Universidade do Minho
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FINAL RESEARCH PROJECT

VOLATILE FATTY ACIDS PRODUCTION FROM FISH WASTEWATER

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ABSTRACT

In this work, two experiments were carried out to investigate the production of volatile fatty acids (VFAs) through the anaerobic degradation of a wastewater collected from a fish processing industry. The first experiment was carried out in batches, at different initial pH values (5, 6, 7, 8 and 9). Acetate and propionate were the main products obtained at the end of the experiment. The highest production of VFAs (5276 mg COD/L) was obtained at pH 8.

The second experiment was performed in an anaerobic continuous reactor at pH 5.5. Several conditions were tested to improve the VFAs production such as different HRT (18 h, 12 h and 8 h), different feed´s dilution (50 x, 25 x, 10 x, 2 x and 1 x) and different feed´s salinity (2.8 g/L and 20 g/L of salt). The increase of feed´s salinity appear to be the best strategy, since the concentration of VFAs increased (457 mg COD/L with 2,8 mg/L of salt to 788 mg COD/L with 20 mg/L of salt) and the percentage of CH4 in the reactor decreased.

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1. INTRODUCTION

Anaerobic digestion is a biological process of degradation of organic substrates (animal and/or vegetable wastes) in the absence of oxygen (O_2). The decomposition of the biodegradable material carried out by several anaerobic microorganisms such as bacteria and archaea. The two main products that originate the biodegradation of organic matter are biogas and bio-fertilizer (Moreno, 2011). Biogas is a biodegradable fuel formed mainly by methane (CH_4) and carbon dioxide (CO_2) although it also contains small proportions of hydrogen (H_2), O_2 and nitrogen (N_2) gases, among others. The bio-fertilizer consists of stabilized effluent after anaerobic decomposition (Moreno, 2011).

Organic raw materials can be used as substrates for anaerobic digestion processes. The substrates can be obtained from a variety of sources such as animal and agricultural wastes, urban solid waste, crop residues, aquatic plants, grass, paper and corn, among others. Since they are usually sub products of other processes they are considered a renewable source (Lorenzo Acosta & Obaya Abreu, 2005). The composition of substrates is important because there are certain substances such as nitrogen (N), sulfur or carbon (C) that are necessary for microbial development. However, there are other substances such as heavy metals or ammonium that can inhibit the anaerobic digestion because they are toxic to the microorganisms involved in the process (Solano, Vargas, & Watson, 2011). Other components can be lipids, proteins or salts, among others. The substrates may require pre-treatments to enable the biodegradation. There are several types of pre-treatment: physical, chemical, biological and combined (Zhang, Hu, & Lee, 2016).

Effluents of animal origin are commonly used in anaerobic digestion processes. They can be of several types: bovine, pig, avian, fish processing industry, among others. The main components of fish processing industry wastewater are lipids and proteins. Effluent pH of fish processing wastewaters is usually close to neutral, with pH values in the range of 5.7 to 7.4. Ammonia emission and proteinaceous matter decomposition is mostly pH dependent, so the pH must be controlled. Excess of N and phosphorous (P) may cause proliferation of algae and can affect the activity of microorganism. A N:P

ratio of 5:1 is suitable for proper growth of the microorganisms (Chowdhury, Viraraghavan, & Srinivasan, 2010).

1.1 Anaerobic digestion stages

Anaerobic digestion is divided into four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. (Figure 1)

