Universidade de Aveiro Departamento de Ambiente e Ordenamento
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Evaluation of anaerobic acidification potential for winery effluent

Avaliação do potencial de acidificação anaeróbica para efluentes vínicos

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Dissertation submitted to the University of Aveiro to meet the requirements to obtain the Master Degree in Environmental Studies, conducted under the scientific guidance of Professor Maria Isabel A. P. Fernandes Capela, Associated Professor of the Department of Environment and Planning at the University of Aveiro and co-supervised by Professor Kerstin Kutcha, Professor of the Hamburg University of Technology.

I dedicate all my work and effort dispensed in preparing this thesis to my husband Flávio and my son Américo.

examinimg committee

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I thank my parents and Celice Nelson Oliveira, Ana Maria
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I likewise thank to my advisor, Prof ª. Maria Isabel Capela.

keywords Acidification, wine, batch reactor, anaerobic treatment.

abstract

Given that the industrial wastewater represent a negative contribution of great significance to environmental, developed this work with a practical study on the behavior of a particular industrial effluent, in this case the wine, when subjected to tests of anaerobic acidification in batch reactors, in order to obtain value-added products from this waste. In this sense we studied the effects of this treatment in three operating parameters: organic load, alkalinity and using a thermal pre-treatment for biomass, in order to inhibit methanogenic activity. In all there were three sets of reactors, with three reactors each, totaling nine reactors were analyzed with varying concentrations of four different organic load, three different concentrations of alkalinity solution of calcium bicarbonate, $NaHCO_3$. It was observed a good acidogenic potential for the wine effluent where it was achieved, for reactors higher organic load, a degree of acidification of 85%, it was found that the alkalinity is related to the organic load used and shows great significance in the formation of the peaks of maximum acidogenic production and diversity of VFAs, higher alkalinities favor acids of longer carbon chain, and the peak is produced earlier. The thermal pre-treatment was not beneficial despite achieved good results in terms of degree of acidification it has not exceeded the values obtained in the reactors were the thermal pre-treatment was not applied to the sludge.

palavras-chave Acidificação, vinho, reactor batch, tratamento anaeróbio.

resumo

Tendo em conta que as águas residuárias industriais representam um contributo negativo de grande significância a nível ambiental, desenvolveu-se com este trabalho de um estudo prático sobre o comportamento de um determinado efluente industrial, neste caso o vínico, quando submetido a ensaios anaeróbios de acidificação em reatores descontínuos, com vista a obtenção de produtos de valor acrescentado a partir deste resíduo. Neste sentido foram estudados os efeitos deste tratamento sob três parâmetros de operação: carga orgânica, alcalinidade e a utilização de um pré-tratamento térmico para a biomassa, a fim de se inibir atividades metanogénica. Ao todo foram realizadas três baterias de ensaios, com três reatores cada, no total foram analisados nove reatores, variando quatro diferentes concentrações de carga orgânica, três diferentes concentrações de solução alcalina de Bicarbonato de Cálcio, $NaHCO_3$. Observou-se um bom potencial acidogénico do efluente vinícola chegando a alcançar para os reatores de carga orgânica mais elevadas graus de acidificação de 85%.Constatou-se que a alcalinidade está relacionada com a carga orgânica utilizada e demonstra grande significância na formação dos picos máximos de produção acidegénica, e na diversidade dos VFAs obtidos. Desse modo, alcalinidades mais elevadas, favorecem ácidos de cadeia carbónica mais longa, e o pico máximo de produção forma-se mais cedo. O pré-tratamento térmico, não se mostrou vantajosos apesar de ter alcançado bons resultados em termos de grau de acidificação, não superou os valores obtidos em reatores que não tiveram as lamas submetidas a este pré-tratamento.

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List of Abbreviations

ACPB	Adega Cooperativa de Ponte da Barca
ALK	Alkalinity
CCDR	Regional Committee of Coordination and Development
COD	Chemical Oxygen Demand
CODs	Soluble Chemical Oxygen Demand
CODt	Total Chemical Oxygen Demand
EGSB	Expanded Granular Sludge Blanket
F/M	Food per Microorganisms
IVV	Vine and Wine Institute
PHAs	Polyhydroxyalkanoates
SIMRIA	Sistema Intermunicipal de Saneamento da Ria de Aveiro
TSS	Total Suspended Solids
UASB	Upflow Anaerobic Sludge Blanket
VFAs	Volatile Fatty Acids
VOAs	Volatile Organic Acids
VSS	Volatile Suspended Solids
WWTP	Waste Water Treatment Plant

1 Introduction

The concept of *sustainability* published by the *Stockholm Conference*, in 1972 and widely discussed among environmentalists, entrepreneurs and managers today, states that development must meet the needs of the present without compromising the ability of future generations to meet their own needs, by maintenance of the planet. From this principle, treatment and subsequent return of wastewater from both the urban and industrial is fundamental, since the water is a natural resource, cyclic, non-renewable and indispensable for all biotic processes.

The economic and financial aspect remains an obstacle to the implementation of an industrial or an urban wastewater treatment plant. To help solving environmental problems like the natural resources degradation, we will need to propose technical alternatives for a more economically viable treatment

It is understood by white (or industrial) biotechnology the development of techniques to valorize a sub-product with little or no aggregated value, which can be from industrial origin or not. This concept is an important tool for the implementation of more sustainable alternatives in wastewater treatment.

In the seventies, the possibility to get a fuel from a wastewater by the biotechnological anaerobic application had stimulated very much the research conducted in this area. However, the expected energetic answer with the methane only was observed in some specific cases: vapor generation and mainly in drying processes. If the final product obtained with the anaerobic treatment processes can be really valorized, probably there we can have an extending in the use of this process (LEITE et al.).

The solution can be contained in the anaerobic process, more specifically in the acidogenic stage where occurs the acidogenic compounds accumulations for further extract of the volatile fatty acids (VFAs) (LEITE et al.).

This work tries to demonstrate the viability of using winery effluents as raw material for biopolymers production though the acidogenic fermentation of wastewater compost. In this sense, the acidogenic products, specially the VFAs, are considered as substrates for the production of polyhydroxyalkanoates (PHAs). That will support the production of value added products like bio-plastics, bio-fuels and other compounds produced by chemical or biotechnology-based developing.

2 Objectives

2.1 Main objectives

The main objective of this essay was to study the acidogenic fermentation of an effluent from a winery industry, making sure that this type of effluents, which are a pollution source, are used as raw material, for the production of value added products. In this sense the production of acidogenesis products, especially the VFAs are an important step to the analyzed and controlled.

The general objectives of this study are:

- Motivate the use of anaerobic processes for the wastewater treatment from the winery industry;
- Turn profitable the treatment of the wastewater from winery industry;
- Foment the concept of white biotechnology.

2.2 Specific objectives

- Evaluate the best optimum conditions for VFAs production;
- To study the effect of the pre-treatment step to prevent the methanogenesis;
- To study the effect of operational parameters on the behavior of the acidogenesis step, such as F/M ratio and initial alkalinity.

3 Literature review

3.1 Relevance of work in the local context

Since wine has always played an important role in almost all civilizations, and is a products with higher expression in agriculture. It is thought that the vines were grown for the first time in the Iberian Peninsula, in the Valley of the Tagus and Sado, in 2000 a.C, by Tartessos, one of the first civilizations to inhabit the Iberian Peninsula. Used wine as a product exchange in metals trading

With the vast expansion of Christianity in the VI and VII centuries a.C, the wine becomes essential for the sacred act of communion, and gains even greater importance in society

In Portugal wine production was stimulated by various historical facts, such as the discoveries in the XVI century period when Lisbon was considered one of the main centers of production and export of wine and Methuen Treaty in XVII century, where it was established that England only buy Portuguese wine while Portugal likewise would only buy English fabrics, with a further increase in exports.

The activities related to wine production, constitute an area of importance for Portugal, due to their significant influence not only in economy and culture, but also in its impact on the environment. With increasing environmental concerns that there has been the concern related to the purpose given to wastewater and solid waste resulting from this activity has increased.

In 2005, Portugal had a utilized agricultural area of 3 679 587ha, 238 647ha of which (6.5%) corresponded to the area of producing vines, being surpassed only by the areas of cereal fields and the olive groves.

The wine sector in Portugal covers all types of businesses from the micro enterprises, large companies and cooperative sector accounts for half of national production.

A study prepared by the Vine and Wine Institute (IVV), referring to wine production campaign 2005/2006, surveyed 116 wine cooperatives, about, and waste treatment system adopted. Among them, only 60 (52%) responded to the survey, with 25 of these wineries admit they have a treatment system.

In the eleven major wine producing countries worldwide campaign of 2005/06, are five countries in the European Union, with Portugal in eleventh place in this ranking (IVV, 2008). Figure 1 presents a graphical representation of wine production in the eleven countries that lead the ranking in this sector of the market.

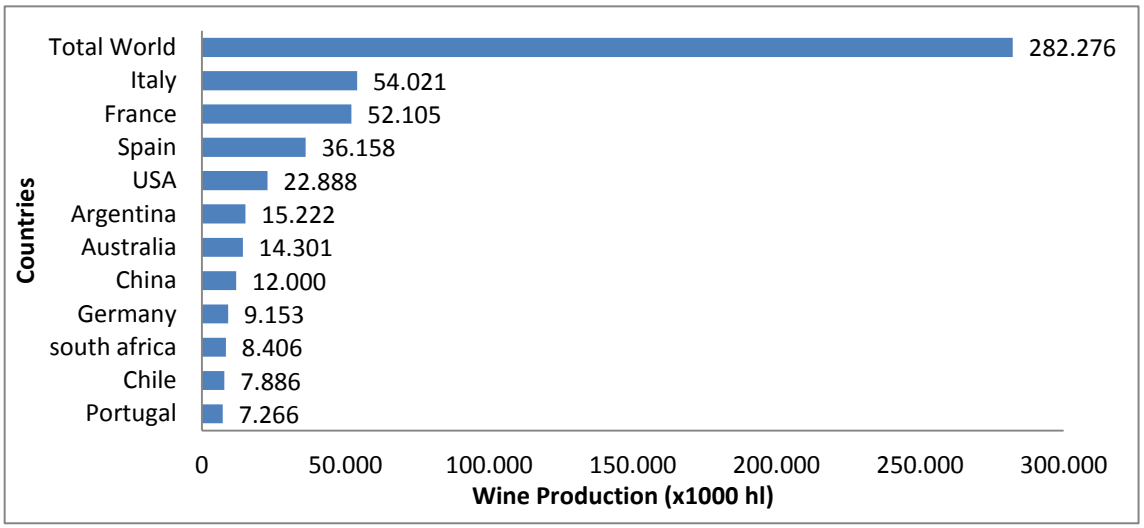

Figure 1 - Major world producers of wine in the 05/06 campaign (IVV, 2008).

3.2 **Winery effluent**

3.2.1 Procedures of wine production

Wine is a product obtained from the total or partial alcoholic fermentation of fresh grape juice, or of grape must (EC, 1999). Producing wine requires the implementation of biotechnological sequence involving several units operations. Although some few products are added to the must and or wine, several residues are rejected, either as liquid or solid waste. White wine is normally produced by the fermentation of a clarified must, which is obtained after grape stem removal, pressing of the resulted grape berries and subsequent clarification. The production of red wine is conducted in non-clarified musts, prepared after grape stems removal and crushing of grape clusters. Musts can also be fermented in the presence of grape stems. After fermentation wines must be clarified and stabilized, chemically and microbiologically, before bottling. Figure 2 shows a schematic process, applied at Adega Cooperativa de Ponte da Barca (ACPB) to produce wine.

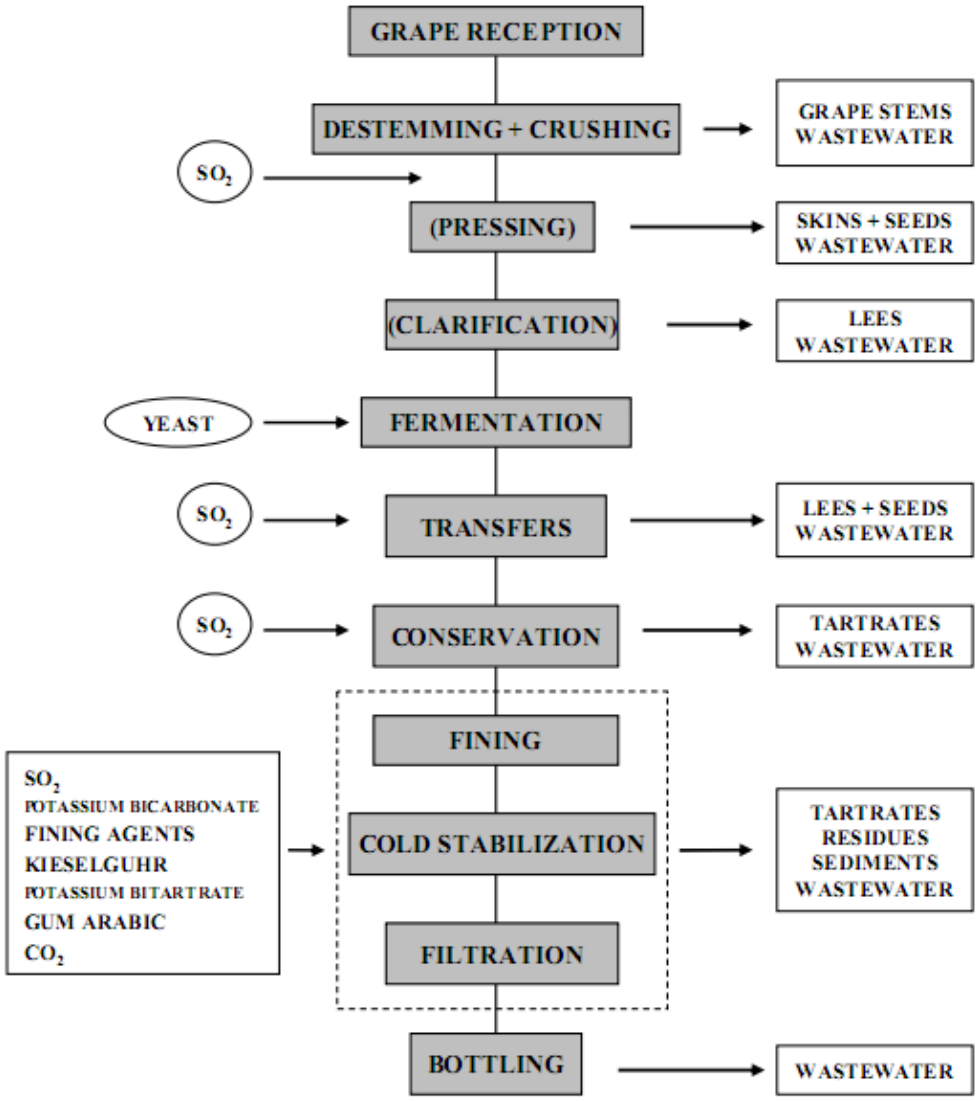

Figure 2 – Technological process adopted at ACPB wine-cellar (BRITO et al.).

Wine distilleries produce large volumes of wastes, called “vinasses”. The wine production is a process that goes through many stages where the quantity and quality of wastewater produced is diverse. This can vary the level of pollution load, hydraulic load, in function of the techniques used in production, operating time, type of wine produced, etc. The same applies to the production of solid waste whose composition varies widely according to the raw material distilled: wine, lies, pressed grapes, etc. (BENITEZ et al., 1999).

Winery effluents contain four types of principal pollutants (BRITO et al.):

- Sub-product residues - stems, seeds, skins, lees, sludge, tartar, etc.;
- Loss of brut products - musts and wines occurred by accidental losses and during washings;
- Products used for wine treatments - fining agents, filtration earths, etc.;
- Cleaning and disinfection products, used to wash materials and soils.

Table 1 provides a breakdown of the steps involved in wine production, which are more significant in terms of waste production.

