Microbial composition assessment of anaerobic biomass through methanogenic activity tests

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Abstract

Maintenance of sufficient methanogenic populations is critical for stable performance of anaerobic systems. The usually monitored parameters like COD removal, VFA levels, quantity and composition of biogas produced etc., do not reflect the composition of biomass under varied operational/environmental conditions. The change in biomass composition in terms of relative population levels of methanogens has been indirectly assessed through methanogenic activity tests using two different substrates at equivalent COD load. The results of activity tests have been well correlated with the performance results of the bench-scale upflow anaerobic filter. This clearly suggests the use of the methanogenic activity test to monitor biomass composition along with usually monitored parameters for obtaining a better insight into the reactor stability and performance.

Abbreviations

AMA	acetoclastic methanogenic activity
AMGPR	average methane gas production rate
BA	bicarbonate alkalinity
EBHRT	empty bed hydraulic retention time
F	final
I	initial
HOM	hydrogen oxidising methanogens
NHOM	non-hydrogen oxidising methanogens
OLR	organic loading rate
SMA	specific methanogenic activity
TMA	total methanogenic activity
UAF	upflow anaerobic filter
VFA	volatile fatty acids
VSS	volatile suspended solids

Introduction

Several anaerobic process variants having specific biomass retention mechanisms are available for field application. Laboratory-, pilot- and full-scale studies have made varied claims regarding applicability and performance of these process variants (Henze and Harremoes, 1983; Stronach et al., 1986; Hickey and Goodwin, 1989; 1991; Lettinga et al., 1980; Pol and Lettinga, 1986). Maintenance of sufficient methanogenic populations in the system is critical for stable performance. Methanogenic species types and their relative population levels in reactor biomass depend on wastewater characteristics as well as operational/environmental conditions maintained (Novaes, 1986). Any imposed stress (intentional or otherwise) may lead to a change in species types and their relative population levels which is ultimately reflected in the reactor performance (Harper and Pohland, 1986). The reactor performance is usually evaluated in terms of process efficiency and stability through estimation of organic matter removal, VFA levels, quantity and composition of biogas produced, etc. However, little effort has been made to assess reactor biomass in terms of relative population levels of methanogenic species under varied operational/environmental conditions.

Counts of methanogens and non-methanogens in reactor biomass have been made by several investigators (Kotze et al., 1969; Hobson and Shaw, 1974; Zeikus, 1980; Gregori et al., 1979; Novaes et al., 1984; Agrawal et al., 1997). These efforts led to the development of well-established laboratory techniques (Ranade and Gadre, 1988). However, these techniques require a high level of skill, advanced equipment, and costly and specific growth media which restrict its application at the plant site. SMA tests on anaerobic sludges (biomass) have been gaining importance. Initially, these tests were mainly used to select an adapted sludge as inoculum (James et al., 1990) but now these tests can also be used for many other purposes such as to:

- Evaluate the behaviour of sludge under the effect of potentially inhibitory compounds (Harada et al., 1994; Perle et al., 1995)
- Establish the degree of degradability of various substances (Stewart et al., 1995)
- Follow the changes in sludge activities due to a possible build-up of inert materials
- Estimate maximum applicable loading rate to a certain sludge (Ince et al., 1995)
- Evaluate batch kinetic parameters, etc.

A number of methods have been proposed for the estimation of maximum methanogenic activity. The summary of experimental conditions is presented in Table 1. Some of these methods are quite simple (Valcke and Verstraete, 1983; De Jong, 1986; Field et al., 1988; Soto et al., 1993) but the sample volume needed is too high (500 mℓ or larger). Several solutions were proposed to reduce the working volume and to automate the monitoring process (Owen et al., 1979; Shelton and Tiedje, 1984; Dolfing, 1985; Bonastre et al., 1987; Concannon et al., 1988; James et al., 1990; Grotenhuis et al., 1991; Rintala and Lepisto, 1992; Soto et al., 1993). Very small working volumes (30 to 125 mℓ) lead to smaller amounts of

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TABLE 1
SUMMARY OF EXPERIMENTAL CONDITIONS FOR MAXIMUM METHANOGENIC ACTIVITY ESTIMATION

