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INITIAL BIOFILM FORMATION OF ACETOCLASTIC METHANOGENIC BACTERIA

by

Eva Andras

A thesis
submitted under the supervision of
Dr. Ronald L. Droste and Dr. Kevin J. Kennedy

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ABSTRACT

Very few data exist concerning the biofilm formation process of mixed anaerobic methane producing cultures, and almost none regarding enriched methanogenic cultures.

In this study attachment properties of acetoclastic methanogenic bacteria were investigated in a downflow system. The experimental conditions were selected to approximate the typical operating conditions of the downflow stationary fixed film reactor (DSFF). The study concentrated on the early stage of biofilm development.

Biofilm development rates as high as 40 $\mu g/cm^2 \cdot h$ were measured. Since film formation rates substantially lower than that were reported for mixed anaerobic cultures, acetoclastic methanogens are likely to have better adhesive properties than acidogens. Attachment rates observed on Tygon surface agree reasonably with the predictions of the dynamic model by Droste and Kennedy (1987).

Increase in liquid flow velocities in the range of 0.05-1.42 cm/s increased biofilm formation rate on silicone surface.

The development of a loosely held biomass was observed in the test sections. This biomass is thought to represent an intermediate stage of biofilm formation at low liquid flow velocities. Increased flow velocities reduced the quantity of this type of biomass.

The ratio of total polysaccharide to COD was increased in both types of biofilm as compared to the reactor suspended biomass. In the attached biofilm the degree of increase was 40-500%. This finding suggests the involvement of polysaccharides in the attachment process. No excess protein was observed in the biofilms.

The material of the support surface affected both biofilm development rates and film density. On silicone tubing surface the biofilm formed faster and was denser than on Tygon surface (PVC).

A maximum biofilm thickness of 250-270 µm was reached on silicone surface regardless of different experimental conditions. This plateau value is likely to be related to the test section material and geometry.

Biofilm development rates were directly proportional to the suspended biomass concentration in the range of 500-850 mg VSS/L.

The metabolic state of the biomass also affected attachment significantly. Attached film development rates were directly proportional to actual specific acetoclastic activity in the range of 0-0.8 mg COD/mg VSS·d. A 4-7 fold increase in attachment rate occurred over that activity range. Biofilm density was also a linear function of specific activity. The results imply that both passive and active attachment mechanisms play a role in the film formation of acetoclastic methanogens.

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CHAPTER 1

INTRODUCTION

Anaerobic waste treatment has been established as a valid biological wastewater treatment option in the last two decades. The advantages of anaerobic treatment over aerobic treatment have been known for some time: relatively low capital and operating costs and recovery of energy in the form of methane. The major disadvantages of anaerobic treatment were thought to be the lack of stability, susceptibility to failure and slow recovery, low loading rates and requirement for specific waste composition. However, with a better understanding of anaerobic microbiology, these negative characteristics have been shown to be mainly due to poor reactor design, and are not inherent features of anaerobic processes. To overcome these limitations a new generation of anaerobic reactors was developed.

These second generation or advanced anaerobic reactors have one common feature in that they all retain biomass, but they differ in the way the biomass is retained. Biomass retention allows a high solids retention time (SRT), independent from hydraulic retention time (HRT), resulting in greater reactor stability. Retained biomass reactors were found to withstand toxic substances or pH shock better

than suspended culture reactors (Costerton et al., 1985; Stephenson and Lester, 1986). In general, all second generation reactors tolerate hydraulic and organic overloading well. Re-start after a long shut-down period is relatively easy. All of them have varying degrees of start-up problems depending on reactor configuration and wastewater composition (Henze and Harremoes, 1983; van den Berg, 1984).

Included in these advanced types of anaerobic reactors are the fixed bed, moving bed, expanded bed, fluidized bed, recycled bed, sludge blanket reactors and their hybrids. They are all different designs, each having its own niche in waste treatment (van den Berg and Lentz, 1979; van den Berg and Lentz, 1980; van den Berg and Kennedy, 1983).

The downflow stationary fixed film (DSFF) reactor has been developed at the National Research Council of Canada. This design eliminated the plugging problems of the anaerobic filter by using large channel oriented media and operating in downflow mode, thereby minimizing the suspended growth. Gas escaping at the top of the reactor provides adequate mixing so an elaborate influent distribution system is not needed (van den Berg et al., 1984).

Despite the fact that the DSFF concept has been implemented in full scale operation, there is still much research needed to fully understand and describe the reactor operation (Hall, 1982; Szendrey, 1983). A dynamic model has been proposed to predict reactor operation during startup period and thereafter (Kennedy, 1985; Droste and Kennedy, 1987).

Among the many variables in this model are the attachment and detachment rates of acidogenic and methanogenic bacteria. An indirect procedure was used to estimate attachment and detachment.

Very little is known about bacterial attachment and detachment in anaerobic treatment systems (Salkinoja-Salonen *et al.*, 1983). Yet, bacterial attachment has primary importance in startup procedures as well as in normal operation. To speed up reactor startup procedures and facilitate the development of a healthy biofilm it is necessary to know what factors play a role in anaerobic biofilm development and their effects on biofilm characteristics and development rate.

The main objective of this study was to establish qualitative and quantitative relationships between operational parameters, biofilm development and biomass characteristics.

This study was limited to experimentation with an enriched acetoclastic methanogenic culture in the initial stage of biofilm development.

The specific objectives were to find the effect of

- flow velocity
- support medium
- metabolic state of bacteria
- suspended biomass concentration

on initial attachment rate and biofilm characteristics.

CHAPTER 2

LITERATURE REVIEW

2.1 Basic Processes of Anaerobic Digestion

During anaerobic digestion organic material is converted into methane and carbon dioxide. This process involves three major steps, which are performed by different groups of coexisting bacteria (Verstraete *et al.*, 1981; Henze and Harremoes, 1982; Kirsop, 1984; Zinder, 1984). Scheme of the process is shown in Fig. 2.1.

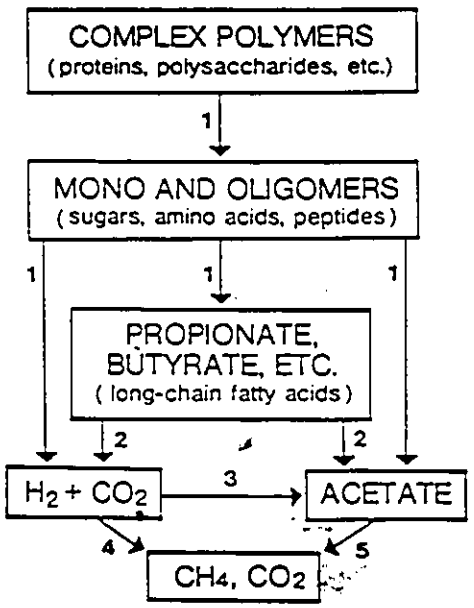


Figure 2.1 Microbial groups involved in the flow of carbon from polymeric materials to methane in an anaerobic bioreactor. 1: fermentative bacteria; 2: hydrogen-producing acetogenic bacteria; 3: hydrogen-consuming acetogenic bacteria; 4: CO_2-reducing methanogens, 5: acetoclastic methanogens (after Zinder, 1984).

The first phase is the liquefaction of insoluble organics and subsequent hydrolysis of biopolymers such as polysaccharides and proteins into simple molecules. In cases where the waste consists of mostly suspended material, this stage can be rate limiting (McInerney and Bryant, 1981; Kennedy and van den Berg, 1982).

In the second phase a group of anaerobic bacteria, the acidogens, convert the simple soluble organic molecules into a variety of products, mostly volatile fatty acids, hydrogen and carbon dioxide. It has been found that the products of acidogenesis are dependent upon the partial pressure of H_2. When H_2 partial pressure was kept low by H_2 utilizing methanogens (below 10^{-3} atm), then acidogenesis resulted in acetate, hydrogen and carbon dioxide. If, however H_2 partial pressure exceeded this level, more reduced compounds (propionate, butyrate, ethanol, lactate) were formed. These reduced compounds then needed to be converted to acetate by other bacteria (McInerney and Bryant, 1981).

Conversion of intermediate products to methane occurs in the third phase, called methanogenesis. Seventy percent of the methane originates from acetate and 30% is formed via reduction of CO_2 by H_2 (Mah *et al.*, 1977; Verstraete *et al.*, 1981) according to the following equations (Daniels *et al.*, 1984; Kirsop, 1984)

$$CH_3COO^- + H_2O \Longrightarrow CH_4 + HCO_3^- \qquad (2.1)$$

$$CO_2 + 4H_2 \Longrightarrow CH_4 + 2H_2O \qquad (2.2)$$

A unique group of bacteria, the methanogens, are responsible for this conversion.

2.2 Methanogenic Bacteria

Since the discovery of coenzyme M in 1971, more and more data have accumulated concerning the unique features of the methanogenic bacteria. They are now known to be truly distinct from the typical bacteria (eubacteria) and are classified in a separate kingdom, the archaebacteria (Fig. 2.2).

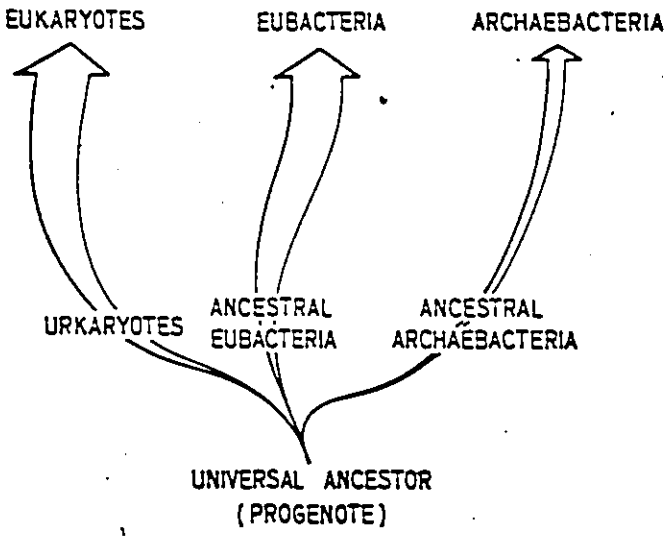


Figure 2.2 The three primary kingdoms proposed by Woese, (1981): Eucaryotes, Eubacteria and Archaebacteria (after Zehnder, 1981).

The establishment of this kingdom is primarily based on a study of the composition of the 16S ribosomal RNA, which is known to be highly conserved and particularly suitable for indicating phylogenic relationships. The archaebacteria

also contain the thermoacidophiles and the extreme halophiles and are believed to represent an extremely ancient bacterial group (Balch et al., 1979; Woese, 1981; Kirsop, 1984).

Classification of methanogens based on 16S rRNA comparative cataloging is shown in Fig. 2.3.

Although the archaebacteria constitute a coherent group, the members of the group are as ecologically and biochemically diverse, as the eubacteria. For this reason while methanogens have a number of major traits in common, the various genera are widely different in many respects.

2.2.1 Distinctive Common Features of Methanogens

Methanogens produce methane from a limited number of substrates at a reducing potential of -330 mV.

2.2.1.1 Unusual Coenzymes

Several coenzymes and factors have been found almost exclusively in methanogens. Coenzyme M is involved in methyl transfer; factor F420 is a low-potential electron carrier; CDR factor is required for carbon dioxide reduction; and, component B is responsible for enzymatic formation of methane from methyl-coenzyme M. Two other factors have been characterized spectrally, F430 and F342, and still others are being discovered (Wolfe, 1979; Daniels *et al.*, 1984).

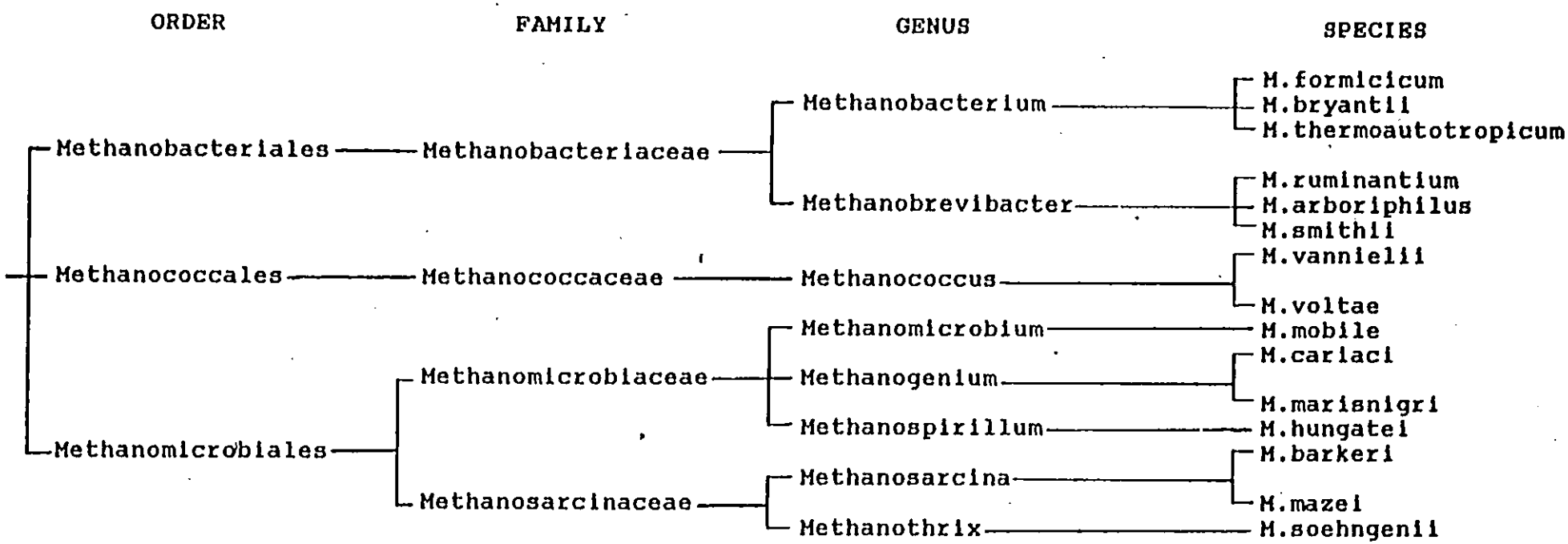


Figure 2.3 Classification of methanogens (combined after Zehnder *et al.*, 1981 and Kirsop, 1984). The phylogenetic position of *Methanothrix soehngenii* is based on the work of Stackebrandt *et al.*

2.2.1.2 Transfer RNA

The tRNA of methanogens differs from that of most eubacteria species, since it does not contain the common arm sequence GTuCG.

2.2.1.3 Cell Wall Composition

The cell wall of methanogens lacks muramic acid, which is a component of cell walls of all eubacteria. It contains no D-amino acids.

There is a great variety of cell wall types among methanogens. For the most important genus in many digesters, *Methanosarcina*, the wall structure is a heteropolysaccharide containing galactosamine, neutral sugars, and uronic acid. In many respects the structure of this wall resembles that of a capsule, in which are embedded the individual cells of *Methanosarcina*. *Methanobacterium* and *Methanobrevibacter* genera have pseudomurein in their cell wall, while the cell walls of other methanogenic genera contain protein subunits (Zeikus and Bowen, 1975; Kandler and Hippe, 1977; Balch *et al.*, 1979; Kirsop, 1984).

2.2.1.4 Lipids

Lipids of methanogens are different from the lipids of typical bacteria. They do not possess saponifiable lipids. Squalene is a major component of the neutral lipids, and C_{20} and C_{40} biphtanyl glycerol ethers are major components of the polar fraction found in the membrane of archaebacte-

ria (Wolfe, 1979). Fig. 2.4 depicts the difference between membrane lipids of archaebacteria and those of other organisms (Woese, 1981).

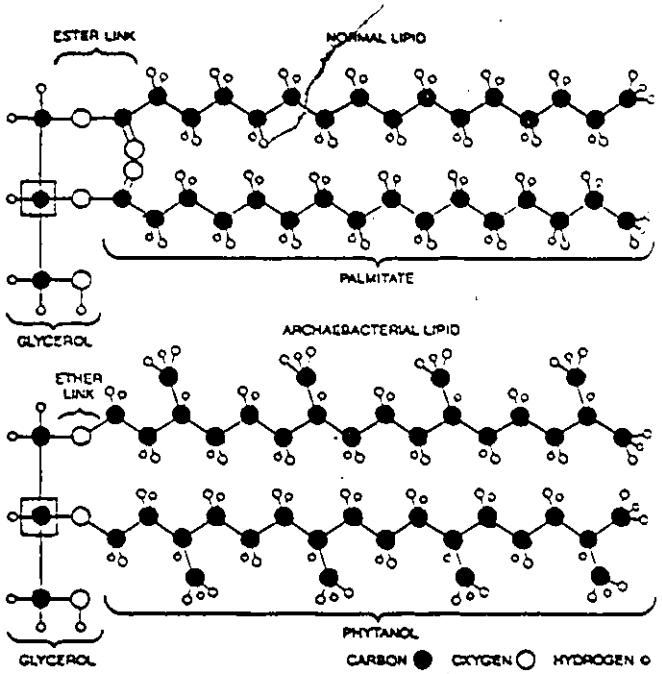


Figure 2.4 Membrane lipids of archaebacteria are different from the lipids found in other organisms (after Woese, 1981)

2.2.2 Characteristics of Substrate Utilization and Growth

All methanogens can utilize H_2 and CO_2 as substrate except for five strains, *Methanosarcina strain* TM1, *Methanothrix soehngenii*, *Methanothrix concilii*, *Methanolobus tindarius* and a coccoid methanogen (Daniels *et al.*, 1984; Patel, 1984). Half of the genera can grow on formate. Only *Methanosarcina* and *Methanothrix* species are able to utilize acetate, but they cannot use formate. *Methanosarcina barkeri* and *Methanosarcina mazei* are more versatile, capable of

utilizing H_2 and CO_2, methanol, methylamines and acetate, while *Methanothrix soehngenii* and *Methanothrix concilii* use acetate as the sole source of energy (Zehnder *et al.*, 1981; Daniels *et al.*, 1984; Patel, 1984). The role of hydrogen utilizing methanogens is not insignificant in anaerobic digestion. Considerable amounts of hydrogen are produced during digestion, and methanogens have been shown to rapidly utilize it, thus maintaining a very low hydrogen partial pressure in the reactor, which is favorable to acetate production (van den Berg and Kennedy, 1982).

Doubling time for methanogens can vary from 30 minutes to weeks. Acetate utilizing methanogens exhibit especially slow growth rates and low cell yields owing to the fact that acetate is a poor substrate energetically (Zehnder *et al.*, 1981; Daniels *et al.*, 1984).

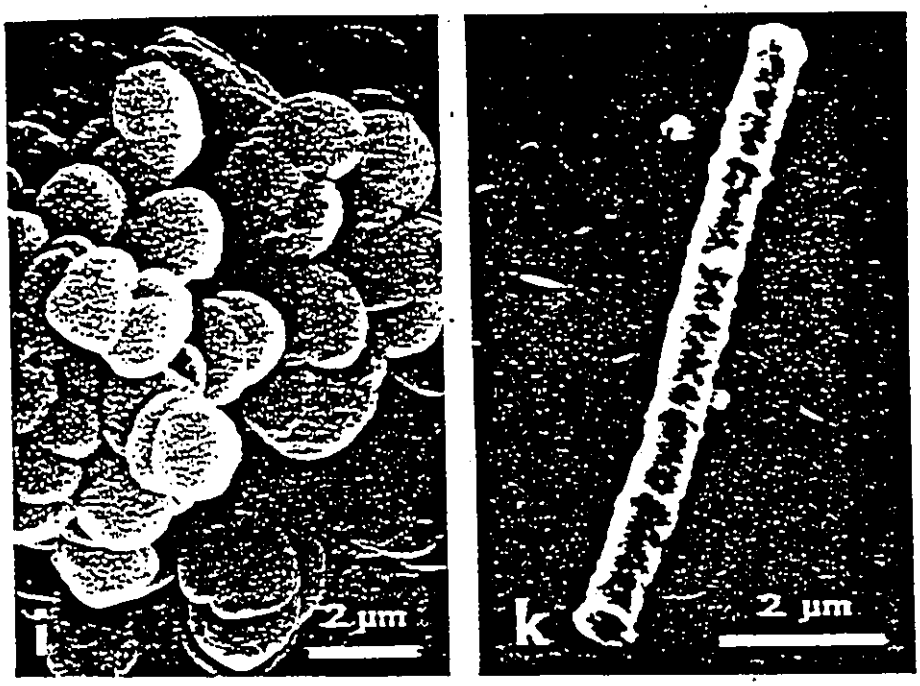
Table 2.1 presents kinetic parameters of pure and enrichment cultures utilizing acetate.

Fig. 2.5 illustrates the morphology of *Methanosarcina* and *Methanothrix*. *Methanosarcina mazei* has recently been transferred from the genus *Methanococcus* to *Methanosarcina* (Mah and Kuhn, 1984).

	ACETATE ENRICHMENT CULTURE	METHANOSARCINA MAZEI	METHANOSARCINA BARKERI	METHANOTRIX SOEHNGENII	METHANOTRIX CONCILII
SUBSTRATE					
Acetate	+	+	+	+	+ (a)
formate		-	-	-	-
methanol		+	+	-	-
methylamines		+	+	-	-
hydrogen + carbon dioxide		+	+	-	-
DOUBLING TIME d	1-3	1	1-6	1-9	1-3
HALF VELOCITY CONSTANT K mg acetate/L	50-150	150-250	300	15-40	70-100
BIOMASS YIELD Y mg VSS/mg COD	0.02-0.04		0.03-0.05	0.017-0.025 (b)	0.018 (b)
SPECIFIC ACTIVITY mg COD/mg VSS.d	2-8		2-12	2.3 (c)	2.2 (c)
MOTILITY		-	-	-	-

(a) presence of CO_2 required
(b) mg TSS/mg COD
(c) mg COD/mg TSS.d

Table 2.1 Properties of acetoclastic methanogenic bacteria in pure and enrichment culture. Compiled from Smith and Mah, 1978; Balch *et al.*, 1979; Zehnder *et al.*, 1980; Zehnder *et al.*, 1981; Huser *et al.*, 1982; Fathepure, 1983; Henze and Harremoes, 1983; Kirsop, 1984; Patel, 1984; Kennedy, 1985.



(a) (b)

Figure 2.5 Scanning electronmicrograph of *Methanosarcina barkeri* (a) and *Methanothrix soehngenii* (b), after Zehnder *et al.* (1981)

2.2.3 Effect of Environmental Factors

2.2.3.1 Oxygen

Common to all methanogens is a sensitivity to oxygen; the degree of sensitivity, however, differs among strains (Patel *et al.*, 1984). For example the bactericidal effect of exposure to air is observed immediately for *Methanococcus vannielii* and *Methanococcus voltae*, while *Methanosarcina barkeri* can survive exposures up to 30 h, although loss of colony-forming units occurs thereafter (Kiener and

Leisinger, 1983). Daniels *et al.* (1984) found methanogenesis extremely oxygen sensitive. *Methanothrix soehngenii* however, appears to be remarkably tolerant to oxygen. After being gassed with O_2 for up to 48 hours, no significant change in methane production was observed (Huser *et al.*, 1982).

2.2.3.2 Temperature

A few thermophilic strains have been isolated: *Methanobacterium thermoautotrophicum*, *Methanothermus* and one *Methanosarcina* strain (Zinder and Mah, 1979). The majority of methanogenic bacteria are active in the mesophilic range, between 20-45°C (Zehnder *et al.*, 1981; Henze and Harremoes, 1983).

2.2.3.3 pH

Studies of Jarrell and Sprott (1980) have demonstrated that methanogens have only a limited ability to maintain a constant internal pH. The pH range of 6-8 has been shown to be acceptable for methanogenesis. All the methanogens tested exhibited maximum methane production in this range. Thus, maintaining a neutral pH in a digester provides an optimum condition for methanogenesis. This is higher than the optimum pH for acidogenesis (pH = 5-6, according to Henze and Harremoes, 1983). However, as long as the two phases occur in the same reactor, the needs of the more pH sensitive methanogens are the governing factor in process opti-

mization (Verstraete *et al.*, 1981). Methanogenesis has also been demonstrated to occur at extreme pH values such as pH = 3 and pH = 9.7 (Zehnder *et al.*, 1981; Daniels *et al.*, 1984).

2.2.3.4 Nutrients

All methanogens seem to require ammonia as the nitrogen source. Additionally, certain specific amino acids may be needed. In general, many individual strains require individual nutrients because of some deficiency in their metabolic equipment. Generalization of these findings is unwarranted. Certain strains have been shown to require sulfide, sodium, magnesium, calcium, trace metals such as nickel, cobalt, iron, molybdenum, selenium, and vitamins (Henze and Harremoes, 1983; Kirsop, 1984).

2.2.3.5 Inhibitors

Many compounds generally known as toxic (such as cyanide) have been shown to be toxic to methanogens too. Nutrients beneficial in small concentration can act as inhibitors in high concentration (metal ions, ammonium). Free hydrogen sulfide is considered to be toxic. Volatile and higher fatty acids are potential inhibitors. Evidence suggests that a high concentration of dissolved CO_2 reduces the rate of acetate utilization by a factor of about 2 (Kirsop, 1984). Anaerobic cultures are able to dechlorinate chlorophenolic compounds (Henze and Harremoes, 1883). They are also able to adapt to toxic substances and recover after

a shock exposure (Henze and Harremoes, 1983; Parkin and Speece, 1983; Kirsop, 1984).

2.3 Microbial Attachment

2.3.1 Introduction

Attachment is termed specific if it involves interactions between complementary molecular structures on the microbe surface and the attachment surface. However, microorganisms are capable of attaching to many different types of surfaces, indicating that some relatively non-specific reaction must occur in those cases (Marshall, 1980). Biofilm formation in an anaerobic reactor certainly belongs to the latter category.

In certain cases of non-specific attachment, classical chemical adsorption equations adequately describe the increase in number of attached bacteria with time, suggesting that simple adsorption and passive attachment may indeed be the governing mechanism (Fletcher, 1977). Supporting evidence is given by the rapid occurrence and the reversible nature of some bacterial attachment (i.e., that elution of bacteria off a solid surface is possible with appropriate reagent) (Fletcher, 1980; Wood, 1980; Marshall, 1985). It was also observed that attachment of nonviable cells does occur to some degree (Fletcher, 1980) and the optimum conditions are very different from those allowing maximum viable cell attachment (for example, optimum pH of 2.4 was found by Stanley, 1983).

In many cases reversible adhesion is followed by irreversible attachment. In this process, active participation by the bacteria is involved.

2.3.2 Events of Biofilm Formation

The following consecutive processes are involved in biofilm formation (Bryers, 1980; Trulear and Characklis, 1982):

1. Transport and adsorption of organic macromolecules to the surface
2. Transport of microorganisms to the surface
3. Microbial attachment to the surface
4. Microbial growth and exopolymer production resulting in growth of biofilm
5. Detachment of biofilm

In the following sections various factors will be discussed which affect biofilm development by affecting one or more of the above mentioned processes. These include properties of the support material, the liquid medium, the microorganism, and environmental factors.

2.3.3 Support Material

Various properties of the support material are known to play an important role in attachment, although it is seldom possible to pinpoint one as solely determining adhesiveness.

2.3.3.1 Surface Charge

In an aqueous environment usually both bacterial and support surfaces are negatively charged. Therefore, bacteria experience a repulsive force as their diffuse double layer overlaps with that of the surface (Fletcher *et al.*, 1980). The success of the attachment strongly depends on the electrolyte concentration. High electrolyte concentrations result in a strong net attraction between bacterium and surface (Rutter and Vincent, 1980). Still at medium electrolyte concentrations the energy barrier is not too high, and motile cells seem to be at advantage in overcoming energy barriers (Fletcher, 1980).

The assessment of repulsive forces between bacterium and solid surface is complicated by the fact that the bacterial surface is not a smooth, homogeneous one with uniform charge distribution, but actually a complex area, comprising several layers, and frequently covered with long-chain capsular polymers (Fletcher and Floodgate, 1973). Furthermore, for glass and silica it was shown that the initial negative surface charge may decrease to zero or even become positive when brought in contact with aqueous solutions of organic salts (Ter-Minassian-Saraga, 1964).

Surface charge may provide a means of attracting essential inorganic nutrients, ions of the opposite charge, suitable for utilization by bacteria (Marshall, 1980).

Studies with ion exchange resins showed that passive or active attachment of the same organism can occur under different circumstances. *Staphylococcus aureus* cells at pH around their isoelectrical point attach equally well to anionic, cationic or uncharged resin, but stronger attachment was demonstrated to anionic exchange resin above that pH and to cationic exchange resin below that pH. Salt composition and concentration of the medium were found to have an important role in inhibiting, facilitating or reversing the adsorption. Different organisms behaved very differently (Wood, 1980).

Fletcher and Loeb (1979) compared a variety of materials for adhesiveness. Attachment of a marine pseudomonad was measured after 2 hours of exposure. The smallest number of bacteria was found on oxidized germanium and negatively charged glass and mica. The number of bacteria attached was an order of magnitude higher on positively charged platinum and reduced (neutral) germanium.

2.3.3.2 Surface Texture

Studies were conducted with variously textured materials of otherwise identical composition or surface treatment. Surface inhomogeneities about 1 micrometer (centerline average roughness) made thrombus formation a certainty regardless of the material used when test rings were implanted into the cardiovascular system of animals. The experiments showed surface textural variations to be major determinants

of cell-adhesion phenomena (DePalma et al., 1972; Baier, 1980).

Copp and Kennedy (1983) compared the biofilm development and performance of anaerobic reactors containing unaltered, physically or chemically treated Cloisonyl as support material (Cloisonyl is an extruded PVC material). Best results were obtained with physically surface roughened Cloisonyl. They found that biofilm formation started earlier and the film formed was thicker, more robust and more stable than on the untreated or chemically treated surfaces. Reactor performance was also superior to the others.

Messing et al. (1979) investigated the relationship between the pore size of support material and the accumulation of stable and viable biomass. In the case of microorganisms reproducing by fission they found that the optimum pore diameter should be 5 times the larger dimension of the microbe. When experimenting with molds they noted that the optimum pore size for spore adsorption was not the one which allowed the highest mycelial growth later. It was also found that during the stage of spore adsorption certain microorganisms preferred certain materials more than others of the same pore size. Later, in the stage of mycelial growth this difference disappeared, or even preference was expressed towards another type of material. The phenomenon was explained on the basis that the first deposition or monolayer of cells will be affected via direct contact with the carrier surface. Subsequently as growth continues and

becomes more remote from the surface, the dimensions of the carrier pores exert a much greater effect on bioaccumulation than do other surface properties. In the case of enzymes where reproduction did not play a role, Messing (1974) found that a carrier with a pore size twice the major axis of the enzyme was optimal for immobilization.

Beeftink (1987) observed that in a gas-lift anaerobic reactor the colonization of sand particles started in surface irregularities. The author attributed the phenomenon to local quiescent conditions.

2.3.3.3 Hydrophobicity

Short-range forces (operating at separation distances smaller than about 2 nm) are particularly important in aqueous systems and may be repulsive or attractive depending on the nature of the surfaces involved. According to Rutter and Vincent (1980) this may be discussed in terms of the displacement of water molecules from the interaction zone as a particle approaches a microscopic surface. If both surfaces are "hydrophilic", the local ordered water structure near the surfaces has to be broken down, causing a net increase in free energy of the displaced water molecules. This results in a short-range repulsive force. When both surfaces are "hydrophobic", the displaced water molecules decrease their free energy as they increase their H-bonding in bulk solution. Therefore a short-range net attraction results.

Most microorganisms are hydrophilic, and the above analysis predicts that their adsorption to a hydrophobic surface (for example, Teflon) will be stronger than to a hydrophilic surface (for example, clean glass).

Hydrophobicity is related to wettability in that solids having critical surface tension below the surface tension of water are considered hydrophobic (Zisman, 1962).

In contrast to the theory, Fletcher (1980) found no absolute preference on the part of all the bacteria in her experiments for either a hydrophilic (tissue culture dish) or a hydrophobic (polystyrene) substratum. The experiment involved five different bacterial species.

Fletcher and Loeb (1979) suggested that electrical charge has more importance for attachment on hydrophilic substrata and wettability is more important on hydrophobic substrata.

2.3.3.4 Surface Wettability

Wettability is a property of the solid surface which is often considered suitable to predict whether a microorganism will adhere to the surface or not. The parameter used to characterize wettability is the critical surface tension. Liquids with surface tension higher than the critical surface tension of the solid will form drops on the solid surface, while liquids with lower surface tension will spontaneously spread on the surface (Dexter *et al.*, 1975). Spreading implies that the interactions between the solid

and liquid molecules are equal to or stronger than those between the liquid molecules themselves (Zisman, 1964; Dexter *et al.*, 1975; Fletcher *et al.*, 1980).

The relationship between composition of the surface and wettability was investigated (Shafrin and Zisman, 1960; Zisman, 1964). Compared to hydrocarbon surfaces, progressive fluoridation results in lower, while progressive chlorination in higher wettability. In fact, a condensed monolayer of perfluorolauric acid is the least wettable surface ever reported. Polymethylsiloxanes, highly fluorinated fatty acids and alcohols; and fluorocarbon resins are commonly used as "abhesive" coatings to prevent the adhesion of two solids that are in intimate contact. Such abhesive coating converts the solid into a low energy surface (Zisman, 1962; Zisman, 1964; Baier *et al.*, 1968)

Critical surface tensions of surfaces of interest are shown in Table 2.2 (combined after Zisman, 1964; Dexter *et al.*, 1975; Baier *et al.*, 1968).

Critical surface tensions of all the surfaces listed (except glass) are well below the surface tension of water (72.8 dyn/cm at 20°C), and therefore all are hydrophobic.

Many high-energy solids, such as glass, silica, metals readily adsorb water, and the adsorbed water greatly decreases their surface energy, in fact, converts them into low-energy surfaces (Baier *et al.*, 1968).

Table 2.2 Critical surface tension of various surfaces

Material	dyn/cm (20°C)
Perfluorolauric acid monolayer	6
Polytetrafluoroethylene (Teflon)	18
Bulk water surface	22
Polymethylsiloxane film	24
Polyethylene	31
Polystyrene	33
Polyvinyl chloride (PVC)	39
Starch	39
Copper (99.9%)	45
Nickel (99.97%)	45
Cellulose	45
Glass	46-70

Several attempts were made to correlate critical surface tension and bioadhesiveness of surfaces. Baier (1972) using human blood cells, found that the range of 25-30 dyn/cm critical surface tension corresponds to a minimum rate of attachment. Dexter *et al.* (1975) using seawater organisms arrived at a similar conclusion; they found the least attachment between 20-30 dyn/cm. This critical surface tension range is termed "biocompatible". The term "biocompatible" refers to the fact that materials falling to that critical surface tension range constitute the best implant materials owing to their low adhesiveness. The authors also found glass outstandingly superior to the low energy surfaces tested concerning the rate of attachment.

Superiority in supporting adhesion of glass over Teflon and siliconized surface was demonstrated by Weiss and Blumenson (1967) as well. In a dental plaque formation study by Glantz (1969) the smallest amount of plaque formed

on the least wettable surface, Teflon. Fowler and McKay (1980) found the order of increasing adhesiveness to be siliconized glass < plate glass < Pyrex glass < stainless steel measured in a radial flow growth chamber. The authors noted that the surface texture of the materials was not identical, and this fact may have contributed to the results.

Mohandas *et al.* (1974) investigated the minimum shear stress for detachment of cells from clean surfaces. They found that it correlated with the initial surface tension of the material (Teflon < siliconized glass < polyethylene < glass). Again, surface texture differences existed, Teflon and polyethylene being rougher than the other two surfaces. In other studies (Fletcher and Loeb, 1979) however, a progressive decrease in the number of attached cells with increasing wettability was indicated. The highest number of attached bacteria was found on Teflon, polyethylene, polystyrene and polyethylene terephtalate, followed by the more wettable epoxy and nylon 66. When the above surfaces were modified for increased wettability, less bacteria were found attached to them. Powell and Slater (1982) noted that the critical shear stress required for detachment of *Bacillus cereus* cells in peptone medium was about 30% higher on silicone-coated glass than on glass surface alone.

More elaborate thermodynamic models reviewed recently by Marshall (1985) appear to predict adhesion more consistently than surface wettability.

2.3.4 Liquid medium

When searching for correlations between surface properties and microbial attachment, there is only one parameter, surface texture, which is truly independent of the properties of the liquid medium. Firstly, water alone has been shown to change the wettability of high-energy surfaces, believed to be preferred in attachment. Secondly, all real media (blood, seawater, sewage) contain a number of organic and inorganic components which are very likely to modify the surface properties of the support or the bacteria. This has been demonstrated by various authors.

Most solids assume a net negative charge when immersed in water. As a result, very soon after exposure, cations and a variety of macromolecular and colloidal materials are attracted to the solid surface altering the original surface charge. The modified surface charge will be a reflection of the ionogenic groups exposed at the surface of the conditioning film (Marshall, 1980). Small molecules, serving as nutrients, can provide an important advantage for attached bacteria, especially in dilute solution such as seawater (ZoBell, 1943). The adsorbed material was found to consist of mainly glycoproteins and their residues (DePalma *et al.*, 1972; Baier, 1980). Coating a surface with adsorbed organics can effectively lower the critical surface tension of surfaces, towards the biocompatible region. Baier (1980) showed that a protein layer lowered the critical surface

tension of the high-energy plasma-cleaned glass to about 32 dyn/cm, while materials already in the biocompatible range did not undergo significant surface tension change, although presence of the same amount of protein coating was evident.

The adsorbed protein layer is generally viewed as beneficial to adhesion, however facts pro and con alike can be found in the literature.

Weiss and Blumenson (1967) observed that the number of erythrocytes adsorbed to glass exceeded the number of cells attached to silicone by ten fold when salt solution served as a suspending medium. This was in contrast to suspension in human serum, when the number of cells attached to glass was as low as on silicone surface. Similar results were obtained using RPMI no.41 cells (derived from a human sarcoma) on glass and siliconized glass surfaces. The results compare favorably with Baier's findings.

In vitro experiments have shown that serum may, on one hand, promote cell adhesion to glass and to other cells, or, on the other hand, inhibit cell adhesion to glass or to other cells (Weiss and Blumenson, 1967).

Fletcher (1976) examined the effect of six different proteins on bacterial attachment on polystyrene dishes (critical surface tension 33 dyn/cm). Bovine serum albumin, gelatin, fibrinogen and pepsin impaired the attachment of a marine pseudomonad, while basic proteins, protamine and histone did not markedly affect attachment.

In a study of blood compatibility of various metals DePalma et al. (1972) concluded that the formation of a low-critical-surface-tension, proteinaceous coating is responsible for "passivating" metal as well as pyrolitic carbon surfaces so that improved thromboresistance results. They found no predictive correlation between electrical rest potentials of the metals and ultimate thromboresistance.

Heukelekian and Crosby (1953) experimented with 35 coatings, with or without special inhibitors. Complete inhibition of slime growth was not obtained even for short periods with any of them.

From the above review the impression is that ultimately it is the proteinaceous coating which determines the adhesive properties of the surface, not the original material. This proteinaceous coating does not improve adhesiveness, but it seems to convert initially highly bioadhesive surfaces into less bioadhesive ones. Since in more cases change in wettability and adhesiveness occurred simultaneously, it is reasonable to assume a relationship between the two.

Baier (1980) investigated the possibility that the properties of the original surface make a difference in attachment, even though the protein-film covered surfaces seem to exhibit no differences in cell adhesion if measured by the number of cells attached per time. The parameters measured were the spread areas of tissue-culture cells and the number of filopodia (irregular projections) on the protein-

covered surfaces. The cells exhibited 50% greater spread areas on glass then on siliconized material. The number of filopodia was also higher on glass, especially compared to dichloromethylsilane surfaces (3 fold). The strength of attachment was examined by subjecting the apparently attached cells to a modest impulse. While glass held the attached bacteria almost completely, nearly all cells detached from highly siliconized surfaces. From the results he drew the conclusion that properties of the original surface continue to affect the attachment after they have been covered by proteinaceous film.

Another interesting difference, as Baier pointed out, is that cells adsorbed to siliconized substrata appeared to be residing on, rather than embedded within, the uniformly dispersed glycoproteinaceous matrix. According to Dexter *et al.* (1975) bacterial populations adsorbed to low energy surfaces of about the same critical surface tension have the poorest rate of colonization. Weiss and Blumenson (1967) found that cells in serum-containing medium adhered and spread on both glass and Teflon. In salt solution they tended to adhere to each other forming small aggregates, rather than spreading on Teflon. Cells adhered and spread on glass in salt medium.

2.3.5 Microorganisms

Experiments are carried out and results are compared in the hope of accumulating a coherent body of knowledge, from

which final and general conclusions can be drawn. It has been noticed, however, that phenomena occurring with one species might not be observed for another one. Bacterial species differ considerably in their quality and quantity of cell surface polymers, thus it is not surprising that they also vary in their ability to attach substrata (Fletcher, 1980).

Messing et al. (1979), for example, noted that in pore size experiments the initial growth of *Streptomyces olivochromogenes* was substantially greater on glass than on cordierite. To the contrary, growth of *Penicillium chrisogenum* was greater on cordierite than on glass.

In a study by Wood (1980) on the interaction of bacteria with ion exchange resin the adsorption of *Staphylococcus aureus* to anion exchange resin was progressively inhibited by increasing concentrations of either sodium or calcium chloride. No effect of sodium chloride was observed on the adsorption of *E. coli*. Also, the salt-dependent recovery of *Staphylococcus aureus* varied greatly with pH, whereas *E. coli* was not affected. Even different strains of a species may differ in attachment properties (Fletcher, 1980). It is conceivable that similar differences might underlie the conflicting reports correlating adhesiveness with wettability or other surface properties.

An attempt was made to relate bacterial adhesion to the Gram reaction, but upon examination of a total of 96 differ-

ent marine bacterium cultures, ZoBell (1943) found no such general relationship.

Both Gram-positive and Gram-negative bacteria are negatively charged mainly owing to an excess of carboxyl and phosphate groups (Ward and Berkeley, 1980). It was found that cell wall compositions of chemostat-grown *Bacillus polymyxa* were markedly different from those of the batch-grown organism (Ellwood and Tempest, 1972). Data reviewed by Ellwood and Tempest show that the walls of bacteria can and do vary phenotypically with nutrient deficiency or amino-acid composition of the growth medium as well as with culturing conditions (batch or continuous flow system). Such changes in wall composition might have substantial bearing on adhesive characteristics.

It is not known whether the unique methanogen wall composition makes those bacteria fundamentally different from eubacteria concerning attachment properties. Kirsop (1984) noted that pure cultures of methanogens tend to be dispersed except for *Methanosarcina barkeri*, which characteristically occurs in aggregates of cells. There is also little indication that pure cultures adhere to glass surfaces of culture vessels or to metal components present within them. However, mixed cultures of organisms are found in general to adhere strongly to supports such as anthracite, glass, plastic, or stone which are commonly used in effluent treatment plants.

Intact cell structure and metabolism is assumed to play an important role in active attachment. Motility has been shown to be a factor in attachment of *Pseudomonas aeruginosa* to stainless steel by Stanley (1983). According to Ottow (1975) specific surface organelles such as fimbriae or pili may facilitate attachment by overcoming the initial electrostatic repulsion barrier. There is some supporting evidence for their involvement in actual attachment, but no clear picture can be drawn (Ward and Berkeley, 1980; Corpe, 1980).

It has been observed by several authors (ZoBell, 1943; Costerton *et al.*, 1978; Fletcher, 1980) that after initial adsorption most cells synthesize or make use of a polymeric material which glues them more tenaciously to the surface. A study by Fletcher (1980) indicated that in some instances polymer synthesis in the bulk liquid phase was required for irreversible attachment, while in other cases a time-dependent physiological activity at the surface was needed. Although investigations have generally implicated polysaccharides (dextran, cellulose), other materials such as nucleic acids and proteins frequently have been shown to play a significant role in bacterial aggregation (Harris and Mitchell, 1973; Marshall, 1985). Such materials may also be introduced to the medium via cell lysis.

In a review of the role of polymers in microbial aggregation Harris and Mitchell (1973) concluded that the generally favored mechanism for aggregate formation is polymer bridging, given the hydrophilic nature of the biocolloid

surface and the similarity in charge of biopolymers and biocolloids. The polymer attaches to a surface as a result of chemical interaction (complex formation, hydrogen bonding, proton transfer). A portion of its length extends into the liquid, and by interacting with vacant bonding sites of nearby particles, bridging results. As high polymer concentration causes restabilization, it is assumed that the saturation of the surface prevents optimal bridging of particles. Under proper experimental conditions all polymers investigated (anionic, cationic or nonionic) could aggregate negatively charged bacteria. The same model may apply to biofilm formation except that one participant is a solid surface until it is covered with bacteria or polymers.

Polymers that have a higher proportion of -OH and -COOH groups in their structure proved to be most effective in enhancing aggregation. In a study of anaerobic bacterial aggregates Beeftink (1987) found that sugar was an important component of the fibrous material that connected the rod-shaped cells to the substratum and to each other (Fig. 2.6). Tago and Aida (1977) discovered a mucopolysaccharide, which was shown to contribute to floc formation of a *Pseudomonas* strain. This fraction constituted only 10% of the total polysaccharides isolated from the floc, and the other 90% could not be shown to play a role in floc formation. The above finding helps to explain why the possession of mucoidness does not always coincide with adhesiveness (Marshall, 1985).

