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ENHANCEMENT OF ANAEROBIC DIGESTION OF MICROCRYSTALLINE CELLULOSE (MCC) USING NATURAL MICRONUTRIENT SOURCES

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Abstract - The effect of adding four minerals as micronutrient sources to stimulate the anaerobic process of microcrystalline cellulose (MCC) was investigated. Three mineral doses (5, 10 and 15 mg/L) were evaluated and the response to trace metal addition was monitored by the methane yield (Y_{CH4}) and specific methanogenic activity (SMA). A clear stimulation of the Y_{CH4} and SMA on MCC due to the presence of minerals with a high content of trace elements was observed, respectively: the Y_{CH4} and SMA of the sludge with 5 mg/L of high-Fe-Mg mineral increased to 397 $NmLCH_4/gVS_{substrate}$ and 0.267 $gCOD_{NmLCH4}/gVS_{inoculum}/d$ compared to 303 $NmLCH_4/gVS_{substrate}$ and 0.205 $gCOD_{NmLCH4}/gVS_{inoculum}/d$ in a medium without adding mineral. An increase in the doses of high-Ca mineral highly decreased methane production and process stability, due to a possible inhibition of the anaerobic digestion by calcium ions.

Keywords: Microcrystalline cellulose; Trace metal; Mineral; Inhibition; Anaerobic digestion.

INTRODUCTION

Anaerobic digestion (AD) is a process that involves a large number of chemical, biochemical and microbiological reactions taking place in series or series-parallel, associated with the metabolism of numerous organisms and intermediates that transform complex organic matter into more easily degradable substrates for anaerobic microorganisms. As a biological process, AD requires macro and micronutrients. Trace metals are essential for optimal growth and development of microorganisms. A proper nutrient balance, as well as nutrient bioavailability, is indispensable for bioreactor improvement and process stability. Micronutrients like iron, nickel, cobalt, selenium, molybdenum, manganese, and tungsten contribute to membrane stability, nutrient transfer and energy saving as in methane-producing and sulfate-reducing bacteria (Patidar and Tare, 2006). Several studies have reported the addition of one or more trace metal salts in anaerobic digestion on the laboratory scale. Positive effects have been obtained mainly because the trace elements are essential co-factors of the enzymes involved in the biochemical pathway of methane production (Zandvoort *et al*., 2006). Trace metals were also applied to substrates such as acetate, methanol or cellulose. In all cases, a clear stimulating effect on the methanogenic activity was observed when using Ni-Co-Fe salts combining more than one trace metal (Kida *et al*., 2001; Pobeheim *et al*., 2010b). Microcrystalline cellulose and starch were used as carbon source in the study of the influence of nickel and cobalt on anaerobic degradation (Pobeheim *et al*., 2010b). The limitation of nickel and cobalt

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*To whom correspondence should be addressed showed a negative impact on the process stability. An appropriate amount of these elements enhanced conversion of the substrate into intermediates and finally biogas.

Moreover, metals are significant natural components of all soils, where their presence in the mineral fraction constitutes a pool of potentially mobile metal species, many essential nutrients for plants and microbes, and important solid components that can have a fundamental effect on soil biogeochemical processes, e.g. clays, minerals, iron and manganese oxides (Huang *et al*., 2004). Nevertheless, there are few researches dealing with natural and/or modified minerals (with Ni^{2+}, Co^{2+}, Mg^{2+} deposition) as the source of trace metals and/or as ion exchangers in anaerobic systems. Acetate, methanol and a mixture of volatile fatty acids were used as the carbon source in order to prove the stimulation of the anaerobic process when minerals were used as a nutrient source (Milán *et al*., 2003; Pereda *et al*., 2006).

In Cuba there are some mineral clays characterized by a varied composition of macro and microelements which can be used as nutrient suppliers to the anaerobic process. Those minerals have a high metallic content which can be promoters, as previously mentioned. Therefore, the aim of this study was to evaluate the influence of adding different minerals on the anaerobic fermentation of microcrystalline cellulose.

METHODS

Inoculum, Model Substrate and Mineral Clays

Inoculum from a full-scale biogas plant (KTG Biogas AG, Germany) fed with maize silage was used for batch experiments. No trace elements have been added to this plant during its operation (it is becoming a practice in Germany to add trace metal solution into operating biogas plants). The inoculum was filtered to eliminate particles larger than 4 mm. Microcrystalline cellulose (MCC) from Sigma Aldrich was used as unique carbon source in all batches.

