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"Gheorghe Asachi" Technical University of Iasi, Romania

INFLUENCE OF INNOCULUM ACCLIMATION IN THE BIODEGRADATION RATE AND ESTIMATED BIODEGRADBILITY OF COW MANURE, FOOD WASTE AND OIL

Lúcia Neves, Raquel Ferreira, Rosário Oliveira, Maria Madalena Alves*

IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal

Abstract

Two different inocula - acclimated and non-acclimated to fat- were used to evaluate the methane production of cow manure, food waste and oily waste in batch assays. The inoculum adapted to fat had a better performance in the methanisation of substrates with significant lipids content. Furthermore, it is demonstrated that an increase in the ratio inoculum/substrate can enhance the initial methane production rate of oily waste when using a non-adapted inoculum, improving also the ultimate methane production. Additionally, this work also reveals that changing from mesophilic to thermophilic temperature conditions an inoculum can overcome adaptation setbacks to a substrate, while another one, that displayed good mesophilic performance, can become unproductive. As the results demonstrate, the microbial consortium present in each inoculum can bring about different outcomes while degrading different organic wastes, especially in anaerobic digestion of oily waste.

Key words: anaerobic digestion, cow manure, food waste, oily waste inoculum source

1. Introduction

Anaerobic degradation of organic matter is a complex dynamic process where products from a microbial group are the substrate of the subsequent groups involved in the global mineralization process. For an efficient degradation process of complex substrates, the consortium must have proper activity and all microbial groups involved should be present in the right fraction, since an imbalance between hydrolysis/acidogenesis and methanogenesis can hinder methane production. Previous studies have shown that in dry-thermophilic digestion the inoculum source and the percentage of total solids are responsible for the accomplishment of a rapid onset of a balanced microbial population (Forster-Carneiro et al., 2007). It has also been reported that the use of a highly active anaerobic inoculum or from animal waste origin significantly reduces the experimental time of batch assays, specifically influencing the lag time (Chudoba et al., 1992). Within this context, Neves et al. (2004) reported some advantages of using a granular inoculum in batch anaerobic biodegradation assays of kitchen waste, when compared to suspended sludge, since it prevented acidification, in case of waste composition fluctuations. However, in several countries, granular sludge is not easily available even in the small amounts required to inoculate laboratory reactors. In fact, suspended sludge collected in anaerobic sludge digesters from municipal wastewater treatment plants (MWWTP) is generally much more easily accessible.

The knowledge on anaerobic co-digestion has significantly expanded in the recent years. However, more research is needed on the effects of various compositions of co-substrates and their influence on the process stability (Gelegenis et al., 2007; Murto et al., 2004).

Accordingly, this work aim was to study the biochemical methane potential of the co-digestion of cow manure, food and oily waste, using two different inocula collected in two mesophilic anaerobic

* Author to whom all correspondence should be addressed: e-mail: madalena.alves@deb.uminho.pt; tel: +351 253 604 4417, fax: +351 253 678 986

digesters from MWWTP, one adapted to lipid content and the other non-adapted.

2. Experimental

2.1. Inocula

The inocula were collected from two different mesophilic municipal sludge anaerobic digesters in Portugal. One inoculum was collected in Oporto MWWTP, where fat is not fed henceforth also designated NAI (from non adapted inoculum). The other one was collected in Coimbra MWWTP, where the fat collected from the raw influent is periodically fed to the digester. So, the latter has some degree of acclimation to lipids degradation, when compared to the former, and will be referred to as AI (adapted inoculum). The characterization of both mesophilic inocula used is depicted in Table 1.

Table 1. Characterization of inocula collected in Oporto (NAI) and Coimbra (AI) MWWTPs. Data are expressed as mean ± standard deviation of three replicates

	NAI	*AI*
Total solids (TS) (gL^{-1})	22±1	37±1
Volatile solids (VS) (gL^{-1})	16±1	22±1
Soluble chemical oxygen demand (COD) (gL^{-1})	0.64 ± 0.02	0.49± 0.01
SMA in acetate ($mLCH_{4(STP}$**$)(gVSday)^{-1}$)	39 ± 5	20± 7
SMA* in H_2/CO_2 ($mLCH_{4(STP}$**$)(gVSday)^{-1}$)	178 ± 5	184± 9
Myristic acid (C14:0) ($gCODkgTS^{-1}$)	1.9± 0.8	-
Palmitic acid (C16:0) ($gCODkgTS^{-1}$)	6.5± 1.5	3.2+1.0
Palmitoleic acid (C16:1) ($gCODkgTS^{-1}$)	2.5± 0.9	-
Stearic acid (C18:0) ($gCODkgTS^{-1}$)	1.9± 0.8	-
Oleic acid (C18:1) ($gCODkgTS^{-1}$)	3.9± 1.0	-
Linoleic acid (C18:2) ($gCODkgTS^{-1}$)	0.9± 0.5	AI

*SMA- Specific Methanogenic Activity;
**STP- Standard Temperature and Pressure conditions.