Volume (useful volume) mℓ	Inoculum size g VSS/ℓ	Substrate type (quantity, g/ℓ)	Monitoring method/ technique	Reference
50 (30)	10% vol.	1 (2.7)	S, M	Van den Berg (1977)
125 (100)	10% vol.	1, 2	SD	Owen et al. (1979)
2 000 (1 000)	5	1 (variable)	AMF	Valcke and Verstraete (1983)
160 (125)	10% vol.	(0.05 g TOC/ℓ)	PT+M	Shelton and Tiedje (1984)
130 (50)	0.8-8	1, 1+2, H_2/CO_2	PLS (M)	Dolfing (1985)
---- (5 000)	1.5-3	1+2+3 (0.6 each)	AMF	De Jong (1986)
120 (50)	100% vol.	1 (1.5)+2 (0.5)	PT	Bonastre et al. (1987)
20 (10)	3-4	1, 2 (1.85)	PT	Concannon et al. (1988)
1 160 (500)	0.27-5.12	1 (0.12-0.6)	PLS (M)	Rinzema (1988)
500-1 500 (>90%)	1-5	1+2+3 (0.6 each)	AMF	Field et al. (1988)
500 (450)	<1	1 (3-4)	MM	Chiang and Dague (1989)
45 (35)	2.5	1 (0.6-5)	WR	James et al. (1990)
160 (80)	0.2-0.7 g	1, 2 (1.25-4.5 g COD/ℓ)	1, 2	Grotenhuis et al. (1991)
118 (65)	10-15 mℓ	VFA (1.85 g COD/ℓ)	SD+M	Rintala and Lepisto (1992)
600 (500)	1-2	1 (2.5 g COD/ℓ)	AMF	Isa et al. (1993)
600 (500)	1.4-1.6	VFA (3 g COD/ℓ)	AMF	Alphenaar et al. (1993)
2 000 (1 500)	1.26	1 (2)+2 (0.5)+3 (0.5)	ASD+M	Soto et al. (1993)
126 (100)	1.26	1 (2)+2 (0.5)+3 (0.5)	AMF	Soto et al. (1993)
126 (50)	1.75	1 (2)	AMF	Soto et al. (1993)

AMF = alkaline mariotte flask; ASD = acid solution displacement; M = % methane
MM = manometric measure; PLS = pressure lock syringe; PT = Pressure transducer
S = substrate; SD = syringe displacement; WR = Warburg respirometer
1 = HAc (acetic acid); 2 = HPr (propionic acid); 3 = HnBu (n-butyric acid)

methane gas production. It necessitates careful measurements of methane gas with sophisticated techniques and hence its application at the plant site becomes even more restricted.

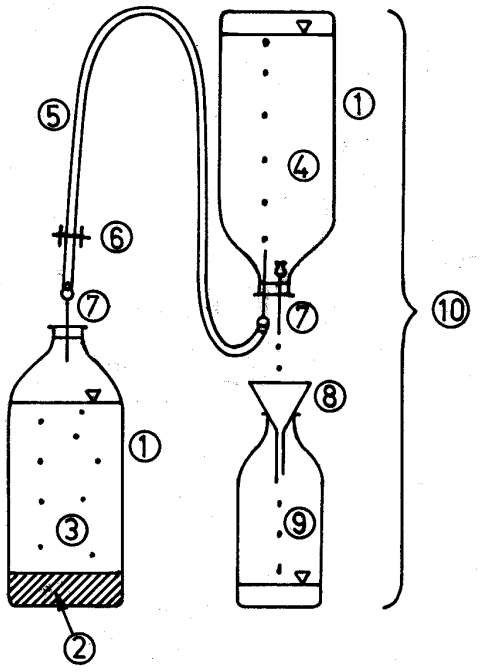
Dolfing and Bloemen (1985) proposed activity measurements as a tool to monitor the microbial composition of methanogenic environments using H_2, formate, acetate and propionate as test substrates. The anaerobic biomass was obtained from a digester and maintained for three months on synthetic growth substrates comprising a mixture of acetate and propionate (50% each on COD basis) and mainly sucrose (95% on COD basis and the rest 5% on COD basis augmented by acetate and propionate). Dolfing and Bloemen (1985) observed 30 to 70% reduction in methanogenic activity of biomass maintained mainly on sucrose compared to that maintained on acetate and propionate. It is not clear from this study whether these reductions were due to experimental limitations (such as lack of acclimatisation of test biomass, short monitoring period for methane production, etc.) or otherwise.

