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Acetification of Porto wine. A preliminary step for vinegar making.

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Author’s Declaration

Under the terms of the “Decreto-lei nº 216/92, de 13 de Outubro” is hereby declared the following original articles were prepared in the scope of this thesis.

Under the terms of the referred “Decreto-lei”, the author declares that she afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in the thesis.

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Dr Pablo Kreimer, in his book "El científico también es un ser humano. La ciencia bajo la lupa", Siglo XXI Editores, exposed that scientific work depends not only of a scientific community, but also from many other actors that are not even related directly to science. He called them transepistemologyc arenas of science. Seeing backwards, it is difficult to list every person that directly or indirectly contributed to make this work. But it is necessary to mention some of them. First of all, my thesis director, Olivia Pinho, who has gone farer from what her function demands helping not only with academic work but also with my general wellbeing. Secondly, to my co-directors Duarte Torres and Olga Nunes. Thirdly, to Ana Rita Lopes. Also must be mentioned the constant assistance of the people in the laboratory E009B of Faculty of Engineering: Ana Catarina Reis, Vera Sousa, Ivone Vass Moureira, Joana Bondoso and Patricia Reis, who where not directly related to my work but have supported it. Also many thanks to Sara and Armindo, who helped in the work in Faculty of Farmacy.

Abstract

Vinegar is a liquid, adequate for human consumption, produced from products containing starch and/or sugars by the process of double fermentation, firstly alcoholic and then acetous. It has been produced in many places and by different ways, since the ancient times. Vinegars can be cheap and easy to do, and others like gourmet vinegar, are traditionally elaborated. Nowadays vinegar is firstly produced and then analyzed or optimized if its value justifies it. It has not been found any research that aims to create new kinds of vinegars, nor cheap neither expensive.

This work aims to give a first step for the developing of a new kind of gourmet vinegar: Porto wine vinegar. To achieve that objective, three main tasks were raised: To evaluate the acetic fermentation for producing Porto wine vinegar: selection of proper bacteria and obtainment of fermentation data; To characterize the profile of the product obtained; and To characterize the inoculum used for fermenting Porto wine vinegar: quantity of bacteria, strains involved and evolution of the inoculum in time.

To accomplish these tasks, two seed vinegars were tested in Douro wine that is similar to Porto wine but less aggressive for bacterial development. They were a "cider seed vinegasr" and a "wine seed vinegar". Working conditions were a commitment between the optimal ones according to bibliography and technical capacity: 30 ºC, 1.4 L/min of air, stirring of 4000 rpm, in 1 L bioreactor filled with 600 mL. One of the "seed vinegar" was selecte and bacteria were adapted to Porto wine vinegar, by adding progressively small aliquots of Porto while fermenting in Douro wine. After that, media was progressively replaced by Porto wine. Working regime was achieved using successively two different brands of Porto wine. Fermentation was analyzed in three points: initiation, middle and at the end of three cycles of fermentation with each raw material. Parameters tested were acetic acid, ethanol, glucose and fructose. In both cases determinations were made by HPLC, and in one of them, it also were evaluated the effectiveness of enzymatic tests. Composition in volatile compounds was analyzed by CG- mass. Also, it was determinate the quantity of bacteria in the three points of fermentation, the species in every stage, and the evolution of strains through successive cycles of fermentation.

“Wine seed vinegar” was selected for vinegar making not only because it oxidizes acetic acid to water and carbon dioxide but also because produced a more regular fermentation. Working regime was achieved with both Porto wines, with similar characteristics but different pH. In the tested conditions (30 ºC, 2 L/min of air, stirring of 4000rpm, in 1 L bioreactor filled with 600 mL, approximately 10^7 bacteria/mL), a cycle of fermentation takes a media of 3 days. Initial and final pH dependent on the pH of the wine used as raw material, being final pH the 83% of pH of raw material and 92% of initial pH. The values of ethanol and acetic acid reveals that fermentation is stable and very similar with both raw material. There is a loss of ethanol or acetic acid, or both, during fermentation process. Glucose and fructose are not consumed nor degraded appreciably during fermentation, bacteria prefers to consume ethanol and even acetic acid before them. The change of concentration trough a cycle of fermentation is due to the volume losses for volatilization and allows to calculate it as being between 4% and 18% of initial volume in bioreactor. According to legislation, the concentration of acetic acid is too low and the ones of ethanol, glucose and fructos are high. The fermentation process could possibly be optimized adding technology: using a more complex bioreactor for a better aeration and control of process. Fermentation can be successfully monitored by pH. The enzymatic method is accurate for acetic acid determination, but not in the case of the ethanol, glucose and fructose. During fermentation, the total amount of volatile compounds increases 2x and 3x for wine vinegars B and C, respectively, in comparison with their raw materials. Esters were the most conspicuous class of compounds detected. This clear enrichment is probably related to synthesis during bacterial metabolism. Although high aeration rate, a high fraction of volatile compounds remain in the product.

