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Mesophilic and thermophilic anaerobic digestion of biologically pretreated abattoir wastewaters in an upflow anaerobic filter

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ABSTRACT

The hydrolysis pretreatment of abattoir wastewaters (AW), rich in organic suspended solids (fats and protein) was studied in static and stirred batch reactors without aeration in the presence of natural microbial population acclimated in a storage tank of AW. Microbial analysis showed that the major populations which contribute to the pretreatment of AW belong to the genera *Bacillus*. Contrary to the static pretreatment, the stirred conditions favoured the hydrolysis and solubilization of 80% of suspended matter into soluble pollution. The pretreated AW, in continuous stirred tank reactor (CSTR) at a hydraulic retention time (HRT) of 2 days, was fed to an upflow anaerobic filter (UAF) at an HRT of 2 days. The performance of anaerobic digestion of biologically pretreated AW was examined under mesophilic (37 °C) and thermophilic (55 °C) conditions. The shifting from a mesophilic to a thermophilic environment in the UAF was carried out with a short start-up of thermophilic condition. The UAF ran at organic loading rates (OLRs) ranging from 0.9 to 6 g COD/L d in mesophilic conditions and at OLRs from 0.9 to 9 g COD/L d in thermophilic conditions. COD removal efficiencies of 80–90% were achieved for OLRs up to 4.5 g COD/L d in mesophilic conditions, while the highest OLRs i.e. 9 g COD/L d led to efficiencies of 70–72% in thermophilic conditions. The biogas yield in thermophilic conditions was about 0.32–0.45 L biogas/g of COD removed for OLRs up to 4.5 g COD/L d. For similar OLR, the UAF in mesophilic conditions showed lower percentage of methanization. Mesophilic anaerobic digestion has been shown to destroy pathogens partially, whereas the thermophilic process was more efficient in the removal of indicator microorganisms and pathogenic bacteria at different organic loading rates.

1. Introduction

Slaughterhouses and meat processing plants produces a large volume of effluents. The wastewaters generated at meat processing industry usually contain high amounts of biodegradable organic matter, with soluble and insoluble fraction. The insoluble fraction is formed by colloidal and suspended matter, in forms of fats, proteins and cellulose. In comparison to their treatability with other wastewater from many agro-processing industries, the abattoir wastewater has encountered significant problems. The high suspended solid content in the wastewater causes severe problems, due to their insolubility which slows the rate of degradation, and its tendency to form scums.

Physical–chemical methods and aerobic processes have been used for the treatment of this type of wastewater [1,2] and they are not regarded as suitable treatment options because of odours, high energy requirements for aeration, large quantities of sludge production and flotation sludge caused by denitrifying bacteria. Anaerobic digestion is becoming the subject of current research of organic waste management for several reasons, it helps to convert a large part of degradable organic carbon to biogas to be used for energy, and it reduces pathogens and minimises odours [3]. The advantages of anaerobic processes are biogas production, low generation of sludge, no aeration costs and elimination of pathogens [4,5].

Anaerobic treatment of abattoir wastewaters is not new and the use of systems for research, demonstration and full scale application has been reported since the 1950s [6]. However, traditional anaerobic processes are also limited by low rates of organic matter removal, long hydraulic retention time, accumulation of excessive residual organic matter and large reactor volume requirements [7]. The developments of high rate anaerobic biological reactors have overcome many of these previous objections. Mesophilic digestion usually requires a long retention time, but is not so efficient in the reduction of volatiles solids and the deactivation of pathogenic organisms. To overcome these limitations, interest in thermophilic

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digestion, using the higher metabolic rate of thermophilic microorganisms has increased. Thermophilic digestion is a little more sensitive to operational conditions, such as temperature, and the organic loading rate, as well as to the characteristics of the influent [8]. However, the application of the technology for the treatment of wastewaters generated in meat processing plants is still incipient, due to the problems with the accumulation of suspended solids and floating fats in the reactor, which lead to a reduction in the methanogenic activity and biomass washout. The success of the technology thus depends on an efficient primary treatment to reduce fats and suspended solids. Many studies have shown that the form of pollutants (suspended, colloidal or soluble) in the influent wastewater greatly affects the performance of high-rate anaerobic systems [9]. Removing solids before treatment becomes a common practice to remove solids, so that only the soluble part of the wastewater with perhaps small amounts of residual solids (up to 500 mg/L) is admitted to the digester without any problems related to clogging of solids [10]. Therefore, one way of improving the performance of digesters treating wastewaters with high content of suspended solids is to promote the hydrolysis of organic matter by pretreatment of the substrate. Several pretreatment methods of AW have been reported: physical–chemical methods including, mechanical [11] or thermo-chemical treatment [12] and biological methods such as thermophilic bacterial treatment [13] and enzymatic hydrolysis pretreatment [14].

In addition, abattoir wastewater carries high levels of pathogenic microorganisms that may constitute a serious risk to the human and animal health. Generally, anaerobic processes can be characterized from the digestion environments, microorganisms and process configuration, and each process has its unique advantages. Although, better performance at high strength with reduction or deactivation of pathogenic organisms can be obtained from thermophilic digestion [15].

The aim of this research was the combination and the evaluation of the performance of an efficient biological pretreatment for solubilization of the suspended solids (proteins and fats) and the anaerobic digestion of biologically pretreated AW under mesophilic and thermophilic conditions in an upflow anaerobic filter (UAF).

2. Materials and methods

2.1. AW sampling

The AW used in this study was collected from an abattoir factory in Tunisia. Abattoir wastewater arises from different steps of the process such as washing of animals, bleeding out, skinning, cleaning of animal bodies, cleaning of rooms. The wastewater contains blood, particles of skin and meat, excrements and other pollutants. It also contained varying amounts of wastewater from the washing of equipment and premises, which caused a big variation in the concentration of organic matter. The characteristics of the abattoir wastewaters before biological pretreatment are presented in Table 1.

