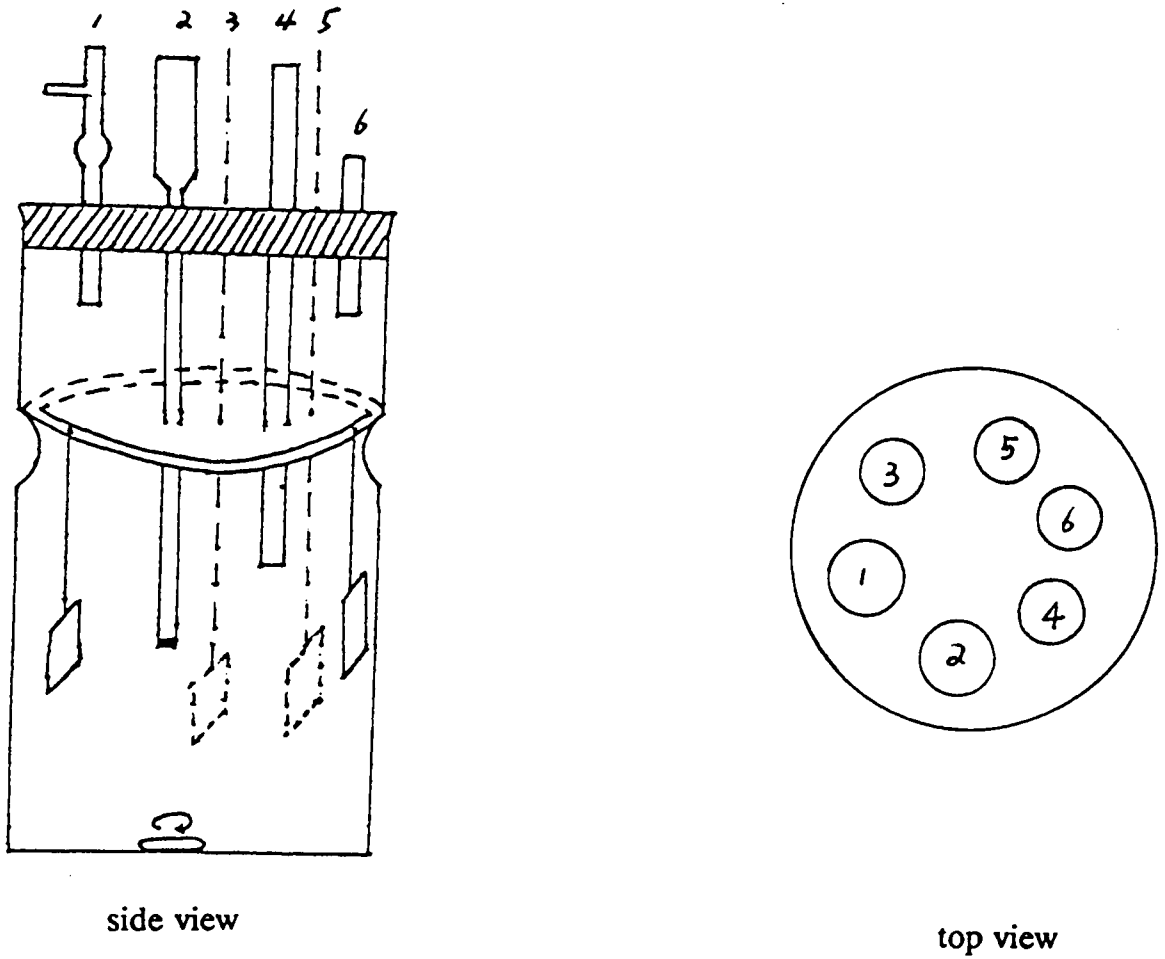


Fig. 6. The schematic diagrams of the electrochemical cell and the flow system.

a. The electrochemical cell: (1) inlet drip tube; (2) ref. electrode; (3) working electrode; (4) outlet tube; (5) counter electrode; (6) ventilation tube.

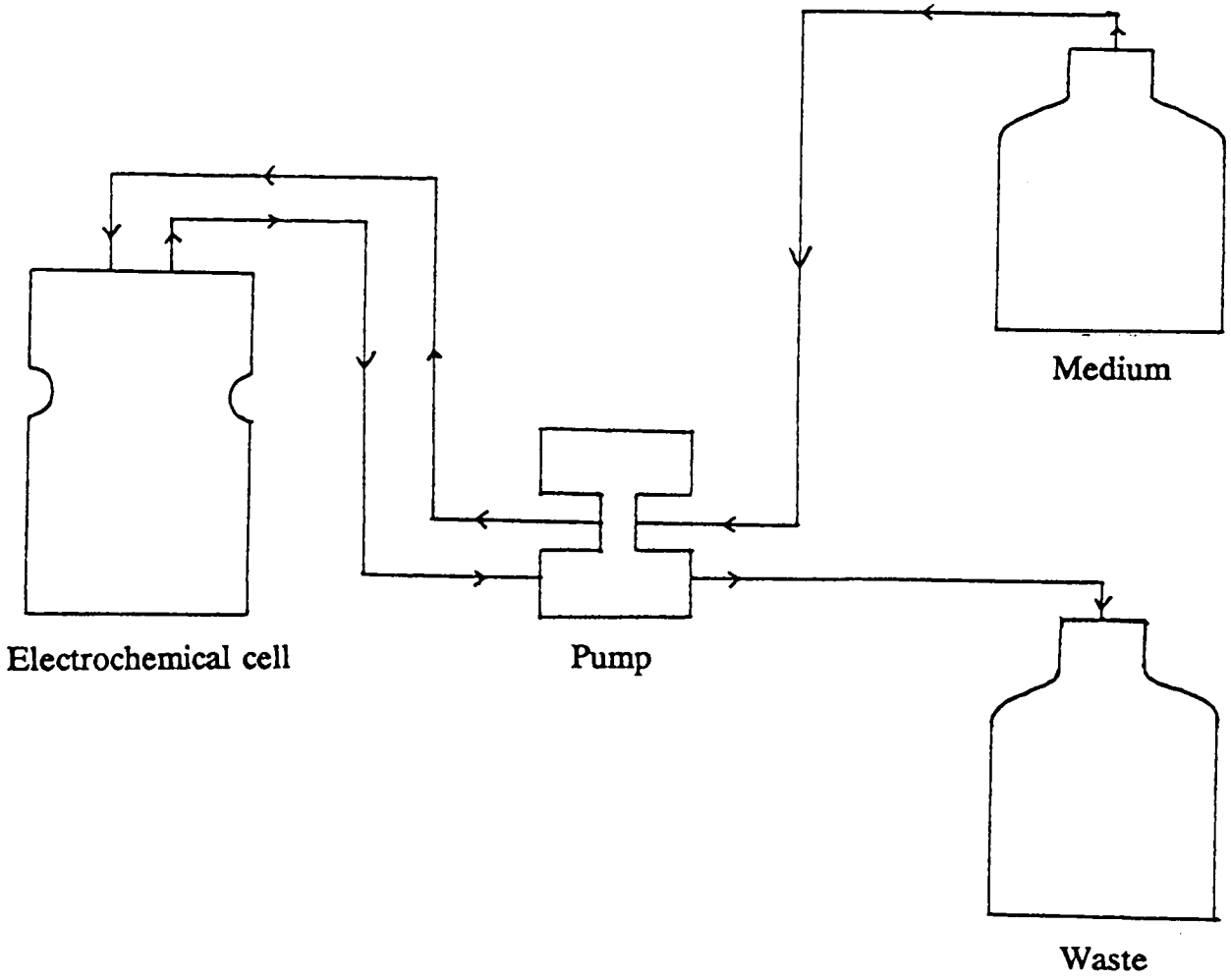


Fig. 6. (continued). b. The flow system.

Each individual gold electrode was a piece of Au foil (0.1 mm thick, 1.6 cm^2, 99.9 %, Aldrich), spot welded to a gold wire (0.5 mm diameter, 4 cm long, 99.9 %, Aldrich), and suspended on a plastic O-ring in the flask. Usually 5-6 working electrodes were placed onto the O-ring at the same time so that different electrochemical treatments could be performed in situ in the growth medium. A piece of platinum foil (2 cm^2), spot welded to a tungsten wire, was used as the counter electrode. A commercial saturated calomel electrode (SCE) was the reference electrode.

A multi-purpose electrochemical analyzer (BAS-100) was employed for cyclic voltammetry studies; and a PAR-173 potentialstat/galvanostat was used for electrolysis studies.

B. Bacteria.

The bacteria used in this study were *Caulobacter crescentus*. They were obtained from the culture collection in the laboratory of the Institute of Applied Microbiology (IAM) at Knoxville, TN. They were chosen because they had been well characterized at IAM (see Chapter IB).

C. Medium.

The medium used in this study contained (per liter of deionized water from Milli-Q system, Millipore) ingredients as follows: 175 mg/L KH_2PO_4, 25 mg/L glucose, 6.25 mg/L NH_4Cl, 1.25 mg/L $CaCl_2$, 6.25 mg/L $MgSO_4$, and 0.25 mL/L

Wolfe's mineral solution. Potassium phosphate and glucose were analytical grade, whereas the rest were laboratory grade. The Wolfe's solution was obtained from IAM, which contained trace amounts of Mg, Mn, Na, Fe, Co, Ca, Zn, Cu, B, Mo, Se and nitrilotriacetic acid (NTA), pH 6.5. Each time 12 L of the medium was prepared for the experiments.

The 0.05 M phosphate buffer solution contained (per liter of deionized water as mentioned above) 6.8 g KH_2PO_4 and 8.7 g K_2HPO_4. The solution was filter sterilized prior to use (detailed later).

The pH of the medium and the buffer solution was adjusted to pH 7.2 with NaOH, using a glass electrode and an ORION model SA 720 pH meter.

