FACTORS AFFECTING THE ADHESION OF *PHANEROCHAETE CHRYSOSPORIUM* TO SURFACES

By

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ABSTRACT

FACTORS AFFECTING THE ADHESION OF *PHANEROCHAETE CHRYSOSPORIUM* TO SURFACES

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The white-rot fungus *Phanerochaete chrysosporium* naturally adheres to many surfaces and forms a biofilm. In this work, many factors were found to influence the irreversible attachment of these cells to surfaces including characteristics of the substratum, physiological activity of the cells, and non-specific physicochemical interactions between the cells and surface. This fungus has the ability to degrade a wide variety of environmental pollutants by the production of extracellular peroxidases. A better understanding of the adhesion process will facilitate the design of immobilized bioreactors for decontamination of soil and water and other applications.

To better understand the role of cellular physiological activity during adhesion, mycelial cells were exposed to various chemical treatments. Adhesion to glass microscope slides was determined using laser scanning microscopy (LSM) to measure the percentage of surface area covered with cells. Results showed that cells had to be viable for adhesion to occur. Protein synthesis, the presence of extracellular proteins and polysaccharides, and a functioning cell wall were also required for adhesion. These results indicate that both metabolic activity and the integrity of the cell surface are important for adhesion.

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In addition, the adhesion of *P. chrysosporium* to various roughened polymers was correlated to theoretical predictions of adhesion based on thermodynamic considerations. The fungus was cultured in agitated flasks containing a polymer coupon. The coupons were made of various polymers and represented a range of surface free energies. Adhesion and growth of the fungus on the polymer coupons were measured by biomass dry weight. The surface energy approach was used to calculate free energy of adhesion. Surface free energies of the cells and polymer surfaces were calculated using an empirical equation of state. Adhesion of this organism is enhanced on materials with low surface free energy and high roughness values.

Few methods are available to easily quantify adhesion of filamentous organisms to solid substrates. Traditional methods of measuring biomass of single-celled organisms are not applicable. The use of LSM in this work demonstrated that it is a fast, convenient method to quantify microbial adhesion to surfaces and to characterize cell and substratum surfaces.

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NOTATION

ΔF_{adh}	interfacial free energy of adhesion
R_a	average roughness (μm) (See Equations 3.4 and 5.2)
R_z	average roughness (μm) (See Eq. 5.3)
l_m	length of measurement for surface roughness determination (μm)

Greek Symbols

β	constant used in the equation of state; value is 0.0001247
γ_L	surface free energy of liquid (dynes/cm)
γ_S	surface free energy of solid (dynes/cm)
γ_C	surface free energy of cell (dynes/cm)
γ_{CS}	cell-substrate interfacial free energy (dynes/cm)
γ_{SL}	substrate-liquid interfacial free energy (dynes/cm)
γ_{CL}	cell-liquid interfacial free energy (dynes/cm)
γ^d	nonpolar component of surface free energy (dynes/cm)
γ^p	polar component of surface free energy (dynes/cm)
θ	contact angle (degrees)
π	equilibrium spreading pressure (decrease in surface tension due to vapor adsorption on surface)

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CHAPTER 1.

INTRODUCTION

The process of microbial adhesion is a complex phenomenon that is not yet fully understood. Interest in microbial adhesion spans an array of detrimental phenomena including marine biofouling, oral pathogenesis, contamination of food preparation surfaces, human implants, and cancer cell growth (Mafu *et al.*, 1990; Rogers *et al.*, 1984; Dexter *et al.*, 1975). Beneficial adhesion and biofilm growth occurs in natural environments as microorganisms decontaminate lakes and rivers. Immobilized biofilms are used in industrial and pharmaceutical applications for the production of chemicals. Adhesion characteristics are unique for each organism and differ even between strains (Loosdrecht *et al*, 1987). Most adhesion studies have focused on plant cells, single-celled bacteria, or yeast with little attention given to filamentous organisms. This work provides a unique approach to the study of adhesion of the filamentous fungus *Phanerochaete chrysosporium* to surfaces in the context of immobilization and bioreactor design which can be extended to other filamentous organisms.

P. chrysosporium is a naturally occurring fungus that mineralizes lignin and a diverse array of toxic environmental pollutants including polychlorinated biphenyls (PCBs), polychlorinated dioxins, alkylhalide

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insecticides, aromatic hydrocarbons, nitroaromatics, and Kraft bleach plant effluents (Boominathan and Reddy, 1992). Some of these compounds are degraded under ligninolytic conditions (i.e. carbon, nitrogen, or sulfur limitation) and require the production of two families of extracellular peroxidases, lignin peroxidase and manganese peroxidase. Other compounds are degraded during nonligninolytic conditions when no peroxidases are produced (Yadav *et al.*, 1993). No other single organism has demonstrated the ability to degrade such a wide array of compounds. For this reason, *P. chrysosporium* has potential as an industrially important immobilized biocatalyst system for the decontamination of soil and water and other applications.

A critical step in the design of a large-scale reactor system for this organism is to immobilize and grow the fungus on a support matrix and prevent undesirable attachment on surfaces such as piping and instrumentation. Immobilization can offer several benefits over suspension cultures including maintaining a viable culture with higher cell concentration and productivity over extended time periods, and a more gentle hydrodynamic environment for the cells. Research in recent years has provided an understanding of the metabolism and physiology of *P. chrysosporium* and its lignin degradation system by using small-volume agitated cultures in which the fungus adopts a free pellet morphology. Alternative reactor systems such as stirred tank reactors, air-lift fermentors, and various types of immobilized film reactors have been explored only to a

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small extent as a means of increasing enzyme production. There are almost no studies which have specifically examined the factors involved in the adhesion of *P. chrysosporium* to surfaces.

The white-rot fungus *P. chrysosporium* naturally adheres to many surfaces and forms a biofilm. Many factors influence the attachment of these cells to a surface including characteristics of the substratum, physiological activity of the cells, and non-specific physiochemical interactions between the cells and the surface. In this work, many factors were evaluated using surface science techniques to determine which ones have the largest effect on the adhesion of *P. chrysosporium* to surfaces and to better understand the process of irreversible fungal adhesion.

The effect on adhesion of various physiological factors of the cells was studied by exposing cells to various treatments to alter the cell surface or metabolism. Several factors significantly affected adhesion including metabolic activity, a functioning cell wall, and protein and polysaccharide synthesis. Properties of the substratum surface, including roughness and surface energy, also had an important role in adhesion. The trend of adhesion on polymer supports predicted from thermodynamic considerations using a surface energy approach correlated to experimental measurements. Laser scanning microscopy was an important tool for measuring fungal biomass adhesion and characterizing the cell and polymer surfaces.

This dissertation is presented in six chapters including a literature review and objectives of the research in Chapter 2 and Materials and

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Methods in Chapter 3. The next three chapters describe results in the format of research papers including the physiological factors of adhesion (Chapter 4), the thermodynamic aspects of adhesion (Chapter 5), and the use of laser scanning microscopy in adhesion studies of *P. chrysosporium* (Chapter 6). Finally, overall conclusions and direction for further research are found in Chapter 7 followed by supporting information in the appendices.

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CHAPTER 2.

BACKGROUND

2.1. The Biocatalytic System of Phanerochaete Chrysosporium

2.1.1 Introduction

The organism used in this work, *Phanerochaete chrysosporium*, is a member of the white-rot basidiomycetes which are able to degrade lignin to carbon dioxide and water more rapidly and extensively than any other known organism (Kirk and Farrell, 1987). Lignin forms a matrix surrounding the other major components of wood, cellulose and hemicellulose, which limits the rate of depolymerization of wood by other organisms (Leisola and Fiechter, 1985). The degradation of lignin by white-rot fungi opens up woody substrates to further decomposition by other organisms. Potentially important industrial applications exist for *P. chrysosporium* since the mechanism it uses to degrade lignin also allows it to degrade toxic environmental pollutants.

The ligninolytic system of the fungus is composed of a family of extracellular enzymes including lignin peroxidases (ligninases) (Odier and Delattre, 1990; Tien and Kirk, 1988) and manganese peroxidases (Paszczynski *et al*, 1988, Gold and Glenn, 1988). *P. chrysosporium* is the most widely studied of the white-rot basidiomycetes because it is an efficient lignin degrader, prolifically produces conidia, and can be successfully cultured in laboratory conditions (Boominathan and Reddy,

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1992). Several reviews provide background about the lignin biopolymer, *P. chrysosporium*, and potential applications of biocatalytic systems for lignin degradation (Gold and Alic, 1993; Buswell and Odier, 1987; Kirk and Farrell, 1987; Leisola and Fiechter, 1985; Kirk and Chang, 1981; Crawford and Crawford, 1980; Eriksson, 1978;).

2.1.2 Structure of P. chrysosporium

P. chrysosporium is a member of the class of basidiomycetes which includes a wide assortment of fungi such as edible mushrooms, wood-destroying fungi, puffballs, stinkhorns, smuts, and rusts. Reviews of the lifecycle mating system, and molecular biology of the fungus are given by Gold and Alic (1993), Alic *et al.*, (1987) and Smith (1975). The most commonly used wild-type strains are BKM-F-1767 and ME-446. The primary vegetative structures of the organism are hyphae which are microscopic, filamentous, branched structures. The mass of hyphae is referred to as mycelium. The hyphae are divided into "cells" by perforated cross-walls called septa. The cell wall is a complex structure made of polysaccharides, lipids, and proteins.

Vegetative asexual propagation is carried out by the formation of conidiospores (asexual spores) within conidiophores (hyphae which produce conidia). The conidia are usually heterokaryotic (two or more genetically distinct nuclei) (Gold and Alic, 1993). Sexual propagation occurs by the formation of basidiospores (sexual spores) externally on a structure called a basidium. The strain is maintained in the laboratory through the formation of conidia. Basidiospore production requires special culture conditions.

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2.1.3 Ligninolytic System of P. Chrysosporium

Lignin is a complex, high molecular weight, three-dimensional, aromatic polymer that has no precise chemical structure but contains 12 dominant types of linkages (i.e. C_α-C_α bonds) which are not easily hydrolyzable. Therefore, lignin degradation must occur by an extracellular and relatively nonspecific mechanism. Studies using lignin model compounds showed that the mechanism of lignin oxidation by *P. chrysosporium* involves many nonspecific side-chain cleavage and aromatic ring opening reactions (Gold and Alic, 1993).

The lignin-degrading system (LDS) of *P. chrysosporium* is produced during secondary metabolism (Leisola *et al*, 1983) which occurs as a result of nitrogen, carbon, or sulfur limitation in the medium (Kirk and Farrell, 1987; Jeffries *et al*, 1981). A cosubstrate such as glucose is required for the LDS since lignin is not a substrate for the organism (Keyser, Kirk, and Zeikus, 1978). High oxygen tension increases the titer and activity of the LDS (Reid *et al*, 1985; Bar-Lev and Kirk, 1981). Several inorganic nutrients are required in the culture medium for growth of the organism and LDS expression including a trace element solution containing Mn, Mg, F, Co, Ca, Zn, Cu, Mo, and Al (Tien and Reddy, 1988; Jeffries *et al*, 1981). The fungus is cultured most commonly as mycelial pellets under agitated conditions in medium containing buffer and a surfactant (Asther *et al*, 1987; Jager *et al*, 1985; Kirk *et al*, 1978). Extracellular and cell-bound substances are produced during nitrogen starvation which have been

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identified as a mixture of polysaccharides containing 92% glucose and 8% arabinose (Buswell, 1991). Mucopolysaccharide sheaths have been observed on hyphae that were degrading woody tissue. This glucan may help to preserve a favorable environment for the fungus, regulate extracellular glucose concentration, and serve as a reserve energy source.

The major components of the LDS are the two extracellular families of isoenzymes called lignin peroxidases and manganese peroxidases (Dass and Reddy, 1990; Leisola *et al*, 1987; Gold *et al*, 1984; Kuwahara *et al*, 1984). Ligninases are heme protein peroxidases with molecular weights ranging between 38-43 kDa (Farrell *et al*, 1989). Manganese peroxidase isoenzymes have molecular weights ranging between 45-47 kDa. Both families of enzymes require H_2O_2 for activity (Tien and Kirk, 1984) which is generated primarily by the activity of glucose oxidase (Eriksson *et al*, 1986). Other oxidases may also be present which play a role in the ligninolytic activity. The production of veratryl alcohol during secondary metabolism may act as an inducer and regulator of the lignin degradation system (Tonan and Odier, 1988; Faison and Kirk, 1985). Lignin peroxidase concentrations in the extracellular fluid are low, usually in the range of 300-1000 units per liter. The enzyme system degrades lignin in a nonspecific manner (Ulmer *et al*, 1983) which includes an oxidative cleavage of C_α-C_β linkages in the lignin polymer (Boominathan and Reddy, 1992).

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2.1.4 Applications of the Lignin Degrading System of P. chrysosporium

Several potential applications exist for the LDS of *P. chrysosporium* including the treatment of bleach plant effluents (Mueller, 1974), biopulping and biobleaching (Pellinen *et al*, 1988), the conversion of lignocellulosic materials into feeds, fuels, and chemicals (Zadrazil *et al*, 1981), and the bioremediation of contaminated soil. In addition, white rot fungi are capable of degrading toxic aromatic pollutants such as dichlorophenol, dinitrotoluene, and dichlorodibenzodioxin which have chemical and structural features similar to the lignin polymer (Lin *et al*, 1990; Kennedy *et al*, 1990; Klecka *et al*, 1988). Detoxification and decolorization of waste effluents from pulp and paper mills and other sites hold the greatest promise for future applications (Huynh *et al*, 1985; Sundman *et al*, 1981; Eaton *et al*, 1980).

Various studies have attempted to develop fermentation systems for the production of ligninolytic enzymes. Different types of reactors have been used including batch reactors, stirred tank fermenters (Michel *et al*, 1990), air-lift fermenters (Prouty, 1990), ceramic supports (Cornwall *et al*, 1990) and rotating biological contactors (Pellinen *et al*, 1988). The fungus has been immobilized on agarose beads, polyurethane foam, nylon, and other plastic supports with varying degrees of success of attachment and/or ligninase production (Linko, 1988; Linko and Zhong, 1987-8; Kirkpatrick and Palmer, 1987; Linko *et al*, 1986). A continuous process, MyCoR, has been developed at North Carolina State University to decolorize bleach plant

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effluents using *P. chrysosporium* immobilized in a rotating biological contactor (Campbell, 1983; Yin *et al*, 1989; Smith, 1981). Although all of these efforts represent a good beginning in developing systems for commercial application, considerable work lies ahead. Almost no research exists which describes a systematic study of the immobilization of the fungus to various supports with subsequent enzymatic production.

2.1.5 Objectives of Research

The overall objective of this research was to better understand fungal adhesion to surfaces. The focus was to investigate the relative importance of various factors in the immobilization and growth of *P. chrysosporium* on solid surfaces while maintaining the metabolism and desired level of enzyme expression in the fungus. One part of the work focused on physiochemical interactions between the cell surface and substratum, as well as the effect of various metabolic processes on fungal adhesion. The remaining efforts of the study applied the surface energy approach to determine the importance of short range forces on cell adhesion (Figure 2.1). Although the proposed work focused on *P. chrysosporium*, a general approach was developed to characterize cell-support interactions and resultant cell productivity for other industrially important microorganisms.

Specific objectives of the research were to: (1) identify physicochemical factors that have the greatest effect in either promoting or inhibiting adhesion of the fungus to surfaces, (2) determine if adhesion to various surfaces can be predicted based on a characteristic property of the

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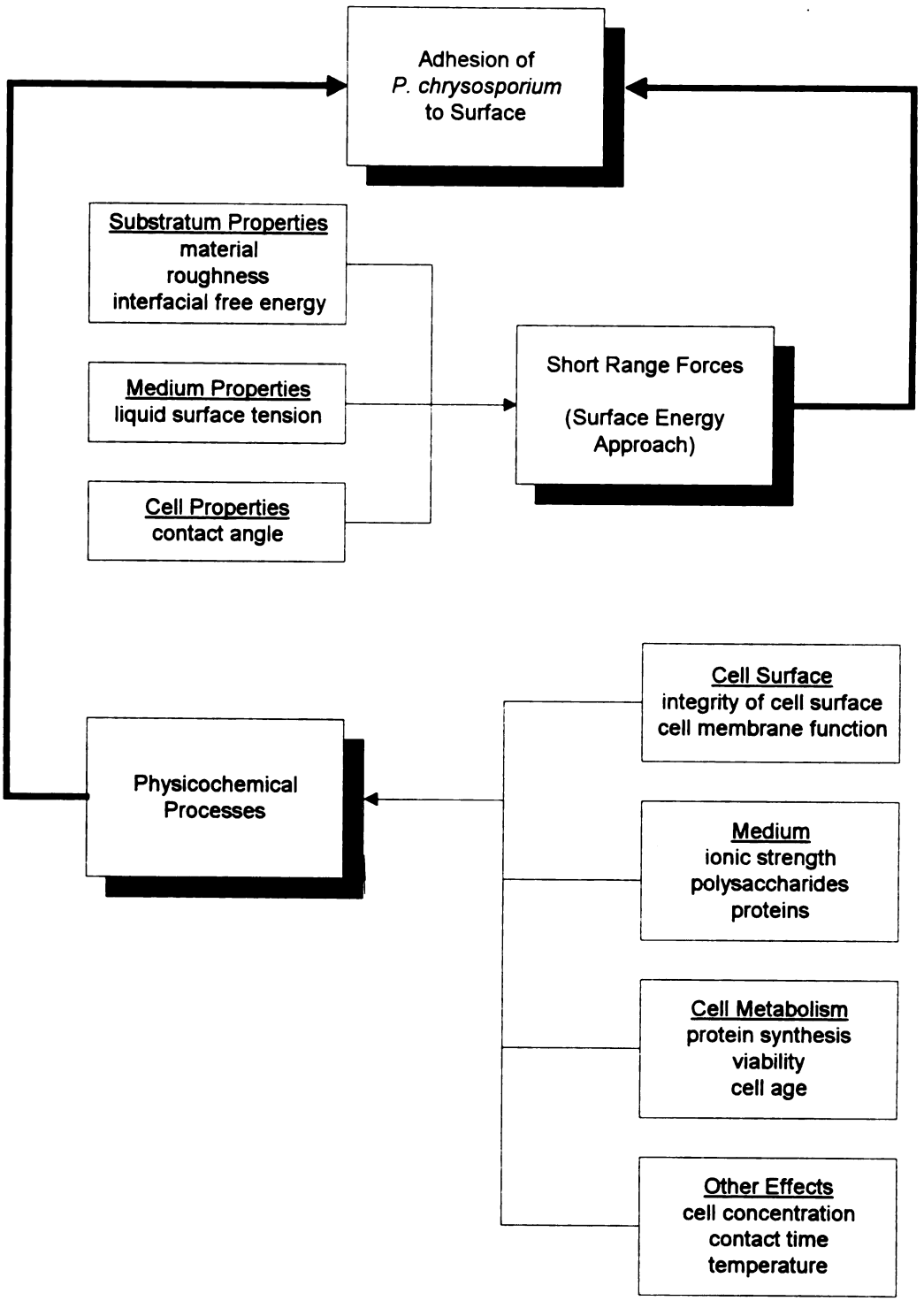

Figure 2.1 Flowchart of approach used in study of *P. chrysosporium* adhesion.

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surface, and (3) determine the role of substratum roughness in promoting cell adhesion. These are described in the following chapters.

2.2. Cell-Surface Interactions

2.2.1 Introduction

Cell adhesion and subsequent biofilm formation on a surface are relevant to a diverse group of phenomena including fouling of marine equipment, cancer cell growth, and microbial immobilization for biocatalytic conversions (Dexter *et al.*, 1975). In the past decade, microbiologists, engineers, and medical researchers have begun a systematic study of biofilm accumulation and activity. The presence of biofilms may be beneficial or detrimental, depending on the situation. Biofilms occurring in lakes, rivers, and oceans remove a significant amount of contaminants from the water. Microbial populations growing in plant root systems aid the plant in obtaining nutrients. Biofilms are also useful in sewage systems to remove pollutants, and in fermentation and pharmaceutical applications. In contrast, cell attachment to tissues such as the oral cavity or prosthetic devices is often a prelude to pathogenesis (Gerson and Scheer, 1980; Marchant, 1986; Rogers *et al.*, 1984). Biofilm accumulation on equipment reduces its effectiveness and can accelerate deterioration of the surface.

Cells may attach to a surface by processes which involve physiological activity of the microorganism such as attachment by organelles, production of extracellular substances that serve as adhesins, or by growth and division on the support. Attachment of microorganisms may also be attributed to nonspecific physicochemical interactions involving

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"long range" electrostatic or "short range" hydrophobic interactions between the surface of the cell and the substratum. These concepts are effectively reviewed by Marshall (1986) and Little *et al* (1986).

2.2.2 Bacterial Cell Communication

At one time, microbial biofilms were considered to be composed of an accumulation of individual cells which functioned independently of the surrounding community. In recent years, a more sophisticated picture of cell populations has emerged in which individual members of a cell population communicate with other members, plant cells, or animal cells using chemical signals. Examples of the diverse and sometimes remarkable results of these chemical discussions include gene activation, the assembly of multicellular structures, the activation of organ development of a higher organism, and simultaneous developmental changes in both a higher organism and the bacterial population (Losick and Kaiser, 1997).

Communication between cells of the marine bacteria *Vibrio fischeri* activates genes responsible for luminescence only when the cell population reaches a high density. In addition, the concentration of *V. fischeri* cells within the light organ of hatchling squid activates the full maturation of the squid's light organ. In another example, chemical signaling in myxobacteria during periods of starvation triggers a dramatic alteration in the structure and activity of the cells. The cells differentiate themselves and assemble complex multicellular structures called fruiting bodies (i.e. packages of spores). A final example of cell communication is the exchange between the nitrogen-fixing bacteria *Rhizobium* and legume plants which results in the simultaneous regulation of developmental changes in both organisms. The

legume roots form nodules to house the bacteria. Free-living *Rhizobium* cells produce the machinery to become nitrogen-fixing microbes after colonizing the nodules.

Biofilms develop different morphological and physiological structures than free-living individuals (Davies *et al*, 1998). Bacterial cells in biofilms are embedded within an extracellular polysaccharide (EPS) matrix which binds the biofilm structure together. In biofilms of *Pseudomonas aeruginosa*, the EPS matrix forms pillar-like structures which are separated by spaces filled with water. These biofilms are more resistant to antibiotics and other biocides. Understanding intercellular signal molecules which activates biofilm formation provides a possible mechanism to control biofilm accumulation on catheters and in the lungs of cystic fibrosis patients (Davies *et al*, 1998).

Knowledge of the myriad of receptors involved in intercellular signaling is rapidly increasing. Stone (1998) presented a useful review of the structure and function of various classes of receptors based on the mechanism by which they signal their targets. For instance, one category of receptors has a domain with enzymatic activity (e.g. the tyrosine kinase activity of insulin receptors). Another class of receptors contains G proteins which are activated by ligand binding (e.g. receptors that bind adrenaline and vasopression). A third class of receptors regulates ion movement across cell membranes upon activation by ligand binding (e.g. acetylcholine receptors of the synapse). In another class, steroid hormone receptors communicate with molecules involved in regulating transcription in the cytosol and nucleus, rather than the plasma membrane. These examples demonstrate the wide diversity of compounds involved in cell communication.

2.2.3 Role of Integrins as Adhesion Receptors in Humans

The structure and function of integrins as adhesion receptor molecules on the surfaces of cells was reviewed by Horwitz (1997). These molecules are involved in holding tissues together by helping cells stick to each other and to the extracellular matrix surrounding them. Integrins are also critical in other roles including embryonic development and regulation of processes such as blood clotting, wound healing, and fighting infections.

The extracellular matrix of tissues contain sugar chains and fibrous proteins such as laminin , fibronectin, and collagen. Integrins located on the surfaces of cells interact with the extracellular matrix and span the cell membrane into the cytoplasm. This facilitates signal transduction into the cell and across the cytoplasm and regulates processes such as gene expression, cell division, and cell aging. Integrins are also involved in changing the shape of cells, or enabling cells to migrate (Lauffenburger and Horwitz, 1996), proliferate, or differentiate during embryo development.

Integrins are also part of unwanted events in the body such as thrombus formation, inflammation, restenosis (the formation of thrombus in vessels after treatments such as balloon angioplasty), osteoporosis, infectious disorders, and cancer. Understanding the structure and function of integrins in the body is critical in advancing better health practices and developing new ways to fight disease (Hynes, 1992).

2.2.4 Reversible and Irreversible Cell Adsorption on a Surface

So far, the phenomena governing the cell response at an interface have not been completely understood. Recent work has attempted to provide a more detailed understanding of the mechanisms cell-surface interactions

which include both physicochemical factors and specific cell biological factors. Busscher and Weerkamp (1987) proposed that macroscopic surface properties control the reversible initial step of adhesion followed by microscopic, specific molecular interactions of irreversible adhesion. They suggest that the specific cell-surface interactions are enabled by the dehydrating effects of hydrophobic groups which allow close proximity of the surface components. Useful models with a broad capacity for predicting cell adhesion have not been developed (Schamberger and Gardella, 1994).

During the adsorption process, cells are transported to the substratum surface and adsorbed. Reversible or physical adsorption is characterized by weak, nonspecific interactions between the cell and the substratum and low heat of adsorption per chemical bond (20-50 kJ/mol) (Characklis, 1990). These interactions involve long range interaction forces between the cell and surface such as London-van der Waals forces, double-layer (electrostatic) interactions, and steric interactions. After some time, the cell either desorbs back into the bulk liquid or is irreversibly adsorbed to the surface.

Irreversible adsorption or "chemisorption" is a chemical interaction (ionic or covalent) between the particle and the substratum characterized by high heat of adsorption (60-600 kJ/mol) (Characklis, 1990). The chemical short range interactions involved include dipole-dipole interactions, dipole-induced dipole interactions, ion-dipole interactions, hydrogen bonds, hydrophobic interactions, and polymeric bridging (Ratzsch *et al*, 1990). When a cell initially adsorbs to a surface, the bonds are weak and the cell may desorb. If the bonds resist shear forces long enough for the bonds to

strengthen, the probability of desorption becomes zero and irreversible adsorption occurs. Reversible and irreversible adsorption are sometimes distinguished experimentally by rinsing the surface on which cells have adhered. Cells which are removed by rinsing are considered reversibly adsorbed (Characklis, 1990).

2.2.5 Acid-Base Theory of Adhesion

Adhesion between materials is determined by contributions from chemical bonding, polar-dispersive interactions, mechanical interlocking, and acid-base interactions. Acid-base interactions involve an electron donor (Lewis base) and an electron acceptor (Lewis acid). The classic example of acid-base interactions is hydrogen bonding.

A better understanding of acid-base interactions in promoting solubility, adsorption, and adhesion of polymers to other materials is beginning to be used to improve adhesion properties. Improved methods allow acid-base properties of polymers, solvents, and inorganic fillers and substrates to be determined. These techniques include inverse gas chromatography, microcalorimetry, ellipsometry, Fourier Transform Infrared (FTIR) spectroscopy, NMR, and X-Ray photoelectron spectroscopy (XPS) (Fowkes, 1991; Fowkes, 1987). The surface concentration and strength of acidic and basic surface sites can be measured using contact angles (Fowkes, 1991).

The acid-base interactions of material surfaces can be manipulated to alter the mechanical properties of various interfacial systems (Fowkes, 1987). For example, silane coupling agents were used to modify the surface

of glass and polymeric substrates (Dwight *et al.*, 1991). The mechanical properties of fiber-reinforced composites were enhanced by modifying the acid-base character of the surfaces (Dwight *et al.*, 1991). Egitto and Matienzo (1994) reviewed the use of plasma treatment to modify polymer surfaces and improve adhesion without compromising properties of the bulk material.

A food industry example of acid-base surface modification to improve adhesion involves polymer coatings applied to aluminum beverage containers. Finalayson and Shah (1991) found that adhesion between aluminum foil and acrylic polymer resin improved by increasing either the acidic sites of the polymer or the basic sites on the foil. This indicates that the wettability of aluminum is much less important than chemical bonding for polymer adhesion.

Kaczinski and Dwight (1991) prepared Teflon surfaces with two types of acidic surfaces and two types of basic surfaces. Adhesive bonds were measured by peel adhesion tests between pairs of each type of film. Interfacial failures occurred in acid-acid pairs while the interfacial bonds remained intact for acid-base pairs. This, again, demonstrates the importance of acid-base interactions in adhesion between surfaces.

2.2.6 Theoretical Approaches to Describe Initial Cell-Surface Interactions

Two approaches have been applied to describe the nonspecific interactions of reversible adsorption between cells and surfaces. The double layer, or DVLO (Derjaguin, Landau, Verway, and Overbeek), theory considers the long range forces of electrostatic forces and London-van der Waals forces (Lips and Jessup, 1979). The second approach considers short

range attractive forces including dipole interactions, chemical (electrostatic, covalent, hydrogen) bonding, and hydrophobic interactions. Hydrophobic effects refer to the interaction of nonpolar groups on the two surfaces and are indirectly determined by measuring surface tension or surface free energy between cells and the substratum. This method is referred to as the surface energy approach. The effect of microbial organelles such as flagella, fimbriae, and pili, which influence transport and adsorption of cells to surfaces if they are present, is not considered in either theory for long range or short range forces.

2.2.7 Long Range Forces (Electrostatic Interactions)

Electrostatic and ionic forces are long range interactions between cells and other charged surfaces. These forces play a dominant role in, for example, cell adhesion to highly charged surfaces and cell dispersal in blood (Gerson and Akit, 1980). The Derjaguin-Landau-Verway-Overbeek (DLVO) theory describes these long range interactions as the sum of an attraction term due to dispersion (London/van der Waals forces) and electrostatic interactions which can be either attractive or repulsive (Overbeek, 1984). The electrostatic interactions result from overlapping electrical double layers on the surfaces. Two positions for attraction are predicted by this theory, one at small separations and another at larger distances. Repulsive forces reach a maximum value at some point between the two attractive positions. The equations of the DLVO theory are plotted in Figure 2.2 as

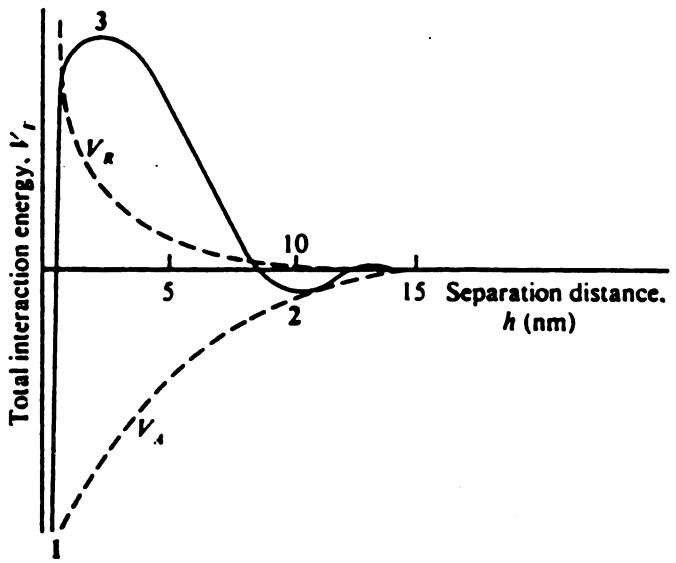

Figure 2.2 Diagrammatic representation of total interaction energy (V_T) as a function of separation distance (h) between a particle and a planar surface (Abbott *et al*, 1983).

the van der Waal's attraction energy (V_A), electrostatic repulsion energy (V_R), and the total interaction energy (V_T) between a spherical colloidal particle and a planar surface. The numbers in the figure refer to the following: (1) primary minimum; (2) secondary minimum; and (3) the electrostatic repulsive energy barrier.

Although the DLVO theory was developed for colloidal particles which are smaller than bacterial cells (cells average 0.15-2 microns in length or diameter), the theory has been applied to interactions of bacterial cells with surfaces. Modifications are made to the theory because viable cells exhibit some differences from colloidal particles such as pH gradients across the cell wall. Some of the problems associated with this approach include determining the value for a parameter called the Hamaker constant and accounting for cell and substratum surface geometry and roughness as well as cell deformation upon adsorption. The theory suggests that reversible adsorption can occur at a range of 5-10 nm. The time spent at this position may be sufficient for other adhesive forces to become effective. Application of this theory to microbial adsorption has led to the hypothesis that long range forces may control the time a cell spends in close proximity to a surface (reversible adsorption) while other forces determine whether irreversible adsorption occurs (Characklis, 1990). The significance of long range forces on adhesion is determined by varying the electrolyte concentration and pH of the suspending medium.

2.2.8 Short Range Forces (Surface Energy Approach)

Gibb's free energy of adhesion between two surfaces provides a thermodynamic framework for exploring short range forces of interaction which may include dipole interactions, chemical bonding (electrostatic, covalent, and hydrogen bonding), and hydrophobic interactions. Although long range forces may have an overall repulsive effect, cell adhesion to a surface may occur if short range attractive forces are sufficient to overcome the repulsive barrier.

Cell adsorption to a surface is predicted to occur if the total free energy of the system is reduced when the cell contacts the surface. The free energy of adhesion (also called work of adsorption or Gibb's free energy of adhesion) is based on an interfacial free energy balance for small particles. If electrical charge interactions and specific biochemical interactions are neglected, adhesion may be described by (Spelt *et al.*, 1982):

$$\Delta F_{adh} = \gamma_{CS} - \gamma_{SL} - \gamma_{CL} \qquad (2.1)$$

where ΔF_{adh} is the interfacial free energy of adhesion, γ_{CS} is the cell-surface interfacial free energy, γ_{SL} is the surface-liquid interfacial free energy, and γ_{CL} is the cell-liquid interfacial free energy. Adhesion will be thermodynamically favorable if

$$\Delta F_{adh} < 0 \qquad (2.2)$$

Interfacial tensions involving a solid phase cannot be measured directly and, as a result, various methods have been presented to determine solid interfacial tensions by indirect methods using liquid contact angle data. A discussion and comparison of the methods of interpreting contact angle data is given by Morra (1990), Neumann (1974), and Good (1973). Interfacial tensions in this work were determined using the equation of state approach (Li and Neumann, 1992; Morra, 1990; Spelt, 1990; Neumann et al, 1974). Other methods to calculate interfacial tensions include the critical surface tension approach (Morra, 1990) and the geometric mean approach to determine the polar and dispersion interactions (Morra, 1990; Busscher *et al.*, 1983; Owens and Wendt, 1969). (See Appendices 4-6 for equation development and EXCEL program.)

2.2.9 Equation of State Approach to Determine Solid Interfacial Tension

A liquid drop placed on a surface will modify its shape until a stable three-phase equilibrium between the liquid, substratum surface, and surrounding vapor is obtained (Figure 2.3). The Young equation describes equilibrium at this three-phase boundary (Morra, Occhiello, and Garbassi; 1990):

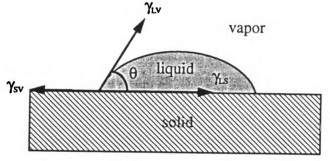

Figure 2.3 Measurement of contact angle, θ, on liquid drop deposited on substrate.

$$\gamma_{LV}\cos\theta = \gamma_{sv} - \gamma_{SL} \qquad (2.3)$$

where γ_{LV} is liquid surface tension in the presence of vapor, θ is the contact angle, and γ_{sv} and γ_{SL} are the solid surface tension with the vapor and liquid, respectively. The liquid surface tension and the contact angle are measurable quantities and the two solid surface tensions are unknowns. Therefore, a second equation is required in order to determine the two unknowns. From thermodynamic considerations, an equation of state relation has been shown to exist such that (Neumann *et al*, 1974):

$$\gamma_{SL} = f(\gamma_{sv},\ \gamma_{LV}) \qquad (2.4)$$

Spelt (1990) presented two proofs for the existence of the equation of state. The first method applied the Gibbs-Duhem equations for a three-phase interfacial system while the second approach was based on the phase rule for surface systems and the associated degrees of freedom. The analogous relations of Equation 2.4 may be written for γ_{CS} and γ_{CL} and substituted into Equation 2.1:

$$\Delta F_{adh} = f(\gamma_{CV},\ \gamma_{sv}) - f(\gamma_{CV},\ \gamma_{LV}) - f(\gamma_{sv},\ \gamma_{LV}) \qquad (2.5)$$

The equation of state relation of Equation 2.4 can be formulated by either a statistical thermodynamics or an empirical approach. Neumann and coworkers (1974) formulated the equation empirically:

$$\gamma_{SL} = \frac{\left(\sqrt{\gamma_{LV}} - \sqrt{\gamma_{SV}}\right)^2}{1.0 - 0.015\sqrt{\gamma_{LV}\gamma_{SV}}} \tag{2.6}$$

Equation 2.6 can be used to calculate the interfacial tension between any two solid or liquid phases from previously determined interfacial tensions (i.e. γ_{12} can be found from the known values of γ_{13} and γ_{23} where the subscripts 1, 2, and 3 refer to different phases). The equation of state is combined with the Young equation (Equation 2.3) with the following result:

$$\cos\theta = \frac{(0.015\gamma_{SV} - 2.00)\sqrt{\gamma_{LV}\gamma_{SV}} + \gamma_{LV}}{(0.015\sqrt{\gamma_{LV}\gamma_{SV}} - 1)} \tag{2.7}$$

This equation can be used to determine the solid-vapor surface tension, γ_{SV}, using the measured values for the liquid surface tension, γ_{LV}, and the contact angle, θ. The solid-liquid interfacial tension can then be found from either the Young equation or the equation of state.

A refinement of the equation of state based on more accurate contact angle measurements and a better understanding of surface thermodynamics has been presented by Li and Neumann (1992):

$$\gamma_{SL} = \gamma_{LV} + \gamma_{SV} - 2\sqrt{\gamma_{LV}\gamma_{SV}}e^{-\beta(\gamma_{LV}-\gamma_{SV})^2} \qquad (2.8)$$

where β is a constant equal to 0.0001247. This constant was determined empirically from curve-fitting a large quantity of experimental contact angle data using various liquids and polymer surfaces. Combining Equation 2.8 with the Young equation yields the following equation which may be used for any solid-liquid system (i.e. cells-liquid or polymer-liquid):

$$\cos\theta = -1 + 2\frac{\sqrt{\gamma_{SV}}}{\sqrt{\gamma_{LV}}}e^{-\beta(\gamma_{LV}-\gamma_{SV})^2} \qquad (2.9)$$

Equations 2.8 and 2.9 contain mathematical limitations in the form of discontinuities (Neumann *et al.*, 1974) which can be avoided by using either computer programs or tables to obtain the values of various parameters. Li and Neumann (1992) presented the logic for a computer program to calculate the solid-vapor interfacial tensions from liquid-vapor interfacial tensions and contact angles. Neumann and coworkers (1980a) provided a computer algorithm for the calculation of solid-liquid and solid-solid interfacial tensions from the corresponding solid-vapor and liquid-vapor surface tensions. Alternatively, tables containing contact angles and the interfacial tensions γ_{LV}, γ_{SV}, and γ_{SL} can be used to find the value of any two

of the parameters by knowing the value of the other two (Neumann *et al.*, 1980b).

2.2.10 Validity of the Equation of State

Considerable discussion has taken place in the literature about the theoretical validity and predictive ability of the equation of state method for determining the interfacial tension of a solid. Spelt (1990) compared the theoretical background and accuracy of the equation of state method to the geometric mean approach. He concluded that predictions based on the equation of state approach give closer agreement with experimental data than the geometric mean approach.

The equation of state and the geometric mean approach have both been applied for the determination of the surface energy of microorganisms. Van Loosdrecht and coworkers (1987) measured contact angles of eight strains of bacteria using 1-bromonaphthalene and 0.1 M NaCl solution on layers of cells. They calculated the surface free energy using the equation of state and the geometric mean approach and found similar results from both methods.

2.2.11 Adhesion Studies Using the Surface Energy Approach

The surface energy approach has been applied to a variety of systems including microbial adsorption to oral surfaces (Van Pelt *et al,* 1984), marine fouling (McEldowney *et al.*, 1986b), and plant cell adhesion to solid

substrates (Facchini *et al.*, 1988). The results are useful in predicting the trend of adsorption onto different materials. Many studies have revealed a correlation between adhesion of various strains of bacteria and substratum surface energy, γ_{SV}, and liquid surface tension γ_{LV} (Absolom *et al.*, 1983; Gerson and Sheer, 1980). These trends agree with thermodynamic predictions for the free energy of adhesion, ΔF_{adh}, indicating that short range hydrophobic interactions are a significant influence in the adhesion process.

Theoretical calculations for ΔF_{adh} reveal that three different cases may exist: (i) when $\gamma_{LV} < \gamma_{CV}$, then ΔF_{adh} decreases with increasing γ_{SV} and adhesion is predicted to increase with increasing γ_{SV}, (ii) when $\gamma_{LV} > \gamma_{CV}$, then ΔF_{adh} increases with increasing γ_{SV} and adhesion is predicted to decrease with increasing γ_{SV}, (iii) when $\gamma_{LV} = \gamma_{CV}$, then $\Delta F_{adh} = 0$ and is independent of the value of γ_{SV} (Absolom *et al.*, 1983). Theoretical values of ΔF_{adh} are shown in Figure 2.4 for a bacterium (γ_{BV} in the figure is equivalent to γ_{CV} where B=bacteria and C=cell). The trends predicted by the theoretical model were seen experimentally in the adhesion of five types of bacteria as a function of liquid surface tension and substratum surface energy (Figure 2.5) (Absolom *et al.*, 1983). The liquid surface tension was manipulated by the addition of dimethylsulfoxide (DMSO) to the suspension medium.

Another series of plots, Figure 2.6, shows predicted values of ΔF_{adh}, and the experimentally measured bacterial adhesion of five species on

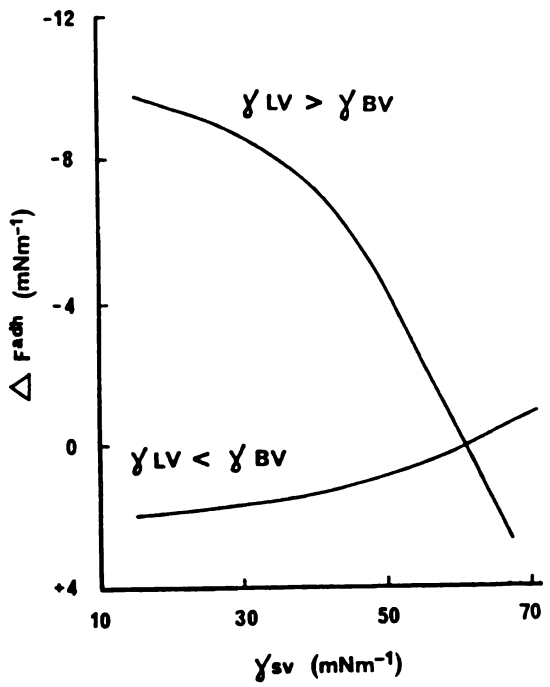

Figure 2.4 Theoretical values of ΔF_{adh} for a bacterium (γ_{BV} = 67.8 dynes·cm·1, γ_{LV} = 72.8 and 64 dynes·cm^{-1}) (Absolom *et al.*, 1983).

