EFFECTS OF CALCIUM ON ANAEROBIC ACIDOGENIC BIOFILMS

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ABSTRACT

Calcium has been found to be involved in formation and development of the biofilms for many species of bacteria, but effects of calcium on anaerobic biofilms for industrial application have rarely been reported. In this study, a mixed-culture of anaerobic bacterial biofilms were grown in lactose cultural medium with various calcium concentrations -- 1.2, 80, 100, 120, 170 and 230 mg/l. Specially designed CSTR reactors were used. The temperature and pH in the reactors were controlled at 35 °C and pH 4.5 for optimal growth of acidogenic bacteria. The influence of calcium on biofilm dry mass, total organic carbon, immobilized calcium concentration and biofilm specific activity were measured.

The biofilm mass accumulation was increased by the presence of calcium in the growth medium when calcium concentration was not higher than 120 mg/l. Calcium accumulated in the biofilms increased in proportion to the calcium level in the feed. The biofilms for an increased input calcium concentration showed a trend of decrease in specific activity. The biofilms with a thickness of less than 0.5 mm had the highest specific activity. The optimum calcium concentration for the substrate consumption by the biofilms was 100 -120 mg/l.

The biofilms transferred from higher calcium medium to lower calcium medium were more susceptible to sloughing from their support surfaces, indicating calcium's role in the stability of the biofilm structure.

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INTRODUCTION

Bacteria adhere to solid surfaces, grow and proliferate, and form a thin film on the surfaces. This film consists of bacteria immobilized in the highly hydrated exopolymers and is called biofilm. It is a common form of life in bacterial ecology.

Biofilm reactors that use biofilms as catalysts offer advantages over the traditional suspended cell reactors in maintaining a higher concentration of bacterial cells, which leads to a faster reaction rate, and immobilization of the cells, which makes separation of the biocatalysts and the desired products much easier. Biofilm reactors are also more advantageous than the other immobilized cell reactors in their simpler cell immobilization processes, higher system stability and reduced diffusional resistance from the bulk liquid to the immobilized cells. Because of their advantages, biofilm reactors have drawn a great deal of attention in the past ten years. Substantial advances have been made in the technology and industrial application of biofilm reactors, in particular, anaerobic biofilm reactors.

Several types of anaerobic biofilm reactors have found their use in treatment of wastewater from many industries, such as food processing, beverage, pulp and paper industries, and agricultural wastes. Such advanced anaerobic digestion systems provide a higher efficiency and stability in wastewater treatment operations than the traditional anaerobic digestion systems. However, the major problems remain with these systems, including slow start-up, lack of control over the biofilm thickness or long term operational stability, and difficulties in development of reliable models for scale up. This is not only

because of the nature of the anaerobic bacteria but also due to lack of understanding of the mechanisms existing in the biofilm formation and development processes.

In order to overcome these problems, many studies have been carried out on the influence of environmental factors, such as substrate concentration, pH, temperature and nutrients on performance of the anaerobic biofilm reactors. But little work has been done so far on the effects of calcium on anaerobic biofilms for wastewater treatment, although calcium is found to be important for the biofilms of many species of bacteria.

Calcium is involved in biofilm formation and activities on three levels. First, on cell-environment; calcium may 'condition' the surfaces of support and bacterial cells. For example, calcium cations may alter the surface charge or electrostatic character of the surfaces, thus facilitating bacterial adhesion to the support surface or bacterial aggregation. Second, on cell-cell; calcium plays a role in buildup of biofilm structures. Typically, calcium ions act as 'cation bridges' between polysaccharides originating from different cells. Third, within cells, calcium is required for certain biochemical reactions in bacteria and some bacterial physiological activities.

Therefore, a study on the influence of calcium on anaerobic biofilms will be beneficial for an understanding of anaerobic biofilm mechanisms and may be useful for control of biofilm processes and practical operations in anaerobic wastewater treatment.

This thesis includes five parts. In the first chapter, the general characteristics, structure and functions of biofilms, biofilm processes and influencing factors are outlined. Then the anaerobic biofilm characteristics and anaerobic digestion processes are briefly discussed. Finally, the previous work on calcium related to biofilms is reviewed. In the second chapter, the objectives and research scope of this study and the methodology used in the experiments are introduced. The experimental results are presented and discussed in the third chapter. The conclusions are given in the fourth chapter with some

recommendations for the further study. The raw experimental data related to the results in the third chapter are attached in this thesis as an appendix.

CHAPTER 1

LITERATURE REVIEW

Biofilm formation and development comprises a series of processes resulting from the interactions between biological, chemical and physical parameters concerning the organisms, the support material and the environment. An understanding of the structure and function of biofilms is essential for the prediction and control of biofilm processes. Research aimed at the understanding and predicting of biofilm structures and functions has been advanced by scientists and engineers in many disciplines, in particular in general microbiology, limnology, soil science, dental and medical sciences, and control of industrial biofouling and biocorrosion. Utilization of biofilms in up-to-date biological wastewater treatment technology has also contributed to the knowledge of biofilms and their mechanism. In this chapter, the general structure and function of a biofilm, biofilm processes and the factors influencing them will be first introduced. Then the studies regarding properties of anaerobic biofilms important for waste water treatment will be reviewed. Finally, calcium's role in bacteria and its effect on adhesion and biofilm development of bacteria, especially those used for anaerobic treatment of waste water, will be discussed.

1.1 The structure and functions of biofilms

Biofilms consist mainly of water (79-95%), extracellular polymer substances EPS (70-95% of the organic matter of the dry biofilm mass), the microorganisms, and the embedded solid particles as well as dissolved substances (Flemming, Hans-Curt, 1993). Although the microorganisms account for only a minor part of the biofilm mass and volume, they are the centre of the biological activities in biofilms. They excrete the EPS and control the physical and chemical properties of the biofilm. The EPS include polysaccharides and glycoproteins. The mass of tangled fibres of polysaccharides or branching sugar molecules extending from the bacterial surfaces forms the very adsorptive and porous structure, or gel-like structure featured by biofilms in aquatic environments. Materials absorbed or entrapped in EPS include solute and inorganic particles - most often, calcium and magnesium. These materials play roles in the biofilm structure by influencing the growth and metabolism of cells, and/or are involved in bridging between cells, polysaccharides and the substratum (Costerton, J.W., Geesey, G.G., & Cheng, K.J., 1978) or in maintenance of the tertiary structure of EPS (Turakhia, M.H., Cookey, K.E., & Characklis, W.G., 1983). The limited growth due to the diffusional transport of nutrients from bulk liquid into the gel matrix is a major characteristics of biofilms. The water content of a biofilm is important for the enzyme activities and the transport of nutrients and metabolites within the biofilms. Biofilms may be heterogenous in space and time. The same biofilm can provide a variety of microenvironments for microbial systems. For example, in one-phase methanogenic biofilm reactors, the biofilm may contain an upper layer dominated by acidogenic bacteria and a base layer dominated by methanogenic bacteria. The biofilm surface is hydrophillic, rough, soft and 'sticky' (Characklis, W.G. & Marshall, K.C., 1990).

1.1.1 The biofilm system

According to Wilderer and Characklis (Wilderer, P.A. & Characklis, W.G., 1989). a biofilm system includes five compartments: 1) the substratum, 2) the base film, 3) the surface film, 4) the bulk liquid and 5) the gas. Each compartment can be described in terms of its thermodynamic and transport properties as well as by the transport and transformation processes that dominate within the compartment.

The substratum plays a major role in biofilm processes during the early stages of biofilm accumulation and may influence the rate of cell accumulation as well as the initial cell population distribution. The base film consists of a rather structured microbiology accumulation, having relatively well-defined boundaries. Molecular (diffusive) transport dominates in the base film. The surface film provides a transition between the bulk liquid compartment and the base film. Gradients in biofilm properties in the direction away from the substratum are most important in the surface film. Advective transport dominates the surface film.

The biofilm compartment contains at least two phases: 1) a continuous liquid phase which fills a connected fraction of the biofilm volume and contains different dissolved and suspended particulate materials. The suspended material consists of particles which can move in space independently of one another; 2) a series of solid compartments each composed of specific particulate materials, such as species of bacteria, extracellular and inorganic particles. The solids can not move freely, because the are attached to each other. Movement of attached particles within one solid compartment causes displacement of neighbouring particles. Thus, each type of attached solid constitutes a different solid phase, which in addition may contain other compartments. Transport of a suspended

particle from the liquid phase to the solid phase within the biofilm has characteristics of a reaction process, since educt and product do not belong to the same phase. Thus the interfacial transfer processes must be distinguished from the transport processes within the biofilm compartment.

Bulk liquid compartment processes affect biofilm system behaviour primarily as a result of the mixing or flow patterns resulting from the system's geometry. Mass and heat transfer from the bulk liquid compartment to the biofilm compartment is dependent on the liquid dynamic regime: mass transfer in laminar flow will be much slower than in turbulent flow systems. The reactor or system geometry also influences mixing and, consequently , the mass transfer processes. Therefore, the reactor geometry and flow regime frequently determine the progression of biofilm accumulation. The gas compartment provides for aeration and removal of gaseous metabolites, such as CO_2, CH_4, H_2 in anaerobic biofilms.

The biofilm system compartments interact with each other via transport and interfacial transfer processes, which play a critical role in biofilm systems and generally, are rate-controlling (Characklis, W.G. & Marshall, K.C., 1990).

1.1.2 The functions of biofilms

Biofilms represent a unique form of bacterial life. When adsorbed to surfaces, bacteria may change greatly in cellular physiological features (Gilbert, P., Evans, D.J. & Brown, M.R.W., 1993). It is the way in which bacteria adapt to the environment under certain circumstances. The advantages of bacterial colonization on surfaces over discrete cells, which can also be considered, to some extent, as the driving forces for biofilm

formation, are summarized (Fletcher, M., 1990; Flemming, Hans-Curt, 1993; Gilbert, P., Evans, D.J. & Brown, M.R.W., 1993) as follow:

I) Nutrient enrichment Nutrients, especially surface-active substrate such as cations, fatty acids and macromolecules, are adsorbed to the surfaces and thus concentrated and localized at the interface, facilitating utilization by attached bacteria. On the other hand, the biofilm functions as a filter in adsorption and uptake of nutrients from the flowing water phase and benefits the cell growth in the gel matrix.

II) Enhancement of survival The biofilm structure provides some protection to bacteria so that they may become more likely than free-living cells to survive potentially lethal conditions, such as short term pH-fluctuations, salt- and biocide concentration shocks; shear forces and dehydration, etc..

III) Development of microconsortia for improved growth, -symbiosis, - utilization of less readily biodegradable substrate by specialized organisms, - creation of ecological niches.

IV) They are not washed from the reactor by high flow rates of influent and have long residence times.

V) Pool and preservation of genetical information because of the long retention times of the microorganisms and promoted genetic exchange brought about through close proximity of other cells.

VI) Modulation of the physicochemical environment of the cells through the establishment and maintenance of pH and electro-potential gradients across the biofilm.

1.1.2 The processes of biofilm formation and development

Although different views exist in the processes involved in biofilm formation (Characklis, W.G., 1990; Gilbert, P., Evans, D.J. & Brown, M.R.W., 1993; Bryers, J.D., 1993; Bryers, J.D. & Banks, M.K., 1990; Bryers, J.D. & Characklis, W.G., 1981), the biofilm formation and development can be divided into three stages: 1) bacterial adhesion to the surface, 2) the competitive success of colonizers and their subsequent growth, and 3) detachment and dispersal from biofilms.

Bacterial adhesion to surfaces This stage includes three processes: 1. cell-particle transport to the substratum 2. reversible adhesion and 3. irreversible adhesion or attachment.

Bacterial cells are transported to the substratum from the bulk liquid because of fluid dynamic forces, gravity, Brownian motion, and bacterial tendency to adhere at the nutritious surfaces, etc. When the cells strike the substratum, they may adsorb to it, and then desorb. This reversible process primarily involves long range interaction forces between the cell and the substratum such as London - van der Waals force, double layer electrostatic interactions, steric interactions, and possibly polymer bridging. Since this step is mainly dependent on the thermodynamic properties of the related surfaces, it is also referred as the non-specific adsorption.

Some of the reversibly adsorbed cells may bond to the substratum firmly, thus the adsorption becomes irreversible. This step is frequently mediated by bacterial surface polymers, and is regarded as the result of specific interactions between cells and substratum. It involves short range force such as dipole-dipole (Keesom) interactions, dipole-induced dipole (Debye) interactions, ion-dipole interactions, hydrogen bonds, hydrophobic interactions, or polymeric bridging (Marshall, K.C., 1985).

Here the colloid chemical theory - Derjaguin-Landau and Verwey Overbeek

(DLVO) theory, which describe the changes in the Gibbs energy of interaction as a function of the distance between two particles, is utilized to explain the adsorption processes (Characklis, W.G., 1990; Gilbert, P., Evans, D.J. & Brown, M.R.W., 1993). The DLVO theory has been successfully applied to microbial adsorption in describing long-range forces while it remains a problem for short-range forces due to the changes in electrochemical states resulting from physiological activities of viable microbial cells (Bryers, J.D. & Characklis, W.G., 1981; Rutter, P.R. & Vincent, B., 1984). For example, at low concentrations (<0.1M) the affinity of *Vibrio Alginolyticus* for an hydroxyapatite surface increased with ionic strength, in agreement with the D.L.V.O. theory. At higher concentrations, bacterial affinity for the surface decreased with increasing concentration of cations and was not related to ionic strength changes in the medium (Gordon, A.S. & Millero, F.J., 1984).

Another basic theory for the quantitative description of the interactions of bacterial cells with substrata surfaces is the "wettability" theory, in which cell adsorption is considered as a function of the total interfacial free energy. This theory is often used for the evaluation of surface characteristics concerning bacterial adsorption (Kozlyak, E.I. & Yakimov, M.M.; 1992; Characklis, W.G., 1990; Gilbert, P., Evans, D.J. & Brown, M.R.W., 1993).

Competitive success and growth Following attachment to the substratum, bacteria will grow, modify the surface and propagate if conditions are suitable, resulting in the development of microcolonies. At this stage, the production and accumulation of extracellular polymers, usually polysaccharides, is often apparent. With time, growth and further attachment can lead to coalescing of microcolonies and complete coverage of the surface by intracellular films of bacteria embedded in a highly hydrated polymeric matrix.

Thus the cells are immobilized with neighbouring cells in close proximity and with little room for growth. The dynamics and interactions with such biofilm communities are poorly understood and almost untouched experimentally. Presumably, there are the following situations: 1) cometabolism and mutualism, exchange of metabolites, protons and hydrogen between functionally different bacteria, which may further stimulate growth of microorganisms (Kent, C.A., 1988). 2) competition for nutrients, which may determine the nature of the mature biofilm community. For a binary bacterial biofilm system, the faster-growing bacteria will rapidly become dominant. However, the slower growing bacteria remain established within the biofilm and continue to increase in number over time (Banks, M.K. & Bryers, J.D., 1991). 3) production of antimetabolites which could allow a strain to resist adjacent colonization by newly attaching organisms.

Detachment and dispersal Detachment from biofilms can be divided into three distinctly different processes: erosion, the continuous removal of small particles from the surface of biofilms, primarily caused by the shear stress created by water flow past the biofilm; abrasion , caused by the collision and / or rubbing together of particles, some of which are covered with biofilm; sloughing, periodic loss of large patches of biofilm. Detachment is often considered as the loss of biofilm resulted from the influences of foreign forces or the decay of the biofilm. To distinguish it from detachment, dispersal is used to describe the process of microorganisms spreading from the biofilm, which is brought about through enzymatic or chemical cleavage control by microorganisms themselves (Gilbert, P., Evans, D.J. & Brown, M.R.W., 1993). Dispersal is a self-adaption mechanism of bacteria in order to survive and colonize new niches. Some studies suggested that changes occurred in the adhesive properties of the cell surfaces, associated with the cell division process, and were able to bias the reversible adhesion in favour of

dispersal (Eighmy, T.T, Maratea, D., & Bishop, P.L., 1983).

The rate of detachment is influenced by the foreign forces to which biofilm is exposed. The surface of the substratum also provides physical conditions which affect detachment although the impacts may be more subtle than for the hydrodynamic effects of shear stress and abrasion. Similarly, the physiological factors of bacteria, such as specific growth rate, extracellular polymer production may play an important role in determine the detachment rate.

1.1.3 The factors affecting the biofilm processes

The factors governing biofilm formation include (Fletcher, M., 1990; Wilderer, P.A. & Characklis, W.G., 1989):

- Inherent genetic characteristics, which ultimately determines the bacterial surface characters
- Genetic expression mechanisms, which control the adhesiveness of bacteria in response to different environments
- Physiological factors such as growth rate, cell concentration, age
- Nutritional factors such as carbon, nitrogen, minerals
- Physicochemical factors such as pH, temperature, ionic strength, cations
- Surface characteristics of substrata such as surface chemical properties, roughness
- Physical conditions such as flow pattern, mixing, reactor geometry

Considering their importance to practical biological operations, only some of the above factors were selected for discussion. Other factors may be covered in discussions of

other topics if related.

Surface characteristics

The DLVO theory and wettability theory provide guidelines for the analysis of the effects of surface characteristics of both substrata and bacteria.

[Substratum surfaces] The properties, namely surface characteristics, of support materials affect mainly the formation of the first layer of biofilm. The surface charge and thermodynamic parameters used to measure substratum differences include interfacial or surface free energy, critical surface tension, water wettability, water contact angle, etc. As far as surface tension is concerned, in the range of 20 -30 dyne/cm, the adhesion of microorganisms is less favourable of. Most engineering materials and coatings show a high capacity for adsorbing the first layers of biofilm.

Higher surface roughness favours the anchorage of microorganisms due to the increase in contact area. However this effect is limited to the first layers of biofilm and therefore to the induction of the overall process. Thus, the use of small size porous granulated carriers and porous fibre carriers was recommended for higher capacity of bacterial adsorption. The minimum pore size of five times larger than the major dimension of the cells reproducing by division was noted (Messing, R.A., 1988). The toxicity of some metallic ions depend on the dominant microbial species and on the environmental conditions. It is thought that the toxic effect of the metallic ions ceases after several layers of biofilm are formed. In this case, this effect may be responsible for retarding the overall process without eliminating it completely.

[Bacterial surfaces] The effect of bacterial surface characteristics are significant not only

at the initial stage of attachment, but also during community development. The cell surface characteristics are greatly variable, depending on genetic determinants and the cellular physiological and morphological status as a result of phenotypical responses to changes in environmental conditions. However, similarity in cellular surface characters still exists among different types of bacteria and are beneficial for general considerations of bacterial adsorption. The bacterial surfaces and fibres of polysaccharides are usually considered to be negatively charged. Therefore, use of positively charged surfaces would facilitate the adhesion of bacteria. Many studies suggested that slightly more-hydrophobic cells are preferable for adsorption on surfaces, especially on hydrophobic surfaces (Kozlyak, E.I. & Yakimov, M.M., 1992; Gilbert, P., Evans, D.J. & Brown, M.R.W., 1993).

Nutrient concentration

The effect of growth limitation on bacterial adhesion was investigated under conditions of limitation of various nutrients, mainly C- and N- sources and oxygen (Applegate, D.H. & Bryers, J.D., 1990; Kozlyak, E.I. & Yakimov, M.M., 1992; Kjelleberg, S., 1984). It was found that growth under C- and N-limitation led to drastic changes in cell surface properties and adhesion patterns. In some cases, C-limitation resulted in the maximum adsorption of bacteria whereas growth limitation due to N-source worsened the adsorption. Possibly, under such conditions bacteria switched metabolism to synthesize reserve polymers, adhesive polysaccharides for C-limitation (Takii, S., 1977) and non-adhesives for N-limitation (Kozlyak, E.I. & Yakimov, M.M., 1992). In some cases the incubation of cells in a starvation medium or tap water remarkably increased the cell adhesiveness.

As the microorganisms become attached their growth will depend on the diffusion

of the nutrient through the film. The limit for this diffusion depends on the thickness and on the structure of the film. As a rule, when a biofilm thickness is more than 50 μm, diffusion limitation occurs (Peyton, B.M. & Characklis, W.G., 1993). The low level of nutrients in the deeper zones of these films may result in a decrease in the production of excreted polysaccharides thus rendering the films more vulnerable to shear forces. Wanda et al. found that a more complex substrate causes a better biofilm development and populations with a high tendency to adhesion showed a high total exopolysaccharide content (Wanda, U., Wollersheim, R., Diekmann, H. & Buchholz, K., 1990). The study of biofilm activity in a three phase fluidized bed showed that the substrate flux and the detachment rate controlled the amount of biofilm colonization. However, only the flux affected the biofilm activity, observed yield, and O_2 consumption. The higher the flux, the less inactive is the biomass, because substrate concentration is higher in the biofilm and which allows the cells to maintain a higher specific growth rate. The amount of biofilm colonization also increased when the biofilm became more active because of having a larger substrate flux (Rittmann, B.E., Trinet, F., Amar, D., & Chang, H.T., 1992).