Figure 2.6 Rod-shaped bacteria on sand surface, linked by a fibrous material. Sugar was found to be an important component of the polymeric substance (Beeftink, 1987).

Mohandas *et al.*, (1974) examined the critical shear stress above which cells would eventually detach. They found that the critical force for detachment increased with time of cell-surface contact, as would be expected from the time-dependent, biological nature of adhesion stabilization. Powell and Slater (1982) found that the critical shear stress increased with cell-surface contact time up to 1 hour and remained constant thereafter. It must be noted that techniques based on cell detachment do not measure the adhesiveness of the cell or the material, but the strength of a fully stabilized biological joint. According to Crouch *et al.* (1985) critical shear forces causing detachment of cells are 1000-fold greater than those permitting attachment. The smallest critical shear stress required to detach human ery-

throcytes was 0.02 N/m² (Mohandas et al., 1974), obtained with fibrinogen-coated substrata. For bacteria, the smallest value observed by Powell and Slater (1982) was 1.1 N/m² in peptone medium.

The production of polymeric material may greatly increase in case of nutrient deficiency. Duguid and Wilkinson (1953) studied polysaccharide formation by *Aerobacter aerogenes*. Limiting concentration of carbon and energy source resulted in a minimum production of polysaccharide, whereas if the concentration of nitrogen, sulfur or phosphorus was growth-limiting, polysaccharide production reached a maximum (Fig. 2.7). Potassium seemed to be essential for polysaccharide synthesis. The effect of nitrogen limitation on excess polysaccharide production was observed by other authors as well (Harris and Mitchell, 1973; Characklis and Dydek, 1976; Williams and Wimpenny, 1978). Shaw et al., (1982) observed that bacterial cells produce extracellular polymeric substances which can reduce the permeability of a porous medium to effectively zero in a matter of a few days. Using dead cells, plugging occurred to a much lesser extent.

Precoating of a glass surface with an exopolysaccharide layer isolated from *Pseudomonas atlantica* resulted in greatly increased bacterial attachment from seawater (Corpe, 1970). Polymer synthesized by cell free *Streptococcus mutans* enzymes would not induce attachment when added to heat killed cells, but when the polymer was synthesized from sucrose *in situ*, attachment did occur (Mukasa and Slade,

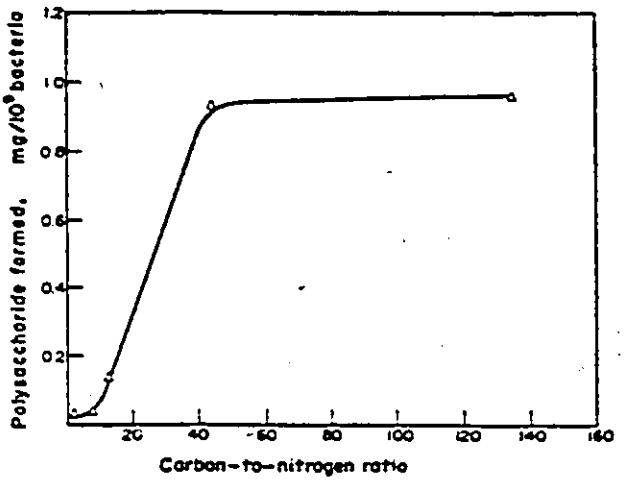


Figure 2.7 Effect of carbon:nitrogen ratio on polysaccharide production by *Aerobacter aerogenes* (Characklis and Dydek, 1976; after Duguid and Wilkinson, 1953)

1973). The authors conclude that participation of a binding site on the cell surface is required for the process.

A relationship exists between the age (activity, metabolic state) of the cells and their attachment properties. Duguid and Wilkinson (1953) found that the rate of polysaccharide synthesis was highest during the logarithmic growth phase and diminished progressively thereafter. However, the major part of the production occurred after the end of the logarithmic phase. Several other authors observed the same phenomenon (review by Harris and Mitchell, 1973). In the review it was also concluded that although there were exceptions, most of the microorganisms had a greater tendency to aggregate under conditions of declining growth than under conditions of prolific growth. Experience

in biological waste treatment operations also led to the conclusion that the higher the growth rate of the microorganisms, the less the tendency of floc formation. Assuming the polymer bridging mechanism of aggregation, it was suggested that rapidly growing cells contained insufficient quantities of polymers required for aggregation. The polymer concentration per cell is expected to increase after the logarithmic growth phase and may eventually reach the concentration necessary for aggregation. Powell and Slater (1982) found that stationary phase cells resisted initial removal by 2.2 N/m^2 wall shear stress much better than did exponential phase cells. The cells arrived at the surface by gravity and the flow was not commenced until one hour thereafter.

On the other hand, in Fletcher's study (1977) with a marine pseudomonad, logarithmic phase cultures had the greatest facility for attachment, followed by the stationary and death-phase cultures, respectively. For a possible explanation, the author presented the observation that logarithmic-phase cultures had the largest proportion of motile cells and there followed a decline in motility with the onset of the stationary phase. Also, as an extracellular polymer plays an important part in the attachment of this pseudomonad, variation in cell surface polymers (as a result of variation in growth conditions) would be expected to affect adhesion. Bryers (1980) in studies on biofouling found increasing attachment rate with increasing biomass growth

rate. High biomass growth rate is indicative of high biomass metabolic activity in a continuous reactor system.

2.3.6 Environmental Factors

2.3.6.1 Shear stress

Effects of shear stress on attachment and detachment of bacteria have been extensively investigated; since most of the systems of interest are mixed or liquid is recirculated, no quiescent conditions exist.

In the initial stage of biofilm development when attachment is the dominant factor rather than microbial growth, increase of flow velocity increases the rate of attachment by increasing the transport of microorganisms to the surface, but also increases the detachment rate by increasing the shear along the surface. The highest biofilm development rate is achieved at velocities where the gain by attachment outweighs the loss by detachment. In the second phase, when growth is the main contributor to biofilm development, velocities lower than the optimum are unable to ensure adequate supply of nutrients at the surface, thus impairing growth while higher velocities increase detachment of biofilm.

Sanders and Characklis obtained maximum growth at a velocity of 0.3 m/s. Characklis found that biofilms grown at higher velocities adhere to the surface more firmly (after a review by Characklis, 1973). According to Heukelekian and Crosby (1956) high velocities (0.45 m/s) retarded primary

film formation, but after a film was established, higher velocities resulted in greater growth. The largest accumulation of slime also produced the widest variations due to sloughing.

Trulear and Characklis (1982) conducted a study in an aerobic annular reactor (width of the annular gap was 0.4 cm) examining the effects of fluid velocity and substrate loading on biofilm accumulation. The study was carried out on a developed biofilm, therefore only growth and detachment contributed to biofilm accumulation, attachment did not. The amount of biomass was characterized by dry biomass weight; biofilm density was also determined. They found that biofilm accumulation rate as well as plateau biofilm accumulation was a function of rotational speed; the maximum accumulation was at 150 rpm. In the plateau phase the biofilm thickness remained constant, new growth being sheared off from the surface by the fluid. Biofilm detachment rate increased with increasing rotational speed and also with increasing mass of attached biofilm. The authors noted that species distribution of the biofilm can vary with the level of turbulence.

A major aerobic study was conducted by Bryers, using Pyrex glass as test material (1.27 cm i.d.). Chemical oxygen demand (COD) characterized biofilm biomass. In the examined range (0.7-1.85 m/s) an increase in Reynolds (Re) number (velocity) resulted in a linear decrease in initial biofilm accumulation rate. Attached biofilm fibers grown at

low Re varied in length and were randomly distributed on the surface. Attached fibers grown at high Re were more uniform in length and surface coverage. Biomass concentration of 12 mg/L total suspended solids (TSS) and specific growth rate of 0.28 h^{-1} was used in this experiment. A biofilm characteristic, polysaccharide to COD ratio was found to be more or less constant (0.2 mg/mg) in various runs and during the course of one experiment, except for a short initial period, when it was substantially higher.

Norrman *et al.* (1977) found that flow velocities 6-7 m/s did not significantly affect film attachment and growth. Roughened acrylic tubing (1.27 cm i.d.) was the support material in this study, the bacteria originated from primary treated sewage.

In the experiments of Bott and Miller (1983) velocities of 0.8-1.0 m/s were optimal. Higher velocity did increase the amount of biofilm accumulated when no substrate and nutrients were present (i.e., only attachment occurred), but to a much lesser degree than in the presence of feed. The authors also suggest that at higher velocities deposition may play a greater role than the growth within the biofilm. Sloughing was more prevalent in thicker films. A pure culture of *Pseudomonas fluorescens* and aluminum support material (1.25 cm i.d.) were used.

Harty and Bott (1979) examined biofilm formation on a brass surface (cross section 1x2 cm) using *E. coli* and *Klebsiella aerogenes*. The velocity range studied was 0.1-0.55

m/s and a steady decrease of both biofilm growth rate and maximum thickness was observed with increasing velocity at both 37 and 42°C. A high carbon to nitrogen ratio was maintained in the medium to facilitate polysaccharide production.

McCoy et al. (1981) studied the relationship between friction factor and biofilm development using bacteria from activated sludge. At 2.65 m/s flow velocity the induction phase (time elapsed prior to increase in friction factor) was 45 h longer than at 1.38 m/s. The authors suggested that at high velocity a biofilm matrix richer in extracellular materials is required before filamentous bacteria are able to colonize.

Turakhia and Characklis (1983) compared biofouling in smooth and finned 70:30 Cu/Ni tubes (i.d. 1.135 cm). Since the extent of fouling in the finned tube section was somewhat less than that of the smooth tube, the authors concluded that increased turbulence in the region between the two fins may decrease attachment rate or increase detachment rate or both.

2.3.6.2 Biomass Concentration

Studies employing various suspended biomass concentrations confirmed that biofilm attachment rate is linearly proportional to suspended biomass concentration as it is predicted by models describing molecular adsorption from solutions onto surfaces (Fletcher, 1977). Bryers (1980) ar-

rived at the same conclusion working in the range of 2-24 mg TSS/L. He was able to describe the overall biofilm accumulation with the empirical equation

$$\frac{dB}{dt} = B\,K\left(\frac{X\,D}{Re}\right) \qquad (2.5)$$

where

X is the suspended biomass concentration [mg TSS/L]
D is the dilution rate [h^{-1}]
B is the attached biofilm amount [$\mu g/cm^2$]
K is the specific rate constant [L/mg TSS]

2.3.6.3 Substrate Concentration and Loading Rate

Trulear and Characklis (1982) studied among other parameters the effect of the reactor glucose concentration on biofilm formation. The possibility of attachment was ruled out so biofilm growth was due only to microbial growth and exopolymer production. Active biofilm thickness increased with higher reactor glucose concentrations (active thickness refers to the biofilm thickness beyond which substrate removal rate remains constant). Specific biomass production rate also increased with reactor glucose concentration in a fashion which may be described by a Monod-type equation. The maximum net biofilm accumulation rate ($mg/m^2 \cdot min$) and plateau biofilm accumulation (mg/cm^2) increased with glucose loading rate ($mg/m^2 \cdot min$). Plateau biofilm thickness (μm), however, decreased sharply with glucose loading rate initially, and remained constant thereafter. These two phenomena occurring together mean that the biofilm formed be-

came denser as glucose loading rate increased. Glucose reaction rate (mg/mg·h) increased with glucose loading rate and the authors suggested that this might be due to the density increase.

Bott and Miller (1983) working with *Pseudomonas fluorescens* found that the presence of substrate greatly increased the rate of initial biofilm accumulation compared to starving state accumulation. Also, biofilm development in the fed reactor started after 1 day, as compared to 4 days with starving culture. In both cases the bacteria were fed until the experiment commenced. Short HRT assured that suspended growth would not occur in the test system. The possibility of attached growth however was not ruled out in the 18-day experiment. The shorter lag phase must be attributed to the effect of substrate availability on the attachment process, which is reasonable recalling that carbon source limitation was shown to decrease polysaccharide production. On the other hand, it is very likely that reproduction in the biofilm contributed to the increase in accumulation rate observed with the fed culture.

2.3.7 Survey of Anaerobic Studies

2.3.7.1 Support Material

Most of the studies on anaerobic biofilm attachment were initiated in an effort to minimize startup difficulties of fixed film reactors. They were mainly concerned about finding the most suitable support medium on an empirical ba-

sis, that is, by trying different supports and monitoring reactor performance and some biofilm characteristics. The following studies have been done with mixed methane producing cultures (i.e., containing both acidogenic and methanogenic bacteria) unless otherwise indicated.

Copp and Kennedy (1983) found that plasticized needle punched polyester (PNPP) support material resulted in best reactor performance demonstrated by highest loading rate, highest methane production rate and fastest film development, compared to surface treated or untreated Cloisonyl (PVC). Biofilm grown on PNPP was more stable. The experiment was completed in 160 days.

Van den Berg *et al.* (1981) showed that biofilms became established much more rapidly on clay (1-3 months) than on glass (10-14 months) or PVC plastic (7 months). Biofilms on clay were also less subject to failure. The maximum activities ultimately reached were only slightly higher (about 10%) on clay than on glass or plastic. Wet film thickness varied from 1 to 3 mm (van den Berg and Lentz, 1980).

Murray and van den Berg (1981) used an enriched methanogenic culture (composed almost exclusively of bacteria converting acetic acid to methane) in their studies. They obtained the highest acetate conversion rate when fired clay support was used. Film development did not become evident on etched glass or PVC until after 10 days and slowed down after about 25 days. Film activity on an area basis was approximately three fold greater on clay than on either

PVC or glass. Substantially more film, which also was more uniform and thicker was present on clay than on the other supports. Attachment was very poor and spotty on glass, and much thicker on PVC. Filament length was shortest on etched glass and longest on clay support. Some clay slides were refired and polished smooth to assess the effect of porosity and roughness on biofilm development. On these slides film development was about 20% slower and leveled off sooner (maximum development was 30% less) compared with the rougher, more porous slides. It was also confirmed, that, among other properties, the slow leaching of nutrients (especially soluble iron) from the clay contributed substantially to its success as a support material for anaerobic biofilms. Tests were completed in 70 days.

Van den Berg and Kennedy (1981) found that when successful support materials were compared, it was needle punched polyester (NPP) and red draintile clay which resulted in best reactor performance. Rates of film development were close to the maximum growth rates of acetate converting methanogens.

Harvey et al. (1984) also experimented with NPP material. They found that bacteria were entrapped between the fibers rather than being attached to the surface. Since phosphorus and calcium were the major elements detected in both the bacterial cell and the extracellular material, they concluded that part of the biofilm material was probably

calcium carbonate and calcium phosphate precipitate. The experiment was terminated after 7 months.

Switzenbaum *et al.* (1985) studied the influence of support materials and precoating on initial biofilm development. Accumulations were more rapid on stainless steel and Teflon (about 400 µg COD/cm² at 15 days) than on PVC and aluminum. Precoating of PVC or aluminum with denitrifying bacterial biofilm, starch, carboxymethyl cellulose, xanthan gum, polyacrylamide, alum or Magnifloc 573c did not increase the initial biofilm accumulation rate. The duration of the study was 15 days.

Kennedy (1984) found stainless steel to support biofilm growth better than plexiglass. The experiment was conducted with approximately 1000 mg/L reactor volatile suspended solids (VSS), 48 cm/s flow velocity and 17 day SRT. A mixed culture was used, fed with 50% sugar-50% acetate. COD was the indicator of biomass concentration. When the experiment was terminated after 40 days, 700 µg COD/cm² was measured on stainless steel, while 450 µg COD/cm² was observed on plexiglass. The difference was characteristic throughout the experiment. The results are shown on Fig. 2.8.

From the above studies it is rather clear, that as far as attachment of anaerobic bacteria is concerned, glass is the poorest support material experimented with, not only with respect to biofilm development rate, but also in holding the film tenaciously. This is contradictory to most of the experiments mentioned in Section 2.3.3. Considering the

success of the porous clay and the fibrillous needle punched polyester it seems that surface texture is a more important

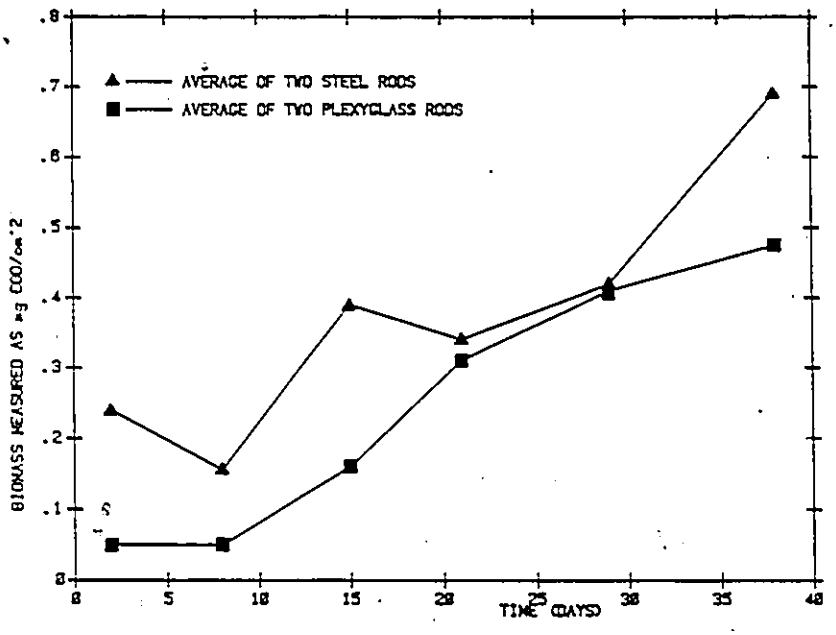


Figure 2.8 Biomass accumulation on steel and plexiglass for 40 days (Kennedy, 1984)

factor than the other surface properties in supporting fast growth and stable, strongly attached anaerobic biofilm.

2.3.7.2 Specific Surface Area

Specific surface area (that is, surface to volume ratio in a reactor) is considered to be an important parameter influencing reactor performance. Song and Young (1985) working with a corrugated modular medium found that the performance of an upflow anaerobic filter was only slightly affected by the specific surface area. Results of Copp and Kennedy (1983) have also shown that the effect of this pa-

rameter can easily be masked by other media characteristics such as surface roughness.

2.3.7.3 Operational Parameters

An extensive study has been conducted by Shapiro and Switzenbaum (1984) on the initial anaerobic biofilm development as a function of certain operational parameters. The parameters chosen were bulk liquid SRT, organic loading rate and flow velocity. The experiments were conducted at 35°C with mixed anaerobic culture and sucrose/nutrient broth media. For support material, silicone tubing (0.795 cm i.d.) was chosen. Runs terminated at 25 days. To monitor the attachment of methanogens, coenzyme F420 was measured, while COD accumulation characterized the total biofilm development. Organic loading rates fell in the range of 70-1500 mg COD/L·d for 15 day SRT and 200-4400 mg COD/L·d for 5 day SRT. Reactor VSS concentrations varied with organic loading rates, ranging from 40-3000 mg VSS/L for 15 day SRT and 40-1900 mg VSS/L for 5 day SRT. The ratio of methanogens to the total biofilm was constant during one run and very similar for all conditions. Biofilm accumulation rate increased with organic loading rate and with suspended solids concentration. Higher initial rates were observed for 15 rather than 5 day SRT. The highest rate for both 5 and 15 day SRT was observed at the highest organic loading rate and VSS concentration, being 2.5 µg COD/cm²·h and 3.33 µg COD/cm²·h respectively. No significant change was found in biofilm

attachment with varying flow velocity in the studied range (2.6-5.4 cm/s), but at either end of this range an increase in biofilm accumulation occurred.

The results of this study agree with data obtained in aerobic studies concerning the effects of reactor bacterium concentration (VSS) and organic loading rate. As for the effect of flow velocity, aerobic experiments themselves do not draw a conclusive picture and velocities employed by these authors were in a much lower range than those used in aerobic studies. Figs. 2.9, 2.10 and 2.11 show some of their results.

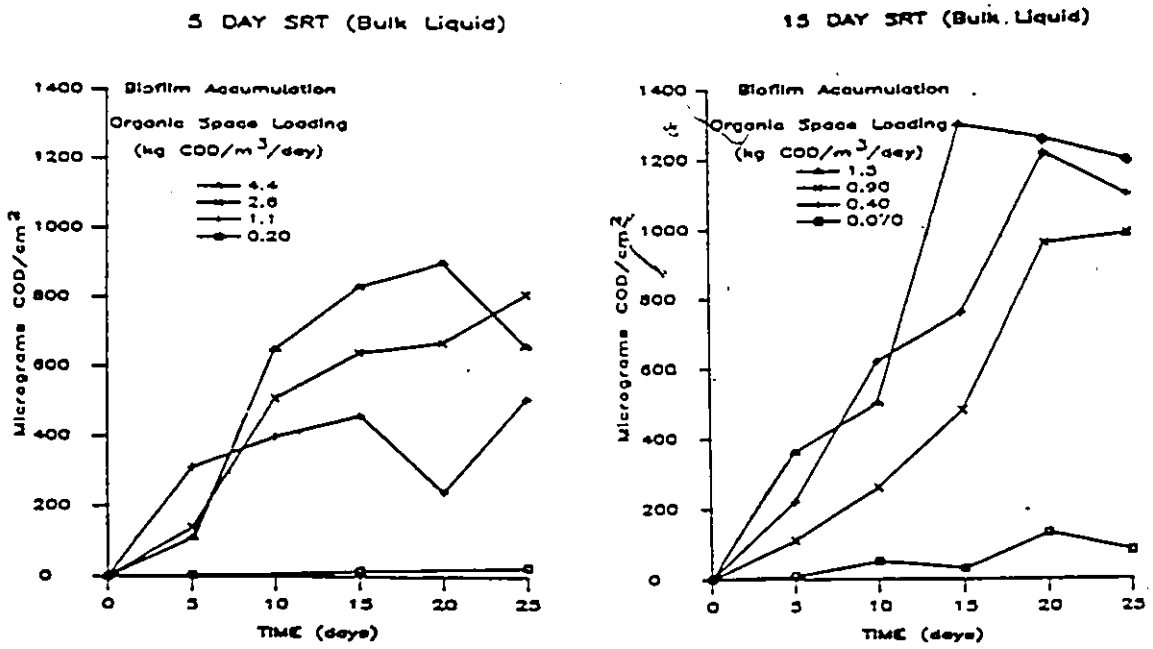


Figure 2.9 Methanogen and anaerobic biofilm accumulation for 25 days (5 and 15 day SRT, four organic loading rates) (Shapiro and Switzenbaum, 1984)

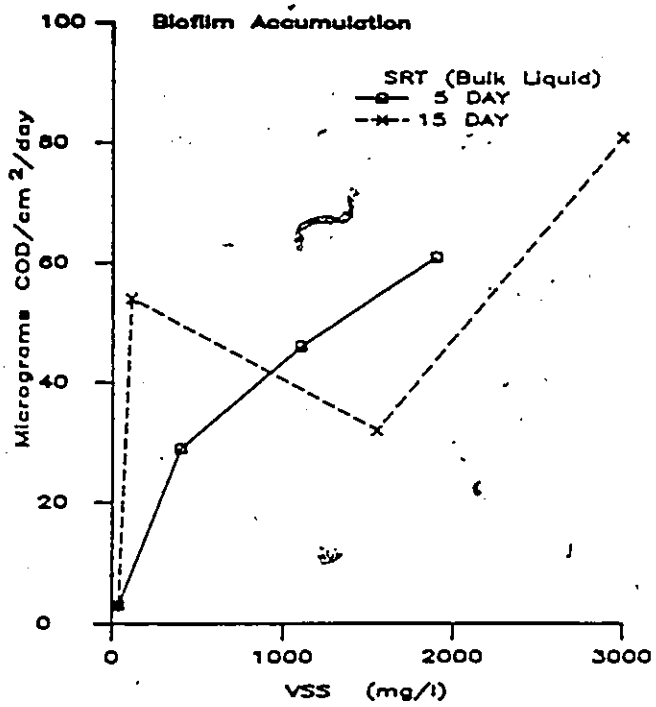


Figure 2.10 Initial rates of anaerobic biofilm accumulation vs. bulk liquid VSS (Shapiro and Switzenbaum, 1984).

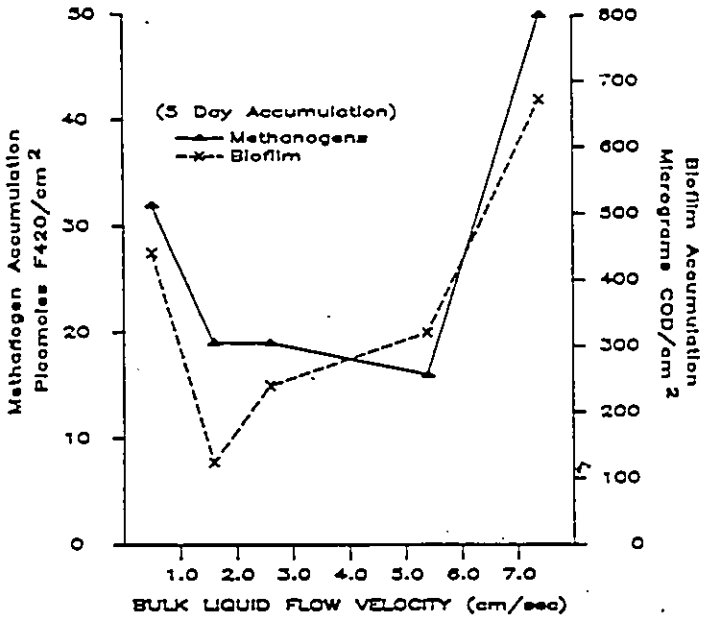


Figure 2.11 Effect of bulk liquid flow velocity on methanogen and anaerobic biofilm accumulation (5 day SRT, 2600 mg COD/L·d, after 5 days).(Shapiro and Switzenbaum, 1984)

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Setup

The experimental system is shown in Fig. 3.1. It consisted of the following components:

Reactor

A 2 L continuously stirred Sovirel model Pyrex glass reactor was used, which permitted a fast change of culture between experiments. The reactor top was equipped with 4 ports which were used for liquid recycle, gas sparging and gas exit. Ports for liquid recycle and gas sparging had glass dip tubes extending into the liquid phase, while the gas exit port opened into the headspace. No attached growth was observed on the glass walls of the reactor.

Recycle pump

A variable speed Masterflex pump (model R-7549-19) recirculated the reactor liquid through the experimental loop at 2 L/min. This ensured identical liquid supply for the variable speed pumps.

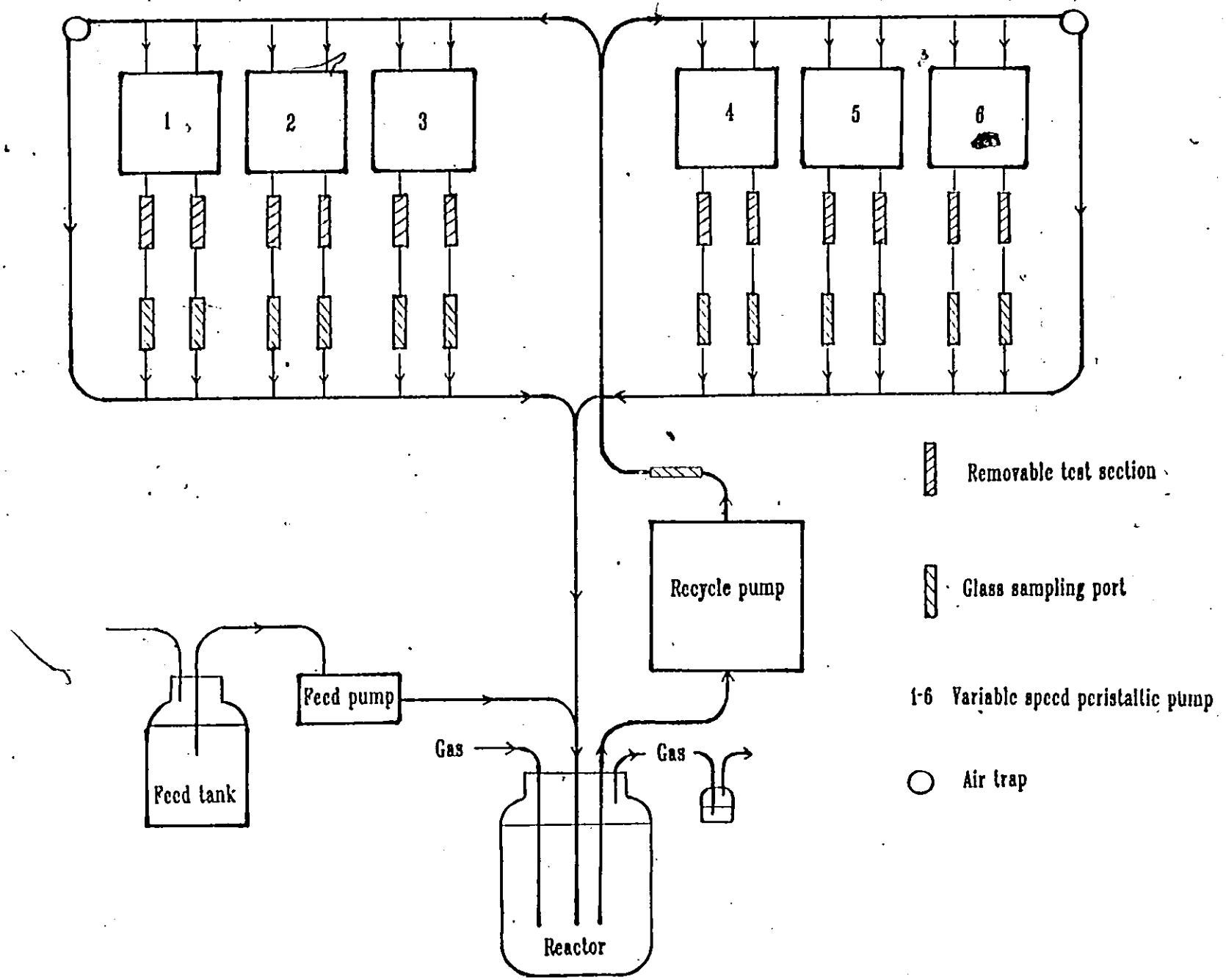


Figure 3.1 Experimental system

Pumps

Six, variable speed (0.004 - 114 mL/min) Harvard peristaltic pumps (model 1203) were installed with 2 lines each. These provided for a study of the effect of different flow velocities. The pumps were operated to cause a downflow mode in the test sections to avoid accumulation of suspended solids in lines.

Feed Tank

A 1.0 L glass bottle served as feed tank. Feed was kept at 35 °C. No precautions were taken to maintain the feed anaerobic or sterile. No bacterial growth was observed in the feed tank due to the acidic pH (4-5).

Feed Pump

This pump was used for continuous feeding during the experiment. A one rpm Masterflex pump (model R-7543-01) with 7016 pump head was employed, capable of delivering a volume of 0.845 mL/min. The feed was supplied at a rate of 36-42 mL/d. This corresponded to an organic loading rate of 510-620 mg COD/L·d. The feed line joined the recycle line close to the point of return to the reactor.

Test section

Approximately 8-10 cm long pieces of plastic tubing were used as supports for biofilm formation. Plastic tubing (Cole-Parmer Inc.) was chosen for the ease of insertion and

removal (by means of connectors and clamps) and its ready availability. The surface of plastic tubing was shown to promote high bacterial attachment rates in preliminary experiments. One test section was inserted per line.

Silicone tubing (0.794 cm i.d., 1.11 cm o.d., 0.16 cm wall thickness: R-6411-18, Cole-Parmer) was used in all experiments. In experiment #1 it was used in parallel with Tygon tubing (1.27 cm i.d., 1.588 cm o.d., 0.16 cm wall thickness: R-6408-18, Cole-Parmer) for comparison of attachment.

Sampling port

Small glass tees fitted with a septum were installed in each line below the test section to provide a means of sampling the liquid that passed through the test section. One additional sampling port was inserted into the main recycle line. Samples representative of the reactor mixed liquor were taken from the main recycle line. No growth was observed on the glass surfaces.

Loop

Tygon tubing (0.953 cm i.d.) connected all parts of the system. Biofilm formation on these tubing surfaces contributed to the depletion of biomass in the reactor. Removal of this film was possible, however, this involved the risk of disturbing the biofilm in the test section. In the main recycle line biofilm removal was performed regularly

and it was done after every sampling in the lines containing test section. The loop volume was 1 L, resulting in 3 L total system volume.

Air Traps

It was unavoidable that a small amount of air entered the system during sampling. One trap on either side of the loop was installed to collect air entrained in the system. Air was removed with a syringe after sampling.

No effluent was produced during operation of the experiment, instead, liquid volumes removed via sampling were replaced to keep the system volume constant.

The system was kept anaerobic. When no feed was delivered or gas production was not sufficient to keep the pressure higher than atmospheric inside the reactor, a gas mixture (20% CO_2 - 80% N_2) at 24 mL/h was sparged through the reactor.

All experiments were performed in a temperature controlled room maintained at 35 $\pm$ 1 °C.

3.2 Sampling Schedule

Each experiment started with the anaerobic transfer of a 3 L portion of the stock culture to the system. The sludge was then circulated overnight under the conditions used in the experiment and biofilm formation was observed the next morning. At this time new test sections were placed in the loop and the sampling schedule was set.

The experiments concentrated on the early stages of anaerobic biofilm formation. The longest experiment lasted 3 days and the shortest 11 hours.

The length of a run was partly determined by the speed of biofilm formation. To prevent biomass depletion in the reactor, the frequency of sampling and the termination of the experiment was scheduled according to the visually observed rate of attachment.

3.3 Sampling Procedure

1) Approximately 5 mL of liquid sample was taken from the moving liquid through the appropriate glass sampling port.

2) Tubing immediately above and below the test section was clamped. Simultaneously, the pump controlling the flow through that particular section was stopped. A needle was inserted at the top of the test section to let air in during sampling. First the liquid residing in the upper plastic connector was removed to prevent dripping later. This liquid was then discarded. Next, liquid filling the test section itself (2-3 mL) was removed slowly at the bottom of the section.

The test section was then removed from the loop. Ends fitting to the connectors were discarded and the volume of the plastic support plus attached biofilm was measured via a displacement technique (Appendix A) in 50 mL double distilled water. Test sections were cut in half to fit in the

measuring dish. Following the volume measurement the test section and the liquid were transferred from the volume measuring dish into a 140 mL glass beaker. The measuring dish was then rinsed with 5 mL of double distilled water, which was added to the 50 mL in the beaker.

At this point a new sample section was inserted into the loop, filled with liquid of the same origin as the reactor contents, clamps were removed and the pump was started again.

3) A sample of the reactor liquid was taken from the main recycle line.

4) Air accumulated in the traps was removed.

Biofilm samples were treated as follows:

Following the volume measurement, the attached biofilm was removed from the tubing surface by sonication in 55 mL double distilled water for 3 minutes, or until the test section appeared to be free of biofilm upon visual inspection. Samples were cooled with ice during sonication. The 55 mL liquid containing the biofilm was analyzed for COD, protein (PR) and polysaccharide (PS) in order to characterize the biofilm. The volume of the clean, dry test section was then remeasured 3 times by the volume displacement method, at the same temperature as the experiment (35°C). The volume of the biofilm was obtained as the difference between the volume of the biofilm carrying test section and the average volume of the clean test section. Since biofilm samples

were not allowed to air dry, the measured film thickness values are thought to be somewhat overestimated. The presence of biofilm was detected by biochemical indicators prior to its detection by the volume measurement technique, due to the greater imprecision of the latter method.

To account for outside contamination, blank test sections were exposed to the atmosphere for 72 hours. Blank test sections were processed in the same way as the biofilm containing sections. This yielded blank COD, PR and PS values. Biofilm values were then corrected with the blank values.

The biomass indicators and parameters monitored during the experiments are summarized in Fig. 3.2.

3.4 Feed Solution

Composition of the feed solution is shown in Table 3.1.

A high acetic acid concentration was chosen to minimize the dilution of reactor contents by feed flow. Components of the solution were dissolved in tap water and kept in refrigerated containers at 4°C. Henze and Harremoes (1983) presented a review of the desirable COD:N ratio for different organic loading rates. The theoretical minimum was found to be 100:2 for high-loaded processes (>1 mg COD/mg VSS·day). For low loading rates this ratio should be higher. In this study organic loading rate varied between 0.3-0.9 mg COD/ mg VSS·day, and a COD:N ratio of 100:0.65 was maintained. A high COD:N ratio also favors polysaccha-

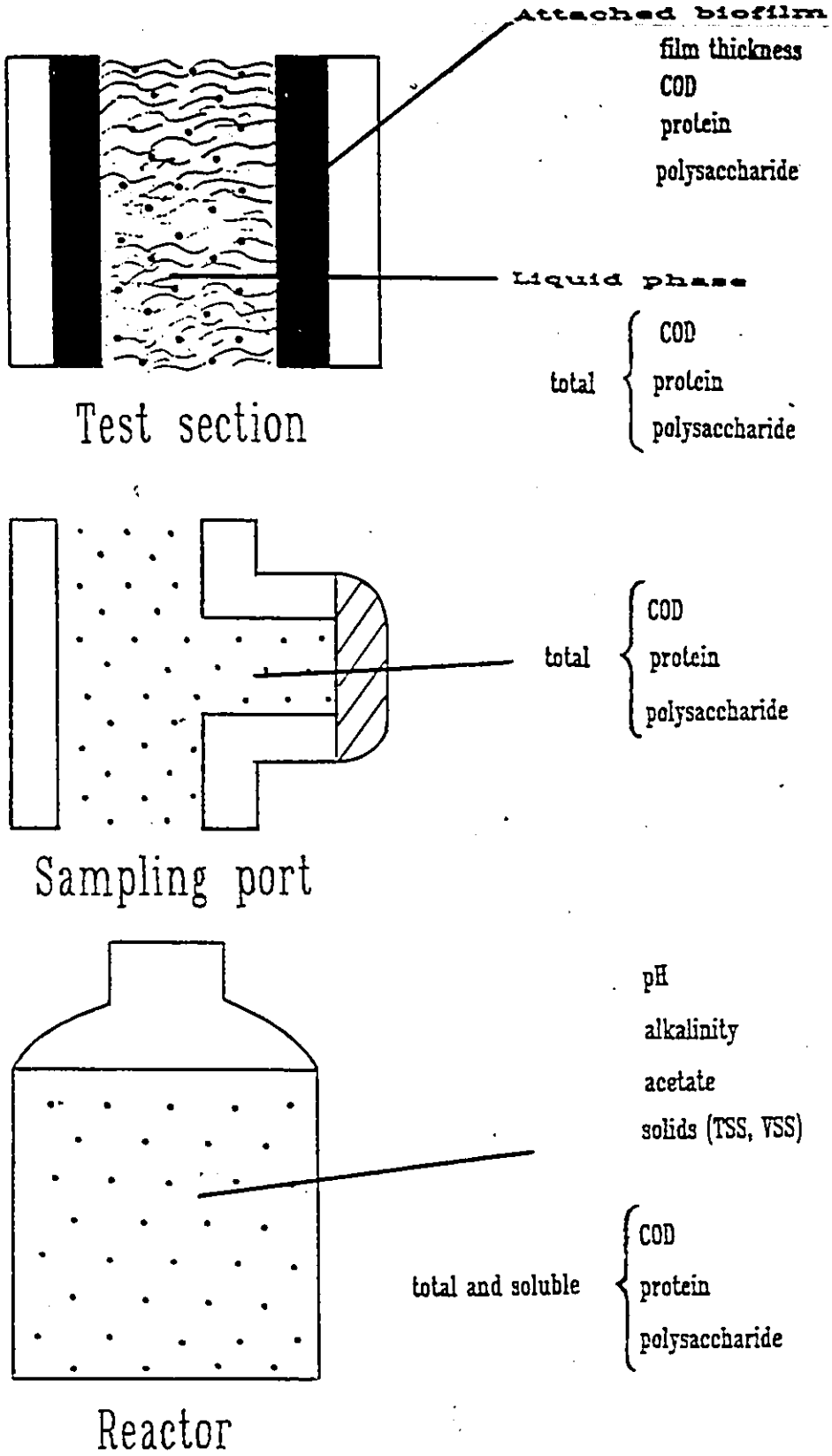


Figure 3.2 Parameters monitored

ride formation (Wilkinson, 1958), which may facilitate attachment (Harris and Mitchell, 1973; Marshall, 1985).

The N:P ratio was kept at 3:1, lower than the usual 7:1 ratio. More phosphate was added for buffering purposes instead of more bicarbonate. When bicarbonate concentrations were adjusted to compensate for the high acetate concentration, an undesirable pH increase was experienced in preliminary experiments.

Table 3.1 Composition of 40,000 mg/L acetate feed solution

Component	Concentration mg/L
NH_4HCO_3	1000
$NaHCO_3$	2000
$KHCO_3$	2000
$(NH_4)_2SO_4$	500
K_2HPO_4	260
KH_2PO_4	100
Yeast extract	100
Acetic acid	40,000

3.5 Reactor Characteristics

Reactor biomass was characterized by VSS, suspended COD (SCOD), suspended protein (SPR) and suspended polysaccharide (SPS) which are referred to as biomass indicators. Reactor biomass was also described by the general metabolic state of the biomass (previously starved or active) and the actual

metabolic state of the biomass (starved or active during the experiment). Further measured characteristics of reactor contents were acetic acid concentration, pH and alkalinity. Acetic acid concentration was set to either 0 mg/L (starvation) or 1000-1100 mg/L (active). A constant pH was maintained during the experiments due to the sludge's own buffer capacity and the buffered feed solution; pH was approximately 7.8 in experiment #1 and 7.4-7.6 in later experiments. Alkalinity was checked occasionally and was found to be 4000-5000 mg/L as $CaCO_3$. Gas production was observed but not measured.

The potential specific acetoclastic activity of the biomass was measured by batch serum bottle activity tests described in Section 3.8.9. The tests were performed on aliquot samples of the reactor mixed liquor. Potential acetoclastic activity is indicative of the capability of the biomass to utilize acetate. The actual activity of the biomass was measured by the specific acetate consumption rate in the reactor during the attachment experiment (Appendix I). The two activities may be different. In absence of substrate in the reactor, actual acetoclastic activity is zero, even if the biomass is capable of utilizing acetate, when it is supplied (such as in serum bottle test). In other words, the existing capability may or may not be expressed, depending on substrate availability and concentration.

3.6 Preparation of Stock Cultures

Two stock cultures were used.

Stock culture I was prepared by concentration of an actively metabolizing enriched acetoclastic methanogenic culture via sedimentation. Stock culture I had a VSS concentration of 610 mg/L and potential specific acetoclastic activity of 0.94 mg COD/mg VSS·d. This culture was used in Experiment A.

Stock culture II was prepared by concentrating the contents of a 60 L reactor to 15 L by sedimentation and centrifugation. The culture had been starving for about 6 months but previously had been continuously fed with acetate. Stock culture II had a VSS concentration of 800 mg/L and zero potential specific acetoclastic activity. Experiment E was completed using this inactive biomass. For experiments F and G, 6 liters of stock culture II were removed and activated. Stock culture II itself was then activated. Acetate feed was supplied continuously at a rate of 450-600 mg COD/L·d for 90 days. Experiments B, C and D were performed using this culture. At the time experiments B, C and D were performed, the culture had a potential and actual specific activity of 0.8-0.9 mg COD/mg VSS·d and a VSS concentration of 500-550 mg/L. For run D the culture was concentrated by sedimentation to a VSS concentration of 850 mg/L.

The culture was maintained in a 15.5 L continuously mixed reactor with internal dimensions of 45 cm height and

21 cm i.d.. The reactor was manufactured from 1 cm thick plexiglass and was fitted with a stainless steel top (New Brunswick Scientific Co., model MF-348S).. One hole opening to the headspace was utilized as an outlet for biogas, while another was used for feed supply. One dip tube was used as inlet for N_2/CO_2 mixture sparging gas. Effluent was removed through a port positioned 5 cm above the reactor bottom. Mixing was provided by a DC servo motor turning a stainless steel shaft with three six-flatblade impellers at 60 rpm. Reactor biogas was vented into a fumehood. A small glass tee fitted with a septum was placed in the gas line to facilitate gas sampling. The reactor was operated at 50 d SRT.

3.7 Experimental Plan

Table 3.2 summarizes the aims of the different experiments performed and some properties of the cultures used.

3.8 Frequently Used Terms and Calculations

General concepts that will be referred to throughout this work to characterize the biofilm or the reactor mixed liquor are discussed in this section.

3.8.1 Attached, Loose and Total Biofilm

a) When the biofilm was removed from the support surfaces into 55 mL double distilled water by sonication, the concen-

Experiment #	Experiment #1						Experiment #2			Experiment # 3		
Parameter varied	Flow velocity and support material						Suspended biomass concentration			Biomass activity		
										Potential	Actual	
Reactor (culture) code	A1	A2	A3	A4	A5	A6	B	C	D	E	F	G
Flow velocity, cm/s	0.054	0.64	1.42	0.056	0.76	1.08	1.05	1.05	1.05	1.18	1.18	1.18
Support material	Tygon			silicone			silicone	silicone	silicone	silicone	silicone	silicone
VSS, mg/L	610						510	522	848	793	790	654
SCOD, mg/L	1084						685	747	1176	1154	1147	986
Potential activity mg COD/mg VSS.d	0.94						0.89	0.89	0.79	0	0.38	0.81
Actual activity mg COD/mg VSS.d	0.94						0	0	0	0	0.38	0.81
Reactor acetate conc. mg/L acetate	1120						0	0	0	0	1000	820
Organic loading rate mg COD/L.d	564						0	0	0	0	300	570
SRT, d	20						7.7	7.7	9.7	36	25	15.8
Duration, h	72						11	11	11	58	20	11

Table 3.2 Experimental plan

trations of different biomass indicators in this liquid were determined in mg/L. Knowing the inner surface area of the test section, these results were converted to $\mu g/cm^2$ of support surface (see Appendix C for a sample calculation).