2.2. Substrates

The three co-substrates used in the batch assays were: (1) Cow manure, collected in a dairy farm in the suburbs of Braga (Portugal) and stored in a refrigerator (4 °C) until use to minimize the decomposition of substrate. (2) Food waste which was a composite sample (one week based) from the waste produced in the canteen of the University of Minho, located in "Campus de Gualtar", Braga. Portugal. The food waste was crushed to 1-3 mm particle size and stored at 4°C during 5 days, until the end of the collecting process. Then it was mixed and stored at -18°C. (3) Oily waste was collected in a canned fish processing industry. The characterization of each substrate is presented in Table 2.

To buffer the medium, 3gL-1 of NaHCO3 were added to each 2.4 L vials already containing the inoculated substrate(s). It should be noted that when cow manure and food waste were co-digested the two wastes were always added in a 1:1 ratio of TS and the oily waste concentration (when applied) was always 1.8 gL-1.

2.3. Biochemical methane potential batch assays.

Three sets of biochemical methane potential batch assays were performed (Table 3): (1) in the first trial all the three co-substrates in different combinations were assessed using 6 and 4 gL^{-1} of TS and VS, respectively, of both inocula. For this trial only, a second set of assays was also carried out to assess, at defined time points, the soluble COD, volatile fatty acids (VFA) and LCFA; (2) the second trial was similar to the first one, but only performed with NAI at 16 and 11 gL^{-1} of TS and VS, respectively; (3) the third trial, was carried out with only one substrate, 1.8 gL^{-1} of oily waste, at thermophilic temperature (55°C), using both inocula at 6 and 4 gL^{-1} of TS and VS, respectively. All batch experiments were performed in triplicate.

The vials were sealed with butyl rubber stoppers and the gas headspace flushed with N_2/CO_2, followed by the addition of 0,1mM of $Na_2S.9H_20$ as reducing agent. The vials were incubated at 37°C and the methane production was regularly measured by gas chromatography. The ultimate methane production was calculated per gCOD in the beginning of the assays. The maximum methane production rate (MMPR) was determined using the initial slope of the methane production curve. Blank assays were performed with both inocula, in order to correct for the residual methane production.

Table 2. Characterisation of the cow manure, food and oily waste used in the batch assays

Substrate	*Cow Manure* (gL^{-1})	*Food Waste* (gkg_{waste}^{-1})	*Oily Waste* (gkg_{waste}^{-1})
COD	35±8	327±73	2690 ± 61
TS	28±5	238±1	971±5
VS	21±4	214±7	972±4
Fat content	-	20±8	877±32
LCFA	Cow Manure ($gCODkgTS^{-1}$)	Food Waste ($gCODkgTS^{-1}$)	Oily Waste ($gCODkg_{waste}^{-1}$)
C14:0 acid	3±1	0	19± 1
C16:0 acid	14±4	14 ± 4	260± 7
C16:1 acid	0	0	27± 1
C18:0 acid	26±9	6 ± 2	75± 2
C18:1 acid	0	16 ± 5	891± 17
C18:2 acid	0	8 ± 2	790± 33
gCOD-LCFA $kgCOD^{-1}$	31	32	767

Whenever applied data are expressed as mean ± standard deviation of three replicates

2.4. Analytical methods

Chemical oxygen demand (COD), total solids (TS), volatile solids (VS) and pH were performed according to Standard Methods (APHA et al., 1989).

The total fat content was extracted with diethyl ether in a Soxtec System HT2 1045 extraction unit produced by Tecator (Official Methods of Analysis 2003.05, 2007).

Table 3. Summary of the batch assays performed with different substrates.