Cellular physiological status

An understanding of the effect of the growth states of suspended cells on initial formation is important for the practical application of biofilms. In a number of experiments, it was observed that bacterial cultures at the exponential phase of growth had the greatest facility for adhesion, followed by stationery and then death-phase cultures (Kozlyak, E.I. & Yakimov, M.M., 1992; Fletcher, M., 1977). However, the adhesiveness of certain bacteria which remained unchanged at all their growth stages were also reported. On the other hand, it was also found that the detachment rate of bacteria, such as marine *Pseudomonas aeruginosa*, is directly related to biofilm growth rate and

that factors which limit growth rate will also limit detachment rate (Peyton, B.M. & Characklis, W.G., 1993). That the adhesiveness and surface hydrophobicity of *Staphococcus epidermidis* and *E. coli* decreased in early- and mid- exponential phase (Gilbert, P., Evans, D.J., Evans, E., Duguid, I.G., & Brown, M.R.W., 1991) provided another example of the effect of physiological status on bacterial attachment. In this case, cell surface charge became more electro-negative for *E. coli* but electro-neutral for *Staphococcuc epidermidis* as the cells proceeded to divide.

Fluid velocity

Low velocities tend to favour initial colonization of surfaces due to the characteristics of the laminar layer that facilitates the anchorage of microorganisms. But the laminar layer constitutes an obstacle to the diffusion of the nutrients and oxygen indispensable to the metabolism of the attached cells. Higher velocities are more favourable to the renewal of nutrients near the surfaces, allowing a faster growth of the microorganisms. However, high velocities produce higher shear stress which would induce the removal of biofilms - erosive effect. The results from experiments in an outdoor open channel, simulating natural river conditions indicate that biofilm biomass accumulation was substantially reduced as flow shear stress increased and that the maximum accumulation occurred under very low flow conditions (Lau, Y.L. & Liu, D., 1993). In another case, the rate of cell removal by fluid shear for a species was found to be a function of biofilm cell number only if the species concentration was uniform with depth; in essence, only the upper layers of the biofilm were sheared off (Banks, M.K. & Bryers, J.D., 1991). No significant influence of shear stress on detachment rate was observed in the experiments with *Pseudomonas aeruginosa* (Peyton, B.M. & Characklis, W.G., 1993). In general, higher velocities tend to produce thinner films than lower velocities but might increase the

microbial activity in the film. Higher velocities lead to compaction of the biofilm, or higher biofilm densities for higher velocities.

The structure of a biofilm (also dependent on the fluid velocity) has a relevant effect on the growth and reproduction of the attached microorganisms. The structure of biofilms is not uniform along the thickness of the biofilms resulting in a more compact layer near the deposition surface and a somewhat looser one near the solid-liquid interface. A more compact structure may be a limiting factor for diffusion of the nutrients throughout the biofilms. Thus, for moderate velocities, cell growth may increase in the presence of higher fluid velocities, but for much higher velocities the "active" layer becomes limited to the superficial layer of the biofilm (Kozlyak, E.I. & Yakimov, M.M., 1992).

pH of the fluid

The influence of fluid pH is associated with the growth rate of the microorganisms and the adhesion forces at the surfaces. a). Greater amounts of deposits are formed at the pH coinciding with the best values for growth and reproduction of the microorganisms present in the film. The pH conditions in the solid- liquid interface may be altered by the cellular metabolism which may produce acids or alkalis. b). pH can affect the distribution of electrical surface charges of materials present, and thus, their zeta potentials. The adhesion between these materials is increased when the corresponding zeta potentials have opposite signs. Since most usually, the majority of bacteria have negative electrical surface charges, it can be said that the coating of solid surfaces with materials that increase the electronegativity, would reduce the possibilities of adhesion of the great majority of bacteria.

Temperature

The effect of temperature is usually concerned with the influence of fluid temperature on the growth of the microorganisms and for some cases, the production of exopolymers by the cells. Therefore, the optimum temperature for the bacteria growth is mostly taken as that to promote adhesion of the cells to surfaces.

Presence of inorganic suspended particles

Microbes have a tendency to cover suspended inorganic particles, forming aggregates. This would enlarge the induction period of biofilm formation. On the other hand, higher quantities of biofilm are obtained when the asymptotic values of the bacteria attachment is reached. This may due to the new type of biofilm structure or to the stimulation of attached cell growth and metabolism in the presence of inorganic particles. The growth and reproduction of cells in the film is increased due to the presence of the inorganic particles. This stimulation is related to the structure formed in the biofilm that tends to be less compact and therefore more favourable to the diffusion of nutrients. It can also constitute a positive factor for microbial growth, inorganic particles may function as a source of nutrients when entrapped in the film. Inorganic particles, such as kaolin particles tend to become not only colonized by microbe but also coated with organic molecules when in suspension.

1.2 Biofilms for anaerobic wastewater treatment

Wastewater treatment with biofilm reactors is one of the most large-scale and oldest applications of biofilms in industry. In this area, anaerobic biofilm reactors have

become more and more important in the last decade. This reflects the recognition of their advantages over the traditional biological treatment processes and the advancement in research and development of biofilm technology.

1.2.1 Anaerobic digestion and related biofilm reactor technologies

The anaerobic digestion process of organic wastes is divided into three stages or phases, based on the metabolic reactions characterized by different groups of bacteria: 1). acidogenesis - hydrolysis of macromelecular compound so that they can be transported into the cell through cytoplasmic membrane, and conversion of these initial degradation products into intermediates, namely small molecular weight organic acids, carbon dioxide and hydrogen 2) acetogenesis - further degradation of the intermediates into acetate, carbon dioxide and hydrogen, and 3) methanogenesis - production of methane by decarboxylation of acetate and reduction of carbon dioxide (Gujer, W. & Zehnder, A.J.B., 1983; Marty, B., 1986; Boone, D.R., 1985; Price, E.C., 1985; Large, P.J., 1983). This model is widely accepted because it better reflects the interactions of different bacterial species in anaerobic digestion (Thiele, J.H., 1991). However, in practical applications, the traditional two-phase model, which combines the phase two and three in the three-phase model as a single methanogenic phase is more often used. This is because the two-phase model emphasizes the important distinction between the microorganisms of each phase, which are vital for design and control of the digestion process in terms of reaction kinetics. For instance, the acidogenic bacteria generally grow faster and are more resistant to inhibition, compared with the slow growing and fastidious methanogens. The reactor loading for acidogenesis can be 4 - 6 times higher than that for methanogenesis (Henze, M. & Harremoes, P., 1983).

The basic types of anaerobic biofilm reactors applied to wastewater treatment include: fluidized bed, expanded bed, upflow anaerobic sludge blanket (UASB), fixed film/filter, rotating biological contactor, and hybrids. Their configurations, advantages and performances can be found in the excellent reviews (Henze, M. & Harremoes, P., 1983; Bhamidimarri, S.M.R., 1990).

1.2.2 Characteristics of biofilms in anaerobic biofilm reactors

Directly measured data on the structure and properties of biofilms for anaerobic wastewater treatment are still quite limited. The anaerobic bacteria may follow the general patterns of biofilm formation and consequently exhibit similar biofilm characteristics although diversity exists.

In a study on the activity and the structural characteristics of methanogenic biofilms on needle punched polyester supports in a downflow-stationary fixed-film reactor (Harvey, M., Forsberg, C.W., Beveridge, T.J., Pos, J., & Ogilvie, J.R., 1984), few bacteria were found to be tightly adherent to the support surfaces. However, there was a morphologically complex, dense population of bacteria trapped within the polyester matrix. Frequently large microcolonies of a uniform morphological type of bacteria were observed. These were particularly evident for *methanosarcina*-like bacteria which grew forming large aggregates of unseparated cells. Leafy deposits of electron-dense, calcium- and phosphorus-enriched material coated the polyester matrix and some cells. As the biofilm matured there was a more extensive material deposition which completely entrapped cells. The trapped cells appeared to autolyse, and many were partially degraded. The results from energy dispersive X-ray (EDX) analysis of the dense precipitate and the

cells indicates that calcium and phosphorus concentrations in the extracellular matrix were 92 and 28 times higher respectively than in the cells. Calcium carbonate and calcium phosphate are probably the major components of the precipitate. It was postulated that further impregnation of the matrix with minerals and cell death may eventually have a deleterious effect on the methanogenic activity of the biofilm. The formation of biofilm is thought to begin by adhesion of single cells, with the gradual formation of a monolayer of cells. This leads to colonization by other organisms and the subsequent development of a thick film. The driving force of colonization is believed to be the adsorption of nutrient to the surfaces. As the population density increases on the support, there is an enhanced opportunity for cross-feeding, cometabolism, interspecies hydrogen transfer, and interspecies proton transfer, which may further stimulate growth of microcolonies.

This postulation coincides with what was suggested by the results from electronic microscopic observation of the ultrastructure of bacterial granules in an UASB and filter reactor (Macleod, F.A., Guiot, S.R., & Costerton, J.W., 1990). SEM (scanning electronic microscopy) and TEM (transmission electron microscopy) studies have revealed that the granular aggregates were three-layered structures. Based on these observations, a structure model to explain the granule development was proposed. The aggregate consists of mostly *Mathanothrix* in the centre core of the granule, acetogens and hydrogen consuming bacteria in the middle layer, and acidogens and hydrogen consuming bacteria in the exterior layer. *Methanothrix* might function as nucleation centres that initiate the development of the granule. The loose mat of *Methanothrix* filament provided a framework for colonization of other organisms. The first colonizing bacteria such as H_2-producing acetogens provided the methane producing bacteria with the required substrate, acetate. Because the high concentrations of H_2 generated by the H_2-producing acetogens inhibit degradation of propionate and butyrate, the syntropic association with H_2-using

bacteria would be required. The existence of this group of bacteria in the granule was confirmed. The metabolism of fermentative bacteria in the exterior layers of the granule would produce the substrate for underlying acetogens. The presence of H_2-using bacteria could consume free hydrogen before it penetrated into the second layer.

The results from the investigation of irreversible attachment to glass slides and biofilm development of anaerobic bacteria showed that the slow rate of development of the methanogenic consortium attached to a surface (probably due to long doubling times of the methanogenic bacteria) is a more significant, ultimate limiting factor in the start-up of a methane producing biofilm reactor than the rate of bacteria attachment, as external mass transfer resistance of substrate may also limit the developing biofilm following bacterial attachment. On the other hand, proper preparation of support surfaces is important for bacterial attachment (Robins, J.P. & Switzenbaum, M.S., 1990).

To study the effect of solid supports on adhesion of methanogenic and acidogenic bacteria, many experiments have been conducted. For example, Yu and Pinder (1992) examined quantitatively the selectivity of attachment of acetogens and methanogens on inert support surfaces. They found that the surface preference of the bacteria species used (degrading acetate, propionate and butyrate) decreased in the sequence of wood, ceramic, PVC and stainless steel, mainly due to the different hydrophilicity and wettability of these materials. Kuroda et al. submerged test specimens into bacteria slurries cultured in different media and found that bacteria adhered to the moderately rough surfaces with pores measuring a few tenths of a micron in diameter better than to the polished surfaces and very rough surfaces, and preferably adhered to the solid supports made of carbohydrate material. The accumulation rates of biomass on the solid supports submerged in the mixed slurry of acid-producing bacteria and methane-producing bacteria are higher than those on the solid supports submerged in a slurry of methane-producing

bacteria (Kuroda, M., Yuzawa, M., Sakakibara, Y., & Okamura, M., 1988). Therefore, the formation of the biofilm depends not only on the characteristics of the bacteria and fluid regimes but also on the characteristics of the solid supports. In another similar experiment with different types of support materials in down flow stationary fixed film anaerobic digesters, wood chips were found to be a more effective support material than charcoal and ceramic Rasching rings. The acid conversion efficiency improved with higher retention times, but the productivity declined (Scharer, J.M., Bhadra, A. & Moo-Young, M., 1988).

Also, film development, indicated by the rate of converting acetate to methane and CO_2, was 3 times faster on fired clay than on either PVC or etched glass. SEM photographs showed that the bacterial film on clay was thick and uniform, while the film attached to PVC plastic was thin and still uniform. Attachment to etched glass was Spotty. The characteristics of clay which made it a superior support appeared to be its rough, porous surface which offered attachment sites to the microorganisms and the presence of minerals in the clay, particularly Fe which is known to stimulate methanogenesis and growth (Murray, W.D. & van den Berg, L., 1981).

A study on the population dynamics of attached biofilms in an anaerobic fluidized bed pilot plant showed that the biofilm biomass contributed to more than half the amount of total biomass in the reactor while the suspended sludge was susceptible to hydraulic washing-out. Filamentous networks and thrix-like anaerobic bacteria grew initially in the deep holes or crevices of the medium. After this initial biofilm network formation, certain types of short rods and small cocci were embedded in the biofilm. High organic loading and high VFA (volatile fatty acids) concentration provided rapid growth of discrete rods which were washed out of the fluidized bed (Chen, S.S., Huang, S.Y., Lay, J.J., Tsai, P.S., & Cho, L.T., 1992).

In summary, in addition to possession of some general biofilm characteristics such as gel matrix structure due to highly hydrated polysaccharides, diffusional limitation, cell adhesion subjected to changes in support properties, etc., the anaerobic biofilm has the following characters: 1). very slow growing due to the nature of the anaerobes 2). the layered structure as the result of the mixed-culture 3). cavities in the biofilm brought about by the presence of gases 4).the interactions between species in the biofilm tend to be more commensal than inhibitory. 5). the biofilm contains a large amount of mineral deposit, especially calcium salts.

1.2.3 Modelling of anaerobic digestion in biofilm reactors

Due to the complexity of anaerobic digestion by biofilms and the difficulties in experiments with anaerobic microbes, modelling the biofilm processes has been of great interest in research and development. Many models for the quantitative description of anaerobic biofilm processes have been established on the basis of the kinetics of bacterial growth and biochemical reactions involved in anaerobic digestion, principles of colloidal particle thermodynamics, principles of mass transfer processes and fluid hydrodynamics, and the characteristics of anaerobic biofilm structures (Bouwer, E.J., 1987; Kitsos, H.M., Roberts, R.S., Jones, W.J., & Tornabene, T.G., 1992; Yu, J. & Pinder, K.L., 1993; Furumai, H. & Rittmann, B.E.,1992), and quite often were made by modifying those for aerobic bacteria (Wanner, O. & Gujer, W., 1986; Chang, H.T. & Rittmann, B.E., et al., 1991; Williamson, K. & McCarty, P.L., 1976; Rittmann, B.E. & McCarty, P.L., 1978; W.G. Characklis & K.C. Marshall , 1990). Since the substrate removal rate is the factor of greatest concern in the evaluation of the performance of an

anaerobic biofilm digester, early work focused mostly on kinetic models regarding substrate consumption, biofilm growth and product production.

Most recent kinetic models can be classified in four levels: I). including a simple mass balance equation on the substrate and the logistic equation or the Monod equation or its modified form such as one incorporating the product inhibition factor. In this case, it is often assumed that all the biomass exists in the form of biofilm and is viable. II). incorporating the Fick's equation for diffusional transport of substrate, into level 1. III). considering the interaction of the bacteria in two different phases or biofilm layers (Fang, M., Howell, J.A. & Canovas-Diaz, M., 1989). IV) incorporating the loss of biofilm as a function of shear force and/or the decay of biomass in the models. The models above level 2 become very complicated and some of the parameters are difficult to measure. In practice, some simplified models are more useful for analysis of substrate consumption and biofilm growth under specific conditions.

1.3 Calcium and biofilms

As mentioned above, calcium is one of the most often found inorganic elements in natural biofilms. Whether this is due to its abundance and wide distribution or because of its roles in the biofilm system, the effect of calcium on biofilm formation and biofilm activity is an interesting unknown with respect to the control of biofilm processes.

1.3.1 The bacterial roles of calcium

The biological roles of calcium have been clearly defined as: 1) structural - Structure of soft tissues (cell adhesion, membrane permeability), 2) electrical - electrical activity across some membranes, 3) cofactor for extracellular enzymes and proteins, 4) intracellular regulator (Campbell, A.K., 1983). However, these theories are established mainly on the basis of the studies on human and animal cells. For bacteria, calcium's functions are far less understood, although calcium is always used in bacterial cultural media (Silver, S., 1977).

Norris et al. summarized a large number of diverse processes in bacteria in which calcium is involved, including chemotaxis, sporulation, phosphorylation, heat shock, the initiation of DNA replication, septation, nucleoid structure, nuclease activity and recombination, the stability of the envelope, and phospholipid synthesis and configuration. They pointed out that since such varied processes have a common factor, calcium, suggesting that there are some major underlying principles of calcium metabolism which have not yet been discovered (Norris, V., Chen, M., Goldberg, M., Voskuil, J., McGurk, G., & Holland, I.B., 1991). Another author mentioned that Ca^{2+} might also function as an intracellular messenger (secondary messenger) in a microbial system, and be involved in processes such as ionic inducing bacterial mating (J.L. Reissig, 1977). Sprott, G.D. examined the structure and function of some methanogens' cell surfaces. He found that the sheaths of the methanogens contain predominant amount of Ca^{2+} and that Ca^{2+} played a role in countering the toxic effects of high concentration of ammonia (Sprott, G.D., 1986). The Ca^{2+} concentrations for maximum growth of methanogenic bacteria in batch culture are 1 mM for *Mc. voltae* in CO_2/H_2 substrate, 13.6 μM for *Ms. thermophile* in methanol, 0.25 mM for *Mtx. concilii* in acetate, and 2.5 μM for *Mtx.concilii* in acetate (Sprott, G.D., 1989).

Calcium is normally transported out of bacterial cells and the intracellular level of

calcium is very low. For example, the intracellular level of free calcium in *E. coli* is very tightly regulated to 10^{-7} M (Rosen, B.P., 1984). The amount of Ca^{2+} required to give 100 g of dry biomass when Ca^{2+} is growth limiting is about 0.1 g, much lower than that of K^{+} and Mg^{2+} (Beveridge, T.J., 1989). According to Campbell (1983), calcium concentration in the cytoplasm is usually 1-10 mMole/l cell water. Takashima and Speece (1990) reported that calcium is required for stability of methyltransferase in methanogens and bacterial aggregation (Takashima, M. & Speece, R.E., 1990); Calcium content in methanogens belonging to order 1 is 85 -550 μg/g dry mass, and order 2 & 3 1000-4500 μg/g dry mass. The optimum calcium concentration for *Mc. voltae* (H_2/CO_2) is 40 mg/l and *Ms.thermophila* (methanol) >0.45 mg/l.

It is believed that all microbial calcium functions are at the cellular membrane or external to the membrane. There is no required intracellular role for calcium in bacterial cells (Silver, S., 1977).

In biological systems, cations Na, K, Mg and Ca are distributed selectively, with K and Mg concentrated inside the cell and Ca and Na outside the cell. The systems are membrane bound and transport Ca^{2+} against a four orders of magnitude concentration gradient, out of the cytoplasm into extracellular fluids or specialized intracellular compartments. The mechanisms involved are based on the operation of Ca^{2+}-activated ATPase utilizing the chemical potential of ATP, or countertransport systems utilizing the potential of other primary electrochemical gradients for transport of Ca^{2+} against its own gradient. Another and complementary aspect of Ca^{2+} regulation is the controlled flux of Ca^{2+} back into the cytoplasm, when Ca^{2+}-dependent systems in the cytoplasm must be activated. These Ca^{2+} fluxes occur down the electrochemical gradient and, in this respect, may be considered 'passive'. Therefore, they are regulated basically by exchanges in membrane permeability. A large body of evidence indicates that these passive fluxes occur

through specific channels, whose gating is voltage dependent, as well as regulated by hormones and drugs ("Calcium blockers") (Rosen, B.P., 1984). Relatively little is known about bacterial transport of Ca^{2+}. Some mechanisms proposed for calcium cation movement across prokaryotic cytoplasmic membranes include secondary cation/proton antiport activity and other antiporters: calcium/proton, calcium phosphate/proton (in *E. Coli*), and sodium/calcium (in *Halobacterium halobium*) (Sprott, G.D., 1989).

1.3.2 Previous studies on the effects of calcium in bacterial biofilms

The studies on the effect of calcium on granule development in UASB reactors (Hickey, R.F., Wu, W.-M., Veiga, M.C., & Jones, R., 1991; Hulshoff Pol, L.W., de Zeeuw, W.J., Velzeboer, C.T.M., & Lettinga, G., 1982) showed that low concentrations (80 -200 mg/l) of calcium appeared to be beneficial for the development of granules (Lettinga et al., 1980; Hulshoff et al., 1983). However, lab-scale experiments to form granules from a digested sludge demonstrated that a calcium level of 450 mg/l increased sludge wash-out (Hulshoff Pol et al., 1983). High calcium concentrations (800 - 1000 mg/l) induced a decline in specific activity of granular sludge (Lettinga et al., 1985; Thiele et al., 1990).