Table 1- Steps of wine production take into account the residual production (PIRRA, 2005)

Process step	Process description
Grape reception	Occurs during the grape harvest.
Crushing and Stalking	Consists of passing the berries by overwhelming, crushing the grapes tearing them without the pips or stalks. The Stalking is the separation of stem (woody part) of the rest of the cluster, and takes place before and after crushing. Too often is performed on a device that combines the two operations. The Stalking is a process recommended as influence the quality of wine, and can be partial (white wines and roses) or total (red wines).
Clarification	Immediately after the crushing, during grape pressing or during decanting but before fermentation a certain quantity of disinfectant is added, usually sulfur. The application of increasing concentrations of sulfur dioxide (SO_2) leads to inhibition in first bacteria, followed by yeasts (Kloeckera apiculata) and finally elliptical yeasts (Saccharomyres ellipsoideus) that are more resilient. Delaying the start of fermentation, the SO_2 favors deposit more or less rapid suspended solids in the wort. The application of SO_2, retards the oxidation of wine, paralyzing tirocinase and lactase enzymes present in rotting grapes. It is important to avoid these pre-fermentative transformations harmful to the quality of the wine. The SO_2 reacts with water producing strong acid which attacks the plant cells favoring the dissolution of the organic acids present. Moreover, opposes the development of bacteria capable of attacking acids mainly malic acid. Thus contributes to the acidification of the wort.

Fermentation	The wort is sent to tanks or vats where the fermentation takes place. The alcoholic fermentation is a phenomenon by which the sugars are converted into ethanol and carbon dioxide, by the action of yeast. The solid parts of the grape tend to go to the surface, and it is necessary to mix it with the remaining liquid which is at the bottom of the fermentation tanks. The mixing is carried out by pumping that also homogenizes the distribution of yeasts and temperature in the fermentation vessels. Can be resorted to leavening, adding selected yeasts and fully activity, in order to cause its multiplication in the mass of force must and alcoholic fermentation. The maceration takes place during fermentation, and is to promote contact of solids with bark and wine, where the alcohol acts as a solvent to extract color, and aroma of the bark tannins.
Transfers	The racking of the fermented mash fermentation tanks for settling tanks where it intends to separate the clear wine of deposits that form on the bottom of the casks or vats. The deposit is not instantaneous, since depending on the diameter and weight of the particles, the nature of wine and the container. The press is made by the compression of the wine that is retained by the mulch and it is considerable (on average 100 kg of bagasse retain 55L of wine). In white grapes usually takes place after crushing the color is done after fermentation.
Bottling	This process consists of depositing a precise amount of wine in bottles which are properly labeled and sealed with cork stoppers normally.

The effluent is mainly originated from various washing operations during the crushing and pressing of grapes, as well as rinsing of fermentation tanks, barrels and other equipment or surfaces. Over the year, volumes and pollution loads greatly vary in relation to the working period (vintage, racking, bottling) and to the winemaking technology used, e.g., in the production of red, white and special wines.

Winemaking is seasonal with high activity in autumn (at north hemisphere), which corresponds to vintages and fermentations, a notoriously less important activity in spring on the occasion of transfers (racking period) and filtrations, and a weak activity during winter and summer (BRITO et al.). The seasonal variation all over the year is illustrated in the Figure 3.

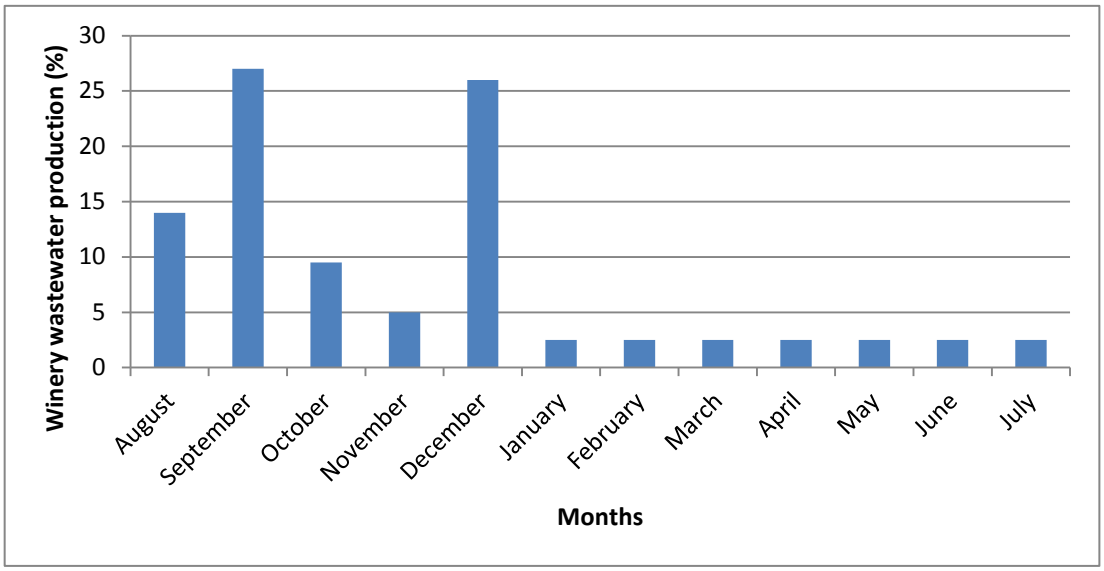

Figure 3 – Distribution of wastewater production in the winery process all over the year (VLYSSIDES et al., 2005).

3.2.2 Characterization of winery effluent

The winery effluents are rich in organic matter, are acidic and contain different microorganisms, mainly bacteria and yeasts (DONOSO-BRAVO et al., 2009a). Usually these effluents are disposed of into evaporation ponds or eliminated through public courses, and cause a large-scale environmental problem to which little attention has been paid by this industry until recently (BENITEZ et al., 1999).

Rejected volumes per volume of produced wine vary from one wine cellar to another, with extreme values comprised between 0.1 m^3/m^3 and 2.4 m^3/m^3. For the ratio of water consumption to produce wine, l.0 m^3/m^3 is the rule of thumb, while (PÉVOST et al., 2003) refer to values between 0.3 m^3/m^3 and 2.5 m^3/m^3.

Washing operations, carried out during different winemaking steps, are at the origin of the rejection of fully charged wastewaters, and may be distributed as follows (BRITO et al.):

- During vintage preparation - washing and disinfection of materials;
- During grape reception - washing of reception materials (hoppers, destemmers, crushers, presses, dejuicers, conveyors and transport pumps); cleaning the floors, with or without addition of cleaning products;
- During vinifications - rinsing of fermentation and clarification vats; cleaning the floors, with or without addition of cleaning products;
- During transfers - rinsing vats after transfers; cleaning the floors, with or without addition of cleaning products;
- During filtrations - rinsing diatomite and earth filters.

Musts and wines constituents are present in wastewaters, in variable proportions: sugars, ethanol, esters, glycerol, organic acids (e.g., citric, tartaric, malic, lactic, acetic), phenolic compounds (coloring matter and tannins) and a numerous population of bacteria and yeasts. They are easily biodegradable elements, except for polyphenols which make biodegradation more difficult and requiring an adapted culture.

Table 2 shows some examples of the main characteristics of winery effluents.

Table 2 - Compilation of the characteristics of winery wastewater produced in vintage period and low season, including that of ACPB (BRITO et al.); (PINHO, 2007).

	ACPB	Vintage period	Low Season
Production (m^3/year)	250	3000	-
pH	5,7	4 – 5	5 - 11
COD (mg/L)	1200 – 10266	2 000 – 20000	1000 – 4000
BOD (mg/L)	130 – 5320	4500 – 18000	500 – 2000
TSS (mg/L)	385 – 5200	5 00 – 15000	1 00 – 2000
Total N (Kjejdahl) (mg/L)	12– 93	20 – 40	5 – 15
Total P (mg/L)	23	1 – 15	0 – 5

Wine as well as winery effluent have some complex chemical compounds that can work as inhibitors of the microbial activity and consequently they may influence the treatment

that the wastewater will be subjected to. Two relevant examples of this potential inhibition are the polyphenols and the SO_2.

3.2.2.1 **Polyphenols**

One of the most complex compounds present in winery effluents are polyphenols, with literature values ranging from 290–1,200mg/l (DONOSO-BRAVO et al., 2009a). Polyphenols are known inhibitors of the growth of microorganisms in treatment plants, and it is understood that these compounds are resistant to biodegradation, impairing the wastewater treatment. Furthermore, these compounds are rapidly oxidized in water, substantially reducing the dissolved oxygen content available for normal development of flora and fauna (MACHADO, 2005)

The toxicity of polyphenols for the microorganisms may be associated with different mechanisms such as inhibition of enzymes, substrate deprivation, and the loss of metal ions. In some cases it may also induce changes in cell morphology (ACAMOVIC et al., 2000).

Polyphenols are responsible for strong inhibitory effects on vinasses microbial activity, as well as their antibacterial activity, affecting the anaerobic digester performance used for biological treatment.

Among the most common types of poly-phenols present in the winery wastewater there are gallic acid, tannic acid, r-coumaric acid and gentisic acid (DONOSO-BRAVO et al., 2009a).

Some mechanisms for degradation of hydrolysable tannins and condensed have been understood and described. For example, tannic acid can be hydrolyzed to gallic acid and glucose by acid hydrolysis under anaerobic conditions (MACHADO, 2005).

3.2.2.2 **Sulfur Dioxide, SO_2**

Another chemical compound present in wine production process, which likewise possesses inhibition to microbial activity is the SO_2. The SO_2 is a sulfite widely used in enology due to its antioxidant, antiseptic and antibacterial properties.

The SO_2 assist in protecting the wine through the toxic action of yeast and bacteria by interfering with the biochemical processes of microorganisms. This toxicity is more effective in bacteria and in certain species of yeast. It also prevents oxidation reactions caused by yeasts that develop more rapidly at the beginning of fermentation, which is the reason for which the musts require addition of SO_2, with the goal of protecting anthocyanins, tannins and aromatic compounds.

The chemical oxidation of the wine caused by contact with oxygen in the air is a slow phenomenon that causes the destruction of compounds that are important factors for the quality of wines (LUCAS et al., 2001).

The total of SO_2 content of wines, other than sparkling wines and liqueur wines, may, on their release to the market for direct human consumption, not exceed: 160 mg/L for red wines; and 210 mg/L for white and rosé wines (EC, 1999).

The maximum SO_2 content shall be raised, as regards wines with a residual sugar content, expressed as invert sugar, of not less than 5 g/L, to: 210 mg/L for red wines and 260 mg/L for white and rosé wines;

Where climatic conditions have made this necessary it may be decided that the Member States concerned may, in certain wine-growing zones of the Community, authorize, for wines produced within their territory, the maximum total SO_2 levels of less than 300 mg/L referred to in this point to be increased by a maximum of 40 mg/L.

3.3 **Anaerobic digestion**

Anaerobic digestion, one of the oldest processes used for the sludge stabilization, is the transformation of organic matter by a consortium of anaerobic micro-organisms. The main product is biogas. It is composed of a mixture of methane and carbon dioxide as main components, and di-hydrogen, carbon monoxide and di-hydrogen sulfur as minor components (MOLETTA, 2005). This biological reaction is widespread in natural environment. It happens in anaerobic environment such as marshes, digestion guts of rumen, landfills, subsoil etc. Anaerobic digestion of industrial wastewater is commonly used all over the world. It is used as a depollution tool but also to produce energy.

Effluents have a pronounced demand in nitrogen and phosphorous when submitted to anaerobic treatment, with a BODs/N/P relation often near 100/1/0,3 (TORRIJOS et al., 1997). Additionally, effluents have a daily great variability, in both quantity and quality, making evaluation of daily pollution complex. Generally, the production of 1 m^3 of wine generates a pollution load equivalent to 100 persons. The pH is usually acidic but, punctually, it may display basic values as the occasion of the cleaning operations (with alkaline products and organochlorides) and on the occasion of chemical detartaration.

The major applications have been, and still remain, in the stabilization of concentrated sludge produced from the treatment of wastewater and in the treatment of some industrial wastes. More recently, it has been demonstrated that dilute organic wastes can also be treated anaerobically (METCALF & EDDY, 2003)

Nowadays, most of the organic effluents and wastes coming from industrial, municipal, or agricultural activities can be effectively treated with anaerobic digestion (Naveau et al., 1979). The anaerobic digestion can be present as a good option for treatment of wastewater, urban and industrial, is a technique widely used around the world. Mainly by its advantages as low energetic necessity, has a small sludge production, needs few nutrients, can produce pipeline methane like an energy source, the dimensions can be

controlled and has a fast answer when the food was stopped for a long period of time ((METCALF & EDDY, 2003) and (MACCARTY, 2001)).

The metabolic pathway of anaerobic digestion is shown in Figure 4.

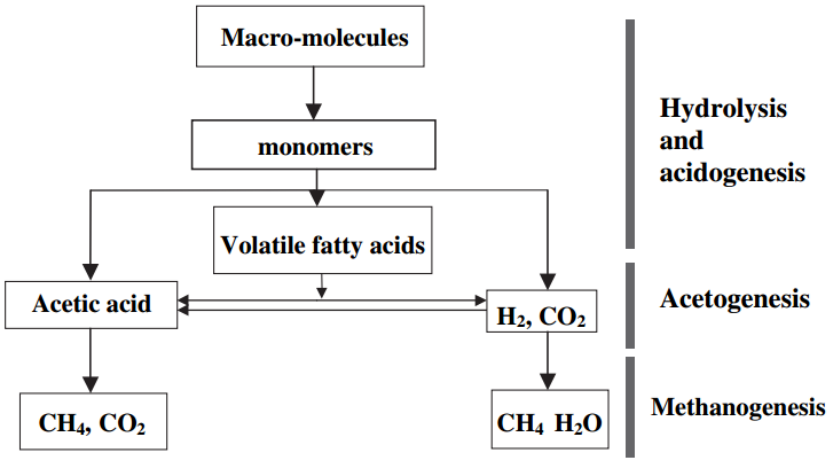

Figure 4 - Anaerobic digestion process.

The first step is the fermentation of organic matter into volatile fatty acids, alcohol, acetic acid, di-hydrogen and carbon dioxide by fermentative and acidogenic bacteria. The second step is the transformation of volatile fatty acids and alcohol into acetic acid, hydrogen and carbon dioxide by acetogenic bacteria; the last step is methane production from acetic acid by acetoclastic methanogens, and from hydrogen and carbon dioxide by hydrogenophilic methanogens (MOLETTA, 2005).

The temperature could be psychrophilic (5 to 25 degrees Celsius), mesophilic (20 to 45 degrees Celsius) or thermophilic (50 to 70 degrees Celsius). The mesophilic temperature range is generally used for industrial wastewater treatment.

The redox potential in the medium is very low (under –300 mV) and the pH range is between 6,5 to 8. Growth of anaerobic bacteria is low and only a little amount of sludge is produced compare to activated sludge processes.

In general for anaerobic treatment the winery effluents can be a good options in terms of methanogenic treatment of winery effluent (VIEIRA, 2009). In this present work, was choose simulate winery effluent as effluent used. To mitigate the effects that can be adversely caused by seasonal feature of this activity it was decided to use a simulated winery effluent. This way it can be achieved a result of the behavior of standard compounds wineries. If this methodology was not adopted probably the process would be subjected to variations of factors in the effluent, such as COD, during the period of this work which could actually mask the real acidification potential of this compound.

Despite the success of aerobic treatment of phenolic wastewater, anaerobic digestion has grown to become a successful technology due to its advantages over aerobic treatment, such as low energy consumption (aeration is not required), less sludge production, generation of biogas (methane and hydrogen) that can be exploited as a source of renewable energy, among others. Most of the anaerobic wastewater treatments of winery effluents have been tested in batch reactors in two phases, to divide the biomass and enable a consortium of more microorganisms adapted the acidogenic phase. (DONOSO-BRAVO et al., 2009b).

- Hydrolysis

The vast majority of the waste consists of macromolecules (proteins, carbohydrates and lipids) that priori cannot be used by fermentative bacteria. Hydrolysis consists of conversion of complex molecules on their monomers (amino acids, carbohydrates, long chain fatty acids and glycerin), these components may be transported into the cell and undergo metabolism. In the hydrolysis are part of the microbial population, the primary fermentative bacteria that normally belong to the families of Streptococcaceae and Enterobacteriaceae. Some authors refer to existence of other microorganisms, such as some flagellate protozoa and fungi can produce enzymes important in the breakdown of molecular bonds of compounds of lignin and cellulose. The solubilization of insoluble compounds such as lignin and cellulosic material constitutes one of the limiting steps of the process of anaerobic digestion due to high energy requirements of the microorganisms involved. It should be noted that there are fractions in particulate matter and/or soluble which cannot be degraded because not all organic matter is biodegradable (SOUSA, 2011).

- Acidogenesis

The second step is acidogenesis. In the process of acidogenesis the amino acids, some sugars and fatty acids are degraded. The organic substrates serve as electron donors and acceptors. The main products of acidogenesis are ethyl, hydrogen, carbon dioxide (CO_2), propionate and butyrate. The propionate and butyrate are then fermented to produce also hydrogen, CO_2 and acetate.