Initial conditions	*Type of substrate*				
	*OW**	*CM**	*CM + OW**	*CM + FW**	*CM + FW + OW**
COD ($g\ COD_{added}L^{-1}$)	4.8	8.7	13.6	16.9	21.7
LCFA ($gCODkgCOD_{added}^{-1}$)	752	31	276	31	187
C14:0 ($gCODkgTS^{-1}$)	5	2	4	1	3
C16:0 ($gCODkgTS^{-1}$)	74	7	42	9	34
C16:1 ($gCODkgTS^{-1}$)	8	0	4	0	2
C18:0 ($gCODkgTS^{-1}$)	21	14	24	11	18
C18:1 ($gCODkgTS^{-1}$)	255	0	120	5	87

*OW- oily waste; CM- cow manure; FW –food waste.

Volatile fatty acids (VFA) (acetate, propionate, iso-butyrate and n-butyrate) were determined by high-performance liquid chromatography using a Chrompack column (300x6.5 mm) and a mobile phase of sulphuric acid 5 mM at 0.7 $mLmin^{-1}$. The column temperature was set at 60°C and the detection was by spectrophotometry at 220 nm.

Long Chain Fatty Acids (LCFA) (lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids) analyses were performed as described in Neves et al., 2009.

Methane content of the biogas obtained in the biochemical methane production assays was measured by gas chromatography, using a Porapack Q (180 to 100 Mesh) column, with He as carrier gas at 30 $mLmin^{-1}$ and a thermal conductivity detector. Temperatures of the detector, injector and oven were 110°C, 110°C and 35°C, respectively.

Specific methanogenic activity tests (SMA) of both inocula were performed in the presence of 30mM of acetate and pressurized with H_2/CO_2 (80/20 (v/v)) at 1 bar. Blank controls were used for acetate (no added substrate) and for the gaseous substrate (pressurized with N_2/CO_2-80/20 (v/v) at 1 bar). Strict anaerobic conditions were maintained by using an anaerobic basal medium composed of cysteine-HCL (0.5 gL^{-1}) and $NaHCO_3$ (3 gL^{-1}), with the pH adjusted to 7.0-7.2. Resazurin was added as an indicator of redox potential. This basal medium was prepared by boiling the medium before adding the bicarbonate. SMA values were determined by dividing the initial linear slope of the methane production curve by the VS content of each vial at the end of the experiment.

The volume of methane produced was corrected to the Standard Temperature and Pressure conditions (STP - 1 atm and 273 K).

3. Results and discussion

3.1. First experiment: inoculum influence

Cumulative methane production profiles at mesophilic temperature obtained with both inocula at $4g_{VSinoculum}L^{-1}$ are illustrated in Fig. 1, without discounting the residual methane production obtained in the blank assays.

The foremost difference between the results obtained with the two inocula was observed in the assay of oily waste methanisation. The microbial consortium present in NAI, presented a lag-phase of 10 days (Fig. 1) in order to acclimatize to lipids. However, the AI presented a higher maximum methane production rate (MMPR) along with 16% increase in maximum methane production (Table 4), indicative of the previous adaptation to lipids, as expected, due to the occasional fat addition to the anaerobic digester of Coimbra WWTP. Table 4 presents the maximum methane production rate (MMPR) and the ultimate methane production after correcting for the residual methane production attained in the blank assays.

Table 4. Methane production ($gCOD\text{-}CH_{4(STP)}gCOD_{added}^{-1}$) corrected against the residual methane production obtained in the blank assays, MMPR ($gCOD\text{-}CH_4(gCOD_{added}.day)^{-1}$), from both inocula assayed at $4g_{VSinoculum}L^{-1}$ and mesophilic temperature. Data are expressed as mean ± standard deviation of three replicates.

	MMPR		*Methane production*	
	$gCOD\text{-}CH_4(gCOD_{added}.day)^{-1}$		*$gCOD\text{-}CH_{4(STP)}gCOD_{added}^{-1}$*	
	Inocula		*Inocula*	
Co-Substrates	NAI	AI	NAI	AI
OW*	≤0.01	0.046±0.01	0.75±0.01	0.87±0.03
CM*	0.064±0.003	0.063±0.01	0.78±0.03	0.76±0.01
CM + OW*	0.040±0.001	0.060±0.01	0.75±0.01	0.80±0.01
CM + FW*	0.037±0.001	0.044±0.04	0.66±0.01	0.76±0.01
CM+FW+OW*	0.027±0.001	0.047±0.03	0.76±0.01	0.79±0.01

*OW- oily waste; CM- cow manure; FW –food waste; NAI – inoculum non-adapted to lipids; AI – inoculum adapted to lipids.