For expanded/fluidized bed reactors, wastes containing high level of calcium are particularly difficult to treat. Wastes with 2.5 g/l calcium were able to be effectively treated on a laboratory scale, however, after 150 days calcite precipitates were observed to be about 30% of the total weight of sand/biomass particles (Jordening, et al., 1988). Greater than 90% of the calcium in a waste containing 0.9 - 3.0 g/l calcium was reported retained in a 10-litre laboratory-scale reactor. The accumulation of calcium resulted in

increased particle density, loss of fluidization, clogging and 40% dead space (Vogel and Winter, 1988) There is serious doubt whether the expanded/fluidized bed systems can function effectively over a long time frame with high-calcium wastes.

A distinct improvement in sludge settleability and specific activity was observed after replacing Na_2CO_3 as a neutralizing agent by $Ca(OH)_2$ in the treatment of potato-processing wastes in a 6 m^3 pilot plant (Versprille, A.I., 1978). A similar result was obtained when $Ca(OH)_2$ was utilized for neutralization during the start-up period of UASB reactors (Salkinoja-Salonen, M.S., Nyns, E.-J., Sutton, P.M., van den Berg, L., & Wheatley, A.D., 1982). These results may imply the positive effect of calcium.

Guiot, S.R., Gorur, S.S. and Kennedy, K.J.(1988) investigated the effects of the presence of calcium ions (80 mg/l) on microbial aggregation during upflow anaerobic sludge bedfilter reactor start-up. The results show that calcium cations had no significant effect on granulation, at least as an inducer of granulation at low substrate consumption rates. They thought that certain specific conditions were required before calcium ions could accelerate the granulation process, because two factors might impair the Ca^{2+} action. Firstly, in bicarbonate-buffered systems, calcium partly precipitates. Secondly, sodium cations abundantly present in the medium might compete for binding sites with Ca^{2+} without bridging occurring.

Turakhia and Characklis investigated the effect of calcium on the biofilm activity of *Pseudomonas aeruginosa* (Turakhia, M.H. & Characklis, W.G., 1988). The results indicated that specific activity in the biofilm was the same as that measured in a chemostat and was not influenced by changing calcium concentrations. However, increasing calcium concentration increased the cohesiveness of the biofilm.

Applegate and Bryers studied bacterial biofilm (pure culture of the obligate aerobe *Pseudomonas putida* ATCC 11172) removal processes due to shear and castrophic

sloughing in a turbulent flow system under conditions of carbon versus oxygen substrate limitations and varying aqueous phase free calcium concentrations. The results showed that increasing free aqueous phase calcium concentration increases the amount of biofilm-bound calcium. The rate of calcium binding in O_2-limited biofilms increases with increasing free calcium concentrations over the entire range studied (5 - 13 mg/l) while the rates of calcium binding in C-limited biofilms are independent of free calcium concentrations above 8.0 mg/l. O_2-limited biofilms, with higher extracellular polymer content and bound calcium, exhibit a much lower shear removal rates than the C-limited biofilms. However, they always experience catastrophic sloughing events. They proposed that reduced shear removal and the susceptibility to sloughing in the O_2-limited biofilm were attributed to their denser, more rigid crystalline structure brought about by excessive polymer production and concomitant binding of calcium (Applegate, D.H. & Bryers, J.D., 1990).

Experiments with a marine bacteria showed that omission of Ca^{2+} and Mg^{2+} from the artificial seawater prevented growth, polymer production and sorption to surfaces by the organism (Marshall, K.C., Stout, R., & Mitchell, R., 1971). In another case, addition of Ca^{2+} (as $CaCl_2$) in concentrations exceeding 128 μm produced significant increase in the adhesion of the test strains to plastic (Dunne, W.M., Jr., & Burd, E.M., 1992).

The importance of Ca^{2+} (and Mg^{2+}) in adhesion was also clearly demonstrated in an electron microscope study of the marine *Pseudomona* species. The cells formed the 'primary' material; then after adhesion, the 'secondary' polymer. When transferred to cation-deficient medium, the secondary polysaccharide was greatly disrupted in a very short time, indicating a role for the ions in maintenance of the adhesive structure. Under conditions of divalent cation deficiency, the polysaccharide-containing polymers

associated with irreversible adsorption were not detected. Some marine bacteria remain attached when washed with sea water but can be removed by treatment with tap water. This may indicate a requirement for critical concentrations of certain ions if adsorption is to be maintained and could also involve highly charged polysaccharides (Sutherland, I.W., 1983).

Many researchers believe that Ca^{2+} may participate in the formation of the cation bridge which linked two polysaccharide fibres of two cells (Costerton, J.W., Geesey, G.G., & Cheng, K,J., 1978). On the other hand, some researchers think that it is unlikely that calcium is involved in direct bridging to a negatively charged substratum. Instead, calcium maintains the tertiary structure of extracellular polymer substances so that the interactions between the adjacent sugars on different chains are promoted (Turakhia, M.H., Cookey, K.E., & Characklis, W.G., 1983).

Calcium cations are also likely to be involved in the modification of the substratum surface and facilitate bacterial adhesion in the initial formation of biofilms because of its adsorption to surfaces (Hermesse, M.P., Dereppe, C., Bartholome, Y., & Rouxhet, P.G., 1988). Furthermore, the presence of calcium may alter the ionic strength of the bulk liquid phase and subsequently affect the biofilm formation process (Kahane, I., Gat, O., Banai, M., Bredt, W., & Razin, S., 1979).

To sum up, the presence of Ca^{2+} in most cases had a positive effect on biofilm processes due to the roles of calcium in bacterial cells and in the biofilm formation mechanisms. The data on the measurement of such effects are very limited. Further studies in this area would be useful for analysis and control of biofilm processes in wastewater treatment, biofouling and the biotechnology applications of immobilized cells.

CHAPTER 2

EXPERIMENTAL

2.1 Experimental objectives

In this study, the effects of calcium on the biofilms in anaerobic fixed-bed reactors for the acidogenesis of lactose were investigated. While the study mainly focuses on the relationship between the biofilm accumulation and the calcium concentration in the culture medium, the influence of calcium on the biofilm activity, density and immobilized calcium content are also examined.

Acidogenesis of lactose under anaerobic conditions is selected for this study because lactose is the major component of cheese whey, a dairy plant waste, which is generated in large quantities worldwide. Anaerobic biofilm reactor technology has been used to treat cheese whey at both laboratory and industrial scales. In the methanation of lactose, acidogens usually grow much faster than methanogens, and the accumulation of excess inactive biomass in acidogenic biofilm reactors was thought to be responsible for the reactor efficiency decline. Anaerobic digestion of lactose by acidogens has been well studied in the chemostat (Murray, W.D. & van den Berg, L., 1981) and biofilm reactors (Barthakur, A., Bora, M., & Singh, H.D., 1991).

In order to achieve the above objectives, the biofilms were grown in a reactor system, where only the calcium concentration in the culture medium was varied in the experiments while the rest of the experimental parameters were kept constant. To observe

the influence of changes in the calcium concentration on established biofilm development, some of the biofilms formed in the culture medium with one calcium concentration were moved into the culture medium with a different calcium level for their further development.

2.2 Experimental apparatus and conditions

2.2.1 Experimental set-up

The experimental set-up used in this study is illustrated in figure 1. It was originally designed by Yu and Pinder (1991). The system consists of the following elements:

1) two parallel continuous flow reactors for the biofilm growth (biofilm reactors). Each reactor, with an effective volume of 1.5 litres, was made of a half Plexiglass tube, which provides the reactor with a curved bottom so that deposition of bacterial sludge in the reactor can be minimized. The reactor was equipped with a circulation pump (Cole Parmer Masterflex pump) for mixing and control of fluid flow velocity, a pH controller (Cole Parmer Series 7142 pH/Pump System) with 1 N caustic solution (50% NaOH and 50% KOH), a water bath thermostat (COLORA) for temperature control through an external heat exchanger, and a nitrogen gas disperser for displacing oxygen in the reactor.

2) biofilm supports and sampling ports. 1.587 millimetre thick PVC slides (4.5x1.5 cm) were utilized as the biofilm supports in the reactors. They were held at one end in rubber stoppers and immersed into the culture medium through the sampling ports arrayed in the top of the reactor.

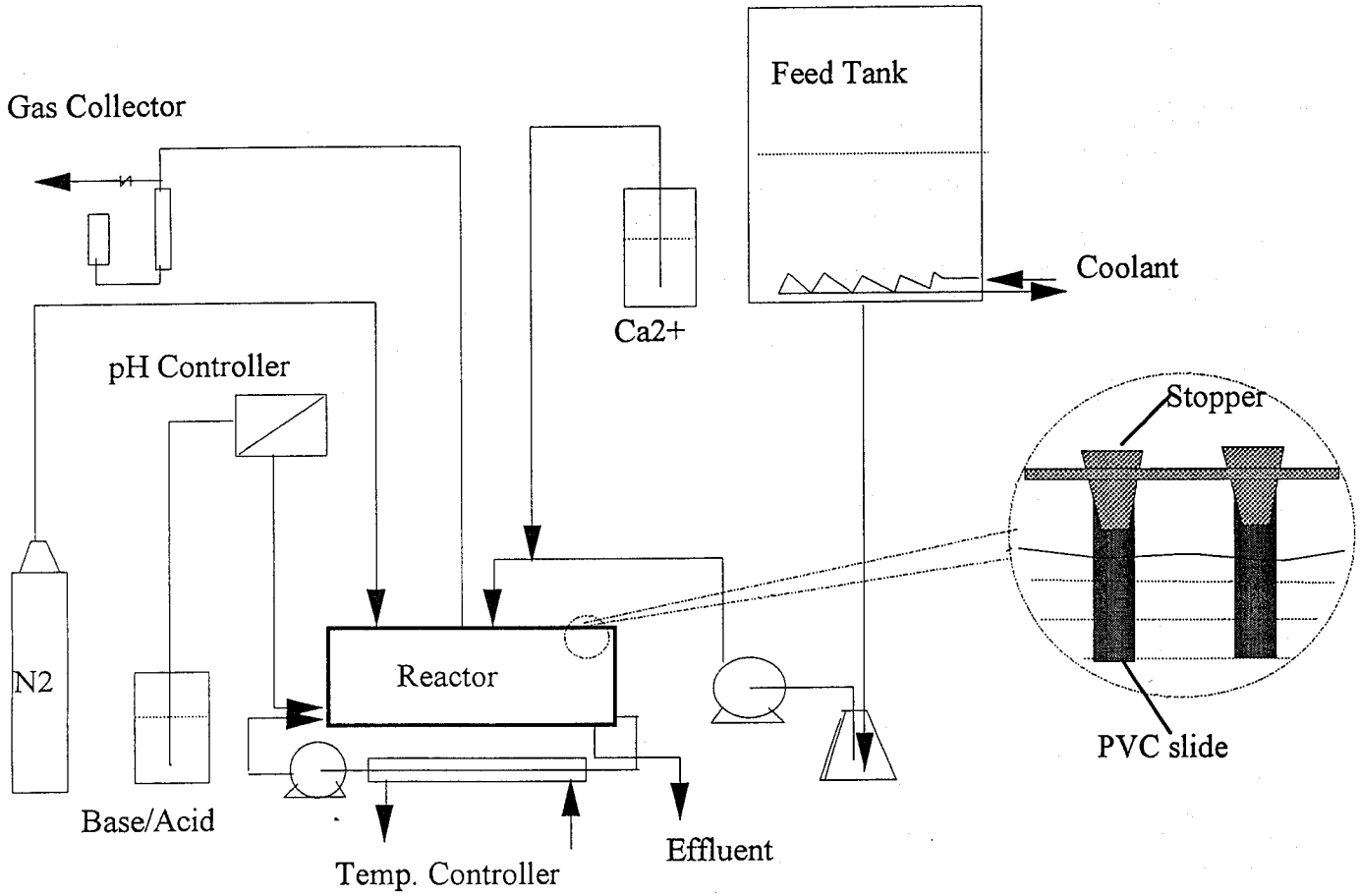


Figure 1 **Illustration of the Experimental Set-up**

3) feed supply system. The culture medium for both of the reactors was stored in the feed tank and kept at 1 - 4 °C by the coolant circulating from the cooling bath (NESLAB) through an immersed coil. Each loading of the feed in the tank was limited to that needed to supply the reactors for three to four days. Nitrogen was introduced into the tank to purge oxygen from the medium. The flow rate of the feeds was controlled with two pumps (Cole Parmer Masterflex). Two sampling ports were installed before the beak tubes, which prevent the feed in the storage tank being contaminated. The sampling ports were also used for monitoring the feed flow rate by measuring the fluid volume over a period of time.

4) calcium addition. Additional calcium solution was added into the feed for the specified reactor by using a precise dosing pump (miniPump, Milton Roy).

2.2.2 Experimental conditions

In each of the biofilm growth experiments, the reactor operating conditions were set to optimize the bacterial growth in the reactors and consequently promote the attachment of cells to the support surfaces.

Generally the optimal pH for acidogenesis is 4 - 8, the optimal temperature for the mesophilic bacterial growth is 32 -39 °C. According to Kisaalita, Pinder and Lo (1988), a lower pH value (4.5) in the range of 4 - 6.5 for acidogenesis of lactose would result in a product distribution which might favour the methanogenesis process. From the stimulation tests, Yu and Pinder (1991) concluded that to ensure a completely mixing pattern in the reactors and to minimize the external mass transfer resistance on substrate utilization in the biofilms, the feed flow rate should be below 1.7 l/hr while the recycle rate should be

kept above 14 l/hr. And in this case, the substrate concentration should be about 10 g/l in order to eliminate growth limitation due to the substrate. Therefore, the reactor operation conditions were set as shown in table 1.

The composition of the basic culture medium is shown in table 2. This formula was used by Kisaalita, Pinder and Lo (1988) and Yu and Pinder (1991) in the previous studies on acidogenesis of lactose. It was believed that such a formula should satisfy the requirements of the balanced growth of acidogenic bacteria for the substrate and nutrients. The lactose concentration was varied depending on whether the medium was used for the microbial inoculum preparation, biofilm growth or the assay of the biofilm activity. The concentrations of the organic salts were then adjusted proportionally according to the formula.

Table 1 The reactor operating conditions

pH	4.5 ± 0.2
temperature	35 ± 1 °C
feed flow rate or dilution rate	150 ml/hr 0.15 hr^{-1}
recycle rate	230 ml/min.
substrate concentration in the feed	5 - 10 g/l

Based on the findings from the previous studies (see 1.3.2), the calcium concentrations in the feed for the first run of the experiment were set as follow: high level

-- 100 mg/l; control -- no added calcium. In the following experiments the selection of calcium concentrations was based on the results of the earlier experiments. They are shown in table 3. It should be noted that these calcium concentrations are much lower than the calcium level reported to inhibit suspended anaerobic bacteria in batch or chemostat culture (Kugelman, I.J. & McCarty, P.L., 1965).

Table 2. The composition of culture medium

Components*	Concentration (g/l)
Lactose	12
NH_4Cl	0.42
$(NH_4)_2HPO_4$	0.8
KCl	0.7
$MgSO_4.7H_2O$	0.35
$Fe(NH_4)_2SO_4$	0.2
$ZnSO_4.7H_2O$	0.005
$MnCl_2.4H_2O$	0.005
$CuSO_4.5H_2O$	0.002
$NaB_4O_2.10H_2O$	0.002
$NaMoO_4.2H_2O$	0.002

* All are CSA reagent grade chemicals.

2.2.3 Preparation of the bacterial inoculum

The bacteria seeds used in this study were a mixed-culture of various anaerobic

bacteria dominated by acidogens. They were originally obtained from municipal sewage (Iona Sewage Treatment Plant) and stored in a cheese whey substrate. Before the experiments started, the bacterial inoculum was prepared and acclimated by the following procedures:

1) 200 g of the bacterial culture with dense cheese whey is dispersed with a homogenizer in 500 ml of cold water, which was previously saturated with nitrogen. Then the solution was quickly collected and loaded into two batch reactors (500 ml glass reactor, Fisher Scientific) (figure 2), containing 250 ml of culture medium diluted to give 4 g/l of lactose. Nitrogen was continuously introduced into the reactors in order to remove oxygen. The remaining solids were discarded. After 5 minutes the nitrogen supply was stopped and the bacteria were incubated at 35 °C.

During the whole experimental process, the bacteria must be protected from oxygen.

Table 3 The calcium concentrations in the feed (mg/l)

Reactor	Run 1	Run 2	Run 3
A with high calcium	100	120	230
B with low calcium	0*	80	170

* No calcium added to the culture medium. The actual calcium concentration is about 1.15 ppm, mainly resulting from calcium in the tap water.

2) When the lactose was almost completely consumed by the bacteria, half of the

bacterial suspension solution in each reactor was removed and centrifuged at 15,000 x g relative centrifugal force for 10 minutes to harvest the bacterial cells. The cells were dispersed in 250 ml of fresh culture medium with an increased substrate concentration (increase by 4 g/l of lactose) and returned into the reactors for a new cycle of cultivation.

3) The above step was repeated twice. When the bacterial growth rate tends to decrease, the bacterial suspensions from the reactors were pooled together and used as the inoculum for the biofilm experiments.

2.2.4 Start-up and maintenance of the reactors

1). Preparation of the solid supports. The PVC slides used for all the experiments were newly prepared in order to eliminate the errors due to the support surfaces. The slides were cut from a 3.175 mm thick PVC sheet and have a size of 15.0 ± 1.0 by 45.0 ± 0.1 millimetres. The slides' surfaces were thoroughly cleaned in an ultrasonic bath, diluted hydrochloric acid (100 ml concentrated HCl/1 *l* distilled water) and distilled water, and then dried at about 70 °C and carefully stored.

2) Start-up of the reactors. The two biofilm reactors with 1000 ml of the culture media (containing 10 g/l of lactose but different concentrations of calcium) were seeded with 500 ml of the bacterial inoculum. Initially the reactors were run in batch mode. When the substrate has been used up, the reactor operation was switched to continuous mode with the desired dilution rate. When the reactors reach their steady state, the PVC slides were inserted into the reactors and the time was set as zero time.

3) Maintenance of the reactor system. To ensure the proper operation of the reactor system, regular maintenance was carried out by periodically cleaning the pH

probes, temperature sensors, feed tubing and effluent outlets, and replacing the pump head tubing.

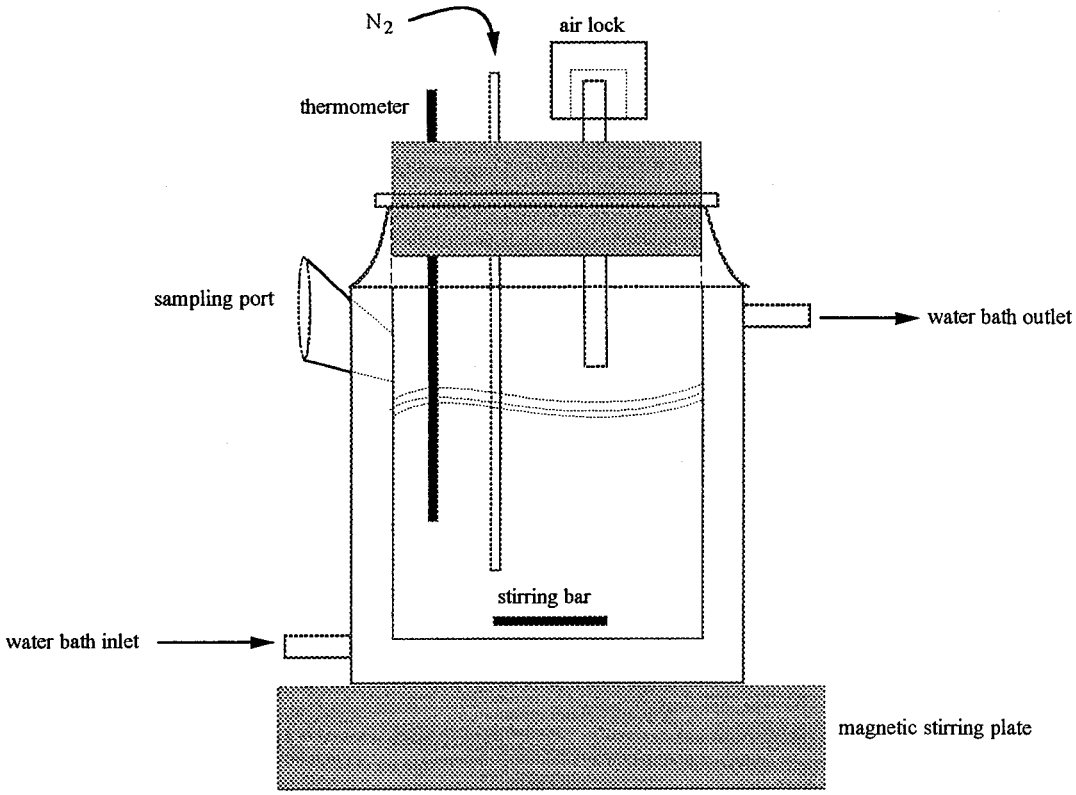


Figure 2 **The batch reactor for biofilm specific activity analysis**

2.3 Monitoring the reactor performance and sampling

[Sampling of biofilms] 2 - 3 of the slides (biofilms) were removed from each reactor every 5 - 8 days for analysis for biofilm area, wet and dry biofilm biomass, biofilm carbon, biofilm specific activity, the immobilized calcium concentration, and biofilm volume. In cases where biofilms were exchanged between the two reactors in order to observe the effect of changes in calcium concentration on the biofilm development, 1 or 2 more biofilm were sampled at the same time.

