



Multi-element hollow fiber membrane bioreactors for cultivation of *Pseudomonas putida*
by James V Odasso

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

The goal of this research was to examine growth patterns of *Pseudomonas putida* immobilized within multi-element hollow fiber membrane (HFM) bioreactors. Understanding bacterial growth patterns provides important information for modeling and scale up of a bioreactor system and will lead to an improved understanding of cell physiology under possible mass transport limitations. Such information will allow one to also set reasonable performance expectations. Three objectives existed for this project: (1) construction of a multi-element HFM bioreactor system and development of methods for cell inoculation, cultivation, and harvesting, (2) development of methods for obtaining glucose, oxygen, DMA, RNA, and protein profiles within the HFM bioreactor, (3) map the accumulation of biomass within the bioreactor as an indication of reactor performance.

Evaluation of reactor performance through examination of glucose removal rate, RNA/Protein ratio of entrapped cells, and total cell protein within the fibers indicated very poor bioreactor performance. Additionally, cell breakout could not be prevented and the physical dynamics of the HFM bioreactor used for this project were unpredictable. The combined evidence of low biomass retention, limited glucose removal, and small RNA to Protein ratio led to the conclusion that very limited to no growth was taking place in 3 of 4 HFM reactor systems examined for this project. The disadvantages of the hollow fiber reactors used in the project far out number the advantages of such a system at this time.

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APPROVAL

of a thesis by

James V. Odasso

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. James D. Bryers

James D. Bryers 8 Dec. 95
(Signature) (Date)

Approved for the Department of Chemical Engineering

Dr. John Sears

John T. Sears 8 Dec 95
(Signature) (Date)

Approved for the College of Graduate Studies

Dr. Robert Brown

R. Brown 1/8/96
(Signature) (Date)

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ACKNOWLEDGMENTS

The following statement has a special meaning to me because it summarizes the frustrations that a person with Dyslexia faces every day. The author and I share this problem so I understand that to get anywhere in life you must never give up because with enough work things will come out right.

*"I think for months and years
ninety nine times the conclusion is false*

The hundredth time, I am right."

Albert Einstein

A very special thanks goes out to Mrs. Mary Huenergardt, my life would be very different if not for the kindest and most caring woman I have ever met other than my mom.

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ABSTRACT

The goal of this research was to examine growth patterns of *Pseudomonas putida* immobilized within multi-element hollow fiber membrane (HFM) bioreactors. Understanding bacterial growth patterns provides important information for modeling and scale up of a bioreactor system and will lead to an improved understanding of cell physiology under possible mass transport limitations. Such information will allow one to also set reasonable performance expectations. Three objectives existed for this project: (1) construction of a multi-element HFM bioreactor system and development of methods for cell inoculation, cultivation, and harvesting, (2) development of methods for obtaining glucose, oxygen, DNA, RNA, and protein profiles within the HFM bioreactor, (3) map the accumulation of biomass within the bioreactor as an indication of reactor performance.

Evaluation of reactor performance through examination of glucose removal rate, RNA/Protein ratio of entrapped cells, and total cell protein within the fibers indicated very poor bioreactor performance. Additionally, cell breakout could not be prevented and the physical dynamics of the HFM bioreactor used for this project were unpredictable. The combined evidence of low biomass retention, limited glucose removal, and small RNA to Protein ratio led to the conclusion that very limited to no growth was taking place in 3 of 4 HFM reactor systems examined for this project. The disadvantages of the hollow fiber reactors used in the project far out number the advantages of such a system at this time.

GOALS AND OBJECTIVES

The goal of this research is to examine growth patterns of *Pseudomonas putida* immobilized within a multi element hollow fiber membrane (HFM) bioreactor. Understanding bacterial growth patterns provides important information for modeling and scale up of a bioreactor system. Information on development of the immobilized cells in HFM bioreactors will lead to an improved understanding of cell physiology under possible mass transport limitations. Such information will allow one to also set reasonable performance expectations. Three objectives exist for this project. First, construct a multi element HFM bioreactor system and develop methods for cell inoculation, cultivation and harvesting. Second, develop methods for obtaining glucose, oxygen, DNA, RNA and protein profiles within the HFM bioreactor. Third, map the accumulation of biomass within the bioreactor as an indication of reactor performance.

CHAPTER 1

Introduction

Interest in whole cell biocatalysts has steadily increased over the past two decades and, more recently, due to advances in recombinant DNA and cell fusion technologies (Belford, 1988). Man has used biocatalyst capabilities in nature to improve his well-being throughout history, by collecting, screening, selecting and domesticating living organisms (Akin, 1987). Molecular biology is rapidly creating new tools to design, engineer and modify biocatalytic activities. Whole cell biocatalysis are capable of complex multiple species chemical reactions which no single catalysis, biological or other, could accomplish individually. Application of this technology to any system requires a mode of cell cultivation. Evolution of cell cultivation systems has involved suspended cell bioreactors (i.e., chemostats), biofilm reactors (i.e., Annular reactors), and porous matrix biofilm reactors (i.e., HFM reactors). Each bioreactor type has distinct and inherent advantages and disadvantages when applied to whole cell biocatalyst systems. Cultivation of a biocatalyst is a specialized application where large quantities of biomass accumulates in the smallest reactor volume possible. Immobilization of the whole cells upon a support matrix provides an avenue through which to minimize the bioreactor volume required for a specific reaction. In some phase of their life cycles, most cells tend to locate or attach themselves to a solid surface (Messing, 1985). This attachment and growth of cells is referred to as biofilm development. Biofilms will form on any surface

although in some fermentors and in waste treatment systems flocculation, pellet formation, can also be viewed as a form of immobilization. For a formal definition, immobilized cell, biofilm, biocatalysis can be designated as, "cells which are physically confined or localized in a defined region or space with retention of their catalytic activities or selected portions thereof, for repeated and continuous use" (compare with Klein and Wager, 1983). The application of a bioreactor for treatment of a contaminated waste stream or production of a specialty chemical involves cultivation of biomass as well as production of effluent devoid of cellular material. HFM bioreactors have the potential to effectively remove the pollutant while effectively retaining the biocatalyst in the reactor.

Suspended vs. Immobilized Cell Bioreactor Systems

Immobilized cell reactor systems have distinct advantages over suspended (or planktonic) cell cultivation systems including improved product yield and process efficiency. The use of cheap, easily obtainable materials for construction of the reactors is another advantage. Support material for cell immobilization can be composed of almost anything. The "quick vinegar" process, invented in 1823 by Scheulzenback (Biofilms, P.736), makes use of wood chips as the biofilm support substance. A list of support materials and the bacteria used for ethanol production is provided in Table 1. Immobilized cells allow the reactor to be operated at residence times well below the generation

time of the microbial species. Suspended continuous flow bacterial cultures, i.e., chemostats, are limited by the growth rate of the cells; when the dilution rate of a

Table 1
Immobilized Cells for Ethanol Production

Immobilization Method	Organism	Reference
Adsorption to wood chips	<i>Saccharomyces cerevisiae</i>	Gencer, 1983
Adsorption to ion exchange resins	<i>S. cerevisiae</i>	Daugulis, 1981
Adsorption to glass fiber filters	<i>Zymomonas mobilis</i>	Arcuri, 1982
Adsorption to gelatin-coated supports	<i>S. cerevisiae</i>	Sitton, 1980
Entrapment in polyurethane foam or stainless steel mesh	<i>S. cerevisiae</i> <i>S. uvarum</i>	Black, 1984
entrapment in κ -carrageenan	<i>S. cerevisiae</i>	Wanda M, 1980
co-entrapment in Ca alginate with β -D-glucosidase	<i>Z. mobilis</i>	Lee, 1983
co-entrapped in Ca alginate with magnetite	<i>S. cerevisiae</i>	Lasson P.O., 1981
entrapped in polyacrylamide	<i>S. formosensis</i>	Furusaki, 1983
entrapped in Na alginate	<i>Kluveromyces marxianus</i>	Margaritis, 1982
entrapment in polyacrylamide or in κ -carrageenan	<i>Kl. fragi</i>	King, 1983
entrapped in pectin	<i>S. cerevisiae</i>	Navarro, 1983
containment by hollow fiber membranes	<i>S. cerevisiae</i>	Inloes, 1983
containment of cells with cellulase and cellobiose by means of a liquid-liquid phase boundary	<i>S. cerevisiae</i>	Hahn-Hagerdal, 1982
Flocculation of yeast	<i>Schizosaccharomyces pombe</i>	Hsiao, 1983

suspended cell bioreactor exceeds the maximum culture growth rate, the cells

will wash out of the bioreactor. Immobilized cell bioreactors physically retain the cells and thus they can handle higher flow rates than suspended cell reactors.

Immobilized cell bioreactor systems also provide an avenue through which multiple bacterial species can be cultured in a synergistic environment.

Advantages of cell immobilization also include reduction in cost of bioprocessing because of repeated and continuous use of the biocatalyst, reduction in required separation processes and protection of shear-sensitive cells such as plant and animal. Recent advances in genetic engineering and cell fusion technologies have produced a class of biocatalyst which benefit from immobilization through improved plasmid stability (de Taxis du Poet, Dhulster, Tomas, 1984 and C.T. Huang, 1992). Immobilized cell bioreactors do have serious limitations including: (1) excessive internal mass transfer resistance due to excessive cell densities, (2) destruction of the cell support system by growing cells and, (3) cell sloughing from the cell support. A challenge of immobilized bioreactor systems is to develop a versatile, general cell support capable of retaining a wide variety

Table 2
Categories of Support Systems

Table 2	
Categories of Support Systems	
1	Solid supports or matrices for adhesion or adsorption of cells.
2	Solid supports or matrices for cross linking with cells.
3	Directly cross linked cells.
4	Gel and other polymers for entrapment or encapsulation of cells.
5	Combinations of gels and other polymers for encapsulation or entrapment of cells.
6	Composite immobilization matrix.
7	Hollow structures, fibers and plates (membrane reactors) for physical retention of cells.

of cells with applications to differing bioprocesses. A summary of the basic types of cell support systems was prepared by Akin (1987) and is presented in Table 2. Criteria for cell supports and matrices were developed by Klien and Wagner (1983) and Brodelius (1985) and are presented in Table 3.

Table 3 Criteria for Cell Supports and Matrices	
1	Not reduce the desired biocatalytic activity of the cell.
2	Not react with the substrates, nutrients or products.
3	Retain their physical integrity and be insoluble under bioprocess reaction conditions.
4	Be permeable to reactants and products.
5	Have large specific surfaces.
6	Have high diffusion coefficients for substrates, nutrients and products.
7	Provide appropriate hydrophilic-hydrophobic balance for nutrients, reactants and products.
8	Be resistant to microbial degradation.
9	Retain chemical and thermal stability under bioprocess and storage conditions.
10	Be elastic enough to accommodate growing cells.
11	Have functional groups for cross linking.
12	Be generally available in adequate quantities with consistent quality and acceptable price.
13	Be generally recognized as safe for food and pharmaceutical bioprocess applications.
14	Be easy and simple to handle in the immobilization procedure.
15	Be environmentally safe to dispose of and/or be capable of recycling.

Types of Immobilization Systems

There are six types of immobilization systems which can be separated into two major categories, chemical immobilization and physical immobilization.

Chemical methods include cross-linking, chelation and covalent bonding.

Flocculation may also be included in this category but is thought to be caused by natural poly electrolytes (Biological Wastewater Treatment, Grady and Lim, 1980). Table 4 is a partial list of organisms which are prone to flocculation through addition of cathodic polyelectrolyte agents to a bioreactor system (Lee and Long, 1974). Physical immobilization methods are cell entrapment and adsorption of cells to a surface. A comparison of attributes for chemical and physical immobilization techniques are summarized in Table 5 and Table 6 (compare with Kennedy, 1983). Table 7 is a partial list of microbial systems which make use of chemical immobilization techniques (compare with Kennedy, 1983). Figure 1 shows *Pseudomonas aeruginosa* bacteria entrapped within an agar bead. This type of immobilization is common, although often injures the cells lowering their biocatalytic ability. When these cells are subjected to a growth environment, development of micro colonies can result in the release of cells from the gel bead. The cells are retained by a polymer matrix which surrounds the cells with nutrients diffusing into the polymer so the cells replicate. The highest rate of growth is near the surface of the matrix where the nutrient concentration is greatest. The existence of micro colonies within the polymer matrix cause weak points to develop. The weak points rupture releasing the whole cells into the environment. This type of process is referred to as detachment in natural biofilms and is a continuous process. Rapid detachment of cells from a surface is known as sloughing and is a response to a process

problem such as excessive mass transport limitations. When the cells are encapsulated the release is called breakout. This process can be seen as it occurred in a gel bead in figure 2a and figure 2b. Breakout is a major disadvantage through two avenues. First, loss of the biocatalyst reduces the effectiveness of the reactor. Second, release of the cells into the reactor effluent creates the need for a separation process down stream. Separation processes are expensive, cumbersome and require frequent maintenance.

Many types of gel polymers have been developed in order to immobilize a wide variety of cells (Table 8). Figure 3 shows a porous carrageenan bead for cultivation of *P. urticae* cells grown in nitrogen free medium. Note the heavy growth throughout the interior surface of the bead.

Table 4
Examples of Whole Cells Immobilized by Flocculation

Cross-linking agent	Cells	Reference
Cationic Polyelectrolyte	<i>Aspergillus niger</i> <i>Streptomyces olivaceous</i> <i>Arthrobacter sp.</i>	Lee & Long, 1974

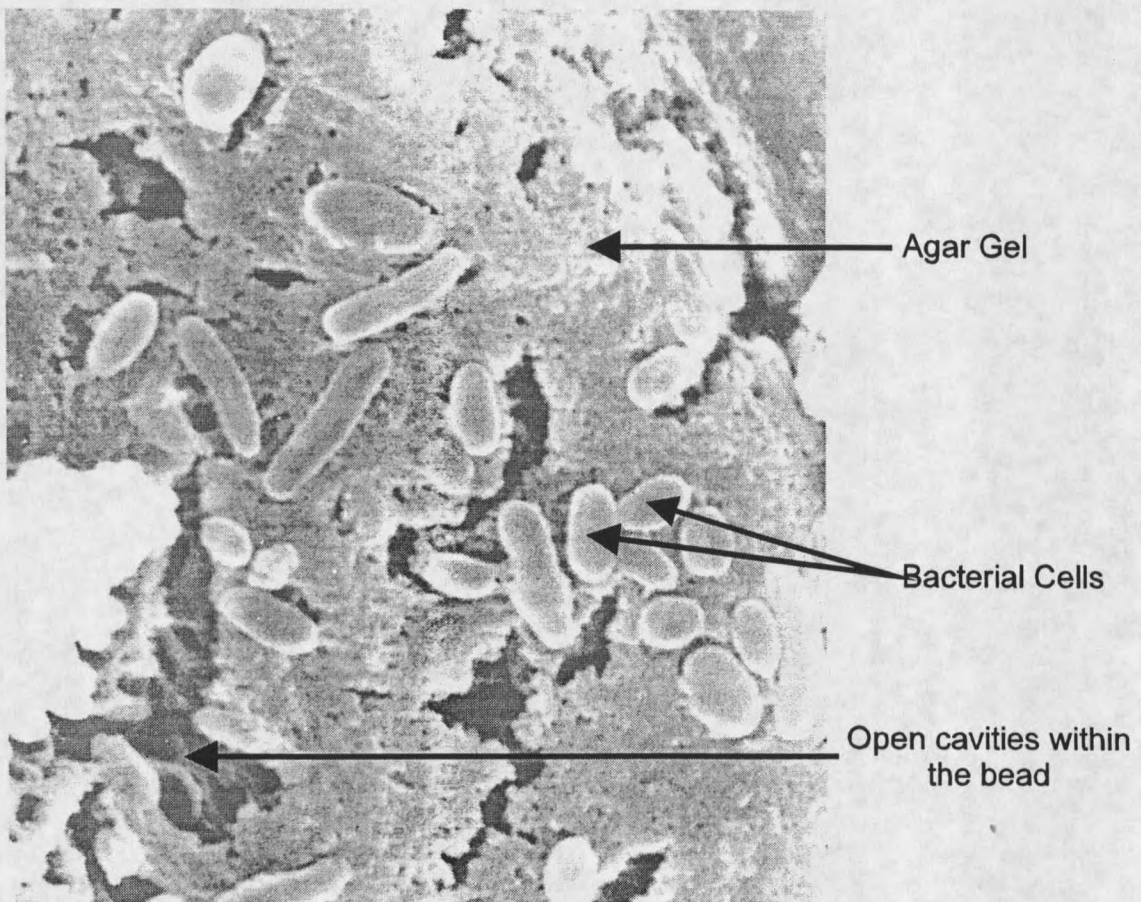
Table 5
Attributes of Various Classes of Chemical Immobilization Techniques

Characteristic	Cross-linking	Chelation	Covalent Bonding
Preparation	Intermediate	Simple	Difficult
Binding Force	Strong	Intermediate	Strong
Retention of Activity	Low	Intermediate	Low
Regeneration of carrier	Impossible	Possible	Rare
Cost of immobilization	Intermediate	Low	High
Stability	High	Intermediate	High
General applicability	No	Yes	No
Protection from microbial attack	No	Yes	No
Viability	No	Yes	No

Table 6
Attributes of Various Classes of Physical Immobilization Techniques

Characteristic	Adsorption	Entrapping
Preparation	Simple	Intermediate
Binding Force	Weak	Intermediate
Retention of Activity	High	Intermediate
Regeneration of carrier	Possible	Impossible
Cost of immobilization	Low	Low
Stability	Low	High
General applicability	Yes	Yes
Viability	Yes	Yes
Protection from microbial attack	Yes	Yes

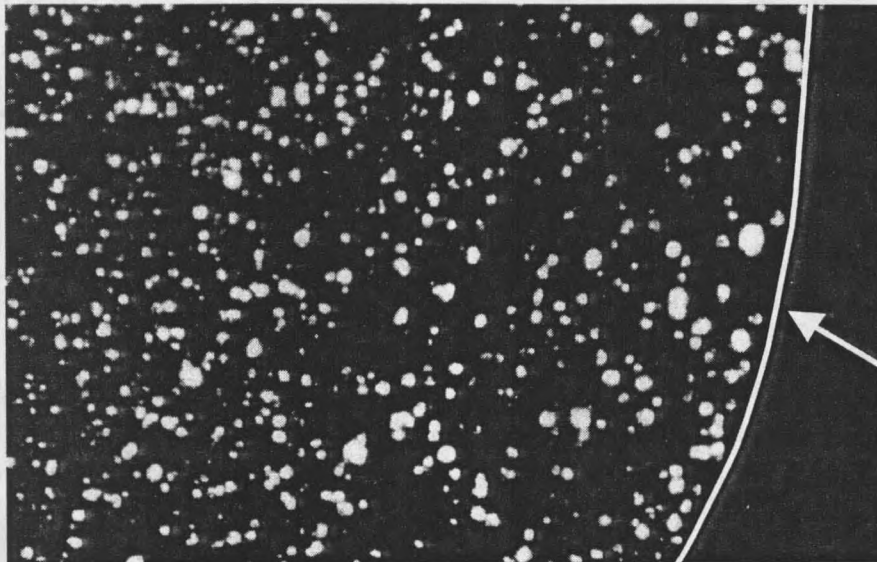
Figure 1
Pseudomonas aeruginosa Cells Immobilized within a Agar Bead



Photograph was obtained from: J. Sui-Lung Lam, 1983

Figure 2a
E.coli DH5 α , Immobilized Within Sr-Alginate
Stained For Total DNA With Mithramycin-A

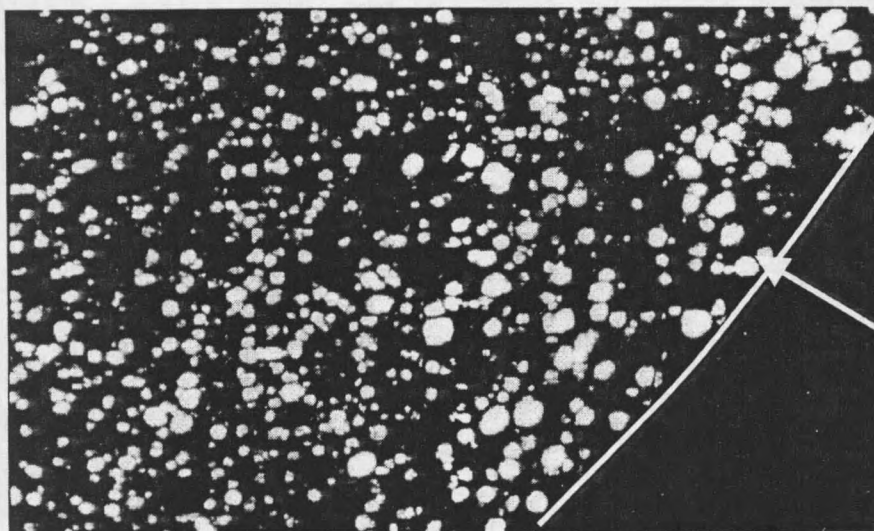
Time =15 hours



Gel Bead Surface

Uniform distribution of bacterial colonies

Time =28 hours



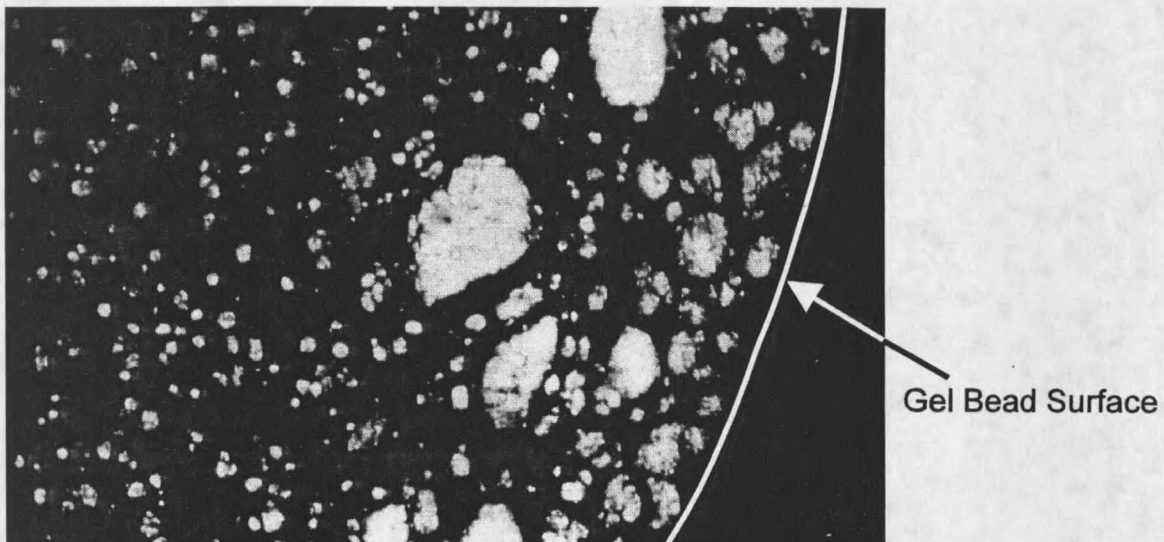
Gel Bead Surface

Uneven development of the bacterial colonies

Photograph series was obtained from: D.F. Ollis, *et.al.*, 1990

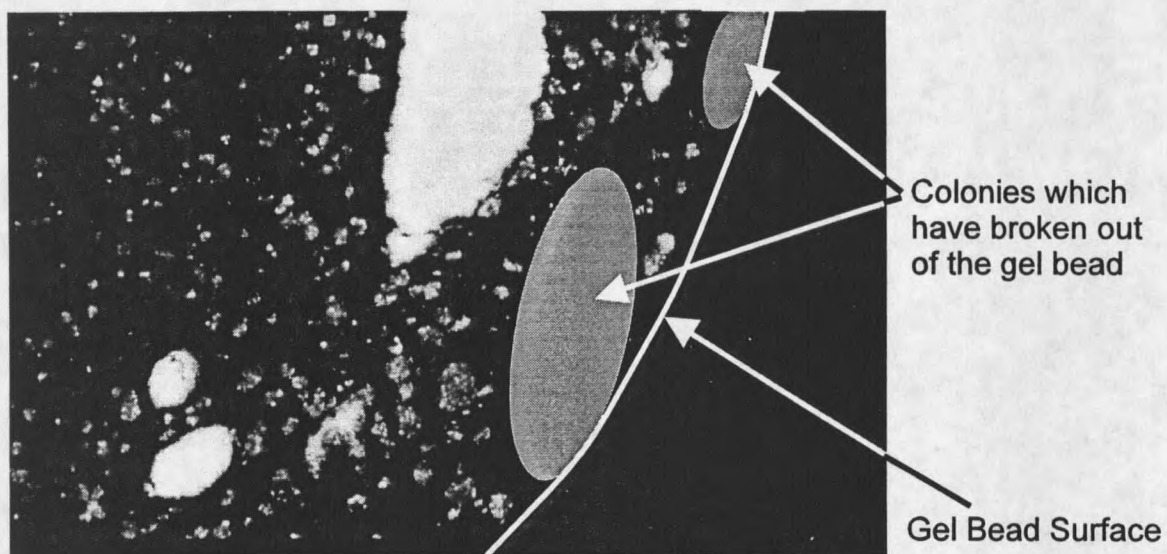
Figure 2b
E.coli DH5 α , Immobilized Within Sr-Alginate
Stained For Total DNA With Mithramycin-A

Time = 52 hours



Continued uneven growth of bacterial colonies

Time = 70 hours



Break out of bacterial colonies

Photograph series was obtained from: D.F. Ollis. *et. al.*, 1990

Table 7

**Examples of Whole Cells Immobilized
by Chemical Intracellular Cross-Linking**

Chemical cross-linking agent	Cells	Reference
Diazotized Diaminies	<i>Streptomyces</i> sp.	Lartigue & Weetal, 1976
Glutarahyde	<i>Bacillus coagulans</i> <i>Escherichia coli</i>	Poulson & Zittan, 1976 Chibata et al., 1974
Toluene Diisocyanate	<i>E. coli</i>	Chibata et al., 1974
Toluene Diisocyanate + 1,6-diaminohexane	<i>E. coli</i>	Chibata et al., 1974

Examples of Whole Cells Immobilized by Chelation

Hydrous metal oxide	Cells	Reference
Hydrous titanium(IV) oxide	<i>Acetobacter</i> sp. <i>E. Coli</i> <i>Saccharomyces cerevisiae</i> <i>Serratia marcescens</i>	Kennedy et al., 1980 Kennedy et al., 1979 Kennedy et al., 1979 Kennedy et al., 1979
Hydrous zirconium(IV) oxide	<i>E. coli</i> <i>S. marcescens</i>	Kennedy et al., 1979 Kennedy et al., 1979

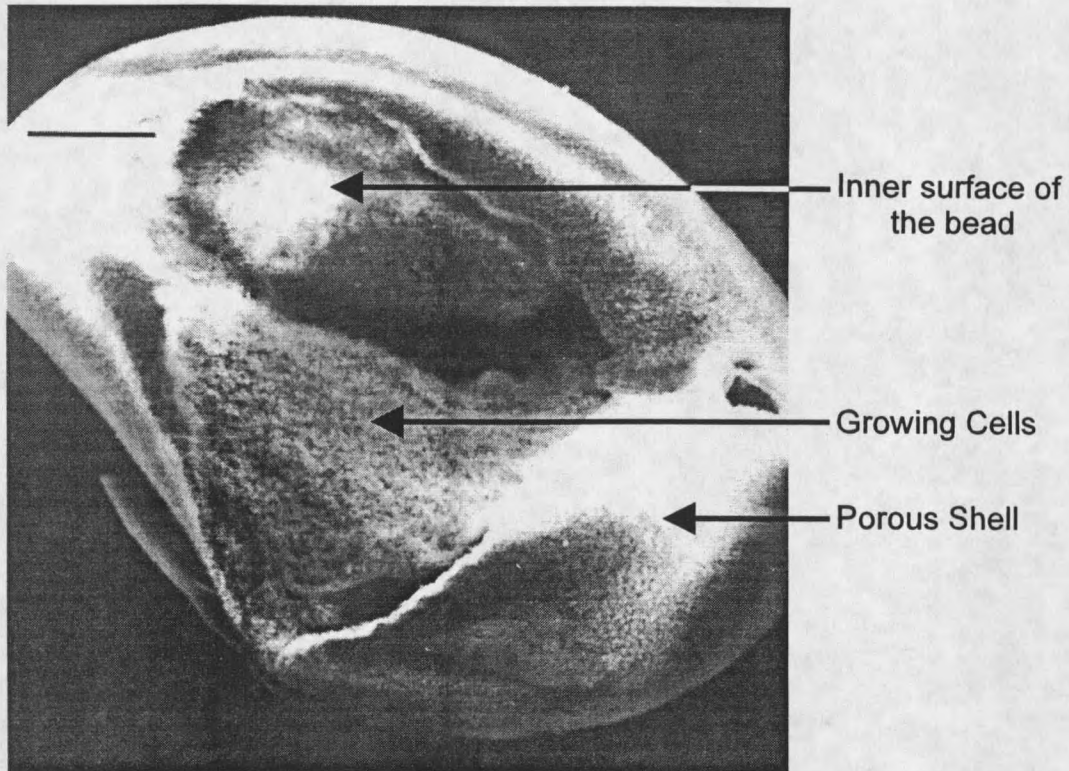
Examples of Whole Cells Immobilized by Covalent Bonding

Carrier	Coupling Section	Cells	Reference
Hydroxyalkyl methacrylate activated with epichlorhydrin + glutaraldehyde	Schiff's base formation	<i>Saccharomyces cerevisiae</i>	Jirk'u et al., 1980
Cellulose activated with cyanuric chloride	Alkylation	<i>Azotobacter vinelandii</i> <i>S. cerevisiae</i>	Gainer et al., 1981
Carboxymethyl-cellulose activated with carbodiimide	Peptide binding	<i>Micrococcus luteus</i>	Jack and Zajic, 1977

Table 8		
Selected Examples of Gel Entrapped Living Whole Cells		
Material	Cells	Reference
Agar	<i>Azotobacter chroococcum</i> <i>Catharanthus roseus</i> <i>S. cerevisiae</i>	Karube et al., 1981 Brodelius & Nilsson, 1981 Margalith & Holcberg, 1981
Agarose	<i>C. roseus</i>	Brodelius & Nilsson, 1981
Albumin + glutaraldehyde	<i>E. coli</i>	Petre et al., 1978
κ -Carrageenan	<i>C. roseus</i> <i>Penicillium urticae</i> <i>Serratia marcescens</i>	Brodelius & Nilsson, 1981 Deo & Gaucher, 1983 Wada et al., 1980
Collagen + glutaraldehyde	<i>K. pneumoniae</i> <i>Penicillium chrysogenum</i>	Venkatasubramanian & Toda, 1981 Morikawa et al., 1979
Crude egg white + glutaraldehyde	<i>Caldariella acidophila</i>	De Rosa et al., 1981
Gelatin + glutaraldehyde	<i>Arthrobacter X4</i>	Tramper et al., 1979
Polycondensation of epoxides	<i>E. coli</i>	Klein & Wagner, 1980
Silica hydrogel	<i>S. cerevisiae</i>	Rouxhet et al., 1981
Examples of Gel and Polymer Combination Entrapped Living Whole Cells		
Gel With Other Polymers		References
Calcium Alginate and Silicone		Kawakami, 1990
Agar and Polyacrylamide		Kuu, 1985
Calcium Alginate and Polyamine-sulphone		Sakata, 1985
Calcium Alginate and Photo Cross-linkable Polymer		Mitani, 1984
Carrageenan and Locust bean gum		Mitani, 1984
Carrageenan and Photo Cross-linkable Polymer		Mitani, 1984
Co-polymers of hydrophilic and hydrophobic monomers		Kumakura, 1984

Figure 3

Encapsulation - Entrapment Method

Penicillium urticae Cells Cultivated within a Carrageenan Porous Bead

Photograph obtained from: Y.M. Deo, 1982

m.cdr

Hollow Fiber Immobilized Cell Bioreactor

Hollow fiber membranes (HFM) are artificial matrixes used for cell immobilization. Entrapment of enzymes and whole cells within the HFM support matrix has been used since the early 1970s for production of speciality chemicals and cell cultivation. Immobilized whole cell reactor systems have several advantages over suspended whole cell systems such as: greater biomass retention and independence of growth rate from reactor dilution rate. Table 9 provides a partial list of applications which have employed hollow fiber

bioreactor technology. Use of an immobilized whole cell reactor system in which

Table 9
Selected Applications of HFM Bioreactor Technology

Organism	Process	Reference
<i>Ps. fluorescens</i>	L-histidine Production	Webster <i>et al.</i> , 1979
<i>E. coli</i>	B-lactamase Production	Inloes <i>et al.</i> , 1983
<i>Acetobacter rancens</i>	Vinegar Production	Nanba <i>et al.</i> , 1985
<i>Xanthobacter Py2</i>	Air purification	Reij <i>et al.</i> , 1994
<i>Z. mobilis</i>	Cell cultivation	Belfort, 1988
<i>C. acidophila</i>	Cell cultivation	Belfort, 1988
<i>S. cerevisiae</i> (Yeast)	Cell cultivation	Belfort, 1988
Liver Cells (Rat, Dog, Pig)	Cell cultivation	Rozga <i>et al.</i> , 1993

a synthetic porous matrix is used for entrapment of a biocatalyst and has the potential to eliminate release of cells and the need of down stream separation processes.

