INFLUENCE OF CO-SUBSTRATES IN THE ANAEROBIC DEGRADATION OF AN ANIONIC SURFACTANT

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Abstract - The removal of linear alkylbenzene sulfonate (LAS) was evaluated in a UASB reactor using short-chain alcohols (ethanol and methanol) and complex co-substrate (yeast extract). Using only methanol and ethanol as co-substrates resulted in removal of LAS between 30 and 41%. At the end, addition of a complex substrate (yeast extract) increased the removal of LAS to 50%. During the assay, water supply aeration increased the volatile fatty acid of the effluent (70 mg HAc.L^{-1}) and decreased the removal of LAS (from 40 to 30%). According to the fluorescence *in situ* hybridization (FISH) results, the amount of *Archaea* decreased due to water supply aeration (from 64 to 48%). Furthermore, addition of complex co-substrate increased the total anaerobic bacteria and methanogenic archaea content (three and four log units, respectively), which were estimated using the most probable number technique.
Keywords: Linear alkylbenzene sulfonate; Acetic acid; Methanogenic archaea; UASB; FISH.

INTRODUCTION

Linear alkylbenzene sulfonate (LAS) is a surfactant that contains a sulfonated aromatic ring attached to a linear alkyl chain with 10 to 14 carbon atoms. In wastewater treatment plants (WWTP), the LAS concentration varies from 1 to 18 mg.L^{-1} (Morita and Santana, 2005; Mungray and Kumar, 2009).

Only primary biodegradation was found under anaerobic conditions; complete degradation was still not observed (Angelidaki *et al*., 2004). Many studies have assessed LAS degradation using anaerobic reactors, such as upflow anaerobic sludge blankets (UASB) (Almendariz *et al*., 2001; Sanz *et al*., 2003; Lobner *et al*., 2005), horizontal-flow immobilized

biomasses (Duarte *et al*., 2008; Oliveira *et al*., 2009), stirred sequencing batches (Duarte *et al*., 2010) and fluidized beds (Oliveira *et al*., 2010). Among these methods, UASB reactors have been widely assessed and are commonly used in WWTP.

In UASB reactors, degradation of LAS varied from 13 to 85% with the highest efficiency due to withdrawal of co-substrates, at an influent concentration of 5 mg.L^{-1} (Sanz *et al*., 2003). Despite the greater efficiency, a loss of biomass (11.5%) was reported in this condition (Sanz *et al*., 2003) and the requirement of other nitrogen and carbon sources for degradation of LAS was reported in facultative anaerobic conditions (Khleifat, 2006; Abboud *et al*., 2007). Some complex co-substrates were used in

studies with LAS, such as glucose, maltose, or sucrose (Sanz *et al.*, 2003; Abboud *et al.*, 2007), but none used only short-chain alcohol (e.g., ethanol, methanol) which could reduce costs. Furthermore, short-chain alcohols can be used to reactivate bacteria with the ability to degrade LAS (Schörberl, 1989).

In the present study, LAS removal was evaluated in a UASB reactor fed with short-chain alcohols (ethanol and methanol). In addition, yeast extract was added to the reactor to compare the LAS removal using a complex co-substrate.

MATERIAL AND METHODS

LAS

The linear alkylbenzene sulfonate used in the present study was a commercial mixture of C_{10}-C_{13} homologues and was provided by Aldrich (CAS no. 25155-30-0, technical grade).

Inoculum

The inoculum consisted of granular sludge from a UASB reactor used to treat effluent from a poultry slaughterhouse (Dacar Poultry, Tietê-SP, Brazil).

Adsorption Experiment

Adsorption assays were conducted to evaluate the adsorption of LAS onto inoculum. Batches were carried out using 250 mL of mineral medium (the concentration of $MgCl_2.6H_2O$ was adjusted to 25 mg.L^{-1}) (Angelidaki *et al.*, 1990), LAS and inoculum. The initial LAS concentration varied from 5 to 45 mg.L^{-1}. The inoculum was sterilized (Mogensen *et al.*, 2003) and was used at a total solid (TS) concentration of 6 g TS.L^{-1}.

