Biodegradation (2010) 21:411–424
DOI 10.1007/s10532-009-9311-x

ORIGINAL PAPER

Influence of incubation conditions on the specific methanogenic activity test

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Received: 17 June 2009 / Accepted: 26 October 2009 / Published online: 10 November 2009

Abstract The main objective of this work was to evaluate the influence of incubating conditions on the specific methanogenic activity (SMA) test in order to harmonize the test protocol. For this serum bottles were incubated with anaerobic sludge (from UASB reactor treating domestic sewage) in factorial planned experiments which assessed the influence of the temperature, substrate concentration, food/microorganism (*F*/*M*) ratio, presence of yeast extract in the medium, as well as type of carbon and nutrient solution. The results showed that the tested methane measuring methods (volumetric with biogas characterization, volumetric with gas wash in alkaline solution, and manometric by using the OxiTop® system) presented a similar performance. The maintenance of a small gaseous phase volume (e.g. 10% of the total volume) resulted in higher SMA values; and the ideal substrate concentration for the SMA test ranged from 0.5 to 3.0 gCOD/l since higher acetate concentration caused sludge inhibition. The suggested temperature for the test is 35°C and the best *F*/*M* ratio varied from 0.125 to 0.750 gCOD/gVS, and this seemed to be the most influent parameter for the SMA test. Finally tests performed with nutrient solution complemented by yeast extract resulted in the highest SMA values.

Keywords Specific methanogenic activity (SMA) · Anaerobic digestion · Reactor control operation · Wastewater treatment

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Introduction

The anaerobic digestion is characterized by a sequence of metabolic processes carried out by specific microorganisms. The complex organic compounds are firstly hydrolyzed by fermentative bacteria, and the by-products from this process (sugars, amino acids, monoglycerides, long-chain fatty acids, among others) are turned into smaller organic acids (or volatile fatty acids) by acidogenic microorganisms. The products from acidogenesis (for instance, propionic and butyric acids) can be converted into acetate and hydrogen by the acetogenic population, which has the role of converting longer chain organic

acids into substrates for the methanogenesis step. The last biological phase of the anaerobic conversion is performed by methanogenic archaea, which transform acetate, hydrogen and carbon dioxide, formate and other one-carbon compounds into methane, which escapes to the gaseous phase (Aquino and Chernicharo 2005).

In this complex interaction amongst the anaerobic microorganisms, the methanogenic community is usually regarded as the most sensitive to the environmental and operational conditions undergone by the treatment system (Silveira et al. 2000). So, monitoring the methane-producing microorganisms is important to determine the capability of anaerobic biomass in treating certain types of effluents or wastes; and the measurement of the maximum methane production rate has been an useful tool for this. Such quantification is normally assessed by the well known specific methanogenic activity (SMA) test, which evaluates the anaerobic sludge capability to convert an organic substrate into methane, which escapes easily to the gas phase carrying with it reducing equivalents that cause chemical (COD) or biochemical oxygen demand (BOD) in the liquid phase.

The SMA indicates the efficiency of the anaerobic treatment because it measures the velocity of the methanogenic phase (Dolfing and Bloemen 1985; Valcke and Verstraete 1983) and, altogether with the quantification of the active biomass, it also evaluates the reactor methanogenic capacity. Indeed, the methanogenic activity is used to calculate the maximum methanogenic potential, which represents the maximum capacity of a reactor, operating under ideal conditions, to convert volatile fatty acids into methane (Leitão 2004; Monteggia 1997).

Besides being a parameter for evaluation of the efficiency of the treatment, the SMA is also used to evaluate the sludge activity during different operational steps of an anaerobic system. The seed sludge activity is an important parameter to assess the evolution of the anaerobic reactor start-up (Muxi et al. 1991), besides determining the maximum organic load to be applied to a reactor during any operational phase of the treatment system (Alves et al. 2005; Aquino et al. 2007; Chernicharo 1997; James et al. 1990; Jawed and Tare 1999; Soto et al. 1993). In addition, during the disposal of the exceeding sludge from a reactor, the SMA can be used to define the minimum sludge mass to be maintained in the reactor, taking into account that, regarding anaerobic reactors treating domestic sewage, sludge disposal is usually performed without discretion, and the sludge volume sent to the dehydration step is established by experience in the routine operation of wastewater treatment plants (Aquino et al. 2007).

Monitoring the methanogenic activity is also important to evaluate the behavior of the sludge under the effect of potentially inhibiting or toxic compounds, and to determine their threshold values (Chernicharo 1997; James et al. 1990; Penna 1994; Soto et al. 1993). In fact, the analysis of the SMA was used in several researches for evaluation of inhibition or toxicity of the anaerobic sludge by oxygen (Estrada-Vazquez et al. 2001; Silva et al. 2005), sulfide and sulfate (Guerrero et al. 2005), sodium, ammonia and iron chlorides (Borja et al. 1996; Dolfing and Bloemen 1985; Lema et al. 1991; Santos 2001), non-ionic detergents (Cohen 1991) and chlorinated compounds of low molecular weight (Colleran et al. 1991).

The SMA test can also be used to verify changes to the anaerobic sludge under long storage periods (Colleran et al. 1991; Penna 1994; Valcke and Verstraete 1983), or to determine the microbial composition of sludges by using specific substrates (Araújo 1995; Colleran et al. 1991; Dolfing and Bloemen 1985; Jawed and Tare 1999; Soto et al. 1993; Valcke and Verstraete 1983). Poetsch and Koetz (1998) emphasized that anaerobic microorganisms are difficult to be isolated and identified, hence the SMA test is a practical solution to verify which specific microbial groups (i.e. acidogenic, acetogenic, methanogenic) prevail under a particular condition. Other applications of the SMA include the quantification of the affinity of a biological group to a certain substrate and the determination of kinetic parameters (Soto et al. 1993); the verification of mass transfer resistances as well as indirect verification of biofilm microbial arrangement (Araújo 1995; Dolfing 1986). As an example, Jing-Song et al. (2005) used the SMA to compare the effect of bioflocculants and synthetic polymers on sludge granulation in UASB reactors operated under low organic loads.