[Monitoring the reactor performance] During the experiments, the pH and temperature in the reactors were recorded three to four times a day; the reactor effluents were sampled daily and measured for biomass concentration. Every two days they were measured for substrate concentration. The effluent flow rates were also measured on a daily basis. The morphology of the bacteria in the effluents were examined with an optical microscope and the components of the gaseous products from the reactors were analyzed twice a week.

[Sampling of feeds] The feeds were sampled every 3 - 4 days at the sampling ports and analyzed for lactose and calcium.

Calibrations or adjustments of the influent flow rates, the reactor liquid surface levels and the influent calcium concentrations were made whenever disagreement of any of these parameters with the desired experimental conditions occurred.

2.4 Sample analysis

Unless specified otherwise, all the chemicals used for the following analysis were

CSA reagent grade. The centrifuge used for sample preparation was the Universal Centrifuge - model UV (IEC).

2.4.1 Turbidity of bacterial suspension

Turbidity measurement is a simple method for quick approximation of bacterial growth in the culture broth. Through the comparison tests, the light wavelength of 660 nm was selected for this study because the absorbance of the samples at this wavelength had a better linear relationship with the dry weight of the cell mass than other wavelengths such as 420 nm. The samples were diluted to give an absorbance between 0.01-0.8 before readings were taken with a spectrophotometer (Baush & Lomb Spectronic 70). Distilled water was used as the blank. The turbidity can be converted into dry biomass concentration with the calibration curve as shown in figure 3a.

2.4.2 Wet weight and dry weight of biofilms and suspended cells

Both the wet weights and dry weights of the biofilms were determined to directly measure the biofilm mass. After their surface areas were measured, the intact biofilms with their support PVC slides were weighed and then dried at 80 °C to a constant weight. The weights of the wet and dried biofilms were obtained by subtracting the slides weight from the total weights. The dry biofilms were kept in the desiccator for analysis of TOC.

For the liquid samples, the dry cell mass was determined by filtering two ml of the bacterial solution using 0.45 μm membrane filters and drying the filtrate at less than 80°c

to a constant weigh.

2.4.3 Total organic carbon (TOC)

Measurement of TOC content of samples is an indirect quantitation of biofilms or suspended bacterial mass. The TOC measurement in this study was conducted with a carbon analyzer (ASTRO 580). The principle is that the sample solution is first treated with phosphoric acid solution to remove the inorganic carbon-containing compounds, then the remaining organic components are oxidized with sodium persulfate solution and ultraviolet light. The carbon dioxide produced is carried by oxygen to an infrared detector, where absorption of infrared light by the carbon dioxide generates a signal proportional to the carbon dioxide concentration.

For the biofilm samples, the dried biofilms were pretreated by dissolving them in sulphuric acid solution with a pH 0.8 to make up 100 ml solution for each sample. An ultrasonic bath was employed to assist the dissolution of the biofilms. For the bacterial culture solutions, two samples were prepared: one was untreated and the other was centrifuged at 15,000 x g for ten minutes to remove the cells. The cellular carbon was obtained by subtracting the carbon content of the soluble organic components from the overall carbon content of the effluents.

Ethylene glycol was selected to make the standard solutions. The carbon analyzer was operated according to the procedures specified by its manufacturer.

2.4.4 Lactose

Lactose in the samples was determined by the phenol sulphuric acid method, which is based on the colour-producing reactions of the sugars consisting of free reducing groups with phenol and concentrated sulphuric acid (Dubois at al., 1956).

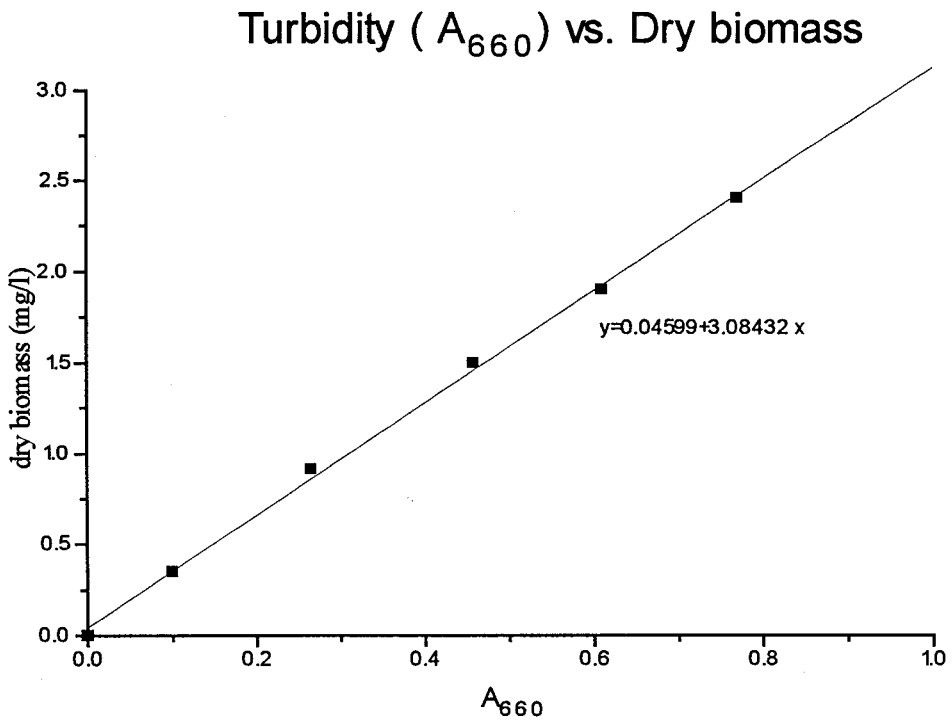


Figure 3a Calibration Curve for Measurement of Bacterial Cell Concentration by Colorimetric Method

Each sample was centrifuged at 15,000 x g for 10 minutes to remove solids and then diluted to 10 -120 mg/l of lactose. One ml of the diluted solution was mixed, in sequence, with one ml of 5% (by weight) phenol solution and five ml of concentrated sulphuric acid in a test tube (15 ml, Kimax). The test tube was placed in the air for ten minutes and then in a water bath at 23-30°C for another ten minutes, before its absorbance at 480 nm was read with the Spectronic 70 spectrophotometer (Baush & Lomb). Then the reading was converted to the lactose concentration by using the calibration curve (figure 3b). Distilled water was used to substitute the sample solution in preparing the blank.

2.4.5 Biofilm volume, density and thickness

A proportion of an intact thick biofilm was scraped into a small test tube (Kimax, diameter 4 mm) with a cap, weighed and centrifuged at 15,000 x g for 10 minutes to separate the biofilm liquid from the solids. The surface level of the biofilm was carefully marked on the tube wall. Then the biofilm water was withdrawn from the tube by using a 1.0 ml syringe with an appropriate needle and the tube was weighed to determine the biofilm water volume. The biofilm water was returned to the tube. A small volume of distilled water used to rinse the inner wall of the syringe was also injected into the tube. The tube was then dried at 80 °C to a constant weight. The dried mass in the tube was thoroughly removed and dissolved in sulphuric acid solution to make 50 ml solution for analysis of TOC (as described in section 2.4.4). The empty tube was cleaned and dried. It was refilled carefully with distilled water to the mark and weighed.

Therefore, the total volume of a biofilm was determined by calculating the volume

of the refilled water from its weight and density. And based on the total volume and weight of the biofilm, the biofilm density was obtained. The biofilm water volume was also measured by subtracting the weight of the biofilm solid from the total weight of the biofilm. The biofilm carbon concentration was equal to the ratio of the measured TOC to the total volume.

The average ratio of TOC/volume of several thick biofilms from the same reactor was used as a constant coefficient to calculate the volumes and thickness of the biofilms for the same calcium level. The volumes of the biofilms from the same reactor were obtained by dividing their TOC values by the average TOC/volume ratio; and their biofilm thicknesses were determined by dividing their TOC by their areas and by the average TOC/volume ratio, or they were calculated from their volumes and areas. This technique will be discussed in section 3.2..

2.4.6 Assay of biofilm specific activity

The biofilm specific activity was expressed as the maximum substrate consumption rate per unit of biofilm area in the batch culture of the resuspended cells from a biofilm. It represents the sum of the number of viable cells in a biofilm and the reproduction and metabolic activity of these cells, and reflected the activity of the biofilm in situ.

To determine the specific activity of a biofilm, the sampled intact biofilm after its area was measured was quickly transferred to and dispersed in 120 ml of the fresh culture medium containing 5 g/l of lactose, and cultivated in a batch reactor (Kimax 125 ml glass reactor, as shown in figure 2) at 35 °C. During this process nitrogen gas was introduced to both the culture medium and the reactor to protect the biofilm from exposure to oxygen.

The turbidity and lactose concentration of the bacterial culture in the reactor was determined periodically until the bacterial growth reached the stationary phase. Then the lactose concentration and turbidity as functions of time were plotted and the maximum substrate consumption rate was measured from the graph (an example is shown in figure 3). The biofilm specific activity was obtained by dividing this rate by the biofilm area.

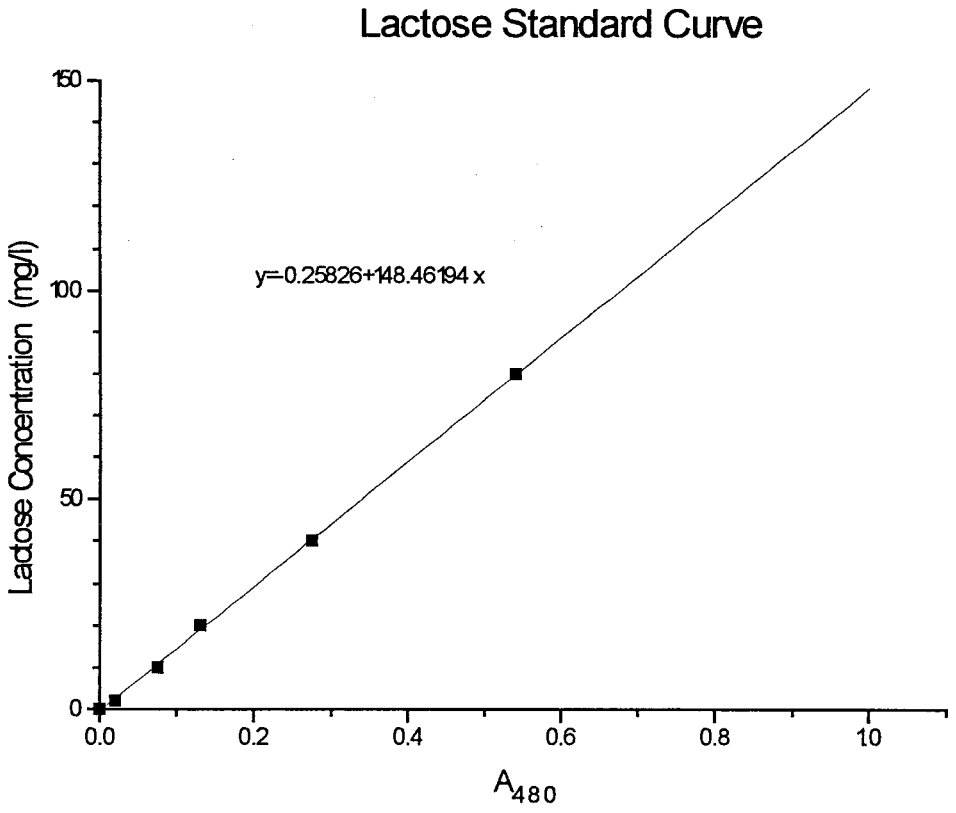


Figure 3b Standard Curve for Determination of Lactose Concentration

2.4.7 Total calcium

The total calcium in the samples was measured with an atomic adsorption spectrophotometer (Vedeo 220 aa/ae spectrophotometer, Thermo Jazzell Ash). When a calcium sample is aspirated in the flame and atomized, the calcium absorbs the light with a certain characteristic wavelength. The amount of light absorbed is proportional to the amount of the calcium atomized. Therefore, the calcium concentration is detected.

The samples, homogenized with an ultrasonic homogenizer when necessary, were acidified to a pH below 2.0 with concentrated hydrochloric acid. The sample solution was then diluted to 5-30 ppm calcium with lanthanum solution containing 58.65 grams of La_2O_3 and 250 ml concentrated hydrochloric acid per litre. The spectrophotometer with a three-slot Boling head was operated in the automatic mode and the operating conditions were set as following: hollow cathode lamp for calcium; wavelength 422.7 nm, band width 2.0 mm, fuel (acetylene) 5 psi., air 28 psi, current 5 mA. The standard solutions were diluted from the 1000 ppm calcium ($CaCl_2$) stock solution for atomic adsorption spectrophotometry (Fisher Scientific). Distilled water was used as the blank and a distilled water containing 1.5 ml concentrated nitric acid/l was used for rinsing the atomizer.

2.4.8 Biofilm minerals

The dried biofilms were first ashed at 600 °C overnight and then cooled to room temperature in a desiccator and weighed.

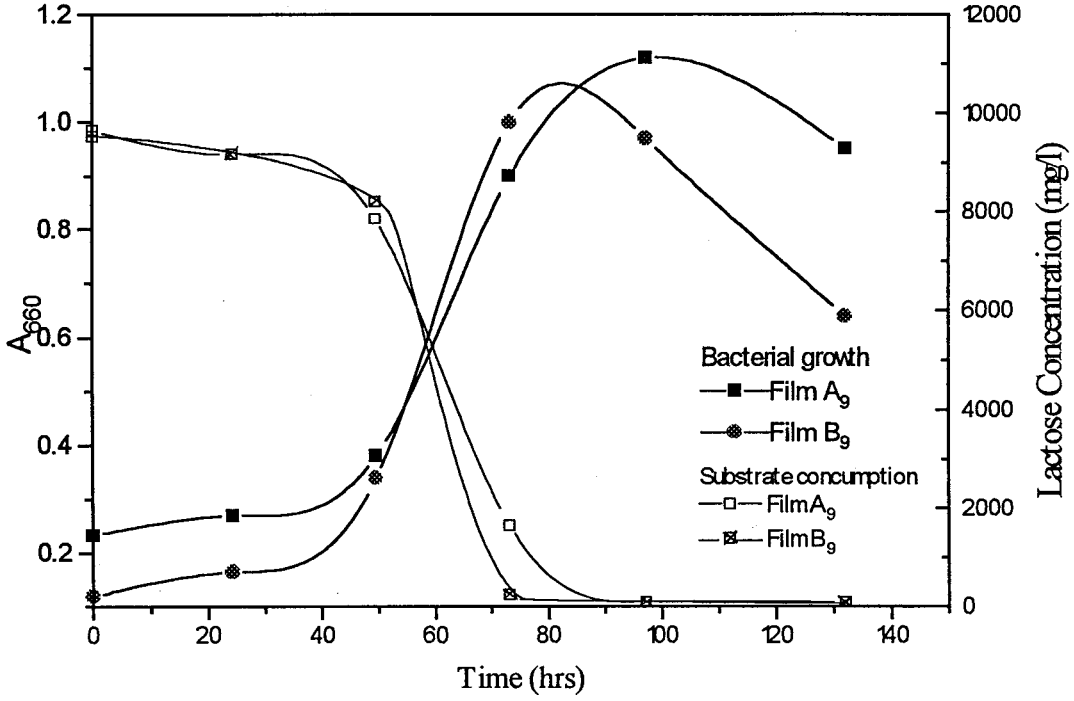


Figure 3 A typical relation curve of cell growth and substrate consumption for biofilm specific activity analysis

2.4.9 Gaseous products

The gas samples from the reactors were analyzed for the major gaseous products, carbon dioxide and hydrogen, by using a gas chromatography (CARLE model 311) which was equipped with a thermal conductivity detector and a 1/8" x 20' Paropack Q column.

The column temperature was set to 35°C. The flow rate of the carrier gases, helium for analysis of carbon dioxide (and methane) and nitrogen for hydrogen, was 20-25 ml/min at 30 psi.

CHAPTER 3

RESULTS AND DISCUSSION

Six different calcium concentrations were applied to the fixed-bed reactors for three runs of experiments in this study. Each of the experiments was run for more than 70 days to let the biofilm mass reach a constant level or a maximum. Some of the biofilms in Run 1 and 3 were transferred from one reactor to the other with a different input calcium concentration after they had grown for 30 - 35 days. The measurements described in the last chapter were made in each experiment. The results will be presented and discussed in the following sections in order of their importance for the experimental objectives.

3.1. Biofilm biomass

Biofilm biomass was expressed as biofilm total organic carbon (TOC). The accumulation of biofilm biomass for the different calcium concentrations is presented in figure 4. In the early stage of the experiments, the biofilms grew very slowly and the biofilm biomass accumulation did not show much difference for different calcium levels. After about twenty days, however, the biofilm growth speeded up and the growth rates reached their maximum in thirty to forty days for the calcium concentrations in 0 - 120 mg/l. For the higher calcium levels, the lag phase lasted longer. After the rapid growth stage, the biofilm growth tended to stop and the biofilm biomass was maintained at its

maximum level. It can be seen that the biofilm biomass accumulation follows the general growth pattern of free cells.

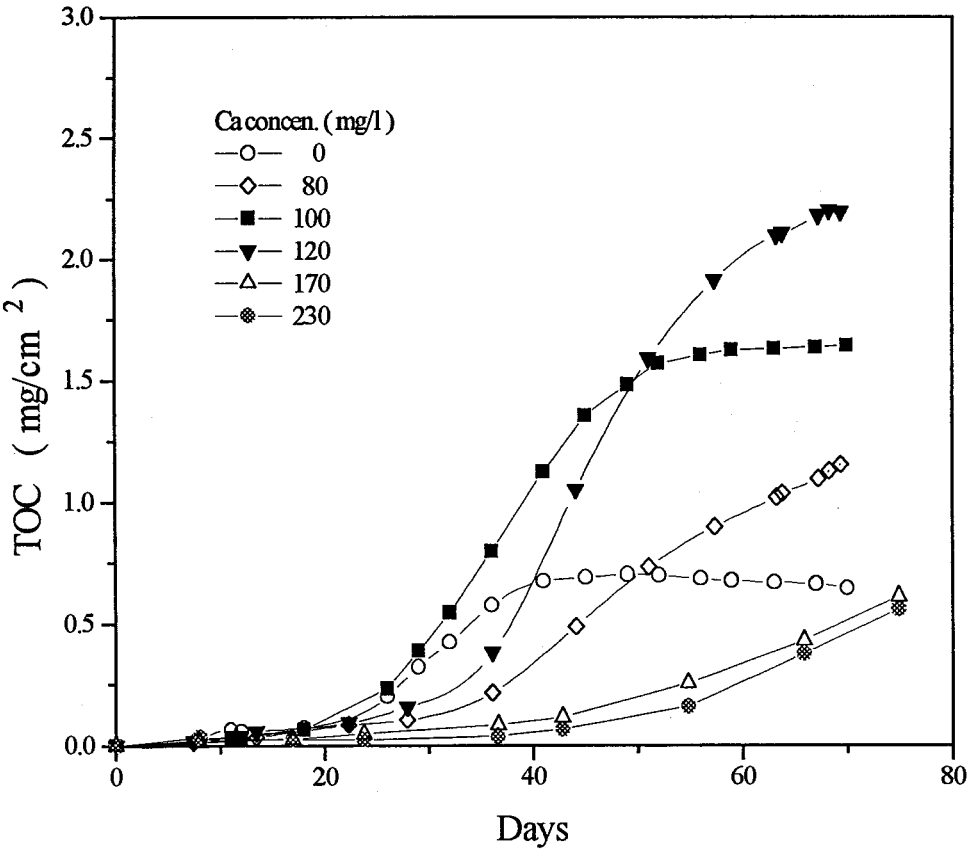


Figure 4. Biofilm TOC vs. Time

Importantly, the results indicate that for the range of low calcium concentration, biofilm biomass accumulation increased with increasing calcium concentration and reached a maximum for 120 mg/l of calcium. At higher calcium concentrations, both the rate and extent of biofilm biomass accumulation decreased (figure 5). Calcium in the bulk liquid influences the biofilm formation and development in two ways. Firstly, it plays a role in conditioning the support and bacterial surfaces so as to effect bacterial adsorption to the surfaces. Secondly, it is involved in the formation of the biofilm structure. It may be required for bacterial cell growth as well, e.g., in *A. xylinum*, cellulose synthesis is regulated indirectly by the level of intracellular calcium ion concentration (Withfield, C., 1988), but the calcium concentration in the bacterial cells usually is very low, e.g., 10^{-7} M in *E. coli* (Rosen, B.P., 1984) so that it is thought that calcium present in the tap water would be sufficient for the needs of bacteria. Therefore, in these experiments, where the low pH and the presence of many other cations might offset the effect of calcium on the bacterial adsorption to the support surfaces and the biofilm accumulation for various calcium levels did not appear significantly different at the beginning, calcium's role in the formation of biofilm structure was important for the development of the biofilms.