An additional indication of the previous adaptation of the AI to lipids is given by the 50 and 74% higher MMPR in the assays cow manure + oily waste and all three co-substrates, respectively, when compared to the assays performed with NAI. The exception was cow manure, which presented similar results independently of the inoculum tested, which could be expected due to its low fat content. Once more the exception was cow manure which attained similar results in the presence of both inocula.

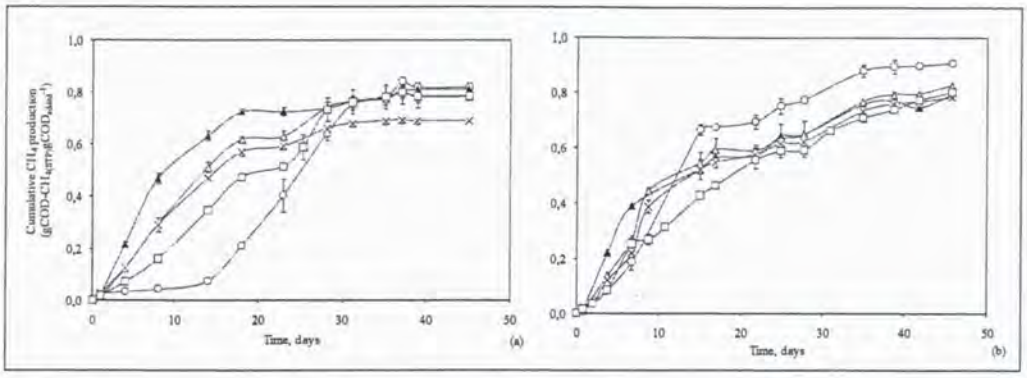

Fig. 1. Cumulative methane production ($gCOD\text{-}CH_{4(STP)}gCOD_{added}^{-1}$) at mesophilic temperature, without discounting the residual methane production obtained in the blank assays, at $4g_{VSinoculum}L^{-1}$. (a) Inoculum non-adapted to lipids, NAI; (b) Inoculum adapted to lipids, AI. Vertical bars represent the standard deviation of average values [-○- oily waste, -▲- Cow manure, -Δ-Cow manure+ oily waste, -x- Cow manure + food waste, -□- Cow manure + food waste + oily waste].

Moreno-Andrade and Buitrón (2004) showed that there was a great variability in anaerobic biodegradability tests due to the use of different sources of inocula, even for an easy-to-degrade substrate such as glucose. However in the methane production of cow manure, the inoculum source appears to be inconsequential. Nevertheless, the present results confirm that methane production from fatty substrates can be enhanced with the use of an inoculum adapted to lipids, suggesting that, as expected, start-up times of continuous reactors should be shorten when acclimated inocula are used.

The biomethanisation values achieved were between 66 and 87% of the theoretical biochemical methane potential expected due to the stoichiometry, which is $350LCH_{4(STP)}kgCOD_{added}^{-1}$, with the highest value attained for the oily waste degradation with the adapted inoculum. Furthermore, the LCFA content to be degraded was much higher in the assay cow manure + oily waste (Table 3) when compared to the cow manure+food waste. Nevertheless, the former combination of substrates displayed a higher biomethanisation in the presence of both inocula, indicating that it is not the LCFA content of food waste that hinders methane production of this substrate, as reported by Carucci et al., 2005.

The hydrolysis rate constants (*kh*) for each assay were calculated assuming a first order kinetics (Eastman and Ferguson, 1981) and following the procedure described by Sanders et al. (2003) and detailed in Neves et al., 2008 (WM), using the values of the initial COD of particulate matter (Table 2), soluble COD at different time intervals (data not show) and the cumulative methane production expressed as COD (Fig. 1). Briefly, the slope of the linear plotting of the ln (particulate COD_{added}-$COD_{hydrolysed}$) versus time for the first 6 days, stands for the *kh* (day^{-1}) of each assay (Fig. 2). The six day period was considered in the calculations because most of the COD solubilisation was accomplished until day 5. The calculated hydrolysis constant values obtained with both inocula ranged between 0.05 and $0.354day^{-1}$, the lowest values were obtained for the oily waste assays and, similarly to the MMPR, the highest values were attained in the cow manure assays, as depicted in Fig. 2.

