Chemically induced biofilm detachment
by Xiao Chen

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

Abstract:
Biofilm detachment induced by various chemicals, including metal salts, surfactants and depolymerization agents, was investigated using an experimental system consisting of a two-species bacterial biofilm grown in continuous flow annular reactors. Two types of experiments were performed: 1) in situ environmental step change experiments conducted in annular biofilm reactors to examine detachment and 2) determination of viscometry of biofilm collected from these reactors. Experimental results showed that biofilm detachment could be induced by addition of various chemicals. Monovalent and divalent salts (including NaCl, CaCl2, MgCl2) were the most effective chemicals in changing biofilm structure, reflected by an average of 73% viscosity reduction. Directly performing step changes in biofilm reactors with these salts detached 40% of the biofilm in 75 minutes. Chelants showed similar results, e.g. 19.6% viscosity reduction and 26.3% bilfilm detachment for EDTA. Surfactants (including sodium dodecyl sulfate, Triton X-100, Tween 20) also altered the structure of biofilm (e.g. viscosity reduction was 8.7% for Tween 20, 41.9% for Triton X-100, -12.6% for SDS) and caused a much larger amount of biofilm to detach (average of 61.7%). Addition of chlorine, monochloramine and some enzymatic lyases (including lysozyme and protease) caused viscosity reduction and biofilm detachment also. We found that 1) electrostatic (e.g., cation bridging) and hydrophobic interactions were two major forces that maintain the integrity of biofilm structure; 2) cells and EPS were the structural components of biofilms. Disruption of biofilm crosslinking forces and destruction of structural biofilm components could cause biofilm detachment.

CHEMICALLY INDUCED BIOFILM DETACHMENT

by

Xiao Chen

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

of

Doctor of Philosophy

in

Chemical Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 1998

APPROVAL

of a thesis submitted by

Xiao Chen

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

Dr. Phil Stewart Philip J. Stewart April 17, 1998

Chairperson, Graduate Committee Date

Approved for the Department of Chemical Engineering

Dr. John Sears John T. Sears April 21, 1998

Head, Department of Chemical Engineering Date

Approved for the College of Graduate Studies

Dr. Joseph J. Fedock Joseph J. Fedock 4/28/98

Graduate Dean Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University-Bozeman, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Signature Xiao Chen

Date April 17, 1998

ACKNOWLEDGMENTS

First of all I would like to express my gratitude to my academic and research advisor Dr. Phil Stewart for his guidance, encouragement and support that enabled me to achieve my goal. Also, I would like to thank all my committee members. Dr. Gordon McFeters and Dr. Anne Camper counseled and assisted in experimental design. Dr. Max Deibert and Dr. Jay Rotella also gave me much help. I want to particularly thank Dr. John Sears for his guidance and admittance to the Ph.D. program in the Chemical Engineering Department as well as to the Center for Biofilm Engineering. I have learned so much in such a short time from the unique interdisciplinary nature of the Center that I would like to thank everybody in the Center for his/her friendship and sharing of his/her expertise.

I especially acknowledge my wife, Xiaobei Wang and my parents, TianChun Chen and FenMin He, with my love and gratitude for their endless support and care for all time.

My research activities have been supported by National Science Foundation and the Center for Biofilm Engineering, a National Science Foundation-sponsored Engineering Research Center.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Biofilm detachment induced by various chemicals, including metal salts, surfactants and depolymerization agents, was investigated using an experimental system consisting of a two-species bacterial biofilm grown in continuous flow annular reactors. Two types of experiments were performed: 1) in situ environmental step change experiments conducted in annular biofilm reactors to examine detachment and 2) determination of viscometry of biofilm collected from these reactors. Experimental results showed that biofilm detachment could be induced by addition of various chemicals. Monovalent and divalent salts (including NaCl, $CaCl_2$, $MgCl_2$) were the most effective chemicals in changing biofilm structure, reflected by an average of 73% viscosity reduction. Directly performing step changes in biofilm reactors with these salts detached 40% of the biofilm in 75 minutes. Chelants showed similar results, e.g. 19.6% viscosity reduction and 26.3% bilfilm detachment for EDTA. Surfactants (including sodium dodecyl sulfate, Triton X-100, Tween 20) also altered the structure of biofilm (e.g. viscosity reduction was 8.7% for Tween 20, 41.9% for Triton X-100, -12.6% for SDS) and caused a much larger amount of biofilm to detach (average of 61.7%). Addition of chlorine, monochloramine and some enzymatic lyases (including lysozyme and protease) caused viscosity reduction and biofilm detachment also. We found that 1) electrostatic (e.g., cation bridging) and hydrophobic interactions were two major forces that maintain the integrity of biofilm structure; 2) cells and EPS were the structural components of biofilms. Disruption of biofilm crosslinking forces and destruction of structural biofilm components could cause biofilm detachment.