A crosssectional view of a asymmetric macro void Amicon HFM is shown in figure 4 and in contrast figure 5 shows a uniform porous matrix HFM produced by AGT Corporation. Both fibers have thin inner cylindrical membranes forming the lumen which is surrounded by a porous support structure. Hollow fiber membranes have been fabricated from various polymer materials including: cellulose acetate, polycarbonate, polyvinylchloride, polyamides, modacrylic copolymers, polysulfones, halogenated resins, and various polyelectrolytes (ref. Handbook of Separation Techniques for Chemical Engineers, 2nd ed, 1988, P.2-

Figure 4
Amicon Corporation's Macro-void Hollow Fiber Membrane

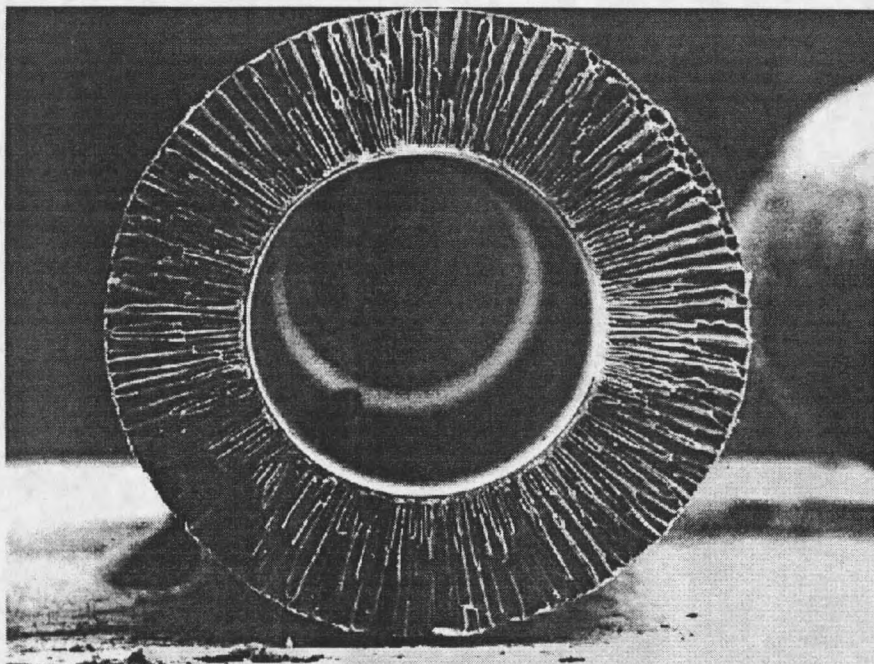
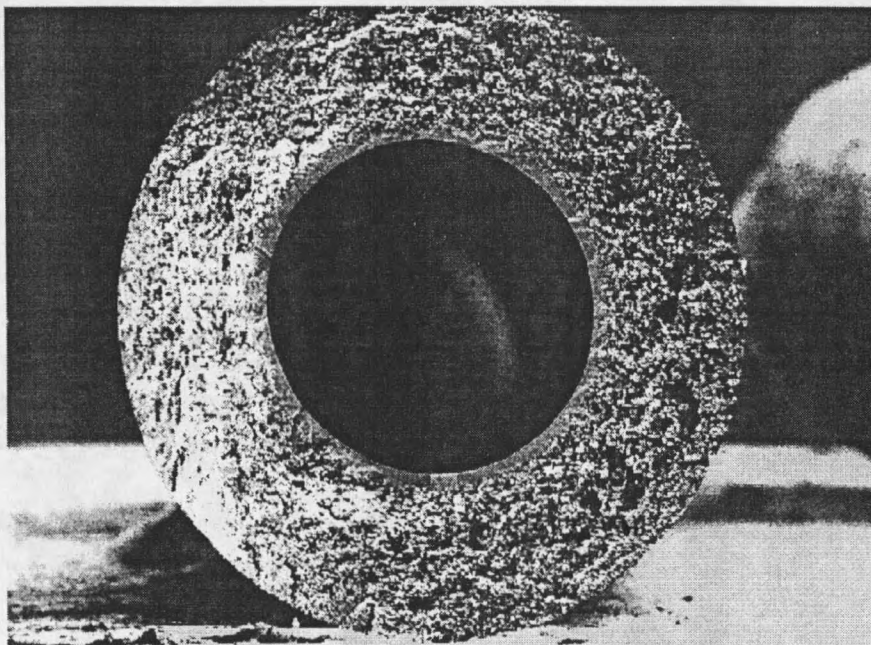


Figure 5
AGT Corporation's Hollow Fiber Membrane



Photographs courtesy of AGT Corporation, Needham, MA

24). The inner membrane can be designed to repel 90 % of molecules over a specific molecular weight (Amicon, 1994). Membrane fibers are designed to separate particulate and colloidal material from aqueous solutions although HFM such as Ucarsep produced by Union Carbide are designed for solvent separations. The Amicon H1P30-43 polysulfone HFM is constructed with a 30,000 molecular weight (MW) cut off interior membrane so only soluble components with a MW below 30,000 will pass through this membrane.

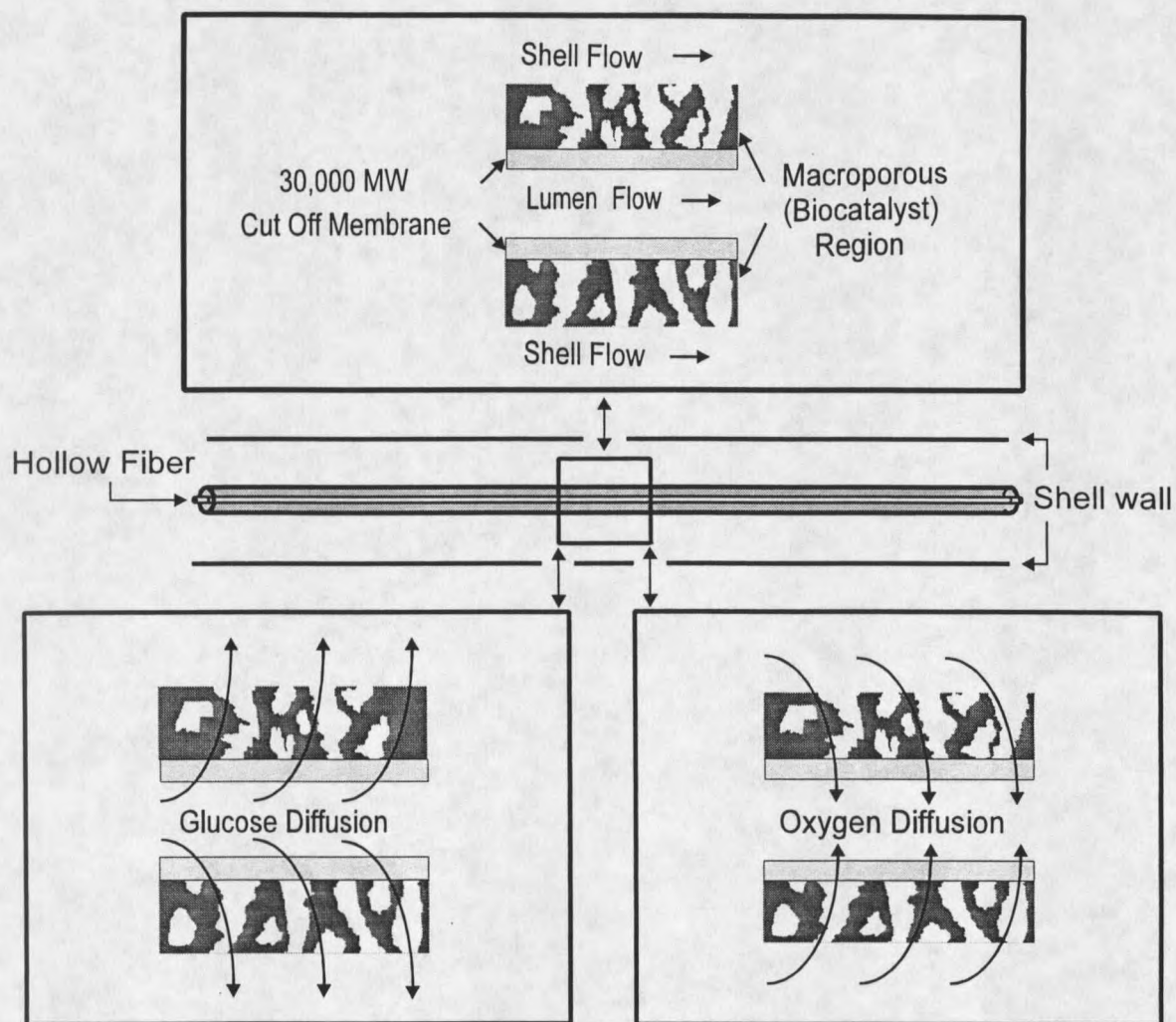
In experiments carried out here, HFMs were used as a support system for a single species whole cell bacterial culture. The bacterial culture used in this system was *Pseudomonas putida* (*Ps. putida*). Cells were seeded into the macro porous support structure surrounding the inner lumen membrane. Two specific challenges arise when using HF membranes as bioreactors. First, the bacterial culture must be supplied all the nutrients necessary for their survival. An exception to this criterion is when the cells are maintained in a non-growth state. Second, they must have enough space to grow. If cells grow within a confined space, they can exert considerable pressure on the fibers, Stewart et al (1989) reports. *Escherichia coli* growing in a confined space can create 3 atm in static pressure. This pressure could be sufficient to break the lumen membrane allowing cells to enter the lumen fluid. This problem is common when all the growth nutrients are supplied from the lumen side of the membrane. The approach taken for these experiments maybe one scenario which minimizes excessive cell growth. Delivering, for example, the electron donor and the

electron acceptor in separate streams requires each to diffuse into the macro porous region from opposite sides. Figure 6 illustrates electron acceptor and donor concentration profiles in a hypothetical HFM uniformly filled with bacterial cells in the macro porous structure. Variances in flow rates can change the quantity of substrate supplied to the reactor and transmembrane pressure gradients could develop if the volumetric shell and lumen flows are not balanced. Pressure gradients result in convective flows through the membranes which have the potential to wash biomass from the fibers. Convective flows also can pollute the anaerobic glucose stream with oxygen or the shell side nutrient stream with glucose.

Figure 4 is a Amicon #H1P30-43 fiber and Figure 5 is a AGT hollow fiber. Both fibers are constructed of polysulfone and have a 30,000 molecular weight (MW) cut off membrane on its inner (Lumen) surface. The primary difference between these two types of HFM is in the membrane support structure. Amicon uses a macro void support structure which allows the immobilized whole cells to reside very close to the membrane itself. AGT uses a uniform porosity support structure which prevents cells from coming in intimate contact with the membrane. Intimate contact of living cells with the fragile 30,000 MW cut off membrane increases the possibility of cells breaking through the lumen membrane. Both fiber types can be inoculated in the same manor. Whole cells are supplied from the outer edge of the fiber and drawn toward the center (lumen) of each fiber by a transmembrane pressure drop. The membrane acts,

as a barrier to prevent release of the cells to the lumen fluid.

Figure 6
Fiber Structure And Diffusion Patterns



CHAPTER 2

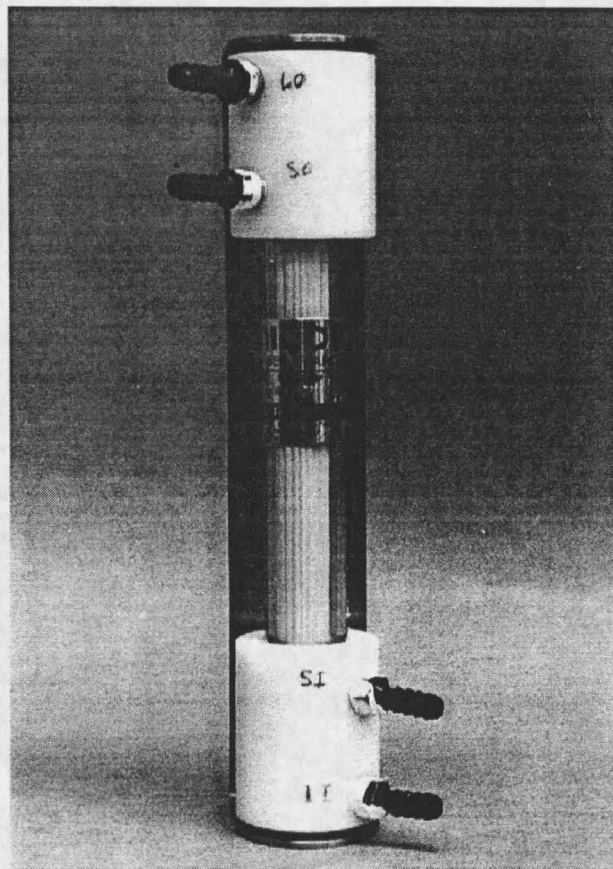
Experimental Design

The goal of this research is to examine growth patterns of *Pseudomonas putida* (*Ps. putida*) immobilized within a multiple hollow fiber membrane (HFM) bioreactor. Three objectives exist for this project which include design of the experimental HFM bioreactor system, development of immobilized cell culturing methods and procedures for substrate sampling. This project involved use of two, multiple fiber, HFM bioreactors. Two experiments involved a commercially available HFM cartridge manufactured by Amicon Corporation. The H1P30-43 HFM cartridge contained 55 fibers bundled together in a configuration similar to a non-baffled heat exchanger with its primary intended application being filtration. Experiments 3 and 4 made use of a, multiple fiber, HFM reactor which was designed and constructed in house. This reactor used a configuration similar to a baffled shell and tube heat exchanger. The alteration in the reactor design was made in an effort to reduce mass transfer limitations so as to increase cell growth within the reactor. The operating conditions were changed also in an effort to improve reactor performance. The analytical methods for determination of biomass and substrate concentrations remained the same for both types of reactors and evaluation of the HFM bioreactor performance was made by using the various assay results.

Experimental System - Amicon Hollow Fiber Cartridge

The Amicon H1P30-43 hollow fiber cartridge as pictured in Figure 7 is described in Table 10. The bioreactor was operated under the conditions outlined in Table 11. The lumen and shell flows were co-current as shown in Figure 8 with the nutrients, required for growth, diffusing through the membrane as shown in Figure 6. The environmental support system for the Amicon cartridge is pictured in Figure 12 and described in Table 14.

Figure 7
Amicon Hollow Fiber Membrane Cartridge
Model H1P30-43



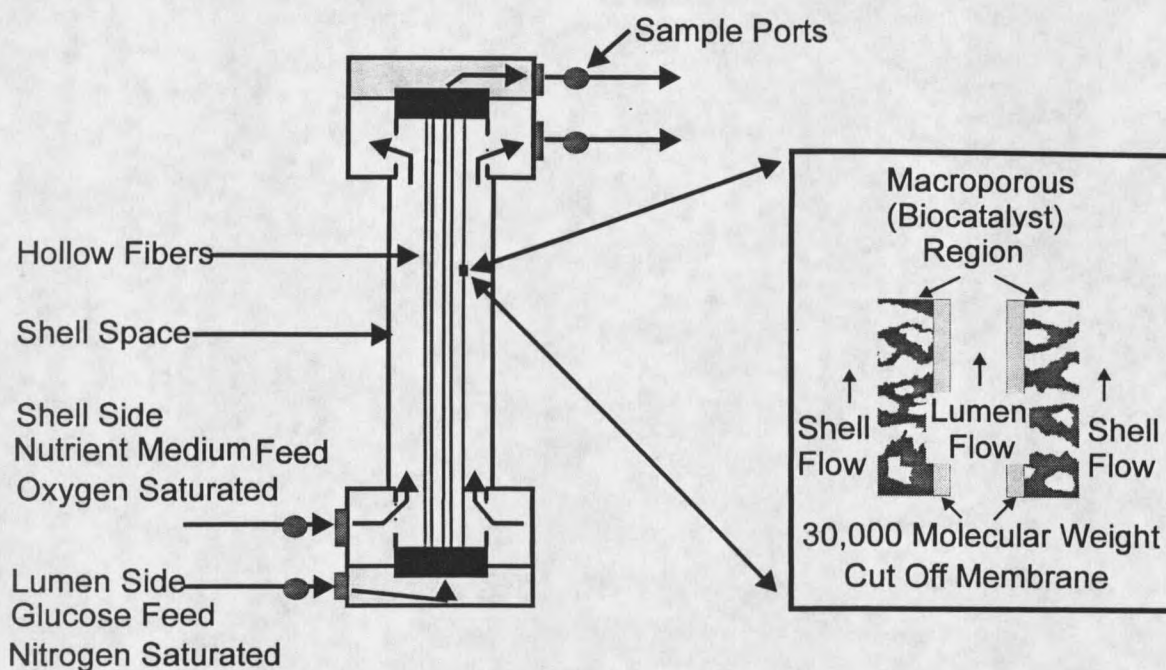
A lumen volumetric flow rate which was easily measurable using standard flow meters was selected as the basis for determination of the bioreactor operation parameters. The shell volumetric flow rate was determined, using Equation 1, that would prevent convective flow through the HFM fibers. These flow rates resulted in a reactor residence time of 2.1 minutes which corresponds to a dilution rate of 28.7 hr⁻¹.

$$Q_s = \frac{Q_L * A_s}{A_L}$$

Equation 1

Table 10 Amicon HFM Cartridge - Model H1P30-43	
Lumen Volume	8.36 cm ³
Shell Volume	18.3 cm ³
Cartridge Length	20.3 cm
Membrane Surface Area (Total)	0.03 m ²
Membrane M.W. Cut Off	30,000 MW
Fiber I.D.	1.1 mm
Fiber O.D.	1.875 mm
Number of Fibers per Cartridge	55
Water permeability Rate	0.15 to 0.20 L/min
Reactor Configuration	Shell and Tube
Fiber Configuration	Bundle
Designed Application	Filtration

Figure 8
Prefabricated Hollow Fiber Reactor



f8.cdr

Table 11
Amicon Cartridge H1P30-43 - Operation Parameters

Lumen Volumetric Flow Rate	4.0 mL/min
Shell Volumetric Flow Rate	20 mL/min
Composition of Shell Flow	Medium: 4.0 mL/min Dilution Water: 16 mL/min
Glucose Concentration	25 mg/L
Re_s	90
Re_L	27
Flow Configuration	Co-current

Experimental System, In-house Fabricated HFM Reactor

The in-house fabricated HFM (IHF-HFM) reactor was developed in response to the results obtained from experiments conducted using the Amicon HFM cartridge. The configuration of this new reactor was similar to a baffled shell and tube heat exchanger which was a significant departure from the Amicon HFM cartridge. The IHF-HFM reactor is pictured in Figure 9. The baffles served three distinct purposes; (1) to physically separate the HFM fibers, (2) to create cross-current flow in the shell side of the reactor and (3) physically support the fibers over the length of the reactor. Baffles were incorporated into the design of the IHF-HFM reactor with the intent that it would improve mass transport and a higher biomass yield would be obtained. Figure 10 shows the baffles with fibers glued in place as they are orientated within the reactor during operation. Figure 11 shows the shell side flow and lumen flow patterns within the reactor. The fibers used in the IHF-HFM reactor are the same as those used within the Amicon HFM cartridge. HFM fibers used in the IHF-HFM reactor were extracted from a Amicon H5P30-43 HFM cartridge. The fibers are the same macro void construction and molecular weight cut off as in the H1P30-43 cartridge. The reactor is described in Table 12 and its operating conditions are described in Table 13.

New operating conditions were developed for experiments 3 and 4 using the IHF-HFM reactor to increase the accumulation of biomass within the reactor. Selection of operating parameters took a different approach than for

Figure 9
In-house Fabricated Hollow Fiber Membrane Reactor

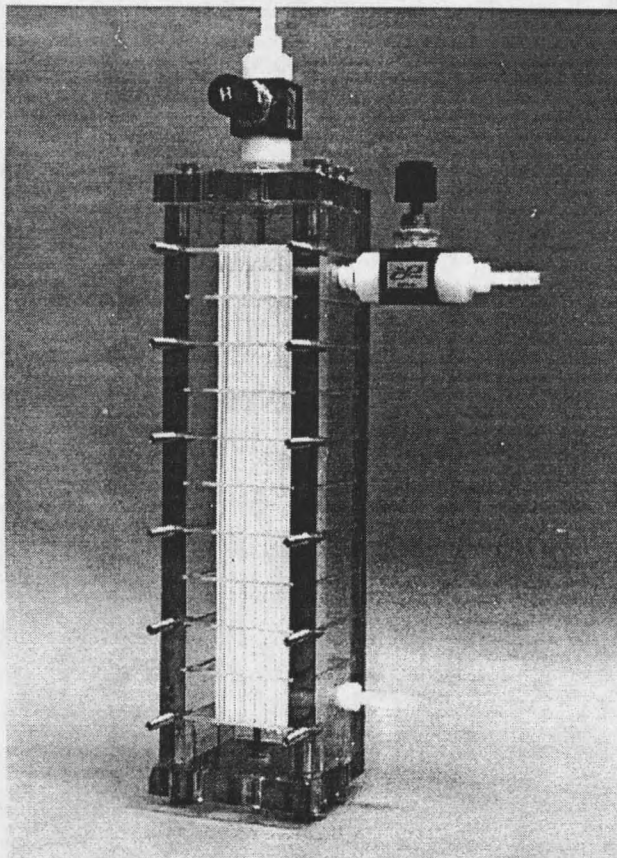
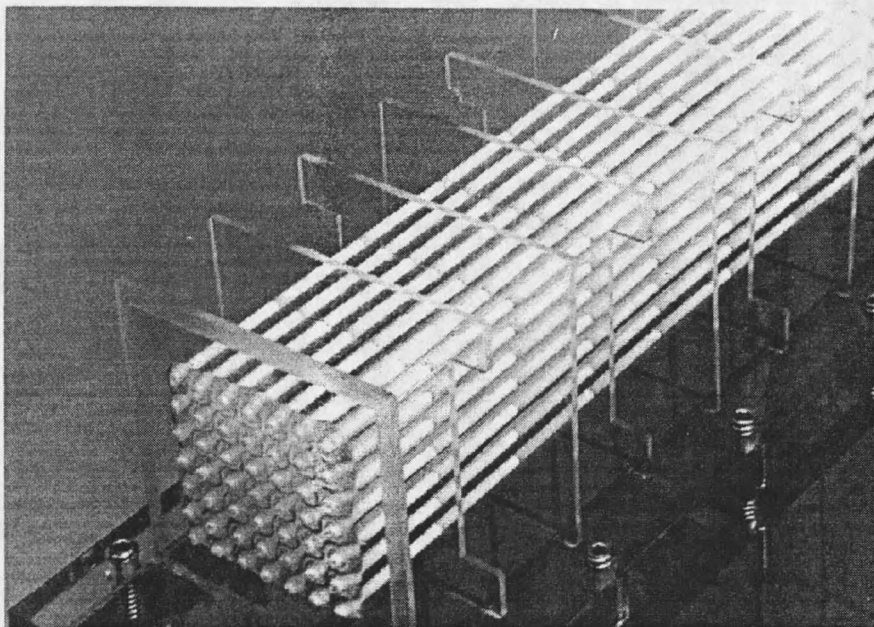


Figure 10
In-house Fabricated Hollow Fiber Membrane Reactor
HFM Bundle with Baffles



f10.cdr

Amicon cartridges. Growth rate of *Ps.putida* was the primary factor in selection of the volumetric flow rates for the IHF-HFM reactor. Drummond, 1993 reported a $\mu_{\max} = 0.8 \text{ hr}^{-1}$ for *Ps.putida* grown planktonically so a reactor dilution rate of 3 times $\mu_{\max}$ was used in order to prevent suspended cells from accumulating in the reactor. The resulting reactor dilution rate was 2.4 hr^{-1} with a residence time of 25 minutes, for the IHF-HFM reactor, using the volumetric flows defined in Table 13.

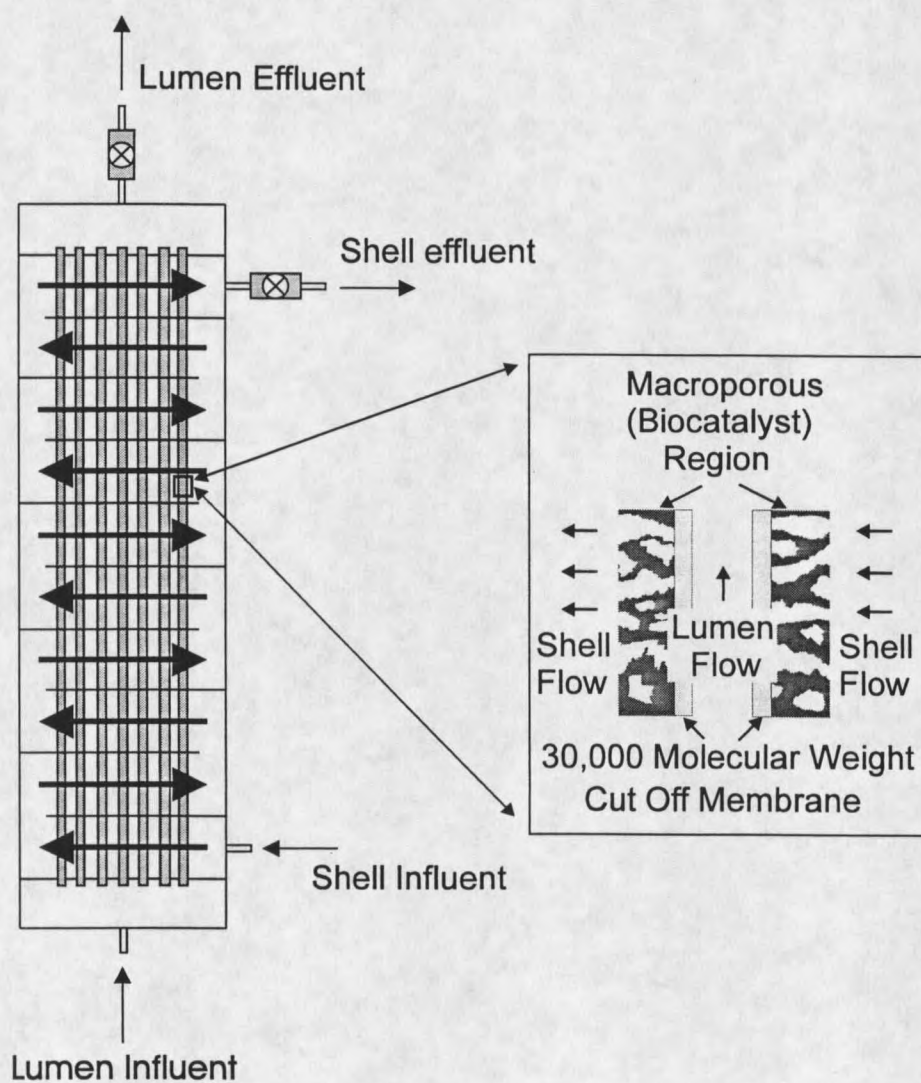
Table 12
In-house Fabricated HFM Reactor

Lumen Volume	7.66 cm ³
Shell volume	489 cm ³
Cartridge Length	28.6 cm
Fiber Length	26 cm
Membrane Surface Area (Total)	0.037 m ²
Membrane M.W. Cut Off	30,000 M.W.
Fiber I.D.	1.1 mm
Fiber O.D.	1.875 mm
Number of Fibers per Cartridge	49
Water Permeability Rate	0.15 to 0.20 L/min
Reactor Configuration	Baffled Shell and Tube
Fiber Configuration	Spaced
Designed Application	Bioreactor

Table 13
In-house Fabricated HFM Reactor - Operating Parameters

Lumen Volumetric Flow Rate	0.3 mL/min
Shell Volumetric Flow Rate	20 mL/min
Composition of Shell Flow	Medium: 4.0 mL/min Dilution Water: 16 mL/min
Glucose Concentration	50 mg/L
Re _s	0.0756
Re _L	0.0013
Flow Configuration, Reactor	Co-current
Flow Configuration, Around Fibers	Cross Current

Figure 11
In-House Fabricated Hollow Fiber Membrane Reactor
Flow Patterns

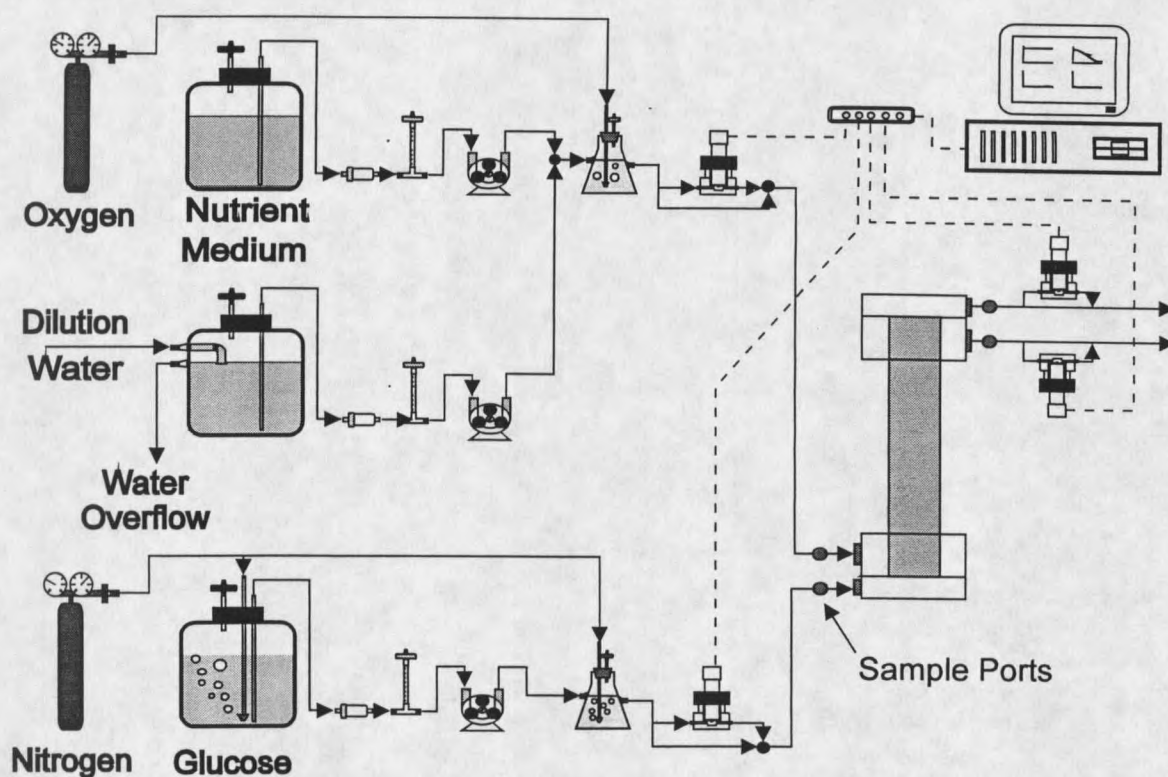


Environmental Support System

Two similar environmental support systems were used over the course of this experiment. Experiments 1 and 2 used the system shown in Figure 12 and described in Table 14. There are several unique features of this system which differ from the support system used for experiments 3 and 4. Figure 12 shows a data acquisition interface which was used for collection of dissolved oxygen concentration data. This apparatus allowed for continuous and simultaneous sampling of all streams flowing into or out of the reactor system. The supply of deionized dilution water comes from the "house" supply and was the only stream filter sterilized. The dilution water, nutrient and glucose flows were measured through the use of in line flow meters located just up stream of each pump.