The hydrolysis constant rate was always smaller for the oily waste assays when compared with all the assays without oily waste, independently of the inocula used, indicating that lipids lowered the hydrolysis rate. Although the *kh* values were very similar for the oily waste assays, the assays performed with the adapted inoculum displayed a much higher MMPR. It should be stressed that *kh* is strongly dependent on the experimental conditions, namely temperature, pH, particle size, stirring conditions, inoculum/substrate ratio which makes very difficult any comparison with existing literature values. Moreover, most of the co-substrates used herein are scarcely referred in the literature.

The individual LCFA reduction profiles adsorbed/accumulated into the solid matrix are depicted in Fig. 3. The most detected and in higher concentration LCFA adsorbed onto the solid matrix was C16:0, in all the assays and for both inocula. However, in the assays with oily waste as co-substrate the two major LCFA fed to the batch were C18:1 and C18:2. Nonetheless, the latter was detected in a very small concentration after only 5 days, in most cases the reduction attained was more than 90%, meaning that this acid is mineralized to shorter acids faster and easily than C16:0.

The results demonstrate that, generally, in the assays with AI, after 5 days, the amount of adsorbed/accumulated LCFA onto the solid matrix and also the difference between the detected C18:1 and C16:0 is somewhat lower than with the NAI. Probably, the developed consortium could better convert the adsorbed LCFA into methane, avoiding the temporarily accumulation of C16:0. In the assays without oily waste, non significant amounts of LCFA were detected, which supports the statement that inhibition of food waste methanisation is not related with the hydrolysis of the LCFA content. Liquid samples were also analyzed for LCFA at the same time of operation but none was detected.

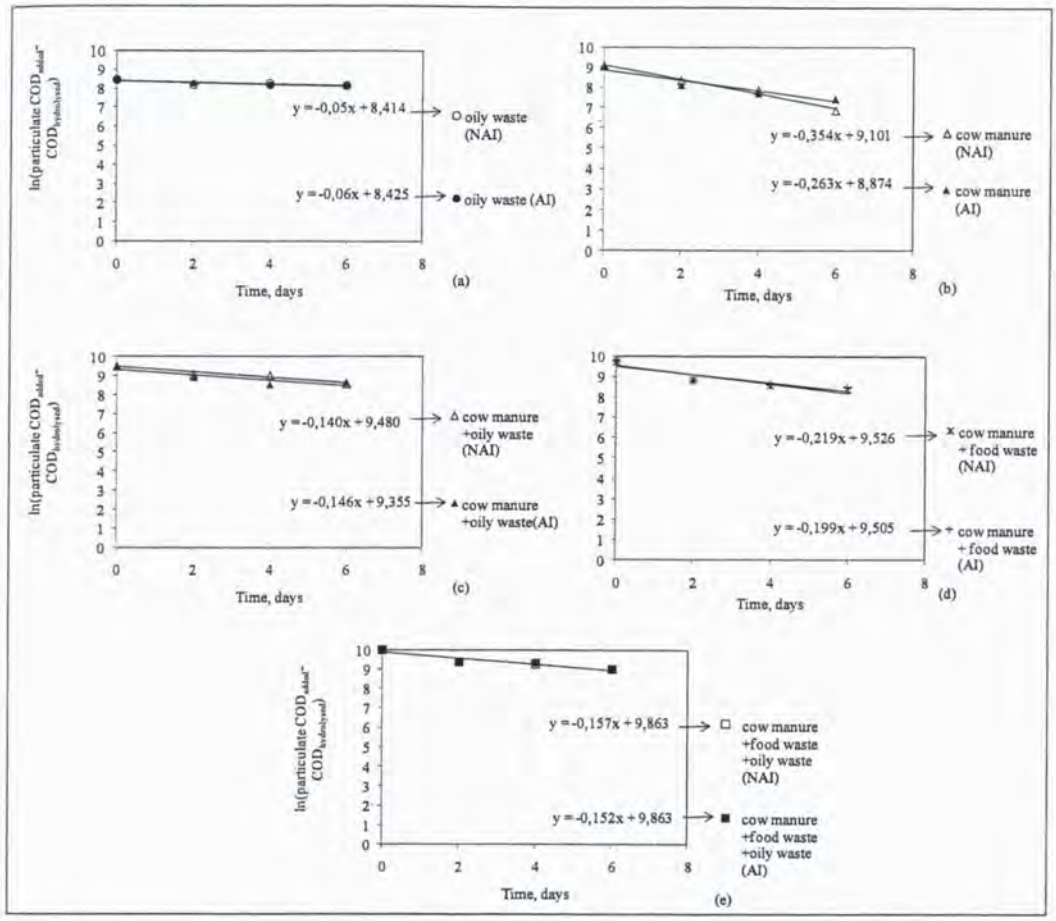

Fig. 2. ln (particulate COD_{added} - $COD_{hydrolysed}$) versus time, in the first six days. The calculated slope represents the hydrolysis constant rate, kh (day^{-1}).