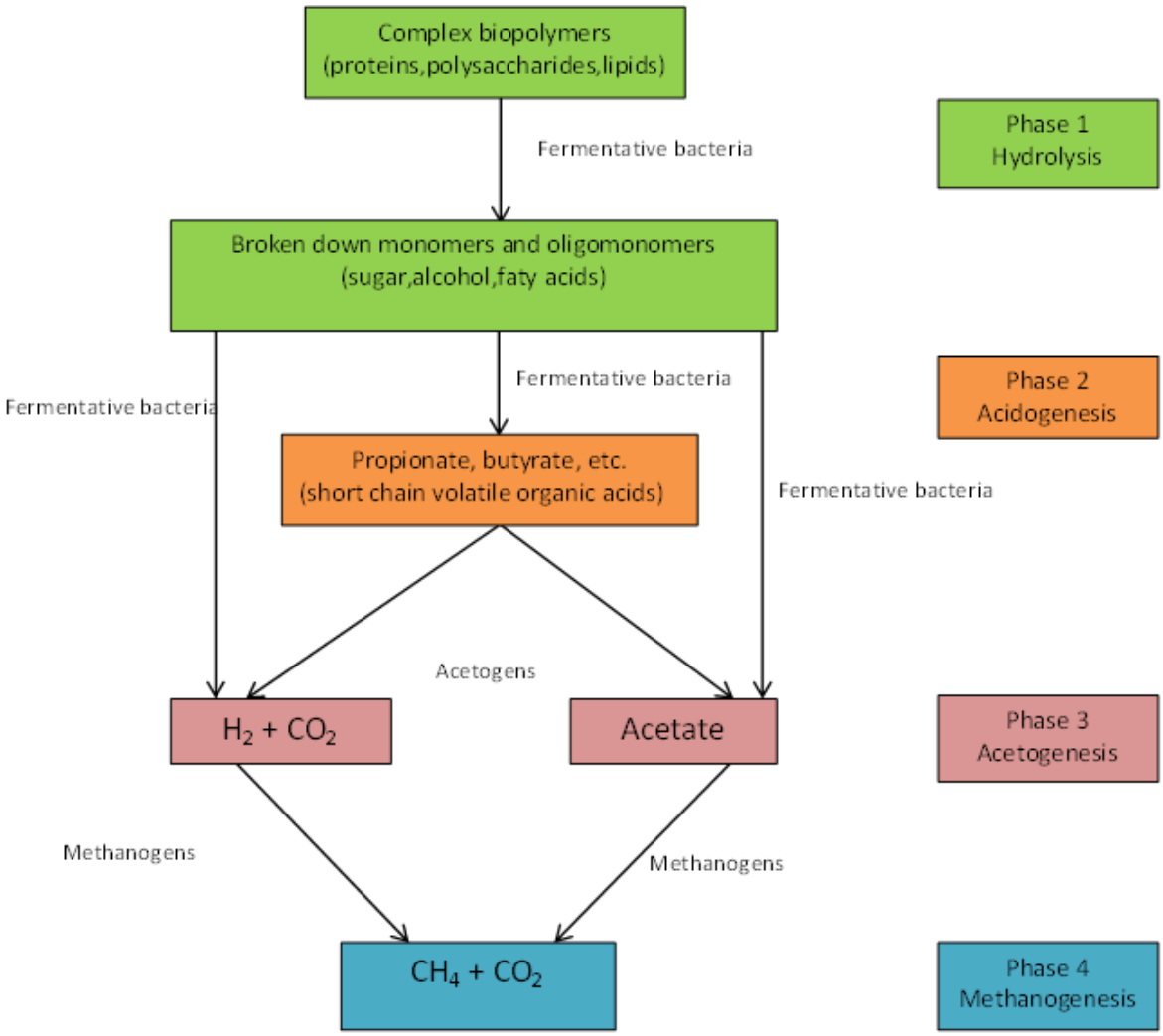


Figure 1 - Diagram of different stages of anaerobic digestion process (adapted from Moreno, 2011).

During the hydrolysis stage, the polymers are converted into simpler and more soluble substances that can be digested by the microorganisms in the following steps. In the

hydrolysis step, the compounds with higher molecular weight such as proteins, lipids and carbohydrates are breaking down into simpler substances such as sugars, alcohols, fatty acids, among others.

After the hydrolysis occurs the acidogenesis stage. Acidogenic bacteria that are responsible by the fermentation of the soluble organic molecules carry out this stage. This fermentation produces volatile fatty acids (VFAs) such as acetic acid, propionic acid, butyric acid, formic acid, valeric acid, among others.