D. Optical density measurements.

When bacteria start to grow, they tend to absorb light in the visible region. The greater the bacteria population, the higher the absorbance. Turbidity can be observed at high absorbance, e.g., absorbance higher than 0.4. This is used as an indirect way to monitor microbial growth.

There were two kinds of growth media used in this part: (1) rich medium of glucose: 500 mg/L glucose, 175 mg/L KH_2PO_4, 125 mg/L NH_4Cl, 25 mg/L $CaCl_2$, 125 mg/L $MgSO_4$, and 5 mL/L Wolfe's solution; (2) rich medium of yeast extract/proteose peptone: 1 g/L yeast extract, 2 g/L proteose peptone, and 0.2 g/L $MgSO_4$. Each time 100 mL of the medium was made. Rich growth media were used instead of the general medium mentioned in the previous part because they were

dense enough to grow the bacteria to a population sufficient to obtain a detectable optical response.

Two 300 mL optical flasks were used for each kind of medium. They were sterilized together with 100 mL medium in each of them prior to use (detailed later). *Caulobacter crescentus* was inoculated into one flask, while the other one was kept in sterile condition. Both flasks were maintained at 25 ^{0}C in a water bath. The optical absorbance was measured every 3 hrs for 2 days at the wavelength of 660 nm on a Baush and Lomb Spec 20 spectrophotometer.

E. Acridine orange direct counting (AODC).

Acridine orange, which is 3,6- bis(dimethylamino)acridine zinc chloride double salt, is a fluorescent dye used to stain microbial cells and increase their contrast to the background. When stained with acridine orange, *Caulobacter crescentus* can be visualized in the field of a low-power microscope. This results from dye molecules that intercalate into the DNA helix of the microbial cells and become intensely fluorescent. The microbial population can be measured this way by counting the number of individual cells with a fluorescence microscope[31].

In the following study, each sample was removed from the electrochemical cell at the end of the experiment, and fixed with 3 % formaldehyde solution, so that the cells stopped growing. Then the sample was stained with 0.01 % acridine orange, and rinsed with 0.05 M buffer solution to get rid of any excess stain. The cells were counted directly off the Au surface. Usually each sample was counted at 10 -15 fields

under the fluorescence microscope (Zeiss, West Germany), the average was taken and multiplied by a factor of 10^4 (the factor was calculated from the parameters of the microscope), to give the cell population per cm^2. Photographs were taken using Kodak Ektar 1000 film.

F. Scanning electron microscopy.

Scanning electron microscopy (SEM) is a highly developed art, providing a greatly increased resolution compared with conventional microscopy. The material to be studied is coated with a thin film of heavy metal such as gold. The electron beam is directed down on the specimen and scanned back and forth across it. Electrons scattered by the heavy metal are collected and activate a viewing screen to produce an image, and the surface of the object can be visualized [2]. SEM in this research was performed at the Department of Microscopy, UTK.

The gold electrodes for SEM studies were removed from the electrochemical cell after the experiment, fixed with 2.5 % glutaraldehyde, and rinsed with filter sterilized buffer solution. Samples were then dehydrated through an acetone series (25, 50, 75, 95, and 100 %) at 15 minute intervals, followed by specimen critical point drying in liquid carbon dioxide with a Ladd critical point dryer #28000. Then samples were mounted onto aluminum specimen mounts with silver paint, and coated with approximately 100 Angstroms palladium-gold alloy in Denton vacuum evaporator, DV-515. After these procedures, the electrodes were observed in an ETEC autoscan scanning electron microscope. Photographs were taken using type 55

P/N polaroid film.

G. Sterilization.

Sterilization is a necessary procedure to kill all the bacteria prior to inoculation of any specific kind of bacterium, so that no other type of microbial cells can grow during the experiment period except the one of interest, i.e., no "contamination" in terms of microbiology. All components of the electrochemical cell and the medium supply apparatus were carefully sterilized before each experiment.

Two sterilization methods were employed. (1) Sterilization in a AMSCO autoclave at 250 ^{0}F (121 ^{0}C) under 20 LBS per square inch for 20 min was used for the electrochemical cell, the Masterflex tubing, and the inlet and outlet reservoirs. (2) The medium and the buffer solution were filter sterilized, using Gelmen Acro 50 A filters with 0.2 micron pores to prevent bacteria from entering the reaction system. The effective diameter of common microbes is in the range of 0.5-10 micron.

H. Procedure.

The electrochemical cell was prepared so that it contained a Teflon magnetic stirring bar, the O-ring to hold working electrodes, and the rubber stopper as described before. One working electrode served as a control to monitor the bacteria growth, and no electrochemistry was performed on it. All the other working electrodes were compared with the control in a given experiment. The electrochemical cell, the Masterflex tubing, the inlet and outlet reservoirs, and the

medium were sterilized as mentioned above. *Caulobacter crescentus* was inoculated from the solid media obtained from IAM into the starter culture (same concentration as the medium). This treatment can minimize or even eliminate the lag phase of bacteria growth (Fig. 1a.) to produce a more efficient biofilm formation. The starter culture was kept in a water bath shaker at 25 ^{0}C for 36 hrs to allow the bacteria to develop, and then it was inoculated into the electrochemical cell in a 1:100 volume ratio, i.e., 5 mL starter culture. It was maintained in a batch culture mode for 12 hrs (i.e., no medium flow) with effective stirring. This permitted the bacteria to grow and to develop their population to a certain size without being washed out at early stages, otherwise there would not be enough population for a good biofilm to be seen. The medium then started to flow into and out of the flask for 50-60 hrs with stirring on. During this time(12 hrs in batch and 50-60 hrs in chemostat), the bacteria grew and attached to the Au foil surfaces.

Cyclic voltammetry and electrolysis studies were then conducted, followed by AODC or SEM. Photographs were taken at that time. After each experiment was done, Au foils were immersed in concentrated nitric acid for 5 minutes, rinsed with distilled water, and sterilized in the autoclave. They were then ready for the next experiment.

CHAPTER III

RESULTS AND DISCUSSION

A. Background studies.

The cyclic voltammogram of a gold foil electrode in 0.1 M NaOH is shown in Fig. 7. The potential region was from +0.7 V to -1.0 V, and the scan rate was 0.1 V/sec. It resembles the one from the literature (see Fig. 4). The peak at ca. - 0.2 V was assigned to be oxygen reduction, since the solution was air saturated. The cyclic voltammogram in the growth medium is shown in Fig. 8, which looks somewhat different from that in the literature (see Fig. 5). The potential region was + 1.2 V to 0.6 V with the scan rate of 0.1 V/sec. The principal difference between conditions for the literature CVs and the CV in Fig. 8 is the solution composition: 0.14 mM glucose in the medium compared to 10 mM in the literature, and the addition of necessary electrolytes and minerals. In the CV of Fig. 8, the oxidative formation and stripping peaks of the gold oxide could be seen easily, along with a cathodic wave due to O_2 reduction. The successive addition of medium ingredients did not affect the CV very much.

The growth curve of *Caulobacter crescentus* is shown in Fig. 9, which followed the general pattern of growth phases of microbial cells. Yeast extract is another type

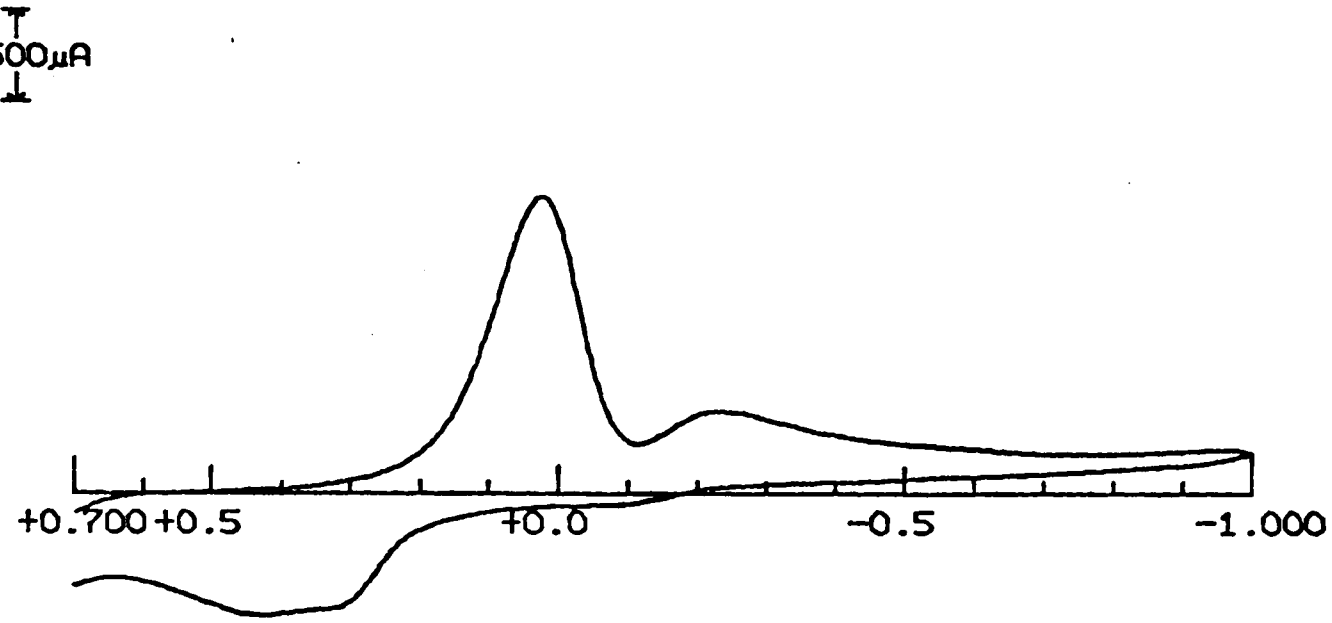


Fig. 7. CV of gold foil electrode in air-saturated 0.1 M NaOH solution.

No stirring during the procedure. SCE was the ref. electrode.

