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Short communication

Real evidence about zeolite as microorganisms immobilizer in anaerobic fluidized bed reactors

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Abstract

Using the scanning electronic microscopy, it was observed that natural zeolite possesses excellent physical characteristics as a support medium in anaerobic fluidized bed reactors (AFBR). Samples for biomass analysis were taken from two identical laboratory-scale AFBR (R-1 and R-2), which were operated with 25% of fluidization. These reactors treated distillery wastewaters (vinasses) at mesophilic temperature (30 ± 2 °C). The experiments were carried out with 0.25–0.50 and 0.50–0.80 mm zeolite particle diameter in reactors R-1 and R-2, respectively. The biomass concentration attached to zeolite in both reactors was found to be in the range of 40–45 g volatile solids/l. COD removal efficiencies as high as 90% were achieved at organic loading rate (OLRs) of up to 20 g COD/l day. The volatile fatty acid (VFA) levels were always lower that the suggested limits for digester failure. The yield coefficient of methane production was 0.29 l CH_4(at STP)/g COD consumed and was virtually independent of the OLR applied. A hybridization technique (fluorescence in situ hybridization, FISH) helped determine the predominant anaerobic microorganisms that colonized the natural zeolite, which were found to be Methanosaeta and Methanosarcinaceae, observing a reduced number of sulphate reducing bacteria. The results obtained for reactors R-1 and R-2 were very similar, showing that the particle size did not significantly influence the microbial community immobilized on zeolite.

Keywords: Zeolite; Anaerobic fluidized bed reactors (AFBR); Scanning electronic microscopy (SEM); Microbial communities; Fluorescence in situ hybridization (FISH) technique; Vinasses

1. Introduction

Zeolites are three-dimensional microporous, crystalline solids with well-defined structures that contain aluminium, silicium and oxygen in their regular framework; cations and water are located in the pores. They can be shape-selective catalysts either by transitional state selectivity or by exclusion of competing reactants on the basis of molecular diameter. Therefore, the most common use for zeolites is as an ion exchange material in many applications [1].

Natural and modified zeolites had been used for increasing process efficiency in the anaerobic treatment of agricultural wastes [2,3]. Milán et al. studied the proper doses of natural zeolite for batch anaerobic digestion of piggery waste [4]. They also found that zeolite reduces the concentrations of ammonia and ammonium ion that are present in raw piggery wastewater and those produced during anaerobic degradation of proteins, amino acids and urea.

The purification of wastewaters using zeolite by an ion exchange mechanism has been performed in many countries all over the world [5–8]. At the same time, zeolite has shown a great capacity for metal adsorption (Cu, Cd, Pb and Zn) and that property can be useful for removing toxics for microorganisms in anaerobic digestion [9]. In addition, it has been found that

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zeolite can be useful as a microbial support both in aerobic and anaerobic processes of different wastewaters [10,11].

The use of natural zeolite as support material in an anaerobic fluidized bed reactor for vinasses treatment has been recently described working at laboratory scale [12]. The fluidized bed reactor is a digester configuration which has been demonstrated in various studies to be feasible for the treatment of both low and high strength industrial wastewaters [4,10,12,13]. The use of small, porous, fluidized media enables the reactor to retain high biomass concentrations and thereby to operate at significantly reduced hydraulic retention times (HRT). Fluidization also overcomes operating problems, such as bed clogging and high pressure drop which would be encountered if such high surface area media were used in a packed bed reactor. A further advantage of using media to retain the biomass within the reactor is the possible elimination of the secondary clarifier [14]. However, the structural physical characteristics of the material as well as the predominant microflora in the colonization step are not totally clarified at this moment and have never before been reported in literature. The present study, therefore, is focused on the determination of the physical properties of zeolite connected with the anaerobic microorganisms that colonized it, considering the special case of anaerobic treatment of vinasses.

2. Materials and methods

2.1. Equipment

Two anaerobic fluidized bed reactors (AFBR): R-1 and R-2 with volumes of 1.64 l and 25% fluidization were used for the experiments. These reactors have been described in greater detail elsewhere [13]. The operating temperature of the reactors (30 ± 2 °C) was maintained virtually constant by placing them in a room with controlled temperature.