In the acetogenesis stage, acetogenic bacteria transforms the VFAs into acetate and H_2. This transformation is necessary because the VFAs cannot be metabolized by acetoclastic methanogens.

Finally, methanogenesis stage is carried out, where the methanogens transform the acetate produced in the previous stage into CH_4 and CO_2. Hydrogenotrophic methanogens also participate by using H_2 in the medium, to reduce CO_2 to CH_4.

1.2 Anaerobic acidogenesis stage

The acidogenic bacteria can be divided into anaerobic facultative and anaerobic strict. Many of these microorganisms also appear in the hydrolysis stage. Depending of the experimental conditions, there are different bacteria that appear in this stage such as, *Clostridium, Paenibacillus and Ruminococcus* (Moreno, 2011), *Bacteriocides, Clostridia, Bifidobacteria, Streptococci and Enterobacteriaceae* (Lee, Chua, Yeoh, & Ngoh, 2014) or *Clostridia, β-Proteobacteria and Bacteroidetes* (Feng, Chen, & Zheng, 2009). In the acidogenesis stage, the VFAs produced are mainly propionic, butyric, valeric and acetic. However, several factors affect the production of VFAs during the acidogenesis step. The most important factors are pH, temperature, hydraulic retention time (HRT) and concentration of the substrate.

1-pH

The pH value in the reactor is important for the production of VFAs, because many of the acidogenic microorganisms cannot survive in extreme conditions of acidity (pH 3) or alkalinity (pH 12). pH can also affect the types of VFA produced in the acidogenic fermentation, especially acetic, propionic and butyric acids. The ideal pH conditions

are between 5.25 and 11, although it also depends on the type of substrate used (Lee et al., 2014). However, in general the low pH values favour the formation of propionic and butyric acids and the high pH values the formation of acetic acids acid. For example, with substrates from dairy industry, propionic acid production is favoured at pH 4-4.5 while acetic and butyric acids are favoured at pH 6-6.5. On the other hand, with substrates rich in glucose, the production of butyric acid is favoured at pH 6 and production of acetic and propionic acids is favoured at pH 8 (Lee et al., 2014).

2-Temperature

The temperature also affects the production of VFAs although less than the pH. Temperature affects the growth rate of microorganisms and therefore the speed of reaction. This parameter affects the concentration of VFAs produced, the rate of VFAs production and the VFAs yield. The temperature should be selected according to the types of acidogenic organisms present in the process (Lee et al., 2014).There are three temperature ranges in which microorganisms can growth, psychrophilic (under 25 °C), mesophilic (25-45 °C) and thermophilic (45-65 °C). The mesophilic regime is the most used because it has a higher reaction rate than the psychrophilic regime and greater stability than the thermophilic regime (Moreno, 2011).

3-Retention time

The hydraulic retention time (HRT) in the reactor is another critical operational parameter (Lee et al., 2014). The HRT indicates the time that a volume of organic substrate remains in the reactor, which means the time that a certain volume of substrate takes since it enters in the reactor until to leave from the reactor. The HRT is inversely proportional to the inlet flow rate (Q) and also depends of the working volume of the reactor (V) (Equation 1):

$$HRT = \frac{V}{Q} \qquad Equiation\ 1$$

In general low ranges of HRT (10-24 hours) promotes the formation of butyric acid, while increasing the HRT promotes the formation of propionic acid (Lee et al., 2014).

4-Concentration of solids

The concentration of solids of the substrate applied to the acidogenic fermentation has a significant influence on the distribution of the VFAs, but this influence is less than that of other parameters. The solid concentration can be expressed in different ways (chemical oxygen demand (COD), organic loading rate (OLR), among others). Acidogenesis is carried out in better conditions with a substrate with a small concentration of solids (less than 10%), since a high concentration (greater than 10%) hinders the degradation of organic matter, reducing the efficiency of the process. Therefore, the organic matter most appropriate for anaerobic degradation process is a substrate with high moisture content (Lee et al., 2014) (Lorenzo Acosta & Obaya Abreu, 2005).

