Growth kinetics of Pseudomonas aeruginosa
by Suet Nee Chen

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
The growth kinetics of Pseudomonas aeruginosa in continuous culture was investigated. A chemostat was used to grow Pseudomonas aeruginosa, and steady state glucose, dissolved oxygen concentrations and utilization rates, and microorganism concentrations were measured. The continuous system experiments were carried out at 25°C, pH = 7.2, with a stirrer agitation rate of 350 rpm. The growth of Pseudomonas aeruginosa was found experimentally to be limited by glucose and dissolved oxygen concentrations in the chemostat. The data taken from the steady state chemostat measurements were used to calculate growth kinetic parameters through nonlinear analysis using a multiple-substrate growth model of Pseudomonas aeruginosa. A dual-substrate growth kinetics model, using Tessier kinetics for both oxygen and glucose, was found to show good agreement with the experimental data. Respective growth kinetic parameters were evaluated to be μmax = 0.29 h-1, KgT = 26.9 mg/L, Kot =1.18 mg/L, Yx/g = 0.628 mg microorganism/mg glucose, and Yx/0 = 0.635 mg microorganism/mg oxygen. Maintenance factors for both glucose and oxygen were found to be mg = 0.0078 g glucose consumed/g microorganism hour and m0 = 0.014 g oxygen consumed/g microorganism hour. The coefficient for oxygen and maintenance factors for glucose and oxygen were evaluated from the constructed model for Pseudomonas aeruginosa.

The oxygen coefficients for Pseudomonas aeruginosa biofilms were calculated from the dissolved oxygen concentration profiles, measured by dissolved oxygen microelectrodes in the artificial Pseudomonas aeruginosa colony biofilms. Both the Tessier and Monod kinetic models were used to calculate the coefficients for oxygen. The Tessier coefficient for oxygen was 0.012 ± 0.003 mg/L and the Monod coefficient for oxygen was 0.18 ± 0.02 mg/L.

The oxygen coefficients calculated using both the Tessier and Monod kinetic models for Pseudomonas aeruginosa colony biofilms and for planktonic cultures were compared. The results from either Tessier or Monod kinetic model showed that the oxygen coefficient for a colony biofilm was lower than the oxygen coefficient for a planktonic culture. In conclusion, the growth kinetic parameters derived from planktonic cultures should not be applied in biofilms.

<

GROWTH KINETICS OF

PSEUDOMONAS AERUGINOSA

by

Suet Nee Chen

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

of

Master of Science

in

Chemical Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

November 2001

APPROVAL

Of a thesis submitted by

Suet Nee Chen

This thesis has been read by each member of the thesis 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.

Dr. Zbigniew Lewandowski (Chair Person) _____ (Signature) 9/20/01 Date

Approval for the Department of Chemical Engineering

Dr. John T Sears John T. Sears (Signature) 9/26/01 Date

Approval for the College of Graduate Study

Dr. Bruce McLeod Bruce R. McLeod (Signature) 11-28-01 Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirement for a master's degree at Montana State University, 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 allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. 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 12/3/01

ACKNOWLEDGMENTS

First of all, I would like to express my gratitude to my research advisor Dr. Lewandowski for his guidance and support that enabled me to achieve my goal. I would like to particularly thank Dr. Beyenal for all his guidance, support, counsel, "patience" and assistance in experimental design. I would also like to thank Dr. Sears and Dr Stewart for their counsel.

I would especially like to acknowledge my parents, Kok Kong Chen and Mei Chin Kuan for their boundless support and love across the sea from Malaysia. "Dad and mum, thank you for your sacrifices and love in sending me to school in the United States."

Last but not least, Nurdan Beyenal, Gary Jackson, Kevin Braughton, Frank Roe and everyone at the Center for Biofilm Engineering, that has made my years at the Center a WONDERFUL time.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

A	Preexponential factor or frequency factor
b	Dimensionless half saturation constant
B_g	Constant in Contois model for glucose
B_i	Constant in Contois model for substrate *i*
B_o	Constant in Contois model for oxygen
c	Constant in Equation 2.20 (h^{-1})
CO_2	Carbon Dioxide
$C_5H_7NO_2$	Biomass
$C_6H_{12}O_6$	Glucose
C_{Nf}	Influent ammonium concentration (mg/L)
C_{Ne}	Effluent ammonium concentration (mg/L)
D	Dilution rate (h^{-1})
De	Diffusion coefficient of oxygen in water (cm^2/sec)
E	Activation energy (J/mol)
H_2O	Water
K_{gT}	Tessier coefficient for glucose (g/L)
K_{oT}	Tessier coefficient for oxygen (g/L)
K_{SM}	Monod coefficient (g/L)

K_{ST}	Tessier coefficient (g/L)
K_{SMz}	Mozer coefficient (g/L)
K_{SA}	Andrews coefficient (g/L)
K_{SE}	Edwards coefficient (g/L)
K_{SL}	Luong coefficient (g/L)
K_{STW}	Tseng and Wayman coefficient (g/L)
K_{Si}	Coefficient for substrate i (g/L)
K_{iA}	Andrews substrate inhibition constant (g/L)
K_{iE}	Edwards substrate inhibition constant (g/L)
L_f	Biofilm thickness (μm)
m_g	Maintenance factor for glucose (h^{-1})
m_i	Maintenance factor for limiting substrate i (h^{-1})
m_o	Maintenance factor for oxygen (h^{-1})
mo	Microorganism
n	Exponential constant for Equation 2.19
na	not available
NH_4^+	Ammonium
O_2	Oxygen
OUR	Oxygen uptake rate (mg oxygen/h)

Q	Flow rate (L/h)
R	Gas constant, 8.314 J/mol·K
Rr	Reaction rate, (g/h)
S	Substrate concentration (g/L)
S_{ei}	Substrate concentration in effluent stream (g/L)
$S_{experiment}$	Experimental substrate concentration (g/L)
S_{fg}	Concentration of glucose in fresh feed (g/L)
S_{fi}	Substrate concentration in influent stream (g/L)
S_{fn}	Concentration of ammonium sulfate in fresh feed (g/L)
S_g	Concentration of glucose in chemostat (g/L)
S_i	Concentration of substrate i (g/L)
S_m	Substrate concentration above which growth is completely inhibited (g/L)
S_o	Concentration of dissolved oxygen (g/L)
S_{os}	Concentration of dissolved oxygen at the surface of the colony biofilm measured using dissolved oxygen microelectrode (g/L).
SOUR	Specific oxygen uptake rate (g oxygen/g microorganism/h)
$S_{predicted}$	Predicted substrate concentration (g/L)
S*	Substrate concentration under which microorganism can not grow in equation 2.20 (g/L)

S^*	Dimensionless substrate concentration
t	Time (sec)
T	Temperature (C^o)
V	Reactor volume (L)
X	Microorganism concentration in chemostat (g/L)
X_f	Biofilm density (g microorganism/L)
$Y_{x/g}$	Yield coefficient for glucose (g microorganism/g glucose)
$Y_{x/o}$	Yield coefficient for oxygen (g microorganism/g oxygen)
$Y_{x/si}$	Yield coefficient for limiting substrate *i* (g microorganism/g limiting substrate)
z	Space coordinate in biofilm (μm)
z*	Dimensionless space coordinate in biofilm

Greek letters

μ	Specific growth rate (h^{-1})
$\mu_{experimental}$	Experimental specific growth rate (h^{-1})
μ_i	Specific growth rate for limiting substrate *i* (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})
μ_{model}	Theoretical specific growth rate (h^{-1})

λ_g	Mozer's constant for glucose (g/L)
λ_i	Mozer's constant for substrate i (g/L)
λ_o	Mozer's constant for oxygen (g/L)
Φ	Dimensionless Thiele Modulus

ABSTRACT

The growth kinetics of *Pseudomonas aeruginosa* in continuous culture was investigated. A chemostat was used to grow *Pseudomonas aeruginosa*, and steady state glucose, dissolved oxygen concentrations and utilization rates, and microorganism concentrations were measured. The continuous system experiments were carried out at 25°C, pH = 7.2, with a stirrer agitation rate of 350 rpm. The growth of *Pseudomonas aeruginosa* was found experimentally to be limited by glucose and dissolved oxygen concentrations in the chemostat. The data taken from the steady state chemostat measurements were used to calculate growth kinetic parameters through nonlinear analysis using a multiple-substrate growth model of *Pseudomonas aeruginosa*. A dual-substrate growth kinetics model, using Tessier kinetics for both oxygen and glucose, was found to show good agreement with the experimental data. Respective growth kinetic parameters were evaluated to be μ_{max} = 0.29 h^{-1}, K_{gT} = 26.9 mg/L, K_{oT} = 1.18 mg/L, $Y_{x/g}$ = 0.628 mg microorganism/mg glucose, and $Y_{x/o}$ = 0.635 mg microorganism/mg oxygen. Maintenance factors for both glucose and oxygen were found to be m_g = 0.0078 g glucose consumed/g microorganism·hour and m_o = 0.014 g oxygen consumed/g microorganism·hour. The coefficient for oxygen and maintenance factors for glucose and oxygen were evaluated from the constructed model for *Pseudomonas aeruginosa*.

The oxygen coefficients for *Pseudomonas aeruginosa* biofilms were calculated from the dissolved oxygen concentration profiles, measured by dissolved oxygen microelectrodes in the artificial *Pseudomonas aeruginosa* colony biofilms. Both the Tessier and Monod kinetic models were used to calculate the coefficients for oxygen. The Tessier coefficient for oxygen was 0.012 ± 0.003 mg/L and the Monod coefficient for oxygen was 0.18 ± 0.02 mg/L.

The oxygen coefficients calculated using both the Tessier and Monod kinetic models for *Pseudomonas aeruginosa* colony biofilms and for planktonic cultures were compared. The results from either Tessier or Monod kinetic model showed that the oxygen coefficient for a colony biofilm was lower than the oxygen coefficient for a planktonic culture. In conclusion, the growth kinetic parameters derived from planktonic cultures should not be applied in biofilms.

CHAPTER 1

INTRODUCTION

Literature Review

Pseudomonas aeruginosa is often used in biofilm studies and in modeling biofilm accumulation (Robinson et al., 1984; Bakke et al., 1984; Wanner et al., 1997; Wirthanem et al., 1999), probably because microbial geneticists have been studying this organism intensively, and its physiology and genetics are well known. Growth kinetic parameters for microbial growth of *Pseudomonas aeruginosa* have been determined in biofilms by Bakke et al., (1984), and in planktonic cultures by Robinson et al., (1984). However, in both papers, the growth parameters of *Pseudomonas aeruginosa* have been determined at relatively low glucose concentrations, less than 7.5 mg/L in the chemostat (Bakke et al., 1984) and less than 1.4 mg/L in the biofilm reactor (Robinson et. al., 1984). The only reasonable conclusion as to why both authors used such low glucose concentrations was to assure that glucose – NOT oxygen – was the limiting substrate.

A number of studies have shown that biofilm accumulation is related to the growth rate of the planktonic microorganisms before their attachment to the surface (Anwar et. al., 1991). Bakke et. al., (1984) has shown that *Pseudomonas aeruginosa* does not behave differently in biofilms than in suspension at steady state. In contrast, it has also been suggested by Fletcher et. al., (1983), and Brown et. al., (1990), that the growth kinetic parameters in biofilms are different from the growth kinetic

parameters derived from planktonic cells. Nonetheless, there are no consistent results predicting how the microbial growth would be different between planktonic and biofilm cells.

It is well known that substrate concentrations decrease in the deeper parts of biofilms, due to mass transfer limitations and substrate consumption by the microorganisms. Therefore, in biofilm, the growth of *Pseudomonas aeruginosa* may be simultaneously limited by more than a single substrate, (Livingston et. al., 1989, Bailey and Ollis, 1986; Keen and Prosse, 1987). In this case, multiple-substrate growth kinetics of microorganisms must be employed to describe and to represent the growth of the microorganism.

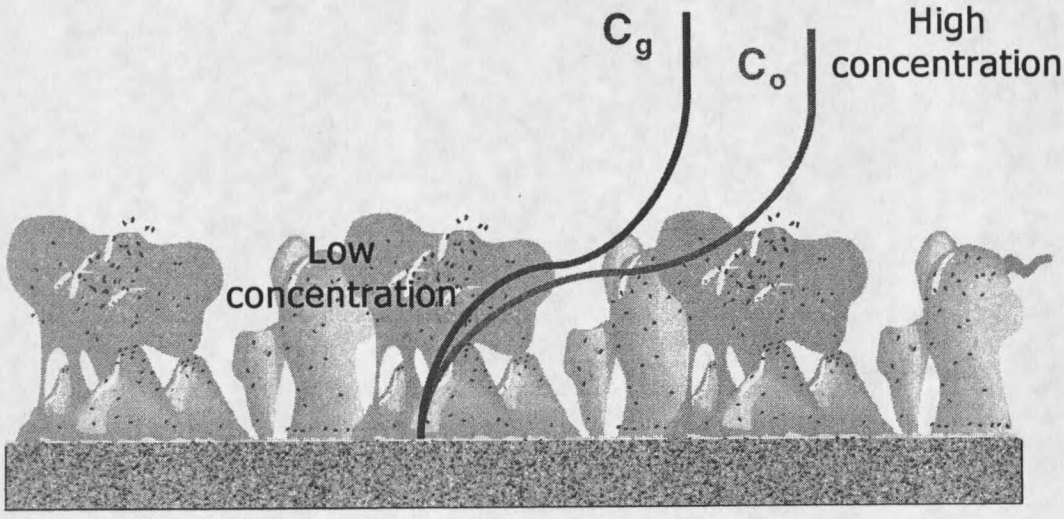

Figure 1.1. Concentration distribution in biofilms.

However, there are inherent difficulties associated with developing relevant multiple-substrate growth models. These difficulties stem from the necessity of providing relevant experimental data and solving non-linear equations. Appropriate techniques to build such models are available (Venkatesh et al., 1997; Beyenal and Tanyolac, 1997). A model can be provided and solved with a reasonable amount of experimental data. The growth dependence of microorganisms on both substrates can be predicted from independent data sets where only a single substrate is limiting.

Several techniques have been proposed in the literature for determining the growth kinetic parameters of biofilms. A published computer simulation program for biofilms, AQUASIM, has been used by several authors to compare experimental data to computer-simulated data (Wanner et. al., 1995; Arcangeli et. al., 1999; Horbel et. al., 1999). However, models derived from AQUASIM are applicable only when the given influent substrate concentration is changing slowly and dissolved oxygen concentration is treated as a state variable. Another technique involved in the measurement of growth kinetic parameters using biofilm cultures is to treat the biofilm as a pseudo suspended culture. This is done by disrupting the biofilm culture (Jih et. al., 1994; Cao et. al., 1995). However, it was shown by de Beer et. al., (1993), that the constituents and the structure of biofilm, such as extracellular polymeric substances (EPS), cell structure, channels and voids, all greatly affected the substrate distribution in the biofilm. In the 1970's and 1980's, dissolved oxygen microelectrodes were introduced into the field of microbial ecology, and became the popular tools for *in situ* analysis of oxygen distribution and microbial respiration in

biofilms (Bungay et. al., 1969; Revsbech et. al., 1986). Dissolved oxygen microelectrodes are able to measure very low concentrations. Their high sensitivity allows them to detect small oxygen concentration changes in the biofilms and has provided investigators with quality in situ experimental data with minimal mass loss and disruption to the structure of the biofilms (Riefler et. al., 1997). In addition, the application of dissolved oxygen microelectrodes in measuring the dissolved oxygen concentration in biofilms has resolved inherent experimental errors from chemical specific analyses and increased the accuracy of growth kinetic parameters estimation. Therefore, microelectrodes are the recent most accurate instruments for evaluating the growth kinetics of biofilms.

From an engineering perspective, a mathematical growth model would have significant importance in predicting the rate and extent of biofilm growth in bioreactors and the feasibility of instruments used in chemical industry. In order to predict the rate and extent of biofilm growth correctly, one must address the question: Could the growth kinetic parameters calculated from planktonic cultures be used in predicting the growth rate and extent of biofilm cells? Thus, the research objectives, hypotheses and methods of this study were designed to answer the above question.

Research Objectives

The objectives of this study were:

1) To produce experimental growth data of *Pseudomonas aeruginosa* in planktonic form.
2) To derive a multiple-substrate growth model for the planktonic cultures of *Pseudomonas aeruginosa.*
3) To measure dissolved oxygen concentration profiles in *Pseudomonas aeruginosa* biofilms using dissolved oxygen microelectrodes.
4) To calculate the coefficient of oxygen in *Pseudomonas aeruginosa* biofilms from the measured dissolved oxygen concentration profiles.
5) To compare the coefficient of oxygen for *Pseudomonas aeruginosa* in planktonic cultures and in biofilms.
6) To answer the question: Can we apply the growth kinetic parameters derived from planktonic cultures in biofilms?

Hypotheses

My hypotheses are:

1.) Glucose (also a limiting substrate to the growth of *Pseudomonas aeruginosa*) should be included in kinetic models to better describe the growth of planktonic cultures of *Pseudomonas aeruginosa.*

2.) The coefficient of oxygen in *Pseudomonas aeruginosa* colony biofilm is different from the half rate coefficient of oxygen in planktonic cultures.

Research Methods

A continuous system chemostat was used to measure glucose, dissolved oxygen concentrations and consumption rates, ammonium sulfate, and microorganism concentration at steady state. These results were used to derive multiple-substrate growth model for *Pseudomonas aeruginosa*. Chemostats have long been utilized in the laboratory to grow bacteria. The pH and the temperature in the chemostat were changed to calculate the optimum growth condition for *Pseudomonas aeruginosa*. Under steady state conditions, the dilution rate is equal to the growth rate of the bacteria. The experimental data collected at steady state, at optimum pH and temperature, and at different dilution rates were then used to develop a multiple substrate growth model for *Pseudomonas aeruginosa* using non-linear solution techniques.