Since the biofilm accumulation is mainly the result of biofilm growth and detachment, that the presence of calcium increased in the biofilm accumulation can be attributed to two mechanisms.

1. The biofilm structure was strengthened by calcium ions and the biofilms became more resistant to the erosion effect caused by fluid shear stress. The bacterial cell surfaces and EPS are usually negatively charged and their association with each other to form a biofilm often requires cations, mostly divalent cations to act as a bridge between the components (Visser, J., 1988; Costerton, J.W., Cheng, K,J., Geesey, G.G., et al., 1987; Lam, S.S., Thompson, J.B., & Beveridge, T.J., 1993; Beveridge, T.J., 1988).

Therefore, the presence of calcium ions would facilitate the linkage of cell-polysaccharide and polysaccharide-polysaccharide. Certainly, calcium ion concentration may also contribute to the stability of the conformation of the polymer network in the biofilm because of its interactions with the secondary functional groups in the polysaccharides like OH^-, and consequently its influence on the folding of the polymers.

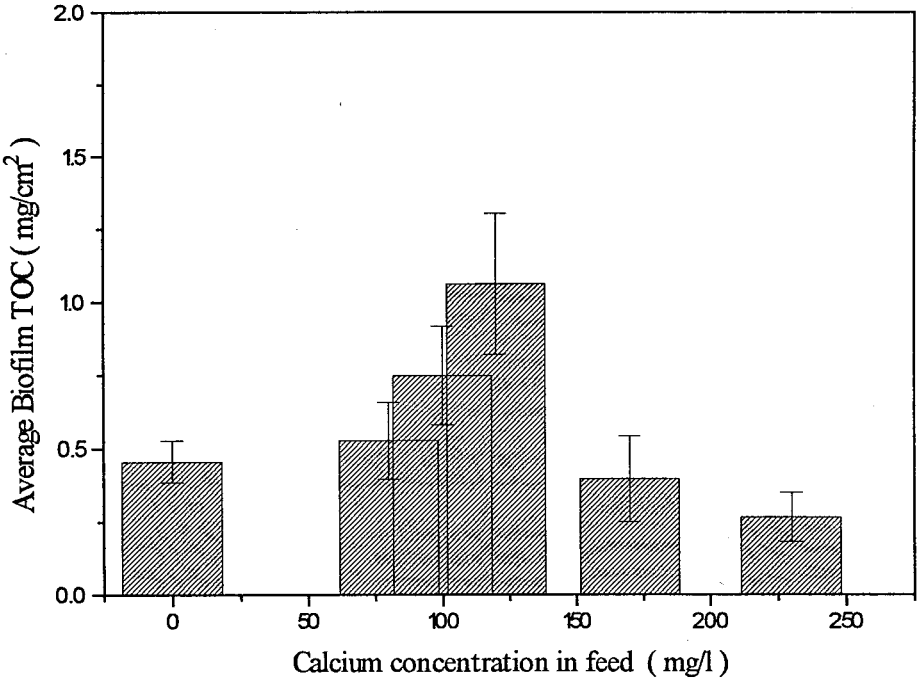


Figure 5 Biofilm biomass vs. Ca concentration

2. An increased calcium concentration in the bulk liquid or calcium accumulation within the biofilms might create, to some extent, an environment which influences the physiological and biochemical aspects of the cells. As a result, the synthesis of extracellular or intracellular polymeric substances by the biofilm cells was stimulated or inhibited. a). Various metal ions including calcium have been known to be required as cofactors in polysaccharide synthesis (Larsen, B. & Haug, A., 1971). Experiments showed that for many bacteria, the presence of metal ions could increase the production of exopolymers that make up the biofilms or change the exopolymer compositions and even the capsule morphology. For example, the polysaccharide synthesis by *Enterobacter aerogenes* was stimulated by calcium, magnesium and potassium ions in the culture medium. The presence of ferrous and calcium ions enhances the polysaccharide production in *Chromobacterium violaceum* (Couperwhite, I. & McCallum, M.F., 1974). An increase in the concentration of Cr(III) led to increased polymer production by a coryneform bacterium isolated from Cr-polluted marine sediments (Lam, S.S., Thompson, J.B., & Beveridge, T.J., 1993). Removal of calcium from the culture medium resulted in an increase of mannuronic acid in the alginate produced by *Azotobacter vinlandii* (Couperwhite, I. & McCallum, M.F., 1974; Larsen, B. & Haug, A., 1971). b). Calcium ions can increase the permeability of the cellular outer membranes of Gram-negative bacteria (Doyle, R.J., 1989) that include most of the species involved in the anaerobic acidogenesis. The outer membrane permeabilizing effect of cold calcium ion solution at 20 mM or more was described by Brass (1986) and Nikaido and Varr (1987). It is suggested that the excessive binding of calcium ions to the lipopolysaccharides which construct the surfaces of outer membranes of Gram-negative bacterial cells "freezes" the lipopolysaccharide monolayer by raising its melting temperature and this easily creates outer membrane cracks, through which

macromolecules can diffuse.

On the other hand, the presence of too much calcium in the biofilms could damage the environment required for maintenance of the biofilm structure or the biofilm cellular activity and limit the biofilm development. Calcium toxicity will be examined in section 3.4 where calcium concentrations in the biofilms are presented.

3.2. Biofilm thickness

The changes in biofilm thickness with time directly reflect the progression of the biofilm development (figure 6).

Because of the rough surfaces and gel-like characteristics of biofilms, thickness measurement of thin biofilms remains a difficult subject in biofilm research although many methods have been developed (Pavlostathis, S.G. & Giraldo-Gomez, E., 1991). The methods for measurement of biofilm thickness can be classified into three principal categories, based on the principles applied: 1. by optical microscopy, 2. by means of electronic conductance and 3. by measurement of biofilm volumes through volumetric displacement. In optical microscope measurements, errors often occur as a result of irregularities in the biofilm surfaces. These methods require a optical microscope with very fine stage adjustments and the results should be corrected for the refractive index of the biofilm. For the methods in the second category, specially designed probes with electronic circuits and electrometers are required.

The author contrived a device for the case of this study (see figure 7). When it is used, probe B is first placed on the substratum-biofilm interface and then probe A was slowly inserted into the biofilm surface by turning the knob. Both of the probes have two

layers of metal coatings which are separated by insulating materials and allowed to acquire data on two levels for a measuring point. The bodies of the probes, which formed two electrodes of a circuit, could be used to signal the improper placement of probe A. The major difficulties would be encountered in coating the probes and calibrating the device.

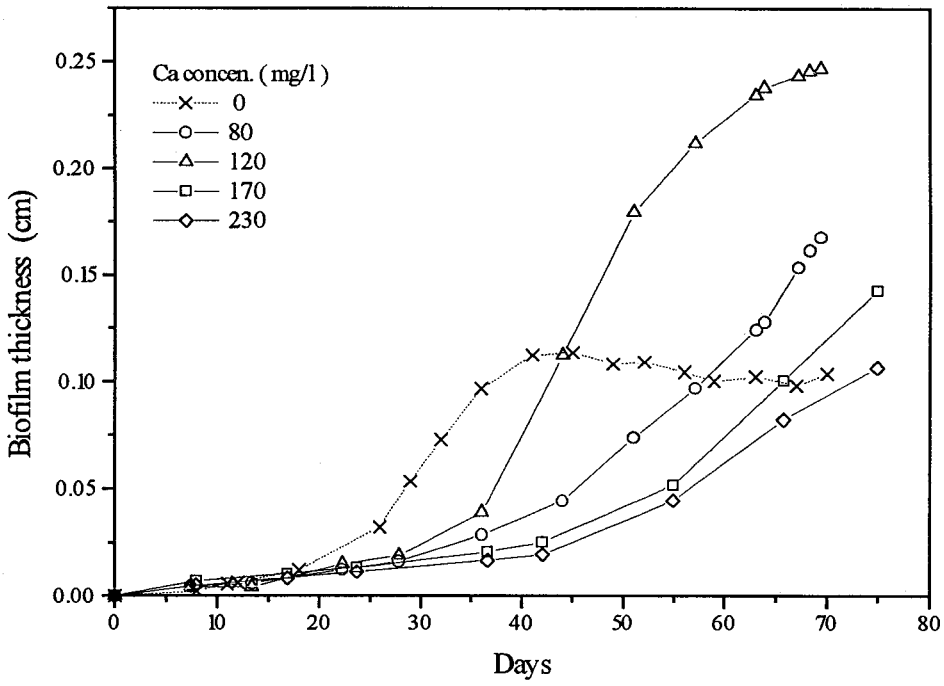


Figure 6. Biofilm Thickness vs. Time

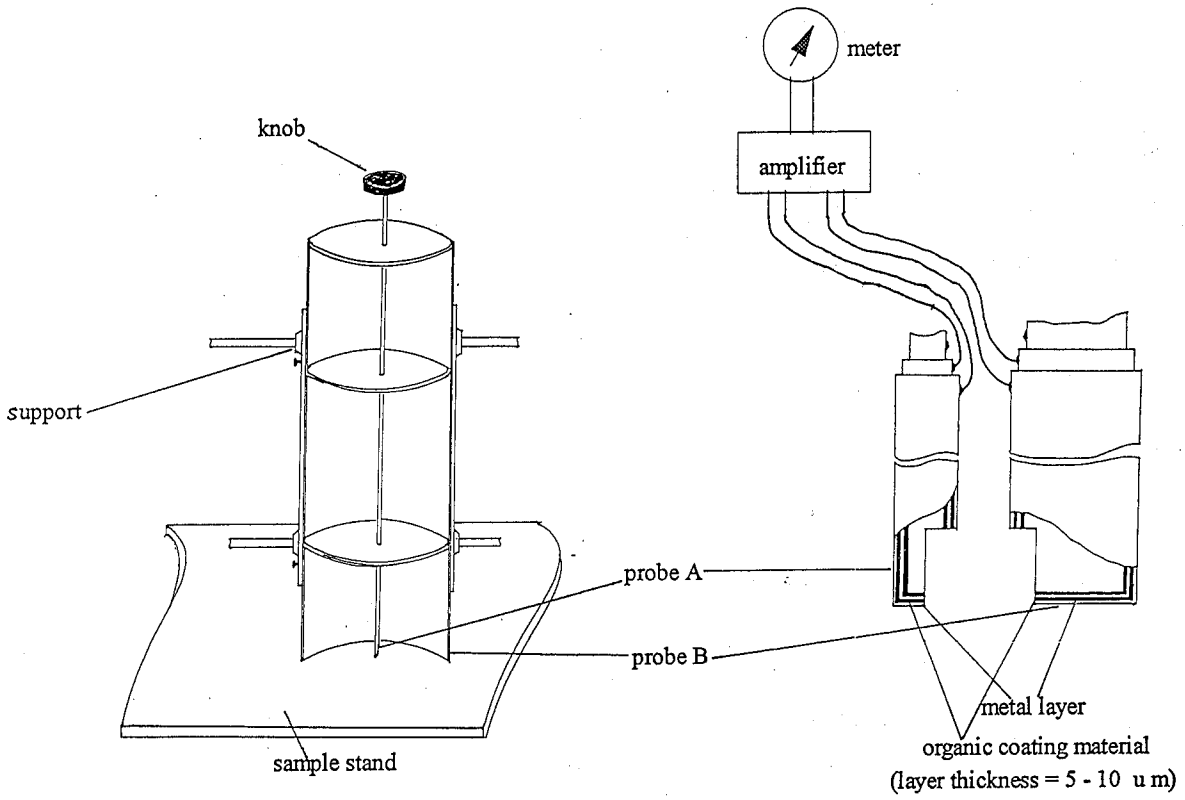


Figure 7. A concenptial device for direct measurement of biofilm thickness by determining contuctance

For the volumetric displacement methods, problems often occur in replacing an exact area of biofilm with water or reading the water volume because the biofilm is not only very small in volume but also sticky.

The method used in this study provides another technique for the estimation of biofilm thickness. It was modified from the method used by Yu and Pinder (1991), in which the thickness of thick biofilms was obtained from measurement of the biofilm volume by volumetric displacement, while thin biofilm thickness was calculated from the density measured from the thick biofilms, the mass and area of the thin biofilm.

It was assumed that the biofilms for a given calcium concentration were homogenous, had the same structure, the same component distribution and the same cellular activity in any portion at any time. Therefore, the carbon content per volume of biofilm for a given calcium level could be considered to be constant, or

$$\left(\frac{C}{V}\right)_1 = \left(\frac{C}{V}\right)_2 = \left(\frac{C}{V}\right)_3 = \cdots = k = constant \qquad (1)$$

or

$$\left(\frac{C}{S\cdot\theta}\right)_1 = \left(\frac{C}{S\cdot\theta}\right)_2 = \left(\frac{C}{S\cdot\theta}\right)_3 = \cdots = k = constant$$

Where:

C -- the biofilm carbon (mg)

V -- the biofilm volume (cm^3)

S -- the biofilm area (cm^2)

θ -- the biofilm thickness (cm)

k -- constant, or the biofilm carbon concentration for a given calcium level (mg/cm^3)

The subscript number stands for the biofilm samples at different times.

In order to obtain the value of the coefficient k, several pieces of intact thick biofilms for the same calcium level were sampled and measured for biofilm volume and carbon content. The process is briefly described as follows. For details, refer to section 2.4.5 in Chapter 2. A small mass from a piece of biofilm was scraped into a small test tube with a diameter of 4 millimetres and centrifuged at 15,000 x g to draw all of the biofilm mass into the bottom of the tube. Then the test tube with the biofilm mass was weighed to determine the biofilm density, and the total volume of the biofilm was determined by replacing the biofilm mass with the same volume of water and calculating the volume of the water from its weight and density. The biofilm mass was analyzed for total organic carbon after it was withdrawn from the test tube. The average ratio of the biofilm carbon to the biofilm volume of these thick biofilms was then used as the k value for the given calcium concentration.

For the biofilms with known carbon contents and corresponding areas, their thickness could be obtained by performing the following calculation:

$$\theta = \frac{1}{k} \cdot \left(\frac{C}{S} \right) \qquad (2)$$

Where:

$\left(\frac{C}{S} \right)$ -- biofilm carbon per unit of biofilm area (mg / cm^3)

θ and k are the same as in equation (1).

Compared with the previous technique used by Yu and Pinder, this technique has the advantage that it eliminated the need to collect the mass of an exact area of biofilm during the measurement of volumes of thick biofilms in small test tubes, which was a great

difficulty in practice.

The k value in the above equations is a reflection of change in the biomass concentration of biofilms. As shown in figure 8, the relationship between the carbon concentration of the biofilms and the input calcium had a similar trend to that of the biofilm mass and the calcium, although changes in the biofilm carbon concentration at low calcium concentrations are less striking than that in the biofilm mass. This indicates that the bacterial biofilms tended to build a structure with denser organic mass when the input calcium was increased, but when the calcium concentration was beyond a certain level, the organic mass concentration of the biofilms declined dramatically.

It is not known whether this was because calcium influenced the polysaccharide production in the bacteria, altered the biofilm structure, or because the build-up of biofilm mass increased the resistance to diffusion of the substrate, nutrients, electron acceptors and the bacterial metabolic products between the bulk liquid and the cells in the lower portion of the biofilms and resulted in changes in the physiological states of these cells. Possibly, both of these causes existed, that is, the presence of calcium stimulated production of the polymers by the bacteria, or calcium interacted with exopolymers in the biofilms and caused a change in the physical state of the polysaccharides (such as gel formation in the case of alginic acid -- Geesey, G.G. & Jang, L., 1989), which further induced the bacterial cells to synthesize polymers. A simulation study on naturally immobilized *Anabaena azollae* cells suggested that confinement of cells in a restricted space could induce the development of a mucilaginous envelope and attachment mechanisms. This in turn would lead to changes in the physiological and biochemical behaviours of the cells. Another possibility is that the development of the polysaccharide mucilage has an effect on the water activity and as a consequence on the metabolism of

the cells (Hamer, G., 1990).

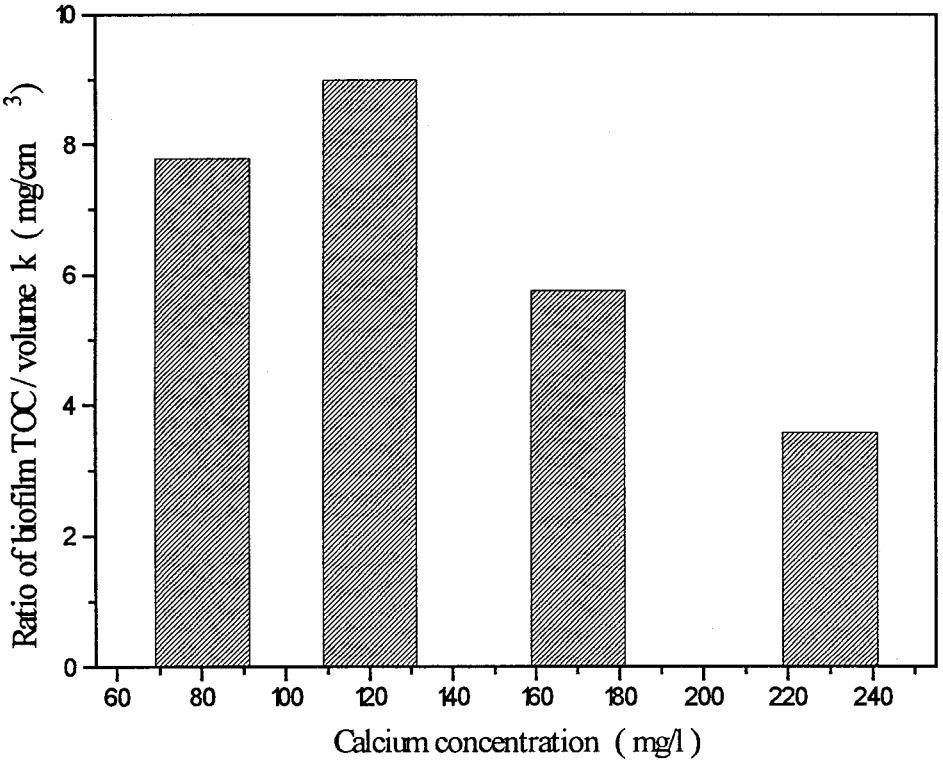


Figure 8. Biofilm carbon concentration vs. calcium concentration in feed

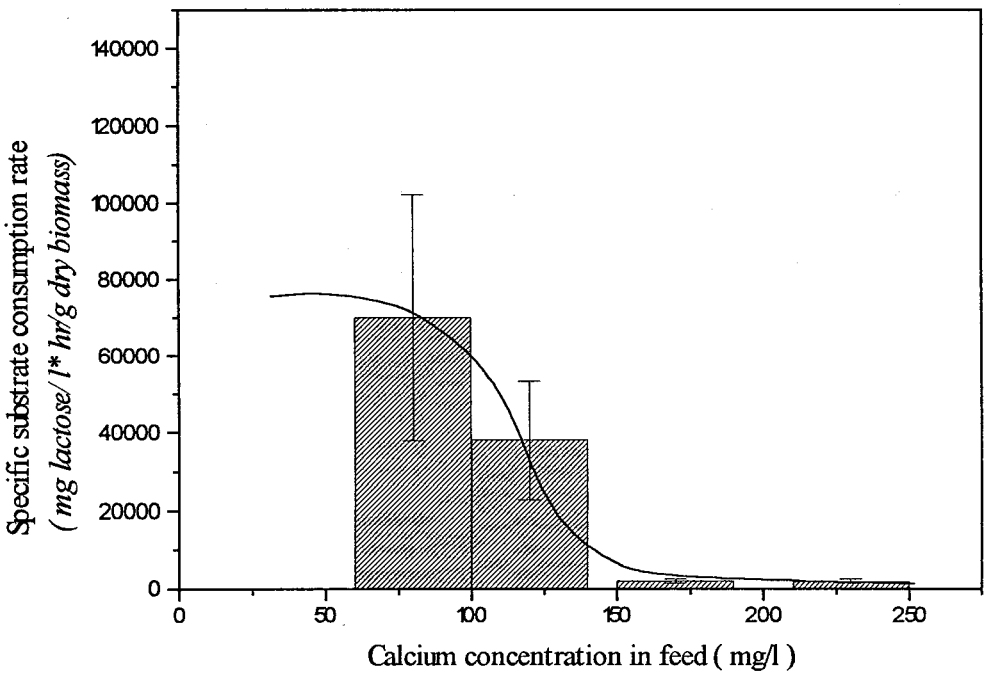
Here it is also worth mentioning the observation by Haug and Larsen (Larsen, B. & Haug, A., 1971) that acetate could stimulate the production of extracellular polysaccharide in *Azotobacter vinilandii*. For the case of acidogenesis of lactose by anaerobic bacteria in this study, acetate is one of the major products. This could be the basis for another mechanism for the biofilm biomass production associated with calcium.