2.2. Zeolite used

The natural zeolite used as biomass support was obtained from a deposit located in the Province of Villaclara, Cuba. The chemical composition (%, w/w) of the zeolite used were—SiO_2, 66.62%; Al_2O_3, 12.17%; Fe_2O_3, 2.08%; CaO, 3.19%; MgO, 0.77%; Na_2O, 1.53%; K_2O, 1.20%; ignition wastes, 11.02%; its phase composition being: clinoptilolite, 35%; mordenite, 15%; montmorillonite, 30%; and others (calcite, feldespate and quartz), 20%. Finally, other characteristics of the zeolite used were: framework density (FD), 20.6 T-atoms/1000 Å^3; porosity, 32.03% and grain density, 2.16 g/cm^3. Zeolite particles of between 0.25 and 0.50 mm in diameter were used in R-1. Meanwhile, zeolite particles of between 0.50 and 0.80 mm in diameter were employed in R-2. As zeolite is heavier than activated carbon, a smaller particle diameter was tested in order to reduce the power consumption for fluidization of the process. Therefore, a whole range of particles of between 0.25 mm and 0.80 mm in diameter was assayed as was previously recommended by Heijnen et al. [14]. 2.4 kg of zeolite were added to each reactor at the start-up of anaerobic digestion processes.

Table 1
Probes used for the microbial communities analysis

Test	Specification	FA (%)[a]	Position[b]	Reference
SRB385	δ-Proteobacteria	35	16S 385–402	Amann et al. [16]
	Sulphate reducers			
MX825	Methanosaeta	20	16S 847–825	Raskin et al. [19]
MSMX860	Methanoplanaceae	35	16S 860–880	Raskin et al. [19]
MG1200	Methanomicrobiaceae	5	16S 1220–1200	Raskin et al. [19]
	Methanocorpusculaceae Methanosarcinaceae			

[a] FA (%): formamide percentage in the hybridizing buffer.
[b] Reference position respecting *S. coli*.

2.3. Wastewater

The wastewater used for anaerobic treatment was a typical vinasses sample. The average characteristics of the waste used in the experiments with their corresponding standard deviation were: pH, 4.4 ± 0.5; chemical oxygen demand, COD (g/l), 81 ± 21; biochemical oxygen demand, BOD_5 (g/l), 35 ± 10; total solids, TS (g/l), 8550 ± 1200; and sulphates (mg/l), 3100 ± 600. The raw wastewater was diluted until the proper value to achieve an HRT of 11 h and the corresponding organic loading rate, according to the process requirements, was reached.

2.4. Experimental procedure

The start-up of the reactors began with the inoculation stage, which was carried out with well-digested pig sludge, obtained from a laboratory-scale batch digester after 40 days' digestion time and with a concentration of volatile solids (VS) of 104 g/l. The reactors operated initially in batch mode for 60 days. During this period the pH was measured daily and ranged from 7.0 to 7.1. After this period, the reactors operated in continuous mode at an OLR of 2 g COD/l reactor day. In both reactors, the OLR increased progressively without varying the hydraulic retention time (HRT), which was maintained constant at a value of 11 h and increasing the influent concentration. The value

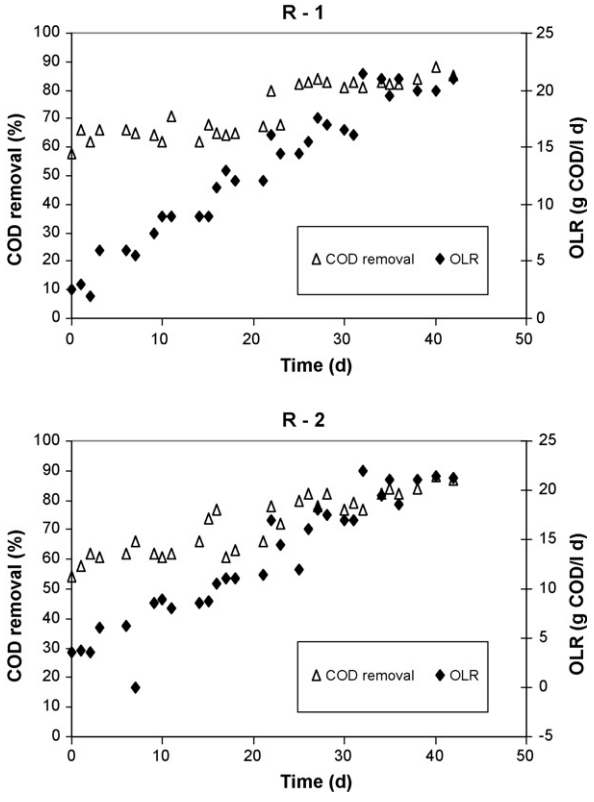


Fig. 1. Variation of the COD removal efficiency with the OLR throughout the operation time in both AFBR studied.

of OLR was increased after a removal of more than 65% COD and a pH of between 7.0 and 8.0 were achieved. Five samples were taken from the effluents of each reactor for every OLR studied after the reactors achieved the steady-state conditions. The steady-state value of a given parameter was taken as the average of the consecutive measurements analyzed for at least 5 consecutive days when the deviations between the observed values were less than 5% in all cases.