2.2. Pretreatment procedure

Pretreatment assay was performed in closed glass flasks with a total volume of 250 ml containing 50 ml of AW previously acclimated in a storage tank in the presence of natural microbial population of the raw AW. Cultivation was conducted at 30 °C in a rotary shaker at 100 rpm and in static conditions. Samples were collected for analysis after 1–3 days of growth. Firstly, the effect of stirring on the hydrolysis and solubilization of AW rich in organic suspended solids will be determined, and on the basis of the obtained results, the optimum HRT will be determined to operate in continuously anaerobic tank.

Table 1
Physico-chemical and microbiological characteristics of raw abattoir wastewater (AW) used in this work.

Parameter	Abattoir wastewater
pH	6.8–7.4
Conductivity (ms/cm)	1.98–2.9
TS (mg/L)	5060–5400
TSS (mg/L)	1500–2500
TCOD (mg/L)	5800–6100
SCOD (mg/L)	1800–2500
Total kjeldhal nitrogen (mg/L)	530–810
N–ammoniacal (mg/L)	130–280
Phosphorous (mg/L)	15–50
Total soluble protein (mg/L)	1950–3600
Fats (mg/L)	40–410
Total aerobes (CFU/ml)	7×10^7 to 8×10^8
Lactic acid bacteria (CFU/ml)	2×10^2 to 6×10^4
Bacillus (CFU/ml)	4×10^5 to 4×10^6
Total coliforms (MPN/ml)	11×10^6 to 20×10^8
Faecal coliforms (MPN/ml)	45×10^3 to 85×10^4
Total Streptococci (MPN/ml)	1.6×10^3 to 2.3×10^3
Faecal Streptococci (MPN/ml)	90–300
*Pseudomonas**	+
*Staphylococcus aureus**	+
*Salmonella**	+

T: total solids; TSS: total suspended solids; SCOD: soluble COD; TCOD: total COD.
*(+) detected.

2.3. Experimental unit

A schematic representation of the anaerobic digestion systems used for the experiments are shown in Fig. 1. A continuously stirred tank reactor (CSTR) with variable working volume (2–15 L) was used to feed the UAF with a biologically pretreated AW. Mixing was assured by the continuous rotation of the magnetic stirrer. A settler (20 cm in diameter and 37 cm in height) was used to remove the total suspended solids (TSS). The mesophilic and thermophilic anaerobic digestion of the AW was carried out in a 5 L continuous upflow anaerobic filter consisting of glass column of 30 cm in height and 20 cm in diameter. The UAF was filled with Flocor (Φ3L3, porosity 95%, specific surface 230 $m^2\ m^{-3}$) as a media support entities for the growth of microorganisms. The anaerobic filter was initially operated during 120 days at the optimal mesophilic temperature range (37 ± 1 °C) and during 140 days at the optimal thermophilic temperature range (55 ± 1 °C). The temperature was maintained constant at each condition by circulating water through the water jacket of the reactor. The mesophilic digester was fed initially with an organic loading rate of 0.9 g COD/L d and at hydraulic retention time of 5 days. Then, the organic loading rate (ORL) was increased gradually by varying the HRT, from 2.5 days (ORL = 1.8 g COD/L d) to 18 h (ORL = 6 g COD/L d). The start-up of the thermophilic UAF was brought by increasing the temperature of the mesophilic UAF from 37 to 55 °C in a single step with a simultaneous decrease of the OLR from 6 to 0.9 g COD/L d. The organic loading rate was increased gradually by varying the HRT, from 2.5 days (ORL = 1.8 g COD/L d) to 12 h (ORL = 9 g COD/L d) at thermophilic condition. The system was fed by a peristaltic pump connected to a programmable timer.

2.4. Analytical methods

The effluent from the anaerobic filter was collected daily, centrifuged at 7000 rpm for 10 min and analysed for SCOD. The total and soluble COD were measured spectrophotometrically [16]. Total solids (TS), TSS, total nitrogen, nitrogen–ammonium ($N–NH_4^+$) and fats were determined according to the procedure listed in Stan-

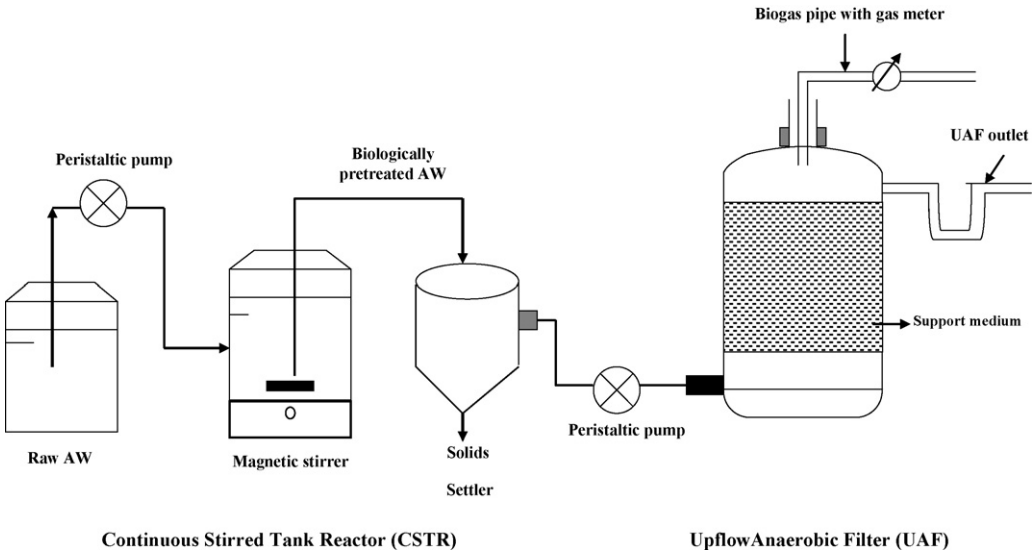

Fig. 1. Schematic diagram of the laboratory experimental set-up used for abattoir wastewater treatment in two stage process: Biological pretreatment in CSTR and mesophilic and thermophilic anaerobic digestion in UAF.

dards Methods for the Examination of Water and Wastewater [17]. The total soluble proteins were determined by the method of Bradford [18]. The dissociation constant for the ammonium ion (p*K*a) was calculated based on this equation [19,20] as follows:

$$pK_a = 0.09018 + \frac{2729.92}{T + 273.25};\ T \text{ is the temperature in } ^\circ C$$