3.2. Second trial: effect of the inoculum/substrate ratio

In the second set of assays, the inoculum was increased 2.7 times in VS concentration, when compared to the identical assays described previously. The increase in the ratio inoculum/substrate was performed trying to overcome the adaptation problems, especially in the assay with oily substrate with NAI, since it is reported that the amount of bacteria present determines the biodegradability rates (Simpkins and Alexander, 1984).

The cumulative methane production profiles at mesophilic temperature obtained with $11 g_{VS inoculum} L^{-1}$ of the NAI are illustrated in Fig. 4, without discounting the residual methane production attained in the corresponding blank assays. The improved MMPR of the oily waste due to the increase in VS inoculum content was noticeable, since the initial 10 days lag-phase presented in the first trial was overcome (Fig. 1(a) and Fig. 4).

In fact, the MMPR calculated values (Table 6) increased considerably from almost null to 0.054 $gCOD\text{-}CH_4 gCOD_{added}.day^{-1}$). Moreover, the methane production, presented an enhancement of 17%, attaining values similar to the assays with the adapted inoculum.

In addition, the assays with the other co-substrates improved the MMPR about 51%. The exception was the cow manure assay, showing no improvement in MMPR or maximum methane production due to the increase in VS inoculum content (Table 4 and Table 6). These results suggest that the source and amount of inoculum used in the batch assays appear to be more essential to some substrates than to others.

The increase of MMPR is accepted to be related with the inoculum/substrate ratio. However, the ultimate methane yield is not expected to be directly related, hence, it is considered a measurement of waste biodegradability, which along with biogas potential are related with the biodegradable carbohydrates content (Angelidaki and Sanders, 2004).

Nevertheless, the ultimate methane production was enhanced in the cow manure + food waste assay in 20%. Hashimoto (1989) showed that the ultimate methane yield in batch anaerobic digestion of ballmilled wheat straw was drastically lower for small inoculum/substrate ratios and increased at a gradual rate as the inoculum/substrate ratio increased up till two (VS basis), after which it remained relatively

constant. Likewise, Lopes et al. (2004) used inoculum/substrate ratios of 0.17, 0.11 and 0.05 (w/w wet weight) to methanise the organic fraction of municipal solid waste, revealing that the amount of inoculum used substantially improved the performance of the process in ultimate biogas production.

