Microbial activity of biofilm during start-up period of anaerobic hybrid reactor at low and high upflow feeding velocity

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Abstract
With an aim to shorten start-up time of an Anaerobic Hybrid Reactor (AHR), initial biofilm development was studied, particularly at different upflow feeding velocities. At a low (0.01 m h⁻¹) upflow velocity, initial biofilm was found to develop via the attachment of suspended biomass in the packed zone, while microbial growth on the film was insignificant. Contrarily, with higher (1.0 m h⁻¹) upflow velocity, initial biofilm development was from both microbial attachment and growth on supporting media. Biofilm thickness was determined using confocal laser scanning microscopy (CLSM), which indicated that the biofilm developed faster with the higher velocity, due to the contribution of the microbial growth on supporting media. When operated beyond the initial biofilm development with the lower velocity, both the activity of acetogens and the methanogens increased, although there was a lower amount of attached biomass on the supporting media. Whereas, both groups were found to decrease with higher upflow velocity, but acidogenic activity increased. It can be concluded that higher upflow velocity positively affected the initial stage of biofilm development and has the potential to accelerate attached biomass on supporting media during the initial phase. Subsequently, the upflow velocity should be reduced to the normal rate to enhance the methanogenic activity.

Keywords Anaerobic hybrid reactor; biofilm; CLSM; microbial activity; start-up; upflow feeding velocity

Introduction
The anaerobic hybrid reactor (AHR) is a high rate anaerobic reactor (HRAR) that combines the advantages of the anaerobic filter (AF) and the upflow anaerobic sludge blanket reactor (UASB). This type of reactor yields a better treatment efficiency compared to the UASB (Elmitwalli et al., 1999). Moreover, the supporting media in the AHR can help to increase the solids retention time by diminishing the short circuiting, improving gas/solids/liquid separation, and providing a surface for the attachment of biomass, resulting in a comparable performance with an AF (Oleszkiewicz and Thadani, 1988; Chung and Choi, 1993; Jahren et al., 1999). Many researchers found that higher amounts of media in the reactor positively affected the performance of the AHR (Oleszkiewicz and Thadani, 1988; Kennedy et al., 1989; Chung and Choi, 1993; Suraruksa et al., 1998). Biofilm systems have several advantages above conventional activated sludge systems due to their ability to support a variety of microbial populations.

In Thailand, this reactor is developed for use in treating wastewater from tapioca factories, which contain high amounts of organic substances and suspended solids. However, a problem occurs when starting the system due to the slow microbial growth on
the supporting media. Many researchers found that most of the substrate was utilized in the sludge zone (Fang and Kwong, 1995; Borja et al., 1995; Suraruksa et al., 1998). Noicleub et al. (1998) reported that the biomass concentration on the supporting material was increased with increasing upflow recirculation velocity, however, the acceleration of the attached biomass growth was not described. Therefore, this research has the purpose to understand the acceleration of attached biomass growth when using high upflow velocity. The basic process of biofilm formation is: microbial cell and organic substance transportation, adsorption of conditioning film, microbial adhesion on surface, maintenance of biofilm, and detachment of biofilm (Characklis and Cooksey, 1983). This work focuses to study the aspects of the initial stage of biofilm development, which concerns the microbial attachment and the microbial growth, assuming that there is no microbial detachment in this stage. The experiment of microbial attachment did not use substrate supplement in order to avoid the effect of microbial growth. As an alternative, glucose was used as a substrate supplement for studying the microbial growth. To monitor the initial biofilm development, confocal laser scanning microscopy (CLSM) was used to determine the structural heterogeneities in the biofilm. Several researchers have used CLSM to analyze the biofilm (Lawrence et al., 1994; De Beer et al., 1994a; Massol-Deya et al., 1994; Murga et al., 1995; De Beer et al., 1997; Lewandowski et al., 1999). The advantages of CLSM are the penetration to the interior of the biofilm, and the ability to image the biofilm in situ and in real time (Stewart et al., 1995). However, the limitation of CLSM is that it is not applicable to thick biofilm because the penetration of the laser light is generally less than 200 µm (Bishop, 1997).