UASB Reactor

The UASB reactor was operated with a hydraulic retention time (HRT) of 24 h (flow 0.44 L.d^{-1}) under mesophilic conditions (30±1 °C). The reactor consisted of acrylic and steel and possessed a volume of 10.5 L.

The feed consisted of mineral medium (the concentration of $MgCl_2.6H_2O$ was adjusted to 25 mg.L^{-1}) (Angelidaki *et al.*, 1990), vitamin solution (Touzel and Albagnac, 1983), sodium bicarbonate (400 mg.L^{-1}), LAS (14 mg.L^{-1}), and various co-substrates (methanol, ethanol and yeast extract). The Figure 1 resumes the conditions employed in each stage.
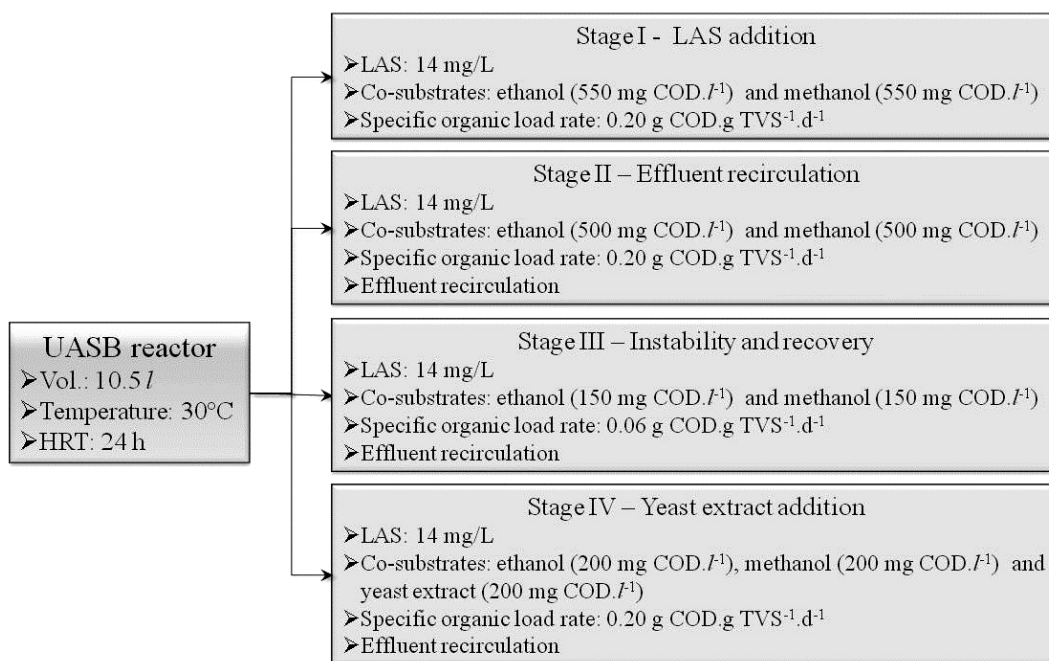

Figure 1: Stages employed in the UASB reactor.