Figure 13 shows the environmental support system used for experiments 3 and 4. This system is described in Table 15. Several modifications were made to the system shown in Figure 12 to reduce flow upsets and provide accurate dissolved oxygen concentration measurements. The data acquisition system shown in figure 12 failed to provide useful data so it was replaced with a single dissolved oxygen (DO) probe with a dedicated meter. This apparatus was physically moved between streams for collection of DO data. Another change was the elimination of flow meters. Flow rate was determined by weight of liquid samples collected from each stream per 10 minute interval. Recycle loops were established to improve flow stability by reducing flow resistance. These changes provided a stable system for support of the HFM reactors.

Figure 12
Amicon HFM Cartridge Environmental Support System



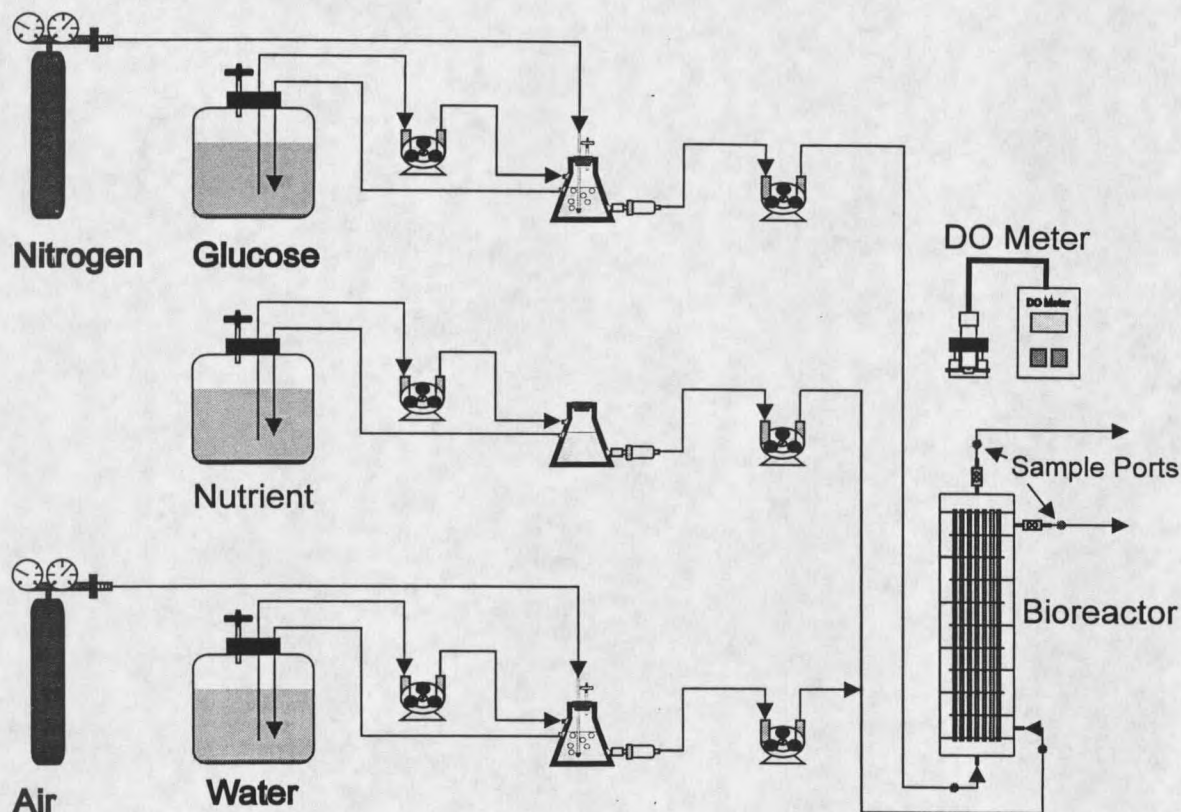
f12.cdr

Table 14
Amicon H1P30-43, Environmental Support System

3	Cole - Palmer, Peristaltic Pumps
1	Compressed Nitrogen Gas Cylinder
1	Compressed Oxygen Gas Cylinder
5	Air Filters, Whatman Vacu-Guard L#7352
3	Solution Filters - Cole-Palmer, Ground Water Filters # H-29600-00, Pore size 0.45 micro meter
4	Dissolved Oxygen Micro electrodes, Microelectrodes Inc.
1	Labworks Data Acquisition Interface, Labworks Inc.
1	Zenith 286 Personal Computer
Tubing	Masterflex: Tygon Tubing, Sizes 13 and 14

Figure 13

In-house Fabricated HFM Reactor Environmental Support System



F13.cdr

Table 15
In-house Fabricated HFM Reactor Environmental Support System

6	Cole - Palmer, Peristaltic Pumps
1	Compressed Nitrogen Gas Cylinder
1	Compressed Air Cylinder
5	Air Filters, Whatman Vacu-Guard L#7352
3	Solution Filters - Cole-Palmer, Ground Water Filters # H-29600-00, Pore size 0.45 micro meter
1	Dissolved Oxygen Probe/Meter, Jenway Inc.
Tubing	Masterflex: Tygon Tubing, Sizes 13 and 14

Design of Experimental System

The experimental system required monitoring specific variables in order to determine reactor performance. These variables are listed in Table 16 for both the Amicon #H1P30-43 hollow fiber membrane cartridge and In-house fabricated HFM reactor systems. Samples of the influent and effluent were periodically collected for determination of volumetric flow rate and glucose concentrations. Knowing the quantity of glucose consumed within the bioreactor allowed an estimation of biomass produced. The volumetric flow rate for both influent and effluent streams was monitored in order to determine if a process problem exists.

Table 16
Process Variables

Variable	Process
$Q_{L,in}$	Lumen - Inlet- Volumetric Flow
$Q_{L,out}$	Lumen - Outlet - Volumetric Flow
$Q_{S,in}$	Shell - Inlet- Volumetric Flow
$Q_{S,out}$	Shell - Outlet - Volumetric Flow
$[G]_{L,in}$	Glucose - Lumen Inlet - Concentration.
$[G]_{L,out}$	Glucose - Lumen Outlet - Concentration.
$[G]_{S,in}$	Glucose - Shell Inlet - Concentration.
$[G]_{S,out}$	Glucose - Shell Outlet - Concentration.
DO all streams	DO probes were employed for this measurement. Probes failed and no useful data was obtained.

Microbial System

The bacterial species chosen for these experiments was *Pseudomonas putida* Bio-type A (ATCC #11172). The culture was purchased from American Type Culture Collection (ATCC) in freeze dried form. The culture was rehydrated using the ATCC recommended procedure and maintained on slants at approximately 5°C. Slants were made from Difco Plate Count Agar and new slants were made every 90 days. Batch experiments and seed cultures were cultivated using a synthetic growth medium recommended by ATCC and described in Table 16 (ATCC, 1992).

Table 17
***Ps. putida* Nutrient Medium and Trace Metal Solution**

Medium:	Quantity:	Trace Metals:	Quantity:
NH ₄ Cl:	1.0 g/L	CuSO ₄ - 5H ₂ O	5.0 g/L
Na ₂ HPO ₄ :	2.6 g/L	ZnSO ₄ - 7H ₂ O	5.0 g/L
KH ₂ PO ₄ :	2.1 g/L	FeSO ₄ - 7H ₂ O	5.0 g/L
CaCl ₂ :	0.1 g/L	MnCl ₂ - 4H ₂ O	2.0 g/L
MgSO ₄ :	0.5 g/L	Concentrated HCl	10 g/L
Trace Metals:	0.02 ml/L		

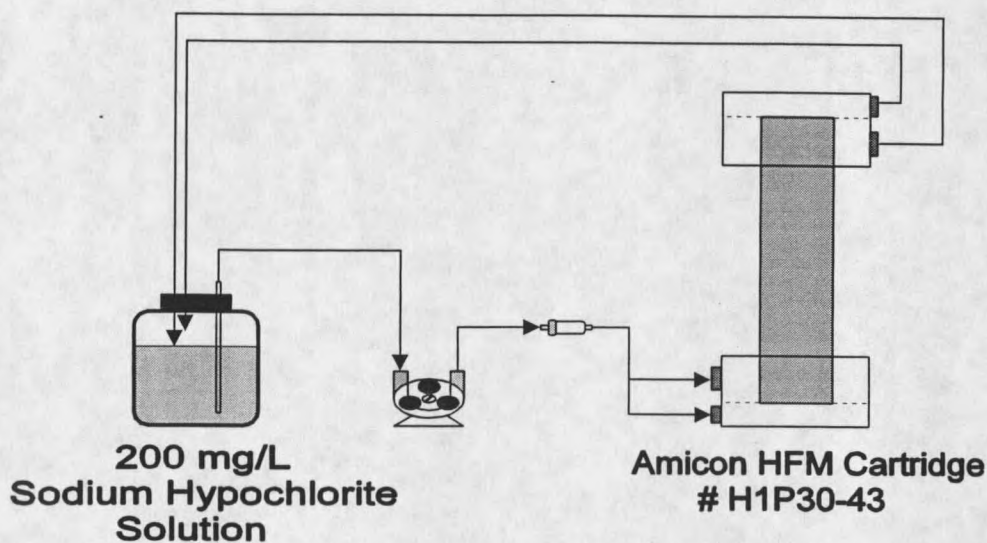
Start - Up and Operation

The Amicon HFM cartridge #H1P30-43 was used as a bioreactor within which a pure culture of *Pseudomonas putida* was cultivated for experiments 1 and 2. Figure 8, describes the construction of the Amicon HFM cartridge and

Table 10 provides a physical description. Figure 12 and Table 14, describe the support system designed for the Amicon HFM cartridge and Table 11 provides a description of the systems operating parameters. The third and fourth experiments used a In-house Fabricated HFM (IHF-HFM) reactor. Figure 11 shows the reactor and describes the lumen and shell flow patterns. Table 13 describes the operating parameters for the reactor and the environmental support system is pictured in Figure 13 and described in Table 12.

Cultivation of *Ps. putida* cells within the Amicon HFM cartridge involved: (1) system sterilization, (2) seed culture development, (3) reactor inoculation, (4) flow regulation and (5) reactor maintenance. Sterilization of the HFM cartridge consisted of passing a 200 mg/L solution of Sodium Hypochlorite through the membrane (method recommended by Amicon). Figure 14, shows the HFM cartridge system as assembled for cleaning. The recycle of hypochlorite solution was maintained for 6 hours, with 4 hours in a convective flow mode (shell to lumen), to remove all fiber preservative and sterilize the tubing as well as the HFM cartridge. The remaining bioreactor system components (i.e., filters, flow meters, tubing, etc) were autoclaved along with the nutrient medium carboys. Concentrated glucose solution was filter sterilized then added to autoclaved ultrapure water in order to obtain concentrations of 25 mg/L and 50 mg/L. The system was completely assembled and flushed with the glucose, nutrient medium and dilution water which were to be used during the experiment. Washing was maintained for 8 to 12 hours. There was no change made to the

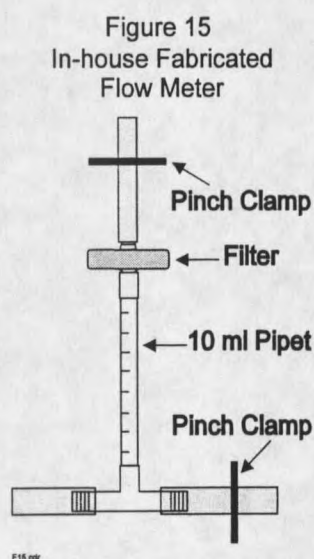
Figure 14
Amicon Hollow Fiber Membrane Cartridge
Cleaning System



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cleaning and sterilization methods when the IHF-HFM reactor was used in experiments 3 and 4. While the cleaning process is underway, *Ps. putida* was cultivated for use as the seed culture for both reactors. A 200 ml culture was cultivated in a batch reactor for 18 to 20 hours (5×10^{10} cell/mL) at an initial glucose concentration of 500 mg/L; the nutrient medium was defined in Table 17. A new procedure for inoculation of the IHF-HFM reactor was developed in order to improve distribution of bacteria along the length of the bioreactor and reduce the physical trauma that the cells experienced. The Amicon cartridge was inoculated using a cell culture concentrated by centrifugation at 10,000 rpm for 10 minutes. To inoculate the Amicon HFM cartridge, twenty ml of phosphate buffer was used to dissolve the pellet and 10 ml of this was injected, using a

syringe, into the shell influent stream. Flows were co-current and cells migrated from bottom to top of the bioreactor during inoculation. The shell side effluent was closed as the plume of cells reached the top of the HFM cartridge and a transmembrane pressure drop was used to entrap cells in the membrane support structure. Convective transmembrane flow was maintained for 1 hour then all flows were adjusted to the operating levels specified in Table 11. In contrast to the method used to inoculate the Amicon HFM cartridges the 200 mL seed culture was recycled through the shell side of the reactor while a transmembrane pressure drop was maintained in order to embed cells within the fibers. The lumen effluent was recycled into the seed culture flask and this



process was maintained for 1 hour. Then the influent and effluent streams were adjusted to the operating parameters shown in Table 13.

Flow rates of all streams were checked twice daily to ensure consistent operation of the Amicon HFM bioreactor. Influent and effluent flows were monitored by using custom flow meters pictured in figure 15. Flow meters were not used as part of the IHF-HFM bioreactor

environmental support system, pictured in Figure 13. Volumetric flows of each stream (i.e., nutrient, glucose, and dilution water influents as well as lumen and shell effluents) were checked daily by collecting samples and determining flow rate by weight of sample divided by time of collection. Glucose samples were

collected at the same time by extracting, with a syringe, 2.0 ml of solution from each stream. Samples were filter sterilized and stored at -20°C for analysis. The IHF-HFM bioreactor glucose samples were collected by diverting each stream to collect 2.0 mL samples which were filter sterilized and stored at -20°C . Dissolved oxygen measurements were made using a Jenway DO probe fitted with a flow cell by diverting each stream until a constant concentration value was obtained.

Maintenance of the HFM bioreactor involved: (1) monitoring and adjusting flows, (2) replacing compressed gas cylinders, (3) repairing plugged tubing, (4) removing air bubbles, (5) and replacing nutrient medium carboys. Flow adjustments were made by either changing pump speeds or adjusting needle valve settings for the effluent of the HFM bioreactor. Replacement of the compressed gas cylinders took place when the cylinder pressure dropped below 500 psi. When tubing replacement was required, because of precipitation of nutrient salts, a new section was autoclaved and inserted into the bioreactor system. Often the flow rate of the stream required adjustment due to the reduction in flow resistance once the new tubing was in place. Repairs to the system often caused air bubbles to become trapped within the tubing and bioreactor. Bubbles were removed by tapping the tubing or bioreactor wall until the air was removed. Leaks in the system were sealed with food grade, silicone sealant (Dow Corning). Replacement of nutrient medium carboys was done using aseptic procedures. New carboys were required when the total volume of the carboy dropped below 5 L of solution.

An experiment was terminated when a irreconcilable problem developed; such as, bacteria breaching the MW cut off membrane and entering the lumen effluent. Once the HFM bioreactor was dismantled, the fibers were removed from the plastic housing, sectioned and the cell mass extracted. Extracted cellular material samples were then pH adjusted to neutral, separated, purified and analyzed for RNA, DNA and protein content.

CHAPTER 3

Analytical Methods

A summary of the analytical methods used for this project is presented in Table 18.

Table 18 Analytical Methods		
Parameter	Purpose	Assay Method
Glucose	Substrate Balance	Sigma Glucose Assay #510A
Dissolved Oxygen	Substrate Balance, Condition, Maintenance	DO probes - Micro electrodes, Inc with a Labworks, Inc Data Acquisition Interface and a Digital , 316sx Computer
Protein	Biomass Indicator	Modified Lowry Assay
DNA	Biomass Indicator	Phenol Extraction with Ethanol precipitation
RNA	Biomass Indicator	Sodium Chloride Extraction with Ethanol precipitation

Extraction of Cellular Material From Fibers

An extraction solution of boiling 1.0 N NaOH was used with the Amicon cartridge, experiments 1 and 2, to remove cellular material from the Hollow Fiber Membranes (HFM). The procedure started with cleaning all glassware in 50 % Nitric Acid for 12 to 24 hours then rinsing three times with double distilled water. When the HF membrane bioreactor was dismantled, the HF membrane fibers are removed from the plastic cartridge using a fine saw blade to cut away the outer cartridge housing. The fibers were placed in clean aluminum foil and stored in the -70 °C freezer. Once frozen, the fibers were sectioned in uniform

lengths and each section labeled with its axial location. Several fibers were taken from each section, 25 is typical, and placed in a test tube. Five mL of 1.0 normal NaOH was added and boiled for 45 min. Each test tube was covered during boiling and samples were allowed to cool before proceeding.

Experiments 3 and 4 used a new extraction solution to process fibers obtained from the In-house Fabricated HFM reactor. This change was made to improve recovery of the cellular material. The new extraction procedure used 0.2 N NaOH, 1 % Sodium Dodecyl Sulfate (SDS) and 0.01 % Diethyl Pyrocarbonate (DEPC) as the extraction solution. Additional benefits of using this procedure were milder process conditions, did not affect the accuracy of the DNA, RNA or protein assays, and the DEPC prevented destruction of RNA.

Two experiments were performed in order to examine the efficiency of cell lysing and material extraction from polysulfone membranes. Hollow fiber membrane samples were obtained from experiment 2 which involved the Amicon HFM cartridge. These HFM fibers were subjected to three consecutive extraction procedures using the boiling 1.0N NaOH extraction solution. Extraction solution samples were assayed for protein content then a determination of the effectiveness of the extraction process made. The results from this test are presented in Table 19. Evaluation of the extraction solution used in experiments 3 and 4 was made by growing a 200 mL seed culture and inoculating a DIAFLOW flat plate polysulfone membrane with 10 mL. The membranes were very similar in structure to the HFM and retain cells well.

The flat membranes were placed upon R2A plates and incubated 48 hours. A 2 cm section was cut from the center of each membrane and placed in 5 mL of the extraction solution. The samples were then sonicated for 30 seconds. The membrane samples were then removed from the extraction buffer and placed in a clean test tube. This process was repeated 2 more times with the same set of membrane samples. The results of this analysis are shown in Table 20.

Table 19
% Recovery of Cell Protein

Section cm	Total Protein Extracted (mg)	% Recovery Extraction 1	% Recovery Extraction 2	% Recovery Extraction 3
0 to 2	0.006156	61.0	27.0	12
2 to 4	0.006356	66.9	22.5	10.6
4 to 6	0.004695	60.2	24.1	15.7
6 to 8	0.004828	59.9	24.8	15.3
8 to 10	0.004263	57.7	25.8	16.5
10 to 12	0.003732	56.2	25.0	22
12 to 14	0.003499	57.0	21.0	22

Table 20
% Recovery of Cell Protein

Trial #	Total Protein Extracted (mg)	% Recovery Extraction 1	% Recovery Extraction 2	% Recovery Extraction 3
1	1.364296	78.0	13.9	8.1
2	1.179324	73.6	18.2	8.2
3	1.308244	75.0	16.0	9

DNA Assay

This procedure was used to isolate and purify DNA from extraction solution samples for specific regions of an HFM bioreactor. This is a 2 step method using phenol to isolate the DNA from the extraneous cellular material then ethanol to precipitate the DNA. This procedure was shown to be sensitive to the presence of polysulfone within the extracted cellular material samples. The presence of polysulfone increased the absorbance of DNA in T.E. buffer at 260 nm.

An experiment was performed to investigate the impact polysulfone had upon the DNA analysis. Clean fibers were cut and placed in an extraction buffer of 1% SDS and 0.2N NaOH. Liquid samples were collected and subjected to the DNA extraction and precipitation procedure described in this section. The phenol extraction with ethanol precipitation procedure produced an absorbance at 260 nm of 2.8 ± 0.2 nm for samples which contained no bacteria. Analysis of the Polysulfone fibers which were not subjected to the DNA assay procedure were analyzed by ultra violet scanning over the range of 250nm to 300 nm and the maximum absorption for the polysulfone sample was located at 271 nm with an absorbance of 2.975 ABS units. The conclusion is that the phenol extraction and ethanol precipitation procedure used for the DNA assay was ineffective for extracting and purifying DNA from samples containing polysulfone.

DNA Extraction Procedure

Equal volumes of Phenol / Chloroform / Isoamyl Alcohol solution and the cellular material extraction solution sample (0.4 mL of each) were added to a polypropylene micro centrifuge tube. The solution was then mixed vigorously for

Table 21
DNA Extraction Materials

Materials:	Notes:
25:24:1 Phenol : Chloroform : Isoamyl alcohol.	Purchase distilled Phenol and then melt it in a vented oven at 60C and place it into the 24 : 1 Chloroform : Isoamyl alcohol solution
3 Molar Sodium Acetate	Dissolve 408g Sodium Acetate in 600 ml of distilled water, Adjust pH to 5.2 with # molar Acetic Acid and fill to 1L with distilled water
100 % Ethanol	ice cold
70 % Ethanol	ice cold
TE Buffer, pH 8.0	1000 ml of TE buffer, Use 10 ml of 1M Tris-Cl and 2 ml of 0.5M mM EDTA
0.5 M EDTA	18.6g EDTA in 70 ml distilled water
1M Tris-Cl, pH 8.0	Dissolve 121 g Tris base in 800 ml distilled water add 58.4 ml of 0.1 M HCl and fill to 1000 ml.

10 seconds. Samples are micro-centrifuged 2 minutes at room temperature and the top aqueous layer (0.2 ml of clear liquid) was transferred to a new micro centrifuge tube .

DNA, Ethanol Precipitation Procedure

The DNA sample obtained from the previous procedure was combined with 0.02 mL 3M Sodium Acetate, pH 5.2. The samples were mixed for 15 seconds and combined with 1.0 ml of ice cold ethanol. The -70°C freezer was used over night to precipitate the DNA. Fifteen minutes of centrifugation at 0°C was used to separate the DNA precipitate from the supernatant liquid. Washing of each sample was required to remove all extraneous material from the sample by adding 1.0 mL of 100% ethanol to the micro centrifuge tube, mixing until the pellet was dissolved and centrifuged another 10 minutes at 0°C. The DNA sample was then dried in a vacuum desiccator. The pellet was dissolved in 1 mL of filter sterilized TE buffer and analyzed at a wavelength of 260 nano meters. DNA quantities were determined by programming the UV-Vis spectrophotometer for a ratio test at wave lengths of 260 to 280 nanometers. The result of the absorbance ratio 260/280 must be greater than 1.5 to prove purity. All dilutions must be compensated for when calculating quantity of DNA present. Quantity of DNA in solution was calculated by using 50 micrograms per absorbance unit for DNA (Reference: Molecular Cloning: A Laboratory Manual).

RNA Assay

This procedure was used to isolate and purify RNA from the biomass extraction solution samples taken from specific regions of the HFM bioreactor. This assay is a 2 step method using an NaCl extraction of the RNA from the extraneous cellular material then precipitation with ethanol.

Table 22
RNA Extraction Materials

Materials:	Note:
Diethyl Pyrocarbonate solution (Neat). DEPC	DEPC is very reactive with Carbon Dioxide take great care when handling.
Saturated NaCl	Ice cold.
100% Ethanol	Ice Cold

RNA Extraction Procedure

A 0.5 mL sample of the 1.0 N NaOH extraction solution was combined with 15 micro liters of DEPC in a micro-centrifuge tube. The sample was incubated at 35°C for 5 minutes and chilled at -20°C for 30 minutes. Two hundred and fifty micro liters of saturated NaCl solution was added to the sample, mixed and chilled at -20°C for 10 minutes. The supernatant liquid was removed after 10 minutes of centrifugation at 0°C. The liquid sample was placed into two micro centrifuge tubes (0.2 ml each tube). Additions to each tube of 1 ml ice cold 100% ethanol were made and the samples placed in

the -20°C freezer overnight (About 8 hours). Micro-centrifuging 15 minutes at 10,000xg at 0°C precipitated the RNA. Pellets were washed with 500 micro liters of ice cold 70% ethanol and centrifuged 15 minutes at 10,000xg at 0°C. The pellet was dried in a vacuum desiccator before being dissolved in 100 micro liters of filtered and DEPC-Treated TE buffer (Table 21). Analyses of the RNA samples were made by programming the UV-VIS spectrophotometer for a ratio test at wave lengths of 280 to 260 nanometers. The result of the absorbance ratio 280/260 must be greater than 1.5 to prove purity. All dilutions were compensated for when calculating quantity of RNA present. Quantifying the concentration of RNA in solution used 40 micrograms per absorbance unit for RNA (Reference: Molecular Cloning: A Laboratory Manual).

Protein Assay: Modified Lowry Procedure

This procedure prepared samples for examination using UV-VIS Spectrometric techniques. This analysis was pH sensitive and was run at or near a neutral pH. Adjustment of the sample pH was done by titrating a known volume of 1N NaOH extraction solution with HCl to a neutral pH. The extraction solution used in experiments 3 and 4 did not interfere with this analysis. All the glassware used in this analysis was cleaned by acid washing in 50% nitric acid.