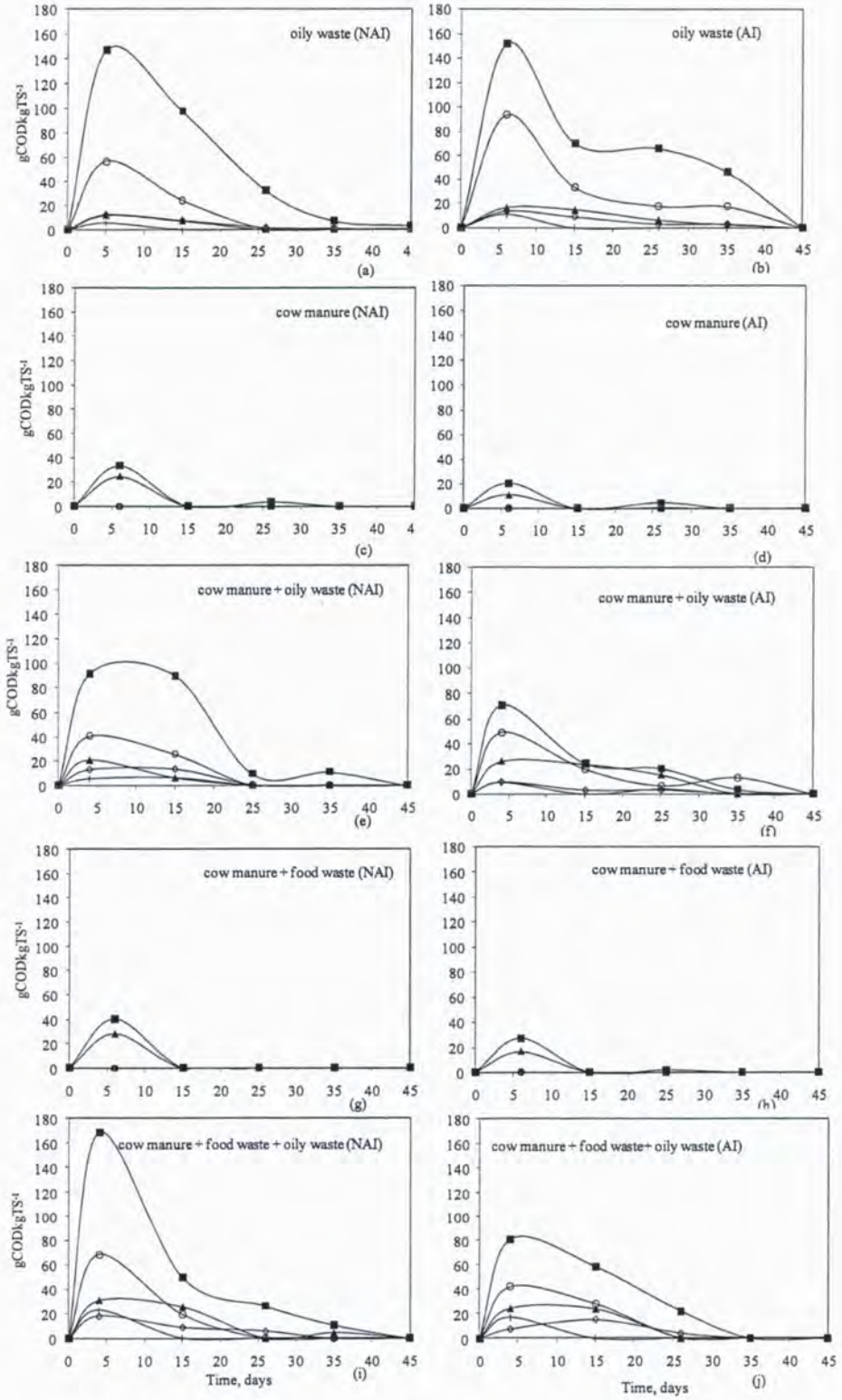

Fig. 3. Individual LCFA adsorbed/accumulated onto the solid matrix in the assays (oily waste (a, b), cow manure (c, d), cow manure +oily waste (e, f), cow manure +food waste (g, h) and cow manure+ food waste + oily waste (i, j)]; [(a, c, e, g, i) non-adapted inoculum (NAI), (b, d, f, h, j) adapted inoculum (AI)) [-◊- C14:0; -■- C16:0; -▲- C18:0;-○- C18:1; -+- C18:2]

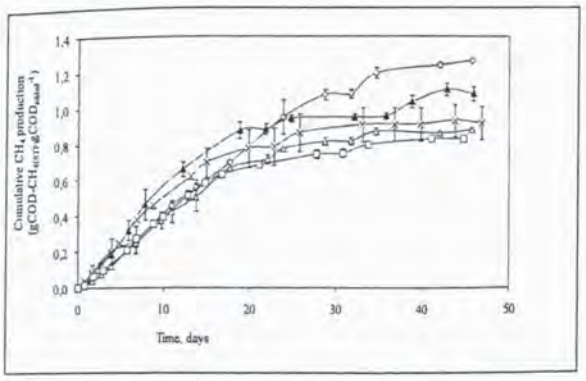

Fig. 4. Cumulative methane production (gCOD-$CH_{4(STP)}gCOD_{added}^{-1}$) at mesophilic temperature, without discounting the residual methane production from blank assays, with $11g_{VSinoculum}L^{-1}$ of NAI. Values represent averages and y-bars represent the standard deviation [-○- oily waste, -▲- Cow manure, -Δ-Cow manure+ oily waste, -x- Cow manure + food waste, -□- Cow manure + food waste + oily waste].

Possibly, when low inoculum/substrate ratios are used the pathway to obtain methane from high loads can be compromised. On the other hand, Hansen et al. (2004) reported that a large amount of inoculum will increase the uncertainty on the results.Similarly, Vedrenne et al. (2008) stated that the incubation conditions used affect the ultimate methane production. In fact, according to their results, the methane production from the inoculum itself ought to be lower than 20% of the total methane production from the assay with inoculum plus substrate, in order to ensure correct results.