INTRODUCTION

Biofilm detachment refers to the transfer of biomass and other particulate constituents of a surface-attached microbial film to the fluid phase surrounding the biofilm. Detachment is one of the fundamental phenomena governing biofilm accumulation and activity. As the primary process balancing microbial growth in most biofilm systems, detachment is a key determinant of the extent of biofilm accretion. Detachment probably plays a role in the development of the heterogeneous structures observed in some biofilm systems (Murga et al., 1995) and it may also influence biofilm ecology (Rittmann, 1989; Stewart et al, 1997). Despite its fundamental importance in virtually every biofilm system, detachment is one of the least well understood processes in biofilms.

Detachment is also interesting as an alternative strategy for controlling unwanted biofilms such as those that foul cooling water towers, oilfield produced water pipelines, or food processing plants. Biocides and antibiotics have been the principle weapons used to combat biofouling. These agents work by killing microorganisms. This strategy is invariably frustrated by the universally observed reduced susceptibility of biofilm microorganisms to disinfection (Costerton et al, 1987; Brown and Gilbert, 1993). Furthermore, in many biofilm fouling problems the desired end result is a clean surface rather than an inactive, yet physically intact, biofilm. Antimicrobial agents achieve this

indirectly by stopping growth and allowing the natural detachment process to slowly remove the biofilm. Promoting the detachment process directly would appear to be an attractive and obvious alternative approach. This approach could have the added advantage of reducing reliance on inherently toxic control agents whose continued use is fundamentally limited with the trend towards increasingly restrictive environmental regulations.

LITERATURE REVIEW

Earlier research on detachment of biofilm has investigated the influence of shear stress, growth rate, limiting nutrient, oxidizing regents, and enzymes. Trulear and Characklis (1982) first determined a relationship between biofilm detachment rate and shear stress. Subsequent analyses have not arrived at a consensus regarding the significance of shear stress (Rittmann, 1982; Bakke et al., 1990, Peyton and Characklis, 1993). Detachment in fluidized bed reactors is thought to occur predominantly by an abrasion mechanism (Chang et al., 1991; Gjaltema et al., 1995). A few analyses suggest that detachment can be growth-associated; that is, specific detachment rates depend on the net growth rate in the biofilm (Speitel and DiGiano, 1987; Stewart, 1993; Peyton and Characklis, 1993; Tijihuis et al., 1995). Howell and Atkinson (1976) proposed that sloughing occurs when the substrate concentration at the substratum falls below a critical value. Applegate and Bryers (1991) found that *P. putida* biofilm grown under oxygen limitation exhibited detachment rates 20-40% of those measured in a biofilm grown under carbon limitation. The oxygen-limited biofilm contained more extracellular polymeric substance (EPS) and more calcium than did the carbon-limited biofilm. Characklis (1980) found that biofilm detachment resulting from chlorination was much higher between pH 7.5 and 8.5 than it was between 6 and 7. An enzyme blend of cellulase, amylase and protease was claimed effective in digesting microbial slime

(Wiatr, 1991). Johansen et al. (1997) reported that a complex mixture of polysaccharide-hydrolyzing enzymes, including protease, cellulase, pectinase, β-glucanase, and xylanase, was able to remove bacteria from steel and polypropylene substrata.