The biogas produced was collected daily in plastic bags at room temperature. The total volume was later determined with a wet gas meter and time to time the methane content was estimated using an ORSAT apparatus. In this way the biogas volume productions of mesophilic and thermophilic reactors were directly comparable. Dissolved volatile fatty acids (VFA) in digested effluents were measured by HPLC (Waters) equipped with a polypore H column (250 mm by 7.8 mm [inside diameter]) connected to a detector (RI-401 Waters). The mobile phase was 0.02N H_2SO_4 at a flow rate of 0.6 ml min^{-1}. It was centrifuged 15 min at 13,000 rpm and filtered through 0.22 μm filter (Millipore) before use. The volume of injection was 20 μl. Bacterial growth was monitored by direct counting of colony forming units (CFU/ml) determined by plating 0.1 ml of serial dilutions on MRS agar (Man Rogosa and Shapman), plate count agar (PCA) and brain heart infusion agar (BHI) (Merck) for the enumeration of the *Lactobacillus* strains, the total aerobes and the *Bacillus* strains, respectively. API 50 CHB and API 20 E galeries (Biomerieux) were used to test biochemical characteristics of *Bacillus* strains. For both mesophilic and thermophilic conditions and at each OLRs, the anaerobic treatment was evaluated for its effectiveness in reducing indicator microorganisms and pathogens (total and faecal coliforms, total and faecal *Streptococcus*, *Pseudomonas*, *Staphylococcus aureus* and *Salmonella*) using the most probable number technique (MPN) [21]. Based on dilutions down to nearly 1 remaining bacterium per test tube (three-fold setups repeated two times), the exit concentration can be estimated statistically.

2.5. Statistical analysis

The analyses of the different parameters during the running of anaerobic process were done daily in triplicate at steady-state. Steady-state conditions were assumed when the coefficient of variation for measured parameters was less than 10%. Average steady-state data and the standard error presented in the paper were calculated as a mean value. The data were analysed using ANOVA with statistically significant differences for $p < 0.05$ [22]. The statistical program used was STATISTICA 6.0. The ANOVA analysis was performed in order to evaluate the influence of the operating conditions during the pretreatment (static and stirred conditions), the anaerobic treatment and the disinfection (OLRs and temperature).

3. Results and discussion

3.1. Microbial pretreatment of abattoir wastewaters

The main contributors of the TSS forming in the AW are bloods and colloidal materials and usually during storage, the precipitation phenomena and the coagulation process of these components take places in the feeding tank. To overcome this problem, the pretreatment of abattoir wastewater was conducted in order to standardize the effluent and to solubilize the TSS into more soluble COD by the natural bacterial population present in AW without aeration at 30 °C. The main results obtained with batch pretreatment of AW under static and stirred conditions in terms of SCOD, TCOD and TSS are given in Fig. 2.

It was observed that during pretreatment of AW at static conditions, total COD decreased with the increase of TSS as a result of the precipitation phenomena induced by protein coagulation and bacterial growth associated with low biodegradation of organic matter (Fig. 2a). Under stirred conditions, the TCOD remained practically constant and the SCOD increased by 64%.

The bacterial populations present in AW are total aerobes, lactic acid bacteria (LAB), *Bacillus* and coliforms (Table 1). The biological systems are complex, and the natural microflora present in AW is competing for nutrients according to the environmental conditions. It is likely that rapid growth of a dominant population could restrict the growth of others organisms simply by uptake of the easily metabilizable nutrients or even by physical occupation of available space. In addition, the stirring would provide the facultative strains with oxygen and as consequence result in more favourable growth conditions. Using the morphological and biochemical characteristics, the microbial dominant populations during the pretreatment under stirred condition were identified as *Bacillus* species. The different *Bacillus* species detected were *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. amyloliquifaciens* and *B. subtilis*.

Table 2
Bacillus spp. and lactic acid bacteria (LAB) count during the pretreatment of AW in static and stirred batch reactors at 30 °C.

CFU/ml	Operating conditions						*p* value
	Static condition			Stirred condition			
	$T=0$ h	$T=24$ h	$T=48$ h	$T=0$ h	$T=24$ h	$T=48$ h	
Bacillus spp.	$5\times10^5\pm0.5$	$6.21\times10^5\pm2$	$6.7\times10^6\pm1$	$5.10^5\pm0.5$	$4.37\times10^7\pm2$	$9\times10^8\pm3$	0
Lactic acid bacteria	$3\times10^3\pm1$	$4.62\times10^4\pm1$	$6.84\times10^4\pm1$	$3.10^3\pm1$	$1.11\times10^3\pm2$	$4.2\times10^3\pm1$	0
p value		0.001			0.001		
p value		0.001			0.001		

Results of each experiment are averages of three samples. *p*-values were determined during the pretreatment and between static and stirred conditions.

In fact, the agitation stimulates *Bacillus* growth (10^7–10^8 CFU/ml) and inhibits LAB growth (10^3 CFU/ml) (Table 2) because of oxygenation and shear stress [23]. Statistical analysis of the data indicated also that the growth of *Bacillus* and LAB strains was statistically significant ($p<0.05$) between the static and stirred conditions and during the pretreatment (Table 2).

These results support our hypothesis that stirring improved proteins and polymeric carbohydrates degradation efficiency by hydrolytic enzymes produced by *Bacillus* such as proteases and lipases to break down and solubilize the macromolecular structures into monomers such as amino acids and glycerol and long-chain fatty acids [24,25]. In fact, bacteria of the genus *Bacillus* are active producers of different enzymes. The strains of *Bacillus* presenting a proteolytic activity included *B. subtilis*, *B. licheniformis* and *B. circulans* [25–28]. Under defined and optimized conditions, *B. circulans* and *B. subtilis* and *B. coagulans* were also able to produce lipases [29,31].