Protein Assay Procedure

A 0.5 mL sample was placed in a micro centrifuge tube and mixed with 0.7 mL of reagent A and 0.1 mL reagent B as defined in table 23 and mixed. After 45 minutes a UV-VIS Spectrophotometer was used to analyze the sample at a wave length of 750 nano meters. The blank was 0.5 mL distilled water with 0.7 mL of reagent A and 0.1 mL of reagent B. A typical calibration curve for

Table 23
Protein Assay Compatibility

0.1m Tris-pH = 8.0	0.5% (NH ₄) ₂ SO ₄	0.05% CaCl ₂
10% SDS	1% Triton X-100	1% thesit
1% Brij	0.5m NaOH	0.25m EDTA 4.0m Urea
1% Tween-20	0.5m HCl	
Protein analysis is not compatible with: 2-mercapthoethanol		

Table 24
Materials for Protein Analysis

Materials:	Make Up:
Reagent A	Make this solution up just before use with distilled water.
Bottle #1	142.8mM NaOH with 269.5 mM Na ₂ CO ₃ , add 5.712g NaOH and 28.564g Na ₂ CO ₃ to 1000 ml water
Bottle #2	57.2 mM CuSO ₄ , 1.428g in 100 ml water
Bottle #3	124 mM Na-Tartrate, 2.853g in 100 ml water
Reagent A, Make up.	Mix bottles 1, 2 and 3 in 100:1:1 proportion and this is Reagent A.
Reagent B	Folin Ciocalteus Reagent diluted 5:6 with distilled water

Figure 16
Protein Calibration Curve

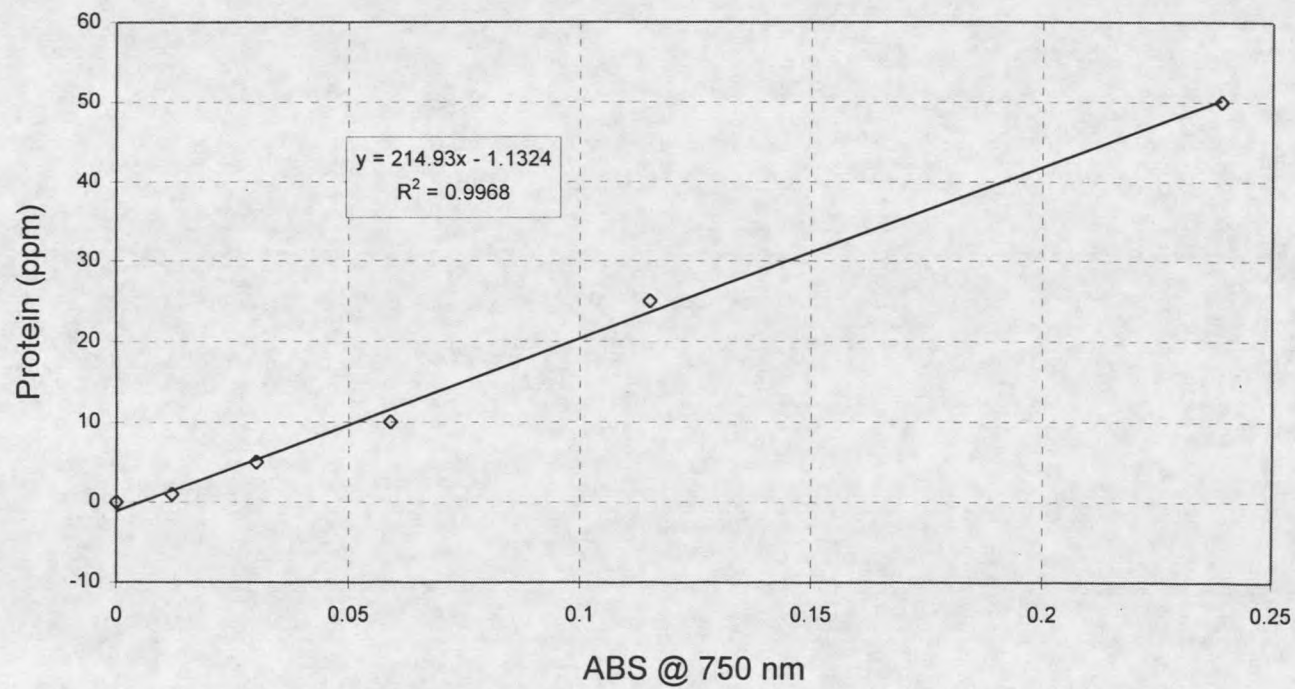
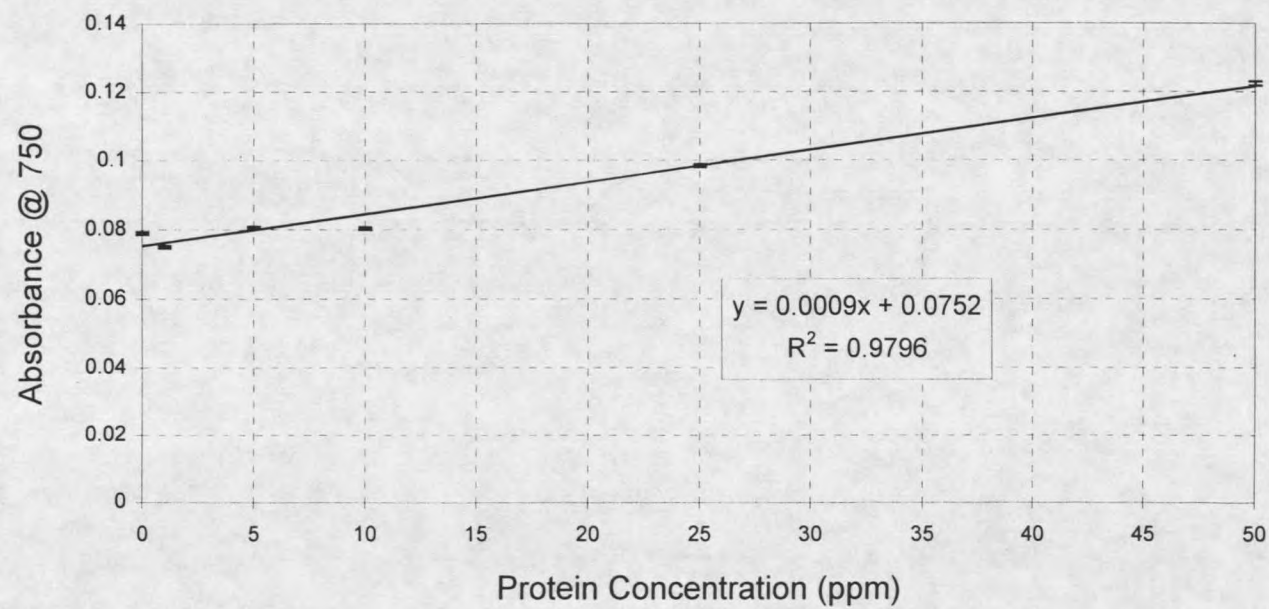


Figure 17
Protein Curve Error Analysis, 5/18/95
Maximum % Deviation: 0 to 50 ppm is 0.5%



conversion of protein absorbance to bovine albumin protein concentration in mg/L is shown in Figure 16. An error analysis was performed by comparing 5 separate calibration curves and determining the experimental error. The results of this experiment are shown in Figure 17.

Glucose Assay: Sigma Kit #510 A

This procedure prepared samples for examination using UV-VIS Spectrometric techniques: Sigma Kit 510A. This was an enzyme specific glucose assay based on two principle coupled reactions. The first reaction oxidized glucose to form Gluconic Acid and Hydrogen Peroxide. Then o-Dianisidine was oxidized by the Hydrogen Peroxide to produce a brown color. The combined enzyme-color reagent solution is defined in Table 25. The samples of 0.5 mL glucose sample with 5.0 mL enzyme-color reagent were incubated for 45 minutes at room temperature and analyzed at 450 nm. Conversion of the absorbance values to glucose concentration is performed using a calibration curve. A typical calibration curve is shown in Figure 18. An error analysis was performed by comparing 5 calibration curves and determining the experimental error. The results of this experiment are shown in Figure 19.

Figure 18
Glucose Calibration Curve
Created, 10/29/95 with the Y-Intercept set to zero.

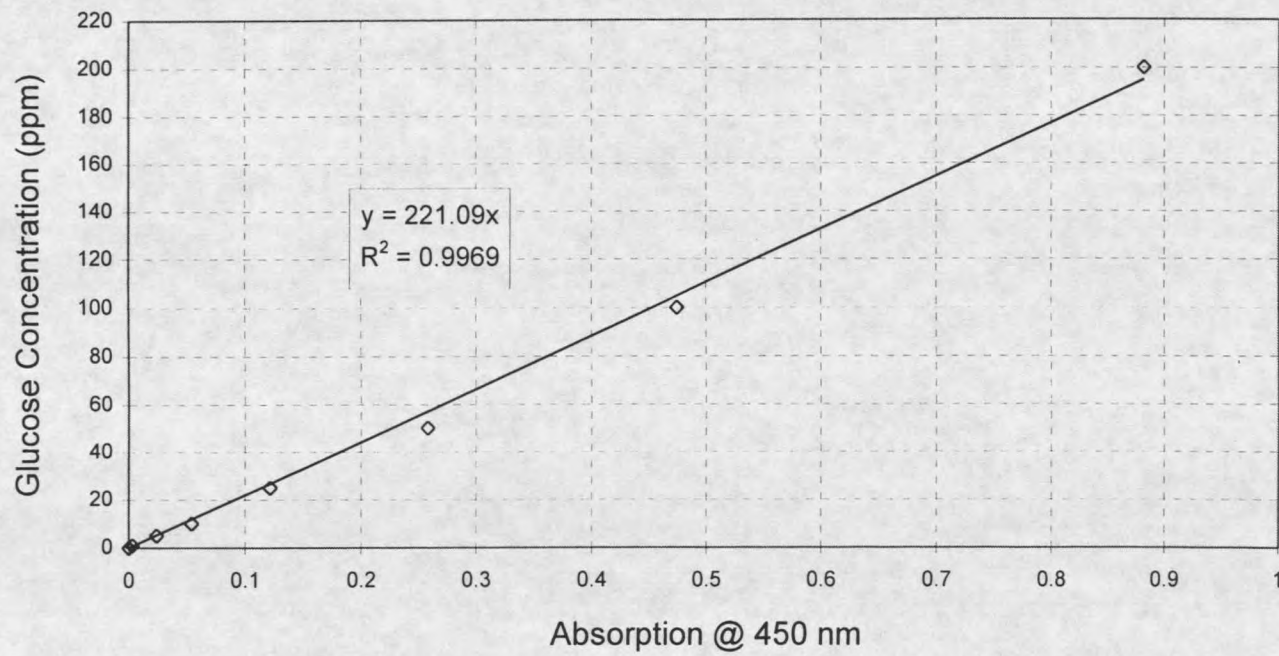


Figure 19
Glucose Curve Error Analysis, 5/16/95
Maximum % Deviation: 0 to 50 ppm is 0.7%
and: 0 to 200 ppm is 2.6%

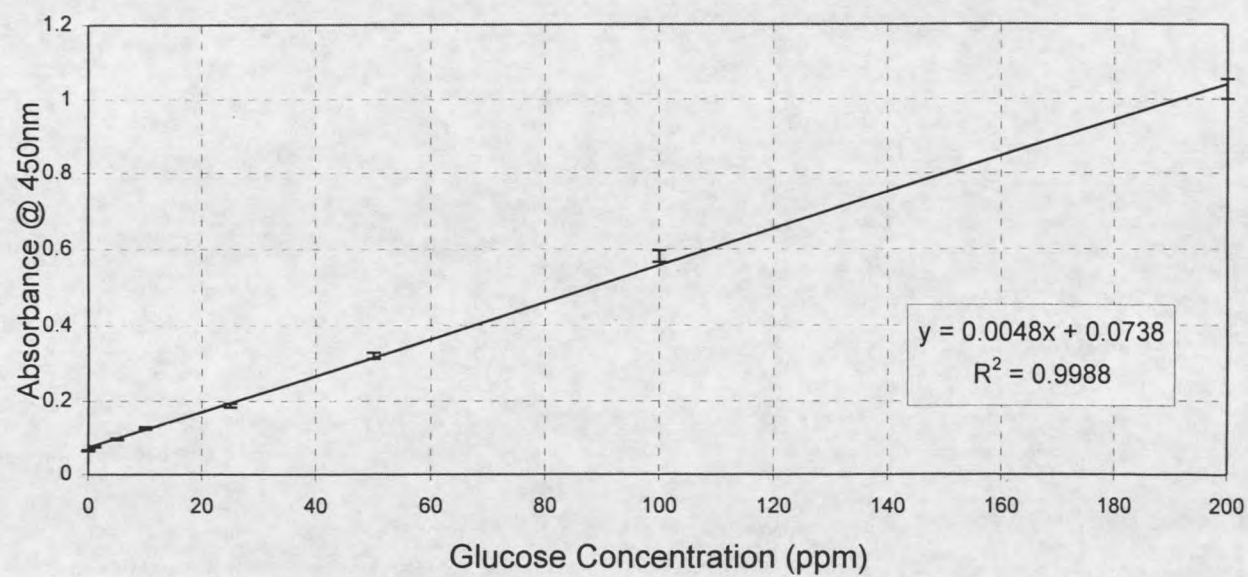


Table 25
Materials for Soluble Glucose Analysis

Solution #	Materials	Make Up
1	Enzyme Solution	One capsule of Sigma chemical #510-6 to 100 mL of Double Distilled Water
2	Color Reagent Solution	One vial of Sigma Chemical #510-50 reconstituted with 20 mL of Double Distilled Water
3	Combined Enzyme-Color Reagent Solution	100 mL solution #1 combined with 1.6 mL of solution #2.

Epifluorescent Cell Count

This procedure was used to determine cell concentrations in suspended cultures and bioreactor effluent. The stain selected for this analysis was Acridine Orange (AO) which stained cellular nucleic acids. AO was fluorescent at wave lengths of 450 to 490 nanometers. Stained cells were fluorescent as either orange/red or green. Protocols for the staining procedure have been published by T.Y. Yeh (1987), R. Zimmerman (1978) and McFeters (1991). Materials which are required for this analysis are listed in Table 26. Samples were obtained from the HFM bioreactor for the following procedure.

AO Staining Procedure

One mL of 0.05% Acridine Orange stain was added to 4 mL of suitably diluted sample. Determination of the correct dilution for each sample was a trial

and error procedure. One order of magnitude sample dilutions were made until the prepared slide contains between 30 and 300 cells per field. The stained sample incubated at room temperature for 1 to 2 minutes then was filtered through a 25 mm diameter black polycarbonate (PC) membrane. The PC membrane was placed on a microscope slide with addition of one drop immersion oil before the cover slip was placed over the membrane. Slides were then placed under a 100X oil objective using a UV light filter (FITC, 400 - 500 nm λ exciter filter) and 10 different grid fields were counted and the average cell count was used to determine cell concentration. Equation 2 was used to calculate cell concentration.

$$\frac{\text{Cells}}{\text{mL}} = \text{Count} * \frac{\left(\frac{\text{Cell Occupied Field Diameter}}{2} \right)^2 * (\pi)}{1e-3} * \text{Dilution Factor}$$

Equation 2

Table 26
Acridine Orange Cell Count Materials

0.05 Acridine Orange Stain Make Up: 0.02g Acridine Orange power in 400 mL double distilled water
Black polycarbonate membranes 0.2 micro meter pore size, 25 mm diameter, Poretics Corporation
Standard microscope slides and cover slips
Immersion Oil, Type FF Cargille Laboratories
Reichert, Diastar/Microstar 4 Microscope Accessories: 1. Etched Grid eye piece 2. 100W mercury arc lamp 3. FITC filter (400 - 500 nano meter wave lengths) 4. 100X oil objective

Dissolved Oxygen Probe

The probe used was an Jenway Model 9071 and was fitted with a flow cell for data collection from the reactor influent and effluent streams. Calibration of the DO probe involved purging ultra pure water with high purity nitrogen for 15 minutes and inserting the DO probe plus its thermal couple into the water until a stable concentration value was obtained. The probe was calibrated in percent saturation mode with the scale adjusted to 0% in the nitrogen purged ultra pure water. The DO probe was calibrated to 84% of saturation, accounting for the 4900 ft elevation of Bozeman, MT, by suspending the probe tip 1 cm above oxygen saturated water until a stable percent saturation value was obtained.

CHAPTER 4

Experimental Results

This project consisted of four experiments which examined *Ps. putida* cell growth within HFM bioreactors. Experiments 1 and 2 used a commercially available HFM cartridge, Amicon H1P30-43, for cell cultivation. Experiments 3 and 4 used an In-house Fabricated HFM (IHF-HFM) reactor. The two reactors were compared based on glucose and oxygen assays performed on reactor influent and effluent streams as well as protein, and RNA assays performed on HFM samples extracted from each reactor.

Experiment 1, Amicon Hollow Fiber Membrane Cartridge

This was the first experiment run for this project. The reactor system ran for 8 days and the run was terminated because of blocked nutrient feed tubing. At the time of termination, the glucose feed line contained bubbles, but was intended to be anaerobic. Whether the bubbles were nitrogen or air was not determined. Cells were detected escaping from the lumen effluent within two days after inoculation. Severe fluctuations in the voltage output of the dissolved oxygen (DO) probes prevented acquisition of useable data and consequently the system failed completely after 2 days of operation. The DO probes and data acquisition system both required repair.

A glucose concentration profile presented in Figure 20 shows essentially

no change in lumen effluent glucose concentration for more than 140 hours.

Under the Amicon HFM cartridge operating conditions outlined in Table 11, the glucose mass loading to the bioreactor was supplied through the lumen influent. Figure 21 shows the consumption rate of glucose within the HFM reactor.

Cellular material extracted from HFM samples at time = 170 hours was analyzed for total protein and total RNA and presented in Figures 22 and 24, respectively, as a function of axial distance. Total protein is shown in Figure 22 with no corrections made for the efficiency of the extraction procedure. Figure 23 is an estimate of the cell numbers per 2 cm section of fiber, calculated from the total protein assay over the length of the cartridge. The cell number profile does account for the efficiency of the cellular material extraction procedure and uses protein per cell and the mass of a single bacterium for an estimation of the cell concentration. An RNA profile is presented in Figure 24 with no corrections made for the efficiency of the extraction procedure. Latter samples were extracted with diethyl pyrocarbonate (DEPC) added to the material extraction solution which may serve to improve the amount of RNA recovered. Figure 25 is the ratio of total RNA to total protein and is used as an indication of growth rate specific regions of the HFM reactor.

Figure 20
Amicon HFM Cartridge, Experiment 1
Glucose Concentration as a Function of Time

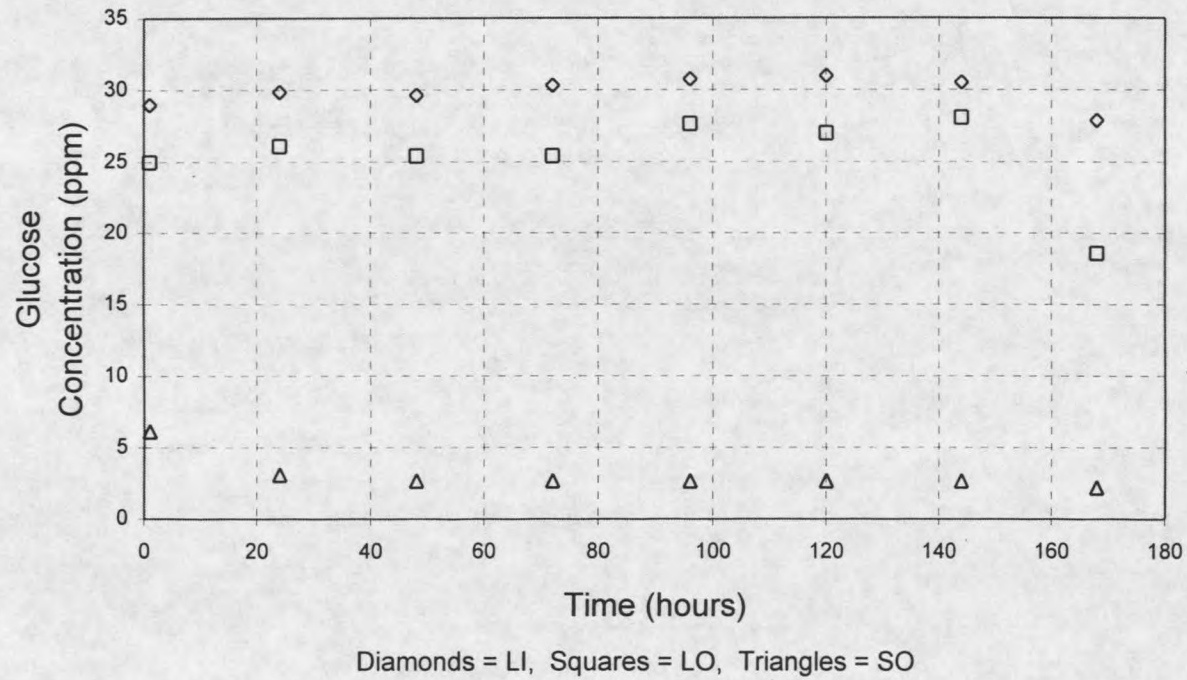


Figure 21
Amicon HFM Cartridge, Experiment 1
Glucose Consumption, Mass Balance

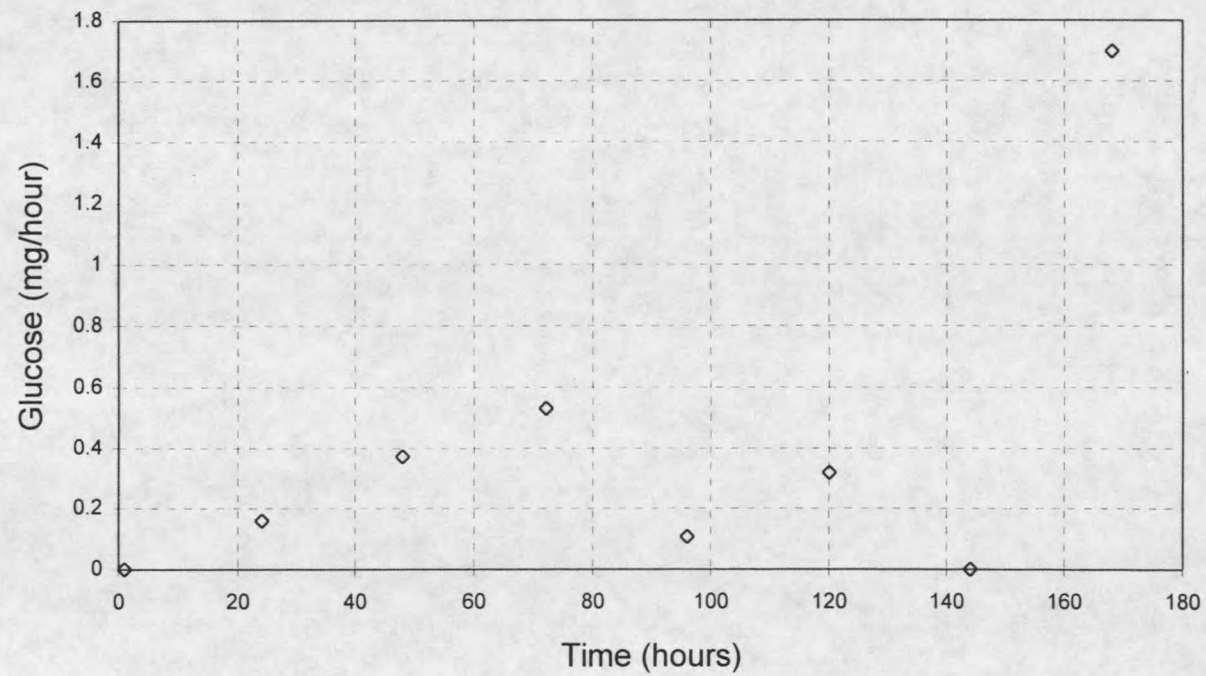


Figure 22
Protein Profile, Amicon HFM, Experiment 1
Samples were harvested after 170 hr. of reactor operation.

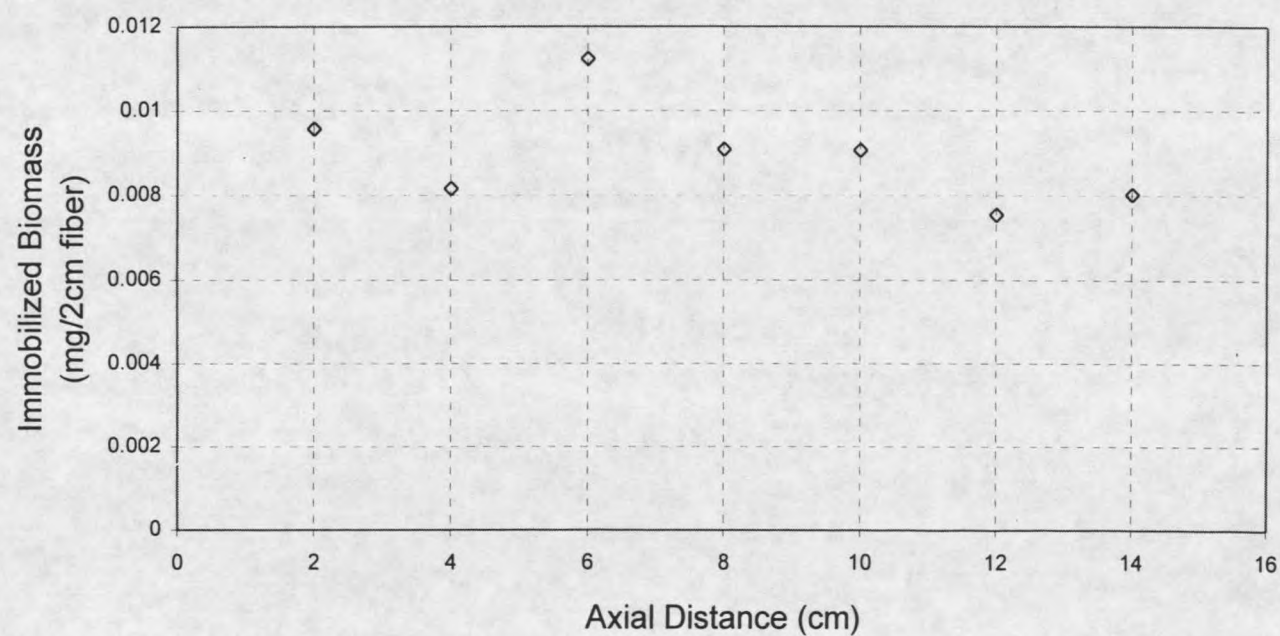


Figure 23
Amicon HFM Cartridge, Experiment 1
Ps. putida Cells Estimated From Protein Data
Samples were harvested after 170 hr. of reactor operation.

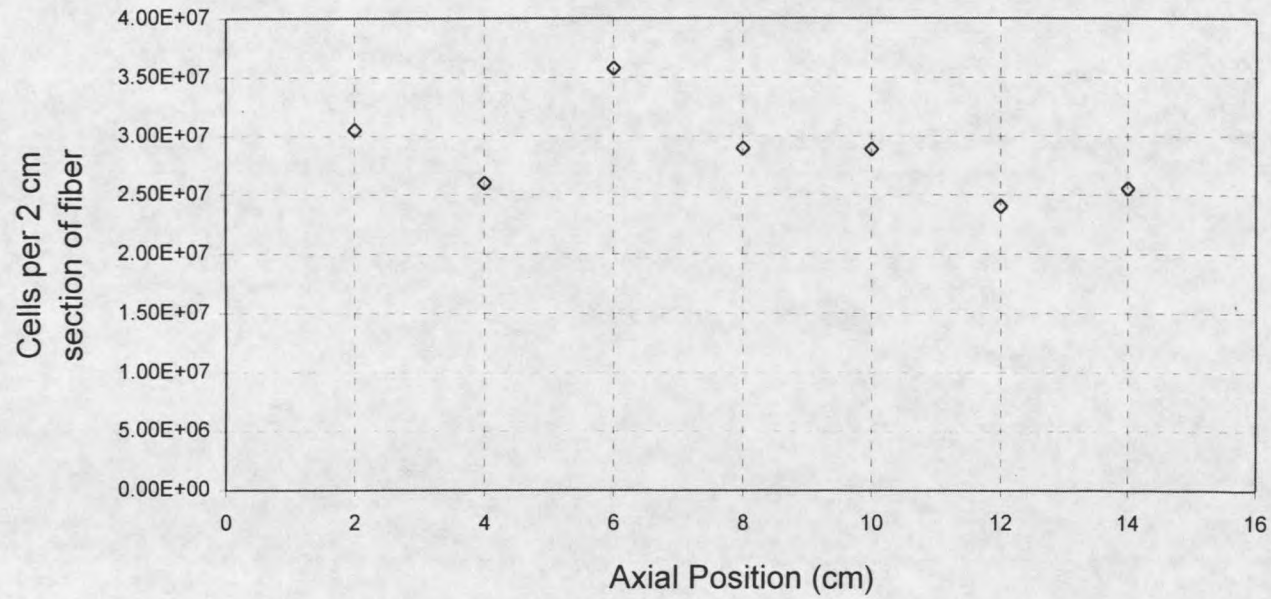


Figure 24
RNA Profile, Amicon HFM Cartridge, Experiment 1
Samples were harvested after 170 hr. of reactor operation.

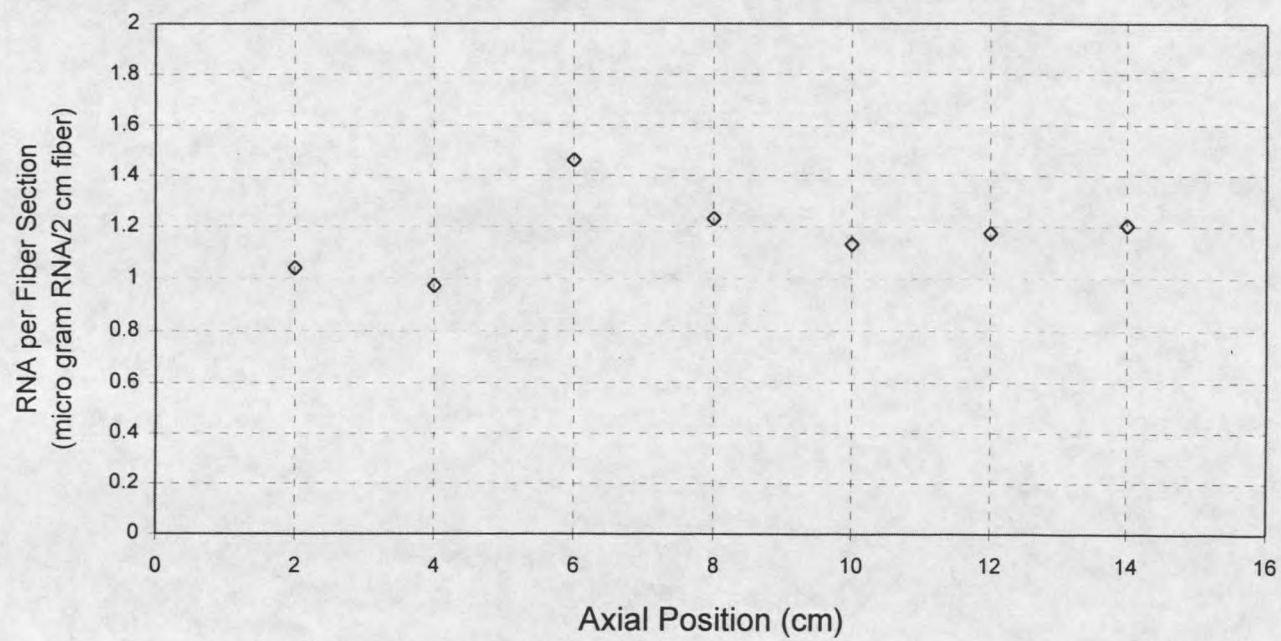
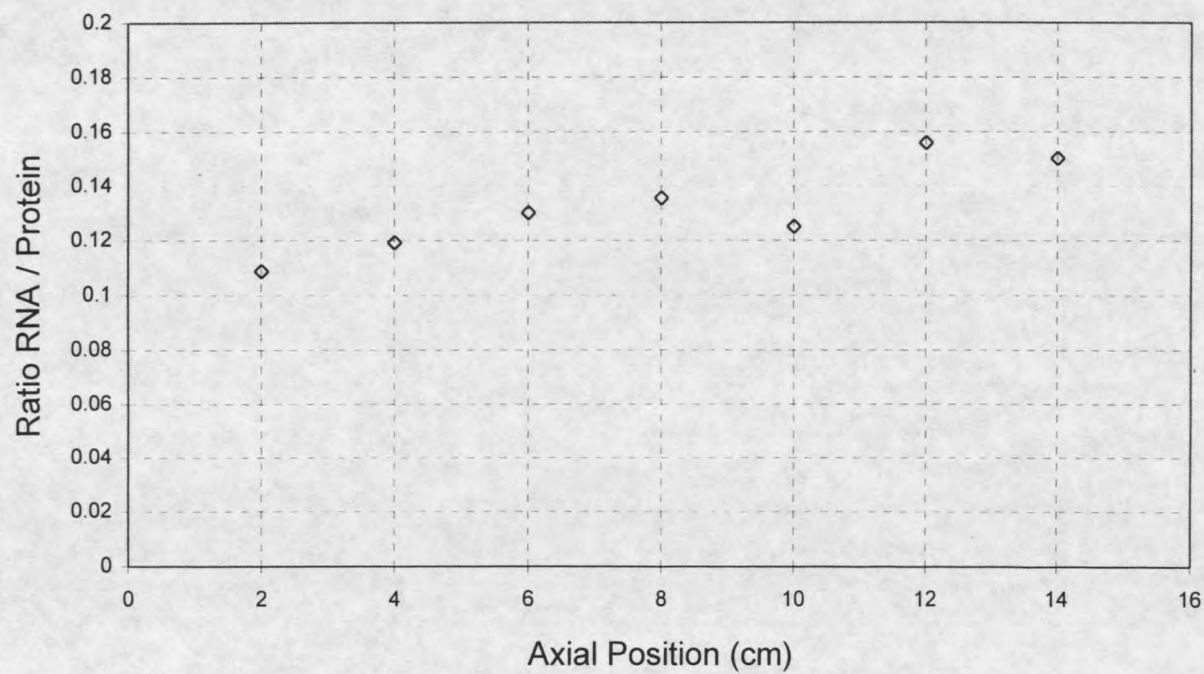


Figure 25
RNA / Protein Ratio, Experiment 1, Amicon HFM Cartridge
Samples were harvested after 170 hr. of reactor operation.