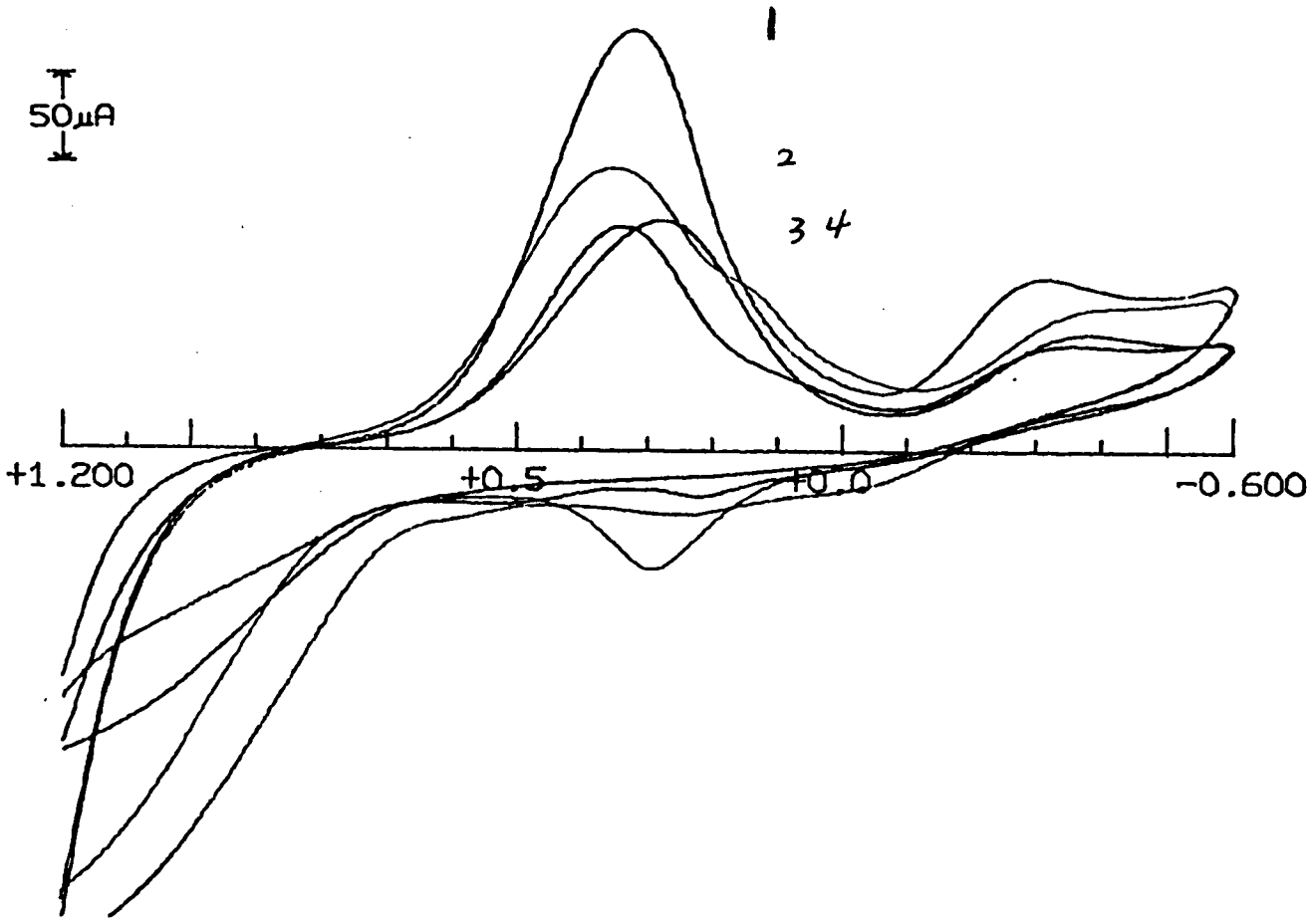


Fig. 8. CV of a gold foil electrode in medium. (1) KH_2PO_4; (2) KH_2PO_4 + Mg + Ca + NH_4; (3) KH_2PO_4 + Mg + Ca + NH_4 + glucose; (4) KH_2PO_4 + Mg + Ca + NH_4 + glucose + Wolfe's solution.

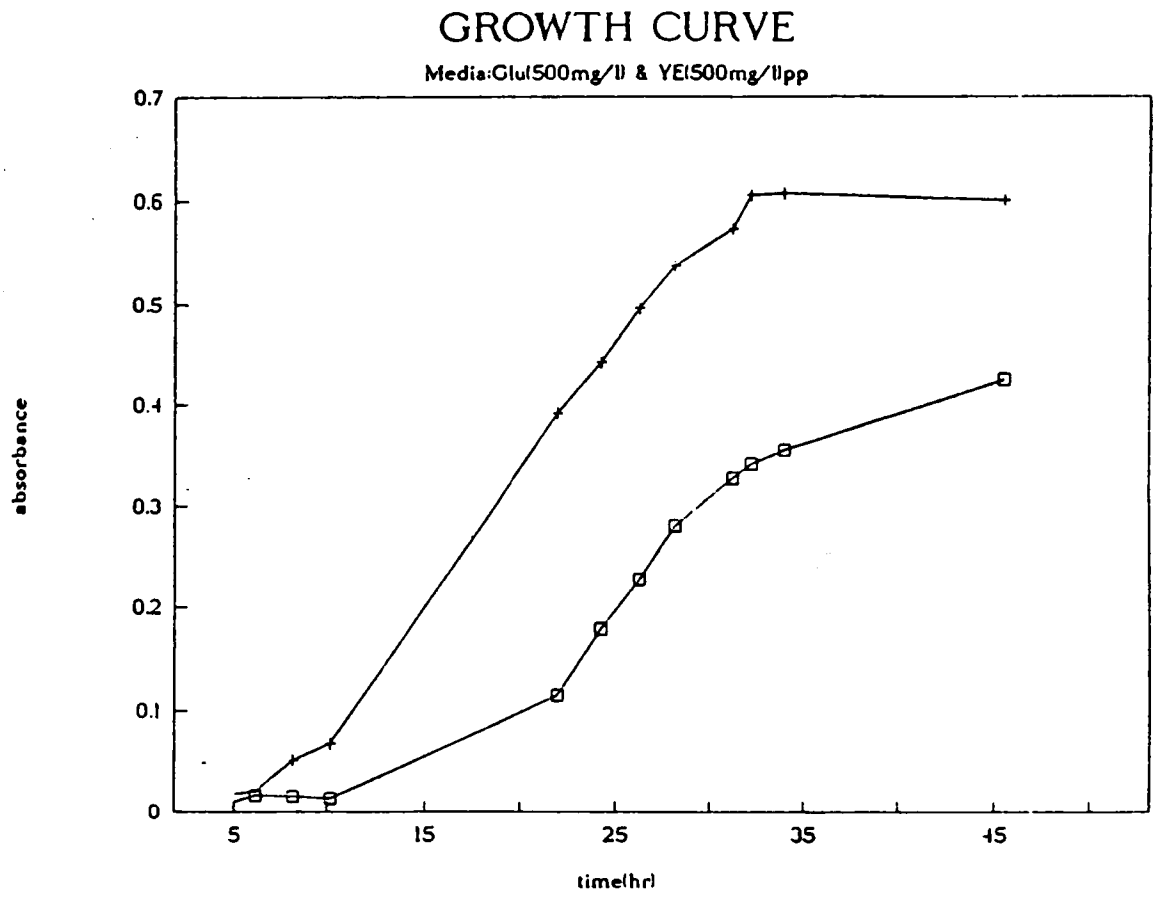


Fig. 9. Growth curve of *Caulobacter crescentus*. Upper: glucose; lower: YEPP (see text).

of medium suitable for the growth of the bacteria. This can be seen from the growth curve, since it gives good optical absorbance, i.e., good bacteria population.

B. The studies of bacteria detachment.

(i). Continuous cyclic voltammetry studies.

One of the gold electrodes was subjected to continuous triangular cyclic potential sweep in the potential region of + 1.0 V to - 0.8 V vs. SCE at the scan rate of 2 or 5 mV/sec, with stirring during the entire period of biofilm growth.. One scan cycle is shown in Fig. 10. The bacteria surface population was measured using AODC, and the results are shown in Table I. Photographs are shown in Fig. 11 and Fig. 12. These two photographs were from different experiments and can not be directly compared. For example, the electrode for the experiment of Fig. 12 had not been subjected to fully continuous CV since the software capacity for the BAS 100 was exceeded for a short period of time (ca. 6 hrs). This could be the reason that the cell population of the treated electrode was not reduced to a significant extent of the control. Sometimes when the frit connecting the reference electrode to the growth medium was not in good connection, bubbles could be seen around the electrodes, and what really happened was the electrolysis of water (shown in Table I as *). However, the population on the gold electrodes in these cases were also significantly lowered than the controls. This implies that continuous electrolysis could be an effective way, too, to inhibit the growth of bacteria on gold electrodes. It can be seen clearly that the population of the bacteria was greatly reduced upon these treatments

