Metabolic response of biofilm to shear stress in fixed-film culture

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Aims: In a biofilm reactor, detachment force resulting from hydraulic shear is a major factor that determines the formation and structure of steady state biofilm. The metabolic response of biofilm to change in shear stress was therefore investigated.

Methods and Results: A conventional annular reactor made of PVC was used, in which shearing over the rotating disc surface was strictly defined. Results from the steady state aerobic biofilm reactor showed that the biofilm structure (density and thickness) and metabolic behaviour (growth yield and dehydrogenase activity) were closely related to the shear stress exerted on the biofilm. Smooth, dense and stable biofilm formed at relatively high shear stress. Higher dehydrogenase activity and lower growth yield were obtained when the shear stress was raised. Growth yield was inversely correlated with the catabolic activity of biofilm. The reduced growth yield, together with the enhanced catabolic activity, suggests that a dissociation of catabolism from anabolism would occur at high shear stress.

Conclusions: Biofilms may respond to shear stress by regulating metabolic pathways associated with the substrate flux flowing between catabolism and anabolism. A biological phenomenon, besides a simple physical effect, is underlying the observed relation between the shear stress and resulting biofilm structure.

Significance and Impact of the Study: A hypothesis is proposed that the shear-induced energy spilling would be associated with a stimulated proton translocation across the cell membrane, which favours formation of a stronger biofilm. This research may provide a basis for experimental data on biofilm obtained at different shear stresses to be interpreted in relation to energy.

INTRODUCTION

Biofilm is a promising biotechnology for wastewater treatment. Intensive research has shown that the morphological characteristics of biofilm are important for the stability and performance of a biofilm reactor. Detachment force is considered a dominant factor that strongly influences the biofilm structure and performance. In a biofilm reactor, detachment force may result from hydraulic shear. Much research has been dedicated to the effects of the detachment force on the morphological characteristics and structures of biofilm (Rittmann 1982; Chang et al. 1991; Chen et al. 1998; Kwok et al. 1998). In the environmental engineering literature, it is generally considered that a higher detachment force makes the biofilm denser and thinner. It has been hypothesized that a physical rather than a biological phenomenon is responsible for the observed relation between detachment force and the resulting biofilm structure (Vieira et al. 1993; Chen et al. 1998). So far, little attention has been paid to the interrelationship of the metabolism of fixed bacteria to detachment force. There is evidence that suspended cells can respond to hydraulic shear by altering their growth rate, morphology, cell size/density and metabolism (Nollert et al. 1991; Meijer et al. 1993; Moreira et al. 1995; Chen and Huang 2000). However, a clear correlation between the detachment force
and the resulting biofilm metabolism is still lacking for fixed-film culture. The performance of a biofilm reactor is directly associated with the metabolic activity of fixed bacteria under given operational conditions. Similar to suspended culture, the metabolic network of fixed bacteria basically includes interrelated catabolic and anabolic reactions. As one of the most important aspects, the relation between the detachment force and the energy metabolism of biofilm has been little studied so far. Therefore, an attempt to show the effects of detachment force on energy metabolism and the structure of biofilm is reported, and related mechanisms are discussed.

**MATERIALS AND METHODS**

**Experimental apparatus**

A conventional annular reactor made of PVC was used. It consists of two coaxial cylinders, a stationary outer cylinder and a rotating inner cylinder made up of a stack of rectified removable PVC discs. The liquid volume of the reactor is 4 l. This type of reactor has the advantage of providing constant shearing over the rotating disc surface. The diameter of the rotating disc is 23 cm. Polystyrene films (20 × 3 cm) were attached to the side surfaces of the discs to serve as support material for biofilm development.

**Operation of reactor**

Bacteria from a steady state, laboratory-scale, aerobic-settling-anaerobic reactor, fed with peptone as sole carbon source, were used as inoculum. The reactor was started under identical seeding conditions for each rotation speed of the discs. After the seeding period, the reactor was switched to once-through continuous flow at a constant hydraulic retention time of 2 h. The synthetic substrate, containing peptone as sole carbon source, KH2PO4, Na2HPO4, NH4Cl, MgCl2, CaCl2 and FeCl3, was continuously fed into the reactor by a peristaltic pump. The influent chemical oxygen demand (COD) concentration was kept at 200 mg l⁻¹, equivalent to a volumetric loading rate of 2.4 kg COD m⁻³ day⁻¹ for the experiments. The shear stress on biofilm was controlled by the rotation speed of the disc in the range of 40–120 rev min⁻¹ and was expressed as the tip velocity of the rotating disc in terms of meters per second. The temperature was kept constant at 25 ± 1°C, while dissolved oxygen (DO) was supplied by pre-aeration of the substrate solution. In order to maintain the DO concentration at above 4.0 mg l⁻¹ in all experimental runs, a DO controller was employed to function as follows. Once the DO concentration of the reactor dropped below 4.0 mg l⁻¹, the DO probe located at the middle of the reactor transmits a signal to the DO controller to activate the electro-valve controlling the oxygen supply.