Although the importance of the SMA test is already acknowledged in the literature, there is no standardized protocol for executing the test. In fact, the literature reports different protocols for the SMA test, and they differ from each other both in the procedures adopted for sludge incubation (e.g. type of mixing,

temperature, biomass concentration, substrate type and concentration, food/microorganism ratio, nutrient type and concentration) as well as in the methodology used to quantify the methane produced (e.g. volumetric methods using or not chromatography; manometric methods). As there is no consensus about the best protocol, it is difficult to compare the SMA results achieved by different research groups. In this context, the main purpose of this work was to evaluate the influence of the main variables associated with the sludge incubation and with the monitoring of the methane produced, in order to contribute to a harmonization of the SMA test.

Materials and methods

Source of sludge

The sludge samples (seed) used in the SMA tests were collected from two demo-scale upflow anaerobic sludge blanket (UASB) reactors ($V = 15$ m^3) built in the Sanitation Research and Training Center of *Universidade Federal de Minas Gerais* and *Companhia de Saneamento de Minas Gerais*. The reactors were fed domestic sewage and were operated at a 9-h hydraulic retention time (HRT). The sludge-seed was taken from the first two sampling ports of the UASB digestion compartment (bottom of the reactor). Although the research was carried out in different periods, it is worth of mention that in the tests that evaluated a certain parameter, all bottles of that experiment were always inoculated with the same sludge.

Sludge incubation

The tests were performed in 110, 220 or 270 ml glass bottles closed either with rubber seals and aluminum seals or with highly resistant adhesive tapes. After sealed, the bottles were purged with a N_2:CO_2 mixture (70%:30% v/v) to allow an anaerobic environment. All the analyses were made in triplicate and followed by a control ('blank flask') which reproduced the test with distilled water in lieu of the substrate solution.

Sodium acetate (2.0 g COD/l) was used in all tests as the single source of carbon and energy, except for the step that evaluated the type of substrate, in which the glucose, sodium formate and raw sewage were also tested. Sludge preserved at 4°C, for a maximum period of 7 days, was used as seed sludge for all experiments, without any adaptation period. The sludge volume added to the bottles was calculated based on the volatile suspended solids content (VS), in order to maintain an initial *F*/*M* ratio of 0.4 g COD/gVS in the bottles, except for the step which evaluated the influence of the *F*/*M* ratio on the SMA test. A nutritional solution (see Table 1) containing macro (100 ml/l nutritional solution ratio) and micronutrients (1 ml/l nutritional solution ratio) were added into the bottles in order to maintain the gaseous phase volume (headspace) constant at 10%, except for the experiments that evaluated the influence of this parameter, in which 30 and 50% headspace values were also used. In this case the higher headspace volume was obtained lowering the liquid volume proportionally reducing the volume of seed, substrate and nutrient solutions and keeping constant the *F*/*M* ratio.

In order to ensure a greater contact between the biomass and the media the bottles were submitted to an orbital continuous stirring, performed by a shaker (Marconi MA420) which imposed a variable stirring (200 rpm during most of the study) and controlled the temperature (30°C, except as otherwise specified). Manual stirring (three times a day, except on weekends) and axial stirring by magnetic bars were also assessed in the tests that evaluated the stirring effect. The 15, 20 and 35°C temperatures were also tested in the experiments that investigated this incubation variable.

Methane quantification

The methane produced during the tests was quantified by the volumetric method of biogas characterization by chromatography, except for the step that compared the different methane measuring methods. In this case, the biogas production was simulated by periodic injection of a known volume of a standard gas containing methane and carbon dioxide, and the methane was quantified by three methodologies: (1) volumetric method with biogas characterization by chromatography; (2) volumetric method with biogas wash in alkaline solution; (3) manometric method with the Oxi-top® equipment.

Table 1 Nutrient stock solution used to prepare the medium added to serum bottles

Macro-nutrients (tenfold concentrated)	Concentration (g/l)	Micro-nutrients (1,000-fold concentrated)	Concentration (g/l)
KH_2PO_4	6.50	$FeCl_3 \cdot 6H_2O$	2.00
K_2HPO_4	1.50	$ZnCl_2$	0.05
NH_4Cl	5.00	$CuCl_2 \cdot 2H_2O$	0.03
$Na_2S \cdot 9H_2O$	0.50	$MnCl_2 \cdot 4H_2O$	0.50
$CaCl \cdot 2H_2O$	1.00	$NiCl_2 \cdot 6H_2O$	0.05
$MgCl_2 \cdot 6H_2O$	1.00	$AlCl_3 \cdot 6H_2O$	0.05
		$CoCl_2 \cdot 6H_2O$	2.00
		H_3BO_3	0.01
		HCl	1 ml/l

Source: Aquino et al. (2007)

In the volumetric biogas characterization method, the produced biogas was first quantified by wetted glass syringes (10, 20 or 50 ml) connected with needles by means of three-way valves. After reading the volume, a biogas sample was transferred to a plastic syringe, also connected with a three-way valve, so that the sample could then be analyzed in a gas chromatograph coupled to a thermal conductivity detector (GC-TCD). The methane content of the biogas was gauged by external calibration with standard gas containing methane and carbon dioxide.