3.3. Biofilm specific activity

The biofilm specific activity, expressed as maximum substrate consumption rate per unit of dry biofilm mass when the cells were cultivated in discrete states (see section 2.4.6 in chapter 2), is the sum of the activities of biofilm cellular reproduction and metabolism. It reflects the viability of bacterial cells in a biofilm, or the number of viable cells and their activity in a biofilm. The biofilm specific activity as a function of calcium concentration is presented in figure 9. It can be seen that the specific activity of the biofilm tends to decrease with an increase in calcium concentration in the medium although it appears quite stable for a low calcium concentration range, indicating that the biofilms at high calcium concentrations had fewer viable cells or a lower cellular metabolic activity. The biofilms for lower calcium levels reached their highest specific activity earlier than do biofilms for higher calcium levels (figure 10), indicating that the cells in the biofilms at the high calcium concentration took more time to recover their cellular activity.

Compared with the results on biofilm mass accumulation (see 3.1) and biomass concentration within biofilms (see 3.2), this implies that at a low calcium concentration, loss of the biofilm specific activity resulted from the increasing transport resistance due to the increase in the biofilm thickness and from the lower viable cell count or lower water

activity because of the higher polymer concentration. For the high calcium concentration range, the biofilm specific activity decreased due to the increase in calcium inhibition to the biofilm cellular activity or enzyme activity (toxic effects), or damages to the bacterial cells caused by calcium accumulation within the biofilms.



Firgure 9. Biofilm specific activity vs. calcium concentration

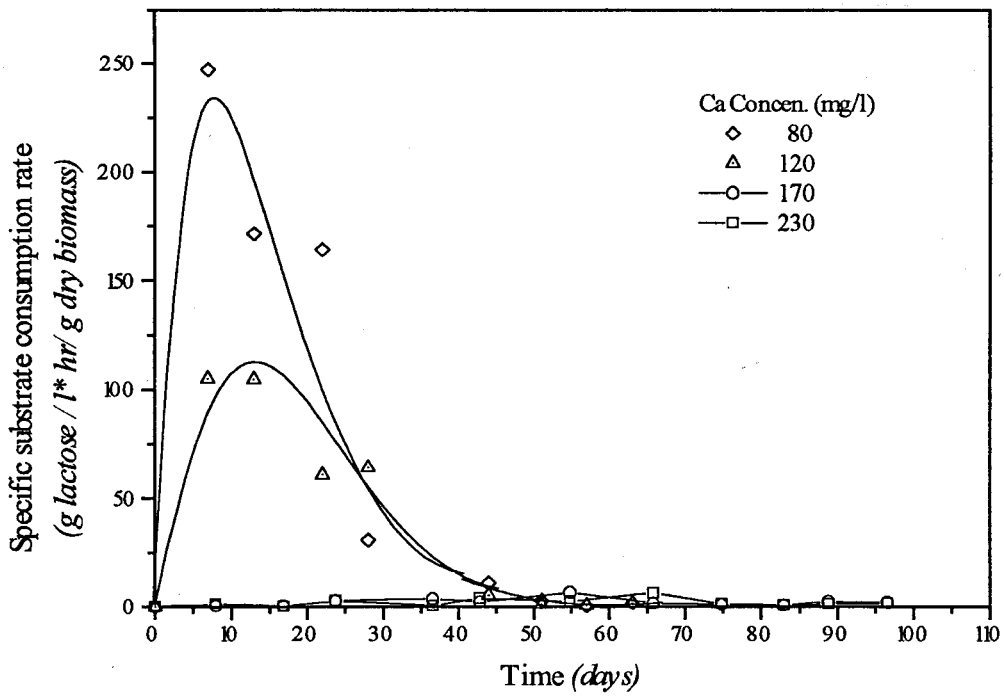


Figure10. Biofilm specific activity vs. Time

In biofilms, the bacteria cells rely entirely on diffusion from their immediate local environment to obtain nutrients and to disperse metabolic waste products. Hence, water content in biofilms is very important for carrying necessary solutes to the cells. Moreover, water is also required by the bacteria to maintain cellular integrity and proper metabolic functioning (Beveridge, T.J., 1988). Therefore, the higher polymer accumulation both in total amount and in concentration would either deny the access of nutrients to the bacterial

cells or decrease the water activity, resulting in changed metabolic activity and product formation rates of the cells in biofilms. It was reported that the presence of polymers even at a low level had a substantial effect on biochemical reactions which are water-dependent (Brouers, M., Shi, D.J. & Hall, D.O., 1990).

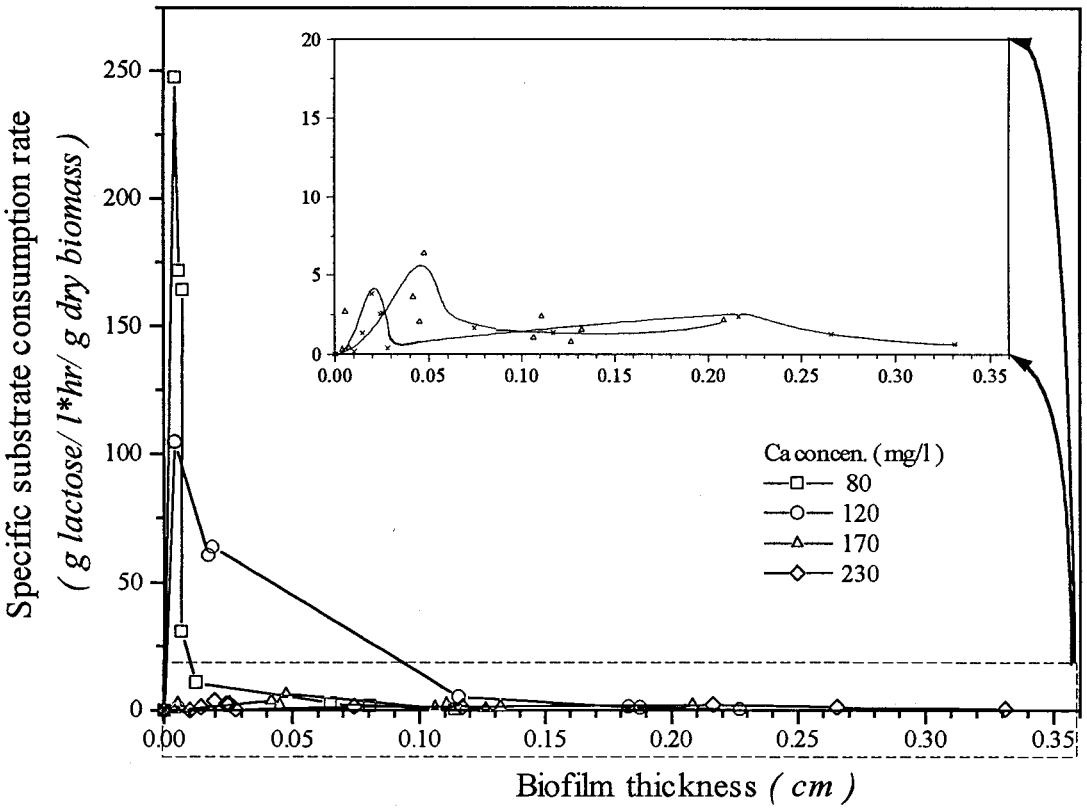


Figure 11. Biofilm specific activity vs. thickness

For similar reasons, too high a calcium concentration would seriously influence the

bacterial growth and metabolism in biofilms. Besides, calcium ions may alter the surface permeability of the bacterial cells (see 3.1). A large quantity of calcium immobilized in the biofilms may cause autolysis of the bacterial cells as well. This was suggested by the observations of Harvey et. al.(1984), in which the mature methanogenic bacterial biofilms formed on the polyester supports, contained extensive deposits of electron-dense, calcium- and phosphorus-enriched material completely entrapped cells. The trapped cells appeared to autolyse, and many were partially degraded. They postulated that further impregnation of the matrix with minerals and apparent cell death may eventually have a deleterious effect on the methanogenic activity of the biofilm.

The mechanisms of metal interactions with bacterial cells and polysaccharides will be further discussed in the next sections (see 3.4 and 3.5).

From the curve of biofilm specific activity and thickness (figure 11), it can be seen that biofilms with a thickness less than 0.5 millimetres have the highest specific activity. Since the overall efficiency of a fixed film reactor depends on the mass of biofilm and the biofilm specific activity, the substrate removal rate per unit of biofilm surface area was plotted as a function of calcium concentration in the feed to find the optimum input calcium level for acidogenic biofilms (figure 12). It can be seen that the optimum calcium concentration is 100 - 120 mg/l.

3.4. Immobilized calcium

As shown in figure 13, the total calcium concentration in the biofilm was proportional to the calcium concentration in the external medium. Calcium accumulation within biofilms mostly resulted from the interaction of calcium ions with the

exopolysaccharide polymers and bacterial surfaces because calcium concentration in bacterial cells normally is very low. Calcium accumulation is a non-specific process, which is driven by the calcium ion gradient from the bulk liquid phase into the biofilms.

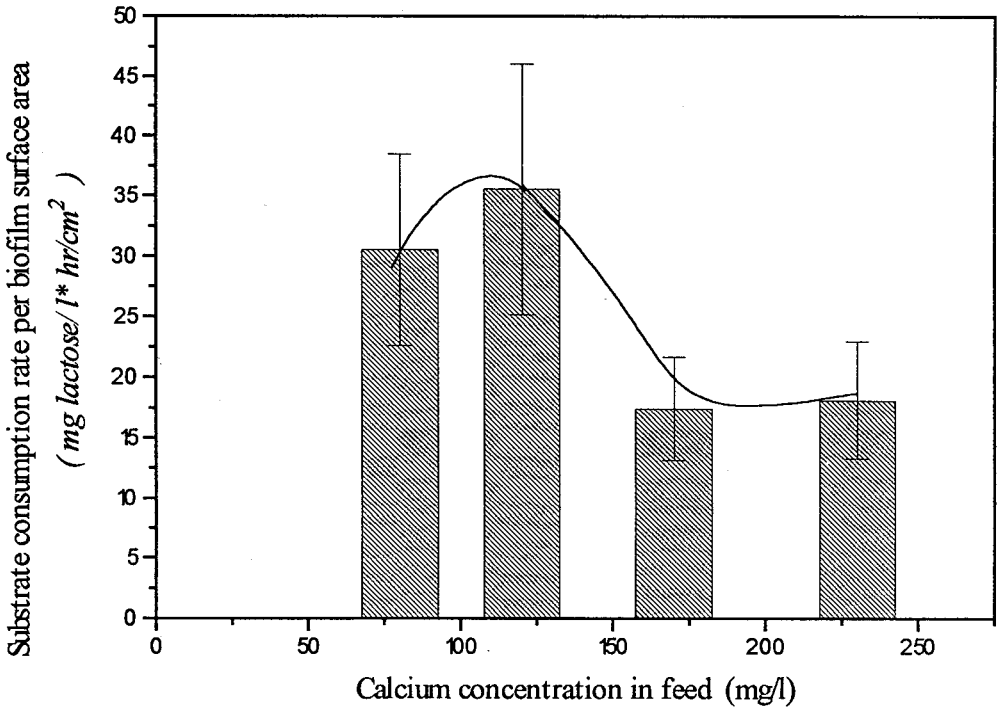


Figure 12 Biofilm activity vs. Calcium concentration in feed

Non-specific binding of metal ions to the cell wall, capsule and extracellular slime

layer is one of the two main mechanisms of metal accumulation by bacteria. The bacterial capsules are composed of the exopolymers or polysaccharides that are intimately associated with the cells, while the slime layers are formed by the polysaccharides that are loosely associated with the cells (Withfield, C., 1988). In bacterial biofilms, these polysaccharide polymers 'knit' the extensive network that embraces all the other components of the biofilms. They create a buffer zone between the surfaces of cells and their external environment, which is developed in response to the external environmental conditions. When the nutrient concentration in the surrounding environment is low, they extract and accumulate nutrients, especially metal ions from the environment so that the bacteria would gain a better control over concentrations of nutrients that actually reach the cell surfaces, and when metals exist at toxic levels in the environment, they function as a barrier to protect the cells (Lam, S.S., Thompson, J.B., & Beveridge, T.J., 1993).

Capsules and slimes are the first structures encountered by metal ions when they are present around the cells. Due to their chemical nature, they are ideal cation scavengers, resembling cation exchange resins, and can accumulate large quantities of metal ions. For example, a capsule-forming bacteria, *Zooloea ramigera* was found to contain up to 25% of metals by weight after growing in sewage sludge (Geesey, G.G. & Jang, L., 1989). The chemical compositions of capsular and slime polysaccharides are usually similar when both capsules and slimes are produced (Geesey, G.G. & Jang, L., 1989). These polysaccharides contain uronic acids and other substituted sugars that possess acidic functional groups important for metal binding, mainly carboxyl and hydroxyl groups. Of the two chemical groups, carboxyl groups are the most active. Most polysaccharides are negatively charged, and metals are usually bound by cross-bridging between anionic groups. This neutralization of charge by the metal ions often results in coprecipitation of the metal-polymer composite resulting in floc formation (Geesey, G.G. & Jang, L.,

1989). This is considered as an important mechanism of metal sedimentation from the fluid phase in natural aqueous environments, brought about by organisms. In case of uncharged polysaccharides, weak electrostatic interaction between metals and hydroxyl groups become important. The affinity of uncharged polysaccharides for metal ions generally decreases with increasing radius of the hydrated metal ions. In general, an acid-base reaction is involved which results in the liberation of protons. Such reactions are significant in the biocorrosion of metal surfaces (Geesey, G.G. & Jang, L., 1989).

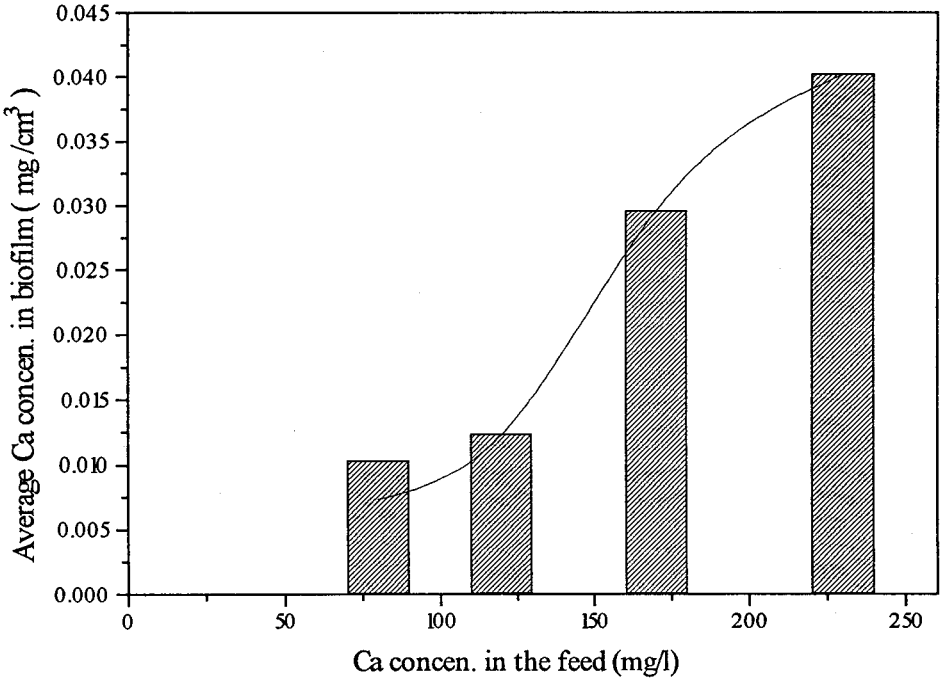


Figure 13. Biofilm calcium content vs. calicium concentration in culture medium

Bacterial cell walls tend to have a net negative charge at circumneutral pH mainly

because of the acidic groups of their polymer components. In some cases, the bacteria's high capacity to accumulate metal is attributed to this charge character (Beveridge, T.J., 1984). For Gram-positive bacteria, the prime site of metal binding in the cell wall seems to be the carboxyl residues of peptidoglycan and teichuronic acids, and the phosphoryl groups of teichoic acids. The contribution of each group to overall metal binding capacity of the cell depends on the amount of each polymer present, which varies with the strains and their culture conditions (Doyle, R.J., 1989). For Gram-negative bacteria, the major functional groups of the cell wall responsible for metal binding, in sequence of importance are phosphoryl groups and carboxyl groups of lipopolysaccharide molecules in the outer membrane and the same groups in the peptidoglycan layer (Lam, S.S., Thompson, J.B., & Beveridge, T.J., 1993). Generally, Gram-positive cell walls bind more metal than do gram-negative walls (Beveridge, T.J., 1984; Lam, S.S., Thompson, J.B., & Beveridge, T.J., 1993). In both cases, the quantities of bound metal are greater than the number of available anionic sites in the walls. To explain this phenomenon, a two-step mechanism for metal deposition in the bacterial wall was proposed by Beveridge and Murry (Beveridge, T.J., 1984). The first step involves the stoichiometric interaction between metal ions and active sites within the wall. Next, this initial bound metal would act as nucleation sites for the deposition of more metal from the solution. The metal aggregates grow within the wall until they are physically limited by the available space within the wall fabric. In this way, metals deposited in the wall are not easily redissolved by water or replaced by protons or other metal ions. Therefore, the matrix of the bacterial wall provides a special environment for the nucleation and growth of metal aggregates.

Binding of calcium ions to the exopolymers and cells in the biofilms would influence the exopolymer production and the physical state of the exopolymers (see section 3.1 and 3.2), and the polymers in turn will affect the calcium adsorption to the

biofilms. The changes in the composition and behaviour of the biofilms for different calcium concentrations reflect the results of the interactions of calcium ions with the cells and exopolymers in the biofilms.

From figure 13, we can find that the calcium concentrations in the biofilms were more than 10 times greater than were present in the feeds. They ranged from less than 1 to higher than 4 g/l. The data on calcium toxicity for the acidogenic bacteria, both for the suspended cells or for the immobilized cells, are not available. But according to Kugelman and McCarty (1965), the optimum calcium concentration for methanation of acedic acid was 0.005 M (0.2 g/l) and the upper limit was from 0.05 M to 0.125 M (2.00-5.00 g/l), which might be higher, depending on the antagonist cations present in the culture medium, the method of cation introduction, the organic loading, and the biological solid retention time (Kugelman, I.J. & McCarty, P.L., 1965). It was also reported (Hamer, G., 1990) that for expanded/fluidized bed reactors, wastes containing high levels of calcium are particularly difficult to treat. Waste with 2.5 g/l of calcium were effectively treated on laboratory scale. However, after 150 days calcite precipitates were observed to be about 30% of the total weight of the sand/biomass particles (Jordening, et al., 1988). Greater than 90% of the calcium in a waste containing 0.9 - 3.0 g/l calcium was retained in a 10-litre laboratory-scale reactor. The accumulation of calcium resulted in increased particle density, loss of fluidization, clogging and 40% dead space (Vogel and Winter, 1988). Based on these relevant facts and the results from this study, as indicated by the measured biofilm specific activity and biomass accumulation (see section 3.1 and 3.3), we postulate that an immobilized calcium higher than 2 g/l in the biofilms would imposed a significant restraint to the cellular activity of the biofilms.

It is interesting that EDX analysis and SEM analysis of the dried biofilm samples showed that calcium was distributed quite evenly on the biofilms, despite the earlier

anticipation that a non-uniform calcium distribution on the biofilms would occur and large calcium deposits might exist in the biofilms. Because the calcium content of the dried biofilms was not high enough to meet the minimum concentration level requirement for the SEM, the imagine of calcium distribution on the biofilms could not be produced. However, the EDX graph for calcium indicated that there was a even calcium distribution in the biofilms. This perhaps can be attributed to the low pH environment for biofilm development which facilitated the dissolution of any calcium deposit. The EDX analytical results do not indicate whether calcium distribution was also uniform in depth in the biofilms.

3.5. Influence of calcium fluctuation

Thirty five days into the runs some of the biofilms were switched from one reactor to the other with a different calcium concentration in the influent. By then the biofilms had formed a relatively thick, uniform layer over the submerged area of the plastic slides. The subsequent progression of the biofilm biomass development is shown in figure 14. Only the biofilms transferred from the reactor with 100 mg/l of calcium to that without the addition of calcium showed a sharp decrease in biomass. The other transferred biofilms showed little difference in biomass growth from those which remained in the original environments. That the biofilms moved from 0 to near optimum (100 mg/l) calcium concentration did not show an improved accumulation of biomass indicates that, perhaps a lack of calcium during biomass attachment to the slide surface causes a structurally weak biofilm which can't support development of further layers.