The bacteria that produces Porto wine vinegar are *Acetobacter pasteurianus subsp paradoxus,* being around 10^7 total cells/mL in every stage of fermentation. The cultivable cells constituted 9% of the total cells. Two morphotypes were distinguishable among the cultivable cells, but they were not possible to distinguish by the tests performed. Based on DGGE-profiling, these two isolates seem to be the only microorganisms present in wine vinegar fermentation trough successive acetification cycles.

In conclusion, the production of Porto wine vinegar is possible and the inocculum obtained is adequate. The improvement of the product would be achieved adding technology to production. Further, legislation adjustments can be made to facilitate the production and commercialization of this unique product as “vinegar”. Instead, this product may be commercialized under other designation like “Porto wine seasoning sauce“.

Resumo

O vinagre é um líquido adequado para o consumo humano, produzido com matérias primas que contenham amido ou açúcares pelo processo de dupla fermentação, primeiro alcoólica e depois acética. Tem sido produzido em muitos lugares e de diferentes maneiras desde a antiguidade. A maioria dos vinagres são baratos e fáceis de produzir, e estão em pobre relação com a sua matéria prima. No entanto existem vinagres gourmet, elaborados tradicionalmente. Por estes motivos, o vinagre é primeiramente produzido e depois avaliado ou otimizado se o seu valor o justificar. Não tem sido encontrada qualquer pesquisa que vise a criação de novos tipos de vinagres, nem baratos nem caros.

Este trabalho tem como objetivo dar um primeiro passo para o desenvolvimento de um novo tipo de vinagre gourmet: vinagre de vinho do Porto. Para conseguir esse objectivo, três tarefas principais foram levadas a cabo: avaliar a fermentação acética para a produção de vinagre de vinho Porto: seleção de bactérias apropriadas e obtenção de dados de fermentação; caracterizar o perfil do produto obtido e caracterizar o inócuo utilizado para fermentar vinagre de vinho Porto: quantidade de bactérias, estirpes envolvidas e a evolução do inóculo no tempo.

Para realizar essas tarefas, duas mães do vinagre foram testados em vinho do Douro, que é semelhante ao vinho do Porto mas menos agressivo para o desenvolvimento bacteriano. Eles eram denominados por "mãe vinagre de cidra" e uma "mae do vinagre de vinho". As condições de trabalho eram um compromisso entre os ideais de acordo com a bibliografia e capacidade técnica: 30 ºC, 1,4 L / min de ar, mexendo de 4000 rpm, num 1 L biorreator preenchido com 600 mL. Um dos "maes do vinagre" foi selecionada e as bactérias foram adaptadas ao vinagre de vinho do Porto, adicionando progressivamente pequenas alíquotas de Porto enquanto fermentavam em vinho do Douro. Depois disso, o meio foi progressivamente substituído por vinho do Porto. O regime de trabalho foi conseguido usando sucessivamente duas diferentes marcas de Vinho do Porto. A fermentação foi analisada em três pontos: o início, meio e fim de três ciclos de fermentação com cada matéria-prima. Os parâmetros testados foram o ácido acético, etanol, glicose e frutose. Em todos os casos determinações foram feitas por HPLC, e num deles, também foram

avaliados a eficácia dos testes enzimáticos. A composição em compostos voláteis foi analisada por CG massa. Além disso, ele foi determinar a quantidade de bactérias nos três pontos de fermentação.