Prior to LAS addition, the UASB reactor was fed with ethanol as co-substrate for 30 days. In stage I, LAS was added and the co-substrates were ethanol and methanol. Effluent was recirculated in stage II (according to the scheme shown in Figure 2), employing a recirculation flow about five times greater than the influent flow. In stage III, an aeration of the water supply provoked instability in the anaerobic process. This aeration led to decreasing consumption of volatile fatty acids (VFA) since oxygen is lethal to methanogenic archaea. Therefore, to remove excess VFA, the sludge blanket was placed under water, and the reactor medium was drained. Furthermore, the pH was buffered by adding sodium bicarbonate, and VFA production was controlled by decreasing the co-substrates concentration (specific organic load was 0.06 g COD.g $TVS^{-1}.d^{-1}$ while in another stage was 0.2 g COD.g $TVS^{-1}.d^{-1}$; TVS = total volatile solids; Figure 1). In stage IV, yeast extract was added to compare the removal of LAS fed with complex co-substrate to previous stages with only short-chain alcohols.
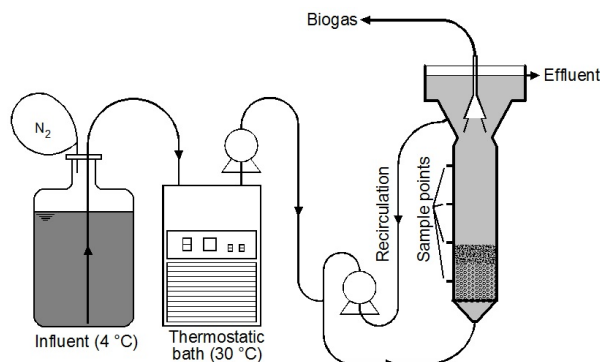

Figure 2: Scheme of the UASB reactor at the stages with effluent recirculation.

Analytical Methods

The chemical oxygen demand (COD), pH, and solids content were determined according to the *Standard Methods of Wastewater Examination* (2005), and the alkalinity was quantified by titration (Ripley *et al.*, 1986). The total solids content of the UASB reactor was measured at the end of stages I, III and IV.

VFA were quantified using a Shimadzu GC-2010 gas chromatograph coupled to an HP-Innovax column (30 m x 0.25 mm x 0.50 μm) (acetic, butyric, isobutyric, caproic, propionic, valeric and isovaleric) (Moraes *et al.*, 2000).

LAS was determined by high performance liquid chromatography, and a *Shimadzu* system (LC-$10AD_{VP}$ pump, CTO-10A oven and RF-10AXL

fluorescence detector) equipped with a reverse-phase C8 column (Supelco) was employed (Duarte *et al.*, 2006). LAS adsorbed on anaerobic sludge was determined according to the protocol described by Duarte *et al.* (2008).

The granule size was measured at inoculation, the end of adaptation, stages I and IV and the beginning of stage III, according to the protocol described by Alphenaar *et al.* (1993). Granules from the sludge blanket were transferred to a Petri dish to acquire images. The images were analyzed with Image Pro-Plus 4.5 software to determine the mean diameter and granule size distribution.

Microbial Community Analysis

Fluorescence *in situ* hybridization (FISH) analysis was performed to quantify the microbial composition of *Bacteria* and *Archaea*. Samples were collected from the sludge blanket at the end of adaptation, stages I and IV and the beginning of stage III. Oligonucleotide probes ARC915 (Stahl and Amann, 1991), EUB338 (Amann *et al.*, 1990) and NON338 (negative control) (Manz *et al.*, 1992) were used to determine the microbial composition of *Bacteria* and *Archaea*. The probes were 5'-end-labeled with rhodamine or CY3, and the fixation and hybridization conditions described by Araújo *et al.* (2000) were applied. An Olympus BX60 microscope was used to acquire the images, and filter sets for DAPI, rhodamine or CY3 were employed, along with *Image Pro-Plus* 4.5 software. To quantify *Bacteria* and *Archaea*, Daime 1.3.1 software (Daims *et al.*, 2006) was used to analyze the images.

The most probable number (MPN) technique was performed to estimate the population of total anaerobic bacteria and methanogenic archaea at the end of stage I and IV.

Samples were homogenized with a sterile mortar and pestle. To dilute the samples, phosphate buffer solution (0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4) was added under an atmosphere of N_2 (100%). Samples were diluted from 10^{-1} to 10^{-20}. One milliliter of each sample was transferred in triplicate to flasks under aseptic and sterile conditions. The flasks contained the feed used in each stage. Counting flasks were incubated at 30±1 °C for 30 days. The turbidity of the medium was determined to evaluate the presence of total anaerobic bacteria (Vazoller, 1995). Methanogenic archaea was quantified by monitoring methane production (GC) (Vazoller, 1995). Populations were estimated according to the MPN table in *Standard Methods of Wastewater Examination* (2005).