Moreover, the microbial population in the biofilm is the essential parameter to determine the biofilm development at different upflow velocities. Several researchers have studied microbial characteristics and populations in sludge in AHRs (Fang and Kwong, 1995; Chang and Choi, 1993; Imai et al., 1998). Imai et al. (1998) studied the characteristics of sludge granules in the sludge bed zone of AHR. They found that the AHR possessed the features of a two-phase anaerobic process in which the two groups of bacteria were separated in different parts of the reactor. The upper part was the methane forming species; Methanothrix-like bacteria were found. The lower part was the acid forming species; bacilli- and cocci-like bacteria were found. There is, however, less research concerning the microbial population and characteristics of biofilm on supporting media, especially anaerobic biofilm.

Therefore, this research aims to study the quantification, morphology and the microbial population in initial biofilm during the start-up period of an AHR, in order to propose possible conditions to accelerate biofilm development when using different upflow velocities.

**Methods**

**Operation of AHR reactors**

Two acrylic-cylindrical AHR reactors, with 5.55 litres working volume, were used in the study. The upper part of the reactor was packed from middle to top height with nylon fiber as the supporting media with the rest of the reactor acting as a sludge zone. Anaerobic digestion sludge (5.5 g VSS l⁻¹) obtained from a tapioca starch factory was used as a starter. The AHR reactors were continuously fed with synthetic wastewater, which contained 1 g l⁻¹ of glucose as substrate (Smolders et al., 1994). Two AHR reactors were operated at 0.01 and 1.0 m h⁻¹ upflow feeding velocities. The upflow feeding velocity of 0.01 m h⁻¹ was generally used to operate the AHR reactors. An upflow velocity of 1.0 m h⁻¹ was used to transport microbial sludge of 0.1 mm diameter size from the sludge zone to the supporting materials (Noicleub et al., 1998).

Two experiments were separated to study the initial biofilm development when using
0.01 and 1.0 m h\(^{-1}\) upflow feeding velocity: I) Study of microbial attachment – without substrate supplement to avoid the effect of microbial growth, and II) Study of microbial development – with substrate supplement. All the experiments were performed at ambient temperature (35°C).

**Biomass estimation in AHR reactors**

The attached biomass on supporting media was sloughed off by sonication for 10 minutes before quantification. The amounts of suspended and attached biomass in the reactors were determined by measuring the volatile suspended solids (VSS). The biomass was dried at 105°C overnight and then placed in a furnace at 550°C for 3 hours. The VSS was estimated as the difference between the dried and burned masses (APHA, 1995).

**Visual technique for analyzing the biofilm**

The biofilm samples were removed from the reactor and were gently washed with phosphate-buffered saline (PBS). The whole biofilm was then fixed overnight at 4°C with 4% paraformaldehyde in PBS. Subsequently, the fixed biofilm was washed with PBS for 5 minutes, and dehydrated by successive passages through 50, 80, and 100% ethanol (three times) and dried at room temperature. The biofilm structure was made visible using CLSM. Thus, the biofilm was slightly autofluorescent when excited with an argon laser of 488 nm and could be imaged without staining (DeBeer et al., 1997). This technique can measure the thickness of the biofilm on the supporting media as the difference between the mean diameter of the media and bioparticle.

**Microbial population in biofilm**

The acidogens, acetogens, and acetoclastic methanogens are the main groups of microorganisms in anaerobic digestion. The ratio of these groups is the essential factor for monitoring the stabilization of an anaerobic reactor. There are a number of different methods for determining the microbial population in an anaerobic reactor. The specific substrate utilization activity of each group is one of the favorite methods to determine the microbial population. Biofilm samples were taken from the reactor every 6 hours and the activity was tested in 75 ml glass bottles sealed with rubber septums retained with screw caps. Each bottle contained 50 ml of mineral medium (pH 7) and a known amount of sludge VSS. The following substrates were used to determine microbial sludge activities:

- 0.1% (w/v) of glucose for acidogenic activity
- 0.1% (v/v) of propionic acid for acetogenic activity
- 0.1% (v/v) of acetic acid for acetoclastic methanogenic activity

The biomass sample was left overnight to eliminate any remaining substrate before activity testing. After inoculating the biofilm into the specific media, the gas phase of the bottles was flushed with nitrogen gas. The bottles were then placed in a thermostat at 37°C. Samples from each bottle were taken to determine the substrate utilization rate; at intervals of 6 hours for acidogens, and intervals of 24 hours for acetogens and acetoclastic methanogens. Glucose concentration was measured by using a lactate-glucose analyzer (model YSI 2300 STAT PLUS, QUASAR). Propionic and acetic acid concentrations were measured by using a gas chromatographer (model GC 14B, SHIMADZU). All activity tests were carried out in three replicates (Kalyuzhnyi et al., 1996).