A "mãe do vinagre de vinho" foi selecionada para produzir vinagre porque oxida o ácido acético a água e o dióxido de carbono e porque produziu uma fermentação mais regular. O trabalho em regime foi obtido com ambos os vinhos do Porto, com características semelhantes, mas diferentes pH. Nas condições testadas (30 °C, 2 L / min de ar, agitação de 4000 rpm, em 1 L biorreactor cheio com 600 ml, aproximadamente 107 bactérias / ml), um ciclo de fermentação tem em média 3 dias. O pH inicial e final dependem do pH do vinho usado como matéria-prima, sendo o pH final de 83% do pH da matéria-prima e 92% do pH inicial. Os valores de etanol e ácido acético revelam que a fermentação é estável e muito semelhante com ambas as matérias-primas. Existe uma perda de etanol ou ácido acético, ou de ambos, durante o processo de fermentação. A glicose e frutose não são consumidas nem degradadas sensivelmente durante a fermentação, as bactérias preferem consumir etanol e ácido acético. A mudança da concentração através de um ciclo de fermentação é devido às perdas de volume por volatilização e permitem estimar valores que podem variar entre 4% e 18% do volume inicial no biorreactor. De acordo com a legislação, a concentração de ácido acético é muito baixa e as de etanol, glucose e fructos são elevados. O processo de fermentação pode, possivelmente, ser otimizado adicionando tecnologia: usando um biorreactor mais complexo para um melhor arejamento e controlo de processo. A fermentação pode ser monitorizada pelo pH com sucesso. O método enzimático é preciso para a determinação de ácido acético, mas não no caso do etanol, glicose e frutose. Durante a fermentação, a quantidade total de compostos voláteis aumenta 2x e 3x vinagres de vinho para B e C, respetivamente, em comparação com as matérias-primas. Os ésteres foram a classe mais conspícua de compostos detectados. Este claro enriquecimento está provavelmente relacionado à síntese durante o metabolismo bacteriano. Embora a taxa de arejamento seja alta, uma fracção elevada dos compostos voláteis permanecem no produto.

As bactérias que produzem o vinagre de vinho do Porto são *Acetobacter pasteurianus subsp paradoxus*, sendo aproximadamente de 107 células totais/mL em cada etapa de fermentação. As células cultiváveis constituem 9% das células totais. Dois morfotipos foram distinguidos entre as células cultiváveis, mas eles não são distinguíveis pelos testes realizados. Baseado em DGGE-profiling, estes dois isolados parecem ser os únicos microorganismos presentes na fermentação do vinagre de vinho do Porto a través de ciclos sucessivos acetificação.

Resumindo, a produção de vinagre de vinho do Porto é possível e o inoculo obtido é adequado. A melhoria do produto seria conseguida adicionando a tecnologia de produção. Além disso, podem ser feitos ajustes de legislação para facilitar a produção e comercialização desse produto único como "vinagre". Em vez disso, este produto pode ser comercializado sob outra designação como "tempero de vinho do Porto ".

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Abbreviations

IVDP: Instituto dos Vinhos do Douro e do Porto

FAO: Food and Agriculture Organization of the United Nations

WHO: World Health Organization

GAP: Good Agricultural Practices

GHP: Good Hygiene Practices

GMP: Good Manufacturing Practices

AAB: Acetic Acid Bacteria

16S rDNA: 16S ribosomal DNA

Ga.: Gluconacetobacter

A.: Acetobacter

G.: Gluconobacter

ITS: internally transcribed spacer

5.8S rRNA: 5.8S ribosomal RNA

TBV: Traditional Balsamic Vinegar

g A.A./L: mass (in grams) of acetic acid by liter

YTGD agar: Yeast extract- Triptone-Glycerol-Dextrose agar

DAPI: 40-6- diamidino-2-phenylindole

PCR: Polymerase Chain Reaction

dNTP's: deoxyribose nucleoside triphosphates

GYC: Glucose – Yeast extract- $CaCO_3$ culture media

CTAB: cetyltrimethylammonium bromide

Tris: tris(hydroxymethyl)aminomethane $(HOCH_2)_3CNH_2$

EDTA: Ethylenediaminetetraacetic acid

DGGE: Denaturing Gradient Gel Electrophoresis

HS-SPME: Headspace solid microextraction

HPLC: High-performance liquid chromatography

GC-MS: Gas Chromatography-Mass Spectrometry

RM: Raw material

nd: not detected

Scope and aims

Douro river valley is the first region demarked in the world, being settled in 1756 by Marquêz de Pombal. Nowadays, two regions of protected designation of origin (DOC, by its acronym in Portuguese *denominação de origem controlada*) coexist in the valley, overlapping one another: the one that produces DOC Porto Wine and the one of DOC Douro Wine. The difference between both wines is in wine making procedure, having the same raw material. DOC Douro wine is elaborated as most wines, in DOC Porto wine the alcoholic fermentation is stopped at a certain moment by the addition of vinous liquor, obtaining wine with high content of alcohol and sugars. Although Douro wine can be produced with more breeds of grapes than DOC Porto wine, some berries can be used in elaboration of both.