1.3 VFAs applications

Among several applications, such as the production of bioenergy and biological nutrient removal, VFAs can be used for biodegradable plastics production (e.g. polyhydroxyalkanoates (PHAs)). PHAs are linear polyesters produced in nature by the action of different bacteria. There are 3 metabolic routes to obtain PHA: i) by degradation of sugars; ii) by degradation of VFAs; iii) by degradation of lipids (Serrano Riaño, 2010). PHAs synthesis from the degradation of VFAs occurs through β-oxidation of the VFAs (Federico, 2011). Numerous bacteria have the ability to synthesize PHAs, such as *Ralstonia eutropha, Pseudomonas aeruginosa, Allochromatium vinosum, Bacillus megateriu, Azotobacter vinelandii, Azotobacter beijerinckii, Rhodospirillum rubrum, Rhodobacter sphaeroides, Caulobacter crescentus* and *Pseudomonas oleovorans* (García, Carlos, Contreras, Reynoso, & Córdova, 2013). The most important PHAs are polyhydroxybutyric acid (P3HB), polyhydroxybutyrate (PHB) and polyhydroxyhexanoate (PHH) (Federico, 2011).

2. MATERIALS AND METHODS

Acidogenesis is the most important stage of this project, because it produces the volatile fatty acids (VFAs) that is the objective of the process. In this work, two different assays were performed:

i) Anaerobic batch assays - at different pH values to investigate the effect of pH on the VFAs production (Experiment 1)

ii) Anaerobic continuous reactor - at pH 5.5 to investigate the VFAs production in continuous mode by decreasing the dilution of the substrate and decreasing the hydraulic retention time (HRT) (Experiment 2)

2.1. Inoculum and substrate

Anaerobic granular sludge was used as inoculum in all the experiments. This inoculum was collected from a wastewater treatment plant, which treats brewery's wastewater located in the north of Portugal (Unicer, Matosinhos). The inoculum was previously boiled at 100 ºC during 15 minutes to eliminate and/or inhibit the methanogenic activity to avoid the conversion of VFAs into methane (CH_4).

The effluent used as substrate in this work was collected from a fish processing industry in the north of Portugal (Poveira, Póvoa do Varzim). This effluent was formed by cleaning (125 m^3/d), cooking (15 m^3/d) and brine (2 m^3/d) wastewaters. The effluent was previously filtrated (500 µm) to remove solids with large dimensions that were present in the effluent such as papers and plastics. The fish processing wastewater had a chemical oxygen demand (COD) of 16 g/L and pH of 5.5. This effluent was used as substrate in the Experiment 1 (anaerobic batch assays) and in the Experiment 2 (anaerobic continuous reactor) until day 2/5/18. After this day, the effluent was directly collected after a physical pre-treatment of gradation and flotation to remove the solids and fat with large dimensions, thus changing the composition of the effluent (COD of 1.06 g/L and pH of 6.5).

2.2. Experiment 1 (anaerobic batch assays)

2.2.1Experimental procedure:

In this experiment, the effect of the pH value on the production of VFAs was investigated for initial pH of 5, 6, 7, 8 and 9. The pH of the effluent was corrected until obtaining the desired pH by adding hydrochloric acid (HCl, 10M) for acid pH values or by adding sodium hydroxide (NaOH, 10M) for basic pH values. The desired initial pH was maintained using a buffer solution of potassium phosphate monobasic (KH_2PO_4) and potassium phosphate dibasic (K_2HPO_4). The amount of each component according to the pH is present in the Table 1.

For the anaerobic batch assays were used 17 bottles of 120 mL prepared with 15 mL of inoculum (anaerobic granular sludge), 30mL of buffer (K_2HPO_4/ KH_2PO_4) and 30 mL of substrate (fish wastewater) to a final volume of 75 ml. For each pH tested, the assay was performed in triplicate and incubated at 37 °C. A blank assay was performed in duplicate these assays allowed to determine the concentration of VFAs from the residual substrate present in the inoculum. Blank was formed by 15 mL of inoculum and 60 mL of buffer.