Results expressed in $\mu g/cm^2$ are referred to as attached biofilm (AF), or more precisely attached film COD (AFCOD), attached film PR (AFPR), or attached film PS (AFPS).

Considering the 1.5-8 d doubling times observed for acetate enrichment cutures (Henze and Harremoes, 1983), it may be safely assumed that the contribution of microbial growth to biofilm development was small during the 11-72 hour long experimental runs. Thus, attached film development rates reported in this study are net attachment rates (attachment minus detachment).

b) It was observed that the liquid inside the test section was richer in biomass than liquid obtained from the glass sampling port below the test section. Since a downflow mode was employed, it could not be floating biomass caused by buoyancy. The additional biomass was being held in place (mechanism unknown) while the liquid was flowing through the test section at a constant rate. At sampling, this excess biomass exited with the liquid when it was removed from the test section. To estimate the quantity of this type of biomass, the liquid from the test section was carefully collected and analyzed for COD, PR and PS. The concentrations of COD, PR and PS determined from the test section liquid were corrected for COD, PR and PS concentra-

tions measured from the sampling port below the test section. In this way COD, PR and PS concentrations in excess of those in the liquid flowing through the section were obtained.

The excess biomass in the test section divided by the support surface area (Appendix D) is called loose biofilm (LF), and described as loose film COD (LFCOD), loose film PR (LFPR), loose film PS (LFPS). Expressing LFCOD, LFPR and LFPS as $\mu g/cm^2$, loose film can be compared to attached biofilm.

c) The sum of attached and loose biofilm is termed total biofilm (TF), or total film COD (TFCOD), total film PR (TFPR) and total film PS (TFPS).

3.8.2 Concentration in Biofilm

Knowing the thickness of attached biofilm and the $\mu g/cm^2$ values of biomass indicators, it is possible to compute (Appendix E) the concentrations of those indicators in the biofilm as mg/mL biofilm (such as mg COD/mL biofilm). These values are referred to as biofilm COD, biofilm PR or biofilm PS concentration.

The concentration values allow a comparison of the density of biofilms of different origin.

3.8.3 Ratios

The ratio of the various biomass indicators to each other was used to further analyze the characteristics of the biofilm.

In the reactor, SCOD, SPR and SPS values were accepted as values characteristic of the bacteria. Reactor data throughout an experiment were summarized and the ratios of SPR/SCOD, SPS/SCOD, SPS/SPR were established as reference numbers.

The same ratios for the attached biofilm were computed and they were compared to the reactor values. Loose biofilm ratios were also compared.

This comparison provided information about the composition of the biofilm as opposed to the composition of the non-attached, suspended biomass.

An example calculation is shown in Appendix F.

3.9 Analytical Methods

3.9.1 pH

The pH was measured using a Radiometer/Copenhagen pH meter model 26 employing a glass electrode. Sensitivity of the unit was 0.05 pH units.

3.9.2 Alkalinity

Alkalinity was measured using a Radiometer/Copenhagen pH meter equipped with an automatic titrator. A pH of 3.75

marked the end point of titration. The titrant used was 0.1 N hydrochloric acid.

3.9.3 Biofilm Volume

Biofilm volume was measured by a volume displacement technique described in detail in Appendix A. Calculation of biofilm thickness (FT) is shown in Appendix B.

3.9.4 Chemical Oxygen Demand (COD)

COD was measured with the method described by Knechtel (1978) except that samples were heated to 150°C in an oven for 3 hours rather than 2 hours. For samples containing less than 145 mg/L COD the method was modified. A 0.03 N potassium dichromate digestion solution was used and after digestion in the culture tube, samples were titrated with suitably dilute ferrous ammonium sulfate (0.011 N).

The method suitable to dilute samples was used for attached biofilm samples (Appendix G), all other samples were processed with the regular method of Knechtel.

3.9.5 Protein

Lowry's method (Lowry *et al.*, 1951) was employed to determine protein concentration. For attached biofilm samples the suggested modification for low protein concentrations was used. Appendix H contains a detailed description of the method.

3.9.6 Polysaccharide

Dubois' method (Dubois et al., 1956) was used for all samples. Dextrose was used as the standard.

3.9.7 Suspended Solids

TSS and VSS were measured according to Standard Methods (APHA, 1985).

3.9.8 Acetic Acid

Acetate concentration was measured by the method of Ackman (1972) with a Hewlett Packard gas chromatograph model 5730A with an automatic sampler model 7671A and integrator model 3380A. The Chromosorb 101 glass column was kept at 180°C while the injection port temperature measured 200°C. Helium passing over formic acid at a flow rate of 15 mL/min was used as the carrier gas. Samples for analysis were centrifuged and 0.5 mL of supernatant was mixed with 0.5 mL of internal standard containing 1000 mg/L isobutyric acid.

3.9.9 Microbial Activity

Potential acetoclastic activity (the ability of methanogens to degrade acetate) was measured in the following manner. Approximately 10-15 mL of reactor fluid were transferred anaerobically into serum bottles. A 0.1 mL volume of 200 g/L acetate solution was then added to each bottle. Bottles were placed on a New Brunswick 4556 shaker ro-

tating at 40 rpm. The shaker was in a room maintained at 35°C. Samples were withdrawn for acetic acid determination at the time of substrate addition and at prescribed intervals thereafter. The sampling schedule depended on the anticipated activity. The rate of acetate consumption was computed and related to the biomass (VSS) present, yielding the specific acetoclastic activity. Acetoclastic activity tests and VSS determination were performed in triplicate. Data indicating an initial lag period were excluded from the rate computation.

Actual acetoclastic activity was measured by the rate of acetate consumption during the attachment experiment. An example calculation is shown in Appendix I.

CHAPTER 4

RESULTS

4.1 Experiment #1 - Flow Velocity and Support Material

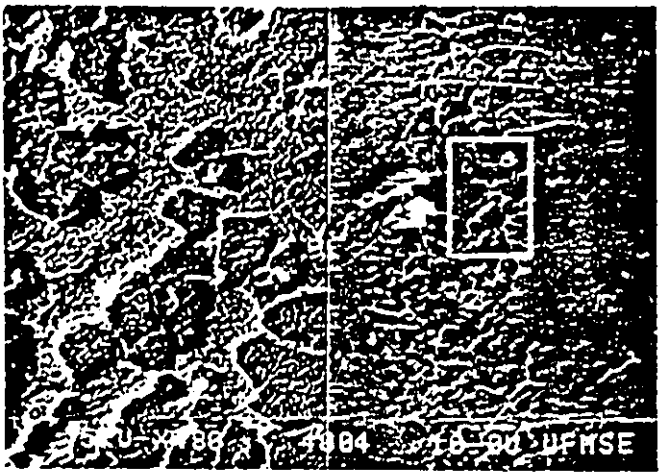
4.1.1 Introduction

In this experiment the effects of support material and liquid flow velocity on biofilm formation and biofilm characteristics were investigated. Table 4.1 shows the materials and velocities employed. The flow velocity range was chosen to approximate the operational conditions of the DSFF reactor. Reynolds numbers corresponding to the flow velocities fall in the laminar range. Reynolds number and velocity calculations for both 0 and 200 μm biofilm thicknesses are shown in Appendix I/b. The data listed below correspond to zero film thickness (clean test section).

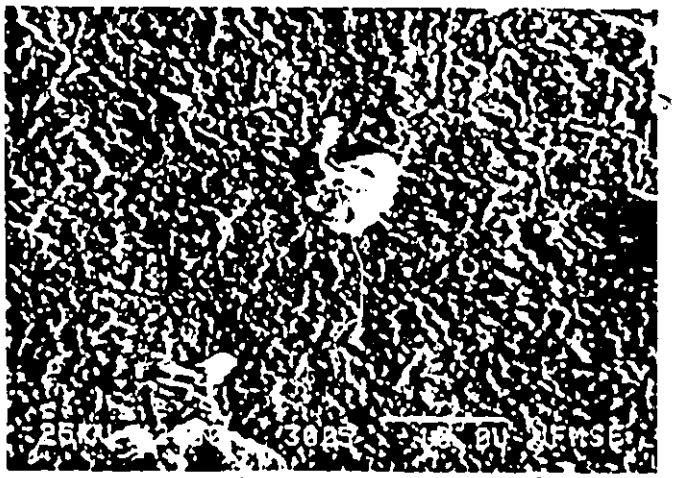
Support material	Tygon tubing	Silicone tubing
Flow velocity cm/s	1.42 0.64 0.054	1.08 0.76 0.056
Reynolds number	247 111 9.4	117 82.7 6.1

Table 4.1 Supports and velocities employed (A)

Scanning electron micrographs of the two plastic tubings provided by the distributor (Cole-Parmer Inc.) are shown in Fig. 4.1.



STANDARD SILICONE RUBBER



PVC (TYGON)

Figure 4.1 Scanning electron micrograph of silicone and Tygon surface

Test sections were inserted in the loop for 1, 2, 3, 4, 6, 12, 18, 24, 48 and 72 hours.

Data and figures related to this experiment and not shown in the text are summarized in Appendix J.

4.1.2 Reactor Characteristics

The average values of the reactor mixed liquor characteristics for the experiment are shown in Table 4.2.

Table 4.2 Characteristics of reactor A

VSS	mg/L	609.5
SCOD		1084
SPR		513
SPS		57.4
SCOD/VSS	mg/mg	1.760
SPR/VSS		0.795
SPS/VSS		0.090
SPR/SCOD	mg/mg	0.481
SPS/SCOD		0.054
SPS/SPR		0.112
pH		7.2
Alkalinity mg/L as $CaCO_3$		4600
Volumetric loading mL/d		43.2
Feed concentration mg COD/L		40140
Organic loading rate mg COD/L·d		564
Specific activity mg COD/mg VSS·d		0.943
Acetate concentration, mg/L		1121
SRT, d		20

Note: 1 mg acetic acid represents 1.066 mg COD

4.1.3 Attached Biofilm Development

Figs. 4.2-4.4 show the development of attached COD, PR and PS versus time.

The notation of F = fast (highest velocity), S = slow (lowest velocity) and M = medium (intermediate velocity) was used on the graphs.

The three biochemical indicators (COD, PR and PS) in AF followed a similar course. They increased linearly for the first 48 hours of the experiment for both materials and all velocities. Curves for silicone tubing started to deviate from linearity after 48 hours.

Six distinctive curves can be observed depending on the velocity and the support material employed. The final concentration and rates of change for silicone support were higher than for Tygon in all cases. The highest value of AFCOD reached at 72 hours was 1500 $\mu g/cm^2$ on silicone and 370 $\mu g/cm^2$ on Tygon. For AFPR, the values were 720 $\mu g/cm^2$ and 200 $\mu g/cm^2$ for silicone and Tygon respectively. For AFPS, maximum values of 125 $\mu g/cm^2$ for silicone and 25 $\mu g/cm^2$ for Tygon were recorded.

Fig. 4.5 shows the change in biofilm thickness with time.

For attached biofilm thickness it was more difficult to distinguish the six slopes of the curves, mainly due to the imprecision of the method during early biofilm development.

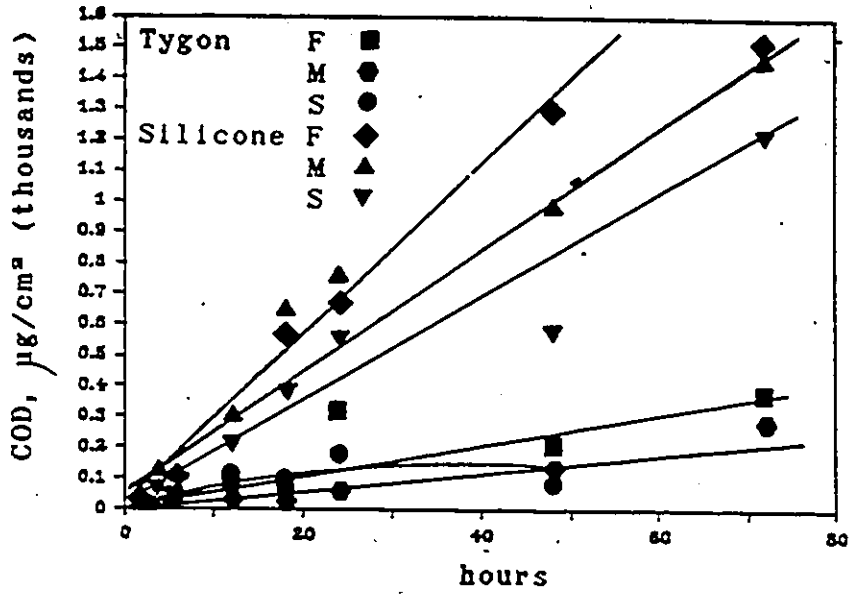


Figure 4.2 Attached COD vs time (A)

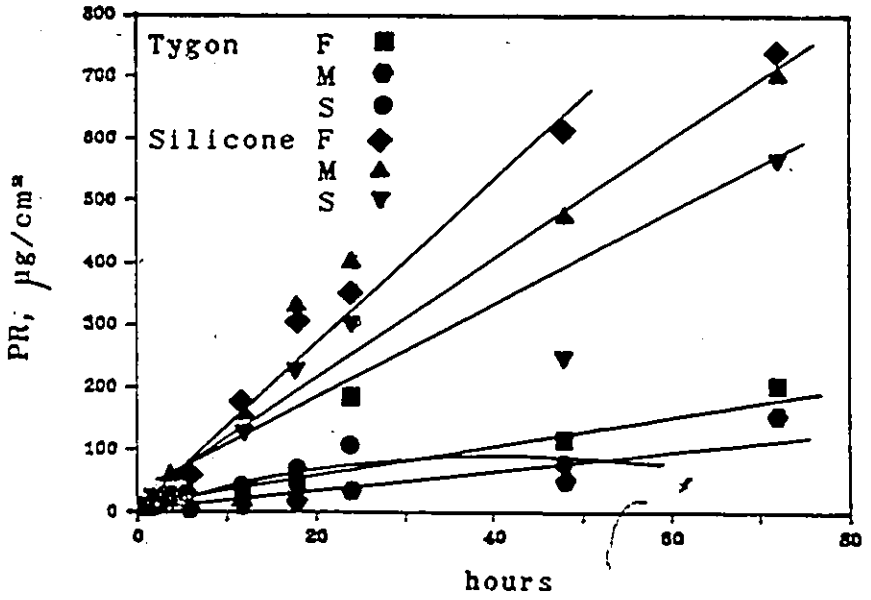


Figure 4.3 Attached PR vs time (A)

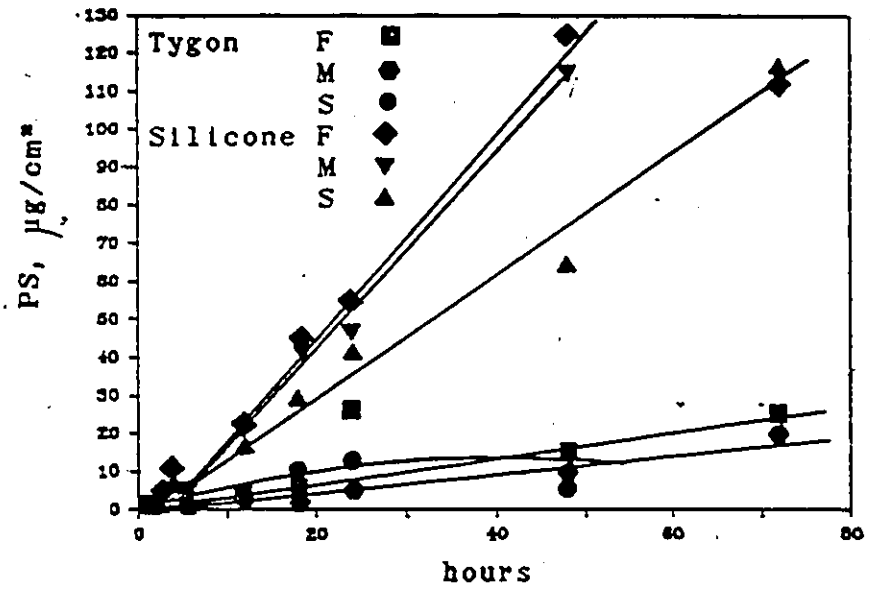


Figure 4.4 Attached PS vs time (A)

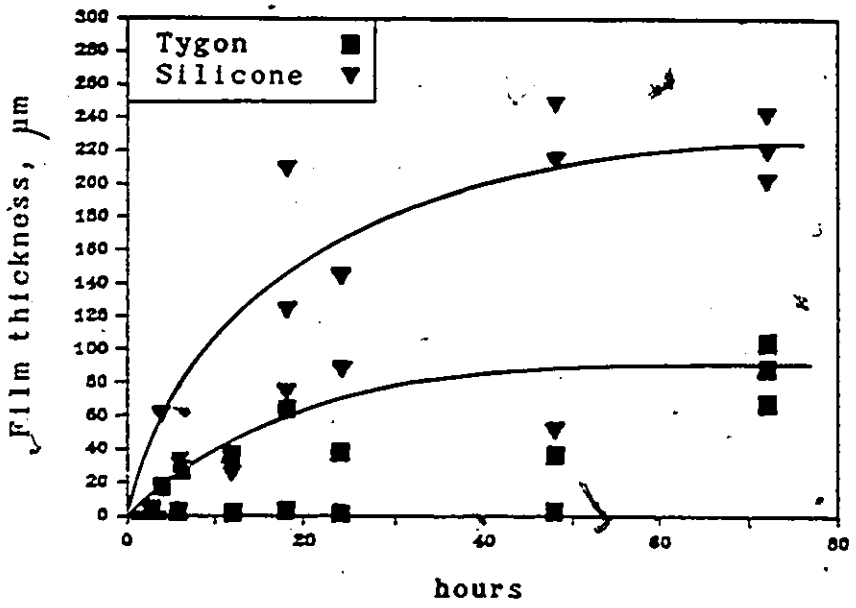


Figure 4.5 Biofilm thickness vs time (A)

Only slight differences in the rate of increase were found for different flow velocities. Basically, the data constituted two curves, one for silicone and one for Tygon, as shown in Fig. 4.5. The greatest thickness reached at 72 hours was 250 µm for silicone and 100 µm on Tygon. The saturation-type curves in Fig. 4.5 significantly deviate from linearity after 24 hours.

The following exponential expression (Eq. 4.1) provided the best fit to the data

$$FT_t = FT_m (1-e^{-kt}) \qquad (4.1)$$

where

FT_t is the film thickness at time t [µm]
FT_m is the maximum film thickness reached [µm]
k is the rate constant [h^{-1}]
t is the time elapsed [h]

The solid curves for Tygon and silicone in Fig.4.5 were obtained using Eq. 4.1. In the case of Tygon tubing, the maximum film thickness predicted by Eq. 4.1 was 92 µm, and the rate constant was 0.05 h^{-1} (correlation coefficient c: 0.956). For silicone tubing 272 µm plateau film thickness and a rate constant of 0.028 h^{-1} were obtained (c: 0.937).

4.1.4 Loose Biofilm Development

Figs. 4.6 and 4.7 show the development of attached, loose and total biofilm (represented by COD) at the highest velocity employed, on Tygon and silicone tubing respectively.

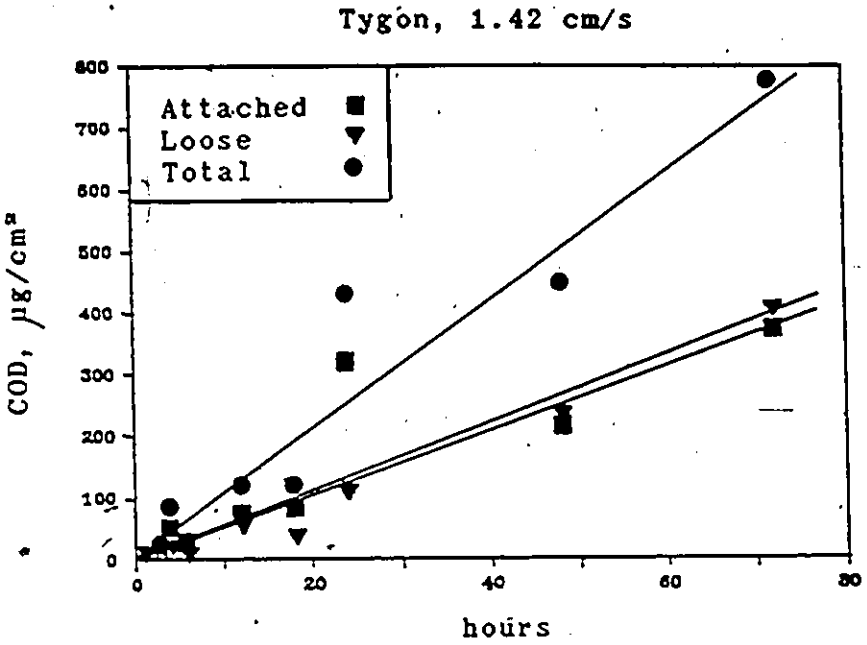


Figure 4.6 AF, LF and TF COD vs time (Tygon tubing, 1.42 cm/s velocity) (A)

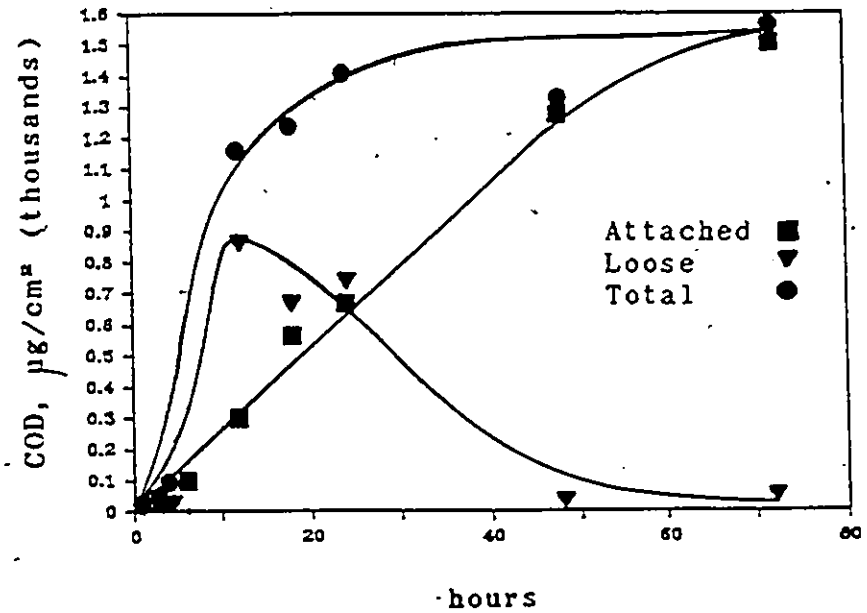


Figure 4.7 AF, LF and TF COD vs time (silicone tubing, 1.08 cm/s velocity) (A)

On Tygon tubing, AFCOD, LFCOD AND TFCOD increased steadily with time, while on silicone loose COD increased very fast at first and decreased to zero subsequently, after which attached COD wholly constituted total COD.

Figs. 4.8 and 4.9 show the loose COD development for the different velocities on Tygon and silicone tubing, respectively.

It is noticeable on Fig. 4.8 that LFCOD formed much faster at a velocity of 0.054 cm/s. After a steady increase for 48 hours, a decrease in LFCOD was observed at this velocity. For the other two velocities, the development was slower and linear until the termination of the experiment.

On silicone tubing (Fig. 4.9), a fast initial increase and gradual decrease in LFCOD is evident for all three velocities. With a lower velocity, however, the period of time for increase in LFCOD became longer. The maximum values of LFCOD reached were higher and the decrease was slower. Loose PR and PS exhibited the same characteristic behavior (Figs. J.1-J.4). Considering the highest values of LF reached at velocities of 0.054-0.056 cm/s, there was very little difference between silicone and Tygon tubing. These values were 1140 $\mu g/cm^2$ and 1115 $\mu g/cm^2$ (LFCOD), 458 $\mu g/cm^2$ and 437 $\mu g/cm^2$ (LFPR) and 64 $\mu g/cm^2$ and 77 $\mu g/cm^2$ (LFPS) for silicone and Tygon, respectively.

The development of total (attached + loose) biofilm measured by all three indicators was linear on Tygon tubing.

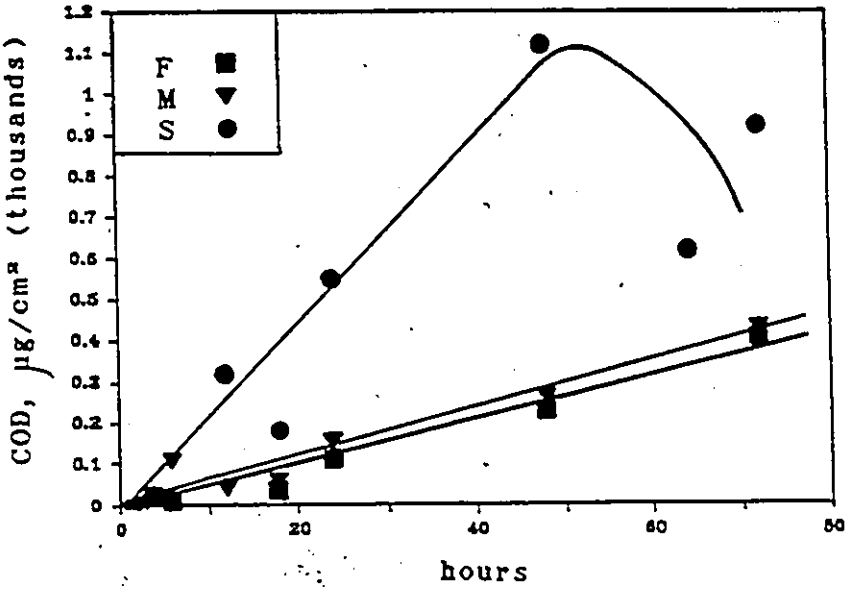


Figure 4.8 Loose COD development, Tygon tubing (A)

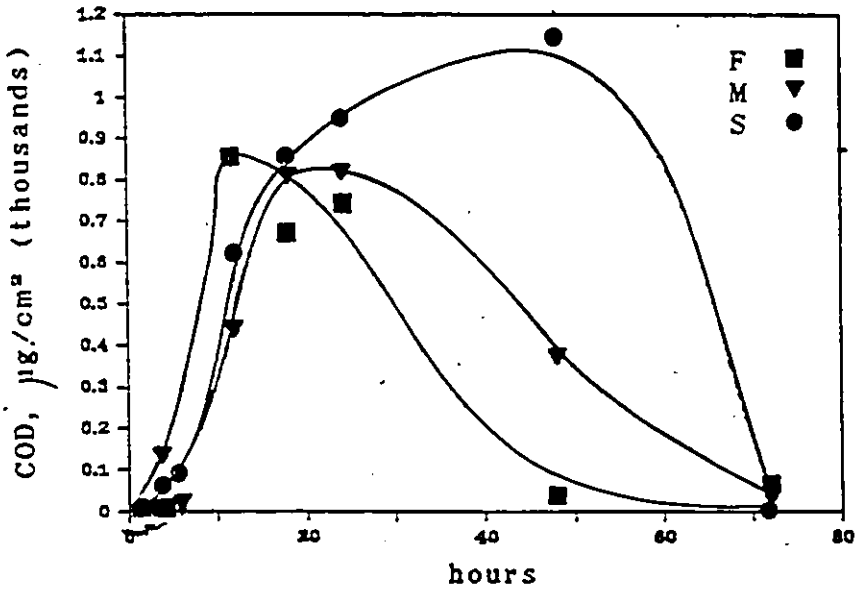


Figure 4.9 Loose COD development, silicone tubing (A)

On silicone surface a phase of very rapid increase was followed by decrease or very slow further accumulation (Figs. J.7-J.9, Table J.2A). TFCOD and TFPR data indicate the onset of the plateau phase at 18-24 hours, while stagnation of TFPS becomes evident at 48 hours. The slowdown of total biofilm accumulation on silicone surface coincided with the disappearance of the loose biofilm (Fig. 4.7).

4.1.5 Percentage of Biofilm Attached

The percentage of the total biofilm indicators found in attached form was averaged for the whole experimental time period. The values are shown in Table 4.3.

The attached percentages of TFCOD and TFPR were higher than that of TFPS, except in the case of the lowest velocity

Table 4.3 Percentage of biofilm attached (A)

COD	Tygon	Silicone
F	67.3	79.7
M	58.2	67.7
S	55.5	58.5
Protein		
F	63.9	73.4
M	43.0	72.1
S	36.0	40.6
Polysaccharide		
F	32.0	67.2
M	22.8	49.3
S	40.6	47.7

for both tubings. For the lowest velocity, the attached percentage of TFPR was lower than that of TFPS.

With higher liquid velocity, AF constituted a higher percentage of the total biofilm. The only one exception is that the highest percentage of TFPS attached (40.6%) for Tygon tubing occurred at the lowest flow velocity.

For the corresponding velocities, the attached percentage of TFCOD, TFPR and TFPS was always higher on silicone tubing than on Tygon. This difference was very pronounced for PS values. Only 23-41 percent of the total PS was attached on Tygon tubing, whereas 48-67 percent was attached on silicone tubing.

4.1.6 Biofilm Development Rate

Biofilm development rates for the different velocities and supports are summarized in Table 4.4. More detailed calculations are shown in Table J.7.

On Tygon tubing the AF development rate ranged from 0.2-1.3 μm/h, 3.1-4.9 μg COD/cm²·h, 1.7-2.7 μg PR/cm²·h and 0.2-0.35 μg PS/cm²·h in terms of FT, AFCOD, AFPR and AFPS, respectively. On silicone tubing the corresponding values were 4.7-5.4 μm/h, 16-23 μg COD/cm²·h, 7.3-11.1 μg PR/cm²·h and 1.5-1.9 μg PS/cm²·h, respectively.

AF development rates are plotted against flow velocity in Figs. 4.10-4.13.

Figs. 4.10-4.13 show that over the liquid velocity range studied the effect of flow velocity on AF development

Table 4.4 Biofilm development rates (A)

Flow velocity cm/s	Attached biofilm µg/cm²·h		Loose biofilm µg/cm²·h	
	COD			
	Tygon	Silicone	Tygon	Silicone
0.054	3.13		14.7	
0.056		15.95		47.48
0.644	3.34		5.81	
0.760		20.80		41.02
1.078		22.93		40.45
1.420	4.87		5.53	
	Protein			
	Tygon	Silicone	Tygon	Silicone
0.054	1.68		8.64	
0.056		7.30		28.53
0.644	1.90		2.55	
0.760		10.06		25.64
1.078		11.15		18.19
1.420	2.67		2.31	
	Polysaccharide			
	Tygon	Silicone	Tygon	Silicone
0.054	0.19		1.63	
0.056		1.54		3.18
0.644	0.24		0.49	
0.760		1.92		3.97
1.078		1.82		2.41
1.420	0.33		0.38	
	Film thickness µm/h			
	Tygon	Silicone		
0.054	0.886			
0.056		4.719		
0.644	1.314			
0.760		5.121		
1.078		5.361		
1.420	0.883			

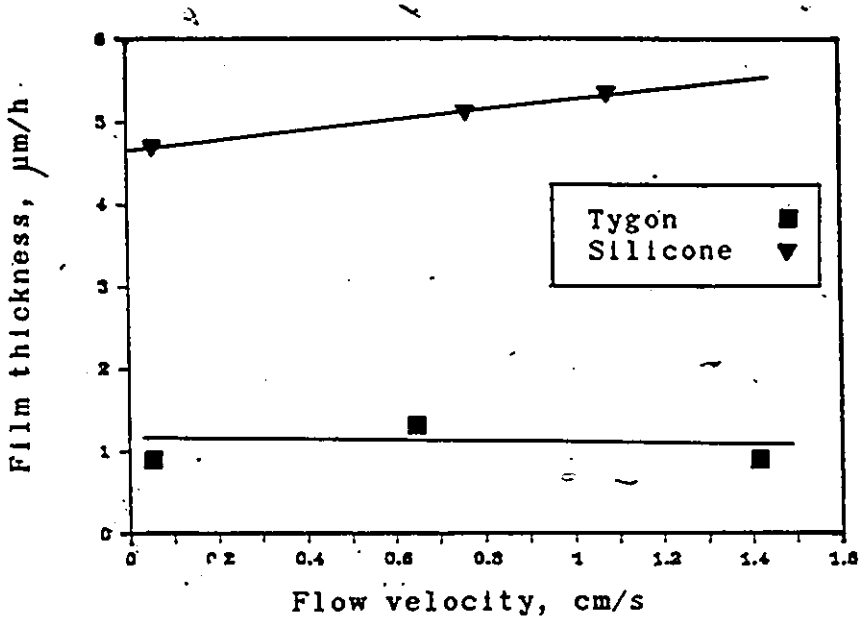


Figure 4.10 Rate of biofilm thickness increase vs flow velocity (A)

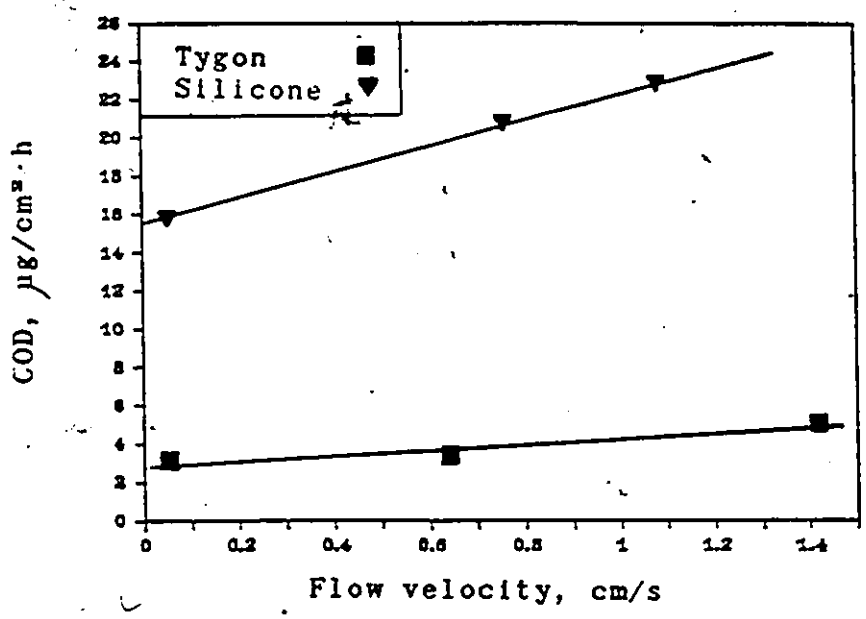


Figure 4.11 Rate of attached COD increase vs flow velocity (A)

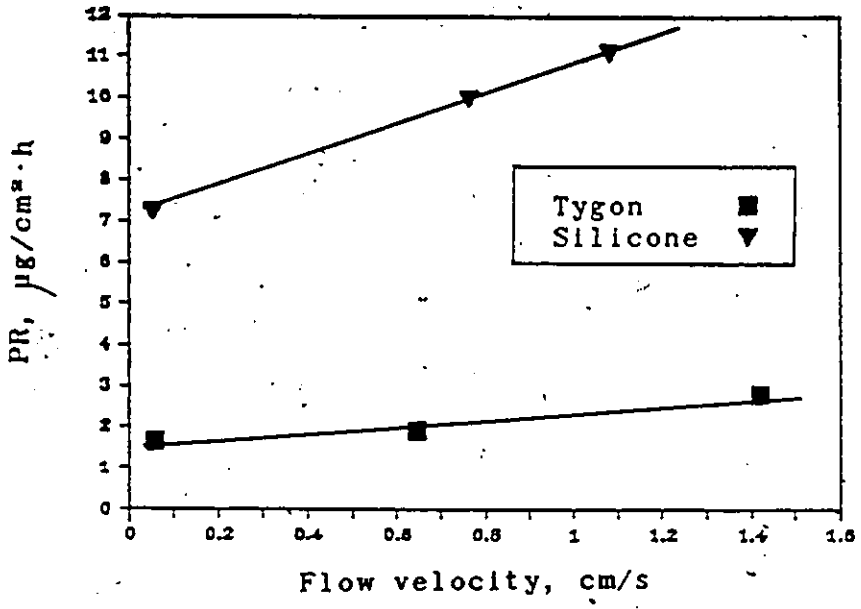


Figure 4.12 Rate of attached PR increase vs flow velocity (A)

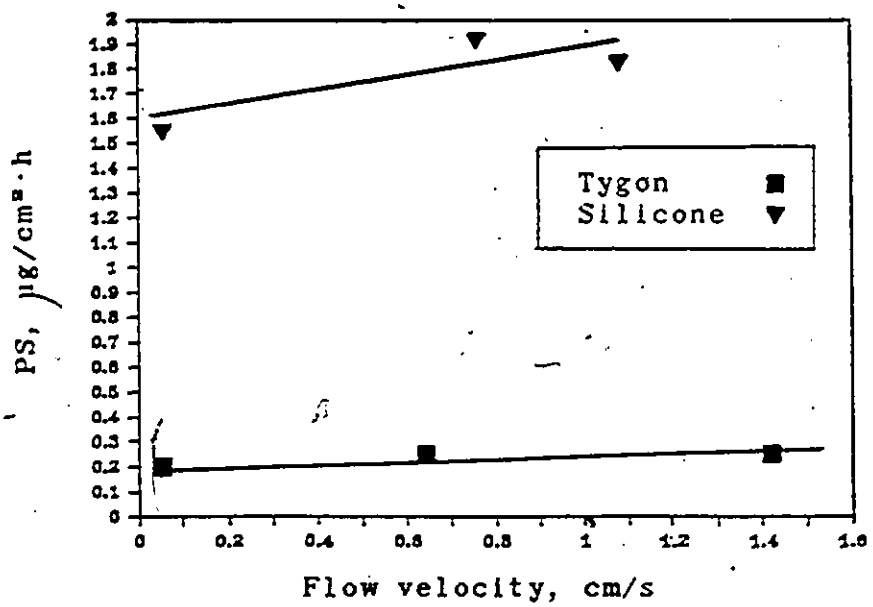


Figure 4.13 Rate of attached PS increase vs flow velocity (A)

rates was more pronounced on silicone tubing than on Tygon. On Tygon tubing, there was very little increase in the rates of AF development. On silicone tubing, AF development rate was higher for higher flow velocity.

The same figures clearly show the difference in rates of AF development for Tygon and silicone tubing respectively. In the studied range AF development rate for all the measured indicators averaged 5-6 times higher on silicone tubing than on Tygon tubing (Table J.9).

Correlation between flow velocity and initial AF development rate was computed. Results are shown in Table J.8.

Fig. 4.14 shows LF development rate (LFCOD) as a function of flow velocity. For silicone tubing only data points constituting the initial linear part of the curve were included in rate calculations. Note that after reaching a maximum value, LF eventually disappeared from silicone test sections. LF development rate was found to decrease with higher velocities. A negative linear correlation for LFCOD and LFPR was found for silicone tubing while for LFPS the pattern was not clear. The relationship between LF development rate and increased flow velocity was nonlinear for Tygon tubing. The initial rate of LF formation was 4-6 times higher on silicone tubing surface.

Figs. 4.15 and 4.16 depict both AF and LF development rates for Tygon and silicone, respectively. Fig. 4.15 illustrates that on Tygon the LF development rate was much

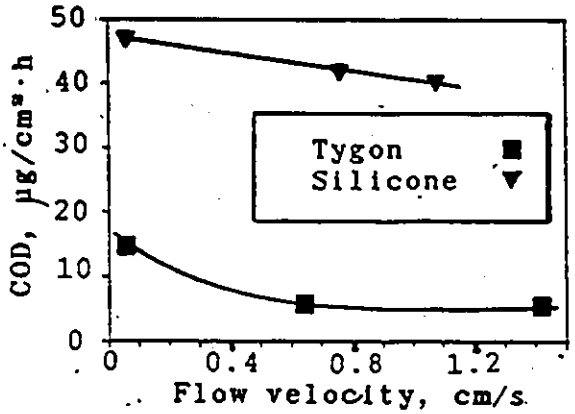


Figure 4.14 Rate of loose COD increase vs flow velocity (A)

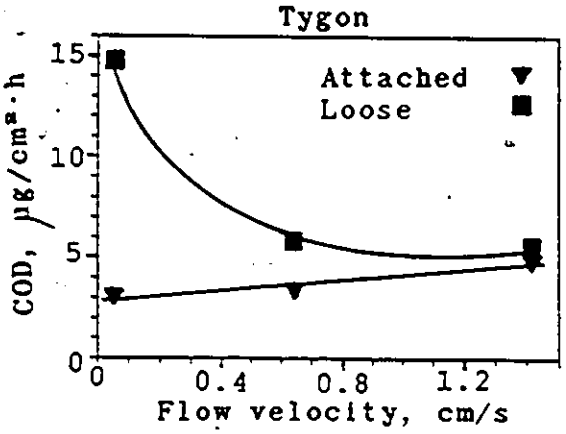


Figure 4.15 Rate of attached and loose COD increase vs flow velocity, Tygon tubing (A)

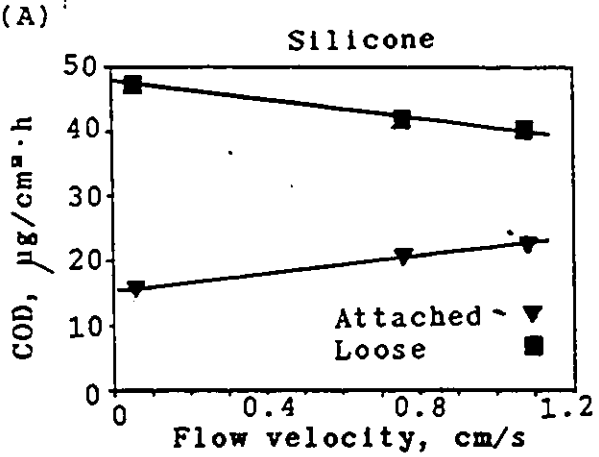


Figure 4.16 Rate of attached and loose COD increase vs flow velocity, silicone tubing (A)

higher than the AF development rate at a low liquid velocity, but they were almost identical for high liquid velocities. On silicone tubing (Fig. 4.16) the initial rate of LF formation was much higher than rate of AF formation within the velocity range studied. As with Tygon tubing, rates of AF and LF developments on silicone began to converge as the liquid velocity increased.

4.1.7 Biofilm Concentration

Fig. 4.17 shows the change in biofilm COD concentration with time.

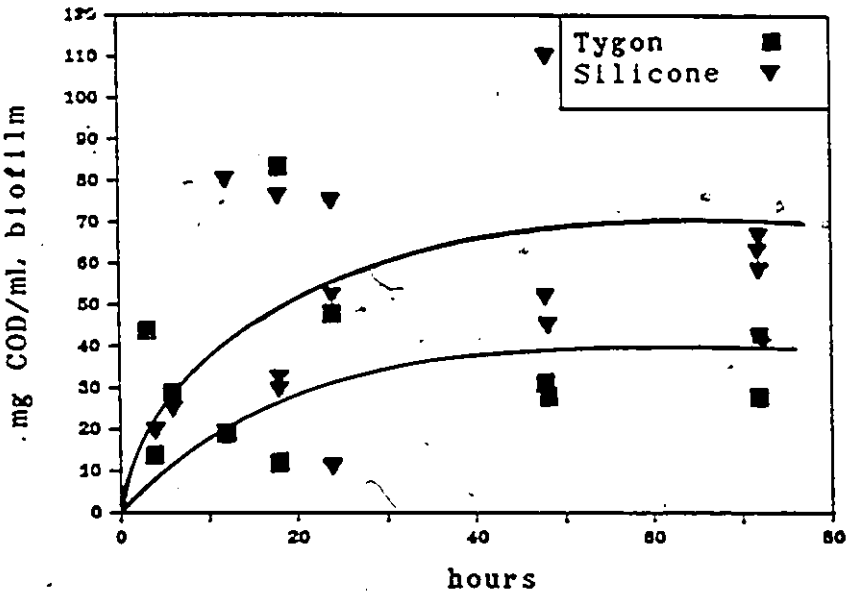


Figure 4.17 Biofilm COD concentration vs time (A)

Although COD concentration data are too scattered to draw individual lines, general trends for silicone and Tygon

can be seen. For both support materials, COD concentration in the biofilm increased initially, then reached a plateau. The rate of increase was faster and the plateau value reached was substantially higher on silicone tubing. PR and PS concentration curves were very similar to the COD graph (Figs. J.5 and J.6).

Averages of the concentration values obtained during this 72-hour experiment are given in Table 4.5.

COD and PR concentrations of biofilm on a silicone surface were 1.8 times higher than on Tygon tubing, and PS concentration was 2.4 times higher.