For *Pseudomonas aeruginosa* biofilm, the coefficient for oxygen was extracted from dissolved oxygen concentration profiles measured with a dissolved oxygen microelectrode. Artificial *Pseudomonas aeruginosa* colony biofilms were grown on the surface of the black polycarbonate membrane filters placed on enriched agar. After the colony biofilms reached maturity, the dissolved oxygen concentration was measured using a dissolved oxygen microelectrode. These dissolved oxygen

concentration profiles were then used to extract the coefficients of oxygen in *Pseudomonas aeruginosa* colony biofilms.

CHAPTER 2

BACKGROUND

Physiology of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative, aerobic, rod-shaped, environmentally adaptable bacterium, belonging to the bacterial family *Pseudomonadaceae,* and comprise the informal group of bacteria known as **Pseudomonads**. *Pseudomonas aeruginosa* is an opportunistic pathogen of humans that causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia and a variety of systemic infections, particularly in the victims of severe burns, and in cancer and AIDS patients who are immunosuppressed.

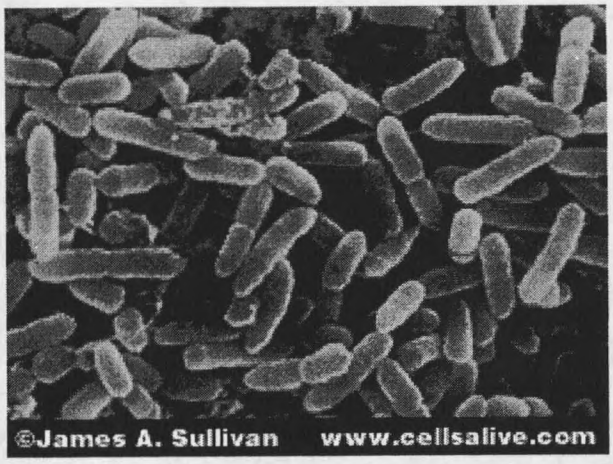

Figure 2.1. *Pseudomonas aeruginosa.*

The typical *Pseudomonas* bacterium in nature might be found living in a biofilm formed on some surface or in a planktonic form (free swimming cells). *Pseudomonas aeruginosa* is actively motile by means of a single polar flagellum (Hoiby et.al, 2001).

Pseudomonas aeruginosa isolates may produce three colony types. Natural isolates from soil or water typically produce a small, rough colony. Clinical samples, in general are present in two appearance forms, smooth and mucoid. The smooth form has a fried-egg appearance, which is large with flat edges and an elevated appearance. The mucoid form is attributed to the production of alginate slime. Both types of colonies are presumed to play a role in colonization and virulence. *Pseudomonas aeruginosa* produces two types of soluble pigments, pyoverdin (fluorescent) and pyocyanin (Schalk et. al., 2001).

Pseudomonas aeruginosa is an environment-adaptable bacterium, as this organism can be isolated from soils and water, particularly in wastewater treatment plants. *Pseudomonas aeruginosa* is often found in "distilled water" due to their ability to grow with minimal nutrition requirements (Tamagnini et. al, 1997). Moreover, this organism has a high tolerance to a wide variety of physical conditions; including temperature that contributes to its ecological success as an opportunistic pathogen (Brown et. al., 1990).

Microbial Growth

Microbial growth is defined as an increase in either cell numbers or total cell mass. The batch and continuous cultures are the two most common techniques used in microorganism growth kinetics studies.

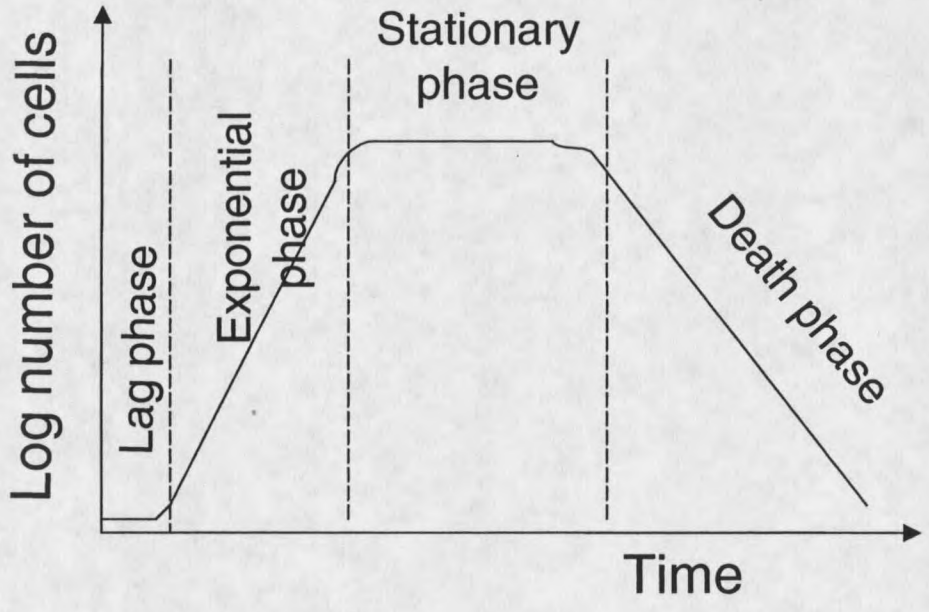

Figure 2.2. The growth curve for batch cell cultivation.

Batch Culture

When cells are inoculated into a reactor containing a fixed amount of nutrient medium is called a batch culture. In batch culture, the growth of microorganisms undergoes several characteristic phases; the lag phase, log phase (exponential phase), stationary phase and death phase (Figure 2.2). In lag phase, cell metabolism is directed towards synthesizing enzymes necessary for growth in the particular

medium. Thus, the lag phase is the longest phase if the inoculum consists of slow-growing cells or if the cells came from a medium of very different composition. Exponential phase is the period of growth, where cells undergo binary fission (a cell reproduce by splitting into two daughter cells) to logarithmically increase in population size. This explosive rate of growth cannot be maintained indefinitely if the amount of nutrients is limited. At some point, an essential nutrient is depleted or a toxic metabolic product accumulates to an inhibitory concentration. The growth rate slows down and the population size reaches a plateau in what is called the stationary phase of growth. The death phase begins at the end of stationary phase. The rapidly changing environment (due to either depletion of one or more essential nutrients or the accumulation of toxic by-products of growth) in batch culture results in unbalanced growth and changes in microbial metabolic activities (Shuler et. al., 1992).

The disadvantages of batch culture mentioned above complexify the computation of the growth kinetics model since the microbial growth kinetics is greatly affected by environmental conditions. Although it is possible to calculate the specific growth rate of the *Pseudomonas aeruginosa* using batch culture, the rapidly changing growth environment in batch culture require the investigators to make loose assumptions and generally produce results with high sum of square differences (Whitely et. al, 1997). Therefore, the continuous culture system such as chemostat is used to study the growth kinetics of *Pseudomonas aeruginosa*.

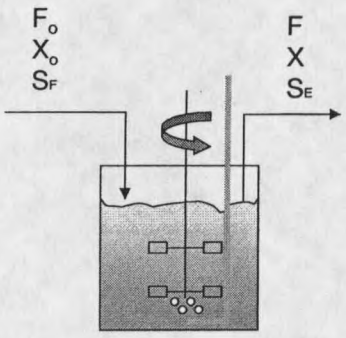

Figure 2.3. Continuous cultures.

Continuous Culture

Continuous culture is one of the alternative ways of growing microorganisms. It differs from a batch culture in that it is an open system, in which fresh medium is continuously fed, and growth medium and culture is removed so that a constant volume is maintained. In this system, cells grow exponentially for extended periods. The continuous culture system has the property of reaching steady state, in which the concentration of limiting nutrient and the cell number do not vary with time (Shuler et. al., 1992, Whitely et. al., 1997). At steady state, the growth rate (μ) of the cells in a continuous culture system is equal to the dilution rate (D), and dilution rate is equal to the ratio of volumetric flow rate (Q) to the reactor volume (V) as shown in equation 2.1 (Bailey and Ollis, 1986).

$$\mu = D = \frac{Q}{V} \tag{2.1}$$

Therefore, a continuous culture was used in this study to determine the growth-limiting substrate(s) for *Pseudomonas aeruginosa.* Since the cell density at steady state is controlled by the concentration of limiting nutrient in the inflow medium, only so much biomass can be constructed from a given amount of a nutrient. Hence, the growth limiting substrates (oxygen and glucose) can be determined by controlling the concentration of the growth limiting substrates (one at a time) of the inflow medium such that it is in relatively low concentration.

Microbial Metabolism for *Pseudomonas aeruginosa*

Metabolism is the term that pertains to all the chemical reactions in a cell. These chemical reactions can be divided into those that synthesize new cell material (anabolism) and those whose purpose is the release of energy from the chemical energy source (catabolism). These two categories are linked, in that catabolic reactions provide the energy necessary to drive the anabolic reactions that result in growth. Respiration, biosynthesis and maintenance are the main chemical reactions categories in the cells.

The overall general stoichiometric equation for microbial growth is given as the following.

$$\text{Carbon source} + \text{mo} + O_2 \rightarrow \text{mo} + CO_2 + H_2O \qquad (2.2)$$

Respiration

Respiration is an energy-producing process in which organic or reduced inorganic compounds are oxidized by inorganic compounds. The following equation, 2.3, shows the typical aerobic respiration using glucose as the carbon source or catabolite.

$$C_6H_{12}O_6 + O_2 + mo \rightarrow 6CO_2 + H_2O + mo \qquad (2.3)$$

Cellular respiration is divided into two phases. In the first phase, organic compounds are oxidized to CO_2, and pairs of hydrogen atoms (electrons) formed are transferred to Nicotinamide Adenine Dinucleotide (NAD), which occurs in Tricarboxylic acid cycle (TCA). The second phase, which is also known as the respiration chain, occurs when the hydrogen atoms combine with oxygen to form water.

Biosynthesis

Biosynthesis influences the substrate or nutrient utilization, cell growth, and product release and uses energy derived from respiration for the necessary processes in the cell. Equation 2.4 is the chemical reaction for biosynthesis.

$$5C_6H_{12}O_6 + 6NH_4^+ \rightarrow 6mo + 18H_2O \qquad (2.4)$$

Maintenance

Cellular maintenance is represented by the energy require for microorganisms to repair damaged cellular components, to transfer nutrients and products in and out of the cell, for motility, and to adjust the osmolarity of the cells' interior volume. Equation 2.5 shows the stoichiometric equation for cell maintenance.

$$mo + H^+ + 5O_2 \rightarrow 5CO_2 + NH_4^+ + 2H_2O \qquad (2.5)$$

Factors Affecting Growth

It is evident that the environment conditions such as the availability of a carbon source, oxygen, micronutrients, pH and temperature will affect the growth rate of *Pseudomonas aeruginosa*. In order to determine the growth limiting substrate(s), one must ascertain the factors affecting microbial growth that are explained briefly below, for detail please refer to Atkinson and Mavituna (1991).

Carbon Source

Carbon compounds are major sources of cellular carbon and energy. Microorganisms are classified in two categories on the basis of their carbon source; *Heterotrophs* and *Autotrophs*. *Heterotrophs* use organic compounds such as carbohydrates, lipids, and hydrocarbons as a carbon and energy source. *Pseudomonas aeruginosa* uses carbohydrates such as glucose as the carbon source for

growth. Therefore, *Pseudomonas aeruginosa* is classified as *heterotrophs*. In this study, the carbon source, glucose, is the electron donor.

Oxygen

In most cases, microorganisms need oxygen for respiration and biosynthesis. Oxygen is present in all organic cell components and cellular water, and constitutes about 20% of the dry weight of the cells. Oxygen affects the redox potential during the oxidation of substrate, generating energy from respiration that is necessary to drive biosynthesis reactions in cells. During oxidation of substrate catalyzed by oxidase, electrons are released and transported out across the cytoplasmic membrane to the terminal electron acceptor – oxygen. Oxygen is the terminal electron acceptor under aerobic conditions. The transfer of electrons across the cytoplasmic membrane establishes a proton gradient that causes a diffusive counterflow of protons across the membrane. This proton counter-flow drives the synthesis of Adenosine Triphosphate (ATP) from Adenosine Diphosphate (ADP) and inorganic phosphate. ATP is the energy source for biosynthesis in microorganisms.

Nutrients and Micronutrients

The availability of nutrients is an important requirement in sustaining biological cell growth. For biosynthesis to occur, nutrients are considered as those elements that are required in large amounts (C, H, O, N, P, and S) and various minerals that are required in minor amounts (K, Na, Mg, Ca and Fe). Nutrients are required by microorganisms to synthesize cell material, protein and nucleic acids.

Nutrients are also needed by microorganisms to stabilize ribosomes, cell membrane and cell wall for the activity of many enzymes.

Micronutrients consist of trace amounts of certain metals (Mn, Zn, Cu, Co, Ni and Mo). Although are required in tiny amounts, micronutrients are as critical as nutrients for microbial growth. Manganese serves as enzyme activator, Zinc as structural role for many enzymes, Cu plays a role in certain enzymes for respiration, Co is needed for vitamin B_{12} formation, Ni is present in the enzyme, *hydrogenases,* that functions to take up or to evolve H_2 and Mo is needed for nitrate reduction assimilatory.

pH

pH affects the growth of a microorganism in such a way that it can change the side chains of the amino acids that make up the enzymes. The side chains of the amino acids may possess basic, neutral or acidic groups. As a result, at any given pH, the intact enzyme may contain positively or negatively charged portions of the active site of the specific enzyme. Consequently, pH can directly affect the ionization state of the active site of the specific enzyme, and the activities of the microorganisms. Therefore, the fraction of catalytically active enzyme to the total enzyme present in the cell greatly depends on the pH. The optimum pH for cell growth varies with the microorganism, as it is based on the specific enzyme mechanism of the microorganism. The optimal pH is likely to be around pH 7, because that is the optimal pH for most of the physiological functions.

Temperature

In chemical reaction kinetics study, the growth kinetic constant is strongly dependant on temperature and could be related to the following Arrhenius equation (2.6).

$$k(T) = Ae^{-E/RT} \qquad (2.6)$$

The optimum temperature for cell growth varies with microorganisms as cells may have different forms of enzymes present, for example, complexes of the enzyme, ionization states, etc.

Microbial Growth Kinetics

Growth kinetics models are the mathematical expressions for the rates of enzyme-catalyzed reactions in the cell. These mathematical equations represent the behavior of the microorganisms and are derived from experimental data. The final growth kinetic models are derived based on all of the factors that influence the growth of microorganism. Including, taking into account the spatial distribution of the microorganisms by observing the growth and decline in cell number as a whole under constant experimental conditions. Therefore, it is important for the investigators or engineers to understand the factors affecting the growth of a specific microorganism through a series of experiments to determine the rate limiting steps. In order to determine the rate limiting steps, it is also important to use an appropriate laboratory

reactor that would provide constant environmental conditions for the cells growth, such as a chemostat.

Cell Growth

A general cell growth balance for microorganisms grown in a chemostat is as the following equation 2.7.

$$\frac{dX}{dt} = \mu X \tag{2.7}$$

Substrate Consumption

The mass balance for microbial growth in a chemostat is equal to the following equation 2.8.

$$D(S_{fi} - S_{ei}) = \frac{\mu X}{Y_{X/Si}} + m_i X \tag{2.8}$$

Single Substrate Growth Kinetics

Several mathematical models have been developed for the effect of a single substrate on the growth rate of a microorganism. Generally, these mathematical models are the adaptations of equations for substrate utilization or inhibition in enzymatic reactions.

Monod (1949) proposed that the effect of substrate concentration on specific growth rate could be governed by the equation 2.9.

$$\mu = \mu_{max} \frac{S}{K_{SM} + S} \tag{2.9}$$

The assumption of diffusion-controlled substrate supply leads to equation 2.10, which was originally derived by Tessier (1942);

$$\mu = \mu_{max} (1 - e^{\frac{S}{K_{ST}}}) \tag{2.10}$$

Mozer (1949) presented equation 2.11 for the specific growth rate.

$$\mu = \mu_{max} (1 + K_{SMz} S^{-\lambda})^{-1} \tag{2.11}$$

Contois (1952) suggested equation 2.12, where the constant in the Monod equation should be proportional to the microorganism concentration. According to Contois' equation, the specific growth rate of a microorganism decreases with increasing microorganism concentration.

$$\mu = \mu_{max} \frac{S}{BX + S} \tag{2.12}$$

Andrews (1968) proposed that the effect of substrate concentration on specific growth rate could be governed by equation 2.13.

$$\mu = \mu_{max} \frac{S}{(K_{SA} + S)(1 + \frac{S}{K_{iA}})} \tag{2.13}$$

Equation 2.13 has been used by several researchers to provide adequate fit plots of experimental data for growth at high substrate levels.