**Statistical analysis**

Mean rates of biomass quantification data were compared using a single factor analysis of variance (ANOVA). Significance was determined at the 95% level \((P = 0.05)\). The assessment of biofilm development on supporting media was significantly different to upflow
feeding velocity, wherein the analysis of variance was followed by a t-test (95% confidence limit).

**Results and discussion**

**Quantification of the initial biofilm development on supporting media**

When the low and high flow velocities were operated without substrate, the quantities of biomass on the supporting media in the two reactors were significantly different ($P<0.0003$) (Table 1). The high upflow feeding velocity resulted in a considerably higher biomass in the bulk near the surface of the supporting media than at the low feeding velocity. Table 1 shows the correlation between the attached biomass on the supporting media and the suspended biomass in the bulk at the packed zone ($r^2 = 0.98$). It shows that the suspended biomass in the packed zone related to the attached biomass on the supporting media at both upflow feeding velocities. This result related to the study of Shapiro and Switzenbaum (1984) that showed that the biomass concentration in the bulk liquid could be a very important parameter affecting initial anaerobic biofilm development. This experiment was run without carbon substrate (assuming no growth), therefore, this correlation can be used to represent the attachment of suspended biomass to supporting media. From these results it can be concluded that the upflow velocity greatly affected the microbial transportation from the suspended zone to the packed zone, resulting in high microbial attachment on the supporting media at high upflow velocity.

When supplemented with glucose as a substrate, the attached biomass was increased at both upflow feeding velocities (Table 1). The glucose concentration was required to activate the metabolism (Reynolds and Fink, 2001). Moreover, glucose is a substrate that is easy to use for microbial growth. Therefore there was a greater microbial development on the supporting media than with no glucose supplement. At low upflow velocity, most substrate was utilized in the sludge zone, and therefore, glucose in the packed zone was not found. However, a glucose concentration was detected in the packed zone at high upflow velocity (data not shown). Higher biomass development, therefore, was found at 1.0 m h$^{-1}$ upflow feeding velocity ($P<0.003$) due to sufficient substrate.

By operating for a long time, the correlation between the suspended biomass in the packed zone and biomass development on supporting media was different at the low and the high upflow velocity (Table 1). The patterns of initial biofilm development on supporting media in the low and the high upflow velocity were also different. At low upflow velocity, the initial biofilm development depended on the microbial attachment of suspended biomass in the packed zone ($r^2 = 0.98$) and no microbial growth on the supporting media due to deficiency of glucose in this zone. Contrarily, initial biofilm development at high upflow velocity depended on microbial attachment during 24 hours of operational time. After biomass had attached onto the supporting media, and glucose was available in this zone, biofilm development did not depend on the suspended biomass in the bulk of the

<table>
<thead>
<tr>
<th>Upflow velocity (m h$^{-1}$)</th>
<th>Operational time (hours)</th>
<th>Without substrate supplement</th>
<th>With substrate supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Microbial development$^a$</td>
<td>Correlation$^b$</td>
</tr>
<tr>
<td>0.01 (low)</td>
<td>24</td>
<td>0.202 ± 0.013</td>
<td>0.9849</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.341 ± 0.023</td>
<td>0.9912</td>
</tr>
<tr>
<td>1.0 (high)</td>
<td>24</td>
<td>0.524 ± 0.023</td>
<td>0.9889</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.618 ± 0.036</td>
<td>0.9760</td>
</tr>
</tbody>
</table>

$^a$ Results expressed as means ± standard deviation

$^b$ Correlation between suspended biomass in bulk at packed zone and attached biomass on supporting media
packed zone \( r^2 = 0.18 \), and there was also microbial growth on supporting media. The yield of microbial growth on the supporting media was 0.024 g VSS g COD\(_{re}^{-1}\) at high upflow velocity. The low microbial growth yield might be due to the short contact time between the microorganisms and substrate. The overall results implied that the initial biofilm development at low feeding velocity relied mainly on the attachment of biomass from the sludge zone with no growth involved, whereas at high feeding upflow velocity there was microbial attachment and growth on supporting media.