Thus, the end products of acidogenesis (acetate, hydrogen and CO_2) are precursors to the formation of methane in the next step, the methane formation (TCHOBANOGLOUS et al., 1993).

- Acetanogenesis

In the acetanogenesis, acetanogenic bacteria, also known as acid formers, convert the products of hydrolysis to simple organic acids, CO_2 and hydrogen. The main acids produced are acetic acid (CH_3COOH), propionic acid (CH_3CH_2COOH), butyric acid ($CH_3CH_2CH_2COOH$), and ethanol (C_2H_5OH). The products formed during acetogenesis are due to a number of different microbes, e.g., syntrophobacter wolinii, a propionate decomposer and sytrophomonos wolfei, a butyrate decomposer. Other acid formers are clostridium spp., peptococcus anerobus, lactobacillus, and actinomyces (Microbes in AD) An acetanogenesis reaction is shown below:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

- Methanogenesis

Finally, in the third stage methane is produced by bacteria called methane former (also known as methanogens) in two ways: either by means of cleavage of acetic acid molecules to generate carbon dioxide and methane, or by reduction of carbon dioxide with hydrogen. Methane production is higher from reduction of carbon dioxide but limited hydrogen concentration in digesters results in that the acetate reaction is the primary producer of methane (OMSTEAD et al., 1980). The methanogenic bacteria include methanobacterium, methanobacillus, methanococcus and methanosarcina. Methanogens can also be divided into two groups: acetate and H_2/CO_2 consumers. Methanosarcina spp. and methanothrix spp. (also, methanosaeta) are considered to be important in AD both as acetate and H_2/CO_2 consumers. The methanogenesis reactions can be expressed as follows:

$$\underset{\text{(acetic acid)}}{CH_3COOH} \rightarrow \underset{\text{(methane)}}{CH_4} + \underset{\text{(carbon dioxide)}}{CO_2}$$

$$\underset{\text{(ethanol)}}{2C_2H_5OH} + CO_2 \rightarrow CH_4 + 2CH_3COOH$$

$$CO_2 + \underset{\text{(hydrogen)}}{4H_2} \rightarrow CH_4 + \underset{\text{(water)}}{2H_2O}$$

The microbiology and biochemistry of anaerobic degradation process is much more complex than those of the aerobic process, due to increased diversity of paths Metabolic available for anaerobic community. In fact, the anaerobic degradation of organic matter to methane and carbon dioxide involves a sequential chain of metabolic pathways and requires the coordinated combined action of different groups of trophic anaerobic bacteria (HENZE et al., 1983).

The anaerobic digestion processes have been mainly applied to high strength waste and wastewaters, such as winery effluents, brewery slurries or sludge from wastewater treatment plants (DONOSO-BRAVO et al., 2009b).

3.4 **Acidification process**

The application of anaerobic treatment of industrial and municipal effluents can be increased if the end product obtained from the treatment contains a value that commercially stimulates or at least minimize investment. The solution may be in the anaerobic process itself, specifically in the acid phase (LEITE et al.).

Research with the goal of enhancing the techniques of anaerobic treatment of industrial wastewater, has been promoting a broader approach acidogenic step of the digestion process.

Several authors have been pointing to acidogenesis as a key step, both the influence that this has on the quality of methanogenesis, but also the value to by-products generated by acidogenic fermentation.

However, little is known about the bacteria involved in the processes of acidogenic metabolism, such as fermentation and reductive acetogenesis (LEITE et al.). Acidification is one of the most common and serious problems inducing process failure in anaerobic digesters.

The acidogenic stage favors some trophic groups (such as acidogenic microorganisms), but is inhibitory to others (methanogens microorganisms), compromising the proper functioning of anaerobic systems. Once the actual production of VFAs, mainly causes shock acid (LEITE et al.).

As a result, came the anaerobic digestion process into two stages: 1st stage, pre-treatment associated with hydrolysis/acidogenesis and 2nd stage associated with acetogenesis/methanogenesis (FANG et al., 2002).

When considering acidogenesis as a first step of anaerobic digestion formed by hydrolysis step itself and the acidogenic stage, it enables a variety of biological applications without being exclusively referred to as a further reaction step this mechanism.

The acidogenesis can thus be used as a unit operation intervening in a production process, where there are conditions of accumulation of VFAs for later retrieval by extraction. The anaerobic degradation steps provide enough allowance for the identification of key intermediates and the imposition of operating conditions for a specific product (LEITE et al.).

Figure 5 shows the destinations that have been suggested through the development of research focusing on profitability of VFAs market, they define two major groups of biochemical destination, biochemical (liquids) and biocombustion (gases).

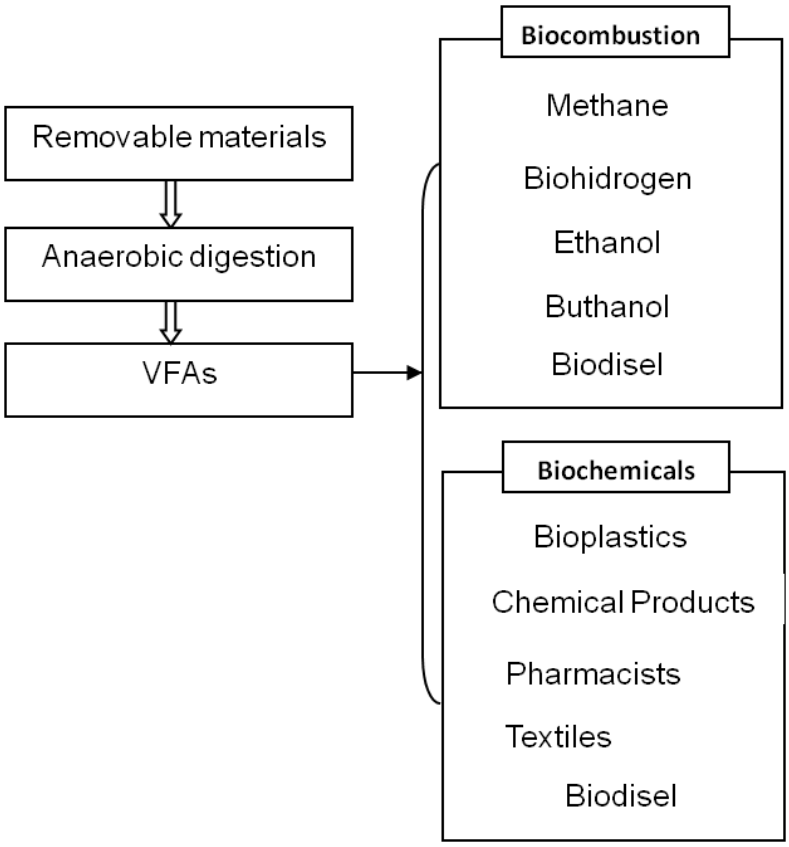

Figure 5 - Valorization potential to acidification by anaerobic digestion

In recent years the study of the acidogenic stage to produce alternative to fossil fuels has increased exponentially, in particular regarding the production of hydrogen (CHEONG et al., 2006). As H_2 is one of the products resulting from the anaerobic acidification, the interest in the study of this biochemical mechanism, in order to define optimum conditions for application to a biological H_2 production on an industrial scale has also increased.

4 Methodology

4.1 Experimental set up

For this study nine reactors, of five liters each, were developed, varying two parameters the ratio F/M (food to microorganisms) and the alkalinity content expressed in $gCaCO_3/L$

The reactors were operated as batch type, i.e., with a single food supply and regular monitoring by manual sampling during each activity, and submitted to a thermal bath, keeping the temperature 35 °C (±1 ºC). In this case, it was considered an operated period of fifteen days, which corresponded to an average of nine samples per reactor. Were performed three batteries of tests, each one with three reactors in operation.

Figure 6 present the global composition of each reactor: biomass; substrate, wine, alkaline solution, $NaHCO_3$, nutrients and water.

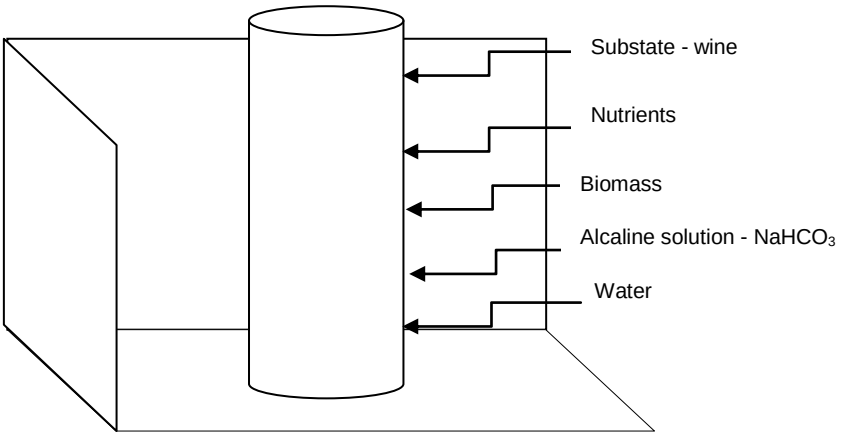

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Figure 6 – The global content composition of each reactor.

As can be demonstrated, each reactor's food was prepared synthetically and its composition as follows: substrate (concentration ranging between 1 gCODs/L and 8 gCODs/L); macro and micronutrients (volume 2 ml/L and 1 ml/L respectively), microorganism (concentration equal to 2 mg/L), alkalinity solution (concentration ranging between 0,2 and 4 $gCaCO_3$/L) and finally the reactor was completed with water to complete the volume of 5,1 L.

4.1.1 Macro and micronutrients

In all reactors were added 5 ml of micronutrients and 10 ml macronutrients, to stimulate the metabolic activity of microorganisms. The composition of these nutrients is presented in Table 3.

Table 3 - Chemical formula and concentrations of the nutrients added to the reactors

	Chemical formula	Concentration
Macronutrients	NH_4Cl	0.074 g/L
	KH_2PO_4	0.01 g/L
Micronutrients	$FeCl_3 \cdot 4H_2O$	4 µg/L
	$ZnCl_2$	0.1 µg/L
	$MnCl_2 \cdot 4H_2O$	1 µg/L
	$CuCl_2 \cdot 6H_2O$	4 µg/L
	$CuCl_2 \cdot 2H_2O$	0.06 µg/L
	$NiCl_2 \cdot 6H_2O$	0.1 µg/L
	H_3BO_3	0.1 µg/L
	$Na_2SeO_3 \cdot 2H_2O$	0.2 µg/L
	$(NH_4)6MoO_2 \cdot 4H2O$	0.18 µg/L

4.1.2 Alkaline solution

The alkaline content of the reactor is quantified in $gCaCO_3/L$. The alkaline solution used in this test was made with $NaHCO_3$, conversion was calculated using the molar concentration of $CaCO_3$ equal to 100.9 g/mol and $NaHCO_3$ equal to 84.01 g/mol.

4.1.3 Substrate

It was used three containers of wine bag-in-box of 5 liters of red wine of brand “Festão”. To reduce possible interference of sulphites present in the wine, which could come to

inhibit the acidification process, the wine was subjected to an aeration session and then frozen in portions.

Characterization tests performed involved the analysis of: TSS, VSS, CODt, CODs and pH (with the exception of the analysis of solids, the other parameters were analyzed before and after aeration).

4.1.4 Microorganism

The consortium of methanogenic sludge to be used in this study was obtained in the local sanitation company Sistema Intermunicipal de Saneamento da Ria de Aveiro (SIMRIA), from the conventional mesophilic anaerobic digester of the South WWTP, designed to digest this surplus activated sludge unit treating the urban wastewater.

In order to dispose of inert and coarse solids, which could cause a false result for the characterization of this biomass, sludge was washed and decanted before perform the TSS and VSS.

4.1.4.1 **Thermal pre-treatment**

Due to the nature of biologic sludge received, it was decided to use a thermal pre-treatment to inhibit the activity of the methanogenic biomass. However, at the end of the first battery and consequent data analysis, it was found that the thermal pre-treatment could also have caused a decrease of the acidogenic activity, this process was discarded for the other essays.

The sludge was characterized after a heating pre-treatment of 30 minutes in an oven at 90ºC. The analyzed parameters were the TSS and VSS. Results of the characterization are presented in Chapter 4.3**Erro! A origem da referência não foi encontrada.**.

4.1.5 Reactor volume

The volume of wine used in each reactor was calculated based on the CODs value of aerated wine, obtained through a characterization previously presented, and the previously determined F/M relation based on the literature review. The volume of wine that was used is presented in Chapter **Erro! A origem da referência não foi encontrada.**.

The concentration of the variables food to microorganisms and alkalinity that were added to the reactors were defined based on literature review and grouped as can demoted in Table 4 whereas only a single reactor without the addition of buffer solution had a pH of 5.37.

Table 4 – Identification of experimental runs.

Alkalinity ($gCaCO_3/L$)	F/M (gCODs/ gVSS)			
	0.5	**1**	**2**	**4**
0	-	-	*A0FM2*	-
2	*A2FM0,5*	*A2FM1*	*A2FM2*	*A2FM4*
4	*A4FM0,5*	*A4FM1*	*A4FM2*	*A4FM4*

4.2 Analytical Methods

At all it was performed three sets of reactors each one with three reactors, the parameters to be evaluated were the pH, alkalinity, VFAs, TSS, VSS, biogas and CODs. In the first reactor set it was operated the A2FM0,5, A2FM1 and A4FM0,5. In the second the A0FM2, A2FM2 and A4FM2 reactor. In the third the A2FM4, A4FM1 and A4FM4 reactor.

The samples were taken discontinuously from the reactors respecting a pattern of parameters analysis that can be showed in Table 5.

Table 5 – Schedule of reactor analysis

	sample	A0	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
	Time (h)	*0*	*2*	*4*	*24*	*26*	*46*	*118*	*165*	*216*	*288*	*336*
Parameters	pH	x	x	X	x	x	x	x	x	x	x	x
	Alkalinity	x										x
	VFA´s	x	x	X	x	x	x	x	x	x	x	x
	TSS	x										x
	VSS	x										x
	CODs	x	x	X	x	x	x	x	x	x	x	x
	Biogas	x	x	X	x	x	x	x	x	x	x	x

4.2.1 pH

The pH was measured in a bench apparatus termed *Consort C-350*.

4.2.2 Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS)

The determination of TSS, VSS was performed according to the methods 2540 B, 2540 D and E and G, respectively of APHA Standard Methods (1998). A volume of 5 ml of sample was filtered with fiber glass membrane with a pore of one micrometer (Whatman) which was subsequently dry in oven at 105ºC for 24 hours for determination of TSS. Subsequently, the same filter containing the dried biomass was calcined in muffle at 550ºC to determine the VSS. The VSS match biomass (organic matter) suspended in the sample, while the TSS represents the total organic and inorganic matter suspended in the sample.

4.2.3 Chemical Oxygen Demand (COD)

The value of COD is a measure of the oxygen equivalent of the organic fraction of sample that can be oxidized by an oxidant under controlled conditions.

To determine this parameter it was used the closed reflux spectrophotometric determination method (Method D 5520 described in APHA (1998)) to quantify the COD of the samples. In this method potassium dichromate (in an excess amount, under an acidic condition) was used as oxidant due to its higher oxidation capacity, applicability to a wide variety of samples and is easy of handling. Most of the organic compounds can be oxidized to 95% - 100% of the theoretical value.

Digestion was performed for two hours at 150°C and the sample contained dichromate potassium along with sulfuric acid (acid conditions so provide) sulfate Silver (to the oxidation of alcohols and long chain acids) and mercuric sulfate (to eliminate chloride interference). After the digestion of the samples, and cooling to room temperature, it was determined spectrophotometrically the unreacted amount of potassium dichromate. The absorbance of samples was measure using a spectrophotometer (Aqualytic brand, model PC023212), COD concentration is obtained from the measured absorbance in accordance with the respective calibration line which was stored in the apparatus. To determine soluble COD, samples were filtered through filter paper (trade Reeve Angel; grid 403) and the filtrate was collected and analyzed in the same procedure as total COD..

4.2.4 Alkalinity

The alkalinity was measured by classical titration.

4.2.5 Volatile Fatty Acids (VFAs)

The VFAs were determined by liquid-gas chromatography, using a chromatograph (Chrompack brand, model CP9001) shown in Figure 7. The samples used for analysis were first filtered, acidified with formic acid (1:10 (v/v)) and chilled to 4°C in polyethylene bottles until they are analyzed.