The use of microbial population and its hydrolytic enzymes for the pretreatment of particles-rich wastewater to increase the rate of solubilization of particulate matter and to improve the anaerobic treatment has been demonstrated [32,33]. However, there are no much works which elucidate the action of *Bacillus* sp. and its enzymes on the pretreatment of agroindustrial wastewaters. Previously, Okuda et al. [34] studied the treatment of lipid-containing wastewater using *Bacillus* sp. which assimilates lipids and Vasala et al. [35] reported the contribution of proteolytic microbes (*Bacillus megaterium*) on the pretreatment of cheese whey in order to improve lactic acid production by *Lactobacillus salivarius*.

Biological pretreatment of raw AW can also reduce the adverse impact of the high content of suspended and colloidal components on the performance of UAF, leading to clogging the installation and deterioration of the microbial activity and washout of active biomass. Saddoud and Sayadi [36] also reported that the pretreatment of abattoir wastewater in an acidogenic step reduced the membrane fouling of the anaerobic membrane bioreactor (AMBR) successive methanogenic step.

According to the obtained results, the pretreatment of raw AW using natural microflora for solubilization of suspended solids into soluble organic matter could be conducted in 2.5 L stirred tank reactor in order to feed continuously the UAF. The pretreated AW had an average SCOD, TSS concentration and soluble protein content of approximately 4.5, 0.4 and 1.044 g/L, respectively, obtained after biological pretreatment and settling (Table 3). From the conditions tested, the best results were obtained when the hydrolysis was performed for 2 days, reaching important SCOD and stable TSS. The enhancement of pretreatment efficiencies was obtained at an HRT of 2 days. This HRT was applied in continuous system because the system was able to maintain above 80% of solubilization of organic matter into soluble COD.

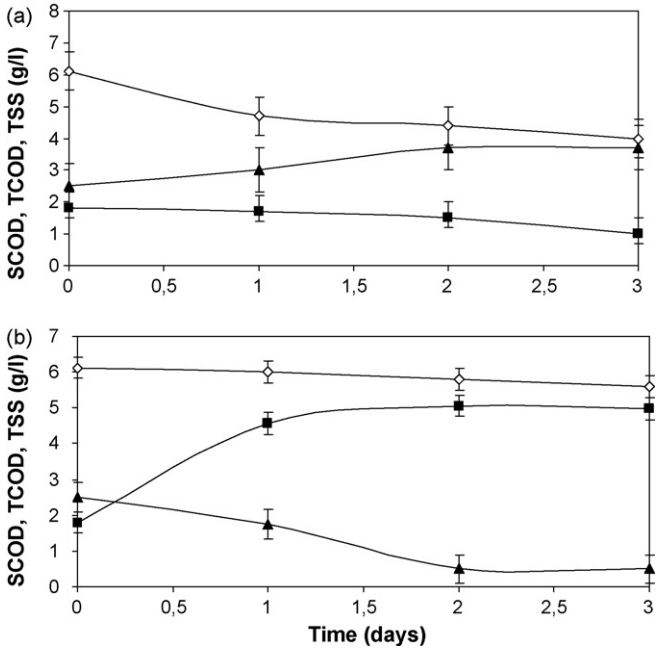

Fig. 2. TSS (▲), SCOD (■) and TCOD (◊) of AW after pretreatment using a natural bacterial population present in the raw AW in static (a) and stirred (b) batch reactors at 30 °C.

3.2. Mesophilic anaerobic digestion of AW in UAF

Pretreated wastewater was applied initially at an OLR of 0.9 g COD/L d corresponding to a hydraulic retention time (HRT) of 5 days. The OLR was progressively increased by varying the HRT, from this value to 6 g COD/L d (HRT = 18 h). The results for the different loading regimes and hydraulic retention time are presented in Fig. 3.

The UAF showed a stable behaviour up to an OLR of 2.8 g COD/L d reaching COD removal efficiencies between 90% and 92%. During this operational period (the first 80 days), the biogas production rate was increased from 0.24 to 0.95 L/L d by decreasing the HRT from 5 to 1.66 days, respectively. As the digester loading rate was increased from 2.8 to 4.5 g COD/L d (80–120 days), the COD removal decreased slightly and ranged between 80% and 85%. Biogas production was improved by the increase of the OLR until 4.5 g COD/L d; it averaged from 0.24 (77% of methane) to 1.1 L/L d (68% of methane) (Table 3, Fig. 3). However, the biogas yield declined from 0.30 to 0.15 L/g COD removed. Ruiz et al. [37] showed that the decrease of OLR above 3 g COD/L d dropped the COD reduction below 65% in an anaerobic mesophilic digestion of slaughterhouse wastewater. However, Tritt [38] reported that a decrease of COD removal from 80% to 30% was observed by increasing the OLRs from 2.5 to 18 g TCOD/L d in an anaerobic filter treating raw slaughterhouse wastewater. Anaerobic digestion of the same wastewater after 2 h settling period improved COD reduction by additional 10–15%. These results supported that the pretreatment step showed a significant improvement in process efficiency as measured by COD removal, and eventually biogas conversion.

Table 3
pH_{outlet}, TSS_{inlet}, TSS_{outlet}, $SCOD_{outlet}$, methane content, total VFA and N–ammonium obtained with mesophilic and thermophilic anaerobic digestion of AW at different OLRs and HRTs.