2.5. Biomass and chemical analyses

Samples for biomass analysis were taken after 60 days' stable operation. The quantification of the biomass attached to any support is performed by the difference between the estimated dried solids at 105 °C and the estimated ones by calcination at 550 °C, which can be related to the concentration of volatile solids [15]. Chemical determinations were carried out according to the recommendations of the Standard Methods for the Examination of Waters and Wastewaters [15].

Gas chromatographic analyses were carried out to determine the concentration of the individual volatile fatty acids (acetic, propionic and butyric acids). These analyses were carried out using a gas chromatograph equipped with a 15 m × 4 mm Nukol-silica capillary column and a flame ionisation detector. The oven temperature was gradually increased from 100 to 150 °C at a rate of 4 °C/min. Helium (28.6 kP), nitrogen (28.6 kP), hydrogen (14.3 kP) and air (28.6 kP) were used as carrier gas at a flow-rate of 50 ml/min.

Scanning electronic microscopy (SEM), visible optic and epifluorescence microscopy were used to characterize the attached biomass. To analyze the different microbial populations, samples were prepared using the molecular technique of fluorescence in situ hybridization (FISH) according to the protocol described by Amann et al. [16,17]. The samples were fixed just after their collection, in 4% *para*-formaldehyde, washed in phosphate buffer saline (PBS) and preserved in PBS–ethanol at −20 °C until its use. For the hybridization procedure, the samples were fixed on a multi-dish slide at 46 °C and dried in ethanol. The probes marked with fluoresceine (5,(6)-carboxy-fluorescein-*N*-hydroxysuccimide ester) were purchased from Genotek (Barcelona, Spain). All the probes carried out were summarized and specified in Table 1. The dyeing 4′,6-diamidino-2-phenilindol (DAPI) was used to corroborate that the observed fluorescence with the FISH technique corresponded to bacteria cells [18].