After calibrating the device with mixed standards of known concentration, we get a relationship between the area of each peak in the chromatogram and the corresponding

concentration of VFAs, thus allowing the identification and quantification of each VFA. To each standard was added formic acid at a ratio of 1:10 (v/v) to allow the array of patterns to be identical to the array of the samples.

Figure 7 - Chromatograph (Chrompack brand, model CP9001).

4.2.6 Biogas

The composition of biogas was determined by gas chromatography in a gas chromatograph detector (SRI brand, model 8610 C) equipped with a TCD (Thermal Conductivity Detector) shown in Figure 8. This device gives values for the fraction (v/v) of methane (CH_4), carbon dioxide (CO_2) and other gaseous components (N_2, H_2, H_2S, etc.).

Figure 8 - Chromatograph (SRI brand, model 8610 C)

4.3 **Characterization of used materials**

The volumes added to each reactor for each experimental assay, presented in the previous chapter, were calculated using the characterization of biomass (anaerobic sludge) and substrate (wine) based on the results of physicochemical analyzes.

- ***Biomass characterization***

The biomass used was always renewed before the start-up of each set of reactors, as justified in the previous chapter. The characterization of the biomass was just performed for TSS and VSS content

Table 6 shows the values obtained for biomass characterization before starting each set of reactors, according to the chosen pre-treatment thermal and washing and just washing. It was performed a thermal pre-treatment as an inhibitory of methanogenesis and it was applied just to one set. The other two sets used sludge which had been just washed to remove suspended solids and gross materials.

Table 6 – TSS and VSS content in biological sludge.

Sludge pre-treatment	TSS (g/L)	VSS (g/L)	%VSS
Thermal	36,74	24,48	67%
Wash	19,00	12,00	63%
Wash	21,97	17,64	80%

It can be seen that the sludge which were not subjected to thermal pre-treatment showed a decrease in the concentrations of TSS and VSS, probably the wash process increased the amount of water in the sludge, even through a decanting process.

The concentration of TSS and VSS, 37 and 25 g/L respectively, in the sludge that was thermally treated, were higher than the values obtained for the sludge which was just wasted.

- ***Substrate characterization***

As detailed in the previous Chapter, it was used as substrate a simulated winery effluent prepared from commercially available red wine, thereby avoiding adverse variations caused by the seasonality of the production process of wine. The simulated winery effluent was performed with industry. However, the bottled wine contains compounds that may function as biological inhibitors. These compounds may come from the raw material, such as tannins, which are present in grapes, even more evident in red grapes, on are produced during the wine producing process.

The presence of SO_2 that was referred to earlier in this work, throughout Chapter 3 has antiseptic, anti-bacterial and antioxidasic properties, which may inhibit the activity of microorganisms. It is usual to add to the grape must before fermentation in order to reduce risks of bacterial contamination that could compromise manufacturing and to add it before bottling the wine to protect chemical or enzymatic oxidation and microbial growth, and camouflage also the flavor of ethanol, then improving the taste of the wine.

The amount of SO_2 added before the fermentation process is consumed almost entirety during the process. However the second dose, that succeeds the fermentation process, maintain a certain amount of residual SO_2 incorporated to bottled wine, within acceptable limits in order to 160 mg/L for red wines; and 210 mg/L for white and rosé wines (EC, 1999), prevent the risks to consumer health.

As in this work is was used a final product, with significant SO_2 level and considering that in fact a winery effluent the influence of SO_2 would be less significant, it was adopted as a palliative measure a previous pre-treatment step, consist in an aeration process in order to remove SO_2.

This pre-treatment step consisted on subjecting the wine to aeration during for approximately two minutes.

Characterization tests performed involved the analysis of: TSS, VSS, CODt, CODs pH and VFAs. With the exception of the analysis of solids, all the other parameters were analyzed before and after the pre-treatment. The result of the wine characterization can be seen in Table 7

Table 7 - Substrate physiochemical characterization.

Substrate	CODt (g/L)	CODs (g/L)	TSS (g/L)	VSS (g/L)	pH
Wine without aeration	266,3	235.5	1,01	0,57	3,44
Wine with aeration	265,2	228.8	-	-	3,44

Given the result of physicochemical parameters analyzed, little or nothing has proved relevant in the comparison of the results with and without aeration step, i.e. for CODs, CODt and pH the aeration did not influenced the results. .

Between the first set of reactors and the others, the wine was kept frozen in a freezer and before each essay, after defrosting, the wine was subjected to a new characterization and the obtained results for the CODs, CODt and pH parameters are presented in Table 8.

Table 8 - Variation of the substrate characterization along the essays.

Substrate	CODt (g/L)	CODs (g/L)	pH
Wine with aeration - 1st Essay	265,2	228,8	3,44
Wine with aeration - 2nd Essay	231.0	215.2	3,50
Wine with aeration - 3rd Essay	218,3	207,3	3,50

The wine's VFAs were measured in two different dilutions, 20 ml/L and 40 ml/L in order to replicate more faithfully the concentrations that will be present in the startup of used in the reactors. For this was used a one liter flask and distilled water, and 20 ml de red wine for first dilution and 40 ml for second dilution thus it was obtained the following values shown by Table 9**Erro! A origem da referência não foi encontrada.**.

Table 9 - Wine VFAs characterization

Wine (mL)	VFAs dilution results (mgCODs/L)	Total of wine VFAs (mgCODs/L)
20	**66**	**3300**
40	**152**	**3800**

4.4 **Calculations**

4.4.1 Volume of sludge used for each reactor

The volume of biological sludge to be use in each reactor was calculated based on the value of VSS, obtained in the characterization of pre-treated sludge, previously presented, and the concentration to be used in the experimental setup (2g VSS/L).

4.4.2 Volume of wine used in each reactor

The volume of wine used in each reactor was calculated based on the value of CODs of aerated wine, obtained in the characterization previously presented, and the F/M ratio previously chosen based on the literature review. In Table 10 it was assumed an M concentration of 2g VSS/L and the VFAs concentration of the wine equal a 3500 mgCODs/L.

In F/M ratio, F corresponds to the value of the food in gCODs/L to be added for a volume of 5,1 L. The F amount and the wine correspondent volumes added to each reactor are presented the concentration of VFAs estimated for these volumes are shown in Table 10.

Table 10 - Volumes of wine added to each reactor

F/M	F (gCODs/L)	Wine (ml)	VFAs (mgCODs/L)
0,5	1	20	14
1,0	2	45	31
2,0	4	90	62
4,0	8	200	137

4.4.3 Volume of alkaline solution used for each reactor

The amount of $NaHCO_3$ to be used, measured as $CaCO_3$, was calculated using the molar mass of $CaCO_3$, that is equal to 100,09 g/mol and $NaHCO_3$, that is equal to 84,01 g/mol. This corresponds to a relation of 1,19g $NaHCO_3$ for 1g $CaCO_3$. The volumes of alkaline solution added to each reactor are presented in Table 11.

Table 11 - Volume of $NaHCO_3$ added to each reactor.

Alkalinity ($gCaCO_3/L$)	$NaHCO_3$ (g/L)	Volume of $NaHCO_3$ solution 50g:1L (mL)
0	0,0	0
2	2,5	240
4	5,0	480

5 Results and Suggestions

To determine the amount of substrate and biomass to be added to each experimental set up it was necessary to perform physicochemical characterization of each material to be used.

5.1 Reactor behavior with time

The behavior of the reactors was evaluated on the basis of the analysis of several physicochemical parameters, according to the operating time. This methodology analyzes the curves obtained for each parameter, in order to identify some trends regarding the evolution of the acidification process.

5.1.1 pH in function of time

All reactors had a similar behavior, as regarding pH evolution during the process, where it can be observed a decrease with time. It was also possible to see two groups of reactors. A bigger group where the final pH was mainly higher than 6, and another group where the pH reached values lower than 5,5.

During the first 24 hours of operation it was observed an unstable period with a high pH variation, probably due to adsorption reactions that might have occurred immediately after mixing the substrate with the biomass. After this period it started to gradually decline, reaching an average pH value between 6.5 and 7.5 for most essays. There were two reactors (A0FM2 and A2FM4) which reached pH values below 5, as can be observed in Figure 9.

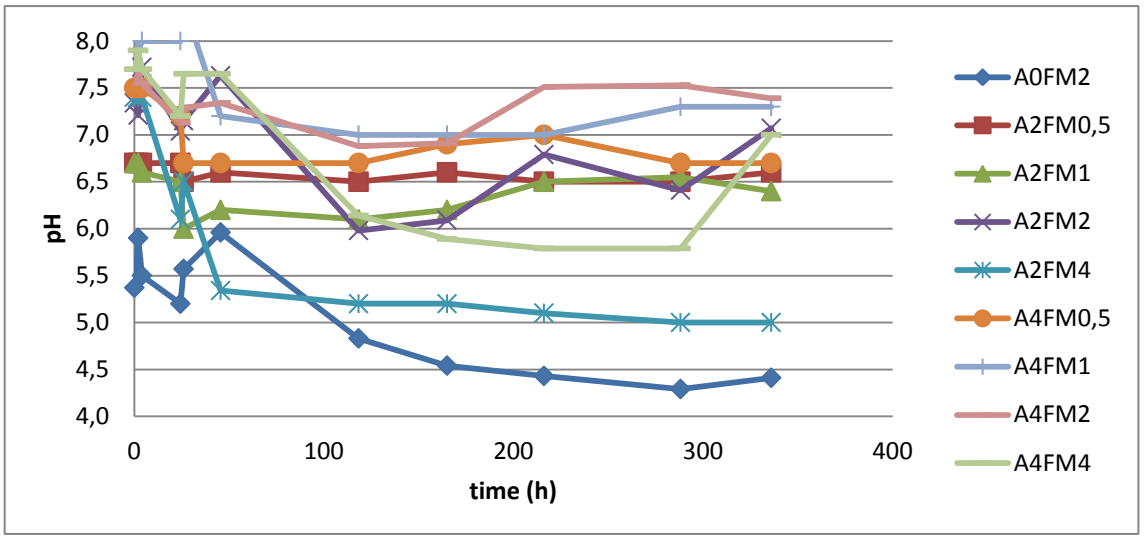


Figure 9 – pH behavior in reactors along the essays.

These two reactors had a lower initial alkalinity when compared to their organic load: A0FM2 reactor (alkalinity=0 $CaCO_3$/L and F/M=4g CODs/L / 2g VSS/L); and A2FM4 reactor (alkalinity=2g $CaCO_3$/L F/M=8g CODs/L / 2g VSS/L), that may have caused the pH to reach such low values.

So, in conclusion, initial alkalinity added to the batch test affects directly the performance of the reactor. Then, to prevent pH to reach values lower than 5.0, it's necessary to add significant alkalinity at reactor start-up. The amount to be added depends on the load applied to the reactor.

5.1.2 CODs removal rate in function of time

The performance of the reactors in terms of COD removal along their activity periods presented similar pattern when comparing the curves obtained for the nine reactors. It can be observed for all reactors a initial period (up to 120 hours) where the removal was lower than 20%. The reactors charged with thermal pre-treated sludge present a higher initial period (A2FM0.5 and A2FM1)

Although the degree of removal varies with organic load and alkalinity levels adopted, the time where it can be observed an increase or decrease are shown similar as can be demonstrated by Figure 10.

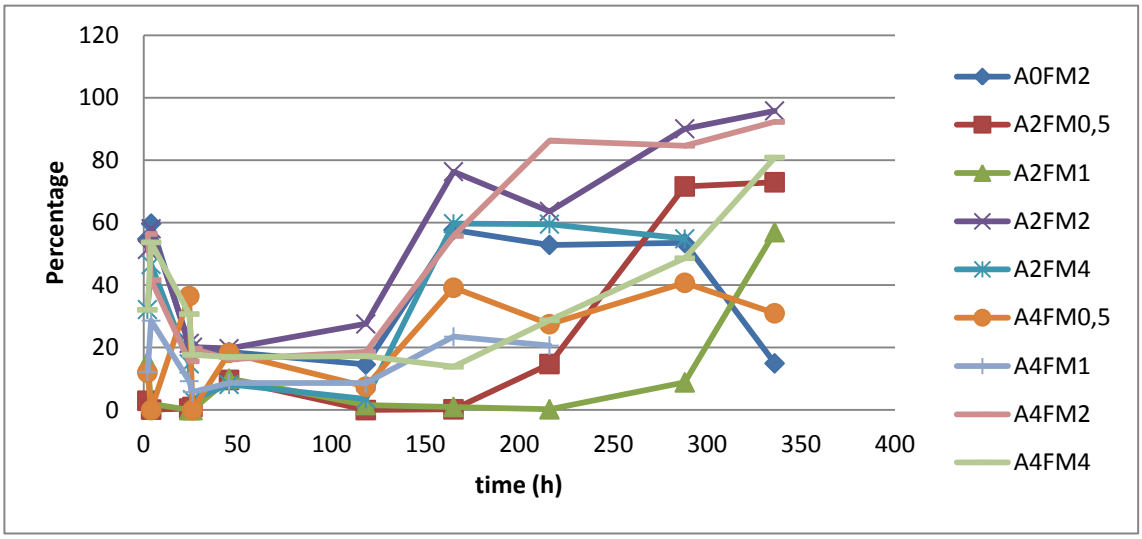


Figure 10 – CODs absolute removal percentage along the essays.

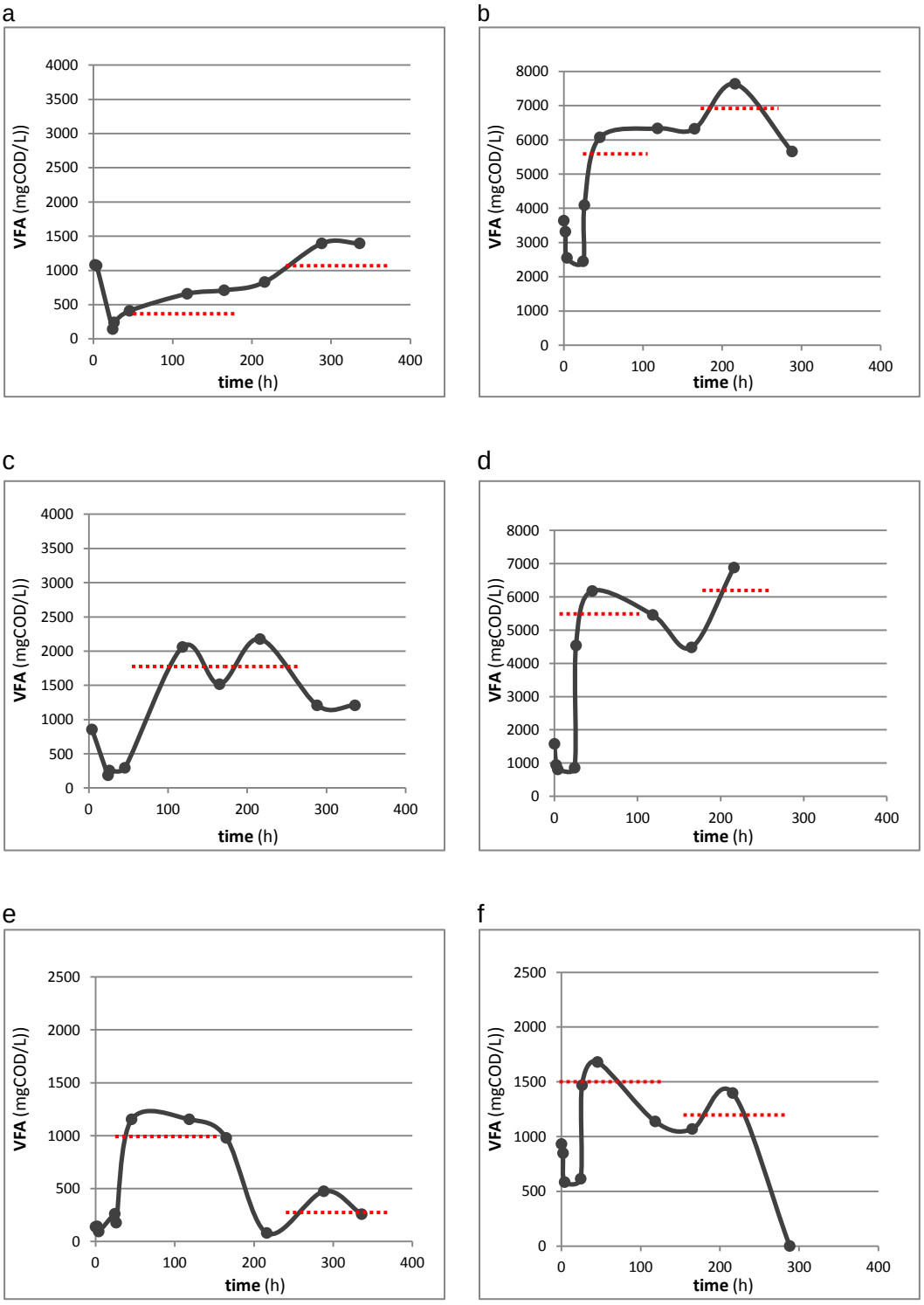
Having the first 24 hours of operation a more intense activity and high degree of instability, most probably due to other mechanisms besides biodegradation which tends to regress, followed by a period of decrease which extends to approximately 120h (or 5 days) of operation of the reactors were the total removal of CODs reached the 0% in reactors A2FM0.5 and A2FM1. At the end of this phase it begins a considerable increase in the removal of CODs.