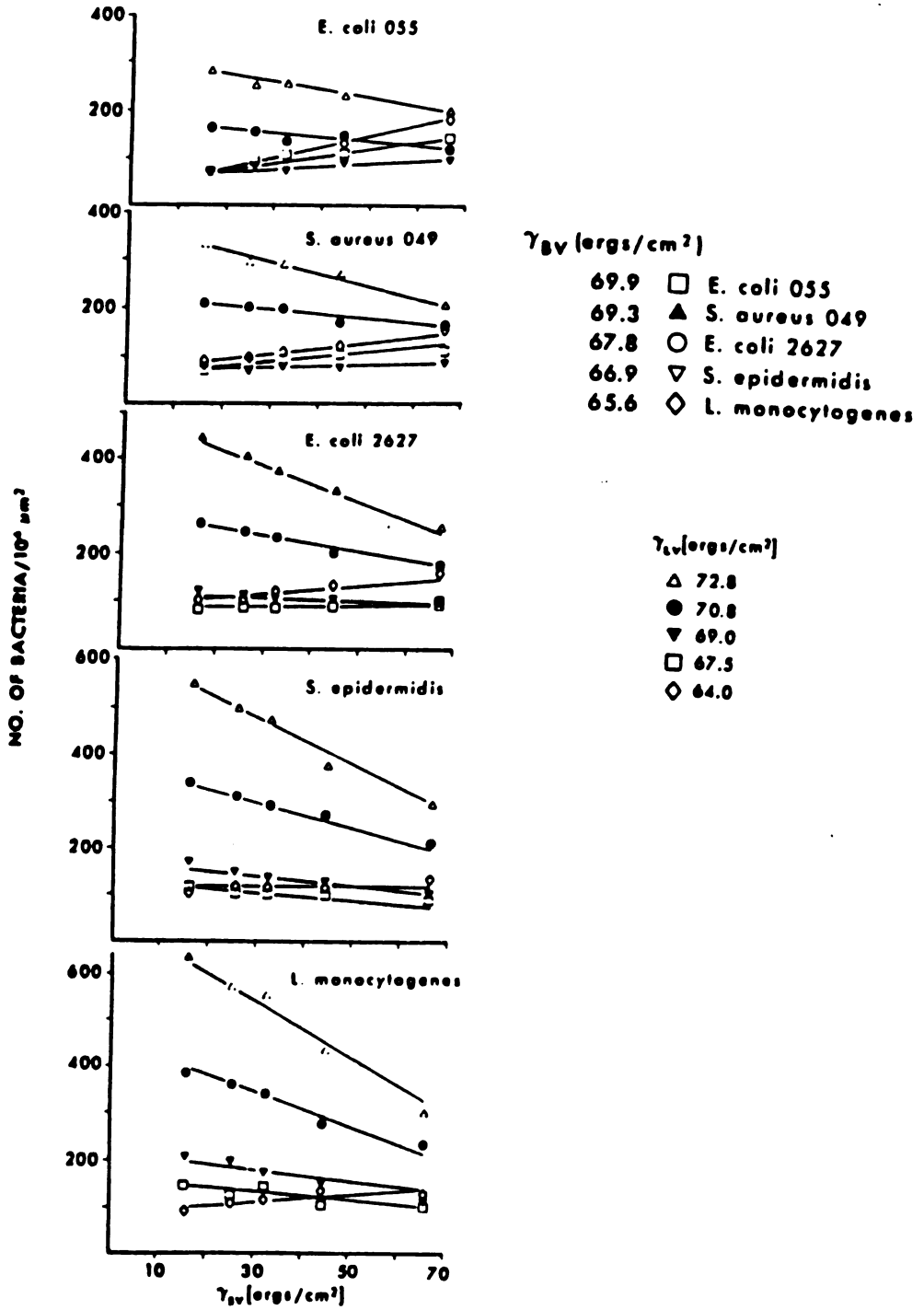

Figure 2.5 Bacterial adhesion in liquids of various γ_{LV} as a function of γ_{SV} (Absolom *et al.*, 1983).

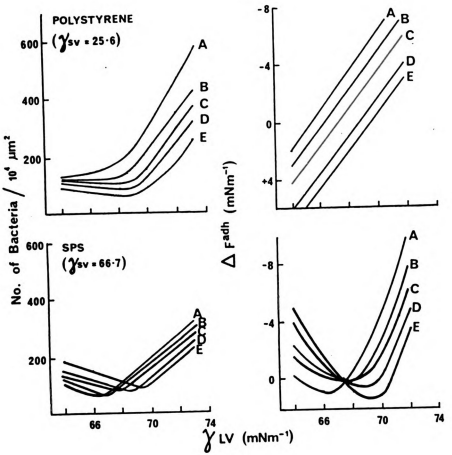

Figure 2.6 Value of ΔF_{adh} as a function of liquid surface tension for five bacterial species (right hand side) and the experimentally measured adhesion (left hand side) on two polymer substrata, polystyrene and sulfonated polystyrene (Absolom *et al.*, 1983).

polystyrene (PS) and sulfonated polystyrene (SPS) as a function of liquid surface tension (Absolom *et al.*, 1983). The thermodynamic model shows a high correlation with actual adhesion for the high surface energy material, SPS. For the low surface energy material, PS, adhesion follows the thermodynamic trend for values of $\gamma_{LV} > \gamma_{CV}$. At lower values of γ_{LV}, the observed adhesion becomes independent of γ_{LV}. Possible explanations of this are that electrostatic interactions or conditioning films become more important at this point.

2.2.12 Adhesion of Filamentous Fungi To Surfaces

Filamentous fungi are widely distributed in soil, water, and the surfaces of plants, insects, and animals. Although the attachment of filamentous organisms has been observed in various systems, little work has been done to understand the actual mechanism of adhesion and the factors which influence the adhesion process. Most microbial adhesion studies have involved single-celled organisms such as bacteria, viruses, and yeasts. In the last ten years, interest in understanding the relationship between fungal hydrophobicity and surface adhesion has increased. The most studied genera are the single-celled yeasts *Candida*, a pathogenic organism associated with immunocompromised patients, and *Saccharomyces*, a common organism of the fermentation industry.

When wood-decaying fungi attach to wood, they produce bore holes by enzymatic digestion which are then penetrated by hyphae (Corpe, 1980).

The organism may be anchored to the surface by the hyphae although surface irregularities and extracellular polysaccharides may also play a role in attachment. Corpe also studied the attachment of saprophytic fungi, which obtain nutrients from decayed organic matter, to leaves and the colonization of fungi in cooling water towers and marine environments but suggested no mechanisms for adhesion.

Asther and coworkers (1990) presented the first study of the adhesion of *P. chrysosporium* to four support surfaces of different hydrophobicities. They used the surface component approach to determine the surface free energy of the cells and surfaces. Although experimental results showed the same general trend as theoretical predictions for adhesion, the carriers exhibited a wide range of porosity, rugosity, surface area, and surface geometry (i.e. from Raschig rings to foam cubes). These factors are all known to affect the extent of adhesion.

2.3 Methodology of Fungal Adhesion Studies

2.3.1 Introduction

When performing cell adhesion studies, an appropriate method must be available for measuring adhesion of cells to a surface. In addition, the surface energy of the cells, substratum surface, and the suspending liquids must be determined. The various methods used to measure these parameters are presented in this section. Surface roughness is known to alter the properties of a surface and, therefore, influence cell adhesion. Methods to measure roughness and contact angles on rough surfaces are also discussed.

2.3.2 Methods for Measurement of Fungal Biomass

A major problem in the study of filamentous fungal cultures adhered to solid substrates is the difficulty of measuring the amount of mycelial biomass (Matcham and Wood, 1988). Fungal hyphae are somewhat heterogeneous in nature as they vary in width, length, and cellular age along a hypha (Hazen, 1990). Some of the common biomass measurement techniques are useful only for single-celled organisms and can not be used for mycelial organisms (i.e. cell counting or light scattering methods). Other methods are time-consuming or are not quantitative in nature.

The method chosen is crucial in the design of the experiment. Some factors which influence the choice are the accuracy and sensitivity required, the required speed of measurement, the properties of the culture medium (viscosity, color, amount of solids), the characteristics of the organism (filamentous or particulate, age, growth rate, and ease of separation from the medium), and whether the culture is a single-species or mixed population (Pirt, 1975). Techniques for biomass measurements are summarized in Table 2.1.

Two compounds specific to fungi which are used for biomass estimation are chitin and ergosterol (Seitz *et al*, 1979). Chitin is found in fungal cell walls and insects but not in plants or other microorganisms. This assay gives total fungal biomass living and dead. Ergosterol is the primary sterol of most fungal cell membranes and can be assayed by a multistep extraction procedure followed by GC, HPLC or UV detection. A

Table 2.1 Methods for biomass measurement.

Method	Comments	Ref.
Mass	Determine dry or wet weight. Prevent lysis by using isotonic saline.	1
Volume	Density of biomass varies little. Use hematocrit (small volume) or graduated cylinder (large volume)	1
Cell or Organelle Counts, Light Scattering	Useful for conidia; not appropriate for mycelial organisms.	1
Linear Extent	Growth of colonies or pellets by linear spread or hyphal length.	1,2,3
Mass of Cell Component	Nitrogen by Kjeldahl method; protein; DNA, RNA, ATP; chitin; ergosterol .	1,2,3, 4,5
Mass of Substrate Consumed or Product Formed	Glucose, wood depolymerization, O_2, CO_2, extracellular growth-linked enzymes. Proportionality to mass may vary during culture.	1,2,3
Staining	Vital stains i.e. fluorescein diacetate for fungal mycelium, eosin for yeast.	1,6
Metabolic Rate	Rate of dye reduction or gas production can be related to amount of biomass i.e. iodonitrotetrazolium (INT).	7
Antibodies	Fluorescent antibodies; enzyme-linked immunosorbent assay (ELISA); radioimmunoassay (RIA).	2,3

[1]Pirt, 1975
[2]Matcham, Jordan, and Wood, 1984
[3]Matcham and Wood, 1988
[4]Lundin, 1988
[5]Lundin, 1982
[6]Soderstrom, 1977
[7]MacDonald, 1980

difficulty with these assays is that the chitin and ergosterol content may not remain constant during fungal growth (Matcham, Jordan, and Wood, 1984).

Other methods of biomass measurement include assays for nitrogen, nucleic acids, adenosine triphosphate (ATP), respiration rate (CO_2 evolution), and substrate consumption (Matcham *et al*, 1984; Lundin, 1988; Lundin, 1982). Hyphal length measurements estimate biomass based on total hyphal length per unit area and average hyphal diameter. Fluorescent antibodies, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) are diagnostic tools for detecting the presence of microbes. Some of the disadvantages with these methods are that they are labor-intensive or may not be quantitative in nature. If a method is quantitative, many samples must be measured to achieve statistically meaningful results.

For quantitatively estimating mycelial biomass, dry weight measurements are fast, inexpensive, require the least amount of sample preparation, and are appropriate for many replicates. The method requires larger amounts of biomass than many of the other techniques. Cell counts or light scattering methods can not be used to measure mycelia but are useful to quantify conidia, the asexual reproductive body of some mycelial fungi including *P. chrysosporium*.

2.3.3 Determination of Surface Free Energy of Biological Surfaces

The determination of the surface energy of biological surfaces poses unique experimental challenges. Several methods have been used for qualitative and quantitative assessment. Cells are considered to be solids, rather than liquids, for the purpose of surface energy determinations

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(Absolom, 1986). This is an important distinction because the techniques used to determine the surface energy of liquids and solids are different. Liquid surface tension can be measured directly while indirect methods must be applied for the determination of solid surface tension. A liquid is defined as having a shape which is dependent only on surface tension and external forces such as gravity. This is not the case for cells which, although deformable, possess shapes not influenced by surface tension or gravity alone. Some cells have internal cytoskeletons or form pseudopods in response to stimuli.

Hydrophobicity of cells varies from one strain to another and is dependent on factors such as medium composition, aeration, and cellular age (Mozes and Rouxhet, 1987). Although a cell surface may contain hydrophobic surface groups, the overall surface may tend to be relatively hydrophilic due to extracellular polymeric substances (Marshall, 1986). Various methods have been used to determine cell surface energy. A brief description, less the theoretical background, of these methods is presented next.

Determination of Cell Surface Energy Using Adhesion Experiments

Cell adhesion on polymer substrates is a function of both substrate surface energy and surface tension of the surrounding liquid. Adhesion is measured using substrates representing a range of surface energies and a series of liquids with a range of surface tensions. Adhesion can be quantified using light microscopy as the number of cells adhering to the

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surface per unit surface area. The adhesion is plotted as a function of substrate surface tension (Figure 2.7). The slope of each substrate surface tension line is then plotted as a function of liquid surface tension (Figure 2.8). Cell surface tension is then determined from this plot as the value of liquid surface tension where the slope equals zero (i.e. γ_{CV} is equal to γ_{LV} where the line crosses the x-axis). This follows the theoretical predictions which indicate that cell adhesion becomes independent of substrate surface energy for a specific value of liquid surface tension.

Hydrophobic Microsphere Attachment Assay (HMA)

Surface hydrophobicity of an individual cell can be detected by observing the attachment of hydrophobic microspheres to the cell surface. Low-sulfate polystyrene microspheres, which are hydrophobic in nature, are mixed with microbial cells (Hazen and Hazen, 1987). Microspheres with a diameter smaller than the diameter of the cell are used. The microspheres adhere to hydrophobic areas of the cell surface and can be observed using microscopy. For single-celled organisms, the percentage of cells with a minimun number of attached microspheres is determined, and the level of hydrophobicity is reported as this percentage. This measure of cell hydrophobicity is influenced by the concentration of microspheres. Hydrophobicity increases with increasing microsphere concentration up to a saturation value in a manner similar to positive cooperativity in enzyme binding (Hazen and Hazen, 1988).

Number of Neutrophils / mm²

Figure 2.7
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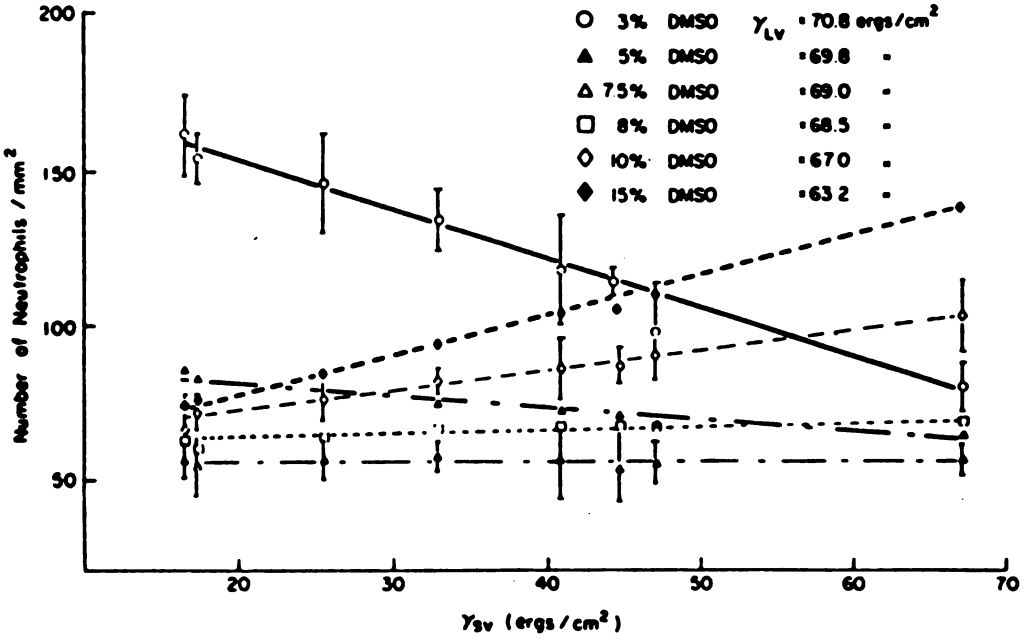

Figure 2.7 Human granulocyte adhesion as a function of substratum surface tension, γ_{SV}, for various concentrations of dimethylsulfoxide concentrations (Absolom, 1986).

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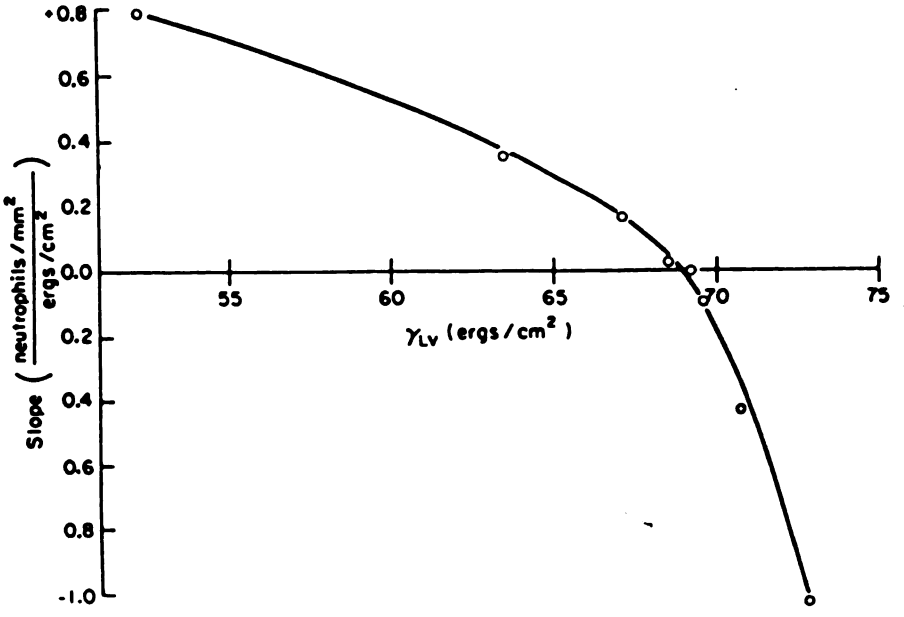

Figure 2.8 Slopes of the straight lines of Figure 2.7 as a function of the suspending liquid surface tension (Absolom, 1986). Cell surface tension, in this case, equals about 69 ergs/cm^2.

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This method is fast, inexpensive, easy to perform and offers the unique advantage of determining the distribution of hydrophobic sites on a cell, whether uniformly arranged or in patches. For example, the hydrophobic sites of *S. cerevisiae* grown at 23°C are randomly dispersed over the entire cell surface while the sites of cells grown at 37°C are located on polar ends (Hazen, 1990). The method can be used for hyphae and germ tubes although not in a quantitative manner.

Other Methods

Other quantitative methods for determining cell surface tension include elution of cells from substrates using liquids with various surface tensions, phagocytic ingestion (ingestion of bacteria by cells) (Absolom, 1986; Absolom *et al*, 1982; Neumann *et al*, 1982), freezing front techniques (Absolom *et al*, 1985), and suspension stability techniques (Absolom, 1986; Neumann *et al*, 1984). Qualitative methods for determining cell surface tension include hydrophobic interaction chromatography (measuring the amount of cells retained by a hydrophobic gel) (Mozes and Rouxhet, 1987), two-phase partition methods (observing the partition of cells between a hydrophobic liquid and water) (Mozes and Rouxhet, 1987; Rosenberg, 1984; Gerson and Akit, 1980; Gerson, 1980; Rosenberg et al, 1980), and the salt aggregation test (promoting cell flocculation by increasing the salt concentration of the medium) (Moses and Rouxhet, 1987).

Contact Angle Measurements

The surface free energy of cells may be obtained from contact angle measurements as described by Van Loosdrecht *et al.* (1987), Klotz *et al.* (1985), and Van Pelt *et al.* (1984). A drop of saline solution is deposited on a layer of cells. The angle formed between the cells and the tangent to the drop at the solid-liquid-air intersection is measured and used in thermodynamic relations to calculate the cell surface tension (Figure 2.3). Van Pelt (1984) used contact angle measurements to determine the surface free energies of several strains of oral streptococci. The approach was used in the work presented here and is described in more detail in the following section.

2.3.4 Contact Angle Measurements on Cells

Biological surfaces are highly hydrated and must be handled in an aqueous medium to preserve structural integrity of the membrane components. These physiological considerations suggest water or buffer solutions as the liquid of choice for contact angle measurements. In addition, cells have a relatively large surface tension and using these liquids, which have a surface tension larger than that of the cells, insures that the equilibrium spreading pressure is negligible (Absolom, 1986). Equilibrium spreading pressure is the decrease in surface tension due to vapor adsorption.

The importance of measuring contact angles on biological surfaces as a function of time has been strongly emphasized by Absolom, Zingg, and Neumann (1986). Cells prepared for contact angle measurements initially carry an aqueous layer and a drop placed on the cells will produce a contact angle of zero (complete spreading). As time progresses, the aqueous layer evaporates and the contact angle increases until a plateau is reached as shown in Figure 2.9. Upon further dehydration, the contact angle rapidly increases, decreases, or becomes erratic. This sequence of events has been repeatedly observed for a variety of highly hydrated systems including bacteria, leukocytes, erythrocytes, mammalian cells, plant cells, hemicellulose, and polyacrylamide gel.

Absolom, Zingg, and Neumann (ibid) suggest that the thermodynamic contact angle is the contact angle corresponding to the plateau value. The time dependency of the contact angle on drying cells is interpreted as follows: the contact angle is initially zero on the hydrated surface and increases as the moisture evaporates until a constant angle is observed. This plateau value is determined only by the properties of the cell layer. Beyond this point, the contact angle is the result of structural and conformational changes of the surface and possible denaturation of proteins. Scanning electron microscopy studies confirm that the cell structure is maintained during the plateau region but is subsequently lost upon further dehydration. Changes in experimental conditions may alter initial slope and duration of the plateau of the data plots while the plateau value remains

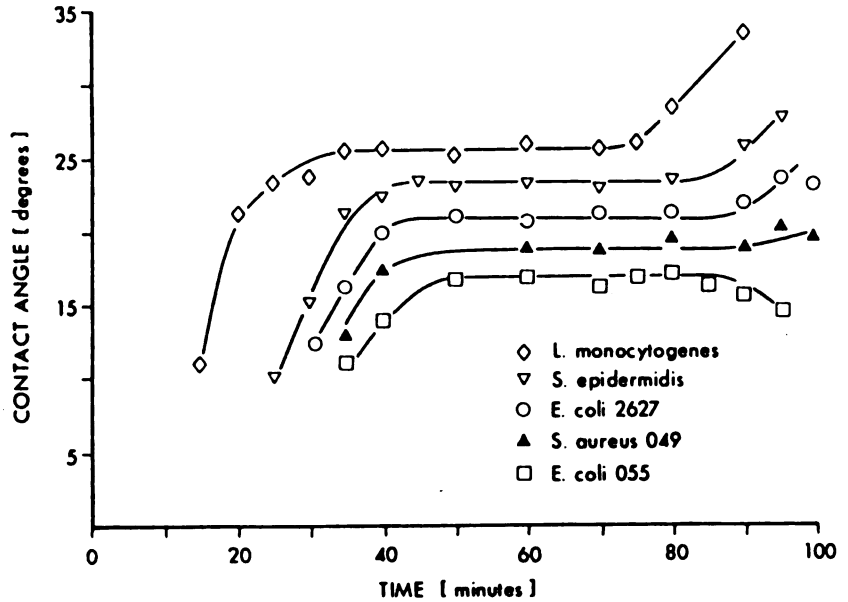

Figure 2.9 Contact angle as a function of time for water evaporation from the wet cell layer surface for various bacteria (Absolom, 1986).

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unchanged. In addition, the plateau value is unique for each biological system and can even be used to distinguish strains of microorganisms (van Pelt et al., 1984).

Many of the contact angles for microorganisms reported in the literature are not true plateau contact angles but are angles measured on dried cell layers and represent misleading data. The method used to obtain the data must be noted. The values of contact angles for cells are generally within the range of about 15° to 60°. Krekeler and coworkers (1991) classified bacterial cells as very hydrophilic (<20°), hydrophilic (20°-30°), and hydrophobic (>30°), although this is not a standard convention.

2.3.5 Cell Surface Hydrophobicity of Fungi

Although a variety of methods have been developed for the determination of bacterial cell surface hydrophobicity, only a few have been applied to fungal systems. Certain characteristics of fungi render many of the methods inappropriate. Almost all of the fungal systems that have been studied exhibited single-cell morphology, not the mycelial form, which is the typical cell morphology for the organism studied in this work..

The morphology of fungi can be complex. Some fungi may exist in two vegetative forms, such as yeast or mold, depending on the environmental conditions. In mycelial fungi, hyphal length and width can vary within a particular strain. Growth of the hypha occurs apically (at the tip) resulting in a gradient of cellular ages (Smith, 1975). The cell wall of fungi is a complex structure composed primarily of polysaccharides but also

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proteins and lipids (Hazen, 1990). The surface may be smooth, rough, or contain microfibrils. The reproductive propagules of fungi exhibit a variety of sizes, shapes, and compositions. All of these factors affect the cell surface hydrophobicity. It is necessary to decide which forms of the fungus are the most important to study for a particular application. In addition, other factors affect the relative hydrophobicity of cells including growth temperature, growth medium, and growth phase (e.g. exponential, stationary, death).

Of the variety of methods described earlier for assessing cell surface hydrophobicity, only adhesion studies, contact angle, and the hydrophobic microsphere attachment assay (HMA) are useful for filamentous fungi. In adhesion studies, fungal adhesion is analyzed using microscopy with image analysis and quantified either as the percentage of surface area covered with cells or as total hyphal length adhered per unit surface area. These methods require that the cells exist as a monolayer on the substratum surface. HMA, while not a quantitative technique, provides information on the distribution of hydrophobic areas on the cell surface and can be used on hyphae.

Contact angle measurements may not be reliable for hyphal cells because a smooth, uniform cell layer may not be obtained on which to deposit a liquid drop. Conidia may be potential candidates for the successful application of contact angle measurements due to their spherical shape, although conidia are larger then bacteria and appear to form a rougher layer. Contact angle measurements (Hazen, 1990) and adhesion experiments, unlike other methods, provide information on the cumulative

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surface free energy of a cell population rather than the number of individual cells that are hydrophobic.

Few studies have compared yeast hydrophobicity results from two or more methods although several such studies have been performed for bacterial populations. In one study using *Candida* yeasts, contact angle measurements and a two-phase partition method provided similar results but could not distinguish between species (Hazen and Hazen, 1987). Another study which compared the hydrophobic microsphere assay, contact angle measurements, hydrophobic interaction chromatography, and the salt aggregation method gave inconclusive evidence about the agreement of hydrophobicity results (Hazen, 1990). Because research groups perform assays differently, results obtained from various methods cannot be easily compared.

2.3.6 Effect of Substratum Roughness on Microbial Adhesion

Substratum roughness plays an important role in the rate and extent of microbial adhesion and growth, although little research is available which quantitatively explores this effect. Roughness may increase both the transport and adsorption of macromolecules and cells to surfaces for the following reasons: (1) convective mass transport is higher near rough surfaces compared to smooth surfaces, (2) cells near the surface are protected from shear forces which reduces the desorption rate, and (3) higher surface area is available for cell-surface contact (Characklis,

McFeters, and Marshall, 1990). Hydraulic roughness is characterized by surface peaks which extend above the viscous sublayer and can be quantitatively measured by frictional resistance methods (Figure 2.10). Microroughness occurs when surface elements are smaller than the viscous sublayer. Microroughness is difficult to measure although this scale of surface roughness does influence microbial adhesion. Figure 2.11 illustrates the size of roughness for various stainless steel sheets relative to the typical dimensions of a bacterium.

Different types of cells colonize rough surfaces differently. Characklis and coworkers (1990) reported that some microorganisms selectively attached to grooves and crevices rather than smooth, flat areas while other microbes showed no preference in adhesion sites even though roughening still increased the rate of colonization. Surface irregularities may serve as anchoring points for polymer bridging or as sites for "entangling" cells.

2.3.7 Measurement of Surface Roughness

Methods for surface texture analysis have been developed to aid in manufacturing quality parts in production processes and have become an important part of quality control procedures. Roughness measurements are commonly obtained using a stylus instrument (i.e. Mitutoyo Surftest or Sloan Dektak surface profile measuring systems) to trace a surface and produce a roughness profile. In recent years, the software of laser scanning

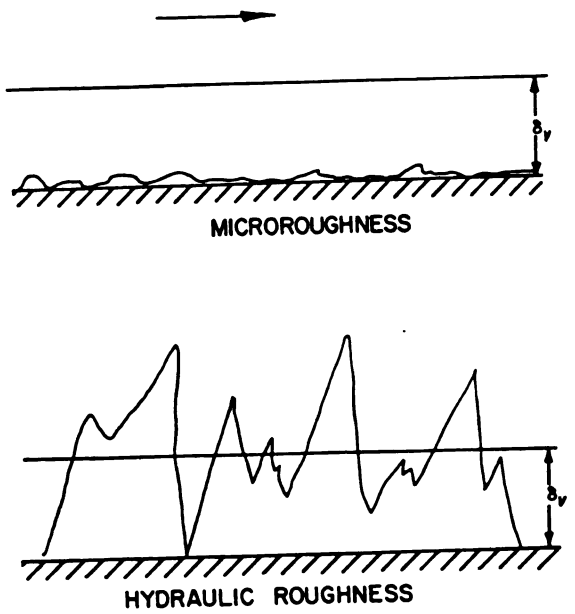

Figure 2.10 Comparison of microroughness and hydraulic roughness relative to viscous sublayer thickness, δ_v (Escher and Characklis, 1990).

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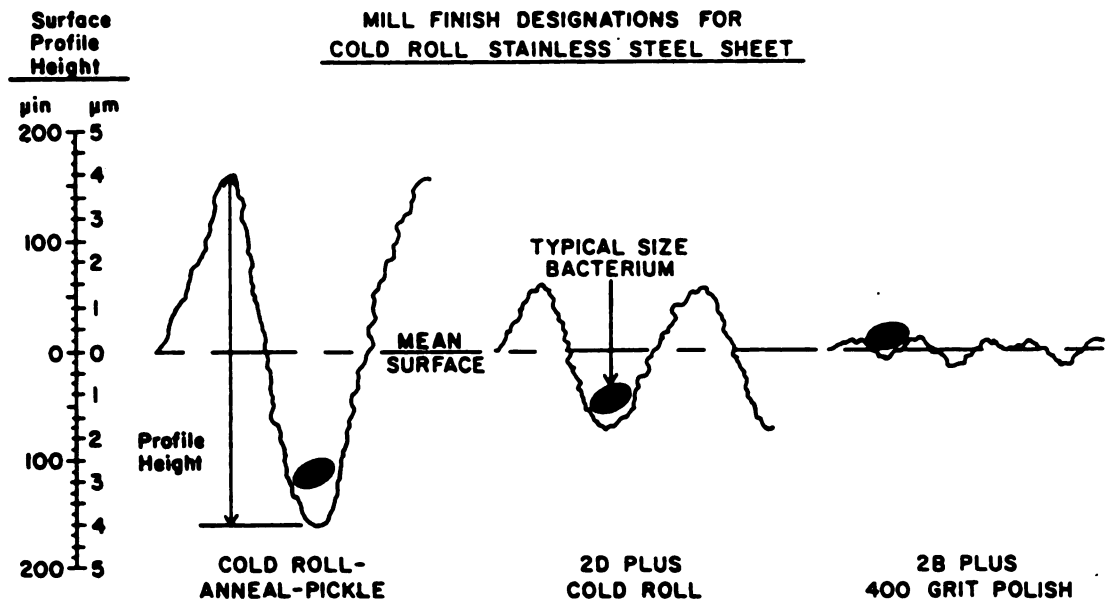

Figure 2.11 Comparison of the size of microroughness on stainless steel tubing with the size of a microbial cell (Characklis, 1990)

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and atomic force microscopes has included topography routines which are able to perform measurements for several roughness parameters. These optical techniques offer the advantage of determining the roughness of an area of the surface instead of only a line. Informative discussions of stylus techniques and surface roughness are presented by Mummery (1990) and Whitehouse (1974).

The primary roughness parameter is average roughness, R_a, which is also known as center line average and arithmetic average and is expressed in microinches or micrometers. R_a is the average distance of the profile to the mean center line over the length of assessment and is determined by the following equation (Mummery, 1990):

$$R_a = \frac{1}{l_m} \int_0^{l_m} |y(x)| dx \qquad (2.10)$$

where l_m is the length of assessment, x is the direction of assessment, and y is the distance of the profile from the mean line as shown in Figure 2.12.

In addition to R_a, an array of other surface texture parameters have been defined to quantify different aspects of a rough surface (Mummery, *ibid*). One or more of these parameters can provide useful information about a surface and distinguish it from another surface of different characteristics. Sometimes one parameter alone is not sufficient to reflect differences between surfaces, as illustrated in Figure 2.13. This figure

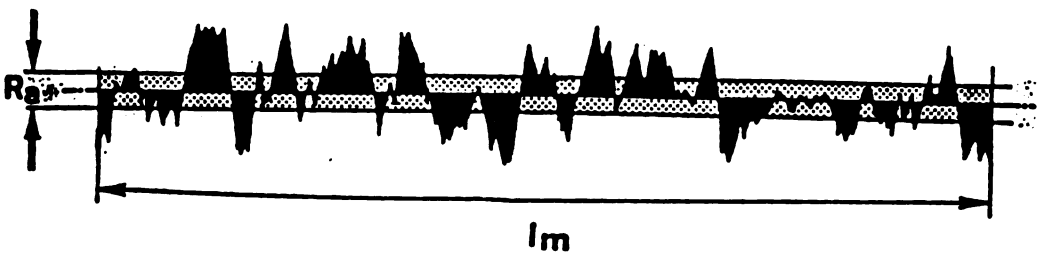

Figure 2.12 Illustration of average roughness, R_a (Mummery, 1990)

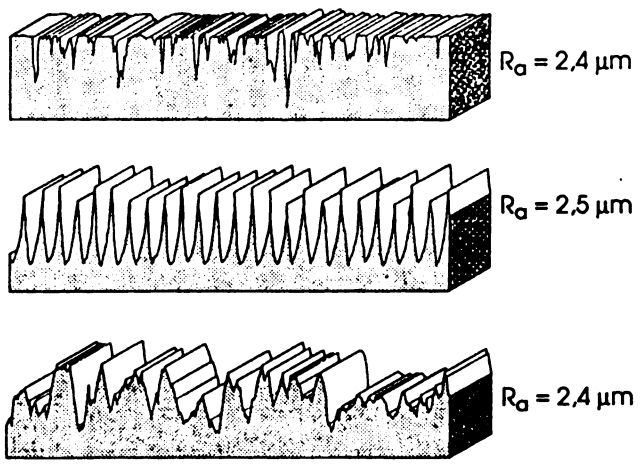

Figure 2.13 Comparison of average roughness, R_a, values for different profiles (Mummery, 1990).

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shows three surface profiles that have the same average roughness, R_a, values even though the profiles are clearly different in roughness character.

2.3.8 Contact Angle Measurements on Roughened Surfaces

Contact angle measurements provide a simple, inexpensive method to obtain the surface tension of a solid in different working environments. A drop placed on a surface will alter its shape until equilibrium is reached between the surface, the liquid drop, and the surrounding vapor or a second immiscible liquid. Young's equation is a thermodynamic description of the 3-phase boundary equilibrium for "ideal" surfaces (Morra, Occhiello, and Garbassi; 1990)

$$\gamma_{LV}\cos\theta = \gamma_{SV} - \gamma_{SL} \qquad (2.11)$$

An "ideal" surface is a smooth surface on which a liquid drop will have no physical or chemical interactions. The surface can be characterized by a particular value of surface tension. More than one stable contact angle may exist, and this time-independent phenomenon is called thermodynamic hysteresis. The maximum stable angle is called the advancing angle, and the minimum stable angle is the receding angle. The contact angle is sensitive to the top few angstroms of the surface.

Real surfaces may contain roughness or heterogeneous areas which will exhibit different contact angles. The Wenzel equation attempts to model the effect of substrate roughness on the contact angle (Morra *et al.*, 1990; Busscher *et al.*, 1984a):

$$\cos\theta_w = r \cos\theta_y \qquad (2.12)$$

where θ_w, θ_y, and r represent Wenzel's angle (angle on rough surface), Young's angle (angle on smooth surface), and the roughness factor, respectively. Roughness is defined as the ratio of the actual (rough) surface area to the geometric (smooth) surface area. The effect of roughness is to increase the wetting properties of the solid. As shown in Figure 2.14, the Wenzel angle will increase with increasing roughness if the Young's angle is greater than 90°, and will decrease if the Young's angle is less than 90°. Busscher and coworkers (1984a) validated the trend predicted by Wenzel's equation for contact angles using five liquids on twelve different commercial polymers roughened by various procedures.

Contact angles on rough surfaces usually exhibit a range of values which represent metastable equilibria (i.e. a range of allowed contact angles exist). For this reason, the Wenzel angle cannot be determined as a distinct value for a surface with a particular roughness value nor can the corresponding Young angle be accurately determined from contact angle

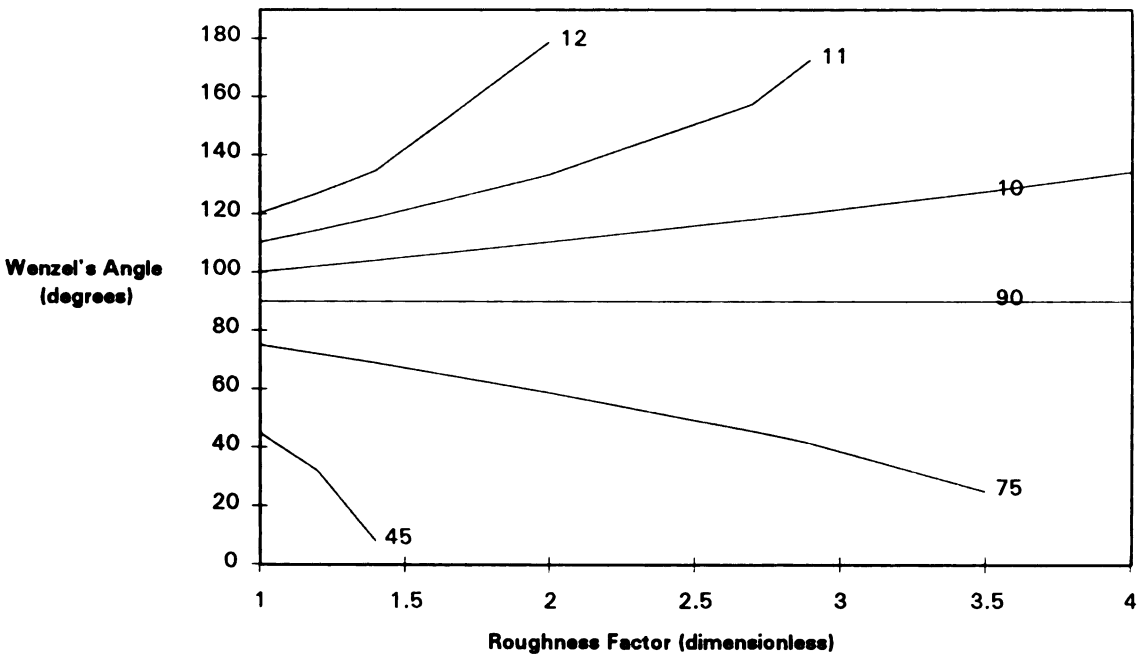

Figure 2.14 Wenzel's angle as a function of surface roughness for different values of Young's contact angle. The number next to each line on the plot refers to the Young's contact angle.

data of rough surfaces. Another characteristic of rough surfaces is that they are usually heterogeneous in nature and contain domains of different compositions and, therefore, different wetting properties. The surfaces of polymers are not rigid but are able to reorient side chains and chain segments. Rough surfaces exhibit greater hysteresis than smooth surfaces, although when roughness is less than about 0.1-0.5 microns, hysteresis is negligible (Morra, Occhiello, and Garbassi, 1990). Hysteresis due to inhomogeneities is negligible when the hetergeneous phase is less than about 0.1 micron.

2.3.9 Liquid Surface Tension Measurements

Measurements for liquid surface tension can be made directly using one of several methods. Du Nouy (1918) and Harkins and Jordan (1930) discussed the fundamental theory and apparatus of the ring method. The Wilhemy plate method, used in this work, measures the force required to pull a plate of known dimensions from a liquid. The force can then be related to the property of liquid surface tension. Other methods include the capillary rise method and the drop weight method.

2.4 Physiological Factors of Microbial Adhesion

2.4.1 Introduction

Microbial adhesion to a surface is a complicated process influenced by many factors including physiological aspects of the cell. Parameters

such as cell viability, metabolic activity, protein or polysaccharide synthesis, cell wall function, and others may play a role in adhesion. The mechanism of adhesion differs between species of organisms and types of substratum surfaces. In order to understand the adhesion of a particular organism in a specific environment, a series of parameters must be systematically examined and their relative significance determined.

2.4.2 Effect of Ionic Strength and pH

Bacteria and most solid surfaces have a net negative charge. The electrostatic repulsion between surfaces that have a like charge inhibits the surfaces from approaching each other closely enough for adhesion to occur (< 0.5 nm). Adhesion of cells to a surface is possible if attractive forces are greater than the repulsive forces. An increase in electrolyte concentration has been correlated to a decrease in the electrophoretic mobility of particles as a result of compression of the electrical double-layer surrounding the particle. Therefore, adhesion that is controlled by electrostatic forces is expected to increase as the electrolyte concentration increases. Beyond a maximum value of electrolyte concentration, the adhesion remains constant.

Electrolytes may affect the molecular interactions governing the adhesion mechanism in several ways including: altering the physiological processes of the cell by influencing growth and, therefore, cell surface components; influencing short range electrostatic interactions by changing

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the conformation of the cell surface adhesives; and influencing hydrophobic interactions since higher electrolyte concentrations increase hydrophobic interactions (McEldowney and Fletcher, 1986a). Ionic strength and pH are known to influence the conformation of bacterial surface macromolecules (Abbott, 1983).

The influence of electrostatic interactions on adhesion is determined by measuring adhesion in solutions of various ionic strengths and pH values. Some electrolytes that have been used include NaCl, $MgCl_2$, $AlCl_3$, in concentrations between 0.001-0.5 M (McEldowney and Fletcher, 1986a; Abbott, 1983). Typical pH values range between 3.5-9, usually in half or whole step increments (Roger, 1990; Abbott, 1983). Studies indicate that electrolyte concentration does influence cell adhesion to surfaces, although no clear correlation has emerged between electrolyte concentration, valency, and adhesion for some bacterial species (McEldowney, 1986). The presence of electrolytes may inhibit, promote, or have no affect on adhesion depending on the organism used. This indicates that other mechanisms may also be contributing to adhesion, and the relative significance of these interactions is determined by the properties of the cell, surface, and liquid.

2.4.3 Hydrophobic Interactions

Hydrophobicity occurs when hydrophobic groups of a material, protein, macromolecule, or cell become concentrated in an aqueous environment (Gerson, 1980). The hydrophobic effect is recognized as an

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important factor in the organization of living matter (Tanford, 1978) and is involved in many phenomena including phagocytosis (ingestion of bacteria by cells), cell-cell and cell-substrate interactions, cellular immunity, motility, agglutination, and morphogenesis (Gerson, 1980). Hydrophobic interactions between surfaces are the result of the interaction of nonpolar groups on the surfaces. Hydrophobicity is known as the property of surface tension, and is quantitatively determined using surface energies (Absolom, 1986).

McEldowney and Fletcher (1986a) tested the role of hydrophobic interactions by evaluating the effect of various surfactants on the detachment of bacteria from polystyrene surfaces. The surfactants used included sodium dodecyl sulfate, Tween 80, and RBS 25. Bacteria were allowed to adhere for 60 minutes on polystyrene or tissue culture dishes, the adhesion was quantified, detergent was added and left in the dish for 60 minutes, and number of adhering bacteria was again counted. Detachment was either promoted or not affected by the presence of detergents depending on which organism and detergent was tested. In general, greater detachment was observed on polystyrene dishes, the more hydrophobic surface, indicating that hydrophobic interactions are important in some adhesion mechanisms.

2.4.4 Viability and Integrity of the Cell Surface

The surface of the cell wall is recognized as an important factor in the adhesion of some microorganisms to surfaces. Treatments to determine if

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adhesion is affected by the cell wall include exposure to UV radiation, heat, and formalin. UV exposure kills the cell by inactivating the DNA but leaves the cell otherwise unaltered. Heat and formalin treatments kill the cell and destroy the cell wall. Cell adhesion that is dependent on only cell wall integrity should be inhibited by heat and formalin treatment but unaffected by UV exposure.