In the volumetric method with biogas wash in alkaline solution, a system that carried the biogas through the 0.1 M NaOH solution was assembled, so that the carbon dioxide could be absorbed and the resulting volume measured (by wetted glass syringes) would be mainly due to methane. In its turn, the manometric method employed the Oxitop® pressure transducer, Control AN-6 model with OxiTop-C measuring heads by WTW. Pastilles of NaOH were placed in a holder assembled inside the OxiTop flask below the transducer head, so that the CO_2 could be absorbed and the pressure readings be assigned mainly to methane. Further details of the methane measuring methods can be found in Aquino et al. (2007). It should be emphasized that in this paper only the methane quantified in the gas phase was taken into account for calculation of the SMA, i.e., the results do not take into account the methane eventually dissolved in the liquid phase.

Factorial planning

For the planning of the first experiments, the variables considered the most influential ones on the SMA test, defined by preliminary experiments, or considered the most polemic ones, that is, those which assumed values were not consensual among different researchers, were chosen. This way, the parameters chosen were as follows: type of stirring, substrate concentration, *F*/*M* ratio, and presence of yeast extract in the nutritional solution. Table 2 shows the experimental outline made with these four variables, assuming two levels ($4^2 = 16$ experiments).

In order to evaluate in more detail the influence of the *F*/*M* ratio and substrate concentration on the SMA test, these variables were changed in 4 levels, according to the factorial planning shown in Table 3.

To evaluate the correlation amongst several types of substrate and nutrient solutions and their influence on the SMA test, another factorial planning was made where two variables, "type of substrate" and "nutrient solution", assumed four levels ($2^4 = 16$ experiments), according to Table 4. For the variable "type of substrate", two more complex substrates (raw sewage and glucose) and two simpler substrates (acetate and formate) were chosen. The choice of raw sewage is important since it is the source of carbon and energy during the operation of real systems. The use of glucose evaluated the behavior of acidogenic, acetogenic and methanogenic microorganisms as a whole, without interference of the hydrolysis step. On their turn, the acetate and formate evaluated mainly the activity of the aceticlastic and hydrogenotrophic methanogenic microorganisms, respectively. The substrate concentration was stoichiometrically calculated considering the desired concentration of 0.3 gCOD/l, which matches the average concentration of raw sewage in Brazil.

Regarding the nutrient solution, the raw sewage was also tested since it represents the actual source of

Table 2 Experimental planning to assess the influence of main parameters on the SMA

Variables	Condition used during test	
Mixing (MIX)	Continuous (cont.)	Intermittent (interm.)
Substrate concentration (SUB) in gCOD/l	0.5	3.0
Food/Microrganism (*F*/*M*) ratio in gCOD/gVS	0.2	2.0
Yeast extract in the medium (YE)	With	Without

Run	Combination				Run	Combination			
	MIX	SUB	*F*/*M*	YE		MIX	SUB	*F*/*M*	YE
1	Cont.	0.5	0.2	With	9	Interm.	0.5	0.2	With
2	Cont.	0.5	0.2	Without	10	Interm.	0.5	0.2	Without
3	Cont.	0.5	2.0	With	11	Interm.	0.5	2.0	With
4	Cont.	0.5	2.0	Without	12	Interm.	0.5	2.0	Without
5	Cont.	3.0	0.2	With	13	Interm.	3.0	0.2	With
6	Cont.	3.0	0.2	Without	14	Interm.	3.0	0.2	Without
7	Cont.	3.0	2.0	With	15	Interm.	3.0	2.0	With
8	Cont.	3.0	2.0	Without	16	Interm.	3.0	2.0	Without

* Fixed conditions: no sludge adaptation period; headspace of 10%; temperature of 30°C; sodium acetate as substrate; methane measurement by biogas quantification followed by chromatography

Table 3 Experimental planning to quantify the influence of substrate concentration and food/microorganism (*F*/*M*) ratio on the SMA

Variables	Conditions used			
Substrate concentration (SUB) in gCOD/l	0.5	2.0	3.0	5.0
Food/microrganism (*F*/*M*) in gCOD/gVS	0.2	0.8	1.4	2.0

Run	Combination		Run	Combination	
	SUB	*F*/*M*		SUB	*F*/*M*
1	0.5	0.2	9	3.0	0.2
2	0.5	0.8	10	3.0	0.8
3	0.5	1.4	11	3.0	1.4
4	0.5	2.0	12	3.0	2.0
5	2.0	0.2	13	5.0	0.2
6	2.0	0.8	14	5.0	0.8
7	2.0	1.4	15	5.0	1.4
8	2.0	2.0	16	5.0	2.0

* Fixed conditions: no sludge adaptation period; headspace of 10%; temperature of 30°C; sodium acetate as substrate; intermittent mixing; addition of yeast extract in biomedia; methane measurement by biogas quantification followed by chromatography

nutrients to the biomass of full scale anaerobic reactors treating domestic wastewater. The solution regarded as standard was that presented in Table 1, and the addition of yeast extract to reinforce the mineral nutrients was also evaluated. Besides that, some bottles were inoculated in which the nutrient solution was replaced with phosphate buffer solution (KH_2PO_4/K_2HPO_4).

In order to verify any significant differences amongst the parameters, the variance test was performed using the Statistica® software. As the number of samples was reduced, it was not possible to verify their normality. For this reason, two tests were applied: parametric test, for samples following the normal distribution; and non-parametric test, applied to samples that exhibit any distribution. The evaluation has been made from the *P* value, which should be lower than 0.05 so that the hypothesis of equal samples is rejected with a 95% confidence interval.