In addition, it is observed that reactors A4FM2 and A2FM2 are the reactors which reached the highest CODs removal rate (higher than 90%), followed by reactors A2FM0.5 and A2FM1 which reached a maximum of 80% of COD removal. The remaining reactors always remained below 40% of removal of CODs.

5.1.3 Total VFAs production in function of time.

VFAs production remained uneven during the first 24 hours, so it was observed an unstable profile during this period.

After this initial phase, the VFAs production started to increase for all reactors. The curve of the total acids measured in these experiments is characterized by the formation of two distinct peaks of productivity as is shown in Figure 11.



Reactors: a:A0FM2; b: A2FM4; c:A2FM2; d:A4FM4; e:A2FM1; f: A4FM1

Figure 11 – Variation of the total VFAs with time.

As it can be observed in Figure 11, the appearances of the two maximum peaks are different.

After analyzing this behavior it can be suggested that the order of appearance between the higher and lower peaks is related to the alkalinity concentration in relation to the organic load applied. The reactors fed with lower levels of alkaline, when compared to their organic loads, tended to have a smallest peak first, followed by higher peak (reactors A0FM2 and A2FM4), as presented in Figure 11 a and b. In the opposite reactors highest levels of alkalinity in relation to their organic loads tended to have the highest peak first followed by the smallest (reactors A4FM1 and A2FM1), as presented in Figure 11 e and f. When the ratio of alkalinity inserted into the reactor and the organic load to which it was subjected is equal or close to one, i.e., the values of alkalinity and organic load are equal or similar, the trend is to decrease the difference between the peaks, Figure 11 c and d are an example.

5.1.4 Biogas accumulated in function of time

The biogas production in the different reactors showed a similar behavior for most reactors, although all presented a low production compared with production levels of VFAs as can be seen in Figure 12.

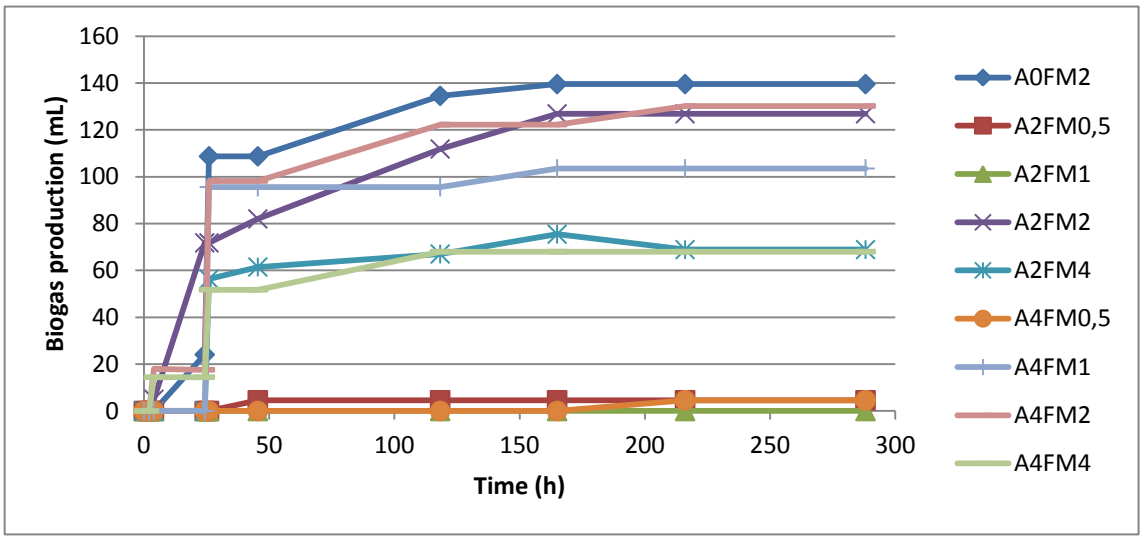


Figure 12 – Biogas accumulated production along the essay (mL).

The exception are the reactors A2FM0.5; A2FM1 and A4FM0,5, who presented a very low production of biogas, next to zero, but it can be emphasized that these three reactors were operated with biomass that has been subjected to thermal pre-treatment.

5.2 **Reactors analysis based on the organic load effect**

The organic load is a determinant factor for the microbial activity, so, in this chapter it will be done a comparison between reactors that run with the same alkalinity but different F/M ratios, in order to evaluate the maximum VFAs production and individual acid composition for each one. At all, it will be discussed the behavior of eight reactors grouped according to their initial alkalinity added: 2 or 4g $CaCO_3$/L.

5.2.1 Volatile Fatty Acids (VFAs) production

The VFAs production was sensitive to the increase in organic load expressed in terms of the ratio F/M, as can be seen in the Figure 13.

Figure 13 presents the maximum total VFAs concentrations and pH variation, obtained in the four reactors with an alkalinity of 2g $CaCO_3$/L, and F/M ratios of 0.5, 1, 2 and 4g CODs/gVSS. Comparing the results obtained in Figure 13, it can be seen an increase with the organic load, and be observed a growth trend. It can also be observed that the pH drop increased with the increase in the VFAs production, where the highest variation was obtained for the highest F/M ratio of 4g CODs/gVSS (reactor A2FM4).

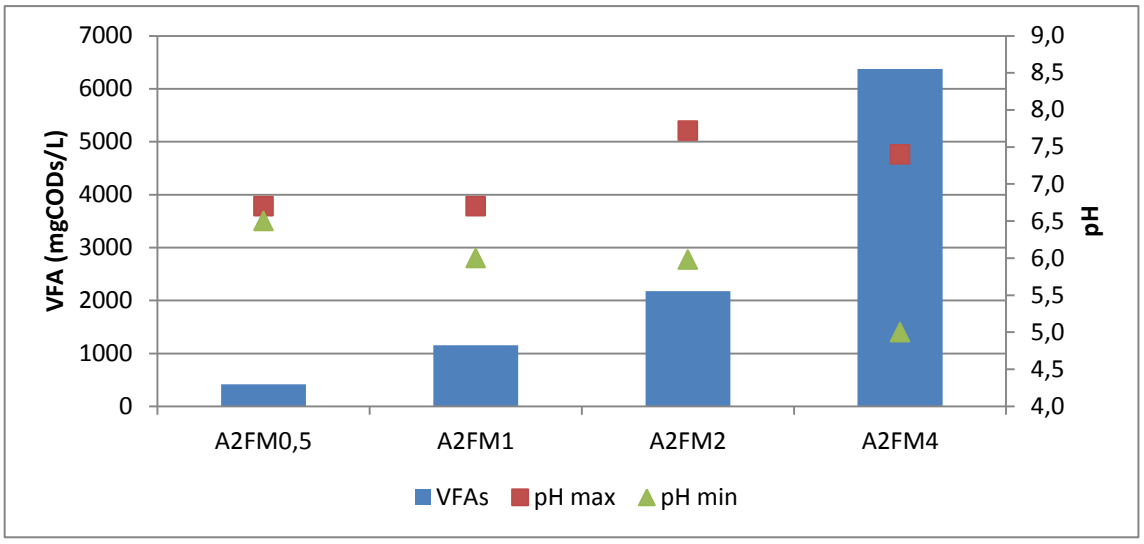


Figure 13 - Total VFAs production and pH variation for reactors with 2g $CaCO_3$/L.

Figure 14 shows the same analysis for the reactors with a highest alkalinity of 4g $CaCO_3$/L, and similar F/M ratios of 0.5, 1, 2 and 4g CODs/gVSS.

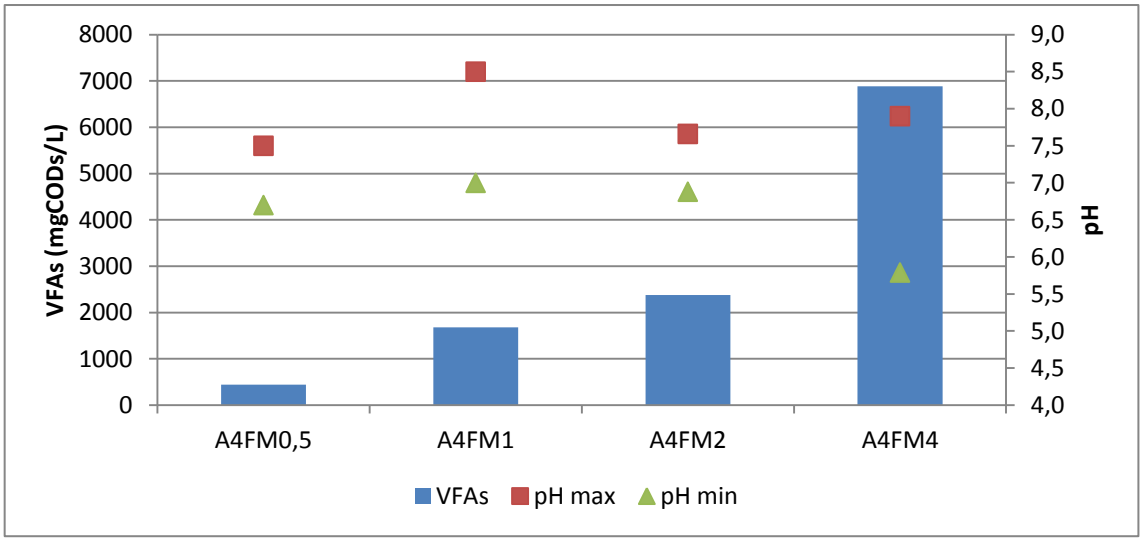


Figure 14 - Total acid production and pH variation for reactors with alkalinity equal to 4g $CaCO_3$/L

As the previous analysis, reactors with an alkalinity equal to 4g $CaCO_3$/L presented a significant VFAs production increase when submitted to increase on the F/M ratio.

Comparing Figure 13 and Figure 14 it is possible to observe that the total productions of VFAs are very similar. However, in Figure 14 the behavior of pH has presented some difference, although only the reactor with the height load reached a pH lower than 6. The greatest differences between maximum and minimum values were observed in two reactors (A4FM4 and A4FM1).

The minimum values for pH were lower for the assays with lower alkalinities (Figure 13) where it was obtained a pH of 5,0 for the highest load (F/M of 4g COD/VSS), when the pH for a similar load but a higher alkalinity (4g $CaCO_3$/L) was 5.7.

5.2.2 Composition of total VFAs production.

In Figure 15 and Figure 16 are presented the compositions of acidogenic products for reactors with the lowest alkalinity (2g $CaCO_3$/L), expressed in gCODs/L, and as a percentage of total VFAs, respectively.

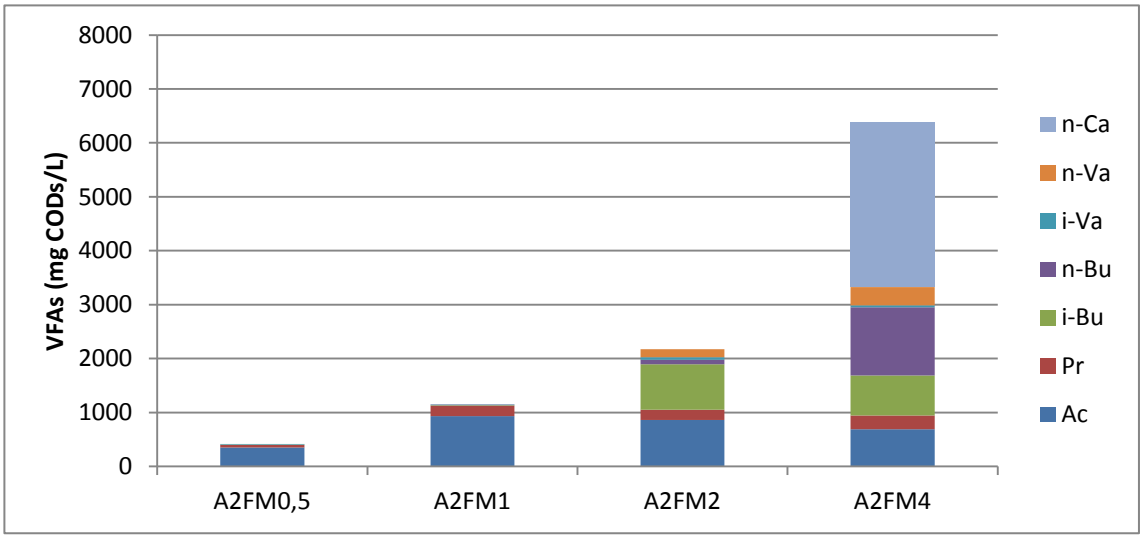


Figure 15 – Total of VFAs composition in terms of mgCODs/L for reactors with alkalinity 2g $CaCO_3$/L.

It is possible to observe in Figure 15 that the increase of the organic load favors not only the quantitative increase in the total VFAs production, but in the same way, it favors the diversification of the acidogenic products.

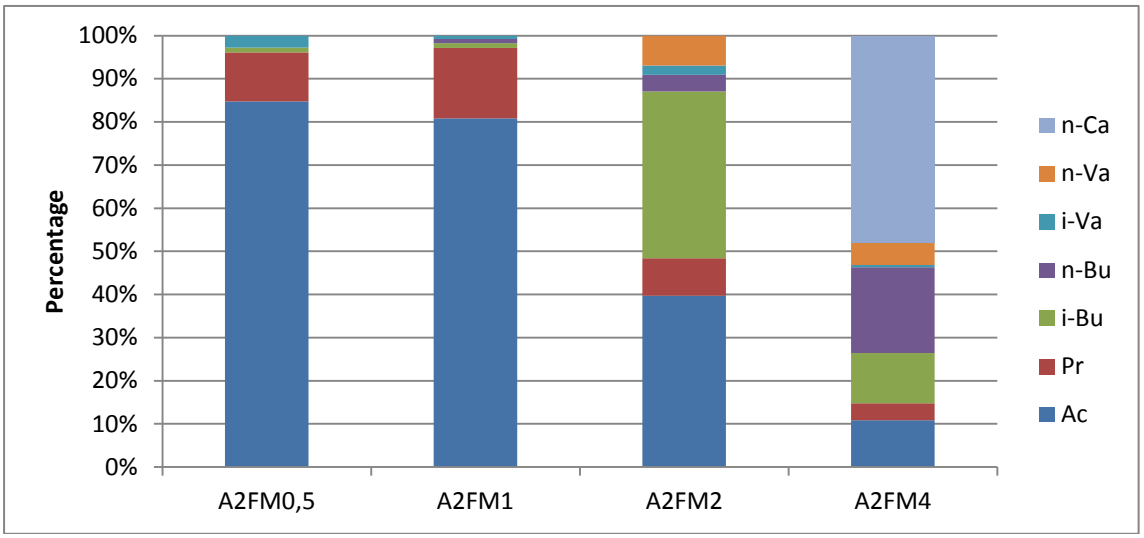


Figure 16 – Percentage distribution of the VFAs production in reactors with alkalinity 2g $CaCO_3$/L

With respect to the proportionality of each VFA, for each reactor with a alkalinity of 2g $CaCO_3$/L, it can be observed in Figure 16 a notorious predominance of the acetic acid production (80% and 85%) in the reactors with lower F/M ratios, A2FM0,5 and A2FM1 respectively followed by the production of propionic acid (10% and 18%). This situation changed with the increase in the F/M ratio, where it can be seen the predominance of VFAs with higher molecular weight (iso, n-butyric and n-caproic).

Hence, for reactor with highest F/M (A2FM4) the production of acetic acid corresponds only to 10% of the total VFAs production and leading to the appearance of acids with a longer carbonic chain (13% iso-butyric, 20% n-butyric and 49% n-caproic).

The same methodology was applied to reactors with the addition of a highest value of the alkaline solution (4g $CaCO_3$/L), as can be seen in Figure 17 and Figure 18.