Meadows (1971) reported that the adhesion of three strains of motile bacteria killed by heat or formalin was reduced while UV had little effect. This implies that the integrity of the cell surface is important for adhesion but an active metabolism is not necessary. Seow and coworkers (1987) studied the adherence to nylon fibers of *Candida albicans*, a yeast responsible for many infections of skin and mucous membranes. They reported that adhesion of heat and formalin treated cells was reduced compared to control samples. Mammalian cells killed by heat or formalin adhered to glass equally as well as living cells (Meadows, 1971). These findings confirm that viability and/or cell coat integrity is a necessary requirement of some cells, but not others, in order for adhesion to occur.

2.4.5 Substratum Conditioning Films and Protein Adsorption

Organic molecules in the bulk liquid transfer to the substratum within minutes of exposure to form a conditioning film which alters the surface properties of the substratum. This process is much faster than other biofilm processes. The organic molecules, usually polysaccharides or glycoproteins,

form a dynamic layer as individual molecules adsorb, desorb, and are replaced by others even though the total material remains the same or increases in volume. Research has not addressed the homogeneity of the film on the surface and so the film may adsorb in a nonuniform distribution. The film influences substratum properties by changing the hydrophobicity, altering both positively and negatively charged surfaces to net negative charges, and either increasing or decreasing zeta potentials and critical surface tensions depending on the initial surface energy. Although a conditioning film forms before microbial adsorption occurs, there is no evidence that indicates that the film is necessary for cell adsorption.

Meadows (1971) studied bacterial adhesion onto protein-coated slides using different proteins (salmine, albumin, casein, and gelatin) representing a range of isoelectric points. Although attachment either increased or decreased in the presence of proteins, there was no apparent correlation with the isoelectric point or chemistry of the protein. Fletcher (1976) reported a similar study using a marine bacterium and the proteins albumin, gelatin, fibrinogen, pepsin, protamine, and histone. She suggested that inhibition of attachment by protein films is probably due to a non-electrostatic means such as steric exclusion on the surface by the absorbed protein. A comparison of microbial adhesion on surfaces conditioned with similar protein layers shows that initial substratum surface energy is still influential in the adhesion process. For example, Baier (1980) showed a correlation between mammalian adhesion and initial substratum surface energy on

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protein-coated surfaces. These and other studies indicate that proteins of different types influence microbial adhesion in different ways.

2.4.6 Role of Extracellular Polymeric Substances

Extracellular polymeric substances (EPS) are thought to be an important component of irreversible adhesion although they do not appear to be necessary for reversible adsorption. Polymeric bridging between irreversibly adhered cells and the substratum has often been observed using electron microscopy. Cells may produce different types of EPS with various compositions of polysaccharides, lipids, and/or proteins. The chemical compositions and rate of production of EPS may change during the lifecycle of the microorganism (Characklis, 1990).

EPS may account for 50-90% of the organic carbon of a biofilm (Christensen and Characklis, 1990). EPS may exist either as a hydrated capsule surrounding a cell or as a viscous, soluble slime. The physical properties of the extracellular polymers, which are mostly polysaccharides, are important in understanding the behavior and properties of biofilms. The polysaccharides may exhibit an amphophilic character due to the hydrophilic hydroxyl groups on the carbohydrates and the hydrophobic groups such as methyl and acetyl groups. EPS may protect the cell from external forces, provide a means of communication between and within cells, serve as an energy storage site, and retard chemical toxins.

Marshall (1986) proposed that if EPS are made in response to a surface, then irreversible adhesion would be a time-dependent function. If EPS exist before the cell approaches the surface, then irreversible adhesion is probably a rapid process. His experiments indicated that irreversible adhesion of bacteria was time-dependent suggesting that EPS synthesis is a response of the organism to the surface.

2.4.7 Metabolic Inhibitors and Other Factors

Roger and coworkers (1990) examined a wide series of physicochemical factors to determine their effect on the adhesion of two species of rumen bacteria to cellulose avicel. The factors included metabolic, hydrophobic, and electrostatic factors. The metabolic inhibitors used in Roger's study included N,N'-dicyclohexylcarbodiimide (inhibitor of membrane ATPases) and antimycin A, hydroxyquinoline-N-oxide, and sodium azide (inhibitors of electron transfer chains).

Their conclusions suggest that the adhesion of each species was affected by a different combination of factors indicating that different mechanisms of adhesion exist for each organism. Adhesion of *Ruminococcus flavefaciens* was not dependent on metabolism or high temperature but was influenced by the interaction of the glycocalyx and the divalent cations Ca^{2+} and Mg^{2+}, and hydrophobic interactions. Adhesion of *Fibrobacter succinogenes* subsp. *succinogenes* appeared to require a

functioning metabolism and was mediated by proteins and bacterial cellulases.

Cell exposure to a variety of other chemicals has aided in understanding the influence of the cell surface and, more specifically, components of the cell surface or its extracellular coat on adhesion. The activity of periodate, which denatures exopolysaccharides on a cell surface, and pepsin on the glycocalyx of ruminal bacteria indicates that cell coat integrity is essential for adhesion (Latham, 1980). Treatment of cells with chloramphenicol, an inhibitor of protein synthesis, did not cause detachment of aquatic bacteria from petri dishes whereas detachment was increased by protease, which non-specifically denatures protein (McEldowney and Fletcher, 1986b). This indicates that protein synthesis after adhesion has occurred is not important for sustaining adhesion. In another study, adhesion was suppressed when fungal cells were treated with amphotericin B, an antibiotic that binds to ergosterol and other sterols of fungal cell membranes and disrupts cell membrane function (Seow *et al.*, 1987). Other work has shown that the influence of treatments to alter proteins, polysaccharides, or other components of the cell surface either promoted, inhibited, or had no consistent effect on adhesion. This shows that the cell response to various treatments is organism-specific and can not be generalized for microbial adhesion.

Many other factors have an effect on microbial adhesion including culture temperature (Roger, 1990; Seow, 1987; Fletcher, 1977), cell age

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(Roger, 1990; Fletcher, 1977), concentration of cells used for adsorption (Seow, 1987), time of contact (Roger, 1990; Mozes, 1987; Seow, 1987; Uyen *et al.*, 1985; Fletcher, 1977), and the presence of metabolic inhibitors, electron transport chain inhibitors, monovalent and divalent cations such as Na^{+}, Mg^{+2}, or Ca^{+2}, or carbohydrates (Roger, 1990).

2.4.8 Adsorption Kinetics

The kinetics of microbial adhesion to surfaces has been described in various ways. Neumann and coworkers (1980) reported that platelet adhesion onto various surfaces followed Langmuir isotherm kinetics and reached equilibrium in 120-180 minutes. The adhesion of oral streptococci to solids reached equilibrium in 20 minutes with a characteristic lag time of 1-15 minutes (Uyen *et al.*, 1985). Others have reported sigmoidal adsorption curves with a characteristic lag time. For example, the adhesion of mouse tumor cells to coverslips exhibited a lag time of 25 minutes which was proposed to be the time necessary for the modification of the cell surface in response to the substratum and/or the production of an adhesin (Weiss and Harloss, 1972). A two-step mechanism for attachment was proposed for the adhesion of oral bacteria to polymer surfaces in which the first step is determined by macroscopic properties of the system, such as substratum surface energy (Busscher *et al.*, 1986). Step 2 was postulated to depend on the response of the cell to the surface. One important point of kinetics research is to establish the equilibrium conditions of a system. This

is important for thermodynamic adhesion studies, such as the surface energy approach, which are applicable in theory only to systems in thermodynamic equilibrium.

Mueller and coworkers (1992) measured the rate coefficients for each process of bacterial accumulation, including adsorption, desorption, growth, and erosion, in a flow system in order to determine the net accumulation. Their goal was to model initial colonization and combine these models with other models for mature biofilms in order to predict the accumulation and activity of a biofilm in various environmental conditions. Their results indicate a positive correlation of the adsorption rate coefficient with cell hydrophobicity, surface free energy, and substratum roughness. Because cell and substratum characteristics vary with time, adhesion experiments should be conducted as a function of time.

In view of this background, adhesion behavior must be determined specifically for *P. chrysosporium*. Cell metabolism, the expression of particular compounds including proteins and polysaccharides, the environment of the cell (e.g. type of reactor, adhesion surface, hydrodynamics, oxygenation, and nutrient supply), and cell adhesion to specific surfaces are all interrelated. Therefore, the applications for which this fungus would be used should be considered in order to design relevant experiments.

CHAPTER 3.

MATERIALS AND METHODS

3.1. Maintenance and Cultivation of Organism

P. chrysosporium BKM-F 1767 (ATCC 24725) was maintained on 2% malt agar slants as described by Jager *et al.*, (1985). The organism was subcultured in petri dishes containing 2% malt agar until an even mat of conidia was produced. The conidia were harvested using 0.1 M phosphate buffer saline and frozen for up to three months. The optical density (OD) of the conidia was measured at 650 nm in 1.5 ml polystyrene cuvettes. An absorbance of 1.0 is approximately equal to 5 x 10^6 conidia per ml (Tien and Kirk, 1988).

Mycelial inoculum for experiments was prepared by inoculating one Fernbach flask containing 75 ml medium with thawed conidia to 1.0 OD at 650 nm (Kirk *et al.*, 1986; Tien and Kirk, 1988). The Fernbach flask was incubated at 37°C for 48 hours to form a thin mycelial mat on the surface of the medium. The contents of the flask were homogenized using a Virtis blender (Gardiner, NY; catalog #6301 0001 0C) at the highest speed for five minutes. An ice-water bath was used during homogenization to prevent heat damage to the mycelia. Homogenized mycelia were then prepared for adhesion experiments as described below.

3.2 Adhesion Tests on Microscope Slides

The microscope slides used for most of the adhesion tests were Superfrost® Plus treated glass slides (Fisher Scientific, Catalog #12-550-15). The microscope slides were cleaned by soaking overnight in 10% nitric acid solution, copius rinsing with distilled water, and air drying. Nunc Lab Tek® Chamber Slide™ culture chambers (Baxter Diagnostics Inc., Catalog #T4136-8 for Permanox® treated slides; Catalog #T4135-8 for glass slides) were used for adhesion tests. These culture slides consisted of a removable plastic chamber which was divided into eight wells (Figure 3.1). The chamber was attached to a microscope slide with a silicone gasket to prevent leaking.

To prepare inoculum for adhesion tests, homogenized mycelia were poured into a 50 ml disposable centrifuge tube and centrifuged for three minutes at a setting of seven (the highest setting). The supernatant was discarded, and the cells were resuspended in distilled water and centrifuged again. The centrifuge steps were repeated three times. Finally, the desired cell concentration, usually a 10% of the original cell concentration, was prepared. The effect of various factors on adhesion was tested by exposing the cells to chemical treatments to alter either the cell metabolism or cell surface. Controls for each experiment consisted of untreated cells which had been washed and resuspended in distilled water.

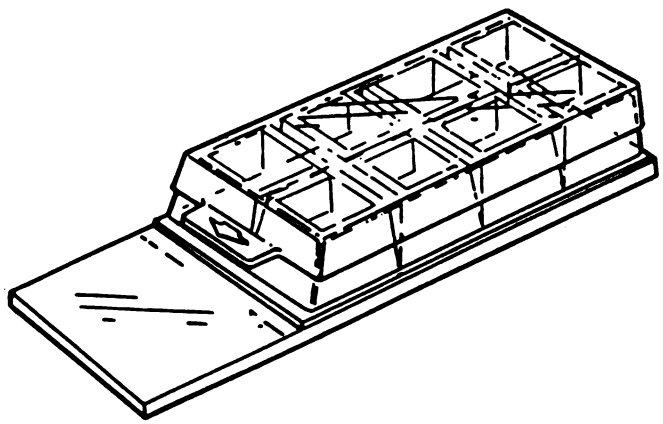

Figure 3.1 Culture chambers used for adhesion tests.

Adhesion tests were conducted by pipeting 0.3 ml of cell inoculum into each well of the culture chamber. Cells were contacted with the slides for 30 minutes. The cell medium was then discarded, and the chamber and gasket were removed from the slide. The slide was gently dipped three times in distilled water to remove unattached cells, fixed in 2% gluteraldehyde, stained with crystal violet, rinsed once with water, and air dried. Permanent specimen slides were prepared by mounting two coverslips on each slide using Accu-Mount 60™ mounting medium (Baxter Diagnostics, Inc., Catalog #M7630-1).

3.3 Use of Laser Microscopy to Measure Adhesion on Slides

Cell adhesion on microscope slides was determined using a Zeiss 10 laser scanning microscope (LSM) to measure the percent area of the slide surface covered by mycelia. This measurement is a feature of the LSM computer software as part of the histogram command. Samples viewed with the laser microscope transmit light at various wavelengths which the microscope computer converts into a histogram. The computer then determines the percent of the total area which is covered by the sample. A well-defined is obtained by increasing or decreasing the contrast and brightness.

Images were viewed in transmittance mode using a 10x objective and a zoom factor of 20. The total area of each measurement was 0.7549 mm^2.

Five to seven measurements were taken of each sample at random points on the slide. The mean and standard deviation of the replicates were calculated and compared to a control using the statistical t-test.

3.4 Methods Used To Determine Role of Physiochemical Factors

This section describes the various treatments used for adhesion studies of *P. chrysosporium* on microscope slides (Chapter 4). Mycelial cells were exposed to chemicals which affected either the metabolic activity or the surface of the cells. The amount of adhesion of treated cells was then compared to a control (cells which had not been exposed to a particular treatment.)

3.4.1 Viability and Integrity of the Cell Surface

In order to focus on the adhesion of cells to surfaces, it was necessary to determine whether the suspending fluid affected the adhesion process. The extracellular fluid of the inoculum may contain many components including proteins and polysaccharides which may spontaneously adhere to a substratum surface. To determine if the suspending fluid affected adhesion, cells were centrifuged and resuspended in distilled water three times. Adhesion of the washed cells was compared to adhesion of cells that were not washed (i.e. homogenized mycelial suspension was diluted with distilled water and then used directly for adhesion tests without centrifuging).

To establish whether viable cells were necessary for cell adhesion to a surface, cells were exposed to UV radiation and heat. UV exposure kills the cell by inactivating the DNA but leaves the cell surface unaltered. Heat kills the cell and destroys the cell wall. Cells were prepared for adhesion tests on microscope slides by pipeting 3 ml of washed cells into several small petri dishes. Cells were exposed to heat at 212°F for 60 minutes or to UV radiation from a UV lamp for 60 minutes. The cells from each treatment were gently vortexed and then contacted with SuperfrostPlus® microscope slides for 30 minutes. Adhesion was measured as the percent area covered by mycelia as described earlier.

Preliminary work was done to establish the level of UV or heat exposure required to kill the cells used for adhesion tests. Cells were exposed to UV radiation or heat for various time periods. The cells were then used to inoculate 125 ml shake flask cultures (3 ml of cells per 30 ml medium) containing one preweighed roughened polyethylene coupon (grit 40, ¾ inch diameter, 1/8 inch thick) and cultured for 24 hours. Biomass dry weight on the coupons was measured and compared to the biomass dry weight of cells that were not exposed to heat or UV. From this testing, a 60 minute exposure time to heat or UV radiation was found to kill the majority of the cells.

3.4.2 Effect of Contact Time, Cell Concentration and Age, Medium, and Temperature

In these experiments, the effect on adhesion of various factors such as cell contact time with the microscope slide, cell concentration, suspending medium, age of the cells, and temperature of the surrounding environment was determined. Adsorption isotherms for a range of cell concentrations and contact times indicate whether adhesion is a time-dependent process or occurs instantaneously. A series of adhesion experiments used four dilutions of inoculum (10%, 5%, 2%, and 1% of the initial inoculum concentration) and contact times of 5, 10, 20, 40 and 90 minutes. A separate culture chamber/microscope slide was used for each test.

Adhesion using various suspending media were tested using distilled water, 0.15 M NaCl, phosphate buffered saline (0.01 M, pH 7.4), and *P. chrysosporium* culture medium (Kirk *et al.*, 1986) with and without the detergent Tween 80 (1.0% and 0.5% v/v Tween 80). The purpose of testing different media was to choose an appropriate fluid for subsequent adhesion studies.

In order to test the effect of temperature, adhesion was measured at -20, 4, 22, 37, and 55°C. Cells and culture chambers were placed at each temperature for 20 minutes before the chambers were inoculated. Washed cells were diluted by a factor of 5 and suspended in distilled water. Cells were contacted with the slides for 30 minutes. Adhesion studies were also conducted using inoculum of two different ages. Adhesion was compared

using cells cultured in Fernbach flasks for 48 hours (the usual time period) and for 96 hours.

3.4.3 Determination of Cell Growth During Adhesion Tests

In order to distinguish cell adhesion from growth, an experiment was performed to determine if significant mycelial growth had occurred during the adhesion test. Cell inoculum was diluted to 10% of the original concentration. For Case 1, cells were contacted with a microscope slide for 15 minutes. The slide was rinsed gently with distilled water to remove any unattached cells and then stained. The amount of cell adhesion (percent area of slide covered by cells) was compared to two other cases. In both cases, cells were contacted with a slide for 15 minutes and then the slide was rinsed to remove unattached cells. The culture chamber was refilled with either distilled water (Case 2) or Fernbach medium (Case 3). After 45 minutes of additional contact time, the slides were rinsed again, stained, and then adhesion was measured.

For Case 2, cell growth would not be expected in distilled water, and adhesion should be equivalent to Case 1. A higher value of adhesion for Case 3 compared to Case 1 would indicate that cell growth occurs with the Fernbach medium during the 45 minute contact time. If significant cell growth does occur under these conditions, then it would need to be distinguished from irreversible attachment, which is the focus of the adhesion experiments of this study. As a control, adhesion was measured on

cells contacted with a slide for 60 minutes (time when amount of adhesion has reached equilibrium) with no intermediate rinsing step after 15 minutes.

3.4.4 Preparation of Metabolic Inhibitors

The effect of various metabolic inhibitors on the adhesion of mycelia to Superfrost® glass slides was tested. The following metabolic inhibitors were purchased from Sigma Chemical Company: 2,4-dinitrophenol (#D-7004), N,N'-dicyclohexylcarbodiimide (DCCD) (#D-3128), sodium azide (#S-2002), antimycin A (#A-8674), lasalosid (#L-1021), and monensin (#M-5273). The effect of each of these chemicals is listed in Table 3.1. A stock solution of each inhibitor was prepared using ethanol as shown in Table 3.2 except for sodium azide for which distilled water was used. The specified volume of each inhibitor was added to a cell suspension to give a total volume of five ml. The cells were incubated with the metabolic inhibitor at room temperature for 20 minutes before the adhesion tests were begun. The method is described below.

Metabolic Inhibitor Adhesion Tests

1. Microscope slides were cleaned in 10% HNO_3.
2. The contents of one Fernbach was homogenized five minutes and 15 ml was poured into two 15 ml centrifuge tubes.
3. The cells were centrifuged at a setting of 7, the supernatant was discarded, and the cells were resuspended in distilled water. This step was repeated three times.
4. A 5x dilution of cells was prepared (1 ml cells + 4 ml distilled water).
5. The cells were centrifuged 3 minutes and the supernatant was discarded.
6. The specified volume of metabolic inhibitor was added to the cells, the volume was brought to 5 ml with distilled water, and the suspension was vortexed and then incubated for 20 minutes at room temperature.

Table 3.1

Table 3.1 Effect of Metabolic Inhibitors

Chemical	Metabolic Effect
2,4-dinitrophenol	proton ionophore
DCCD	membrane ATPase inhibitor
Sodium azide	electron transport inhibitor
Antimycin A	electron transport inhibitor
Lasalocid	metal ionophore
Monensin	metal ionophore

Table 3.2 Preparation of Metabolic Inhibitor Stock Solutions

Metabolic Inhibitor	Conc. (mM)	Formula Weight	Stock Solution		Volume used per 5 ml (ml)
			Chemical (g)	Solvent† (ml)	
2,4-D	1.6	184	0.0108	1	0.15
DCCD	0.02	206.3	0.0069	50	0.15
NaN_3	40	65.01	0.0130	2	2
Antimycin A	0.1	500	0.0033	2	0.15
Lasalocid	0.02	612.8	0.0102	25	0.15
Monensin	0.02	692.9	0.0115	25	0.15

†The stock solution of NaN_3 was made with distilled water. All other stock solutions were made with ethanol.

7. 0.3 ml of the cell suspension was pipeted into each cell well of the culture chamber. A separate culture chamber was used for each metabolic inhibitor.
8. The culture chambers were incubated 40 minutes at room temperature before they were disassembled and the slides were rinsed, fixed, stained, and viewed using LSM.

3.4.5 Preparation of the Cell Treatment Actinomycin, Periodate, Protease, and Amphotericin B

The role of proteins, exopolysaccharides, and the cell membrane in adhesion was examined using chemicals which alter these components. Actinomycin D (Sigma, #A-5156), a chemical which limits the protein synthesis of eukaryotic organisms, was prepared at concentrations of 0.1, 0.01, and 0.001 μg per ml. A 1% w/v solution of sodium m-periodate (Sigma, #S-1878), which denatures exopolysaccharides, was prepared by adding 0.1 g to 10 ml of distilled water. Protease (Sigma, #P-0384), which degrades proteins, was prepared in a concentration of 1 unit of activity per 5 ml (i.e. 0.0043 g protease to 15 ml of distilled water for the particular batch used). Cell inocula for adhesion tests on microscope slides were prepared as described for the metabolic inhibitor adhesion tests, except that the cells were incubated for 30 minutes when resuspended in the cell treatment.

A chemical which disrupts fungal cell membrane function, amphotericin B (Sigma, #A-9528, solubilized), was prepared at concentrations of 0.45, 0.045, and 0.0045 μg per ml using distilled water. Mycelial inoculum was prepared by adding 15 ml of homogenized mycelia

into four 15 ml centrifuge tubes. The cells were centrifuged and resuspended in distilled water two times. The cells were centrifuged once more and resuspended in amphotericin B solution. After incubation for 60 minutes at room temperature, the cells were centrifuged and resuspended in distilled water to remove the amphotericin B. A dilution was made to give a cell concentration equivalent to 10% of the initial value. Adhesion on microscope slides was measured after 30 minutes.

3.4.6 Preparation of Electrolytes

Solutions of NaCl at various ionic strengths (0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 M) were prepared. Solutions of $MgCl_2$ and $AlCl_3$ at 0.005 and 0.001 M concentrations were also prepared. Aliquots of cells (5 ml) were centrifuged and resuspended in 5 ml of the appropriate solution three times before the cells were diluted with distilled water to 10% of the original concentration. Adhesion studies were performed using Superfrost Plus glass microscope slides and a contact time of 30 minutes. Cells washed in deionized distilled water were used as a control.

3.4.7 Adhesion to Polymer Coupons in Shake Flask Cultures

Cell response to a stimulus may or may not be affected by the environment. Adhesion studies were conducted in shake flask cultures containing polymer coupons and compared to adhesion on glass microscope slides. Shake flask cultures provide a means to simulate the hydrodynamic

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and oxygenation conditions of immobilized cell bioreactors such as, for example, rotating biological contactors used in treatment of waste effluents. Cells were cultured in 125 ml flasks containing 30 ml of medium and a 10% inoculum (Kirk *et al.*, 1986). Chemical treatments were added to each flask to provide a final concentration equivalent to that used for adhesion studies on microscope slides. Controls were prepared using distilled water. Each flask contained one preweighed polyethylene coupon (3/4 inch diameter and 1/8 inch thickness) roughened by hand with 40 grit sandpaper. Flasks were incubated at 37°C on an orbital shaker (190 rpm) and oxygenated with pure oxygen once each day. The coupons were removed after one week and rinsed with distilled water. Biomass dry weight on each coupon was determined.

3.5 Methods to Determine Role of Polymer Surfaces on Adhesion

Adhesion of *P. chrysosporium* was measured on polymer coupons in shake flask cultures and correlated to theoretical predictions of adhesion based on the equation of state approach (Chapter 5). The steps of this process are outlined in the flowchart of Figure 3.2. Cells were cultured and maintained as described in Section 3.1.

3.5.1 Surface Energy Determination of Liquids

Several liquids with various values of surface tension were used in this work. For cell adhesion studies, the surface tension of cell medium was

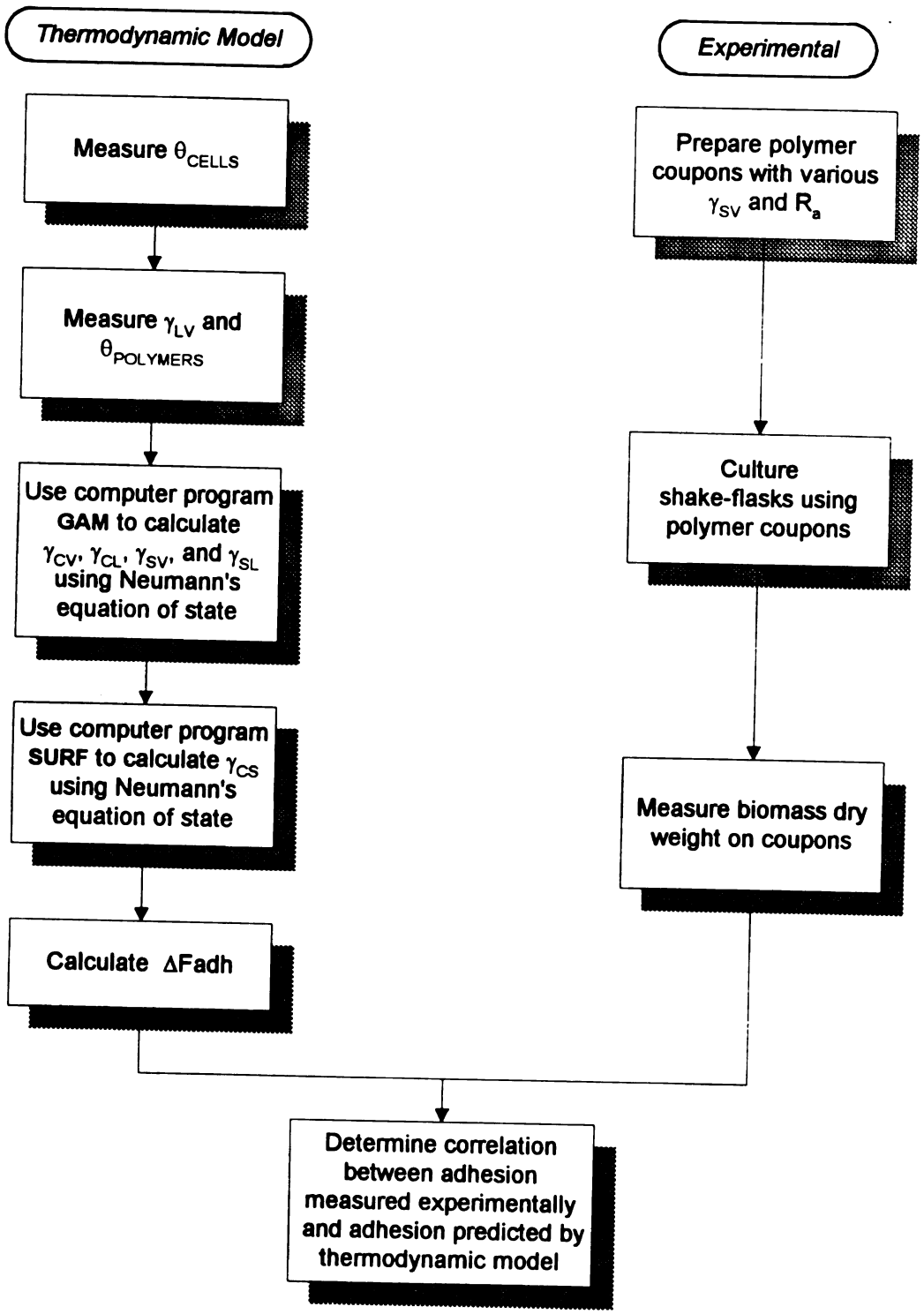


Figure 3.2 Flowchart for thermodynamic approach to predict adhesion of *P. chrysosporium* to polymer surfaces.

adjusted by the addition of the surfactant polyoxyethylene monooleate (Tween 80). Tween 80 is a normal component of *P. chrysosporium* culture medium (Kirk and Tien, 1988; Kirk *et al*, 1986) and provided a convenient way to adjust the surface tension of the medium. Contact angle measurements were made using deionized distilled water on polymer surfaces and 0.15 M sodium chloride on films of biomass.

The surface free energy of the liquids, γ_{LV}, was determined using the Wilhemy plate technique. This technique correlates the force required to withdraw a flat plate (i.e. glass coverslip) from a liquid to the surface tension of the liquid. Measurements were made using a Cahn Dynamic Contact Angle Analyzer (model DCA-322) in the Composite Materials and Structures Center of Michigan State University. Approximately 50 ml of the sample liquid was poured into a 100 ml beaker and placed on the stage of the analyzer. Moisture and contamination were removed from glass coverslips by passing them through the flame of a propane torch before placement in the instrument. Operating settings included a platform speed of 22.02 microns/second and a 10 mm cycle depth. The glass beaker was cleaned before use with Nochromix and then rinsed with distilled water.

3.5.2 Preparation of Polymer Surfaces

Low density polyethylene (LPDE), polytetrafluoroethylene (PTFE), and polystyrene (PS) were obtained from McMaster-Carr Company,

Chicago, IL. Acetal was purchased from Almac Plastics, Detroit, MI. The polymers were purchased as sheets of 1/8 inch thickness. Sulfonated polystyrene (SPS) was prepared by sulfonating polystyrene sheets at the Composite Materials and Structures Center of Michigan State University. Details of the sulfonation procedure are found in Appendix 3. Round disks, or coupons, with a 3/4 inch diameter were prepared using a punch press. The coupons were roughened using an electric hand-held sander and sandpaper with grit size 40.

Before use, the coupons were handled with Teflon-coated forceps and cleaned. PTFE, LPDE, and acetal were cleaned by sonication in methanol for 15 minutes. The methanol was discarded and replaced by fresh methanol and the sonication step was repeated for another 15 minutes. The polymers were removed from the methanol, blotted with tissue, and allowed to thoroughly air dry. PS was placed in a beaker of methanol for five minutes. The methanol was replaced and the step repeated. The PS was removed, blotted with tissue, and air dried. SPS was immersed in hexane for ten seconds, blotted with tissue, and air dried.

3.5.3 Characterization of Polymer Surfaces

Contact angles on the polymer surfaces were measured using a goniometer (Model #100-00 115, Rame-Hart, Inc., Mountain Lakes, NJ). A Pipetman pipet (volume of 0-200 µl) was used to deliver 4 µl drops (drop

diameter of about 2.5 mm) of deionized distilled water on the polymer surface. A minimum of 25 contact angle measurements were made to obtain an average value.

The roughness of the polymer surfaces was measured using a laser scanning microscope in reflection mode with a 50x objective. Three-dimensional images of the polymer surfaces were produced using sectioning techniques. The optical sections were 0.8 microns thick and the dimensions of the imaged sample was 189 x 284 microns. Photographs were taken using Kodak T-max 100 film for black and white prints and Kodak Ektachrome 100 for color slides. The topography software of the LSM calculated the mean roughness, R_a, and the averaged surface roughness, R_z, according to the following formulae.

Mean value (mean height) is calculated from

$$z_m = (z_1 + z_2 + \ldots)/n \qquad (3.1)$$

where n is the number of points and is chosen by the computer software and z is the vertical distance from a peak to a valley at a particular point on the surface. Mean roughness (arithmetic mean deviation) is given by

$$R_a = (|z_1-z_m| + |z_2-z_m| + \ldots)/n \qquad (3.2)$$

Averaged surface roughness is calculated according to

$$R_z = (z_{max1}-z_{min1}+z_{max2}-z_{min2}+\ldots+z_{max5}-z_{min5})/5 \qquad (3.3)$$

where the numbers 1 to 5 refer to the 5 sections into which the image is split by the computer software.

3.5.4 Preparation of Mycelia for Contact Angle Measurements

The following procedure to prepare homogenized mycelia of *P. chrysosporium* for contact angle measurements was adopted from Klotz *et al.* (1985), Van Loosdrecht *et al.* (1987), and Van Pelt *et al.* (1984):

Contact Angle Measurements on Mycelia

1. Fernbach flasks were inoculated with thawed conidia at a concentration of 0.1 OD. After incubation at 39°C for 48 hours, the mycelial mats from three flasks were homogenized in a blender (Virtis Co., Inc., Gardiner, NY, catalog #6301 0001 OC) at high speed for 5 minutes. The blender was placed in an ice-water bath during homogenization to prevent heat damage to the cells.
2. The homogenized mycelia were transferred into four 50 ml centrifuge tubes and centrifuged for 10 minutes at a setting of 7.
3. The supernatant was discarded. 30 ml of distilled water was added to each tube. The tubes were centrifuged again for 10 minutes at the highest setting.
4. Step 3 was repeated twice.
5. The supernatant was discarded. The cells were transferred to a graduated cylinder and diluted to 150 ml with distilled water. The cell suspension was stirred using a magnetic stir bar.
6. The cell suspension was filtered (30 ml per filter) using GA-6 triacetate metricel membrane filters (0.45 μm, 47 mm diameter, plain, catalog #09-730-20, Fisher Scientific, Itasca, IL). The filters were placed in a glass fritted filter unit and filtered under a gentle vacuum.

7. The filters were placed onto agar plates (1% agar in 10% v/v glycerol in water) and allowed to come to an equilibrium moisture content (about 20 minutes).
8. The filters were removed from the agar and placed on a Whatman 41 filter for 5 minutes to remove excess moisture.
9. The filters were cut in quarters and each quarter was mounted on a microscope slide using poster tape (adhesive on both sides, removable).
10. Contact angles on cells were measured as a function of drying time using a goniometer equipped with a 10x objective lens. A green filter was used to enhance to contrast between the liquid drop profile and the substrate. 4 µl drops (drop diameter equal to 2.5 mm) of 0.15 M NaCl were delivered with a 0-200 ml Pipetman. A minimum of five measurements was taken at each time increment.

3.5.5 Calculation of Surface Free Energy of Polymers and Cells

The measured values for liquid surface tension and contact angles on cells and polymer surfaces were used to calculate the interfacial tensions γ_{CS}, γ_{SL}, and γ_{CL} using the equation of state approach. The strategies presented by Li and Neumann (1992) and Neumann *et al.* (1980) were used to develop two FORTRAN programs for a personal computer. Program *GAM* calculated γ_{SV} and γ_{SL} from θ_s and γ_{LV} where the subscript "s" refers to a solid phase such as, for example, polymers or cells. The Wegstein method was used in the program for root-finding. Program *SURF* calculated γ_{12} from γ_{1V} and γ_{2V} where the subscripts "1" and "2" refer to two different phases such as cells and polymers or cells and liquid. The logic and accuracy of the programs were tested by entering various values for parameters and comparing the output with values obtained from the conversion tables of Neumann *et al.* (1980). FORTRAN code for programs *GAM* and *SURF* is presented in Appendices 1 and 2, respectively.

The programs were applied in the following sequence:

1. Liquid surface tensions were measured using the Cahn instrument.
2. Contact angles on cells, θ_C, and polymers, θ_S, were measured using various liquids.
3. Program *GAM* was used to find γ_{CV} and γ_{CL} from θ_C and γ_{LV}.
4. Program *GAM* was used to find γ_{SV} and γ_{SL} from θ_S and γ_{LV}.
5. Program *SURF* was used to find γ_{CS} from γ_{CV} and γ_{SV}.

The free energy of adhesion, ΔF_{adh}, was then determined according to the equation

$$\Delta F_{adh} = \gamma_{CS} - \gamma_{SL} - \gamma_{CL} \qquad (3.4)$$

3.5.6 Shakeflask Adhesion Studies to Polymer Coupons

Homogenized mycelial suspensions were used as inocula for agitated cultures in nitrogen-limited medium according to the method described by Kirk *et al.* (1986) and Kirk and Tien (1988). The buffer was 10 mM 2,2-dimethylsuccinate (DMS) (pH 4.5). Agitated submerged cultures were grown in 125 ml Erlenmeyer flasks containing 30 ml medium (Kirk *et al.*, 1986) and 3 ml inoculum at 37°C on a G50 orbital shaker (New Brunswick Scientific Co., Inc.) at 190 rpm. Each flask contained one preweighed polymer coupon. The flasks were oxygenated once daily with pure oxygen. After 24 hours, the medium in each flask was filtered and then returned to the flask in order to remove any mycelia that had not adhered to the polymer coupon. Flasks were cultured for seven days to allow the biofilm

to complete the growth phase, secondary metabolic phase with production of lignin peroxidase, and to enter the death phase. After seven days, the coupon was removed from each flask, rinsed gently with distilled water, and dried. Biomass on each coupon was determined on a dry weight basis. Samples of culture fluid were collected daily for measurement of lignin peroxidase activity (Tien and Kirk, 1988).

3.6 Cell Characterization Using Microsphere Attachment Studies

Hydrophobicity of *P. chrysosporium* hyphae was observed using microspheres from Interfacial Dynamics Corporation (Portland, Oregon). Fluorescent sulfate polystyrene latex microspheres (Interfacial Dynamics Corp., catalog #L-5081) with a diameter of 1.0 μm were used to adhere to hydrophobic areas. The microspheres were yellow-green fluorescent with an excitation/emission wavelength at 490/515 nm.

Microsphere suspensions were prepared in small glass test tubes using phosphate-urea-MgSO4 buffer (PUM buffer, pH 7.1, Rosenberg, *et al.*, 1980) at a concentration of 7×10^8 beads/ml. The suspensions were gently vortexed and placed in an ice-water bath. A mycelial suspension was prepared by homogenizing the contents of one Fernbach flask at high speed for five minutes. The cells were then centrifuged in microcentrifuge tubes and resuspended in medium. Equal volumes of mycelial and microsphere suspensions were mixed by gently vortexing 30 seconds on a flat surface

vortexer. After allowing the microspheres to contact the cells for five minutes, the mixture was mounted onto a glass microscope slide with a coverslip.

The mycelia were imaged with the laser scanning microscope in transmission mode using a 40x objective. The microspheres were imaged in fluorescent mode using a section series with 1 micron section thickness and 5-20 sections, as needed. The images of the mycelia and the microspheres were combined using the "color overlay" feature.

CHAPTER 4.

FACTORS THAT AFFECT ADHESION OF *PHANEROCHAETE CHRYSOSPORIUM* TO SURFACES

Abstract

Microbial adhesion studies on bacteria, yeasts, and other types of cells have established that adhesion is influenced by many types of interactions among the cells and surface, the physiology of the cell, and other factors. The purpose of this work was to better understand the process of irreversible cell adhesion of the white-rot fungus *Phanerochaete chrysosporium* to polymer surfaces through the study of the physiological activity of the organism. Mycelial cells were exposed to various chemical treatments which affected either metabolic activity or the surface of the cells. The amount of adhesion to treated glass microscope slides was determined using laser scanning microscopy (LSM) to measure the percentage of the surface area of the slide covered with cells. A comparison of adhesion between treated and untreated cells indicated whether the treatment significantly affected the adhesion process. Several factors influenced adhesion including cell viability, metabolic function, cell wall function, protein synthesis, and the presence of proteins and polysaccharides. Electrostatic interactions did not appear to be significant. Subsequent experiments were conducted in shake flask cultures

containing a roughened polymer coupon to simulate the hydrodynamic and oxygenation conditions of immobilized cell bioreactors. The most significant factor required for adhesion in shake flasks was the presence of exopolysaccharides. These results will be useful in developing a better understanding of fungal adhesion and improve the design of bioreactors for the treatment of toxic environmental contaminants.

Introduction

The adhesion of cells to surfaces is a complex process that involves interactions between the cells, the surface, and the suspending medium as well as physiological processes of the cell. The focus of this work was to identify factors that affect the irreversible adhesion of *Phanerochaete chrysosporium* to surfaces. The role of specific physiological processes in the adhesion process was studied by altering those processes and observing the resulting response. The factors studied included various aspects of cell metabolism, the surface properties of the cell, and protein and polysaccharide production. Factors relating to the substratum material such as surface free energy or roughness were not considered in this study.

Microbial adhesion can be influenced by long range electrostatic interactions as well as short range attractive forces due to dipole interactions, chemical bonding, and hydrophobic interactions. Electrostatic interactions can be investigated by measuring adhesion in electrolyte solutions of different concentrations and valencies (McEldowney and Fletcher, 1986a). The effect

of hydrophobic interactions can be determined by measuring adhesion in solutions prepared with surfactants to provide different surface tensions in the suspending medium (McEldowney and Fletcher, 1986a).

Physiological processes of cells at surfaces, which may differ from those in the bulk aqueous phase, may either promote or inhibit adhesion (Mozes *et al*, 1987b). Roger and coworkers (1990) found that various metabolic inhibitors affected the adhesion of two species of rumen bacteria to cellulose differently. The integrity of the cell coat can sometimes be a critical factor for adhesion. Seow and coworkers (1987) reported that amphotericin B, an antibiotic that binds to ergosterol and disrupts fungal cell membrane function, suppressed adhesion of *Candida albicans* to nylon fiber. By the use of periodate, Latham (1980) found that exopolysaccharides on the cell surface were required for the adhesion of ruminal bacteria. Although protein synthesis during adhesion was not required for attachment of aquatic bacteria to solid surfaces, the presence of extracellular proteins was needed (McEldowney and Fletcher, 1986b).

Cell age (Roger, 1990) and culture temperature (Seow *et al*, 1987) are other critical factors that influence microbial adhesion. Kinetics of microbial adhesion has been explored by many groups for various organisms and related to steps in the adhesion process (Neumann *et al*, 1980; Uyen *et al*, 1985; Busscher *et al*, 1986). The mechanism of adhesion differs among organisms and must be investigated individually for each.

In this work, mycelial cells of *P. chrysosporium* were treated, usually with a chemical, for a specified amount of time and then contacted with a glass microscope slide. The amount of adhesion was measured by determining the percentage area of the slide covered by a monolayer of cells using laser scanning microscopy. The adhesion of treated and untreated cells was compared to determine the relative importance of each factor.

P. chrysosporium has the ability to degrade a wide range of toxic environmental contaminants by the extracellular production of lignin peroxidases (Boominathan and Reddy, 1992). The results of this study will help in understanding the adhesion behavior of *P. chrysosporium* and aid in bioreactor design for potential applications of enzyme production and effluent treatment. In addition, the approach presented here, including the use of laser scanning microscopy, to quantify the amount of fungal adhesion, is applicable to other mycelial adhesion studies.

Materials and Methods

Maintenance and Cultivation of Organism

P. chrysosporium BKM-F 1767 (ATCC 24725) was maintained on 2% malt agar slants as described by Jager *et al.*, (1985). The organism was subcultured in petri dishes containing 2% malt agar until an even mat of conidia was produced. The conidia were harvested using 0.1 M phosphate buffer saline and frozen for up to three months. The optical density (OD) of the conidia was measured at 650 nm in 1.5 ml polystyrene cuvettes. An

absorbance of 1.0 is approximately equal to 5 x 10^6 conidia per ml (Tien and Kirk, 1988).

Mycelial inoculum for experiments was prepared by inoculating one Fernbach flask containing 75 ml medium with thawed conidia to 1.0 OD at 650 nm (Kirk *et al.*, 1986; Tien and Kirk, 1988). The Fernbach flask was incubated at 37°C for 48 hours to form a thin mycelial mat on the surface of the medium. The contents of the flask were homogenized using a Virtis blender (Gardiner, NY; catalog #6301 0001 0C) at the highest speed for five minutes. An ice-water bath was used during homogenization to prevent heat damage to the mycelia. Homogenized mycelia were then prepared for adhesion experiments as described below.