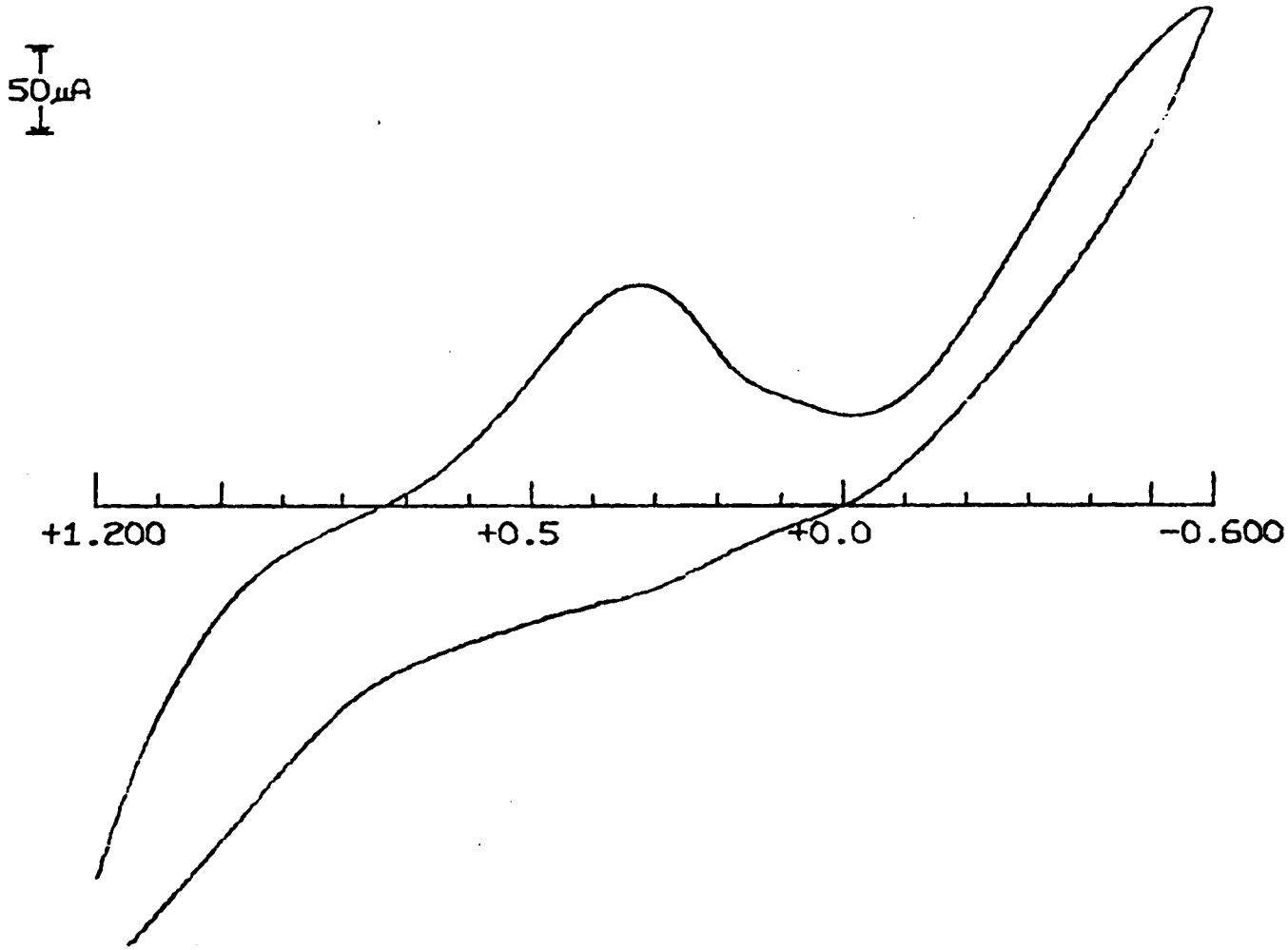


Fig. 10. CV of a gold electrode at the first cycle during continuous sweep.

Table I: AODC results of bacteria surface population

in the condition of continuous CV

expt. no.	time in flow cell (hrs)	population of control [cells/cm^2]	population of treated electrodes [cells/cm^2]	relative population [treated]/[control]
1	55	2.5×10^6	1.5×10^5	0.06
2	52	1.0×10^7	1.0×10^6	0.10
3*	64	3.0×10^6	1.0×10^5	0.03
4	50	3.0×10^6	0.5×10^5	0.02
5*	55	5.0×10^5	0.5×10^5	0.10

(*) Open circuit condition; see text.

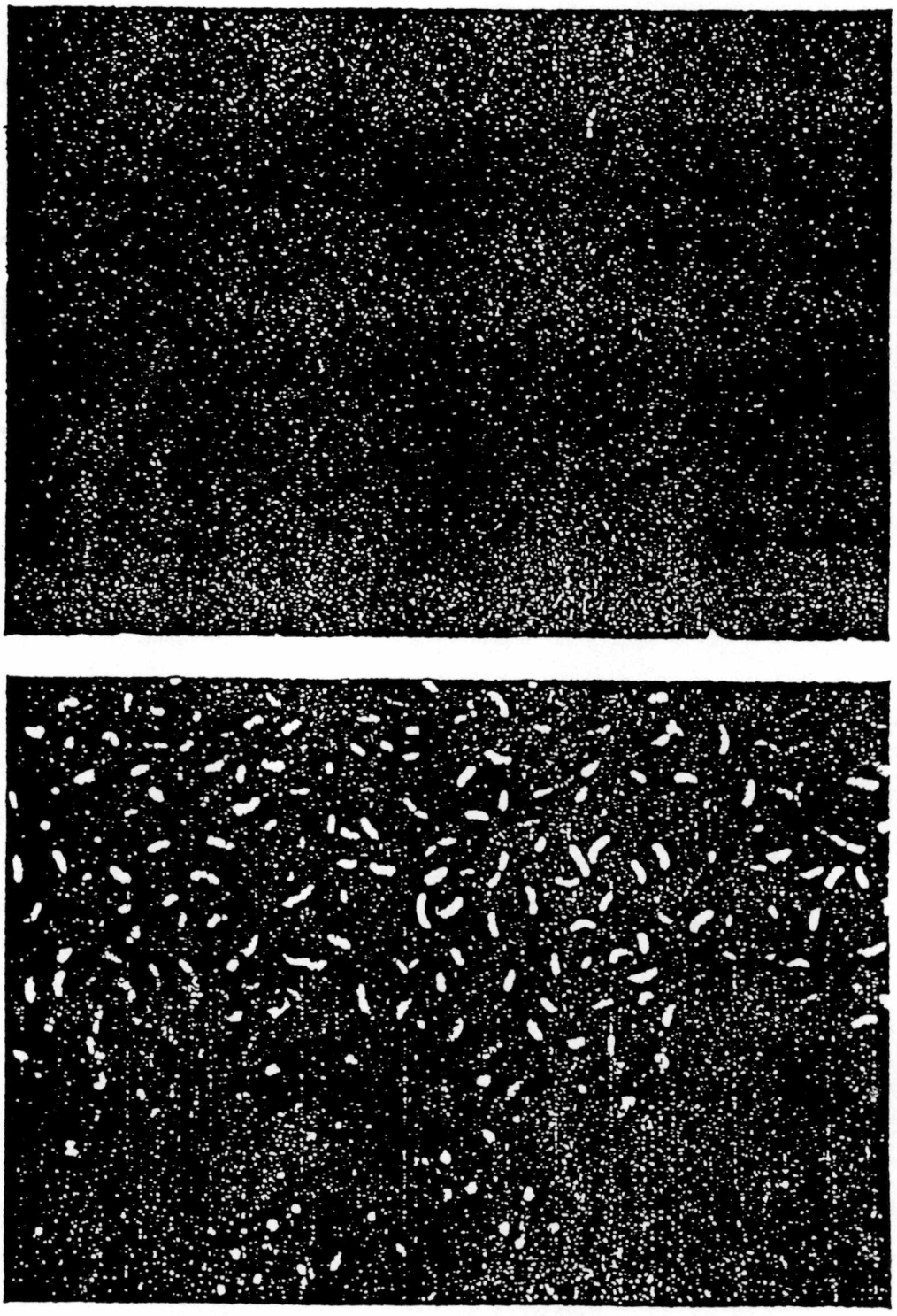


Fig. 11. AODC photographs in the condition of continuous CV. Upper: treated electrode. Lower: control. Magnification: 1000 x.

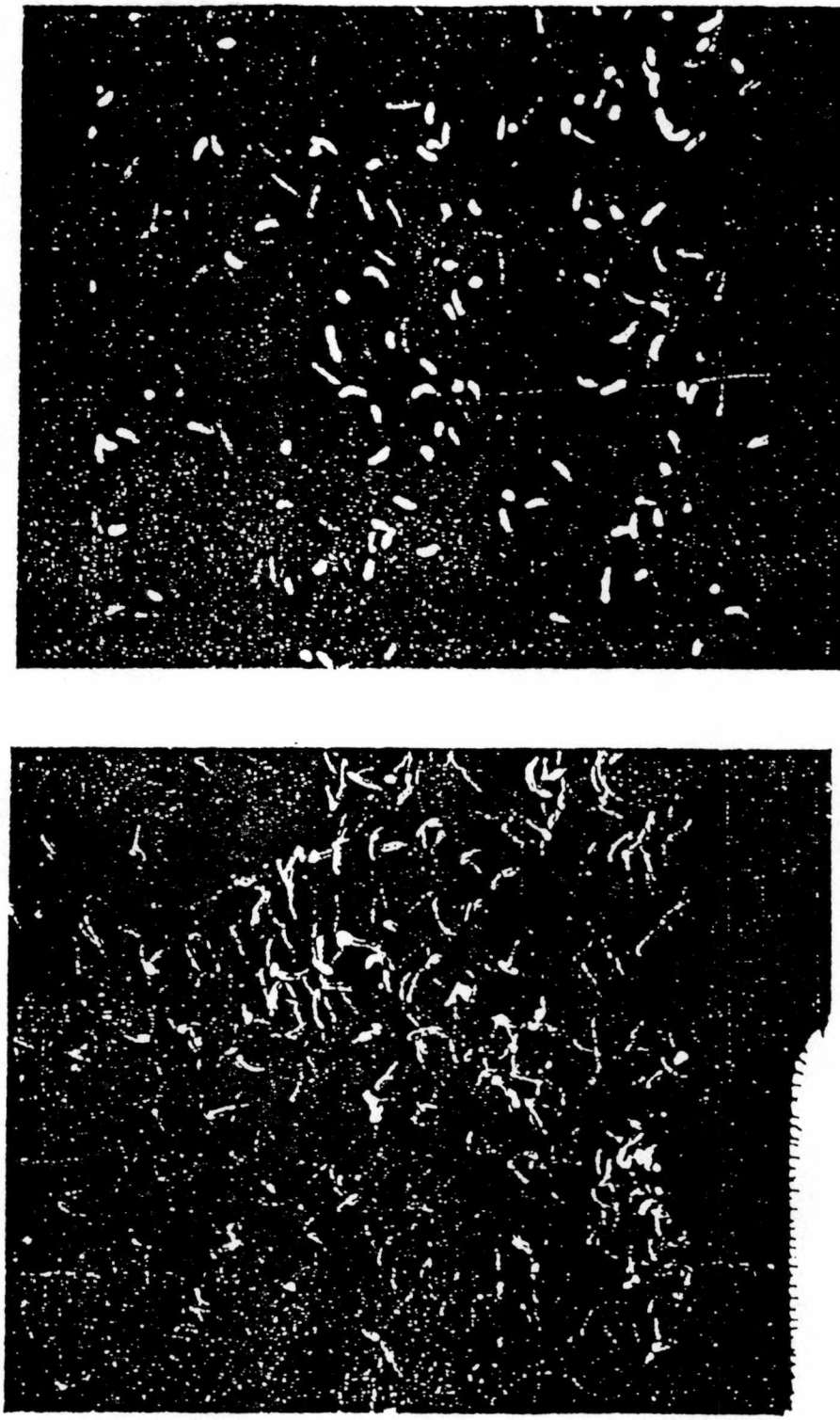


Fig. 12. SEM photographs in the condition of continuous CV. Upper: treated electrode. Lower: control. Magnification: 1500 x.