Table 4.5 Biofilm concentrations (A)

		mg COD/mL biofilm	mg PR/mL biofilm	mg PS/mL biofilm
Tygon	F	30.81	15.67	1.91
	M	27.79	15.53	2.08
	S	30.42	15.46	1.69
	ave.	29.67	15.55	1.89
Silicone	F	66.34	34.04	5.07
	M	42.91	21.52	3.69
	S	53.38	27.41	4.99
	ave.	54.21	27.65	4.59
Silicone/Tygon		1.83	1.78	2.43

4.1.8 Ratios in Biofilm and in Reactor

Examining the ratios of indicators in the biofilm and in the reactor mixed liquor with time, no general trends were found. Initial scatter was greater and data were more homogeneous in later stages. The effect of different velocities was not apparent in the case of silicone tubing. In the case of Tygon tubing, higher flow velocities resulted in lower PR/COD but higher PS/COD and PS/PR ratios in the AF. Ratios for LF showed a similar dependence on flow velocity. PS/COD and PS/PR ratios were somewhat lower for AF than for LF with both types of tubing. For Tygon tubing the average PR/COD ratio for LF was lower than for AF, while for silicone tubing it was reversed. The average ratios for the two kinds of tubing were similar to each other with the exception of LF PR/COD ratio.

AF on either tubing had about the same PR/COD ratio as the suspended solids in the reactor. LF PR/COD ratio was approximately equal to the ratio found in the reactor for silicone tubing. For Tygon, PR/COD ratio in LF remained below the reactor value. Both AF and LF contained more PS per unit of COD or PR than did the reactor biomass.

The values for the two kinds of tubing are summarized in Table 4.6. Average values normalized to the reactor data are shown in Table 4.7. The data are graphed in Figs. 4.18 and 4.19.

Table 4.6 Ratios of indicators in biofilm (A)

	Attached			Loose		
Tygon	PR/COD mg/mg	PS/COD mg/mg	PS/PR mg/mg	PR/COD mg/mg	PS/COD mg/mg	PS/PR mg/mg
F	0.407	0.087	0.204	0.290	0.117	0.301
M	0.496	0.072	0.166	0.398	0.129	0.249
S	0.509	0.063	0.123	0.449	0.077	0.195
ave.	0.471	0.074	0.164	0.380	0.108	0.248
Silicone	PR/COD mg/mg	PS/COD mg/mg	PS/PR mg/mg	PR/COD mg/mg	PS/COD mg/mg	PS/PR mg/mg
F	0.450	0.075	0.145	0.575	0.102	0.148
M	0.482	0.065	0.130	0.466	0.114	0.212
S	0.442	0.094	0.194	0.494	0.093	0.149
ave.	0.458	0.078	0.156	0.511	0.103	0.170

Table 4.7 Comparison of biofilm and reactor ratios (A)

	Attached		Loose	
	Reactor suspended		Reactor suspended	
	Tygon	Silicone	Tygon	Silicone
PR/COD	0.979	0.952	0.790	1.062
PS/COD	1.370	1.472	2.030	1.907
PS/PR	1.473	1.393	2.214	1.518

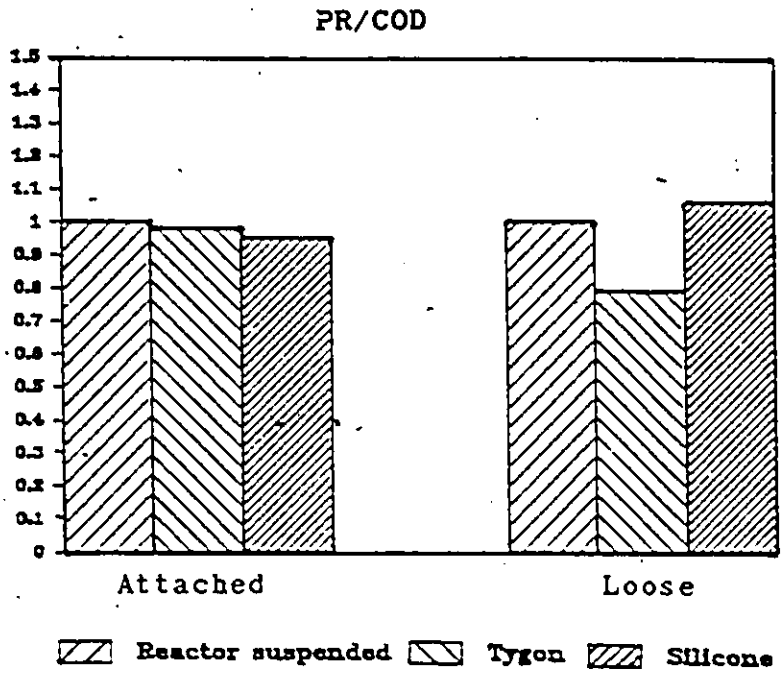


Figure 4.18 PR/COD in attached and loose biofilm, normalized to the reactor suspended value (A)

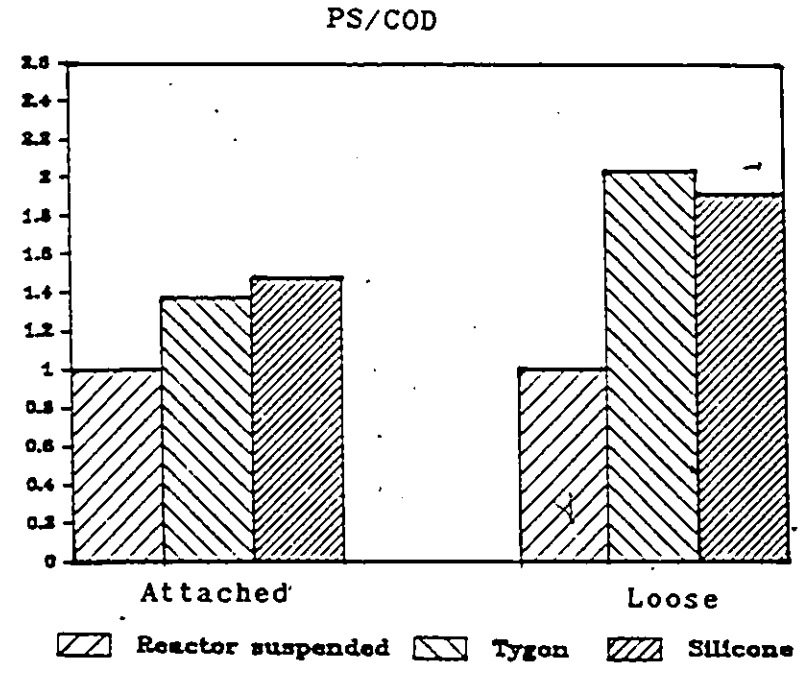


Figure 4.19 PS/COD in attached and loose biofilm, normalized to the reactor suspended value (A)

4.2 Experiment #2 - Suspended Biomass Concentration

4.2.1 Introduction

In this experiment the effect of suspended biomass concentration on biofilm formation and biofilm characteristics was investigated. Table 4.8 shows the reactor suspended biomass concentrations employed.

Table 4.8 Suspended biomass concentrations

		B	C	D
VSS	mg/L	510	522	848
SCOD	mg/L	685	747	1176
SPR	mg/L	348	375	578
SPS	mg/L	36.7	42.2	76.7

Samples were taken in duplicate at 2, 4, 6, 8 and 11 hours (B) or 3, 5, 7, 9 ard 11 hours (C and D).

Since the reactor suspended biomass decreased slightly during the experiment, the values in Table 4.8 are weighted averages of measurements taken during the experiments.

The results were evaluated in a fashion similar to the first experiment. After film development rates were obtained, the correlation between film development rate and the corresponding reactor suspended biomass indicator was determined (film thickness - VSS; AFCOD, LFCOD - SCOD; AFPR, LFPR - SPR; AFPS, LFPS - SPS).

Biofilm characteristics were also compared.

Detailed data and figures related to this experiment can be found in Appendix K.

4.2.2 Reactor Characteristics

Reactor characteristics other than suspended biomass concentration are listed in Table 4.9. Ratios of biomass indicators in the reactors are also summarized in Table 4.9. Although the numbers differ slightly, they were judged sufficiently close so that no major effect on biofilm characteristics was assumed.

Table 4.9 Characteristics of reactors B, C and D

	B&C	D
SCOD/VSS	1.383	1.403
SPR/VSS	0.737	0.720
SPS/VSS	0.078	0.091
SPR/SCOD	0.534	0.513
SPS/SCOD	0.056	0.065
SPS/SPR	0.105	0.126
Support surface	silicone	silicone
pH	7.65	7.50
Alkalinity mg/L as $CaCO_3$	4450	4500
Potential specific activity mg COD/mg VSS·d	0.89	0.79
Actual specific activity mg COD/mg VSS·d	0	0
Acetate concentration, mg/L	0	0
SRT, d	7.7	9.7
Flow velocity, cm/s	1.044	1.044
Reynolds number	114	114

Ratios SPR/SCOD and SPR/VSS were slightly higher in reactors B&C, whereas ratios SPS/SCOD, SPS/SPR and SPR/VSS were all slightly higher in reactor D.

No feed was supplied during the experiment, concomitantly actual activity of the biomass was zero.

4.2.3 Attached and Loose Biofilm Development Rate

Fig. 4.20 shows the steady increase of AFCOD with time. The other indicators followed a similar course (Figs. K.1-K.5).

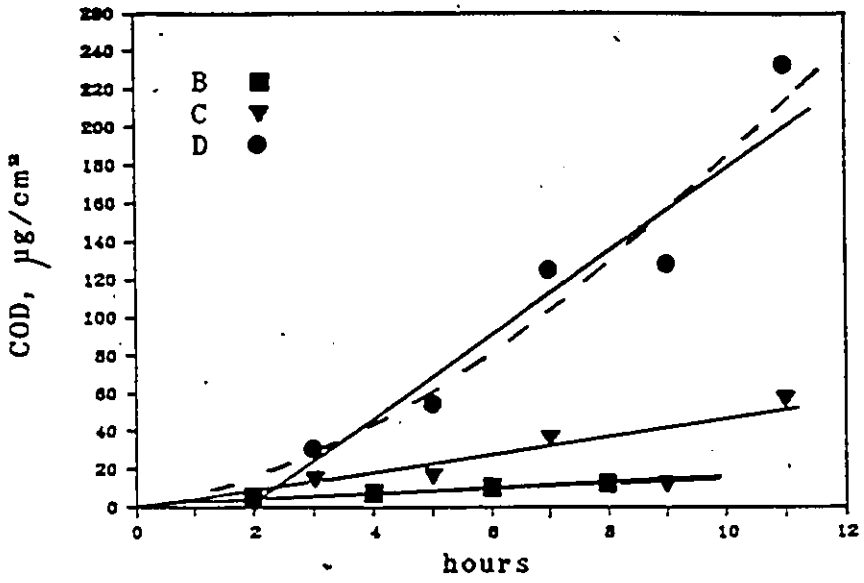


Figure 4.20 Attached COD vs time (B,C,D)

AF and LF development rates are summarized in Table 4.10. Both AF and LF development rates increased with increasing reactor suspended biomass concentration.

Biofilm formation rates obtained with the highest biomass concentration (reactor D) were similar to those obtained in the first experiment with 1.076 cm/s flow velocity (Table 4.4). Biomass concentration was slightly lower in reactor A (Experiment #1), but feed was supplied and the biomass was active in that experiment (Table 4.2). The sim-

ilarity of results indicates reproducibility of the experiment.

Table 4.10 Biofilm development rates (B,C,D)

	Reactor suspended biomass mg/L	Attached biofilm µg/cm²·h	Loose biofilm µg/cm²·h
	SCOD	COD	
B	685	1.26	4.87
C	747	5.53	12.12
D	1176	23.90	46.79
	SPR	Protein	
B	348	1.515	2.624
C	395	3.166	8.331
D	578	12.085	23.470
	SPS	Polysaccharide	
B	36.7	0.712	0.830
C	42.2	0.790	1.135
D	76.7	1.890	4.043
	VSS	Film thickness, µm/h	
B	510	4.73	
C	522	2.96	
D	848	12.18	

It was hypothesized that the rate of PS attachment was related to the soluble, rather than the suspended PS concentration in the reactor. The assumption was warranted by the fact that a significant portion, 12-30% of the total reactor polysaccharide was in soluble form. In comparison, soluble PR accounted for only 1.5-3.5% of the total reactor PR. However, the relationship between AFPS and LFPS development rates, and soluble PS concentration was found to be nega-

tive, as shown in Fig. 4.21. It was concluded that increased soluble PS concentration does not enhance the formation of AFPS and LFPS.

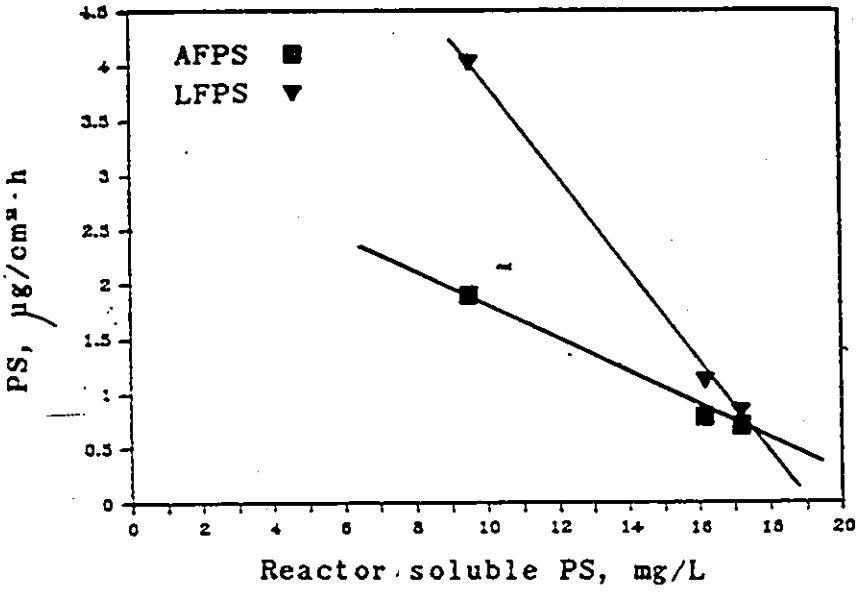


Figure 4.21 Rate of AFPS and LFPS increase vs reactor soluble PS (B,C,D)

Table 4.11 shows the correlation between biofilm development rates and suspended biomass concentration. The correlation was lower in the case of film thickness than for the other biomass indicators.

Comparison of the corresponding slopes for LF and AF biomass parameters gives an indication of the sensitivity of each type of film development to suspended biomass concentration. Table 4.11 shows that the rate of LF development was twice as sensitive to changes in biomass concentration compared to the rate of AF development. The ratio of corresponding slopes for LF and AF development were 1.86 (COD), 1.89 (PR) and 2.70 (PS).

Table 4.11 Correlation between film development rate and suspended biomass concentration (B,C,D)

	Slope	y-intercept	Correlation coefficient	
FT-VSS	0.0248	-8.92	0.953	(Fig.4.22)
AFCOD-SCOD	0.0449	-28.79	0.998	(Fig.4.23)
AFPR-SPR	0.0467	-14.99	0.999	(Fig.4.24)
AFPS-SPS	0.0303	-0.44	0.998	(Fig.4.25)
LFCOD-SCOD	0.0837	-51.50	0.998	(Fig.4.23)
LFPR-SPR	0.0884	-27.45	0.995	(Fig.4.24)
LFPS-SPS	0.0817	-2.24	0.998	(Fig.4.25)
Ratio of slopes				
LFCOD/AFCOD	1.866			
LFPR/AFPR	1.894			
LFPS/AFPS	2.695			

The relationships between AF and LF development rates and reactor biomass are illustrated in Figs. 4.22-4.25.

It is seen that the biofilm development rates increased linearly with increasing reactor suspended biomass concentration. This relationship was seen for all biomass indicators describing both AF and LF development. The rate of increase was less in the case of AFPS than for AFCOD and AFPR.

Plotting LF development rates vs AF development rates for the different biomass indicators shows the relationship between the development of both types of biofilm when ex

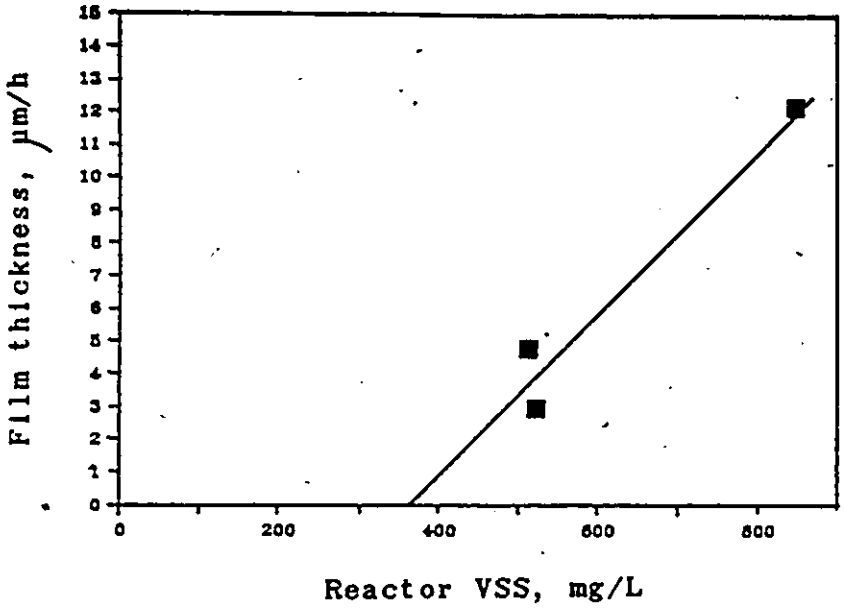


Figure 4.22 Rate of biofilm thickness increase vs reactor VSS (B,C,D)

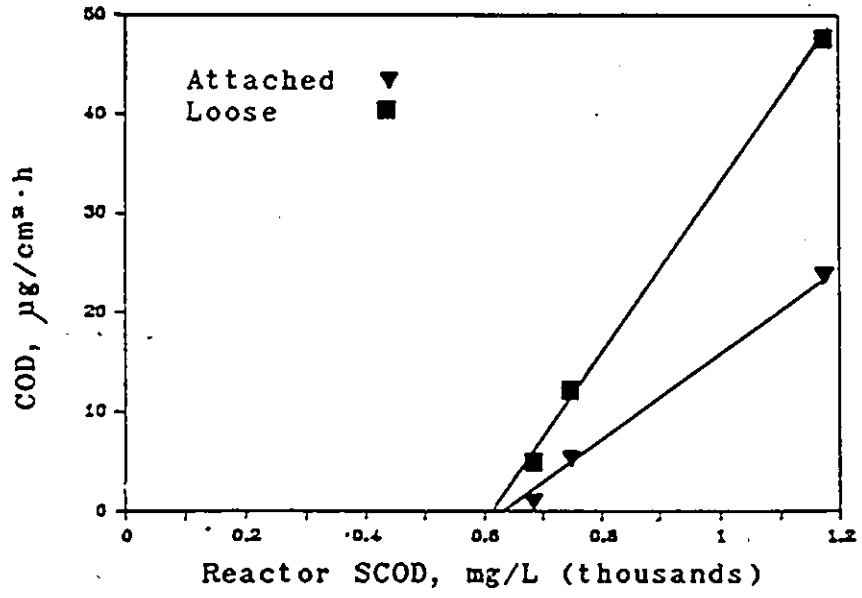


Figure 4.23 Rate of AFCOD and LFCOD increase vs reactor SCOD (B,C,D)

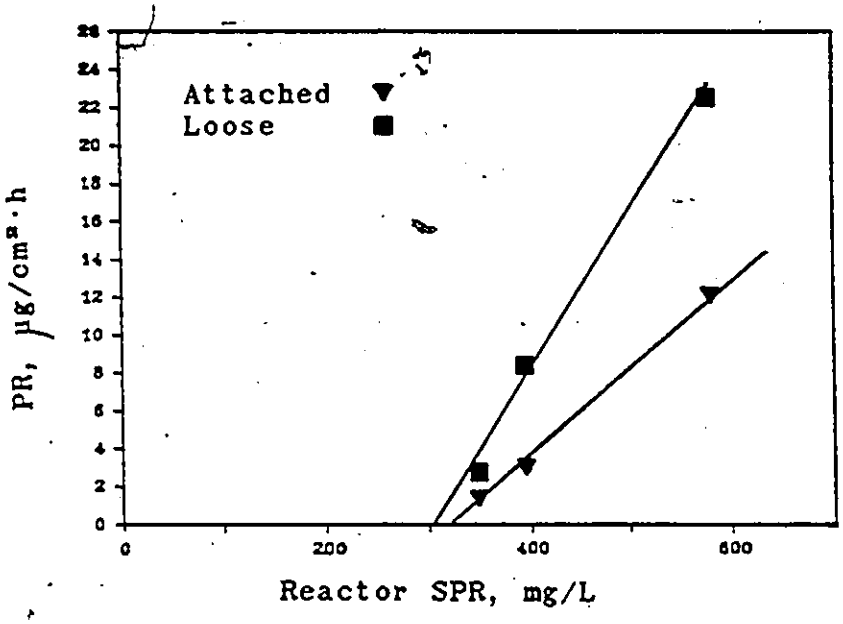


Figure 4.24 Rate of AFPR and LFPR increase vs reactor SPR (B,C,D)

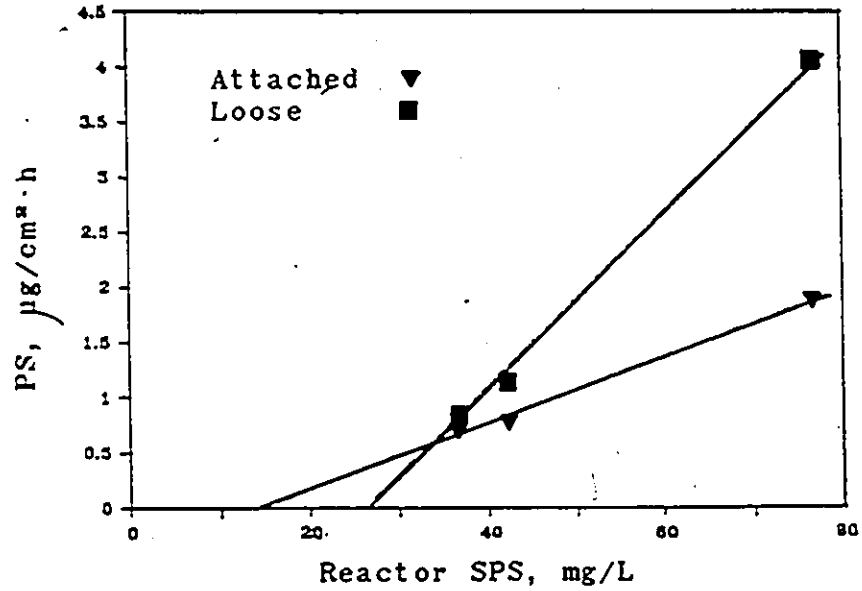


Figure 4.25 Rate of AFPS and LFPS increase vs reactor SPS (B,C,D)

posed to different reactor biomass concentration. Figs. 4.26-4.28 show that when AF development rates are plotted against LF development rates, straight lines result. This indicates a direct relation between the development of both biofilm types. The negative y-intercepts obtained for COD and PR in Figs. 4.26 and 4.27 show that zero or very small LFCOD and LFPR development rates coincided with zero AFCOD and AFPR development rates. This indicates a parallel development of LF and AF, as far as COD and PR are concerned. On the other hand, when LFPS development rate was zero, there was still a 0.37 µg PS/cm²·h AFPS development rate, according to Fig. 4.28. This suggests that AFPS development may take place while there is no LFPS development.

The linear regression lines relating biofilm formation rate to suspended biomass concentration do not go through zero (Table 4.11, Figs. 4.22-4.25), indicating that for biomass concentrations less than a certain limit there would be no or very slow film formation. From the equation of the regression lines these concentrations were computed in terms of the appropriate indicator. The average COD/VSS, PR/VSS and PS/VSS ratios for the reactors were determined and the limiting concentrations were then expressed as VSS. These data are shown in Table 4.12.

The scatter of raw data permits the assumption that the increase of biofilm indicators was exponential ($y=a\cdot e^{bt}$) rather than linear (see Fig. 4.20). Values of b are indicative of the speed of film development. If values of b are

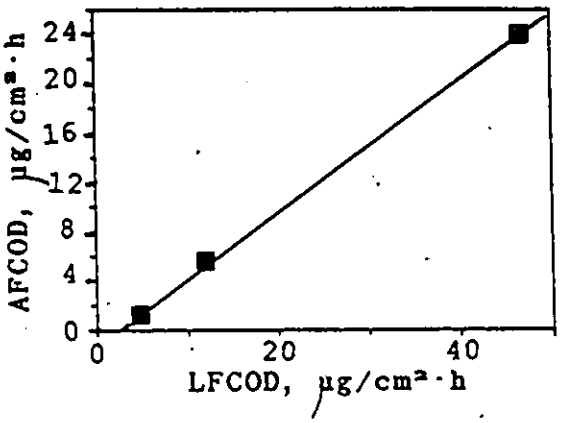


Figure 4.26 AFCOD vs LFCOD (B,C,D)

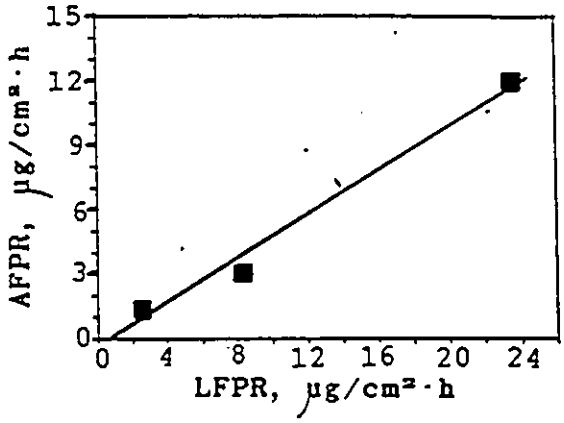


Figure 4.27 AFPR vs LFPR (B,C,D)

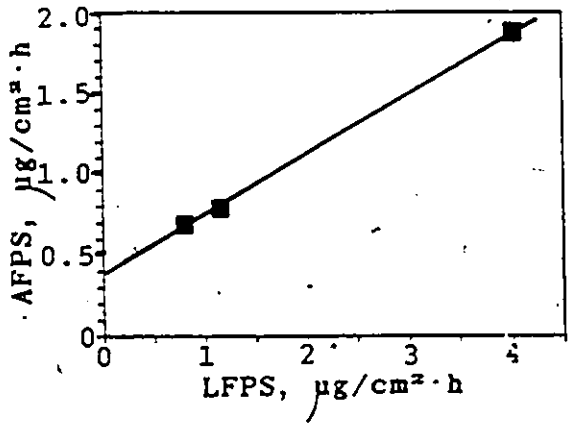


Figure 4.28 AFPS vs LFPS (B,C,D)

plotted against the appropriate suspended biomass indicator, the limiting biomass concentration below which b would be zero can be approximated from the graph. One such graph is shown in Fig. 4.29. Limiting values obtained in this manner and converted into VSS are also shown in Table 4.12.

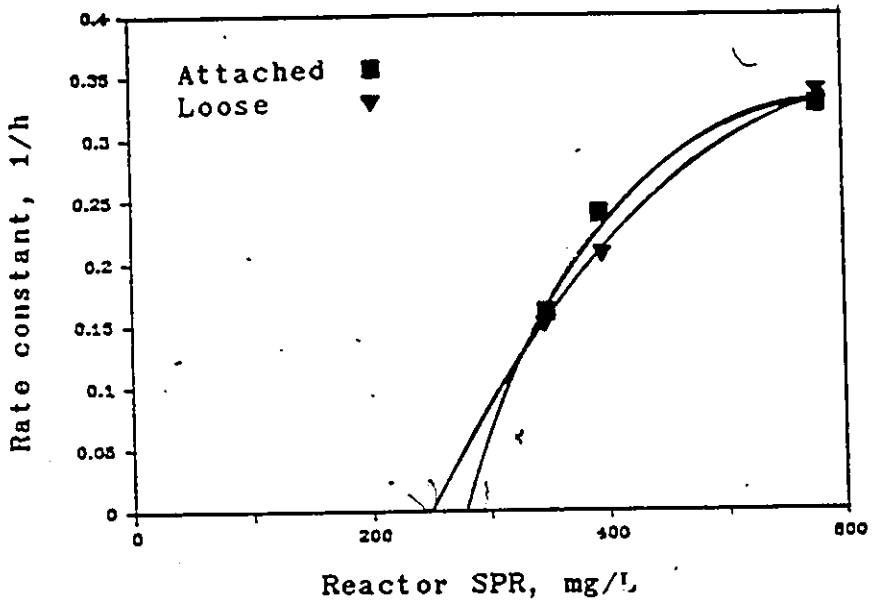


Figure 4.29 b values for AFPR and LFPR vs SPR (film development is assumed to be exponential) (B,C,D)

The limiting biomass concentration values calculated from PS were very similar for both linear and exponential treatment of the data. Values computed based on COD and PR were 50-60 mg/L VSS lower when exponential development of biofilm is assumed. The results indicate the existence of a suspended biomass concentration below which the rate of biofilm development would be very slow.

It is seen from Table 4.12 that the presence of the least suspended biomass (140 mg/L VSS) would support AFPS accumulation. LFPS will start to develop when reactor VSS exceeds 320 mg/L. The rate of increase in AFPR and LFPR is predicted to be zero if the reactor biomass concentration is less than about 370-380 mg/L VSS. AFCOD and LFCOD will increase with a measurable rate at about 390-410 mg/L reactor VSS concentration.

Table 4.12 Biomass concentrations limiting biofilm formation, mg VSS/L (B,C,D)

Calculated from	Linear	Exponential
Film thickness	364	-
AFCOD	460	410
AFPR	440	380
AFPS	172	140
LFCOD	442	390
LFPR	421	364
LFPS	316	320

Note: The values used in conversion were

$$\frac{SCOD}{VSS} = 1.393 \qquad \frac{SPR}{VSS} = 0.7285 \qquad \frac{SPS}{VSS} = 0.0845$$

It must be kept in mind, that the above findings are valid only for these experimental conditions: absence of substrate, 1.044 cm/s flow velocity and enriched culture.

4.2.4 Biofilm Characteristics

4.2.4.1 Ratios in Biofilm and in Reactor

Attached PR/COD increased in time until a plateau was reached at a PR/COD ratio of 0.5 mg/mg (Fig. 4.30). Attached PS/COD and PS/PR showed a tendency to decrease in time, although the data are rather scattered for Runs B and C (Table K.6, Figs. K.8 and K.9). Other ratios did not show definite change with time.

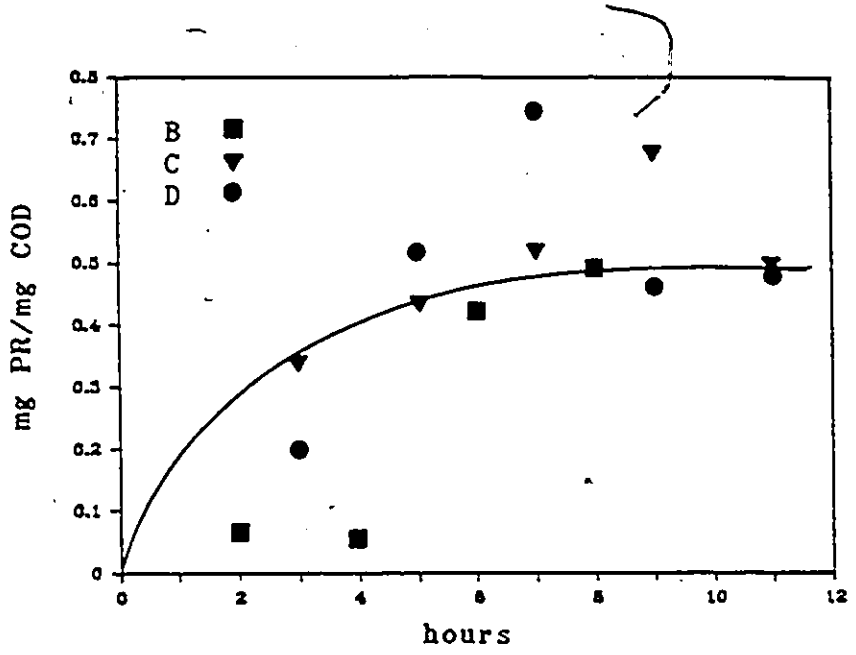


Figure 4.30 AF PR/COD ratio vs time (B,C,D)

Average values of the ratios of indicators in the biofilm are contained in Table 4.13.

Attached biofilm PS/COD and PS/PR decreased as suspended biomass concentration increased. Loose PR/COD in-

creased with higher VSS. The other ratios did not show characteristic change with biomass concentration.

Table 4.13 Ratios of indicators in biofilm (B,C,D)

	B	C	D
Attached			
PR/COD	0.305	0.494	0.479
PS/COD	0.366	0.248	0.117
PS/PR	0.537	0.493	0.240
Loose			
PR/COD	0.366	0.467	0.572
PS/COD	0.164	0.081	0.111
PS/PR	0.198	0.167	0.196

Ratios normalized to the corresponding reactor values are summarized in Table 4.14.

A low value of AF PR/COD was obtained with reactor B (Table 4.14). In all three experiments there was less PR/COD in AF than in the respective reactors. This was also true for LF except that in Run D the value was slightly higher than one. LF PR/COD ratios increased with biomass concentration. Normalized AF and LF PR/COD ratios in biofilms and in the reactor are graphed in Fig. 4.31.

In all three runs PS/COD and PS/PR ratios were higher in AF and LF than in the reactor. For AF, the lower the biomass concentration, the greater the PS/COD and PS/PR ratios in the film exceeded those ratios for the reactor. Normalized AF and LF PS/COD ratios in the biofilms and in the reactor are graphed in Fig. 4.32.

Table 4.14 Comparison of biofilm and reactor ratios (B,C,D)

	B	C	D
Attached/Reactor suspended			
PR/COD	0.572	0.926	0.933
PS/COD	6.497	4.403	1.803
PS/PR	5.114	4.675	1.902
Loose/Reactor suspended			
PR/COD	0.686	0.875	1.114
PS/COD	2.911	1.438	1.718
PS/PR	1.877	1.583	1.557

4.2.4.2 Percentage of Biofilm Attached

With respect to biofilm concentration and attached percentage there was no major difference between Runs B and C, therefore they were treated together. Table 4.15 shows the attached percentages of TFCOD, TFPR and TFPS.

It is seen that a higher percentage of TFPS than of TFCOD or TFPR was found to be attached, in all cases. AFCOD and AFPR percentages were less in Runs B&C compared with Run D (highest biomass concentration), while the percentage of AFPS was smaller for Experiment D.

Table 4.15 Percentage of biofilm attached (B,C,D)

	B&C	D
AFCOD	29.1	46.2
AFPR	28.9	36.4
AFPS	58.1	50.9

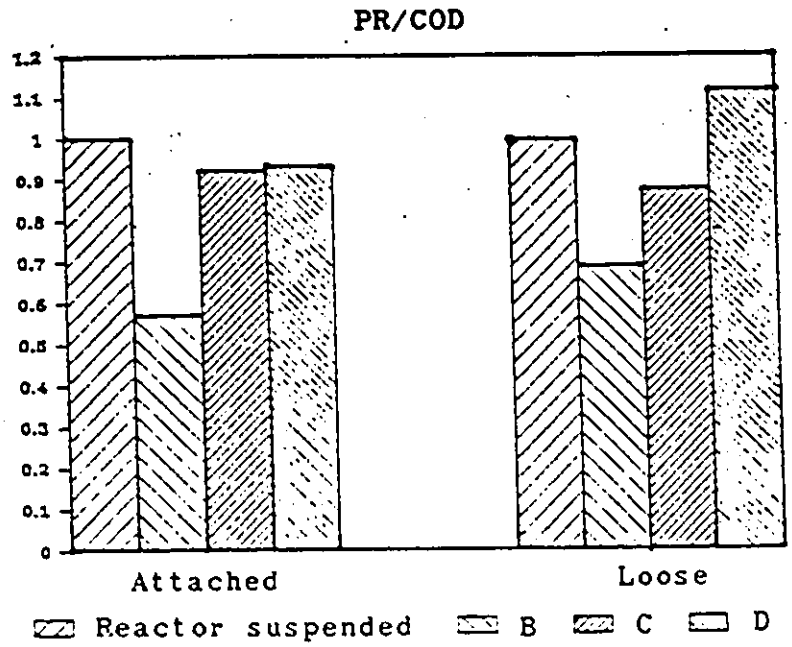


Figure 4.31. Normalized PR/COD ratios in reactor, AF and LF for Runs B, C and D.

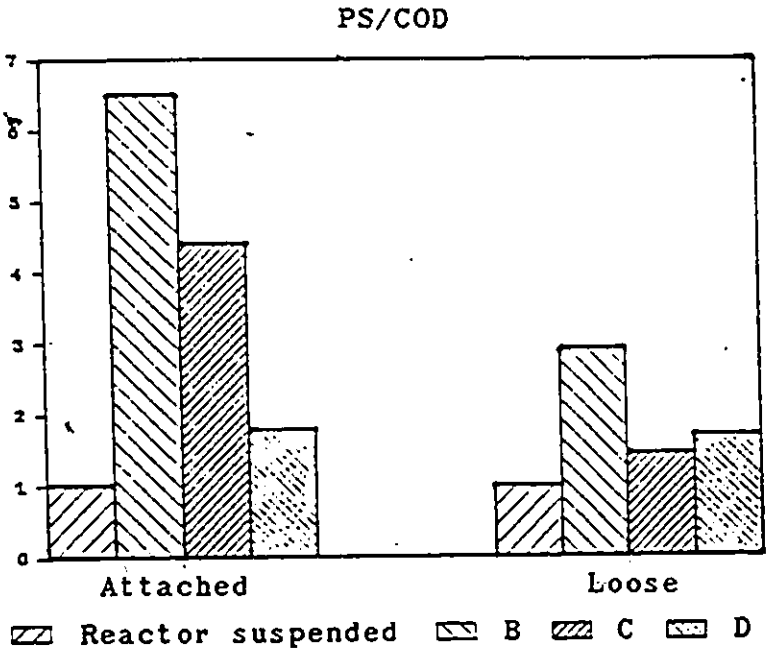


Figure 4.32. Normalized PS/COD ratios in reactor, AF and LF for Runs B, C and D.

4.2.4.3 Biofilm Concentration

Concentrations of COD and PR in the biofilm showed an initial increase, then a plateau value was reached (Figs. 4.33, K.6). PS concentration in the biofilm was constant throughout Experiment D, and it showed a decrease in Runs B&C (Fig. K.7).

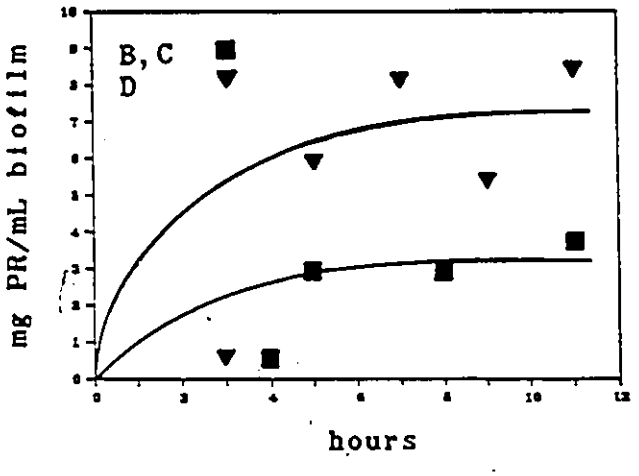


Figure 4.33 Biofilm PR concentration vs time (B,C,D)

Table 4.16 summarizes the average concentrations of indicators in the biofilm. The COD and PR concentrations of the biofilm were higher when higher suspended biomass concentration was present (Run D), but the opposite was observed for PS concentration.

Table 4.16 Biofilm concentrations (B,C,D)

mg/mL of biofilm	B&C	D
COD	8.53	11.16
PR	2.54	5.73
PS	2.33	1.26

4.3 Experiment #3 - Potential and Actual Specific Activity

4.3.1 Introduction

In this experiment the effect of biomass metabolic activity on biofilm formation and biomass characteristics was investigated. Table 4.17 summarizes the experimental conditions for the two parts of this experiment.

Table 4.17 Experimental conditions (D,E,F,G)

Culture	Substrate concentration	Potential activity	Actual activity	Organic loading rate
	Acetate mg/L	mg COD/ mg VSS·d	mg COD/ mg VSS·d	mg COD/ L·d
Part I.				
D	0	0.79	0	0
E	0	0	0	0
Part II.				
E	0	0	0	0
F	1000	0.383	0.383	300
G	820	0.813	0.813	570

In all experiments samples were taken in duplicate. Data and figures related to this section can be found in Appendix L.

4.3.2 Part I - Potential Specific Activity

Biofilm formation using two cultures (D and E) was compared. Culture D was active before starting the experiment. During the experiment substrate concentration and actual ac-

tivity were zero, while potential activity (as determined before the experiment via batch serum bottle acetoclastic activity test) was 0.79 mg COD/mg VSS·d. Samples were taken at 3, 5, 7, 9 and 11 hours.

Culture E was a 3 L portion of stock culture II. The culture had been starved for 6 months before the experiment, preceded by a 6 month continuous feeding period on acetate. During the experiment substrate concentration, potential and actual activity were zero (calculations in Appendix I/a). Samples were taken at 10.5, 23, 32.5, 48 and 57.5 hours.

The experiments were conducted to determine whether a difference in the general metabolic state of the bacteria is capable of affecting biofilm development rate, when the actual metabolic activity is the same (zero).

4.3.2.1 Reactor Characteristics

The important reactor properties are listed in Table 4.18.

The concentration of SPR in reactor E was higher than in reactor D even though VSS concentration in reactor E was lower. SPS concentration was higher in reactor D than in reactor E. These differences may originate from the prolonged starved state of reactor E.

Table 4.18 Characteristics of reactors D and E

		D	E
VSS	mg/L	848	793
SCOD		1176	1154
SPR		578	683
SPS		76.7	65.8
SCOD/VSS	mg/mg	1.403	1.441
SPR/VSS		0.720	0.862
SPS/VSS		0.091	0.085
SPR/SCOD	mg/mg	0.513	0.593
SPS/SCOD		0.065	0.057
SPS/SPR		0.126	0.096
Support surface		silicone	silicone
pH		7.50	7.46
Alkalinity mg/L as $CaCO_3$		5100	4500
SRT, d		9.7	36
Flow velocity, cm/s		1.044	1.18
Reynolds number		114	128

4.3.2.2 Biofilm Development Rate

To compare results obtained with cultures D and E, all experimental conditions except the examined one should be identical for the two cultures. However, slight differences occurred between the suspended biomass concentrations of the two reactors. To take these differences into account, predicted film development rates for culture E were calculated from the equations in Table 4.11, relating film development rate to reactor biomass. The equations in Table 4.11 were obtained using active cultures (0.8-0.9 mg COD/mg VSS·d). If the lack of potential specific activity does not have any effect on biofilm formation, than the predicted rates (based

solely on biomass concentration) should be close to the measured rates. Table 4.19 shows the measured and predicted film development rates for culture E.

Table 4.19 Measured and predicted biofilm development rates, culture E

$\mu g/cm^2 \cdot h$	Measured(M)	Predicted(P)	M/P
AFCOD	9.05	23.02	0.393
AFPR	3.18	16.92	0.188
AFPS	0.47	1.55	0.303
LFCOD	8.88	45.09	0.197
LFPR	4.42	31.93	0.138
LFPS	0.36	3.13	0.115

The measured AF development rate was 20-40% of the predicted, and the LF development rate was 10-20% of the predicted. Therefore it must be concluded that a certain suspended biomass concentration does not result in the expected film formation rate when the biomass is inactive. Inactivity slowed down LF development more than AF development in comparison to the active culture.

4.3.2.3 Biofilm Characteristics

No prediction is available relating reactor biomass to biofilm characteristics, therefore biofilm developed with culture E is compared to biofilm developed using culture D.

Ratios of indicators in the biofilm are compared in Table 4.20. All biofilm ratios were higher for reactor D except LF PR/COD. Table 4.21 compares ratios normalized to the corresponding reactor ratios.

Table 4.20 Ratios of indicators in biofilm (D,E)

	D	E
Attached		
PR/COD	0.479	0.461
PS/COD	0.117	0.101
PS/PR	0.240	0.216
Loose		
PR/COD	0.572	0.585
PS/COD	0.111	0.066
PS/PR	0.196	0.129

PR/COD ratio in the film did not exceed that of the reactor except for LF for reactor D. PS/COD and PS/PR ratios in AF and LF were higher than the corresponding ratios of reactors D and E. All normalized ratios were higher for the active culture (D) with the exception of AF PS/PR ratio.

Table 4.21 Comparison of biofilm and reactor ratios (D,E)

	D	E
Attached/Reactor suspended		
PR/COD	0.993	0.778
PS/COD	1.803	1.769
PS/PR	1.905	2.241
Loose/Reactor suspended		
PR/COD	1.114	0.986
PS/COD	1.717	1.166
PS/PR	1.555	1.342

Table 4.22 contains the attached percentage of the total biofilm indicators .

Slightly higher percentage of PR and PS was found attached in the case of the inactive culture E. The percentage of TFPS attached was higher than that of TFCOD and TFPR for both cultures.

Table 4.22 Percentage of biofilm attached (D,E)

	D	E
COD	46	46
PR	36	42
PS	51	56

Table 4.23 shows the concentration of indicators in the attached biofilm. The concentrations of all indicators were slightly higher for reactor E.

Table 4.23 Biofilm concentrations (D,E)

mg/mL of biofilm	D	E
COD	11.16	14.62
PR	5.73	6.50
PS	1.26	1.38

4.3.3 Part II - Actual Specific Activity

Biofilm formation using three cultures with different potential and actual specific activities was compared.