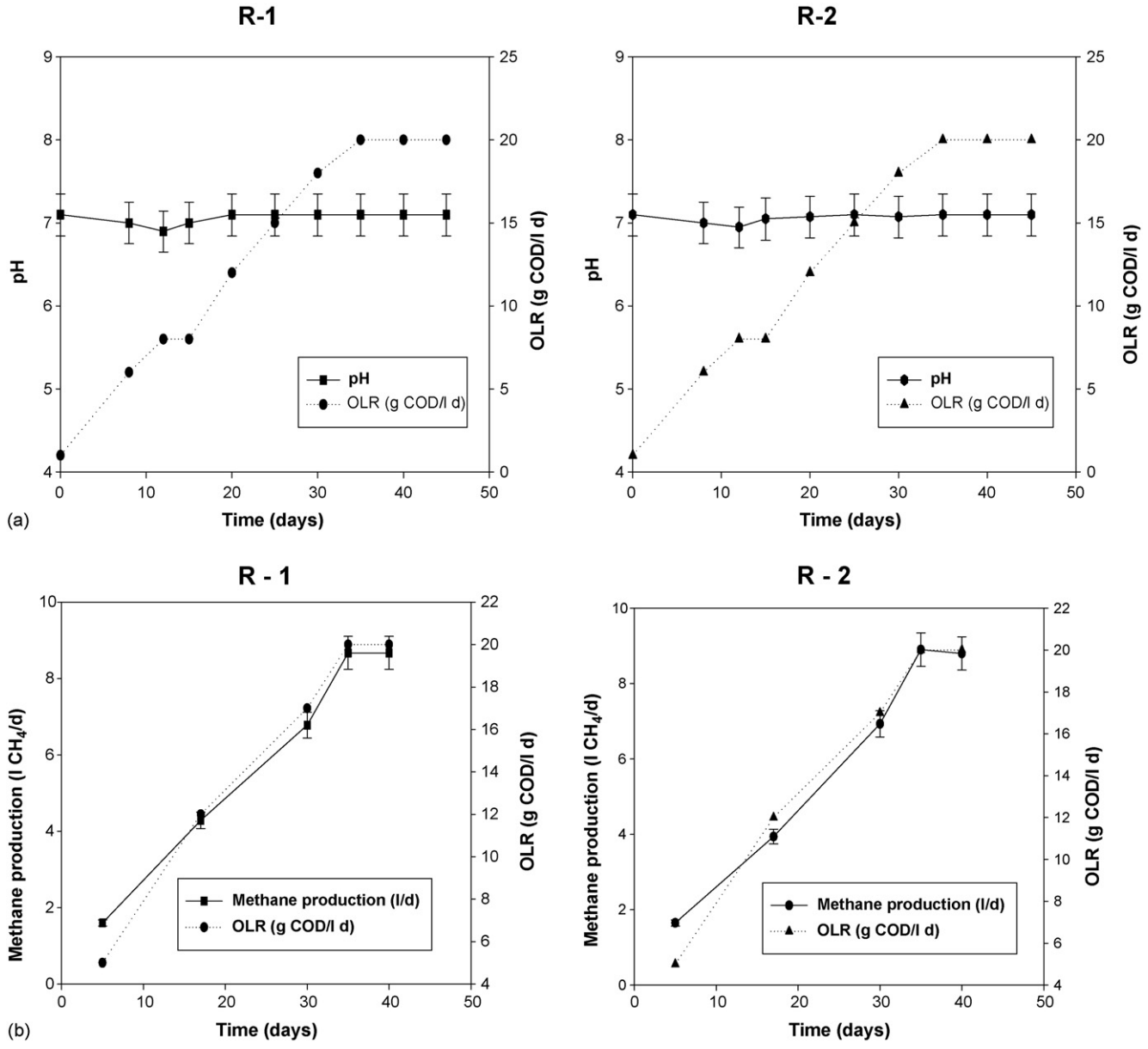


Fig. 2. Variation of the effluents' pH (a) and methane production (b) with the OLR throughout the operation time in the two AFBR studied.

Table 2
Variation of the different volatile fatty acids contained in the influent and effluent of the reactors 1 and 2 with the OLR

OLR (g COD/l day)	Acetic acid (mg/l)				Propionic acid (mg/l)				Butyric acid (mg/l)			
	Influent		Effluent		Influent		Effluent		Influent		Effluent	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Reactor 1 (R-1)												
0–5	801	205	66	32	94	32	ND*	–	62	43	ND*	–
6–12	1147	350	122	95	101	50	32	16	32	11	ND*	–
13–17	1220	91	125	85	158	16	62	29	39	7	ND*	–
18–20	1315	80	130	69	219	30	98	57	45	8	ND*	–
OLR (g COD/l day)	Acetic acid (mg/l)				Propionic acid (mg/l)				Butyric acid (mg/l)			
	Influent		Effluent		Influent		Effluent		Influent		Effluent	
	x	s	x	s	x	s	x	s	x	s	x	s
Reactor 2 (R-2)												
0–5	784	236	60	35	89	35	ND*	–	57	44	ND*	–
6–12	1054	368	117	120	96	58	28	19	29	12	ND*	–
13–17	1215	86	128	99	161	12	59	34	37	3	ND*	–
18–20	1304	85	131	75	213	28	95	62	46	7	ND*	–

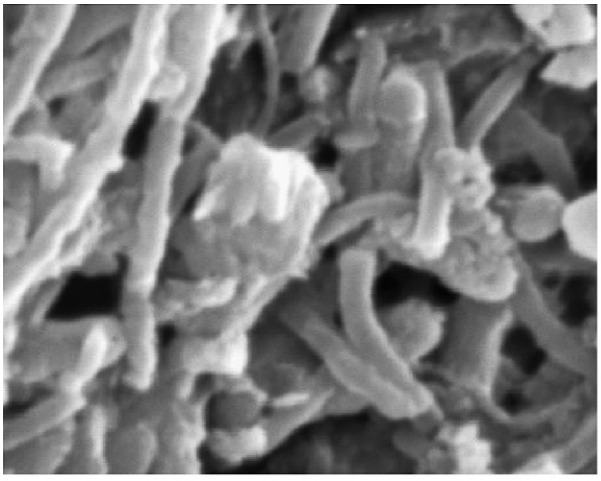
x: mean value; s: standard deviation; *ND: not detected.