Different organic loading rates; different hydraulic retention time (OLRs: g/L d; HRT:d)									
	Mesophilic								
Runs (d)	(0.9; 5) 1 → 30	(1.8; 2.5) 31 → 63	(2.8; 1.66) 64 → 84	(3.6; 1.25) 85 → 106	(4.5; 1) 107 → 121	(6; 0.75) 122 → 140			*p* value
pH_{outlet}	7.49 ± 0.37	7.55 ± 0.41	7.60 ± 0.36	7.62 ± 0.34	7.73 ± 0.28	7.86 ± 0.19			0.03071
TSS_{inlet} (g/L)	0.4 ± 0.05	0.4 ± 0.05	0.45 ± 0.05	0.4 ± 0.02	0.4 ± 0.05	0.4 ± 0.03			nd
TSS_{outlet} (g/L)	0.012 ± 0.001	0.03 ± 0.005	0.05 ± 0.01	0.068 ± 0.01	0.077 ± 0.01	0.083 ± 0.01			0
$SCOD_{outlet}$ (g/L)	0.39 ± 0.01	0.42 ± 0.02	0.45 ± 0.05	0.65 ± 0.03	0.8 ± 0.02	0.9 ± 0.02			0
CH_4 content (%)	77 ± 2.1	75 ± 2	72 ± 2	70 ± 1.4	68 ± 2.3	65 ± 3.1			0
Total VFA (mg/L)	90 ± 5	118 ± 10	124 ± 8	180 ± 14	265 ± 11	390 ± 23			0
N–ammonium (mg/L)	579 ± 28	643 ± 40	752 ± 77	814 ± 71	995 ± 147	1270 ± 180			0.00004
	Thermophilic								
Runs (d)	(0.9; 5) 1 → 30	(1.8; 2.5) 31 → 60	(2.8; 1.66) 61 → 86	(3.6; 1.25) 87 → 108	(4.5; 1) 109 → 122	(6; 0.75) 123 → 129	(7; 0.66) 130 → 135	(9; 0.5) 126 → 141	*p* value	*p* value
pH_{outlet}	7.58 ± 0.44	7.72 ± 0.64	7.77 ± 0.29	7.97 ± 0.25	8.03 ± 0.16	8.16 ± 0.11	8.26 ± 0.02	8.32 ± 0.35	0.00001	0.00001
TSS_{inlet} (g/L)	0.5 ± 0.01	0.4 ± 0.02	0.45 ± 0.03	0.4 ± 0.05	0.35 ± 0.05	0.45 ± 0.02	0.4 ± 0.05	0.45 ± 0.05	nd	nd
TSS_{outlet} (g/L)	0.023 ± 0.002	0.033 ± 0.001	0.067 ± 0.05	0.07 ± 0.02	0.081 ± 0.05	0.135 ± 0.022	0.142 ± 0.01	0.165 ± 0.01	0	0
$SCOD_{outlet}$ (g/L)	0.30 ± 0.01	0.31 ± 0.04	0.32 ± 0.03	0.32 ± 0.01	0.5 ± 0.1	0.8 ± 0.2	1.08 ± 0.1	1.26 ± 0.5	0.0003	0
CH_4 content (%)	78 ± 1.7	76 ± 0.5	75 ± 1.1	74 ± 1.4	74 ± 1.2	70 ± 5	65 ± 2.01	63 ± 5.1	0	0
Total VFA (mg/L)	121 ± 11	153 ± 20	175 ± 14	253 ± 9	320 ± 26	442 ± 17	730 ± 13	965 ± 20	0	0
N–ammonium (mg/L)	627 ± 14	756 ± 21	843 ± 66	917 ± 49	1020 ± 120	1388 ± 70	1570 ± 180	2060 ± 225	0	0

Protein content of the effluent from CSTR (g/L) = 1.044 ± 0.09; SCOD (g/L) = 4.5 ± 0.5; TCOD (g/L) = 6.1 ± 0.2; nd: not determined. Results of each experiment are averages of three samples. *p* values were determined for each OLRs in mesophilic and thermophilic conditions and between mesophilic and thermophilic conditions.

At an OLR of 6 g COD/L d, the UAF performance declined (Fig. 3). Consequently, a decrease of biogas yield was observed (0.20–0.15 L biogas/g COD removed) and the COD removal efficiency ranged between 77% and 80%. The reason of the biogas decrease was due to the inability of mesophilic bacterial biomass to survive at low HRT. It seems that the protein hydrolysis and the amonification rates were higher than the methanization rate, which affects the methanogenic bacteria activity, resulting from a high level of ammonium nitrogen ($N–NH_4^+$) (995–1270 mg/L).

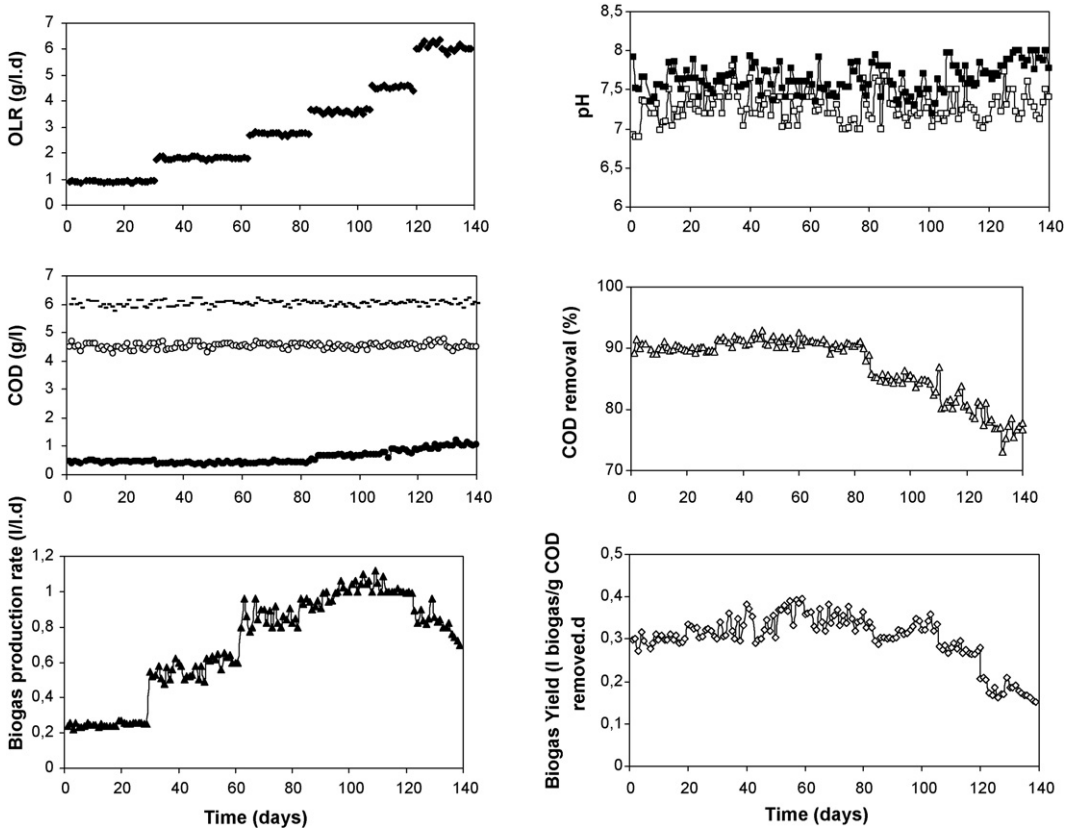

Fig. 3. pH variation of the influent (□), effluent (■), $TCOD_{inlet}$ (–), $SCOD_{inlet}$ (○), the $SCOD_{outlet}$ (●), COD removal (△), the biogas production rate (▲) and the biogas yield (◊) at different OLRs (♦) during anaerobic digestion of the mixture AW in UAF at mesophilic condition.