Culture E was described in Section 4.3.2.

Culture F was obtained by activating a 3 L portion of stock culture II. The starving culture was subjected to a

high acetic acid concentration (2800 mg/L) in batch mode. The experiment was started on the 4th day, when the concentration of acetic acid decreased to 1000 mg/L. Samples were taken at 3, 6, 8, 14.5 and 20 hours.

Reactor G operated with a less concentrated 3 L portion of stock culture II. The culture was subjected to 1100 mg/L acetate concentration for 11 hours to activate it. Samples were taken at 2, 3, 4, 5, 7, 8, 9 and 11 hours.

The aim of the experiment was to determine the effect of increasing actual specific acetoclastic activity on biofilm formation.

4.3.3.1 Reactor Characteristics

Table 4.24 summarizes the important characteristics of the reactors. Biomass concentration in reactor G was somewhat lower than in reactors E and F. The ratios of indicators in each of the reactors were slightly different. However, they were close enough to allow comparison of biofilm development.

For cultures with higher specific acetoclastic activity (reactors F and G) more frequent sampling was necessary to obtain reliable data from the initial stage of biofilm development. Frequency of sampling affected the duration of the experiments and reactor SRT. Additionally, higher organic loading rates were required to maintain similar levels

of substrate in each reactor when specific acetoclastic activity was higher. Acetate concentration in reactor G was somewhat lower than in reactor F.

Table 4.24 Characteristics of reactors E, F and G

		E	F	G
Activity mg COD/mg VSS·d		0	0.3833	0.8135
VSS	mg/L	793	790	654
SCOD		1154	1147	986
SPR		683	645	534
SPS		65.8	69.1	53.3
SCOD/VSS	mg/mg	1.441	1.477	1.515
SPR/VSS		0.862	0.816	0.818
SPS/VSS		0.085	0.086	0.082
SPR/SCOD	mg/mg	0.593	0.552	0.542
SPS/SCOD		0.057	0.059	0.054
SPS/SPR		0.096	0.107	0.100
Acetate concentration, mg/L		0	1000	820
Organic loading rate mg COD/L·d		0	300	570
SRT, d		36	25	15.8
Flow velocity, cm/s		1.18	1.18	1.18
Reynolds number		128	128	128

4.3.3.2 Biofilm Development Rate

Film thickness data vs time from the three experiments were similar enough that one curve could be used to describe the results (Fig. 4.34). It should be noted that data from reactor G showed greater scatter than values from the other two reactors. The increase in biofilm thickness became non-

linear after about 11 hours. Eq. 4.1 provided the best fit to the data. A maximum film thickness of 246 µm and a rate constant of 0.0628 h^{-1} was obtained (c: 0.947).

Table 4.25 summarizes development rates of the different indicators. AF development determined by AFCOD, AFPR

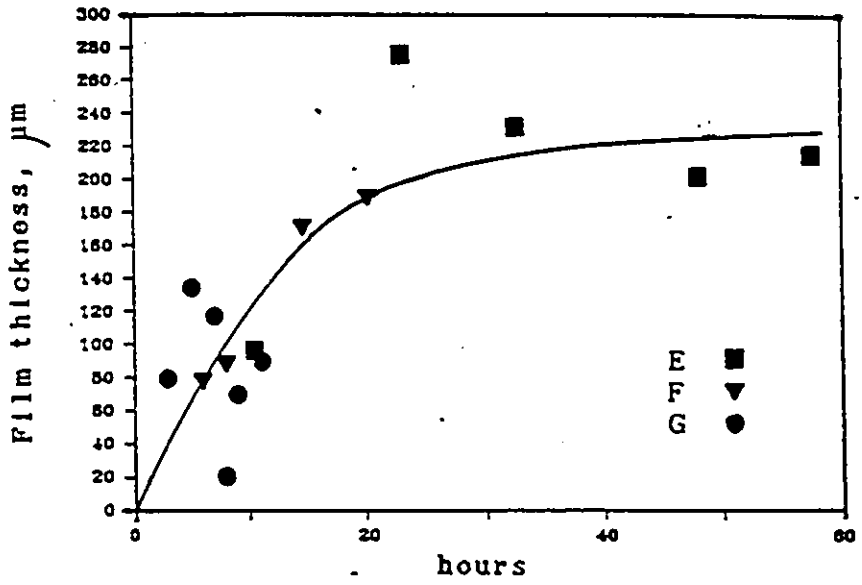


Figure 4.34 Biofilm thickness vs time (E,F,G)

and AFPS occurred faster as the biomass became more active. LF development rates were very close to AF development rates in reactors E and G. In reactor F, however, LF development rates were much higher than AF development rates. The development rates of LF in reactor F (highest activity) were very similar to those found for reactor G.

Figs. 4.35 and 4.36 show AFCOD and LFCOD increases for the different reactors, respectively. AF and LF PR and AF

Table 4.25 Biofilm development rates, $\mu g/cm^2 \cdot h$ (E,F,G)

	E	F	G
AFCOD	9.05	24.50	40.00
AFPR	3.18	10.28	21.15
AFPS	0.47	2.09	3.57
LFCOD	8.89	46.57	43.65
LFPR	4.42	24.26	23.00
LFPS	0.36	3.29	3.07

and LF PS increases with time are shown in Figs. K.1-K.4. The results of the linear regression analysis are summarized in Table 4.26.

Strong positive linear correlation was found between specific acetoclastic activity and AF development rates (Figs. 4.37, L.5 and L.6). The relation between specific acetoclastic activity and LF development was nonlinear. The almost identical LF development rates measured for reactors F and G indicate a plateau on a saturation-type curve (Figs. L.7, L.8 and L.9). Concomitantly, the correlation coefficients in Table 4.26 were lower for LF than for AF.

Table 4.26 Correlation between biomass specific activity and biofilm development rate (E,F,G)

	Slope	Intercept	Correlation coefficient
AFCOD	37.86	9.38	0.999
AFPR	22.08	2.71	0.996
AFPS	3.79	0.53	0.998
LFCOD	41.73	16.39	0.810
LFPR	22.20	8.35	0.816
LFPS	3.25	0.94	0.811

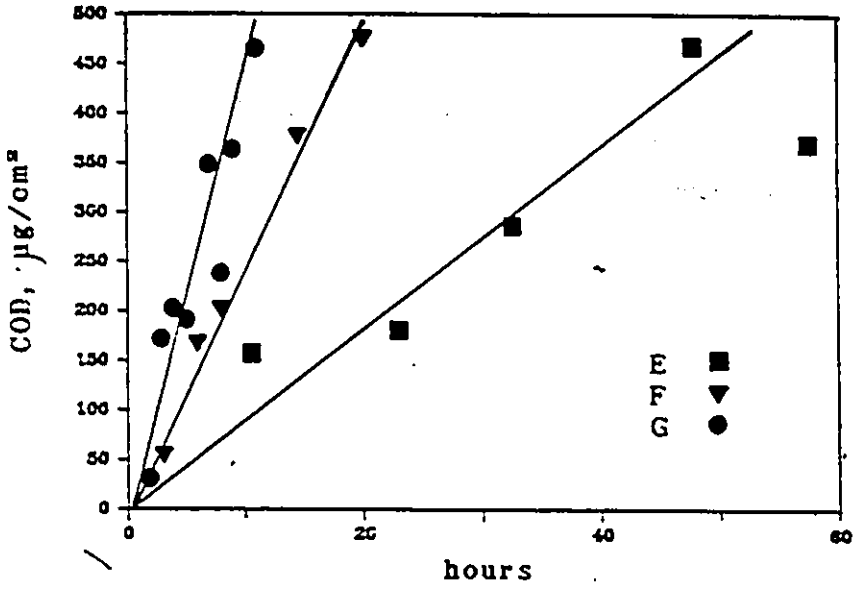


Figure 4.35 AFCOD development vs time, reactors E, F and G

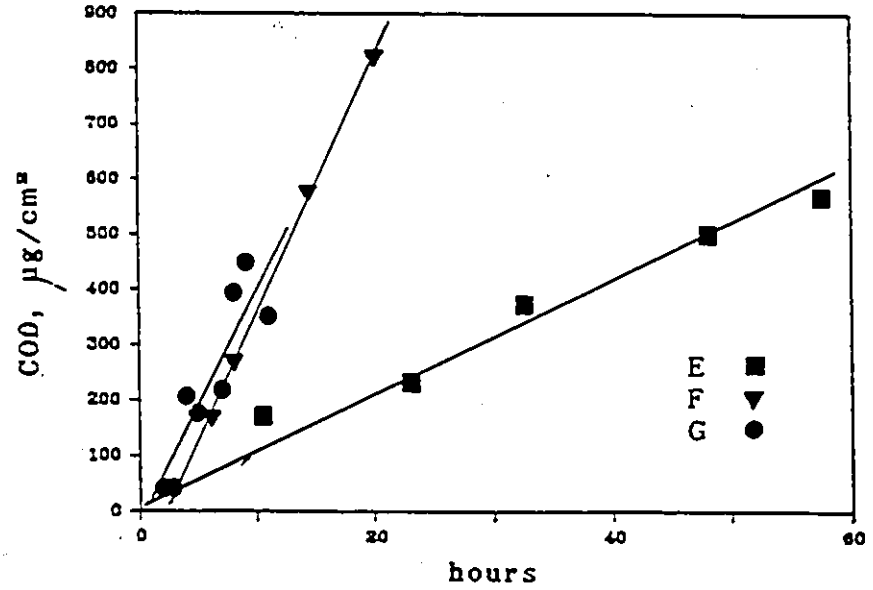


Figure 4.36 LFCOD development vs time, reactors E, F and G

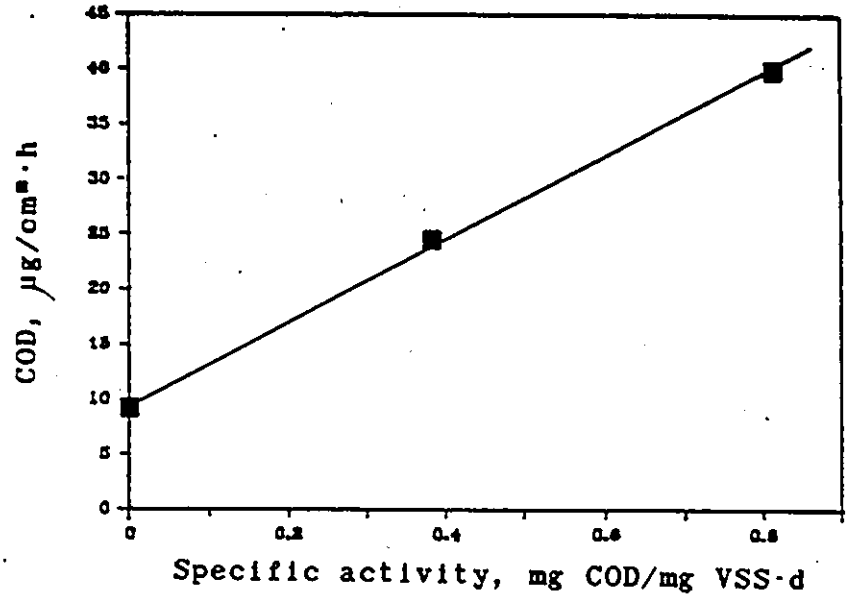


Figure 4.37 AFCOD deposition rate vs biomass specific activity (E,F,G)

4.3.3.3 Biofilm Characteristics

Table 4.27 shows the ratios of indicators in the biofilm. Table 4.28 summarizes the ratios of indicators in the biofilm normalized to the corresponding reactor ratios.

Indicator ratios did not show a characteristic dependence on biomass activity. Ratios for reactor E (inactive culture) tended to be somewhat smaller than for the other two reactors; exceptions were AF PS/PR and LF PR/COD.

It was only in the case of culture G that AF and LF PR/COD ratios slightly exceeded that of the reactor. PS/COD and PS/PR ratios in both AF and LF exceeded those in the corresponding reactors, more in the case of AF than for LF.

Fig. 4.38 shows normalized values of AF and LF PS/COD for the three reactors.

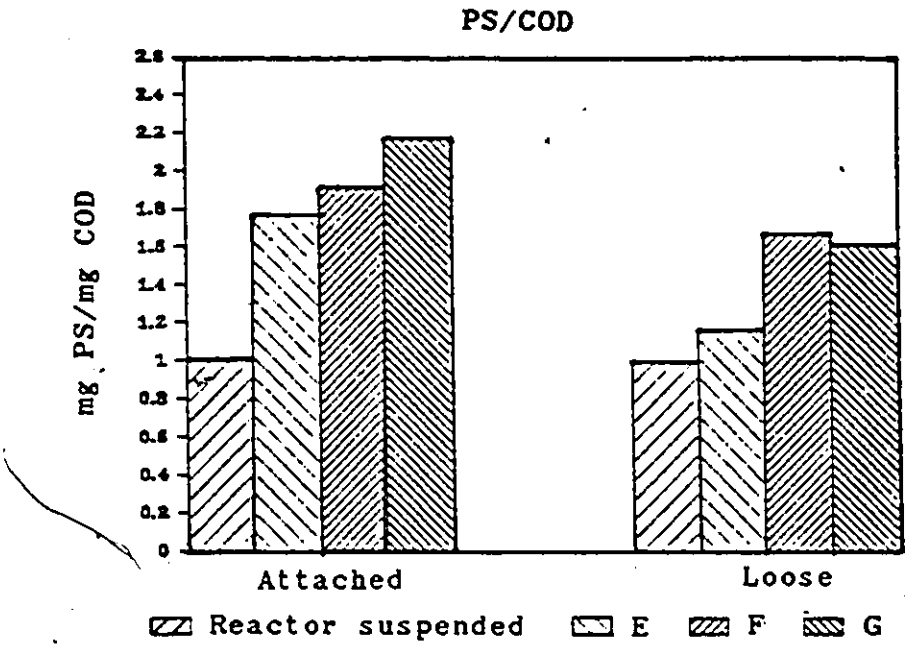


Figure 4.38 Normalized PS/COD ratios in reactor, AF and LF for runs E, F and G.

Table 4.29 summarizes the effect of specific acetoclastic activity on the percentages of attached biomass indicators. The higher the biomass activity, the higher the percentage of the total biofilm found attached, with the exception of PS for reactor F.

The concentrations of all three indicators in AF increased with higher biomass activity (Table 4.30).

An increasing tendency in the measured AF concentration values with time was also observed, especially in the case of highest activity (reactor G) (Figs. 4.39, L.10 and L.11).

A strong positive linear correlation was found between the average concentration of indicators in biofilm and the specific acetoclastic activity of biomass (Figs. 4.40, L.12 and L.13). These data are summarized in Table 4.31.

Table 4.27 Ratios of indicators in biofilm (E,F,G)

	E	F	G
Attached			
PR/COD	0.461	0.539	0.560
PS/COD	0.101	0.112	0.117
PS/PR	0.216	0.208	0.217
Loose			
PR/COD	0.585	0.528	0.590
PS/COD	0.066	0.098	0.087
PS/PR	0.129	0.185	0.146

Table 4.28 Comparison of biofilm and reactor ratios (E,F,G)

	E	F	G
Attached/Reactor suspended			
PR/COD	0.778	0.976	1.033
PS/COD	1.769	1.908	2.167
PS/PR	2.241	1.953	2.170
Loose/Reactor suspended			
PR/COD	0.986	0.956	1.088
PS/COD	1.166	1.670	1.611
PS/PR	1.342	1.737	1.460

Table 4.29 Percentage of biofilm attached (E,F,G)

	E	F	G
COD	45.6	47.6	53.3
PR	41.5	46.6	52.6
PS	55.5	52.3	65.9

Table 4.30 Biofilm concentration (E,F,G)

mg/mL	E	F	G
COD	14.62	25.09	33.50
PR	6.50	12.95	18.26
PS	1.38	2.66	3.95

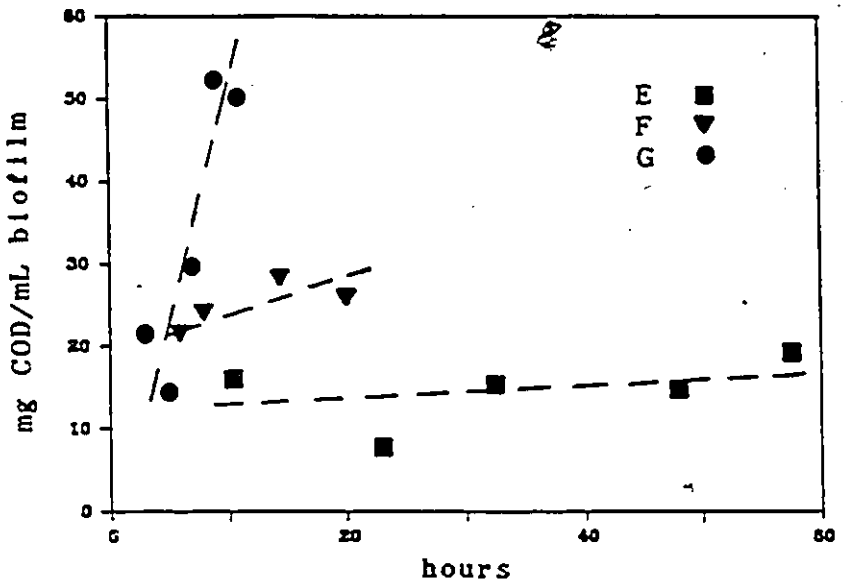


Figure 4.39 Biofilm COD concentration vs time, reactors E, F and G

Table 4.31 Correlation between biomass specific activity and biofilm concentration (E,F,G)

	Slope	Intercept	Correlation coefficient
COD	23.13	15.17	0.995
PR	14.41	6.82	0.996
PS	3.16	1.04	0.999

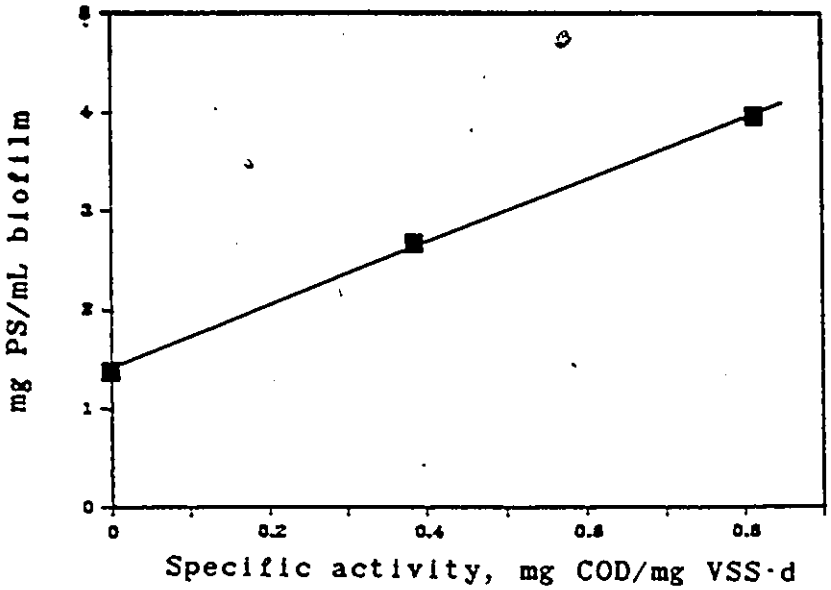


Figure 4.40 Biofilm PS concentration vs biomass specific activity (E,F,G)

CHAPTER 5

DISCUSSION

5.1 Rate of Attached Biofilm Formation

5.1.1 Comparison with Biofilm Development Rates Found in Other Studies

In this study anaerobic biofilm formation was found to be remarkably fast considering the 1-2 months startup times reported for anaerobic reactors (Kennedy, 1985; Henze and Harremoes, 1983).

On Tygon surface 3-5 µg COD/cm²·h biofilm formation rates were observed. On silicone surface the film development was faster with rates between 16-40 µg COD/cm²·h.

Droste and Kennedy (1987) attempted to indirectly determine the value of certain parameters in the anaerobic fixed film process using the dynamic anaerobic fixed film model developed by Kennedy (1985). They assigned different realistic values to chosen parameters and compared the model's prediction to experimental data. The best sets of values were those which led to predictions that had the least deviation from the experimental data. For both acidogens and methanogens values of six parameters were varied: maximum reaction velocity constant, half velocity constant,

biomass yield constant, biomass decay rate, attachment rate and detachment rate. The authors assumed no difference in kinetic constants for liquid and film phase bacteria. Since there has been no evidence suggesting that attachment or detachment rates of acidogenic and methanogenic bacteria should be different, they also assumed the same values respectively for both groups, as a first approximation.

According to Droste and Kennedy (1987) net attachment rates (attachment - detachment) allowing best prediction were in the range of 0-0.1 mg VSS/mg VSS·d. To be able to compare the results of this study with those of Droste and Kennedy, the above attachment rate values have to be converted into mg COD/cm²·d. The experimental system of Kennedy (1985) had a total surface area of 16,800 cm² and a total suspended COD content of 10,685 mg. Assuming that mg VSS attached/mg VSS suspended can be directly substituted with mg COD attached/ mg suspended COD, the following conversion of results can be made.

$$0.1 \frac{\text{mg COD}}{\text{mg SCOD}\cdot\text{d}} \cdot \frac{10685 \text{ mg SCOD}}{16800 \text{ cm}^2} = 0.064 \text{ mg COD/cm}^2\cdot\text{d}$$

$$= 2.667 \text{ µg COD/cm}^2\cdot\text{h}$$

This model predicts a net attachment rate of 0-2.67 µg COD/cm²·h for acidogens and methanogens alike. This result agrees reasonably with data found in this study for Tygon tubing (3-5 µg COD/cm²·h), but substantially lower than rates measured for silicone tubing (16-40 µg COD/cm²·h). The differences in experimental circumstances that have to

be taken into account when comparing the results are listed in Table 5.1.

Table 5.1 Comparison of this study and Kennedy's study (1985)

	Kennedy's study	This study
Culture	mixed	enriched methanogen
Stage	up to mature biofilm	initial
Support material	NPP	plastic tubing
Flow velocity cm/s	0.04	0.054-1.42
Reactor VSS, mg/l	355	610-800
Channel cross-section, cm^2	8-9	0.5-0.7
Surface to volume ratio, m^2/m^3	75-80	4-5
Volatile acids, mg/L	300-1000	1000
SRT, d	variable	16-25

Several factors explain why the model's predicted net attachment rate is lower than those measured in this study. The low velocity and VSS concentration in Kennedy's study favor slower attachment. Also, since biofilm formation is fastest in the initial phase (Shapiro and Switzenbaum, 1984), an average rate taken for the whole period of mature biofilm development will certainly be less than the initial rate. On the other hand, NPP support was found to be a better support than PVC in various experiments (see section 2.3.4.1). Although a large surface area to volume ratio al-

lows the retention of large amounts of biomass in the reactor, it does not affect the rate of attachment per unit surface area. It is not clear, whether the channel cross-sectional area has any role in attachment other than being a factor in plugging. Finally, it must be considered that the assumption that attachment rates for acidogens and methanogens were equal might be wrong. From simulations Kennedy estimated the percentage of methanogens to be 25-33% of the total biomass in the biofilm. If acidogenic bacteria have a low rate of attachment, then the overall biofilm attachment rate will reflect this value more than that of the methanogens. Since acidogens are faster growing bacteria (average of 8 hours doubling time, Henze and Harremoes, 1983), they may still constitute the same proportion of biomass in the biofilm as in the reactor even if their attachment rate is less than that of the methanogens.

Another study, very similar to this study (Shapiro and Switzenbaum, 1984) provides an opportunity for comparison of results. The similarities and differences between the conditions and results of the two experiments are listed in Table 5.2. The comparison is more realistic in this case since the same support (silicone tubing) was used.

Table 5.2 Comparison of this study with Shapiro's study

	Shapiro's study	This study
Culture	mixed	enriched methanogen
Stage	initial	initial
Duration, d	25	1-3
Support	silicone i.d. 7.95 mm	silicone i.d. 7.95 mm
Feed	sucrose	acetate
Reactor VSS mg/L	40-3000	610-800
Organic loading rate, mg COD/L·d	70-4400	300-570
Flow velocity cm/s	3.3	0.054-1.18
SRT, d	5-15	16-25
Biofilm formation rate µg COD/cm²·h	0.1-3.33	16-40

In Shapiro's study coenzyme F420 was used to quantify methanogenic bacteria while COD was used as a measure of total biomass. The ratio of F420 to COD did not differ markedly during the course of the experiments, regardless of differing VSS concentration, organic loading rates or SRT. The highest biofilm development rate measured by Shapiro was 3.33 µg COD/cm²·h. It was observed in an experiment with a 15 d SRT using 3000 mg/L VSS concentration and 1500 mg/L·d organic loading rate.

In Shapiro's study as well as in this study film formation rates increased with higher VSS concentration and or-

ganic loading rate. It would therefore be reasonable to assume that because the VSS concentration they used exceeded concentrations used in this study by 2-3 fold, the biofilm development rate should have been higher than in this study. However, the highest film formation rate measured in their study was 4-12 times less than rates found in this study.

One explanation could be the different flow velocity. According to the authors, COD and F420 accumulations were highest at 0.5 and 7.4 cm/s velocities (the extremes of the range studied), but they were substantially lower (by 40-85%) in the middle velocity range (see Fig. 2.10). The velocity chosen to perform other experiments was 3.3 cm/s, a mid-range value. Considering their data, it is possible that if the experiments were perfqrmed at lower velocities, higher film formation rates would have been observed. This, however contradicts the observation from this study of higher biofilm development rates corresponding to higher velocities in the range of 0.054-1.078 cm/s.

Another explanation is the difference in the anaerobic culture. Here again a constant methanogen:acidogen ratio was indicated in the biofilm, based on actual measurements. It is important to note that given the doubling time difference between acidogens (8 h) and methanogens (1-9 d), preferential attachment of methanogens may contribute to maintaining the constant ratio in the biofilm. Differences in adhesiveness among acidogenic bacteria were in fact observed by Beeftink (1987). In a study of anaerobic bacterial ag-

gregates Beeftink found that propionate forming organisms had better adhesive properties than butyrate forming bacteria. It is conceivable that such differences in adhesiveness can also exist between the acidogens and the methanogens.

Kennedy (1984, unpublished data) measured anaerobic biofilm development rates in a horizontal recirculation system (Fig. 2.8). Conditions and results are listed in Table 5.3.

Table 5.3 Comparison of this study and Kennedy's study (1984)

	Kennedy's study	This study
Culture	mixed	enriched methanogen
Stage	initial	initial
Duration, d	38	1-3
Support	stainless steel (316), plexiglass	plastic tubing
Feed	50% sucrose 50% acetate	acetate
Flow direction	horizontal	vertical downflow
Flow velocity, cm/s	48	0.054-1.42
Reactor VSS, mg/L	680	610-800
Biofilm development rate $\mu g\ COD/cm^2 \cdot h$	0.5	3-40

The low film formation rate observed by Kennedy might be attributed to the high velocity, different support material, mixed culture and possibly to the horizontal arrangement, in which sampling involved moving and inversion of the test section.

5.1.2 Effect of Support Material on Attached Biofilm Formation Rate

Silicone tubing proved to be a better support material than Tygon. Film development measured by film thickness, COD, PR or PS was 4-6 times faster on the surface of silicone tubing (Table J.9).

Film development on the two surfaces was also different in that biofilm accumulation rate on Tygon tubing was less sensitive to velocity change, although a positive correlation between flow velocity and biofilm development rate was observed (Figs. 4.10-4.13, Table J.8). The sensitivity to velocity change is indicated by the slope of the regression line. It was steeper for silicone tubing (COD 4.5, PR 4.7, PS 2.8 times) than for Tygon.

Tygon tubing is a PVC material with a critical surface tension of 39 dyn/cm (20°C). The composition of silicone tubing is polydimethylsiloxane (critical surface tension 24 dyn/cm at 20°C) and silica filling. The results of this study do not support the existence of a universally biocompatible (recall from Section 2.3.3.4 that a biocompatible surface exhibits poor attachment properties) critical surface tension range between 20-30 dyn/cm (Baier, 1972; Dex-

ter, 1975), since excellent biofilm formation occurred on silicone surface. Furthermore, glass, which has a high surface energy (critical surface tension 46-70 dyn/cm) proved to be a very poor support in this study. No attachment was observed on the surface of the inserted glass sampling ports, while abundant film growth was found on the plastic tubing surfaces. The more hydrophobic silicone supported biofilm formation better than the less hydrophobic Tygon.

It is possible that it was the difference in surface texture rather than surface composition that had the most decisive effect on the attachment process.

Examining the scanning electron micrographs (Fig. 4.1) it becomes evident that the Tygon surface is more homogeneous. An average pore size of 0.5 µm was estimated. Silicone surface has a fine roughness, but larger "pores" of 5-10 µm size exist as well. Dealing with an enriched methanogenic culture fed with acetate for over 10 years, it is reasonable to assume that acetate utilizing methanogens constitute the majority of the biomass. The size of these bacteria has been found to be 1-3.5 µm (*Methanothrix soehngenii* 0.8x3.3 µm, Zehnder *et al.*, 1980; 0.8x2 µm, Huser *et al.*, 1982; *Methanothrix concilii* 0.8x3.5 µm, some 2-7 µm long, Patel, 1984; *Methanosarcina mazei* 1-3 µm, 20-100 µm in clumps, Mah, 1980; Mah and Kuhn, 1984; *Methanosarcina barkeri* 1.5-2.5 µm, Balch *et al.*, 1979). According to Messing *et al.* (1979) for microbes reproducing by fission, the maximum accumulation of biomass occurs when the pore diameter is

1-5 times the major dimension of the bacterium. It is assumed that growth of bacteria in biofilms is not likely to contribute significantly to biofilm accumulation during a 3-day experiment, thus the results of another study by Messing (1974) are more applicable. In enzyme immobilization studies Messing found the optimum pore diameter to be twice the major dimension of the enzyme. For the acetate utilizing methanogens this pore diameter is 2-7 μm. Since the pore size of silicone tubing (5-10 μm) is nearly optimal as opposed to the small pore size (0.5 μm) of Tygon, this could be one reason for the observed difference in adhesiveness.

5.1.3 Effect of Flow Velocity on Attached Biofilm Formation Rate

In the range of 0.054-1.42 cm/s the rate of biofilm formation increased with higher velocity. The magnitude of increase was rather small (Figs. 4.10-4.13).

In the range studied, an 1 cm/s increase in liquid flow velocity caused an increase of 1.46 μg COD/cm²·h in COD deposition rate for Tygon tubing, and a 6.84 μg COD/cm²·h increase for silicone tubing (Table J.8).

This finding contradicts Shapiro and Switzenbaum's results which showed a U-shape curve characterizing the relationship between flow velocity and COD attached in 5 days on a silicone surface (Fig. 2.10). Examination of the total biofilm accumulation in that study shows that the amount of attached COD increased with higher velocity in the range of 1.6-7.4 cm/s. It was only the 0.5 cm/s velocity which re-

sulted in an unexpectedly high accumulation. For the 1.6-7.4 cm/s range, an 1 cm/s increase in flow velocity caused an increase of 0.725 µg COD/cm²·h in the film formation rate. Comparison of this number with the 6.84 µg COD/cm²·h found in this study indicates that biofilm development in Shapiro's study was less sensitive to velocity change than it was in this study. The difference may originate from the use of an enriched culture in this study as opposed to a mixed culture in their study.

From this study it can be concluded that for initial attachment in the low velocity range, highest velocity allows better transport of bacteria and nutrients to the surface, thereby facilitating attachment, and unfavorable shear effects do not appear.

5.1.4 Effect of Suspended Biomass Concentration on Attached Biofilm Formation Rate

The increase of biofilm formation rate with higher suspended biomass concentration found in this study has been observed by various authors in aerobic as well as in anaerobic studies (Fletcher, 1977; Bryers, 1980; Shapiro and Switzenbaum, 1984).

The film development rates measured by Shapiro and Switzenbaum are summarized in Table 5.4 along with rates measured in this study.

Shapiro's results do not indicate the existence of a limiting VSS concentration, instead the data constitute a saturation-type curve starting from zero (see Fig. 2.9).

Table 5.4 Biofilm development rates in Shapiro's study (1984) and in this study

	Shapiro' study		This study	
	VSS mg/L	µg COD/ $cm^2 \cdot h$	VSS mg/L	µg COD/ $cm^2 \cdot h$
	40	0.125	510	1.26
	400	1.17	522	5.53
	1100	1.88	848	23.90
	1900	2.50		
Culture	mixed		enriched methanogen	
SRT, d	5		7.7	
Presence of substrate	+		-	
Flow velocity cm/s	3.3		1.04	

The increase in accumulation rate with increasing VSS concentration was very modest in their study (Table 5.4). The difference probably results from the use of a mixed culture in their study.

The indication in the present study of a limiting biomass concentration that affect AF development rates was surprising and was the result of the experimental design, viz., the absence of substrate.

It was found in Experiment #3 that increased biomass metabolic activity had a positive effect on film formation rate. During the experiments investigating the effects of biomass concentration none of the cultures were actually ac-

tive. However, it is not known whether the lack of activity causes a decrease in film formation rate which is proportional to the VSS present or whether attachment is impaired disproportionately at low VSS concentrations. It is possible that the cumulative effect of low biomass concentration and the absence of substrate resulted in an unusually sharp drop in attachment rate for reactors B and C. It is not expected that such a limit should exist in the case of active substrate-metabolizing cultures.

In spite of the limited applicability of the results, certain general conclusions regarding the process of attachment can be derived from them.

AF polysaccharide accumulation rate is less dependent on SPS concentration in the reactor, than COD and PR on SCOD and SPR concentrations, as indicated by the slopes of the regression lines (Table 4.11). These were 0.0303 for AFPS, as compared to 0.0449 for AFCOD and 0.0467 for AFPR. In other words, polysaccharide will accumulate at only a slightly slower rate when there is little biomass present than when the biomass concentration is high. This is reinforced by the fact that PS concentration in the AF was about the same for each of the runs. On the other hand, AFCOD and AFPR deposition rates depend on the reactor biomass more strongly. This fact is also reflected in the higher COD and PR concentrations in the AF observed for Run D (Table 4.16).

5.1.5 Effect of Biomass Metabolic Activity on Attached Biofilm Formation Rate

From the data of Part I it is concluded that the general metabolic state of the biomass influences the rate of biofilm development. Much higher (2-5 times) biofilm formation rates were observed with a previously active culture than with a previously inactive one, even though neither of them exhibited actual activity during the experiment (Table 4.19). In Part II it was found that biofilm accumulation rate increased with higher actual activity.

There are conflicting reports in the literature on the effect of metabolic state on attachment. In some experiments with aerobic bacteria (Fletcher, 1977) logarithmic phase cells attached better than stationary or death phase cells. In an experiment by Bott and Miller (1983) a lag period of 1 day was observed before biofilm formation started in the presence of substrate, as compared to a 4-day lag seen in the absence of substrate. Note that in both cases the bacteria were fed until the experiment commenced, thus they possessed a potential activity. In a continuous flow system, Bryers (1980) found increasing attachment rate with higher biomass growth rate. Harris and Mitchell (1973), however, in their review conclude that most microorganisms have a greater tendency to aggregate under declining growth conditions. It is likely, that this tendency is dependent on the organism and cannot be generalized.

In this experiment, the starting culture was an inactive, dormant culture (E). Because of the absence of substrate for over 6 months, the culture was in a stationary or declining growth phase. It was subsequently activated, and reactors D, F and G are considered to have been in an accelerating growth phase. In the case of an enriched methanogenic culture, it must be concluded that under conditions of prolific growth, attachment occurs much faster. In the activity range studied (0-0.83 mg COD/mg VSS·d) biofilm accumulation rate increased linearly with increasing specific activity. The above observations indicate an attachment mechanism involving active participation of the bacteria (discussed later).

To achieve actual activities of 0.38 and 0.81 mg COD/mg VSS·d, organic loading rates of 300 and 570 mg COD/L·d needed to be employed, respectively. Although it is the higher organic loading rate that allows for a higher specific activity, it is thought that the specific activity rather than the organic loading rate affects film formation rates directly. This conclusion is supported by the data of Shapiro and Switzenbaum (1984). In the experiments with 15 d SRT a drop in biofilm accumulation rate occurred coincidentally with a sharp drop in specific activity, regardless of the steadily increasing organic loading rate and VSS concentration. The sharpest increase in film development rate was observed when specific activity, organic loading rate and VSS concentration increased simultaneously. In

other cases the beneficial effect of VSS increase overrode the adverse effect of the decreasing specific activity.

5.1.6 Biofilm thickness

When summarizing the results of all the experiments, it becomes clear that biofilm thickness does not show the same behavior as the biochemical indicators used to characterize the film. While the indicators increased linearly during most of the experiments, biofilm thickness reached a plateau value and did not increase further over the course of the experiment.

It was found that regardless of the experimental conditions, the approached maximum thickness value was very similar in all experiments (excluding the results with Tygon tubing). This value was 250-270 μm, calculated using Eq. 4.1. The rate at which this plateau was reached however, was different for the different reactors. The highest rate constant was obtained for data from reactors D and G (0.117 1/h), followed by reactors E and F (0.063 1/h), and reactor A yielded the lowest rate constant (0.028 1/h). Data from reactors B and C had a still lower slope, but no curve could be fitted because of the lack of data from the nonlinear part. The curves are shown in Fig. 5.1.

The results suggest that higher rate constants correspond to high VSS-high activity cultures, but the pattern is not fully consistent. It is not readily explainable for instance, why reactor A should produce such a low rate con

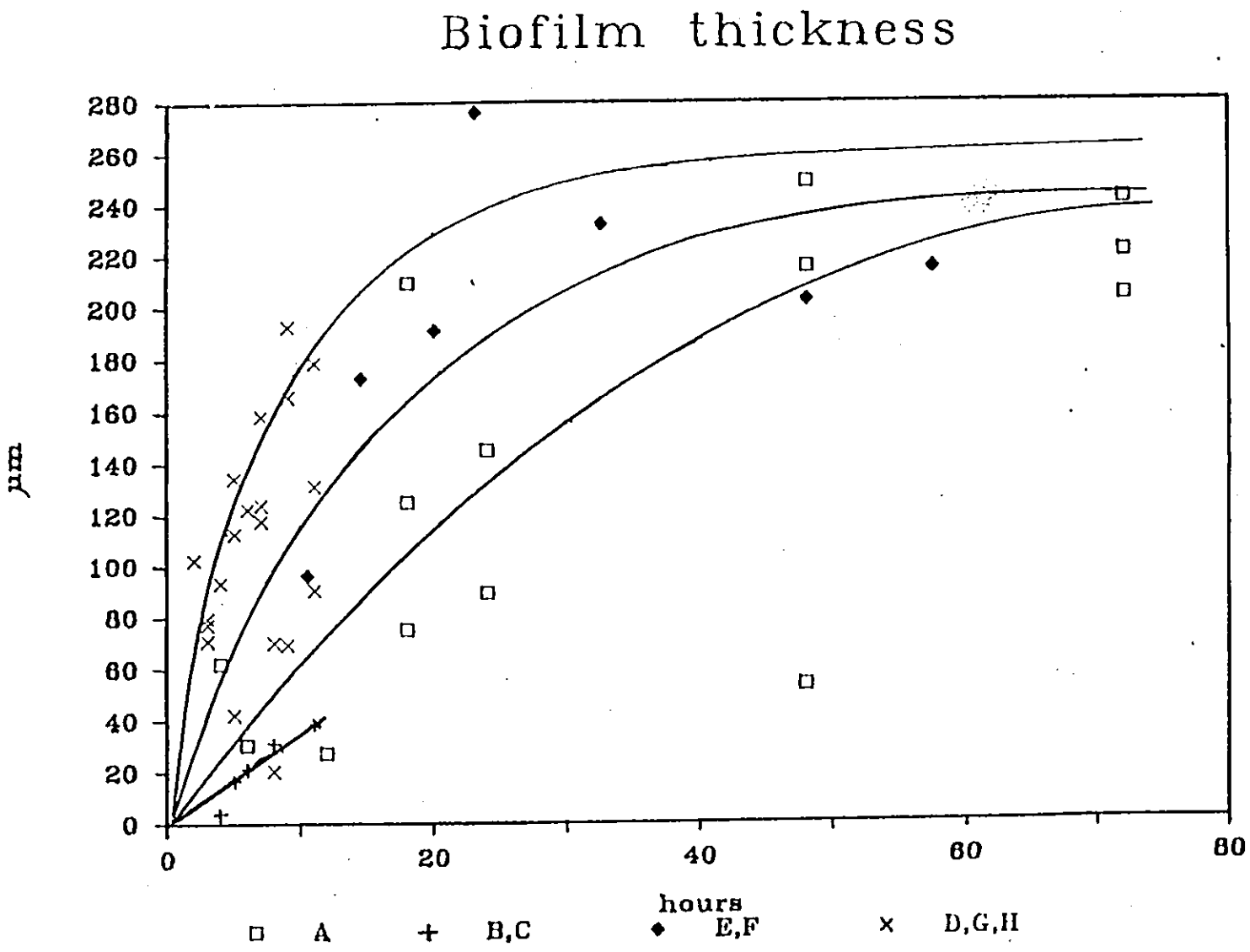


Figure 5.1 Attached biofilm thickness vs time (all experiments)

stant. The different origin of culture A may cause this deviation.

It is suspected that the plateau value is related to the support material and test section geometry, since these were the only factors common to all the experiments.

5.2 Attached Biofilm Characteristics

5.2.1 Effect of Flow Velocity on Attached Biofilm Characteristics

Flow velocity did not have a significant effect on biofilm characteristics in the case of silicone tubing. For Tygon tubing, the biofilm contained the most PS/COD and PS/PR when the flow velocity was lowest (0.054 cm/s). No tendency relating flow velocity to the concentrations of indicators was found (Tables 4.5 and 4.6).

5.2.2 Effect of Support Material on Attached Biofilm Characteristics

Biofilms developed on the two plastic tubing surfaces showed marked differences. As illustrated in Figs. 4.17, J.5 and J.6, biofilm formed on the silicone surface contained much more COD, PR and PS per unit film volume than biofilm formed on Tygon tubing (Table 4.5).

Ratios of indicators in the biofilms were different from those of the reactor suspended biomass, but very similar to each other. The ratios of PS/COD and PS/PR were

found to be substantially higher (1.4 times) in AF on both tubing surfaces than in the reactor (Table 4.7).

It is seen that support media differences significantly affected the density of anaerobic biofilms formed on the surface. The mechanism underlying this phenomenon is not yet clear. The composition of the biofilm was not dependent on the support material.

5.2.3 Effect of Suspended Biomass Concentration on Attached Biofilm Characteristics

Higher reactor biomass concentrations resulted in higher COD and PR concentrations in the biofilm. PS concentration in the biofilm however, was somewhat higher for the reactor with less biomass (Table 4.16). It is an indication of the fact that biofilm PS concentration is not as directly a function of reactor biomass concentration as biofilm COD or PR concentrations are.

PS/COD ratio in the biofilm for reactor D (highest biomass concentration) was half of the value obtained for culture C, and one-third of the number found for reactor B (Tables 4.13 and 4.14, Figs. 4.30 and 4.31). These numbers resulted because there was much more COD and PR attached in the case of reactor D, than for reactors B and C. At the same time, attached PS values for culture D did not differ dramatically from those of the other two reactors (Table 4.2). This observation again supports the conclusion that PS attached is not related to SPS concentration in the reac-

tor as strictly as attached COD and PR are related to SCOD and SPR in the reactor.

A tendency of AF PS/COD decrease (Fig. K.8) and a definite AF PR/COD increase (Fig. 4.29) with time may be the indication of an instant PS deposition and accumulation on the surface as the primary event of biofilm formation, followed by the attachment of the bacteria and possibly more in situ PS production.

5.2.4 Effect of Biomass Metabolic Activity on Attached Biofilm Characteristics

In Part I somewhat higher biofilm concentrations of indicators were found for reactor E (inactive culture). The difference may be an artifact, stemming from the different duration of the experiments. Experiment D lasted 11 hours, during which biofilm COD and PR concentrations showed a steady increase, PS concentration was constant (Table K.4). Experiment E lasted 58 hours, during which biofilm COD and PR concentrations showed only an insignificant increase, PS concentration was constant (Tables K.4 and L.4). COD and PR concentration values reached by 11 hours in experiment D were similar to those measured in experiment E at the first, 10.5 hour sampling. Thus in experiment D samples were taken in a period of biofilm concentration increase, while in experiment E the samples were taken later, when the plateau value of biofilm concentration had already been reached.

Biofilm PS concentrations were constant in time and almost identical for reactors D and E, indicating that in both

cases the amount of PS on the support surface increased concurrently with biofilm volume and thickness from the beginning of biofilm formation.

PS enrichment in biofilm as compared to the suspended biomass was observed for both cultures D and E (Table 4.21). Therefore it was necessary that the extra amount of PS involved in film formation was available or was produced even in the inactive culture. The available quantity of the polymer involved might have determined the corresponding amounts of other substances such as PR that may attach, thus constituting the rate-limiting factor in attachment.

In summary, the lack of potential activity did not have a very pronounced effect on biofilm composition and density when actual activity of the culture was zero.

In Part II the concentrations of PR and PS in the biofilm were doubled for culture F and tripled for culture G compared to culture E (Table 4.30). COD concentration increase was somewhat slower with increase of activity. These findings indicate that as the biomass becomes more active (actual activity), the biofilm formed becomes more dense, more tightly packed.