compared with the control, in one case even to 2%. This could be due to the gold catalyzed oxidation of the adhesive material at the stalk tip of the bacteria. Although the detailed mechanism needs further investigation, the results give us a useful message that when certain potentials are applied, the gold surface is changed in some way, and it becomes difficult for the bacteria to attach. This would also suggest that care should be exercised when using electrochemical detection, such as AC impedance, on the surface microbial activity, since the potential applied may affect the surface condition and change the way bacteria attach.

(ii). In situ cyclic voltammetry studies.

After the bacteria stayed enough time in the flow cell and the biofilm was believed to form on the gold surface, cyclic voltammetry was performed in the original medium, i.e., in situ, to see the effect on the biofilm. The gold electrodes had been immersed in the chemostat for the entire growth period. The potential region was +1.0 V ---> -0.8 V or +1.2 V ---> -0.6 V, and different scan rates and scan segments were applied. One of the cyclic voltammograms is shown in Fig. 13. Only parts of the scan cycles were plotted. These voltammograms did not display distinct peaks, an indication of severely filmed surfaces. A possible hint of a surface oxide stripping process is seen at ca. 0.3 V in the CV of Fig. 13. According to the AODC results (shown in Table II), the surface population of the bacteria was almost the same compared with the control upon these treatments. A possible reason could be that the current generated, usually less than 1 mA, was not high enough to detach

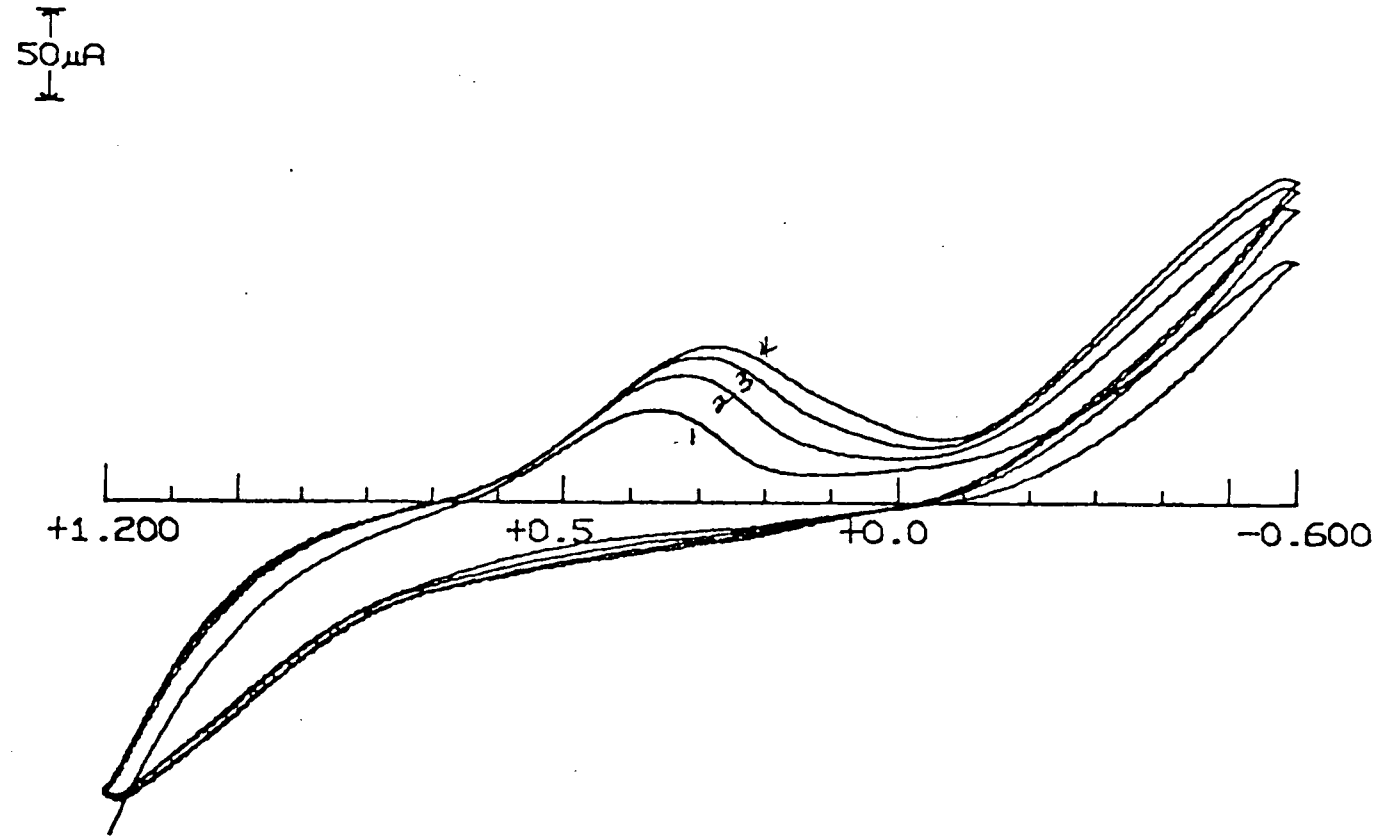


Fig. 13. CV of a gold electrode in the condition of in situ CV. The numbers stand for successive cycles. Only partial cycles are plotted.

Table II: AODC results of bacteria surface population in condition of in situ CV

expt. no.	CV conditions	relative population $[cells/cm^2]_{treated}/[cells/cm^2]_{control}$
1	+1.0 -> -0.8 V, 20 mV/sec, 20 segments	>0.95
2	+1.0 -> -0.8 V, 20 mV/sec, 40 segments	>0.95
3	+1.0 -> -0.8 V, 10,204 mV/sec, 120 segments	>0.95
4	+1.0 -> -0.8 V, 1,000 mV/sec, 40 segments	>0.95
5	+1.2 -> -0.6 V, 100 mV/sec, 20 segments	>0.95
6	+1.2 -> -0.6 V, 10,000 mV/sec, 20 segments, with stirring	0.50
7	+1.2 -> -0.6 V, 10,000 mV/sec, 40 segments	>0.95
8	+1.2 -> -0.6 V, 10,000 mV/sec, 40 segments, with stirring	>0.95

the biofilm, even though the gold surface could catalyze the oxidation of organic compounds. One exception could be seen from Table II, where one population was reduced to half (photograph shown in Fig. 14), but this was not reproducible.

(iii). In situ electrolysis studies.

After the bacteria stayed enough time in the flow cell and the biofilm was believed to form on the gold surface, controlled potential and controlled current electrolysis under different conditions were applied. During constant current electrolysis, bubbles could be observed around the electrodes, which was believed to be the electrolysis of water. In three instances, the surface population was found to decrease, as given in Table III. One photograph is shown in Fig. 15. The reproducibility was poor.

Other electrolysis methods were tried, too, such as constant current at 50 mA/cm^2 for 1 min followed by constant potential at -0.6 V for 1 min for 5 times; constant current at 50 mA/cm^2 for 60 min followed by constant potential at -0.6 V for 10 min; and constant current at 100 mA/cm^2 for 1 min followed by constant potential at -0.6 V for 1 min for 5 times. Constant currents only were tested, i.e., 10 mA/cm^2 for 5 min; 50 mA/cm^2 for 5 and 10 min; and 100 mA/cm^2 for 5 min. In these cases, no decrease in the bacteria surface populations were observed.