Experiment 2, Amicon Hollow Fiber Membrane Cartridge

This second experiment, using the Amicon H1P30-43 HFM cartridge, was the longest of any HFM experiment run for this project. Total running time was thirty one days and encompassed several major process variations. Normal operating parameters are outlined in Table 11. Process upsets included: (1) cells escaping through the lumen side of the reactor, (2) inconsistent flows through the lumen and shell sides of the HFM bioreactor and (3) problems with the house supply of deionized water. The HFM cartridge was inoculated on 3/3/95 with *Ps .putida* and on 3/10/95 the lumen effluent stream was found to be blocked causing convective fluid transport from the lumen to shell regions of the reactor. Flows within the bioreactor may have been disrupted for as much as 14 hours. Flow of nutrients to the reactor was interrupted due to a block in the tubing on 3/26/95 which required replacement of the section. A major upset in the shell side influent flow occurred on 3/28/95 when the building maintenance department turned off the deionized water supply. At the time of this incident all the dilution water for the reactor was being drawn from the building supply. The deionized water system was shut down for replacement of a depleted ion exchange bed for the removal of chloride ion. There is no record of what the chloride concentration was in the DI water fed to the Amicon HFM bioreactor before replacement of the ion exchange bed. Subsequent supply of ultrapure water drawn from a 50 L carboy was used for the remainder of the experiment

and the reactor was dismantled on 4/2/95. The reduction in flow through the shell side of the reactor created convective flow through the HFM fibers which resulted in loss of cells from the fibers.

Seventeen days after inoculation, cells were detected in both the lumen and shell effluent streams using a plate count method. This was the first time since the inoculation of the reactor that the streams were inspected for cells so its possible that cell break out occurred significantly earlier. Cell break out was confirmed for the lumen and shell effluents on 3/23/95. Cells detected in the effluent streams originated within the reactor because no cells were detected within the lumen or shell influent streams. Further investigation of the effluent streams involved the use of a total cell count. The shell effluent contained 6.3×10^6 cells/mL but the cell concentration in the lumen effluent was below detection using a AO cell count method. The size and color of cells stained with AO can be used as an indication of growth phase (McFeters, 1991). Cells escaping from the bioreactor when viewed under a microscope appeared small and green in color.

Figure 26 shows the glucose concentration in the lumen influent, effluent and shell effluent as a function of time. Samples were not taken from the shell influent stream where the glucose concentration is known to be zero. Two samples time = 24 hrs and time = 216 hrs were lost in a laboratory accident. The consumption rate of glucose from the reactor is shown in Figure 27. A block in

the lumen effluent stream produced the peak located at 350 hr. and indicates the presence of convective fluid transport through the HF membranes. Severe fluctuations in the voltage output of the dissolved oxygen (DO) probes continued to prevented acquisition of useable data after the equipment was repaired. The probes did not hold calibrations and the probes failed totally after 4 days of operation. At this point the DO probe system was abandoned. Figure 28 is the total protein extracted from the Amicon HFM cartridge fibers after 720 hrs as a function of distance. The efficiency of the extraction process is not corrected for in this profile. The estimated total cell count per 2cm section of fiber (Figure 29) did take into account the efficiency of the extraction process. RNA results are shown in Figure 30 with no correction made for the efficiency of the extraction procedure. Figure 31 shows the RNA/Protein ratio which is for indication of cell growth.

Figure 26
Amicon HFM Cartridge, Experiment 2
Glucose Concentration as a Function of Time

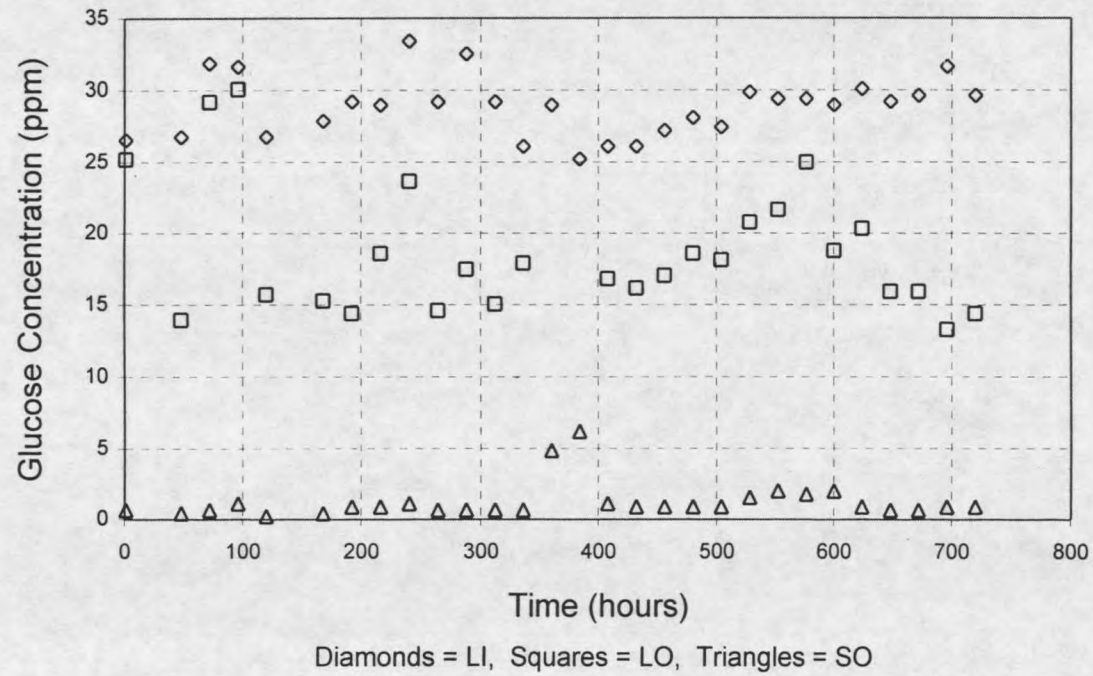


Figure 27
Amicon HFM Cartridge, Experiment 2
Glucose Consumption, Mass Balance

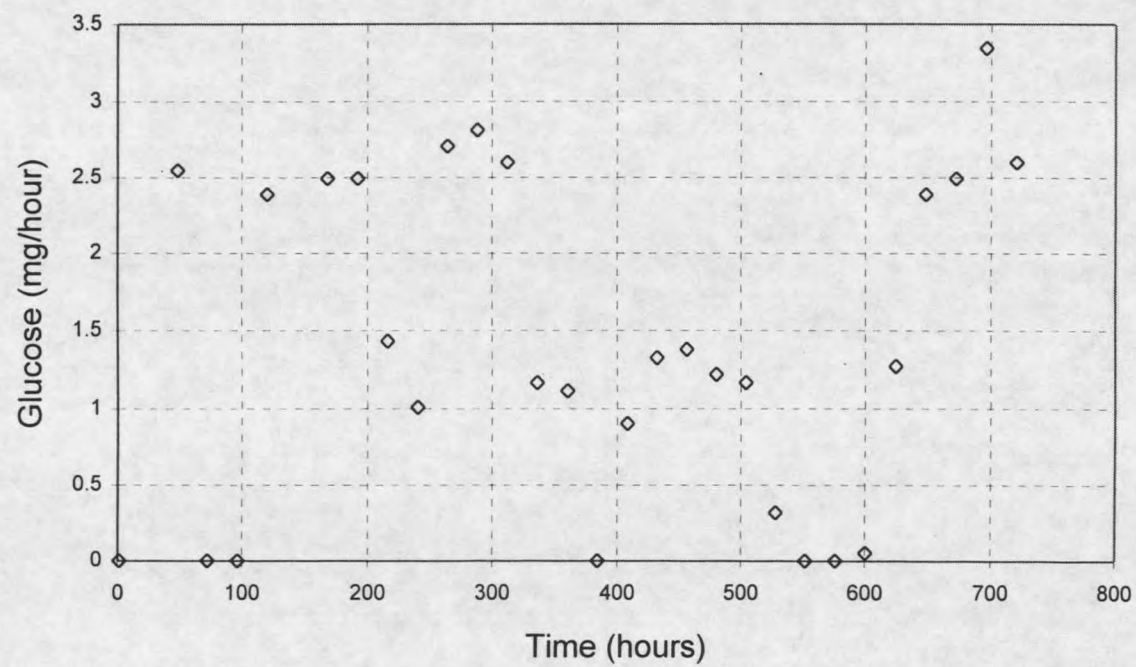


Figure 28
Protein Profile, Amicon HFM Cartridge, Experiment 2
Samples were harvested after 720 hr. of reactor operation.

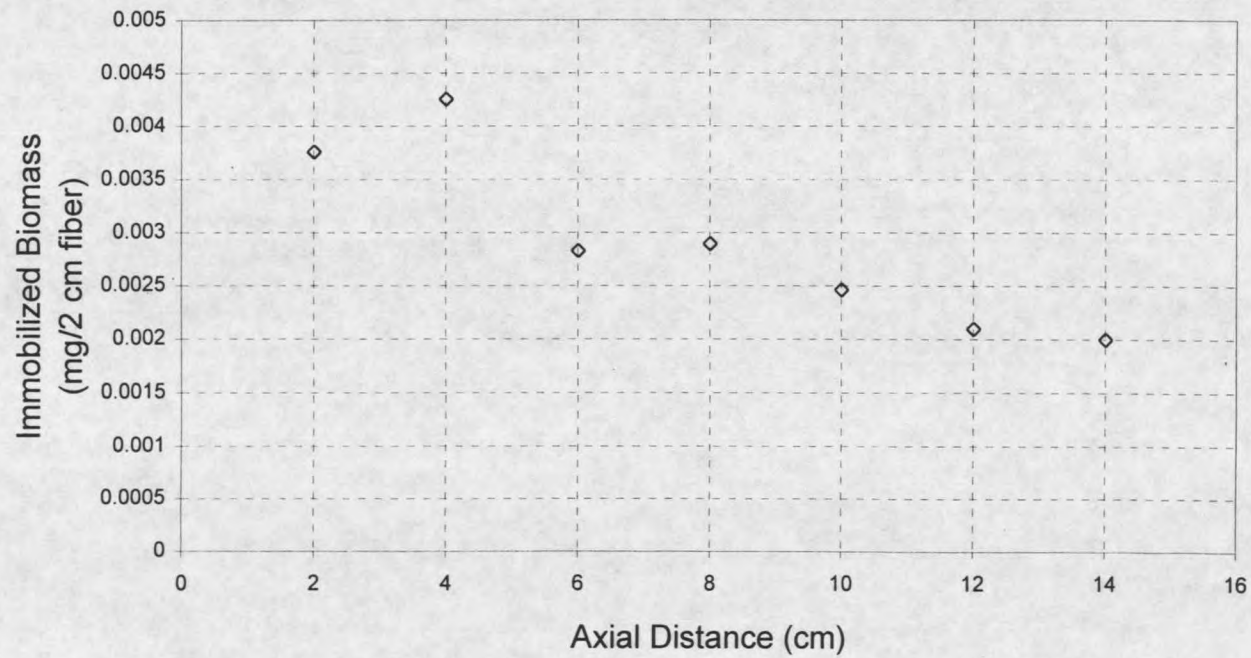


Figure 29
Amicon HFM Cartridge, Experiment 2
Ps. putida Cells Estimated From Protein Data
Samples were harvested after 720 hr. of reactor operation.

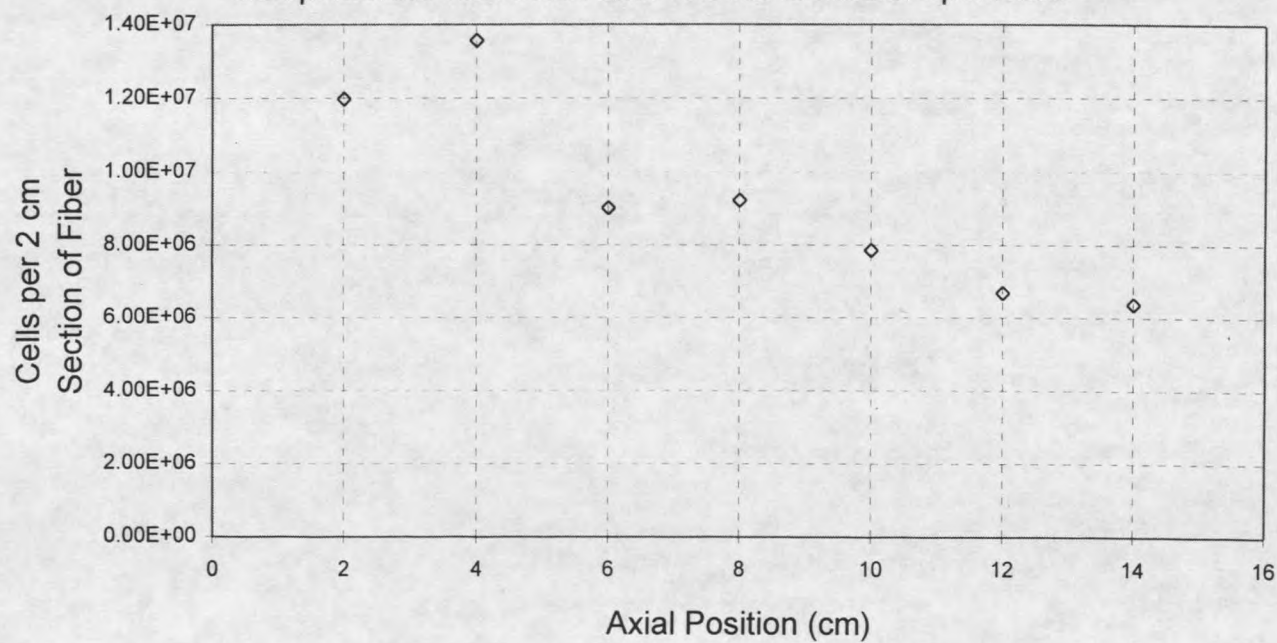


Figure 30
RNA Profile, Amicon HFM Cartridge, Experiment 2
Samples were harvested after 720 hr. of reactor operation.

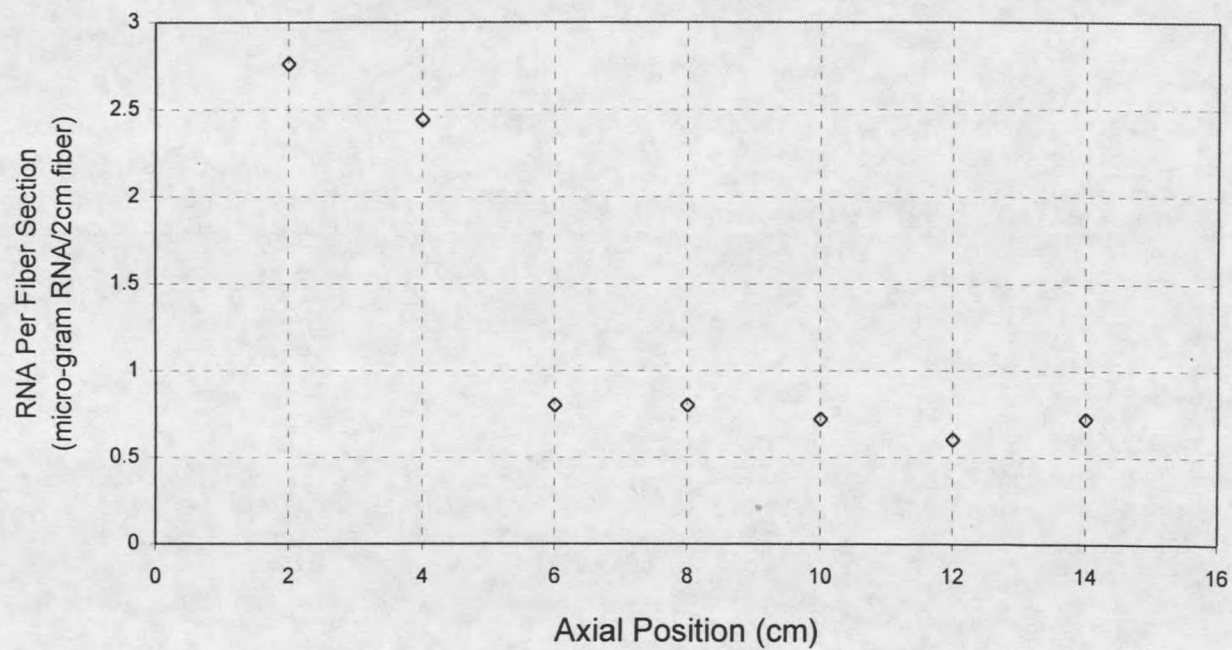
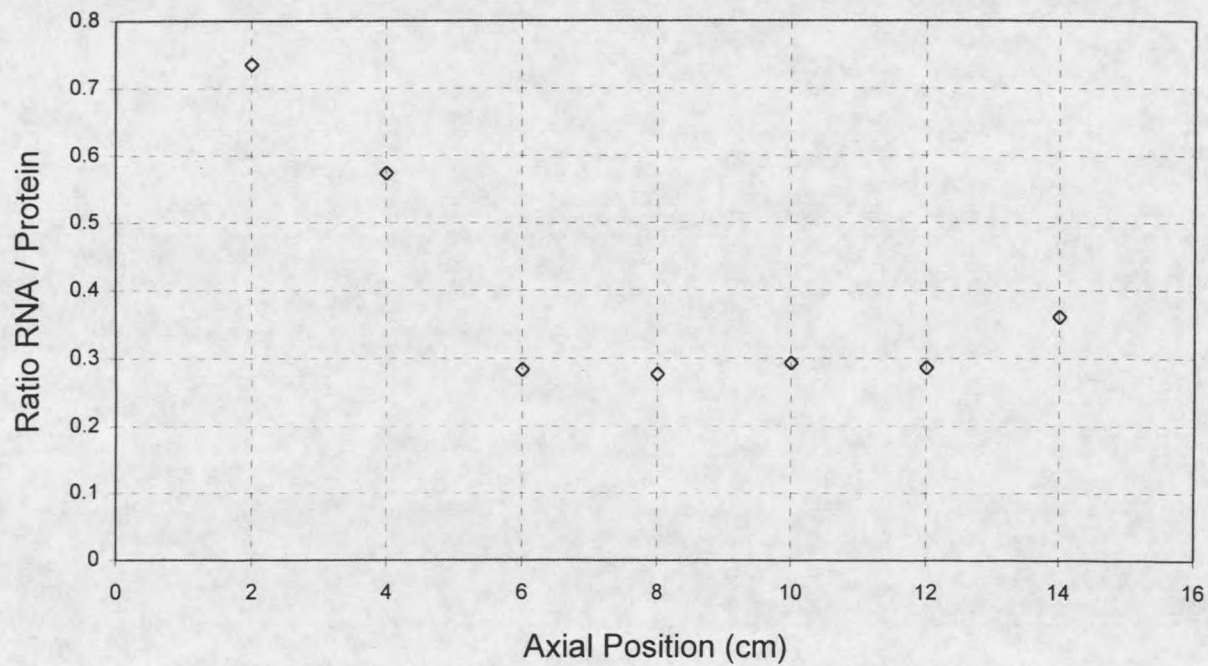


Figure 31
RNA / Protein Ratio, Experiment 2, Amicon HFM Cartridge
Samples were harvested after 720 hr. of reactor operation.



Experiment 3, In-house Fabricated HFM Reactor

This experiment was the first using the In-house Fabricated HFM (IHF-HFM) reactor. The duration of the experiment was 5 days and the first to obtain useful dissolved oxygen concentration data. This reactor was shut down after cells were detected in both the lumen and shell effluent. As the experiment progressed, precipitation of nutrient salts within the Tygon tubing was an increasing problem. The Tygon was replaced with silicone which reduced the precipitation of the nutrient salts and restored flow to initial conditions.

Figure 32 and 33 show the glucose concentrations entering and exiting the lumen and shell as a function of time. Lumen and shell volumetric flow rates were changed for use with the IHF-HFM reactor and the concentration of glucose in the feed stream was increased to 50 ppm. The consumption rate of glucose is shown in Figure 34. The mass loading rates of dissolved oxygen in the lumen and across the shell side of the reactor is shown in Figure 35. Figure 36 is the rate of oxygen removal in the shell side of the reactor. Analysis of immobilized biomass produced an axial total protein profile shown in Figure 37. Estimated cell numbers per 2 cm of fiber are presented in Figure 38. The RNA profile, Figure 39, shows a decreasing amount of cell material with increasing length. The quantity of RNA recovered was lower than expected. The cellular material extraction solution used with this experiment did contain DEPC to protect the RNA. Figure 40 shows an RNA / protein ratio indicates limited cell growth within

Figure 32
In-house Fabricated HFM Reactor, Experiment 3
Glucose Concentration as a Function of Time

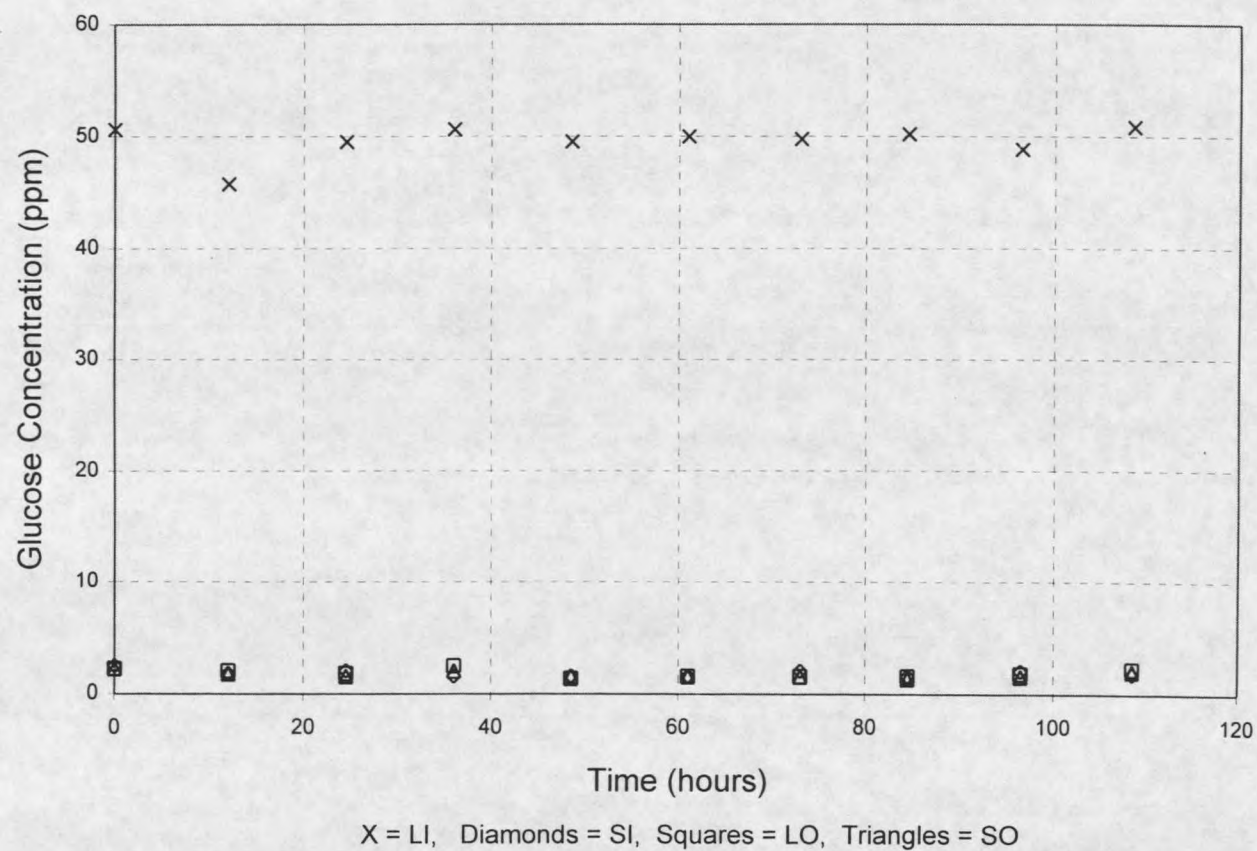
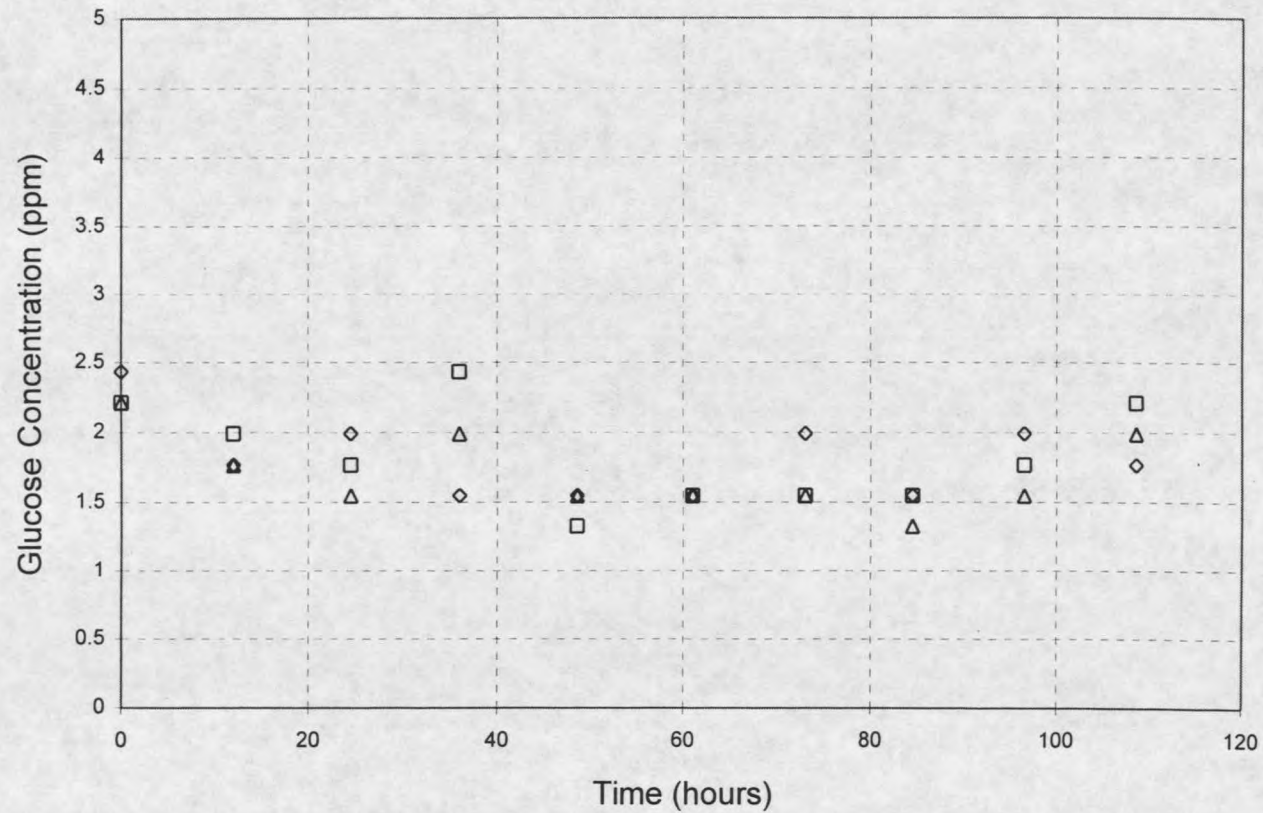


Figure 33
In-house Fabricated HFM Reactor, Experiment 3
Glucose Concentration as a Function of Time



Diamonds = SI, Squares = LO, Triangles = SO

Figure 34
In-house Fabricated HFM Reactor, Experiment 3
Glucose Consumption, Mass Balance

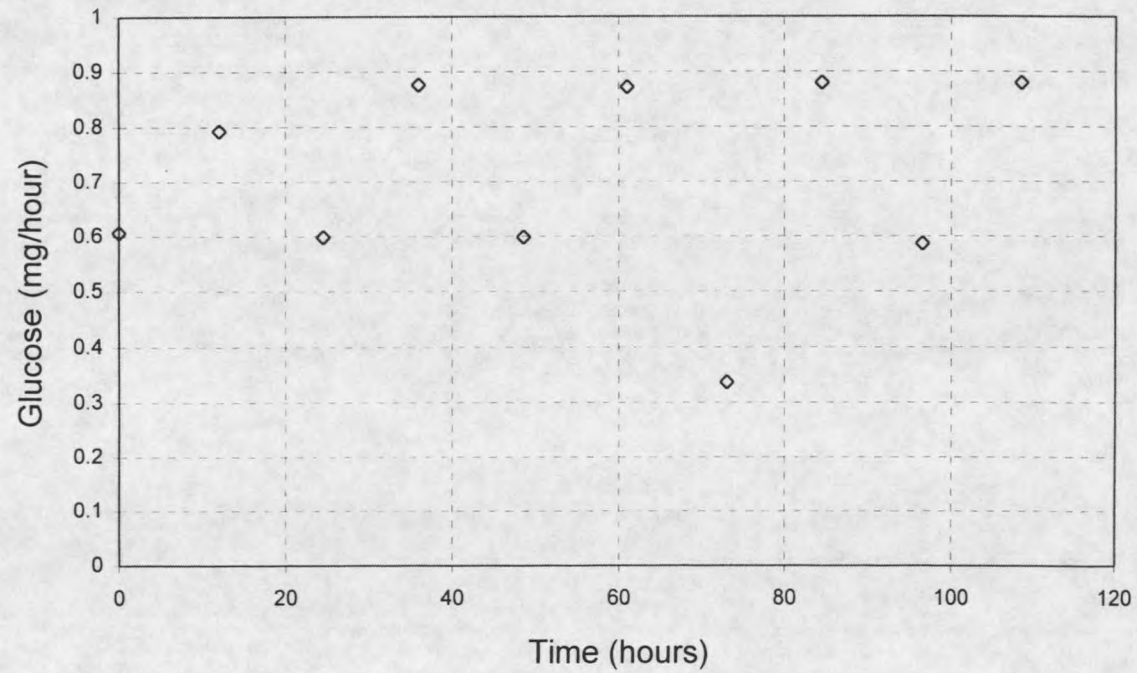


Figure 35
In-house Fabricated HFM Bioreactor, Experiment 3
Dissolved Oxygen as a Function of Elapsed Time