The importance of the wine industry in the region, especially of Porto wine, is undeniable. It has impacts in economy, environment and in social aspects, due to the modification of the landscape, the generation of direct and indirect jobs and the turism industry that is developed around it, among others. Porto wine is one of the most important exportation products of Portugal and its sales moves, on average, €470 millions every year, according to statistics from *Instituto dos Vinhos do Douro e do Porto* (IVDP) from 2006 to 2013 (2013). The quantity of Porto wine produced in a year, is around 118 million liters according to the same statistic, depending on political decisions made by IVDP based on demand, with the aim of maintaining a certain price of product on market. It does not depend on grape production or winery capacity. The surplus on grapes is used for Douro wine making. However, developing new kinds of Porto wine, such as rose Porto wine, not meaning necessary a growth of market, nowadays makes the diversification of market. There is not an intention of giving a further step by developing products based on Porto wine, a post-industrialization of Porto wine. However, developing ~~of~~ new products based on Porto wine, maintaining their characteristics and gourmet character would be a very interesting opportunity. In this context, the production of gourmet vinegar based on Porto

wine would be a valuable opportunity of market growth, engaging to the production chain, using preexisting facilities and adding value to production.

There is a part of Porto wine producers that consider the production of vinegar as a loss in quality of wine. Vinegar is usually considered poor in relation to wine and also an inexpensive product. This is not always like that: in food industry, from good quality raw material can be achieved good quality products. This is the case, for instance, of Sherry vinegar made from Sherry wine.

As Porto wine is a very aggressive environment for bacterial growth, and the lack of data and previous experiences, the initial task for developing this new product is to evaluate the possibility of acetification of Porto wine. This means, to get the bacteria able of growing and fermenting it, to produce proper inoculums and to search the proper conditions of fermentation. It is also important to get some parameters of the fermentation process, as time consumed and quantity of bacteria needed, and determination of a proper parameter for monitoring the reaction. Finally, the resulting vinegar must be chemically analyzed.

The study of it would provide very important hints in fermentation and an initial product from which it will be possible to plan an optimization. In this work is intended to obtain a vinegar with known chemical composition, under specified conditions and with characterized inoculums. Having a developed product and data of fermentation, it would be possible to optimize the composition of vinegar and its production in future works. In this sense, this research aims to be a first step or starting point for future technological development.

The overall aim of this project was to develop the conditions for acetification of Porto wine tending to a future production of Porto wine vinegar.

The specific aims were:

- To evaluate the acetic fermentation of Porto wine by selecting proper inoculums and obtainment of fermentation data.
- To characterize the fermented product and finding suitable parameters for monitoring the process.
- To characterize the inoculum used for acetic fermentation of Porto wine: quantity of bacteria, strains involved and evolution of the inoculums in time.

For accomplishing these objectives, one section of the present thesis is dedicated to each one of them.

The results obtain in the experimental work were organized in three sections according to steps mentioned previously

Section A. To evaluate the acetic fermentation of Porto wine by selecting proper inoculums and obtainment of fermentation data:

- To get proper inoculum for producing acetic fermentation of Porto wine.
- To acetificate Porto wine.
- To obtain parameters of acetic fermentation of Porto wine production such as time needed and pH in several phases of production.

Section B. To characterize the obtained product and find suitable parameters for monitoring the process:

- To evaluate the composition of the obtained product in the parameters usually measured in wine: acetic acid, ethanol, glucose and fructose, the evolution of them during the fermentation process and the evolution across successive fermentation cycles.
- To find the optimal parameters for monitorizing the process and a proper method of measurement.
- To contrast the parameters of the obtained products with the demands of legislation.

Section C. To characterize the inoculums used for acetic fermentation of Porto wine:

- To determinate the number of microorganisms present in different stages of acetic acid fermentation of Porto wine: beginning, middle and end.
- To determinate the strains involved in the acetic fermentation.
- To evaluate the development of the strains mixture during successive fermentation cycles.
- To determinate the difference in quantity of strains recovery between culture-dependent and culture-independent methods.

Chapter 1.

Introduction

1.1 Generalities of vinegar

FAO/WHO defines vinegar as any liquid, adequate for human consumption, produced from products containing starch and/or sugars by the process of double fermentation, firstly alcoholic and then acetous. (FAO/WHO 1998). Vinegar is a cheap product, coming from substandard fruit, seasonal agricultural over products, by-products from food processing, and fruit waste (Solieri and Giudici 2009). It is used as a flavoring agent, as a preservative and, in many countries, also as a healthy drink (Solieri and Giudici 2009).