Later in 1970, Edwards proposed several equations that were adapted from enzyme kinetics to correlate the effect of substrate inhibition to the microorganism specific growth rate.

$$\mu = \mu_{max} \frac{S(1 + S/K_{SE})}{K_{SE} + S + \frac{S^2}{K_{iE}}} \tag{2.14}$$

$$\mu = \mu_{max} \frac{S}{K_{SE} + S + \frac{S^2}{K_{iE}}(1 + S/K_{SE})} \tag{2.15}$$

Edwards also suggested that the exponential relation, proposed by Aiba et. al. (1968), could be used to correlate substrate inhibition to microorganism growth.

$$\mu = \mu_{max} \frac{S}{K_{SE} + S} e^{\frac{S}{K_{iE}}} \tag{2.16}$$

However, when $S/K_i << 1$, equation 2.16 becomes equivalent to the Monod equation, and both of these equations approach equation 2.17 by a Taylor series analysis.

$$\mu = \mu_{max} \frac{S}{K_{SE} + S} (1 - \frac{S}{K_{iE}}) \tag{2.17}$$

Edwards also proposed equation 2.18, which showed that microorganism specific growth rate could be governed by diffusion limitation of high and inhibitory substrate concentrations.

$$\mu = \mu_{max} (e^{\frac{S}{K_{iE}}} - e^{\frac{S}{K_{SE}}}) \tag{2.18}$$

Later in 1986, Luong proposed a generalized nonlinear power equation for substrate inhibition that was adapted from Levenspiel's (1980) proposed equation for product inhibition.

$$\mu = \mu_{max} \frac{S}{K_{SL} + S}(1 - \frac{S}{S_m})^n \qquad (2.19)$$

Tseng and Wayman (1975, 1976) showed that microorganisms could grow under the threshold concentration, S*, without inhibition and could be represented with equation 2.20.

$$\mu = \mu_{max} \frac{S}{K_{STW} + S} - c(S - S^*) \qquad (2.20)$$

Equation 2.20 becomes the Monod equation when $S < S^*$, but equation 2.20 has to be used to calculate specific growth rate when $S > S^*$.

Multiple Substrate Growth Kinetics

Microbial growth depends on the concentration of more than one substrate; here multiple substrate kinetics is often observed. Usually a single substrate proves to be limiting and the Monod kinetic equation or a similar model is adequate to describe the behavior of the system. However, microorganisms metabolize several substrates and nutrients simultaneously. Therefore, multiple substrate models must be taken into consideration when microbial growth is limited by more than a single substrate. There are three types of multiple-substrate growth kinetic models; interactive, additive and non-interactive.

Interactive, also known as multiplicative form:

$$\mu/\mu_{max} = [\mu(S_1)][\mu(S_2)].....[\mu(Si)] \quad (2.21)$$

Additive form:

$$\mu/\mu_{max} = [\mu(S_1)] + [\mu(S_2)] + ...+ [\mu(Si)] \quad (2.22)$$

Non-interactive form:

$$\mu/\mu_{max} = [\mu(S_1)] \text{ or } [\mu(S_2)] \text{ or } ..... \text{ or } [\mu(Si)] \quad (2.23)$$

The determination of the growth kinetic model is based on which substrate is the most limiting.

Diffusion and Reaction in Biofilm

A biofilm is a biologically active population of microorganisms that are attached to a surface and enclosed by an extracellular matrix (Christensen, 1990; Costerton, 1995). Brown et. al., (1990), and Flectcher et. al., (1983), reported that the growth kinetic parameters of biofilm cells are different from the growth kinetic parameters for planktonic cells. Bakke et. al., (1984), showed that there was no difference between biofilm cells and planktonic cells. The two transport processes, diffusion and reaction, produce concentration gradients in the biofilm and can be represented by the following equation 2.24

$$\frac{dS_i}{dt} = D_e \frac{d^2S_i}{dz^2} - Rr \quad (2.24)$$

At pseudosteady state ($dS_i/dt = 0$), equation 2.24 becomes the ordinary differential equation 2.25.

$$D_e \frac{d^2 S_i}{dz^2} = Rr \tag{2.25}$$

The boundary conditions for equation 2.25 are:

$$@\ z = 0 \text{ (Substratum)} \qquad \frac{dS_i}{dz} = 0 \tag{2.26}$$

$$@\ z = L_f \text{ (Biofilm surface)} \qquad S_i = S_s \tag{2.27}$$

Generally, equation 2.26 is formed based on the physical condition of the inert support material of the reactor, while equation 2.27 is the experimental condition and is dependent on the experimental measured concentration profile in biofilms.

CHAPTER 3

MATERIALS AND METHODS

Microorganism and Culture Conditions

A pure cultures of *Pseudomonas aeruginosa* (ATCC# 700829), which were environmental isolates from the stock at the Center for Biofilm Engineering was used as the inoculums in the chemostat and to grow the colony biofilms.

Procedures for Stock Culture

1. Prepared 100 mL growth medium in 6 culture flasks. Then inoculated each flask with 1 mL of the microorganism.
2. Prepared cryogenic vials (Fisher®, Denver, Co) with label.
3. Then, prepared and vacuum filtered a 20% glycerol solution.
4. Pipette 25 mL of culture into centrifuge vials and centrifuge for 15 minutes. (6000 rpm)
5. Poured off the supernate and added 125 mL of both growth medium and 20% glycerol into the centrifuge vials.
6. After that, mixed well with vortex.
7. Finally, pipette 1 mL culture solution into cryogenic vials.
8. The stock culture was stored in the –70°C freezer.

Planktonic Culture Conditions

One ml of the stock culture was inoculated into each 250 ml Pyrex® flasks containing 100 ml of growth medium for 24 hrs as a batch culture. The flasks were placed onto the shaker set at 150 rpm in order to make sure that the growth medium was well mixed and aerated. The batch culture of *Pseudomonas aeruginosa* was then transferred and inoculated into the chemostat. The volume of the inoculums used was 10% of the volume of the reactor.

Growth Medium

All the experiments were conducted with an artificial growth medium as shown in Table 3.1.

Table 3.1. The composition of the growth medium.

Chemical compound	Final concentration, g/L
Na_2HPO_4	1.83
K_2HPO_4	0.35
$MgSO_4 \cdot 7H_2O$	0.01
yeast extract	0.1
$(NH_4)_2SO_4$	0.1-1
glucose	5 -30

The concentration of glucose and ammonium sulfate ($(NH_4)_2SO_4$) varied between 5-30 g/L and 0.1-1 g/L, respectively, according to the experimental conditions. Also, 1 mL of trace elements was added into the growth medium for every 1-liter of growth medium. Trace elements were added into the sterile growth medium by using a disposable sterile syringe filter (0.2 μm, Corning). The trace elements consisted of the following compounds shown in Table 3.2

Table 3.2. The trace element concentrations in 1 liter of 0.1M HCl.

Chemical compositions	Final concentration, mg/L
$MnCl_2 \cdot 4H_2O$	527
$CuCl_2 \cdot 2H_2O$	228
$CoCl_2 \cdot 2H_2O$	317
$(NH_4)Mo_7O_4 \cdot H_2O$	231
$Na_2B_4O_7 \cdot 10H_2O$	127
$ZnCl_2$	363
$FeCl_3$	2160
$CaCl_2$	3700

The growth medium for every set of experiments was prepared with distilled water and sterilized in the autoclave at 121°C and 1 atm absolute pressure for 3 hours. Glucose, yeast extract and $(NH_4)_2SO_4$ were prepared and autoclaved separately.

Experimental Setup for Planktonic Culture – Chemostat

All continuous system experiments were carried out in a New Brunswick (BioFlo 2000) chemostat with a working volume of 2 L and equipped with pH, agitation, temperature, and dissolved oxygen control units. Figure 3.1 shows the chemostat used in this study. The chemostat was autoclaved prior to use, for 30 minutes at 121°C and 1 atm including growth medium but without glucose ammonium chloride and trace elements. The glucose, ammonium chloride and trace elements were added prior to inoculation. The sensitivities of the control units for dissolved oxygen, pH and agitation rate were 0.1%, 0.1 unit and ±1 rpm respectively. The pH of the growth medium was controlled by adding sterile 0.1 N NaOH and 0.1 N H_2SO_4 solutions. The agitation rate was altered between 150 - 550 rpm to maintain a homogeneous culture. The dissolved oxygen concentration was controlled in the range of 0.5 - 7.2 mg/L by sparging filtered air or air + pure oxygen through the chemostat. The airflow rate was controlled between 1 - 5 L/h. For setting up the continuous system, the inoculums cultures was inoculated into the chemostat containing the growth medium, and it was ran as batch cultures before it was ran as continuous cultures.

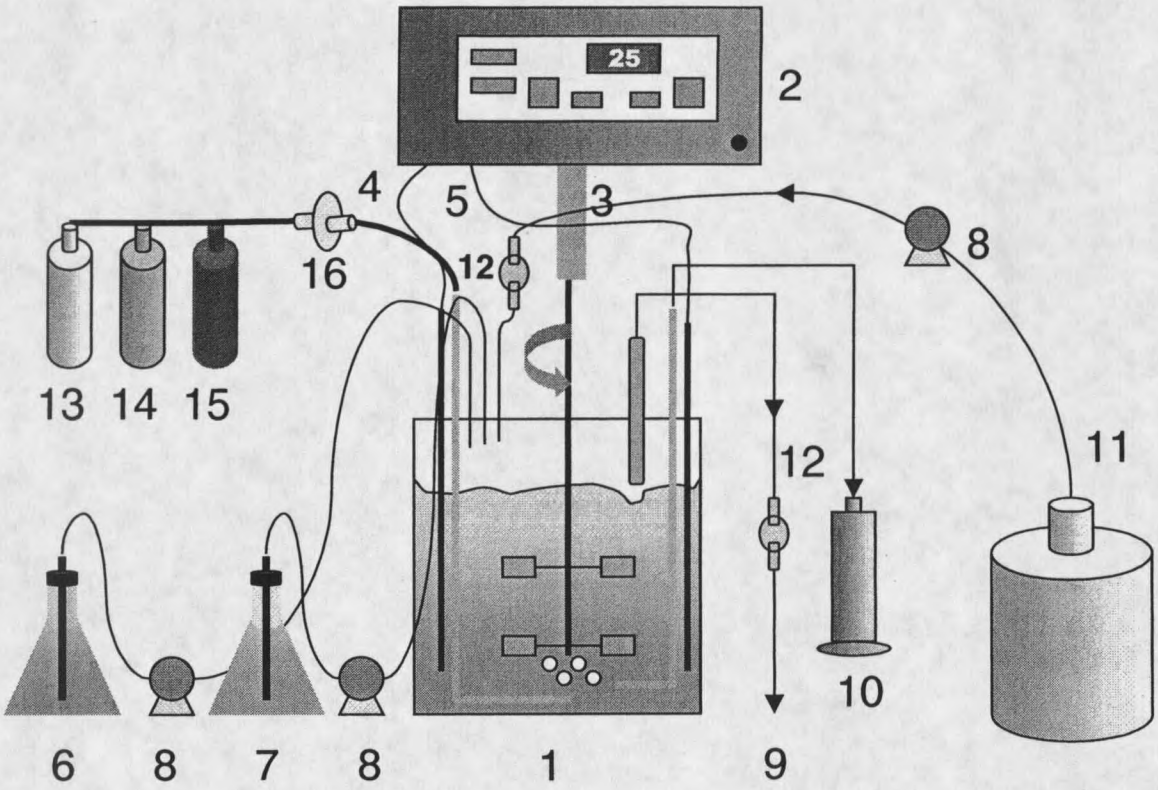

Figure 3.1. Planktonic cultures experimental setup.

1. Chemostat; 2. Controller; 3. Agitation unit; 4. pH electrode; 5. Dissolved oxygen electrode; 6. Base; 7. Acid; 8. Pump; 9. Output (waste); 10. Sampling syringe; 11. Fresh feed; 12. Flow breaker; 13. Air; 14. Nitrogen; 15. Oxygen; 16. Bacterial air filter.

All the continuous experiments were carried out at 25°C with different combinations of influent substrate concentration, medium pH, agitation rate and temperature. Continuous pumping of fresh feed was started after the culture had entered the exponential growth phase (20 – 40 h). In order to establish a steady state, the reactor was left to equilibrate over six to seven retention times and the steady state was assumed if the absolute differences in consecutive measurements of the substrate concentration differed by less than 3%. Several dilution rates up to the washout point were applied and corresponding steady state data were recorded. For a new steady state, the dilution rate was increased carefully by a gradual increase in the feed rate.

Analytical Methods

For each set of experiments, the concentration of the microorganism, glucose, ammonium, dissolved oxygen (DO), pH, temperature, oxygen consumption rate (OCR), glucose consumption rate (GCR), and ammonium consumption rate (NCR) were measured at steady state.

Microorganism Concentration (X)

The microorganism concentration was determined using a standard dry-weight method (APHA, 1962) and optical density measurement. The procedures were as follows:

Standard Dry-weight Procedures

1. 50 mL of sample solution was collected from the 2 L chemostat in a 50 mL centrifuge tube.
2. The above procedure was repeated until a total of 400 ml of sample solution was collected.
3. Each tube was labeled.
4. The samples were centrifuged at 6000 rpm at 4°C for 25 minutes.
5. Then, the supernate was gently poured away in order to avoid detachment of the pellets and cause the loss of microorganisms.
6. Next, approximately 10 mL of water was added into each of the 50 mL centrifuge tubes containing the pellets.
7. The new suspension was vortexed and mixed well to disperse the microorganisms in the water.
8. The liquid containing the microorganism was then poured into the next 50 mL centrifuge tube.
9. Vortex to obtain a well-mixed solution.
10. Steps 7 - 9 were repeated for each sample until all the biomass was collected in **ONE** 50 mL centrifuge tube.
11. Then, steps 5 - 9 were repeated in reverse direction at least 4 times, in order to make sure that all the biomass was collected into one tube.
12. A 100 mL beaker was pre-dried at 105°C for 10 minutes and weighed on the analytical balance and the mass was recorded into the notebook.
13. Finally, the biomass solution was added into the beaker and the total weight of

the beaker and solution was recorded into the notebook.

14. The beaker and cells were left overnight and the weight of the beaker was taken at least 3 times to obtain the average of the total dried-weight.
15. The dried-weight of the biomass was calculated and recorded into the notebook.

Optical Density Measurement

1. 50 mL of sample solution was collected from the 2 L chemostat in a 50 mL centrifuge tube.
2. The sample was centrifuged at 6000 rpm at 4°C for 25 minutes.
3. Then, the supernate was gently poured away in order to avoid detachment of the pellets and cause the loss of microorganisms.
4. Next, approximately 50 mL of water was added into each of the 50 mL centrifuge tubes containing the pellets.
5. The new suspension was vortexed and mixed well to disperse the microorganisms in the water.
6. The sample was again centrifuged at 6000 rpm at 4°C for 25 minutes.
7. The sample was re-suspended with vortex and mixed well.
8. A series of dilution of the samples were prepared and the absorbance for each of the dilution sample was measured.
9. The concentration of the microorganism for each of the dilution sample was calculated.
10. The correlation between microorganism concentration and absorbance was

presented in Figure 3.2.

11. This calibration graph was then used to measure the concentration of microorganisms in the chemostat.

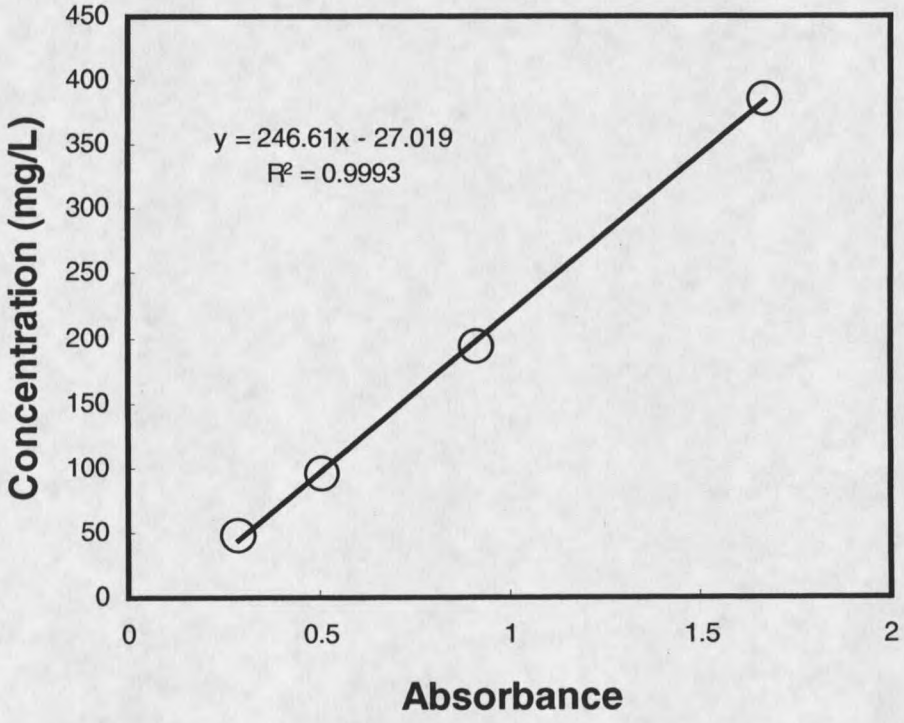

Figure 3.2. Microorganism concentration calibration graph.

Glucose Concentration

The glucose concentration in the chemostat was measured using Sigma® procedure 510 (Sigma® Diagnostics, ST.Loius, MO). The following was the procedure for the glucose concentration measurement.

Combined Enzyme-Color Solution Preparation

1. The **enzyme solution** was prepared by dissolving contents of one capsule of PGO enzymes in 100 mL DI water in an amber bottle.
2. The bottle was inverted several times with gentle shaking to dissolve the PGO enzymes.
3. The **color reagent solution** was prepared by reconstituting one vial of o-Dianisidine Dihydrochloride with 20 mL DI water.
4. Then, combined enzyme-color reagent solution was prepared by combining 100 mL of enzyme solution and 1.6 mL of color reagent solution.
5. The solution was mixed well by inverting several times with mild shaking
6. The enzyme solution, combined enzyme-color reagent solution, and standard glucose solution were stored in refrigerator (2 - 8°C).

Glucose Concentration Measurement Procedures

1. 50 mL of sample solution was collected from the 2 L chemostat in a 50 mL centrifuge tube.
2. The sample solution was centrifuged at 6000 rpm, 4°C for 20 minutes.