Table 1: Amount of reagents for the buffer solutions for each pH

pH	**5**	**6**	**7**	**8**	**9**
K2HPO4 / mg	0.8	7.6	49.8	112.4	128.6
KH2PO4 / mg	101.4	96.1	63.1	14.2	1.6

2.3. Experiment 2 (anaerobic continuous reactor)

2.3.1 Experimental procedure:

The equipment used for this experiment was a glass reactor with temperature control at 37 °C, a peristaltic pump 101 U/R (Watson Marlow, USA) for feed, a peristaltic pump 405 U/R1 (Watson Marlow, USA) for recirculation, a gas counter PMMA/PVDF (Ritter, Germany) a container with the feed and an agitator to keep the feed homogeneous. The recirculation was used to promote the agitation inside the reactor.

The reactor was operated at pH 5.5 in order to inhibit the methanogenic activity. The effluent used in this experiment was previously acidified to pH 5.5 by adding HCl (10M). .The desired pH was maintained by using a buffer solution of KH_2PO_4/K_2HPO_4.

The work volume was 1015 mL, composed by 30 % of inoculum (305 mL) and 70 % of substrate. The concentration of substrate fed to the reactor, in terms of COD, was increased by decreasing the dilution of the substrate (50 x, 25 x, 10 x, 2 x and 1 x). The dilution was obtained by using the buffer solution. Also, different hydraulic retention times (HRT) (18 h, 12 h and 8 h) were tested to improve the production of VFAs.

2.4 Analysis

Various analyses were performed during both experiments. Total COD and soluble COD (after centrifugation (15000 rpm during 15 minutes and filtration (0.22 µm)) were measured using Hach cuvette tests (LCK 514) (Hach, Germany) and a spectrophotometer DR 2800 (Hach, Germany). pH was also measured using a pH meter inoLab pH 7110 (WTW, Germany).

CH_4 in headspace were measured using a gas chromatograph GC-2014 (SHIMAZDU, Japan) equipped with a FID detector and a PoraPak Q (80/100 mesh, 2 m x 1/8 inch, 2 mm, stainless steel) column using nitrogen as carrier gas (30 mL/min). Column, injector and detector temperatures were 35, 110 and 220 °C, respectively.

VFAs were analysed by high performance chromatography (HPLC) (Jasco, Japan) equipped with a UV detector and Chrompack column (6.5 x 30mm^2) at 60 °C. Sulphuric acid (2.5 mM) at a 60 mL/min was used a mobile phase.

3. RESULTS AND DISCUSSION

3.1 Experiment 1 (anaerobic batch assays)

Anaerobic batch assays were performed to investigate the effect of different pH values on the production of VFAs.

The concentration of VFAs produced as a function of the initial pH is represented in Figure 2:

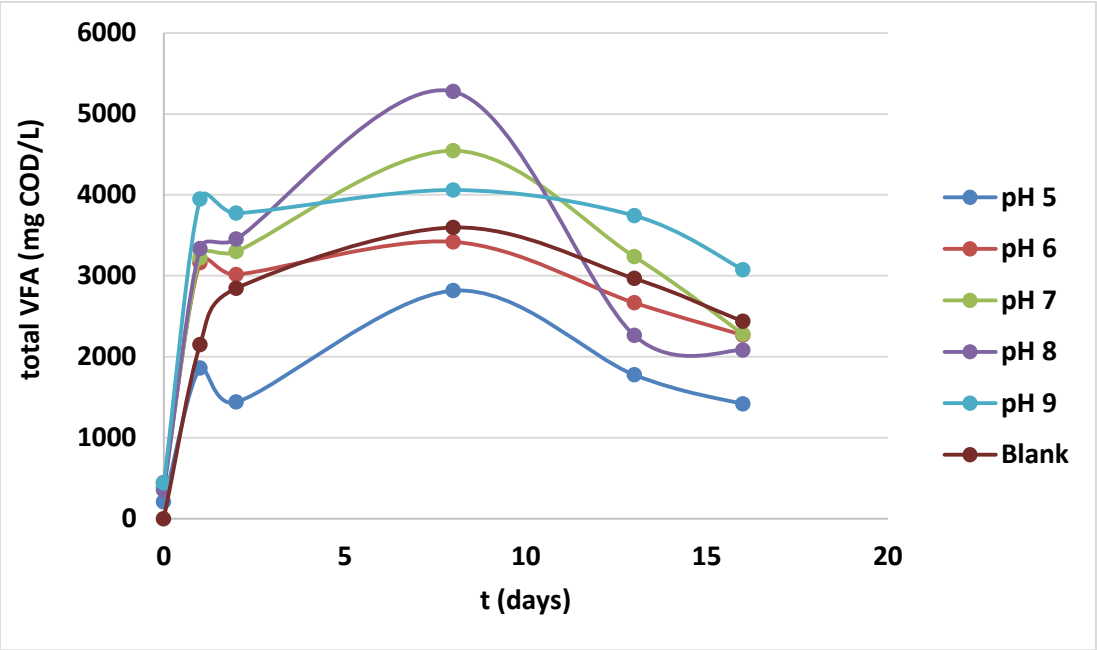


Figure 2- total VFAs concentration according to the different initial pH.

According to the results, the concentration of VFAs increase until day 8 for all batches. At this day, the highest concentration of VFAs was obtained with pH=8 (5276 mg COD/L). After 8 days of operation, the concentration of VFAs decrease for all pH tested suggesting that VFAs were converted to other products such as CH_4.

The effect of pH on the distribution of VFAs obtained at day 8 of operation is shown in the Figure 3.

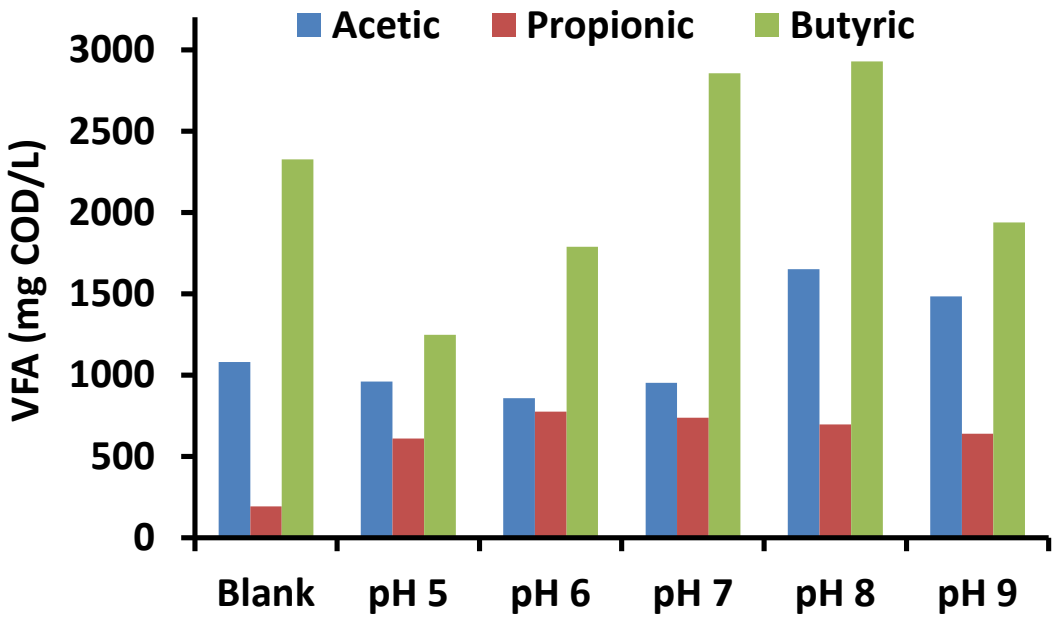


Figure 3- VFAs distribution at day 8 of operation according to the initial pH.