**Analytical methods**

Biofilm samples were collected at steady state, indicated by constant effluent and fixed biomass concentrations, from the removable flexible PS slides (8 × 3 cm). The slides were located on the middle disc of the reactor. The soluble COD concentration was analysed using the standard method (APHA 1995). The dry weight of fixed biomass was also analysed by the standard method (APHA 1995). Biofilm thickness was determined according to the method proposed by Trulier and Characklis (1982). The sample slide was placed on a microscope. The 10× objective was lowered micrometre by micrometre until the biofilm surface was in focus, and the micro-adjustment dial setting was recorded. The objective was then lowered further until the inert plastic surface was in focus. The difference between the two focus lengths was then compared with a calibration curve, and the biofilm thickness was determined. The biofilm thickness was then expressed as a mean value evaluated from at least 10 measurements along the slide for each sample. Ohashi and Harada (1994) also used this method to determine biofilm thickness. The dehydrogenase activity of the biofilm is measured by following the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to iodonitrotetrazolium (INT-formazan), according to the procedure established by Lopez et al. (1986) and then used by Lertpocasombut (1991). The INT-formazan concentration is then determined spectrophotometrically at 490 nm. The specific INT-dehydrogenase activity of the biofilm is expressed in terms of the optical density at 490 nm per milligram of dry weight of biofilm. In fact, the specific INT-dehydrogenase activity closely correlates with electron transport system activity (Trevors 1984). According to Trulier and Characklis (1982), for steady state fixed-film culture, the observed growth yield (Yobs) is calculated as the increase in biomass concentration, including both fixed and detached biomass, divided by the corresponding decrease of COD concentration.

**RESULTS**

A series of experiments was conducted at different tip velocities (Vt) of the rotating disc, ranging between 0-48 and 1-45 m s⁻¹. In the annular reactor, the tip velocity of the rotating disc acts as a major shear stress on biofilm (Rittmann 1982; Trulier and Characklis 1982). The tip velocity vs steady state biofilm thickness and density is presented in Fig. 1. Results indicate that a higher tip velocity leads to a thinner and denser biofilm, i.e., a compact biofilm structure. Figure 2 shows the effects of Vt on the observed growth yield (Yobs) and specific INT-dehydrogenase activity of the biofilm. The reduced Yobs and increased INT-dehydrogenase activity are observed with the increase...
in tip velocity. In a three-phase fluidized bed reactor, Lertpocasombut (1991) also found that the INT-dehydrogenase activity of biofilm was increased from $3 \times 10^5$ to $7 \times 10^5$ Absorbance (Abs) mg$^{-1}$ dry weight as the specific velocity of air was shifted from $5.7 \text{ m h}^{-1}$ to $10.2 \text{ m h}^{-1}$. The interrelationship of the growth yield to INT-dehydrogenase activity is further shown in Fig. 3. The growth yield is apparently inversely dependent on the INT-dehydrogenase activity of the biofilm.

During the aerobic oxidation process, the respiratory activity of cells can be indirectly determined by measurement of the proton translocation activity, and the INT can be used as artificial proton acceptor to quantify the catabolic activity of the biofilm. In order to demonstrate the possible interrelationship of catabolic activity of the biofilm to the resulting biofilm structure, biofilm density vs specific INT-dehydrogenase activity of the biofilm is shown in Fig. 4. A clear correlation between the catabolic activity of the biofilm and the biofilm density can be seen. These results suggest that a higher catabolic activity of biofilm would favour formation of a stronger biofilm. It appears from Figs 1, 2, 3 and 4 that the shear stress not only influences the biofilm structures, but also has a significant effect on the energy metabolism of the biofilm.