3. Results and discussion

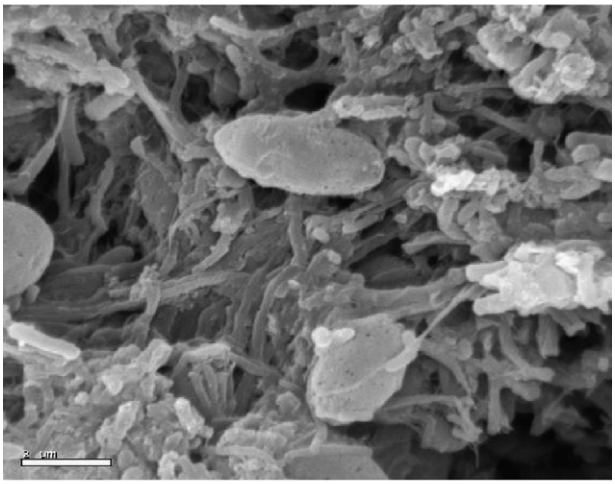
Fig. 1 shows the variation of the organic matter removal efficiency with the applied OLR during the entire experimental period in both reactors used. As can be observed, efficiencies higher than 65% were already obtained at the beginning of the continuous process. As a consequence, the start-up step was accelerated by incrementing the OLR after no more than three times the HRT (11 h). The designed value of OLR (20 g COD/l day) was achieved in 32 days with COD removal efficiencies in the range of 80–85%. These results can be explained as a combination of many factors such as:

(a) The use of an appropriate and methanogenically active inoculum.
(b) Correct starting-up strategy, initially in a batch mode, prior to the beginning of the continuous experiments, which made the development of the appropriate biomass possible.
(c) Favorable behaviour of zeolite as microorganisms support.

COD removal efficiencies of between 80 and 90% were obtained in both reactors for the designed OLR. Ninety percent of the feed COD could be removed at OLR of 20 g COD/l day, which indicates that the anaerobic fluidized bed system is very effective. The average biomass values (with their corresponding standard deviations) attached to the zeolite for reactors R-1 and R-2 measured during the 30th, 40th, 50th and 60th days of operation time were: 40 ± 3, 45 ± 1, 43 ± 2 and 44 ± 1 g/l (R-1) and 41 ± 2, 40 ± 3, 44 ± 1 and 45 ± 1 g/l (R-2), respectively. These values are similar to those reported by Engers et al. [20] in two-stage anaerobic digestion processes of wastewaters carried out in full-scale AFBR. In addition, values of between 0.27 and 0.31 g VS/g zeolite were found in both reactors. The values of biomass also supported the high COD removal efficiencies obtained in both reactors. However, no significant differences between the biomass values in reactors



R-1 (A)



R-2 (B)

Fig. 3. Colonized zeolite. Photographs taken at: (A) 5000× amplification and (B) 16000× amplification.

R-1 and R-2 were observed. The attainment of high reactor biomass hold-up in the anaerobic fluidized-bed system, via the immobilization of the microorganisms on the small, fluidized particles, contributed to such a good system-efficiency. In addition, the production and subsequent release of methane from the biofilms could have had a profound effect on the equilibrium biofilm thickness (and, therefore, equilibrium biomass hold-up) in the reactor, because the resulting effervescence might have sloughed the biofilms off the zeolite particles [21].

Fig. 2a shows the variation of the effluents' pH with the OLR throughout the operation time for the two reactors studied. As can be seen, the pH in both reactors remained approximately constant for all the OLR–OLR interval studied, with 7.1 and 6.9 as extreme values. This stability can be attributed to the carbonate/bicarbonate buffering, which guards against possible acidification of the reactors giving a pH of the same order as the optimal for methanogenic bacteria [22]. In the present work, total alkalinity values in the range of 3400–3850 mg/l (as $CaCO_3$) were sufficient to prevent the pH from dropping to below 7.0 at OLR of up to 20 g COD/l day.

Table 2 summarizes the variation of the different volatile fatty acids (VFA) concentrations of the effluents with the OLR in the reactors 1 and 2. As can be seen, slight increases in acetic and propionic acids concentrations were observed when the OLR increased from 5 to 20 g COD/l day in both reactors. Moreover, the maximum concentrations of VFA achieved at the highest OLR studied were always lower than the maximum tolerable concentration (500 mg/l, as acetic acid) for the methanogenic microorganims, which is clearly indicative of balanced populations of acidogenic and methanogenic microorganisms [22]. In addition, according to Hill and Bolte [23], an acetic acid concentration above 800 mg/l points to the impending failure of swine manure digestion. Hill and Holmeberg [24] also consider that other VFA such as butyric acid is accurate indicator for stress conditions preceding complete failure. The maximum level for impending failure is 5 mg/l (butyric). In the present work, acetic acid concentration was always lower than the above-mentioned value and butyric acid was not detected despite the high OLR studied, which demonstrates the high stability of the process. Other authors [25] have proposed a ratio of 1.4 between the propionic and acetic acid concentrations as the failure criterion, which, taking into account the values from Table 2, was never reached under the present experimental conditions.