Four natural clay mineral mixtures extracted from soils were provided by the Research Center for the Mining and Metallurgical Industry (CIPIMM) and codified as M1, M2, M3 and M4. Minerals were used with a particle size from 0.5-1.0 mm.

Analytical Methods

Macronutrient and trace metal contents of the minerals were determined by ICP–OES (inductively coupled plasma–optical emission spectrometer). The total solids (TS) and volatile solid (VS) were analyzed according to the Standard Methods (APHA, 1995). pH analyses were conducted with a WTW PMX3000 pH-meter. The VFA/TIC ratio (Volatile fatty acids/ total inorganic carbonate) was carried out with the two-point titration technique, according to Burchard *et al*. (2001) by using FOS/TAC 2000, and the biogas composition (CH_4, CO_2, O_2 and H_2S) was determined with a Multitec 540 gas analyzer equipment.

Experimental Procedure

The anaerobic batch digestion tests were carried out in triplicate under mesophilic conditions (37±1 ºC), according to the guidelines VDI 4630 (2006). An inoculum with 77.46 gTS/L and 60.38 gVS /L was used. The initial volatile solid (VS) ratio of substrate to inoculum was kept at 1:2.5 throughout all the experimental setups. Each reactor with 500 mL capacity contained 300 g of inoculum and 6.96 gVS of MCC. The batch tests were conducted during 30 days of digestion, using eudiometers and gas sampling tube apparatus (DIN 38414-8, VDI 4630). The volume of the produced methane was measured in agreement with the liquid displacement method using a NaOH (15%) solution in order to remove CO_2 and H_2S. The corresponding values of methane volumes were normalized according to VDI 4630 (2006).

Dosages of 5, 10 or 15 mg/L per mineral were added according to trials and taking into account previous results obtained by Pereda *et al*. (2006). A control reactor was monitored for comparisons with and without mineral addition. A blank reactor, containing only 300 g of inoculum, was also used for correction purpose of biogas production.

Response Variables

Methane yield (Y_{CH4}), the specific methane rate (r_s CH_4) and the specific methanogenic activity (SMA) were the variables selected to evaluate the anaerobic process performance. Methane yield was calculated according to Equation (1):

$$Y_{CH4} = \frac{V_{CH4}}{gVS} \qquad \left[mL\,CH_4 / gVS_{substrate}\right] \qquad (1)$$

where Y_{CH4} is the methane yield, V_{CH4} is the total amount of methane produced during the digestion time, and VS is the volatile solids content in the substrate added to the reactors.

The SMA is used to estimate the capacity of methanogenic bacteria to convert substrate into CH_4

in any anaerobic system, i.e., to evaluate the kinetics of the system (Pereda *et al.*, 2006). The SMA was obtained according to Equation (2):

$$\mathrm{SMA} = \frac{\mathrm{R}}{\mathrm{CF} \times \mathrm{V} \times \mathrm{VS}} \quad \left[\mathrm{gCOD_{NmLCH4}/gVS_{inoculum}/d}\right] \tag{2}$$

where R (mL CH_4 /d) is the methane production rate and was determined as a mean slope during the maximum production activity of the cumulative methane curve for both feedings; CF is a conversion factor equal to 350 mL CH_4/g COD (VDI 4630). V is the effective digestion volume (mL) and VS is the volatile solids concentration (g/mL) of the seeding sludge.

In order to evaluate the kinetics of the anaerobic digestion process, the specific methane rate was calculated according to the Chapman model, Equations (3) and (4) (Pagés-Díaz *et al.*, 2013):

$$\mathrm{Y_{(t)}} = \mathrm{Y_{max}}\left[1-\mathrm{e}^{\text{-bt}}\right]^{\mathrm{c}} \tag{3}$$

$$\mathrm{r_S}(\mathrm{t}) = \mathrm{Y}'(\mathrm{t}) = \mathrm{Y_{max}}\frac{\mathrm{b}\cdot\mathrm{c}^{\mathrm{b}}\cdot t^{\mathrm{b}-1}}{\left(\mathrm{c}^{\mathrm{b}}+\mathrm{t}^{\mathrm{b}}\right)^2} \tag{4}$$

where $r_s(t)$ is the specific methane rate at time t (m^3CH_4/kgVS*d), Y(t) is the methane cumulative curve (m^3CH_4/kgVS), Ymax is maximum fitting methane yield (m^3CH_4/kgVS), b and c are coefficients of the curve fitting.