**DISCUSSION**

**Effect of shear stress on biofilm structure**

Figure 1 shows that a higher shear stress results in a thinner and denser biofilm. Similar phenomena have also been reported in the literature (Chang et al. 1991; Vieira et al. 1993; Ohashi and Harada 1994; Chen et al. 1998). In addition, biofilm density was found to correlate very closely with the self-immobilization strength of fixed bacteria, which was dependent on the shear stress exerted on the biofilm.
biofilm (Ohashi and Harada 1994; Chen et al. 1998). This, and previous research, suggests that hydrodynamic conditions have a significant influence on the density of biofilm. It has been hypothesized in the literature that a physical phenomenon is responsible for the observed relation between detachment force and biofilm structure (Vieira et al. 1993). However, the results in Figs 2 and 4 exhibit an interrelationship between biofilm metabolism and shear stress.

Response of biofilm to shear stress

To date, the mechanism by which fixed bacteria respond to a shear stress and further produce a compact biofilm has been unclear in a metabolic sense. In Fig. 2, a remarkable reduction in \( Y_{\text{obs}} \) is observed when the shear stress, in terms of tip velocity of the rotating disc, increases, while the high shear stress on the biofilm leads to a significant increase in specific INT-dehydrogenase activity. It has been shown that the specific INT-dehydrogenase activity is very highly correlated with the electron transport system that determines catabolic activity of micro-organisms (Trevors 1984; Lopez et al. 1986). The higher catabolic activity implies that the more organic carbon should go to carbon dioxide. In fact, it has been demonstrated that growth yield is a function of the ratio between carbon channeled into carbon dioxide by catabolism and that converted to biomass through anabolism in suspended culture (Liu 1999). Therefore, the observed variations in growth yield and specific INT-dehydrogenase activity in Fig. 2 may represent stimulated catabolic activity compared with anabolism. This, in turn, implies that a discrepancy between the rate of energy production by catabolism and the rate of energy utilization by anabolism would occur at high shear stress. A similar \( Y_{\text{obs}} \) variation pattern with detachment force has also been observed in a steady state biofilm airlift suspension reactor (Kwok et al. 1998). These observations suggest that biofilms may indeed respond to shear stress by regulating metabolic pathways associated with the substrate flux flowing between catabolism and anabolism.

Literature data

Lertpocasombut (1991) studied the kinetics of biofilm developed in a three-phase fluidized bed reactor coupled to a quadrupole mass spectrometer, and determined the growth yield, dissolved oxygen (DO) utilization rate \( r_{\text{O}_2} \) and total organic carbon (TOC) removal rate \( r_{\text{TOC}} \). The effects of the specific velocity of air on the observed growth yield and \( r_{\text{O}_2}/r_{\text{TOC}} \) ratio are shown in Fig. 5 for steady state biofilm. In the study of Lertpocasombut (1991), only the specific velocity of air varied in the experiments, while the carrier concentration and water velocity were kept constant.

In this case, the specific velocity of air can reflect shear stress in the reactor. Similar to Fig. 2, Fig. 5 shows that higher shear stress results in a lower growth yield and a stimulated DO consumption rate. In fact, the biochemical reactions associated with bacterial metabolism result in an approximately linear relationship between oxygen utilization and carbon dioxide production (Selna and Schroeder 1979). In a metabolic sense, the \( r_{\text{O}_2}/r_{\text{TOC}} \) ratio thus represents carbon distribution in the metabolic network of bacteria. The reduced growth efficiency, together with the increased \( r_{\text{O}_2}/r_{\text{TOC}} \) ratio, seems to suggest energy uncoupling between catabolism and anabolism. A direct relationship between \( Y_{\text{obs}} \) and the \( r_{\text{O}_2}/r_{\text{TOC}} \) ratio is further given in Fig. 6. Similar to Fig. 3, it is observed again that \( Y_{\text{obs}} \) is inversely dependent on the catabolic activity of the biofilm. As noted above, oxygen utilization and cell production oppose one another, and the more oxygen used in carbon dioxide production, the fewer cells are produced. Results reported by Trinet et al. (1991) and Rittmann et al. (1992) also showed that the ratio of carbon dioxide-carbon to biofilm-
carbon increased with increasing shear stress in a steady state fluidized bed reactor. On the other hand, Vanderborght and Gilliard (1981) observed that up to 89% of the organic carbon was oxidized to carbon dioxide in the same type of reactor. This evidence from the literature supports a shear stress-induced metabolic shift in biofilm culture.