3. The liquid solution was poured into a clean 50 mL plastic container.
4. Prepared a standard as the reference
    a. 25 µL **Standard glucose solution** (1000 mg/L)
    b. 0.5 mL DI H_2O
    c. 5 mL Combined Enzyme Reagent
5. Prepared the test solution for sample
    a. 25 µL **Sample solution**
    b. 0.5 ml DI H_2O
    c. 5 mL Combined Enzyme Reagent
6. Then, both the standard and sample were incubated at 37°C in a water bath for 30 minutes.
7. Next, the absorbance of the standard and the sample at 420 nm was measured. The 420 nm was chosen because the maximum absorbance was observed at 420 nm.
8. Finally, the concentration of glucose was calculated from the glucose calibration equation.

$$\text{Glucose Concentration} = \frac{\text{Sample Absorbance}}{\text{Standard Absorbance}} \cdot 100 \qquad (3.1)$$

Ammonium Concentration

Ammonium sulfate concentration was measured by using a Hatch® ion selective electrode (Loveland, CO). The following were the procedures for ammonium concentration measurements.

Ammonium Standard Solution Calibration

1. Prepared a series of ammonium solutions of known concentration: 200 mg/L, 100 mg/L, 50 mg/L, 25 mg/L, 12.5 mg/L, 6.25 mg/L, and DI water.
2. One packet of ammonia ionic strength adjustor powder pillow was added into each of the ammonium solution.
3. The voltage (mV) of each known concentration ammonium solution was recorded into notebook. The voltage of the solution was measured using ammonium electrode attached to a voltage meter.
4. Graph the recorded values as log ammonium concentration (mg/L) vs. Voltage (mV) in order to observe the maximum voltage for the ammonium solution.

Ammonium Concentration Determination

1. A 25 mL of sample solution was added into a small beaker containing a magnetic stir bar.
2. The contents of one packet of ammonia ionic strength adjustor powder pillow were added into the sample solution.
3. The voltage of the solution was measured using ammonium electrode attached

to a voltage meter.

4. Ammonium concentration was calculated based on the ammonium concentration calibration equation.

Dissolved Oxygen Concentration

Dissolved oxygen was monitored by an Ingold® dissolved oxygen electrode that was integrated to the chemostat. The dissolved oxygen concentrations were controlled by a control unit that is attached to the chemostat as shown in Figure 3.1.

Oxygen Uptake Rate (OUR)

The oxygen consumption rate was measured using a dynamic method suggested by Bandyopdhyay et al. (1967). In this method, the overhead volume of the chemostat is flashed with nitrogen gas to remove the oxygen. The drop of dissolved oxygen concentration in the reactor was recorded against time and the value of $-dS_o/dt$ at the linear region yields the oxygen-consumption rate of the culture per unit volume. Thus, the term $(-dS_o/dt \cdot V)/(X)$ gives the specific oxygen consumption rate as shown in equation (3.3).

$$OUR = \frac{d(S_o)}{dt} \cdot V \tag{3.2}$$

$$SOUR = \frac{OUR}{X} \tag{3.3}$$

Example OUR Calculation:

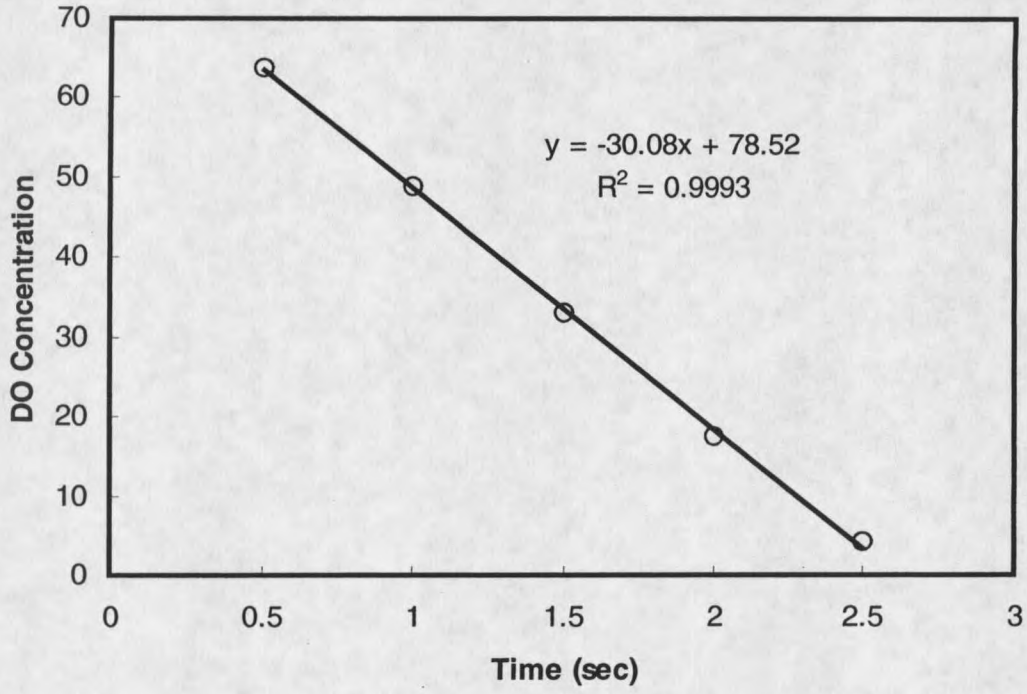

Figure 3.3. The change of dissolved oxygen concentration in the reactor over time.

From Figure 3.3,

slope = 30.08

$$\frac{dS_o}{dt} = \text{slope} \cdot \frac{7.2}{100}$$

$$OUR = \frac{dS_o}{dt} \cdot V$$

Ammonium Consumption Rate (NCR)

The ammonium consumption rate was calculated by using equation (3.4) and the specific ammonium consumption rate (SNCR) was calculated by dividing the NCR by the microorganism concentration (X).

$$NCR = (S_{Nf} - S_{Ne}) \cdot Q/V \tag{3.4}$$

$$SNCR = \frac{NCR}{X} \tag{3.5}$$

Glucose Consumption Rate (GCR)

The glucose consumption rate was calculated by using equation (3.6) and the specific consumption rate was calculated by dividing the GCR by the microorganism concentration (X).

$$GCR = (S_{Gf} - S_{Ge}) \cdot Q/V \tag{3.6}$$

$$SGCR = \frac{GCR}{X} \tag{3.7}$$

pH

pH for the sample solutions were measured using a pH meter. The following procedures were used to calibrate the pH meter and to measure the pH of the sample solutions at steady state.

1. The pH meter was calibrated with standard pH buffer solution at pH 4 and 7

for pH lower than 7. For pH higher than 7, the pH was calibrated with standard pH buffer solution at pH 11 and pH 7.

2. Measured and recorded the pH of the sample solution.

Temperature

The temperature was measured and controlled with the control unit attached to the chemostat as shown in Figure 3.1. The temperature was recorded for every set of measurements.

CHAPTER 4

MATHEMATICAL MODELING

Growth Kinetic Estimation for Planktonic Cells

Multiple substrate growth kinetics can be developed in such a way that each of the single substrate models (Equations 2.9 - 2.20) are combined in a manner as described by Equations 2.21 - 2.23 in order to obtain equations that are consistent with the experimental data (Shuler et al., 1992).

To develop growth models for multiple substrates, the specific growth rate, μ, can be calculated from Equation 2.1 ($\mu = D$) for each steady state. Different growth models, based on single substrate (Equations 2.9 - 2.20) are then inserted into Equation 2.21 or, 2.22, or 2.23 to find the best multiple substrate model. From the same data, the maintenance factor, m_i, and yield factor, $Y_{x/si}$, are then calculated using the substrate consumption equation (2.8).

Non-linear Regression

Microsoft Excel's 2000® Solver® can be used to estimate the growth kinetic parameters from the experimental data. The program solves non-linear regression problems using Newton's method (Larson et al., 1978). The equations are solved to find values of the growth kinetic parameters that minimize the objective function, the

sum of squares of the differences (SSD) between experimental and theoretical data for specific growth rates, as given by Equation 4.1. The goal of this method is to reduce the objective function while staying "on" a subset of the nonlinear constraints. The objective is achieved by adjusting the variables so that the active constraints can be satisfied exactly at each trial point.

$$SSD = \sum_{i=1}^{N} (\mu_{experimental} - \mu_{model})^2 \qquad (4.1)$$

To calculate the maintenance and yield factors (Equation 2.8), the objective function is described as the sum of squares of differences between the substrate consumption rates, which are experimentally measured and theoretically estimated, separately for each of the limiting substrates. Because the solution is sensitive to initial guesses, the search is constrained to a predetermined range, determined as the range of growth kinetic constants for bacteria reported in literature and shown in Table 4.1 (Luong 1982; Beyenal and Tanyolac 1997).

Table 4.1. The search range for growth kinetic constants of *Pseudomonas aeruginosa* in non-linear regression analysis.

Constants	Allowable range
μ_{max} (h^{-1})	0 - 1
K_g (mg/L)	0 - 1000
K_o (mg/L)	0 - 7.2

Best Kinetic Model

The growth kinetic parameters are calculated as described above using the non-linear regression technique and the selected multiple-substrate growth kinetics. The best multiple substrate growth model is then selected from among the different combinations of Equations 2.9 - 2.13 that gives the minimum sum of squares of differences (SSD) between the experimental data and the model solutions.

CHAPTER 5

RESULTS AND DISCUSSIONS

Optimum Operating Conditions

In aerobic systems, dissolved oxygen acts as the electron acceptor during substrate oxidation (Pirt, 1975). Research on suspended cell cultures has revealed that the specific oxygen uptake rate (SOUR) (g O_2 /g dry biomass h^{-1}) is proportional to microbial activity, which makes it suitable as an indicator of microbial activity (Syrett, 1958; Harris, 1979; Volesky et al., 1982; Siegmund and Diekmann, 1989). Therefore, SOUR was selected to determine the optimum operating conditions in the chemostat.

Figure 5.1 shows SOUR for different agitation rates in the chemostat. It was expected that at low agitation rates the growth of the microorganisms was limited by external mass transport. Therefore, when the agitation rates increased, the mass transfer rate to the microorganisms increased, along with the SOUR, which reached a maximum value. Increasing the agitation rate beyond this maximum value actually decreased the SOUR, probably because the agitation was injuring the microorganisms. Based on the results in Figure 5.1, 350 rpm was selected as the working agitation rate. Microscopic examinations showed that the microorganisms were distributed uniformly in the chemostat, and neither flocculation nor agglomerations were observed.

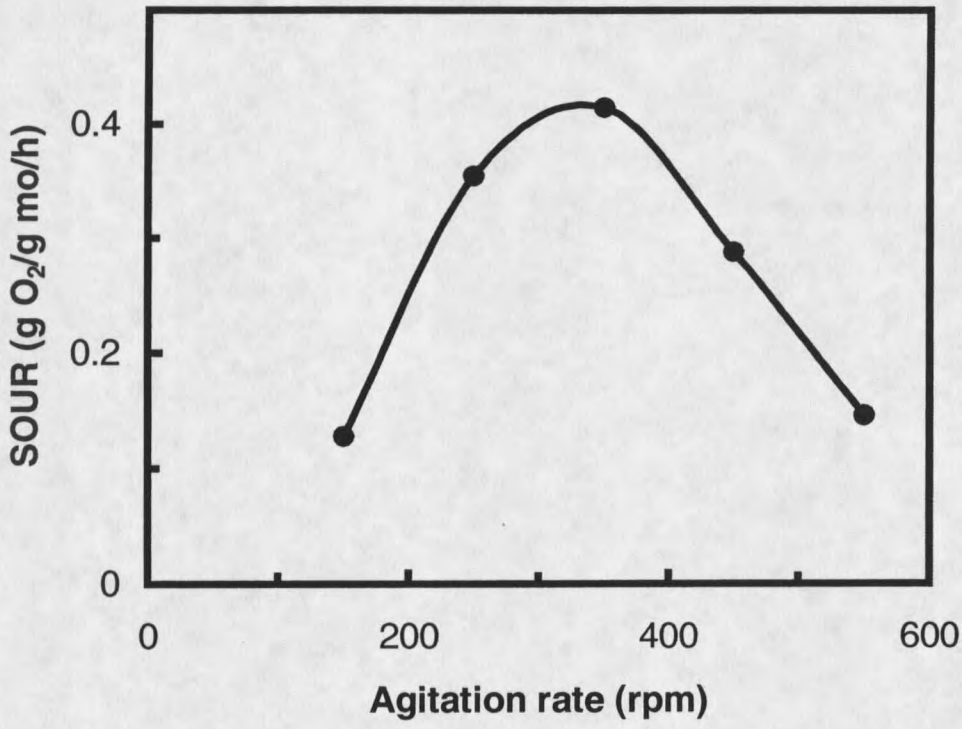

Figure 5.1. The SOUR for different agitation rates in the chemostat ($D = 0.124\ h^{-1}$, pH = 7.2, T =25 C^{o}, $S_{fg} = 5$ g/L, and $S_{fn} = 0.1$ g/L).

The effect of pH on SOUR (Figure 5.2) was measured. The SOUR increased, reached a maximum value, and then decreased. The optimum pH of 7.2 was used in all runs. At pH's lower than 7.2, the activitities of ATPase had increased and caused the flow of protons out of the cell. Subsequently, the SOUR was lower at pH's lower

than 7.2. For pH's higher than 7.2, the tendency of protons to return to the cells was lower due to the collapse of the proton-motive force. Therefore, the synthesis of ATP for biosynthesis was reduced, hence, the SOUR decreased.

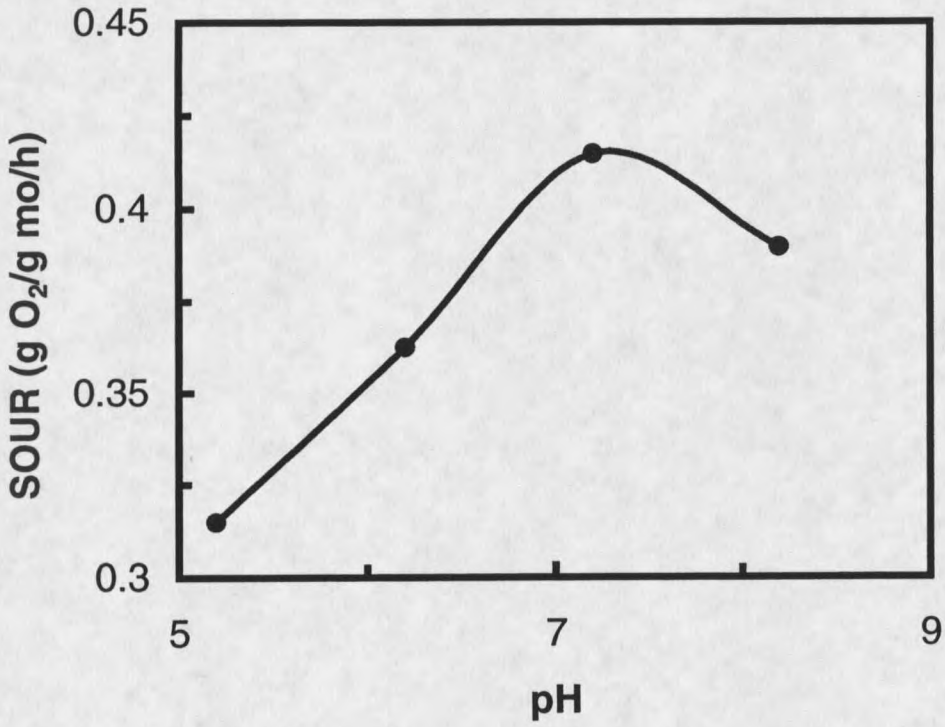

Figure 5.2. The effect of pH on SOUR in the chemostat (D = 0.124 h^{-1}, agitation rate = 350 rpm, T =25 C^o, S_{fg} = 5 g/L, and S_{fn} = 0.1 g/L).

The data shown in Table 5.2 was collected at steady states, and was used for modeling of the growth kinetics.

Table 5.1. The results of steady state experiments (T = 25°C, pH = 7.2, agitation rate = 350 rpm, S_{fg} = 5 g/L, S_{fn} = 0.1 g/L).

D (h^{-1})	S_o (mg/L)	S_g (mg/L)	X (mg/L)	OUR (mg oxygen/h)
0.03	1	5	1725	323
0.04	7.2	3.8	3000	424
0.0556	0.5	19.4	2381	581
0.069	7.2	7.1	2820	719
0.118	0.8	45.7	3150	1212
0.124	7.2	15.1	3070	1273
0.162	7.2	22.7	3225	1658
0.18	7.3	255	2820	1948
0.187	1.5	69.4	3285	1912
0.24	6.6	255	2760	2565
0.24	2.5	87.4	3105	2440
0.275	3.3	112.7	3090	2799
0.299	5.4	217	2850	2599
0.325	5.6	154.9	3045	3306

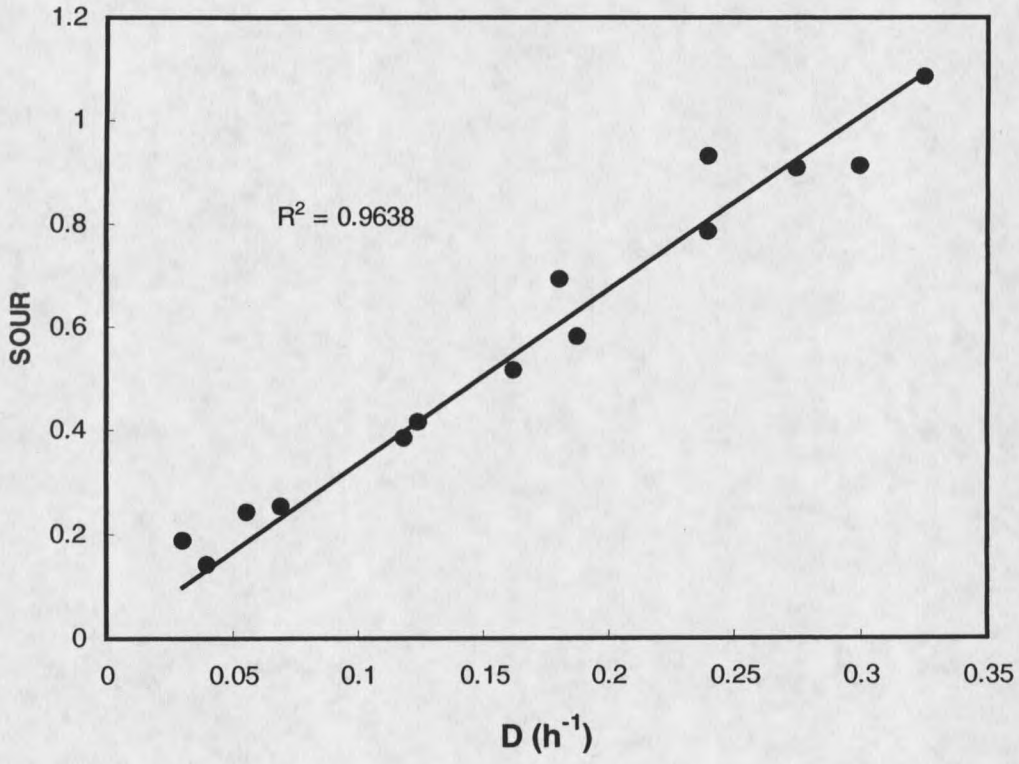

Figure 5.3. The effect of dilution rates on SOUR for *Pseudomonas aeruginosa*.