Statistical Analysis

A statistical analysis of the results was developed using Statgraphics Centurion XV software. One factor ANOVA (Analysis of Variance) to analyze the data and a test for significant effects of mineral addition on selected response variables were used. A multiple range test by Fisher method (with 95% LSD, Least Significant Difference) was carried out to determine the significant differences between average values from each trial.

RESULTS AND DISCUSSION

Anaerobic Digestion Assessment

The effect of mineral addition to the anaerobic process was confirmed to be either stimulatory or inhibitory, depending on the chemical composition of the minerals (Table 1). Table 2 summarizes the results obtained during the experiments conducted. Fig. 1(a), (b), (c) and d show the accumulated methane production for each mineral dose during the 30 days of the anaerobic digestion. The ANOVA of the obtained results confirmed significant differences when applying different minerals at different doses with respect to the control reactor (p =0.005). Mineral 1 at 5, 10 and 15 mg/L showed the lowest values of Y_{CH4}, corresponding to 282, 112 and 98 $mLCH_4/gVS_{substrate}$, respectively. These decreased levels of methane production (Fig. 1(a)) were observed in those batches with higher mineral doses, indicating a possible inhibition due to M1 addition. Only with a dose of 5 mg/L was it possible to obtain an appropriate behavior of the anaerobic process with methane yields of 303 $mLCH_4/gVS_{substrate}$, attaining 93% if compared with the control reactor. Moreover, a gradual increase of M1 up to 15 mg/L resulted in high VFA/TIC values (1.69) and an accumulation of organic acids with concomitant dramatic decreases of the pH value and SMA (Tables 2 and 3). According to Lossie and Pütz (2009), a VFA/TIC ratio at the end of the fermentation process between 0.2-0.3 indicates an appropriate substrate conversion to biogas.

Table 1: Chemical composition of the minerals quantified by ICP–OES.