The specific parametric test employed was the variance analysis for a factor (Drumond et al. 1996; Helsel and Hirsch 2002; Montgomery and Runger 2003; Snedocor and Cochran 1989) whereas the non-parametric test was the Kruskal–Wallis ANOVA and

Table 4 Experimental planning to assess the influence of nutrient and substrate type on the SMA

Variables	Conditions used			
Substrate type	Raw sewage	Glucose	Acetate	Formate
Nutrient solution[a]	I	II	III	IV

Run	Combination		Run	Combination	
	Substrate type	Nutrient		Substrate type	Nutrient
1	Raw sewage	I	9	Acetate	I
2	Raw sewage	II	10	Acetate	II
3	Raw sewage	III	11	Acetate	III
4	Raw sewage	IV	12	Acetate	IV
5	Glucose	I	13	Formate	I
6	Glucose	II	14	Formate	II
7	Glucose	III	15	Formate	III
8	Glucose	IV	16	Formate	IV

[a] Nutrient solution used for dilution: *I* raw sewage as source of nutrients, *II* standard nutrient solution (Table 2), *III* standard nutrient solution with yeast extract, *IV* no nutrients added, just buffer solution (KH_2PO_4 at 1.5 g/l and K_2HPO_4 at 6.5 g/l)

Fixed conditions: no sludge adaptation period; headspace of 10%; temperature of 30°C; intermittent mixing; *F/M* ratio of 0.2 gCOD/gVS, substrate concentration of 0.3 gCOD/l; methane quantification by biogas measurement followed by gas chromatography

the median test (Kruskal and Wallis 1952; Siegel and Castellan 1988). When the experimental results were considered different by ANOVA, multiple comparison tests were employed. In this case the Tukey parametric test ("post hoc" test) was applied following the ANOVA. Likewise, the non-parametric test for multiple comparisons (Siegel and Castellan 1988) was employed after the application of the Kruskal–Wallis ANOVA test and the median test.

Results and discussion

Comparison of the different methane measuring methods

As the SMA depends on the methane production rate, the correct analysis of the methanogenic activity is associated with the accuracy of the measuring methodology (James et al. 1990) and biogas characterization. Regarding the biogas composition, the main gases present in the degradation of domestic effluents are methane and carbon dioxide, therefore the absorption of carbon dioxide and other gases (e.g. H_2S) by using an alkali would allow the purification of the methane produced. However, it is worth of mention that the composition of the biogas resulting from the anaerobic degradation of industrial effluents will depend on the nature of such waste, and in this case the chromatographic characterization of the biogas would be recommended.

Regarding the gas properties, some authors correct the volumes to normal temperature and pressure conditions for the calculation of the SMA results (Aquino et al. 2007; James et al. 1990; Poetsch and Koetz 1998). In addition, several authors consider the methane a little soluble gas (James et al. 1990), disregarding this effect in the SMA calculation, while others believe that a considerable part of the methane produced from the anaerobic degradation is actually soluble in the liquid (Hartley and Lant 2006), specially in systems kept at high pressure (e.g. manometric measuring methods).

In order to verify the similarity between the different methane quantification methods used in the SMA test, the maximum production rate of this gas was calculated in each analysis and compared with the measured data (Fig. 1).

In the evaluation of the different measuring methodologies, both the parametric and non-parametric methods showed no significant divergence amongst the methane production rates, with *P* values ranging from 0.11 to 0.78 for a regular distribution and to 0.083 for any distribution. However, by comparing the methane readings with the theoretical amount injected, a statistical difference was noticed in the case of the normal distribution, with a *P* value of 0.0002.

Figure 1a shows that the three methodologies yielded similar methane measurements, which were lower than the theoretical amount injected in the bottles. Hartley and Lant (2006) questioned the acceptance of methane as a little soluble gas, suggesting that CH_4 is made soluble until it reaches a certain saturation concentration, determined as 38% of total methane. Assuming this saturation value, the theoretical production rate would be 9.0 ml/day, which is very close to the measured methane production (Fig. 1b). In this new scenario, both parametric and non-parametric variance analyses

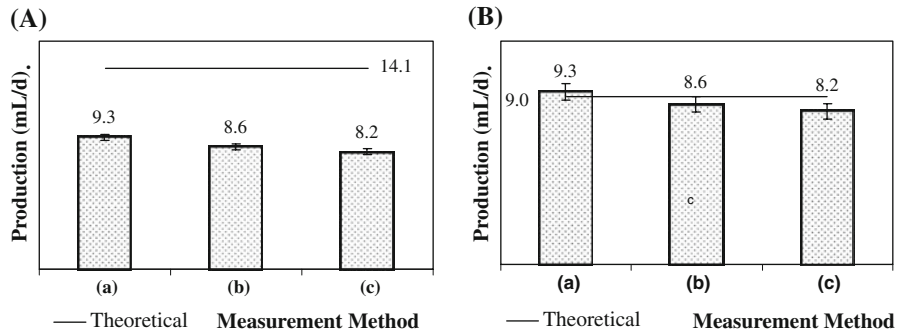

Fig. 1 Comparison of methane measurement techniques: *a* volumetric measurement followed by chromatography; *b* biogas passage through alkaline solution; *c* manometric method using the OxiTop® system.Graph **a** Not considering methane solubility; **b** taking into account that 38% of methane is soluble, according to Hartley and Lant (2006)

showed no significant divergence, with *P* values ranging from 0.0669 to 0.0833, respectively, amongst the measuring methodologies.

These results confirm that the three techniques can be used to quantify the methane produced in a SMA test. Regarding the efficiency of the alkaline solution (biogas washing method) and NaOH pastilles (OxiTop® method) for carbon dioxide absorption, the chromatographic analyses of the samples collected at the end of the tests (data not shown) confirmed the absence of carbon dioxide, evidencing the effectiveness of such methods for methane purification. Therefore, laboratories that lack infrastructure can use the methodology of washing biogas through an alkaline solution, thus simplifying the SMA protocol. The volumetric method followed by chromatographic characterization of biogas, and the manometric method by using the OxiTop® system, demand higher investments for equipment purchase, operation and maintenance. However, such techniques might be important to assess the SMA of anaerobic sludges treating industrial wastewater where the biogas composition might change and a certain degree of automation/instrumentation is encouraged.