Method to Measure Adhesion on Microscope Slides

The microscope slides used for most of the adhesion tests were Superfrost® Plus treated glass slides (Fisher Scientific, Catalog #12-550-15). The microscope slides were cleaned by soaking overnight in 10% nitric acid solution, copius rinsing with distilled water, and air drying. Nunc Lab Tek® Chamber Slide™ culture chambers (Baxter Diagnostics Inc., Catalog #T4136-8 for Permanox® treated slides; Catalog #T4135-8 for glass slides) were used for adhesion tests. These culture slides consisted of a removable plastic chamber which was divided into eight wells (Figure 4.1). The chamber was attached to a microscope slide with a silicone gasket to prevent leaking.

To prepare inoculum for adhesion tests, homogenized mycelia were poured into a 50 ml disposable centrifuge tube and centrifuged for three minutes at a setting of seven (the highest setting). The supernatant was discarded, and the cells were resuspended in distilled water and centrifuged again. The centrifuge steps were repeated three times. Finally, the desired cell concentration, usually a 10% of the original cell concentration, was prepared. The effect of various factors on adhesion was tested by exposing the cells to chemical treatments to alter either the cell metabolism or cell surface. Controls for each experiment consisted of untreated cells which had been washed and resuspended in distilled water.

Adhesion tests were conducted by pipeting 0.3 ml of cell inoculum into each well of the culture chamber. Cells were contacted with the slides for 30 minutes. The cell medium was then discarded, and the chamber and gasket were removed from the slide. The slide was gently dipped three times in distilled water to remove unattached cells, fixed in 2% gluteraldehyde, stained with crystal violet, rinsed once with water, and air dried. Permanent specimen slides were prepared by mounting two coverslips on each slide using Accu-Mount 60™ mounting medium (Baxter Diagnostics, Inc., Catalog #M7630-1).

Measurement of Adhesion Using Laser Microscopy

Cell adhesion was determined using a Zeiss 10 laser scanning microscope (LSM) to measure the percent area of the slide surface covered by mycelia. This measurement is a feature of the LSM computer software as part

of the histogram command. Samples viewed with the laser microscope transmit light at various wavelengths which the microscope computer converts into a histogram. The computer then determines the percent of the total area which is covered by the sample. A well-defined is obtained by increasing or decreasing the contrast and brightness.

Images were viewed in transmittance mode using a 10x objective and a zoom factor of 20. The total area of each measurement was 0.7549 mm^2. Five to seven measurements were taken of each sample at random points on the slide. The mean and standard deviation of the replicates were calculated and compared to a control using the statistical t-test.

Viability and Integrity of the Cell Surface

In order to focus on the adhesion of cells to surfaces, it was necessary to determine whether the suspending fluid affected the adhesion process. The extracellular fluid of the inoculum may contain many components including proteins and polysaccharides which may spontaneously adhere to a substratum surface. To determine if the suspending fluid affected adhesion, cells were centrifuged and resuspended in distilled water three times. Adhesion of the washed cells was compared to adhesion of cells that were not washed (i.e. homogenized mycelial suspension was diluted with distilled water and then used directly for adhesion tests without centrifuging).

To establish whether viable cells were necessary for cell adhesion to a surface, cells were exposed to UV radiation and heat. UV exposure kills the

cell by inactivating the DNA but leaves the cell surface unaltered. Heat kills the cell and destroys the cell wall. Cells were prepared for adhesion tests on microscope slides by pipeting 3 ml of washed cells into several small petri dishes. Cells were exposed to heat at 212°F for 60 minutes or to UV radiation from a UV lamp for 60 minutes. The cells from each treatment were gently vortexed and then contacted with SuperfrostPlus® microscope slides for 30 minutes. Adhesion was measured as the percent area covered by mycelia as described earlier.

Preliminary work was done to establish the level of UV or heat exposure required to kill the cells used for adhesion tests. Cells were exposed to UV radiation or heat for various time periods. The cells were then used to inoculate 125 ml shake flask cultures (3 ml of cells per 30 ml medium) containing one preweighed roughened polyethylene coupon (grit 40, ¾ inch diameter, 1/8 inch thick) and cultured for 24 hours. Biomass dry weight on the coupons was measured and compared to the biomass dry weight of cells that were not exposed to heat or UV. From this testing, a 60 minute exposure time to heat or UV radiation was found to kill the majority of the cells.

Effect of Contact Time, Cell Concentration, Medium, Cell Age, and Temperature

In these experiments, the effect on adhesion of various factors such as cell contact time with the microscope slide, cell concentration, suspending

medium, age of the cells, and temperature of the surrounding environment was determined. Adsorption isotherms for a range of cell concentrations and contact times were used to determine whether or not adhesion is a time-dependent process or occurs instantaneously. A series of adhesion experiments used four dilutions of inoculum (10%, 5%, 2%, and 1% of the initial inoculum concentration) and contact times of 5, 10, 20, 40 and 90 minutes. A separate culture chamber/microscope slide was used for each test.

Adhesion using various suspending media were tested using distilled water, 0.15 M NaCl, phosphate buffered saline (0.01 M, pH 7.4), and *P. chrysosporium* culture medium (Kirk *et al.*, 1986) with and without the detergent Tween 80 (1.0% and 0.5% v/v Tween 80). The purpose of testing different media was to choose an appropriate fluid for subsequent adhesion studies.

In order to test the effect of temperature, adhesion was measured at -20, 4, 22, 37, and 55°C. Cells and culture chambers were placed at each temperature for 20 minutes before the chambers were inoculated. Washed cells were diluted by a factor of 5 and suspended in distilled water. Cells were contacted with the slides for 30 minutes. Adhesion studies were also conducted using inoculum of two different ages. Adhesion was compared using cells cultured in Fernbach flasks for 48 hours (the usual time period) and for 96 hours.

Determination of Cell Growth During Adhesion Tests

In order to distinguish cell adhesion from growth, an experiment was performed to determine if significant mycelial growth had occurred during the adhesion test. Cell inoculum was diluted to 10% of the original concentration. For Case 1, cells were contacted with a microscope slide for 15 minutes. The slide was rinsed gently with distilled water to remove any unattached cells and then stained. The amount of cell adhesion (percent area of slide covered by cells) was compared to two other cases. In both cases, cells were contacted with a slide for 15 minutes and then the slide was rinsed to remove unattached cells. The culture chamber was refilled with either distilled water (Case 2) or Fernbach medium (Case 3). After 45 minutes of additional contact time, the slides were rinsed again, stained, and then adhesion was measured.

For Case 2, cell growth would not be expected in distilled water, and adhesion should be equivalent to Case 1. A higher value of adhesion for Case 3 compared to Case 1 would indicate that cell growth occurs with the Fernbach medium during the 45 minute contact time. If significant cell growth does occur under these conditions, then it would need to be distinguished from irreversible attachment, which is the focus of the adhesion experiments of this study. As a control, adhesion was measured on cells contacted with a slide for 60 minutes (time when amount of adhesion has reached equilibrium) with no intermediate rinsing step after 15 minutes.

Preparation of Metabolic Inhibitors

The effect of various metabolic inhibitors on the adhesion of mycelia to Superfrost® glass slides was tested. The following metabolic inhibitors were purchased from Sigma Chemical Company: 2,4-dinitrophenol (#D-7004), N,N'-dicyclohexylcarbodiimide (DCCD) (#D-3128), sodium azide (#S-2002), antimycin A (#A-8674), lasalosid (#L-1021), and monensin (#M-5273). The effect of each of these chemicals is listed in Table 4.1. A stock solution of each inhibitor was prepared using ethanol as shown in Table 4.2 except for sodium azide for which distilled water was used. The specified volume of each inhibitor was added to a cell suspension to give a total volume of 5 ml. The cells were incubated with the metabolic inhibitor at room temperature for 20 minutes before the adhesion tests were begun. The method is described below.

Metabolic Inhibitor Adhesion Tests

1. Microscope slides were cleaned in 10% HNO_3.
2. The contents of one Fernbach was homogenized five minutes and 15 ml was poured into two 15 ml centrifuge tubes.
3. The cells were centrifuged at a setting of 7, the supernatant was discarded, and the cells were resuspended in distilled water. This step was repeated three times.
4. A 5x dilution of cells was prepared (1 ml cells + 4 ml distilled water).
5. The cells were centrifuged 3 minutes and the supernatant was discarded.
6. The specified volume of metabolic inhibitor was added to the cells, the volume was brought to 5 ml with distilled water, and the suspension was vortexed and then incubated for 20 minutes at room temperature.
7. 0.3 ml of the cell suspension was pipeted into each cell well of the culture chamber. A separate culture chamber was used for each metabolic inhibitor.

8. The culture chambers were incubated 40 minutes at room temperature before they were disassembled and the slides were rinsed, fixed, stained, and viewed using LSM.

Preparation of the Cell Treatment Actinomycin, Periodate, Protease, and Amphotericin B

The role of proteins, exopolysaccharides, and the cell membrane in adhesion was examined using chemicals which alter these components. Actinomycin D (Sigma, #A-5156), a chemical which limits the protein synthesis of eukaryotic organisms, was prepared at concentrations of 0.1, 0.01, and 0.001 µg per ml. A 1% w/v solution of sodium m-periodate (Sigma, #S-1878), which denatures exopolysaccharides, was prepared by adding 0.1 g to 10 ml of distilled water. Protease (Sigma, #P-0384), which degrades proteins, was prepared in a concentration of 1 unit of activity per 5 ml (i.e. 0.0043 g protease to 15 ml of distilled water for the particular batch used). Cell inocula for adhesion tests on microscope slides were prepared as described for the metabolic inhibitor adhesion tests, except that the cells were incubated for 30 minutes when resuspended in the cell treatment.

A chemical which disrupts fungal cell membrane function, amphotericin B (Sigma, #A-9528, solubilized), was prepared at concentrations of 0.45, 0.045, and 0.0045 µg per ml using distilled water. Mycelial inoculum was prepared by adding 15 ml of homogenized mycelia into four 15 ml centrifuge tubes. The cells were centrifuged and resuspended in distilled water two times. The cells were centrifuged once more and resuspended in amphotericin B solution. After incubation for 60 minutes at room temperature, the cells

were centrifuged and resuspended in distilled water to remove the amphotericin B. A dilution was made to give a cell concentration equivalent to 10% of the initial value. Adhesion on microscope slides was measured after 30 minutes.

Preparation of Electrolytes

Solutions of NaCl at various ionic strengths (0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 M) were prepared. Solutions of $MgCl_2$ and $AlCl_3$ at 0.005 and 0.001 M concentrations were also prepared. Aliquots of cells (5 ml) were centrifuged and resuspended in 5 ml of the appropriate solution three times before the cells were diluted with distilled water to 10% of the original concentration. Adhesion studies were performed using Superfrost Plus glass microscope slides and a contact time of 30 minutes. Cells washed in deionized distilled water were used as a control.

Adhesion to Polymer Coupons in Shake Flask Cultures

Cell response to a stimulus may or may not be affected by the environment. Adhesion studies were conducted in shake flask cultures containing polymer coupons and compared to adhesion on glass microscope slides. Shake flask cultures provide a means to simulate the hydrodynamic and oxygenation conditions of immobilized cell bioreactors such as, for example, rotating biological contactors used in treatment of waste effluents. Cells were cultured in 125 ml flasks containing 30 ml of medium and a 10% inoculum (Kirk *et al.*, 1986). Chemical treatments were added to each flask to provide

a final concentration equivalent to that used for adhesion studies on microscope slides. Controls were prepared using distilled water. Each flask contained one preweighed polyethylene coupon (3/4 inch diameter and 1/8 inch thickness) roughened by hand with 40 grit sandpaper. Flasks were incubated at 37°C on an orbital shaker (190 rpm) and oxygenated with pure oxygen once each day. The coupons were removed after one week and rinsed with distilled water. Biomass dry weight on each coupon was determined.

Results and Discussion

Optical Density Measurements of Homogenized Mycelia

The absorbance of homogenized mycelia and various dilutions was measured using a spectrophotometer. The dilutions included samples that contained 2, 5, 10, and 20% of the original cell concentration. The relationship between absorbance and cell concentration was linear with a correlation coefficient of 0.9995 (Figure 4.2).

Effect of Cell Washing and Suspending Medium on Adhesion

Cells which were washed and resuspended in distilled water adhered to glass slides in high numbers while unwashed cells adhered poorly (Figure 4.3). Adhesion was tested using several other suspending media and compared to adhesion of cells suspended in distilled water. Adhesion decreased in solutions of 0.15 M NaCl, phosphate-buffered saline, culture medium with Tween 80, and culture medium without Tween 80 (Figure 4.4). Based on this

result, washed cells suspended in distilled water were used as the control in all subsequent adhesion experiments.

Effect of Cell Concentration and Contact Time on Adhesion

Adhesion was measured as a function of cell concentration and contact time with the slide. Homogenized mycelia were diluted by a factor of 10, 20, 50, and 100 times the initial cell concentration. Adhesion of each cell concentration was measured after 5, 10, 20, 40, and 90 minutes. Adhesion increased with increasing cell concentration up to a maximum value, specific for each concentration. Beyond this point, adhesion remained constant (Figure 4.5). Adhesion was a time-dependent process with most attachment occurring within the first 30-40 minutes. At that time, a plateau was reached and no additional adhesion occurred. This response is consistent with typical Langmuir adsorption kinetics.

Cell Growth During Adhesion Experiments

An experiment was conducted to determine whether measurable cell growth occurred during the adhesion experiment which should then be subtracted from the amount of irreversible cell attachment. Cell growth during adhesion was measured by comparing adhesion of cells after 15 minutes of contact time to cells that were contacted with a microscope slide for 15 minutes, rinsed, and the liquid replenished with either distilled water or Fernbach medium for 45 minutes. The results indicated that measurable cell

growth did not occur during the adhesion test for short contact times of less than one hour (Figure 4.6). Therefore, the measurements of cell adhesion in this work (measured as percent of surface area covered with cells) were considered to be from only cell attachment without the confounding factor of cell growth.

Cell Viability

Results suggest that *P. chrysosporium* cells need to be viable during the attachment process. Adhesion of cells was significantly reduced after exposure to either heat or UV radiation (Figure 4.7). Heat kills cells and denatures cell walls and other cell components. Exposure to UV radiation kills cells by inactivating DNA while leaving the cell surface unaltered. These results indicate that both an active metabolism and an intact cell surface are required for *P. chrysosporium* to adhere to surfaces.

The necessity of viability and/or an unaltered cell coat for adhesion differs between microorganisms. Meadows (1971) reported that mammalian cells killed by heat or formalin adhered equally well to glass as living cells. UV radiation had little effect on the adhesion of three strains of motile bacteria, while adhesion was reduced when the cells were killed by heat or formalin (Meadows, 1971). The yeast *Candida albicans* adhered to glass in lower numbers after exposure to heat and formalin (Seow *et al.*, 1987).

Effect of Treatments Involving the Cell Membrane, Proteins, and Polysaccharides

Further evidence that the cell surface plays a critical role in adhesion is given by amphotericin B. This chemical disrupts fungal cell membrane function and binds ergosterol, which is a component of fungal cell membranes (Seow *et al.*, 1987; Matcham *et al.*, 1988). Adhesion of cells exposed to amphotericin at a concentration of 0.45 ug/ml was statistically different from the control (Figure 4.8). Adhesion of cells exposed to lower concentrations of amphotericin B, 0.045 and 0.0045 ug/ml, was not statistically different from the control. As discussed earlier, the reduced adhesion of heat-killed *P. chrysosporium* cells indicated that the cell surface is a critical component of the adhesion process. Further evidence of this is seen from the results of amphotericin B treatment.

Actinomycin limits the protein synthesis of eukaryotic organisms. Exposing cells to various concentrations of actinomycin resulted in reduced adhesion (Figure 4.9). Adhesion of cells that were exposed to actinomycin and then rinsed before being contacted with the microscope slide was not statistically different from the control. This indicates that protein synthesis after adhesion has occurred is important for maintaining adhesion. As expected, chloramphenicol, which disrupts the protein synthesis of prokaryotes but not eukaryotes, did not alter the adhesion of the fungus.

Cells exposed to protease and periodate adhered in significantly lower numbers compared to the control (Figure 4.10). These chemicals nonspecifically degrade proteins and denature surface polysaccharides,

respectively. The suspending medium was distilled water and the glass microscope slides were acid-cleaned before the adhesion experiment. Thus, the cells themselves were the likely source of proteins and polysaccharides which adhered to the slide surface and inhibited cell adhesion.

Overall, these results suggest that a functioning cell membrane and protein synthesis is required for adhesion. In addition, exopolysaccharides and proteins associated with the cell are involved in the adhesion mechanism. This finding is consistent with microbial adhesion studies of other researchers in which treatments to alter cell surface components, protein synthesis, or the presence of proteins and polysaccharides of other organisms either promoted, inhibited, or had no effect on adhesion (McEldowney and Fletcher, 1986; Latham, 1980; Seow *et al.*, 1987).

Effect of Metabolic Inhibitors

Various metabolic inhibitors reduced cell adhesion indicating that metabolic activity is required for adhesion (Figure 4.11). Sodium azide and 2,4-dinitrophenol, which affect the electron transport chain, had the greatest effect on reducing adhesion. Adhesion was moderately decreased when cells were treated with DCCD, lasalocid, or antimycin A. No statistical difference on adhesion was measured for monensin compared to the control (i.e. no chemical treatment). These results complement the finding that adhesion is affected by treatment with UV radiation which terminates metabolic activity and destroys DNA. Microbial adhesion studies by other researchers have led

to the conclusion that a functioning metabolism is required for adhesion by some organisms but not others (Rogers *et al.*, 1990). It appears that *P. chrysosporium* is among those microorganisms for which a functioning metabolism is necessary for adhesion to surfaces.

Effect of Electrolyte Concentration and Valency

Evidence of the role of electrostatic interactions in adhesion can be determined by observing whether adhesion is promoted by increased electrolyte concentration or valency (McEldowney and Fletcher, 1987a). This was tested for *P. chrysosporium* by suspending cells in sodium chloride solutions ranging from 0.001 to 0.5 M. Adhesion decreased as the concentration of NaCl increased compared to the control (Figure 4.12). Significantly decreased adhesion was observed for other electrolyte solutions including $MgCl_2$ and $AgCl_3$ (Figure 4.13). This indicates that electrostatic interactions, or long range forces, are probably not a significant factor influencing adhesion of this organism.

Effect of Temperature and Cell Age on Adhesion

Temperature affected the amount of adhesion of cells to microscope slides. Adhesion was measured at -20, 4, 22, 37, and 55°C with the optimum temperature for adhesion found to be 22°C (Figure 4.14). Adhesion decreased for temperatures higher and lower than this value. This differs from the optimum temperature of 37°C required for the production of lignin peroxidase and manganese peroxidase.

The effect of cell age on adhesion was determined by comparing inoculum prepared from mycelia grown in Fernbach flasks for 48 hours (the standard length of time) and for 96 hours. No significant difference was observed (Figure 4.15). In another experiment, mycelial pellets cultured in shake flasks were removed each day during a two week period and used in adhesion tests on microscope slides. There did not appear to be a correlation between the amount of adhesion and the lifecycle of the fungus, which includes a growth phase of 1-2 days, a secondary metabolic phase of about eight days, and a death phase (Figure 4.16) .

Shake Flask Adhesion Studies

Potential industrial uses of *P. chrysosporium* for wastewater remediation or other applications include growing the organism in various types of reactors. The hydrodynamic and oxygenation environment of these reactors can be simulated in the lab using shake flask cultures. A comparison between cell adhesion on glass microscope slides and shake flask cultures containing polymer coupons showed that cell behavior can differ depending on the environment. Adhesion studies in shake flasks showed significantly reduced adhesion in cultures to which sodium azide, an electron transport inhibitor, and periodate, which denatures exopolysaccharides, had been added.

These results also occurred on glass microscope slides. No significant effect on adhesion was observed with additions of lasalocid (metal ionophore), amphotericin (disrupts cell wall function), actinomycin (inhibits protein synthesis), or protease (degrades extracellular proteins) although each of these chemicals decreased cell adhesion to glass microscope slides (Figure 4.17).

This indicates that differences exist in adhesion on glass microscope slides versus polymer coupons in shake flasks. The presence of polysaccharides is necessary for adhesion to polymers in shake flasks.

Conclusions

This work identified physicochemical factors involved in the adhesion of *P. chrysosporium* to glass microscope slides. Initial studies indicated that an active cell metabolism is required for adhesion. Subsequent studies showed that inhibiting the electron transport chain reduced adhesion. Protein synthesis and a functioning cell wall was required during adhesion. The presence of extracellular proteins and polysaccharides promoted adhesion. Exopolysaccharides were found to influence adhesion to polymer coupons in shake flask cultures and may be one of the more significant factors influencing adhesion to surfaces. Electrostatic interactions did not appear to play a significant role in adhesion. In addition to the physicochemical factors identified here, it is recognized that properties of the substratum surface and suspending medium also impact cell adhesion.

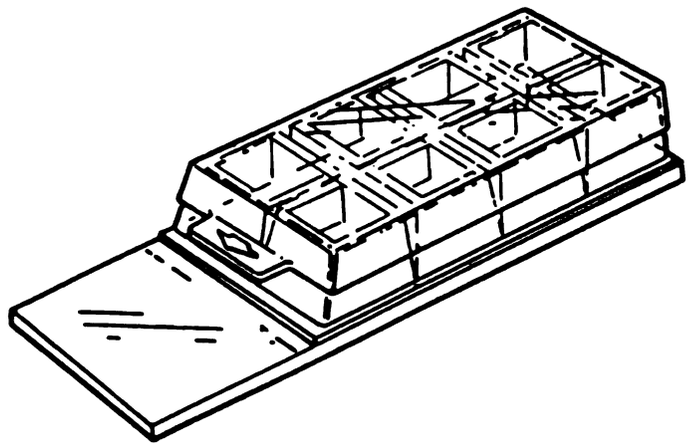


Figure 4.1 Culture chambers used for adhesion tests.

Table 4.1 Effect of Metabolic Inhibitors

Chemical	Metabolic Effect
2,4-dinitrophenol	proton ionophore
DCCD	membrane ATPase inhibitor
Sodium azide	electron transport inhibitor
Antimycin A	electron transport inhibitor
Lasalocid	metal ionophore
Monensin	metal ionophore

Table 4.2 Preparation of Metabolic Inhibitor Stock Solutions

Metabolic Inhibitor	Conc. (mM)	Formula Weight	Stock Solution		Volume used per 5 ml (ml)
			Chemical (g)	Solvent† (ml)	
2,4-D	1.6	184	0.0108	1	0.15
DCCD	0.02	206.3	0.0069	50	0.15
NaN_3	40	65.01	0.0130	2	2
Antimycin A	0.1	500	0.0033	2	0.15
Lasalocid	0.02	612.8	0.0102	25	0.15
Monensin	0.02	692.9	0.0115	25	0.15

†The stock solution of NaN_3 was made with distilled water. All other stock solutions were made with ethanol.

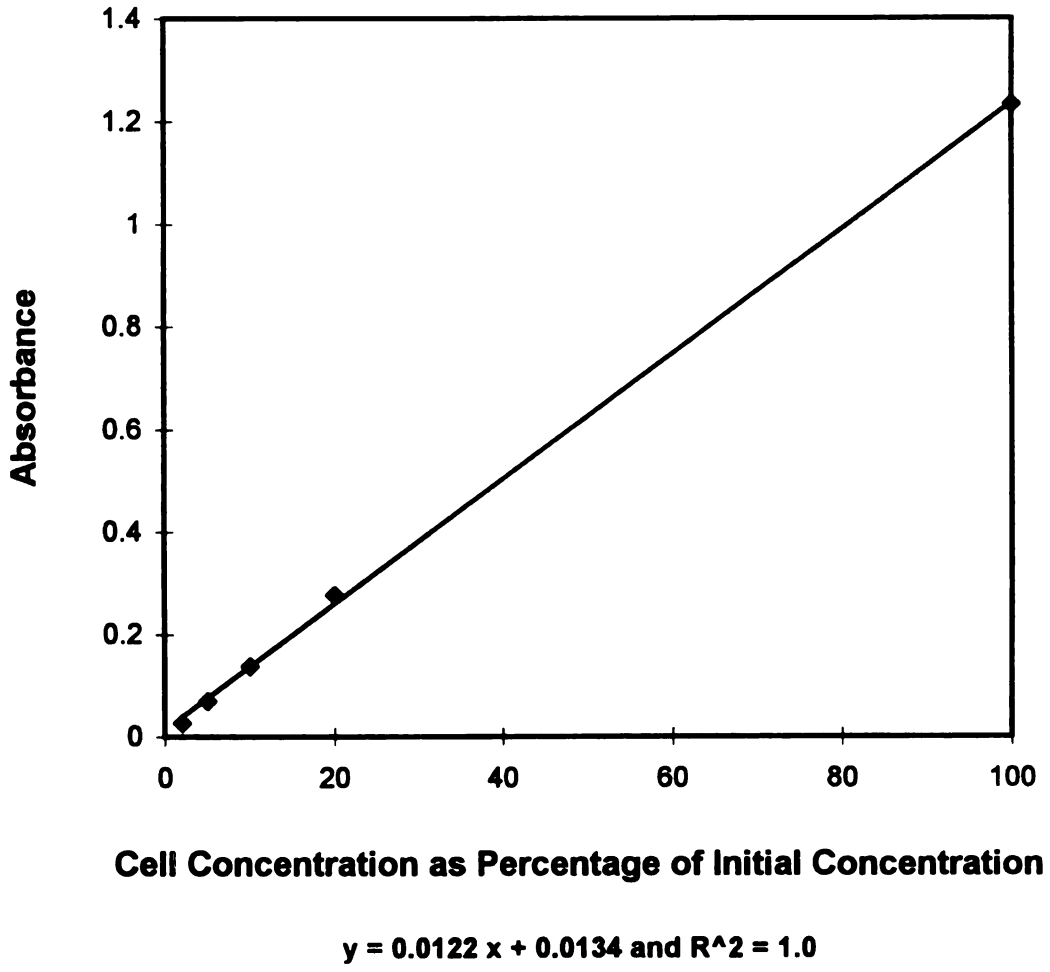


Figure 4.2 Optical density of homogenized *P. chrysosporium* mycelia at 435 nm.

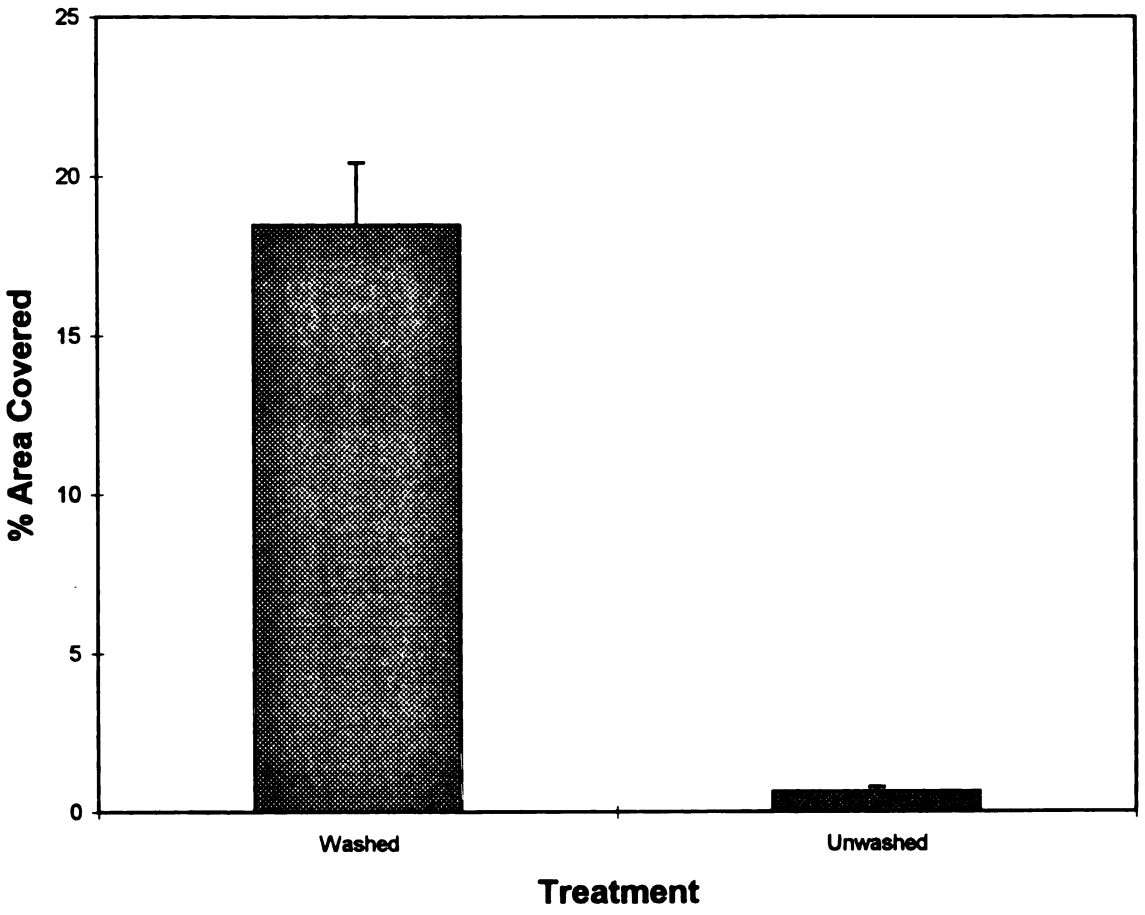


Figure 4.3 Adhesion of washed and unwashed *P. chrysosporium* mycelia.

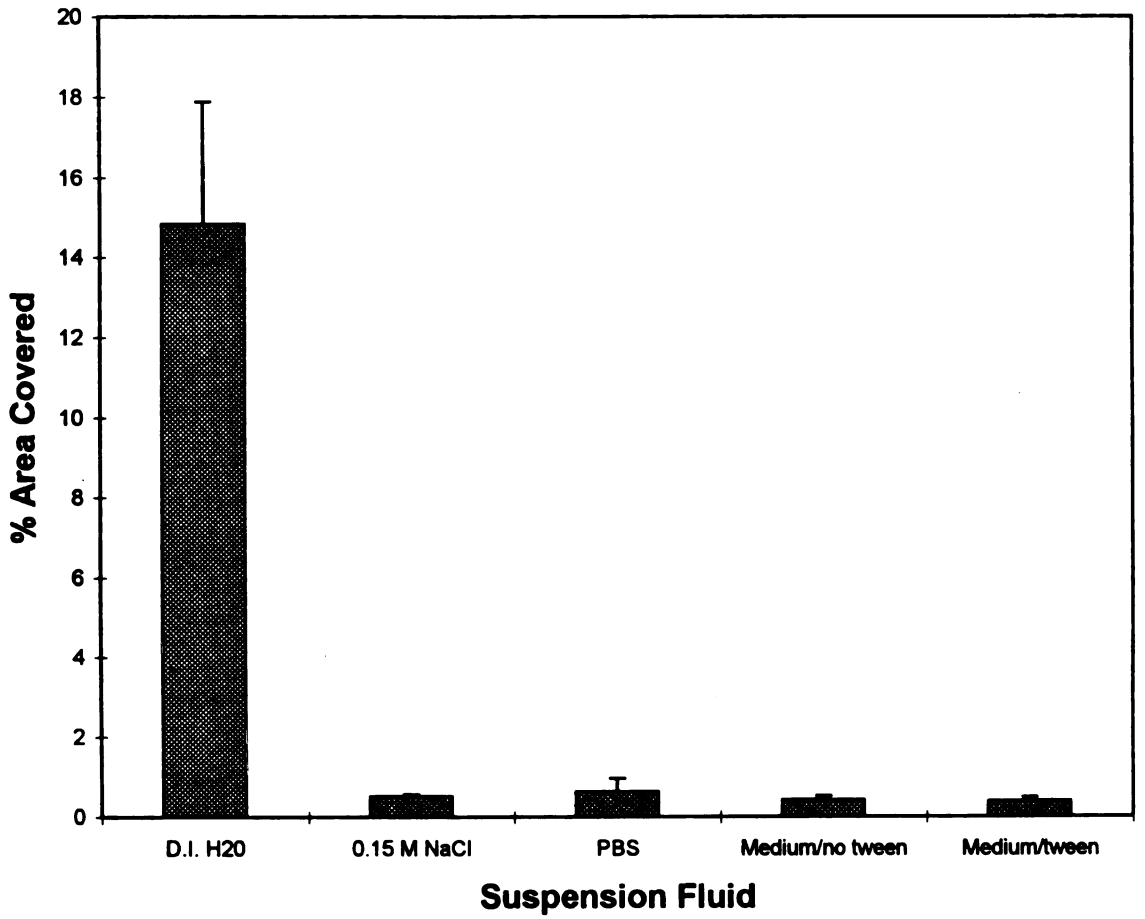


Figure 4.4 Adhesion of *P. chrysosporium* using various suspension fluids.

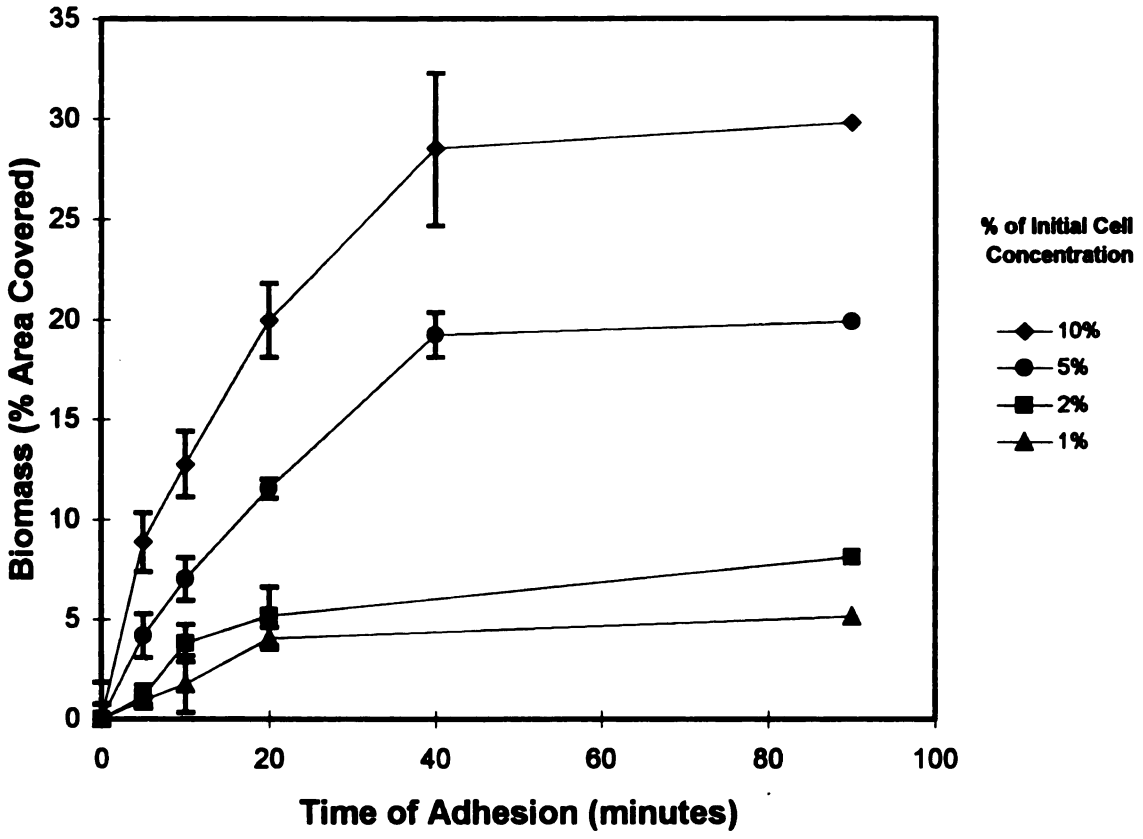


Figure 4.5 Adhesion of *P. chrysosporium* as a function of cell concentration and contact time.

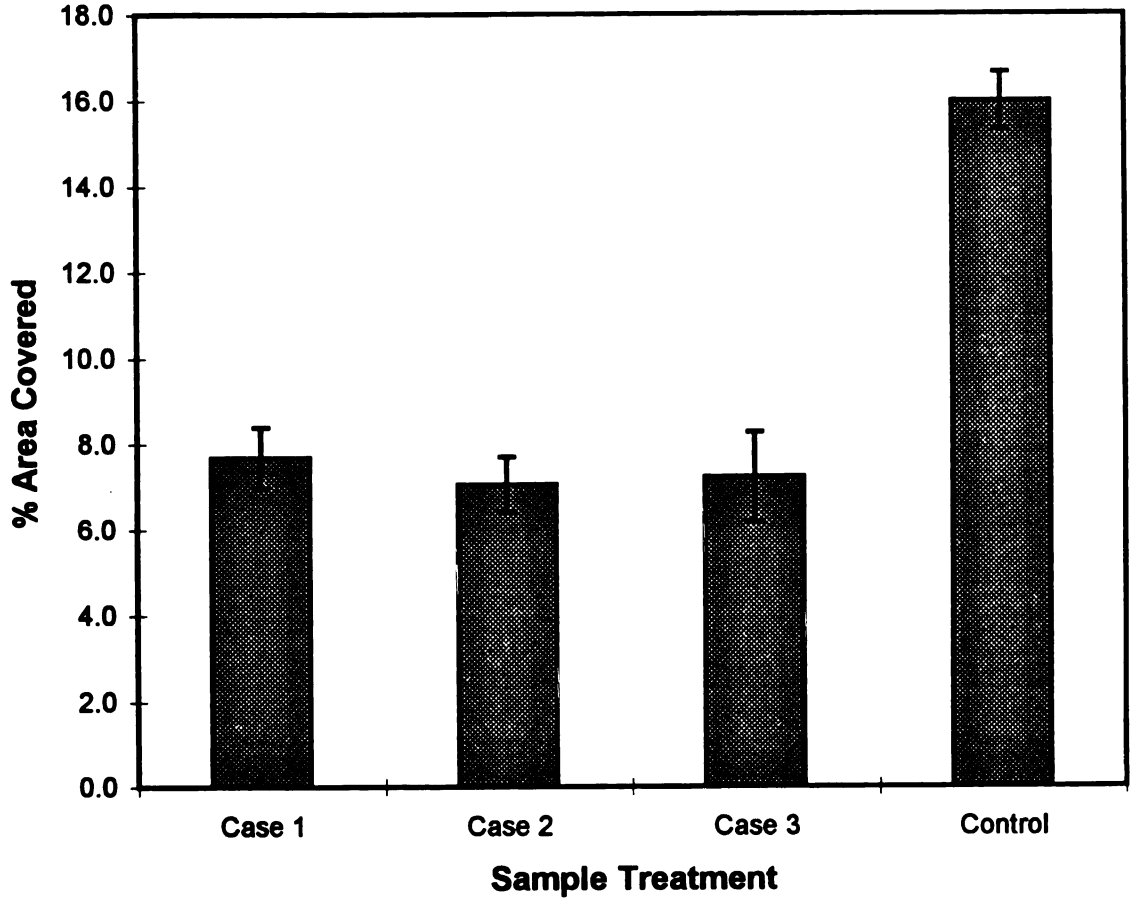


Figure 4.6 Growth of *P. chrysosporium* during adhesion to microscope slide. For Case 1, cells were contacted with a microscope slide for 15 minutes, rinsed, and stained. For Cases 2 and 3, cells were contacted with a slide for 15 minutes, rinsed, and then the culture chamber was refilled with distilled water (Case 2) or Fernbach medium (Case 3). After 45 minutes of additional contact time, the slides were rinsed, stained, and then adhesion was measured.

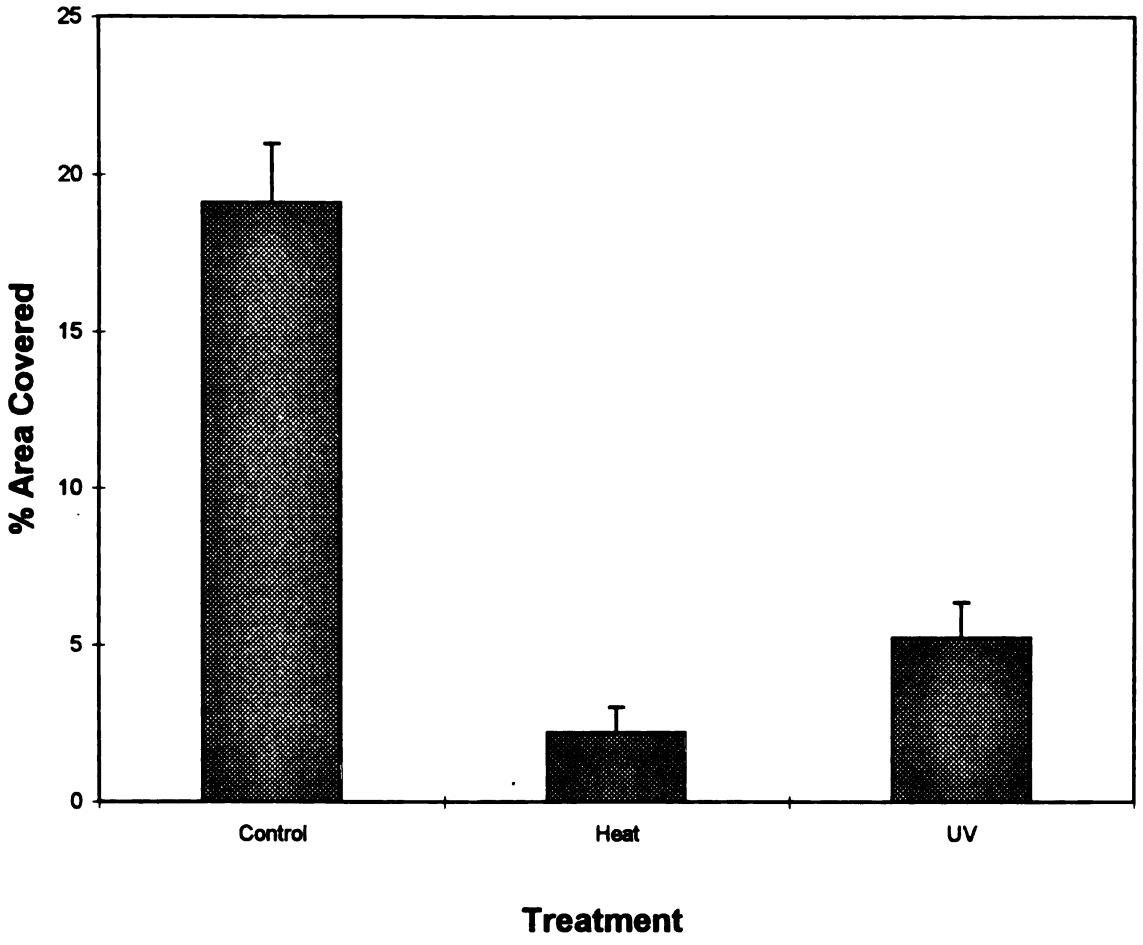


Figure 4.7 Adhesion of *P. chrysosporium* cells exposed to heat and UV radiation.