The traditional elaboration and use of vinegars in different cultures can be followed back to ancient times (Holzapfel 2008). The historical and geographical success of vinegars is due to the low technology involved and the variety of raw materials that can be used as substrate (Holzapfel 2008). Because most vinegars are cheap and easy to do, its scientific and technological manufacture has a slow development through the years (Holzapfel 2008). However, it is important to increase scientific knowledge, to improve the manufacturing technology and to implement higher patterns of quality and safety for the global and increasingly worldwide market (Holzapfel 2008).

One of the most popular vinegars is the one made from wine. The quality of wine vinegar is determined by a set of factors most importantly the raw material, the microorganisms involved, and the acetification process employed (Holzapfel 2008). The attempts to differentiate vinegars are based either on the kind of raw material used or on the manufacturing process involved (Natera et al. 2003).

Generally, basic safe food operating principles, such as Good Agricultural Practices (GAP), Good Hygiene Practices (GHP) and Good Manufacturing Practices (GMP), should be present in all the steps. They are more important before starting fermentation, to avoid the growth of dangerous microorganism's namely aflatoxin-producing moulds and harmful bacteria, since these steps are carried out at room temperature (Solieri and Giudici 2009). After acetification, there is no real danger of spoilage, since acetic acid has strong antibacterial activity at low pH (Solieri and Giudici 2009).

In the case of wine vinegar, first fermentation - alcoholic fermentation - has already occurred, therefore production is focused just in the second one: the acetic fermentation.

1.2 Raw material

Wine vinegar is normally made from red or white wine. In vinegar, as in wine, there is a large range in quality (Sellmer-Wilsberg 2009). Individual varieties of wine, such as Champagne, Sherry or Pinot Grigio, are the raw material of the most expensive wine vinegars (Sellmer-Wilsberg 2009).

1.3 Microbiology of acetic acid fermentation

Vinegar is produced by microorganisms which oxidize ethanol into acetic acid, through the following equation, $C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$ ($\Delta H = 493$ kJ), and are usually called Acetic Acid Bacteria (AAB) (Garcia-Garcia et al. 2009). These bacteria normally resist to lower pH than other bacteria surrounding (Sellmer-Wilsberg 2009). AAB are Gram-negative, polymorphous, ellipsoidal to rod-shaped, straight or slightly curved, 0,6-0,8 µm by 1,0-4,2 µm, occurring single, in pairs, or in chains. They are compulsory aerobic; some produce pigments, and some produce cellulose (Sellmer-Wilsberg 2009).
AAB convert ethanol in acid with high efficiency (95-98% of the stoichiometric value). Due to AAB are strictly aerobic and to the quantity of oxygen used in ethanol oxidation, the acetification process is highly oxygen demanding (Garcia-Garcia et al. 2009). The ability to oxidize ethanol is a main characteristic of AAB, but there is a great variability among strains and species in what concerns the amount of acetic acid produced. (Gullo and Giudici 2008).
AAB are difficult to cultivate and keep in pure culture, especially for strains isolated from high acetic acid level source (Entani et al. 1985; Sievers et al. 1992). According to Sievers

and Teuber (1995), most AAB lose their ability to produce acetic acid when they are plated. These reasons make difficult their study. (Entani et al. 1985; Sievers et al. 1992)

The taxonomy of the acetic acid bacteria is going under extensive and continuous revision at present, and many species and genders may soon be reclassified (Solieri and Giudici 2009). Actual taxonomic studies are based on a polyphasic approach that includes morphological, physiological and biochemical aspects, metabolism, ecology, genome characterization and phylogeny (Solieri and Giudici 2009). Phylogenetically, the *Acetobacteraceae* belong to the α-subclass of the Proteobacteria. All described species of the family are described by a phylogenetic tree based on almost complete 16S rDNA sequences (Cleenwerck and De Vos 2008).Nowadays thirty-three genera are classified and accommodated to the family *Acetobacteraceae*: *Acetobacter, Acidicaldus, Acidiphilium, Acidisoma, Acidisphaera, Acidocella, Acidomonas, Ameyamaea, Asaia, Belnapia, Craurococcus, Endobacter, Gluconacetobacter, Gluconobacter, Granulibacter, Humitalea, Komagataeibacter, Kozakia, Muricoccus, Neoasaia, Neokomagataea, Paracraurococcus, Rhodopila, Rhodovarius, Roseococcus, Roseomonas, Rubritepida, Saccharibacter, Stella, Swaminathania, Tanticharoenia, Teichococcus, Zavarzinia* (Garrity 2005)

The genera and strains that are present in vinegars have been studied in several researches. Table 1 shows a summary of them.