Influence of the gaseous phase volume (headspace)

Figure 2 shows the cumulative methane profile and the SMA values obtained in the tests performed with different gaseous phase volumes. It was noticed that the sludge acclimation period, or lag phase, was shorter when the liquid phase volume was larger (10% headspace), followed by the tests with 30 and 50% headspace values, which presented lag phases longer than 1 day and 4 days, respectively. This behavior is coherent, and it is probable that the shorter lag phase observed in the bottles with lower headspace is due to the larger mass of biomass added to the bottles. This would increase the representativeness of the sludge and increase the methane production, thereby reducing errors associated to its quantification.

Figure 2b shows that the measured SMA seemed to be inversely proportional to the gaseous phase volume, and the variance test confirmed this effect. In the parametric tests, *P* values were 0.0243 for the comparison between 10 and 30% gaseous phase volumes; 0.0009 between 30 and 50% volumes; and 0.0003 between 10 and 50% volumes. For the non-parametric tests, a significant difference was detected only between the tests with 10 and 50% gaseous phase volumes, with *P* values of 0.0219.

It is theoretically expected that the gaseous phase volume would not interfere with the SMA value, since it is obtained per biomass unit present in the bottle, and all bottles were incubated with the same *F*/*M* ratio. However, from the results of the variance tests, it can be concluded that smaller gaseous phase volumes yielded higher activity values, and this probably happened because the smaller headspace was related to a larger mass of sludge being added to the bottles, which was possibly more homogeneous and representative.

It is worth of mention that the gaseous phase volume can have a great influence on manometric tests due to the narrow range of the operational pressure of some respirometers. This way, the

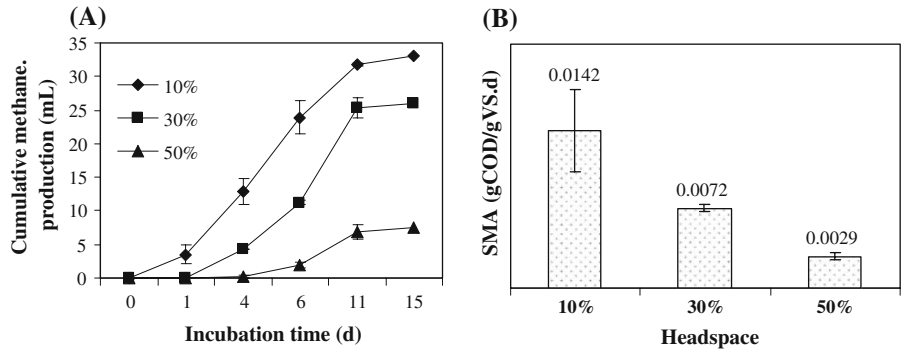

Fig. 2 Cumulative methane production (**a**) and average SMA values (**b**) from experiments that assessed the headspace influence on the test

execution of the SMA test can be preceded by a study of the maximum amount of biogas produced for adequacy of the headspace volume.

Influence of the type of stirring

The SMA results obtained with different forms of stirring were similar. The bottles submitted to manual intermittent stirring presented SMA values as those obtained with orbital continuous mixing (Fig. 3) carried at 200 rpm (graph 3A) or 360 rpm (graph B). Overall, the axial stirring yielded the lowest SMA values, particularly in the second experiment (graph B). One hypothesis is that the high turbulence and the direct contact between the magnetic bar and the sample can result in the rupture of the microbial flocs, thus reducing its activity, as observed by Soto et al. (1993).

The rotational speed of 200 rpm is reported in several studies (Alves et al. 2005; Ghangrekar et al. 1996; Kalyuzhnyi et al. 1996), but the results that originate Fig. 3 indicated that the sludge adaptation period (lag phase) was not influenced by the type of stirring (360 vs. 200 rpm). The parametric variance tests confirmed the difference observed between the axial and the other forms of stirring and showed that there is no difference between the intermittent and the orbital mixing (at 200 or 360 rpm). When considering a not normal distribution, a significant variance was only noticed between the axial stirring and the other two stirrings, with P value of 0.0219.

Another important aspect to be taken into consideration is that the stirring tests were performed with a 100 ml of useful volume. It is expected that larger liquid phase volumes would imply a smaller interference of the magnetic bar stirring with the biomass, hence minimizing biomass deflocculation. Nevertheless, for small reaction volumes, it can be concluded that the orbital continuous stirring is the most suitable form for the SMA test, followed by the intermittent manual stirring which can be used in laboratories that lack the adequate infrastructure.

Influence of the incubation temperature

The experiments that evaluated the temperature on the SMA yielded results that were coherent with the literature, since it was observed an increase in the SMA as the temperature increased. The experiments also indicated that higher temperatures led to shorter acclimation periods (Fig. 4). The test performed at 35°C had an immediate methane production, while the bottles incubated at 30°C showed a 1-day lag phase and the bottles incubated at 20°C had a lag phase that lasted 3 days. When the temperature of the bottles was kept at 15°C the methane production started only in the sixth day.

The variance analysis considering that the SMA follows a normal distribution showed a significant difference amongst the incubation temperatures, with $P = 0.0260$ in the comparison of 15 versus 20°C, 20 versus 30°C, and 30 versus 35°C; $P = 0.0005$ when comparing 15 versus 30°C, and 20 versus 35°C; and $P = 0.0002$ when comparing the temperatures of 15 and 35°C. In a more conservative analysis, which considers any distribution, a statistical difference was only detected between the tests performed at 15 and 35°C ($P = 0.0134$).