RESULTS AND DISCUSSION

Adsorption Experiment

The data were fitted to the Freundlich isotherm (Equation (1)):

$$S = K_f \times C^{1/n} \tag{1}$$

where S is the adsorbed concentration, K_f is the adsorption coefficient (L.g TS^{-1}), C is the equilibrium concentration of LAS (mg.L^{-1}), and 1/n is a measure of the sorption intensity and describes the degree of curvature of the isotherm.

The results showed that $K_f = 1.03 \pm 0.15$ and $1/n = 0.88 \pm 0.09$ ($R^2 = 0.9723$) for a mixture of C_{10-13} homologues (Figure 3). These values agreed with the data reported for anaerobic sludge. Mogensen *et al.* (2003) found that $K_f = 0.796$ and $1/n = 0.35$ for a C_{12} LAS. Garcia *et al.* (2006a) obtained a K_f of 3.24 and a 1/n of 0.92 for a LAS mixture containing C_{10-14} homologues. The observed variation in K_f (0.35-3.24) among studies was attributed to differences in the homologues.

When the value of 1/n is less than 1, adsorption becomes more difficult as sorption sites are filled. In contrast, values greater than 1 are indicative of cooperative adsorption. Values of 1/n close to 1 indicate that the adsorption sites and LAS concentration are independent (Garcia *et al.*, 2002). In the present study, 1/n was less than 1, which suggested decreasing adsorption as sorption sites were filled.
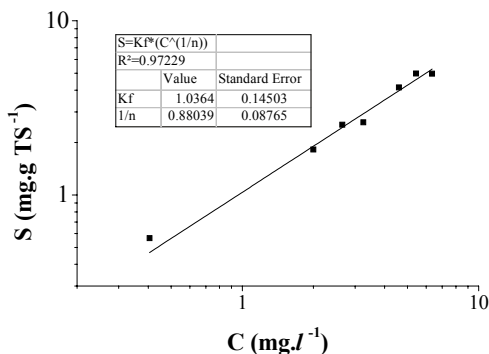

Figure 3: Freundlich isotherm for the adsorption of LAS on inoculum.

UASB Reactor

The granule size distribution exhibited low variations in the inoculums, adaptation (30 d) and stages I (140 d), III (77 d) and IV (44 d), for which the mean varied from 2.9 to 3.6 mm (Figure 4). From stage I to IV, these mean variation decreased to values between 3.2 and 3.6 mm. In stage III, aeration of the water supply provoked a slight decrease in the granule size (3.2±0.4 mm), while the addition of yeast extract (stage IV) increased the granule size to 3.6±0.4 mm. Furthermore, in stage IV, 95% of the granules possessed a diameter greater than 3 mm.
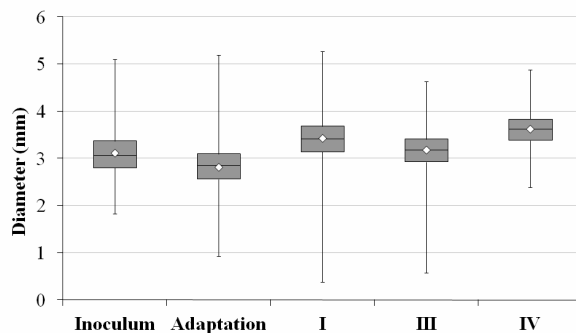

Figure 4: Box plot of the distribution of granule size in the inoculums, adaptation, stage I, III and IV.