Investigation has been showed that some strains that are very close to each other in the phylogenetic tree based on 16S rDNA have different phenotypic data. The genera Asaia, Kozakia, Swaminathania and Neoasaia could be regrouped in only one genera, but when other differentiation methods are applied, they show differences, for instance the ability to grow in average with 0,35% of acetic acid (pH 3,5) (Cleenwerck and De Vos 2008). In Table 2 it can be found the research from several authors that dealt with different segments of DNA to identify the acetic acid bacteria (AAB).

Table 1 Summary of the bacteria strain that were found in different vinegars

substrate	technology implied	microorganisms	author
spirit vinegar	submerged fermentation	*Acetobacter sp.*	Trcek et al. (1997)
		Gluconacetobacter europaeus,	Fernández-Pérez et al. (2010b)
		Ga. oboediens	Andres-Barrao et al. (2011)
cider vinegar	submerged fermentation	*Acetobacter sp.*	Sokollek et al. (1998)
		A. pasteurianus, Ga. xylinus, A. hansenii, Ga. europaeus,	Fernández-Pérez et al. (2010b)
Shanxi aged vinegar	spontaneous solid-state fermentation	*A. pasteurianus, A. senegalensis, A. indonesiensis, A. malorum, A. orientalis, Gluconobacter oxydans,*	Wu et al. (2012)
white wine vinegar	submerged fermentation	*Ga. europaeus,*	Fernández-Pérez et al. (2010b)
red wine vinegar	submerged fermentation	*Acetobacter sp.*	Sokollek et al. (1998)
		Ga. europaeus,	Andres-Barrao et al. (2011) Fernández-Pérez et al. (2010a)
traditional balsamic vinegar	surface fermentation	*Ga. xylinus, A. pasteurianus, A. aceti*	Gullo et al. (2006)
		A. pasteurianus, Ga. Europaeus, Ga. Xylinus, G. oxidans	Vegas et al. (2010)

The sequence that is most used in research is the 16S rRNA genes, being analyzed in at least 22 ways in research works. It has been used for bacterial identification in research since 2006 until nowadays. It was also used to test stability of cultures by Gullo et al. (2012) and to optimize the extraction of bacterial genomic DNA, by Mamlouk et al. (2011). The second mostly used is the 16S–23S internally transcribed spacer (ITS) regions (9 works), and it was used for identification and characterization. The whole genoma was also used for identification by DNA-DNA hybridization, but researches report use it from 1998 to 2006. It is possible that from year 2006 other techniques, such as analysis of 16S rRNA genes and 16S–23S internally transcribed spacer (ITS) regions, have become better options for identification. Finally, the 5.8S rRNA has only been found in one research, by Andorra et al. (2010), for bacterial population monitoring.

Table 2: Researches that have used different segments of DNA to identify the acetic acid bacteria

Technique	Objective	Authors
16S rRNA genes	Bacterial identification	Camu et al. (2008), Castro et al. (2013), Cleenwerck et al. (2008), Dutta and Gachhui (2006), Gulitz et al. (2013),Iino et al. (2012), Ilabaca et al. (2008), Kadere et al. (2008), Karahan et al. (2011), Kregiel et al. (2012), Larcia et al. (2011), Lefeber et al. (2011), Ongol and Asano (2009), Ouoba et al. (2012), Park et al. (2012), Romero-Cortes et al. (2012), Silva et al. (2006), Tanasupawat et al. (2011), Torija et al. (2010), Yuan et al. (2013)
	to test stability of cultures	Gullo et al. (2012)
	optimization of bacterial genomic DNA extraction	Mamlouk et al. (2011)
16S–23S internally transcribed spacer (ITS) regions	Bacterial identification	Castro et al. (2013), Fernández-Pérez et al. (2010b), Greenberg et al. (2006), Ouoba et al. (2012), Ruiz et al. (2000), Tanasupawat et al. (2011), Tokunaga et al. (2009), Torija et al. (2010)
	characterization	Sievers et al. (1996)
5.8S rRNA	bacterial population monitoring	Andorra et al. (2010)
Whole-genome	Bacterial identification	Boesch et al. (1998), Dutta and Gachhui (2006), Schuller et al. (2000), Silva et al. (2006), Sokollek et al. (1998)