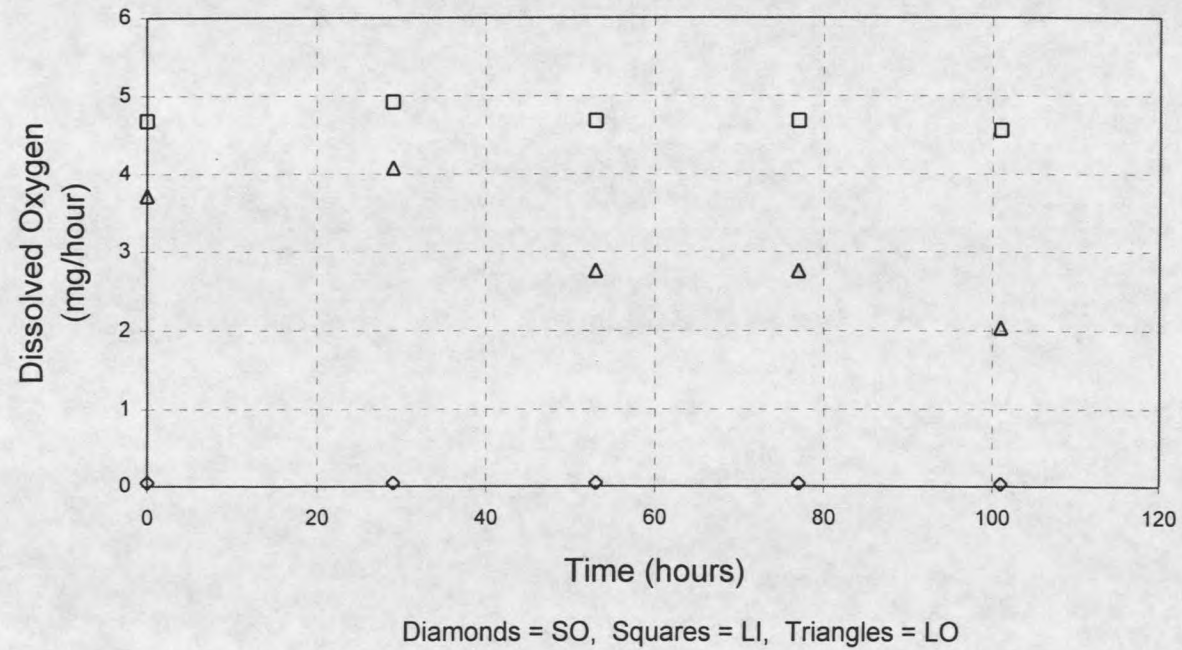


Figure 36
In-house Fabricated HFM Bioreactor, Experiment 3
Oxygen Removal Rate, Shell

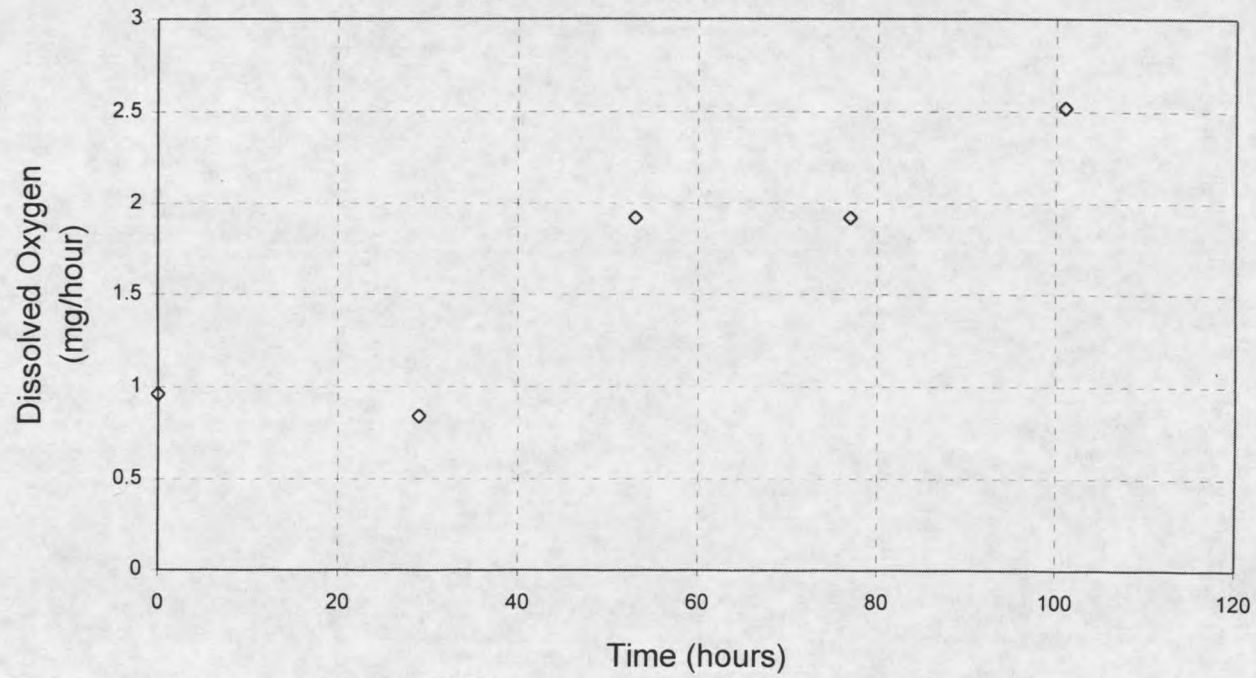


Figure 37
Experiment 3

Protein Profile, In-house Fabricated HFM Bioreactor
Samples were harvested after 110 hr. of reactor operation.

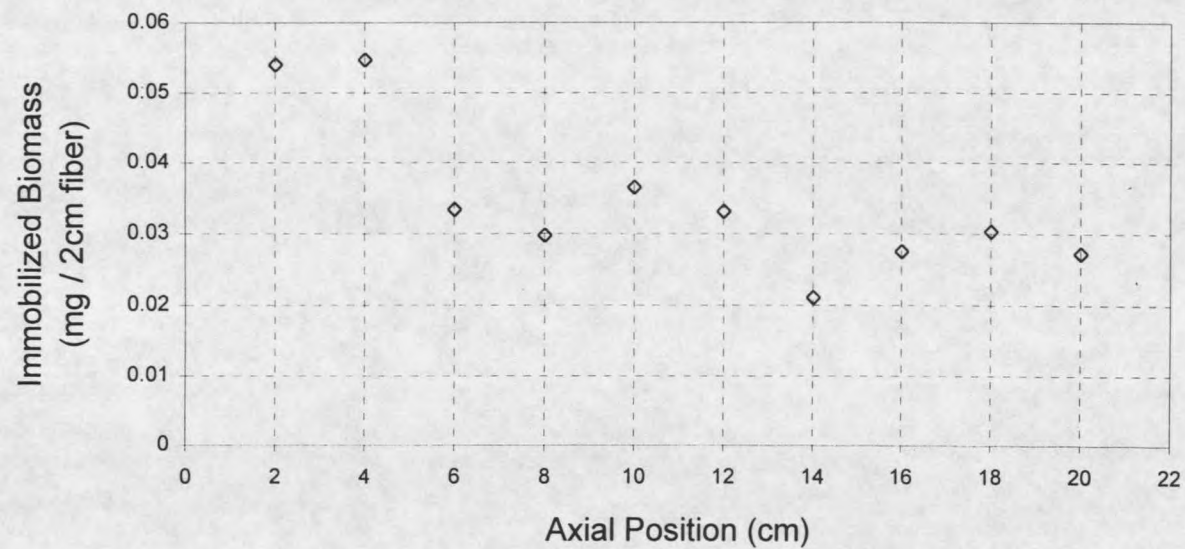


Figure 38
In-house Fabricated HFM Bioreactor, Experiment 3
Ps. putida Cells Estimated From Protein Data
Samples were harvested after 110 hr. of reactor operation.

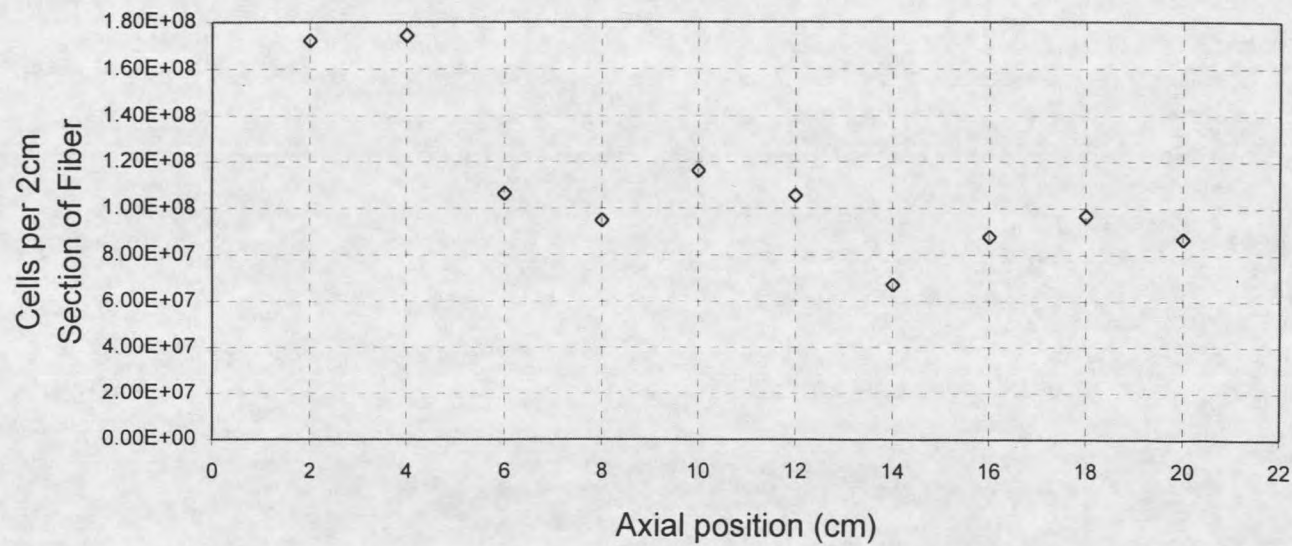


Figure 39
Experiment 3

RNA Profile, In-house Fabricated HFM Bioreactor
Samples were harvested after 110 hr. of reactor operation.

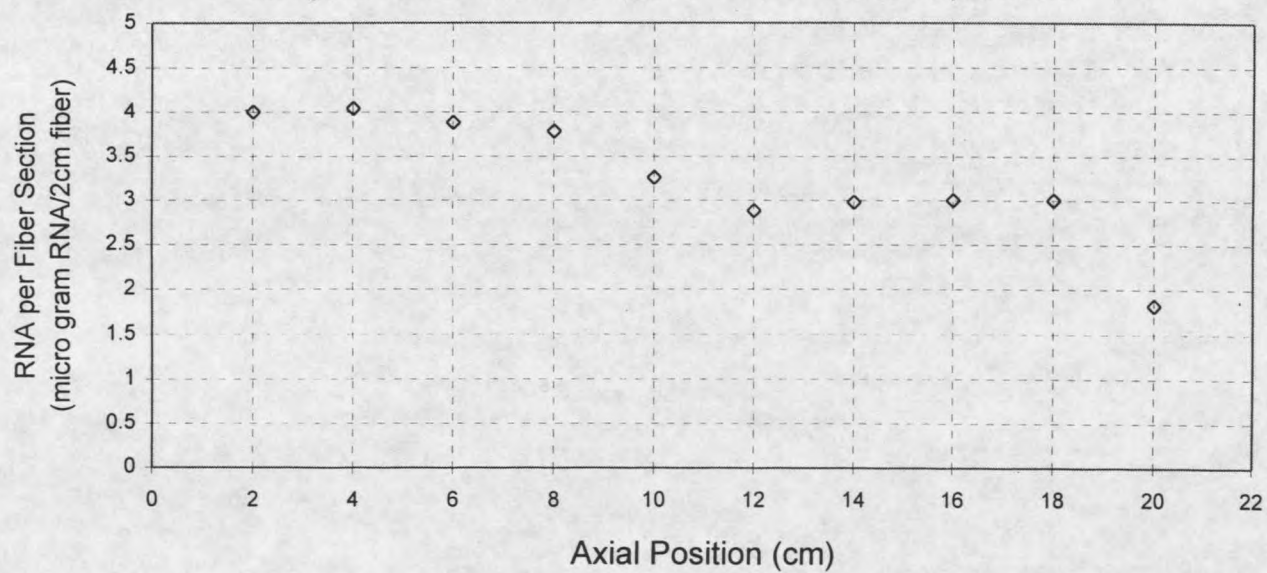
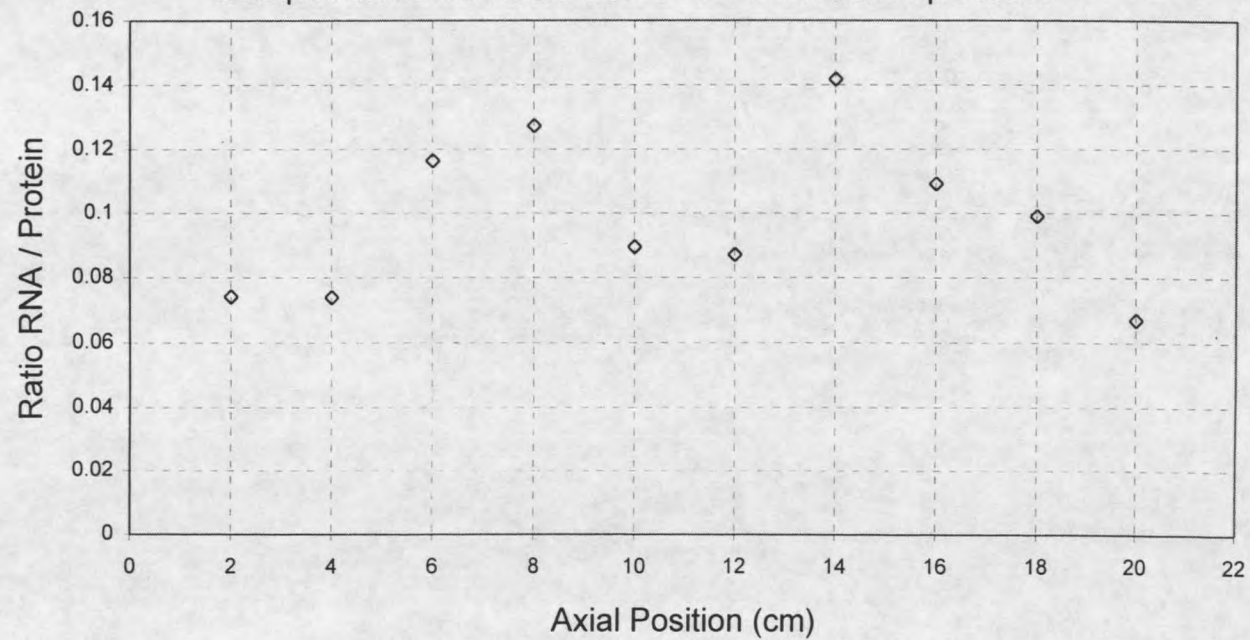


Figure 40
RNA / Protein Ratio, Experiment 3
In-house Fabricated Hollow Fiber Membrane Reactor
Samples were harvested after 110 hr. of reactor operation.



Experiment 4, In-house Fabricated HFM Reactor

The final experiment for this project used the In-house Fabricated HFM (IHF-HFM) reactor and lasted 10 days. The reactor was shut down due to detection of cells in both effluents. There were few upsets in the system over the 10 day run in comparison to the other 3 experiments. At 9pm on 9/1/95 the nutrient influent line was blocked and the tubing as well as the filter required replacement. This problem was corrected quickly and the impact to the system was minimal. The compressed air tank was depleted, due to a leak, on 9/4/95 and was replaced with pure oxygen. A large peak in the shell dissolved oxygen mass loading rate was the result of using the pure oxygen for a short time. The peak returns to normal levels within one day after returning to compressed air for saturation of the dilution water fed to the reactor. Cells were first detected exiting the reactor lumen 2 days after start up of the reactor. The reactor was shut down 8 days later when cells were found entering the reactor lumen stream.

Figures 41 and 42 show the glucose concentration entering and leaving the reactor. The consumption rate of glucose is shown in Figure 43. Figure 44 shows the mass rate of dissolved oxygen for the various flow streams. Note the O₂ excursion at 150 hours. Figure 45 shows the shell O₂ removal rate as a function of time. Note there was little impact of using pure oxygen. At 240 hours, the protein profile, Figure 46 shows the greatest biomass accumulation near the lumen entrance of the reactor. The RNA profile has the expected

shape but the quantity of RNA recovered is less than expected, Figure 48. The RNA / protein ratio, Figure 49, shows that little or no growth was occurring within the bioreactor when the HFM samples were harvested. There was no correction made for the efficiency of the extraction procedure in figures 46, or 48. There was a correction made for the extraction efficiency for calculation of the estimated cell concentration profile shown in figure 47.

Figure 41
In-house Fabricated HFM Rector, Experiment 4
Glucose Concentration as a Function of Time

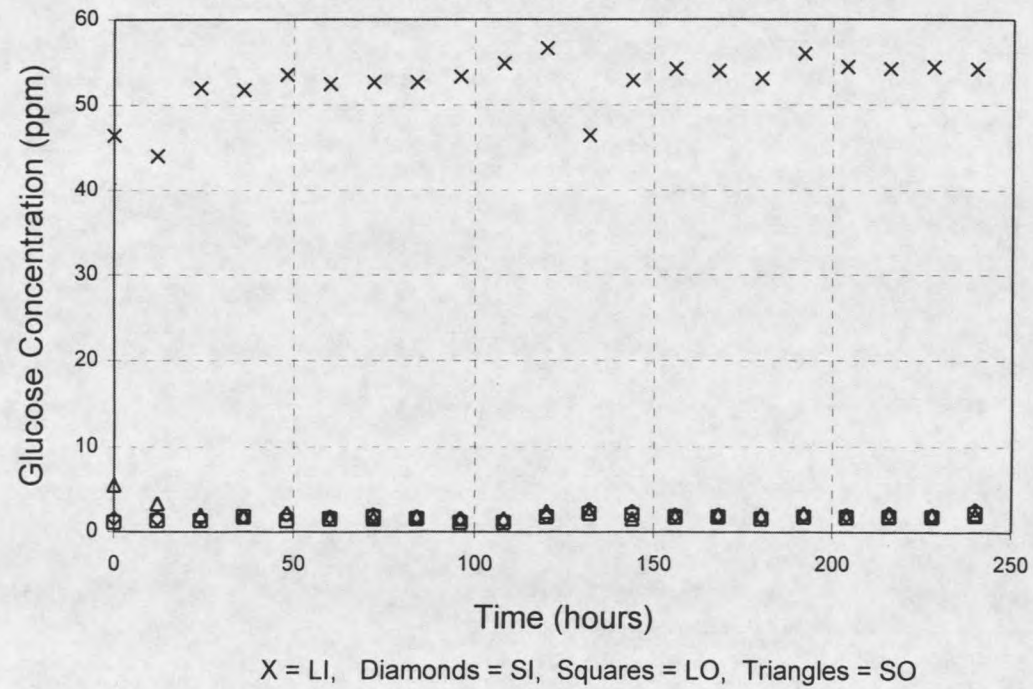


Figure 42
In-house Fabricated HFM Rector, Experiment 4
Glucose Concentration as a Function of Time

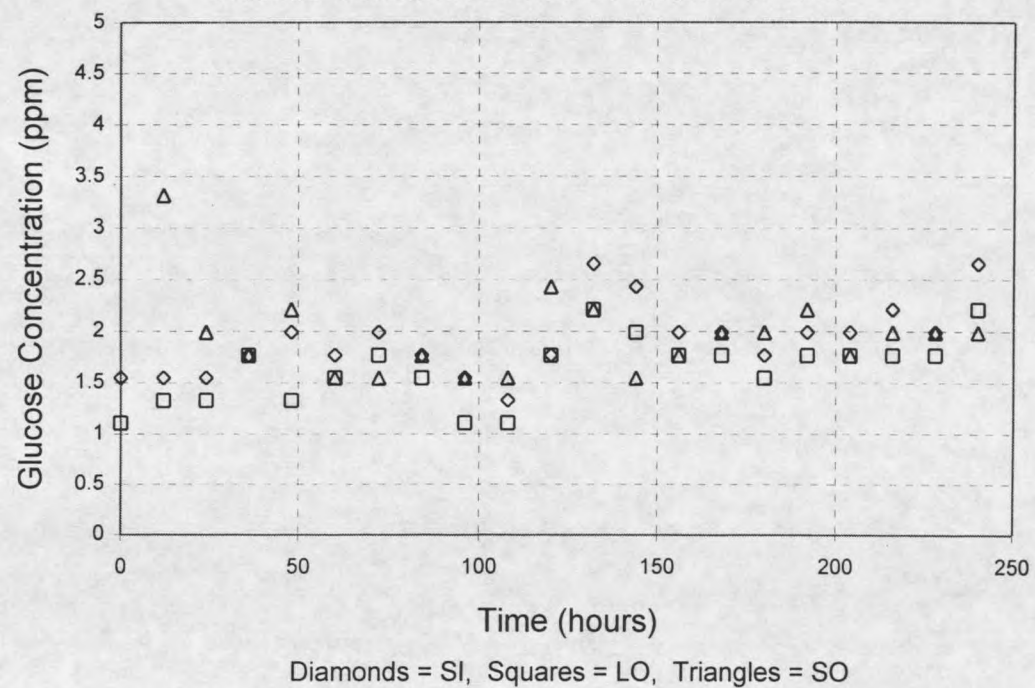


Figure 43
In-house Fabricated HFM Reactor, Experiment 4
Glucose Consumption, Mass Balance

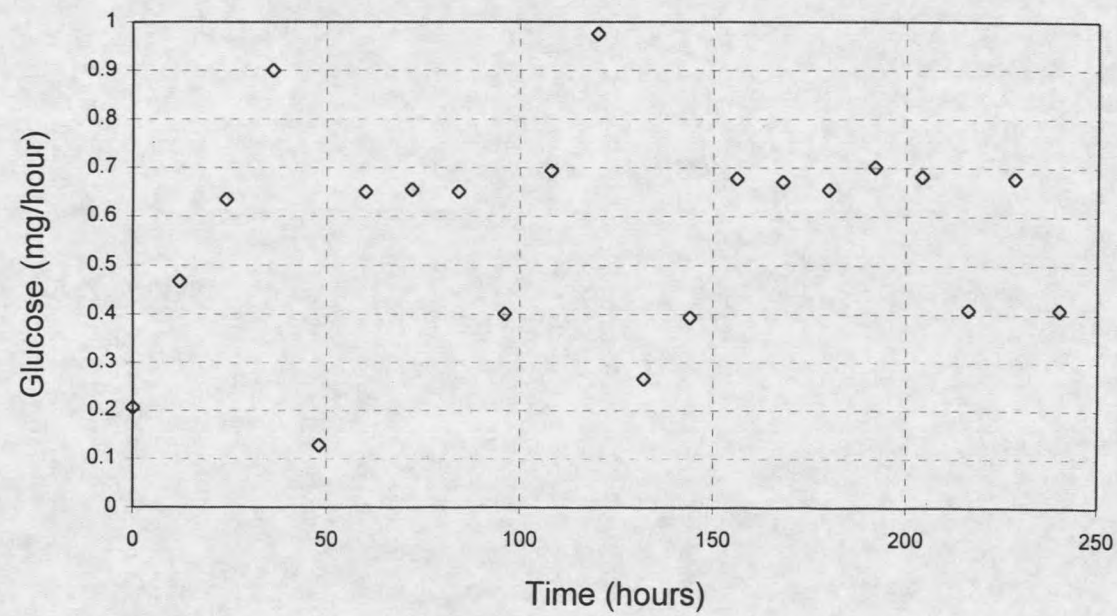


Figure 44
In-house Fabricated HFM Bioreactor, Experiment 4
Dissolved Oxygen as a Function of Elapsed Time

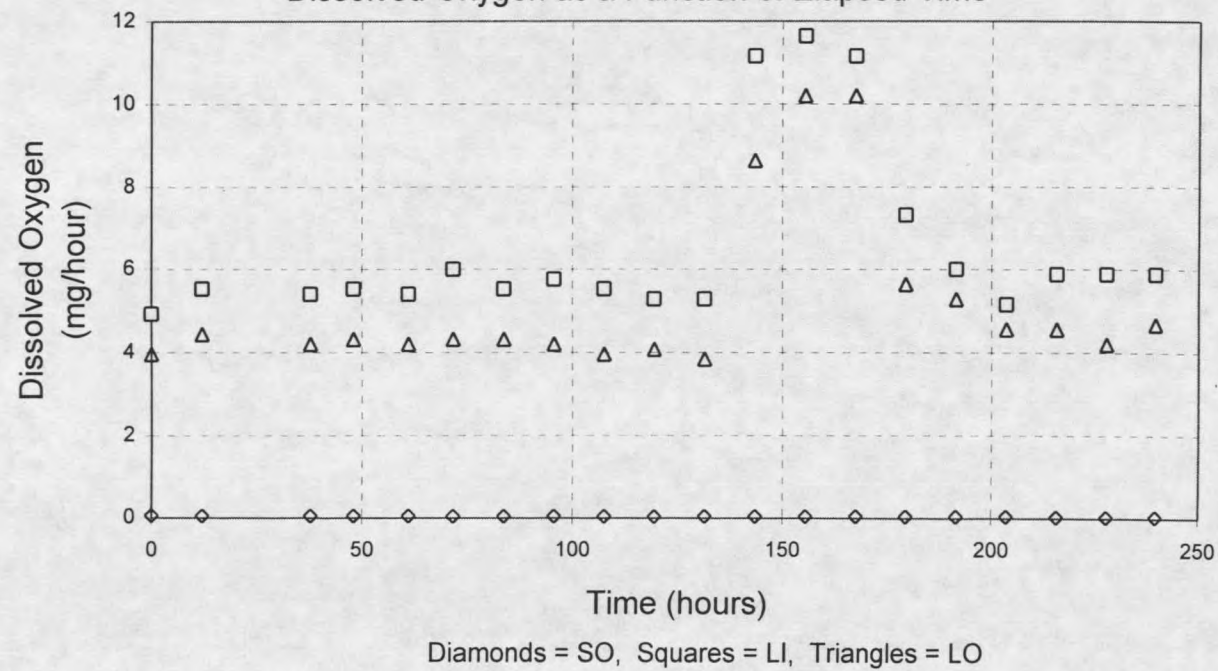


Figure 45
In-house Fabricated HFM Bioreactor, Experiment 4
Oxygen Removal Rate, Shell

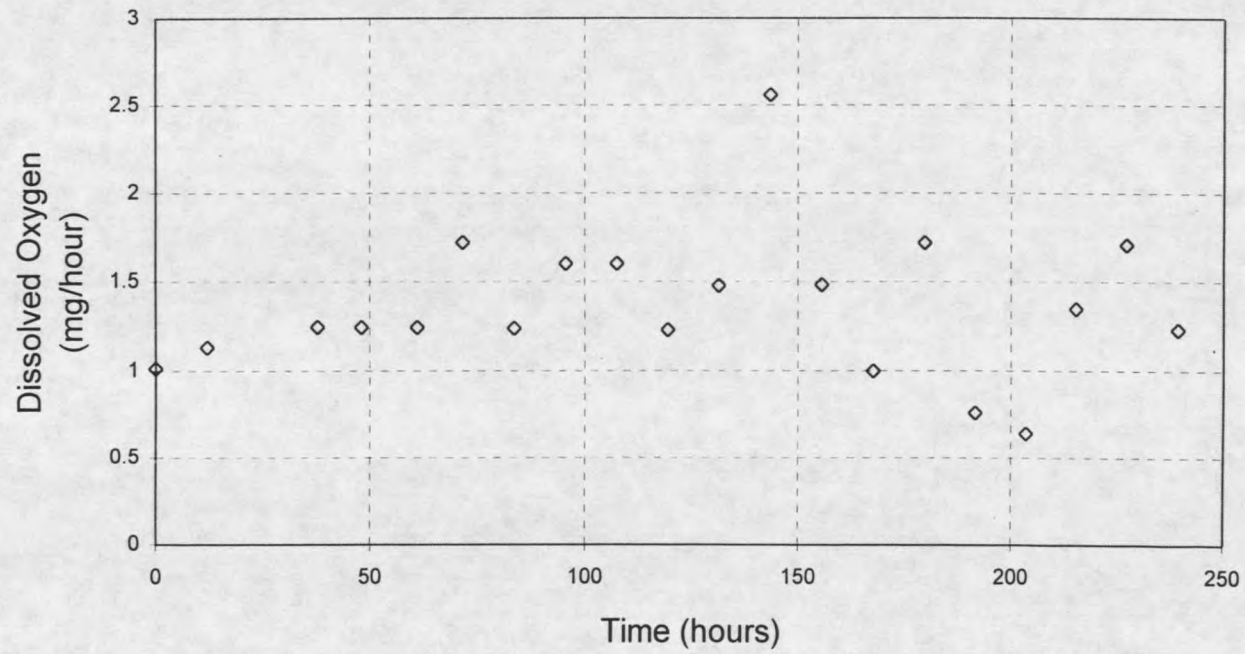


Figure 46
Experiment 4
Protein Profile, In-house Fabricated HFM Bioreactor
Samples were harvested after 240 hr. of reactor operation.

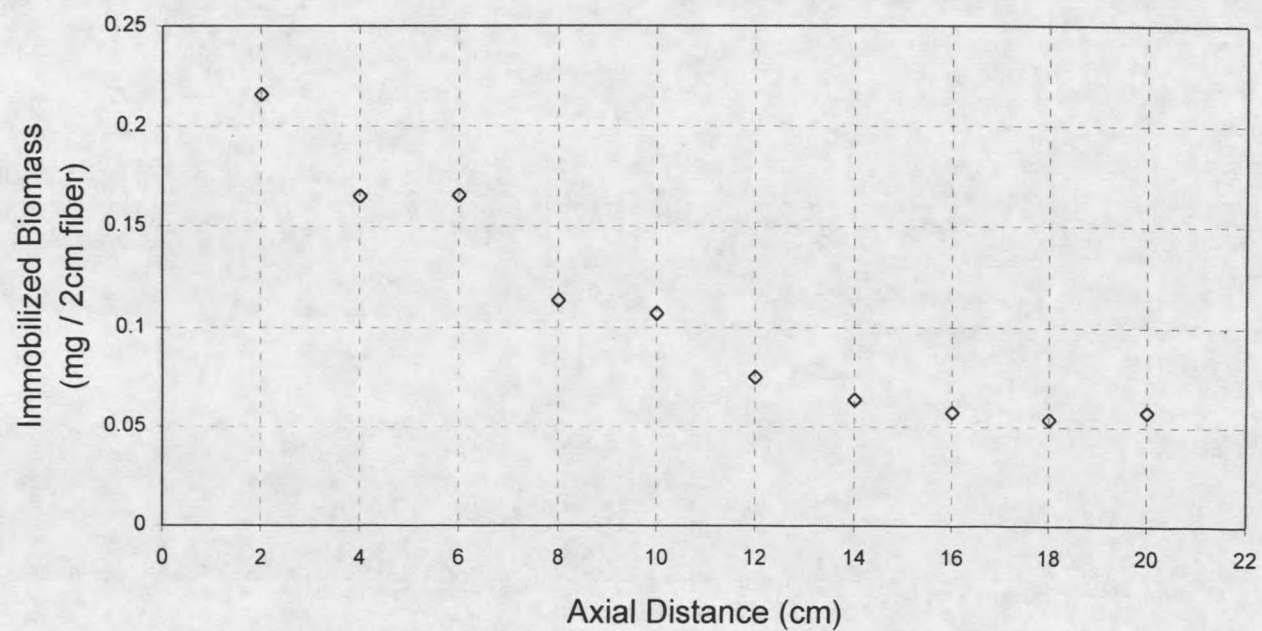


Figure 47
In-house Fabricated HFM Bioreactor, Experiment 4
Ps. putida Cells Estimated From Protein Data
Samples were harvested after 240 hr. of reactor operation.