The combination of constant current electrolysis employed in the experiment used to generate the data in Table III was selected with the following idea in mind. At - 0.6 V the oxide stripping process will proceed at an appreciable rate, thus

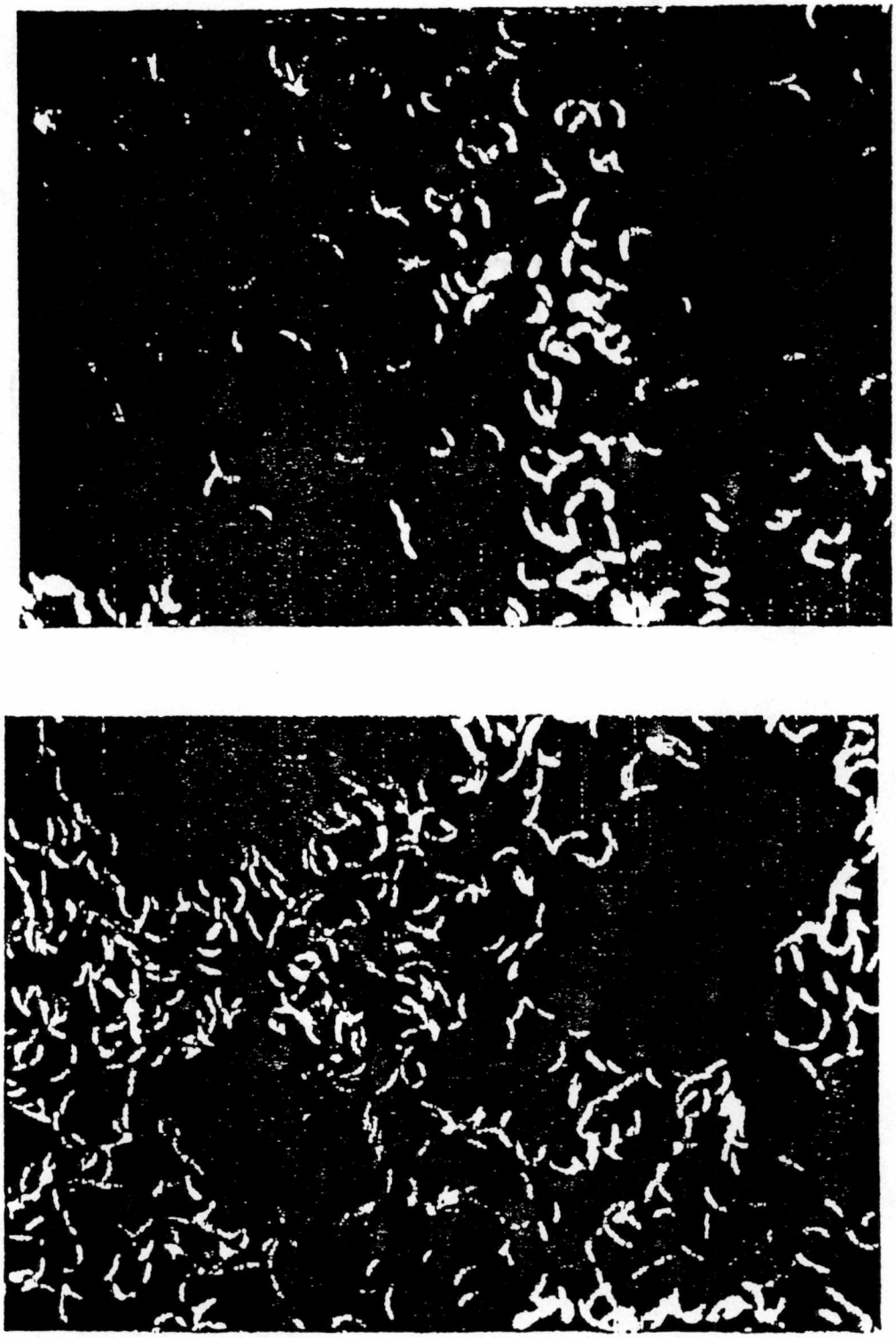


Fig. 14. AODC photographs in the condition of in situ CV. Upper: treated electrode. Lower: control. Magnification: 1000 x.

Table III: AODC results of bacteria surface population in the condition of in situ electrolysis

expt. no.	electrolysis conditions	population of control	population of treated electrodes	relative population [treated]/[control]
1	I = 100 mA/cm^2 0.5 min; E = -0.6 V 0.5 min	3.0 x 10^6	3.0 x 10^5	0.10
2	I = 10 mA/cm^2 5 min; E = -0.6 V 5 min	3.0 x 10^6	3.0 x 10^5	0.10
3	I = 50 mA/cm^2 1 min; E = -0.96 V 1 min; repeat 5 times	1.0 x 10^7	5.0 x 10^6	0.50

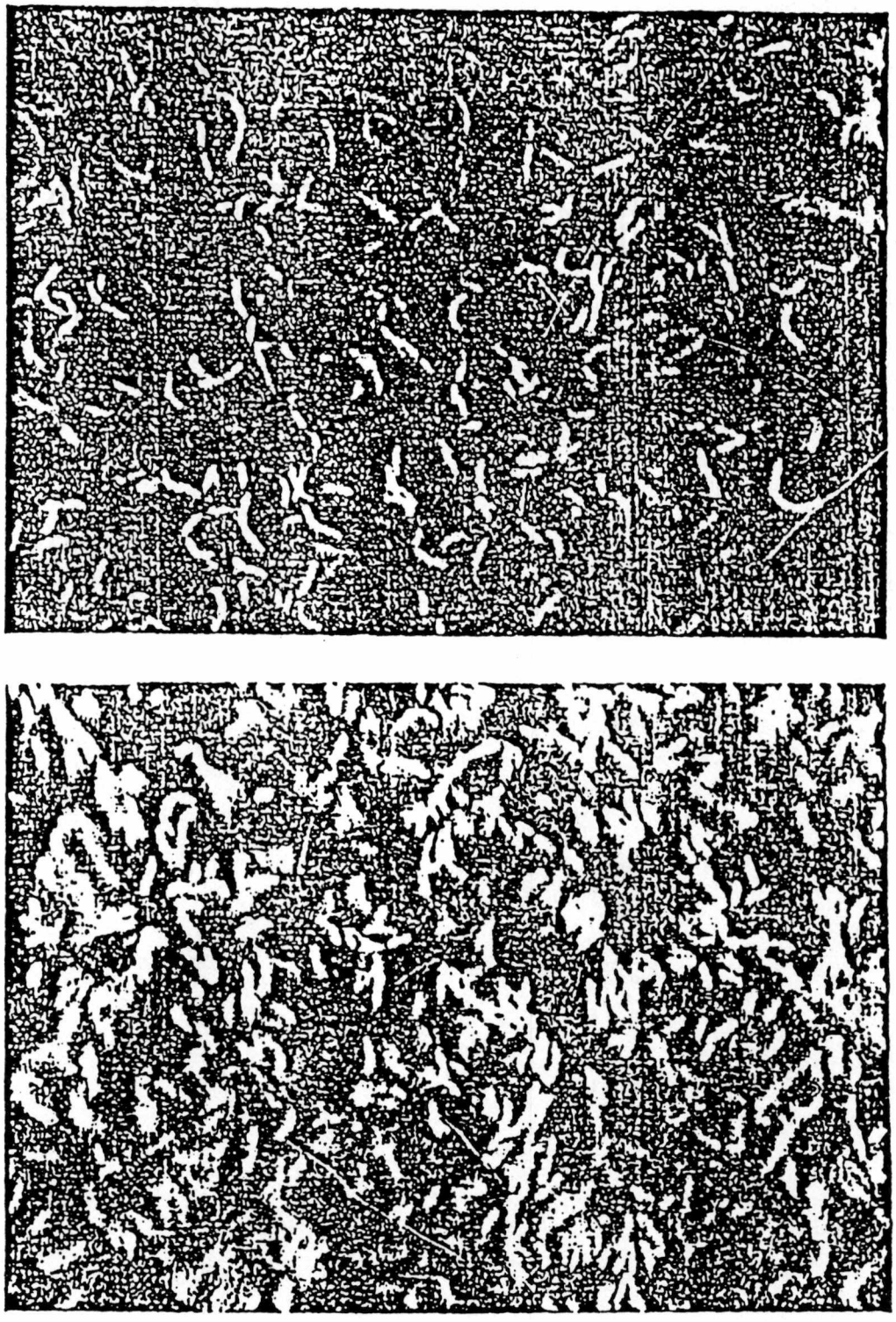


Fig. 15. AODC photographs in the condition of in situ electrolysis. Upper: treated electrode. Lower: control. Magnification: 1000 x.

activating the surface for forced anodic oxidation under constant current conditions. This protocol follows the procedure of Johnson et al. for amperometric detection of carbohydrates [19]. In spite of the inconsistencies, it is believed that further experimentation along these lines should lead to an efficient procedure for the in-situ removal of these biofilms directly in the growth medium.

(iv). Ex situ CV studies.

In view of the inconsistent results described above, conditions were sought for the efficient electrolysis of the surface biofilms. Accordingly, electrodes were performed ex situ by transferring the biofouled electrodes to a 0.1 M NaOH solution. This medium was chosen because the gold surface redox chemistry is extremely rapid in aqueous NaOH solution, a phenomenon Johnson and coworkers have exploited for the oxidation of many organic compounds [19].

After the bacteria stayed enough time in the flow cell and the biofilm was believed to form on the gold surface, the gold foil was removed from the electrochemical cell and placed into 0.1 M NaOH solution. CV was then performed under different conditions and the results are given in Table IV. One photograph is given in Fig. 16. Although decrease of the surface population was obtained in expt. no. 1, 1 M NaOH solution was too concentrate for the flow system, and it was not used later on. No decrease of the surface population was observed in other two cases. The reason proposed for in situ CV studies could also hold here, i.e., too low current to detach the biofilm. It is still too early to come to any conclusion.