The ratios in the biofilm showed some characteristic dependence on biomass activity. PR/COD ratio in the biofilm was much lower and PS/COD ratio somewhat lower for the inactive culture (E) than for the two active cultures (Table 4.27).

It is conceivable, that changes in metabolic processes and possibly in membrane structure occurring as a result of renewed activity are responsible for the above noted differences in biofilm density and composition.

5.2.5 Summary

Summarizing all the results, it is seen that in each experiment PS/COD and PS/PR ratios in the AF were higher than the corresponding ratios of the reactor suspended biomass. PS/COD ratios in biofilms exceeded those of the reactors by 40-500%. On the contrary, AF PR/COD ratios seldom reached the value characteristic of the reactor biomass. PR/COD ratios in biofilms were 50-110% of the SPR/SCOD ratios of the corresponding reactors.

The above results suggest no involvement of proteinaceous materials in the attachment of acetoclastic methanogenic bacteria. The results also indicate a significant role of polysaccharide in the biofilm formation of these bacteria.

The results of this study agree with those of Beeftink (1987), who also found an increased polysaccharide content in anaerobic microbial aggregates compared to free suspended cells. In a study of acidogenic bacterial aggregates he noted that steady-state aggregates contained 15-20% sugar (as glucose, w/w on a dry weight basis), while freely suspended cells of a low dilution rate culture contained three

times less polysaccharide. The origin of the extra amounts of PS is not yet clear.

Tago and Aida (1977) have found a polysaccharide fraction appearing in flocs exclusively. They have shown that after treatment with a deflocculating enzyme, no such polysaccharide was present in the resulting free cells. However, it is not clear, how much of this polysaccharide the freely suspended cells contained prior to floc formation, i.e., whether the presence of this fraction is the cause or the result of floc formation.

Marshall (1985) in his review raises the possibility that some bacteria may produce a polymer for solely adhesive purposes and following adhesion they may produce a second polymer. The function of the second polymer can be either to "cement" the adhesive bond, to provide an amenable surface to other microorganisms or to protect the bacteria from adverse environmental effects and predators.

In this study, the high percentage (12-30%) of soluble PS suggested that it may be the source of the extra PS in the biofilm. Increase of soluble PS concentration, however, coincided with decreasing rates of AFPS formation (Fig. 4.21). Furthermore, increase rather than decrease of soluble reactor PS concentration with time was observed during all experiments, with the exception of reactor E. The above evidence does not support the participation of soluble polysaccharides in biofilm formation. It does not, though, exclude the possibility that a particular fraction of solu-

ble PS is involved in attachment, the quantity of which is not related to the total soluble PS concentration. The importance of suspended PS is emphasized by the positive relationship between SPS concentration and AFPS formation rate (Fig. 4.25). In situ PS production may also play a role in the case of active cultures.

It is concluded that the available information is not sufficient to identify the source(s) of the extra PS amounts found in acetoclastic methanogenic biofilms.

Trulear and Characklis (1982) also observed change in biofilm density. In their experiment biofilm formation was due solely to growth and exopolymer production, not attachment. The increase in biofilm density was observed as a result of increased glucose loading rate. An increased glucose reaction rate of the biofilm biomass was also noted and was thought to be due to the density increase.

In this study, activity increased in parallel with an increase in acetate loading rate. It is believed that loading rate affected biofilm density indirectly, through the increase of biomass activity. As high biomass activity was present prior to the start of the attachment experiment, it is likely to be the cause, not the result of high biofilm density.

5.3 Loose Biofilm Development and Characteristics

The presence of a loosely held biomass within the test section was detected in every experiment. The rate at which the LF developed and its composition was dependent on the experimental conditions.

5.3.1 Effect of Flow Velocity on Loose Biofilm Development and Characteristics

A negative correlation was observed between flow velocity and loosely held biofilm formation rate. This suggests that at sufficiently high velocities no loose biofilm would form, the shear exerted by the liquid flow would be greater than the forces keeping the loose biomass in place. Flow velocity did not affect noticeably the composition of the loose biofilm.

5.3.2 Effect of Support Material on Loose Biofilm Development and Characteristics

On silicone tubing, loose film development rates were much higher than on Tygon tubing. LF development rates decreased linearly with flow velocity on silicone tubing. On Tygon, the LF formation rate at a low velocity was disproportionately higher than rates measured at higher velocities (Figs. 4.14, 4.15 and 4.16).

For silicone tubing the estimated flow velocity above which no loose development would occur was 3.3-7.8 cm/s.

For Tygon tubing, the corresponding range of 4.1-16.8 cm/s was estimated.

In Experiment #1 an interesting phenomenon was observed in the development of the loose biofilm on silicone tubing surface. The amount of loose biofilm increased at a high rate until a certain maximum was reached. With higher liquid flow velocity, the maximum was reached faster and its value was lower. The maximum value was 820-860 µg COD/cm² for the two higher velocities, and 1100 µg COD/cm² for the low flow velocity. After reaching the maximum, the amount of loose biofilm started to decline, more rapidly with higher flow velocity. At the termination of the experiment, almost no loose biofilm could be detected in silicone tubing (Fig. 4.9).

Loose film values on Tygon tubing were lower than those measured on silicone support, except the values obtained with the lowest velocity. These values were almost identical to the maximum amounts obtained on silicone with the lowest velocity. It is seen, that COD values at 64 and 72 hours show a sharp decline (Fig. 4.8). PR value measured at 72 hours indicate that the increase of LF PR had stopped. Unfortunately, the last two PS data are missing.

It is concluded, that the same mechanism has been observed for both support surfaces. It is not clear, why and how the phenomenon happens.

5.3.2.1 A Hypothetical Mechanism of Attachment at Low Flow Velocities

Several possibilities were considered to identify the cause of the decrease and disappearance of LF. It was first suspected that the decline of the loose biofilm is connected with the attainment of the plateau film thickness. However, in all cases, biofilm thickness was well below the maximum value reached, when the decrease of loose biomass started.

The change in flow velocity as a result of decreased cross-sectional flow area was also investigated. It was found, that in the presence of a 200 μm thick biofilm, velocities employed in silicone tubing would increase only slightly, resulting in 1.15, 0.81 and 0.06 cm/s velocities, respectively (Appendix I/b). These velocities are not high enough to account for the disappearance of the loose biofilm.

It was also considered that the commencement of the decline is associated with a certain value of biomass indicators reached in the AF. This reasoning is contradicted by the fact that attached biomass on Tygon tubing was far less than on silicone. For Tygon tubing, AF was actually lowest for the low velocity in which case the commencement of decline was observed.

It seems that it is a maximum level of the loose rather than the attached biomass, which leads to the decline of the loose biofilm, once the maximum level is reached. The maximum value is somewhat dependent on the flow velocity. The

observation that loose biofilm in Tygon tubing started to decline at similar loose COD, PR and PS concentrations as in silicone tubing supports the above explanation. It is also possible, that a critical level of the total biomass has to be reached for the decline of loose biofilm to occur. This possibility is supported by the observation that total biofilm values reached a plateau relatively soon (within 18-24 hours for COD and PR) and stayed at that level thereafter (Figs. J.7-J.9, Table J.2A).

Examining the data of the other experiments, similar decrease or disappearance of loose biofilm was not found. It is thought to be due to the fact that sufficiently high total COD, PR and PS accumulations were not reached, except as the last value in experiment E.

From the above observations a mechanism of biofilm formation at low velocities (0.05-1.42 cm/s) is pictured as follows. Along with a steady attached biofilm formation from the very beginning, a large, loosely held biomass establishes itself rapidly in the test section. The forces allowing this biomass to stay in place are not known. It is possible that polysaccharide products have a role in this attachment, as polysaccharides in excess to the suspended biomass were found in loose biofilms. It is hypothesized, that some and possibly most of the bacteria join the attached film via the loose biofilm, as an intermediate stage. The loose biofilm may be in dynamic equilibrium with the suspended biomass as well as with the attached biofilm. The

loose biofilm can accept bacteria from the suspension as long as bacteria can eventually join the attached biofilm. After a critical total biomass is reached, no more bacteria join the loose biofilm, but the transformation of loose biofilm into attached biofilm continues until loose biofilm almost completely disappears. It is not yet understood what factors determine the magnitude of the critical biomass.

The above mentioned mechanism may exist at low flow velocities, but it is not expected to play any role at sufficiently high velocities. Instead of loose-attached transformation, at higher velocities the bacteria may join the biofilm constantly as they flow by. This process may be a slower one, as attached biofilm development was found to slow down when the loose biofilm disappeared.

5.3.2.2 Loose Biofilm Characteristics

PS/COD and PS/PR ratios in loose biofilm were higher than in the reactor for both support surfaces. Loose biofilm on Tygon tubing contained less PR/COD but more PS/COD than LF on silicone tubing (Figs. 4.18 and 4.19). Also, for Tygon, the loose fraction constituted a higher percentage of the total biofilm than for silicone (Table 4.3).

In the case of Tygon tubing, more polysaccharide was needed per unit of protein to hold the loose biomass in place than was required in the attached biofilm (Table 4.7).

The differences in loose biofilm formation rate and film characteristics between the two kinds of tubing may be

explained by assuming that surface properties can affect processes more remote from the surface, such as loose biofilm formation. The observed difference may also be due to the slightly larger diameter of the Tygon tubing. Perhaps because of the larger diameter more polysaccharide was required to hold the loose biomass in place.

5.3.3 Effect of Suspended Biomass Concentration on Loose Biofilm Development and Characteristics

Loose biofilm development rate increased linearly with the concentration of suspended biomass present in the reactor. The increase was such that the loose biofilm development rate was about twice as high as the attached film development rate at each suspended biomass concentration (Table 4.11). It is concluded that loose biofilm development rate is more sensitive to suspended biomass concentration than attached biofilm development rate. It may be an indication of an intimate connection between suspended and loosely held biomass.

It can be seen in Figs. 4.26-4.28, that while deposition of COD and PR in attached and loose films was a parallel process, PS deposition in attached film occurred in the absence of loose PS formation as well. This finding suggests a sequence of events of biofilm formation at low flow velocities. The starting step would be deposition of PS on the surface, followed by the appearance of loose PS and subsequently loose and attached PR and COD.

A certain limiting concentration of biomass was found to be needed for loose film development to occur. For loose PS this VSS concentration was found to be 320 mg/L, for LF-COD and LFPR it was 370-390 mg VSS/L (Table 4.12).

The PR/COD ratio in the loose film was found to increase with increasing reactor biomass. PS/COD and PS/PR ratios were not affected significantly, although PS/COD was highest in the case of lowest reactor biomass (Figs. 4.31 and 4.32). The results indicate that the protein content of the loose biofilm reflects the suspended biomass concentration, while the polysaccharide content does not.

Higher percentages of the total COD and PR and a lower percentage of PS were found in loose film when suspended biomass concentration was low (Table 4.15). Possibly the loose-attached conversion happens more slowly in the case of low biomass concentration, because the loose biofilm itself is less dense. This possibility is supported by the decreasing LF PR/COD ratio with decreasing biomass.

5.3.4 Effect of Biomass Metabolic Activity on Loose Biofilm Development and Characteristics

In Part I the inactive culture loose film development rates were much slower (5-8 fold) than the rates predicted based on the biomass concentration of the reactor (Table 4.19). It is therefore concluded that lack of activity impairs locse film development more severely than it retards attached film formation.

The above observation is also reflected in loose biofilm characteristics. In the case of the inactive culture, loose biofilm contained less PS/COD and PS/PR than in the case of the previously active culture (Table 4.20). PS/COD and PS/PR ratios in LF exceeded ratios in the reactor only slightly (Table 4.21). A somewhat higher percentage of the total PR and PS was found in attached form for the inactive culture E (Table 4.22).

In Part II it was found that a six-fold increase in loose film development rate occurred with an actually active culture (F) compared to the inactive one (E). However, when biomass specific activity increased further (culture G), no further increase in loose biofilm development rate was seen (Table 4.25). Similar loose biofilm development rates to those measured with cultures F and G were found using reactor D (Table 4.10), but higher rates were not observed in any of the experiments. The stagnation of loose film formation rate did not bring about similar behavior in attached film formation rate. AF development rate remained linearly proportional to biomass acetoclastic activity. The rates of attached and loose film developments obtained with culture G were almost identical.

The above findings suggest that under the experimental conditions employed, there may be a limit to the loose film development rate above which it cannot be increased with increase of metabolic activity. This limit may be a function of VSS concentration, but it may also be a function of the

experimental system (test section surface and geometry). It was also noted that attached film formation rate was not significantly higher than loose biofilm development rate in any of the experiments. This observation supports that in a low velocity system there may indeed be an intimate connection between loose and attached biofilm formation.

In the two active cultures (F and G) PS/COD ratios in loose film were higher than in the case of culture E. As biomass activity increased, a higher percentage of the biomass indicators was found attached.

5.4 Active versus Passive Attachment

Previously it was found that biofilm formation rate was linearly proportional to the suspended biomass concentration. This observation suggests a passive mechanism of attachment. In Experiment #3 however, it was found that regardless of the VSS concentration culture E was unable to produce the expected film development rates because it was inactive. In Part II, higher film formation rates were found as biomass specific activity increased, again regardless of even lower VSS concentration. The results of these experiments suggest that an attachment mechanism assuming actively participating bacteria has to be considered in these cases.

The fundamental role of polysaccharides in the attachment of acetoclastic methanogens was demonstrated in all the experiments. It is therefore the quantity and quality of

polysaccharides present that is expected to affect attachment significantly.

It is known (Ellwood and. Tempest, 1972) that PS molecules produced by the cells may be different depending on nutritional conditions and metabolic state. Tago and Aida (1977) studied the role of exopolysaccharides in floc formation of a *Pseudomonas* strain. They found that it was not the total amount of extracellular polysaccharide which participates in aggregate formation, but only a certain small fraction of it. The PS fraction present in flocs was not detectable in cells released from the floc by a deflocculating enzyme. The film mesh in which the cells of the floc were embedded also disappeared as a result of the treatment with the enzyme. The fraction unique to the floc was identified as a mucopolysaccharide and accounted for only ten percent of the total polysaccharide.

In the light of the above data the results of this experiment may be interpreted in the following way. It is possible that in the case of methanogenic bacteria there is also a specific type of polysaccharide that is involved in attachment. Active cells may produce this polysaccharide continuously or upon stimulus. In a starving culture these molecules may be decomposed and not reproduced, leading to a polymer concentration insufficient for attachment. Some attachment may be possible in this case by making use of cell lysis products (Harris and Mitchell, 1973).

The appearance of substrate provides a stimulus leading to various changes in metabolic activities, which may include the production of a PS for attachment purposes (Marshall, 1985). The presence of these PS molecules greatly increases the rate of attachment, even if the molecules may not be actually produced during the attachment experiment. This is thought to be the case regarding reactor D. Increasing metabolic activity may result in a more rapid production of the specific PS, thereby increasing the concentration of the bridging polymer in solution.

The production of a cementing material (presumably PS) after reversible adhesion may further contribute to the positive effects of high metabolic activity.

Metabolic changes may also involve changes in membrane structure, such as appearance of binding sites necessary for attachment on the cell surface (Fletcher, 1985).

Factors enhancing passive and active attachment are expected to exert a cumulative positive effect on attachment when they occur simultaneously.

CHAPTER 6

CONCLUSIONS

Initial attachment properties of acetoclastic methanogenic bacteria were investigated in a downflow system. The following conclusions have been drawn from the study.

1) Biofilm formation rates found in this study were substantially higher than those reported in the literature with mixed anaerobic cultures. Thus acetoclastic methanogens are likely to have better adhesive properties than acidogens.

2) A loosely held biomass formed in the test sections simultaneously with the attached film development. It is thought to be an intermediate stage of biofilm formation at the low liquid flow velocities (0.05-1.42 cm/s) used in these experiments.

3) The ratio of polysaccharide to COD exceeded that of the reactor suspended biomass in both attached and loose biofilms. PS/COD was 40-500% higher in the attached film than in the reactor. Thus the polymer involved in biofilm formation is most likely a polysaccharide. Proteinaceous materials were not found to play a role in attachment.

4) For silicone tubing the maximum biofilm thickness reached in each experiment was 250-270 μm, regardless of different experimental conditions. The rate of film thickness increase was different for different experimental conditions. The plateau value is likely to be related to the test section material and geometry.

5) Liquid flow velocity changes in the range of 0.05-1.42 cm/s did not significantly affect biofilm characteristics. A small linear increase in attached film formation rate was observed with higher flow velocities. On the contrary, loose film development was increasingly retarded by higher flow velocities.

6) Both attached and loose biofilm developed at a lower rate on Tygon surface than on silicone surface. Attached biofilms on silicone surface were thicker and denser than films on Tygon surface. Thus silicone proved to be a better support material for biofilm formation than PVC (Tygon), likely owing to its larger pore size.

7) Biofilm development rates were directly proportional to the suspended biomass concentration in the reactor. Loose film development rate was twice as sensitive as the attached film development rate to changes in reactor VSS concentration. In absence of substrate a limiting VSS concentration of 120 mg/L was found, below which no or very slow film formation would be expected.

8) Polysaccharide concentration in biofilms did not reflect changes of suspended polysaccharide concentration in the reactor. PS deposition rates depended on the reactor SPS concentration to a lesser degree than PR and COD formation rates depended on SPR and SCOD concentrations in the reactor. The results show a distinct behavior of PS from PR, a measure of the bacterial cells.

9) Lack of general specific acetoclastic activity impaired both attached and loose film development rates. Loose biofilm formation was more severely affected.

10) Attached biofilm development rates were directly proportional to actual specific acetoclastic activities in the range of 0-0.813 mg COD/mg VSS·d. The density of the attached biofilm was also a linear function of actual activity. These results imply active participation of the bacteria in the attachment process.

CHAPTER 7

FUTURE STUDIES

The following topics are suggested as possible subjects for further investigation:

1) Verify experimentally the existence of an attachment limiting VSS concentration in an actually inactive culture.

2) Conduct further experiments to determine the effect of reactor VSS concentration and substrate concentration on the attachment of an active metabolizing methanogenic culture.

3) Isolate the specific polysaccharide compound(s) involved in the film formation of acetoclastic methanogens. Identify the origin of the PS molecules and the way in which their production is induced.

4) Conduct experiments to determine attachment properties of acidogenic bacteria.

5) Recirculating an enriched methanogenic culture prior to the normal procedure of anaerobic reactor inoculation may greatly reduce the startup time. Verify this experimentally.

6) Investigate the effect of channel diameter on biofilm attachment rates, ultimate biofilm thickness and biofilm characteristics.

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APPENDIX A

APPENDIX A

A.1 Biofilm Volume Determination by Volume Displacement Technique

The measurement is based on the fact that the volume of an immersed object equals the liquid volume displaced by it. The volume of liquid displaced was determined by measuring the change of the height of the liquid surface in a measuring dish after the test section was immersed.

Fifty mL of glass distilled water was used as the immersion liquid. A stainless steel dish of 3.8100 $\pm$ 0.0127 cm inside diameter (d_d) and 6.35 cm height was used as a measuring dish. A micrometer capable of measuring a 2.54 cm height difference in 0.0001 inch gradations was used to measure the liquid height. The micrometer was mounted on a plexiglass platform and it was supported by three legs in such a way that the measuring dish could be placed underneath. A circular depression was created in the plexiglass plate to ensure the exact same placement and stability of the dish in each measurement. The distance between the platform and the tip of the micrometer was 3.8 cm when the micrometer was set to the 0" gradation. A photograph of the volume measuring device is shown in Fig. A.1.

A reading was taken when the tip of the micrometer just touched the quiescent water surface. The height of the water surface was recorded before and after a clean or biofilm containing test section was immersed. The height difference

(h_t or h_{f+t}) multiplied by the cross-sectional area (A_d) of the dish yielded the volume of the test section (V_t) or the combined volume of test section and biofilm (V_{f+t}).

$A_d = d_d^2\pi/4 = 1.767$ in^2

One inch height difference corresponds to an 1.767 in^3 or 28.958 cm^3 volume displacement.

Δh_t height difference corresponds to V_t volume displacement.

V_t [cm^3] = $\Delta h_t \cdot 28.958$

The volume of biofilm (V_f) is the difference between the volume of the biofilm containing test section (V_{f+t}) and the volume of the clean test section (V_t).

Example calculation is shown for the duplicate samples taken at 10.5 hours in Run E.

(1) $\Delta h_{f+t} = 0.13075$"; $\Delta h_t = 0.12378$"

$V_{f+t} = 0.13075 \cdot 28.958 = 3.78626$ mL

$V_t = 0.12378 \cdot 28.958 = 3.58442$ mL

$V_f = 3.78626 - 3.58442 = 0.20184$ mL

(2) $\Delta h_{f+t} = 0.11960$"; $\Delta h_t = 0.11449$"

$V_{f+t} = 0.11960 \cdot 28.958 = 3.46338$ mL

$V_t = 0.11449 \cdot 28.958 = 3.31540$ mL

$V_f = 3.46338 - 3.31540 = 0.14798$ mL

Average $V_f = 0.17491$ mL

The volume displacement method had a standard deviation of 0.0019" in terms of Δh_t, which resulted in a standard de-

viation of 28 μm in terms of biofilm thickness (calculation not shown).

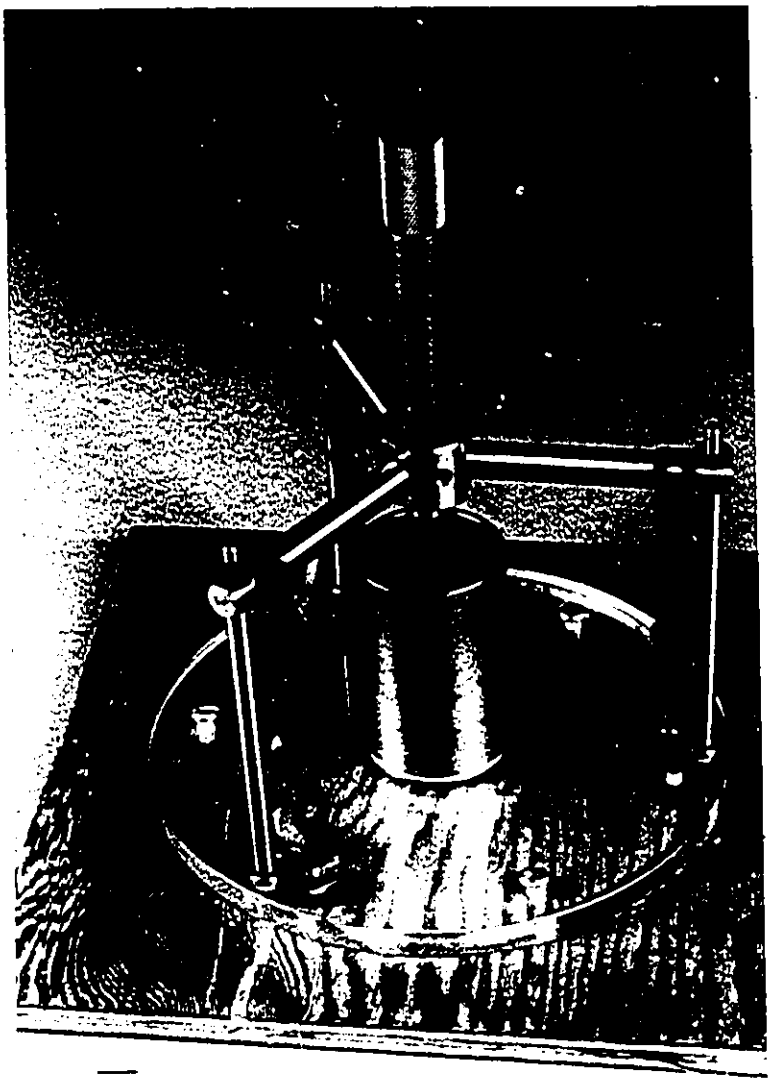

Figure A.1 Photograph of the volume measuring device

APPENDIX B

APPENDIX B

B.1 Calculation of Biofilm Thickness

B.1.1 Calculation of the Inner Surface Area of the Test Section (A_t)

A_t is calculated from the volume of the clean test section measured by the volume displacement technique and from the dimensions of the plastic tubing.

The volume of the test section equals the length of the section (l_t) multiplied by the ring-shape cross-sectional area of the tubing (A_c).

$$V_t = l_t \cdot A_c$$

where

$$A_c = (o.d.)^2\pi/4 - (i.d.)^2\pi/4$$

For silicone tubing of 1.111 cm o.d. and 0.794 cm i.d., A_c is 0.475 cm^2. Then the tubing length is

$$l_t = V_t/A_c = \Delta h_t \cdot 28.958 / 0.475 = \Delta h_t \cdot 60.959$$

The inner surface area equals the inner circumference of the tubing multiplied by the length of the section:

$$A_t = (i.d)\pi \cdot l_t = 0.795 \cdot \pi \cdot \Delta h_t \cdot 60.959$$

$$= \Delta h_t \cdot 152.01$$

An example calculation is shown for the duplicate samples taken at 10.5 hours in run E.

(1) $\Delta h_c = 0.12378"$

$A_c = 0.12378 \cdot 152.01 = 18.816\ cm^2$

(2) $\Delta h_c = 0.11449"$

$A_c = 0.11449 \cdot 152.01 = 17.4036\ cm^2$

B.1.2 Calculation of Biofilm Thickness

Biofilm thickness (FT) is obtained by dividing the biofilm volume (V_f) by the inner surface area of the test section (A_c).

$FT\ [\mu m] = V_f\ [cm^3] / A_c\ [cm^2] \cdot 10{,}000$

The film thickness is then

$FT = V_f/(\Delta h_c \cdot 152.01) \cdot 10{,}000 = (V_f/\Delta h_c) \cdot 65.785$

An example calculation is shown for the duplicate samples taken at 10.5 hours in Run E.

(1) $FT = (0.20184/0.12378) \cdot 65.785 = 107.27\ \mu m$

(2) $FT = (0.14798/0.11449) \cdot 65.785 = 85.03\ \mu m$

Average FT = 96.15 µm (Table L.2)

APPENDIX C

APPENDIX C

C.1 Calculation of Attached Biofilm Indicators

AFCOD (as well as AFPR and AFPS) were determined from the 55 mL of liquid in which the biofilm was dispersed after volume measurement and sonication. Therefore the COD content of the whole biofilm is given by

$$AFCOD\ [mg] = AFCOD\ [mg/L] \cdot 0.055\ L$$

COD values as $\mu g/cm^2$ are obtained when the COD content of the whole biofilm is divided by the inner surface area of the test section (A_t):

$$AFCOD\ [\mu g/cm^2] = AFCOD\ [mg/L] \cdot 55 / A_t$$
$$= AFCOD\ [mg/L] \cdot 55 / (\Delta h_t \cdot 152.01)$$
$$= (AFCOD\ [mg/L] / \Delta h_t) \cdot 0.3618$$

AFPR and AFPS values as $\mu g/cm^2$ were calculated similarly.

The above calculated value needs to be corrected with the blank value. The blank values were: 12.61 µg COD/cm^2, 0.00 µg PR/cm^2 and 2.26 µg PS/cm^2.

An example calculation is shown for the duplicate samples taken at 10.5 hours in Run E.

(1) $AFCOD\ [mg/L] = 71.5\ mg/L$

$$AFCOD\ [\mu g/cm^2] = (71.5/0.12378) \cdot 0.3618 - 12.61$$
$$= 196.38\ \mu g/cm^2$$

(2) AFCOD [mg/L] = 40.8 mg/L

AFCOD [$\mu g/cm^2$] = (40.8/0.11449) · 0.3618 - 12.61

= 116.32 $\mu g/cm^2$

Average AFCOD = 156.36 $\mu g/cm^2$ (Table L.3)

APPENDIX D

APPENDIX D

D.1 Calculation of Loose Biofilm Indicators

COD, PR and PS values as mg/L were determined from the liquor removed from the test section during sampling (COD_{es}). The corresponding values measured in samples taken from the sampling ports (COD_p) were then subtracted from these values. In this way COD, PR and PS concentrations in excess to those in the liquid flowing through the section were obtained and termed loose biofilm.

$$COD_{es}\ [mg/L] - COD_p\ [mg/L] = LFCOD\ [mg/L]$$
$$= LFCOD\ [\mu g/mL]$$

From the length of the test section (l_e) and the cross-sectional area of flow (A_{es}) the volume of liquid inside of the test section is calculated (V_L). For silicone tubing:

$$V_L\ [mL] = (i.d.)^2\pi/4 \cdot l_e = 0.495 \cdot \Delta h_e \cdot 60.969$$
$$= \Delta h_e \cdot 30.164$$

The amount of loose COD present in this volume is

$$LFCOD\ [\mu g] = LFCOD\ [\mu g/mL] \cdot V_L\ [mL]$$
$$= LFCOD\ [\mu g/mL] \cdot \Delta h_e \cdot 30.164$$

LFCOD as $\mu g/cm^2$ is obtained when the above amount is divided by the inner surface area of the test section (A_e):

$$LFCOD\ [\mu g/cm^2] = LFCOD\ [\mu g] / A_e\ [cm^2]$$
$$= LFCOD\ [\mu g/mL] \cdot \Delta h_e \cdot 30.164 / \Delta h_e \cdot 152.01$$
$$= LFCOD\ [\mu g/mL] \cdot 0.1984$$

An example calculation is shown for the duplicate samples taken at 10.5 hours in Run E.

(1) COD_{el} = 2298 mg/L; COD_{p} = 1312 mg/L

LFCOD [mg/L] = 2298 - 1312 = 986 mg/L

LFCOD [$\mu g/cm^2$] = 986 · 0.1984 = 195.62 $\mu g/cm^2$

(2) COD_{el} = 2148 mg/L; COD_{p} = 1413 mg/L

LFCOD [mg/L] = 2148 - 1413 = 735 mg/L

LFCOD [$\mu g/cm^2$] = 735 · 0.1984 = 145.82 $\mu g/cm^2$

Average LFCOD = 170.72 $\mu g/cm^2$ (Table L.3)

APPENDIX B

APPENDIX E

E.1 Calculation of Concentration of Indicators in Biofilm

Concentration in biofilm is obtained when $\mu g/cm^2$ values are divided by the film thickness.

AFCOD [$\mu g/cm^2$] $\cdot 10^{-3}$ = AFCOD [mg/cm^2]

FT [μm] $\cdot 10^{-4}$ = FT [cm]

(AFCOD [$\mu g/cm^2$] / FT [μm]) $\cdot$ 10 = AFCOD [mg/mL]

An example calculation is shown for the duplicate samples taken at 10.5 hours in Run E.

(1) AFCOD = 196.38 $\mu g/cm^2$

FT = 107.27 μm

AFCOD [mg/mL] = (196.38/107.27) $\cdot$ 10 = 18.307 mg/mL

(2) AFCOD = 116.32 $\mu g/cm^2$

FT = 85.03 μm

AFCOD [mg/mL] = (116.32/85.03) $\cdot$ 10 = 13.680 mg/mL

Average AFCOD concentration = 15.99 mg/mL (Table L.4)

APPENDIX F

APPENDIX F

F.1 Calculation of Ratios in the Reactor and in the Biofilm

An example calculation is shown for the duplicate samples taken at 10.5 hours in Run E.

Reactor ratio

SCOD = 1248 mg/L; SPR = 689 mg/L

SPR/SCOD = 689/1248 = 0.552 mg/mg at 10.5 hours

Average for the experiment:

SPR/SCOD = (0.552+0.571+0.646+0.601+0.595)/5 = 0.593

(Tables L.1, 4.18 and 4.24)

AF ratio

(1) AFCOD = 196.38 $\mu g/cm^2$; AFPR = 86.15 $\mu g/cm^2$

AF PR/COD = 86.15/196.38 = 0.4387 mg/mg

(2) AFCOD = 116.32 $\mu g/cm^2$; AFPR = 56.39 $\mu g/cm^2$

AF PR/COD = 56.39/116.32 = 0.4848 mg/mg

Average AF PR/COD = 0.462 mg/mg at 10.5 hours

Average for the experiment:

AF PR/COD = (0.462+0.531+0.469+0.403+0.442)/5 = 0.461

(Tables L.6, 4.20 and 4.27)

AF PR/COD for the experiment normalized to the reactor SPR/SCOD ratio:

(AF PR/COD)/(SPR/SCOD) = 0.461/0.593 = 0.778

(Tables 4.21 and 4.28)

Ratios for loose biofilm were calculated similarly.

APPENDIX G

APPENDIX G

G.1 Chemical Oxygen Demand Analysis for Dilute Samples

The method is suitable to measure COD of undiluted samples up to 145 mg/L concentration.

Reagents

1. Standard solution: 850 mg/L potassium acid phtalate. Represents 1000 mg/L COD.

2. Digestion solution: 0.03 N potassium dichromate solution.

Contains
- 1.4709 g $K_2Cr_2O_7$ (dried)
- 10 g $HgSO_4$
- 100 mL concentrated H_2SO_4

in 1 L glass distilled water.

3. Catalyst: 22 g silver sulfate in a 9 lb bottle of concentrated H_2SO_4 (9 g/L)

4. Titrant: 0.011 N ferrous ammonium sulfate solution.

Contains
- 4.312 g $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$
- 20 mL concentrated H_2SO_4

in 1 L glass distilled water.

Standardized in triplicate daily against 6 mL digestion solution diluted with 54 mL double distilled water after addition of 20 mL concentrated H_2SO_4.

5. Indicator: ferroin. Three (3) drops were used. The sharp color change from royal blue to brown indicated the endpoint.

Procedure

Samples were digested in KIMAX screw-cap culture tubes (25x150 mm; GPI thread 24-410).

Between uses the tubes were cleaned by rinsing with distilled water several times and with double distilled water three times. After rinsing the tubes were dried at 150°C.

Ten (10) mL of sample, six (6) mL of digestion solution and fourteen (14) mL of catalyst solution were placed in a culture tube. The tube was then capped and mixed with a Vortex mixer. Three blanks and duplicate standards containing either 20 mg/L or 100 mg/L COD were prepared simultaneously and analyzed with each set of samples. All the tubes were heated to 150°C in an oven for 3 hours. After cooling, the excess dichromate in each sample was determined by titration with standardized ferrous ammonium sulfate. The titration was performed directly in the culture tube, while the sample was mixed rapidly with a magnetic stirrer.

COD was calculated with Eq. G.1:

$$COD\ [mg/L] = (bl - s) \cdot N \cdot 8000 / V_s \qquad (G.1)$$

where

bl is the volume of titrant required for blank [mL]
s is the volume of titrant required for sample [mL]
N is the normality of the titrant
V_s is the sample volume [mL]

Precision

Standard deviations of ± 2.72 mg/L and ± 1.1 mg/L were found at 100 mg/L and at 20 mg/L COD concentration respectively, using potassium acid phtalate samples. The results are summarized in Table G.1.

Table G.1 COD analysis on potassium acid phtalate samples

COD concentration mg/L	100	20
Measured COD mg/L	100.20	19.70
	98.80	21.05
	100.03	19.10
	97.10	19.08
	97.80	17.70
	91.30	20.09
	99.97	20.45
	98.10	
	98.23	
Mean value, mg/L	97.95	19.60
Standard deviation, mg/L	2.72	1.096
Standard deviation, %	2.78	5.59

APPENDIX H

APPENDIX H

H.1 Protein Determination Method for Dilute Samples

The method is a modification of Lowry's method, suggested for protein values under 25 mg/L in the publication by Lowry *et al.* (1951).

Reagents

1. 2% wt Na_2CO_3 in 0.1 N NaOH
2. 1% wt $CuSO_4 \cdot 5H_2O$
3. 2% wt sodium citrate
4. 1 N Folin reagent
5. Standard: bovine albumin in saline solution, containing 0.05% sodium azide

Procedure

Reagent I was prepared freshly by mixing 1 mL of 1% $CuSO_4$ with 1 mL of 2% sodium citrate and adding 50 mL of 2% Na_2CO_3 in 0.1 N NaOH. A 2.5 mL volume of Reagent I was added to 2.5 mL sample, mixed and let stand for 10 minutes. Thereafter 0.5 mL Folin reagent was added to each sample and mixed rapidly. Absorbance was read after 30 minutes at 750 nm with a Beckman DU-40 spectrophotometer.

Duplicate blanks and standard series were prepared along with each set of samples.

APPENDIX I

APPENDIX I

I.1 Measurement of Actual Acetoclastic Activity

Actual acetoclastic activity was measured during the attachment experiment.

In a continuously fed reactor acetate consumption rate (R_A) is computed according to Eq. I.1.

$$R_A\ [mg/L \cdot d] = (c_{t1} - c_{t2} + c_t) \cdot 24/\Delta t \qquad (I.1)$$

where

c_{t1} is the acetate concentration in the reactor at $t = t_1$, mg/L
c_{t2} is the acetate concentration in the reactor at $t = t_2$, mg/L
Δt is the time elapsed between t_1 and t_2, hours
c_t is the acetate concentration added to the reactor in the t interval, mg/L

$$c_t\ [mg/L] = (c_A \cdot V_A) / V_R \cdot (\Delta t/24) \qquad (I.2)$$

where

V_A is the volumetric feed rate, L/d
c_A is the acetate concentration in the feed solution, mg/L
V_R is the volume of the reactor, L

An example calculation is shown for reactor A.

$V_A = 0.048$ L/d; $c_A = 36725$ mg acetate/L

$V_R = 3$L; $\Delta t = 30$ h

$c_t = (36{,}725 \cdot 0.048) / 3 \cdot (30/24) = 734.5$ mg/L

$c_{t1} = 1100$ mg/L; $c_{t2} = 1176$ mg/L

$R_A = (1100 - 1176 + 734.5) \cdot (24/30) = 526.8$ mg/L·d

Computations for the whole experiment are summarized in Table I.1.

Table I.1. Activity calculations for reactor A

V_A	c_A	V_R	Δt	c_e	c_{e1}	c_{e2}	R_A
L/d	mg/L	L	h	mg/L	mg/L	mg/L	mg/L·d
0.048	36725	3	30	734.5	1100	1176	526.8
0.042	36725	3	17	365.9	1176	1109	611.2
0.042	37325	3	6	131.4	1109	1123	469.6
0.042	37350	3	18	394.4	1123	1080	583.2
0.042	37287	3	24	525.0	1080	1077	528.0
0.042	40500	3	48	1140.5	1077	1183	517.3
							539.4

The average acetate consumption rate during the experiment was 539.4 mg/L·d. Expressed in COD equivalents

R_A = 539.4 · 1.066 mg COD/mg acetate = 575 mg COD/L·d

The average VSS concentration was 610 mg/L during the experiment.

The actual specific activity of the biomass (U) during the experiment was

U = 575/610 = 0.943 mg COD/mg VSS·d (Table 4.2)

The reactor volume was kept constant during the experiment.

APPENDIX I/a

I/a.1 Calculation of Zero Potential Specific Acetoclastic Activity

In a batch activity test performed in a manner described in 3.9.9. the following results were obtained for stock culture II:

Time elapsed (hours)	Specific Acetoclastic Activity (mg COD/mg VSS·d)
17.30	0.181
29.55	0.597
42.25	0.917

The data show an increase of specific activity with time. Linear regression analysis of the data yielded a correlation coefficient of 0.996, a slope of 0.0295 and an y-intercept of -0.31. The negative intercept indicates that at t=0 the specific acetoclastic activity of the culture was zero, and the activity started to increase after a lag period.

APPENDIX I/b

I/b.1 Calculation of Reynolds Numbers and Liquid Flow Velocities

$$Re = \frac{\rho \cdot v \cdot d}{\mu}$$

where the density and viscosity of the water at 35°C were used to approximate those of the reactor contents

$\rho = 993.95\ kg \cdot m^{-3}$

$\mu = 0.7255\ E\text{-}3\ Ns \cdot m^{-2}$

d is the internal diameter of the test section

v is the liquid flow velocity through the test section, calculated as the flow rate divided by the cross-sectional area of the test section

In absence (FT=0 µm) and in presence (FT=200 µm) of biofilm diameter, flow velocity and Reynolds number values differ slightly.

Silicone tubing	FT = 0 µm			FT = 200 µm		
Pump delivery rate mL/min	d cm	v cm/s	Re -	d cm	v cm/s	Re -
30.78	0.794	1.08	117	0.754	1.15	119
21.66	0.794	0.76	82.7	0.754	0.81	83.7
1.60	0.794	0.056	6.09	0.754	0.060	6.20

Tygon tubing	FT = 0 µm			FT = 200 µm		
Pump delivery rate mL/min	d cm	v cm/s	Re -	d cm	v cm/s	Re -
107.95	1.270	1.42	247	1.230	1.51	254
48.65	1.270	0.64	111.3	1.230	0.68	114.6
4.10	1.270	0.054	9.40	1.230	0.058	9.77

APPENDIX J

Table J.1 Reactor data

A

d	SCOD mg/l	SPR mg/l	SPS mg/l	VSS mg/l
1	1351	594	57.2	655
2	990	580	61.8	-
3	1191	511	58.2	586
4	1017	522	46.9	-
5	1299	587	75.2	-
6	929	450	56.2	541
7	809	374	46.0	656
ave.	1084	517	57.4	610

d	SCOD/VSS mg/mg	SPR/VSS mg/mg	SPS/VSS mg/mg
1	2.06	0.907	0.087
2	-	-	-
3	2.03	0.872	0.099
4	-	-	-
5	-	-	-
6	1.72	0.832	0.104
7	1.23	0.570	0.070
ave.	1.76	0.795	0.090

d	SPR/SCOD mg/mg	SPS/SCOD mg/mg	SPS/SPR mg/mg
1	0.440	0.042	0.096
2	0.586	0.062	0.107
3	0.429	0.049	0.114
4	0.513	0.046	0.090
5	0.452	0.058	0.128
6	0.484	0.060	0.125
7	0.462	0.057	0.123
ave.	0.481	0.054	0.112

Table J.2 Attached and loose film development vs time

TYGON

hours	AFCOD µg/cm2	AFPR µg/cm2	AFPS µg/cm2	LFCOD µg/cm2	LFPR µg/cm2	LFPS µg/cm2
	v=1.420 cm/s					
1	11.84	3.40	1.47	2.82	2.63	3.90
2	7.14	0.68	0.00	0.00	0.00	1.60
3	8.05	3.31	1.42	16.93	2.59	2.08
4	55.65	25.15	3.67	28.22	64.20	6.15
6	31.54	6.09	0.00	0.00	0.00	0.78
12	65.91	29.64	3.50	55.25	11.51	4.15
18	82.68	43.49	5.10	36.83	37.46	5.10
24	322.49	189.40	25.95	108.27	-	-
48	213.26	112.92	14.67	233.36	84.13	21.71
72	372.38	199.24	24.66	404.81	188.62	27.84
	v=0.644 cm/s					
1	12.69	3.20	1.47	0.00	0.00	0.85
2	9.87	3.12	0.00	0.00	2.05	3.15
3	27.07	12.14	3.96	5.64	14.45	8.73
4	14.80	11.30	0.32	0.00	38.70	3.68
6	19.67	9.96	0.63	110.49	40.64	7.56
12	33.02	17.36	1.77	41.28	45.88	7.15
18	35.97	19.36	2.03	50.80	52.45	11.20
24	67.39	32.95	4.73	159.07	-	-
48	106.01	58.87	8.08	259.40	77.26	23.43
72	279.73	157.39	20.43	425.45	224.69	39.72
	v=0.054 cm/s					
1	7.28	1.65	0.26	0.00	3.84	0.00
2	33.96	0.00	2.10	0.00	9.90	5.57
3	16.24	7.17	0.17	0.00	-	0.95
4	22.12	9.47	1.47	28.22	19.23	1.56
6	71.98	28.60	4.17	0.00	0.00	1.34
12	71.34	34.67	3.91	317.50	145.79	26.73
18	75.37	70.09	10.63	178.12	54.81	18.15
24	181.79	105.27	13.63	542.29	-	-
48	110.15	64.61	6.86	1115.70	412.20	76.97
64	247.99	-	-	605.79	-	-
72	-	-	-	913.13	437.52	-
Blank	5.39	0.00	1.37			

Table J.2 Attached and loose film development vs time (cont.)