The mean COD removal rate was approximately 90% in all stages, except stage III where the rate decreased to 80% (Table 1). During stage III, COD removal decreased to 9% due to aeration of the water supply and, subsequently, the efficiency increased to 80% after 26 days. In stage IV, the mean COD removal rate increased to 90%.

In all of the stages, the alkalinity and pH exhibited slight variations. The total alkalinity of the effluent varied from 290 to 360 mg CaCO$_3$.L^{-1}, and the pH of the effluent varied from 7.2 to 7.4 (Table 1).

The total suspended solids (TSS) content of the effluent suggested a remarkable loss of solids over time, and values ranged between 30 and 67 mg.L^{-1}. The amount of biomass in the reactor decreased until stage III (from 5.7 to 4.6 g TS.L^{-1}; Table 1). In stage IV, the solids content of the reactor decreased slightly to 4.5 g TS.L^{-1}.
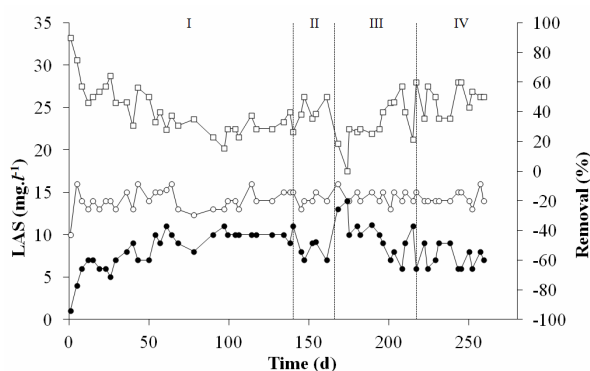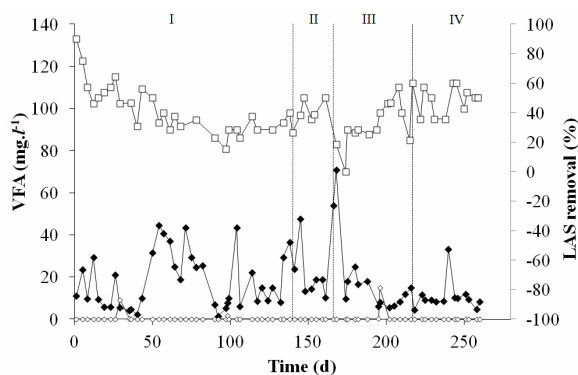
The LAS removal rate was approximately 40% in stages I and II (Table 1). Due to adsorption of LAS onto biomass, the LAS removal rate was high at the beginning of stage I (between 50 and 90%, Figure 5). In stage I, the LAS content of the effluent increased until the 60th day and stabilized at values around 8-11 mg.L^{-1} (Figure 5), which indicated decreasing adsorption. When the effluent stabilized (between the 60th day and the end of stage I), the LAS removal rate was 30±7%. In stage II, the LAS removal rate increased to 41 %, due to recirculation of the effluent. Aeration in the water supply decreased LAS removal to 31% (stage III). Subsequently, the LAS removal rate increased to 50% in stage IV (addition of yeast extract).

Table 1: Mean and standard deviation of the parameters analyzed in the UASB reactor.

Parameter	I	II	III	IV
Alkalinity (mg CaCO$_3$.l^{-1})				
Effluent				
Partial	230±20	210±20	230±30	270±20
Total	300±20	290±10	310±30	360±30
COD				
Influent (mg.l^{-1})	1200±100	1000±100	300±200	600±200
Effluent (mg.l^{-1})	130±60	100±40	70±60	60±20
Removal (%)	89±5	89±3	80±20	90±2
pH				
Influent	7.6±0.1	7.5±0.1	7.5±0.1	7.5±0.1
Effluent	7.2±0.1	7.2±0.2	7.4±0.1	7.3±0.1
LAS				
Influent (mg.l^{-1})	14±1	14±1	14±1	14±1
Effluent (mg.l^{-1})	8±2	9±2	10±2	7±1
Removal (%)	40±20	41±9	31±15	50±10
Total suspended solids effluent (mg.l^{-1})	44±8	31±9	54±8	67±9
Solids				
Total (g.l^{-1})	5.7±0.5	-	4.6±0.5	4.5±0.5
Volatile (g.l^{-1})	4.5±0.4	-	3.6±0.4	3.2±0.4
Fixed (g.l^{-1})	1.2±0.2	-	1.0±0.3	1.3±0.2
Stage time (d)	140	26	51	44