In all batches, acetic, propionic and butyric were the VFAs detected. The two mayor products obtained were acetic and butyric acids, being the butyric acid the major product in all pH values tested. Higher concentrations of acetic acid were obtained at pHs 8 (1652 mg COD/L) and 9 (1483 mg COD/L), whereas higher concentrations of butyric acid were obtained at pHs 7 (2855 mg COD/L) and 8 (2928 mg COD/L). The concentration of propionic acid was similar for all pH studied (approximately 700 mg COD/L).

The production of methane for the different initial pH is shown in the Figure 4.

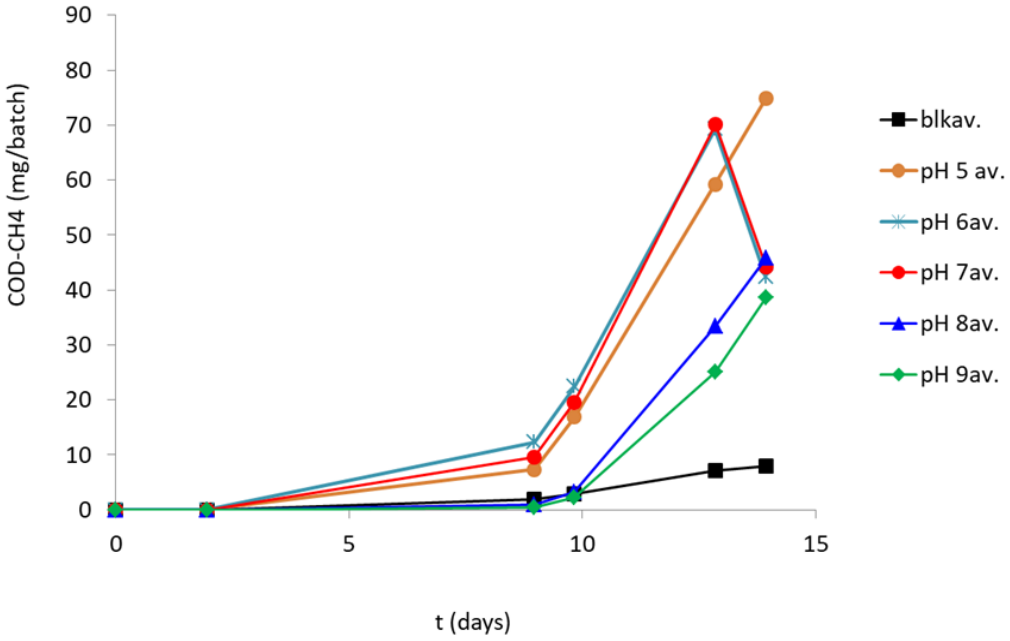


Figure 4- The production of methane according to the initial pH value.