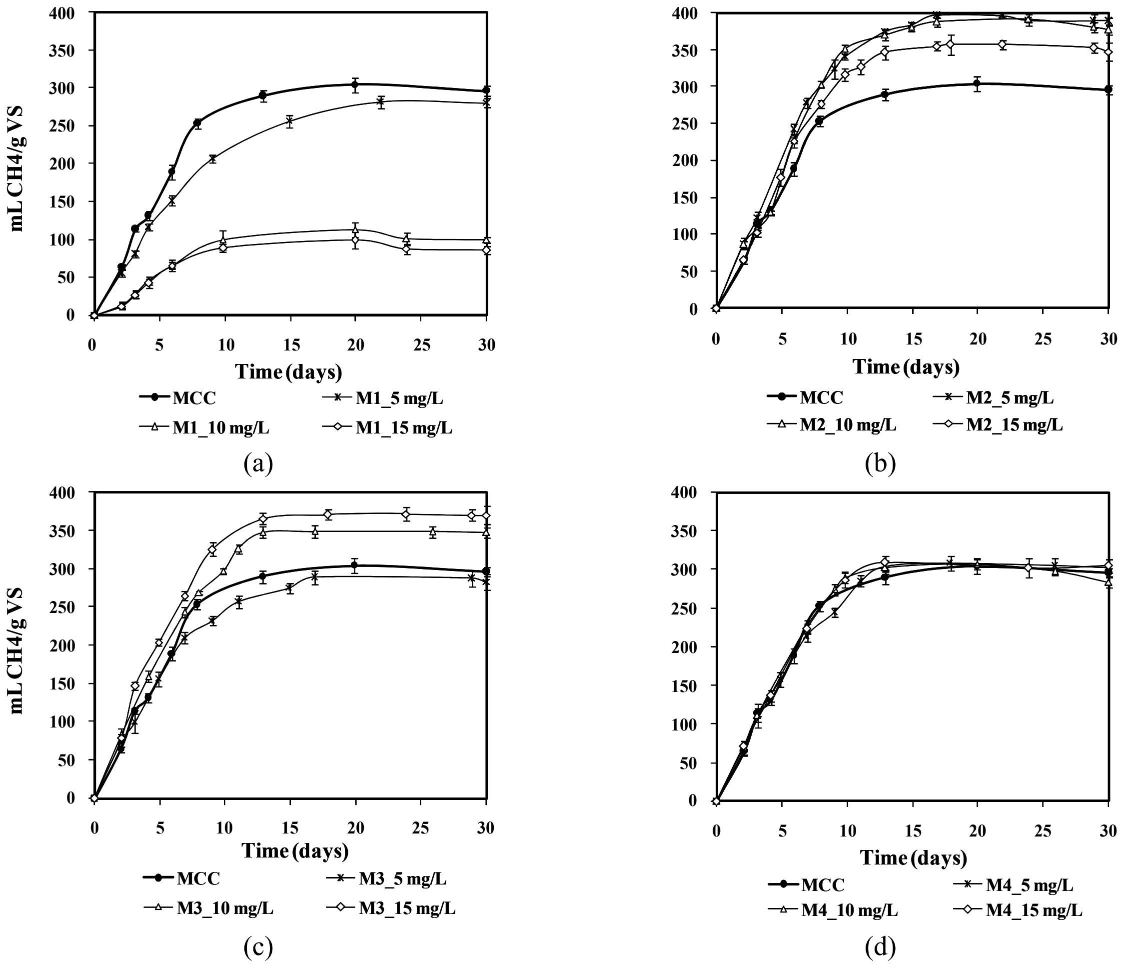
	Chemical Composition (mg element/g mineral)								
Mineral	**Na**	**Ca**	**Mg**	**Al**	**Si**	**Cr**	**Mn**	**Fe**	**Ti**
M1	0.1	301.6	2.8	6.0	82.8	0.008	1.2	3.7	0.6
M2	20.1	27.5	30.2	37.6	220.4	0.003	1.4	38.5	4.0
M3	2.9	259.4	13.0	12.0	95.1	0.436	2.8	14.8	1.3
M4	6.9	67.3	9.7	30.9	253.3	0.002	0.6	14.8	1.7

Table 2: Summary of results, for methane yield and specific methanogenic activity (mean values and pooled standard deviations from triplicate samples) with the significant difference of each batch experiment (Fisher 95.0% LSD).

Reactor/Doses (mg/L)		SMA [gCOD $_{NmLCH4}$/ gVS$_{inoculum}$/d]	Pooled standard error	Y$_{CH4\ max}$ [mLCH$_4$/ gVS$_{substrate}$]	Pooled standard error
Model*	MCC	0.205^{a}	0.004	303^{a}	10.0
M1	5	0.171^{b}	0.002	282^{b}	8.3
	10	0.051^{c}		112^{c}	
	15	0.051^{c}		98^{d}	
M2	5	0.267^{d}	0.007	397^{e}	12.7
	10	0.262^{e}		391^{e}	
	15	0.192^{f}		357^{f}	
M3	5	0.231^{g}	0.009	289^{b}	32.0
	10	0.268^{d}		349^{g}	
	15	0.252^{h}		372^{h}	
M4	5	0.211^{i}	0.005	309^{a}	11.0
	10	0.230^{g}		305^{a}	
	15	0.224^{j}		302^{a}	

*Defined model substrate based on microcrystalline cellulose

a-j Values with the same letter are not significantly different from each other.