The variation of the methane production with OLR for the two reactors studied is illustrated in Fig. 2b. As can be seen, the volume of methane produced per day increased linearly with increased OLR during all the OLR range tested. Therefore, the activity of methanogenic microorganisms was not impaired even at OLR as high as 20 g COD/l day because of the adequate buffering capacities provided in the two reactors studied.

The experimental data of methane production and COD removed were used to determine the methane yield coefficient, Y_P, taking into account that the volume of gas produced per day is assumed to be proportional to the amount of substrate consumed [21,22]. From these data, a value of 0.29 ± 0.03 l CH_4(at STP)/g COD consumed (95% confidence limits) was obtained over the substrate concentration range used; this agrees with the data reported in literatures [21,22].

Using SEM it was observed the aspect of the colonized surface (Fig. 3), making it possible to notice a large accumulation of microorganisms in both the interior of the ruggedness and in the superficial zones, which were more protected from friction. It is observed that the support surface is formed by a compact mass of microorganisms, principally with bacillary and filamentous forms, that are contained in a matrix which keeps them together. Some amorphous crystalline structures that might be formed by precipitations of salt from inorganic compounds, such as carbonates and struvite ($MgNH_4PO_4$) are also appreciated on the biofilm, which are frequently produced in anaerobic processes [19]. On the other hand, but to a lesser extent, the proliferation of other microorganisms was observed, which by morphological analysis look like *coccus* type microorganisms and their

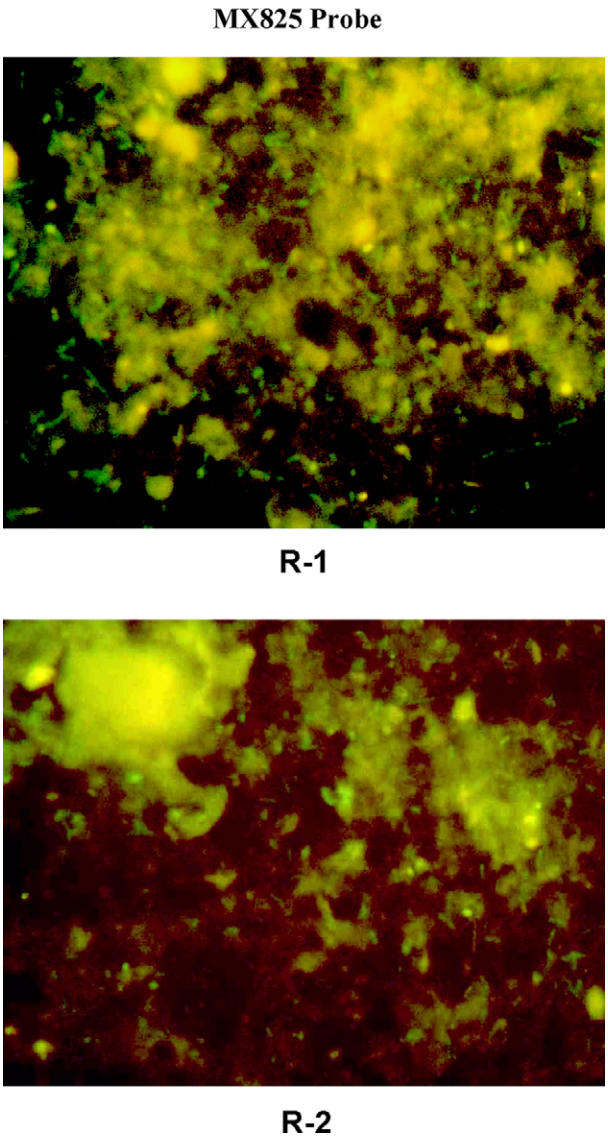


Fig. 4. In situ hybridization of the anaerobic biofilm with the MX825 probe in reactors R-1 and R-2.