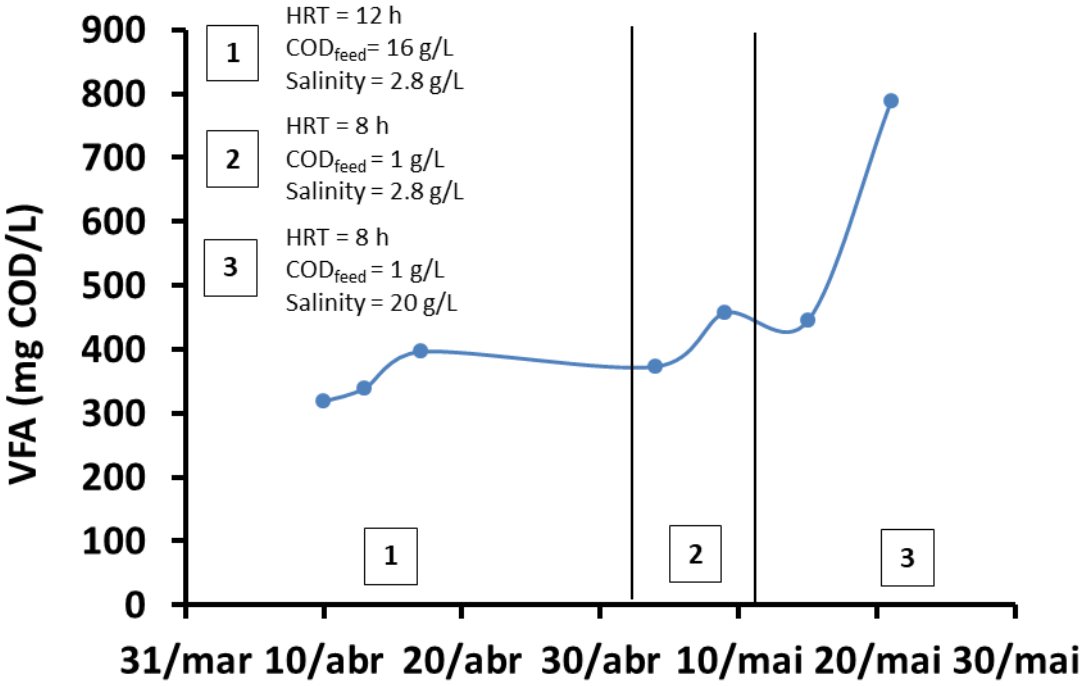
The production of CH_4 increased from day 8 of the experiment, which coincides with the reduction of the acids concentration. This suggest that from that day the VFAs were transformed into CH_4, completing the anaerobic digestion process.

3.2 Experiment 2 (anaerobic continuous reactor)

An anaerobic continuous reactor to produce VFAs from fish wastewater was operated during 69 days. Several strategies were investigated in order to improve the production of VFAs by inhibiting the methanogenic activity such as different HRT and different substrate concentrations. The different HRT tested were 18, 12 and 8 hours and the different substrate concentrations were obtained according to different feed dilutions 50 x, 25 x, 10 x, 2 x and 1 x. For undiluted feed, a VFAs concentration of approximately 400 mg COD/L was obtained, which corresponds to a very low conversion of the initial COD (16 g/L) (Figure 5). CH_4 was also detected (approximately 50 % at day 36 (27/04/2018)), demonstrating that the conversion of VFAs into CH_4 was not inhibited. In addition, the increase of feed´s salinity was also investigated.

At day 11/05, the salinity of the feed was increased from 2.8 mg/L to 20 mg/L by increasing the percentage of brine wastewater in the feed. The increase of salinity

appear to be a good strategy to improve the production of VFAs since an increase of VFAs concentration from 457 mg COD/L to 788 mg COD/L was observed, which corresponds to a conversion of 80 % of the initial COD (1000 mg/L). During this experiment, the percentage of CH_4 in the reactor decrease to less than 10 %.



4. CONCLUSIONS

The objective of the project was the production of VFAs from fish wastewater, through the anaerobic digestion of it.

According to the results obtained during the anaerobic batch assays, most suitable pH for produce VFAs was 8, since at this pH the highest concentration of VFAs was obtained (5276 mg/L). The VFAs obtained were mostly acetic (1652 COD mg/L) and butyric (2928 COD mg/L) acids. Also, propionic acid was obtained, but in a lower concentration (697 COD mg/L). The higher concentration was obtained at 8 days of operation, after which the concentration of VFAs decreased suggesting that VFAs were converted to other products. The increase of CH4 production after day 8, confirm the decrease on VFAs concentration due to their conversion into CH4 thus completing the cycle of anaerobic digestion.

The results obtained in the continuous reactor showed that the increase of salinity at day 11/5 showed to be a good strategy to improve the VFAs production. The increase of feed´s salinity from 2.8 g/L to 20 g/L increased the concentration of VFAs in the reactor. Also the percentage of CH4 decreased in the reactor. Therefore, it is proposed as future experiments, to test higher salinity values and lower HRT to inhibit the transformation of VFAs into CH_4.

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