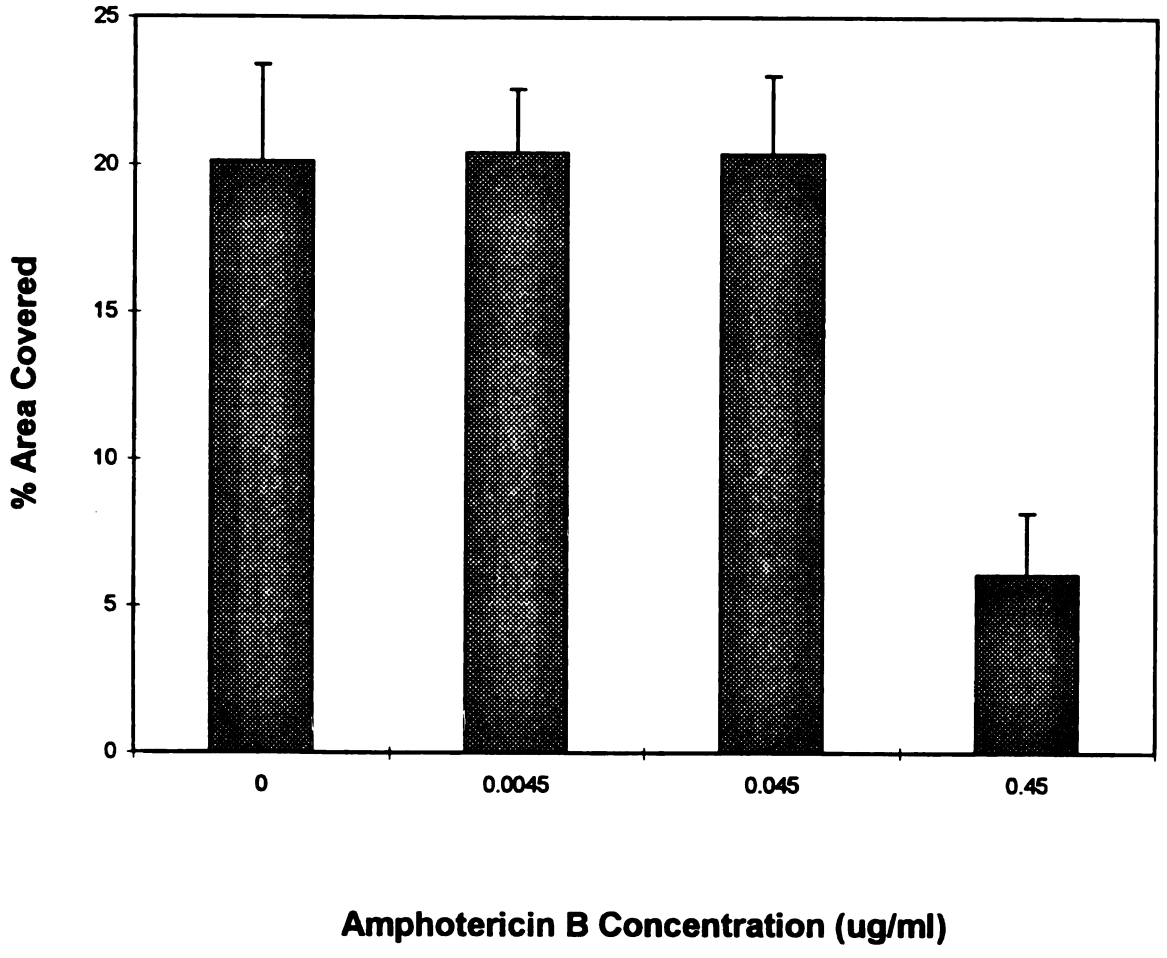


Figure 4.8 Adhesion of *P. chrysosporium* exposed to amphotericin B.

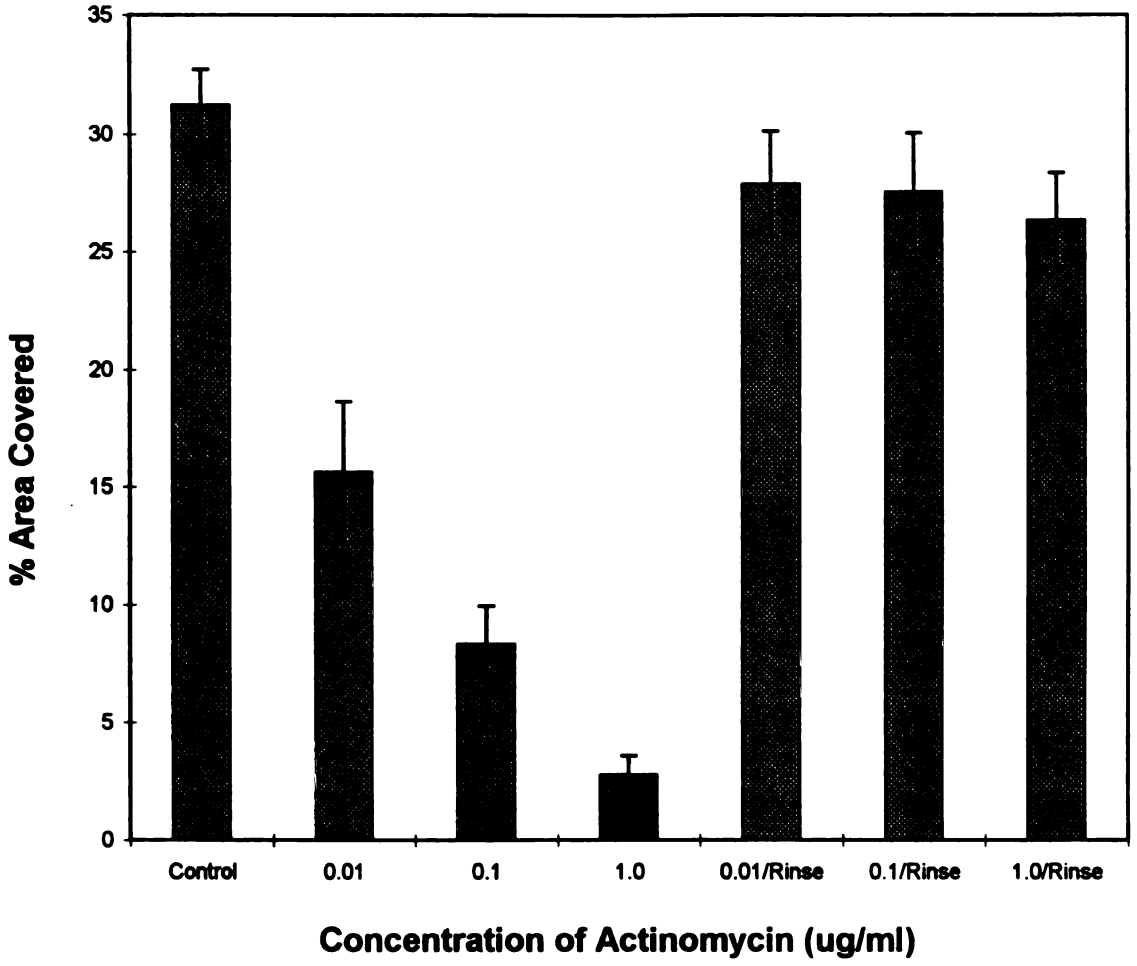


Figure 4.9 Adhesion of *P. chrysosporium* exposed to actinomycin.

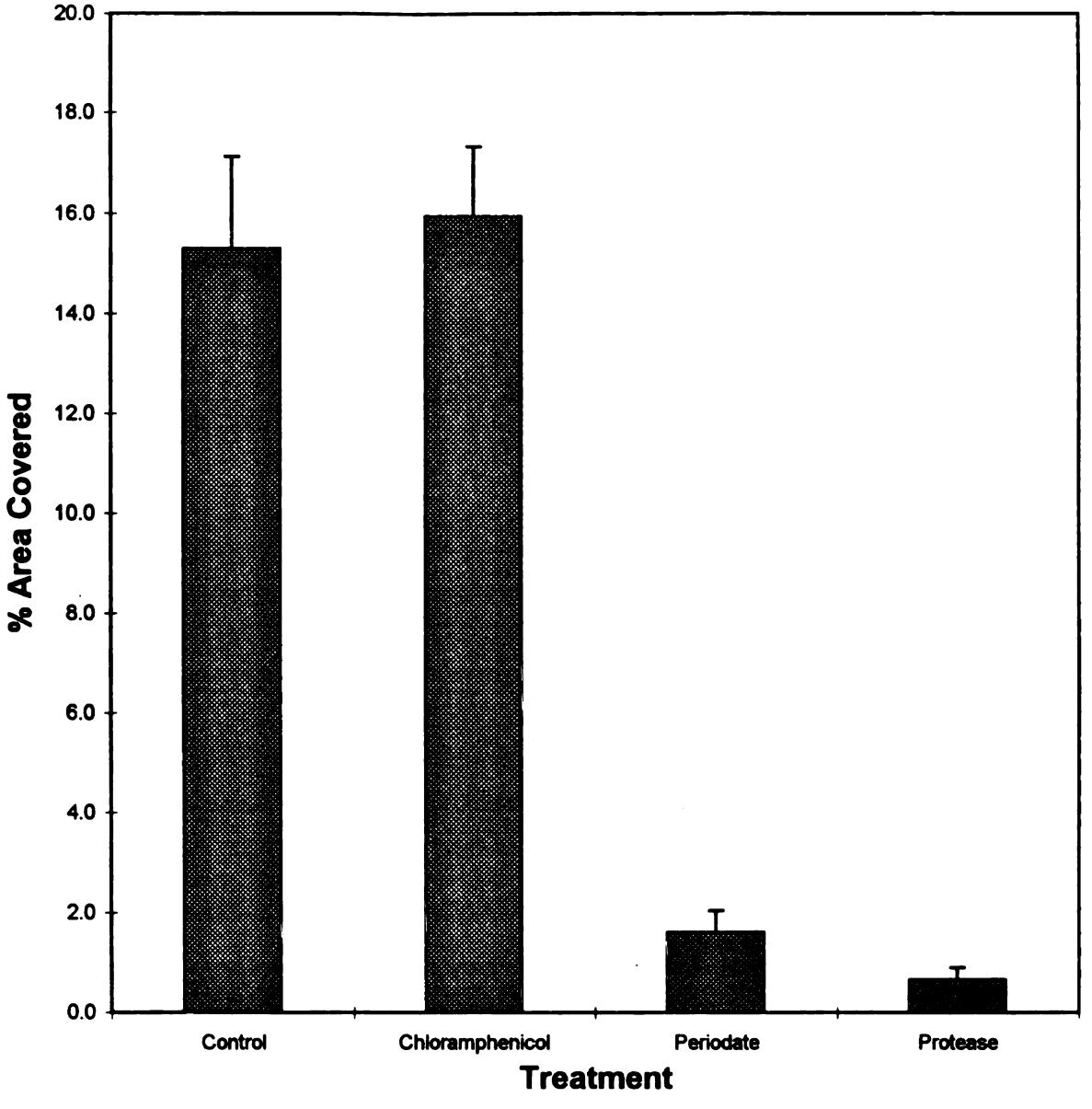


Figure 4.10 Adhesion of *P. chrysosporium* exposed to periodate, protease, and chloramphenicol.

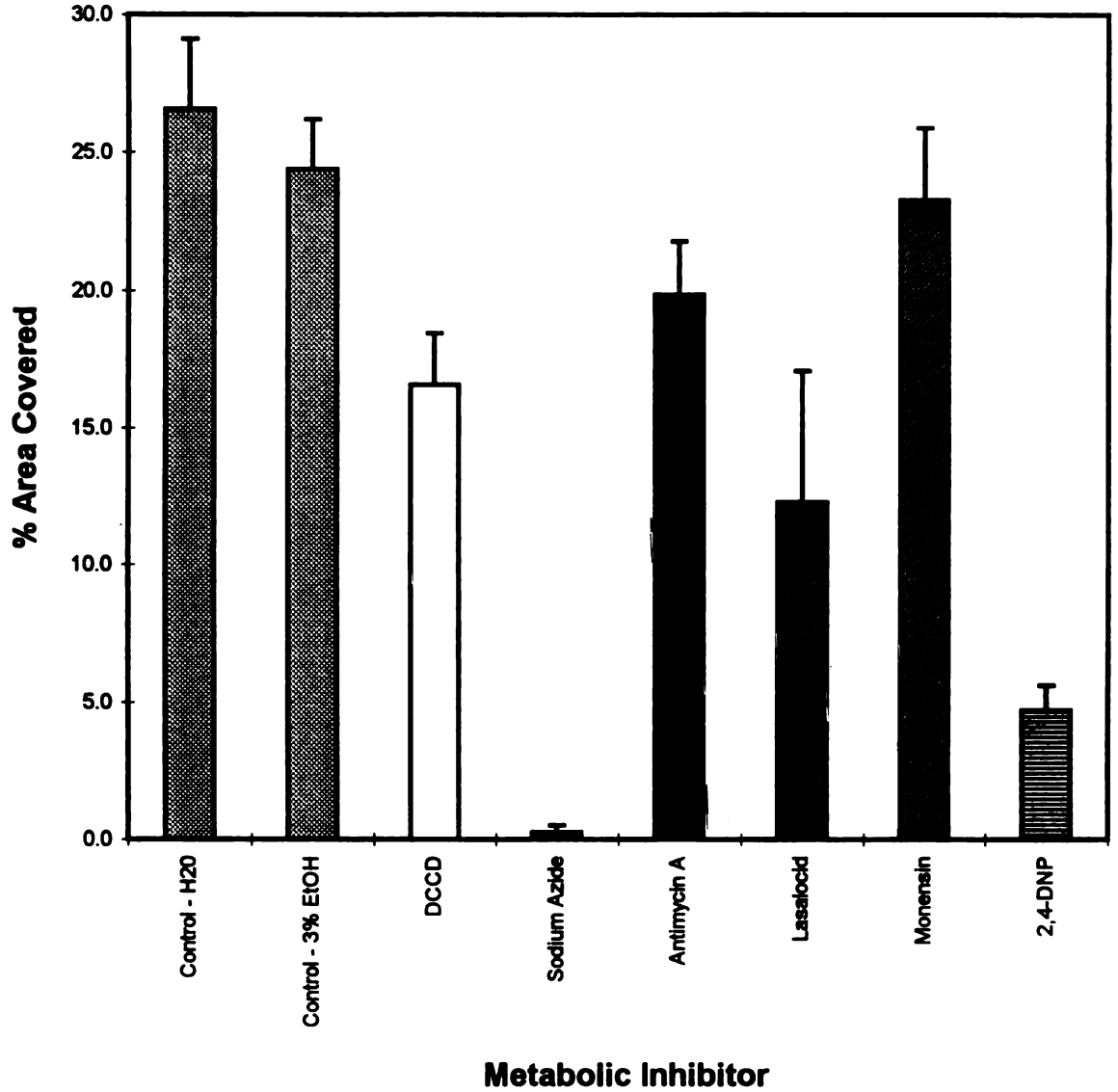


Figure 4.11 Adhesion of *P. chrysosporium* exposed to various metabolic inhibitors.

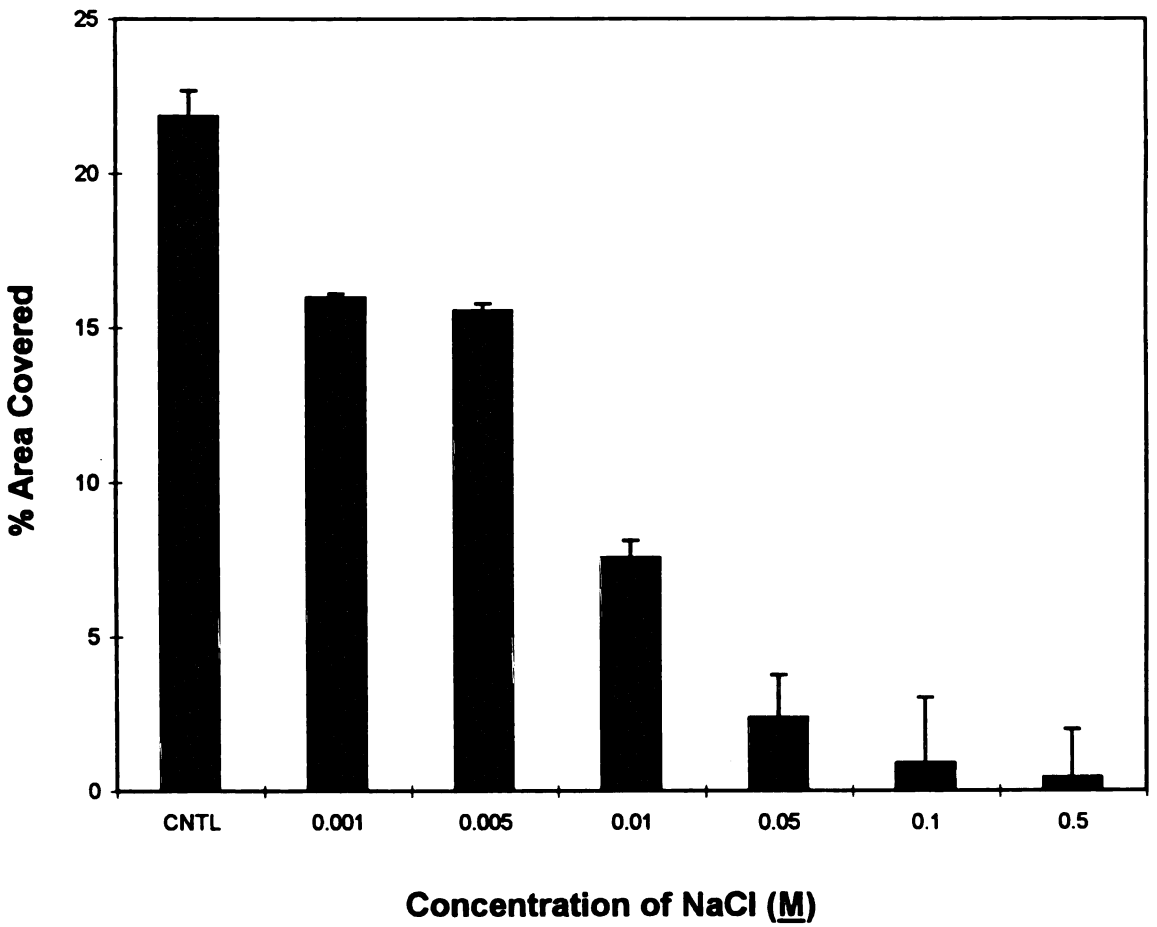


Figure 4.12 Effect of electrolyte concentration (NaCl) of medium on *P. chrysosporium* adhesion.

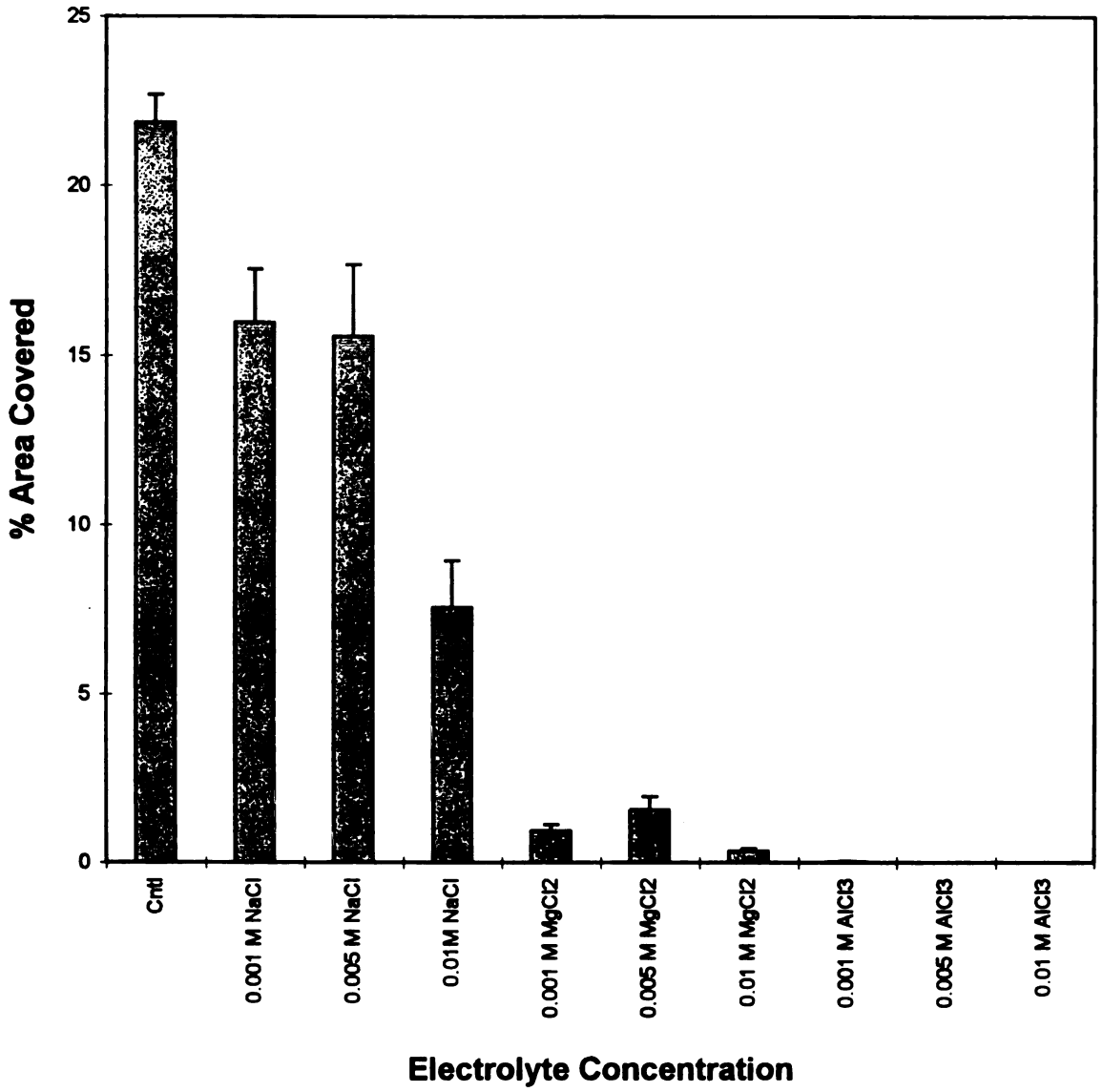


Figure 4.13 Adhesion of *P. chrysosporium* suspended in solutions of various ionic valencies.

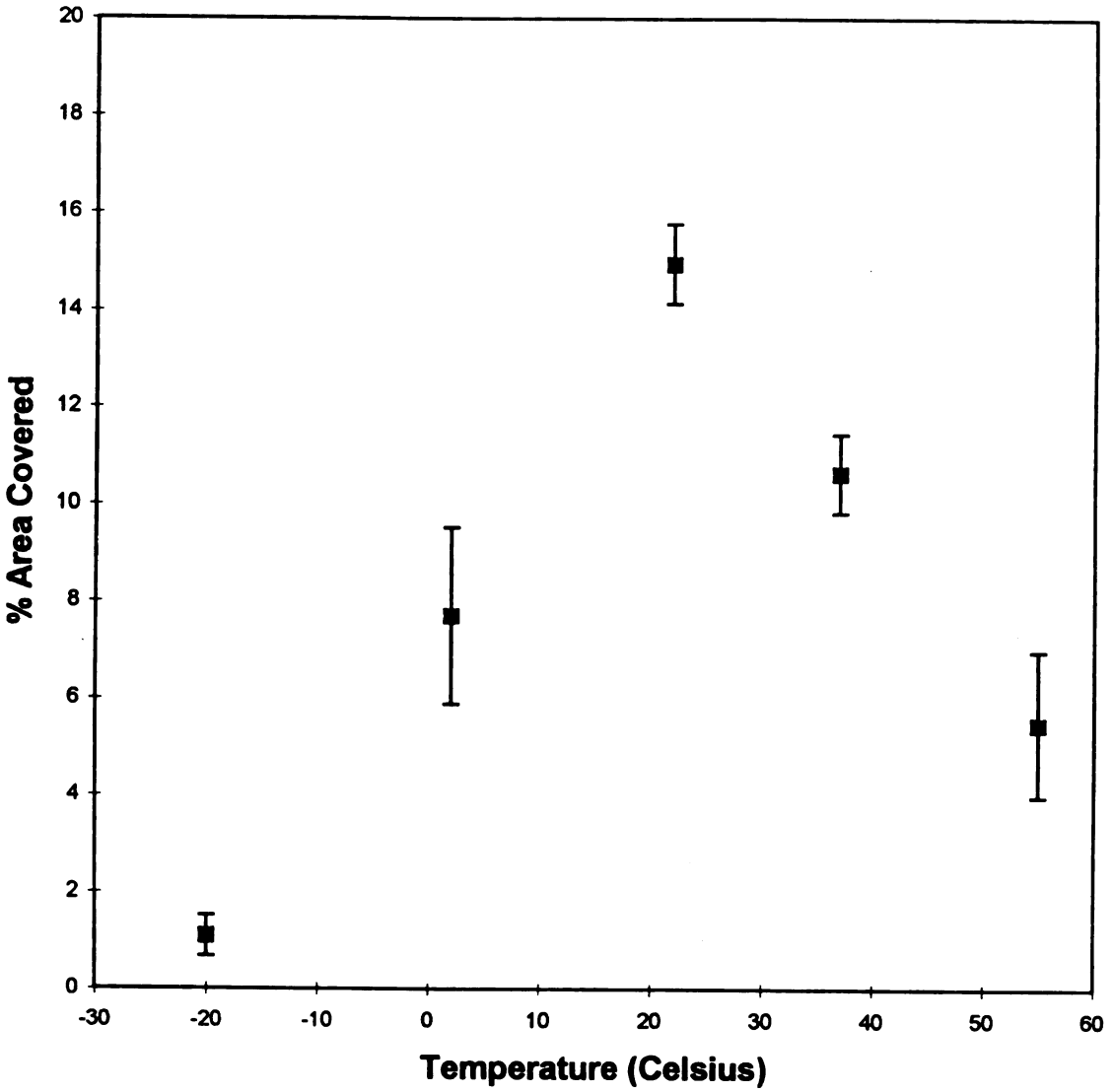


Figure 4.14 Effect of temperature on *P. chrysosporium* adhesion.

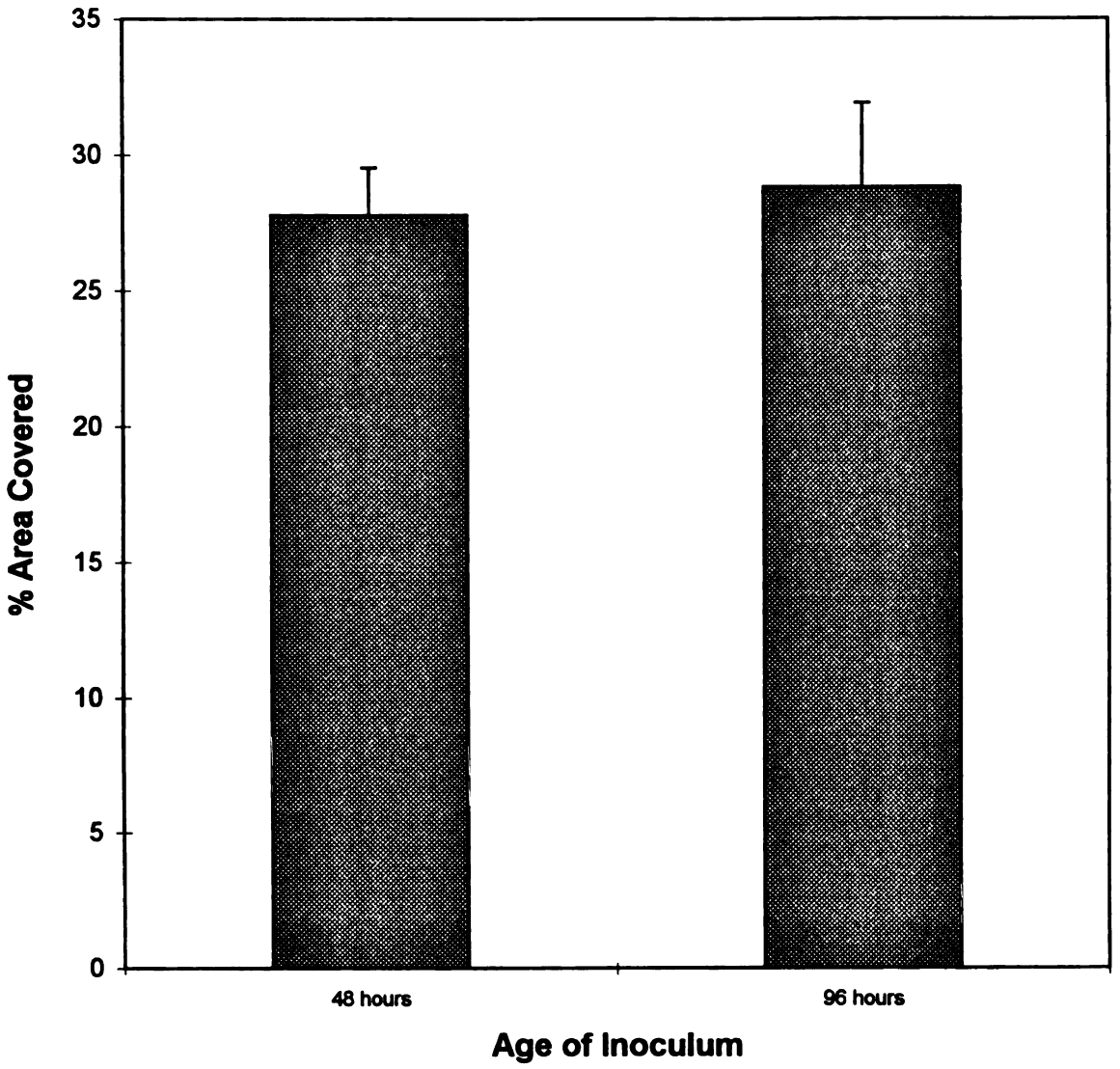


Figure 4.15 Adhesion of *P. chrysosporium* a function of inoculum age.

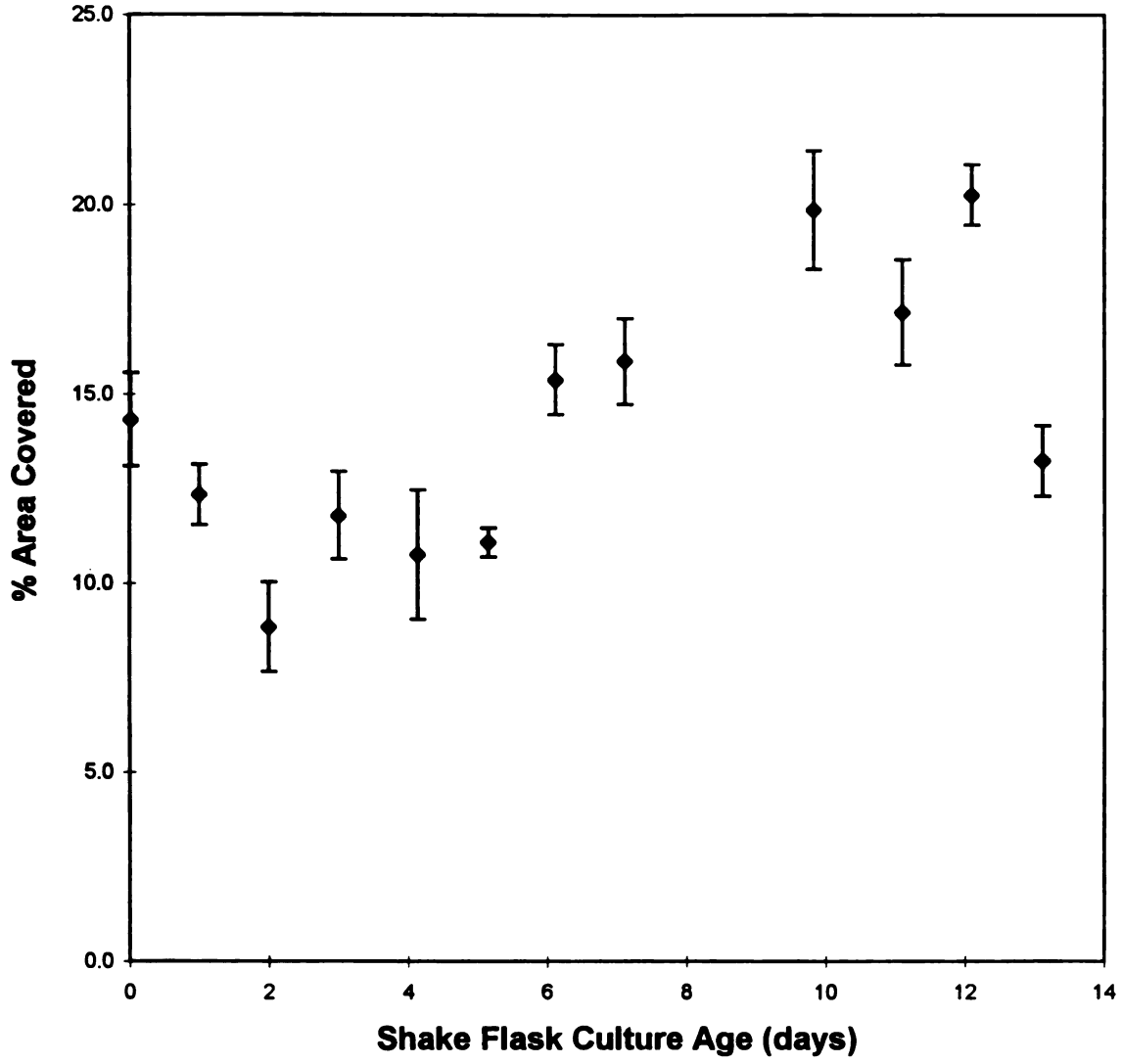


Figure 4.16 *P. chrysosporium* adhesion to microscope slides using cells cultured in shake flasks for various time periods.

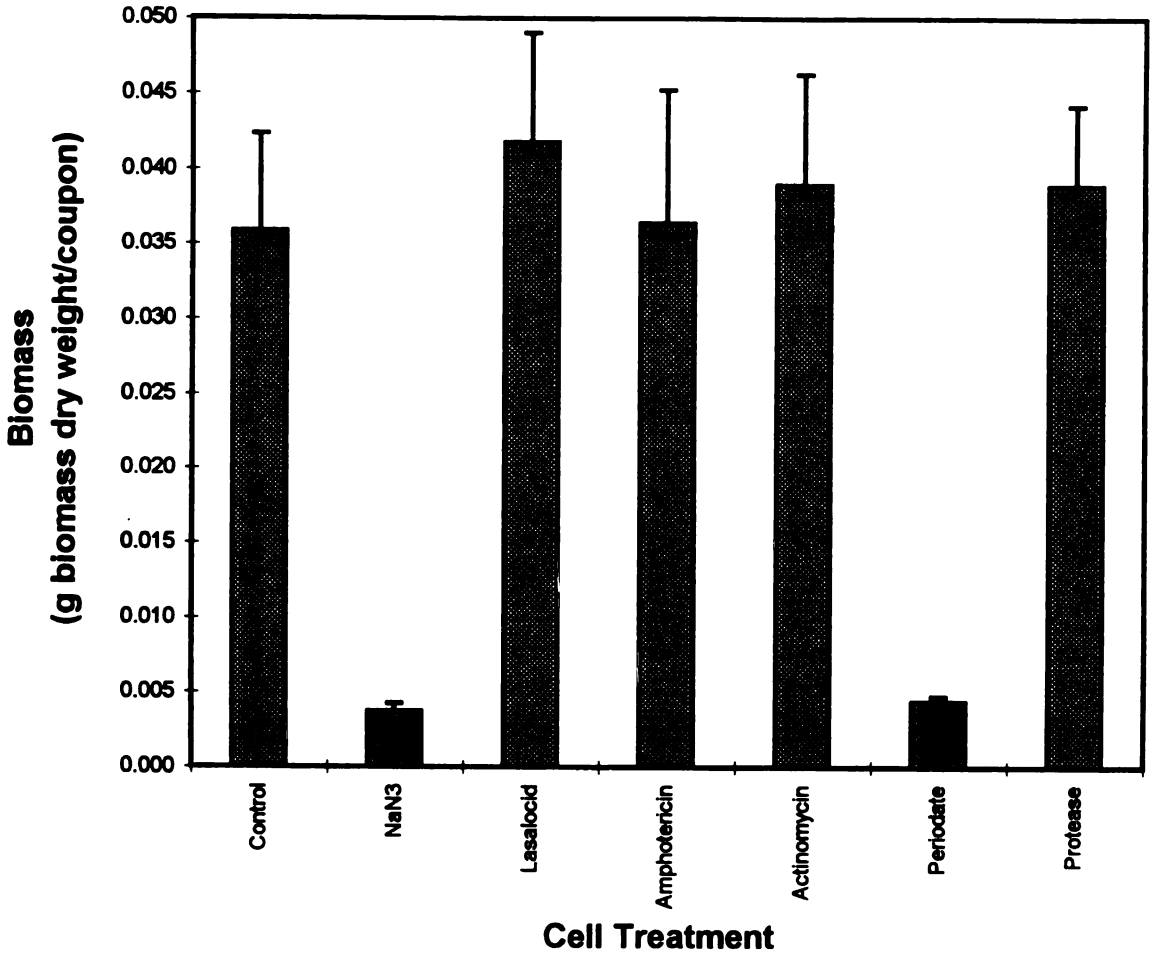


Figure 4.17 Effect of various treatments on *P. chrysosporium* adhesion to polymer coupons in shake flask cultures.

CHAPTER 5.

ROLE OF SURFACE PROPERTIES IN ADHESION OF *PHANEROCHAETE CHRYSOSPORIUM* TO POLYMERS

Abstract

Many factors influence the adhesion of microbial organisms to surfaces including properties of the cell, substratum, and suspending medium. In this study, the adhesion of the white-rot fungus *Phanerochaete chrysosporium* to various roughened polymers was correlated to theoretical predictions of adhesion based on thermodynamic considerations. Adhesion was also correlated to the degree of roughness of the polymer surface. The fungus was cultured in agitated flasks containing a polymer coupon. The coupons were made of polytetrafluoroethylene, polyethylene, acetal, and sulfonated polystyrene and represented a range of surface free energies. Adhesion and growth of the fungus on the polymer coupons were measured by biomass dry weight.

Thermodynamic predictions based on the surface energy approach were used to calculate the free energy of adhesion. Surface free energies of the cells and polymer surfaces were determined using advancing contact angle measurements. Interfacial free energies were calculated using an empirically-derived equation of state. The correlation between experimental and theoretical adhesion results indicates that initial substratum surface energy is a

significant factor in the adhesion process of *P. chrysosporium* to surfaces. Adhesion of this organism is enhanced on materials with low surface free energy and high roughness values.

Introduction

The adhesion of cells to a surface depends on interactions between the cells, the surface, and the suspending liquid. This work explored the effect of surface properties, including roughness and surface free energy, on the irreversible adhesion of the fungus *P. chrysosporium* to polymer surfaces. The significance of thermodynamic considerations to predict adhesion to polymers was evaluated. The focus of this study included only material-induced responses and did not include biological or other factors, such as metabolic activity of the cells or the integrity of the cell surface, which are also known to influence adhesion.

The fungus was cultured in shake flasks containing roughened coupons of various polymer materials. The surface free energies of the cells and polymer surfaces were calculated from contact angle measurements. Interfacial free energies between the cells, polymers, and liquid were determined using an empirically-derived equation of state. Adhesion measured experimentally, as biomass dry weight per coupon, was then statistically correlated to thermodynamic predictions of adhesion. Measurements of lignin peroxidase and manganese peroxidase activity indicated that the lignin

degrading system (LDS) of the fungus was functioning during adhesion experiments.

This work will contribute to the understanding of the adhesion behavior of *P. chrysosporium*. The LDS of this white-rot fungus is comprised of a series of extracellular isoenzymes, lignin peroxidases and manganese peroxidases (Tien and Kirk, 1988; Odier and Delattre, 1990; Gold and Glenn, 1988), that are capable of degrading a wide variety of aromatic pollutants (Lin *et al*, 1990; Kennedy *et al*, 1990). Several promising applications exist to use the LDS for detoxification and decolorization of waste effluents and bioremediation of contaminated soil (Huynh *et al*, 1985). Future development of fermentation systems for enzyme production would require immobilizing the organism on a support surface within a bioreactor. Little research exists to describe the factors which influence the immobilization of the fungus to various supports while maintaining subsequent enzymatic production.

Theoretical Background

Microbial adhesion to surfaces can be predicted using a thermodynamic approach based on an interfacial free energy balance for small particles. If electrical charge interactions and specific biochemical interactions are neglected, adhesion may be described by (Spelt *et al.*, 1982):

$$\Delta F_{adh} = \gamma_{CS} - \gamma_{SL} - \gamma_{CL} \qquad (5.1)$$

where ΔF_{adh} is the interfacial free energy of adhesion, γ_{CS} is the cell-surface interfacial free energy, γ_{SL} is the surface-liquid interfacial free energy, and γ_{CL} is the cell-liquid interfacial free energy. Adhesion will be thermodynamically favorable if

$$\Delta F_{adh} < 0 \qquad (5.2)$$

Interfacial tensions involving a solid phase cannot be measured directly. Several methods have been used to determine solid interfacial tensions by indirect methods using liquid contact angle data. These methods include critical surface tension (Morra, 1990), the geometric mean approach to determine the polar and dispersion interactions (Morra 1990; Busscher *et al.*, 1983; Owens and Wendt, 1969), and the equation of state approach (Li and Neumann, 1992; Morra, 1990; Spelt, 1990; Neumann et al., 1977;).

Equation of State Approach to Determine Solid Interfacial Tension

A liquid drop placed on a surface will modify its shape until a stable three-phase equilibrium between the liquid, substratum surface, and surrounding vapor is obtained. The Young equation describes equilibrium at this three-phase boundary (Morra, Occhiello, and Garbassi; 1990):

$$\gamma_{LV}\cos\theta = \gamma_{SV} - \gamma_{SL} \qquad (5.3)$$

where γ_{LV} is liquid surface tension in the presence of vapor, θ is the contact angle, and γ_{SV} and γ_{SL} are the solid surface tension with the vapor and liquid, respectively. The liquid surface tension and the contact angle are measurable quantities, and the two solid surface tensions are unknowns. Therefore, a second equation is required in order to determine the two unknowns. From thermodynamic considerations, an equation of state relation has been shown to exist such that (Neumann *et al.*, 1974):

$$\gamma_{SL} = f(\gamma_{SV},\ \gamma_{LV}) \tag{5.4}$$

Li and Neumann (1992) formulated this equation as:

$$\gamma_{SL} = \gamma_{LV} + \gamma_{SV} - 2\sqrt{\gamma_{LV}\gamma_{SV}}\, e^{-\beta(\gamma_{LV}-\gamma_{SV})^2} \tag{5.5}$$

where β is a constant equal to 0.0001247. This constant was determined empirically from curve-fitting a large quantity of experimental contact angle data using various liquids and polymer surfaces. Combining Equation 5.5 with the Young equation (Equation 5.3) yields:

$$\cos\theta = -1 + 2\frac{\sqrt{\gamma_{SV}}}{\sqrt{\gamma_{LV}}}\, e^{-\beta(\gamma_{LV}-\gamma_{SV})^2} \tag{5.6}$$

This equation can be used to determine the solid-vapor surface tension, γ_{SV}, of any solid-liquid system (i.e. cells-liquid or polymer-liquid) using the measured values for the liquid surface tension, γ_{LV}, and the contact angle, θ. The solid-liquid interfacial tension can then be found from either the Young equation or the equation of state.

Equations 5.5 and 5.6 contain mathematical limitations in the form of discontinuities (Neumann *et al.*, 1974) which can be avoided by using either computer programs or tables to obtain the values of various parameters. Li and Neumann (1992) presented the logic for a computer program to calculate the solid-vapor interfacial tensions from liquid-vapor interfacial tensions and contact angles. Neumann and coworkers (1980a) provided a computer algorithm for the calculation of solid-liquid and solid-solid interfacial tensions from the corresponding solid-vapor and liquid-vapor surface tensions. Alternatively, tables containing contact angles and the interfacial tensions γ_{LV}, γ_{SV}, and γ_{SL} can be used to find the value of any two of the parameters by knowing the value of the other two (Neumann *et al.*, 1980b). In the work presented here, solid-liquid and solid-solid interfacial tensions were calculated using two FORTRAN computer programs.