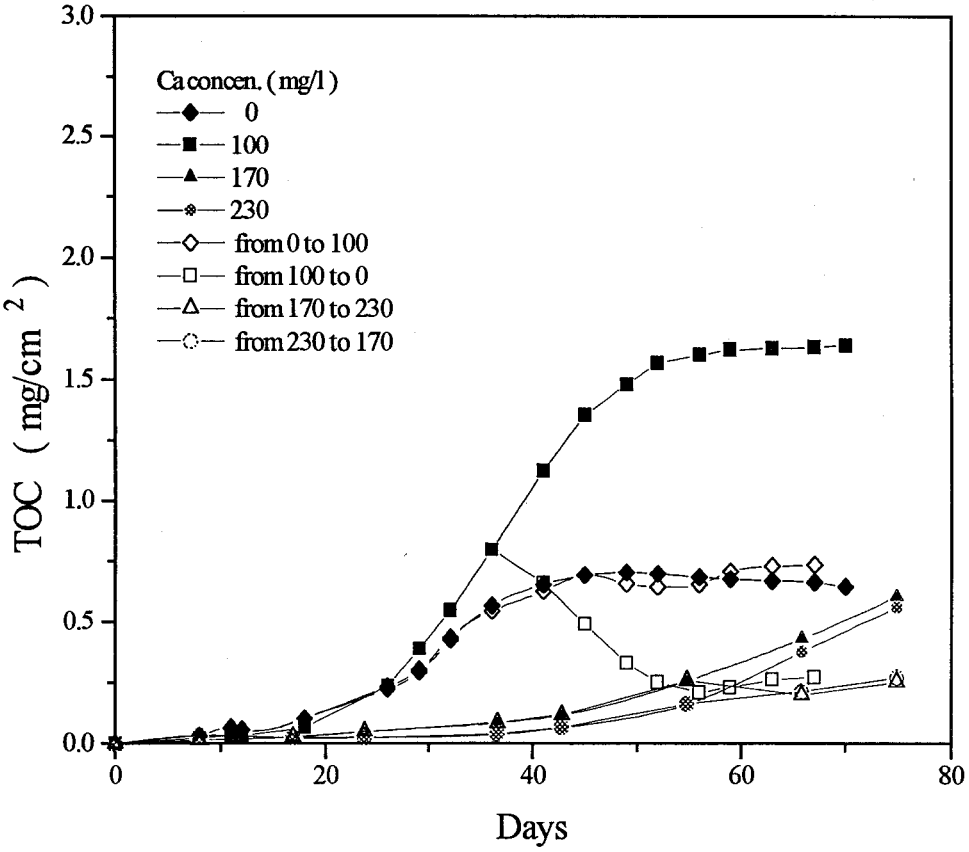
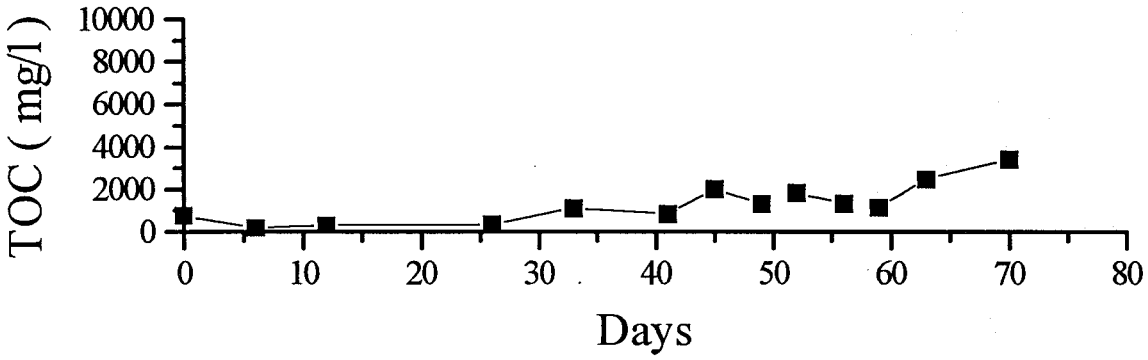
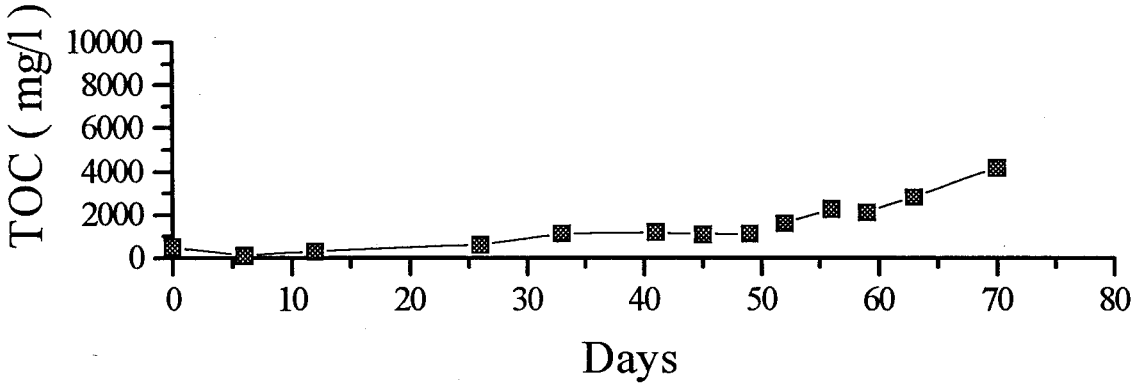


Figure 14. Influence in biomass progression of changes in calcium concentration during biofilm development

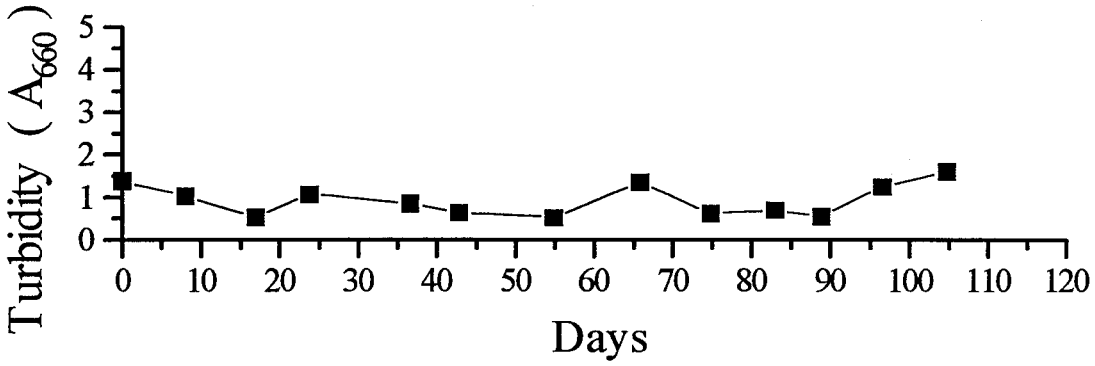


a) in the reactor without addition of Ca to feed

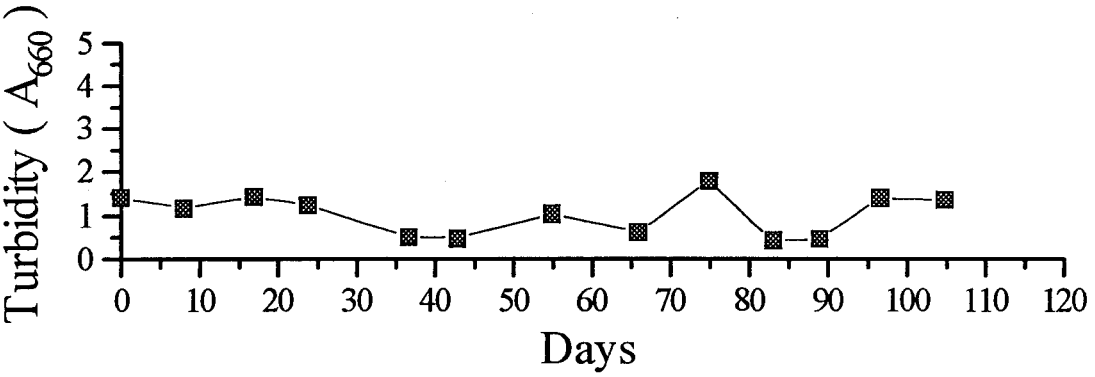


b) in the reactor with 100 mg/l of Ca in feed

Figure 15. Biomass concentration in the effluent



c) in the reactor with 170 mg/l of Ca in feed



d) in the reactor with 230 mg/l of Ca in feed

Figure 15. Biomass concentration in the effluent

On the other hand, it was observed that serious sloughing occurred from the surfaces of the biofilms started at 100 mg/l of calcium after they were transferred to the reactor without added calcium. This was further proven by the fluctuation in the suspended biomass concentration in the effluent (figure 15 a.) after the switch in samples. It is postulated that calcium within the biofilms which had a high calcium concentration, trends to leave the biofilms and enter the external environment which has a much lower calcium level and this would decrease the stability of biofilm structure and greatly speed up biofilm detachment due to the concentration shock.

In a study on the effects of nutrient limitation on biofilm sloughing with the obligate aerobe *Pseudomonas putida*, Applegate and Bryers (1990) found that the biofilms with higher extracellular polymer content and bound calcium exhibit a much lower shear removal rate. However, these biofilms always experience catastrophic sloughing events. They attributed the characters of reduced shear removal and the susceptibility to sloughing in these biofilms to their denser, more rigid crystalline structure brought about by excessive polymer production and concomitant binding of calcium.

3.6. Biofilm compositions and density

The compositions of the biofilms are listed in table 4 . It can be seen that the biofilms contained 87-96% of water and 4-13% of dry mass. Compared with some biofilms formed in aquatic environments or industrial fields, which may contain up to 99% water by weight (Geesey, G.G. & Jang, L., 1989), the biofilms produced in this study have higher solid contents. This is probably because they were cultivated in the nutrient-enriched culture medium.

As illustrated in figure 16, when calcium concentration in the feed was raised, the water content of the biofilms decreased and the total dry mass of the biofilms increased.

Table 4. The composition of dried biofilms

Ca in feeds (mg/l)	Water %	Dry mass %	Ash %	Organic %
80.00	95.73	4.27	1.45	2.82
120.00	94.89	5.11	2.14	2.97
170.00	91.16	8.84	6.04	2.80
230.00	87.40	12.60	9.81	2.79

However, in the dry materials, the proportion of minerals increased significantly while that of organic materials slightly decreased. This indicates that the presence of calcium

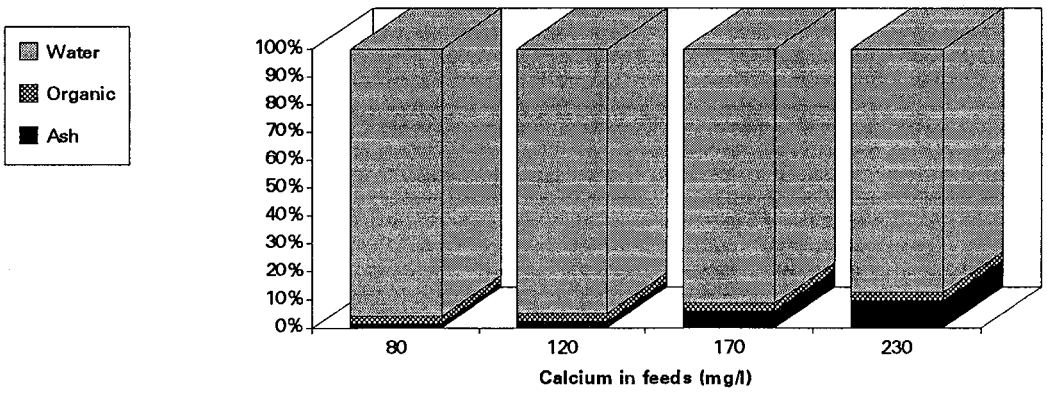


Figure 16. Calcium concentration vs. Biofilm composition

increases the dry mass of the biofilms mainly by increasing the concentration of minerals in

the biofilms. The increased mineral content is very likely the result of more calcium trapped in the biofilm. The density of the biofilms increased with increasing calcium concentration in the feeds (figure 17), reflecting the changes in the biofilm compositions.

At this point, we can probably narrow down the reasons for changes in the biofilm specific activity and the biofilm mass accumulation with calcium concentration. For the high calcium concentrations, a large amount of mineral deposits within the biofilms along

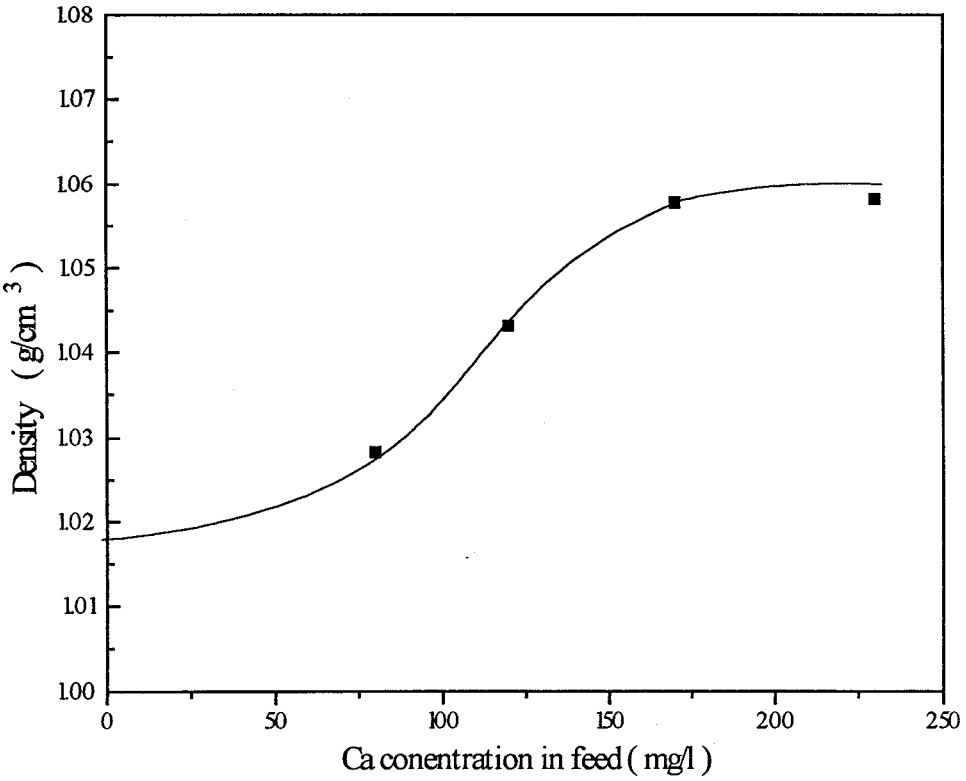


Figure 17. Biofilm density vs. Calcium Concentration in feed

with the significant decrease in the biofilm water content would cause an inhibition of the

cellular metabolism and even damages to the cells, resulting in a lower biofilm specific activity and biofilm mass accumulation. When the calcium concentrations were lower, the stress to the cells that was caused by accumulation of minerals within the biofilms was less, if the calcium did not promote the cellular reproduction and exopolymer production. Therefore, the biofilms showed a higher mass accumulation and when the cells in the biofilms were dispersed in the fresh culture medium, they would exhibit a higher specific activity.

A more important mechanism for the biofilm accumulation at low calcium concentrations is likely that with the increase of the calcium concentration, more bound calcium in the biofilms led to a stronger biofilm structure, resulting in a smaller loss of the biofilm mass. This explanation is also supported by the results reported by Turakhia and Characklis (1988). In their study of the effect of calcium on the biofilms of marine bacteria *P. aeruginosa* in the RotoTorque reactor, they found that in the calcium concentration range from 0.4 to 50 mg/l, increasing calcium concentration in the growth medium significantly increases the biofilm accumulation; the cellular activity, indicated by specific growth rate and biomass yield, was the same as that measured in a chemostat and was not influenced by the calcium concentration; extracellular polymer production rate in the biofilm was unaffected by the calcium concentration but was higher than that determined in a chemostat. They did not explain clearly why the biofilm accumulation increased in the presence of calcium. But they also reported that calcium in the biofilm increased proportionally to the calcium concentration in the culture medium and the cohesiveness of the biofilm, as indicated by a lower relative detachment rate, was increased.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

1. The presence of calcium in the growth medium increases both the mass accumulation rate and ultimate total mass of anaerobic acidogenic biofilm when the calcium concentration in the feeds is in the range from 0 to 120 mg/l. For a higher calcium concentration, the biofilm accumulation may decline.

2. The specific activity of the acidogenic biofilm decreases with increasing calcium concentration in the medium. The biofilm thickness for the highest specific activity is less than 0.5 millimetres. The optimum calcium concentration for the substrate consumption rate/unit area biofilm is from 100 to 120 mg/l.

3. Calcium accumulation in the biofilms is proportional to the calcium level in the liquid phase.

4. Increasing calcium concentration in the culture medium increases the stability of the biofilm structure when the calcium concentration is not higher than the optimum level.

4.2 Recommendations

1. Further study on the relationship between external calcium concentration and EPS and the cellular mass in the biofilms is recommended for a better understanding of the mechanism of the higher acidogenic biofilm mass accumulation promoted by calcium. Comparison experiments with suspended cells would be useful for clarifying the experimental results with the immobilized cells.

2. For the same purpose, a further study on the effect of calcium on the substrate and calcium transport into the biofilms is also recommended. A study on the ultrastructure of the biofilms by utilization of an electronic microscopy may be important for this study.

3. Utilization of a pure culture of the acidogenic bacteria in studies on biofilms would be beneficial for better control of the experiments.

4. Another design of the experimental system for the study of anaerobic biofilms is presented in figure 16. The author believes that it will provide the following advantages over the current reactor system:

- avoid the measurement of each individual biofilm area because there is no head space in the biofilm chamber.

- make biofilm sampling easier and better because the slide bases would be made of the material similar to that for the chamber wall or other hard organic materials so that it would not become deformed even after being used for a long time and would be convenient for inserting and taking out the biofilm supports. The screw threads on the bases would facilitate the installation and removal of the support assemblies.

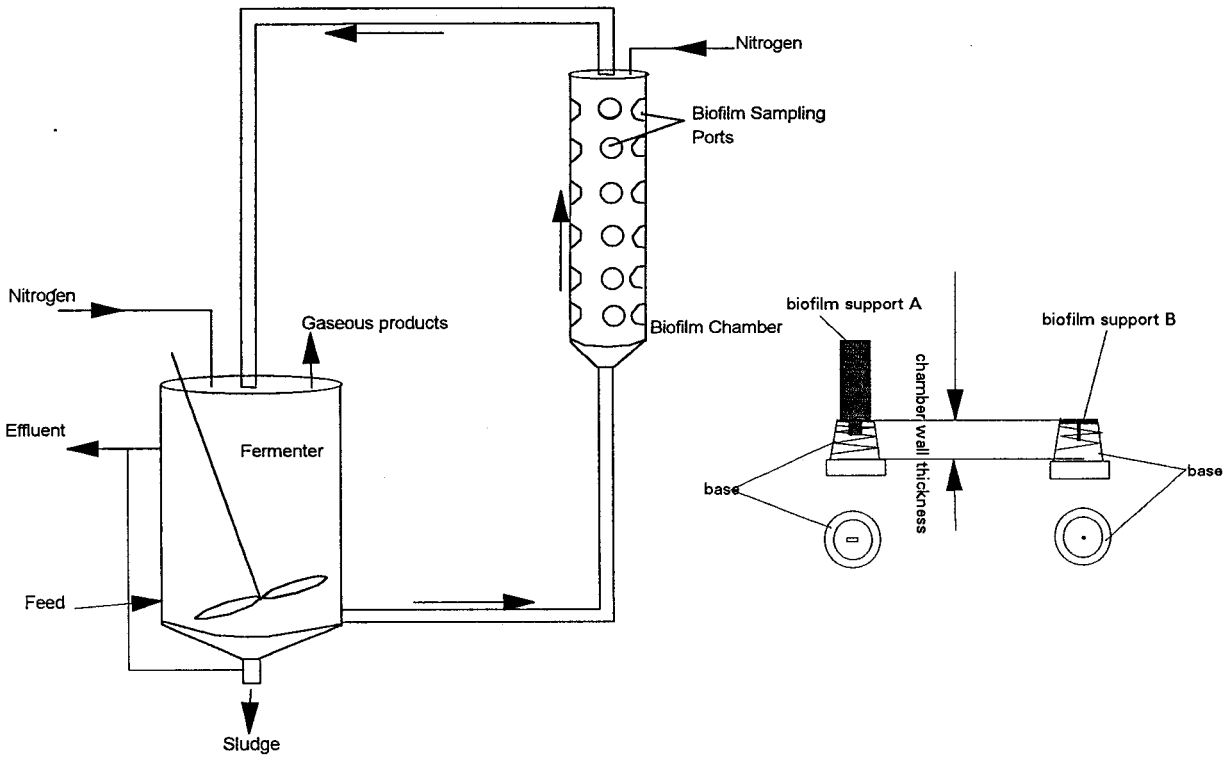


Figure 18. An alternative design of the experimantal system for study of anaerobic biofilms

- simplify the reactor control and maintenance greatly, because control of the reactor liquid surface level would be much easier and would no longer influence the biofilm areas; removing the sludge and cleaning the reactor would become simpler; preparation of the microbial inoculum can be made with the same fermenter.