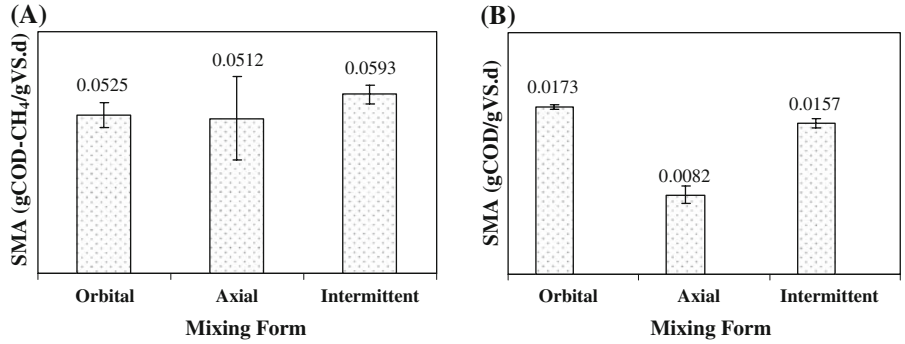

Fig. 3 Average SMA values under different mixing conditions for two different sludges. **a** Orbital mixing at 360 rpm; **b** orbital mixing at 200 rpm

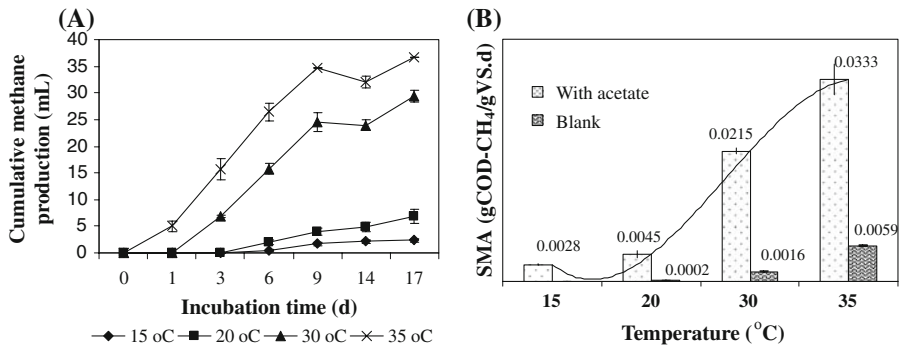

Fig. 4 Cumulative methane production (**a**) and average SMA values (**b**) under different incubation temperature

For purposes of standardization of the SMA test, the temperature of 35°C is suggested, but it should be emphasized that the reactor is operated, in most of the cases, at room temperature, especially in tropical countries. Therefore, with the purpose of associating the methanogenic activity with the temperature, a correlation was made between the SMA value of a sludge kept under a given temperature (SMA_T) and the activity of the same sludge at 35°C (SMA_{35}). Based on the results achieved in this step, a curve associating the SMA_T/SMA_{35} ratio with the temperature (Eq. 1) was proposed and resulted in a high correlation coefficient ($R^2 = 0.999$).

$$SMA_T = 0.0111.SMA_{35} \times e^{0.1307.T} \quad (1)$$

It is noteworthy that Eq. 1 can only be applied for the sludge investigated here, but the same procedure could be adopted for other sludges so that the SMA value determined in laboratory (e.g. SMA_{35} for tests at 35°C) can be converted into the reactor operation temperature (SMA_T). This would avoid the overestimation of the metabolic capacity of the methanogenic microorganisms, thereby minimizing mistakes when calculating the reactor maximum allowable organic load when using the SMA results.

Influence of the substrate concentration

The average results of the SMA tests performed with different acetate concentrations are presented in Fig. 5. Figure 5 shows clearly that the SMA value depends on the substrate concentration; with higher SMA values being observed when the substrate concentration ranged from 0.5 to 3.0 g/l. The test performed with 10 g/l of acetate produced no biogas within the 15-day period, and this probably happened due to biomass toxicity, as observed by Monteggia (1997).

As the hypothesis tests are very sensitive to nil dependent variables, the 10.0 g/l acetate concentration was excluded from the variance analysis. The parametric tests showed a divergence amongst the substrate concentrations being studied, except for the 0.0 and 0.1 g/l concentrations, which had P value of 0.1096. The P values obtained are prevailingly within the 0.0002–0.022 range. It is important to

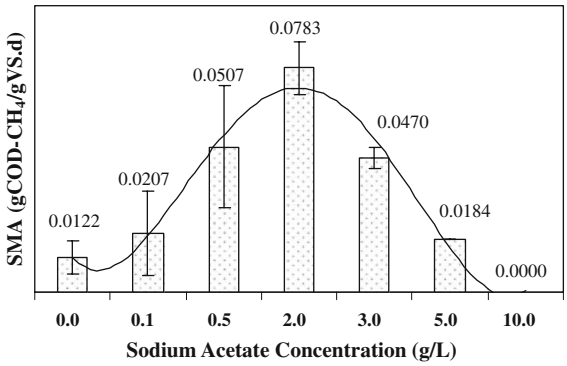

Fig. 5 Average SMA values under different acetate concentration

notice that the *P* value calculated in the evaluation of equality between the 2.0 and 3.0 g/l concentrations was 0.0498—a result very close to the confidence value ($\alpha = 0.05$). If the assumption of a normal distribution is disregarded, the result of the variance analysis becomes very distinct. By applying non-parametric tests, only the SMA results obtained with acetate concentrations of 0.0 and 5.0 g/l were statistically different ($P = 0.0087$).