**Morphology of initial biofilm on supporting media**

The morphology and structure of biofilm at both velocities were determined by using confocal laser scanning microscopy (CLSM). Figure 1 shows the non-uniformity and roughness of the biofilm structure on the supporting media. Moreover, a thicker biofilm was developed at high feeding velocity when compared with low feeding velocity. After 72 operational hours, the biofilm thickness was 13.33 ± 0.25 and 91.91 ± 7.40 µm at 0.01 and 1.0 m .h\(^{-1}\) upflow feeding velocity, respectively. This result supports the data of the quantity of microbial attachment on the supporting media in Table 1. It can be concluded that the upflow feeding velocity positively affected the biofilm development on the supporting media.

The high upflow velocity, moreover, resulted in a rougher biofilm than low upflow velocity. Peyton (1996) noted that the rougher biofilm would likely have higher mass transfer coefficients than the smoother biofilm which would result in a higher growth rate for a given bulk-liquid substrate concentration. Therefore thicker biofilm was found at the high upflow velocity. In addition, biofilm thickness does not only show heterogeneous morphology, but more mixed microbial populations in the biofilm.

**Microbial population in the initial biofilm on supporting media**

For a more in-depth understanding of the biofilm development during the start-up period of an AHR at low and high upflow feeding velocities, the microbial population in the initial biofilm was determined. Thus, microbial groups were separated into three groups; i.e. acidogens, acetogens, and acetoclastic methanogens. These microbial groups are different mainly with respect to their nutritional requirements, physiology, and pH requirements. This study would like to estimate the microbial population by determining the activity of substrate utilization of each microbial group.

Figure 2 shows the microbial activity in each group at 0.01 m .h\(^{-1}\) upflow feeding velocity. The result shows that although the quantity of attached biomass was increased, the acidogenic activity slightly steadied after 60 operational hours. The acetogenic and methanogenic activities continuously increased when operated for a long time. Figure 3

![Figure 1](https://iwaponline.com/wst/article-pdf/48/8/79/423811/79.pdf)
shows the results of microbial activity in each group at 1.0 m·h⁻¹ upflow velocity. The results found that acidogenic activity increased together with the quantity of attached biomass. Contrarily, acetogenic and methanogenic activities decreased when operating longer than 30 hours.

Araki and Harada (1994) introduced the Enrichment Index (EI) of trophic microbial groups to describe the ability of microbial activity enrichment in biofilm. This work modified the EI as the ratio of microbial activity of biofilm and of suspended biomass in the bulk of the packed zone. A high EI represented the high ability of microbial activity enrichment in biofilm. Table 2 shows the EI of three trophic microbial groups. It is shown that the EI of acetogens and methanogens at low upflow velocity was increased, whereas the EI of acidogens was slightly decreased when operated for a long time. At the high upflow velocity, only the EI of acidogens was increased.

These results can be used to explain the microbial population in the biofilm on supporting media at the low and the high upflow feeding velocities. At low upflow velocity, the activity of acetogens and methanogens was higher than that of acidogens for a long operational time. Imai et al. (1998) noted that the main groups of floating biomass were acetogenic and methanogenic bacteria, which resulted in increasing activity of acetogens and methanogens in the biofilm. These results indicate that the quantity of attached biomass at low upflow velocity depended on the attachment of the microorganisms that had grown in the sludge zone. Moreover, this study found that most glucose was utilized in the sludge zone and synthesized volatile fatty acids in the packed zone. Thus, volatile fatty acids were detected as 3 mM at the middle height of the reactor. Therefore, there were substrates avail-
able for acetogenic and methanogenic bacteria in the packed zone resulting in the high EI of these groups. At the high upflow velocity, the microbial population during the initial stage of the biofilm development was similar to that found at the lower velocity. After 30 operational hours, the suspended biomass was washed out from the reactor (data not shown). The activity of acetogens and methanogens were decreased whereas the acidogenic activity was increased. When determining the glucose concentration, a high level of glucose was found remaining in the packed zone. As a result, acidogens on the supporting media could grow by utilizing glucose as a substrate. Nevertheless, the high feeding velocity resulted in a short contact time between substrate and microorganisms. Wanner and Gujer (1984) reported that the fast growing microorganisms tended to predominate near the surface, while slow growers were deeper inside the biofilm, scavenging substrate from the fast growers. Hence acidogens were near the surface of the biofilm. Therefore the activity of acidogens in the biofilm at 1.0 m h⁻¹ upflow velocity was highest among the others, resulting in a high EI of acidogens.