- facilitate the study of the effects of the radial flow rates on the biofilms at different positions of the cross section of the chamber.

Besides, the chamber can be made removable and can be easily replaced with another chamber which may have a different length or diameter to meet the needs of the experiments.

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Appendix Raw Experimental Data

Biofilm TOC

mg/biofilm

Time days	at Ca=100	at Ca=0	from 100 to 0	from 0 to 100	Time days	Ca=120	Ca=80	Time days	at Ca=230	at Ca=170	from 230 to 170	from 170 to 230
0	0.0000	0.0000	0.0000	0.0000	0.0	0.0000	0.0000	0.0	0.0000	0.0000	0.0000	0.0000
8	0.1183	0.0000	0.1183	0.0000	7.4	1.5850	1.4785	8.0	2.8000	1.3000	2.8000	1.3000
11	0.0918	1.8816	0.0919	1.8814	13.4	1.6717	1.8366	16.9	2.3000	2.2000	2.3000	2.2000
12	5.7220	13.3845	5.7218	13.3846	22.3	6.6469	2.1282	23.7	5.7600	1.9000	5.7600	1.9000
12	2.7892	9.2180	2.7891	9.2179	27.9	7.5990	2.0293	36.6	6.4000	15.3340	6.4000	15.3340
18	0.4082	1.6767	0.2394	1.6769	36.1	13.4815	41.3316	42.8	4.5007	17.0600	4.5007	17.0600
26	19.9042	17.2365	19.9041	17.2365	44.0	46.8456	3.7730	54.8	5.7355	18.0000	5.7355	18.0000
29	23.8937	33.6745	23.8935	33.6747	51.0	88.7393	25.1436	65.8	27.1866	13.2915	51.2545	50.1798
32	39.2106	48.2570	39.2106	48.2570	57.4	107.4000	46.6853	74.9	19.9902	22.7566	27.7268	40.3010
36	50.4227	42.3465	53.1722	30.5697	63.3	92.7220	32.9300	83.0	40.5397	38.3838	78.4465	47.9888
41	72.4941	26.9282	29.5279	36.0203	63.9	79.4760	43.4026	88.9	41.0505	40.7571	64.8215	43.3116
45	77.4090	35.5485	10.0043	38.8513	67.2	103.5381	64.5021	96.5	15.6865	83.8964	18.1602	15.6244
49	83.0880	38.2188	14.6745	36.7253	68.3	108.3845	41.1213	104.7	73.3807	198.4854	76.2857	41.0672
52	87.1397	21.0827	6.2403	30.0763	69.4	137.2035	84.9520					
56	64.6085	26.9977	13.3211	23.1014								
59	90.3701	42.0513	15.3406	65.4096								

Dry Biofilm Mass

g

Time days	Ca=120	Ca=80	Time days	Ca=230	Ca=170	from 170 to 230	from 230 to 170 0
0	0.0000	0.0000	0.0	0.0000	0.0000	0.0000	0.0000
7	0.0016	0.0006	8.0	0.0046	0.0179	0.0179	7.9358
13	0.0024	0.0015	16.9	0.0137	0.0136	0.0136	16.8685
22	0.0043	0.0020	23.7	0.0154	0.0170	0.0170	23.6968
28	0.0022	0.0000	36.6	0.0264	0.0104	0.0104	36.5674
36	0.0067	0.0038	42.8	0.0098	0.0158	0.0158	42.7869
44	0.0145	0.0060	54.8	0.0101	0.0412	0.0412	54.7683
51	0.0211	0.0082	65.8	0.0326	0.0799	0.0799	65.6792
57	0.0353	0.0180	74.9	0.0447	0.0777	0.0777	74.7318
63	0.0343	0.0194	83.0	0.0472	0.0482	0.0482	82.8838
63	0.4562	0.3334	88.9	0.0164	0.0174	0.0174	88.8412
67	0.0349	0.0225	96.5	0.0119	0.0111	0.0111	0.0130
68	0.5354	0.3354	104.7	0.0205	0.0162	0.0162	0.0209
70	0.0344	0.0248					

Wet Biofilm Mass

g

Time days	at Ca=120	at Ca=80	Time days	at Ca=230	at Ca=170
0	0.0000	0.0000	0.0	0.0000	0.0000
7	0.0401	0.0486	8.0	0.0943	0.0502
13	0.0601	0.0549	16.9	0.0671	0.0734
22	0.0728	0.0211	23.7	0.2868	0.0907
28	0.0550	0.0191	36.6	0.0860	0.2848
36	0.2179	0.1133	42.8	0.2093	0.1958
44	0.2915	0.0741	54.8	0.1911	0.2163
51	0.3862	0.1861	65.8	0.2370	0.0811
57	0.4862	0.1943	74.9	0.1942	0.1750
63	0.6433	0.3015	83.0	0.2538	0.1982
63			88.9	0.2831	0.2419
67	0.5591	0.3327	96.5	0.2130	0.2486
68			104.7	0.4233	0.4472
70	0.5868	0.4417			

Average ratio of TOC/biofilm volume

Ca level	Sample No.	Density	Wet Weight	Measured Volume	TOC	TOC/biofilm volume	time
ppm		g/ml	g	ml	mg	mg/mm3	days
	1	1.0216	0.4864	0.4761	0.3548	0.7452	
	2	1.0357	0.3983	0.3846	0.3167	0.8236	
	3	1.0273	0.5341	0.5199	0.4004	0.7701	
80	Average	1.0282	0.4729	0.4602	0.3573	0.7796	36
	1	1.0374	0.1167	0.1125	0.0964	0.8569	
	2	1.0407	0.1032	0.0992	0.0908	0.9154	
	3	1.0512	0.0694	0.0660	0.0614	0.9300	
120	Average	1.0431	0.0964	0.0926	0.0829	0.9008	36
	1	1.0528	0.1376	0.1307	0.0632	0.4838	
	2	1.0590	0.1574	0.1486	0.0955	0.6422	
	3	1.0613	0.1450	0.1366	0.0821	0.6012	
170	Average	1.0577	0.1467	0.1387	0.0803	0.5757	38
	1	1.0630	0.1677	0.1578	0.0512	0.3245	
	2	1.0486	0.1963	0.1872	0.0759	0.4052	
	3	1.0627	0.1892	0.1780	0.0619	0.3478	
230	Average	1.0581	0.1844	0.1743	0.0630	0.3592	38

Biofilm Surface Area

cm^2

Time days	at Ca=100	at Ca=0	from 100 to 0	from 0 to 100	Time days	at Ca=120	at Ca=80	Time days	at Ca=230	at Ca=170	from 230 to 170	from 170 to 230
0	0.00	0.00	0.00	0.00	0.0	0.00	0.00	0.0	0.00	0.00	0.00	0.00
8	4.66	4.27	4.66	4.27	7.4	3.98	4.35	8.0	4.05	4.50	4.05	4.50
11	4.14	4.39	4.14	4.39	13.4	4.35	3.98	16.9	4.76	4.02	4.76	4.02
12	3.97	4.76	3.97	4.76	22.3	4.28	3.68	23.7	4.76	4.76	4.76	4.76
12	4.89	4.63	4.89	4.63	27.9	4.50	3.90	36.6	4.80	4.80	4.80	4.80
18	5.17	4.85	5.17	4.85	36.1	4.95	5.10	42.8	4.80	4.95	4.80	4.95
26	4.75	4.68	4.75	4.68	44.0	4.50	3.90	54.8	4.95	4.95	4.95	4.95
29	4.92	4.59	4.92	4.59	51.0	5.25	4.95	65.8	4.95	4.80	4.95	4.95
32	4.68	5.07	4.68	5.07	57.4	5.25	5.25	74.9	4.95	4.95	4.95	4.95
36	4.89	5.26	4.89	5.26	63.3	5.63	5.25	83.0	4.95	4.95	4.95	4.95
41	5.12	5.24	5.51	5.34	63.9	5.55	5.55	88.9	5.10	5.10	5.10	5.10
45	5.08	5.29	5.26	5.03	67.2	5.55	5.55	96.5	5.10	5.25	5.10	4.95
49	5.34	5.18	4.96	5.26	68.3	5.40	5.40	104.7	5.10	5.10	5.10	4.80
52	5.44	5.07	5.27	5.39	69.4	5.55	5.55					
56	5.15	5.39	5.13	5.09								
59	5.52	5.46	5.50	5.46								

Biofilm thickness and volume

Biofilm Thickness (cm)

Time Days	at Ca=120	at Ca=80	Time Days	at Ca=230	at Ca=170
0	0	0	0	0	0
7.3958	0.0044	0.0044	7.9583	0.0144	0.0038
13.396	0.0043	0.0059	16.896	0.0101	0.0071
22.278	0.0173	0.0074	23.729	0.0253	0.0052
27.882	0.0187	0.0067	36.604	0.0278	0.0416
36.097	0.0302	0.1039	42.813	0.0196	0.0449
44.049	0.1156	0.0124	54.82	0.0242	0.0474
51.031	0.1876	0.0652	65.792	0.2162	0.1321
57.354	0.2271	0.1141	74.854	0.117	0.1061
63.257	0.183	0.0805	82.979	0.331	0.1263
63.875	0.159	0.1003	88.875	0.2654	0.1106
67.229	0.2071	0.1491	96.542	0.0744	0.2082
68.292	0.2228	0.0977	104.73	0.3124	0.507
69.417	0.2744	0.1963			

Biofilm Volume (cm^3)

Time Days	at Ca=120	at Ca=80	Time Days	at Ca=230	at Ca=170
0	0	0	0	0	0
7.3958	0.0176	0.019	7.9583	0.0585	0.0169
13.396	0.0186	0.0236	16.896	0.048	0.0287
22.278	0.0738	0.0273	23.729	0.1203	0.0248
27.882	0.0844	0.026	36.604	0.1337	0.1998
36.097	0.1497	0.5301	42.813	0.094	0.2223
44.049	0.5201	0.0484	54.82	0.1198	0.2345
51.031	0.9851	0.3225	65.792	1.0704	0.6339
57.354	1.1923	0.5988	74.854	0.579	0.525
63.257	1.0294	0.4224	82.979	1.6382	0.6252
63.875	0.8823	0.5567	88.875	1.3537	0.5643
67.229	1.1494	0.8273	96.542	0.3792	1.093
68.292	1.2032	0.5274	104.73	1.5931	2.5858
69.417	1.5232	1.0896			

Biofilm TOC Per Unit Area

mg/mm^2

Time days	at C=100	at Ca=0	from 100 to 0	from 0 to 100	Time days	at Ca=120	at Ca=80
0	0.0000	0.0000	0.0000	0.0000	0.0	0.0000	0.0000
8	0.0025	0.0000	0.0025	0.0000	7.4	0.0399	0.0340
11	0.0022	0.0429	0.0022	0.0429	13.4	0.0384	0.0462
12	0.1441	0.2810	0.1441	0.2810	22.3	0.1555	0.0579
18	0.0571	0.1991	0.0570	0.1990	27.9	0.1689	0.0520
26	0.0079	0.0346	0.0046	0.0346	36.1	0.2724	0.8104
29	0.4189	0.3684	0.4189	0.3684	44.0	1.0410	0.0967
32	0.4859	0.7332	0.4859	0.7332	51.0	1.6903	0.5080
36	0.8387	0.9522	0.8387	0.9522	57.4	2.0457	0.8892
41	1.0311	0.8048	1.0874	0.5810	63.3	1.6484	0.6272
45	1.4167	0.5143	0.5363	0.6743	63.9	1.4320	0.7820
49	1.5226	0.6726	0.1902	0.7727	67.2	1.8656	1.1622
52	1.5568	0.7381	0.2956	0.6987	68.3	2.0071	0.7615
56	1.6010	0.4162	0.1184	0.5576	69.4	2.4721	1.5307
59	1.2536	0.5007	0.2599	0.4541			
63	1.6371	0.7700	0.2789	1.1989			
67		0.5174	0.2659				
70		0.6312					

(continued)

Time days	at Ca=230	at Ca=170	from 230 to 170	from 170 to 230
0.0	0.0000	0.0000	0.0000	0.0000
8.0	0.0346	0.0144	0.0346	0.0144
16.9	0.0242	0.0274	0.0242	0.0274
23.7	0.0606	0.0200	0.0606	0.0200
36.6	0.0667	0.1597	0.0667	0.1597
42.8	0.0469	0.1723	0.0469	0.1723
54.8	0.0579	0.1818	0.0579	0.1818
65.8	0.5177	0.5069	0.2746	0.1385
74.9	0.2801	0.4071	0.2019	0.2299
83.0	0.7924	0.4847	0.4095	0.3877
88.9	0.6355	0.4246	0.4025	0.3996
96.5	0.1780	0.7990	0.1538	0.1578
104.7	0.7479	1.9459	0.7194	0.4278

Biofilm Specific Activity Assay

Time days	Initial biofilm dry mass g	Lactose concentration drop mg/l	Time interval hrs	Lactose consumption rate mg/l/hr	Specific activity mg/l/hr/g
at Ca=80 mg/l					
0	0	0.00	0.00	0.00	0.00
7	0.0016	3691.00	22.00	167.77	104857.95
13	0.0024	5521.00	22.00	250.95	104564.39
22	0.0043	6195.00	23.70	261.39	60788.93
28	0.0022	3850.00	27.30	141.03	64102.56
44	0.0145	1009.00	13.20	76.44	5271.68
51	0.0211	477.00	21.00	22.71	1076.51
57	0.0353	239.00	14.80	16.15	457.47
63	0.0343	1072.00	20.20	53.07	1547.21
at Ca=120 mg/l					
0	0	0.00	0.00	0.00	0.00
7	0.0006	3264.00	22.00	148.36	247272.73
13	0.0015	5664.00	22.00	257.45	171636.36
22	0.002	7784.00	23.70	328.44	164219.41
28	0.005	4210.00	27.30	154.21	30842.49
44	0.006	865.00	13.20	65.53	10921.72
51	0.0082	477.00	21.00	22.71	2770.03
57	0.018	120.00	14.80	8.11	450.45
63	0.0194	714.00	20.20	35.35	1821.99

(continued)

Time days	Initial biofilm dry mass g	Lactose concentration drop mg/l	Time interval hrs	Lactose consumption rate mg/l/hr	Specific activity mg/l/hr/g
Ca= 170 mg/l					
0	0	0	0	0.00	0.00
8	0.0046	145	23.5	6.17	1341.35
17	0.0137	148	64	2.31	168.80
24	0.0154	1039	26	39.96	2594.91
37	0.0264	504	51.17	9.85	373.09
43	0.0098	891	23.83	37.39	3815.29
55	0.0101	4187	16.45	254.53	25200.87
66	0.0326	2056	10	205.60	6306.75
75	0.0447	1218	19.9	61.21	1369.26
83	0.0472	376	13	28.92	612.78
89	0.0164	535	25.83	20.71	1262.95
97	0.0119	362	18.47	19.60	1647.00
at Ca=230 mg/l					
0	0.0000	0	0	0.00	0.00
8	0.0179	119	23.5	5.06	282.90
17	0.0136	352	64	5.50	404.41
24	0.017	1188	26	45.69	2687.78
37	0.0104	905	24	37.71	3625.80
43	0.0158	772	23.83	32.40	2050.39
55	0.0412	1841	7	263.00	6383.50
66	0.0799	1244	10	124.40	1556.95
75	0.0777	1773	22.5	78.80	1014.16
83	0.0482	468	12.56	37.26	773.05
89	0.0174	852	20.31	41.95	2410.91
97	0.0111	510	21.32	23.92	2155.06

Cell and substrate concentration in effluent

Cell Concentration

TOC (mg/l)

Time days	Ca=120	Ca=80	Time days	Ca=230	Ca=170
-8	498.3	521.9	0	--	--
0	493.9	759.9	7.95833	0.03457	0.01444
6	103	183	16.8958	0.02419	0.02736
12	268	284	23.7291	0.06057	0.01998
26	454.38	274.5	36.6041	0.06667	0.15973
33	888.24	726.4	42.8125	0.04688	0.17232
41	600	628	54.8427	0.05793	0.18182
45	746.44	1115.9	65.8113	0.51772	0.50687
49	655.88	761.05	74.8672	0.28007	0.40708
52	624.12	704	82.9685	0.79239	0.48474
56	691.38	398.33	88.8555	0.63551	0.42462
59	695.25	383.37	96.5536	0.17804	0.79901
63	704.71	747.79	104.704	0.7479	1.94594
70	831.76	728.84			
77	1137.3	775.58			
85	763.53	877.26			

Lactose concentration

(mg/l)

Time days	Ca=120	Ca=80	Time days	Ca=270	Ca=170
-9	89.5	80.7	0	--	--
-4	855.7	274.5	7.94969	9501.56	8467.92
1	396.8	324.9	16.9056	5131.85	2060.37
6	118.3	55.3	23.7483	1055.72	3305.66
12	61	55.3	36.6283	6878.73	1992.45
14	64.9	74	42.8171	5296.78	7584.90
19	303	5478	54.8427	5138.43	4030.18
21	504.5	1510	65.8113	3615.87	3532.07
26	1106	540.5	74.8672	430.539	4305.39
32	1137.6	1991.8	82.9685	2444.66	2060.37
34	1689.3	1672.3	88.8555	189.701	2286.79
40	1124.4	863.8	96.5536	4123.94	4866.25
45	1780.1	3873.2	104.704	3575.45	1245.28
52	702.2	4115.8			
59	720.1	477.8			

Calcium within Biofilms

	at Ca=120			at Ca=80		
Time Days	total Ca mg	Ca/area mg/cm^2	Ca/volume mg/cm^3	total Ca mg	Ca/area mg/cm^2	Ca/volume mg/cm^3
0	0	0	0	0	0	0
7.3	0.15	0.0019	0.42624	0.2	0.0023	0.52731007
13.3	0.25	0.0029	0.67353	0.3	0.0038	0.63676437
22.2	0.45	0.0053	0.30492	0.3	0.0041	0.54951154
27.8	0.3	0.0033	0.17781	0.3	0.0038	0.57629796
36.1	0.55	0.0056	0.18374	0.5	0.0049	0.0471585
44.0	0.5	0.0056	0.04807	0.1	0.0013	0.10332076
51.0	0.9	0.0086	0.04568	0.2	0.002	0.03100806
57.3	2.2	0.021	0.09226	0.6	0.0057	0.05010059
63.2	2.2	0.0196	0.10686	0.6	0.0057	0.07102833
63.8	2.1	0.0189	0.11901	0.8	0.0072	0.0718533
67.2	2.4	0.0216	0.1044	0.6	0.0054	0.03626181
68.3	2.1	0.0194	0.08727	0.4	0.0037	0.03791973
69.4	2.15	0.0194	0.07058	0.5	0.0045	0.02294397

(continued)

	at Ca=230			at Ca=170			from 230 to 170	from 170 to 230
Time days	total Ca) mg	Ca/area mg/cm^2	Ca/volume mg/cm^3	total Ca mg	Ca/area mg/cm^2	Ca/volume mg/cm^3	total Ca mg	total Ca mg
0	0	0	0	0	0	0	0	0
7.9583	0.3	0.0037	0.2565	0.2	0.0022	0.5904	0.3	0.2
16.896	0.2	0.0021	0.2082	0.2	0.0025	0.3489	0.2	0.2
23.729	0.4	0.0042	0.1663	0.3	0.0032	0.606	0.4	0.3
36.604	0.2	0.0021	0.0748	0.6	0.0063	0.1502	0.2	0.6
42.813	2.3	0.024	1.2235	1.5	0.0152	0.3374	2.3	1.5
54.82	2.4	0.0242	1.0019	3.7	0.0374	0.7889	2.4	3.7
65.792	3.1	0.0313	0.1448	1.03	0.0107	0.0812	0.31	3.73
74.854	8.29	0.0837	0.7159	0.69	0.007	0.0657	0.83	9.53
82.979	3.85	0.0389	0.1175	1.55	0.0157	0.124	1.46	3.75
88.875	2.39	0.0234	0.0883	1.23	0.0121	0.109	1.41	4.1
96.542	3.22	0.0316	0.4245	1.15	0.011	0.0526	2.13	3.53