In an anaerobic treatment system, the low substrate concentration can lead to prevailing microorganisms with low specific consumption rate and great affinity with acetate (e.g. *Methanosaeta* species), while in the SMA test performed in laboratory the high substrate concentration (e.g. 2 g/l) can lead to prevailing methanogens with a high substrate consumption rate (e.g. *Methanosarcina* species). However, microscopic observation revealed that independently of the initial substrate concentration, the predominant morphology was related to the filamentous *Methanosaeta* rather than *Methanosarcina*-like morphology (results not shown). These archaea are characterized by a great affinity with acetate, although they have a low specific growth rate, and this is why they are favored by low substrate concentrations. This observation suggests that the representativeness of the sample was not changed during the SMA test, probably because the incubation time was short to allow an ecological succession. These results show that the SMA is a good tool to assess the actual situation of the sludge collected from an anaerobic reactor in operation. This characteristic is essential to allow the use of the SMA to predict the health of the anaerobic reactor as well as in the calculation of the minimum sludge mass to be kept in the system after periodic discharges.

Correlation among the main variables

Figure 6 shows the results of the experiments planned according to Table 2, which jointly evaluated the variables stirring, substrate concentration, *F*/*M* ratio and presence of yeast extract in the nutritional solution. The result of this step showed that the addition of vitamins, represented by the yeast extract, to the nutritional solution is important to an optimized analysis of the methanogenic activity. The experiments showed that, in general, the SMA had a higher value in the presence of yeast extract and that the *F*/*M* ratio had a large influence on the SMA test, with a high *F*/*M* value being related to greatest SMA results. These results confirm the data presented in Fig. 5, which evidences that higher SMA values have been achieved at higher *F*/*M* values, provided that the acetate concentration is not inhibitory.

Figure 7 shows that the result of the SMA is more impacted by the *F*/*M* ratio, followed by the presence of yeast extract in the nutritional solution. The form of stirring had an intermediate level of influence on the SMA, that is, it will only have a significant influence on the test should the other interfering parameters be in their ideal conditions. According to Fig. 7, the substrate concentration is the incubation condition that interferes the least with the SMA. However, it is emphasized that the substrate concentrations used in the experiment did not inhibit the microorganisms present in the sample.

Figure 8 shows the results of the experimental planning presented in Table 3, which had the purpose of evaluating the interference of the variables substrate concentration and food/microorganism ratio with the SMA test. The charts evaluating the individual influence of the parameters show that, consistently with the previously presented results, higher *F*/*M* ratio values are related to better SMA results. The analysis of the substrate concentration shows that the highest SMA was achieved at lower concentrations, which are 0.5 and 2.0 g/l. In the same manner, these results are also consistent with those previously presented (Fig. 6). Figure 8b also confirms that the *F*/*M* ratio has a more influence in the SMA result than the substrate concentration, hence

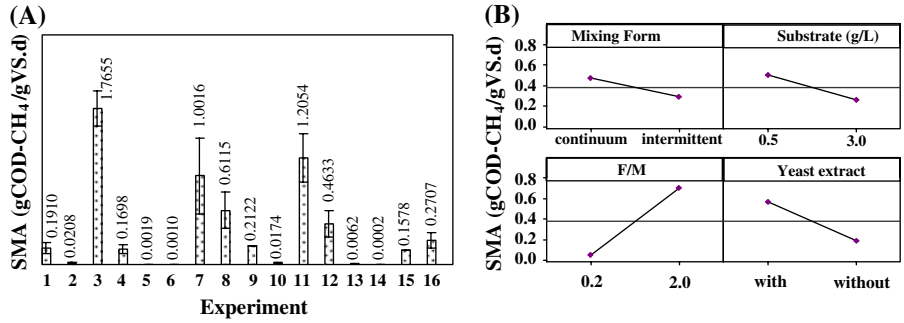

Fig. 6 Average SMA values from the factorial planned experiment that assessed the influence of mixing, substrate concentration, *F/M* ratio and presence of yeast extract on the test

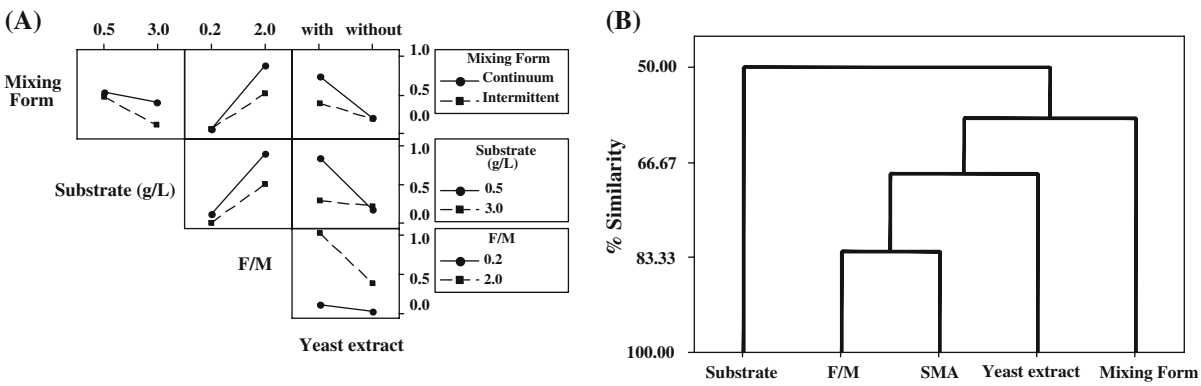

Fig. 7 Combined effect of major variables on the SMA test

this variable should be optimized when planning SMA tests.

Influence of the nutritional conditions

Figure 9a presents the average results of the analyses made according to the experimental planning specified in Table 4. Figure 9b shows that acetate yielded the highest SMA values, followed by glucose, formate and raw sewage. Regarding the nutritional solution, a better biomass performance was observed by using the standard nutritional solution added by yeast extract, followed by raw sewage and standard solution without yeast extract. As expected, the lowest activity was observed in the tests without added nutrient, in which the nutritional solution was replaced with water containing just a source of alkalinity. It is worth of mention that the final pH of all tests was within the ideal range for methanogenesis, that is, between 6.8 and 7.2 (Speece 1996).