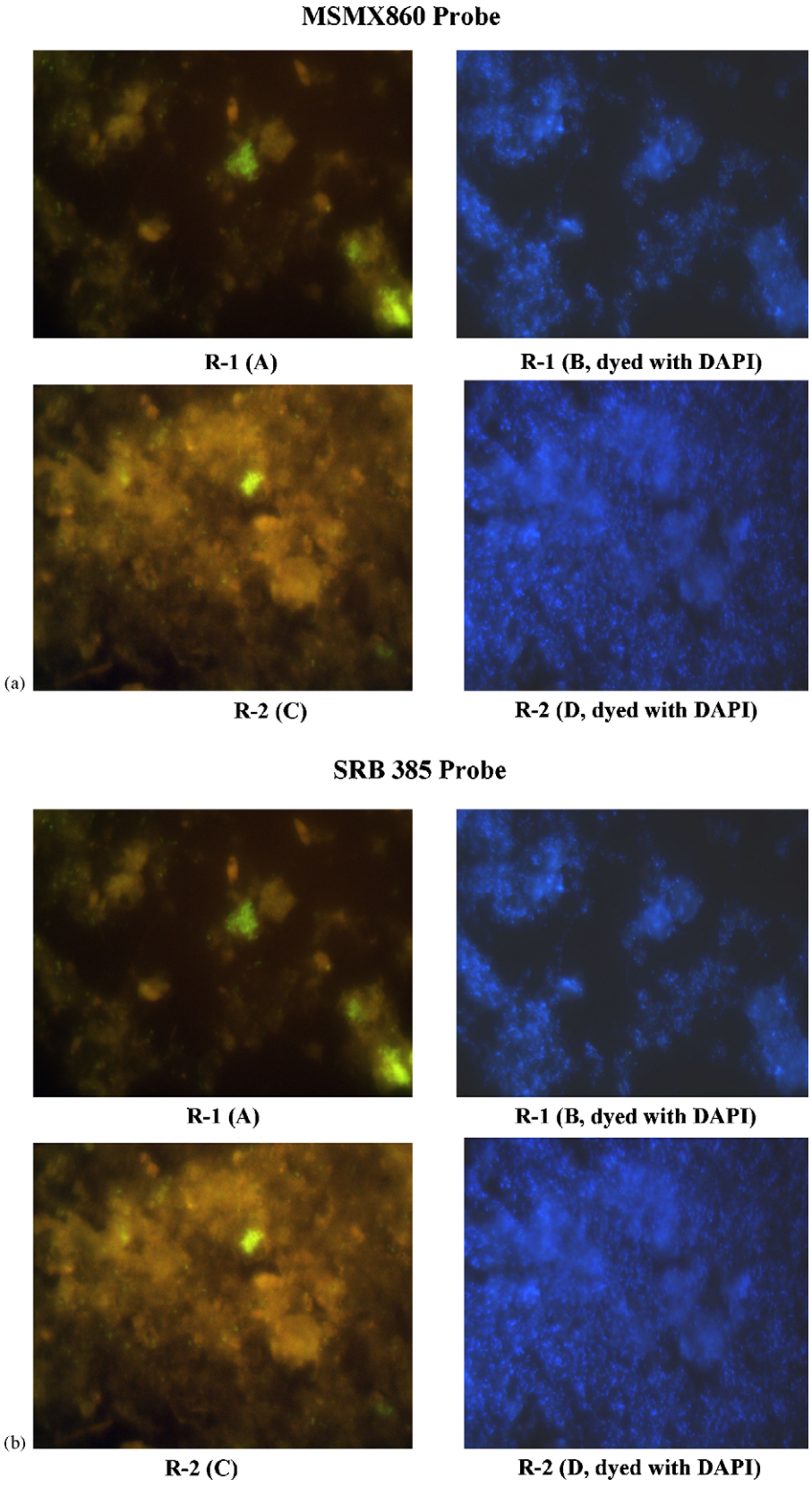


Fig. 5. In situ hybridization of the anaerobic biofilm with the MSMX860 probe in reactors R-1 (A) and R-2 (C) (B and D correspond to the same samples but dyed with DAPI) (a) and in situ hybridization of the anaerobic biofilm with the SRB385 probe in reactors R-1 (A) and R-2 (C) (B and D correspond to the same samples but dyed with DAPI) (b).

peculiar colonies in form of sarcinas, that could presumably be *Methanosarcina* (Fig. 3).