Figure 5.3 shows that the SOUR for *Pseudomonas aeruginosa* increased as the dilution rates ($D = \mu$) increased in the reactor.

Modeling Planktonic Growth Kinetics

In addition to the data in Table 5.1, the growth-limiting substrates and their interaction(s) were also tested. According to these results, the growth rate of *Pseudomonas aeruginosa* in the absence of oxygen or glucose was negligible. Furthermore, changing the NH_4^+ concentration in the feed did not affect significantly the effluent concentrations of glucose, oxygen, and microorganisms (Table 5.2). These observations, combined with the results in Table 5.1, demonstrated that glucose and oxygen influenced the growth kinetics of *Pseudomonas aeruginosa*. Hence, the growth of *Pseudomonas aeruginosa* should be represented by a kinetic expression taking into account the dual-substrate limitations of oxygen and glucose combined.

Table 5.2. Concentration of Glucose, Oxygen and Microorganisms at different Ammonium Sulfate feed concentrations.

Sn-feed mg/L	D 1/h	x mg/L	So mg/L	Glucose mg/L
100	0.36	126.8839	4.4148	271.6792
500	0.36	120.1805	3.0225	248.1188

As shown in Appendix C that none of the growth models using substrate inhibition kinetics well represent the growth data of *Pseudomonas aeruginosa.* Therefore, only Equations 2.9 - 2.12 were used to obtain the best kinetic model. Equations 2.9 - 2.12 were combined in such a manner as described in section 4.1. Table 5.3 shows possible growth models using combinations of Equations 2.9 - 2.12, calculated growth kinetic parameters, SSD, and regression coefficients (R^2). The minimum SSD were found for models #12 and #17, with R^2 between 0.96 – 0.97. Model #17 combined the Mozer and Tessier kinetics. However, according to the Mozer kinetics (model # 17), *Pseudomonas aeruginosa* should grow in the absence of the oxygen, which contradicts experimental results. Consequently, the double Tessier kinetics was selected as the best growth model.

$$\mu = \mu_{max}\left(1 - e^{\frac{So}{K_{oT}}}\right)\left(1 - e^{\frac{Sg}{K_{gT}}}\right) \qquad (5.1)$$

For the selected model, the growth kinetic parameters were μ_{max} = 0.29 h^{-1}, K_{gT} = 26.9 mg/L, K_{oT} = 1.18 mg/L, $Y_{x/g}$ = 0.628 g biomass/g glucose and, $Y_{x/o}$ = 0.635 g biomass/g oxygen. Maintenance factors were m_g = 0.0078 g glucose consumed/g microorganism·hour, and m_o = 0.014 g oxygen consumed/g microorganism·hour. Figure 5.4 shows the growth rates, measured and model, from Equation 5.1. The high correlation coefficient (0.97) demonstrated that the growth

model (Double Tessier kinetic model) accurately represented the growth of *Pseudomonas aeruginosa*.

Table 5.3. Growth models, growth kinetic parameters, SSD, and regression coefficients (R^2). The second and third columns show numbers of the equations that were combined to assemble the double substrate kinetics.

Model number	Equation # for oxygen	Equation # for glucose	λ_o	λ_g	B_o	B_g	K_o	K_g	μ_{max}	SSD	R^2
1	-	2.9	-	-	-	-	-	31.97	0.30	0.0272074	0.78
2	-	2.10	-	-	-	-	-	39.76	0.26	0.0256887	0.80
3	-	2.11	-	1.06	-	-	-	37.42	0.29	0.027141	0.79
4	-	2.12	-	-	-	1.13E-02	-	-	0.30	0.0308807	0.76
5	2.9	-	-	-	-	-	0.65	-	0.21	0.1055993	0.17
6	2.9	2.9	-	-	-	-	0.50	20.32	0.32	0.0192399	0.85
7	2.9	2.10	-	-	-	-	0.51	20.00	0.21	0.1012759	-
8	2.9	2.11	-	1.49	-	-	0.62	66.67	0.30	0.0174575	0.86
9	2.9	2.12	-	-	-	6.81E-03	0.55	-	0.32	0.021275	0.83
10	2.10	-	-	-	-	-	0.98	-	0.19	0.0980918	0.22
11	2.10	2.9	-	-	-	-	0.85	19.13	0.29	0.016236	0.87
12	2.10	2.10	-	-	-	-	1.18	26.89	0.29	0.0041595	0.97
13	2.10	2.11	-	1.49	-	-	0.96	60.30	0.27	0.0146219	0.88
14	2.10	2.12	-	-	-	1.13E-02	9.9E-06	-	0.30	0.0308807	0.76
15	2.11	-	3.32	-	-	-	0.76	-	0.20	0.0956579	0.24
16	2.11	2.9	2.5	-	-	-	0.40	18.78	0.29	0.0163252	0.87
17	2.11	2.10	-	1.51	-	-	0.78	27.30	0.30	0.0045242	0.96
18	2.11	2.11	2.29	1.47	-	-	0.59	105.56	0.27	0.0154927	0.88
19	2.11	2.12	2.56	-	-	6.36E-03	0.44	-	0.29	0.0176969	0.86
20	2.12	-	-	-	1.97E-04	-	-	-	0.20	0.1126745	0.11
21	2.12	2.9	-	-	1.53E-04	-	-	21.14	0.32	0.0202808	0.84
22	2.12	2.10	-	-	2.05E-04	-	-	25.96	0.29	0.0164883	0.87
23	2.12	2.11	-	1.53	2.00E-04	-	-	76.13	0.30	0.0181868	0.86
24	2.12	2.12	-	-	1.64E-04	7.27E-03	-	-	0.32	0.0232337	0.82

Though the double Monod kinetics (#6) is more commonly used for this procedure, the double Tessier (#12) kinetics proved to better fit our needs with a correlation of $R^2 = 0.97$. An R^2 value higher than 0.85 was accepted as a cutoff value to select the kinetic expressions. All models with $R^2 > 0.85$ were considered as options. Double Monod kinetics showed $R^2 = 0.85$, which according to my definition was borderline for the kinetic expressions that was decided to consider.

The comparison of the growth kinetic parameters evaluated in this study with those reported by Bakke et al. (1984) and Robinson et al. (1984) showed that the μ_{max} from this study was slightly lower, but on the same order of magnitude as those reported. However, the K_{gT} value calculated was nearly an order of magnitude higher than those reported. This difference was most likely caused by the chemostat being operated at much higher glucose concentration than the systems used by Bakke et al., (1984) and Robinson et al., (1984). As mentioned earlier, Bakke et al., (1984) and Robinson et al., (1984) used relatively low glucose concentration to assure that glucose - not oxygen - was the limiting substrate. This is to say that the authors did not consider oxygen as a limiting substrate. Also, Bakke et al. (1984) and Robinson et al. (1984) considered only Monod kinetics to describe microbial growth, a model that was not found below the acceptance level ($R^2 \leq 0.85$) in this study.

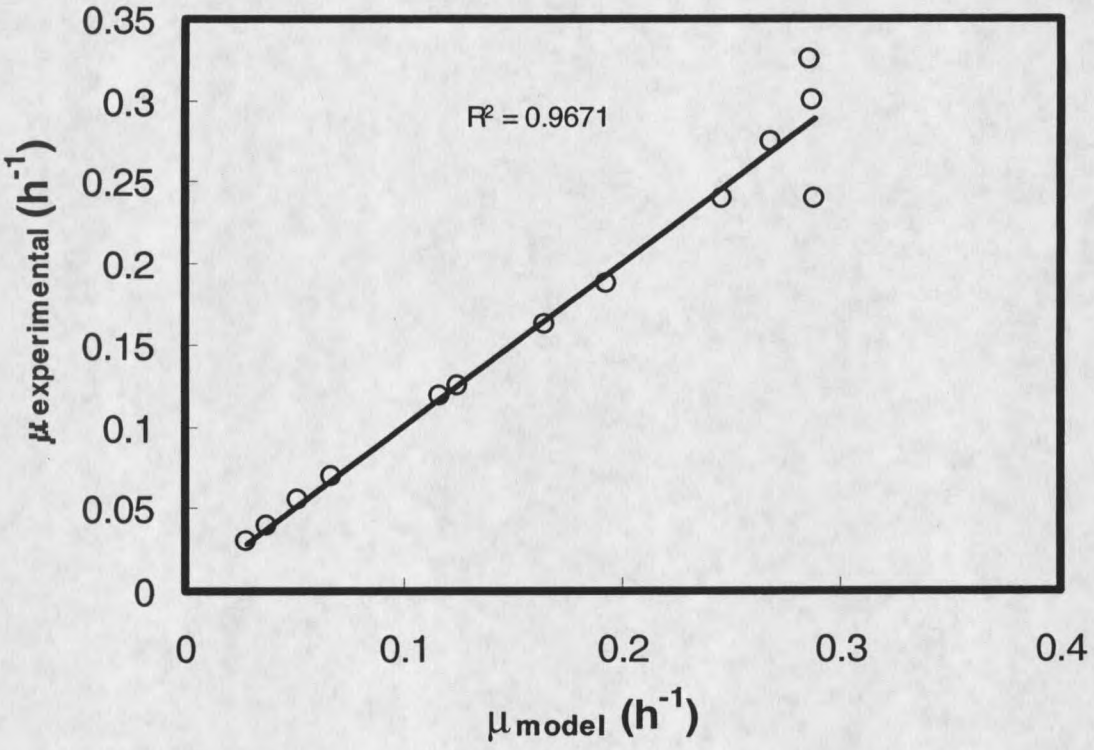

Figure 5.4. The specific growth rates predicted from Equation 2.10 (Double Tessier kinetics) versus measured specific growth rates.

Inhibition Modeling

The results from the glucose inhibition simulation on the growth rate of *Pseudomonas aeruginosa* (Appendix C) indicate that it is possible to have substrate inhibitory effect on the growth rate of *Pseudomonas aeruginosa* when the glucose concentration in the chemostat is above 2000 mg/L. However, the experimentally measured glucose concentrations in the chemostat were approximately 250 mg/L, which were far below 2000 mg/L, the minimum inhibitory glucose concentration. Therefore, substrate inhibition kinetic models were neglected in the mathematical modeling to derive the final double-substrate growth kinetic model of *Pseudomonas aeruginosa*. In addition, none of the substrate inhibition kinetic models adequately represent the experimental growth data of *Pseudomonas aeruginosa* (Table C.1). For detailed explanations please refer to Appendix C.

Colony Biofilms

The results from the mathematical modeling for colony biofilms based on the assumption that the maintenance factor is negligible are shown in Appendix A.

CHAPTER 6

CONCLUSION

A dual-substrate microbial growth kinetics, dual Tessier kinetics for oxygen and glucose, was found to be in agreement with the chemostat data, yielding the following growth kinetic parameters describing growth of *Pseudomonas aeruginosa*: μ_{max} = 0.29 h^{-1}, K_{gT} = 26.9 mg/L, K_{oT} = 1.18 mg/L, $Y_{x/g}$ = 0.635 mg microorganism/mg glucose and, $Y_{x/o}$ = 0.628 mg microorganism/mg oxygen. Maintenance factors for glucose and oxygen were m_g = 0.0078 g glucose consumed/g microorganism·hour, and m_o = 0.014 g oxygen consumed/g microorganism·hour.

For *Pseudomonas aeruginosa* colony biofilms (Appendix A), the half rate coefficient of oxygen calculated using Tessier and Monod kinetic models was 0.012 ± 0.0003 mg/L and 0.17 ± 0.02 mg/L, respectively. It was showed that the half rate coefficient of oxygen for colony biofilm was different from the half rate coefficient of oxygen for planktonic culture. Therefore, the growth kinetic parameters determined from planktonic cultures should not be applied in biofilm study.

CHAPTER 6

CONCLUSION

A dual-substrate microbial growth kinetics, dual Tessier kinetics for oxygen and glucose, was found to be in agreement with the chemostat data, yielding the following growth kinetic parameters describing growth of *Pseudomonas aeruginosa*: μ_{max} = 0.29 h^{-1}, K_{gT} = 26.9 mg/L, K_{oT} = 1.18 mg/L, $Y_{x/g}$ = 0.635 mg microorganism/mg glucose and, $Y_{x/o}$ = 0.628 mg microorganism/mg oxygen. Maintenance factors for glucose and oxygen were m_g = 0.0078 g glucose consumed/g microorganism·hour, and m_o = 0.014 g oxygen consumed/g microorganism·hour.

For *Pseudomonas aeruginosa* colony biofilms (Appendix A), the coefficient of oxygen calculated using Tessier and Monod kinetic models was 0.012 ± 0.0003 mg/L and 0.17 ± 0.02 mg/L, respectively. It was showed that the coefficient of oxygen for either Tessier or Monod kinetic model for a colony biofilm was lower than the coefficient of oxygen for a planktonic culture. Therefore, the growth kinetic parameters determined from planktonic cultures should not be applied in biofilm study.

REFERENCES

Aiba, S., Humprey, E A. and Millis, N.F. *Biochemical Engineering,* 2nd ed., Academic Press, Inc., New York, 1973.

Aiba, S., Shoda, M. and Nagatani, M. Kinetics of product inhibition in alcohol fermentation, *Biotechnol. Bioengng*, 1968. **10**: 845-856.

Andrews, J.F. A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. *Biotechnol. Bioengng*, 1968. **10**: 707-723.

Anwar, H., Strap, J.L., and Costerton, J.W. Growth characterististics and expression of iron-regulated outer-membrane proteins of chemostat-grown biofilm cells of *Pseudomonas aeruginosa. Can. J. Microbiol*, 1991. **37**:737-743.

APHA, Standard Methods for the Examination of Water and Wastewater, Amer, 1962. Public Health Assoc., Washington, DC.

Arcangeli, J.P., and Arvin, E. Modeling the growth of methanotrophic biofilm: Estimation of parameters and variability. *Biodegradation*, 1999. **10**(3):177-191.

Atkinson, B., Mavituna, F. *Biochemical Engineering and Biotechnology Handbook.* 2nd edition. Stockton Press, New York, 1991. p. 199-234.

Bader, F.G. Analysis of double-substrate limited growth. *Biotechnol. Bioeng.*, 1878. **20**: 183-202.

Bailey, J. E. and Ollis, D. F. *Biochemical Engineering Fundamentals*, 2nd ed., McGraw Hill Book Company, New York, 1986. p. 500-507.

Bakke, R,, Characklis, W.G., Turakhia, M.H., Yeh, A.-I. *Modeling amonopopulation biofilm system*: *Pseudomonas aeruginosa*: *Biofilms*. Ed. W.G. Characklis and K.C. Marshall. Wiley, New York, 1989.

Bakke, R., Trulear, M.G., Robinson, J.A., Characklis, W.G. Activity of *Pseudomonas aeruginosa* in biofilms. *Biotechnol. Bioeng.*, 1984. **26**: 1418-1424.

Bandyopdhyay, B., Humhrey, E., Taguchi, N. Dynamic measurement of the volumetric oxygen transfer coefficient in fermentation systems. *Biotechnol Bioeng.*, 1967. **9**: 533-544.

Beyenal, H., Tanyolac, A. A combined growth model of *Zoogloea ramigera* including multisubstrate, pH, and agitation effects Enzyme and Micro. *Technol.*, 1997. **21**: 74-78.

Brown, M. R.W., Collier, P. J., Gilbert, P. 1990. Influence of growth rate on susceptibility to antimicrobial agents-modification of the cell envelope and batch and continuous culture studies. *Antimicrob. Agents Chemother*, 1990. **34**:1623-1628.

Burgary, H. R., 3rd, Whalen, W. J., Sanders, W. M. Microprobe techniques for determining diffusivities and respiration rates in microbial slime system. *Biotechnol. Bioeng.*, 1969. **11**:765-772.

Campa, M., Bendinelli, M. and Friedman, H. *Pseudomonas aeruginosa as an Opportunistic Pathogen*, Plenum Press, New York, 1993.

Cao, Y. S., and Alaerts, G. J. Influence of reactor type and shear stress on aerobic biofilm morphology, population, and kinetics. *Water Res*, 1995. **29**:107-118.

Christensen, B. E., and Characklis, W. G. *Physical and chemical properties of biofilm*. Interscience, New York, N. Y, 1990. p. 83-138.

Contois, D. E. Kinetics of bacterial growth: Relationship between population density and specific growth rate of continuous cultures. *J. Gen. Microbiol*, 1959. **21**:40.