Considering that the sample followed a normal distribution, the variance analysis did not show a significant difference in only 13% of the comparisons. A prevailing presence of raw sewage was observed in these statistically "non-distinct" tests, suggesting that the complex composition of the raw sewage adds important compounds to the samples in their use as both substrate and nutritional solution. The non-parametric tests have also indicated a prevailing statistical divergence amongst the nutritional conditions analyzed.

By evaluating the interaction between the type of substrate and the nutrient solution, it was observed that the acetate was little influenced by the presence of yeast extract. Yet in the experiments in which the glucose was the substrate, the addition of yeast extract was very beneficial, coherently because the glucose favors the growth of a larger amount of microorganisms if compared with acetate and formate. The tests using sewage as substrate achieved a

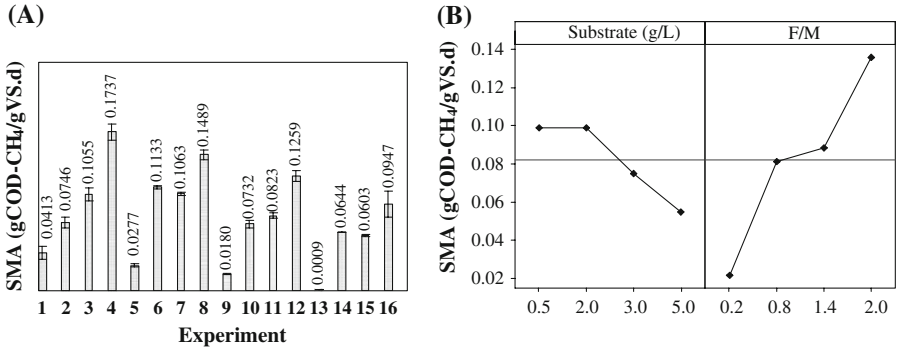

Fig. 8 Average SMA values from factorial planned experiments that quantified the influence of substrate concentration and *F*/*M* ratio on the test

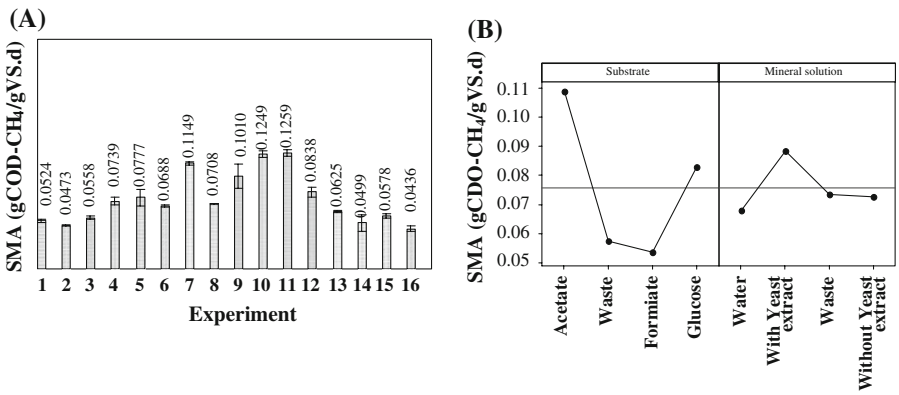

Fig. 9 Average SMA values from the factorial planned experiment that assessed the influence of different types of nutrient and substrate on the test

better performance even when external nutrients (complemented or not with yeast extract) were not added. This confirms that raw sewage already has in its composition the nutritional requirements of the methanogenic archae and could thereby be used in order to measure the activity of the anaerobic reactor treating such wastewater. This would be particularly important to obtain the actual activity of the anaerobic biomass in order to use it as parameter for estimation of allowable load and sludge discharge.

For harmonization of the SMA test, the use of sodium acetate as substrate and the incorporation of yeast extract to the nutritional solution are suggested. In this case, it should be emphasized that the anaerobic sludge activity can be overestimated if compared with the actual operating condition of the reactors, mainly those fed by industrial effluent that lack essential nutrients.

Conclusions

Taking into consideration the results achieved in this investigation of SMA of anaerobic sludge from UASB reactor fed with domestic sewage, it is possible to conclude that:

- The evaluated methane measuring methods (volumetric with biogas characterization, volumetric with gas wash in alkaline solution, and manometric by using the OxiTop® system) had a similar performance. This way, it is possible to simplify the methane quantification process in laboratories that lack the adequate infrastructure;
- The intermittent manual mixing yielded similar results when compared to continuous orbital mixing, and the axial mixing should be avoided in small volume flasks in order not to disrupt microbial aggregation;

- The maintenance of a small gaseous phase volume (ex.: 10% of the total volume) allows the lag phase and the duration of the test to be shortened, and resulted in higher SMA values, probably due to the higher amount and representativeness of the sludge sample;
- The 35°C temperature is suggested for harmonization of the test, although the application of the result of the SMA obtained in laboratory to the sludge management in the reactors operated at room temperature should require correlation studies for correction of the methanogenic activity;
- The ideal acetate concentration for the SMA test ranged from 0.5 to 3.0 gCOD/l with higher values being associated with sludge inhibition. The *F*/*M* ratio that resulted in higher SMA values varied from 0.125 to 0.750 gCOD/VS and this parameter was found to be the most influent on the SMA test;
- The tests performed with nutrient solution complemented by yeast extract resulted in the highest SMA values and should be used when simpler substrates are used in the test. The SMA test can also be carried out using raw sewage without the need of nutrient addition, and this would be appropriate when using the SMA result to support decisions regarding operational routine of anaerobic reactors (e.g. sludge discharge, allowable loads).

Acknowledgments The authors thank CNPq, Fapemig and CAPES for granting master and post-doctor degree scholarships and resources for the development of this research.

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