The results obtained by SEM were contrasted with those obtained by using the FISH technique with the purpose of identifying the microbial groups present in the biofilm attached to the support. The results obtained show differences in the number of hybrid cells with the different specific probes that were used. The identification of the different microbial communities was carried out with different samples taken from intermediate AFBR levels. In both reactors, there was a predominance of the microorganisms which hybridized with the MX825 (Methanosaeta) (Fig. 4). The marked bacteria are in green and were grouped in small micro-colonies which are an important fraction of the methanogenic community. These results support the typical bacillary and filamentous structure of the Methanosarcinaceae family that was observed in the microphotography taken with SEM (Fig. 3).

The presence of small isolated colonies that hybridized with the MSMX860 probes (Methanosarcinaceae) (Fig. 5a) was also observed. This observation is in concordance with the coccus type microorganisms forming sarcina isolated colonies, which are shown in the microphotography taken with SEM (Fig. 3). Fig. 5a-B and -D corresponds to the same samples, but dyed blue with the micro-colonies very well distinguished from the rest of the biomass. With the MG1200 (Methanomicrobiaceae, Methanocorpusculaceae and Methanosarcinaceae) probe, there was no positive hybridization detected in R-1. This also happened in R-2, but with less fluorescent intensity. Because of this, we can deduce that these genera do not have a relevant presence in this case.

Fig. 5b illustrates the hybridization results achieved with the SRB385 (sulphate reducers) probe, observing that there was a much reduced number of hybridized cells, for which it can be stated that there is sulphate reducer bacteria (SRB) present in the support, but a very limited amount. This fact might be mainly due to the existence of a biological mechanism that can be developed in an AFBR, where a part of the sulphate is converted to elemental sulphur by microorganisms different from sulphur-reducing bacteria [26,27].

In many cases, the hybridization fluorescence presented is not strong, even though by observing SEM, an abundant adhesion of biomass to the support can be seen. This may be due, as several authors have pointed out, to the fact that in the colonization process and in anaerobic methanogenic ecosystems, an initial methanogenic bacteria absorption is carried out and acidogenic bacteria, which are in a much greater proportion, are attached to methanogenic bacteria in syntrophic association. This fact can cause interferences [28,29].

Analysis of the microbial communities may be decisive to understand the microbial processes taking place during vinasses decomposition in anaerobic fluidized bed reactors and optimize their performance. During the final stage of this experimental study carried out at high OLRs (20 g COD/l day), in which high COD removal efficiencies (85–90%) were achieved, the most frequently encountered microbial group were Methanosaeta and Methanosarcinaceae. These microorganisms have been frequently reported to be important components of anaerobic processes decomposing a variety of organic wastes [30,31]. Rincón et al. [31] reported recently the presence of these microorganisms in continuous stirred tank reactors treating two-phase olive mill solid wastes (OMSW) at OLRs of up to 3 g COD/l day, which operated adequately with COD removal efficiencies as high as 95%. Therefore, these microbial groups might held a critical role in the anaerobic digestion of vinasses and other high strength wastes. Methanosaeta are acetoclastic methanogens [32]. These are common in stable anaerobic systems [30,33,34] and often represents the major component of the methanogenic community [35], such as the steady-state reactor conditions analyzed in this study. Thus, Methanosaeta represents the major methane producers during the anaerobic decomposition of vinasses at these high OLRs.

4. Conclusions

The results of this study show real evidence about both the excellent physical characteristics and the adequate environment of natural zeolite as an anaerobic microorganism immobilizer in anaerobic fluidized bed reactors, taking into account the high organic removal efficiencies achieved in AFBR, using natural zeolite as support media treating vinasses. Moreover, the irregular rough surface of natural zeolite that was observed by SEM, is a factor that contributes to corroborate this result. A large microorganism accumulation in both the interior of the ruggedness and in the superficial zones of natural zeolite was observed.

Finally, a high VS concentration (40–45 g/l) was attached to the natural zeolite in reactors R-1 and R-2. COD removal efficiencies as high as 90% were achieved at OLRs of up to 20 g COD/l day. The volatile fatty acid levels were always lower that the suggested limits for digester failure. The methane yield coefficient was 0.29 l CH_4(at STP)/g COD consumed. The different anaerobic microbial communities attached to the support were identified in both reactors by using the FISH technique, observing a predominance of Methanosaeta and Methanosarcinaceae and a reduced number of sulphate reducing bacteria. In addition, the particle size did not significantly influence the microbial populations attached to this support.

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