Materials and Methods

Adhesion of *P. chrysosporium* was measured on polymer coupons in shake flask cultures and correlated to theoretical predictions of adhesion based

on the equation of state approach. The steps of this process are outlined in the flowchart of Figure 5.1.

Surface Energy Determination of Liquids

Several liquids with various values of surface tension were used in this work. For cell adhesion studies, the surface tension of cell medium was adjusted by the addition of the surfactant polyoxyethylene monooleate (Tween 80). Tween 80 is a normal component of *P. chrysosporium* culture medium (Kirk and Tien, 1988; Kirk *et al*, 1986) and provided a convenient way to adjust the surface tension of the medium. Contact angle measurements were made using deionized distilled water on polymer surfaces and 0.15 M sodium chloride on films of biomass.

The surface free energy of the liquids, γ_{LV}, was determined using the Wilhemy plate technique. This technique correlates the force required to withdraw a flat plate (i.e. glass coverslip) from a liquid to the surface tension of the liquid. Measurements were made using a Cahn Dynamic Contact Angle Analyzer (model DCA-322) in the Composite Materials and Structures Center of Michigan State University. Approximately 50 ml of the sample liquid was poured into a 100 ml beaker and placed on the stage of the analyzer. Moisture and contamination were removed from glass coverslips by passing them through the flame of a propane torch before placement in the instrument. Operating settings included a platform speed of 22.02 microns/second and a 10

mm cycle depth. The glass beaker was cleaned before use with Nochromix and then rinsed with distilled water.

Preparation of Polymer Surfaces

Low density polyethylene (LPDE), polytetrafluoroethylene (PTFE), and polystyrene (PS) were obtained from McMaster-Carr Company, Chicago, IL. Acetal was purchased from Almac Plastics, Detroit, MI. The polymers were purchased as sheets of 1/8 inch thickness. Sulfonated polystyrene (SPS) was prepared by sulfonating polystyrene sheets at the Composite Materials and Structures Center of Michigan State University. Details of the sulfonation procedure are found in Appendix 3. Round disks, or coupons, with a 3/4 inch diameter were prepared using a punch press. The coupons were roughened using an electric hand-held sander and sandpaper with grit size 40.

Before use, the coupons were handled with Teflon-coated forceps and cleaned. PTFE, LPDE, and acetal were cleaned by sonication in methanol for 15 minutes. The methanol was discarded and replaced by fresh methanol and the sonication step was repeated for another 15 minutes. The polymers were removed from the methanol, blotted with tissue, and allowed to thoroughly air dry. PS was placed in a beaker of methanol for five minutes. The methanol was replaced and the step repeated. The PS was removed, blotted with tissue, and air dried. SPS was immersed in hexane for ten seconds, blotted with tissue, and air dried.

Characterization of Polymer Surfaces

Contact angles on the polymer surfaces were measured using a goniometer (Model #100-00 115, Rame-Hart, Inc., Mountain Lakes, NJ). A Pipetman pipet (volume of 0-200 μl) was used to deliver 4 μl drops (drop diameter of about 2.5 mm) of deionized distilled water on the polymer surface. A minimum of 25 contact angle measurements were made to obtain an average value.

The roughness of the polymer surfaces was measured using a laser scanning microscope in reflection mode with a 50x objective. Three-dimensional images of the polymer surfaces were produced using sectioning techniques. The optical sections were 0.8 microns thick and the dimensions of the imaged sample was 189 x 284 microns. Photographs were taken using Kodak T-max 100 film for black and white prints and Kodak Ektachrome 100 for color slides. The topography software of the LSM calculated the mean roughness, R_a, and the averaged surface roughness, R_z, according to the following formulae:

Mean value (mean height) is calculated from

$$z_m = (z_1 + z_2 + \ldots)/n \qquad (5.7)$$

where n is the number of points and is chosen by the computer software and z is the vertical distance from a peak to a valley at a particular point on the surface.

Mean roughness (arithmetic mean deviation) is given by

$$R_a = (|z_1-z_m| + |z_2-z_m| + \ldots)/n \qquad (5.8)$$

Averaged surface roughness is calculated according to

$$R_z = (z_{max1}-z_{min1}+z_{max2}-z_{min2}+\ldots+z_{max5}-z_{min5})/5 \qquad (5.9)$$

where the numbers 1 to 5 refer to the 5 sections into which the image is split by the computer software.

Preparation of Mycelia for Contact Angle Measurements

The following procedure to prepare homogenized mycelia of *P. chrysosporium* for contact angle measurements was adopted from Klotz *et al.* (1985), Van Loosdrecht *et al.* (1987), and Van Pelt *et al.* (1984):

Contact Angle Measurements on Mycelia

1. Fernbach flasks were inoculated with thawed conidia at a concentration of 0.1 OD. After incubation at 39°C for 48 hours, the mycelial mats from three flasks were homogenized in a blender (Virtis Co., Inc., Gardiner, NY, catalog #6301 0001 OC) at high speed for 5 minutes. The blender was placed in an ice-water bath during homogenization to prevent heat damage to the cells.

2. The homogenized mycelia were transferred into four 50 ml centrifuge tubes and centrifuged for 10 minutes at a setting of 7.
3. The supernatant was discarded. 30 ml of distilled water was added to each tube. The tubes were centrifuged again for 10 minutes at the highest setting.
4. Step 3 was repeated twice.
5. The supernatant was discarded. The cells were transferred to a graduated cylinder and diluted to 150 ml with distilled water. The cell suspension was stirred using a magnetic stir bar.
6. The cell suspension was filtered (30 ml per filter) using GA-6 triacetate metricel membrane filters (0.45 μm, 47 mm diameter, plain, catalog #09-730-20, Fisher Scientific, Itasca, IL). The filters were placed in a glass fritted filter unit and filtered under a gentle vacuum.
7. The filters were placed onto agar plates (1% agar in 10% v/v glycerol in water) and allowed to come to an equilibrium moisture content (about 20 minutes).
8. The filters were removed from the agar and placed on a Whatman 41 filter for 5 minutes to remove excess moisture.
9. The filters were cut in quarters and each quarter was mounted on a microscope slide using poster tape (adhesive on both sides, removable).
10. Contact angles on cells were measured as a function of drying time using a goniometer equipped with a 10x objective lens. A green filter was used to enhance to contrast between the liquid drop profile and the substrate. 4 μl drops (drop diameter equal to 2.5 mm) of 0.15 M NaCl were delivered with a 0-200 ml Pipetman. A minimum of five measurements was taken at each time increment.

Calculation of Surface Free Energy of Polymers and Cells

The measured values for liquid surface tension and contact angles on cells and polymer surfaces were used to calculate the interfacial tensions γ_{CS}, γ_{SL}, and γ_{CL} using the equation of state approach. The strategies presented by Li and Neumann (1992) and Neumann *et al.* (1980) were used to develop two FORTRAN programs for a personal computer. Program *GAM* calculated γ_{SV} and γ_{SL} from θ_S and γ_{LV} where the subscript "s" refers to a solid phase such as, for example, polymers or cells. The Wegstein method was used in the program for root-finding. Program *SURF* calculated γ_{12} from γ_{1V} and γ_{2V}

where the subscripts "1" and "2" refer to two different phases such as cells and polymers or cells and liquid. The logic and accuracy of the programs were tested by entering various values for parameters and comparing the output with values obtained from the conversion tables of Neumann *et al.* (1980). FORTRAN code for programs *GAM* and *SURF* is presented in Appendices 1 and 2, respectively.

The programs were applied in the following sequence:

1. Liquid surface tensions were measured using the Cahn instrument.
2. Contact angles on cells, θ_C, and polymers, θ_S, were measured using various liquids.
3. Program *GAM* was used to find γ_{CV} and γ_{CL} from θ_C and γ_{LV}.
4. Program *GAM* was used to find γ_{SV} and γ_{SL} from θ_S and γ_{LV}.
5. Program *SURF* was used to find γ_{CS} from γ_{CV} and γ_{SV}.

The free energy of adhesion, ΔF_{adh}, was then determined according to Equation 5.1.

Shakeflask Adhesion Studies to Polymer Coupons

Homogenized mycelial suspensions were used as inocula for agitated cultures in nitrogen-limited medium according to the method described by Kirk *et al.* (1986) and Kirk and Tien (1988). The buffer was 10 mM 2,2-dimethylsuccinate (DMS) (pH 4.5). Agitated submerged cultures were grown in 125 ml Erlenmeyer flasks containing 30 ml medium (Kirk *et al.*, 1986) and 3 ml inoculum at 37°C on a G50 orbital shaker (New Brunswick Scientific Co., Inc.) at 190 rpm. Each flask contained one preweighed polymer coupon. The flasks were oxygenated once daily with pure oxygen. After 24 hours, the

medium in each flask was filtered and then returned to the flask in order to remove any mycelia that had not adhered to the polymer coupon. Flasks were cultured for seven days to allow the biofilm to complete the growth phase, secondary metabolic phase with production of lignin peroxidase, and to enter the death phase. After seven days, the coupon was removed from each flask, rinsed gently with distilled water, and dried. Biomass on each coupon was determined on a dry weight basis. Samples of culture fluid were collected daily for measurement of lignin peroxidase activity (Tien and Kirk, 1988).

Results

Characterization of the Cells, Polymer Surfaces, and Liquids

The surface energy of *P. chrysosporium* mycelia was determined by measuring the contact angle of 0.15 M NaCl drops on a filtered layer of homogenized mycelia as the cell layers air-dried. The contact angle as a function of drying time is shown in Figure 5.2. (See Appendix 8 - Figures A-E for contact angle data from five replicate experiments.) The Young's contact angle for mycelia using liquid drops of 0.15 M NaCl was 21±3 degrees. This was found by calculating the average of the plateau contact angles from the five experiments. The surface energy of mycelial cells, γ_{CV}, was 64.8 dynes·cm^{-1} as determined using the equation of state approach and the FORTRAN program "GAM" (Table 5.1).

Characteristics of each polymer, including values of roughness, contact angles, and interfacial free energy, are shown in Table 5.1. Contact angle measurements using distilled water ranged from 128 degrees for PTFE to 0

degrees for SPS. The interfacial free energies, γ_{SV} and γ_{SL}, were calculated using the contact angle and liquid surface tension measurements and the computer program "GAM". The values correlated well with those found in the literature (e.g. J. Brandup and E. H. Immergut, 1989). Surface roughness of the polymers was quantified using the topography feature of a laser scanning microscope. Mean roughness, R_a, and average roughness, R_z, were comparable for PTFE, PE, and acetal with R_a values between 6.2 μm and 7.9 μm and R_z between 40 μm and 47 μm. The roughness parameters were somewhat higher for SPS than the other polymers with R_a=10.4 μm and R_z=59.8 μm. The surface morphology of each polymer is shown in the laser scanning microscope photos (Figures 5.3-5.6).

The surface free energies of deionized distilled water and culture medium with various concentrations of the Tween 80 are listed in Table 5.2 (see plots in Appendix 9). As the concentration of Tween 80 increased, the surface free energy decreased.

P. chrysosporium Adhesion to Polymer Coupons in Shake Flask Cultures

Biomass adhesion measured on various polymer coupons after seven days of growth is shown in Figure 5.7 for 1.0% Tween 80 in the suspending medium. Each data point represents the average and standard deviation of three replicates. Adhesion was inversely proportional to the surface free energy of the polymer. The greatest amount of adhesion occurred on PTFE (γ_{SV}=7.6 dynes·cm^{-1}) and decreased as the polymer surface free energy increased. The least amount of biomass was measured on SPS (γ_{SV}=72.5 dynes·cm^{-1}). The same trend occurred when the suspending medium contained 0.5% Tween 80 and no Tween 80 (Figures 5.8 and 5.9, respectively).

A statistical analysis of variance (ANOVA) was performed on the biomass adhesion data for the independent variables of interfacial free energy of the polymer surface, γ_{PV}, and liquid surface tension, γ_{LV}. Details of the ANOVA are included in Appendix 10. Both factors, γ_{PV} and γ_{LV}, were statistically significant although only γ_{PV} was a statistically important factor for biomass adhesion. (Statistical significance refers to the repeatability of an effect. Statistical importance relates to the magnitude of the effect.) 88% of the variability in the ANOVA model was explained by the factor γ_{PV}. Less than 4% of the variability was explained by γ_{LV}. These results indicate that interfacial free energy of the polymer surface was an important factor for *P. chrysosporium* adhesion to polymer coupons in shake flask cultures. Surface tension of the suspending medium had little effect on adhesion.

The concentration of lignin peroxidase, an extracellular enzyme, was measured in order to insure that this work is externally valid for potential industrial applications. Lignin peroxidase produced by biomass adhering to polymer coupons was compared to biomass growing as free pellets (Figure 5.10). The amount of enzyme activity closely correlated to the amount of biomass adhesion on the polymer coupons. The highest lignin peroxidase activity was measured in flasks containing PTFE (134 U/l). This was slightly less than the activity measured from free pellets (156 U/l). Biomass growing on SPS coupons produced no lignin peroxidase.

Thermodynamic Predictions of P. chrysosporium Adhesion

Predictions of the free energy of adhesion, ΔF_{adh}, based on the equation of state approach are shown in Figure 5.11. The plot shows ΔF_{adh} for three liquids with different values of surface tension using 64.8 dynes·cm^{-1} as the interfacial free energy, γ_{CV}, of *P. chrysosporium* mycelia. The points are

calculated values of ΔF_{adh} at a chosen γ_{PV}. The line represents the best fit through the points. Increasingly negative values of ΔF_{adh} correspond to greater cell adhesion. According to the thermodynamic prediction, as liquid surface tension increases, more cell adhesion will occur. In addition, when γ_{LV} is less than γ_{CV}, the model predicts that more cells will adhere as the interfacial free energy of the polymer surface, γ_{PV}, increases. Conversely, when γ_{LV} is greater than γ_{CV}, adhesion will decrease with higher values of γ_{PV}.

Comparison of Thermodynamic Prediction of Adhesion to Experimental Data

The trend of biomass adhesion predicted by the thermodynamic model was compared to experimental values of adhesion on polymer coupons. In general, experimental results showed that mycelial adhesion decreased as the surface free energy of the polymer increased. The correlation between the model and experimental values was determined using hypothesis testing and the t-test for correlation (Appendix 11). Figure 5.12 shows the free energy of adhesion predicted by the thermodynamic model and experimentally measured fungal adhesion on polymer coupons for the case of 0.5% Tween 80 in the medium.

Hypothesis testing for correlation was performed for the experimental cases of 0, 0.5, and 1.0% Tween 80 in the suspending medium using the thermodynamic prediction for the case of $\gamma_{LV} > \gamma_{CV}$. From this analysis, there was sufficient statistical evidence to infer that a correlation exists between the model prediction and the experimental values. (Although high causation will result in high correlation, high correlation does not necessarily imply high causation.) The trend predicted by the model for $\gamma_{LV} < \gamma_{CV}$ was not observed

experimentally so no correlations of experimental data to this case were performed.

Effect of Substratum Roughness on Adhesion

The degree of roughness of the substratum surface significantly affected the amount of fungal adhesion to polymer coupons in shake flask cultures. Polymer coupons of PTFE, PE, acetal, and SPS were roughened with grit 400, 150, and 40 sandpaper (a lower grit value indicates a higher degree of roughness). Two different average roughness parameters, R_a and R_z, were measured on the polymers using laser scanning microscopy (Figure 5.13). Average roughness was fairly equivalent for all the polymers that were prepared with a specific grit (Table 5.3). The value of R_a was less than 1 μm on smooth polymers and increased to 6-10 μm for the roughest grit. For all polymer materials, fungal adhesion increased with increasing surface roughness (shown in Figure 5.14 for PFTE).

Discussion

The average contact angle of 21±3 degrees determined for *P. chrysosporium* mycelia is reasonable since values of contact angles for cells are generally within the range of 15° to 60°. According to Krekeler's (1991) convention, this organism could be considered hydrophilic since the contact angle falls between 20° and 30°. Asther and coworkers (1990) reported the surface free energy, γ_{CV}, of *P. chrysosporium* as 44 dynes·cm^{-1} based on the geometric mean approach. This is somewhat different than 64.8 dynes·cm^{-1} determined in this work and can most likely be attributed to the different methods used to prepare the cells for contact angle measurements. Asther did

not measure the plateau contact angle as the cell layers dried. This can have a significant impact on the contact angle value. Also, they used a different method to calculate the interfacial free energy of the cells and polymers (i.e. geometric mean approach versus the equation of state approach) which may also account for some of the difference in the values.

Surface free energy of the substratum had a significant effect on the adhesion and growth of *P. chrysosporium* to surfaces. Biomass adhesion was promoted on surfaces with lower interfacial free energy. Liquid surface tension of the suspending medium, on the other hand, was not an important factor in the adhesion process. This suggests that short range hydrophobic interactions influence the adhesion process but that other factors are also involved. In bacterial studies, the role of hydrophobic interactions have been studied by adding surfactants to the medium to change the surface tension (McEldowney and Fletcher, 1986). For some organisms, adhesion increased when a particular surfactant was added to the medium. For other organisms, adhesion decreased or remained unchanged indicating that different factors influence the adhesion of a particular organism.

The free energy of adhesion as a function of substratum surface energy predicted by the thermodynamic model for the case of $\gamma_{LV} > \gamma_{CV}$ correlated well to experimental results. Thermodynamic considerations predicted a different trend for adhesion when $\gamma_{LV} < \gamma_{CV}$ but this trend was not observed experimentally. Actual adhesion behavior was independent of liquid surface tension. Once again, this evidence suggests that short range hydrophobic interactions between the cells and the substratum are involved in the adhesion process of the fungus but that other factors also play a significant role. Although the model does not provide a complete description of adhesion for

this organism during shake flask conditions, it does provide useful insight into fungal adhesion.

The thermodynamic approach to study short range forces is based on an interfacial free energy balance for colloidal particles. The approach has been applied to bacterial systems including marine fouling and microbial adsorption to oral surfaces (McEldowney and Fletcher, 1986b; Van Pelt *et el.*, 1984). Bacterial cells more closely resemble small particles than hyphal fragments of a mycelial fungus. This may account for some of the limits of the model in this study.

An attempt was made in this work to compare adhesion on polymer surfaces which were alike except for the property of surface free energy. The polymers were selected in order to provide a range of surface free energy. The size and geometry of the various coupons were the same. The surfaces of the polymers were roughened to approximately the same value. This is in contrast to previous studies by Aster et al. (1990) which measured adhesion of *P. chrysosporium* in shake flask cultures containing solid carriers with widely different properties including geometry (Rashig rings, foam cubes), weight (24-670 $kg \cdot m^{-3}$), surface area (215-675 $m^2 \cdot m^{-3}$), porosity, and surface roughness (0.5-18 μm).

There were some unavoidable differences in the polymer coupons used in this work which may have had some effect on adhesion. The density difference of the polymer material may have affected the motion of the coupon in the flask and, in turn, the exposure of biomass to oxygen in the headspace and liquid. Visual inspection of the flasks during agitation suggested that this was probably a minor factor since the trajectories of all types of coupons at 190 rpm appeared to be similar. Hardness of the materials is another factor which affected the surface topography when they were roughened. Although

the average roughness values were close, light microscope photographs showed that the actual topography was somewhat different between the materials. This may have affected biomass adhesion due to the increased surface area available for adhesion, altered hydrodynamics on a local level (i.e. protection from shear forces), or physical immobilization of hyphal fragments. Additional research is needed to determine the importance of these factors.

Conclusions

The properties of the polymer surface played a significant role in the adhesion of *P. chrysosporium*. Adhesion and growth were promoted on surfaces with low interfacial free energy which indicated that short range hydrophobic interactions are involved in initial adhesion. Liquid surface tension of the suspending medium was not an important factor in the adhesion process. Fungal adhesion increased as the degree of roughness on the polymer surfaces increased. Lignin peroxidase and manganese peroxidase production were maintained during the adhesion studies. The properties of the support surface on which *P. chrysosporium* adheres should be an important factor in the design of an effective bioreactor for industrial applications.

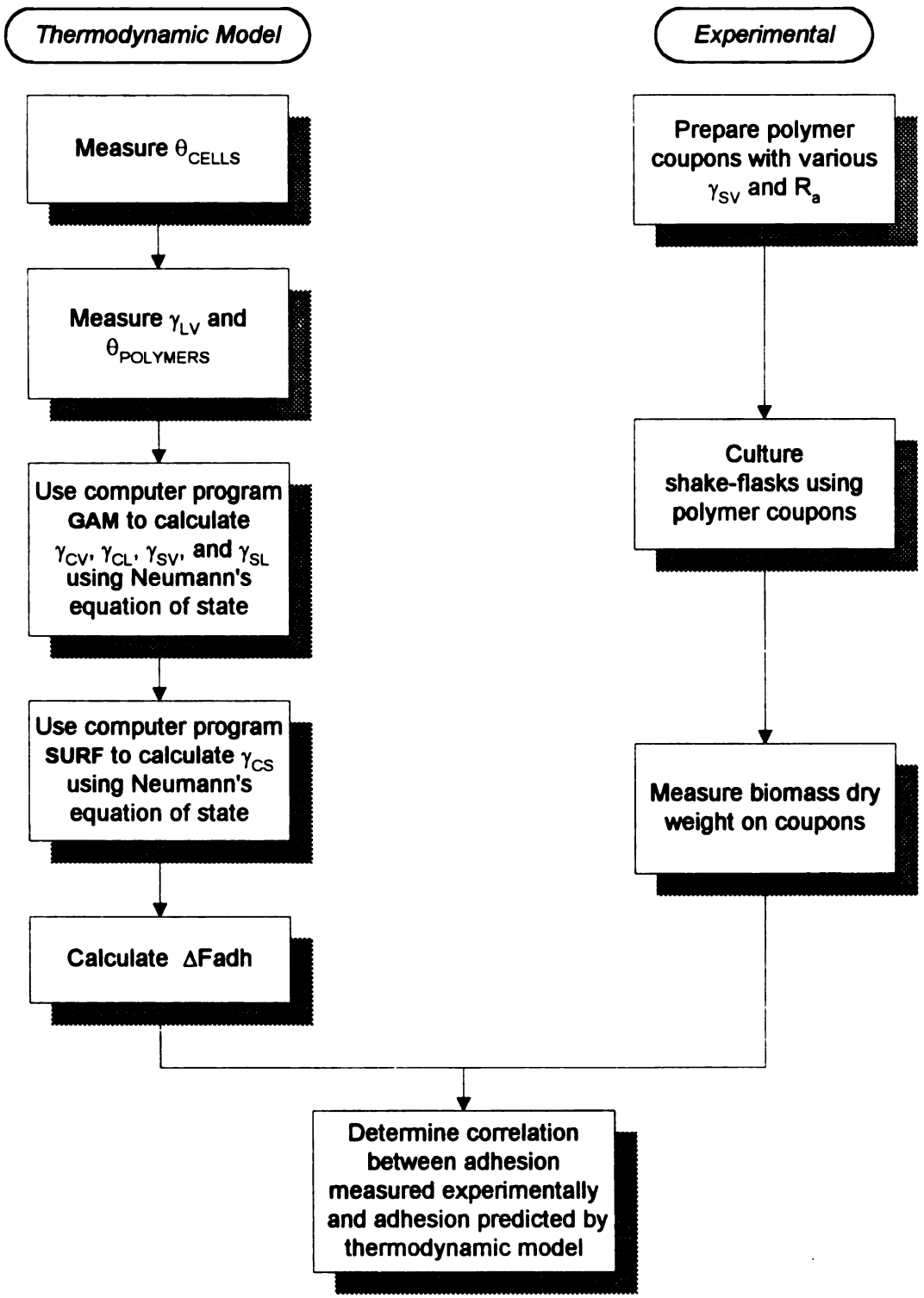


Figure 5.1 Flowchart for thermodynamic approach to predict adhesion of *P. chrysosporium* to polymer surfaces.

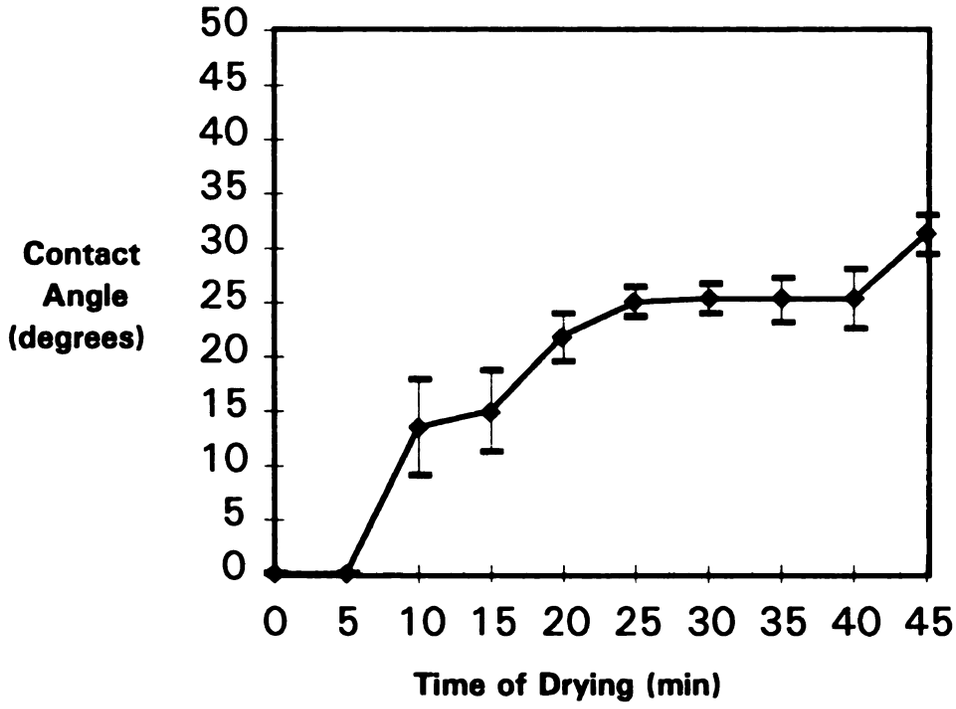


Figure 5.2 Contact angle of *P. chrysosporium* cells as a function of air drying time using 4 µl drops of 0.015 M NaCl.

Table 5.1 Properties of the Cell and Polymer Surfaces

Material	Advancing Contact Angle (degrees)	Surface Free Energy ($dynes \cdot cm^{-1}$)		Surface Roughness of Polymer (grit 40) (μm)	
		γ_{SL}	γ_{SV}	R_a	R_z
Cells	21±3	0.4	64.8	---	---
PTFE	128±7	52.3	7.6	6.24	39.6
PE	107±7	39.9	18.7	5.95	47.0
Acetal	60±6	11.4	47.6	7.39	40.7
SPS	0	0.0	72.5	10.4	59.8

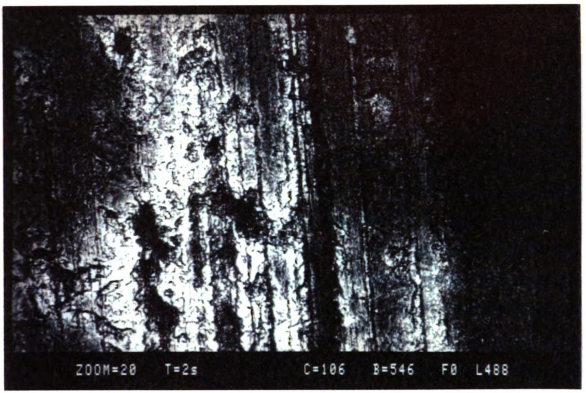


Figure 5.3 Laser scanning microscope photograph of PTFE surface.

Figure 5.4 Laser scanning microscope photograph of PE surface.

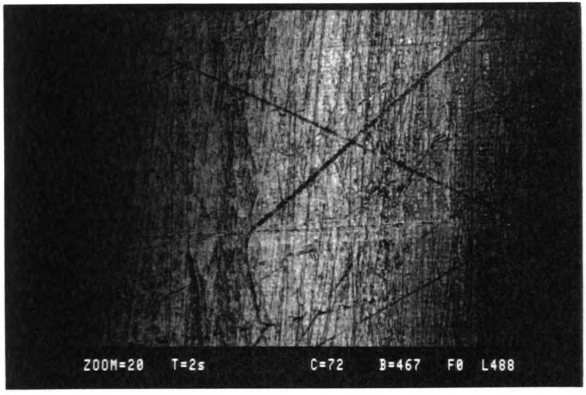


Figure 5.5 Laser scanning microscope photograph of acetal surface.

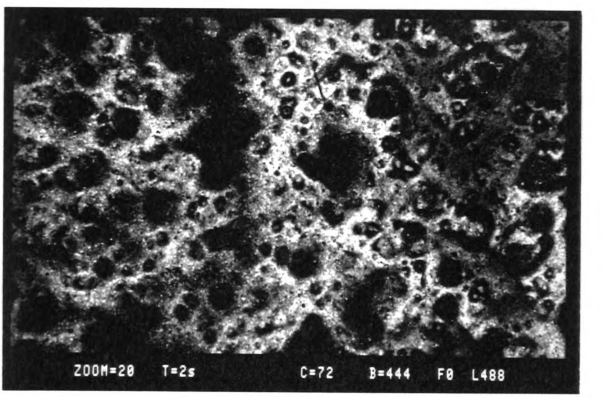


Figure 5.6 Laser scanning microscope photograph of PS surface.

Table 5.2 Surface free energies of liquids.

Liquid	γ_{LV} (dynes·cm^{-1})
DI distilled water	72.5
Medium with no Tween 80	72.5
Medium with 0.5% Tween 80	58.0
Medium with 1.0% Tween 80	36.8
0.1 M NaCl	69.0

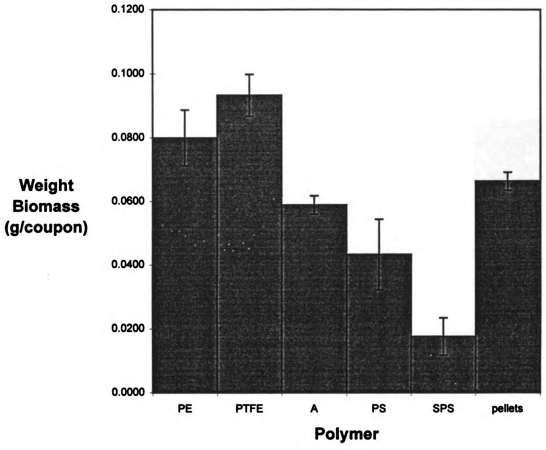


Figure 5.7 Biomass adhesion on polymer coupons using 1.0% tween in the suspending medium.

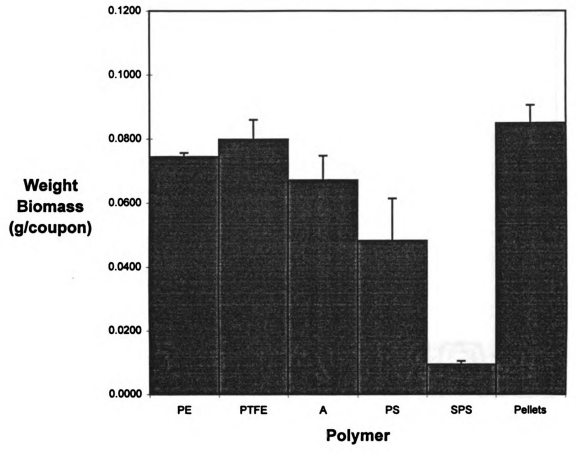


Figure 5.8 Biomass adhesion on polymer coupons using 0.5% tween in the suspending medium.

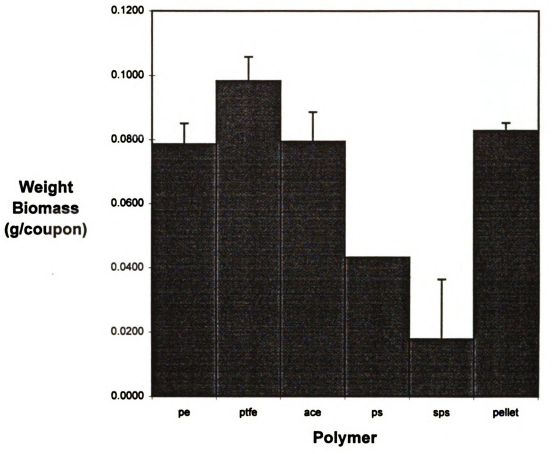


Figure 5.9 Biomass adhesion on polymer coupons using no tween in the suspending medium.

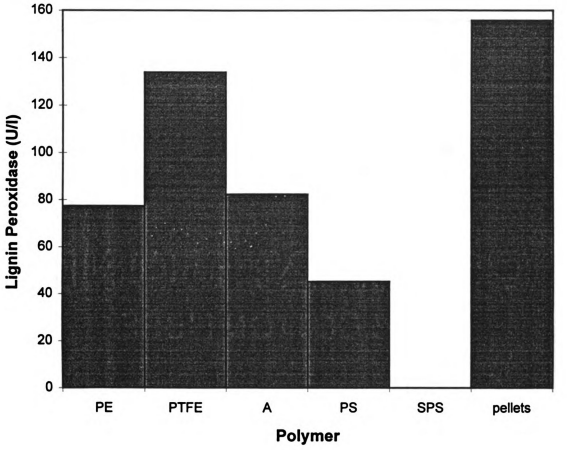


Figure 5.10 Lignin peroxidase activity on polymer coupons using 1% tween in the suspending medium.

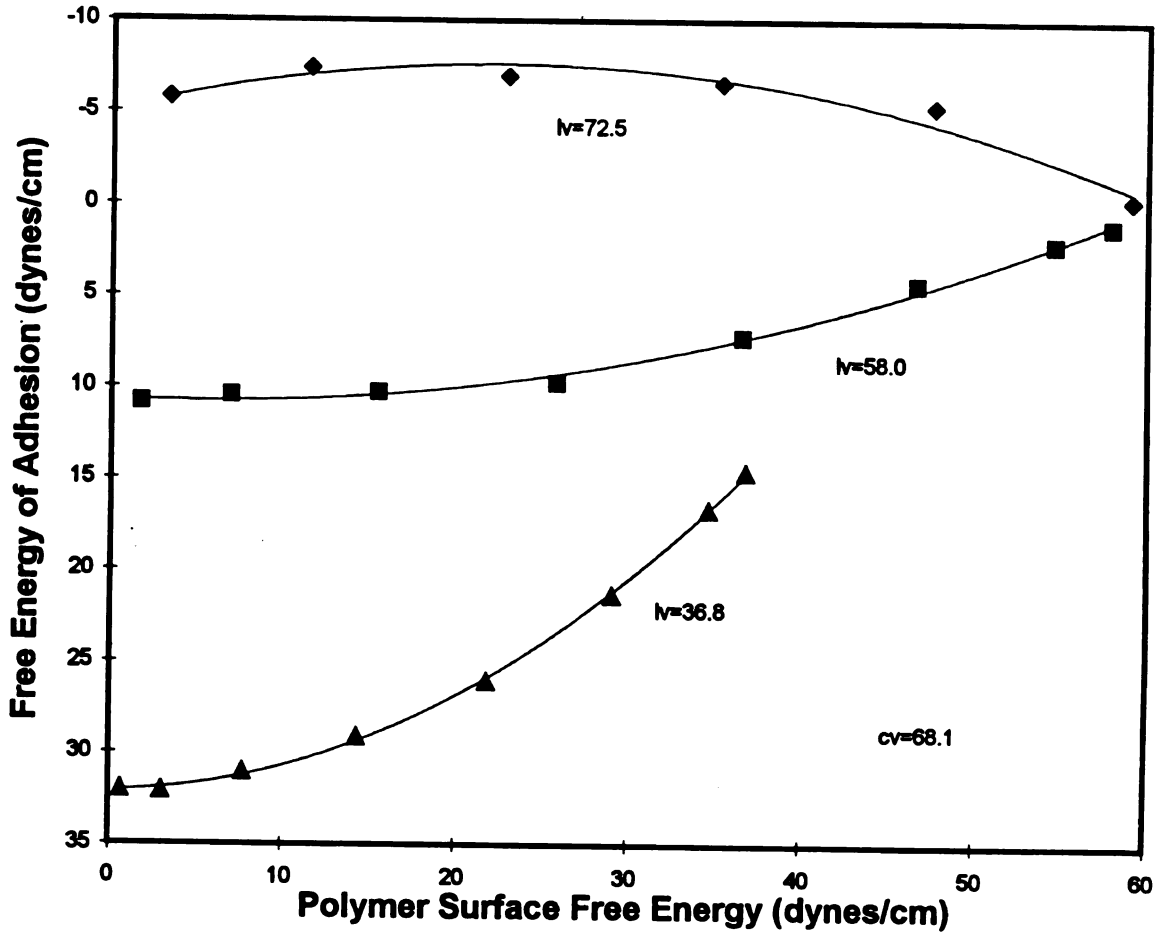


Figure 5.11 Prediction of the free energy of adhesion for *P. chrysosporium* based on the equation of state approach.

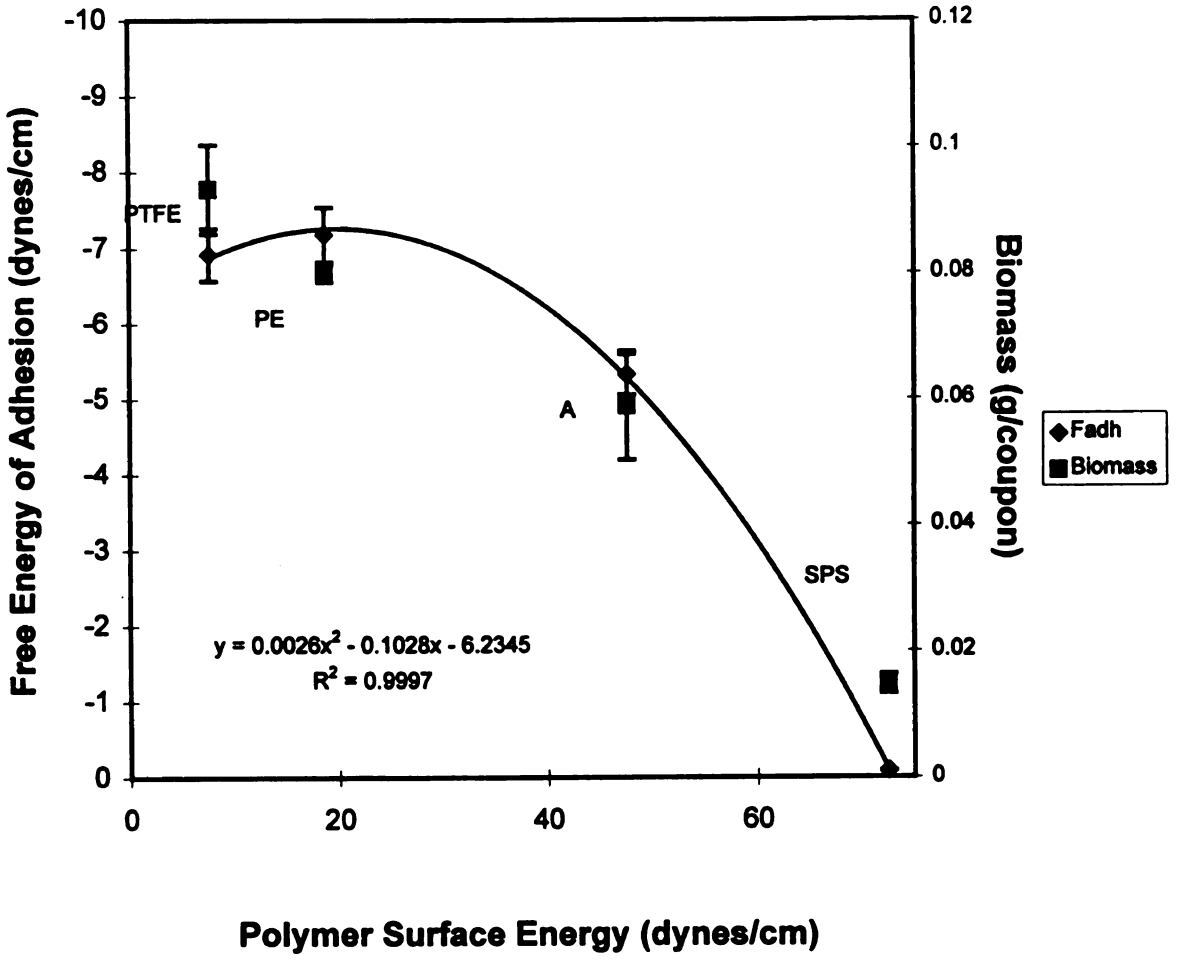


Figure 5.12 Comparison of predicted adhesion to experimental results for 0.5% Tween 80 in the suspending medium.

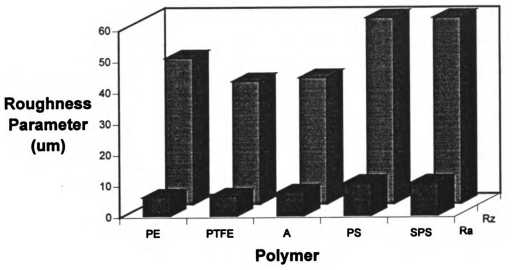


Figure 5.13 Average roughness, R_a and R_z, of roughened polymer coupons.

Table 5.3 Roughness parameters and contact angles for polymer surfaces.

Polymer	Grit	R_a (μm)	R_z (μm)	Contact Angle (degrees)
PE	smooth	0.2	1.8	92±4
	400	1.4	10.9	99±5
	150	2.4	18.4	94±6
	40	5.9	47.0	107±7
PTFE	smooth	0.9	6.0	110±12
	400	0.8	9.1	107±8
	150	2.9	19.4	104±7
	40	6.2	39.6	128±7
Acetal	smooth	0.2	2.5	82±5
	400	1.3	16.3	78±4
	150	3.4	25.6	60±4
	40	7.4	40.7	60±6
PS	smooth	1.0	8.5	84±6
	400	1.1	10.6	95±7
	150	2.2	20.9	95±8
	40	10.4	59.8	107±5
SPS	smooth	1.0	8.5	84±6
	400	1.1	10.6	95±7
	150	2.2	20.9	95±8
	40	10.4	59.8	107±5

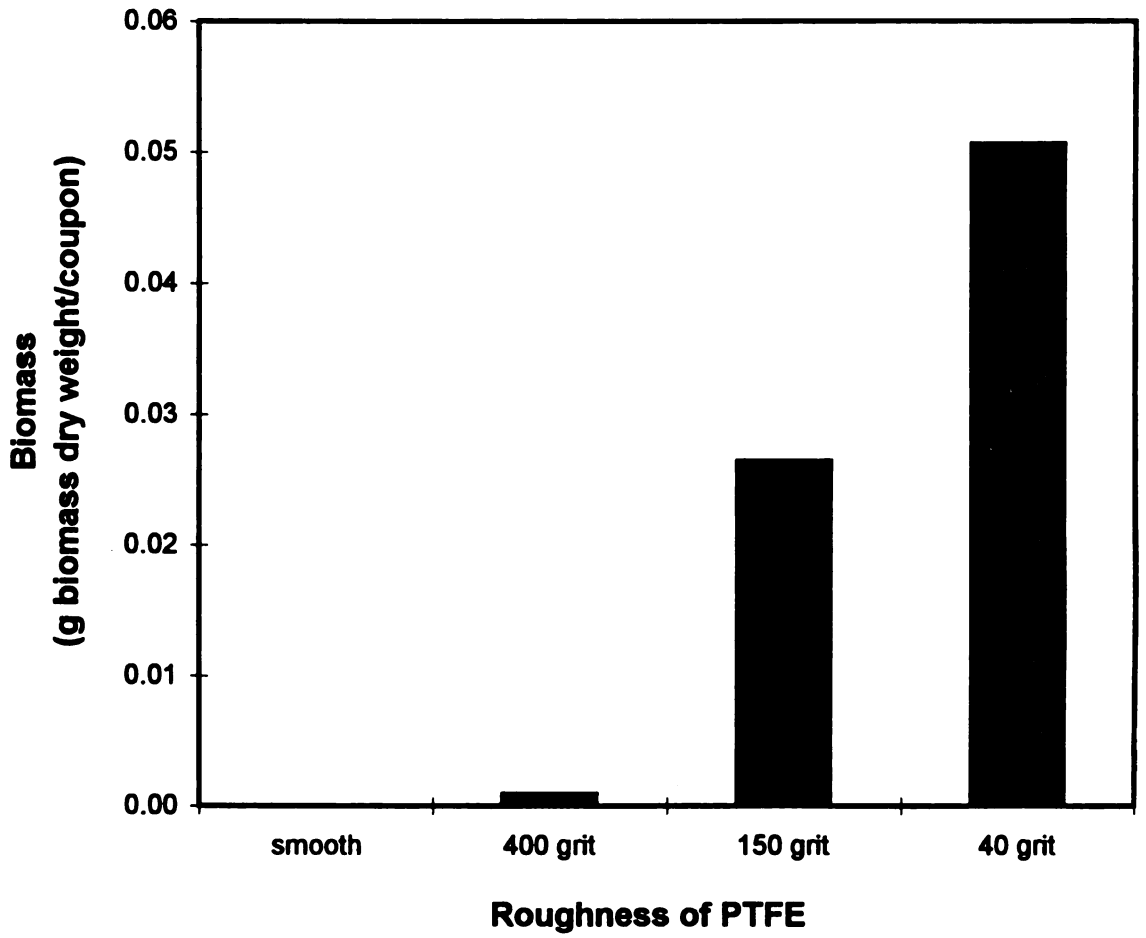


Figure 5.14 Adhesion of *P. chrysosporium* to roughened PTFE.