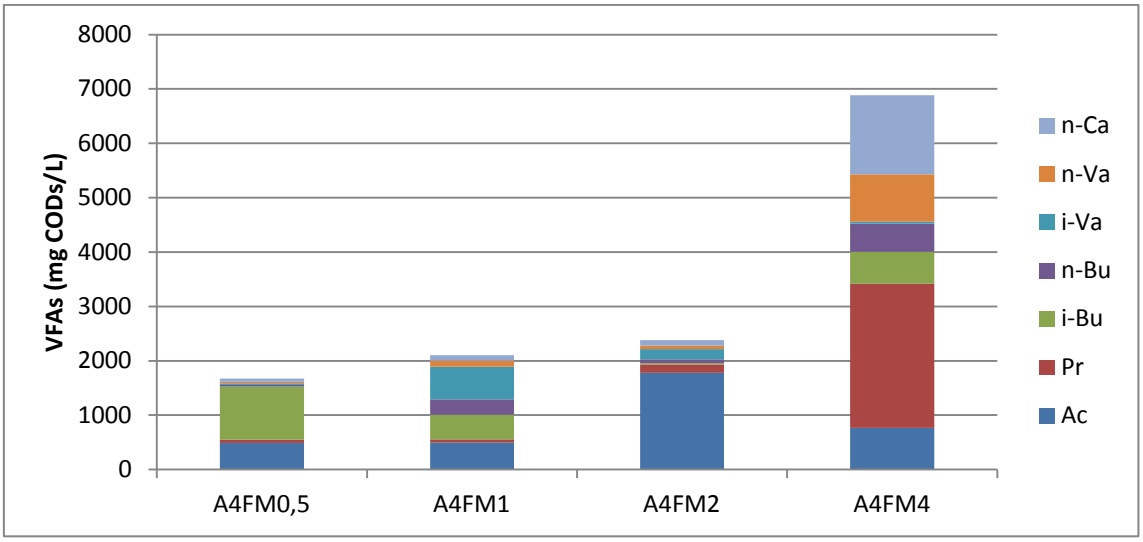


Figure 17 - Total of VFAs composition in terms of mgCODs/L for reactors with alkalinity 4g $CaCO_3$/L

Similar do what happened with the reactors with lower alkalinity, the maximum total VFAs production increase with the increase in F/M ratio, but with values higher for each reactor. When compares with the results obtained with a lower alkalinity.

Figure 17 shows a larger quality of different acids at lower concentrations and the predominance of iso-butyric acid in reactors A4FM0.5 and iso-butyric and iso-valeric for reactor A4FM2.

The reactor with the highest load (A4FM4) presented a predominance of the propionc acid, followed by similar amount of the other VFAs.

Based on the analysis of Figure 18 it is possible to observe that the production of acetic acid is less expressive when compared with reactors with a lower alkalinity. However, the production of this acid, as in the previous example, was inversely proportional to increasing concentrations of CODs, corresponded to F/M increase, except for reactor A4FM2.

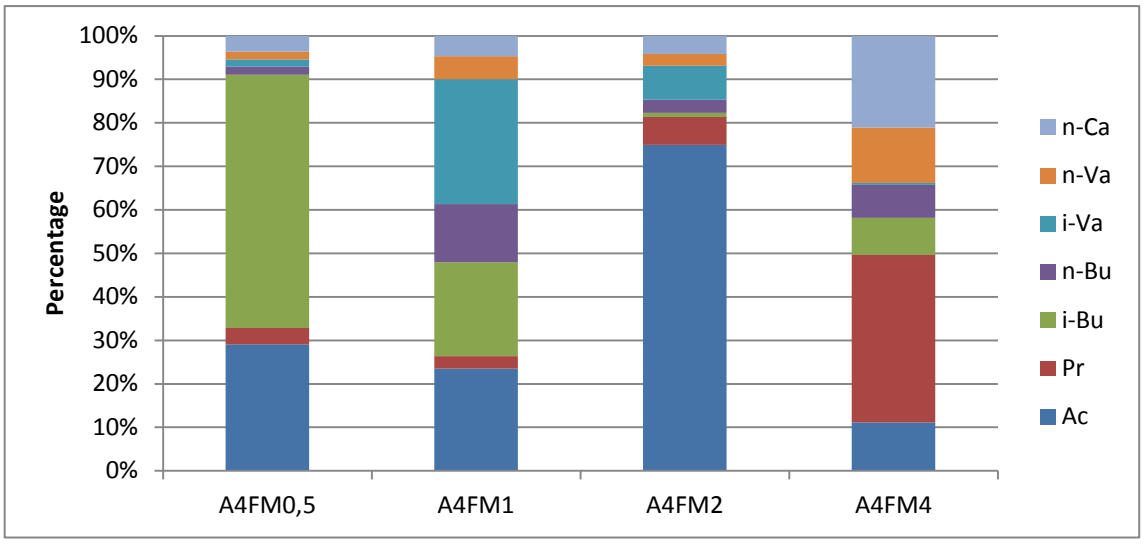


Figure 18 – Percentage distribution of the VFAs composition for reactors with alkalinity equal 2g $CaCO_3$/L

The amount of the iso-butyric acid decrease with the increase in the F/M ratio, whereas the amount of increase of propionic acids increase with F/M ratio, reaches 40% of total VFAs for reactor A4FM4.

For F/M ratio of 0.5, 1 and 2g CODs/gVSS the main VFA were acetic, iso-butyric and iso-valeric. The predominant acid for the reactor with F/M ratio of 0.5g CODs/gVSS is the iso-butyric (60%) and for the reactor with F/M ratio of 1g CODs/gVSS were the acid iso-butyric (23%) and iso-valeric (30%). For the highest F/M ratio, 4g CODs/gVSS, the predominant VFA was propionic (40%), followed by others VFAs with large carbon chain, namely iso-butyric (8%), n-butyric (8%), n-valeric (13%) and n-caproic (21%).

5.2.3 Mass Distribution of CODs

Mass Distribution of CODs allows a better understanding of reactor behavior and presents a schematic estimation of the distribution of each organic load fed to each batch reactor. Once it was added to each reactor a known amount of CODs, and assuming that the reactor is a closed system under anaerobic conditions, there will be nothing lost in terms of CODs during the process. Based on the laboratorial analysis it is possible to distribute

the initial CODs as CH_4 and VFAs produced and non acidified CODs remained in the liquid. The non acidified CODs was quantified by the subtraction of the VFAs obtained from the CODs analyzed. The difference between the initial CODs and the sum of these three components is assumed to be both the growth of biomass and the amount aggregated adsorbed to biomass.

Figure 19 presents the mass distribution for the reactors that were submitted to the lowest alkalinity (2g $CaCO_3$/L).

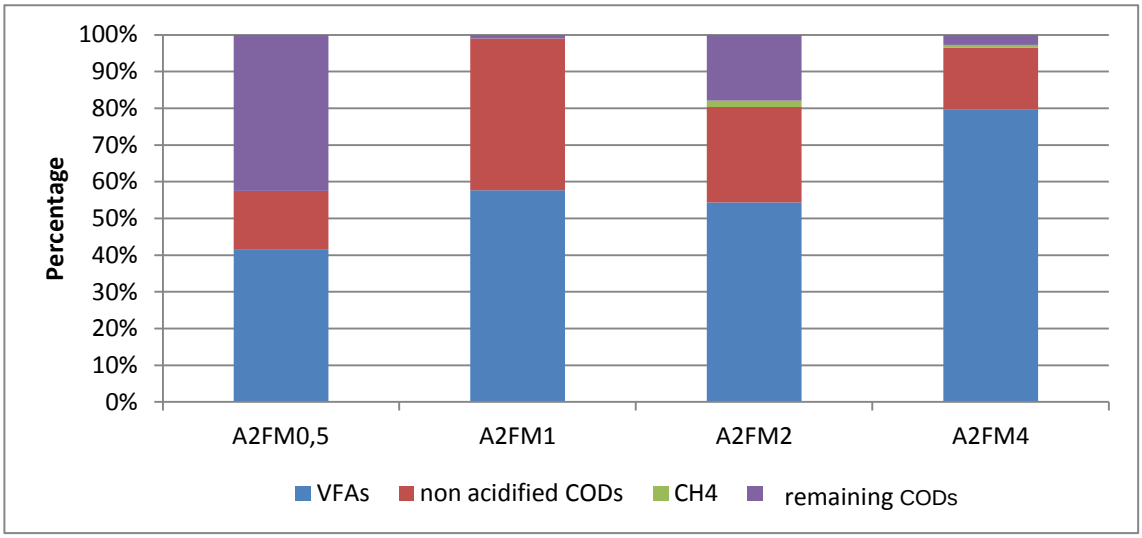


Figure 19 – Percentage distribution of the CODs mass distribution for reactors with alkalinity of 2g $CaCO_3$/L.

The organic load increase had favored the production of VFAs (40-80% of COD load). The lowest VFAs production (40%) was observed in reactor with a lower load (reactor A2FM0.5. The highest VFAs production (80%) was observed in the reactor with the highest load (reactor A2FM4).

The reactor with the lowest load (A2FM0.5) was the only reactor which didn't produce methane, most probably because the biomass had a previous thermal pre-treatment step, which inhibited methanogenesis.

In the mass distribution shown in Figure 20 it is presented the comparison of the CODs distribution in the reactors submitted to an alkalinity 4g $CaCO_3$/L. It is possible to observe that all reactors present a very small contribution of CH_4, although higher than the reactors with a lower alkalinity.

For these conditions, the reactor with the lowest load (A4FM0.5) was the only one which didn't produce methane, similarly to what happened in the previous conditions, due to the fact that this biomass that was also thermal pre-treated.

The reactor A4FM4 with the highest organic load is the reactor which presented the highest acidogenic potential (85% of COD feed was converted to VFAs). The portion of non acidified CODs in this reactor was negligible.

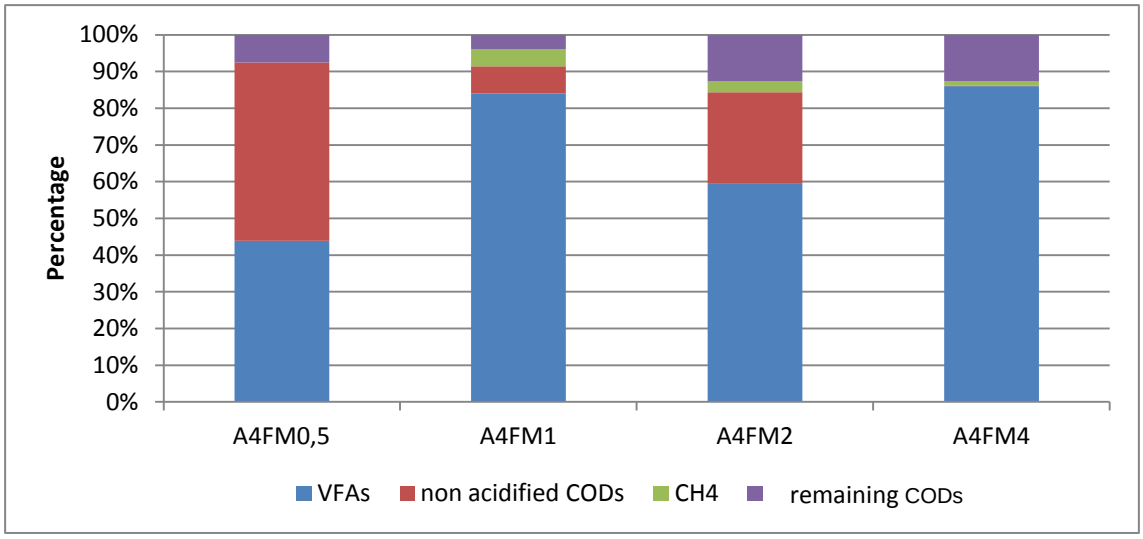


Figure 20 – Percentage distribution of the CODs mass distribution of reactors with alkalinity of 4g $CaCO_3$/L

5.2.4 Acidification degree

The acidification degree is defined as the ratio of the maximum of total VFAs that are Produced in the reactor and the amount of CODs fed. This analysis suggests which conditions had presented higher yields according to the acidification degree.

Figure 21 presents the comparison between CODs input, the respective production of VFAs and also the degree of acidification, for reactors submitted to an alkalinity of 2g $CaCO_3$/L.

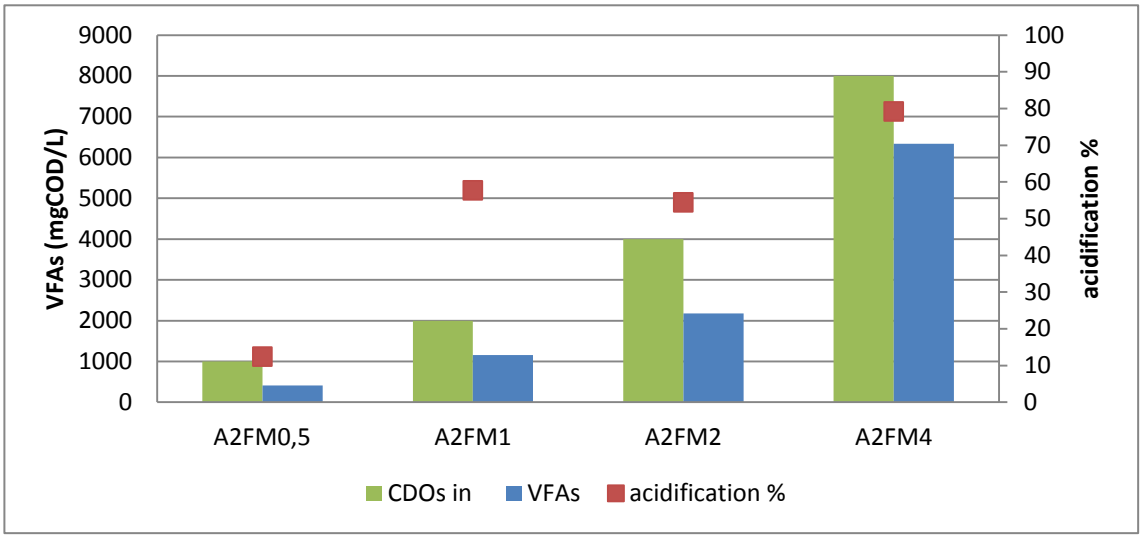


Figure 21 - Relation between acid production, organic load input and acidification degree for reactors with an alkalinity equal to 2g $CaCO_3$/L.

The organic load has revealed to be efficient in the increase of the acidification potential. However, it is noteworthy that only the lowest reactor submitted to the F/M ratio of 0.5 gCOD/gVSS presented the lowest degree of acidification (around 10%) probably because the acidogenic bacteria need more organic matter. All the other reactors presented higher acidification degree, (54 – 80%).

Figure 22 presents the comparison between the CODs input, the respective production of VFAs and also the degree of acidification, for reactors submitted to an alkalinity of 4g $CaCO_3$/L.

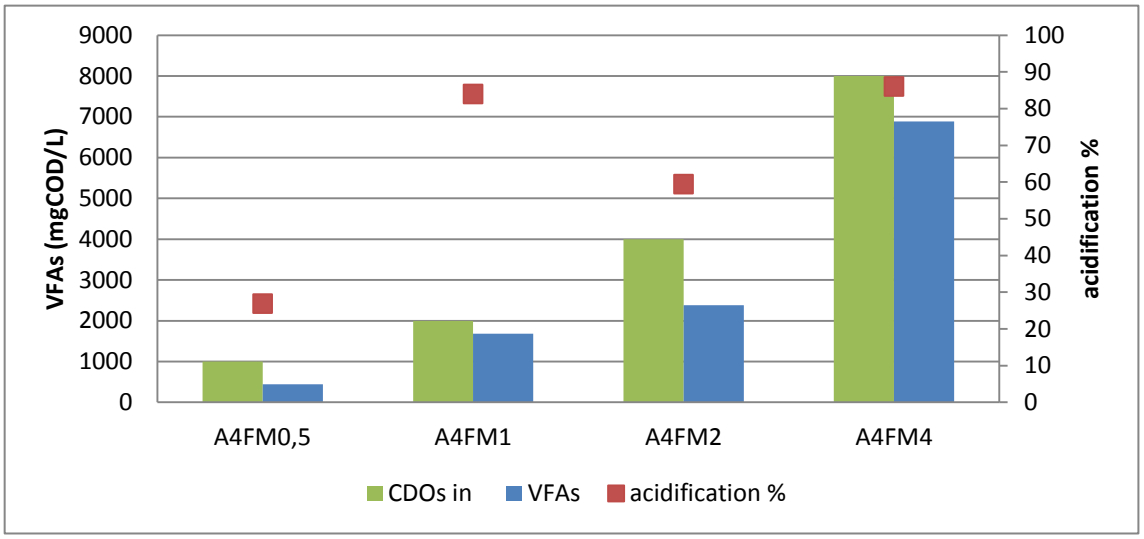


Figure 22 - Relation between acid production, organic load input and acidification degree for reactors with an alkalinity equal to 4g $CaCO_3$/L.