This paper presents the application of a simple methanogenic activity test procedure to monitor reactor biomass in terms of relative population levels of methanogenic species by using two different test substrates. The results so obtained are correlated with the performance of a laboratory-scale reactor.

Experimental

A simple methanogenic activity test procedure as proposed by Isa et al. (1993) was adopted with suitable modifications to suit the requirements of this study. The experimental set-up is shown in Fig. 1. A known amount of sludge (VSS ≈ 1 to 2 g/ℓ) was transferred into a 500 mℓ serum bottle. Tap water (purged of oxygen with nitrogen gas) was added up to the 500 mℓ mark. An appropriate quantity of substrate was added to the serum bottle so as to obtain initial COD levels in the range of 2 to 2.5 g/ℓ. Nutrients were not added with an aim to restrict growth of biomass during the test period (Dolfing and Bloemen, 1985; Soto et al., 1993). Methane gas production was measured by means of the liquid displacement method at a shorter time interval (0.5 to 2 h) in the first 12 h and at longer time intervals (4 h or more) afterwards up to 48 h of feeding. Contents of the serum bottle were mixed by swirling manually after every gas measurement. When gas production for the first feeding had been recorded, supernatant of the serum bottle was decanted. Tap water (purged of oxygen with nitrogen gas) was immediately poured into the bottle again and the volume was again made up to the 500 mℓ mark. The same quantity of substrate was fed as in the first feeding and the bottle was capped and connected to a liquid displacement system. Gas production was recorded. This constituted the second feeding. Likewise the procedure was repeated for the third feeding as suggested (De Zeeuw, 1984; Alphenaar et al., 1993; Soto et al., 1993; Isa et al., 1993). The entire test was conducted at 35±1°C in a temperature-controlled cabin. On completion of the third feeding, the amount of sludge (VSS) remaining in the serum bottle was determined. This VSS and slope of the linear portion of cumulative methane production rate in the third feeding were used to calculate the methanogenic activity. COD and VSS were determined as per *Standard Methods* (1989).

The test sludge (biomass) for this study was obtained from a bench-scale model of the UAF. The UAF was developed and maintained on synthetic feed at 35±1°C and operated at three distinct combinations of EBHRT and influent COD concentration keeping a fixed OLR ≈ 5 kg COD/m^3·d. The test sludge was



1. Serum Bottle
2. Test Biomass
3. Reaction Mixture
4. CO_2 Scrubber (11.2% w/v KOH Solution + Thymol Blue Indicator)
5. Rubber Tubing
6. Pinch-Cock
7. Hypodermic Needle
8. Conical Funnel
9. Displaced Liquid
10. Liquid Displacement System

Figure 1
Schematics of methanogenic activity test set-up

TABLE 2
CHARACTERISTICS OF JAGGERY
(SOURCE: GEHLAWAT, 1996)

Component	Per 100 g jaggery
Sucrose (g)	65-85
Reducing sugar (g)	5-15
Protein (g)	0.4
Fat (g)	0.1
Calcium (mg)	8
Phosphorus (mg)	3-4
Total mineral (g)	0.6-1
Moisture content (g)	3-8
Carotene (Vitamin A, µg)	280
Nicotinic acid (µg)	1
Thiamine (Vitamin B, µg)	20
Colour	Golden yellow to brown
Energy (kcal)	383

withdrawn from the feed inlet point under hydraulic pressure. The synthetic feed was prepared from jaggery, known as Indian sugar or gur, which is a simple energy-rich substrate. The characteristics of jaggery are presented in Table 2. Further details are available elsewhere (Jawed, 1996).

The activity tests were run in two similar set-ups using two different substrates. In one set-up, acetic acid neutralised with approximately 6N NaOH (neutralised acetic acid) was used as substrate for the estimation of AMA whereas jaggery (substrate on which test biomass was grown and maintained in the UAF) was used as substrate for TMA estimation in the other set-up. One gram $NaHCO_3$ was also added along with jaggery to buffer the serum bottle contents to near neutral pH conditions during the TMA test.