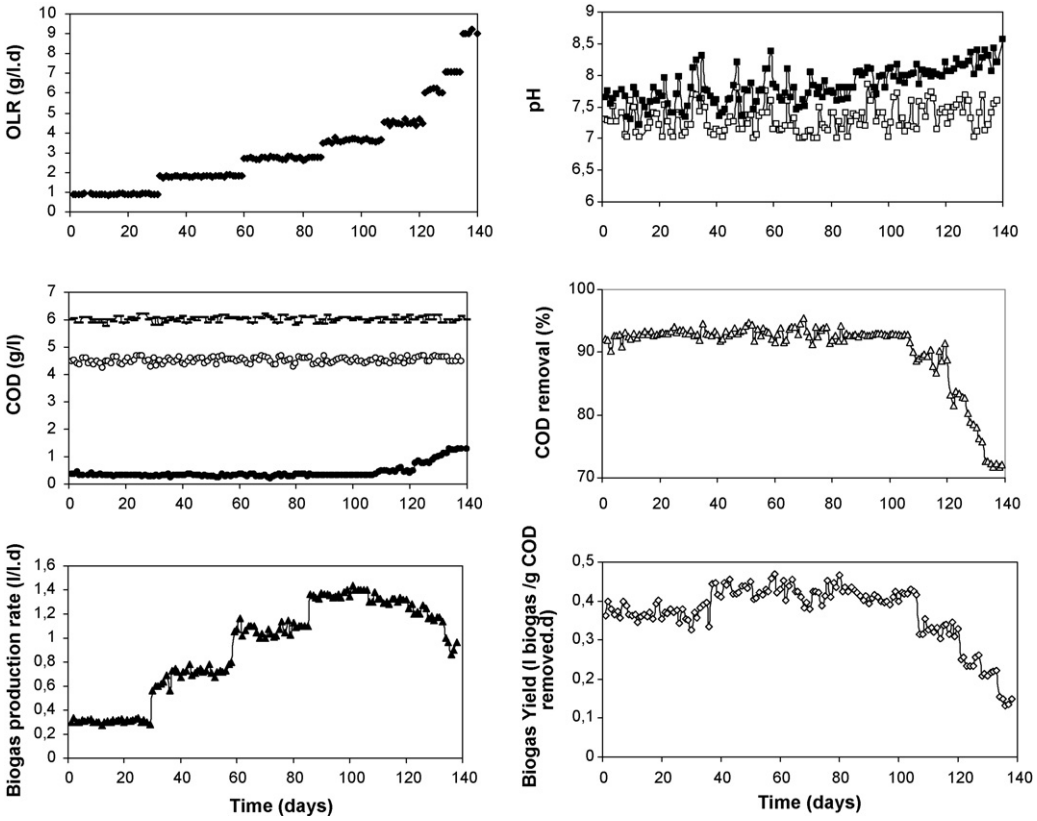

Fig. 4. pH variation of the influent (□), effluent (■), $TCOD_{inlet}$ (−), $SCOD_{inlet}$ (○), the $SCOD_{outlet}$ (●), COD removal (△), the biogas production rate (▲) and the biogas yield (◊) at different OLRs (♦) during anaerobic digestion of the mixture AW in UAF at thermophilic condition.

During the operational period, the effluent pH remained between 7.5 and 7.9 showing a high buffering capacity in the digester. The concentration of total volatile fatty acids (VFA) in the reactor effluent was between 90 and 390 mg/L. These values were much lower than the concentrations reported in other studies of poultry slaughtering wastes, in which their accumulation caused inhibition of the anaerobic process [39,40].

3.3. Thermophilic anaerobic digestion of AW in UAF

The start-up of the thermophilic UAF was brought by increasing the temperature from 37 to 55 °C. When the steady-state was reached, the OLR was gradually increased from 0.9 to 9 g COD/L d by decreasing the HRT from 5 to 0.5 days. Nevertheless, the optimal transition of anaerobic digestion systems from mesophilic to thermophilic conditions is not clearly defined. Several researchers have studied the procedure of the thermophilic start-up in terms of how to increase the temperature from the mesophilic to thermophilic range. Most researchers showed that a one step increasing temperature from mesophilic to thermophilic was the best strategy in changing operational temperature in anaerobic digestion [41,42]. For the treatment of food waste, Ortega et al. [43] mentioned that a fully adapted inoculum was developed by eliminating the initial time-consuming acclimatization stage from mesophilic to thermophilic conditions. The fast adaptation of the mesophilic sludge to the thermophilic conditions indicates the presence of thermophilic microorganisms in the mesophilic inoculum. Mata-Alvarez [44] observed a transition from mesophilic to thermophilic conditions (35–55 °C in 10 days) requiring significant variations in organic loading (from 15% to 40% over 2 days) without permanent effect on the process performance. As expected from the previous reports and also confirming them, the one step increase of temperature coupled to a reduction of OLR was very efficient in our case since stable thermophilic methanogenesis was achieved within a period of 2 weeks.