It can be observed that the behavior of these reactors is very similar to the other group with lower alkalinity, but with higher acidification degrees for the reactors (59 – 86%) for the reactors with F/M ratios of 1, 2 and 4g CODs/gVSS.

It is suggested the use of winery effluent non-simulated, for to evaluate possible effects caused by seasonal , characteristic of the vinification process and consequently this substrate. In order to test for variations of organic loads along of one productive year, and the effect of these variations on this treatment process.

5.3 **Reactors analysis based on the alkalinity**

In this topic it will be discussed the effect of alkalinity in the behavior of the acidogenic reactors operated with winery simulated effluent as substrate for microbial mixed cultures.

An alkaline solution was added at the start-up of the reactors with the purpose of rising the pH value and give some buffer capacity to the system, in order to prevent a strong inhibition to the acidification process. To evaluate the influence of the alkaline solution in the acidification process it was performed two sets of experiments with two different concentrations of alkaline solution in the reactor (2 and 4g $CaCO_3$/L). Each set of experiments was composed by four reactors with different F/M ratios ranging from 0.5 to 4g CODs/gVSS. Finally, to further prove the influence of the addition of the alkaline solution in the acidification process it was performed a reactor with no alkaline solution added and an F/M ratio of 2g CODs/gVSS.

5.3.1 VFAs production

Figure 23 shows a comparison of the maximum production of VFAs among the three reactors subjected to the same organic load, an F of 4g CODs/L. The pH variations are expressed as the maximum and minimum pH values registered along his essays, where the minimum corresponded to the end of the experiment.

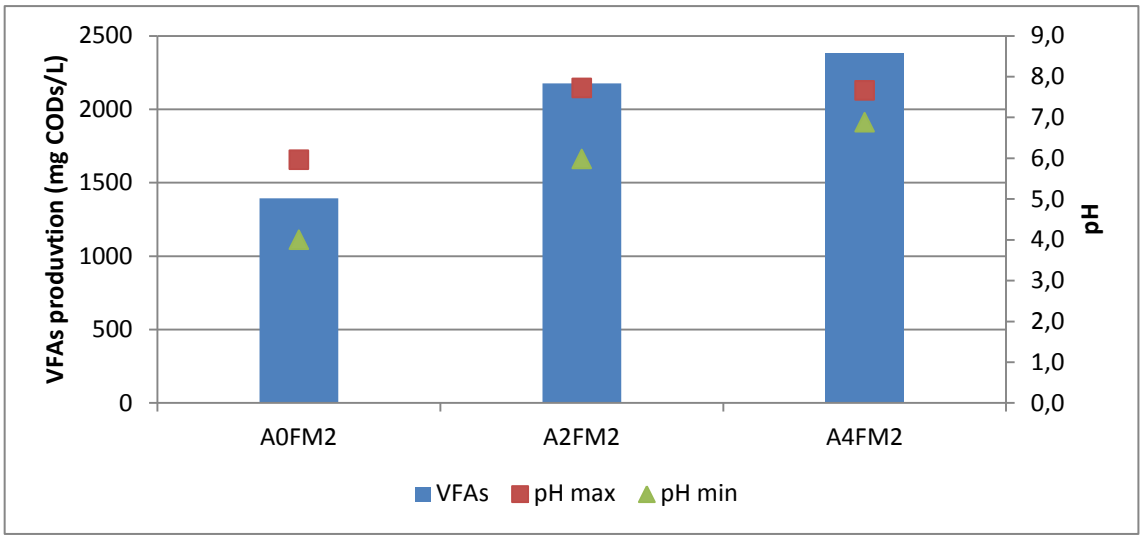


Figure 23 – Comparison of the maximum production of VFAs and pH ranges among three reactors subjected to the same organic load, 4g CODs/L.

The increase of the addition of alkalinity presented advantages when regarding the production of VFAs where it was observed an increase of this parameter with the increase of alkalinity. It can also be observed an increase on alkalinity results in a narrower range of pH variation. So the pH for the highest alkalinity didn't change as much as for the reactor with any alkalinity added.

Hence, Figure 23 shows that, in terms of productivity, the best performance was achieved for reactor A4FM2 presenting a short variability of pH (7,7 – 6,9) and higher VFAs production of 2378mg CODs/L. The reactor A2FM2 presented the second best performance 2176mg CODs/L, with little divergence compared to the first. The reactor which had no alkaline addition presented a wider variability in terms of pH and was not as efficient (1394mg CODs/L of VFAs) as the other acidogenic reactors.

Figure 24 repeats the same analysis, but for reactors with an F/M ratio of 4g CODs/gVSS. It can be observed that the results exhibit the same trend as observed in Figure 23, where the essay with the highest alkalinity shows a slight increase over the one with lower alkalinity, although the pH variation was relatively the same.

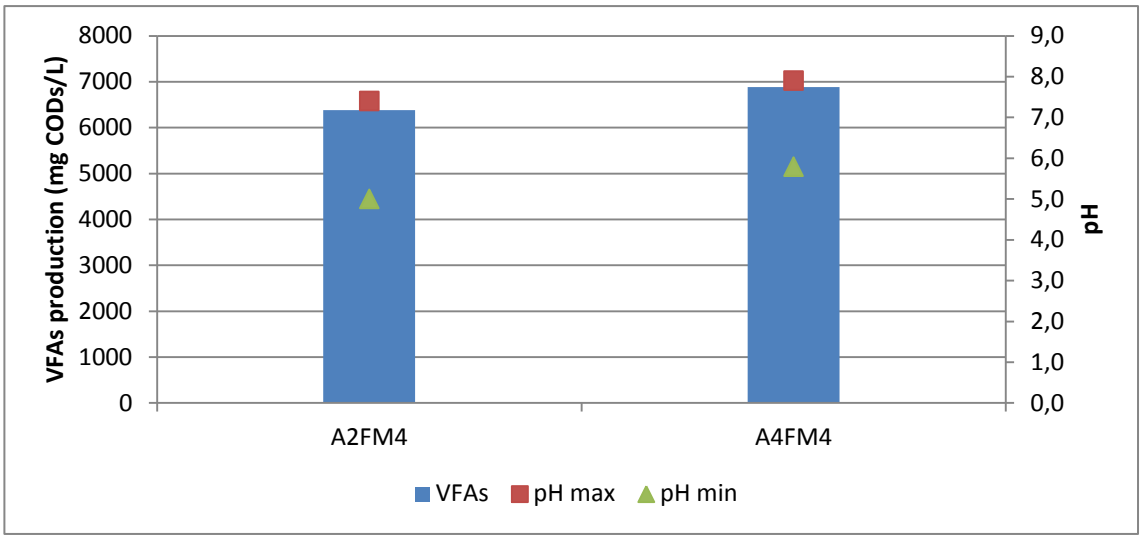


Figure 24 - Comparison of the maximum production of VFAs and pH values between two reactors subjected to the same organic load (2g CODs/L).

5.3.2 Peak formation for VFAs production

As presented in section **Erro! A origem da referência não foi encontrada.**, the VFAs production with time presented two peaks during the reactor operation period. Reactors with higher alkaline profiles present a higher VFAs production in the first peak, whereas in reactors where the value of organic load fed to the reactor was greater than the values of the to alkalinity concentration, the maximum acid production was observed only in the second peak.

It is important to consider that the level of alkalinity needed is directly related to the concentration of organic load to which the reactor was submitted to.

Thereby, to define the level of alkalinity that is necessary in each reactor it should be considered the ratio Alk/F, where Alk is the alkaline concentration in $gCaCO_3/L$, and F is the concentration in gCODs/L to which the reactor was submitted.

Figure 25 shows a schematic representation of maximum concentrations of VFAs produced in the reactors in the two times of higher activity (first and second peaks), associated with the ratio Alk/F.

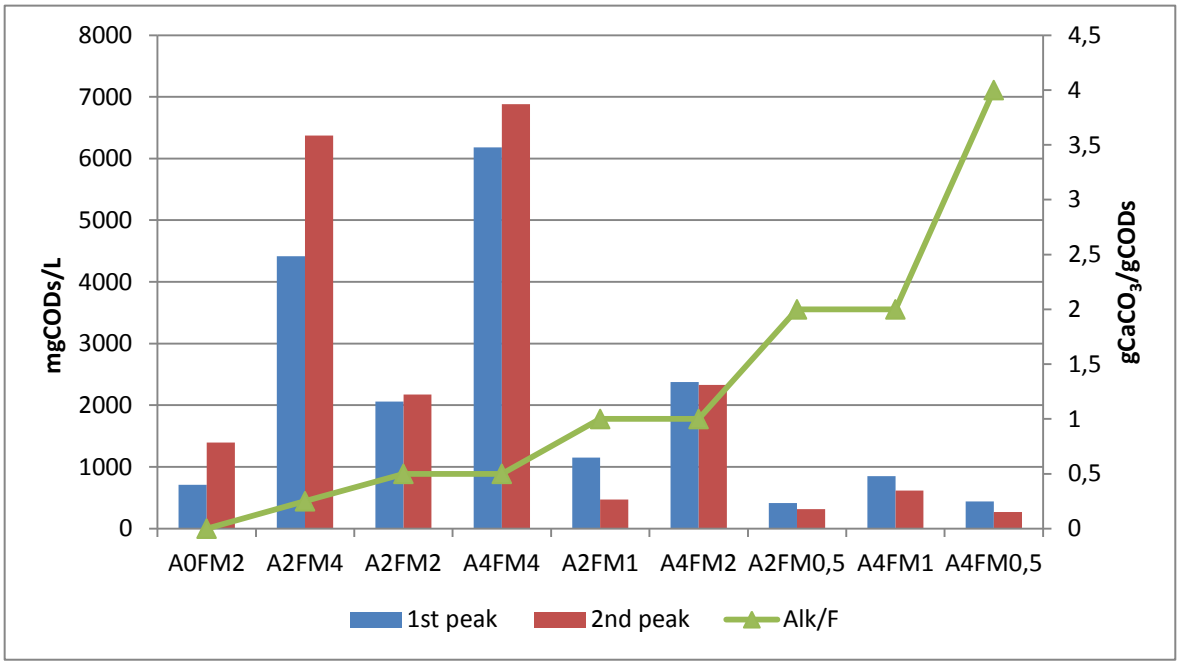


Figure 25 – Peak production (mg CODs/L) of VFAs in the reactors associated with the ratio Alk/F ($gCaCO_3/gCODs$).

It can be seen that when the ratio Alk/F is lower than 1 the acidogenic process has a greater performance in the second productive peak in addition to the higher acidification degree. When the ratio is equal to 1 (which corresponds to the reactors A2FM1 and A4FM2) the condition is reversed and the first peak overtakes the second one. The same happens when the ratio is higher than one.

This behavior appears to be also relevant to the analysis of the composition of VFAs, since acid carbon chain shorter as acetic acid and propionic acid, tend to be formed earlier in the acidogenic process.

The Figure 26 and Figure 27 shows the composition of individual acids formed in the two peaks of reactors with the ratio of organic load to alkaline concentration higher than one, i.e., with the second VFAs production peak greater than the first one.

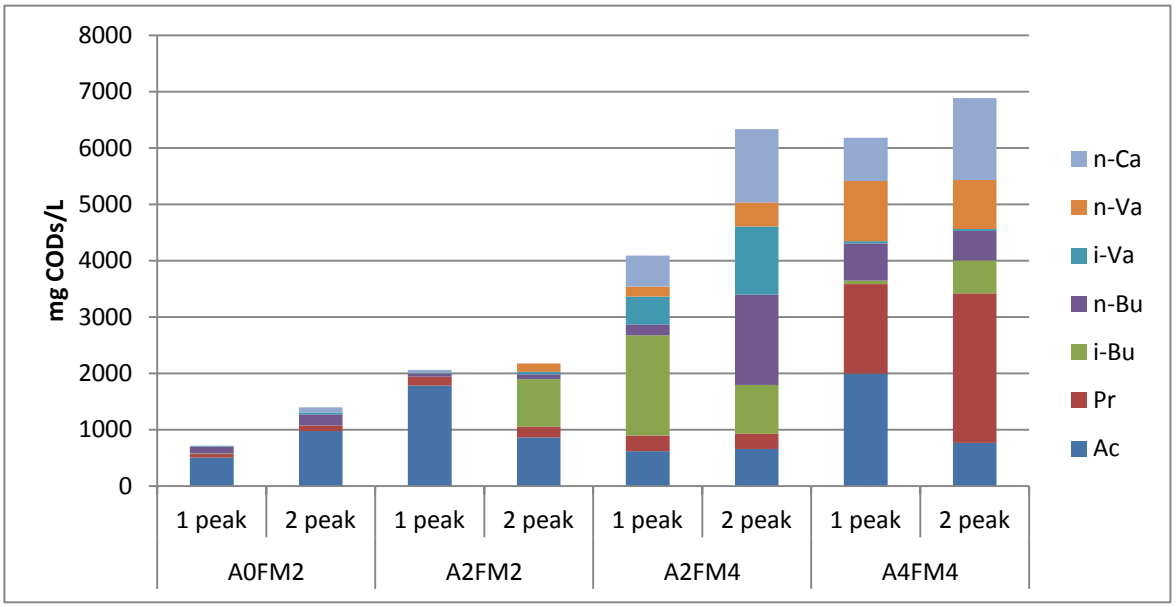


Figure 26 – Composition of the VFAs production (mg CODs/L) in the two peaks of reactors with Alk/F<1.

Figure 26 shows that the production of VFAs in the second peak present a greater number of types of VFAs formed. This behavior is been due to the decrease of acetic acid, and appearance of acid with highest molecular weight between the first and second peak.

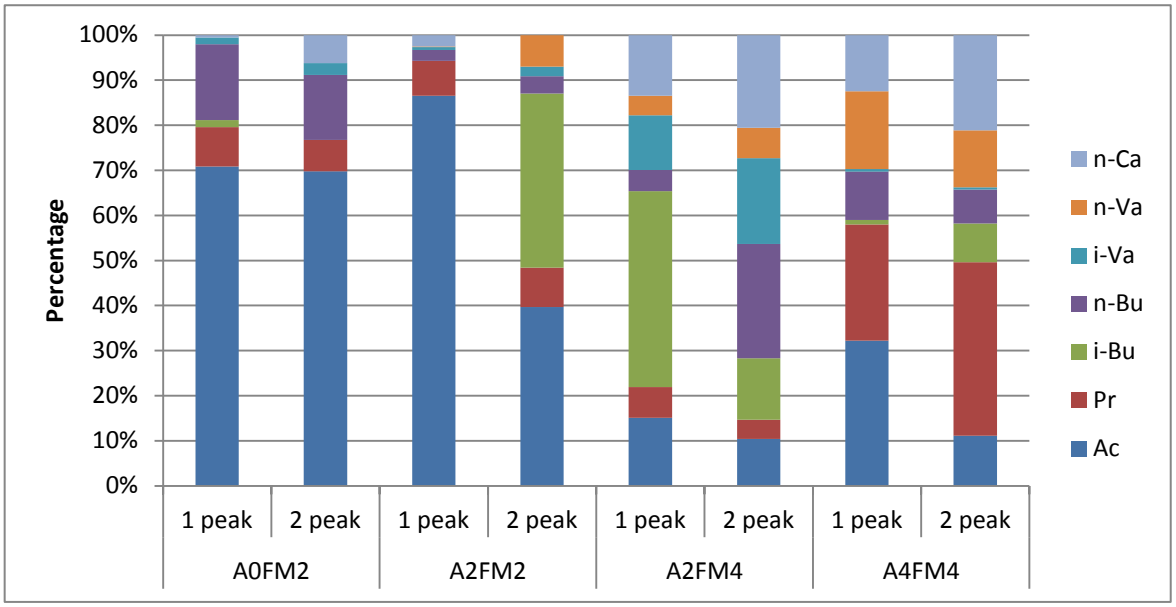


Figure 27 – Percentage distribution of the composition of VFAs production (mg CODs/L) in the two peaks of reactors Alk/F<1.