Results and discussion

Test procedure

In order to monitor biomass composition of anaerobic sludge through the methanogenic activity test, the methane production potential of the test biomass is measured under unlimited substrate and optimal environmental conditions. There are essentially two classes of methanogens namely HOMs and NHOMs. The latter (NHOMs) are substrate-specific and cleave the acetic acid molecule to produce methane. As such, the methanogenic activity test with neutralised acetic acid or acetate as sole substrate reflects activity of NHOMs, also known as acetoclastic methanogens, and it has been referred to as the AMA test. Jaggery, which was the main source of carbon for the test biomass in the UAF, was also used as a substrate in a separate test. In this, both HOMs and NHOMs contribute to methane production and therefore it is referred to as the TMA test.

In an effort to monitor reactor biomass in terms of relative population levels of HOM and NHOM, AMA and TMA tests were carried out on sludge (biomass) samples withdrawn from the UAF operated using three distinct combinations of EBHRT and influent COD concentration and grouped as Region I : Influent COD ≈ 5 g/ℓ, EBHRT ≈ 1 d; Region II : Influent COD ≈10 g/ℓ, EBHRT ≈2 d; and Region III : Influent COD ≈20 g/ℓ, EBHRT ≈ 4 d. The AMA and TMA test results for the sludge biomass withdrawn towards the end of each operating region are presented in Fig. 2a and 2b respectively. It can be observed from AMA tests that a very small amount of methane production takes place in the first 20 h of feeding whereas in the case of TMA tests, almost 50% of cumulative methane production takes place in the first 15 h out of 48 h of monitoring after feeding. Instantaneous addition of acetic acid or acetate as substrate in the AMA test, as against formation of the same by acid formers from the substrate, jaggery, in the TMA test, may retard substrate utilisation and hence, the methane production rate by acetoclastic methanogens. Aguilar et al. (1995) have also reported that sludge acclimatised to the presence of VFA arising from substrate degradation could use them better than un-acclimatised sludge. In the present study, even after acclimatising the test biomass by three feedings of neutralised acetic acid, the methane production rate did not improve during the first 15 h of feeding. This may be the reason for the observed reduction in activity values by Dolfing and Bloemen (1985) in the case of test biomass maintained and grown on mainly sucrose substrate.

Direct addition of jaggery to serum bottles in the TMA test leads to complete conversion to acetate, CO_2 and H_2 within the first 2 h with a large and rapid increase in concentration of acetate and H_2 (Mosey and Fernandes, 1989). Availability of H_2/CO_2 (substrate for HOM) and acetate (substrate for NHOM), both HOMs and NHOMs contribute to methane production during the initial period

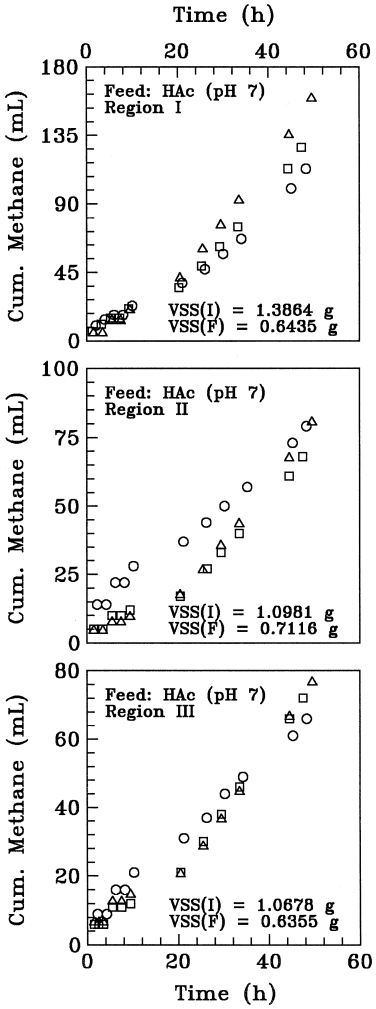


Figure 2a
AMA test results for test biomass maintained at different operating conditions.
VSS(I): Volatile suspended solids (initial)
VSS(F): Volatile suspended solids (final)
Legend: ○ *First feeding*
□ *Second feeding*
△ *Third feeding*