Biofilm is mainly composed of a variety of bacteria embedded in a matrix of extracellular polymeric substances (EPS) of bacterial origin with water channels inside its structure (Costerton and Lewandowski, 1997; Costerton et al., 1995; Christensen and Charaklis, 1990; Siebel, 1987; Bakke, 1986). The adhesion forces that maintain the structural integrity of biofilms or flocs (i.e. biological aggregates from activated sludge) are thought to include long range forces, such as van der Waals forces, and short range forces, such as chemical bonds and hydrophobic interactions (Marshell, 1990; Tadros, 1980; Forter and Lewin, 1972).

The long range adhesion forces can be addressed in part by the theory of Derjaugin, Landau, Verwey, and Overbeek (DLVO theory) (Verway and Overbeek, 1948; Derjaugin and Landau, 1941). Since surfaces of bacterial cells are normally negatively charged (Marshall, 1976), the net interaction of cells in biofilm would be expected to be repulsive at very low electrolyte concentrations. In an ionic milieu, the net charge on the cell surface is counter-balanced by cations. The result is an electrical double layer at the interface between the cell and the aqueous phase. The double layer is made up of the charged cell surface and the layer of opposite charged counter-ions that are attracted electrostatically to the cell surface. In a high ionic strength aqueous medium the double layer is compressed. At electrolyte concentrations corresponding to ionic strength about I=0.001, a shallow secondary minimum may be formed at some separation distance

where the long range attractive van der Waals forces balances the short range repulsive electrostatic forces. The decrease of electrical double layer is insignificant with further addition of electrolytes. Zita and Hermansson (1994) reported that microbial flocs from wastewater activated sludge behaved in close accord with the predictions from DLVO theory. It was found that the floc dissociation coefficient (a measure of the tendency of flocs to disperse) decreased gradually to its smallest value when the ionic strength of the solution increased to about 0.01; then it remained almost unchanged with increasing ionic strength from 0.01 to 0.1. However, it was found in the same study that when ionic strength increased from 0.1 to 1, the dissociation coefficient was drastically increased. The later could not be explained by DLVO theory.

Cation bridging (e.g. Ca^{+}) and hydrophobic interactions can be considered as short range adhesion forces that maintain the structural integrity of biofilms, flocs, and biological gels. Turakhia et al. (1983) showed that immediate and substantial detachment of a *P. aeruginosa* biofilm could be effected by addition of the calcium-specific chelant ethylene glycol-bis-(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA). Gordon et al. (1991) found that the gel strength of the alginate gel separated from mucoid *Pseudomonas aeruginosa* was significantly reduced upon addition of NaCl, EGTA, EDTA, and sodium dodecyl sulfate (SDS). SDS is a surfactant which may disrupt the hydrophobic interactions that maintain gel strength. Singh and Vincent (1987) found that the hydrophobic character of the isolated *Pseudomonas* sp from sewage sludge was associated with the capacity to form aggregates. Valin and Sutherland (1992) even found a correlation between flocculation in activated sludge and hydrophobicity on the basis of

contact angle measurement from sludge sample. The hydrophobicity of the cell surface is also believed to be an important factor in determining the extent of adhesion of bacteria to solid surfaces (Rosenberg, 1986).

The majority of the extracellular polymers in a biofilm are thought to be polysaccharides. Research focused on polysaccharides or polymer matrices may well bear on some phenomena in biofilms, such as biofilm detachment. For example, with regard to the solution properties of polysaccharides, it was found that the viscosity decrease that occurred when salt was added to solutions of different polysaccharides varied considerably with the type of polysaccharide presented (Smidsrod and Haug, 1967; Cox, 1960). It was also found that the viscosity of an exopolymer produced by a *Pseudomonas* sp. decreased by 75% of that in fresh water when 2.7M $CaCl_2$ was added, and by 85% when subjected to 4.4M NaCl (Dasinger et al., 1994). Parker et. al. (1996) determined the influence of a whole range of metal cations on the viscosity of capsular polysaccharide from *Microcystis flos-aquae*. He observed a biphasic effect of metal ion concentration on viscosity of this polysaccharide. Initially the viscosity increased with increasing metal ion concentration until a maximal viscosity occurred at a concentration of 1~10mM. The viscosity decreased with further addition of that ion.