LAS removal was associated with the concentration of VFA and instability in the anaerobic process. Between the 80th and 120th days, the LAS removal rate decreased from 56% to 23%, while the acetic acid content of the effluent ranged from 20-45 mg.L^{-1}. In the beginning of stage III, LAS removal decreased to 20%, and the acetic acid content of the effluent increased to 70 mg.L^{-1} (Figure 6). In a thermophilic UASB reactor submitted to a temperature reduction from 50 to 32 °C (30 h, LAS 10 mg.L^{-1}), LAS removal was null, and the VFA content of the effluent was high (300 mg HAc.L^{-1}) (Lobner et al., 2005). The authors (Lobner et al., 2005) reported that LAS removal rates greater than 0.15 mg LAS.g TS^{-1}.d^{-1} were obtained at VFA concentrations less than 50 mg.L^{-1} in the aforementioned reactor.

Namely, inhibition of acetate and propionate degraders has been observed at LAS concentrations of 3-27 mg.L^{-1} (Mosche and Meyer, 2002; Garcia et al., 2006b), which increased the VFA content and led to instability in the bacterial consortium. Probably, the instability in the bacterial consortium decreased the LAS removal since the surfactant degradation requires the formation of consortium (Khleifat, 2006; Peressutti et al., 2008). Furthermore, the availability of readily degradable co-substrates, such as VFA, contributed to decreasing the removal of LAS. Sanz et al. (2003) observed a high LAS degradation rate (85%) in a UASB reactor after the co-substrates were withdrawn from the medium, while a reactor fed with co-substrates exhibited 64% efficiency.

Figure 5: LAS in the influent (○), effluent (●) and removal rate (□).

Figure 6: LAS removal (□) and the concentration of acetic (♦) and propionic (◊) acid.

According to the mass balance of LAS, the degradation efficiency was equal to 28.5% (Table 2), which was lower than that (50-85%) of other UASB reactors under mesophilic conditions (30-37 °C) (Sanz *et al*., 2003; Lobner *et al*., 2005). Namely, the influent concentration used in previous studies was lower than those employed in the present investigation, which decreased the inhibitory potential of LAS (Mosche and Meyer, 2002; Garcia *et al*., 2006b). Lobner *et al*. (2005) added 10 mg.L^{-1} of LAS, and Sanz *et al*. (2003) added 4-5 mg.L^{-1}. Furthermore, Lobner *et al*. (2005) used a reactor with an HRT of 48 h, while an HRT of 24 h was applied in the present study. The high HRT of the aforementioned reactor favored the degradation of recalcitrant substances such as LAS.

Table 2: Mass balance of LAS in the UASB reactor.

Mass balance (mg)	Final
Added	35,429
Effluent	23,306
Adsorbed on sludge	2,016
Degradation	10,107
(%)	28.5%
Time (d)	261

Using a complex co-substrate (yeast extract), the LAS removal rate was greater (50±10%) than in stages with only short-chain alcohols (30±7% in stabilized stage I and 41±9% in stage II). The absence of effluent recirculation in stage I contributed to the greater difference in the LAS removal rate, around 20%, while in stage II this difference was around 10%. Namely, effluent recirculation increases mass transfer, which enhanced LAS removal in an expanded granular sludge bed (EGSB) and fluidized bed reactors (Delforno *et al*. 2012; Oliveira *et al*., 2010). Moreover, the rate of biomass loss was 2 mg TS.d^{-1} in stage IV (with yeast extract), while this rate was 14 mg TS.d^{-1} in stages I and II. The addition of yeast extract provides micronutrients and vitamins that are needed by methanogenic archaea (Boone and Castenholz, 2001); therefore, this addition could have helped to stabilize the bacterial consortium, as seen in previous studies (Abboud *et al*., 2007; Khleifat, 2006).