Costerton, J.W., Cheng, K-J, Gessey, G.G., Lodd, T.T., Nickel, J.C., Dasgupta, M., and Marrie, T.J. Bacterial biofilm in nature and disease. *Ann. Rev. Microbial*, 1987. **41**: 435-464.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., and Lappin-Scott, H. M. Microbial biofilms. *Annu. Rev. Microbiol*, 1995. **49**:711-745.

Cussler, E.L. *Diffusion: Mass Transfer in fluid systems*. Cambridge University Press, 1984.

de Beer. D., Stoodley, P., Roe, F., and Lewandowski, Z. Effects of biofilm structure on oxygen distribution and mass transport. *Biotechnol. Bioeng*, 1993. **43**: 1131-1138.

Edwards, V.H. The influence of high substrate concentration on microbial kinetics. *Biotechnol, Bioengng*, 1969. **12**: 679-712.

Fletcher, M., Marshall, K. C. *Are solid surfaces of ecological significance toaquatic bacteria*?: *Advances in microbial ecology*. Ed. K. C. Marshall. Plenum Press, New York, 1983. **6**: 199-236.

Hadson, B.K., Sherwood, L. Exploration in Microbiology. *A discovery-based approach*, 1997. p. 129-135.

Hamilton, C. Lawrence. *Regression with Graphics: A Second Course in Applied Statistics*. Brooks/Cole Publishing Company, California, 1992. p. 125.

Harris, D.. Measurement of oxygen uptake by straw microflora using an oxygen electrode: *Straw Decay and Its Effects on utilization and Disposal.* Ed. Grossbard, E. John Wiley, Chichester, 1979. p. 265–266.

Hoiby, N., Johansen, h. K., Moser, C., Song, Z., Ciofu, O., Kharazmi, A. *Pseudomonas aeuginosa* and the in vitro and invivo biofilm mode of growth. *Microbes and Infection*, 2001. **3**: 23-35.

Horber, C., Christensen, N., Arvin, E., and Ahring, B. K. Tetrachloroethene dechlorination kinetics by Dehalospirillum multivorans immobilized in upflow

anaerobic sludge blanket reactors. *Applied Microbiology and Biotechnology*, 1999. **51**(5):694-699.

Jacoby, L.S. Samuel., Kowalik, S. Janusz. *Mathematical Modeling with Computers*. Prentice-Hall, Inc. New Jersey, 1980. p.234.

Jih, C. G., and Huang, J. S. Effect of biofilm thickness distribution on substrate-inhibited kinetics. *Water Res*, 1994. **28**: 967-973.

Jorgensen B., and Revsbech, N. P. *Microsensor: Method in Enzymology*. Academic Press, Inc., 1988. **167**: 639-650.

Keen, G. A., Prosser, J.I. Interrelationship between pH and surface growth of *Nitrobacter*. Soil Niol. *Biochem*, 1987. **19**: 665-672.

Levenspiel, O. The Monod equation: A resvisit and a generalization to product inhibition situations. *Biotecnol. Bioeng,* 1980. **22**:1671-1687.

Luong, J. H. T. 1986. Generalization of Monod kinetics for analysis of growth data with substrate inhibition. *Biotechnol. Bioeng.* **29**:242-248.

Monod, J. The growth of bacterial cultures. *Annual Review of Microbiology*, 1949. **3**: 371-394.

Mozer, A. The dynamic of bacterial populations maintained in the chemostat. Publication 614, Washington, DC: The Carnegie Institution, 1948.

Pirt, S. J. *Principles of Microbial and Cell Cultivation.* J. Wiley & Sons, New York, 1975.

Press, W. H., Teukolsky, S. A., Vetterling, W. T., and Flannery, B. P. *Numerical Recipes in Fotran.* 2nd edition. Cambridge University Press, Cambridge, 1992.

Revsbech, N. P., Jorgensen, B.B. Microelectrodes: Their use in microbial Ecology. Ed. K. C. Marshall. Plenum, New York, 1986.

Riefler, R. G., Ahlfeld, P. D., Smets, F. B. Respirometric assay for biofilm kinetics estimation: Parameter identifiability and retrievability. *Biotechnol. Bioeng*, 1997. 57(1): 35-45.

Rittmann, B. E., McCarty, P. L.. Model of steady state biofilm kinetics. *Biotechnol. Bioeng*, 1980. **22**: 2359-2373.

Robinson, J.A., Truler, M.G., Characklis, W.G.. Cellular reproduction and extracellular polymer formation by *Pseudomonas aeruginosa* in continuous culture. *Biotechnol. Bioeng*, 1984. **26**: 1409-1417.

Schalk, I. J., Hennard, C., Dugave, C., Poole, K., Abdallah, M. A., Pattus, F. Iron-free pyoverdin binds to its outer membrane receptor FpvA in **Pseudomonas aeruginosa**: A new mechanism for membrane iron transport. *Molecular Microbiology*., 2001. **39**(2): 351-360.

Seker, S., Beyenal, H., Salih, B., Tanyolac, A. Multi-substrate growth kinetics of *Pseudomonas pultida* for phenol removal. *Appl. Microbiol Biotechnol*, 1997. **47**: 610-614.

Shuler, M.L., Kargi, F. 1992. *Bioprocess Engineering Basic Concepts*. Prentice Hall, Inc., Englewood Cliffs, New Jessey. 171.

Siegmund, D. and Diekmann, H. Estimation of fermentation bio-mass concentration by measuring oxygen uptake off-line with an oxygen electrode. *Appl. Microbiol. Biotechnol.* 1989, **32**: 32–36.

Syrett, P. E. Respiration rate and internal adenosine triphosphate concentration in *Chlorella. Arch. Biochem. Biophys.* 1958, **75,** 117–124.

Tamagnini, L. M., Gonzalez, R. D.. Bacteriological stability and growth kinetics of **Pseudomonas aeruginosa** in bottled water. *J. Appl. Micro*, 1997. **83**(1): 91-94.

Tessier, G.. Croissance des populations bacterie'nnes et quantite'd'aliment disponible *Rev. Sci.Paris,* 1942. **80**: 209.

Tseng M. C., Wayman, M.. *Can. J. microbial*, 1975. **21**: 994.

Volesky, B., Yerushalmi, L., and Luong, J. H. T. Metabolic-heat relation for aerobic yeast respiration and fermentation. *J. Chem. Eng. Technol,* 1982. **32:** 650–659.

Wanner, O., Cunningham, A. B., and Lundman, R.. Modeling biofilm accumulation and mass transport in a porous medium under high substrate loading. *Biotechnol. Bioeng*, 1995. **47**(6): 703-712.

Wayman, M. Tseng, M.C.. *Biotechnol. Bioeng*, 1976. **28**: 383.

Whitely, M., Brown, E., McLean, R. J. C.. An inexpensive chemostat apparatus for the study of microbial biofilms. *Journal of Microbiological Methods*, 1997. **30**: 125-132.

APPENDICES

APPENDIX A

COLONY BIOFILMS MODELING

Colony Biofilm Experimental Setup

A standard petri dish (Fisher, Denver, Co) was used as the reactor to grow colony biofilm. A 40 µl sample of overnight culture *Pseudomonas aeruginosa* (prepared as described in section 3.1.1) was dropped onto the black polycarbonate membrane filter with diameter = 25 mm and pore size = 0.2 µm, (Poretics, Corp. Livermore, Calif.) placed on an agar plate. The agar plate was enriched with the growth medium (Table 3.1) and trace elements (Table 3.2) used for the planktonic study, except that the glucose concentration was 5g/L. After inoculation, the plates were inverted and incubated at 25°C for 48 hours. The colony biofilms grown on the membrane were transferred to fresh enriched agar every 12 hours.

Before inoculation of the overnight culture onto the polycarbonate filter, both sides of the membrane were sterilized by UV light exposure for 15 minutes in a biological hood.

Clark-type Dissolved Oxygen Microelectrode

The modified Clark-type dissolved oxygen (DO) electrode was used to measure the oxygen concentration in the colony biofilms (Jorgensen et. al., 1988). The DO electrodes consisted of an outer casing, a silicon rubber membrane, a cathode, an internal silver/silver chloride reference electrode and an electrolyte. The following were the procedures for DO microelectrode construction.

Outer Casing

1. Pasteur pipette was cut so that the thick part was about 5 cm long; and fire-polished the cut end.
2. The pipette was clamped to a Micro Puller by Stoelting Co. and supplied current to the heating coil until red-hot.
3. When the pipette began to fall, turned off the current and repositioned the pipette to the original heating point.
4. Then turned the current back on. Repeated steps 3 and 4 until the pipette fell.
5. The casing was mounted under microscope with tip in focus.
6. The tip was then moved slowly to the end of the glass ball to break it off to the desired tip diameter.
7. The glass ball assembly was replaced with the assembly of "100 micron Platinum wire fed by transformer and Variac"
8. The wire was brought into focus next to tip and heated until the cut tip was fire polished, but not sealed.
9. After that, silicone rubber membrane of approximately 5-10 μm was filled to the casing's tip.
10. The silicon rubber membrane was allowed to dry for 24 hours.

Cathode

1. Prepared the capillary pipettes from 2 or 3 mm outside diameter (OD) soft glass tubing. The capillary pipettes was rinsed with acetone then water to remove any trapped dust in the glass tubing.
2. Cut 10 cm sections from a 4' length of glass tube.
3. Heated the middle section of the 10 cm sections of the tube over a propane torch until molten and then pulled thinning the molten section until two glass capillaries formed.
4. Electrochemically etched a 50 or 100 μm Platinum wire in 2 M Potassium cyanide.
5. Cyanide solution: Dissolved 8 g Potassium cyanide in 10 ml DI water.
6. Before etching, cleaned the Platinum wire (about 7 cm in length) in DI water and acetone to remove grease.
7. Supplied 8 VAC to the Platinum wire and continually dipped it into the cyanide solution until the wire tip was tapered to approximately 1-10 μm using a carbon counter electrode.
8. Rinsed the tapered wire in nanopure water to remove any dirt on the tapered wire.
9. Then, the tapered wire was inserted carefully into the wide end of the glass capillary pulled earlier.
10. Mounted the glass capillary with tapered wire on the microscope and brought into focus. Then, marked the end of the tapered wire as the reference point for the next heating and pulling.

11. Clamped the glass capillary with tapered wire in it to the pipette puller with the marked reference point approximately 2 cm above the heating element.
12. Turned the current on until the heating element (coil) was red-hot.
13. When the tube started to melt and fall, followed the melting interface down with the coil until it fell off.
14. Finally, the tip was grounded flat on a Narishige grinder in order to expose about 5 μm of wire at the tip of the electrode.

Silver/silver Chloride Reference Electrode:

1. Cleaned a 5 cm long and 1mm OD silver wire with acetone and DI water.
2. The silver wire was polished with 600 grit carbide paper, and then the wire was dipped into 3 M HNO_3 and rinsed clean with DI water.
3. One end of the silver wire was connected to the anode of the electrometer.
4. The cathode of electrometer was then connected to the carbon rod.
5. Placed the silver wire and carbon rod into a beaker containing 0.1 M HCl.
6. The electrometer was set at a constant potential of 0.6 V with a current maximum of 0.2 mA, and the silver wire was left electroplating overnight.

Components Assembling into Dissolved Oxygen Electrode:

1. The casing was mounted on microscope stage with clay and the tip was brought into focus
2. Then, the open end of the casing was moved out of focus.

3. The cathode was clamped on alligator clip on end of a glass rod held by xyz Positioner
4. The cathode's tip was brought into focus by adjusting xyz positioner
5. Next, the casing was slid over the cathode, while the cathode's tip remained in view until the casing's tip was in view.
6. The tip of the cathode was carefully positioned to approximately10 μm from the silicon rubber membrane. The distance between the tip of the cathode and the silicon rubber membrane must not exceed 10 μm in order to have a fast response time.
7. Then, silver/silver reference wire was slid into casing.
8. The cathode and the silver/silver chloride reference wire were glued in place with drop(s) of 5 minutes epoxy. Let dried for 2 hours.
9. Next, filled in electrolyte and placed under vacuum for approximately 15 seconds to evacuate any air bubbles in the tip.
10. Electrolyte:
    - 0.3 M K_2CO_3 (41.46 g/l)
    - 0.2 M $KHCO_3$ (20.02 g/l)
    - 1.0 M KCl (74.55 g/l)
11. Finally, applied 5 minutes epoxy to glue closed the open end of the electrode.

Figure A.1 shows the finished DO microelectrode.

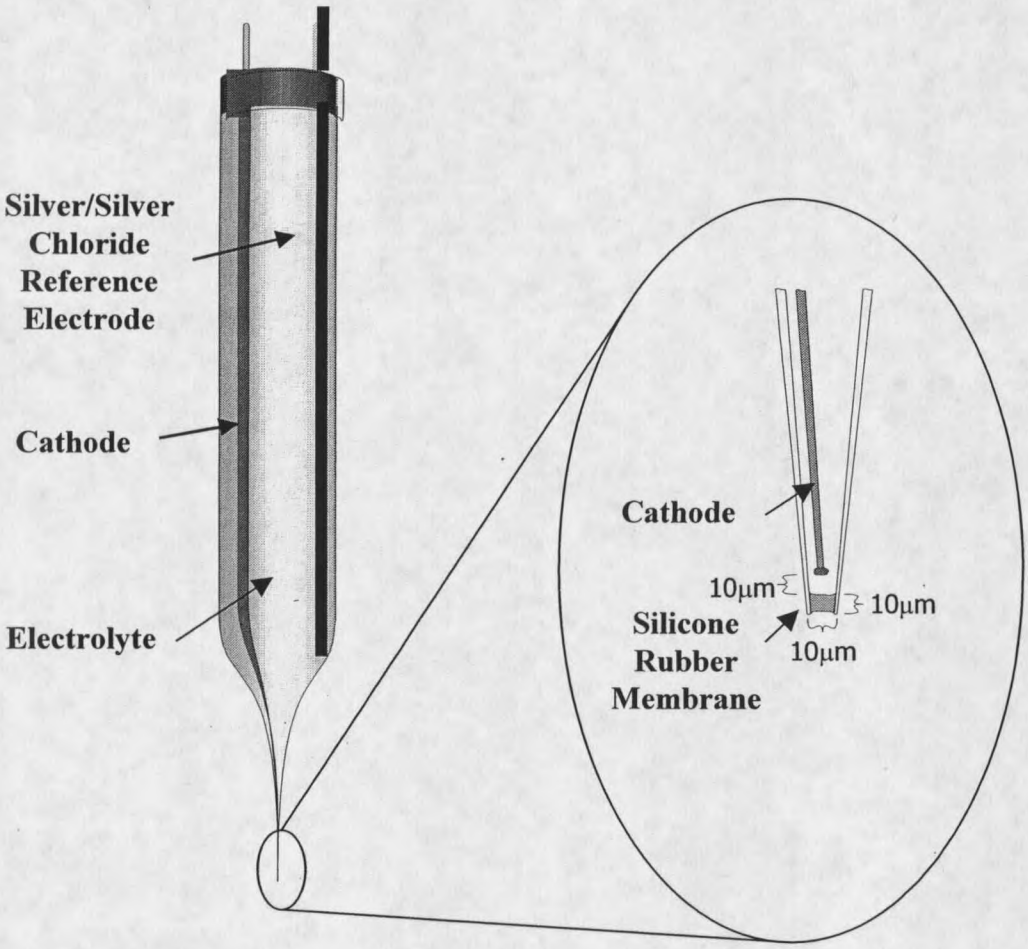

Figure A.1. Dissolved oxygen microelectrode.

Dissolved Oxygen Microelectrode Measurement

Before measurement, the DO microelectrode was calibrated in a beaker containing water saturated with air and nitrogen (sparging nitrogen gas into the water to remove oxygen). The cathode was polarized with a constant potential of –0.8 V. A computer containing a data acquisition system was used to acquire the measurements. A micromanipulator from World Precision Instruments (Sarasota, FL), a computer controller and a stepper motor (Oriel, Stratford, CT) were used to adjust and to control the microelectrode position during measurement. Figure A.2 shows the colony biofilm and dissolved oxygen microelectrode during the dissolved oxygen concentration measurement.

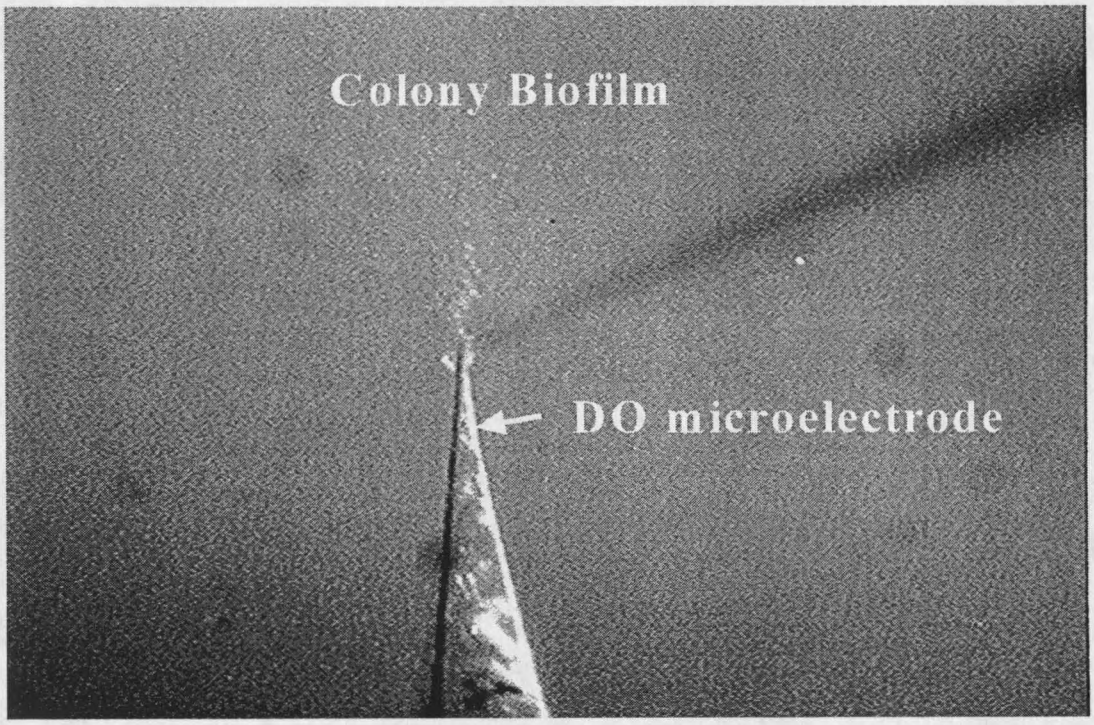

Figure A.2. Colony biofilm and dissolved oxygen microelectrode.