To start vinegar production ~~it~~ is necessary to put in contact the AAB with substrate (wine). It can be achieved by two different methods. The first one is spontaneous fermentation: the raw material is processed and the changed environmental conditions encourage the most appropriate indigenous microflora to grow. The more restrictive the growth conditions are, the greater becomes the selective pressure exerted on the indigenous microorganisms (Solieri and Giudici 2009). These bacteria may come from wine, being the grapes and the winery the main sources. Grapes microflora is very diverse, but during alcoholic fermentation the quantity of strains is drastically reduced due to the change of environment (Gonzalez et al. 2005). Spontaneous acetic fermentation is suitable for small-scale production and only for very specific wines. However, this method is difficult to control and there is a great risk of occurring spoilage (Solieri and Giudici 2009).The second method is adding bacteria, either by back-slopping or by the addition of starter cultures (Solieri and Giudici 2009). Back-slopping consists on using part of a previously fermented

batch to inoculate a new batch. The initial number of desirable microorganisms is increased and the process is more reliable and faster than spontaneous fermentation (Solieri and Giudici 2009). The back-slopping practice is particularly useful for inoculating AAB cultures, because it is difficult to produce true starter cultures (Solieri and Giudici 2009). The aliquot of vinegar used to inoculate a new batch is usually called seed vinegar or vinegar mother, and they are usually unknown mixed-strain cultures. Nowadays, many modern industries use this method (De Ory et al. 2002; Sokollek et al. 1998). A starter culture is a microbial preparation of a large number of cells of one or more microorganisms (single-strain or mixed-strain cultures), which is added to the raw material to produce and accelerating a fermentation process (Leroy and De Vuyst 2004). Single-strain cultures have advantages such as to improve the process control and to predict of metabolic activities. Although, there are also some disadvantages, for instance an increased probability of spoilage through bacteriophage infection, spontaneous mutation, and loss of main physiological properties (Holzapfel 2002). Mixed-strain cultures are less susceptible to deterioration. The use of starter cultures is not being applied on a large scale, for two main reasons: first, the AAB are difficult to cultivate and maintain in laboratory or to preserve as a dried starter; and, second, vinegar is generally an inexpensive commodity therefore an expensive starter culture selection is not a warrant (Solieri and Giudici 2009).

1.4 Conditions for the development of acetic acid bacteria to the vinegar production

The development of the acetic acid bacteria depends on a various list of specific factors. The most important ones are: temperature, dissolved oxygen availability, ethanol concentration, acetic acid concentration and the sum of the concentrations of acetic acid and ethanol. These factors are strongly interdependent (Garcia-Garcia et al. 2009) and

therefore these parameters can only be controlled in fermentation in a bioreactor (Solieri and Giudici 2009; Tesfaye et al. 2002).

1.4.1 Temperature

Usually, vinegar production by acetic bacteria is performed at 30ºC (Erbe and Bruckner 1998). De Ory et al. (2002) have demonstrated that the optimum temperature for AAB is 30.9ºC. As the oxidation of ethanol to acetic acid is an exothermic reaction, a cooling system is need in the quick fermentation (Tesfaye et al. 2002).

It has been found several thermotolerant *Acetobacter* bacteria active between 37 and 41 ºC by Kanchanarach et al. (2010). They are stains of *Acetobacter pasteurianus*, similar to a *A. pasteurianus* SKU1108 previously isolated by the same team. The codes of the isolated stains are MSU10, MSU22, and IFO3191. In this way the operation costs, due to the refrigeration process, decreases. However, a higher temperature also accelerates physicochemical processes, such as evaporation of volatile compounds that are of great value in the final product as they are responsible for the organoleptic properties.

1.4.2 Oxygen availability

Oxygen availability is often the rate limiting factor in acetification (Adams, 1998; cited by Solieri and Giudici 2009). Romero et al. (1994) found that the maximum growth rate of acetic acid bacteria is obtained at oxygen values between 1 and 3 ppm.

When fermentation is operated in a bioreactor, the oxygen concentration can be strictly controlled (Solieri and Giudici 2009). Mass transfer can only be optimized through increasing the coefficient of mass transference across the film and the surface area of the gas-liquid interface. At first, both transferences should be as high as possible (Garcia-Garcia et al. 2009). If the bioreactor is operated with high flow-rates of air to ensure high oxygen transfer coefficient, the lost of volatile compounds will be increased. A compromise must, therefore, be made between the velocity of fermentation and the content of aromas in the final product (Garcia-Garcia et al. 2009)

It had also been an attempt to improve oxygen availability in surface fermentation. It has been made by increasing air-liquid interface ~~in~~ around 30% and also expanding the size of the top hole of the barrel. Oxygen availability wasn't measured, but acetification rate was increased (Hidalgo et al. 2010).