Shear stress-induced proton translocation: an hypothesis

In the aerobic oxidation process, ATP is generated by oxidative phosphorylation in which process electrons are transported through the electron transport system from an electron donor (substrate) to a final electron acceptor (oxygen) (Mitchell 1961; Wolfe 1993). On the other hand, the respiratory activity of aerobic bacteria couples with proton translocation activity, and a clear linkage of oxygen reduction to proton translocation has been shown (Babcock and Wikstrom 1992). Thus, it appears that the respiratory activity of aerobic bacteria can be indirectly determined by the measurement of proton translocation activity, and the INT can be used as artificial proton acceptor to quantify the catabolic activity of biofilm. In this case, the magnitude of the specific INT-dehydrogenase and oxygen uptake activities can be used to describe the activity of proton translocation across the cell membrane, i.e., the INT-dehydrogenase and oxygen utilization activities would be linked to the activity of proton diffusion across the cell membrane.

As discussed earlier, shear stress-induced energy uncoupling can be seen as the increase in specific INT-dehydrogenase activity and oxygen uptake rate, as shown in Figs 2 and 5. In fact, under the energy uncoupling state of cells, stimulated respiration activity was also observed in suspended microbe cultures (Mayhew and Stephenson 1998).

Russell and Cook (1995) reported that futile cycles of protons would operate whenever there was an imbalance of catabolic and anabolic rates, and would be responsible for most of the observed energy spilling which led to lower growth efficiency. Further, a linear correlation between the rate of energy spilling and flux of protons across the cell membrane was shown (Cook and Russell 1994). Therefore, it appears that the shear stress-induced INT-dehydrogenase and oxygen uptake activities could reflect facilitated proton diffusion across the cell membrane. It has been reported that acidification is a common response to the cells subjected to high shear stress in suspended cell cultures (Meijer et al. 1993; Chen and Huang 2000). In fact, Chen and Huang (2000) found that high shear stress could induce proton elicitation in a suspended Stizolobium hassenso culture.

Effect of shear-induced proton translocation on biofilm structure

It has been reported that proton translocation would have the following consequences in relation to surface characteristics of cells. (i) High proton flux across the cell membrane leading to enhanced hydrophobic interaction of cells (Tay et al. 2000). (ii) Proton conductance across the cell membrane could induce surface dehydration (Babcock and Wikstrom 1992; Deamer and Akeson 1994; Tay et al. 2000). (iii) The proton translocating activity could induce the protonation and membrane fusion of the cell surface (Liu 1989; Babcock and Wikstrom 1992). (iv) The biovolume of cells would be condensed by up to 50% when catabolic activity was stimulated (Zubay 1984; Shen and Wang 1991).

In general, the surface of micro-organisms is negatively charged under normal pH conditions. Obviously, increased proton translocation activity would decrease the net negative charge of the cell surface, and further promote cell–cell interaction. As noted above, one of the most important consequences of enhanced proton translocation is an increase in hydrophobic characteristics of the cell surface. In fact, there is strong evidence that the hydrophobicity of the cell surface is an important affinity force in the self-immobilization and attachment of cells (Marshall and Gruickshank 1973; Pringle and Fletcher 1983; van Loosdrecht et al. 1987). It had been reported that biofilm density is proportionally related to the self-immobilization strength of fixed bacteria, which, in turn, is determined by the shear stress on the biofilm surface (Ohashi and Harada 1994; Chen et al. 1998). Recent research on microbe granulation has shown that the proton translocation-induced dehydration of the cell surface could facilitate and strengthen the cell–cell interaction and further, result in a high density of the microbial community (Tay et al. 2000; Teo et al. 2000). The proton translocation-strengthened structure of the microbe community had also been observed in suspended cultures. Low et al. (2000) reported that in media supplemented with an organic protonophore, para-nitrophenol (pNP), cell agglomerates formed dense flocs compared with pNP-free cultures. This suggests that shear stress-induced proton translocation could facilitate and strengthen the cell–cell interaction and further, result in the high density of adhering cells, as observed in Fig. 4.

It appears from the above discussion that when shear stress on the biofilm is high, the biofilm community would have to regulate its metabolic pathway so as to maintain a balance with the external detachment force through consuming non-growth-associated energy. As Russell and Cook (1995) noted, energy spilling is probably not a fortuitous act, and may be a mechanism for protecting bacteria from stressful environmental conditions and potentially toxic schemes of substrate metabolism.
REFERENCES