Growth Kinetic Parameter Estimation for Colony Biofilm

A biofilm reaches steady state when there is no change in the measured growth limiting substrate concentration profiles with time. However, when there is a slightly changes in the measured growth limiting substrate concentration profile over time, but negligible, a biofilm is said to have reached *Pseudosteady state*. Pseudosteady state is assumed when the variation of dissolved oxygen concentration in the colony biofilms varied less than 10% (The difference between the highest and the lowest measured dissolved oxygen concentrations) at different times. For example: Figure A.3 shows that the variation of oxygen concentration profiles taken at different time (48 hrs, 52 hrs, 72 hrs, and 78 hrs) in the colony biofilm was below 10%. The diffusion and reaction in the colony biofilm can then be simplified with the pseudosteady state equation.

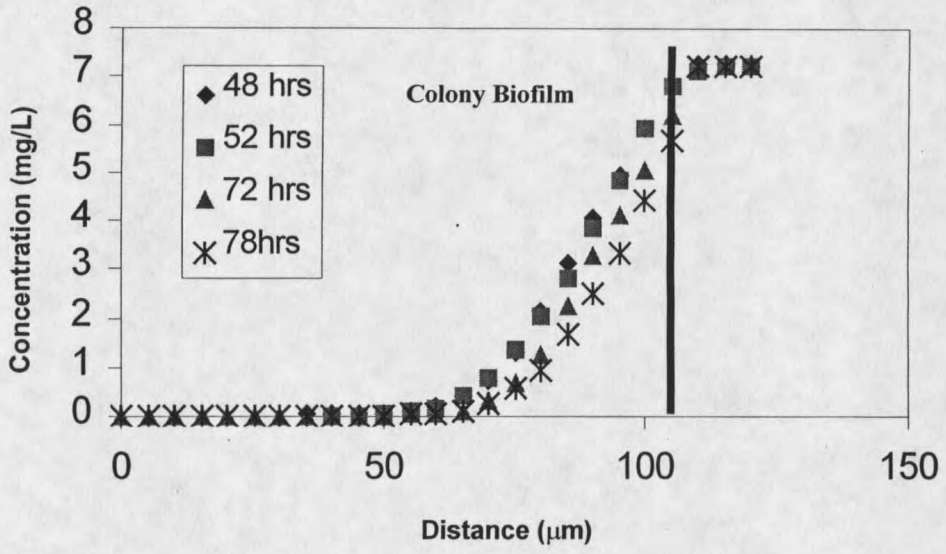

Figure A.3. Dissolved oxygen concentration profiles in the colony biofilms.

At pseudosteady state, the oxygen diffusion and reaction in the colony biofilm can be described in Cartesian coordinate system with the following assumptions:

1) The growth of *Pseudomonas aeruginosa* is limited only by oxygen concentration. The glucose concentration in the agar was 5g/L, where $S_g >>> K_{gT}$,
2) One-directional substrate transport from the agar to the colony biofilm (Walters, 2001).

3) Tessier kinetic model is assumed the best kinetic model describing the microorganism growth in the colony biofilm, based on planktonic studies.
4) The system is at *pseudosteady state* with respect to dissolved oxygen concentration profiles in the colony biofilm (see Figure A.3).
5) The maintenance factor for *Pseudomonas aeruginosa* colony biofilm is negligible.

The diffusion and reaction in the colony biofilm can be represented by the following Equation A.1.

$$\frac{\partial S_o}{\partial t} = D_e \frac{\partial^2 S_o}{\partial z^2} - \frac{\mu_{max} X_f}{Y_{x/o}} \cdot (1 - e^{-\frac{So}{KoT}}) \quad \text{(A.1)}$$

At pseudosteady state, there is no change of dissolved oxygen concentration over time in the colony biofilm, where $\partial S_o/\partial t = 0$. Equation A.1 then becomes the ordinary differential Equations A.2 and A.3.

$$0 = D_e \frac{d^2 S_o}{dz^2} - \frac{\mu_{max} X_f}{Y_{x/o}} (1 - e^{-\frac{So}{KoT}}) \quad \text{(A.2)}$$

$$D_e \frac{d^2 S_o}{dz^2} = \frac{\mu_{max} X_f}{Y_{x/o}} (1 - e^{-\frac{So}{KoT}}) \quad \text{(A.3)}$$

The boundary conditions for Equation A.3 are:

@ $z = L_f$ (Substratum) $\quad \frac{dS_o}{dz} = 0$ (A.4)

@ $z = 0$ (Biofilm surface) $\quad S_o = S_{os}$ (A.5)

Dimensionless Variables Development

Equation A.3 and the boundary conditions (Equation A.4 and A.5) can be made dimensionless by introducing the following dimensionless variables:

$$S^* = \frac{S_o}{S_{OS}} \quad \text{(A.6)}$$

$$z^* = \frac{z}{L_f} \quad \text{(A.7)}$$

$$b = \frac{S_{OS}}{K_{oT}} \quad \text{(A.8)}$$

Differentiate Equation A.6 with respect to S_o and Equation A.7 with respect to z.

$$\frac{dS^*}{dS_O} = \frac{1}{S_{OS}} \quad \text{(A.9)}$$

$$\frac{dz^*}{dz} = \frac{1}{L_f} \tag{A.10}$$

Using these dimensionless variables, Equation A.3 becomes the dimensionless Equation A.11.

$$\frac{d^2S^*}{dz^{*2}} = \frac{\mu_{max} X_f L_f^2}{D_e Y_{x/o} S_{OS}} (1 - e^{-bS^*}) \tag{A.11}$$

Let

$$\Phi = \sqrt{\frac{\mu_{max} X_f L_f^2}{D_e Y_{x/o} S_{OS}}} \tag{A.12}$$

Equation A.11 is then further simplified to Equation A.13.

$$\frac{d^2S^*}{dz^{*2}} = \Phi^2 (1 - e^{-bS^*}) \tag{A.13}$$

The boundary conditions are:

$$@\ z^* = 0 \qquad \frac{dS^*}{dz^*} = 0 \tag{A.14}$$

$$@\ z^* = 1 \qquad S^* = 1 \tag{A.15}$$

Numerical Technique

The shooting method coupled with the fourth-order Runge-Kutta Method was used to integrate Equation A.13 numerically using Matlab (Press et. al., 1992).

To solve the second order differential equation numerically, Equation A.13 can be described mathematically as Equations A.16 and A.17:

$$y = \frac{dS^*}{dz^*} \tag{A.16}$$

$$\frac{dy}{dz^*} = \Phi^2 (1 - e^{-bS^*}) \tag{A.17}$$

To perform the fourth-order Runge-Kutta method in Matlab, the initial y value was estimated with the shooting method until both the boundary conditions are satisfied. The algorithm used in shooting method is the Newton's method as descried in section 4.1.1. Then, the K_{oT} and Φ^2 values are estimated with a trial and error method until the minimum of the objective function (Equation A.18) is achieved. The goal of the objective function is described as the minimum sum of least square value between the experimental (microelectrode data) and the selected kinetics model (solution of the numerical technique) data. Figure A.4 shows the flow chart of the numerical solution.

$$SSD = \sum_{i=1}^{k} (S_{experimental} - S_{model})^2 \qquad (4.18)$$

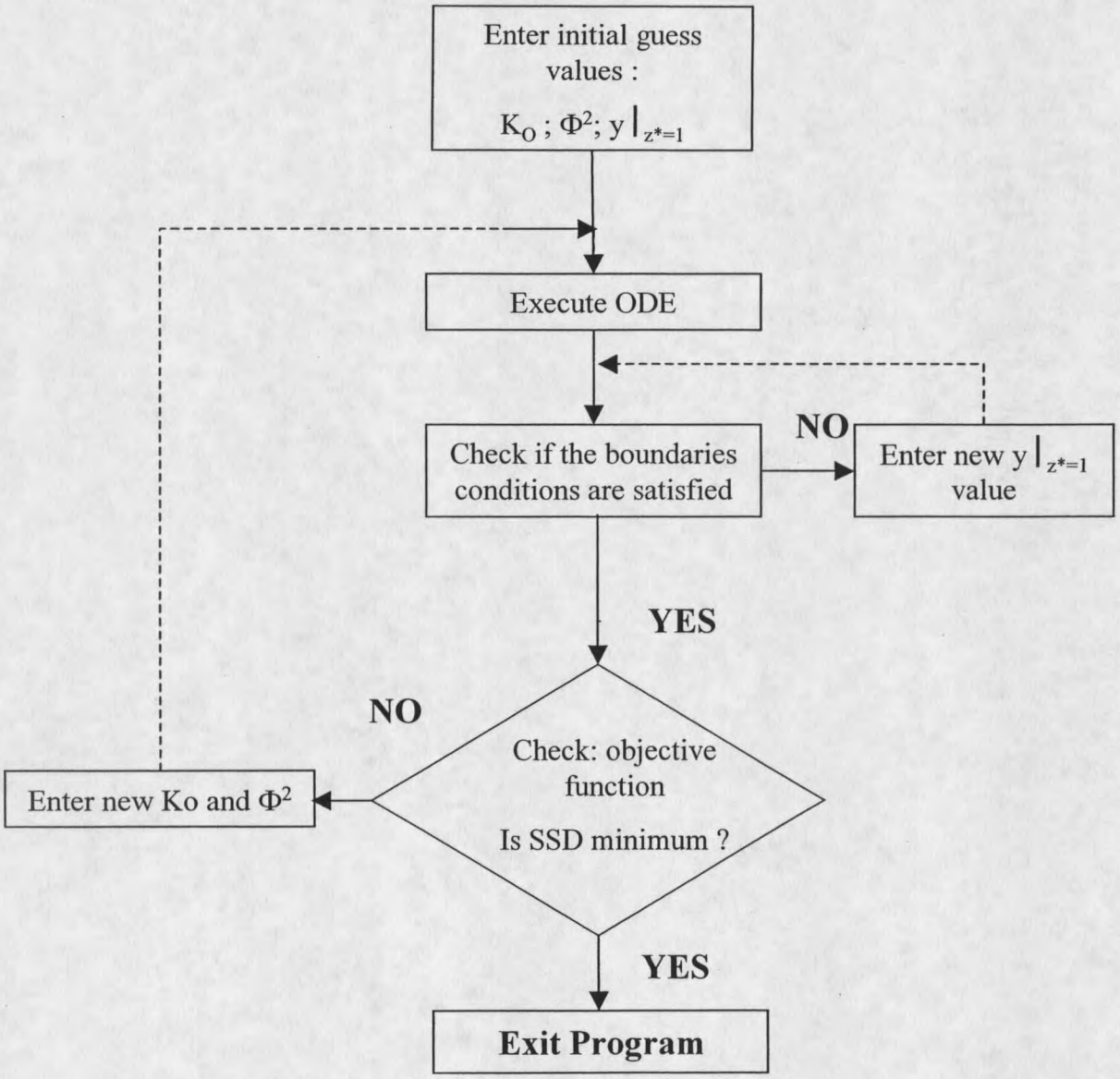

Figure A.4. The flow chart of the numerical calculation.

Growth Kinetic Parameter Estimation for *Pseudomonas aeruginosa* Colony Biofilms

The dissolved oxygen concentration profile was divided into two parts: outside and inside colony biofilm. The concentration profile in the colony biofilm (Figure A.5) was calculated as described in section A.2, while the half rate coefficient for oxygen was extracted using the trial and error method as described in section A.2.2. The coefficient of oxygen calculated using the Tessier kinetic equation for the concentration profile presented in Figure A.5 was 0.0144 mg/L. The K_{oT} calculated using Tessier kinetic was observed to be very small. For comparison, Monod kinetic equation was also used to calculate K_{oM} from the same dissolved oxygen

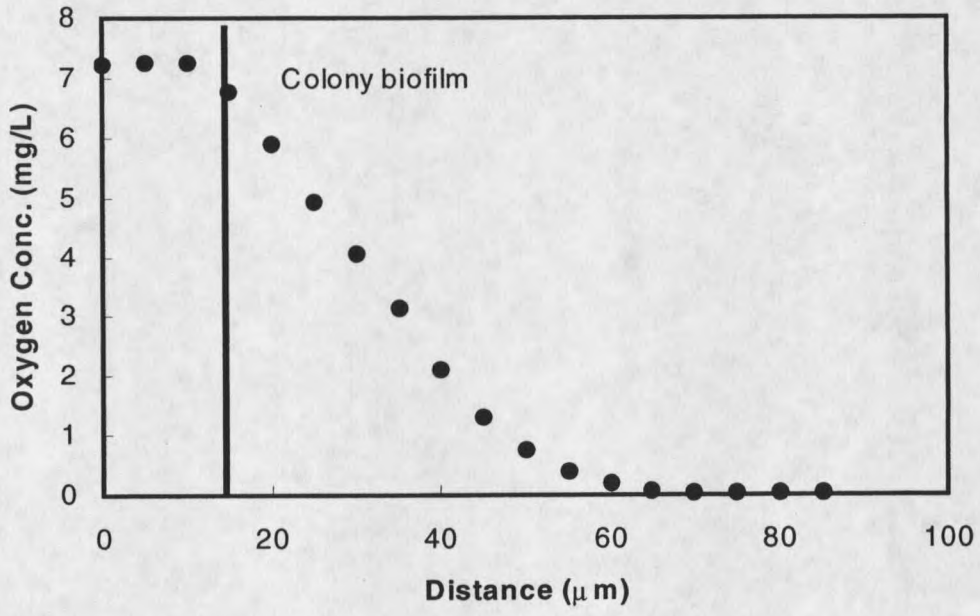

Figure A.5. Oxygen concentration profile in the colony biofilm.

concentration profile. The K_{oM} calculated using the Monod kinetic equation was 0.18 mg/L. Figures A.6 and A.7 show the experimental and the model dissolved oxygen concentration in the colony biofilm determined using Tessier and Monod kinetic equations, respectively.

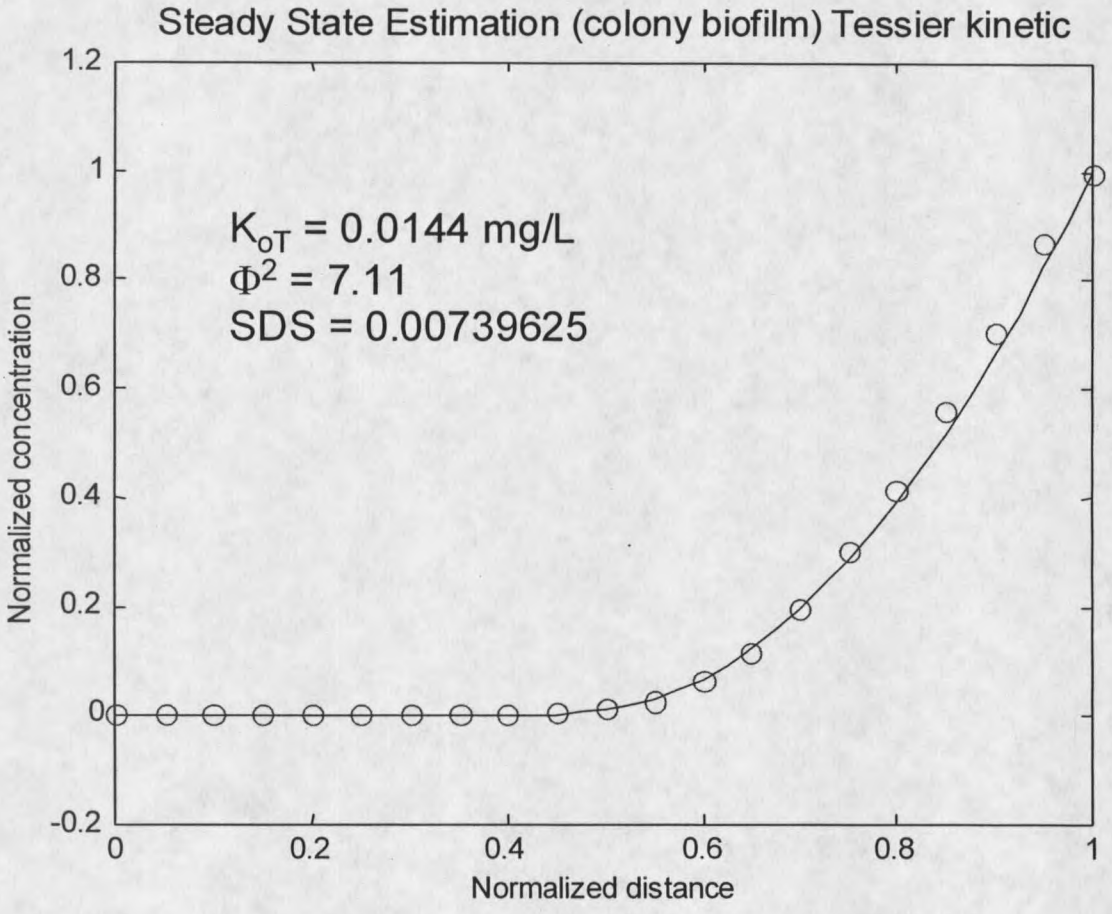

Figure A.6. The model oxygen concentration calculated using the Tessier kinetic equation versus experimental oxygen concentration in the colony biofilm.