Microbial Community Analysis

According to FISH analysis, the predominant microbial community of the inoculum was methanogenic archaea cells. In the inoculum, probe ARC915 attributed 63% to *Archaea*, and probe EUB338 attributed 37% to *Bacteria*. This microbial composition was maintained in adaption and stage I (ARC915 ≈ 64%; EUB338 ≈ 38%) until aeration of the water supply (stage III) decreased the amount of archaea cells (48%) in the microbial composition (Figure 7). After recovery, the microbial composition of archaea cells increased to 60% in stage IV.
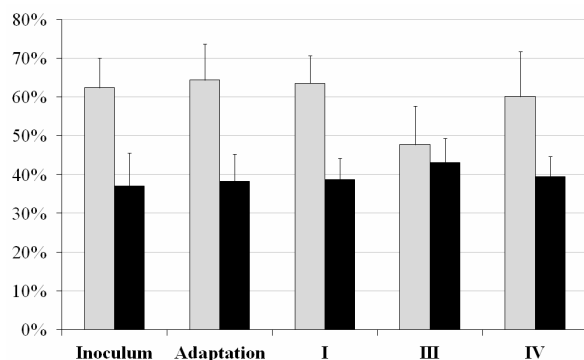

Figure 7: Microbial community composition determined by FISH in the inoculum, adaptation, stages I, III and IV, for probes ARC915 (■) and EUB338 (■). The error bars indicates standard error.

The total anaerobic bacteria and methanogenic archaea content estimated by MPN increased from stage I to IV. The total anaerobic bacteria content increased from 1.9×10^8 (stage II) to 3.8×10^{11} MPN.g TVS^{-1} (stage IV), and the methanogenic archaea content increased from 1.9×10^3 (stage I) to 4.4×10^7 MPN.g TVS^{-1} (stage IV). The primary differences between stages I and IV were the recirculation of effluent, introduction of instability in the anaerobic process, and yeast extract addition. Among these modifications, only effluent recirculation and yeast extract addition could increase the MPN estimates. The observed increase in the granule size in stage IV (95% of granules with a diameter greater 3 mm, Figure 4) suggested that the increase in the MPN estimate was due to yeast extract addition.

CONCLUSIONS

Addition of complex co-substrate (yeast extract) increased the LAS removal to 50% and efficiencies less than 41% were obtained using only ethanol and methanol as co-substrates (stages I and II). Using only short-chain alcohols (ethanol and methanol) as co-substrates, the rate of biomass loss was greater (14 mg TS.d^{-1}) than with yeast extract (2 mg TS.d^{-1}). Furthermore, an increase in the total anaerobic bacteria and methanogenic archaea content (three

and four log units, respectively) was related to addition of yeast extract. Therefore, the feeding with complex co-substrates (yeast extract) resulted in greater LAS removal and less biomass loss than feeding with short-chain alcohols (ethanol and methanol). Despite the greater LAS removal using a complex co-substrate, the use of short-chain alcohols is feasible considering the difference in LAS removal rate between stages II and IV (around 10%), which were maintained similar conditions (effluent recirculation).

FISH analysis suggested that changes in the microbial composition after LAS addition (stage I) were negligible, whereas aeration of water supply (stage III) decreased the amount of methanogenic archaea.

Aerating the water supply increased the acetic acid concentration to 70 mg.l^{-1} and decreased LAS removal to 20%. The observed relationship between the VFA concentration and LAS removal was due to LAS inhibition, which interfered with the bacterial consortium, decreasing LAS removal.

ACKNOWLEDGMENTS

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