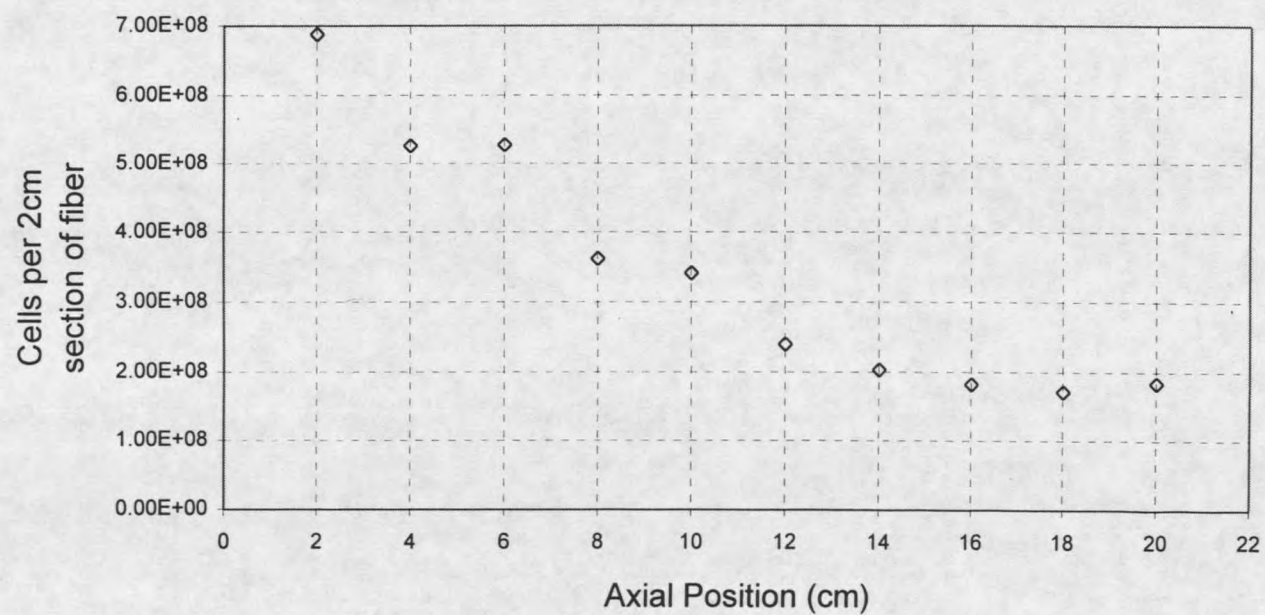


Figure 48
Experiment 4
RNA Profile, In-house Fabricated HFM Bioreactor
Samples were harvested after 240 hr. of reactor operation.

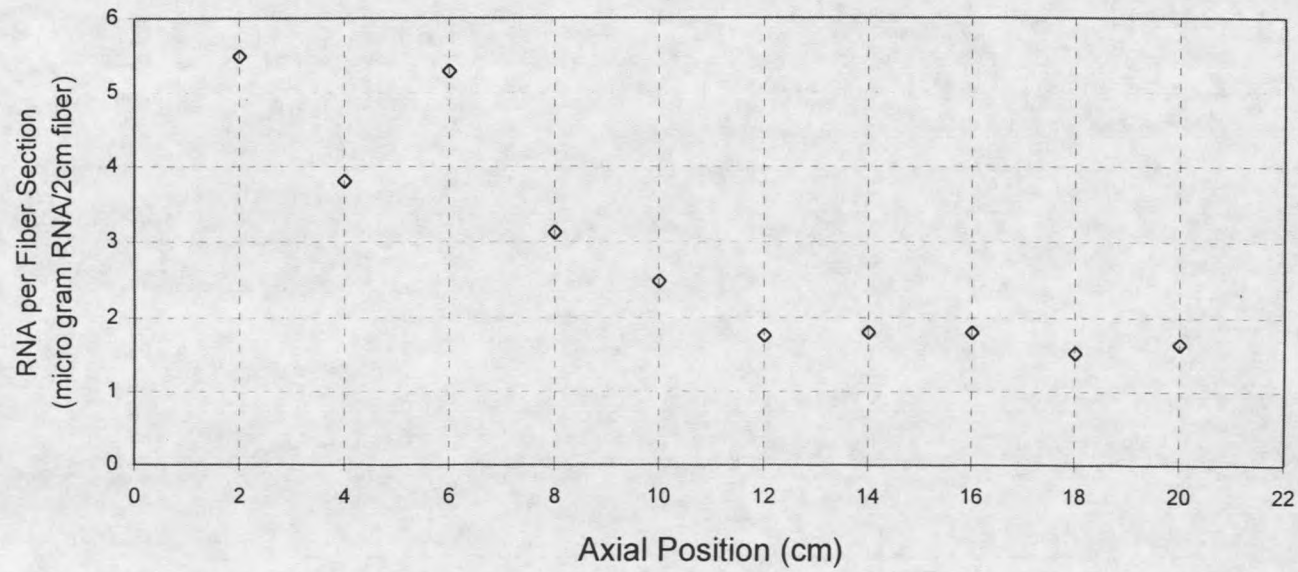
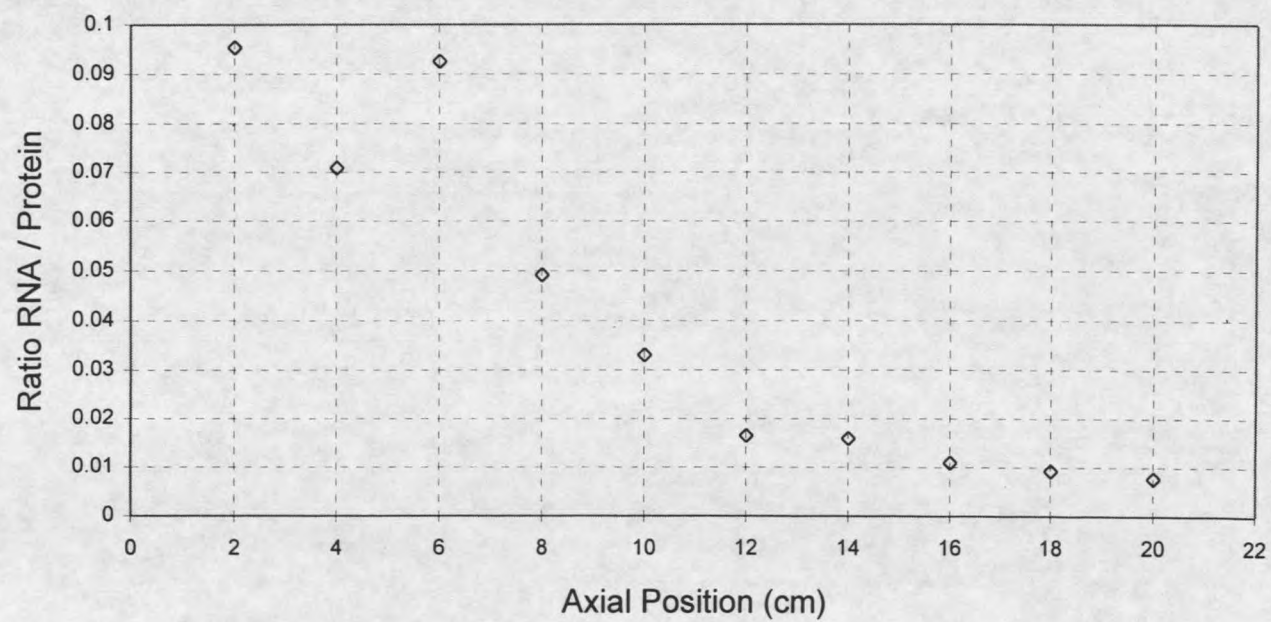


Figure 49
RNA / Protein Ratio, Experiment 4
In-house Fabricated Hollow Fiber Membrane Reactor
Samples were harvested after 240 hr. of reactor operation.

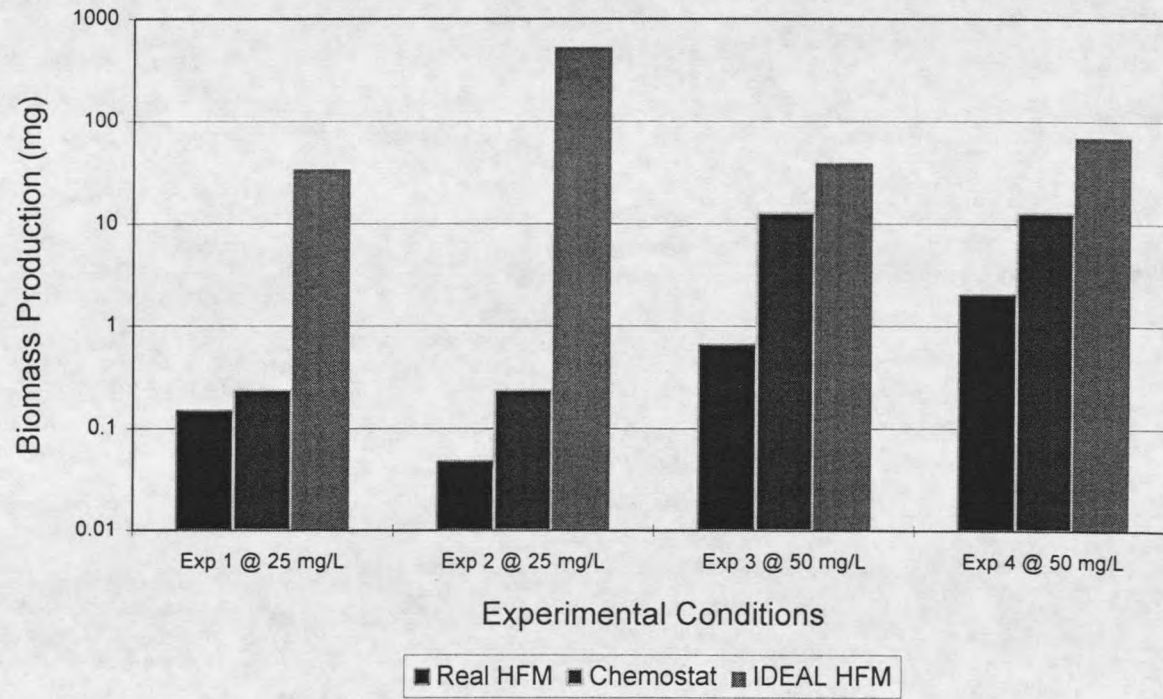


CHAPTER 5

Summary

Evaluation of reactor performance through examination of glucose removal rate, total cell protein, and RNA / Protein ratio of entrapped cells indicates very poor bioreactor performance. The physical dynamics of the HFM bioreactor used for this project were unpredictable; as a result the system was inconsistent. Glucose profiles for each experiment provided useful information as to the quantity of material consumed by the entrapped bacteria. The Amicon HFM cartridges retained little cell mass at the end of each experiment when compared to the In-house Fabricated HFM bioreactor. The In-house Fabricated HFM reactor did improve the retention of biomass within the fibers and also improved the stability of the HFM reactor system. Figure 50, compares three types of bioreactors. An ideal chemostat, operated under the same operating conditions as each HFM, is compared to an ideal HFM bioreactor, 100% biomass retention, and the real HFM bioreactor from each experiment. Theoretical biomass production values for each type of ideal reactor were compared with the lowest quantity of biomass retained by the real HFM reactor. The chemostat contained more biomass than the real HFM reactor but less than an ideal HFM reactor. Expectations for the real HFM reactor were to retain more biomass than a chemostat reactor and completely retain the biocatalyst within the HFM fibers. The real HFM reactors did not meet either expectation.

Figure 50
Bioreactor Comparison



Stability of the Bioreactor System

The stability of the HFM bioreactor system can be evaluated through examination of glucose concentration profiles. Figure 20 is the glucose concentration profile obtained from experiment 1. The lumen influent (LI) and lumen effluent (LO) profiles are smooth and consistent throughout the duration of the experiment. This indicates stable flows within the Amicon HFM cartridge system (Figure 12) in experiment 1 for up to 140 hours of operation. A flow problem developed after 140 hours and is evident in the reduction of the lumen out (LO) glucose concentration. Figure 26 is the glucose concentration profile from experiment 2 which shows a very unstable Amicon HFM cartridge system. The lumen influent (LI) glucose profile is stable when compared to the lumen effluent profile. Drastic swings in the LO profile may indicate convective flow. Experiment 2 also retained a low concentration of biomass at termination of the experiment which was caused by transmembrane convective flow washing cells from the fibers. Stability improves for experiments 3 and 4. Figures 32 and 41 show stable lumen influent glucose concentration but the stability of the effluent streams cannot be determined based on these profiles. The lumen effluent (LO) profile shows a glucose concentration near zero. The influent and effluent flows for this system, Figure 13, were measured by capturing liquid samples and calculating flow rates. Little fluctuation was observed in either the shell or lumen flow rates for duration of the experiment.

Glucose Removal

Figure 51 is a comparison of the glucose consumption rate for each bioreactor used in experiments 1, 2, 3 and 4. The consumption rates for experiments 1, 3 and 4 are similar and each had stable flows for the duration of the experiment. The unstable system of experiment 2 produced a faster and inconsistent glucose consumption rate. Figure 52 shows the average glucose consumption rate for each experiment. This figure shows that experiment 2 had nearly twice the glucose consumption rate as experiments 1, 3, or 4.

Protein Profile Comparison

Figure 53 shows a comparison between the quantities of proteins recovered from 2 cm HFM samples harvested at the termination of each experiment. The In-house Fabricated HFM bioreactor used in experiments 3 and 4 clearly retained more biomass than the Amicon HFM cartridge used in experiments 1 and 2. Profiles from experiments 3 and 4 also show that most of the protein was recovered from the lumen influent end of the reactor and the quantity of protein recovered decreased down the reactor. This was expected as long as cell growth was taking place within the fibers. The quantity of recovered protein was less than expected for all four experiments, thus giving an indication of very limited cell growth within the reactor.

Figure 51
Glucose Consumption Rate Comparison
Experiments 1, 2, 3, 4

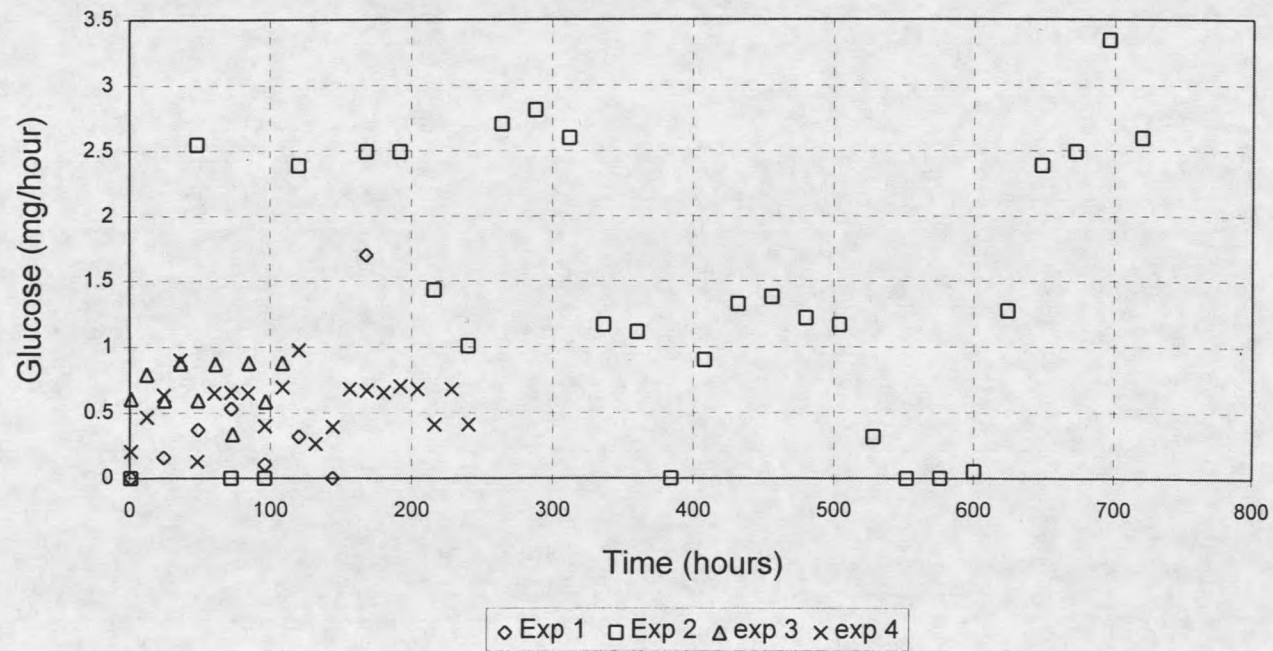


Figure 52
Average Glucose Consumption Rate Comparison
Experiments 1, 2, 3, 4

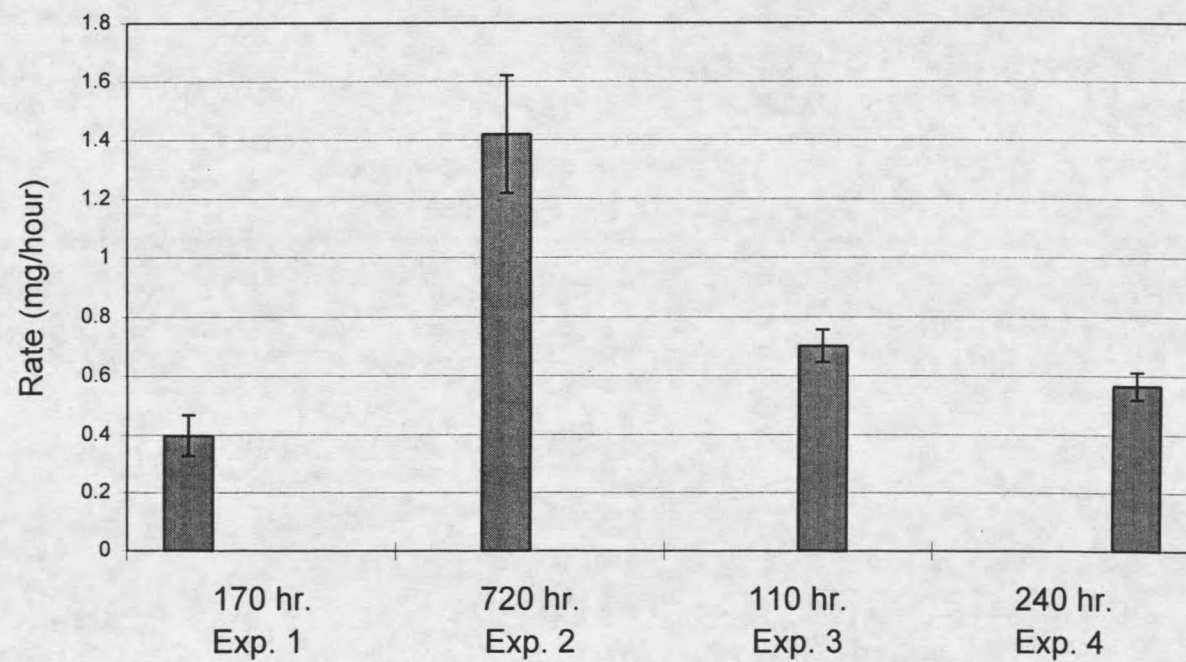
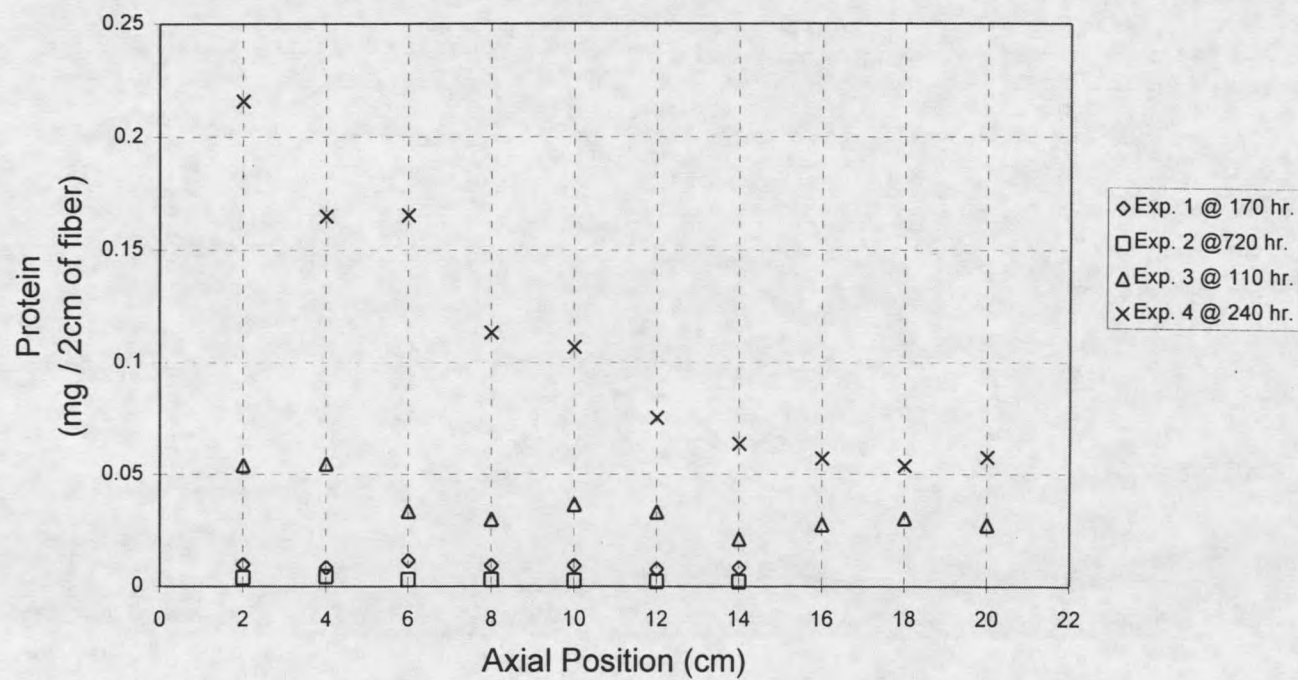


Figure 53
Protein Profiles, Comparison Between Experiments 1 - 4
Samples were harvested at the end of reactor operation.



RNA / Protein Ratio, Indication of Cell Growth

Figure 54 presents the RNA recovered from 2 cm sections of HFM fibers harvested from the same location within the HFM reactors used for experiments 1, 2, 3 and 4. An RNA / protein ratio can be used to indicate cell growth within a bacterial culture. As the ratio increases so does the rate of cell growth as shown in Figure 55. The RNA / protein ratio profile for experiment 2 indicates cells were growing within the HFM cartridge used in experiment 2. However, this is not an indication that all cells retained within reactors 1, 3 and 4 were dead. A portion of the cells in any section of the reactor could be growing or considered active. The method used to assay the quantity of recoverable RNA and protein provided an average value for all cells within that section. E. Wentland, 1995; proved that cells grown within a biofilm did not uniformly exhibit the same activity level. Cells in the center of a biofilm when stained with acridine orange appeared green in color indicating low growth, and the cells along the outer edges of the biofilm were orange/red indicating high growth. Therefore the conclusion which can be made on the basis of RNA / protein ratio alone is that on average the cells retained within reactor 2 were growing and reactors 1, 3 and 4 were not growing.

Figure 54
RNA / Protein Ratio Comparison, Experiments 1, 2, 3, 4

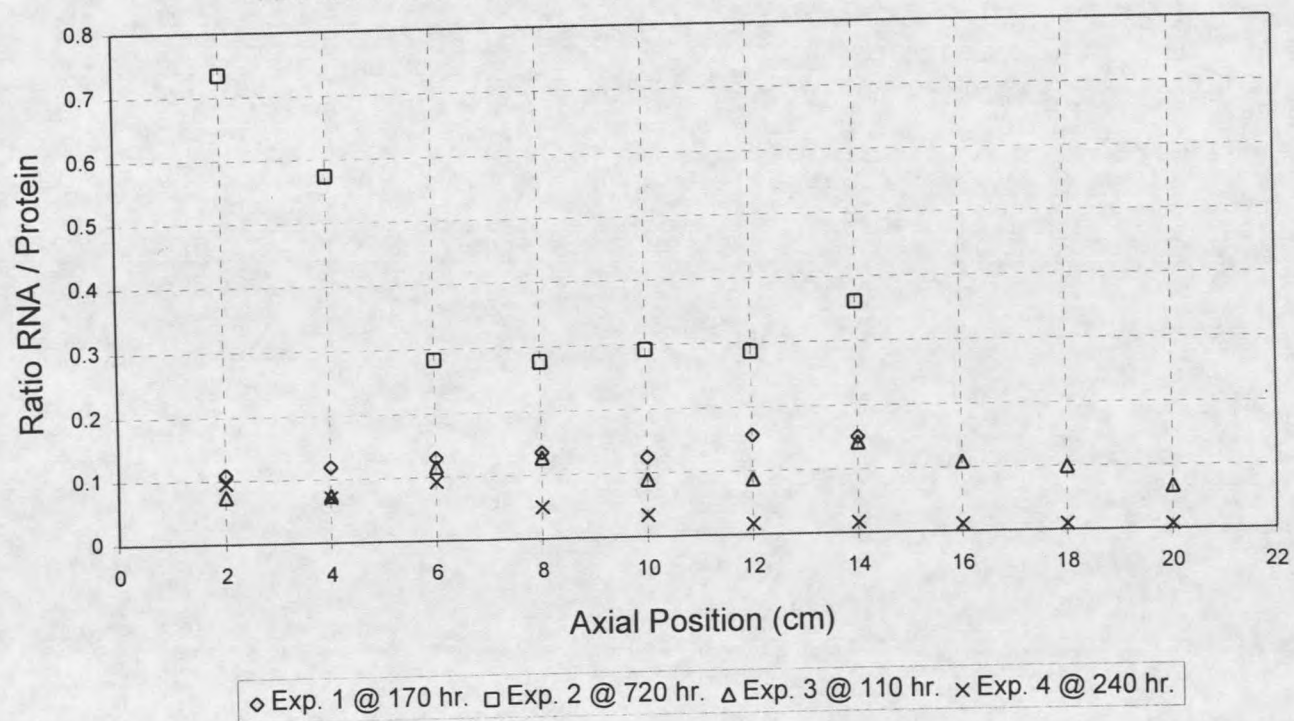
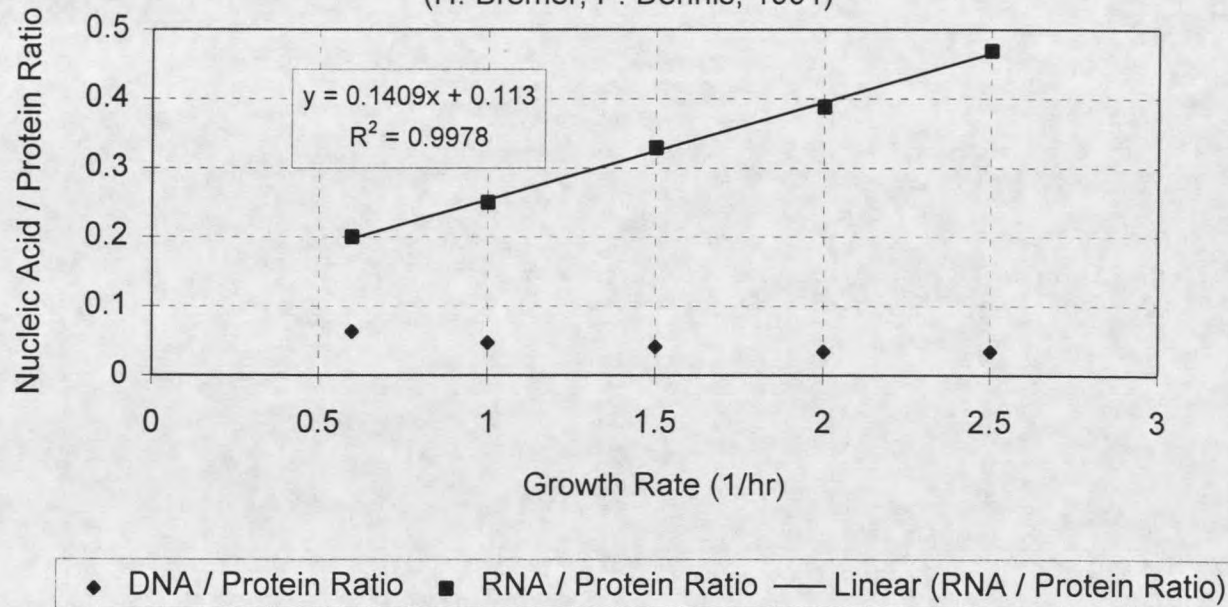


Figure 55
 Nucleic Acid / Protein Ratio for Escherichia coli B/r
 Growing Exponentially as a Function of Doubling Time at 37oC
 Cells were obtained from plank tonic cultures.
 (H. Bremer, P. Dennis, 1991)



Conclusions

The hollow fiber membrane cartridge and reactors used for this project failed to meet expectations for biomass production retention and activity. Although the performance expectations for the HFM reactor systems were not met information useful for design and construction of this type of bioreactor was obtained. The HFM reactor systems failed for several reasons ranging from inadequate environmental control to inherent disadvantages of the Amicon hollow fiber membranes.

The sensitivity of the system to very small trans membrane pressure gradients was a discovery which could be attributed to the environmental support system. Stable influent flows appear to contribute greatly to biomass retention within the fibers and the Amicon HFM cartridges appear to be more sensitive than the In-house Fabricated HFM reactor. Inconsistent flows within HFM reactors appeared to not only disrupt biomass retention but contribute to pollution of the shell and lumen streams with glucose and oxygen, respectively. The resulting conclusions are that the flows entering and leaving the reactor system must be very tightly controlled in order to detect small changes in the volumetric flow rate of each stream. The In-house Fabricated HFM (IHF-HFM) reactor was less susceptible to flow variances and consequently retained more biomass within the HFM. Construction of the IHF-HFM reactor with baffles to space the fibers apart improved biomass retention in experiments 3 and 4.

Closing of mass balances around each reactor provided information on the glucose consumption rate of each reactor system. Figure 56 shows a comparison between the average glucose consumption rate and the total protein recovered from each reactor at the termination of each experiment. Experiments 1 and 2 appear to consume glucose at a rate similar to experiments 3 and 4 but contain much less biomass. In fact, experiment 2 exhibited the fastest rate of glucose consumption but contained the lowest quantity of recoverable protein. This leads to the conclusion that the biomass in experiment 2 is growing and escaping from the HFM reactor. This conclusion is supported by the previous conclusion that inconsistent shell and lumen flows caused trans membrane convective flows which could have washed cells from the fibers.