In summary, cells and extracellular polymers are considered major components of biofilm. The structural cohesiveness of various types of biological aggregates including biofilms, microbial flocs, microbial gels, appears to result from a combination of forces including long range van der Waals forces and short range forces like electrostatic and hydrophobic interactions. These cohesive forces can be disrupted by numerous ways,

such as addition of salts (NaCl, $CaCl_2$), addition of chelants (EGTA, EDTA), and disturbance of hydrophobic interactions by adding surfactants. Treatment of a biofilm with oxidizing reagents (e.g., chlorine) or with enzyme lyases (e.g., protease) can also cause biofilm detachment.

HYPOTHESES

I hypothesize that there are two broad types of crosslinking interactions in the biofilm matrix: electrostatic and hydrophobic. Electrostatic crosslinks could involve direct interactions between the two charged elements or they could be mediated by bridging substances, for example divalent cations or charged proteins. Similarly, hydrophobic interactions could be direct or they might be mediated by a hydrophobic protein.

With this structural model of the biofilm in mind, biofilm detachment could be induced by a number of different treatments, including, for example, 1) degradation of an EPS polymer chain, 2) loss of cellular structural integrity (i.e., cell lysis), 3) disruption of a cell-polymer or polymer-polymer electrostatic crosslinking interactions, and 4) disruption of cell-polymer or polymer-polymer hydrophobic crosslinking interactions.

The overall goal of the work reported in this dissertation was to perform preliminary experimental investigations of these hypotheses in order to understand the mechanisms that cause biofilm detachment.

MATERIAL AND METHODS

Two types of experiments were conducted to investigate the influence of various chemicals on biofilm detachment. The first type of experiment involved directly measuring the amount of biofilm detachment caused by a step change addition of a treatment chemical added directly to a biofilm reactor. Second type of experiment used viscometry to measure changes in the viscous properties of resuspended biofilm after chemical treatment. Biofilms were grown on the inner surface of a continuous flow annular reactor for about 7 days to reach a pseudo-steady state. Then they were challenged with an instantaneous concentration change of certain chemicals which for the most part were not biocidal. Any changes of biomass on the surface and in the effluent of reactor were recorded, analyzed, and quantified using in-line optical density measurement, total protein measurements, and a cell enumeration method. Biofilms scraped from the surface of an untreated reactor were used for viscometry experiments. Biofilm viscosity changes before and after addition of various chemicals were recorded and quantified using a viscometer.

Microorganism and Culture Conditions

Pseudomonas aeruginosa (ERC1) and *Klebsiella pneumoniae* (KP1) were co-cultured on a minimal salts medium to grow binary species biofilms. These two species

were stored in an autoclaved medium of peptone (2%) and glycerol (20%) at -70 ^{0}C after enrichment. Medium was prepared in two 20-liter carboys containing 62-fold concentrated stock solution. One was filled with phosphate buffer alone, i.e. Na_2HPO_4 and KH_2PO_4; the other held the remaining components of medium. The desired final medium concentrations in the annular reactors were achieved by mixing flows of these two carboys with an additional one from a continuously aerated water tank. The medium components were sterilized by autoclaving and dilution water was sterilized by filtration using two filters (0.2 μm capsule filter, Gelman Sciences) in series. The culture preparation protocol and media compositions are listed below.

Culture preparation protocol

1) Pure species isolation
   - Transfer the stock culture onto R2A agar.
   - Streak the liquid in T-shape on the R2A agar using a flame sterilized loop.
   - Incubate the plates at 35^{0}C for 1-2 days.
   - Find a single colony of the target species.
2) Enrichment
   - Pick that single colony and restreak onto TSB agar thoroughly (Tryptic Soy Broth, Difco).
   - Incubate the plates at 35^{0}C for 1-2 days.
3) Transfer 5ml autoclaved solution of 2% peptone and 20% glycerol to each of TSB plates.