Table IV: AODC results of bacteria surface population

in the conditions of ex situ CV

expt. no.	conc. of NaOH	Treatments	relative population $[cells/cm^2]_{treated}/[cells/cm^2]_{control}$
1	1 M	+1.0 --->-0.8 V 100 mV/sec; 40 segments	0.02
2	0.2 M	+0.5 --->-1.0 V 100 mV/sec; 40 segments	> 0.95
3	0.1 M	+0.5 --->-1.0 V 100 mV/sec; 20 and 40 segments	> 0.95

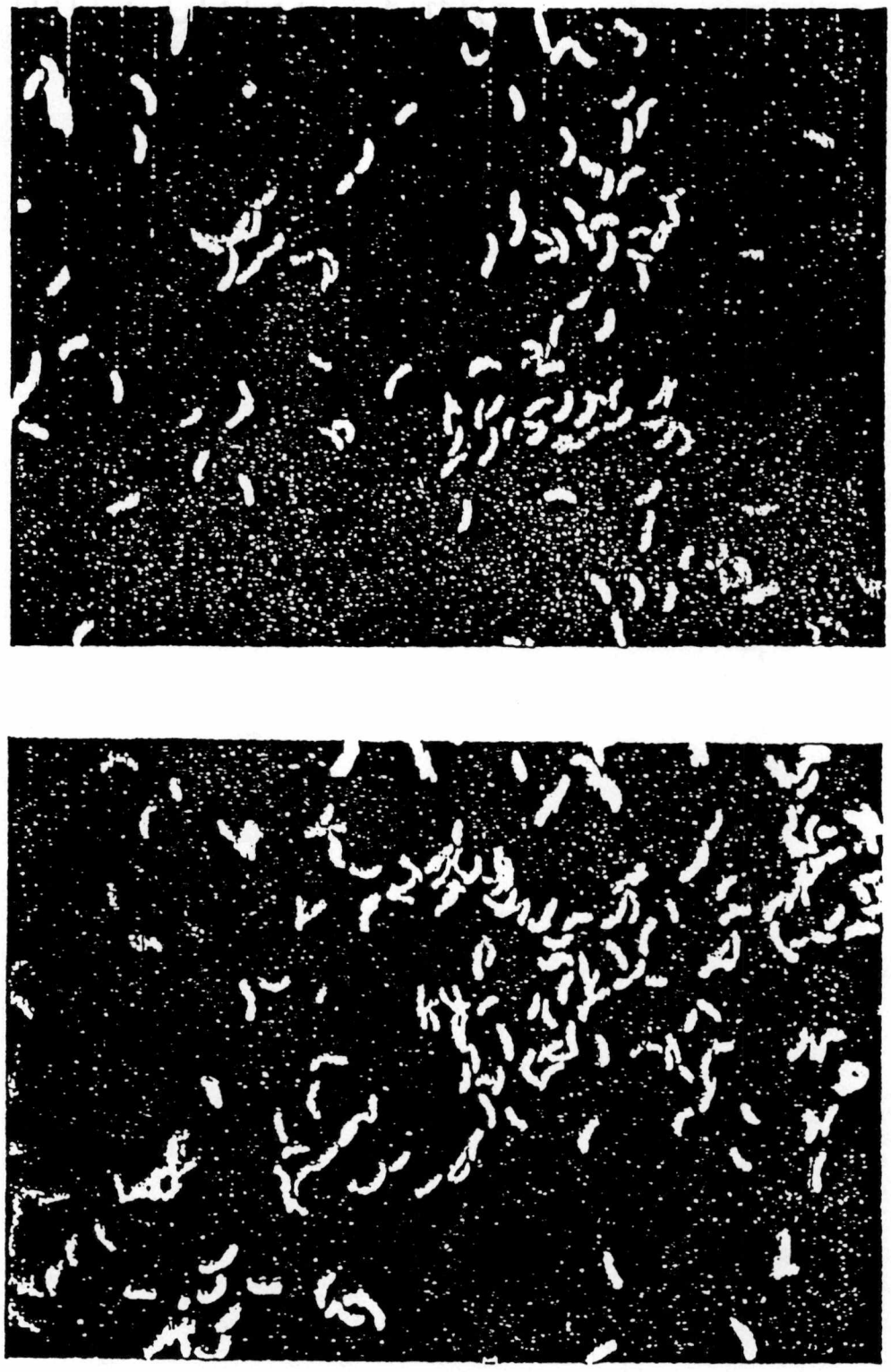


Fig. 16. AODC photographs in the condition of ex situ CV in 0.1 M NaOH. Upper: treated electrode. Lower: control. Magnification: 1000 x.

(v). Ex situ electrolysis studies.

After the bacteria stayed enough time in the flow cell and the biofilm was believed to form on the gold surface, the gold foil was removed from the electrochemical cell and placed into 0.1 M NaOH solution. Electrolysis was conducted under different conditions, and the corresponding results are shown in Table V. One photograph is given in Fig. 17.

The population of the biofilm was greatly decreased under these treatments, and most important, the results were reproducible. Controls were monitored in NaOH solutions within the same time as those under electrochemical treatments, and they appeared to have no effect on the surface population of the bacteria. CVs before and after the treatments are shown in Fig. 18. They both show oxide formation and stripping peaks, which means they are not passivated. However, the CV after the electrolysis shows clear evidence of oxidation of organic compounds on the surface. Although details are not fully understood by now, these organic compounds are of main interest in future study.

It could be possible that the adhesive material at the stalk tip of the bacteria was oxidized during the process and resulted in the bacteria detachment.

Another possible reaction is the generation of hypochlorite ion, a known biocide, at the working electrode as shown in equation (4),

$$Cl^- + 2\ OH^- \text{---> } OCl^- + H_2O + 2\ e \qquad (4)$$

The concentration of hydroxide ion in the growth medium was $10^{-7.2}$, i.e.,6.3 x 10^{-8}, and it could be too low to form in situ OCl^-, and it might not affect the

Table V: AODC results of bacteria surface population in the conditions of ex situ electrolysis

expt. no.	Treatments	population of control	population of treated electrodes	relative population [treated]/[control]
1	I= 50 mA/cm^2; 30 min	3.0 x 10^6	5 x 10^4	0.02
2	I= 50 mA/cm^2; 10 min E= -0.6 V; 10 min	3.0 x 10^5	2 x 10^4	0.07
3	I= 100 mA/cm^2; 1 min E= -0.6 V; 1 min repeat 5 times	1.0 x 10^7	1.0 x 10^5	0.01
4	I= 50 mA/cm^2; 1 min E= -0.6 V; 1 min repeat 3 times	2.0 x 10^7	1.0 x 10^5	0.005
5	I= 100 mA/cm^2; 5 min	1.0 x 10^7	2.0 x 10^5	0.02

Note: [NaOH]: (1) 1 M; (2) 0.2 M; (3), (4), and (5) 0.1 M.

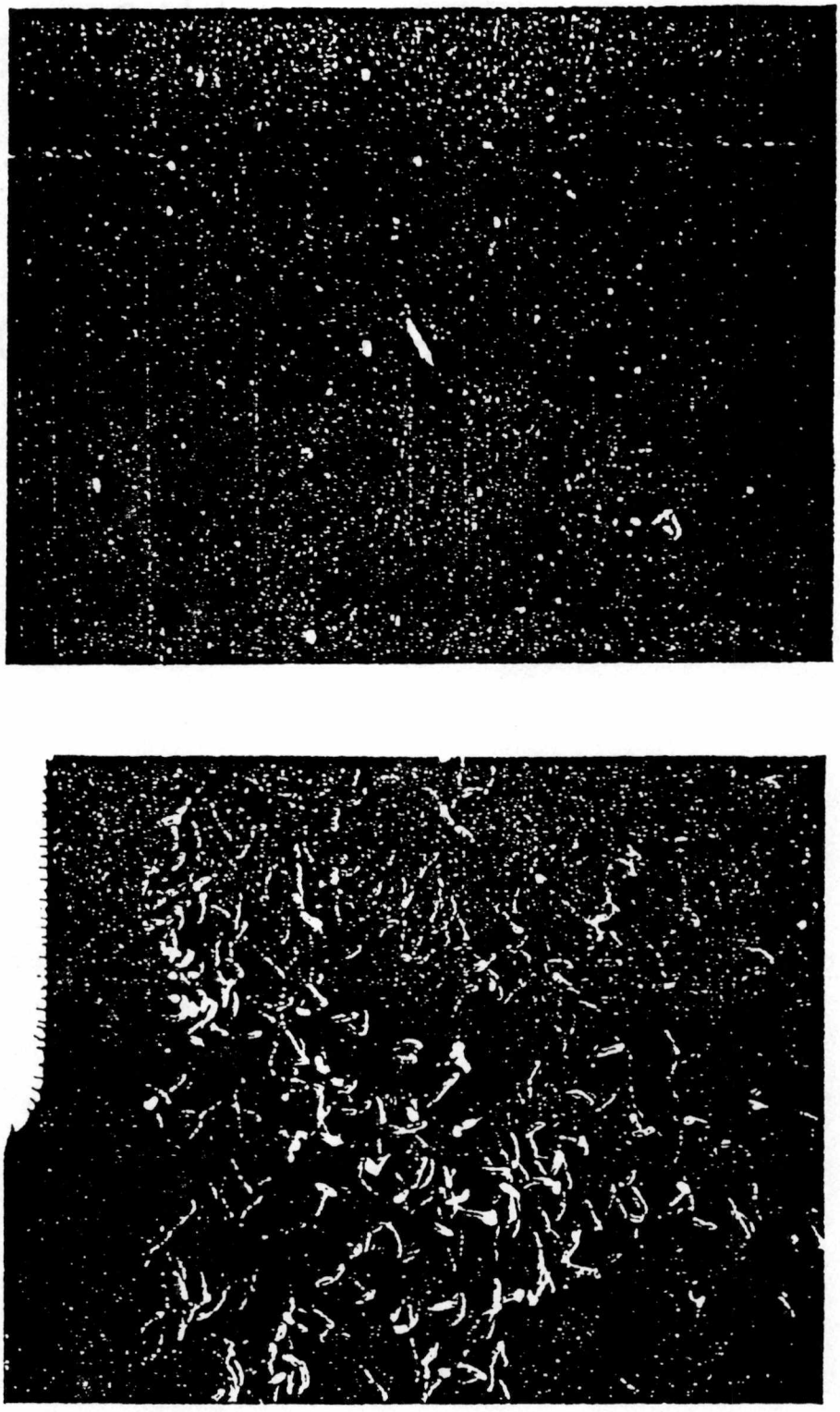


Fig. 17. SEM photographs in ex situ electrolysis in 0.1 M NaOH. Upper: treated electrode. Lower: control. Magnification: 1500 x.