Stable removal COD efficiency in the range of 93% was achieved at OLRs ranging from 0.9 to 3.6 g COD/L d The biogas production rate increased from 0.35 to 1.4 L/L d as the OLR increased from 0.9 to 4.5 g COD/L d. The change of temperature leads to enhance the degradation of organic matter associated with higher biogas production [45]. As the digester OLR was increased from 3.6 to 4.5 g COD/L d, both the COD removal and the biogas yield declined (Fig. 4). From day 120 to 130, a reduction in removal efficiency to as low as 75% was observed, this could be related to the combined effects of high OLR and low HRT.

At the end of the 130-day period at OLR of 9 g COD/L d, the biogas yield decreased dramatically to achieve 0.15 L biogas/g COD removed, indicating the inhibition of methanogenic bacteria. An important decrease in the methane content from 70% to 63% was observed by increasing the OLR from 6 to 9 g COD/L d (Table 3). Under such conditions, the COD removal efficiency decreased and the VFA was above 965 mg/L. The nitrogen ammonium concentration of the effluent during different applied OLRs varied between 627 and 2060 g/L. The pH of the effluent varied in the range of 7.9–8.3 and the highest values were observed at high OLR of thermophilic digestion. The increase in the buffer capacity of the reactor is mainly due to the relatively high concentration of ammonium nitrogen. High concentration of ammonia would decrease the methanogens activity and further accumulation can lead to process failure [46]. Braun et al. [47] working on the anaerobic digestion of liquid piggery manure reported that the pH of the effluent was about 8 and the VFAs accumulated to 316 mg/L. Adjustment of pH to 7.4 led to reutilization of VFAs and lowered VFAs concentrations to 20 mg/L. It should also be noted that both methanogenic and acidogenic microorganisms have their optimal pH. Failing to maintain pH within an appropriate range could cause reactor failure

Table 4
Removal of indicator microorganisms and pathogenic bacteria at different organic loading rates and hydraulic retention times in mesophilic and thermophilic anaerobic digestions.

Removal efficiency (as $\log_{10}$ MPN/ml)	Operating conditions									
	Different organic loading rates; different hydraulic retention time (OLRs: g/L d; HRTs:d)									
	Mesophilic									*p* value
	(0.9; 5)	(1.8; 2.5)	(2.8; 1.66)	(3.6; 1.25)	(4.5; 1)	(6; 0.75)				
Total coliforms	3.1 ± 0.1	2.5 ± 0.01	1 ± 0.1	0.8 ± 0.02	0.4 ± 0.3	0.4 ± 0.05				0
Faecal coliforms	1.5 ± 0.35	1.1 ± 0.02	0.8 ± 0.1	0.4 ± 0.1	0.4 ± 0.04	0.2 ± 0.15				0
Total *Streptococci*	1 ± 0.5	1 ± 0.01	0.8 ± 0.02	0.4 ± 0.2	0.35 ± 0.1	0.3 ± 0.15				0
Faecal *Streptococci*	1 ± 0.5	0.8 ± 1.1	0.8 ± 0.05	0.4 ± 0.3	0.2 ± 0.2	0.2 ± 0.2				0
Pseudomonas	–	–	–	–	–	+				
Staphylococcus aureus	–	–	–	–	–	+				
Salmonella		–	–	–	–	+(*)				
	Thermophilic									
	(0.9; 5)	(1.8; 2.5)	(2.8; 1.66)	(3.6; 1.25)	(4.5; 1)	(6; 0.75)	(7; 0.66)	(9; 0.5)	*p* value	*p* value
Total coliforms	4 ± 0.5	3 ± 0.03	2.3 ± 0.02	2 ± 0.1	1.9 ± 0.04	1.7 ± 0.1	1 ± 0.5	0.8 ± 0.02	0	0
Faecal coliforms	3 ± 0.2	2.5 ± 0.3	2.5 ± 0.2	2.5 ± 0.05	1.7 ± 0.2	1.8 ± 0.1	1.7 ± 0.15	1 ± 0.02	0	0
Total *Streptococci*	1.5 ± 0.02	1.3 ± 0.2	1.5 ± 0.3	nd	1 ± 0.1	0.8 ± 0.05	0.4 ± 0.2	0.4 ± 0.15	0.00004	0
Faecal *Streptococci*	–	–	–	–	–	–	–	–		
Pseudomonas	–	–	–	–	–	–	+	+		
Staphylococcus aureus	–	–	–	–	–	–	+	+		
Salmonella	–	–	–	–	–	–	+(*)	+(*)		

Results of each experiment are averages of three samples and given as $\log_{10}$ MPN/ml. *p* values were determined for each OLRs in mesophilic and thermophilic conditions and between mesophilic and thermophilic conditions. (*): presence of *S. Arizonae*, (–): none detected, (+): detected, nd: not determined.

although ammonia is at a safe level [48]. Zeeman et al. [49] also reported that reducing pH from 7.5 to 7 during thermophilic anaerobic digestion of cow manure increased the methane production by four times.

3.4. Evolution of performances of the UAF treating pretreated AW from mesophilic to thermophilic conditions

The average values of pH_{outlet}, $SCOD_{outlet}$, TSS_{inlet}, TSS_{outlet}, methane content, VFA and nitrogen ammonium parameters for each run are presented in Table 3. At OLRs ranging from 0.9 to 6 g COD/L d, the levels of SCOD in the effluents of mesophilic and thermophilic digesters were comparable, 390–900 mg/L and 300–800 mg/L, respectively. Under these conditions, the UAF showed stable operation for both mesophilic and thermophilic digestions. The VFA levels in thermophilic digester increased with the increase of OLR from 6 to 9 g COD/L d (442–965 mg/L). Organic loads used in this work were considerably comparable with those found in the literature [38,50,51]. Higher COD loadings appear to lead to poorer performance. Hence, effective biological pretreatment of raw abattoir wastewater to remove suspended solids in our study was essential to improve the reactor performance.

Many studies on the anaerobic treatment of slaughterhouse wastewater conducted with anaerobic filter reactors [52], upflow anaerobic sludge blanket reactors [53] and anaerobic membrane bioreactors [36] reported the problems of TSS which must be removed or solubilized. They suggested that a pretreatment to hydrolyse a part of the particles could accelerate the anaerobic treatment of the wastewater.