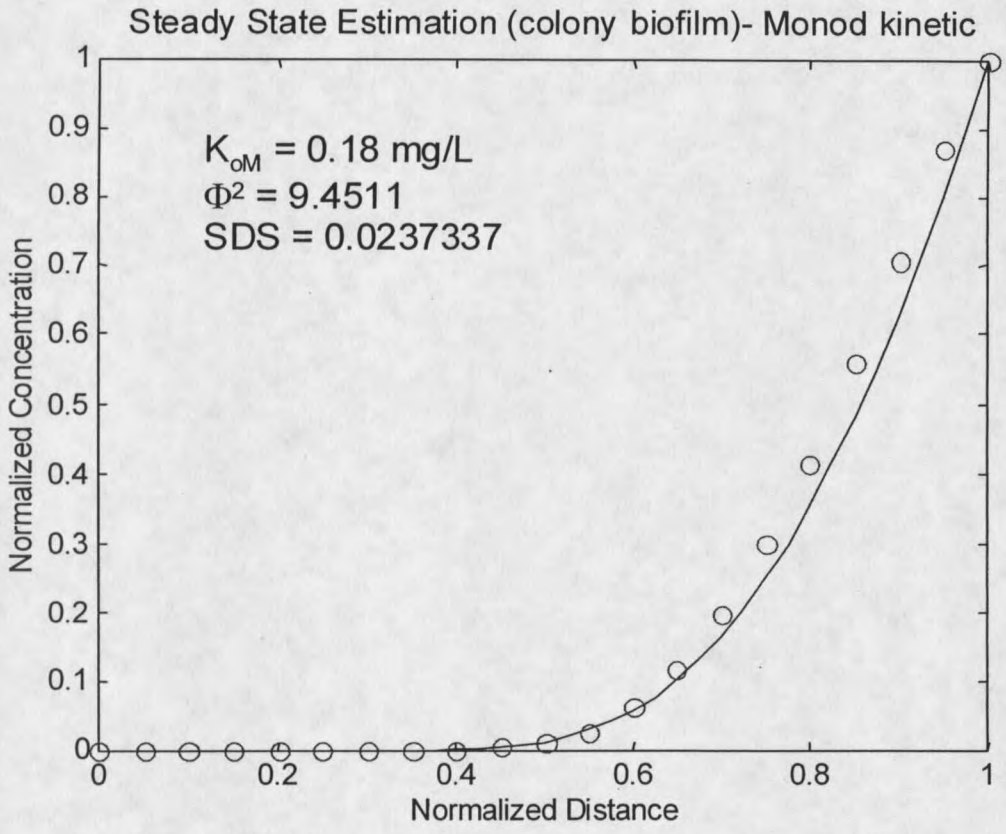

Figure A.7. The model oxygen concentration calculated using the Monod kinetic equation versus experimental oxygen concentration in the colony biofilm.

The SSD value for Tessier kinetic equation was lower than the SSD value for the Monod kinetic equation. However, it is not clear which model to apply to describe the growth behavior of *Pseudomonas aeruginosa* colony biofilm. But the results clearly showed that the K_{oT} and K_{oM} for the colony biofilm were smaller than the K_{oT} and K_{oM} for the planktonic culture.

Table A.1. Comparison of K_{oT} and K_{oM} determined using Tessier and Monod kinetic equations for both the planktonic and the colony biofilm.

	K_{OT}, mg/L Tessier Equation	K_{OM}, mg/L Monod Equation
Chemostat	1.18	0.5
Colony Biofilm	0.0012 ± 0.003	0.17 ± 0.02

The procedures employed to calculate the coefficient of oxygen in colony biofilm were repeated for another three dissolved oxygen profiles. A total of four coefficient of oxygen were determined from four dissolved oxygen profiles measured in colony biofilms (Figure A.3), either using Tessier kinetics or Monod kinetics. The results showed that K_{oT} calculated using Tessier equation was 0.0012 ± 0.0003 mg/L, and K_{oM} calculated using Monod equation was 0.17 ± 0.02 mg/L, as listed in

Table A.1. The results shown in Table A.1 indicate that the growth kinetic parameters determined from the planktonic cultures should not be applied in biofilm.

Figure A.5 shows that most of the oxygen was consumed in the external zone (near surface) of the colony biofilm, which was consistent with the predicted Φ values calculated using Tessier and Monod Kinetics. The Φ^2 values were 7.11 and 9.411, for Tessier kinetic and Monod kinetic, respectively. For $\Phi > 1$, the activity in the biofilm is controlled by diffusion rate; and for $\Phi < 1$, the activity in the biofilm is controlled by reaction rate (Cussler, 1984). The Φ value for Tessier kinetic equation was 2.67, and for Monod kinetic equation, it was 3.07. As a result, the calculated Φ values indicate that the activity in the colony biofilm was mainly limited by diffusion of oxygen into the colony biofilm.

APPENDIX B

MATLAB CODE

Main Function

```
function s_auto

global bguess Vmax Sguess
bguess=0.025;
%Vmax=9.411;
Vmax=3.1;

ODEfile='colonyequation';
x0=1;
xf=0;
deltax=-0.025;
y0=1;
yf=0;
yguess=4.140959301;
order=4;

% solution of the ODE
[x,y,ygu]=shooting(ODEfile,x0,xf,deltax,y0,yf,yguess,order,[],[],Vmax,bguess);
testx=x';
testy=y';
Sguess=ygu;
yexp =[1        .1
0.95    0.871198605
0.9     0.707986147
0.85    0.560610387
0.8     0.414753128
0.75    0.299044525
0.7     0.195209548
0.65    0.116039782
0.6     0.061229968
0.55    0.025298843
0.5     0.012510064
0.45    0.006419842
0.4     2.52958E-05
0.35    0.000329932
0.3     0
0.25    0
0.2     0
0.15    0
0.1     0
0.05    0
0       0];
```

```
% Graphing ----------------------------------------------------------
ko=bguess*7.2;
L=length(testy);
Parameters=[ko Vmax];
x_n=round(0.7*L);  %only for text positioning in figure
x_pos=yexp(x_n);

y_n=round(1/8*L);  %only for text positioning in figure
y_pos=yexp(y_n);

figure(1);plot(testx,testy(:,1),'-',yexp(:,1), yexp(:,2),'o')
 title('Steady State Estimation (colony biofilm)- Monod kinetic', 'FontSize', 12);
 xlabel('Normalized Distance');ylabel('Normalized Concentration')
text(x_pos, y_pos, sprintf('Ko = %g mg/L\nThiele = %g', Parameters), 'FontSize', 14)

figure(2);plot(testx,testy(:,2))
```

Sub-functions

```
function [x,y,ygu] = shooting(ODEfile,x0,xf,h,y0,yf,gamma0,order,rho,tol,varargin)

% Initialization
if isempty(h) | h == 0
  h = (xf - xi)/99;
end

if nargin < 8 | isempty(order)
  order = 4;
end
if nargin < 9 | isempty(rho)
  rho = 1;
end
if nargin < 10 | isempty(tol)
  tol = 1e-6;
end

y0 = (y0(:).')';                    % Make sure it's a column vector
yf = (yf(:).')';                    % Make sure it's a column vector
gamma0 = (gamma0(:).')';% Make sure it's a column vector

% Checking the number of guesses
if length(yf) ~= length(gamma0)
  error(' The number of guessed conditions is not equal to the number of final
conditions.')
end

r = length(y0);                     % Number of initial conditions
n = r + length(yf);                 % Number of boundary conditions
% Checking the number of equations
ftest = feval(ODEfile,x0,[y0 ; gamma0],varargin{:});
if length(ftest) ~= n
  error(' The number of equations is not equal to the number of boundary conditions.')
end
gamma1 = gamma0 * 1.1;
gammanew = gamma0;
iter = 0;
maxiter = 100;

% Newton's technique
while max(abs(gamma1 - gammanew)) > tol & iter < maxiter
  iter = iter + 1;
  gamma1 = gammanew;
```

```
  [x,y] = RK(ODEfile,x0,xf,h,[y0 ; gamma1],order,varargin{:});
  fnk = y(r+1:n,end);

  % Set d(gamma) for derivation
  for k = 1:length(gamma1)
    if gamma1(k) ~= 0
      dgamma(k) = gamma1(k) / 1000;
    else
      dgamma(k) = 0.01;
    end
  end
  % Calculation of the Jacobian matrix
  a = gamma1;
  for k = 1:n-r
    a(k) = gamma1(k) + dgamma(k);
    [xa,ya] = RK(ODEfile,x0,xf,h,[y0 ; a],order,varargin{:});
    fnka = ya(r+1:n,end);
    jacob(:,k) = (fnka - fnk) / dgamma(k);
    a(k) = gamma1(k) - dgamma(k);
  end

  % Next approximation of the roots
  if det(jacob) == 0
    gammanew = gamma1 + max([abs(dgamma), 1.1*tol]);
  else
    gammanew = gamma1 - rho * inv(jacob) * (fnk - yf);
  end
end
ygu=gammanew;
if iter >= maxiter
  disp('Warning : Maximum iterations reached.')
end
```

```
function dsdx=colonyequation(x,y,Vmax,bguess)

%global Vmax bguess

% This is the function !!!!

%b=Sbulk/ko; Definition of b
%Sbulk=7.2; %mg/L

%b=1/bguess; % for tessier
b=bguess; % for monod

% define the equation
%dsdx=[y(2);Vmax*(1-exp(-b*y(1)))]; % tessier
%dsdx=[y(2);Vmax*(y(1)/(b+y(1)))]; % monod
dsdx(1)=Vmax*(y(1)/(b+y(1)));
dsdx(2)=y(1);
dsdx=dsdx';
```

APPENDIX C

INHIBITION MODELING

Equation 2.13 was used to simulate the inhibition effect of glucose concentration on the specific growth rate of *Pseudomonas aeruginosa.* The Lineweaver-Burk plot (Figure C.1), S_g vs. $1/\mu$, using inhibition constants of 200, 250 and 2000 mg/L clearly shows that there is no substrate inhibition effects on the growth rate of *Pseudomonas aeruginosa* at glucose concentrations less than 250 mg/L. However, Figure C.1 shows that it is possible to have substrate inhibition effects on the growth rates of *Pseudomonas aeruginosa* when glucose concentration in the reactor is above 2000 mg/L. The results from this simulation conclude that there is no inhibition on the growth rate of *Pseudomonas aeruginosa* since the experimentally measured glucose concentrations in the chemostat were approximately 255 mg/L, which are far below the minimum inhibitory glucose concentration of 2000 mg/L. In addition, the results (Table C.1) from the inhibition kinetic modeling showed that none of the growth models using substrate inhibition kinetics for glucose described the growth rate of *Pseudomonas aeruginosa* better than the double Tessier kinetic model (SSD = 0.97). Table C.1 shows the results of all the possible growth models using combinatons of Equation 2.9 – 2.12 for oxygen and Equations 2.13, 2.14, 2.15, 2.16, 2.17, 2.18 and 2.20 for glucose.

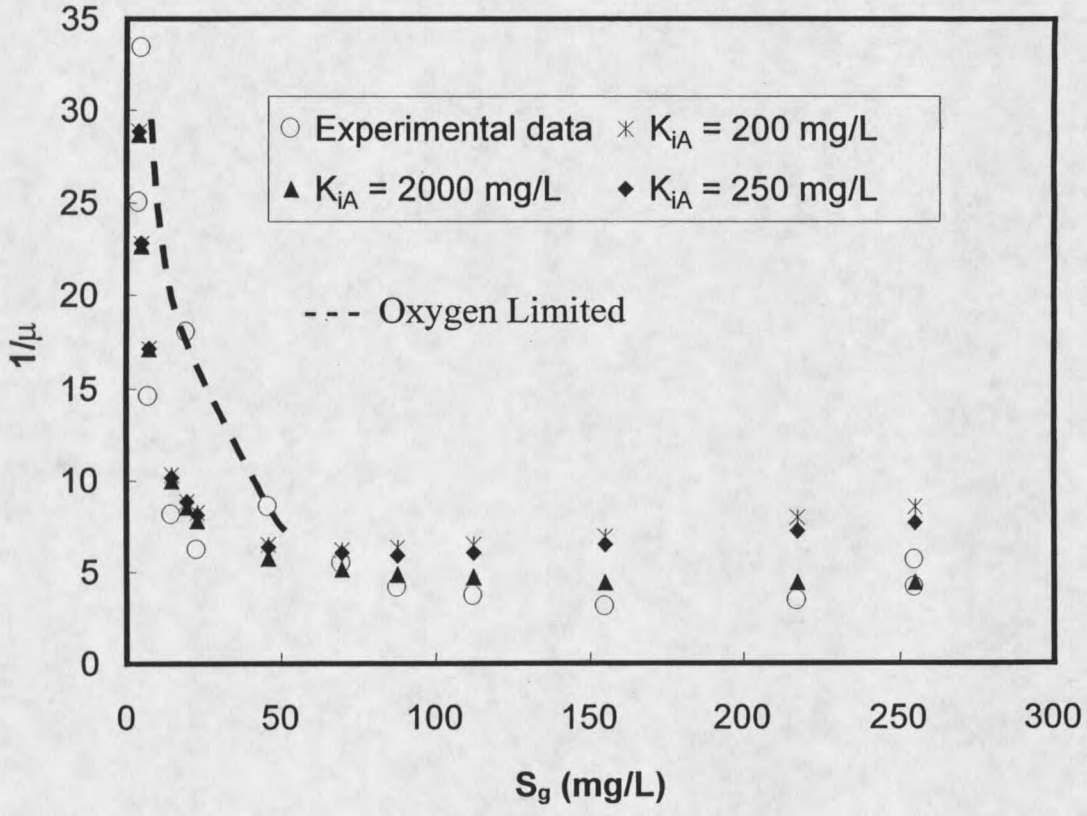

Figure C.1. The Lineweaver-Burk plot, S_g vs. $1/\mu$, using inhibition constants of 200, 250 and 2000 mg/L for glucose using Equation 2.13. ($\mu_{max} = 0.28\ h^{-1}$, $K_{SA} = 26.8$ mg/L, S_g = experimental glucose concentrations).

Table C.1. Mathematical modeling using substrate inhibition kinetic models for glucose (Equations 2.13, 2.14, 2.15, 2.16, 2.17, 2.18, and 2.20).

Eqn. for Oxygen	Eqn. for Glucose	λ_o	λ_g	B_o	B_g	K_o	K_g	μ_{max}	K_{I_g}	c	SDS	R^2
-	2.13	-	-	-	-	-	104.111	0.691178	267.46	-	0.023311	na
-	2.20	-	-	-	-	-	2.6	2.37E-06	-	255	0.33890972	na
2.9	2.13	-	-	-	-	0.8735	53.84138	0.6935	256.84	-	0.01046	na
2.10	2.13	-	-	-	-	0.0001	48.20995	0.4424	239.00	-	0.380314	na
2.11	2.13	1.65	-	-	-	0.7677	52.08591	0.6202	256.91	-	0.009534	na
2.12	2.13	-	-	2.77E-04	-	-	54.17438	0.6898	256.73	-	0.010907	na
2.9	2.14	-	-	-	-	0.2573	7.654599	0.0571	43.12	-	0.024523	na
2.10	2.14	-	-	-	-	1.2451	115.3702	1.0000	22.97	-	0.010684055	na
2.11	2.14	2.25	-	-	-	0.4498	7.716730483	0.1022	21.25	-	0.015923478	na
2.12	2.14	-	-	1.70E-04	-	-	9.43018	0.1144	25.71	-	0.01952	na
2.9	2.15	-	-	-	-	1.4180	69.48268	0.8212	723.90	-	0.006037	na
2.10	2.15	-	-	-	-	2.1146	58.81991	0.9916	256.35	-	0.047132581	na
2.11	2.15	0.89	-	-	-	1.6055	76.1402	0.9434	604.65	-	0.00602	na
2.12	2.15	-	-	5.00E-04	-	-	82.2992	0.9494	501.50	-	0.00574	na
2.9	2.16	-	-	-	-	0.8308	42.11644762	0.5552	401.03	-	0.010268832	na
2.10	2.16	-	-	-	-	1.2330	48.21481931	0.5545	401.03	-	0.007750087	na
2.11	2.16	1.70	-	-	-	0.7248	40.1106743	0.4948	499.91	-	0.009289862	na
2.12	2.16	-	-	2.63E-04	-	-	42.83518711	0.5534	499.31	-	0.010756286	na
2.9	2.17	-	-	-	-	1.0382	55.3848791	0.6877	501.23	-	0.00648799	na
2.10	2.17	-	-	-	-	1.6204	77.5023373	0.7851	408.92	-	0.00503329	na
2.11	2.17	1.30	-	-	-	1.0148	50.016784	0.6253	500.00	-	0.00639264	na
2.12	2.17	-	-	3.38E-04	-	-	55.249144	0.6850	501.60	-	0.00661787	na
2.9	2.18	na	na	na	na	na	na	na	na	na	na	na
2.10	2.18	na	na	na	na	na	na	na	na	na	na	na
2.11	2.18	na	na	na	na	na	na	na	na	na	na	na
2.12	2.18	na	na	na	na	na	na	na	na	na	na	na
2.9	2.20	-	-	-	-	1.0162	20	3.39E-06	-	250	0.334554	na
2.10	2.20	-	-	-	-	1.0055	20	2.89E-06	-	254.9998	0.32812461	na
2.11	2.20	0.1	-	-	-	1.4301	20	6.00E-06	-	255	0.342792	na
2.12	2.20	-	-	2.56E+01	-	1.0000	19.99985	0.02788	-	254.9995	0.406283	na