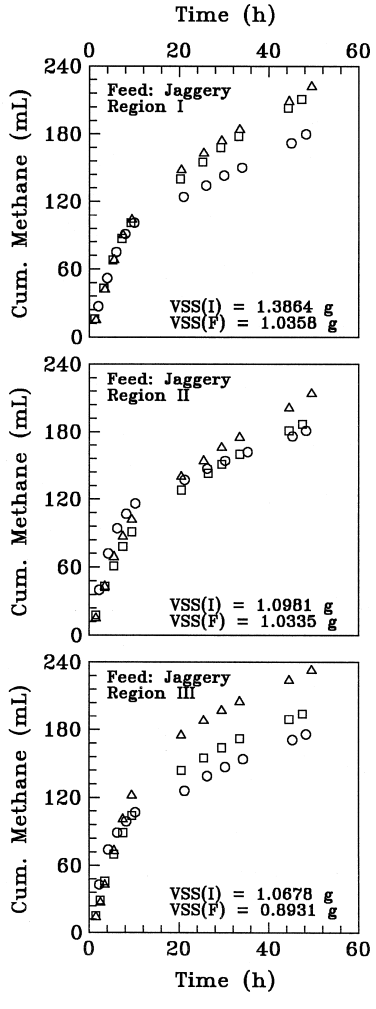


Figure 2b
TMA test results for test biomass maintained at different operating conditions.
VSS(I): Volatile suspended solids (initial)
VSS(F): Volatile suspended solids (final)
Legend: ○ *First feeding*
□ *Second feeding*
△ *Third feeding*

in the TMA test, while in the latter period, essentially NHOMs are expected to contribute to methane production depending upon acetate availability. However, methane production in the AMA test is essentially by NHOMs as no substrate is available for HOMs. As such, it can be stated that the specific methanogenic activity computed from the initial methane production rate in the TMA test reflects the methane production potential of both HOM and NHOM. Hence, TMA is reported on the basis of the initial methane production rate while AMA is reported on the basis of maximum methane production rate after the initial lag phase.

Activity values

The summarised results of AMA and TMA tests are presented in Table 3. The maximum activity for pure or enriched methanogenic cultures is ≈10 g COD/g VSS·d (Harper and Pohland, 1986), while observed activity in industrial and laboratory digesters ranges between 0.1 and 1.0 g COD/g VSS·d (Dolfing and Bloemen, 1985; Field et al., 1988; Guiot, 1991; Soto et al., 1992; Isa et al., 1993).

The estimated values of AMA and TMA in the present case are within the reported range. An analysis of TMA and AMA values for the test sludges reveals that the difference in TMA and AMA values has increased while the ratio of AMA to TMA has decreased with an increase in EBHRT. This indicates an increase in relative population levels of HOM in comparison to NHOM. TMA values of test sludges have remained almost same in Region I and II, while AMA values have decreased in Region II in comparison to that in Region I. Again, AMA values are almost the same in Regions II and III, while TMA values increased in Region III. This certainly signifies the effect of changed operational conditions on the test biomass composition under a fixed OLR. As EBHRT was changed from 1 to 2 d and influent COD levels increased from ≈5 (Region I) to ≈10 g/ℓ (Region II), there seems to be a shift in relative population levels of HOM and NHOM to ward off the stress caused by the changed operational conditions (Harper and Pohland, 1986). The population level of NHOM may have decreased in Region II as compared to Region I since AMA values have decreased. This also suggests that HOM population levels would have increased as the total methanogenic population level, as indicated by almost the same values of TMA in Regions I and II, remains unchanged. There is a net increase in population levels of HOM while maintaining lower population levels of NHOM in Region II. This change in operational conditions between Region I and Region II has resulted in a shift in population level of HOM and NHOM while almost maintaining total methanogenic population levels. Further increases in EBHRT (from 2 to 4 d) and influent COD (from ≈10 to ≈ 20 g/ℓ) in Region III while keeping a fixed OLR has encouraged maintenance of larger population levels of HOM. Grotenhuis et al. (1991) also observed that methanogenic activity of granules increased steadily with increasing influent substrate concentration attributed to an increased fraction of viable organisms in the more heavily loaded granules. This situation may have contributed to more methane production via utilisation of H_2/CO_2 substrate by HOM. This change in biomass composition might have improved filter performance at increased EBHRT at a fixed OLR.