CHAPTER 6.

APPLICATION OF LASER SCANNING MICROSCOPY IN FUNGAL ADHESION STUDIES

Abstract

A major difficulty in the study of the adhesion of filamentous organisms to solid substrates is the lack of methods to easily and accurately quantify adhesion. Most methods of biomass measurement are applicable only for single-celled bacterium, yeast or plant cell adhesion. In this work, laser scanning microscopy (LSM) is shown to be a useful method to measure adhesion of fungal mycelia, and to characterize cells and substratum surfaces. During adhesion studies of the white-rot fungus *Phanerochaete chrysosporium*, cells were exposed to various chemical treatments and then contacted with glass microscope slides. LSM was used to quantify adhesion by measuring the percent surface area of the slide covered by a monolayer of cells. The effect of the treatment was determined by comparing the adhesion of treated and untreated cells.

In a different series of adhesion studies, *P. chrysosporium* adhered to roughened polymer coupons in agitated cultures. Confocal LSM provided a means to measure surface roughness and to image the topography of the polymer coupons. Higher surface roughness was correlated to increased adhesion.

Finally, LSM was used to characterize the hydrophobicity of fungal cells by observing the degree of attachment of fluorescent microspheres to the cell surface. This work has demonstrated the utility of LSM as a fast, easy-to-perform method to quantify adhesion of filamentous organisms to surfaces and to characterize cell and substratum surfaces.

Introduction

Although microbial adhesion is relevant to many diverse fields including marine fouling, pathogenesis, and bioreactor design for fermentation, the mechanisms that drive the adhesion process are not well-understood (Mafu et al., 1990; Dexter et al., 1975; Rogers et al., 1984). Most adhesion studies have focused on single-cell organisms such as bacteria, yeast, and animal cells with little attention given to the adhesion of mycelial organisms. A difficulty in working with mycelial organisms has been the lack of methodology with which to quickly quantify adhesion of small volumes of biomass (Matcham and Wood, 1988). This work describes three uses of laser scanning microscopy (LSM) in adhesion studies of a filamentous organism to a surface. In one application, LSM was used to quantify the irreversible adhesion of the white-rot fungus *Phanerochaete chrysosporium* to glass microscope slides after cells were exposed to various physiochemical treatments. A comparison of adhesion between treated and untreated cells indicated whether the treatment was a significant factor in the adhesion process of the organism.

In another portion of this study, images taken with LSM helped to characterize the surfaces of the polymers and cells. *P. chrysosporium* was cultured in shakeflasks containing roughened polymer coupons. LSM measured the average roughness of the polymer surfaces and provided an image of the surface topography. In addition, the hydrophobic nature of the fungal cell surface was determined by observing the amount of attachment of fluorescent microspheres to the cells. Images of the microspheres on the cell surface were taken with LSM.

Traditional methods of measuring biomass of single-celled organisms such as cell counts and light-scattering techniques are fast and easy to perform but are not appropriate for mycelial organisms because of the long, branched strands of hyphae. Filamentous organisms are often measured using dry weight but large amounts of biomass are needed for this technique (Pirt, 1975). Chitin and ergosterol, two compounds specific to fungal cell walls and membranes, respectively, require multistep assays and may not remain at constant levels during cell growth (Matcham, Jordan, and Wood; 1984). Light microscopy used in conjunction with image analysis to measure hyphal lengths of adhered cells is an accurate yet time-consuming method.

The use of LSM and image analysis has not been utilized to its full potential for quantifying mycelia (i.e. percent area covered by fungus). This approach is especially applicable to cell-surface studies where a monolayer of cells on a surface can be measured. Samples can be prepared and measured quickly, and computer images can be saved or converted into photographs.

Materials and Methods

Cell Cultivation

P. chrysosporium BKM-F 1767 (ATCC 24725) was cultured as described by Jager *et al.*, (1985) and Tien and Kirk (1988). Mycelial inoculum was prepared by inoculating a Fernbach flask containing 75 ml of medium with thawed conidia to give a final optical density of 1.0 at 650 nm (Kirk *et al.*, 1986). The Fernbach flask was incubated at 37°C for 48 hours to form a thin mycelial mat on the surface of the medium. The contents of the flask were homogenized using a blender at high speed for five minutes. An ice-water bath was used during homogenization to prevent heat damage to the mycelia. The homogenized mycelial suspension was used as inoculum for adhesion studies on roughened polymer coupons in shake flask cultures and on microscope slides.

Fungal Adhesion to Treated Microscope Slides

Superfrost Plus treated glass slides (Fisher Scientific; catalog #12-550-15) with Nunc Lab Tek Chamber Slide culture chambers (Baxter Diagnostics Inc.; catalog #T4136-8) were used for adhesion tests. These culture slides consist of a removable plastic chamber divided into eight wells. The chamber was attached to a microscope slide with a silicone gasket to prevent leaking. New microscope slides were cleaned by soaking overnight in 10% nitric acid solution and rinsing in distilled water.

Homogenized mycelia were centrifuged and resuspended in distilled water three times. The cells were diluted to the desired concentration and 0.3 ml of cell suspension was pipeted into each well of the culture chamber. After 30 minutes, the chamber and gasket were removed from the slide. The slide was gently dipped three times in distilled water to remove unattached cells, fixed in 2% gluteraldehyde, stained with crystal violet, rinsed once more with water, and air dried. Cover slips were permanently mounted on each slide.

Measurement of Adhesion Using Laser Scanning Microscopy

Cell adhesion was determined by using a Zeiss 10 confocal laser scanning microscope to measure the percent area of the slide surface covered by mycelia. This measurement is a feature of the LSM computer software histogram command. Samples viewed with the laser microscope transmitted light at various wavelengths which the microscope computer converted into a histogram. The computer then determined the percent of the total area which was covered by the sample. A well-defined histogram was obtained by increasing or decreasing the contrast to move the histogram right or left, respectively, and increasing or decreasing the brightness to decrease or increase, respectively, the width of the histogram. Images were viewed in transmittance mode using a 10x objective and a zoom factor of 20. The total area of each measurement was 0.7549 mm^2. Five to seven measurements were taken of each sample at random points on the slide. The mean and standard deviation of the replicates were calculated.

Characterization of Roughened Polymer Surfaces for Shakeflask Adhesion Studies

In order to study the effect of substratum roughness on cell adhesion, *P. chrysosporium* was cultured in shakeflasks containing roughened polymer coupons. Coupons with a 3/4 inches in diameter and 1/8 inch thick were made from sheets of polyethylene (PE). The coupons were roughened by hand using 40 grit sandpaper and cleaned with methanol in a sonicator.

Shakeflasks containing one preweighed polymer coupon and 30 ml of medium were inoculated with 3 ml homogenized mycelia (Kirk *et al.*, 1986). The cultures were maintained at 37°C, agitated at 190 rpm on an orbital shaker, and oxygenated once daily with pure oxygen. After seven days of growth, the coupon was removed from each flask, rinsed gently with distilled water, and air dried. The dry weight of biomass on each coupon was determined.

The roughness of the polymer surfaces was measured using the laser scanning microscope in the reflection mode. Microscopic sectioning techniques produced three-dimensional images of the polymer surfaces. A z-series was generated using a 50x objective and optical sections of a 0.8 micron thickness. The dimensions of the scanned portion of the sample were 189 x 284 microns. Photographs were taken using Kodak T-max 100 film for black and white prints and Kodak Ektachrome 100 for color slides.

Cell Characterization Using Microsphere Attachment Studies

Hydrophobicity of *P. chrysosporium* hyphae was observed using microspheres from Interfacial Dynamics Corporation (Portland, Oregon). Fluorescent sulfate polystyrene latex microspheres (Interfacial Dynamics Corp., catalog #L-5081) with a diameter of 1.0 μm were used to adhere to hydrophobic areas. The microspheres were yellow-green fluorescent with an excitation/emission wavelength at 490/515 nm.

Microsphere suspensions were prepared in small glass test tubes using phosphate-urea-MgSO4 buffer (PUM buffer, pH 7.1, Rosenberg, *et al.*, 1980) at a concentration of 7×10^8 beads/ml. The suspensions were gently vortexed and placed in an ice-water bath. A mycelial suspension was prepared by homogenizing the contents of one Fernbach flask at high speed for five minutes. The cells were then centrifuged in microcentrifuge tubes and resuspended in medium. Equal volumes of mycelial and microsphere suspensions were mixed by gently vortexing 30 seconds on a flat surface vortexer. After allowing the microspheres to contact the cells for five minutes, the mixture was mounted onto a glass microscope slide with a coverslip.

The mycelia were imaged with the laser scanning microscope in transmission mode using a 40x objective. The microspheres were imaged in fluorescent mode using a section series with 1 micron section thickness and 5-20 sections, as needed. The images of the mycelia and the microspheres were combined using the "color overlay" feature.

Results and Discussion

Fungal Adhesion on Microscope Slides

In the first application, the laser scanning microscope was used to measure the adhesion of mycelial fragments of *P. chrysosporium* to microscope slides (Figure 6.1) The time-dependence of adhesion was observed for a cell concentration equal to 10% of the initial cell concentration (Figure 6.2). Each datum point in the plots represents the average of seven measurements. The adhesion of the fungus followed a typical adsorption isotherm (Busscher *et al.*, 1986) with an initial increase in adhesion followed by a plateau during which little additional adhesion occurred.

The same technique was used to measure the adhesion of cells which had been exposed to various chemical treatments. A greater amount of adhesion of treated cells compared to untreated cells indicated that the treatment affected the adhesion process. For example, amphotericin B, which disrupts fungal cell membrane function, inhibited adhesion of mycelia to slides (Figure 6.3). This suggests the critical importance of the cell membrane during adhesion. This technique was used for many other similar experiments to determine the effect on adhesion of treatments that altered cell functions such as metabolic activity, protein synthesis, or the integrity of the cell surface.

Laser scanning microscopy was a critically important method for quantifying small amounts of *P. chrysosporium*. Few techniques exist for easily quantifying monolayers of mycelial cells. Dry weight measurements

require a large amount of biomass while techniques such as cell counts are useful only for single-celled organisms. Methods such as ATP or ergosterol assays require time-consuming preparation. Because LSM was fast and needed only simple preparation (i.e. staining cells), many experiments could be conducted with the statistically appropriate number of replicate samples.

Characterization of Adhesion Surfaces

In the second application of LSM, various polymer surfaces used in cell adhesion studies were characterized. Images of smooth and roughened surfaces, taken in reflection mode using sectioning techniques, showed the surface topography. Average roughness was determined for each polymer. Figures 6.4 and 6.5 show the image of roughened polyethylene (grit 150) and the corresponding roughness profile, respectively.

LSM offers flexibility and ease of use for imaging surfaces. The transmission, reflection, and fluorescent modes allow a variety of materials to be imaged. Roughness profiles obtained by LSM are determined from a two-dimensional surface area unlike other profile instruments which measure R_a for only a one-dimensional distance. This provides a more complete picture of a surface. Also, since direct contact is not required, roughness measurements using this instrument will not damage or alter surfaces.

Hydrophobicity of Fungal Cells

The attachment of fluorescent sulfate microspheres to washed, homogenized mycelia is shown in the laser scanning micrograph (Figure 6.6). Numerous microspheres were attached along the entire length of the hyphal fragments. The extent of bead attachment indicates that the surface of the fungal cells contains hydrophobic regions. The presence of hydrophobic regions on mycelial cells is consistent with the results of other *P. chrysosporium* adhesion studies on roughened polymer coupons (Jones and Briedis, in preparation for publication). In these studies, interfacial free energy of the substratum significantly influenced cell adhesion (lower interfacial free energy promoted adhesion) which indicates that hydrophobic interactions are involved in cell adhesion.

Microscope attachment studies used in conjunction with LSM provides a method to characterize the hydrophobic nature of individual mycelial fragments. This differs from methods such as contact angle measurements which give information about the average hydrophobicity of a population of cells. Other methods, such as aqueous-hydrocarbon partitioning and hydrophobic interaction chromatography, are appropriate for single-celled, but not mycelial, organisms.

In this work, a transmission image of the cells and a fluorescent image from a series of sections of the microspheres were combined. In these images, the colored microspheres were clearly defined and distinguishable from other cellular material. Quantitative methods could be easily applied in microsphere

attachment studies by using the LSM computer software to count the number of individual microspheres per unit surface area. Hazen and Hazen (1987 and 1988) used brightfield, rather than fluorescence, microscopy in their microsphere attachment studies to determine the surface hydrophobicity of the yeast *Candida albicans*.

Conclusions

One difficulty in adhesion studies of filamentous organisms to surfaces is the lack of methods to quickly and quantitatively measure adhesion. Laser scanning microscopy was a powerful tool for studying adhesion of the fungus *P. chrysosporium* to surfaces. Three distinct applications of LSM in this work included (1) quantifying the amount of mycelia which irreversibly adhered to a surface, (2) imaging the topography of substratum surfaces and measuring the roughness parameters, and (3) determining the hydrophobic nature of the cell surface using fluorescent microspheres. LSM has many potential applications in microbial adhesion studies for characterizing both biological and material surfaces.

Acknowledgments

We gratefully acknowledge of Dr. Joanne Whallon, Crop and Soil Sciences, Michigan State University, for her expertise in laser scanning microscopy and the use of the LSM.

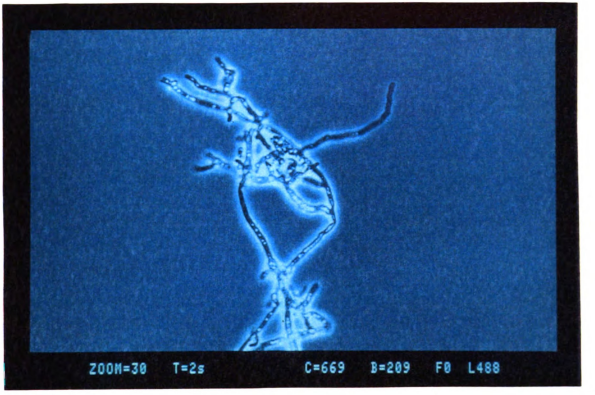


Figure 6.1 LSM photo of *P. chrysosporium* adhesion to a microscope slide.

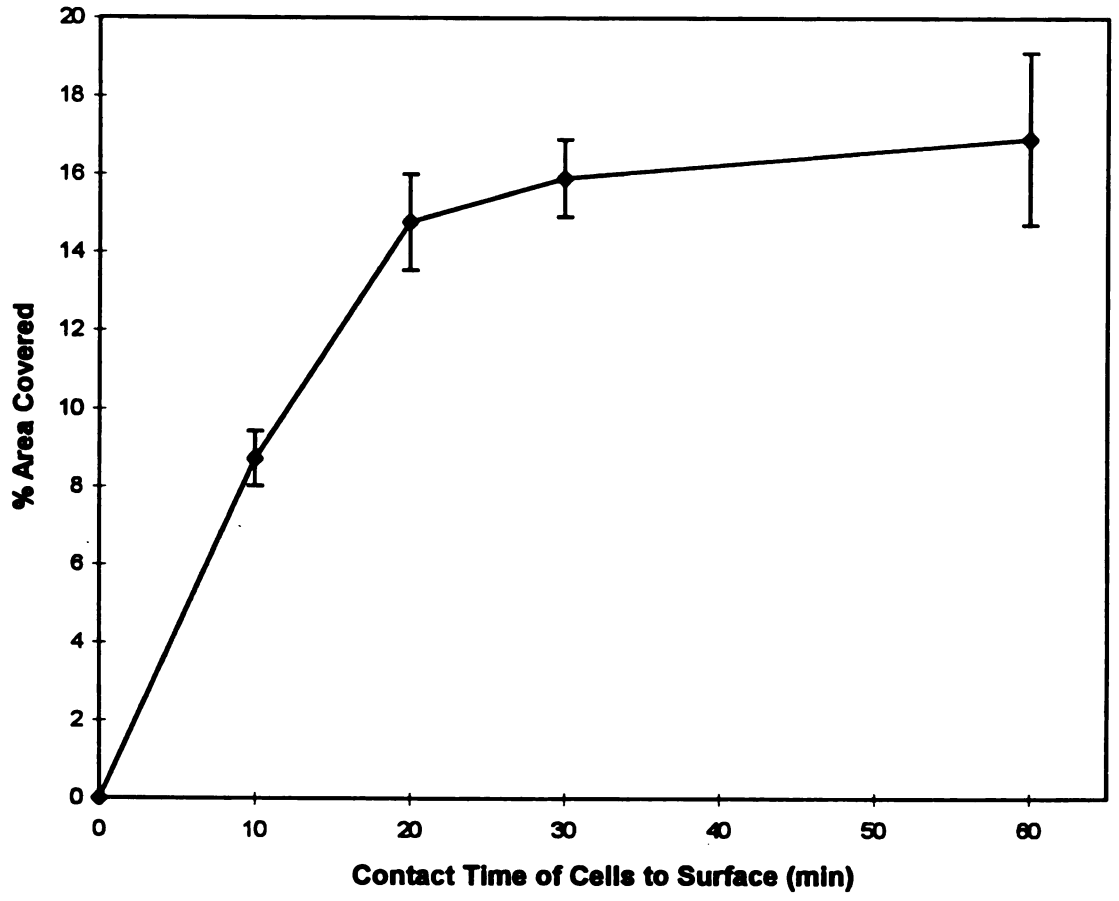


Figure 6.2 Adhesion of *P. chrysosporium* to glass microscope slides as a function of contact time. Cells were diluted to 10% of the initial cell concentration.

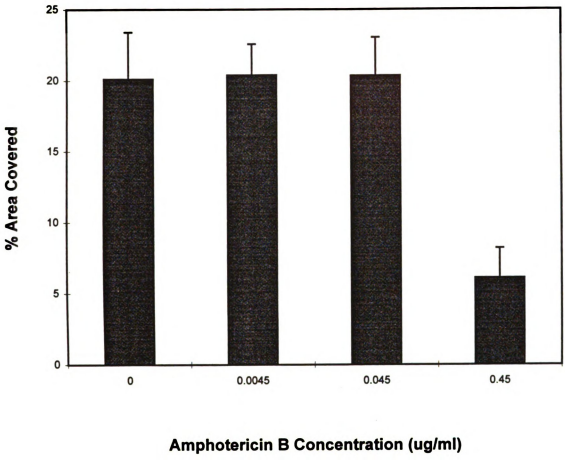


Figure 6.3 Effect of amphotericin B on the adhesion of *P. chrysosporium* to microscope slides.

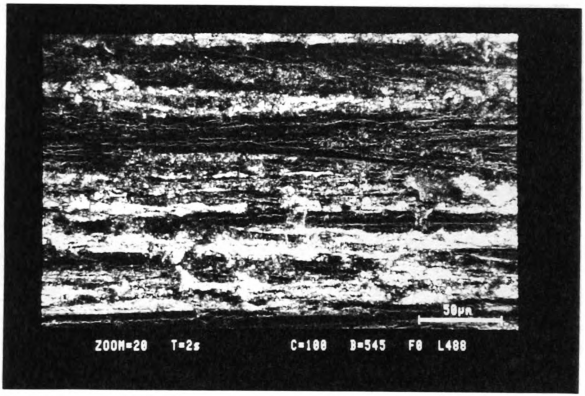


Figure 6.4 LSM photo of polyethylene surface (150 grit).

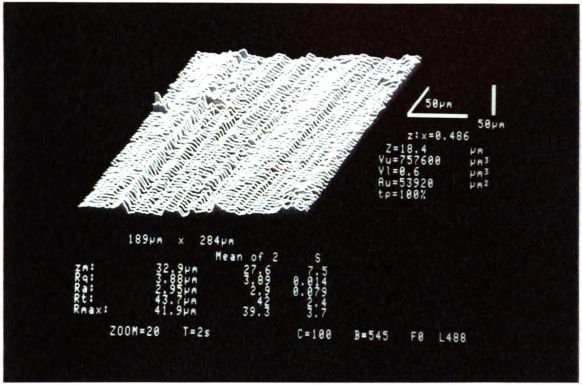


Figure 6.5 Roughness profile of polyethylene surface (150 grit).

Figure 6.6 Attachment of fluorescent sulfate microspheres to *P. chrysosporium*.

CHAPTER 7.

CONCLUSIONS

Overall Conclusions

An overall framework does not currently exist for understanding the phenomena involved in microbial adhesion. Adhesion is a result of many interactions including long range electrostatic forces, short range hydrophobic interactions, properties of the substratum and cell surface, and the physiological activity of the cell. This work represents the most comprehensive study of the adhesion of *P. chrysosporium* to date. The majority of the factors investigated here have not been discussed in previous studies of this organism. This work also includes the first reported use of laser scanning microscopy to easily quantify adhesion of a filamentous organism and to characterize cell and substratum surfaces. These results will provide a means of developing improved bioreactor systems for *P. chrysosporium* and contribute to the understanding of fungal adhesion.

The objective of this research was to investigate the factors that influence the adhesion of *P. chrysosporium* to surfaces, including properties of the cell, substratum, and suspending medium. Experiments using glass microscope slides indicated the following:

- an active cell metabolism is required for adhesion
- a functioning cell membrane is required for adhesion
- protein synthesis occurs during adhesion
- the presence of extracellular proteins and polysaccharides promotes adhesion
- electrostatic interactions (long range forces) do not appear to be significant
- adhesion follows Langmuir isotherm kinetics
- the optimum temperature for adhesion is 22°C, which is lower than the optimum of 37°C required for growth and production of the lignin peroxidase enzyme system

Adhesion studies using polymer coupons in shake flask cultures showed:

- the presence of exopolysaccharides is important for adhesion
- protein synthesis and the presence of extracellular proteins appeared to be less important

Characteristics of the substratum played a key role in adhesion to polymer coupons:

- fungal adhesion and subsequent growth was higher on surfaces with higher values of average roughness, R_a
- thermodynamic predictions of adhesion based on an empirically-derived equation-of-state showed a high correlation to adhesion observed experimentally on various polymer materials
- adhesion increased as the surface free energy of the substratum decreased, irrespective of the surface tension of the suspending medium
- these results suggest that short range forces between the cell and substratum are an important factor in adhesion

In summary, irreversible adhesion of *P. chrysosporium* is complex process involving short range interactions between the substratum and the cells, protein synthesis, the presence of extracellular polysaccharides and proteins, and a functioning cell wall. Adhesion is promoted on surfaces with lower surface free energies. Long range electrostatic interactions do not appear to be a significant factor in adhesion.

Future Directions

Two primary areas should be targeted for further research. First, the influence on adhesion of polysaccharides in the medium and exopolysaccharides excreted by the cell should be better understood. Similarly, the role of compounds adsorbed from the suspending medium, such as proteins, onto the substratum surface should be studied. These experiments should be planned within the framework of typical conditions which may be encountered in commercial applications of this organism in a bioreactor or wastewater treatment system. The ultimate goal of this work is to build a comprehensive understanding of the interactions between microbial organisms and material surfaces during the adhesion process.

APPENDICES

Appendix 1 Algorithm and FORTRAN Code for Program "Gam"

Algorithm for "GAM"

1. Enter value of contact angle, THETA
2. Enter value of γ_{LV}
3. Solve for γ_{SV} using subroutine "WEGSTEIN" to solve for roots. Subroutine calls function "FUNC" to solve for γ_{SV}.
4. Calculate γ_{SL}
5. Print value of γ_{SL}

FORTRAN Code For Program "GAM"

```
   PROGRAM GAM
C
C  REF:  LI AND NEUMANN, J. COLLOID & INTERFACE SCIENCE,148:190-200,1992
C
C  EQUATION-OF-STATE APPROACH TO DETERMINE (OUTPUT):
C     SOLID-VAPOR INTERFACIAL TENSION    (SV IN MJ/M*M)
C     SOLID-LIQUID INTERFACIAL TENSION    (SL IN MJ/M*M)
C
C  FROM THE MEASURABLE QUANTITIES (INPUT):
C     CONTACT ANGLE                      (THETA IN DEGREES)
C     LIQUID SURFACE TENSION.            (LV IN MJ/M*M)
C
C  THE PROGRAM USES EQUATION 24 FROM REF. TO FIND SV.  THE
C  EQUATION HAS BEEN REARRANGED TO SOLVE FOR ONE OF THE ROOTS
C  OF SV.  THE WEGSTEIN ROOT-FINDING ALGORITHM WAS USED TO SOLVE
C  FOR THE ROOTS.  THE INITIAL GUESS FOR SV IS INPUT BY THE USER
C  AND SHOULD HAVE A VALUE OF EITHER 5 OR 50 MJ/M*M.
C
C  EQUATION 4 FROM REF. IS USED TO CALCULATE SL.
C
     REAL THETA, LV,SV,B,SL
     INTEGER IT
     PRINT *, 'ENTER VALUE OF THETA IN DEGREES'
     READ *, THETA
     PRINT *, 'ENTER VALUE OF LV'
     READ *, LV
     PRINT *,'ENTER VALUE OF INITIAL GUESS FOR SV--5 OR 50'
     READ *, SVGUESS
     CALL WEG(SVGUESS,THETA,LV,SV,IT)
     PRINT *,'SV=',SV
     PRINT *,'IT=',IT
     B=-0.0001247
     SL=LV + SV - 2.0*SQRT(LV*SV)*EXP(B*(LV-SV)**2.)
     END
     PRINT *, 'SL=',SL
****************************************
```

```
      SUBROUTINE WEG(SVGUESS,THETA,LV,SV,IT)
      REAL  LV
      TOL=0.00001
      DELTA=1.0
      IT=0
      ILIM=50
      THETA=THETA*3.14159265/180.0
      X1=SVGUESS
      X2=FUNC(THETA,LV,X1)
   10 GX1=FUNC(THETA,LV,X1)
         GX2=FUNC(THETA,LV,X2)
         X3=(X1*GX2-X2*GX1)/(X1-X2-GX1+GX2)
         X1=X2
         X2=X3
         DELTA = ABS(X2 - X1)
         IT=IT+1
         IF((DELTA .GT. TOL) .AND. (IT .LE.ILIM)) THEN
         GO TO 10
         END IF
         SV=X3
         RETURN
         END
*****************************************
      FUNCTION FUNC(THETA,LV,SV)
      REAL LV
      B=-0.0001247
      FUNC=(((COS(THETA)+1.0)*SQRT(LV))/(2.0*EXP(B*(LV-SV)**2.0)))**2.0
      RETURN
      END
```

Appendix 2 Algorithm And FORTRAN Code for Program "Surf"

Algorithm for "SURF"

The equation of state contains a discontinuity for large values of γ_{S2V} which is accounted for in the subroutine. See Neumann *et al* (1980) for more details (note: Equation 5 is incorrect this reference).

1. Enter γ_{S1V}, γ_{S2V}
2. Call subroutine "SUB1" to calculate γ_{S12}
3. Print value of γ_{S12}

Subroutine SUB1

1. Rename the surface tension with the lower value G1V; call the higher value G2V
2. If $0 < \gamma_{S2V} < 30$, then calculate γ_{S12} using the equation of state, function "FUNC1"

   Else if $\gamma_{S2V} > 30$ and $\gamma_{S2V} \leq 50$, then calculate γ_{S12} using the equation of state ("FUNC1") until $\gamma_{S12}=\gamma_{S1V}$. After this point, calculate γ_{S12} using a different equation, which avoids the discontinuity of the equation of state, function "FUNC2".

   Else if $\gamma_{S2V} > 50$, then calculate the slope of the equation of state, $(\partial\gamma_{S12}/\partial\gamma_{S1V})$, FUNC3.
   Calculate γ_{S12} from the equation of state until the slope is equal to -1.0. Beyond this point, calculate γ_{S12} from a straight line with slope -1.0, FUNC4, until the line reaches the curve for γ_{S12} calculated from FUNC2. After this point, begin using the FUNC2 curve.
3. Return the value of γ_{S12} to main program

FORTRAN Code For Program "SURF"

```
      PROGRAM SURF
C
C  REF:NEUMANN, HUM, FRANCIS, J. BIOMEDICAL MATERIALS RESEARCH,
C  14:499-509 (1980)
C
C  THIS PROGRAM FINDS THE INTERFACIAL TENSION BETWEEN TWO
C  PHASES (SOLIDS) USING THE EQUATION OF STATE APPROACH.  THE
C  MEASURABLE QUANTITIES FOR SURFACE TENSIONS ARE KNOWN.
C
C  INPUT:  S1V, S2V = SURFACE TENSION, ERG/CM2
C  OUTPUT:  S12 = INTERFACIAL TENSION BETWEEN THE TWO PHASES,ERG/CM2
C
```

```
      REAL G1V,G2V,G12,S1V,S2V,S12,FUNC1,FUNC2,FUNC3,FUNC4
      PRINT *, 'ENTER VALUE OF S1V'
      READ *, S1V
      PRINT *, 'ENTER VALUE OF S2V'
      READ *, S2V
      CALL SUB1(S1V,S2V,S12)
      PRINT *,'S12= ',S12
      END
C
      SUBROUTINE SUB1(G1V,G2V,G12)
C
      INTEGER ITER
      REAL G1V,G2V,G12,S1V,S2V,S12,GAM1V,GAMS,SLOPE1,SLOPE2,GAMS12
C
      IF (G1V .GT. G2V)THEN
         S1V = G2V
         S2V = G1V
      ELSE IF (G1V .LE. G2V) THEN
         S1V = G1V
         S2V = G2V
      END IF
C
      IF (S2V .GT. 0.0 .AND. S2V .LE. 30.0) THEN
         PRINT *, 'S2V LESS THAN 30'
         S12 = FUNC1(S1V,S2V)
         G12 = S12
      ELSE IF (S2V .GT. 30.0 .AND. S2V .LE. 50.0) THEN
           PRINT *, 'S2V BETWEEN 30 AND 50'
         S12 = FUNC1(S1V,S2V)
C
          IF (S12 .GT. S1V) THEN
            G12 = S12
            PRINT *, 'MESSAGE1'
          ELSE IF (S12 .LE. S1V) THEN
            PRINT *, 'MESSAGE 2'
            S12 = FUNC2(S1V,S2V)
            G12 = S12
          END IF
C
      ELSE IF (S2V .GT. 50.0)THEN
         PRINT *, 'S2V GREATER THAN 50'
         GAMS1 = 10.0
         ITER=0
C
 10        GAMS=GAMS1
           SLOPE1=FUNC3(GAMS,S2V)
           SLOPE2=FUNC4(GAMS,S2V)
           GAMS1=GAMS - (SLOPE1 + 1.0)/SLOPE2
           ITER = ITER + 1
C
            IF (ABS(GAMS1-GAMS).GT. 0.0001 .AND. ITER .LE. 2500.)THEN
               GOTO 10
            ELSE IF(ITER .GT. 2500.)THEN
               PRINT *, 'ITERATIONS USED EXCEEDED 2500= ', ITER
```

```
            END IF
            PRINT *, 'ITERATIONS USED = ',ITER
C
          GAMS2 = FUNC1(GAMS1,S2V)
C
          IF (S1V.LE. GAMS1)THEN
           PRINT *,'CONDITION 3-A'
           S12 = FUNC1(S1V,S2V)
           G12=S12
          ELSE IF (S1V.GE. GAMS2)THEN
           PRINT *, 'CONDITION 3-B'
           S12 = FUNC2(S1V,S2V)
           G12 = S12
          ELSE
           PRINT *, 'CONDITION 3-C'
           S12 = GAMS1 + GAMS2 - S1V
           G12=S12
          END IF
C
      END IF
      RETURN
      END
C
      FUNCTION FUNC1(S1V,S2V)
         FUNC1=((SQRT(S1V)-SQRT(S2V))**2.0)/(1.0 - 0.015*(SQRT(S1V*S2V)))
      RETURN
      END
C
      FUNCTION FUNC2(S1V,S2V)
         FUNC2=(((2.0-0.015*S1V)*SQRT(S2V)-SQRT((2.0-0.015*S1V)**2.0*S2V-
     C        4.0*(S2V-S1V)))**2.0)/4.0
      RETURN
      END
C
        FUNCTION FUNC3(S1V,S2V)
         U=(SQRT(S1V)-SQRT(S2V))**2.0
         V=1.0-0.015*SQRT(S1V)*SQRT(S2V)
         DU=(SQRT(S1V)-SQRT(S2V))/SQRT(S1V)
         DV=(-0.015*SQRT(S2V))/(2.0*SQRT(S1V))
         FUNC3=(V*DU - U*DV)/(V*V)
        RETURN
        END
C
        FUNCTION FUNC4(S1V,S2V)
         U=1.0-SQRT(S2V/S1V)-0.015*(SQRT(S2V*S1V)-(S2V)**1.5/SQRT(S1V))/2.0
         V=(1.0-0.015*SQRT(S2V*S1V))**2
         DU=SQRT(S2V)/(2.0*S1V**1.5)-(0.015*SQRT(S2V/S1V))/4.0-(0.015*
     C        (S2V/S1V)**1.5)/4.0
         DV=-0.015*SQRT(S2V/S1V)+(0.015*S2V)**2
         FUNC4=(V*DU - U*DV)/(V*V)
        RETURN
        END
```

Appendix 3 Procedure for Sulfonating Polystyrene

This is a brief description of the method used for sulfonating polystyrene.

1. Dry polystyrene in oven for several hours to remove moisture.
2. Clip sample by its corners into holding rack and insert into treatment chamber. The chamber is a stainless steel rectangular box with a removable end for sample insertion/removal. It has an inlet and outlet fitting which are regulated by ball valves and three way valves. One of the valves is connected to a vacuum pump. The entire system is hooked to the inlet/outlet of the sulfonator.
3. Sample chamber is flushed with nitrogen for 10 minutes.
4. A vacuum is drawn on the sample chamber to remove any contaminants or water vapor.
5. Sample chamber is flushed again with nitrogen for 10 minutes.
6. The valves are reversed and the system is opened to the sulfonator.
7. Samples is exposed to sulfur trioxide gas for 2 minutes.
8. The valves are reset for nitrogen circulation. Sample chamber is flushed with nitrogen for 10 minutes.
9. Sample is removed from the chamber and placed in a 5% NaOH solution for neutralization.

Appendix 4 Program Listing Of Excel Spreadsheet "Surfcomp"

This spreadsheet calculates the polar and nonpolar components of liquid surface tension. The input parameters are the liquid surface tension the contact angle of the liquid on Parafilm, an apolar material.

The spreadsheet also calculates the polar and nonpolar components of the surface free energy of a solid (cells or substratum). It solves two equations with two unknowns, γ_S^d and γ_S^p, simultaneously using the command OPTIONS/CALCULATION/ITERATION with an chosen iteration limit of 100 and a tolerance of 0.001. The input parameters are the contact angle values for each liquid on the solid.

SURFACE COMPONENT APPROACH MODEL

Characterization of Liquids

ds of parafilm= 25.9 θ= contact angle on parafilm

	Measured quantities		*Calculated Values*	
LIQUID	LV	θ	DL	PL
1-bromonaphthalene	43.9	120	0.0	43.9
water	72.7	64	21.4	51.3

Solve for DS and PS of a solid surface (cell or substratum):

Input values for liquid 1 and liquid 2:

Liquid 1 surface free energies (mJ/m2): water

polar	lp1=	51.3
nonpolar	ld1=	21.4
total	l1=	72.7

Liquid 2 surface free energies (mJ/m2): 1-bromonaphthalene

polar	lp2=	0
nonpolar	ld2=	43.9
total	l2=	43.9

Contact Angles (input degrees)

Liquid	*degrees*	*radians*
liquid 1	20	0.35
liquid 2	25	0.44

Results:

Surface free energy (mJ/m2):

polar=	33.2
nonpolar=	39.9
total=	73.1

Appendix 5 Surface Component Approach

This is a description of how the equations for the surface component approach may be applied to determine the free energy of adhesion of cells to a substratum surface from the polar and nonpolar components of interfacial free energy.

The free energy of adhesion, ΔF_{adh}, is related to the interfacial free energy of each interaction:

$$\Delta F_{adh} = \gamma_{CS} - \gamma_{SL} - \gamma_{CL} \qquad \text{(A5.1)}$$

The Owens and Wendt equation defines the interfacial free energy between two surfaces as:

$$\gamma_{12} = \gamma_1 + \gamma_2 - 2(\gamma_1^d \gamma_2^d)^{1/2} - 2(\gamma_1^p \gamma_2^p)^{1/2} \qquad \text{(A5.2)}$$

The Owens and Wendt equation written for each term of Equation A5.1:

$$\gamma_{CS} = \gamma_C + \gamma_S - 2(\gamma_C^d \gamma_S^d)^{1/2} - 2(\gamma_C^p \gamma_S^p)^{1/2} \qquad \text{(A5.3)}$$
$$\gamma_{CL} = \gamma_C + \gamma_L - 2(\gamma_C^d \gamma_L^d)^{1/2} - 2(\gamma_C^p \gamma_L^p)^{1/2} \qquad \text{(A5.4)}$$
$$\gamma_{SL} = \gamma_S + \gamma_L - 2(\gamma_S^d \gamma_L^d)^{1/2} - 2(\gamma_S^p \gamma_L^p)^{1/2} \qquad \text{(A5.5)}$$

Substitute into Equation A5.1:

$$\Delta F_{adh} = -2\gamma_L + 2[(\gamma_S^d \gamma_L^d)^{1/2} - (\gamma_S^d \gamma_C^d)^{1/2}] + 2[(\gamma_S^p \gamma_L^p)^{1/2} - (\gamma_S^p \gamma_C^p)^{1/2}] + 2[(\gamma_C^d \gamma_L^d)^{1/2} + (\gamma_C^p \gamma_L^p)^{1/2}] \qquad \text{(A5.6)}$$

The polar and nonpolar components of the liquid, cell, and substratum surface must be found.

Young's equation relates the angle of a droplet on a solid surface to interfacial energies by:

$$\cos\theta = (\gamma_{SV} - \gamma_{SL})/\gamma_{LV} \qquad \text{(A5.7)}$$

The Owens and Wendt equation written for each term of the Young's equation:

$$\gamma_{SV} = \gamma_S + \gamma_V - 2(\gamma_S^d \gamma_V^d)^{1/2} - 2(\gamma_S^p \gamma_V^p)^{1/2} \qquad \text{(A5.8)}$$
$$\gamma_{SL} = \gamma_S + \gamma_L - 2(\gamma_S^d \gamma_L^d)^{1/2} - 2(\gamma_S^p \gamma_L^p)^{1/2} \qquad \text{(A5.9)}$$
$$\gamma_{LV} = \gamma_L + \gamma_V - 2(\gamma_L^d \gamma_V^d)^{1/2} - 2(\gamma_L^p \gamma_V^p)^{1/2} \qquad \text{(A5.10)}$$

Substitute into Young's equation and neglect spreading pressure (neglect all "v" terms):

$$\cos\theta = -1 + \frac{2(\gamma_S^d \gamma_L^d)^{1/2}}{\gamma_L} + \frac{2(\gamma_S^p \gamma_L^p)^{1/2}}{\gamma_L} \qquad \text{(A5.11)}$$

Find Polar and Nonpolar Components of Liquids

Use the same liquids that are used to measure contact angles on the cell and substratum surface.

1. Measure γ_L of liquids using Cahn DCA instrument.
2. Measure contact angle, θ, of liquids on Parafilm.
3. Find γ_L^d from Equation A5.11 where $\gamma_S^p = 0$.
   Parafilm is completely apolar so $\gamma_S^p = 0$.
   Since $\gamma_S = \gamma_S^d + \gamma_S^p$ then $\gamma_S^d = \gamma_S = 25.9$ mJ/m^2 for Parafilm at 20° (Asther *et al.*, 1990).

$$\gamma_L^d = \left[\frac{(\cos\theta + 1)\gamma_L}{2\sqrt{\gamma_S^d}}\right]^2 \qquad \text{(A5.12)}$$

4. Find γ_L^p from $\gamma_L^p = \gamma_L - \gamma_L^d$.

Find Polar and Nonpolar Components of Solid Surfaces (Cells And Substratum)

1. Measure contact angle, θ, on the solid surface using two different liquids (i.e. 0.15M NaCl and 1-bromonaphthalene).
2. Write Equation A5.11 for each liquid, for example:

$$\cos\theta_1 = -1 + \frac{2(\gamma_S^d \gamma_{L1}^d)^{1/2}}{\gamma_{L1}} + \frac{2(\gamma_S^p \gamma_{L1}^p)^{1/2}}{\gamma_{L1}} \qquad \text{(A5.13)}$$

$$\cos\theta_2 = -1 + \frac{2(\gamma_S^d \gamma_{L2}^d)^{1/2}}{\gamma_{L2}} + \frac{2(\gamma_S^p \gamma_{L2}^p)^{1/2}}{\gamma_{L2}} \qquad \text{(A5.14)}$$

These two equations contain two unknowns and can be solved for γ_S^d and γ_S^p.

Rearrange Equation A5.13 for γ_S^p:

$$\gamma_S^p = \frac{\left[\gamma_{L1}\cos\theta_1 + \gamma_{L1} - 2(\gamma_S^d \gamma_{L1}^d)^{1/2}\right]^2}{2\gamma_{L1}^p} \qquad \text{(A5.15)}$$

Rearrange Equation A5.14 for γ_S^d:

$$\gamma_S^d = \frac{\left[\gamma_{L2}\cos\theta_2 + \gamma_{L2} - 2(\gamma_S^p \gamma_{L2}^p)^{1/2}\right]^2}{2\gamma_{L2}^d} \qquad \text{(A5.16)}$$

Solve on EXCEL spreadsheet. Use the command OPTIONS/CALCULATION/ITERATION to solve for the unknowns.

3. The steps above must be repeated for each solid surface.

Determine the Free Energy of Adhesion

1. The values of all the parameters have been determined. Calculate ΔF_{adh} using Equation A5.6 for each substratum surface.

Appendix 6 Check of FORTRAN Program "GAM" and Excel Spreadsheet "Surfcomp"

The accuracy of the values calculated for γ_S^d by the EXCEL spreadsheet "SURFCOMP" and γ_{SV} by the FORTRAN program "GAM" was examined. The values were compared to literature values reported for various species of bacteria (Loosdrecht *et al.*, 1987). The values are presented in Table A6.

The following values were used in the programs:

water	$\gamma_{LV} = 72.7$ mJ·m^{-2}
α-bromonaphthalene	$\gamma_{LV} = 43.9$ mJ·m^{-2}

Table A6. Comparison of surface free energy values.