SILICONE

hours	AFCOD µg/cm2	AFPR µg/cm2	AFPS µg/cm2	LFCOD µg/cm2	LFPR µg/cm2	LFPS µg/cm2
	v=1.078 cm/s					
1	17.92	1.78	1.53	0.00	0.00	2.99
2	49.70	21.42	2.68	0.00	0.00	2.09
3	14.19	4.62	5.80	0.00	23.16	2.33
4	69.51	33.93	5.66	14.11	12.36	2.80
6	93.47	52.03	6.30	0.00	12.48	0.87
12	301.49	169.60	22.43	857.09	198.30	29.95
18	568.43	310.33	43.16	664.24	285.20	38.43
24	668.08	350.39	45.71	738.64	-	-
48	1288.11	612.21	115.44	29.16	16.97	4.42
72	1509.95	740.73	114.39	51.98	40.50	3.77
	v=0.760 cm/s					
1	13.95	0.00	0.91	0.00	0.00	0.45
2	29.67	8.87	0.52	0.00	0.00	2.43
3	15.88	6.39	0.12	10.58	0.00	2.14
4	121.44	61.47	9.97	141.08	22.95	8.87
6	52.21	29.07	3.05	23.01	28.53	2.98
12	299.73	166.80	22.37	440.05	361.00	53.94
18	641.79	331.04	43.50	816.81	354.95	58.85
24	758.77	401.95	54.67	823.56	-	-
48	981.36	477.20	125.37	382.52	145.18	23.01
72	1466.90	705.85	114.52	38.89	20.99	6.50
	v=0.056 cm/s					
1	14.18	1.87	3.14	0.00	12.58	0.74
2	11.60	2.56	0.00	0.00	15.19	4.61
3	43.62	18.26	4.68	31.74	7.23	-
4	69.08	31.34	8.31	61.72	67.31	11.33
6	80.17	43.91	7.32	97.81	76.70	9.49
12	219.22	135.27	17.71	621.39	329.80	46.14
18	394.48	235.93	29.66	854.11	455.17	50.04
24	572.07	307.90	41.49	947.16	-	-
48	584.37	251.69	64.54	1144.77	457.93	64.38
72	1224.64	567.12	115.55	0.00	0.00	0.03
Blank	12.61	0.00	2.26			

Table J.2A Total biofilm development vs time

hours	TYGON TFCOD µg/cm2	TYGON TFPR µg/cm2	TYGON TFPS µg/cm2	SILICONE TFCOD µg/cm2	SILICONE TFPR µg/cm2	SILICONE TFPS µg/cm2
	v=1.420 cm/s			v=1.078 cm/s		
1	14.66	6.03	5.38	17.92	1.78	4.52
2	-	0.68	1.60	-	21.42	4.77
3	24.99	5.90	3.50	14.19	27.79	8.14
4	83.87	89.34	9.82	83.61	46.29	8.46
6	31.54	6.09	0.78	93.47	64.51	7.17
12	121.15	41.14	7.65	1158.57	367.90	52.38
18	119.51	80.95	10.20	1232.68	595.53	81.59
24	430.76	-	-	1406.72	-	-
48	446.63	197.05	36.38	1317.27	629.18	119.86
64	-	-	-	-	-	-
72	777.19	387.87	52.50	1561.93	781.23	118.16
	v=0.644 cm/s			v=0.760 cm/s		
1	12.69	3.20	2.32	13.95	0.00	1.36
2	-	5.17	3.15	-	8.87	2.94
3	32.72	26.58	12.70	26.46	6.39	2.25
4	14.80	50.00	4.00	262.52	84.43	18.85
6	130.16	50.60	8.19	75.23	57.59	6.02
12	74.30	63.24	8.92	739.78	527.80	76.31
18	86.77	71.80	13.24	1458.51	685.99	102.35
24	226.45	-	-	1582.32	-	-
48	365.40	136.13	31.51	1363.88	622.39	148.38
64	-	-	-	-	-	-
72	705.18	382.09	60.15	1505.79	726.84	121.02
	v=0.054 cm/s			v=0.056 cm/s		
1	7.28	5.48	0.26	14.18	14.45	3.88
2	-	9.90	7.67	-	17.75	4.61
3	16.24	-	1.12	75.36	25.48	-
4	50.34	28.70	3.02	130.81	98.65	19.63
6	71.98	28.60	5.51	177.98	120.61	16.82
12	388.84	180.45	30.63	840.61	465.07	63.85
18	253.49	124.90	28.78	1248.60	691.10	79.70
24	724.08	-	-	1519.23	-	-
48	1225.85	476.81	83.83	1729.14	709.62	128.91
64	853.78	-	-	-	-	-
72	-	-	-	1224.64	567.12	115.58

Table J.3 Biofilm thickness (μm) vs time

TYGON

h	v=1.420 cm/s	v=0.644 cm/s	v=0.054 cm/s
1	0.00	0.00	0.00
2	0.00	0.00	0.00
3	0.00	0.00	0.37
4	0.00	0.00	15.87
6	0.00	0.00	26.27
12	34.46	0.00	0.00
18	0.00	4.31	65.36
24	0.00	0.00	37.57
48	0.00	37.15	36.35
64	-	-	66.26
72	87.65	103.42	-

SILICONE

h	v=1.078 cm/s	v=0.76 cm/s	v=0.056 cm/s
1	0.00	0.00	0.00
2	0.00	0.00	0.00
3	0.00	0.00	0.00
4	0.00	61.91	0.00
6	0.00	0.00	30.45
12	0.00	0.00	27.26
18	74.73	209.60	124.53
24	89.00	144.67	498.70
48	248.63	215.67	52.94
72	241.92	220.98	204.19

Table J.4 Biofilm concentrations vs time (mg/mL of biofilm)

	Tygon, v=1.42 cm/s			Silicone, v=1.08 cm/s		
hours	COD	PR	PS	COD	PR	PS
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
6	-	-	-	-	-	-
12	19.13	8.60	1.01	-	-	-
18	-	-	-	76.07	41.53	5.78
24	-	-	-	75.06	39.37	5.14
48	-	-	-	51.81	24.62	4.64
72	42.49	22.73	2.81	62.42	30.62	4.73
ave.	30.81	15.67	1.91	66.34	34.04	5.07

	Tygon, v=0.64 cm/s			Silicone, v=0.76 cm/s		
hours	COD	PR	PS	COD	PR	PS
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	19.61	9.93	1.61
6	-	-	-	-	-	-
12	-	-	-	-	-	-
18	83.52	44.94	4.72	30.61	15.79	2.08
24	-	-	-	52.45	27.78	3.78
48	28.54	15.85	2.18	45.50	22.13	5.81
72	27.05	15.22	1.98	66.38	31.94	5.18
ave.	27.79	15.53	2.08	42.91	21.52	3.69

	Tygon, v=0.054 cm/s			Silicone, v=0.056 cm/s		
hours	COD	PR	PS	COD	PR	PS
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	43.93	19.41	0.47	-	-	-
4	13.94	5.97	0.92	-	-	-
6	27.39	10.88	1.59	26.32	14.42	2.40
12	-	-	-	80.42	49.63	6.50
18	11.53	10.72	1.63	31.58	18.95	2.38
24	48.39	28.02	3.63	11.47	6.17	0.83
48	30.30	17.77	1.89	110.39	47.54	12.19
64	37.43	-	-	-	-	-
72	-	-	-	59.97	27.77	5.66
ave.	30.42	15.46	1.69	53.38	27.41	4.99

Table J.5 Biofilm ratios (mg/mg) vs time, TYGON

	Attached biofilm			Loose biofilm		
	v=1.42 cm/s					
hours	PR/COD	PS/COD	PS/PR	PR/COD	PS/COD	PS/PR
1	0.287	0.125	0.434	1.035	1.536	1.484
2	0.095	-	-	-	-	-
3	0.411	0.177	0.430	0.146	0.117	0.802
4	0.452	0.066	0.146	2.198	0.210	0.096
6	0.193	-	-	-	-	-
12	0.450	0.053	0.118	0.208	0.075	0.361
18	0.526	0.062	0.117	1.017	0.139	0.136
24	0.587	0.080	0.137	-	-	-
48	0.529	0.069	0.130	0.361	0.093	0.258
72	0.535	0.066	0.124	0.466	0.069	0.148
ave.	0.407	0.087	0.204	0.295	0.117	0.300
	v=0.644 cm/s					
hours	PR/COD	PS/COD	PS/PR	PR/COD	PS/COD	PS/PR
1	0.252	0.116	0.459	-	-	-
2	0.316	-	-	-	-	1.538
3	0.448	0.146	0.326	2.528	1.528	0.605
4	0.764	0.022	0.028	-	-	0.095
6	0.506	0.032	0.064	0.368	0.068	0.186
12	0.526	0.054	0.102	1.112	0.173	0.156
18	0.538	0.056	0.105	1.032	0.221	0.214
24	0.489	0.070	0.143	-	-	-
48	0.555	0.076	0.137	0.298	0.090	0.303
72	0.563	0.073	0.130	0.528	0.093	0.177
ave.	0.496	0.072	0.166	0.398	0.129	0.248
	v=0.056 cm/s					
hours	PR/COD	PS/COD	PS/PR	PR/COD	PS/COD	PS/PR
1	0.226	0.036	0.159	-	-	-
2	-	0.062	-	-	-	0.563
3	0.442	0.011	0.024	-	-	-
4	0.428	0.066	0.155	0.658	0.053	0.081
6	0.397	0.058	0.146	-	-	-
12	0.486	0.055	0.113	0.459	0.084	0.183
18	0.930	0.141	0.152	0.308	0.102	0.331
24	0.579	0.075	0.129	-	-	-
48	0.587	0.062	0.106	0.369	0.069	0.187
ave.	0.509	0.063	0.123	0.449	0.077	0.196

Table J.5 Biofilm ratios (mg/mg) vs time, SILICONE (cont.)

	Attached biofilm			Loose biofilm		
	v=1.078 cm/s					
hours	PR/COD	PS/COD	PS/PR	PR/COD	PS/COD	PS/PR
1	0.099	0.085	0.859	-	-	-
2	0.431	0.054	0.125	-	-	-
3	0.326	0.409	1.255	-	-	0.101
4	0.488	0.081	0.167	0.853	0.193	0.227
6	0.557	0.067	0.121	-	-	0.069
12	0.563	0.074	0.132	0.231	0.035	0.151
18	0.546	0.076	0.139	0.429	0.058	0.135
24	0.524	0.068	0.130	-	-	-
48	0.475	0.090	0.189	0.582	0.151	0.260
72	0.491	0.076	0.154	0.779	0.073	0.093
ave.	0.450	0.075	0.145	0.575	0.102	0.148
	v=0.760 cm/s					
hours	PR/COD	PS/COD	PS/PR	PR/COD	PS/COD	PS/PR
1	-	0.065	-	-	-	-
2	0.299	0.017	0.058	-	-	-
3	0.402	0.007	0.018	-	0.189	-
4	0.506	0.082	0.162	0.157	0.061	0.387
6	0.557	0.058	0.105	1.240	0.129	0.104
12	0.557	0.075	0.134	0.820	0.123	0.149
18	0.516	0.068	0.131	0.435	0.072	0.166
24	0.530	0.072	0.136	-	-	-
48	0.486	0.128	0.263	0.380	0.060	0.158
72	0.481	0.078	0.162	0.540	0.167	0.309
ave.	0.482	0.065	0.130	0.466	0.114	0.212
	v=0.054 cm/s					
hours	PR/COD	PS/COD	PS/PR	PR/COD	PS/COD	PS/PR
1	0.132	0.222	1.681	-	-	0.058
2	0.221	-	-	-	-	0.303
3	0.419	0.107	0.256	0.219	-	-
4	0.454	0.120	0.265	1.054	0.177	0.168
6	0.548	0.091	0.167	0.784	0.097	0.124
12	0.617	0.081	0.131	0.531	0.074	0.140
18	0.598	0.075	0.126	0.533	0.059	0.110
24	0.538	0.073	0.135	-	-	-
48	0.431	0.110	0.256	0.400	0.056	0.141
72	0.463	0.094	0.204	-	-	-
ave.	0.442	0.094	0.192	0.493	0.093	0.149

Table J.6 Attached percentage (%) vs time

	TYGON			SILICONE		
hours	COD	PR	PS	COD	PR	PS
	v=1.42 cm/s			v=1.078 cm/s		
1	80.75	56.39	27.43	100.00	100.00	33.88
2	100.00	100.00	0.00	100.00	100.00	56.19
3	32.23	56.12	40.68	100.00	16.64	71.33
4	66.35	28.15	37.41	83.13	73.30	66.90
6	100.00	100.00	0.00	100.00	80.65	87.92
12	54.40	72.03	45.71	26.02	46.10	42.82
18	69.18	53.72	50.01	46.11	52.11	52.90
24	74.87	-	-	47.49	-	-
48	47.75	57.30	40.32	97.79	97.30	96.31
72	47.91	51.37	46.97	96.67	94.82	96.81
ave.	67.34	63.90	32.06	79.72	73.44	67.23
	v=0.644 cm/s			v=0.760 cm/s		
1	100.00	100.00	63.40	100.00	-	66.77
2	100.00	60.40	0.00	100.00	100.00	17.52
3	82.75	45.65	31.20	60.02	100.00	5.19
4	100.00	22.60	8.05	46.26	72.81	52.91
6	15.11	19.68	7.74	69.41	50.47	50.60
12	44.45	27.44	19.83	40.52	31.60	29.32
18	41.46	26.96	15.35	44.00	48.26	42.50
24	29.76	-	-	47.95	-	-
48	29.01	43.24	25.65	71.95	76.67	84.49
72	39.67	41.19	33.97	97.42	97.11	94.63
ave.	58.22	43.02	22.80	67.75	72.12	49.33
	v=0.054 cm/s			v=0.056 cm/s		
1	100.00	30.01	100.00	100.00	12.93	81.04
2	100.00	0.00	27.34	100.00	14.42	0.00
3	100.00	-	15.46	57.88	71.64	-
4	43.94	33.00	48.50	52.81	31.77	42.32
6	100.00	100.00	75.67	45.04	36.41	43.55
12	18.35	19.21	12.76	26.08	29.09	27.73
18	29.73	56.12	36.95	31.59	34.14	37.22
24	25.11	-	-	37.66	-	-
48	8.99	13.55	8.19	33.80	35.47	50.06
64	29.05	-	-	100.00	100.00	99.97
ave.	55.52	35.98	40.61	58.49	40.65	47.74

Table J.7 Biofilm attachment rates vs liquid flow velocity

Attached biofilm

Flow velocity cm/s	Rate ug/cm2/h		Intercept		Correlation coefficient	
	COD					
	T	S	T	S	T	S
0.054	3.13	-	26.9	-	0.869	-
0.056	-	15.95	-	18.3	-	0.968
0.644	3.34	-	-2.85	-	0.954	-
0.760	-	20.80	-	43.0	-	0.970
1.078	-	22.93	-	22.3	-	0.982
1.420	4.87	-	4.51	-	0.991	-
	PR					
	T	S	T	S	T	S
0.054	1.68	-	15.0	-	0.710	-
0.056	-	7.30	-	21.3	-	0.935
0.644	1.90	-	-3.5	-	0.953	-
0.760	-	10.06	-	27.6	-	0.958
1.078	-	11.15	-	17.9	-	0.978
1.420	2.67	-	-2.1	-	0.991	-
	PS					
	T	S	T	S	T	S
0.054	0.195	-	2.24	-	0.630	-
0.056	-	1.54	-	-0.04	-	0.993
0.644	0.245	-	-0.32	-	0.937	-
0.760	-	1.92	-	1.08	-	0.951
1.078	-	1.82	-	1.63	-	0.967
1.420	0.328	-	0.00	-	0.987	-
	Film thickness, μm/h					
	T	S	T	S	T	S
0.054	0.886	-	9.00	-	0.739	-
0.056	-	4.72	-	2.95	-	0.929
0.644	1.314	-	-10.48	-	0.928	-
0.760	-	5.12	-	30.64	-	0.83
1.078	-	5.36	-	-24.47	-	0.975
1.420	0.883	-	-4.56	-	0.726	-

T: TYGON S: SILICONE

Table J.7 Biofilm attachment rates vs liquid flow velocity

Loose biofilm

Flow velocity cm/s	Rate µg/cm2/h		Intercept		Correlation coefficient	
	COD					
	T	S	T	S	T	S
0.054	14.70	-	11.11	-	0.848	-
0.056	-	47.48	-	-88.68	-	0.978
0.644	5.81	-	-5.25	-	0.969	-
0.760	-	41.02	-	-83.07	-	0.968
1.078	-	40.45	-	-69.69	-	0.870
1.420	5.53	-	-16.22	-	0.986	-
	PR					
	T	S	T	S	T	S
0.054	8.54	-	-20.1	-	0.954	-
0.056	-	28.53	-	-47.7	-	0.986
0.644	2.55	-	7.99	-	0.931	-
0.760	-	25.64	-	-58.8	-	0.937
1.078	-	18.19	-	-43.6	-	0.972
1.420	2.31	-	0.757	-	0.921	-
	PS					
	T	S	T	S	T	S
0.054	1.63	-	-2.76	-	0.975	-
0.056	-	3.18	-	-2.40	-	0.960
0.644	0.49	-	2.72	-	0.982	-
0.760	-	3.97	-	-7.56	-	0.947
1.078	-	2.41	-	-4.52	-	0.950
1.420	0.38	-	1.16	-	0.972	-

T: TYGON S: SILICONE

Table J.8 Relationship between film development rate (µm/h or µg/cm2.h) and liquid flow velocity (cm/s)

	TYGON			SILICONE		
	Slope	Intercept	Correlation	Slope	Intercept	Correlation
FT	-	1.049	NONE	0.619	4.676	0.997
AFCOD	1.460	2.810	0.940	6.840	15.570	0.999
AFPR	0.837	1.535	0.967	3.793	7.110	0.999
AFPS	0.112	0.183	0.993	0.318	1.560	0.853
LFCOD	-6.388	13.200	0.839	-7.259	47.566	0.972
LFPR	-4.410	7.610	0.843	-9.122	29.880	0.894
LFPS	-0.876	1.450	0.866	-	3.470	NONE

Table J.9 Ratio of biofilm development rates on silicone and Tygon tubing at flow velocities of 0 cm/s and 1 cm/s

	0 cm/s	1 cm/s
FT	4.07	4.46
AFCOD	5.54	5.25
AFPR	4.63	4.60
AFPS	8.52	6.38
	6.23	5.41
LFCOD	3.60	5.92
LFPR	3.93	6.49
LFPS	2.75	5.23
	3.43	5.88

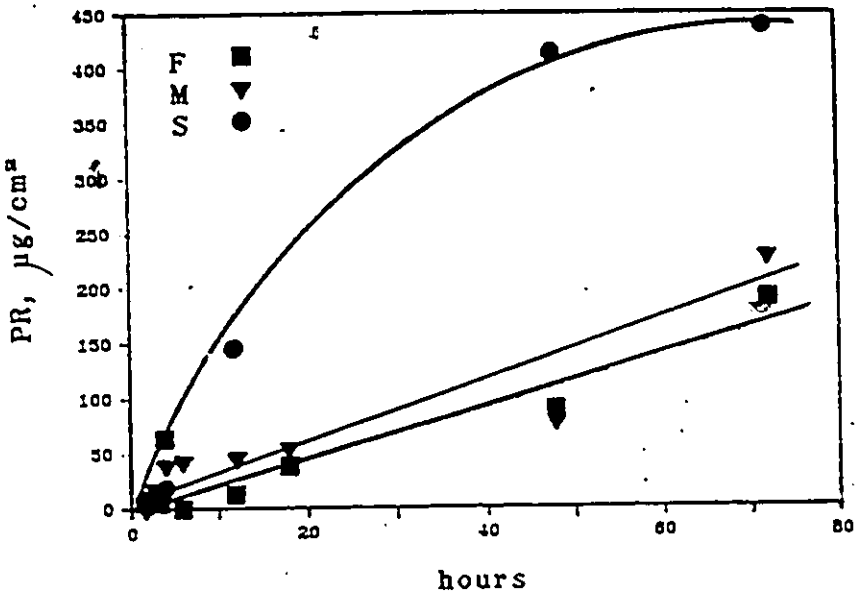

Figure J.1 Loose protein vs time, Tygon

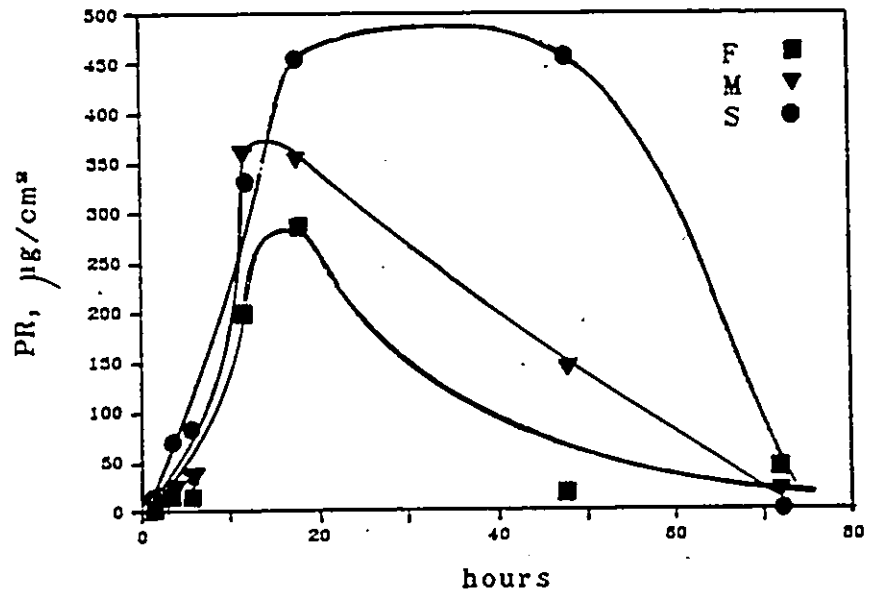

Figure J.2 Loose protein vs time, silicone

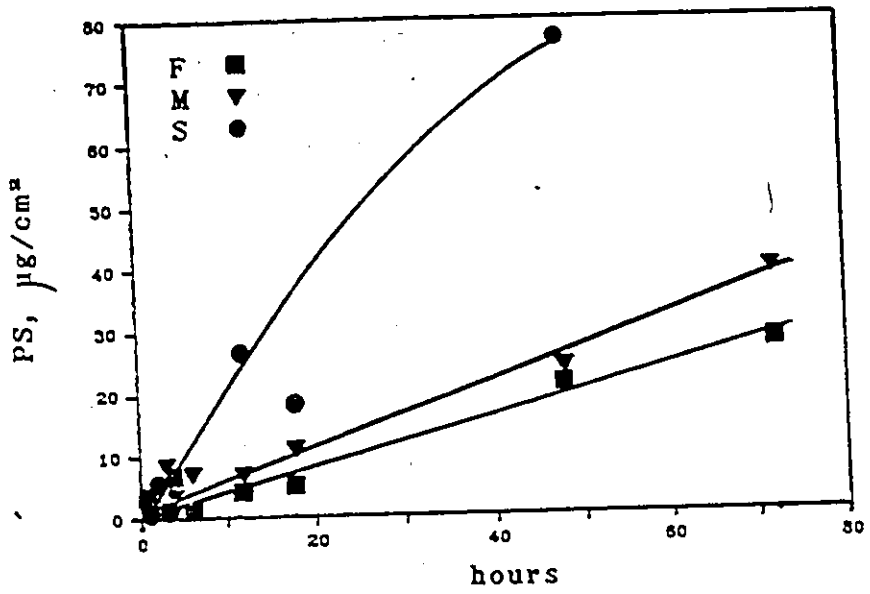

Figure J.3 Loose polysaccharide vs time, Tygon

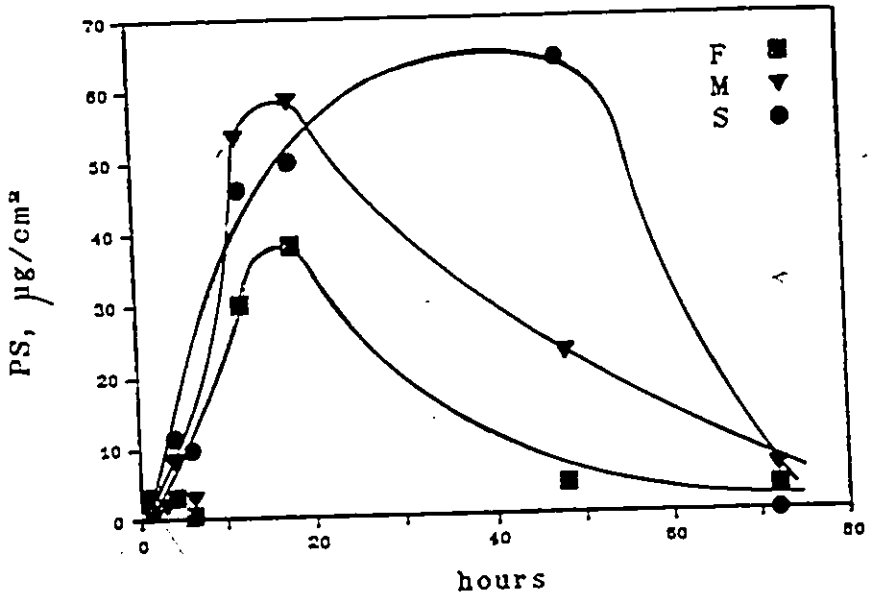

Figure J.4 Loose polysaccharide vs time, silicone

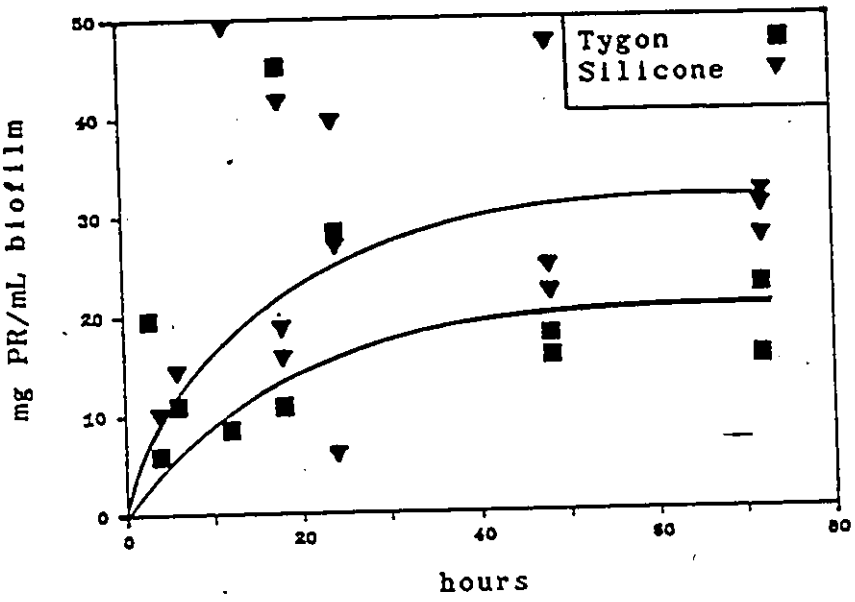

Figure J.5 Biofilm protein concentration vs time

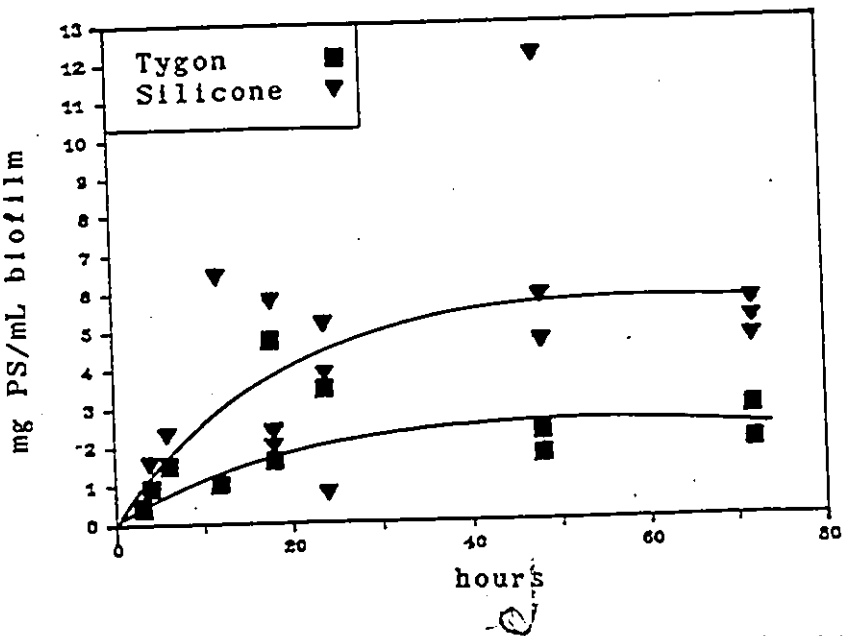

Figure J.6 Biofilm polysaccharide concentration vs time

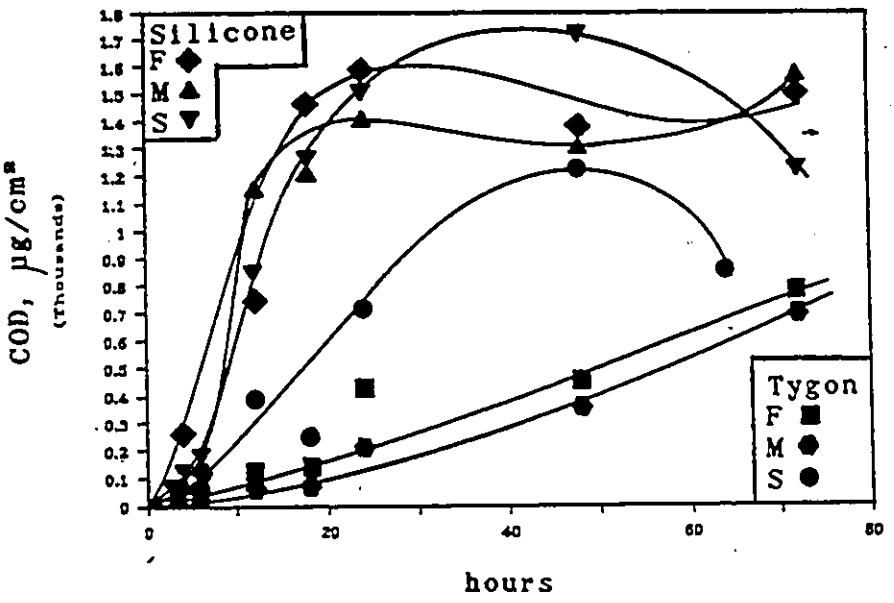

Figure J.7 Total COD vs time (A)

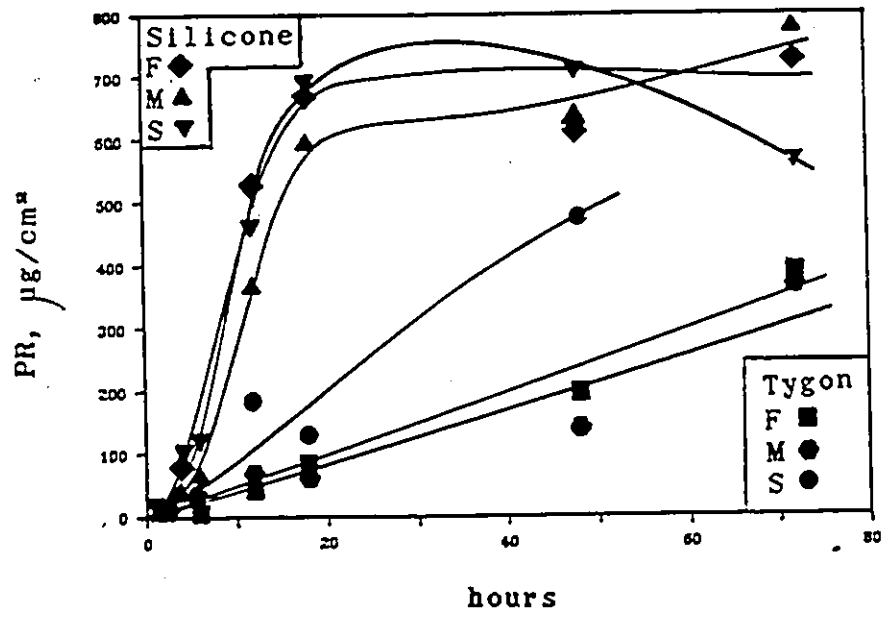

Figure J.8 Total PR vs time (A)

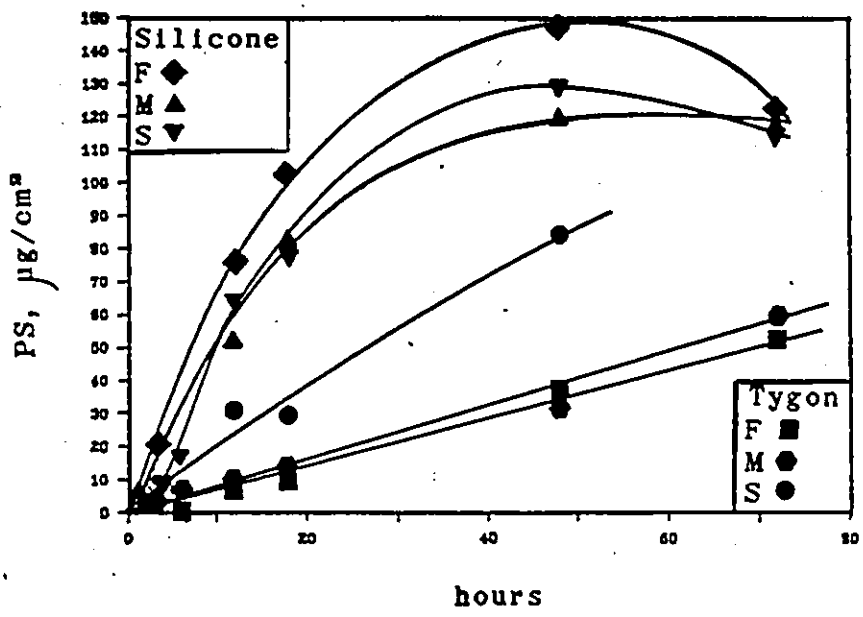

Figure J.9 Total PS vs time (A)

APPENDIX K

Table K.1 Reactor data

B&C

hours	SCOD mg/L	SPR mg/L	SPS mg/L	VSS mg/L
0	-	-	-	645
0	864	467.9	47.6	538
3	711	379.0	43.2	518
5	707	366.4	39.7	528
7	689	358.1	39.8	472
9	638	328.0	28.8	567
11	594	348.4	38.1	484
12	718	374.8	39.7	463
ave.	721	374.7	39.6	510

hours	SCOD/VSS	SPR/VSS	SPS/VSS
0	1.607	0.870	0.088
3	1.372	0.732	0.083
5	1.339	0.694	0.075
7	1.460	0.759	0.084
9	1.125	0.578	0.051
11	1.227	0.720	0.079
12	1.551	0.809	0.086
ave.	1.383	0.737	0.078

hours	SPR/SCOD mg/mg	SPS/SCOD mg/mg	SPS/SPR mg/mg
0	0.541	0.055	0.102
3	0.533	0.061	0.114
5	0.518	0.056	0.108
7	0.520	0.058	0.111
9	0.514	0.045	0.088
11	0.587	0.064	0.109
12	0.522	0.055	0.106
ave.	0.534	0.056	0.105

Table K.1 Reactor data, cont.

D

hours	SCOD mg/L	SPR mg/L	SPS mg/L	VSS mg/L
0	1350	643.7	90.81	959
3	1225	569.7	72.39	892
5	1090	559.6	75.12	778
7	1009	538.1	63.19	767
9	891	497.0	70.99	626
11	978	517.1	69.89	703
12	1289	667.8	56.93	853
ave.	1142	570.4	71.33	797

hours	SCOD/VSS	SPR/VSS	SPS/VSS
0	1.408	0.671	0.095
3	1.374	0.639	0.081
5	1.401	0.719	0.097
7	1.315	0.702	0.082
9	1.424	0.794	0.113
11	1.391	0.736	0.099
12	1.511	0.783	0.067
ave.	1.403	0.720	0.091

hours	SPR/SCOD mg/mg	SPS/SCOD mg/mg	SPS/SPR mg/mg
0	0.477	0.067	0.141
3	0.465	0.059	0.127
5	0.513	0.069	0.134
7	0.534	0.063	0.117
9	0.558	0.080	0.143
11	0.529	0.071	0.135
12	0.518	0.044	0.085
ave.	0.513	0.065	0.126

Table K.2 Attached and loose film development vs time

	AFCOD µg/cm2			LFCOD µg/cm2		
hours	B	C	D	B	C	D
2	5.54	-	-	5.81	-	-
3	-	16.04	31.3	-	34.84	6.07
4	7.53	-	-	-	-	-
5	-	17.88	54.1	-	49.36	106.2
6	10.16	-	-	49.36	-	-
7	-	36.26	124.6	-	65.32	213.9
8	13.08	-	-	29.03	-	-
9	-	13.02	128.2	-	110.33	247.2
11	57.44	57.44	233.2	200.33	200.33	403.5

	AFPR µg/cm2			LFPR µg/cm2		
hours	B	C	D	B	C	D
2	4.28	-	-	1.47	-	-
3	-	4.30	6.1	-	15.55	14.2
4	0.37	-	-	-	-	-
5	-	7.46	28.0	-	21.64	29.3
6	2.47	-	-	12.54	-	-
7	-	18.71	93.3	-	33.29	90.1
8	6.43	-	-	17.07	-	-
9	-	8.83	58.9	-	42.74	121.2
11	28.38	28.38	111.5	88.31	88.31	203.1

	AFPS µg/cm2			LFPS µg/cm2		
hours	B	C	D	B	C	D
2	3.21	-	-	1.87	-	-
3	-	1.90	6.0	-	1.98	0.7
4	2.97	-	-	0.12	-	-
5	-	4.66	5.7	-	3.55	6.2
6	4.26	-	-	5.26	-	-
7	-	8.81	12.7	-	6.55	20.5
8	5.12	-	-	3.44	-	-
9	-	4.91	11.7	-	8.55	21.9
11	9.67	9.67	21.9	13.80	13.80	33.3

Table K.3 Biofilm thickness (μm) vs time

hours	Film thickness μm B	C	D
2	0.0	-	-
3		0.0	70.9
4	3.5	-	-
5		16.8	42.3
6	21.2	-	-
7		0.0	123.8
8	31.2	-	-
9		0.0	165.6
11	38.0	38.0	131.0

Table K.4 Biofilm concentrations vs time (mg/mL biofilm)

	COD		PR		PS	
hours	B	D	B	D	B	D
2	-	-	-	-	-	-
3	-	4.60	-	0.65	-	1.232
4	14.04	-	0.54	-	4.313	-
5	7.60	11.66	2.97	5.96	1.738	1.400
6	-	-	-	-	-	-
7	-	10.32	-	8.17	-	1.041
8	5.30	-	2.90	-	2.139	-
9	-	11.53	-	5.40	-	0.952
11	7.17	17.70	3.77	8.46	1.139	1.668
ave.	8.53	11.16	2.54	5.73	2.332	1.259

Table K.5 Attached percentage (%) vs time

	COD		PR		PS	
hours	B&C	D	B&C	D	B&C	D
2	48.8	-	52.1	-	62.9	-
3	30.4	83.2	21.7	31.6	52.8	88.4
4	-	-	18.4	-	96.1	-
5	27.0	31.7	25.8	28.8	56.5	48.7
6	14.1	-	20.9	-	44.7	-
7	36.2	43.0	36.6	52.6	57.4	41.9
8	31.6	-	27.3	-	61.6	-
9	10.6	36.5	17.1	33.7	36.5	35.6
11	34.2	36.5	39.9	35.5	54.0	40.1
ave.	29.1	46.2	28.9	36.4	58.1	50.9

Table K.6 Biofilm ratios (mg/mg) vs time

	Attached biofilm			Loose biofilm		
	PR/COD					
hours	B	C	D	B	C	D
2	0.064	-	-	0.253	-	-
3	-	0.341	0.199	-	0.450	-
4	0.054	-	-	-	-	-
5	-	0.438	0.516	-	0.600	0.381
6	0.419	-	-	0.221	-	-
7	-	0.519	0.742	-	0.511	0.888
8	0.494	-	-	0.605	-	-
9	-	0.678	0.459	-	0.387	0.515
11	0.496	0.496	0.478	0.385	0.385	0.502
ave.	0.305	0.494	0.479	0.366	0.467	0.572
	PS/COD.					
hours	B	C	D	B	C	D
2	0.579	-	-	0.323	-	-
3	-	0.158	0.189	-	0.060	-
4	0.432	-	-	-	-	-
5	-	0.285	0.106	-	0.105	0.072
6	0.259	-	-	0.141	-	-
7	-	0.252	0.102	-	0.102	0.195
8	0.392	-	-	0.134	-	-
9	-	0.377	0.091	-	0.077	0.095
11	0.168	0.168	0.095	0.059	0.059	0.082
ave.	0.366	0.248	0.117	0.164	0.081	0.111
	PS/PR					
hours	B	C	D	B	C	D
2	0.44	-	-	1.27	-	-
3	-	0.44	0.425	-	0.13	0.061
4	7.95	-	-	-	-	-
5	-	0.64	0.206	-	0.16	0.195
6	1.95	-	-	0.23	-	-
7	-	0.48	0.169	-	0.20	0.244
8	0.83	-	-	0.21	-	-
9	-	0.56	0.200	-	0.20	0.183
11	0.34	0.34	0.199	0.15	0.15	0.163
ave.	0.537	0.493	0.240	0.198	0.167	0.196

Table K.7 Biofilm attachment rates vs suspended biomass concentration

	AFCOD			LFCOD		
	SCOD	Rate ug/cm2/h	Intercept	Correlation coefficient	Rate ug/cm2/h	Intercept	Correlation coefficient
B	685	1.262	2.76	0.996	4.87	2.08	0.683
C	747	5.533	-4.06	0.978	12.12	-7.77	0.957
D	1176	23.895	-52.98	0.957	46.79	-132.17	0.972

	AFPR			LFPR		
	SPR	Rate ug/cm2/h	Intercept	Correlation coefficient	Rate ug/cm2/h	Intercept	Correlation coefficient
B	348	1.515	-6.00	0.985	2.624	-3.63	0.999
C	395	3.166	-5.86	0.982	8.331	-18.01	0.914
D	578	12.085	-25.04	0.871	23.470	-72.65	0.952

	AFPS			LFPS		
	SPS	Rate ug/cm2/h	Intercept	Correlation coefficient	Rate ug/cm2/h	Intercept	Correlation coefficient
B	36.7	0.712	0.63	0.913	0.830	-2.04	0.637
C	42.2	0.790	0.79	0.778	1.135	-1.65	0.993
D	76.7	1.890	-1.63	0.908	4.043	-11.77	0.958

	Film Thickness			
	VSS	Rate um/h	Intercept	Correlation coefficient
B	510	4.78	-10.83	0.971
C	522	2.96	-9.76	0.558
D	848	12.18	-21.50	0.606