TABLE 3
TMA AND AMA VALUES FOR TEST SLUDGE

Activity (g CH_4-COD/g VSS·d)					
Region I		Region II		Region III	
TMA	AMA	TMA	AMA	TMA	AMA
0.635	0.359	0.604	0.175	0.887	0.176

TABLE 4
SUMMARISED PERFORMANCE RESULTS OF UAF REACTOR

Parameters		Region I	Region II	Region III
Monitoring period (d)		21	18	37
EBHRT (d)		1.06 ± 0.12 (21)	1.94 ± 0.18 (18)	4.19 ± 1.22 (37)
Influent COD (g/ℓ)	Soluble COD	4.64 ± 0.68 (16)	9.07 ± 1.14 (14)	19.21 ± 2.78 (20)
	Total COD	4.82 ± 0.36 (16)	9.50 ± 0.66 (14)	20.13 ± 2.54 (20)
OLR (kg COD/m^3·d)	Soluble COD	4.47 ± 0.60 (09)	4.72 ± 0.56 (08)	4.84 ± 1.48 (11)
	Total COD	4.66 ± 0.72 (09)	4.95 ± 0.52 (08)	5.06 ± 1.44 (11)
VFA leakage (g/d·ℓ as HAc)		1.17 ± 0.14 (08)	1.04 ± 0.18 (07)	0.86 ± 0.56 (10)
BA utilised (g/d·ℓ as $CaCO_3$)		0.40 ± 0.42 (08)	0.25 ± 0.28 (07)	0.19 ± 0.32 (10)
AMGPR (kg CH_4-COD/m^3·d)		2.746 ± 0.286 (21)	3.251 ± 0.294 (18)	3.845 ± 0.740 (38)
COD removal (%)	Soluble COD	64.75 ± 3.24 (08)	71.47 ± 2.42 (07)	79.37 ± 10.44 (10)
	Total COD	61.86 ± 4.88 (08)	66.85 ± 3.18 (07)	73.02 ± 9.44 (10)
COD methanisation (#)		0.909 ± 0.054 (08)	0.944 ± 0.122 (07)	1.019 ± 0.240 (10)

= g CH_4-COD produced per g COD destroyed;
( ) = indicates number of data points;
± = values define 95.4% confidence interval range assuming normal distribution.

Filter performance correlation with activity results

The summarised performance results of UAF grouped in three regions are presented in Table 4. The details are available elsewhere (Jawed, 1996). Results indicate that performance of the filter changed significantly with increasing EBHRT even though OLR was fairly constant. The average COD removal increased from 62% at lower EBHRT (1 d) to 73% at higher EBHRT (4 d). The AMGPR increased with an increase in EBHRT which is in agreement with the overall increase in total methanogenic population as obtained from TMA tests. Similar trends for biogas production in a fixed film reactor were observed by Liu et al. (1995) in which EBHRT was decreased from 72 to 6 h at a constant OLR (≈5 kg COD/m^3·d). Also, COD equivalent of methane gas produced increased with an increase in EBHRT and was significantly higher than COD removed as calculated from influent and effluent COD values. This indicates that with increase in EBHRT, biomass yield decreased. The BA utilisation to maintain reactor pH ≈7 decreased significantly with an increase in EBHRT. The leakage of VFA in effluent is more at lower EBHRT and decreased significantly at higher EBHRT which is in agreement with decreased population levels of acetoclastic methanogens as obtained from AMA tests.

Conclusions

The anaerobic biomass composition may be assessed using a simple methanogenic activity test procedure selecting acetate as one test substrate while the other substrate should be one on which the biomass is being maintained. The activities so obtained correlate well with the reactor performance and clearly demonstrate the change in relative population levels of methanogenic species (mainly HOM and NHOM) with changing operational conditions. Therefore, the activity test can be used to monitor the biomass composition along with usual reactor performance evaluation parameters for giving a better insight into the reactor stability and performance.

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