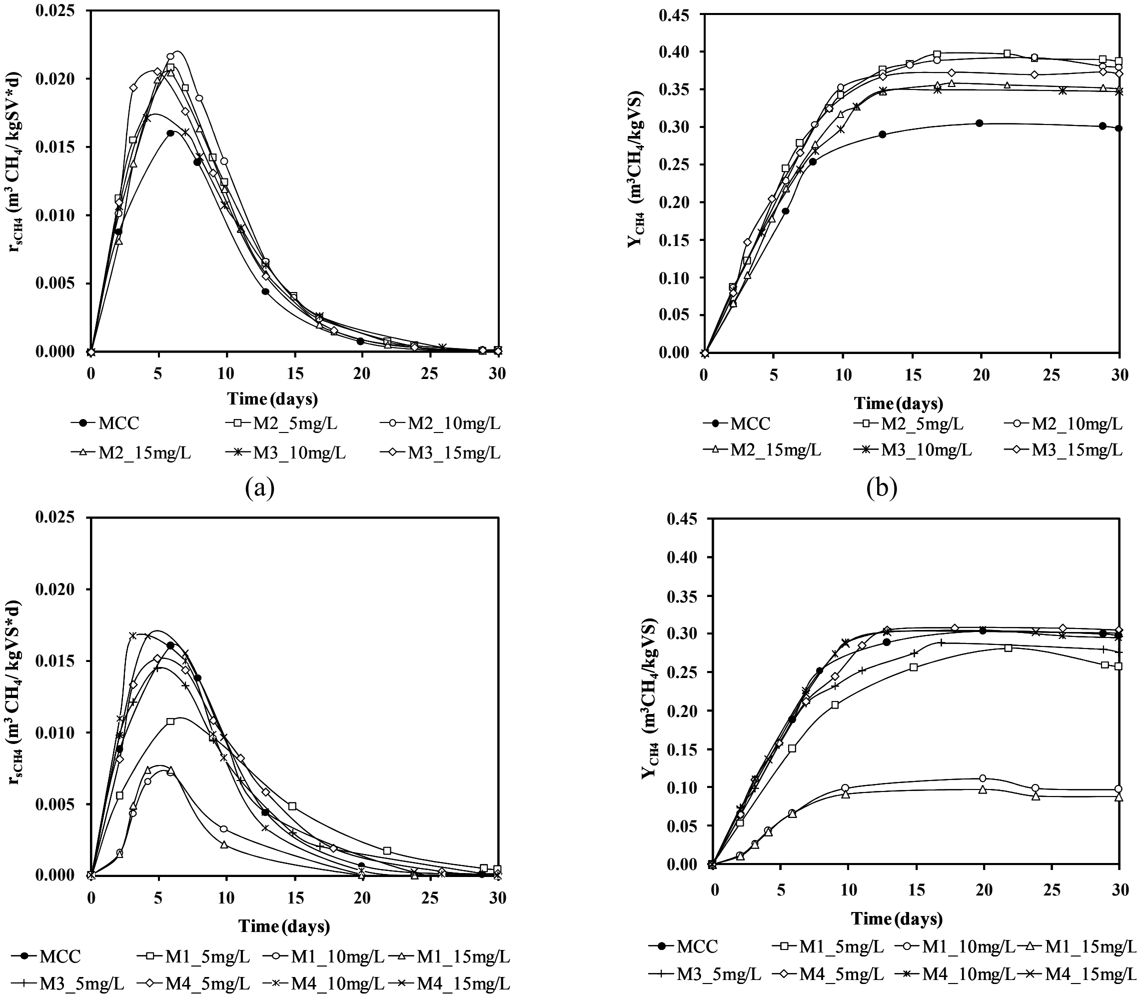
(a) (b)

(c) (d)

Figure 1: Cumulative methane production during the anaerobic process of MCC with and without the addition of clay minerals (mean value from triplicate samples). Mineral 1 (a), mineral 2 (b), mineral 3 (c) and mineral 4 (d).

Excess calcium seems to play an important role in this inhibition. As noted before, M1 has the highest Ca content with respect to the rest of the minerals studied. Even though very little is known about the toxicity of Ca^{2+} in the anaerobic system, it is admitted that excessive amounts of calcium lead to precipitation of calcium carbonate and phosphate, which may result in: (i) the scaling of reactors and pipes, (ii) the scaling of biomass and reduced SMA, and (iii) the loss of buffer capacity and essential nutrients for anaerobic degradation (Chen *et al.*, 2008). In the same way that moderate concentrations of calcium stimulate microbial growth in anaerobic digestion (Tada *et al.*, 2005), excessive amounts lessen the speed of growth and may even can cause a severe inhibition or toxicity in the system (Saifullah *et al.*, 2010) that results in a decrease in the biodegradation process. On the other hand, Liu *et al.* (2011) reported that a high calcium concentration (1600–1800 mg/L) caused a decrease in the methanogenic activity of sludge and the CH_4 production rate. The Ca^{2+} ions released in the present study could have a similar effect. According to Tada *et al.* (2005), it is also possible that the addition of calcium affects not only microorganisms but also the chemical reaction in the reactors.