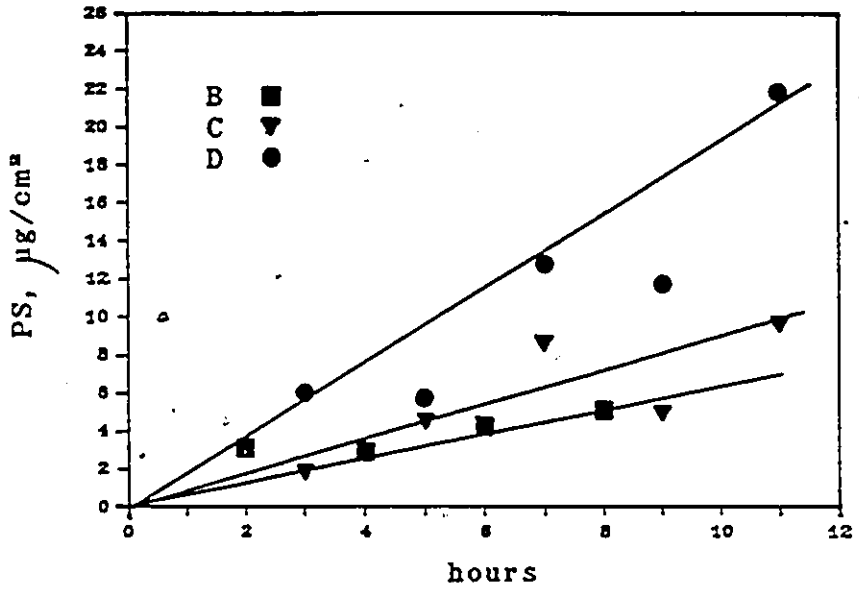

Figure K.1 Attached polysaccharide vs time

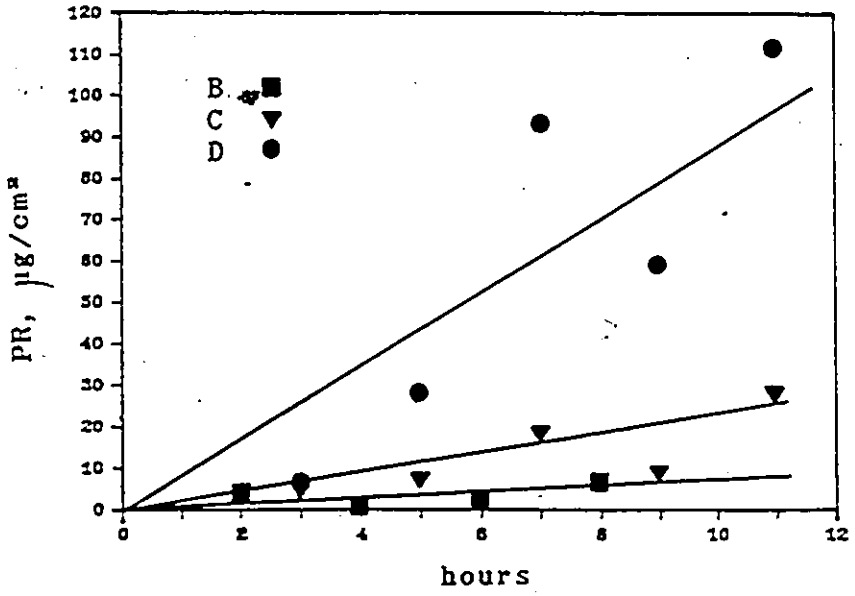

Figure K.2 Attached protein vs time

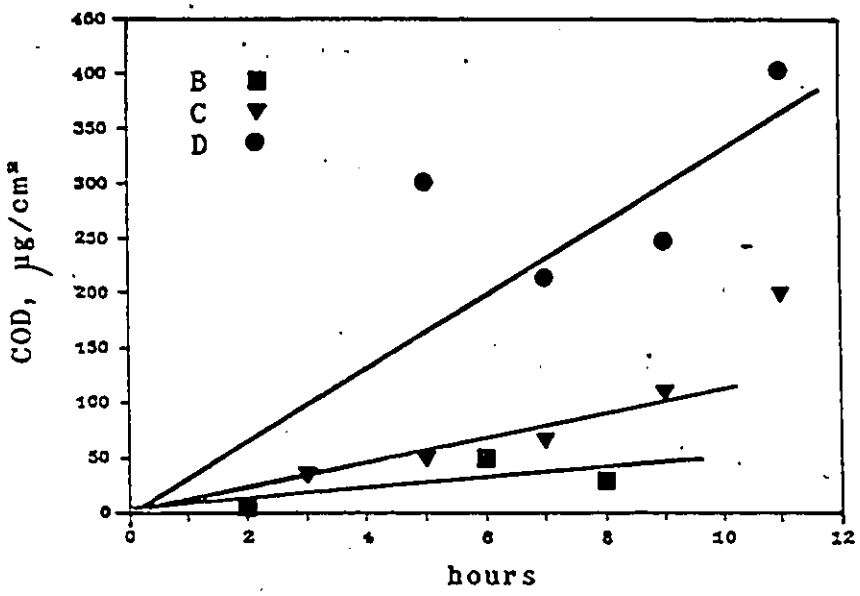

Figure K.3 Loose COD vs time

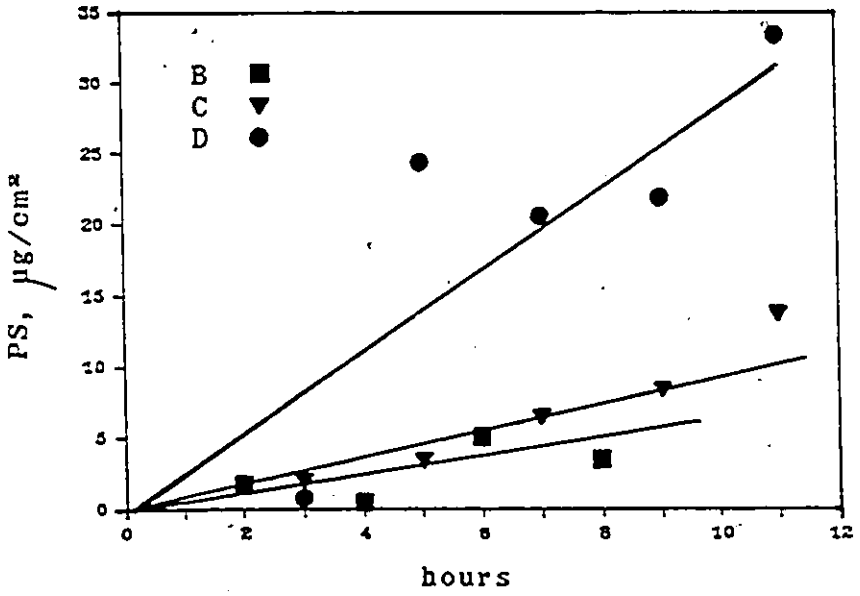

Figure K.4 Loose polysaccharide vs time

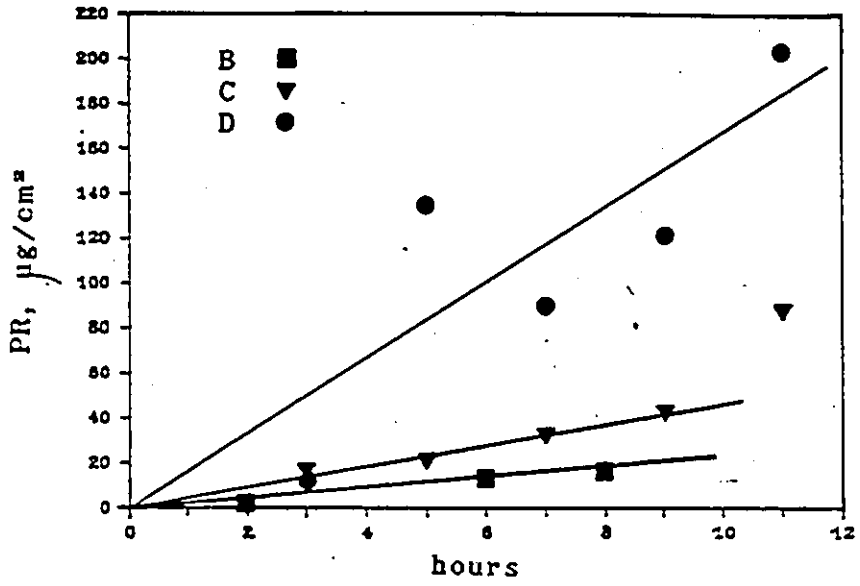

Figure K.5 Loose protein vs time

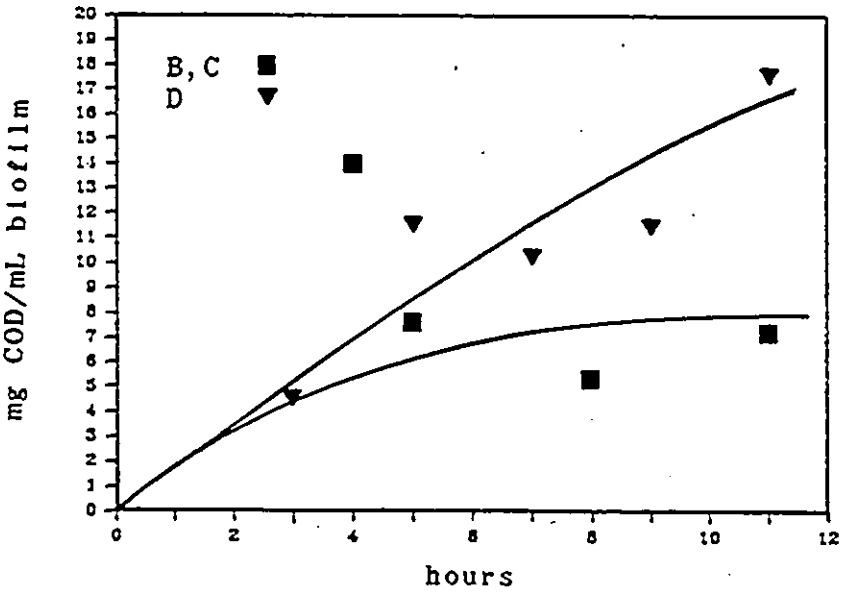

Figure K.6 Biofilm protein concentration vs time

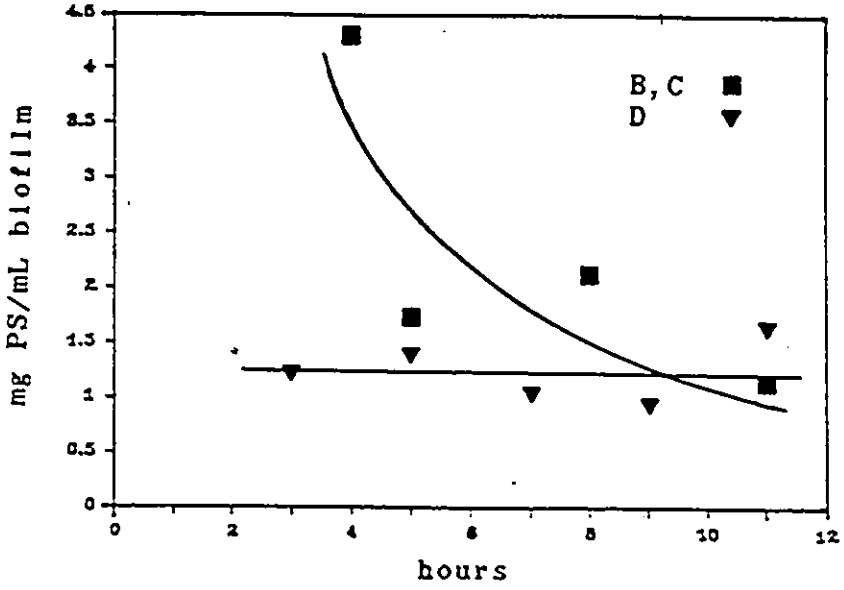

Figure K.7 Biofilm polysaccharide concentration vs time

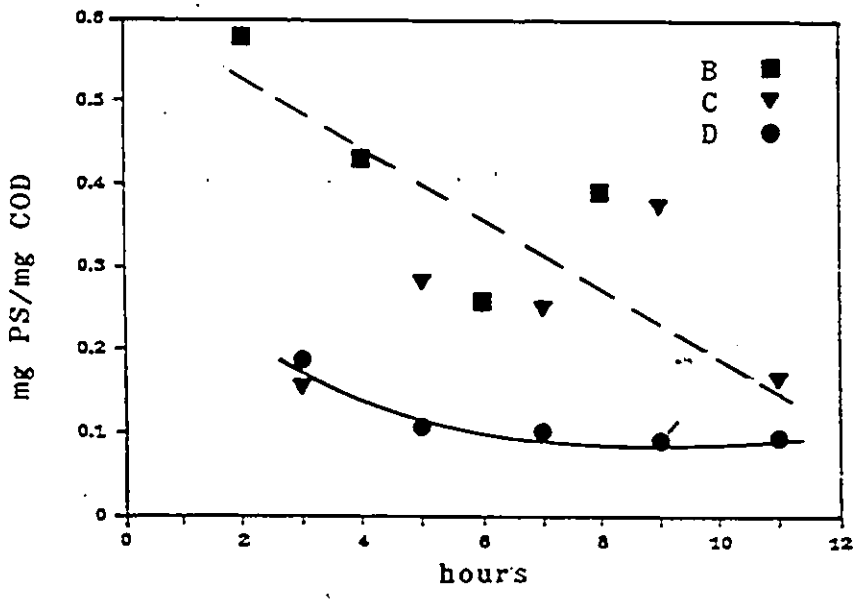

Figure K.8 Attached PS/COD vs time

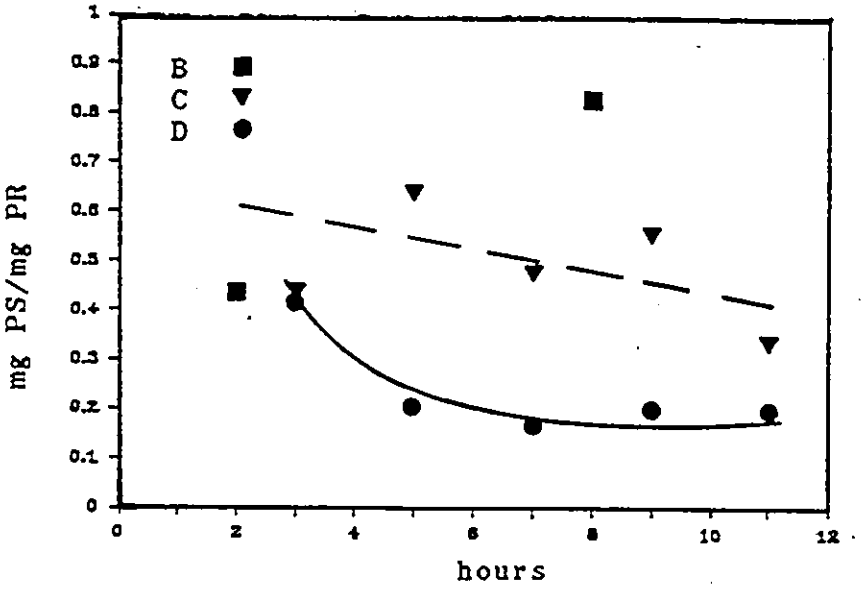

Figure K.9 Attached PS/PR vs time

APPENDIX L

Table L.1 Reactor data

E

hours	SCOD mg/L	SPR mg/L	SPS mg/L	VSS mg/L
10.5	1248.0	689.0	71.76	849
23.0	1203.5	687.0	59.78	1087
32.5	1036.5	670.0	59.35	728
48.0	1117.5	671.6	68.15	795
57.5	1167.5	695.0	69.72	798
ave.	1154.6	682.5	65.75	793

hours	SCOD/VSS	SPR/VSS	SPS/VSS
10.5	1.470	0.812	0.085
23.0	1.107	0.632	0.055
32.5	1.424	0.920	0.082
48.0	1.406	0.845	0.086
57.5	1.463	0.871	0.087
ave.	1.441	0.862	0.085

hours	SPR/SCOD	SPS/SCOD	SPS/SPR
10.5	0.552	0.058	0.104
23.0	0.571	0.050	0.087
32.5	0.646	0.057	0.089
48.0	0.601	0.061	0.101
57.5	0.595	0.060	0.100
ave.	0.593	0.057	0.096

Table L.1 Reactor data, cont.

F

hours	SCOD mg/L	SPR mg/L	SPS mg/L	VSS mg/L
0.0	1163	880.0	87.3	1076
6.0	409	695.7	74.7	819
14.5	1022	565.0	60.5	700
20.0	968	564.0	56.1	629
21.0	1453	754.0	85.3	1015
ave.	1147	645.0	69.1	790

hours	SCOD/VSS	SPR/VSS	SPS/VSS
0.0	1.081	0.818	0.081
6.0	0.499	0.849	0.091
14.5	1.460	0.807	0.086
20.0	1.539	0.897	0.089
21.0	1.432	0.744	0.084
ave.	1.477	0.816	0.086

hours	SPR/SCOD	SPS/SCOD	SPS/SPR
0.0	0.757	0.075	0.099
6.0	1.703	0.182	0.107
14.5	0.553	0.059	0.107
20.0	0.583	0.058	0.099
21.0	0.519	0.059	0.113
ave.	0.552	0.059	0.107

Table L.1 Reactor data, cont.

G

hours	SCOD mg/L	SPR mg/L	SPS mg/L	VSS mg/L
0.0	1227	638.1	71.4	794
7.0	968	558.0	53.6	694
11.0	1004	510.8	53.0	614
ave.	986	534.4	53.3	654

hours	SCOD/VSS	SPR/VSS	SPS/VSS
0.0	1.545	0.804	0.090
7.0	1.395	0.804	0.077
11.0	1.635	0.832	0.086
ave.	1.515	0.818	0.082

hours	SPR/SCOD	SPS/SCOD	SPS/SPR
0.0	0.520	0.058	0.112
7.0	0.576	0.055	0.096
11.0	0.509	0.053	0.104
ave.	0.542	0.054	0.100

Table L.2 Biofilm thickness (μm) vs time

hours	E	F	G
2	-	-	-
3	-	-	79.8
4	-	-	-
5	-	-	134.4
6	-	80.4	-
7	-	-	117.5
8	-	89.6	20.5
9	-	-	69.4
10.5	96.2	-	-
11	-	-	90.5
14.5	-	172.7	-
20	-	191.2	-
23	276.3	-	-
32.5	232.5	-	-
48	202.8	-	-
57.5	215.3	-	-

Table L.3 Attached and loose biofilm development vs time (ug/cm2)

	Attached COD µg/cm2			Attached PR µg/cm2			Attached PS µg/cm2		
hours	E	F	G	E	F	G	E	F	G
2	-	-	31.4	-	-	18.8	-	-	15.5
3	-	56.0	170.5	-	35.1	90.0	-	7.6	18.6
4	-	-	200.6	-	-	111.7	-	-	21.4
5	-	-	192.1	-	-	116.0	-	-	-
6	-	168.6	-	-	94.8	-	-	21.9	-
7	-	-	346.8	-	-	197.1	-	-	34.1
8	-	203.2	237.0	-	113.9	132.2	-	19.2	40.6
9	-	-	361.7	-	-	197.8	-	-	37.8
10.5	156.4	-	-	71.3	-	-	17.0	-	-
11	-	-	466.3	-	-	243.7	-	-	46.8
14.5	-	379.9	-	-	188.8	-	-	39.7	-
20	-	477.9	-	-	213.9	-	-	43.4	-
23	180.5	-	-	95.7	-	-	24.5	-	-
32.5	287.9	-	-	132.2	-	-	25.0	-	-
48	466.9	-	-	188.0	-	-	35.4	-	-
57.5	369.7	-	-	162.7	-	-	31.1	-	-

	Loose COD µg/cm2			Loose PR µg/cm2			Loose PS µg/cm2		
hours	E	F	G	E	F	G	E	F	G
2	-	-	41.1	-	-	19.3	-	-	2.1
3	-	37.1	38.1	-	7.2	26.9	-	11.8	6.0
4	-	-	204.7	-	-	111.2	-	-	16.5
5	-	-	173.8	-	-	135.0	-	-	18.6
6	-	172.3	-	-	92.5	-	-	20.5	-
7	-	-	216.9	-	-	138.4	-	-	16.8
8	-	274.1	392.8	-	125.9	189.9	-	25.2	31.8
9	-	-	448.4	-	-	248.3	-	-	31.5
10.5	170.7	-	-	90.4	-	-	17.7	-	-
11	-	-	351.3	-	-	193.9	-	-	26.6
14.5	-	579.5	-	-	301.6	-	-	47.9	-
20	-	824.8	-	-	417.8	-	-	41.9	-
23	230.4	-	-	124.7	-	-	13.8	-	-
32.5	372.9	-	-	256.7	-	-	24.0	-	-
48	498.9	-	-	258.4	-	-	26.3	-	-
57.5	566.8	-	-	291.8	-	-	33.3	-	-

Table L.4 Biofilm concentrations vs time (mg/mL biofilm)

	COD mg/mL			PR mg/mL			PS mg/mL		
hours	E	F	G	E	F	G	E	F	G
3	-	-	21.4	-	-	11.0	-	-	2.3
4	-	-	-	-	-	-	-	-	-
5	-	-	14.3	-	-	8.6	-	-	-
6	-	21.7	-	-	12.2	-	-	3.1	-
7	-	-	29.5	-	-	16.8	-	-	2.9
8	-	24.1	115.3	-	13.7	64.3	-	2.3	19.8
9	-	-	52.2	-	-	28.5	-	-	5.4
10.5	16.0	-	-	7.3	-	-	1.8	-	-
11	-	-	50.1	-	-	26.4	-	-	5.2
14.5	-	28.6	-	-	14.3	-	-	2.9	-
20	-	26.0	-	-	11.6	-	-	2.4	-
23	7.7	-	-	4.1	-	-	1.0	-	-
32.5	15.3	-	-	6.8	-	-	1.3	-	-
48	14.8	-	-	5.9	-	-	1.2	-	-
57.5	19.3	-	-	8.4	-	-	1.6	-	-
ave.	14.62	25.09	33.50	6.50	12.95	18.26	1.38	2.66	3.95

Table L.5 Attached percentage (%) vs time

	COD			PR			PS		
hours	E	F	G	E	F	G	E	F	G
2	-	-	43.3	-	-	49.3	-	-	88.2
3	-	60.2	81.7	-	39.0	76.6	-	83.0	75.7
4	-	-	49.3	-	-	50.2	-	-	56.5
5	-	-	52.5	-	-	46.2	-	-	-
6	-	54.4	-	-	52.8	-	-	53.2	-
7	-	-	61.5	-	-	58.7	-	-	67.0
8	-	43.2	37.6	-	43.8	41.0	-	50.1	56.1
9	-	-	44.6	-	-	44.3	-	-	54.6
10.5	47.2	-	-	43.6	-	-	51.5	-	-
11	-	-	55.5	-	-	54.6	-	-	63.6
14.5	-	40.0	-	-	48.2	-	-	38.6	-
20	-	40.3	-	-	52.2	-	-	36.6	-
23	44.3	-	-	43.8	-	-	64.1	-	-
32.5	47.2	-	-	38.4	-	-	54.7	-	-
48	49.3	-	-	45.2	-	-	57.7	-	-
57.5	39.8	-	-	36.5	-	-	49.3	-	-
ave.	45.6	47.6	53.3	41.5	46.6	52.6	55.5	52.3	65.9

Table L.6 Biofilm ratios (mg/mg) vs time

	Attached biofilm PR/COD			Attached biofilm PS/COD			Attached biofilm PS/PR		
	E	F	G	E	F	G	E	F	G
2	-	-	0.60	-	-	0.49	-	-	0.83
3	-	0.63	0.52	-	0.14	0.11	-	0.22	0.21
4	-	-	0.56	-	-	0.11	-	-	0.19
5	-	-	0.60	-	-	-	-	-	HA
6	-	0.56	-	-	0.14	-	-	0.24	-
7	-	-	0.57	-	-	0.10	-	-	0.17
8	-	0.56	0.56	-	0.09	0.17	-	0.17	0.31
9	-	-	0.55	-	-	0.10	-	-	0.19
10.5	0.462	-	-	0.119	-	-	0.25	-	-
11	-	-	0.53	-	-	0.11	-	-	0.20
14.5	-	0.50	-	-	0.10	-	-	0.21	-
20	-	0.45	-	-	0.09	-	-	0.20	-
23	0.531	-	-	0.136	-	-	0.26	-	-
32.5	0.469	-	-	0.089	-	-	0.19	-	-
48	0.403	-	-	0.076	-	-	0.19	-	-
57.5	0.442	-	-	0.085	-	-	0.19	-	-
ave.	0.461	0.539	0.560	0.101	0.112	0.117	0.216	0.208	0.217

	Loose biofilm PR/COD			Loose biofilm PS/COD			Loose biofilm PS/PR		
	E	F	G	E	F	G	E	F	G
2	-	-	0.47	-	-	0.05	-	-	0.11
3	-	0.19	0.71	-	0.32	0.16	-	1.65	0.22
4	-	-	0.54	-	-	0.08	-	-	0.15
5	-	-	0.78	-	-	0.11	-	-	0.14
6	-	0.62	-	-	0.16	-	-	0.25	-
7	-	-	0.64	-	-	0.08	-	-	0.12
8	-	0.44	0.48	-	0.09	0.08	-	0.23	0.17
9	-	-	0.55	-	-	0.07	-	-	0.13
10.5	0.536	-	-	0.099	-	-	0.19	-	-
11	-	-	0.55	-	-	0.08	-	-	0.14
14.5	-	0.53	-	-	0.08	-	-	0.16	-
20	-	0.52	-	-	0.06	-	-	0.10	-
23	0.543	-	-	0.060	-	-	0.11	-	-
32.5	0.703	-	-	0.062	-	-	0.09	-	-
48	0.632	-	-	0.053	-	-	0.14	-	-
57.5	0.509	-	-	0.058	-	-	0.11	-	-
ave.	0.585	0.528	0.590	0.066	0.098	0.087	0.129	0.185	0.146

Table L.7 Attached and loose film development rates vs biomass specific activity

	Specific activity gCOD/gVSS/d	Rate µg/cm2/h	Intercept	Correlation coefficient	Rate µg/cm2/h	Intercept	Correlation coefficient
		AFCOD			LFCOD		
E	0.0000	9.05	12.03	0.979	8.89	63.15	0.990
F	0.3833	24.50	4.55	0.994	46.57	-102.10	0.999
G	0.8135	40.00	5.86	0.925	43.65	-34.00	0.891
		AFPR			LFPR		
E	0.0000	3.18	31.32	0.991	4.42	52.88	0.922
F	0.3833	10.28	23.35	0.975	24.26	-60.85	0.999
G	0.8135	21.15	8.70	0.923	23.00	-8.22	0.901
		AFPS			LFPS		
E	0.0000	0.47	12.20	0.974	0.36	10.70	0.894
F	0.3833	2.09	4.87	0.960	3.29	-7.47	0.998
G	0.8135	3.57	8.25	0.986	3.07	-0.06	0.876

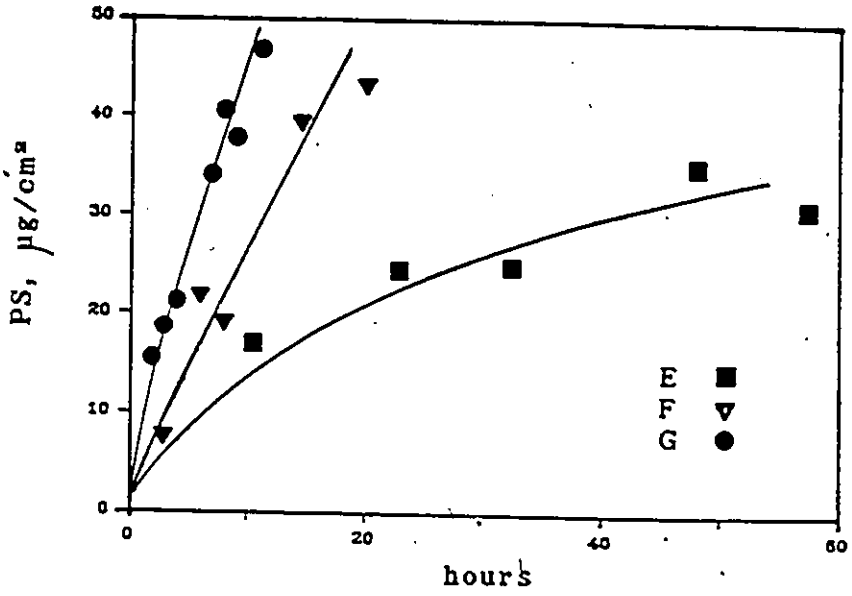

Figure L.1 Attached polysaccharide vs time

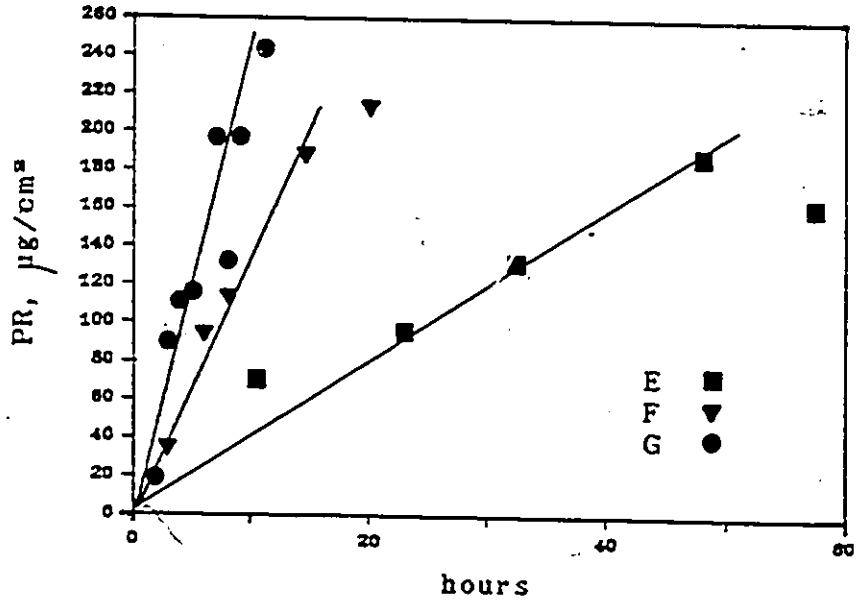

Figure L.2 Attached protein vs time

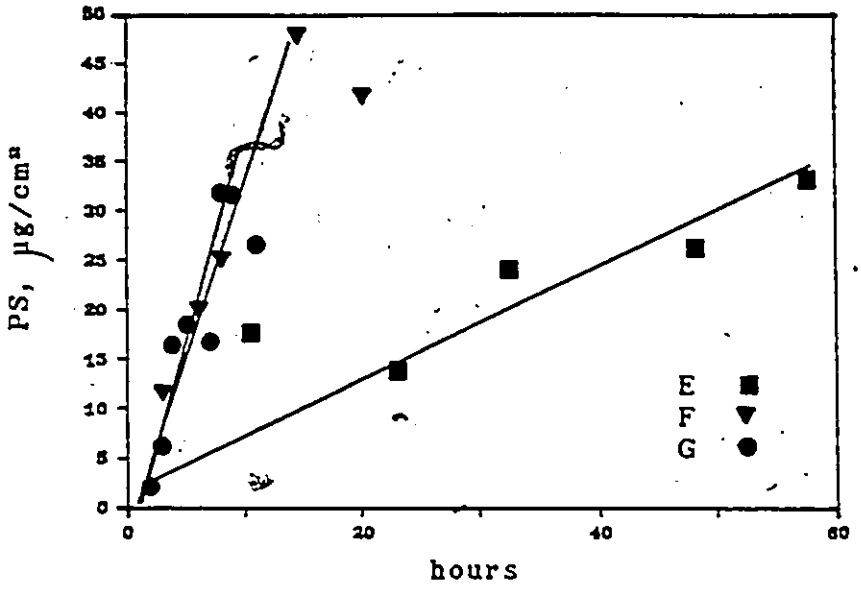

Figure L.3 Loose polysaccharide vs time

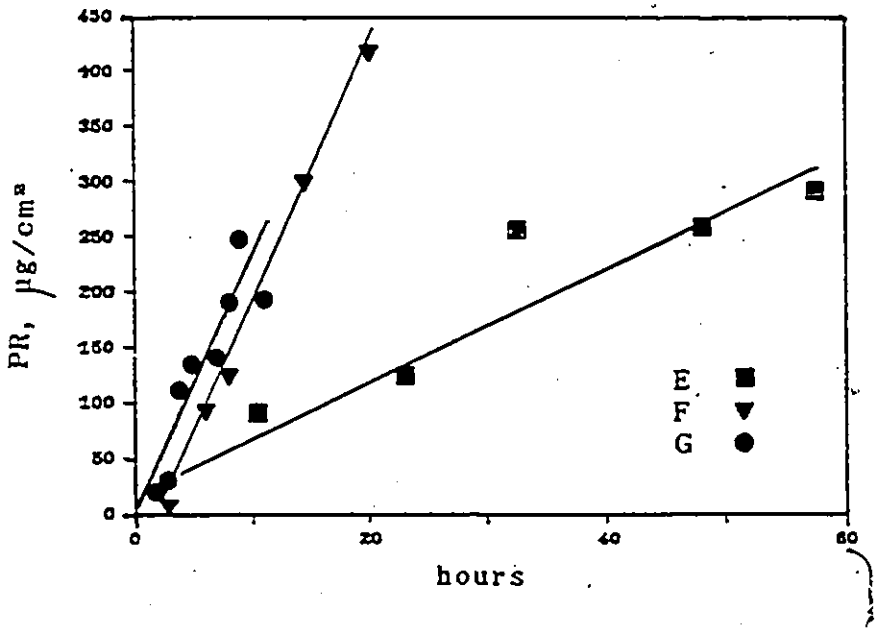

Figure L.4 Loose protein vs time

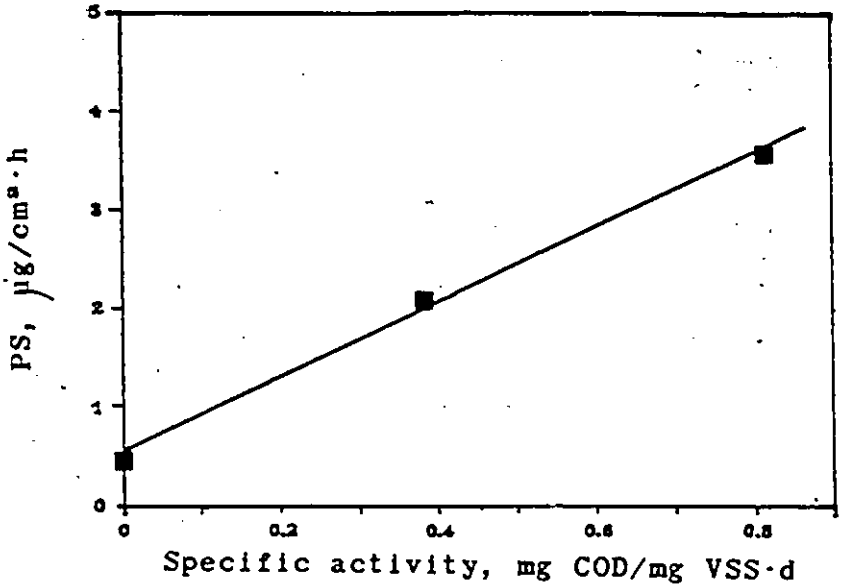

Figure L.5 AFPS deposition rate vs biomass specific activity

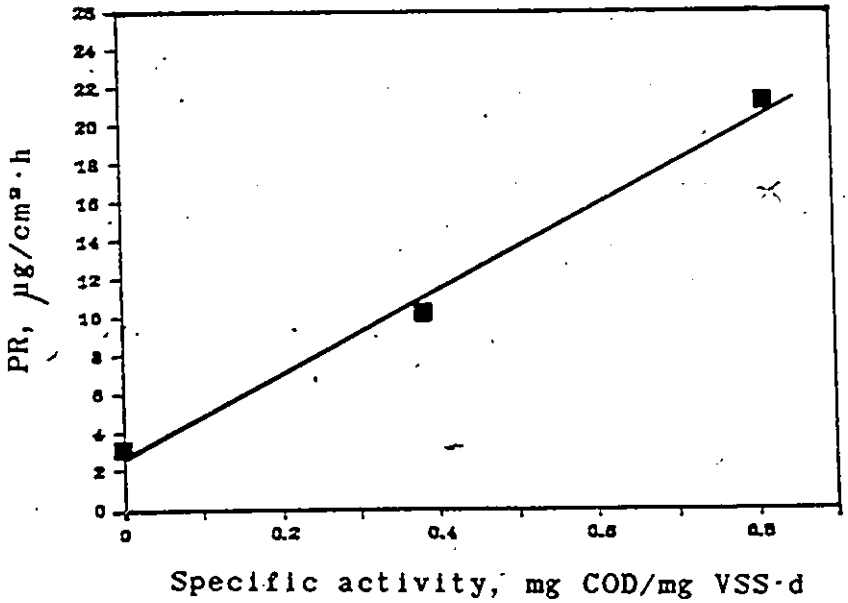

Figure L.6 AFPR deposition rate vs biomass specific activity

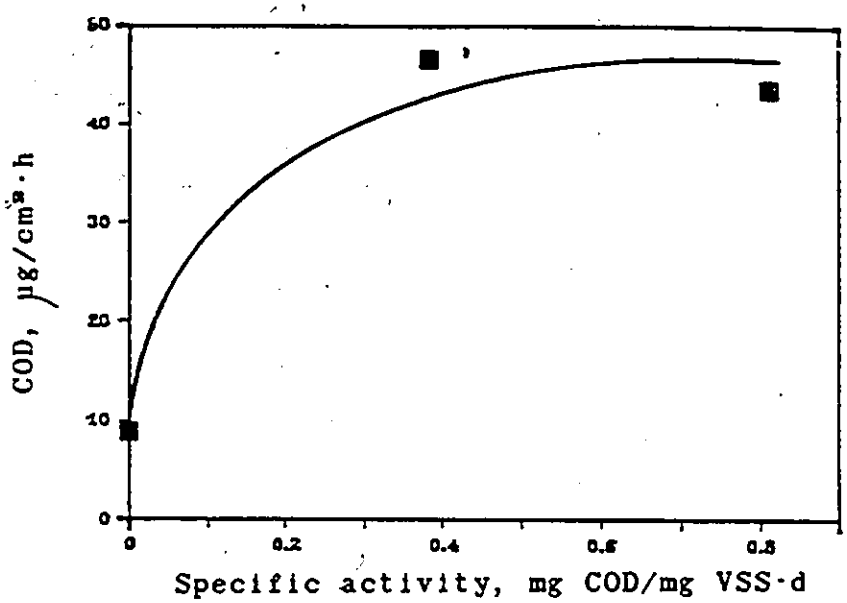

Figure L.7 LFCOD deposition rate vs biomass specific activity

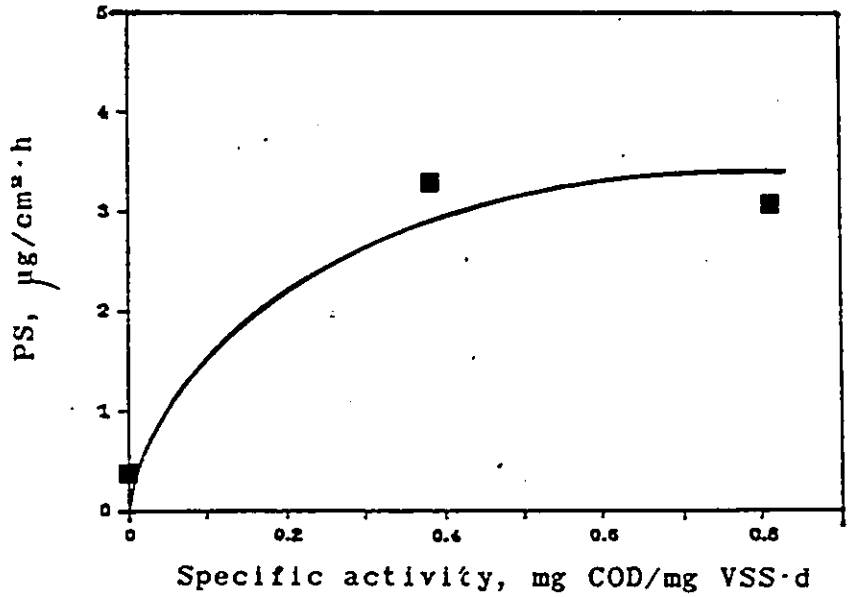

Figure L.8 LFPS deposition rate vs biomass specific activity

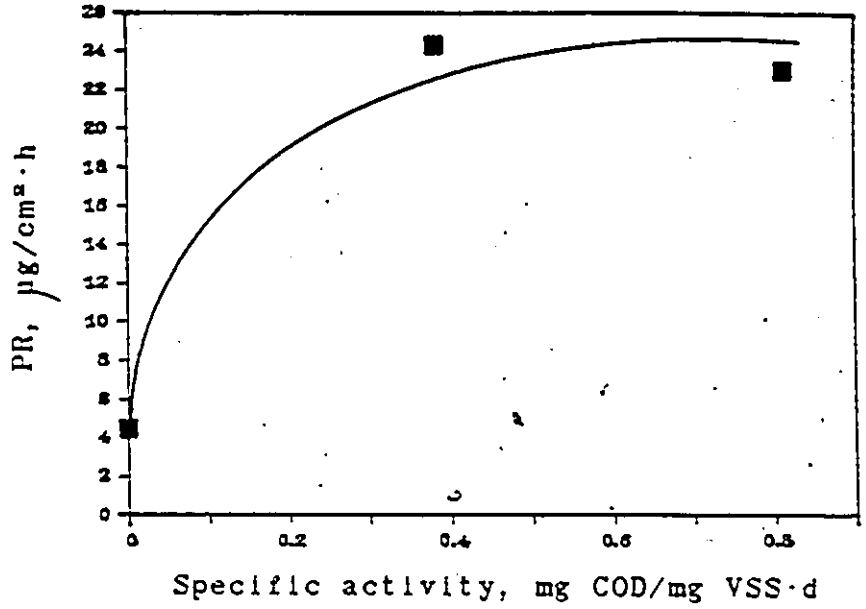

Figure L.9 LFPR deposition rate vs biomass specific activity

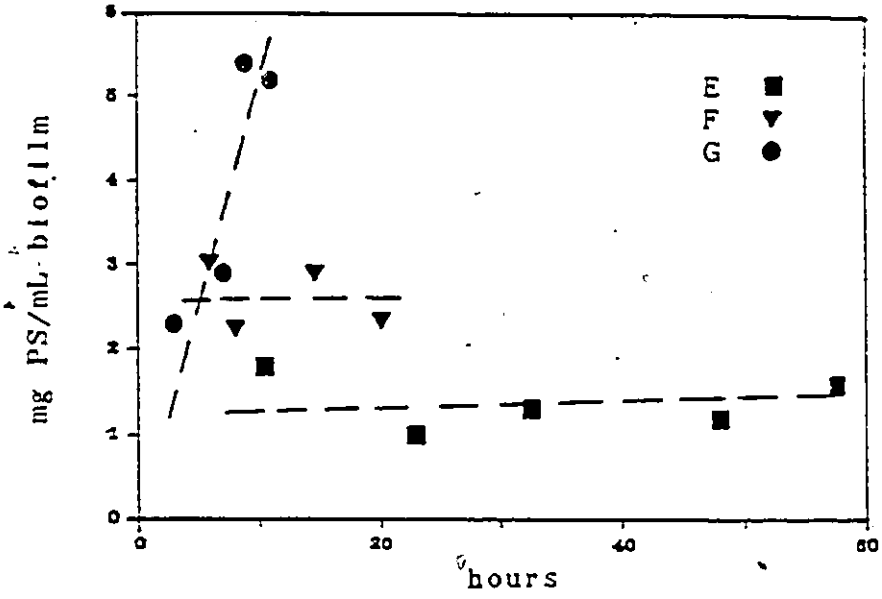

Figure L.10 Biofilm polysaccharide concentration vs time

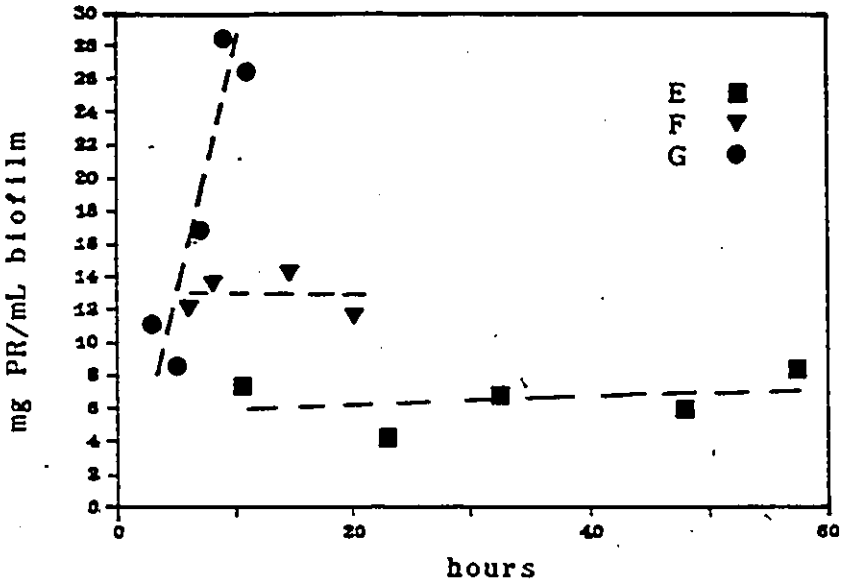

Figure L.11 Biofilm protein concentration vs time

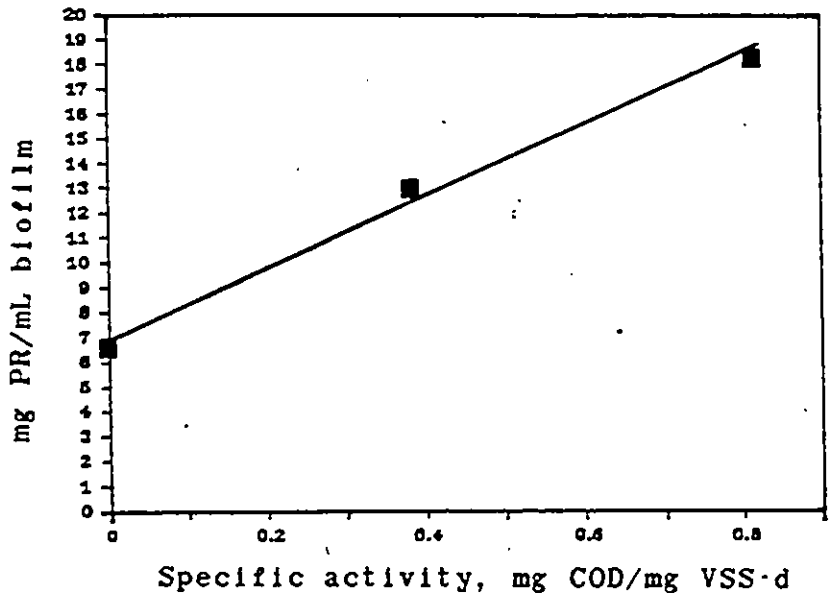

Figure L.12 Biofilm protein concentration vs biomass specific activity

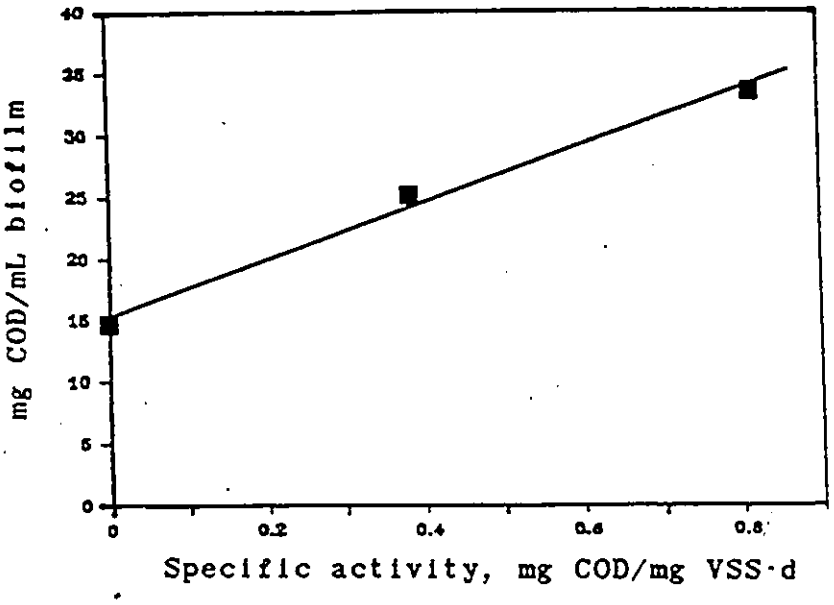

Figure L.13 Biomass COD concentration vs biomass specific activity