Figure 27 emphasizes the discussion presented in Figure 26, illustrating the proportionality of the evolution of the types of VFAs produced between the first and the second peak of acidogenic production. It is observed that there's always a decrease of acetic acid between the first and second peaks. The only exception is the reactor A0FM2, which hasn't the addition of alkaline solution and has a very low VFA production. In all reactors, is noticed a greater amount of the acids with higher molecular weigh in the second peak.

For example reactor A4FM4 acetic acid changed from 32 to 11%; propionic from 26 to 39%; iso-butyric from 1 to 9%; n-butyric from 11 to 8%; iso-valeric from 0.59 to 0.45%; n-valeric from 17 to 13%; n-caproic from 1 to 21%.

Figure 28 and Figure 29 repeat the analysis for the reactors with an alkalinity greater or equal that the concentration of added organic load, i.e., the ratio Alk/F was greater or equal to one.

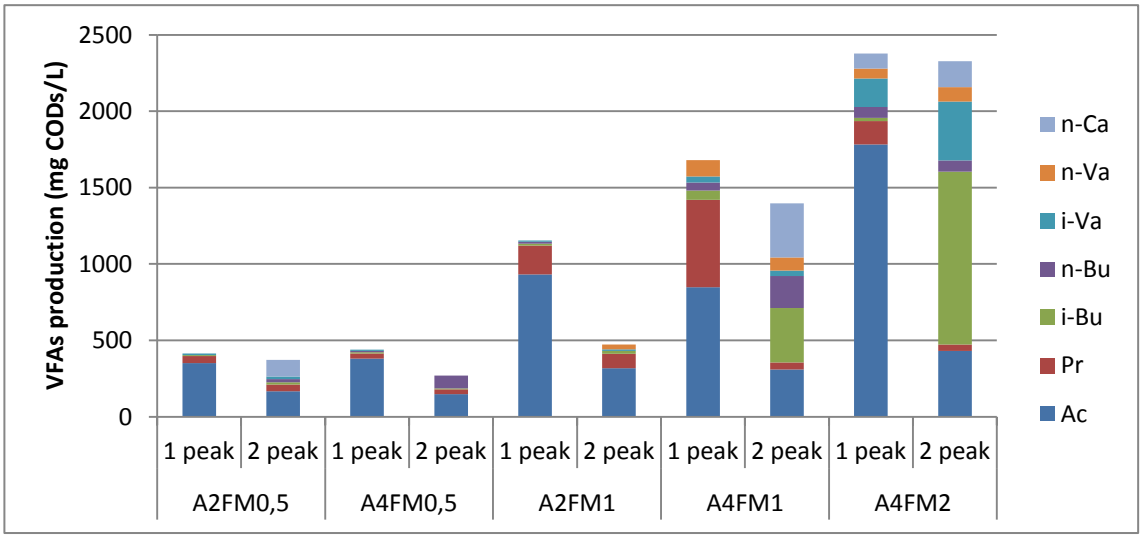


Figure 28 - Composition of the VFAs production (mg CODs/L) in the two peaks of reactors with the Alk/F≥1.

Repeating the trend shown in the previous situation, there is also a decrease of acetic acid between the first and second peaks. However, in this case, it is noticeable a wider

range of acids in the reactors with the increase of the alkalinity. In this case, the formation of propionic acid is more evident in the first peak and tends to decline in the second peak, like in the reactors A2FM1 and A4FM1 with a higher amount in the last reactor. This can be better observed in Figure 29.

In this case the maximum F/M studied was 2g CODs/gVSS, so the amount of VFA product only achieved 2500mg CODs/L, although higher than in the previous situation with the same load (F equal to 4g CODs/L).

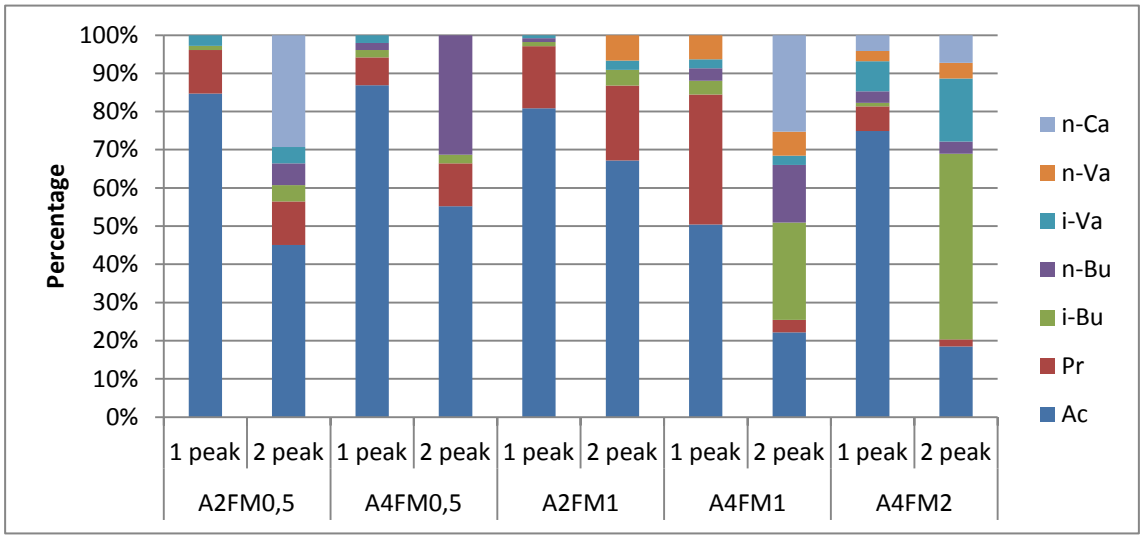


Figure 29 - Percentage distribution of the composition of VFAs production (mg CODs/L) in the two peaks of reactors with Alk/F≥1.

It is suggested retesting using the higher organic load in order to test the process efficiency for reasons Alk/F lower, thus reducing the use of alkaline solutions and thereby minimizing the economic costs of waste treatment process.

5.4 **Effects of sludge thermal pre-treatment**

The thermal pre-treatment of sludge was adopted with the aim of inhibiting the activity of methanogenic bacteria. In this work it has been tested in three reactors, A2FM0.5, A2FM1 and A4FM0.5.

This topic seeks to provide a comparison between reactors with and without the use of thermal pre-treatment, in order to confirm whether or not the significance of this stage for the winery effluents acidification process. The inclusion of a heat source, would result in an increased expense to the treatment system, thus decreasing the environmental advantages that the anaerobic technology offers, so it is important to discuss it.

Given that, in this work, the ratio F/M proved to be a determining factor for the quantitative productivity of VFAs, the reactors submitted to thermal pre-treatment will be compared with the reactor that presents the most similar F/M ratio, i.e., the A4FM1 reactor.

Figure 30 shows a comparison between the reactors A2FM1 (with no pre-treatment) and A4FM1 (with thermal pre-treatment), taking into account the CODs removal rate and the biogas production.

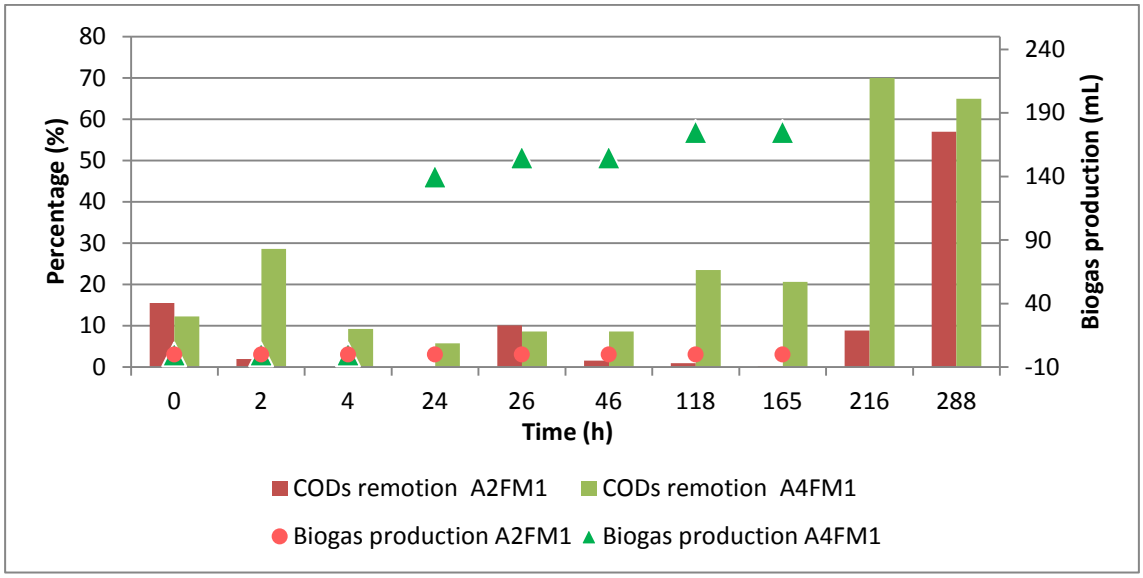


Figure 30 – Evolution of COD removal rate regarding the biogas production to evaluate the thermal pre-treatment effect.

It was observed that the reactor that was not subjected to thermal pre-treatment (A4FM1) presented a higher performance in terms of CODs removal and biogas production. Biogas production achieved in reactor A2FM1 was insignificant, which suggests an inhibition of all bacterial activity. The CODs removal in this reactor only showed an improvement at 288th hour (Day 12), suggesting a high delay on bacterial activity, although achieving a similar removal efficiency to the reactor without thermal pre-treatment in the end of experiment, it is noteworthy however, that reactor A4FM1 has a higher alkalinity and this factor may have been contributing in some way to this result.

The mass distribution shown in Figure 31 compares the three reactors that had thermal pre-treatment for biomass with the reactor without thermal pre-treatment and with the most similar F/M ratio (A4FM1).

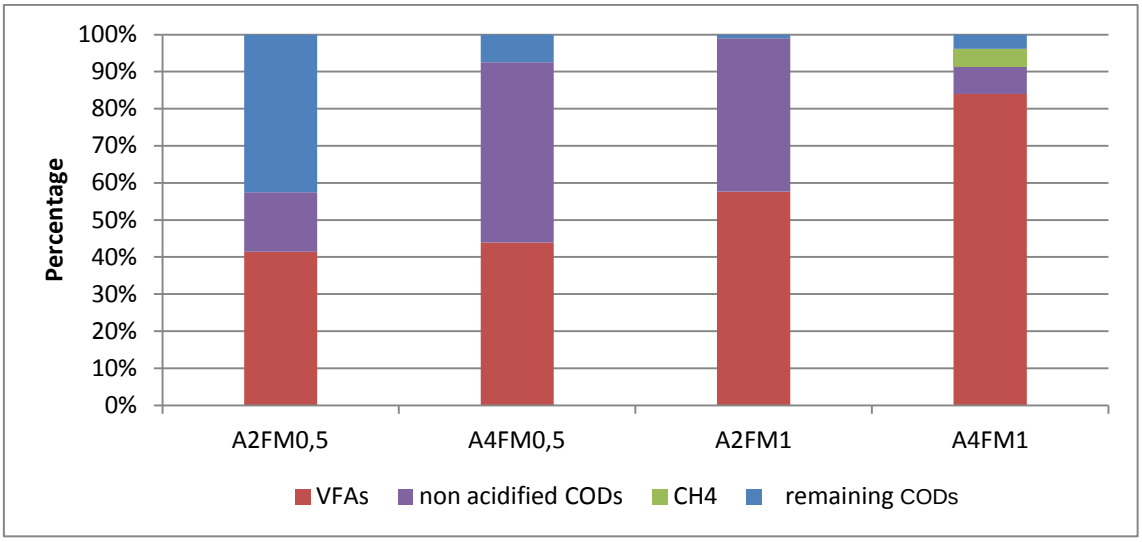


Figure 31 – CODs mass distribution to evaluate the effect of the thermal pre-treatment.

It is noted that the reactors subjected to pre-treatment (A2FM0.5, A4FM0.5 and A2FM1) haven't recorded any CH_4 production, which confirms the efficiency of the pre-treatment in terms of inhibiting the methanogenic activity. However, despite presenting a contribution of CH_4 in the mass distribution of CODs composition, yet the reactor that wasn't subjected to thermal pre-treatment A4FM1 showed higher VFAs conversion (84%) in comparison with the same organic load reactor A2FM1 (56%).

Figure 32 shows the comparison, in terms of acidification degree, between the four previously mentioned reactors. It is emphasized that reactor A4FM1 (with no thermal pre-treatment) showed a degree of acidification higher than 80%, which represented a 20% increase when compared to reactor A2FM1 that had a similarly organic load and sludge thermal pre-treatment.

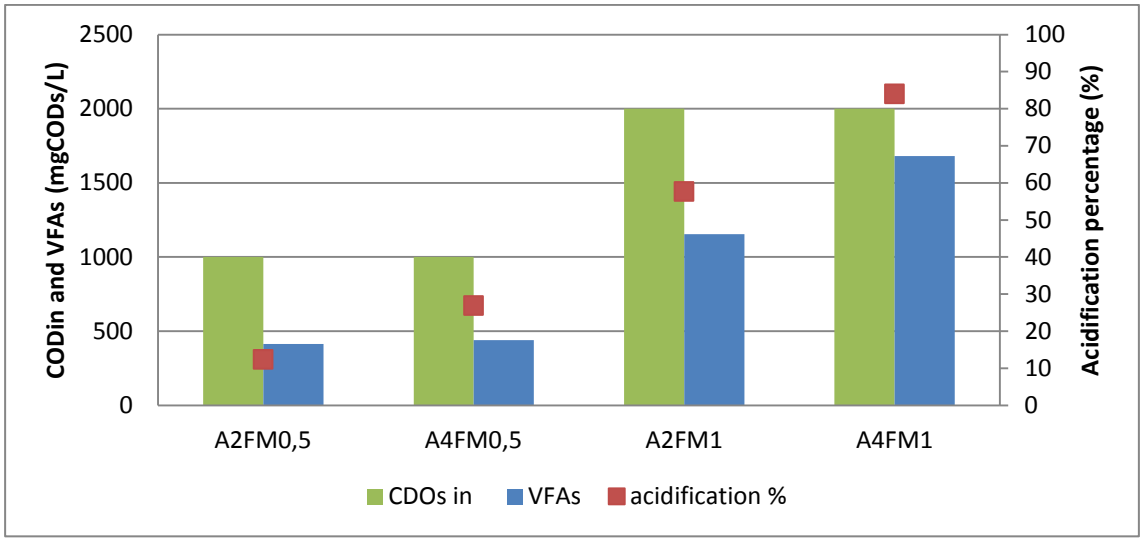


Figure 32 – Acidification degree to analysis of the thermal pre-treatment.

Based on this study, it appears that sludge thermal pre-treatment, at the conditions tested, did not prove to have a significant contribution to VFAs production. Despite having promoted an inhibition of methanogenic activity, the reactors undergoing this process did not achieve significant results when compared to reactors not thermally treated. Most probably because the pre-treatment used inhibit both methanogenic and acidogenic bacteria.

It may be suggested, a the further study of the effects of sludge thermal pre-treatment for reactors subjected to higher loads in order to confirm this contraindication of this stage.

6 Conclusions

The development of this work has reached to the following conclusion:

- For the tested organic load concentrations, the increasing in the load and alkalinity were directly proportional to VFA production in terms of maximum amount and acidification degree.

- It occurred an adaptation period between the substrate and biomass to in reactors which listed to 48 hours, during which there was a high physicochemical instability.
- The diversification of produced acids has benefited from the organic load increase and/or alkalinity.

- The alkalinity also proved to be relevant in terms of acidogenic production decreasing the pH variation and produce a longer chair fatty acids.

- The evolution of the reactors showed two peaks of VFAs production, where the second was the most favorable to volatile organic acids with longer chains.

- The amount of alkalinity added to reactor interfered significantly in the process, mainly the ratio between alkalinity and organic load, affecting the maximum production peak which turned to be a determinant factor in the definition og highest peak and the low peak to the VFA production.

- The absence of alkalinity in reactors turned to be determinant to the acidification process, reached very low VFA production and acidification degree that the increased alkalinity is related to the rate of acidification in the reactors.

- The wine, as simulated wastewater, represented a compound with high acidogenic potential.

- The heat treatment fulfill the objective of inhibit bacterial methanogenic activity, although it was also observed some inhibitory to the acidogenic bacteria, provoking a delay on VFA degree of acidogenic.

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