Evaluation of cell growth was made by comparing the average ratio of recovered RNA (mg/2 cm fiber) per recovered protein (mg/2 cm fiber) to the total recovered protein, Figure 57, and to the average consumption rate of glucose (mg/hr), Figure 58, for each experiment. The conclusion that experiment 2 had growing cells retained within its fibers is supported by the smallest quantity of biomass possessing the highest RNA / protein ratio. Figure 57, leads to the conclusion that the growth of immobilized cells within a HFM reactor could be dependent upon the amount of biomass present. As biomass density increases the growth rate of the biomass decreases. Figure 58, shows that the highest RNA /protein ratio is associated with the highest glucose consumption rate as

Figure 56
Glucose Consumption Rate and Total Recoverable Protein
Comparison for Experiments 1, 2, 3, 4

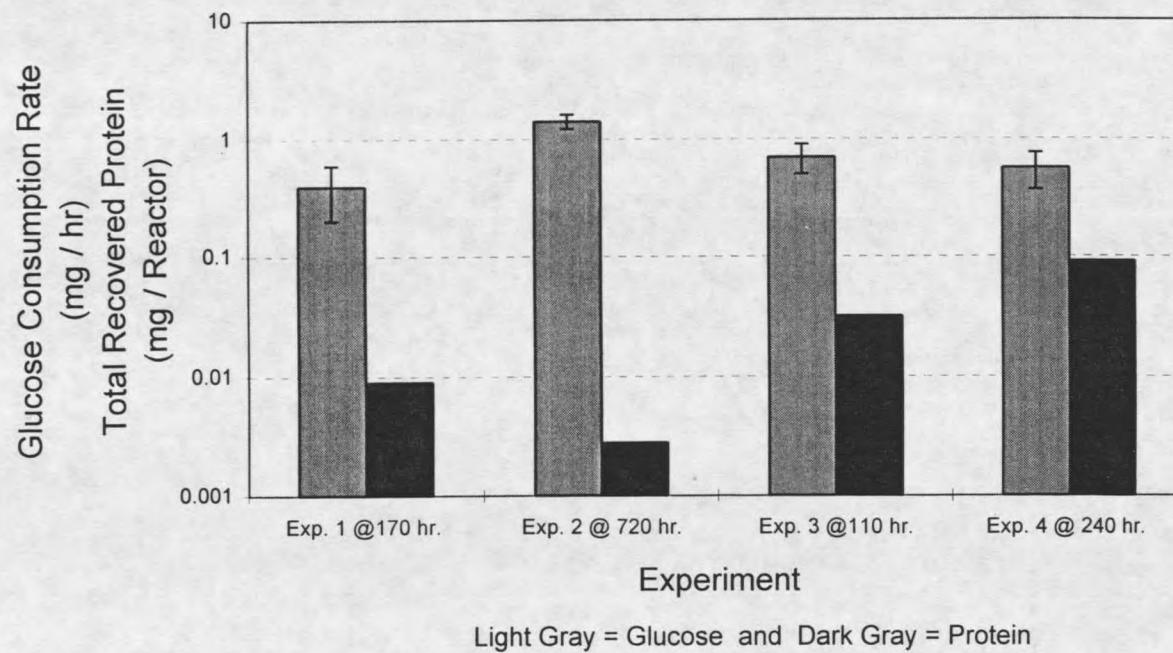


Figure 57
RNA / Protein Ratio and Total Protein Comparison
Experiments 1, 2, 3, 4

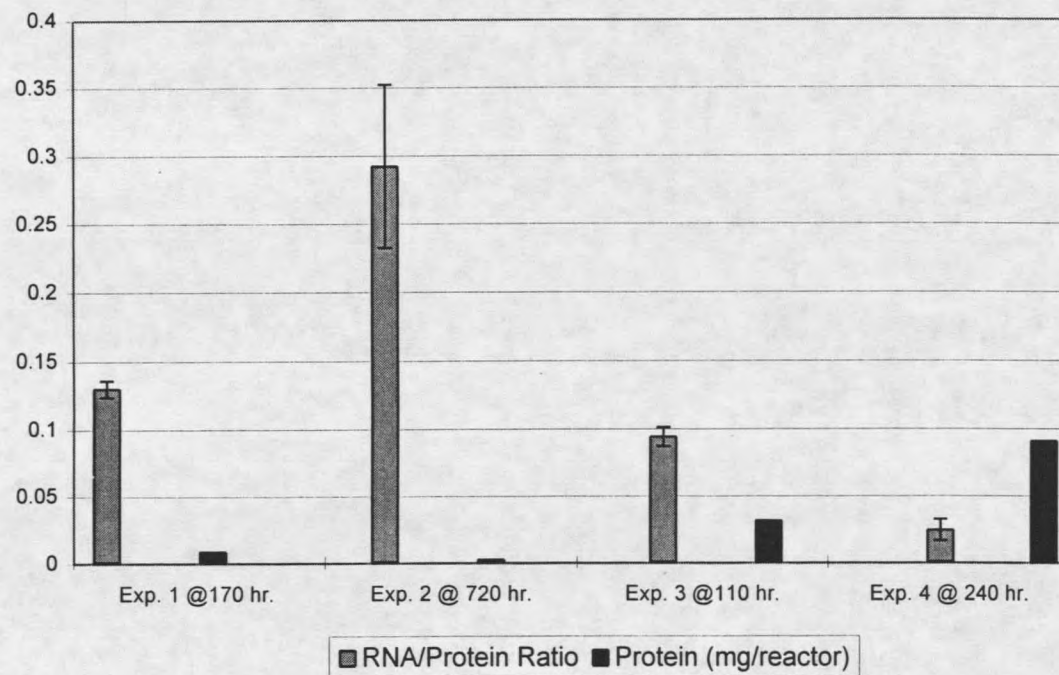
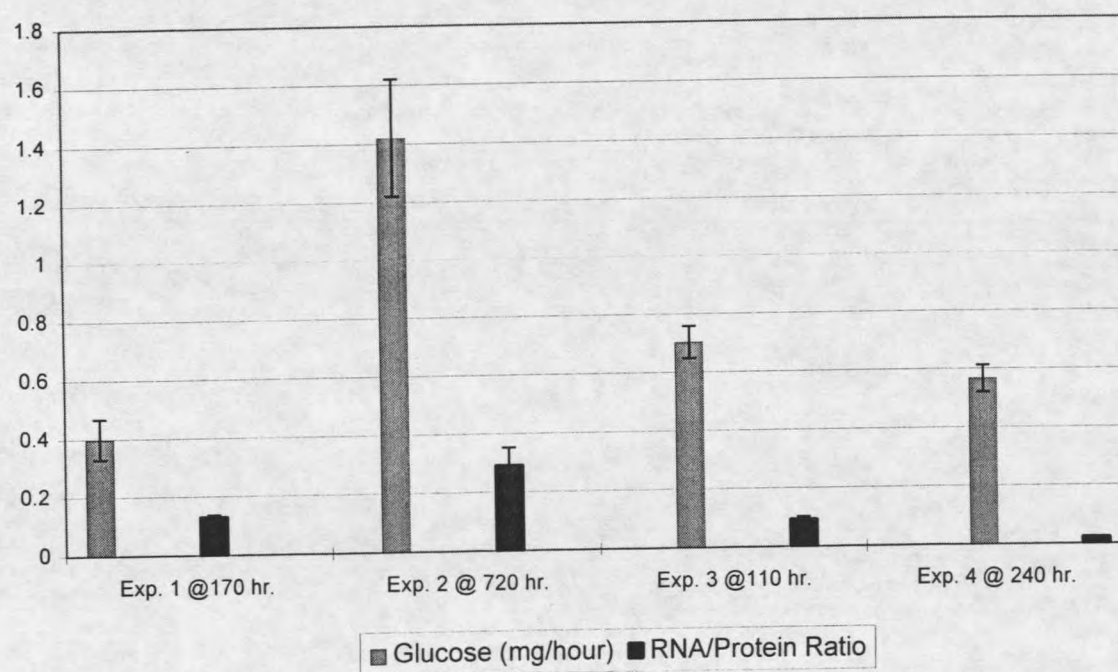


Figure 58
Glucose Consumption Rate and RNA / Protein Ratio Comparison
Experiments 1, 2, 3, 4



expected. But, the lowest glucose consumption rate is not associated with the lowest RNA / protein ratio. This shows that a percentage of the biomass immobilized within each HFM is growing, but as the biomass accumulates increases the fraction of the biomass which is growing decreases. Therefore the biomass appears not to have uniform activity.

The goal of this research was to examine growth patterns of *Ps. putida* immobilized within a multi element hollow fiber membrane (HFM) bioreactor. This was accomplished by construction of 2 multi element reactor systems and development of inoculation, cultivation and fiber harvesting methods. The discovery that the reactor systems were very sensitive to flow variances proved valuable. The second objective of this research was to obtain glucose, oxygen, DNA, RNA and protein profiles which were used to map biomass accumulation within the reactor. Polysulfone was found to interfere with phenol extraction and purification of DNA which was not anticipated initially. Biomass mapping revealed that the majority of biomass accumulated at the reactor influent. Evaluation of bioreactor performance revealed lower than expected substrate consumption rates and biomass retention. Examination of biomass development within the HFM bioreactor showed a dependence of growth on the quantity of biomass retained in the reactor. Although much work remains to refine a multi element HFM bioreactor the potential of this system is tremendous for applications to a wide spectrum of biocatalytic systems.

CHAPTER 6

Future Work

A new design of reactor is recommended. The use of a uniform porous support matrix hollow fiber is recommended for delivery and recovery, convective contaminate transport, of a polluted waste stream. A configuration similar to a baffled shell and tube heat exchanger should be used with influent solution provided to the reactor through 1/4 of the fibers in the reactor and removed through the other fibers. Oxygen should be supplied to the reactor through a hydrophobic membrane with a free form surface biofilm on a support material seeded between the hollow fibers of the reactor for cell cultivation. Substrate analysis should be made using the most sensitive measurement device available (i.e., HPLC or GC). The application of hollow fiber membrane technology is a viable alternative to the standard methods for cell cultivation. Although a great deal more work is required to develop a basic criteria for membrane selection and analytical methods for evaluation of reactor performance.

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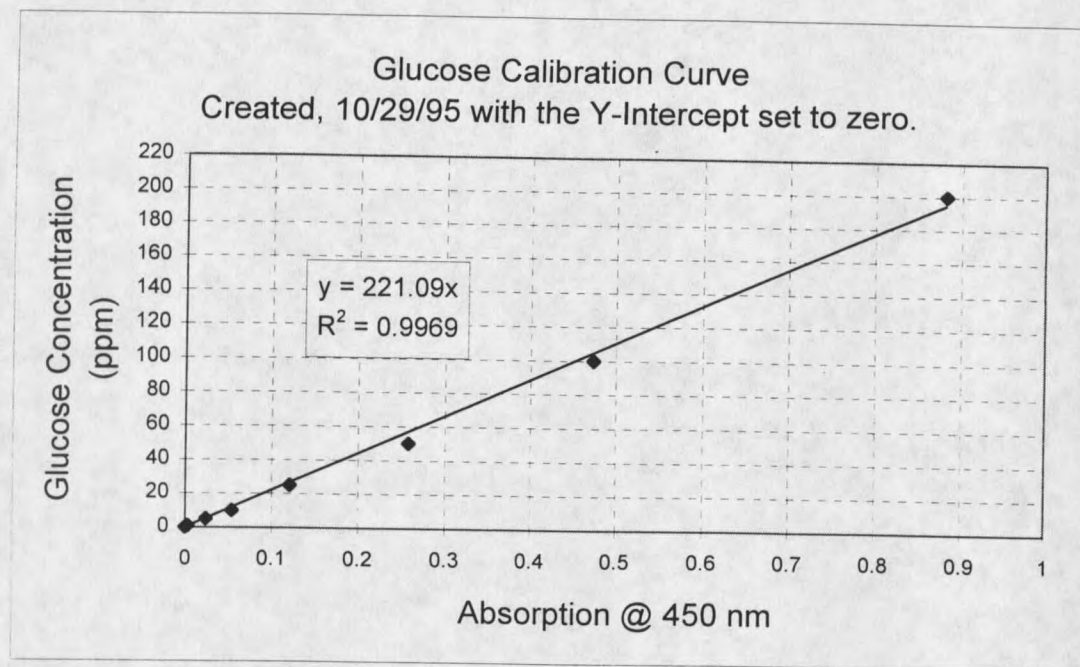
APPENDICES

APPENDIX A

GLUCOSE CALIBRATION CURVES AND ASSAY DATA

Glucose Data

The same glucose calibration curve was used for all four experiments.



Calibration Curve - Data

Retest calibration curves, 10/29/95

These calibration curves used nutrient medium in the place of distilled water

ppm	curve 1	curve 2
0	0.007	0.009
1	0.014	0.012
5	0.033	0.033
10	0.057	0.063
25	0.13	0.13
50	0.243	0.267
100	0.482	0.483
200	0.925	0.891

Recalculation of experiment 1 glucose concentration and material balance
Data from glucose assay 12/22/94

ABS			
Time (hrs)	LI	LO	SO
1	0.131	0.113	0.0277
24	0.135	0.118	0.014
48	0.134	0.115	0.012
72	0.137	0.115	0.012
96	0.139	0.125	0.012
120	0.14	0.122	0.012
144	0.138	0.127	0.012
168	0.126	0.084	0.01

Recalculation of experiment 2 glucose concentration and material balance

Assay Results

Samples taken on dates 3/4 and 3/9 were not able to be retested - small volume

ABS				
Time (hrs)	LI	LO	SO	LI
1	0.12	0.114	0.003	25.0856
48	0.121	0.063	0.002	25.3031
72	0.144	0.132	0.003	30.3056
96	0.143	0.136	0.005	30.0881
120	0.121	0.071	0.001	25.3031
168	0.126	0.069	0.002	26.3906
192	0.132	0.065	0.004	27.6956
216	0.131	0.084	0.004	27.4781
240	0.151	0.107	0.005	31.8281
264	0.132	0.066	0.003	27.6956
288	0.147	0.079	0.003	30.9581
312	0.132	0.068	0.003	27.6956
336	0.118	0.081	0.003	24.6506
360	0.131		0.022	27.4781
384	0.114		0.028	23.7806
408	0.118	0.076	0.005	24.6506
432	0.118	0.073	0.004	24.6506
456	0.123	0.077	0.004	25.7381
480	0.127	0.084	0.004	26.6081
504	0.124	0.082	0.004	25.9556
528	0.135	0.094	0.007	28.3481
552	0.133	0.098	0.009	27.9131
576	0.133	0.113	0.008	27.9131
600	0.131	0.085	0.009	27.4781
624	0.136	0.092	0.004	28.5656
648	0.132	0.072	0.003	27.6956
672	0.134	0.072	0.003	28.1306
696	0.143	0.06	0.004	30.0881
720	0.134	0.065	0.004	28.1306

Recalculation of experiment 3 glucose concentration and material balance
Glucose Analysis 8/1/95

A B S O R B A N C E @ 450 nm						
Hours	Sample #	SO	SI	LO	LI	
0	1	0.011	0.01	0.01	0.229	
12	2	0.008	0.009	0.008	0.207	
24.5	3	0.009	0.008	0.007	0.224	
36	4	0.007	0.011	0.009	0.229	
48.5	5	0.007	0.006	0.007	0.224	
61	6	0.007	0.007	0.007	0.226	
73	7	0.009	0.007	0.007	0.225	
84.5	8	0.007	0.007	0.006	0.227	
96.5	9	0.009	0.008	0.007	0.221	
108.5	10	0.008	0.01	0.009	0.23	

Recalculation of experiment 4 glucose concentration and material balance
Glucose Analysis 9/12/95

ABS @ 450nm

Hours	Sample #	SO	SI	LO	LI	
0	1	0.007	0.005	0.025	0.21	
12	2	0.007	0.006	0.015	0.199	
24	3	0.007	0.006	0.009	0.235	
36	4	0.008	0.008	0.008	0.234	
48	5	0.009	0.006	0.01	0.242	
60	6	0.008	0.007	0.007	0.237	
72	7	0.009	0.008	0.007	0.238	
84	8	0.008	0.007	0.008	0.238	
96	9	0.007	0.005	0.007	0.241	
108	10	0.006	0.005	0.007	0.248	
120	11	0.008	0.008	0.011	0.256	
132	12	0.012	0.01	0.01	0.21	
144	13	0.011	0.009	0.007	0.239	
156	14	0.009	0.008	0.008	0.245	
168	15	0.009	0.008	0.009	0.244	
180	16	0.008	0.007	0.009	0.24	
192	17	0.009	0.008	0.01	0.253	
204	18	0.009	0.008	0.008	0.246	
216	19	0.01	0.008	0.009	0.245	
228	20	0.009	0.008	0.009	0.246	
240	21	0.012	0.01	0.009	0.245	

APPENDIX B

PROTEIN CALIBRATION CURVES AND ASSAY DATA

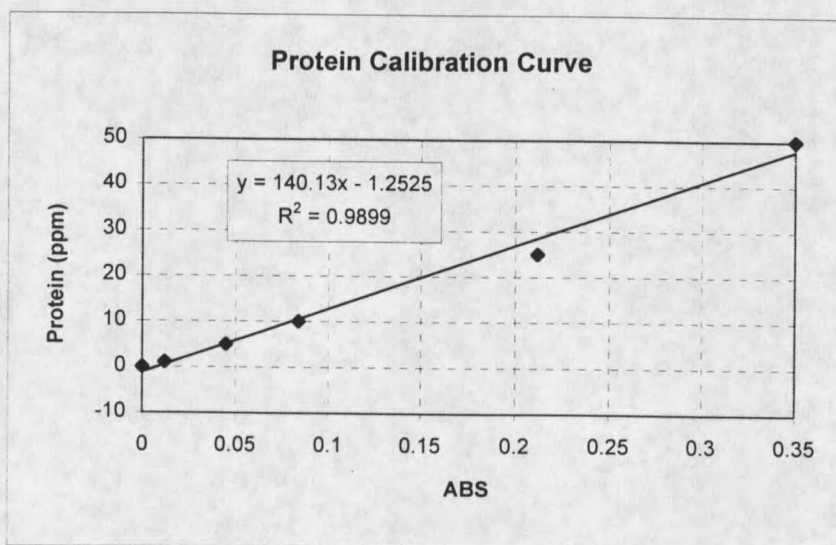
Protein data - experiment 1

Prefab HF Reactor #1

Protein Profile, Amicon HFM Cartridge, 12/4/94

Test was made using the frozen core from reactor #1

Sample #	1cm Sections Per sample	ABS Protein	Protein Standard Curve 7/1/95		
1	51	0.368	Standard Solution		
2	51	0.346			
3	52	0.257			
4	51	0.359			
5	52	0.435			
6	52	0.416			
7	53	0.384			
8	51	0.309	ABS	C-ABS	ppm
9	50	0.365	0.029	0	0
10	52	0.311	0.041	0.012	1
11	38	0.246	0.074	0.045	5
12	47	0.221	0.113	0.084	10
13	36	0.231	0.241	0.212	25
14	30	0.167	0.379	0.35	50
15	45	0.167			



Protein Data - experiment 2

Protein assay results Amicon HFM Cartridge 4/4/95

Protein Assay Results These are 2 cm fiber samples pH adjusted to 6.5

Sample #	Region of HFMR	Extraction #1	Ave ABS	
1	outlet	0.038	0.04	0.039
2		0.04	0.041	0.0405
3		0.045	0.047	0.046
4		0.054	0.051	0.0525
5		0.049	0.054	0.0515
6		0.066	0.08	0.073
7	inlet	0.066	0.065	0.0655

Extraction #2			Ave ABS	Extraction #3			Ave ABS
0.02	0.02	0.02		0.02	0.021	0.0205	
0.021	0.025	0.023		0.02	0.019	0.0195	
0.026	0.025	0.0255		0.02	0.019	0.0195	
0.027	0.027	0.027		0.02	0.02	0.02	
0.026	0.026	0.026		0.02	0.02	0.02	
0.029	0.032	0.0305		0.019		0.019	
0.033	0.035	0.034		0.021	0.019	0.02	

Calibration Curve 7/1/95 was used for analysis of this data.

Linear fit equation: Protein (ppm) = 140.13*(ABS @ 750 nm) - 1.2525

pH adjustment of the samples required a dilution factor of $((0.5+0.685)/0.5)=2.37$
25 fibers per sample

Protein data - experiment 3

Protein Analysis- 8/1/95

Protein Standard Curve

ABS @ 750nm

ABS	ppm
0.029	0
0.041	1
0.074	5
0.113	10
0.241	25
0.379	50

Protein membrane analysis

ABS @ 750nm

Sample #	D0	D10
1	0.645	0.135
2	0.701	0.146
3	0.575	0.136
4	0.773	0.113
5	0.557	0.156
6	0.807	0.168
7	0.7	0.144
8	0.992	0.157
9	0.758	0.233

Protein Data - Experiment 4

Protein Calibration Curve - 9/13/95 experiment 4

ABS @ 750nm		ABS @ 750nm 9/14/95	
ABS	ppm	ABS	ppm
0.023	0	0	0
0.035	1	0.012	1
0.054	5	0.03	5
0.078	10	0.059	10
0.141	25	0.115	25
0.233	50	0.239	50

Protein Samples: D10

Sample # ABS		S1 Sample# ABS	
1	0.363	1	0.227
2	0.083	2	0.17
3	0.176	3	0.207
4	0.073	4	0.111
5	0.203	5	0.115
6	0.065	6	0.083
7	0.113	7	0.08
8	0.07	8	0.071
9	0.102	9	0.07
10	0.078	10	0.09

Retest - Protein Samples 9/16/95

Corrected			
Sample #	ABS D10	abs D10	Axial Distance
1	0.177	0.139	20
2	0.168	0.13	18
3	0.176	0.138	16
4	0.191	0.153	14
5	0.218	0.18	12
6	0.292	0.254	10
7	0.307	0.269	8
8	0.428	0.39	6
9	0.427	0.389	4
10	0.545	0.507	2

APPENDIX C
DNA ASSAY DATA

Experiment 1

DNA Concentrations Amicon HFM Cartridge, 12/4/95

Sample # ABS-DNA, Each sample measured three times.

	280 NM		average	
1	0.417	0.417	0.415	0.416333
2	0.36	0.361	0.361	0.360667
3	0.701	0.698	0.692	0.697
4	0.116	0.115	0.116	0.115667
5	0.397	0.397	0.397	0.397
6	0.318	0.317	0.317	0.317333
7	0.518	0.519	0.518	0.518333
8	0.348	0.348	0.347	0.347667
9	0.614	0.612	0.612	0.612667
10	0.311	0.311	0.312	0.311333
11	0.569	0.569	0.57	0.569333
12	0.087	0.088	0.088	0.087667
13	0.182	0.182	0.181	0.181667
14	0.302	0.301	0.301	0.301333
15	0.368	0.369	0.369	0.368667

	260 NM		average	
	0.263	0.263	0.263	0.263
	0.226	0.227	0.227	0.226667
	0.374	0.372	0.37	0.372
	0.114	0.113	0.114	0.113667
	0.268	0.269	0.269	0.268667
	0.25	0.25	0.251	0.250333
	0.311	0.312	0.312	0.311667
	0.234	0.234	0.234	0.234
	0.349	0.349	0.35	0.349333
	0.202	0.203	0.204	0.203
	0.359	0.362	0.362	0.361
	0.07	0.071	0.071	0.070667
	0.11	0.11	0.11	0.11
	0.177	0.177	0.177	0.177
	0.219	0.219	0.22	0.219333

Experiment 2 - DNA Analysis - 4/6/95

DNA ABS @ 280	Axial Posit		ABS @ 280
Inlet	1	2	0.033
	2	4	0.027
	3	6	0.013
	4	8	0.011
	5	10	0.02
	6	12	0.005
Outlet	7	14	0.011

Experiment 3

DNA- 10/4/95

Sample #	ABS @ 260nm	ABS @ 280nm	Ratio
1	0.225	0.134	1.678
2	0.243	0.141	1.721
3	0.26	0.149	1.743
4	0.337	0.218	1.547
5	0.497	0.291	1.706
6	0.314	0.182	1.728
7	0.406	0.26	1.564
8	0.39	0.225	1.734
9	0.407	0.228	1.786
10	0.425	0.244	1.743

Experiment 4

DNA analysis 9/15/95

Sample #	ABS@260	ABS@280	Ratio
s1	0.466	0.293	1.593
s1	0.444	0.253	1.755
s2	0.31	0.179	1.729
s2	0.34	0.213	1.591
s3	0.383	0.288	1.329
s3	0.426	0.348	1.224
s4	0.297	0.209	1.425
s4	0.373	0.364	1.025
s5	0.228	0.2	1.14
s5	0.278	0.253	1.1
s6	0.137	0.099	1.387
s6	0.167	0.124	1.338
s7	0.152	0.12	1.263
s7	0.175	0.158	1.108
s8	0.173	0.181	0.953
s8	0.191	0.238	0.801
s9	0.209	0.288	0.727
s9	0.146	0.136	1.074
s10	0.175	0.194	0.9
s10	0.169	0.152	1.108

APPENDIX D
RNA ASSAY DATA

Experiment 1

RNA from Amicon HFM Cartridge, 12/4/94

Sample	260 NM		average	
1	0.022	0.022	0.022	0.022
2	0.031	0.031	0.031	0.031
3	0.024	0.023	0.022	0.023
4	0.027	0.027	0.027	0.027
5	0.033	0.034	0.033	0.033333
6	0.043	0.042	0.043	0.042667
7	0.032	0.033	0.033	0.032667
8	0.031	0.031	0.032	0.031333
9	0.03	0.03	0.031	0.030333
10	0.028	0.027	0.027	0.027333
11	0.026	0.026	0.026	0.026
12	0.024	0.022	0.023	0.023
13	0.022	0.021	0.021	0.021333
14	0.019	0.018	0.018	0.018333
15	0.018	0.018	0.018	0.018

280 NM		average		Ratio 260/280
0.012	0.012	0.012	0.012	1.833333
0.02	0.019	0.02	0.019667	1.576271
0.014	0.012	0.012	0.012667	1.815789
0.017	0.016	0.016	0.016333	1.653061
0.02	0.02	0.02	0.02	1.666667
0.028	0.028	0.028	0.028	1.52381
0.018	0.018	0.019	0.018333	1.781818
0.018	0.018	0.019	0.018333	1.709091
0.016	0.015	0.016	0.015667	1.93617
0.016	0.015	0.015	0.015333	1.782609
0.013	0.013	0.013	0.013	2
0.015	0.013	0.013	0.013667	1.682927
0.012	0.012	0.012	0.012	1.777778
0.012	0.012	0.012	0.012	1.527778
0.01	0.01	0.011	0.010333	1.741935

Experiment 2
HFMR #2 - 4/6/95

Sample #	Data: EXTR. #1		EXTR. #2	
	ABS @ 260	ratio 260/280	ABS @260	ratio 260/280
1	0.018 **	1.667	0.017 **	2
1	0.011	2.885	0.017	2.38
2	0.009	3.647	0	
2	0.015 **	3.194	0.009 **	3
3	0.035 **	1.791	0 **	
3	0.018	2.727	0	
4	0.02 **	1.787	0 **	
4	0.009	4.357	0	
5	0.02	1.971	0.017 **	1.883
5	0.026 **	1.978	0	
6	0.061 **	2.2237	0 **	
6	0.029	2.463	0	
7	0.019	3.146	0.021 **	1.959
7	0.069 **	2.075	0.012	3.417

EXTR. #3	
ABS @ 260	Ratio 260/280
0 **	
0	
0 **	
0	
0.028 **	1.703
0.025	1.421
0	
0.01 **	4.333
0	
0.009 **	3.389
0	
0.014 **	2.293
0 **	
0	

** The highest value obtained was used for the analysis as long as the ratio of 260/280 was above 1.5.

Experiment 3
RNA analysis- 8/1/95

Sample #	ABS @ 280nm	ABS @ 260nm
1	0.091	0.178
2	0.069	0.128
3	0.15	0.289
4	0.133	0.263
5	0.15	0.291
6	0.12	0.237
7	0.149	0.3
8	0.143	0.278
9	0.122	0.239
10	0.144	0.225
11	0.163	0.298
12	0.134	0.263
13	0.189	0.353
14	0.149	0.297
15	0.194	0.384
16	0.148	0.289
17	0.202	0.395
18	0.2	0.397
19	0.202	0.395
20	0.2	0.397

Experiment 4
RNA analysis 9/15/95

Sample #	Hour	ABS@280	ABS@260	Ratio
s1		0.137	0.219	0.624
s1		0.127	0.193	0.657
s2		0.083	0.151	0.551
s2		0.095	0.151	0.629
s3		0.124	0.21	0.591
s3		0.132	0.229	0.576
s4		0.078	0.134	0.581
s4		0.077	0.13	0.591
s5		0.062	0.105	0.591
s5		0.056	0.101	0.554
s6		0.036	0.062	0.584
s6		0.044	0.075	0.583
s7		0.042	0.071	0.59
s7		0.045	0.071	0.635
s8		0.045	0.071	0.633
s8		0.037	0.059	0.619
s9		0.036	0.058	0.612
s9		0.038	0.063	0.599
s10		0.041	0.072	0.567
s10		0.037	0.059	0.633

APPENDIX E

OXYGEN CONCENTRATION DATA

Experiment 3

Dissolved Oxygen

Time (hr)	LI ppm	SI ppm	SO ppm
0	2.5	3.9	3.1
29	2.3	4.1	3.4
53	2.2	3.9	2.3
77	1.8	3.9	2.3
101	1.7	3.8	1.7

Experiment 4

Oxygen Data, 8/29/95

	Date	Time	Hours	DO	ppm	SO
				LI	SI	
Air	29-Aug	9 pm	0	3	4.1	3.3
	30-Aug	9 am	12	2.8	4.6	3.7
	31-Aug	10:45 AM	37.75	2.5	4.5	3.5
	31-Aug	9:30 PM	48	2.4	4.6	3.6
	1-Sep	10:30 AM	61	2.4	4.5	3.5
	1-Sep	9:00 PM	71.5	2.1	5	3.6
	2-Sep	9am	83.5	2.1	4.6	3.6
	2-Sep	9pm	95.5	2.1	4.8	3.5
	3-Sep	9am	107.5	2.1	4.6	3.3
	3-Sep	9pm	119.5	1.6	4.4	3.4
Pure O2 *	4-Sep	9am	131.5	2	4.4	3.2
	4-Sep	9:00 PM	143.5	2	9.3	7.2
	* 5-Sep	9am	155.5	2.3	9.7	8.5
	* 5-Sep	9pm	167.5	2.2	9.3	8.5
Air	6-Sep	9am	179.5	2	6.1	4.7
	6-Sep	9pm	191.5	2.2	5	4.4
	7-Sep	9am	203.5	2.2	4.3	3.8
	7-Sep	9pm	215.5	1.5	4.9	3.8
	8-Sep	9am	227.5	1.4	4.9	3.5
	8-Sep	9pm	239.5	1.7	4.9	3.9

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