Organism	Literature Values†						FORTRAN program GAM		EXCEL spreadsheet SURFCOMP	
	Contact angle (°)		Equation of state (mJ·m^{-2})		Geometric mean (mJ·m^{-2})		Equation of state (mJ·m^{-2})		Geometric mean (mJ·m^{-2})	
	γ_S^d α-BN‡	γ_{SV} water	γ_S^d α-BN	γ_{SV} water	γ_S^d α-BN	γ_{SV} water	γ_S^d α-BN	γ_{SV} water	γ_S^d α-BN	γ_{SV} water
Pseudomonas sp. strain 26-3	25	20	41	68	40	70	40.0	68.7	39.9	73.1
Arthrobacter sp. strain 177	37	60	36	47	36	48	36.1	47.8	35.5	49.7
Streptococcus salivarius	44	26	33	65	33	67	33.4	66.2	32.4	67.9
Streptococcus sanguis	41	42	34	57	34	59	34.5	58.2	33.8	59.7
Streptococcus mitior	31	55	38	49	38	53	38.2	50.7	37.9	54.0

† Taken from Loosdrecht *et al;* (1987)
‡ α-bromonaphthalene

The values in the table indicate that the programs calculate γ_{SV} and γ_S^d correctly. In addition, good agreement exists between the equation of state approach and the geometric mean approach in calculating surface free energies.

Appendix 7 Check of FORTRAN Program "Surf" and Calculation of ΔF_{adh}

The accuracy of the values calculated for interfacial free energy, γ_{S12}, by the FORTRAN program "SURF" was examined. Since no values of γ_{S12} could be found in the literature with which to make a direct comparison, values for γ_{S12} were calculated and used to determine ΔF_{adh}. ΔF_{adh} was then plotted as a function of γ_{SV}, as shown in Figure A7.1 (top figure). The plot was compared to Figure A7.1 (bottom figure) taken from Absolom (1986).

A comparison of the two plots indicate that the values calculated by the program "SURF" as well as the method to calculate ΔF_{adh} agree with the literature values. Minor differences in values can be attributed to error in back-calculating the values of γ_{SL} by interpolation from tables (Neumann *et al.*, 1980) (the Absolom source did not provide substratum contact angle data for the direct calculation of γ_{SL}). Also, γ_{SL} was calculated for γ_{LV} = 73 dynes/cm instead of 72.8 dynes/cm (the tables do not contain values for 72.8 dynes/cm).

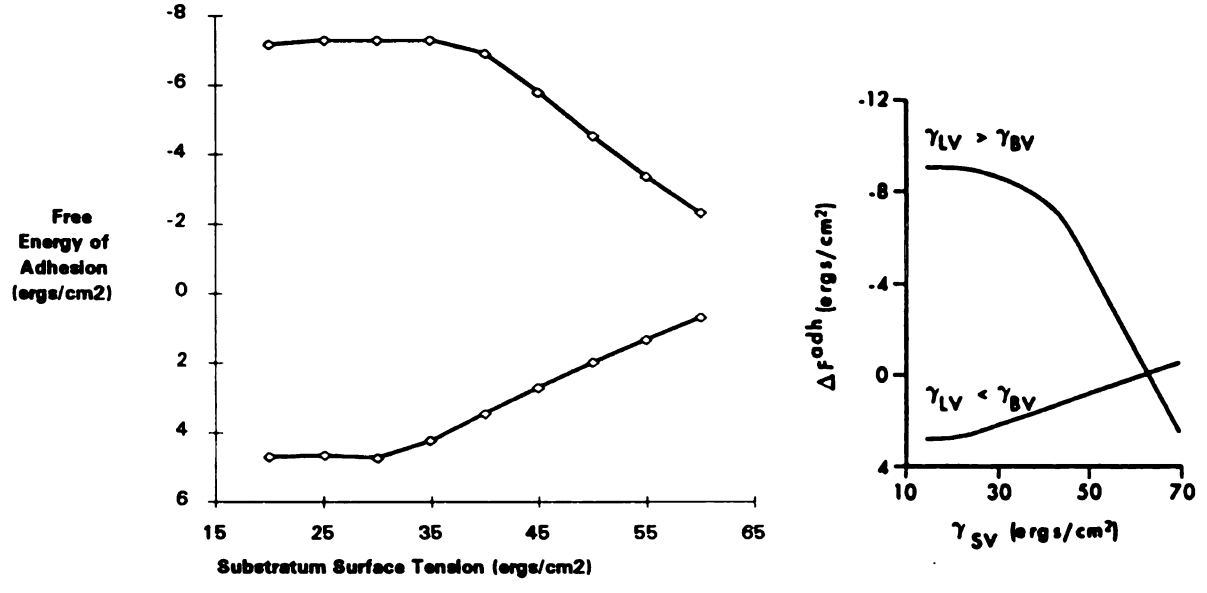


Figure A7.1 Comparison of theoretical plots of ΔF_{adh} as a function of γ_{SV} calculated by methods in this work (left) and Absolom (1986) (right). The bacterium *E. coli* 2627 has a surface tension γ_{CV}=67.8 ergs/cm^2.

Appendix 8 Contact Angle Data

The following tables contain the contact angle measurement data for mycelia using different liquids. Mean contact angles in boldface indicate values used to determine the plateau contact angle (Young's contact angle) of mycelia.

Table A8.1 A-E Contact angles of 0.15 M NaCl drops on mycelia.

Mean values are plotted in Figures A8A-E.

A. 6/1/93

Time (min)	Contact Angle (degrees)	Mean	Std. Dev.
0	0	0	0
5	0	0	0
10	13,12,12,14	12.8	1
15	20,18,17,16,15	**17.2**	1.9
20	14,17,14,20,15	**16**	2.5
25	18,20,21.5,20.5,20	**20**	1.3
30	16,20,16,18,18,18	**17.7**	1.5
35	23,17,19,21,21,21,18	**19.8**	2.2
40	19,22,21,19,19,24	**20.7**	2.1
45	20,20,22,23,21,22	**21.3**	1.2
50	19,15,23,22,21,23	**20.5**	3.1
55	21,23,19,22	**21.3**	1.7
60	21,23,19,22	23	2.9
65	19,23,22,24,27	26.2	1

B. 6/2/93

Time (min)	Contact Angle (degrees)	Mean	Std. Dev.
0	5	5.0	0
5	8	8.0	0
10	13,19,21	17.7	4.2
15	20,22,22,23,23,23	**22.2**	1.2
20	23,24,21,20,20,24	**22.0**	1.9
25	22,24,25,21,23,24	**23.2**	1.5
30	24,25,24.5,24,25,21,22	**23.6**	1.5
35	23,27,26,27.5,26.5	26.0	1.8
40	26,28,25.5,25,27,25,26	26.1	1.1
45	27,27,25	26.3	1.2
50	24,27,29.5,28,28,29,27	28.0	1.0
55	29,29,30,30,30,30	29.7	0.5
60	28,30,30,31.5,28,27	29.1	1.7
65	30.5,31.5,34,34,34	32.8	1.7

C. 6/5/93

Time (min)	Contact Angle (degrees)	Mean	Std. Dev.
0	0	0	0
5	0	0	0
10	8,14,13,19	13.5	4.5
15	10,18,11,18,18,15	15	3.7
20	23,18,23,22,23.5	21.9	2.2
25	23,26,25,26	**25**	1.4
30	24,24,27,24.5,25.5,27	**25.3**	1.4
35	25,27,28,24.5,25,22	**25.3**	2.1
40	22.5,26.5,21,26,27.5,28	**25.3**	2.8
45	30,33,30,33.5,30	31.3	1.8

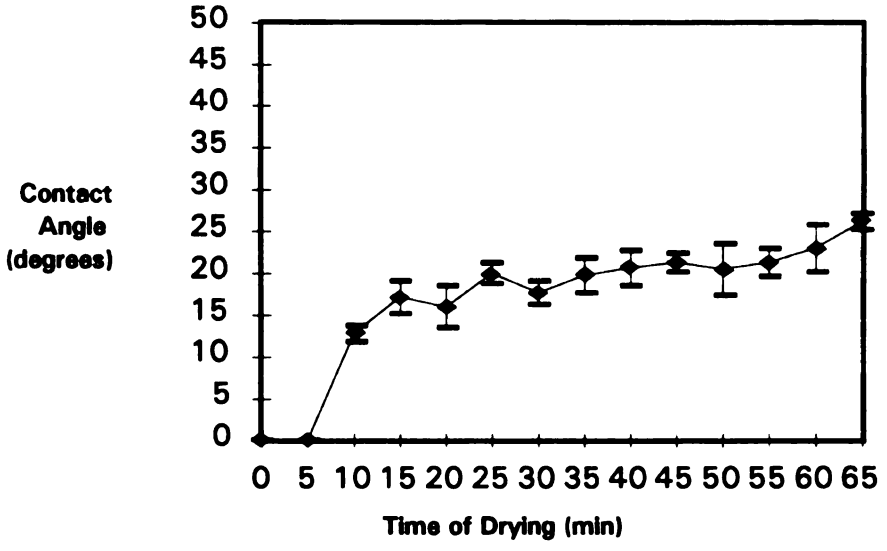
D. 6/10/93

Time (min)	Contact Angle (degrees)	Mean	Std. Dev.
0	0	0	0
5	0	0	0
10	17,16,21.5	**18.2**	2.9
15	17,16,17,17.5	**16.9**	0.6
20	18,20,20,21,19	**19.6**	1.1
25	21,18,19,21	**19.8**	1.5
30	27,28,29.5,26.5,25	27.2	1.7
35	27,29,28,26	27.5	1.3

E. 6/17/94

Time (min)	Contact Angle (degrees)	Mean	Std. Dev.
0	0	0	0
5	0	0	0
10	11,10	10.5	0.7
15	11,13	12	1.7
20	17,13	15	2.8
25	17,19,19	18.3	1.2
30	18	18	0
35	17,17,20	18	1.7
40	15,15,15	15	0
45	14,18,20	17.3	3.1
50	21,26,17,19	20.8	3.7
55	33,36	34.5	2.1

A. 6/1/93



B. 6/2/93

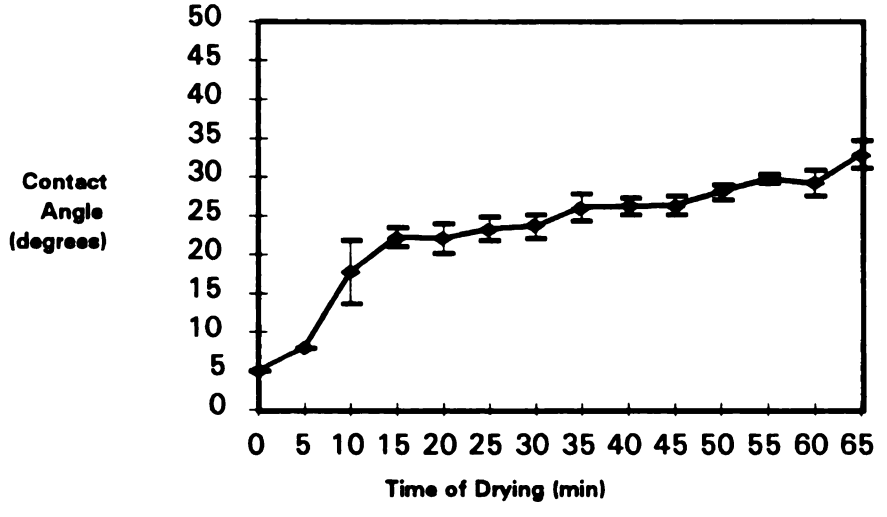
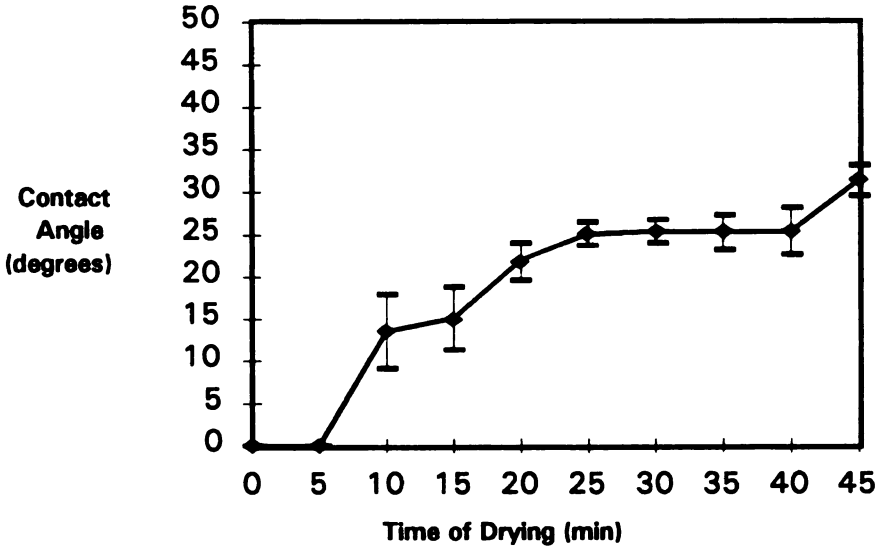


Figure A8.A-E Contact angles on homogenized mycelia as a function of air drying time using 4 µl drops of 0.015 M NaCl.
Each point in the plot represents the mean of 3-7 measurements. Error bars represent one standard deviation of the mean. (Figure continued on next page.)

C. 6/5/93



D. 6/10/93

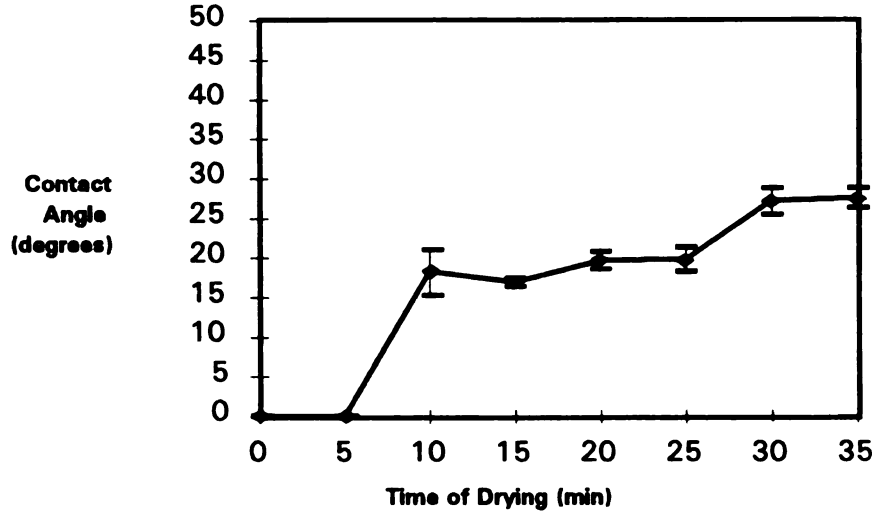


Figure A8.A-E (cont.) See legend on previous page.

E. 6/17/94

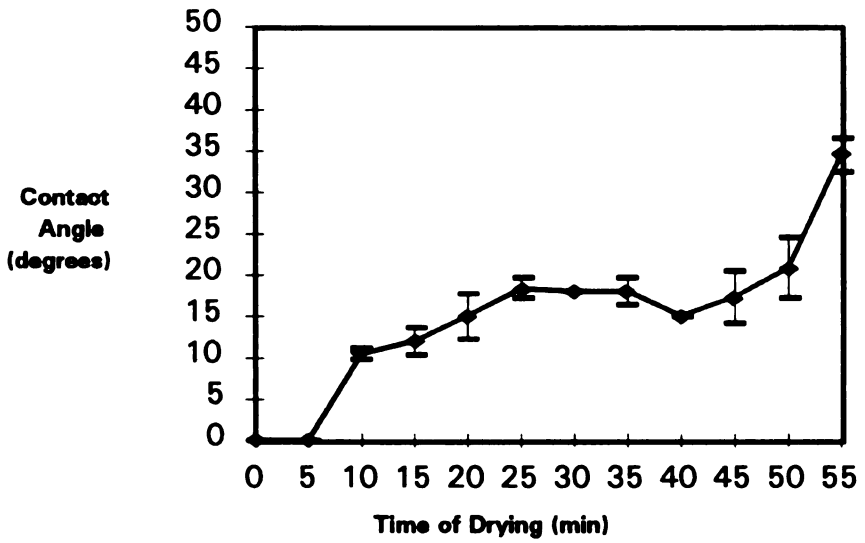
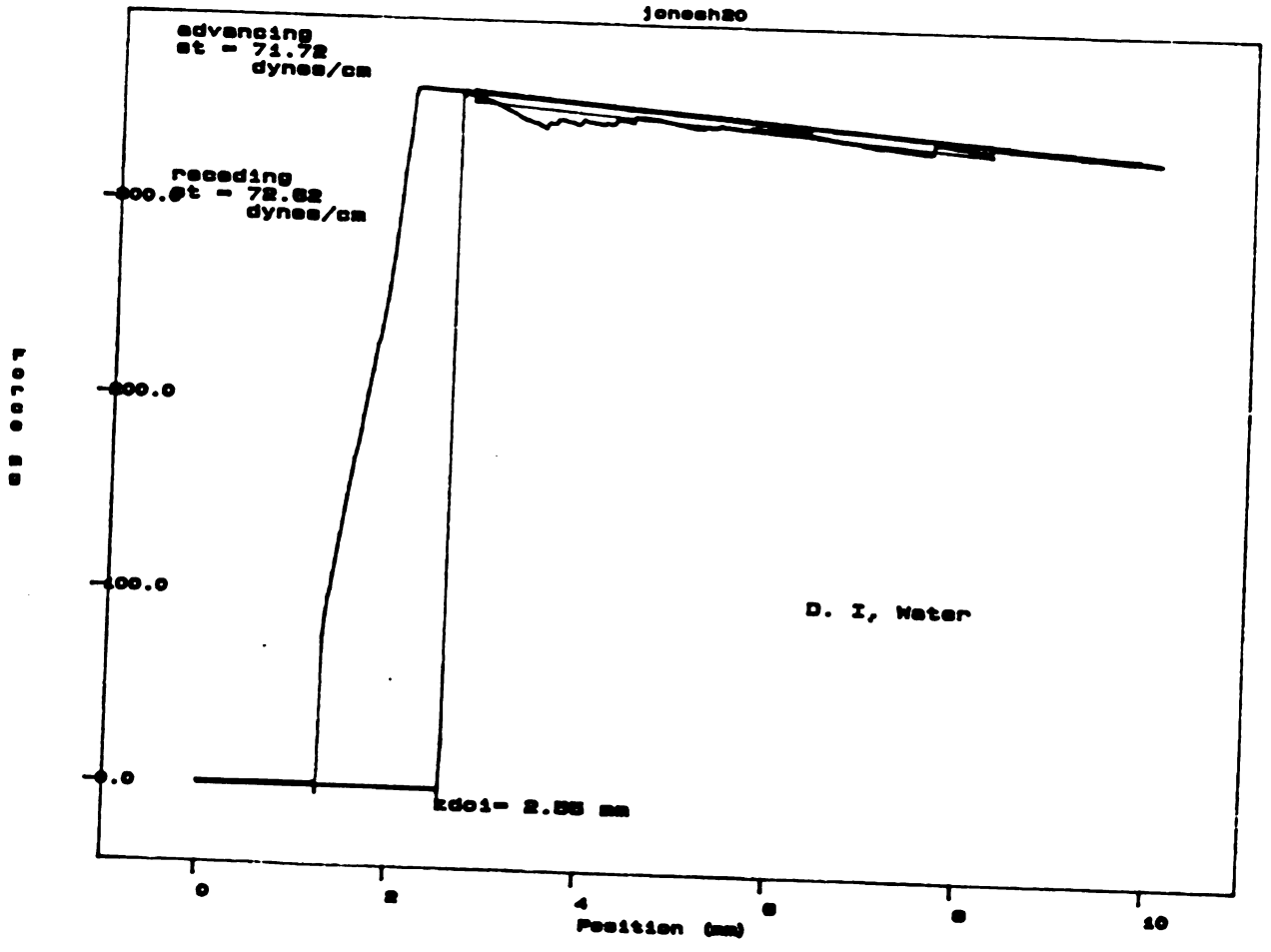


Figure A8.A-E (cont.) See legend on previous page.

Table A8.2 Plateau contact angles of 0.15 M NaCl drops on mycelia

Experiment	Number of Points	Plateau Contact Angle±Std. Dev. (degrees)
6/1/93	9	19.4±1.9
6/2/93	4	22.8±0.8
6/5/93	4	25.2±0.2
6/10/93	4	18.6±1.4
6/17/94	3	18.1±0.2
	Average=	21±3

Appendix 9 Surface Tension Measurements of Liquids



Sample Number: 1
Surface Tension: 0.00 dynes/cm Perimeter: 48.42 mm
Calibration Weight: 500.0 mg Balance Loop: B
Time Interval: 1.0 sec Platform Speed: 22.02 microns/sec

.....

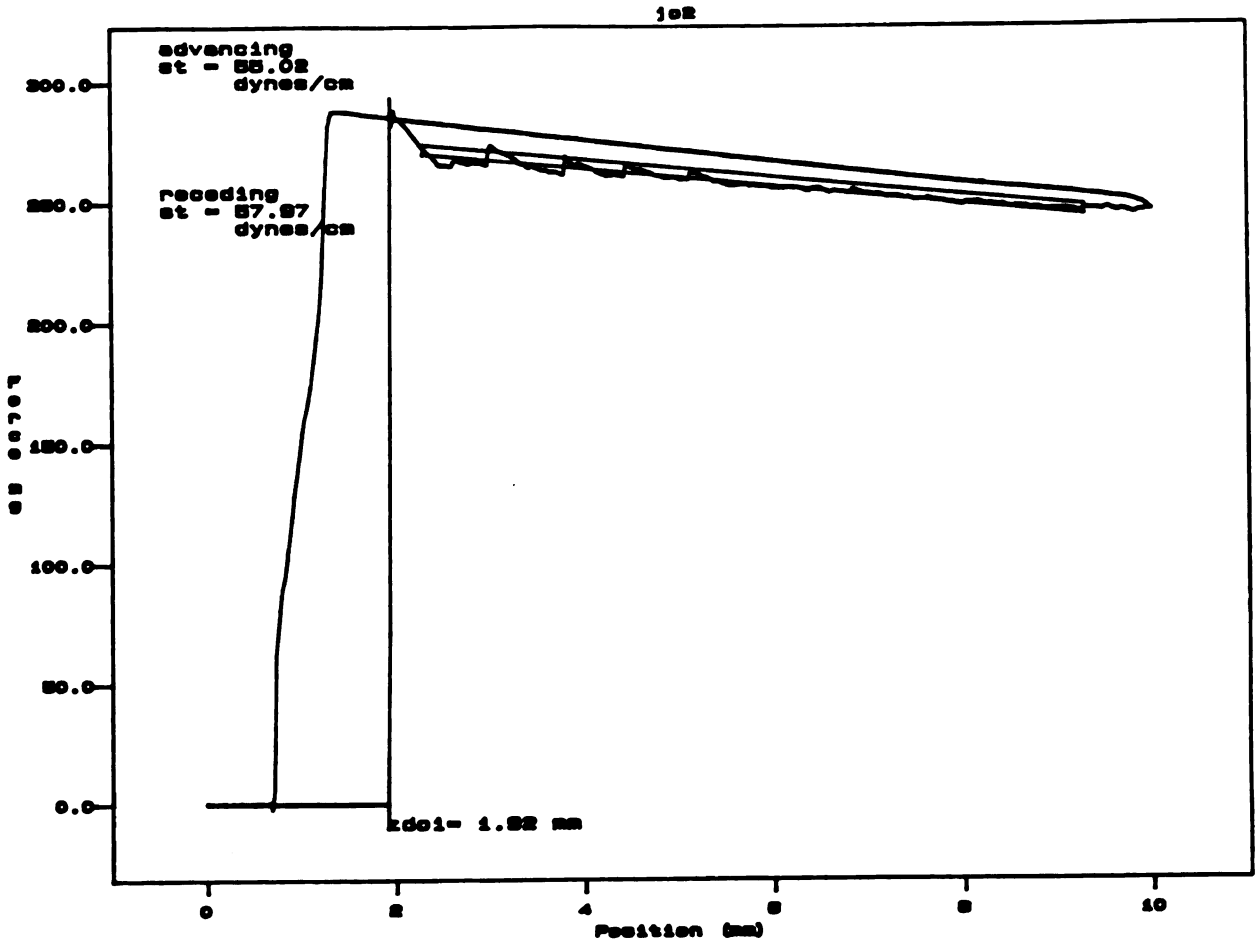
.....

Cycling Data:	Pos 1	Dwell 1	Pos 2	Dwell 2
cycle #1	0.00 mm		10.00 mm	

Zero Depth of Immersion at 2.554 mm

	Surface tension [dynes/cm]	0.95 Confidence [dynes/cm]	coefficient of determination
Advancing cycle #1	71.72	+-0.56	0.9384
Receding cycle #1	72.62	+-0.04	0.9998

Figure A9.1 Surface tension measurement of deionized distilled water.



Sample Number: .....
Surface Tension: 72.6 dynes/cm Perimeter: 48.30 mm
Calibration Weight: 500.0 mg Balance Loop: B
Time Interval: 1.0 sec Platform Speed: 22.02 microns/sec

.....

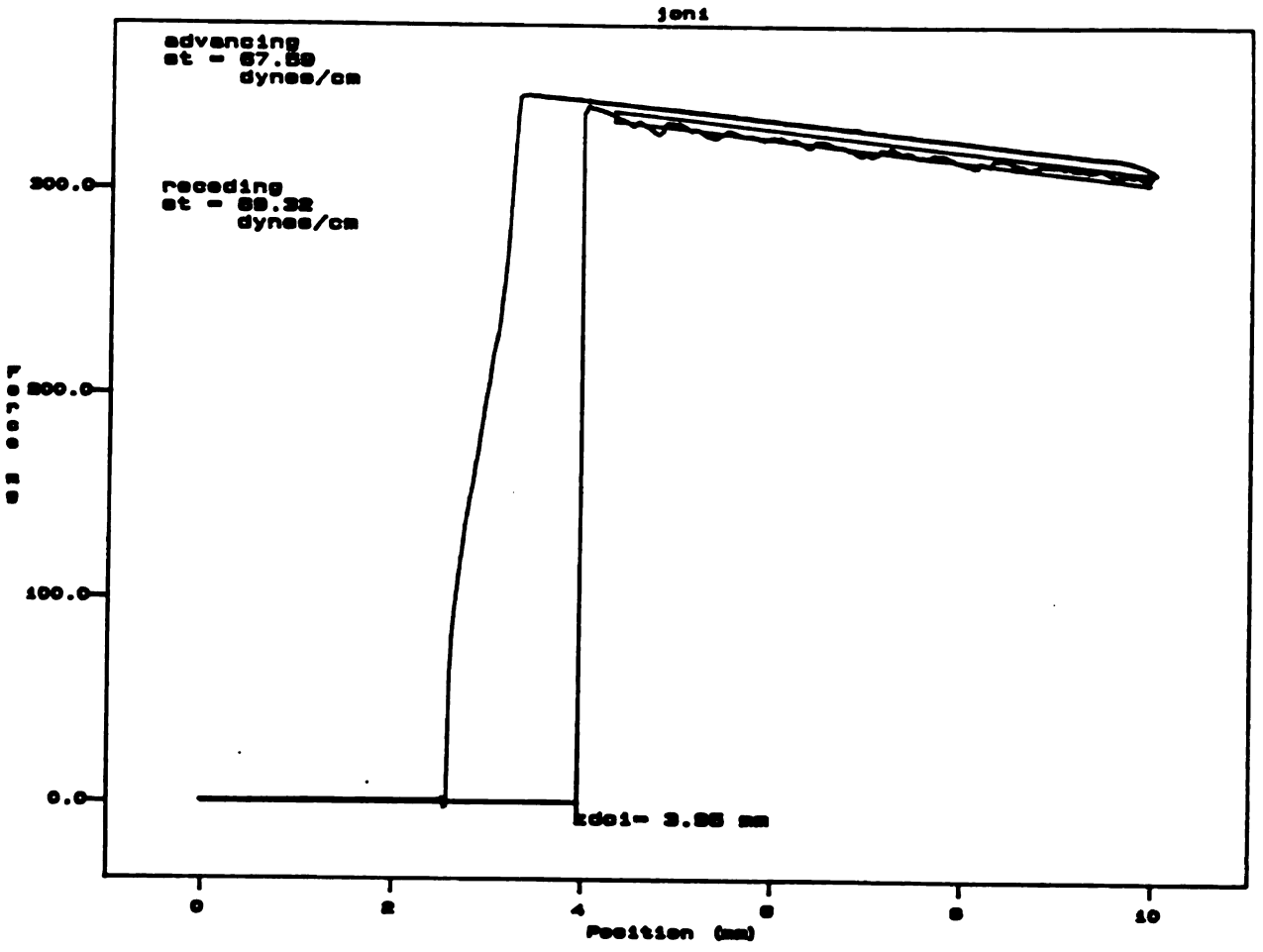
.....

Cycling Data:	Pos 1	Dwell 1	Pos 2	Dwell 2
cycle #1	0.00 mm		10.00 mm	

Zero Depth of Immersion at 1.917 mm

	Surface tension [dynes/cm]	0.95 Confidence [dynes/cm]	coefficient of determination
Advancing cycle #1	55.02	+-0.32	0.9854
Receding cycle #1	57.97	+-0.03	0.9999

Figure A9.2 Surface tension measurement of culture medium with no Tween 80.



Sample Number: .....
Surface Tension: 0.00 dynes/cm Perimeter: 48.68 mm
Calibration Weight: 500.0 mg Balance Loop: B
Time Interval: 1.0 sec Platform Speed: 22.02 microns/sec

.....

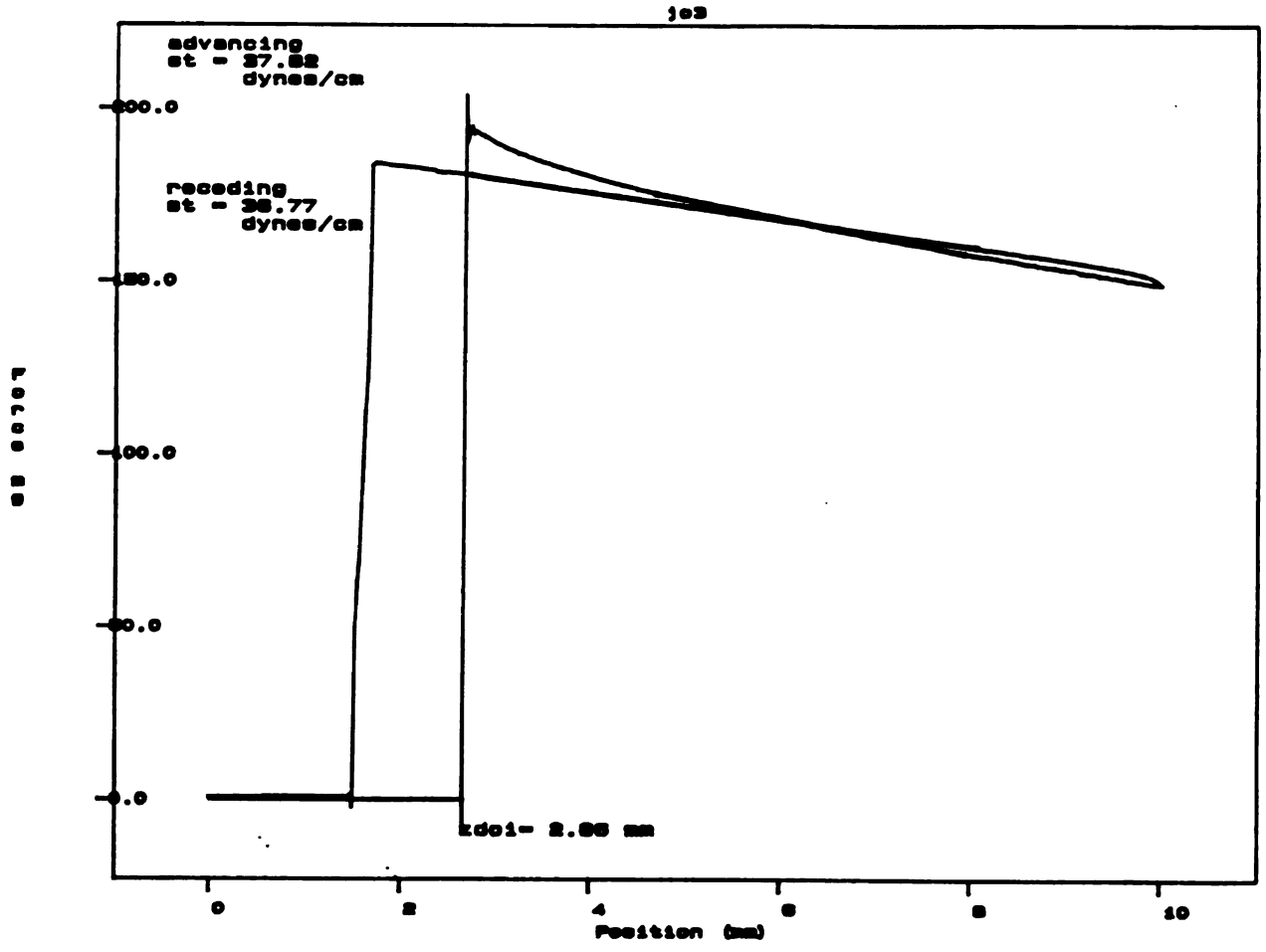
.....

Cycling Data: Pos 1 Dwell 1 Pos 2 Dwell 2
cycle #1 0.00 mm 10.00 mm

Zero Depth of Immersion at 3.951 mm

	Surface tension [dynes/cm]	0.95 Confidence [dynes/cm]	coefficient of determination
Advancing cycle #1	67.59	+-0.47	0.9741
Receding cycle #1	69.32	+-0.03	0.9996

Figure A9.3 Surface tension measurement of culture medium with 0.5% Tween 80.



Sample Number: .....

Surface Tension:	72.6 dynes/cm	Perimeter:	48.30 mm
Calibration Weight:	500.0 mg	Balance Loop:	B
Time Interval:	1.0 sec	Platform Speed:	22.02 microns/sec

.....

.....

Cycling Data:

	Pos 1	Dwell 1	Pos 2	Dwell 2
cycle #1	0.00 mm		10.00 mm	

Zero Depth of Immersion at 2.661 mm

	Surface tension [dynes/cm]	0.95 Confidence [dynes/cm]	coefficient of determination
Advancing cycle #1	37.82	+-0.06	0.9993
Receding cycle #1	36.77	+-0.05	0.9995

Figure A9.4 Surface tension measurement of culture medium with 1.0% Tween 80.

Appendix 10 Anova for Biomass Adhesion Data

Dependent Variable Biomass adhesion

Criterion Measure (g dry weight/coupon) after 1 week of culturing

Factors
1. Interfacial free energy of the polymer, γ_{PV}, at 4 levels
   1 = PE
   2 = PTFE
   3 = Acetal
   4 = SPS
2. Liquid surface tension, γ_{LV}, at 3 levels
   1 = 36.8 dynes·cm^{-1}
   2 = 58.0 dynes·cm^{-1}
   3 = 72.5 dynes·cm^{-1}

ANOVA Results using Statgraphics Software

Statistical Significance

- Since the value of the "sig. of F" from the ANOVA table is less than 0.05, both γ_{PV} and γ_{LV} are *statistically significant.* Statistical significance refers to differences of the means among levels and the presumed repeatability of the results.

Statistical Importance

- Importance calculation $\omega^2 = (SS_i - MS_w)/(SS_T + MS_w)$
  SS_i = sum of squares of a factor
  MS_w = mean square of the residual
  SS_T = total sum of squares

- Importance of polymer interfacial free energy
  $\omega^2 = (0.023 - 0.0)/0.026 = 88\%$

- Importance of liquid surface tension
  $\omega^2 = (0.001 - 0.0)/0.026 = 3.8\%$

- 88% of the variability in the model is explained by γ_{PV}. Less than 4% of the variability is explained by γ_{LV}.

Polymer interfacial free energy is a statistically significant and statistically important factor affecting biomass adhesion to polymer coupons

in shake flask cultures. Liquid surface tension is a statistically significant but not statistically important factor affecting biomass adhesion.

* * * A N A L Y S I S O F V A R I A N C E * * *

BIOMASS
by LIQUID
POLYMER

EXPERIMENTAL sums of squares
Covariates entered FIRST

Source of Variation	Sum of Squares	DF	Mean Square	F	Sig of F
Main Effects	.024	5	.005	92.169	.000
LIQUID	.001	2	.000	8.429	.002
POLYMER	.023	3	.008	146.504	.000
2-Way Interactions	.001	6	.000	1.652	.185
LIQUID POLYMER	.001	6	.000	1.652	.185
Explained	.025	11	.002	42.796	.000
Residual	.001	20	.000		
Total	.026	31	.001		

32 cases were processed.
0 cases (.0 pct) were missing.

* * * C E L L M E A N S * * *

BIOMASS
by LIQUID
POLYMER

Total Population

.07
(32)

LIQUID

1	2	3
.06	.06	.07
(10)	(11)	(11)

POLYMER

1	2	3	4
.08	.09	.07	.01
(7)	(10)	(9)	(6)

LIQUID \ POLYMER	1	2	3	4
1	.08	.09	.06	.01
	(2)	(3)	(3)	(2)
2	.07	.08	.06	.01
	(3)	(3)	(3)	(2)
3	.08	.10	.08	.02
	(2)	(4)	(3)	(2)

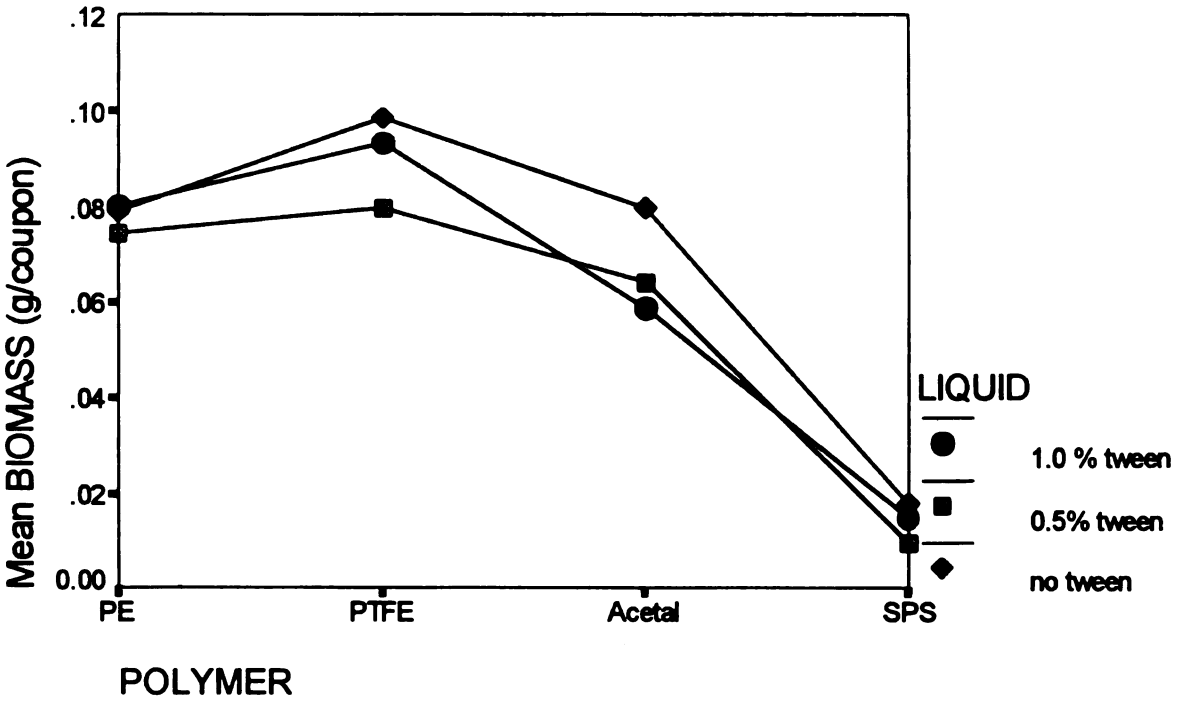

.12
.10
.08
.06
.04
.02
0.00
Mean BIOMASS (g/coupon)
PE
PTFE
Acetal
SPS
POLYMER
LIQUID
1.0 % tween
0.5% tween
no tween

Table A10. Statgraphics Data file for ANOVA

LIQUID	POLYMER	BIOMASS
1	1	0.0861
1	1	0.0738
1	2	0.0858
1	2	0.096
1	2	0.098
1	3	0.0556
1	3	0.0599
1	3	0.0611
1	4	0.0116
1	4	0.018
2	1	0.0758
2	1	0.0734
2	1	0.074
2	2	0.0764
2	2	0.0869
2	2	0.0761
2	3	0.0655
2	3	0.0579
2	3	0.0684
2	4	0.0102
2	4	0.0087
3	1	0.0832
3	1	0.0741
3	2	0.0975
3	2	0.0907
3	2	0.1086
3	2	0.0964
3	3	0.0697
3	3	0.0875
3	3	0.0813
3	4	0.031
3	4	0.0047

Appendix 11 Comparison of Thermodynamic Prediction of Adhesion To Experimental Data

Hypothesis Testing using t-test for Correlation (Luftig, 1991)

Liquid Medium with 0.5% Tween 80

r = correlation coefficient = -0.9784
n = 11 pairs of observations
df = degrees of freedom = 11 - 2 = 9

ρ_{xy} = population correlation coefficient

H_o: $\rho_{xy} = 0.0$

H_1: $\rho_{xy} \neq 0.0$ (2-tail)

Type I error level $\alpha = 0.05$ (α = probability of rejecting H_o when it is true.)

Associated test statistic

$$t = \frac{r - \rho_{xy}}{s_r} = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

IV. RSD of the test statistic when H_o is true

t = t(n-2)df if H_o is true

V. Critical value for rejecting H_o

reject H_o if $| t | > 2.262$

VI. Calculate value of test statistic

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}} = \frac{(-0.9784)(3)}{\sqrt{1-(-0.9784)^2}} = -14.2$$

VII. Make appropriate decision

Since $| t | > | t |_{critical}$, reject H_o

∴There is sufficient statistical evidence to infer that a correlation exists between the model prediction and the experimental values.

Liquid Medium with 1.0% Tween 80

r = correlation coefficient = -0.9510
n = 10 pairs of observations

V. $|t|_{critical} = 2.306$
VI. $|t| = 8.70$
VII. Since $|t| > |t|_{critical}$, reject H_o

∴There is sufficient statistical evidence to infer that a correlation exists between the model prediction and the experimental values.

Liquid Medium with no Tween 80

r = correlation coefficient = -0.9147
n = 11 pairs of observations

V. $|t|_{critical} = 2.262$
VI. $|t| = 6.79$
VII. Since $|t| > |t|_{critical}$, reject H_o

∴There is sufficient statistical evidence to infer that a correlation exists between the model prediction and the experimental values.

Table A11. Data file for correlation between theoretical model and experimental data.

$y_{theoretical}$	$y_{experimental}$		
	1.0% tween	0.5% tween	No tween
-7.248	0.0861	0.0758	0.0832
-7.248	0.0738	0.0734	0.0741
-7.248	---	0.074	---
-6.868	0.0858	0.0764	0.0975
-6.868	0.096	0.0869	0.0907
-6.868	0.098	0.0761	0.0964
-6.868	---	---	0.1086
-5.237	0.0556	0.0655	0.0697
-5.237	0.0599	0.0579	0.0875
-5.237	0.0611	0.0684	0.0813
-0.0212	0.0116	0.0102	0.031
-0.0212	0.018	0.0087	0.0047
r	-0.9510	-0.9784	-0.9147

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