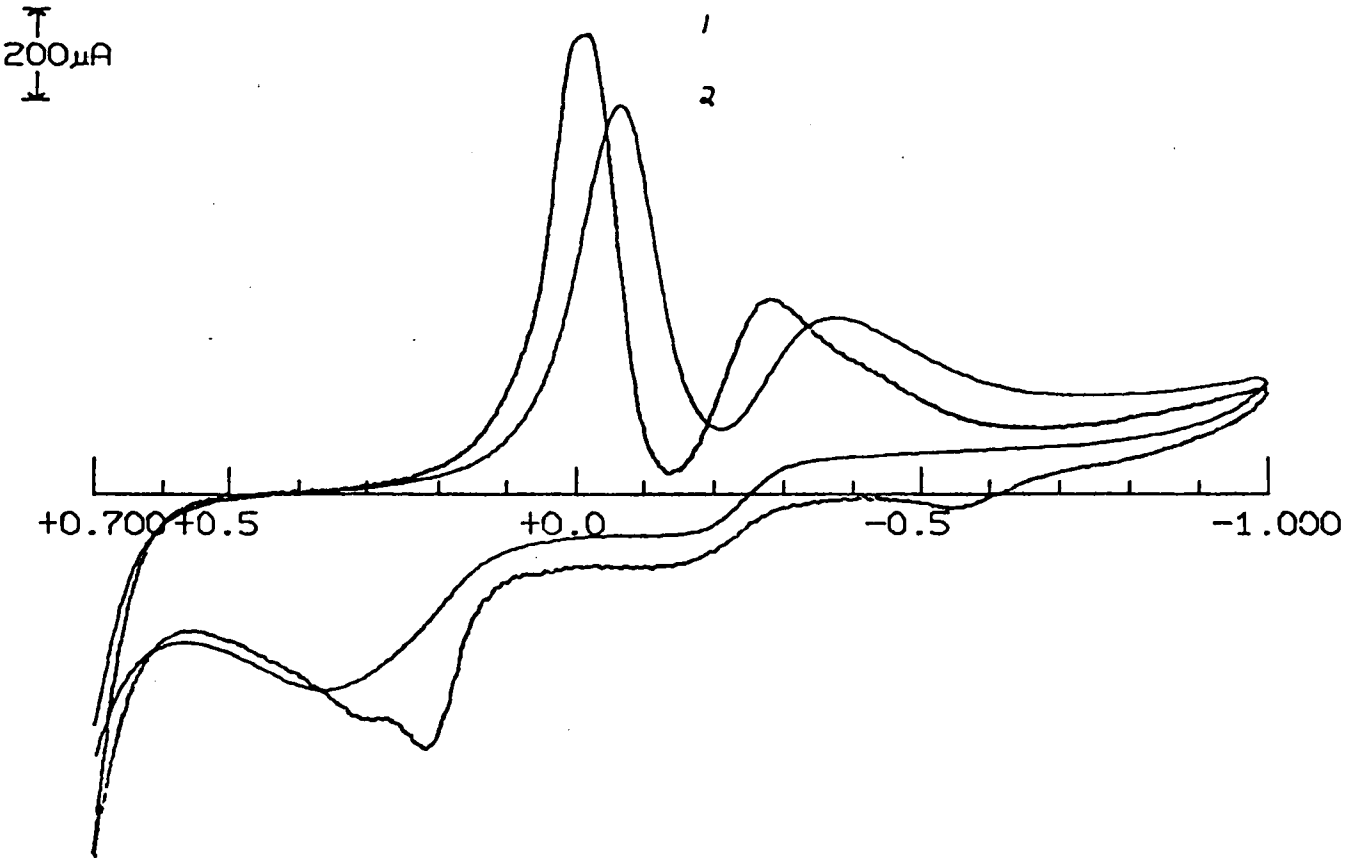


Fig. 18. CV of a gold electrode in the condition of exsitu electrolysis in 0.1 M NaOH. (1) Before and (2) after the treatment.

biofilm to a significant extent. The concentration of hydroxide ion in 0.1 M NaOH was 0.1 M, thus it could be enough to generate biocide OCl^- to detach the biofilm. The two proposed mechanism could be working at the same time.

When combined with QCM in a suitable way, ex situ electrolysis could be a good method to detach the biofilm in an on-line design. Alternatively, direct electrooxidation of the polysaccharide material of the stalk of the bacteria is a possibility.

CHAPTER IV

SUMMARY

The quartz crystal microbalance is a relatively new method to monitor the mass of the biofilm in an on-line configuration. At a certain point when the biofilm gets too thick, however, the sensitivity decreases. In order to search for an effective way to detach the biofilm so that repetitive measurements are possible, *Caulobacter crescentus* was allowed to grow in the growth medium and attach on the gold electrodes. Different electrochemical methods were tried to get them off the gold surface.

From acridine orange direct counting results, it seemed that in situ, i. e., in the growth medium, electrochemical treatments after the growing period, including in situ CV and in situ electrolysis, can not offer a resolution of the problem at the present time. Although detachment can be seen sometimes, the results were not consistent. Same results were found in ex situ electrolysis in 0.1 M NaOH solution.

Continuous CV during the growth period and ex situ electrolysis in 0.1 M NaOH solution proved to be the right choices for the proposed research. The surface population of the bacteria can be reduced to as low as 0.5 % of the control. If combined with QCM in an suitable design, continuous measurements of the surface biomass and bacterial regrowth are possible.

LIST OF REFERENCES

REFERENCES

[1] Davis, B. D.; Dulbeceo, R.; Eisen, H.N.; Ginsberg, H.S. In "Microbiology", 4 th edition, J. B. Lippincott Compony, 1990.

[2] Brock, T. D.; Smith, D. W.; Madigan, M. T. In "Biology of Microorganisms", 4 th edition, Prentice-Hall Inc, 1984.

[3] Characklis, W. G.; Marshall, K. C. In "Biofilms", Wiley-Interscience Publication: , 1990.

[4] Savage, D. C.; Fletcher, M. In "Bacterial Adhesion: Mechanisms and Physiological Significance", Plenum Press, 1985.

[5] Bryers, J. D.; Characklis, W. G. *Biotech. Bioeng.*, **1982**, *24*, 2451.

[6] Zobell, C. E. *J. Bacterial.*, **1943**, *46*, 39.

[7] Characklis, W. G.; Cooksey, K. E. *Adv. Appl. Microbiol.*, **1983**, *23*, 93.

[8] J. S. Poindexter. *Microbiological Reviews*, **1981**, *45*, 123.

[9] Newton, A.; Osley, M. A.; Terrana, B. In D. Schlessinger (ed.), "Microbiology-1975", American Society for Microbiology, Washington D. C. **1975**, 442.

[10] Newton, A.; Allebach, E. *Genetics*, **1975**, *80,* 1.

[11] Umbeit, T. H.; Pate, J. L. *Arch. Microbiol.* **1978**, *118*, 157.

[12] Nivens, D. E. Institute of Applied Microbiology, Knoxville, TN. Private communication.

[13] Cohen-Bazire, G.; Kurisawa, R; Poindexter, J. S. *J. Gen. Microbiol.*, **1966**, *42*, 301.

[14] Bard, A. J.; Faulkner, L. R. In "Electrochemical Methods: Fundamentals and Applications". John Wiley & Sons, **1980.**

[15] Kissinger, P. T.; Heineman, W. R. In "Laboratory Techniques in Electroanalytical Chemistry". Marcel Dekker, Inc., **1984.**

[16] Woods, R. In "Electroanalytical Chemistry", Bard, A. J. (ed.), Marcel Dekker, Inc. **1976,** *9*, 1.

[17] Icenhower, D. E.; Urbach, H. B.; Harrison, J. H. *J. Electrochem. Soc.,* **1970,** *117*, 1500.

[18] Laitinen, H. A.; Chao, M. S. *J. Electrochem. Soc.*, **1961,** *108,* 726.

[19] Johnson, D. C.; LaCourse, W. R. *Anal. Chem.*, **1990,** *62*, 589A.

[20] Johnson, D. C. *Nature* (London), **1986,** *321*, 451.

[21] Burke, L. D.; McCarthy, M. M.; Roche, M. B. C. *J. Electroanal. Chem.*, **1984,** *167*, 291.

[22] Neugen Van Huong, G.; Hinnen, C.; LeCoeur, J. *J. Electroanal. Chem.*, **1980,** *106*, 185.

[23] Brucknstein, S.; Shay, M. *J. Electroanal. Chem.*, **1985,** *188*, 131.

[24] Angerstein-Kozlowska, H.; Conway, B. E.; Barnett, B.; Mozota, J. *J. Electroanal. Chem.*, **1979,** *100*, 417.

[25] Angerstein-Kozlowska, H.; Conway, B. E.; Hamelin, A.; Stoicovicici, L. *J. Electroanal. Chem.*, **1987,** *228*, 429.

[26] Angersten-Kozlowska, H.; Conway, B. E.; Hamelin, A.; Stoicovicici, L. *Electrochic. Acta.*, **1986,** *31*, 1051.

[27] Burke, L. D.; Cunnane, V. J. *J. Electroanal. Chem.*, **1986**, *210*, 69.

[28] Larew, L. A.; Johnson, D. C. *J. Electroanal. Chem.*, **1989**, *262*, 167.

[29] Larew, L. A.; Johnson, D. C. *J. Electroanal. Chem.*, **1989**, *264*, 131.

[30] Nivens, D. E.; White, D. C.; Chambers, J. Q. Paper presented at A. S. M. National Meeting,Anaheim, CA, May, **1990**.

[31] Hobbie, J. E.; Daley, R. J.; Jasper. *Applied and Environmental Microbiology*, **1977**, *33*, 1225.

VITA

Yingbo Guo was born and grew up in Changchun, P. R. China, where she attended elementary and high school, and was graduated from Jilin University with a Bachelor of Science degree in Chemistry in 1988.

After receiving her B. S. degree, she stayed in Jilin University as a graduate student in Department of Chemistry for one semester, then transferred to The University of Tennessee at Knoxville in the fall of 1989, to pursue her M.S. degree. She was married to Mr. Ziyang Zhang of her home town, on Dec. 26, 1990.

The degree of Master of Science, with a major in Chemistry, was awarded in December, 1991.