Table 5. Methane production (gCOD-$CH_{4(STP)}gCOD_{added}^{-1}$), after correction of the residual methane production obtained in the blank assays and MMPR (gCOD-$CH_4(gCOD_{added}.day)^{-1}$) at mesophilic temperature with $11g_{VSinoculum}\ L^{-1}$ of NAI. Data are expressed as mean ± standard deviation of three replicates

	MMPR	***Methane production***
Substrates	***gCOD-CH_4 $(gCOD_{added}.day)^{-1}$***	***gCOD-$CH_{4(STP)}$ $gCOD_{added}^{-1}$***
OW*	0.054±0.002	*0.88±0.01*
CM*	0.063±0.001	*0.75±0.02*
CM + OW*	0.040±0.003	*0.75±0.01*
CM + FW*	0.056±0.001	*0.80±0.09*
*CM + FW + OW**	*0.041±0.003*	*0.75±0.01*

*OW- oily waste; CM- cow manure; FW –food waste.

In the present set of experiments the ratios inoculum/substrate used were 1 and 2.5 (w/w wet weight), in the first and second trials, respectively. Nevertheless, the residual methane production discounted in the cow manure+ food waste assay was 3% and 12%, in the first and second trial, respectively, using the NAI.

3.3. Third trial: Temperature influence.

This trial aimed to assess the performance of both inocula in the methanisation of lipids at thermophilic temperature. The methane production assays, using oily waste as substrate with the original mesophilic inocula at thermophilic temperature (Fig. 5), displayed a different outcome compared to the similar assays at 37ºC (Fig. 1). The NAI presented an improved MMPR, increasing from almost null values at mesophilic temperature (Table 4) to 0.014±0.001 $gCOD\text{-}CH_4(gCOD_{added}.day)^{-1}$. Nonetheless, the ultimate methane production was 0.75±0.04 gCOD-$CH_{4(STP)}gCOD_{added}^{-1}$, attaining similar values for both temperatures.

Conversely, the consortium formerly adapted to lipids, which presented a better MMPR at 37ºC when degrading oily waste, was not able to easily withstand the temperature increase, considering the lag-phase presented in Fig. 5.

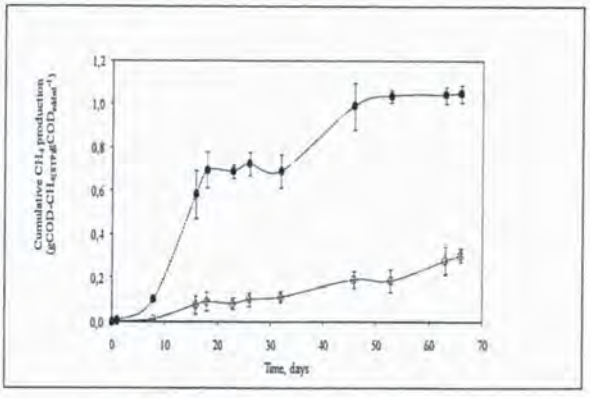

Fig. 5. Cumulative methane production time course (gCOD-$CH_{4(STP)}gCOD_{added}^{-1}$) at thermophilic temperature, with $4g_{VSinoculum}L^{-1}$ and 1.8 $g_{oily\ waste}L^{-1}$, without discounting the residual methane production obtained in blank assays. Values represent averages and y-bars represent the standard deviation (-○- AI + oily waste; -●- NAI+ oily waste).

The calculated MMPR for this assay was almost null and so noticeably lower than at 37ºC (Table 4). In addition, the corrected methane production reached after 67 days was 0.25±0.03 gCOD-$CH_{4(STP)}gCOD_{added}^{-1}$, representing 30% of the value attained in the same assay at 37ºC after 45 days.

Possibly, the microbial population responsible for the better methanisation of the soluble organic matter present in the adapted inoculum is more vulnerable to temperature changes. These results point out that the adaptation of an inoculum should be carried out at the operating temperature to enhance its performance.

4. Conclusions

The present results demonstrate that the consortium present in each inoculum as well as the anaerobic digestion conditions can lead to different

outcomes especially when the anaerobic digestion of fatty wastes is concerned.

The inoculum previously adapted to lipids promoted higher MMPR and ultimate methane production regarding oily waste degradation. However, the previous adaptation to oily waste can be overcome by increasing the inoculum/substrate ratio. Additionally, this work demonstrates that whilst altering the operating temperature, an inoculum can improve or decrease its performance against a given substrate, pointing out to the need of an adaptation to the operating temperature.

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