The pH of the mesophilic and thermophilic process increased with the increase of the OLR in both cases. This was a result of high conversion level of organic nitrogen to ammonia under high OLRs. In fact, high level of free ammonia (FA) would result in increased toxicity. Angelidaki and Ahring [54] and Angelidaki et al. [55] showed that the interaction between FA, VFAs and pH may lead to an "inhibited steady state", a condition where the process is running stably but with a lower methane yield. The pH of the reactor ranged between 7.5 and 8.3 and probably above critical threshold toxicity value, especially under thermophilic conditions. In fact, the dissociation constant for the ammonium ion (p*K*a) decreased from 8.89 to 8.31 by increasing the temperature from 37 to 55 °C. Therefore, the concentration of FA increased by increasing the p*K*a value [18,19].

In comparison to their treatability with other wastewaters from many agro-processing industries, the AW has encountered significant problems. Recent studies have shown that the anaerobic co-digestion of AW with other organic wastewaters has been proposed as a solution to the problems mentioned above. The content of nutrients can thereby be balanced, and the negative effect of toxic compounds on the digestion process may be decreased giving an increased gas yield and energy [56–58].

In fact, the ANOVA analysis showed that pH, TSS, SCOD, VFA, N–ammonium of the effluent and the methane content were statistically significant ($p < 0.05$) between all OLRs in mesophilic and thermophilic conditions. These analyses showed also that the different parameters determined were statistically significant ($p < 0.05$) between the two tested temperatures 35 and 55 °C (Table 3).

The reduction of indicator microorganisms (total and faecal coliforms; total and faecal streptococci) and selected pathogens (*Salmonella*, *Pseudomonas*, *S. aureus*) was examined during the mesophilic and thermophilic anaerobic digestions (Table 4). As may be observed, a satisfactory reduction of total and faecal coliform counts was achieved under mesophilic conditions at OLRs ranging from 0.9 to 3.6 g COD/L d ($3.1\log_{10}$–$0.8\log_{10}$ and $1.5\log_{10}$–$0.4\log_{10}$, respectively). The residual numbers ranged from 10^3 to 10^4 MPN/ml and from 10^2 to 10^3 MPN/ml for total and faecal coliform counts, respectively. At an OLR of 4.5 and 6 g COD/L d, the residual numbers present in the digested effluent still high, it was in the range of 10^5–10^3 MPN/ml for the total and faecal coliform, respectively. However, only a $1\log_{10}$ was obtained for total and faecal *Streptococcus*. The effluent contained less than 10^2 MNP/ml total and faecal *Streptococcus*. The removal of all tested bacteria decreased then with the increase of the OLR from 3.6 to 6 g COD/L d and remained low ($<1\log_{10}$). This difference in removal efficiency of the bacteria tested in mesophilic conditions may be depending on the decrease of HRT and to the inefficient mixing, which can present dead zones and

hydraulic short circuits in the reactor [59]. The ANOVA analysis the removal of the indicator and pathogenic bacteria showed significant differences ($p < 0.05$) at different OLRs in mesophilic conditions.

The thermophilic process was apparently more efficient in the reduction of total ($4\log_{10}$–$1.7\log_{10}$) and faecal coliforms ($3\log_{10}$–$1.8\log_{10}$), total *Streptococci* ($1.5\log_{10}$–$0.8\log_{10}$) at the same OLRs applied in mesophilic conditions (0.9 to 6 g COD/L d). The residual number of total coliform, faecal coliform and total *Streptococci* were in the range of 10^2–10^5, 10^1–10^3, 10^2 MPN/ml, respectively. In addition, the populations of faecal *Streptococci*, *Pseudomonas*, *S. aureus* and *Salmonella* were undetectable. The most resistant microorganisms at relatively high OLRs for both mesophilic (OLR = 6 g COD/L d) and thermophilic conditions (OLR = 7 and 9 g COD/L d) are faecal *Streptococci*, *Pseudomonas*, *S. aureus* and *Salmonella*. Smith et al. [60] demonstrated that *Salmonella* spp. is not damaged by mesophilic temperatures, whereas rapid inactivation occurred by thermophilic digestion. The detected *Salmonell*a was identified as *S. Arizonae*. Although, the Arizona subgroup may be isolated from a wide variety of nonhuman and human sources, the Arizonae are uncommonly recognized as human pathogens, and surprisingly little is known about their epidemiology.

The anaerobic thermophilic digester presents higher efficiency on the removal of pathogens, than the mesophilic digester. The important reduction achieved of indicator and pathogens could be attributed to different factors which can cause pathogen decay during treatment such as temperature, retention time, reactor configuration, microbial competition, pH value and chemical interactions.

The ANOVA analysis of the data indicated that the removal of total and faecal coliforms, total and faecal Streptococci were statistically significant ($p < 0.05$) for each OLR in thermophilic conditions. A comparison of the removal of the bacteria tested between mesophilic and thermophilic conditions showed also significant differences (Table 4).

4. Conclusions

The microbial pretreatment was based on the hydrolysis and solubilization activities of natural microflora present in AW under stirred condition. The dominant bacteria in this wastewater are identified as *Bacillus* species. Maximal removal of 80% of TSS into more soluble COD was obtained under stirred conditions. The ecological pretreatment could be an attractive solution since the TSS considered as a limiting step for the biogas generation and organic matter removal in the anaerobic digestion process.

The mesophilic UAF proved to be efficient for the treatment of pretreated AW with an average organic loading rate of 6 g COD/L d. Under thermophilic conditions, the anaerobic digestion of pretreated AW showed an improvement in terms of COD removal and biogas yield. These higher performances of thermophilic anaerobic digestion might be mainly attributable to selection of the active anaerobic microorganisms, as a result of the sludge exchange between mesophilic and thermophilic digesters. However, the increase of the OLR (9 g COD/L d) caused a decrease of the thermophilic UAF performance, especially due to the inhibition of methanogenic bacteria, resulting from the accumulation of ammonium nitrogen at high OLR. Thermophilic anaerobic digestion of pretreated AW may be considered as an efficient treatment for organic load reduction and biogas production, and also for pathogens removal, being an important contribution for a global and integrated schema of pollution control and environmental protection.

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