Taking into account the significant differences in the results obtained with M1 with respect to the control reactor, as well as the anaerobic digestion performance, it is possible to conclude that M1 had an inhibitory effect on the anaerobic degradation of MCC under the conditions studied (Fig. 2 (c) and (d)), mainly at doses of 10 and 15 mg/L.



(a) (b)

(c) (d)

Figure 2: Effect with and without the addition of minerals during the anaerobic process of MCC. Stimulation (a) and (b) and inhibition (c) and (d).

M4 showed a similar behavior compared with the control (MCC) reactor. There was no significant difference in methane yield in the reactors with or without adding M4 for the doses studied (Table 2 and Fig. 1(d)). The addition of 10 mg/L resulted in a slight increase (12%) in SMA with respect to the control. The effect of the main components had no apparent significance; nevertheless, an antagonism between the relative amounts of metals contained in this mineral is clear. A higher Al/Mn (50) ratio is present in M4. The manganese content of M4 is lower compared with the rest of the minerals studied. This metal is essential in the anaerobic reactions, stabilizing methyltransferase in methane-producing bacteria and participating in redox reactions (Fermoso *et al*., 2009). In addition, Cabirol *et al*. (2003) showed that the mechanism of aluminum inhibition was due to its competition with iron and manganese or to its adhesion to the microbial cell membrane or wall, which may affect microbial growth. Furthermore, aluminum (a non-essential metal) appears to be an exception as it become gradually inhibitory only beyond 1500 mg/L as $Al_2(SO_4)_3$. This high toxicity threshold for aluminum could be attributed to its low solubility in anaerobic conditions (Oleszkiewicz and Sharma, 1990). Due to the "balance" of multiple effects regarding mineral composition, M4 at the doses studied is not relevant for the stimulation and/or inhibition of the anaerobic digestion of MCC.

Even though the iron content is similar to M2, the magnesium content is two-fold lower (Table 1). Magnesium ions at high concentrations have been shown to stimulate the production of single cells (Schmidt and Ahring, 1993). The high sensitivity of single cells to lysis is an important factor in the reduction of acetoclastic activity in anaerobic reactors (Chen *et al*., 2008). The majority of cellular Mg^{2+} has roles as enzyme cofactors and in the stabilization of nucleotides and nucleic acids. The most prominent type of enzymatic reactions where Mg^{2+} is indispensible are those associated with energy transfer and phosphorylation/dephosphorylation. Much of the cell's energy is stored in the high-energy ester and pyrophosphate bonds of phosphosugars and diphosphate and triphosphate compounds such as ADP and ATP. Release of this energy by enzymes like phosphotransferases and ATPases requires the presence of Mg^{2+} that forms a 'bridge' between the oxygen atoms of two adjacent phosphate groups and a nitrogen atom on the protein catalytic site.

Minerals M2 and M3 at the doses of 5 and 15 mg/L were shown to have the best performance, with methane yields of 397 and 372 mL $CH_4/gVS_{substrate}$, an increment of 30% and 23%, respectively (Fig. 1(b) and (c)). The results indicate that Y_{CH4} progressively decreased with the increment of the dose of M2, without significance difference between lowest doses. In contrast, with the M3, the best values of Y_{CH4} were obtained when the highest doses were added; showing significance difference among the studied doses (Table 2). The addition of both minerals led to a continuous increase of methane yields and final methane yield values were reached 3 days earlier, compared to trials with defined substrate only (data not shown). Furthermore, the pH-values and VFA/TIC clearly indicate that appropriate concentrations of M2 and M3 enhance sustainable process stability, apart from faster conversion of the substrate (Table 3). Only with 5 mg M2/L and 15 mg M3/L were VFA/TIC ratio values of 0.27 and 0.30 obtained, respectively. These values are within the range reported by Lossie and Pütz (2009). This is in agreement with the results of Pobeheim *et al*. (2010a) who described an increase of methane yields up to 30% with the addition of a mixture of trace elements in batch digestion of maize silage and a model substrate. An increase of the trace elements from 1 mL to 5 mL decreased the methane yield by 10–13%. Moreover, Pobeheim *et al*. (2010b) reported a negative effect of trace elements (Ni/Co), with limitations on the process stability and biogas production in anaerobic semi-continuous fermentations of a model substrate (50% microcrystalline cellulose and 46% starch). The best methane yield performance was obtained with a nickel concentration of 0.6 mg/kg fresh mass. It is noteworthy that those studies were developed with trace metal solutions and allowed a better bioavailability for microorganisms.