These results supported the above conclusions that the biofilm development at low upflow velocity was influenced by the attachment of microorganisms, which had grown in the sludge zone. At high upflow velocity, the biofilm development depended on the microbial attachment on the supporting media in the early stage, and later microbial growth on the supporting media was the dominant effect.

To compare the specific activity in each microbial group; i.e. acidogens, acetogens, and methanogens, the specific microbial activity at 0.01 m h⁻¹ upflow velocity was determined. It was found that the specific activity in this study was lower than the references (Table 3).

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![Figure 3](https://iwaponline.com/wst/article-pdf/48/8/79/423811/79.pdf)  
Figure 3 The quantity of attached biomass and the activity of acidogens, acetogens, and methanogens during the start-up period at 1.0 m h⁻¹ upflow feeding velocity
This result can be explained by the fact that the previous studies used pure culture or enriched culture whereas this study used a mixed culture. Therefore the specific microbial activity was found to be lower.

**Conclusions**

The results of this work show that the initial biofilm development at the low upflow feeding velocity is dependent on microbial attachment, whereas at the high velocity it is dependent on microbial attachment and growth. From these overall results it can be concluded that the high velocity positively affects the initial stage of the biofilm development due to sufficient substrate for microbial growth. Therefore the thicker biofilm is found at 1.0 m h\(^{-1}\) upflow velocity. Contrarily, the low feeding velocity leads to high methanogenic activity, although attached biomass on supporting media is low. These results suggest that the initial stage of start-up of an AHR should be operated at high upflow velocity in order to transport biomass and substrate to the supporting media. When biomass has been attached onto the supporting media, the upflow velocity should, subsequently, be reduced to increase the contact time between the substrate and microorganisms. The methanogenic population, moreover, will be enhanced. This strategy recommends reducing the start-up period and increasing stabilization of the AHR at long operational times.

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**References**


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**Table 2** Enrichment Index of trophic microbial groups in AHR

<table>
<thead>
<tr>
<th>Microbial groups</th>
<th>At 0.01 m h(^{-1}) upflow velocity</th>
<th>At 1.0 m h(^{-1}) upflow velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
<td>48 hours</td>
</tr>
<tr>
<td>Acidogens</td>
<td>0.63</td>
<td>0.50</td>
</tr>
<tr>
<td>Acetogens</td>
<td>0.73</td>
<td>1.10</td>
</tr>
<tr>
<td>Acetoclastic methanogens</td>
<td>0.53</td>
<td>0.81</td>
</tr>
</tbody>
</table>

**Table 3** The specific activities of anaerobic trophic groups

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Substrate</th>
<th>Maximum specific activity (a) (g COD g VSS(^{-1}) D(^{-1}))</th>
<th>Specific activity (b) (g COD g VSS(^{-1}) D(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidogens</td>
<td>Glucose</td>
<td>77</td>
<td>17.01</td>
</tr>
<tr>
<td>Acetogens</td>
<td>Propionic acid</td>
<td>7.1</td>
<td>4.01</td>
</tr>
<tr>
<td>Acetoclastic methanogens</td>
<td>Acetic acid</td>
<td>2.45</td>
<td>1.72</td>
</tr>
</tbody>
</table>

\(a\) Reference from Araki and Harada (1994)

\(b\) Data obtained from the 72nd operational hour of the experiment at 0.01 m h\(^{-1}\) upflow feeding velocity