Table 3: VFA/TIC values at the end of the MCC anaerobic digestion at different concentrations of minerals.

Reactor/Doses (mg/L)		pH	VFA/TIC
Model		8.4	0.39
M1	5	7.3	0.97
	10	6.9	1.20
	15	6.4	1.69
M2	5	8.4	0.27
	10	8.4	0.29
	15	8.4	0.32
M3	5	8.4	0.37
	10	8.4	0.35
	15	8.4	0.30
M4	5	8.3	0.65
	10	8.3	0.63
	15	8.2	0.72

Iron content in M2 and M3 seems to play the most important role in the anaerobic degradation of the MCC. In the presence of iron, the rate of conversion of biomass to methane increases because iron acts as cofactors of enzymes that are involved, like cytochrome and ferredoxin in methylotrophic methanogens (Oleszkiewicz and Sharma, 1990; Chen *et al*., 2008) in an electron transport chain of metabolism, consequently increasing methane productivity (Hoban and Van Den Berg, 1979). Likewise, Raju *et al*. (1991) described a 40% increase of biogas production after addition of 4 g/L of iron in a fruit waste digester. Pretti and Seenayya (1994) reported a faster conversion of accumulated volatile fatty acids to methane in the presence of iron due to the increased activity of the methanogens. In a previous study, Patidar and Tare (2006) reported a maximum stimulation of the methanogens and sulfate reducing bacteria with the supplementation of iron and cobalt.

An interesting trend was observed in SMA values in the presence of both minerals. Doses of 5 and 10 mg M2/L showed the highest increase of 30% and 28%, respectively, compared to the control reactor, although the values share the same homogeneous group (Table 2). These results correspond to those reached in the methane yield. On the other hand, with the addition of 10 mg M3/L the highest SMA value was obtained (Table 2), the ANOVA analysis revealing significant differences in the doses studied. However, both mineral doses have a clear stimulating effect on methanogenic activity, the methane yield in the reactors (Fig. 2(a)). These results are in accordance with those of Zandvoort *et al*. (2003). They found that only iron had a significant effect on the methanol degradation rate. The addition of iron at 10 µM almost doubled the SMA of the sludge sampled.

In a previous study with halophilic and mixed cultures, Krongthamchat *et al*. (2006) observed that iron slightly stimulated both the initial and maximum SMA of both methanogens. Iron had the capacity to stimulate the mixed culture to achieve a higher value of maximum SMA at a shorter time. Taylor and Pirt (1977) also reported that iron is a growth-limiting factor for *Methanobacterium thermoautotrophicum*, and omission of iron prevented the increase in the final biomass.

Taking into account the chemical formula of MCC ($C_6H_{10}O_5$), it is possible to obtain 0.415 L CH_4/g VS of theoretical methane yield. Minerals M2 and M3 were able to approach experimental methane yields of up to 95.7% and 89.6%, respectively, of the theoretical value. In summary, taking into account the mineral composition, it can be concluded that the results obtained during the anaerobic process of MCC are due to the highest values in iron and magnesium content in mineral 2, regarding the rest of the minerals. With M3 a higher concentration was required to provide enough iron and magnesium content to stimulate the process like M2. Due to the relevance of the present results it is recommended to evaluate the speciation of metals throughout the process.

CONCLUSIONS

Mineral 2 and 3 have been shown to be micronutrient sources for anaerobic digestion of MCC. These minerals, at doses of 5 mg/L and 10-15 mg/L, stimulated both methane yield and specific methanogenic activity by up to 30%, respectively. An increase of the concentration of mineral 1 resulted in the accumulation of organic acids, indicating a clear inhibitory effect on the process. An appropriate content of trace metals combined with the choice of mineral dose could be a strategy for anaerobic bioreactors limited by micronutrients. Long-term effects of minerals on the biogas process should be studied in continuous or semi-continuous reactors.

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