1.4.3 Ethanol concentration, acetic acid concentration and the combination of both

High concentration of ethanol has negative influence on bacterial growth (Ebnar et al., 1996; cited by Garcia-Garcia et al. 2009; Romero et al. 1994). At an industrial level, ethanol concentrations above 50 g/L have an adverse affect in the acetification rate. However, the low concentration of ethanol leads to the death of part of the culture (De Ley et al. 1984). As a rule, the medium ethanol concentration should never be reduced below 1.58 g/L (Garcia-Garcia et al. 2009). When all the substrate is totally consumed, some strains of AAB start over-oxidizing de acetic acid to carbon dioxide and water as a carbon source (Garcia-Garcia et al. 2009; Parrondo et al. 2009; Saeki et al. 1997). The resistance of acetic bacteria to acetic acid makes possible the production of high acetic vinegar. Despite this, it is not possible to produce vinegar with more than 8-9% acetic acid in a continuous process (Garcia-Garcia et al. 2009). In a semi-continuous method, the acetification rate and acetic acid production can be improved when there is an increase in the final ethanol concentration. The use of two serially arranged reactors were suggested: the first one to ensure a high acetification rate and the other one to finish the fermentation (Baena-Ruano et al. 2010).

The vinegar production cannot just contemplate the ethanol concentration or the acetic acid concentration, but both. Therefore the overall medium concentration of ethanol as well as the final metabolic product (acetic acid) must be controlled and maintained within certain limits since an elevated concentration will inhibit bacterial growth (Romero et al. 1994).

Research in this area has clearly shown that the previous variables are strongly interdependent. Therefore, it cannot be examined the influence of any individual variable in isolation. Thus, the sensitivity to oxygen deficiency depends on the total concentration

of ethanol and acetic acid in culture medium and the specific stage in the bacterial cycle (Garcia-Garcia et al. 2009).

1.5 Technology of wine vinegar production

The systems of production can be grouped in two main types: surface fermentation (also known as static, traditional or slow) and submerged fermentation (also known as industrial or fast) (Fernández-Pérez et al. 2010b; Natera et al. 2003; Tesfaye et al. 2002; Tesfaye et al. 2009a; Vegas et al. 2010). In another classification, Solieri and Giudici (2009) excluded the solid state fermentation from the surface fermentation.

1.5.1 Surface fermentation

In surface fermentation, acetic acid bacteria (AAB) are placed on the air–liquid interface (Fernández-Pérez et al. 2010b; Tesfaye et al. 2002) in a barrel-shaped container (Tesfaye et al. 2009a; Tesfaye et al. 2009b). The size of barrels vary according to the vinegar that is being produced: to make Traditional Balsamic vinegar (TBV) sets of barrels of different sizes between 66L to 15L are used (Giudici et al. 2009); to produce Sherry vinegar 500L barrels are used, previously used to contain wine (Tesfaye et al. 2009b). These containers are filled two-thirds of their total capacity to create a big air-liquid interface. Room temperature is normally used; it has been reported to be 25±1ºC in an experience made in Spain (Hidalgo et al. 2010).The quantity of inoculum varies according to the system of production: the use of 10% of inoculum was reported to make sherry vinegar. In Traditional Balsamic Vinegar (TBV), aliquots of vinegars (around one-third of barrel content) called “soleras y criaderas” and “rincalzo” are transfered from a barrel to the next were the acetification process is more advanced (Giudici et al. 2009). This process allows simultaneous acetification and ageing, and also the accumulation of metabolites produced by AAB, that increase volatile aromatic compounds of the vinegar and therefore a vinegar of high quality is obtained (Tesfaye et al. 2002). The disadvantages are: high

installation costs, the long production time, lack of control of the process, which results in a product of high commercial value (Tesfaye et al. 2002). Traditional and gourmet vinegars are made by this method (Tesfaye et al. 2002). There is a variable that increases the acetification surface contact by the use of wood shavings as a bacterial supporting material. It is known as solid state fermentation (Llaguno, 1991; cited by Tesfaye et al. 2002). It increases the velocity of acetification but there is accumulation of dead AAB over the wood shavings, it may occur growth of cellulose producing bacteria on the shaving woods, and infection of the vinegar with anguillulas (vinegar eels) (Solieri and Giudici 2009; Tesfaye et al. 2002). A similar technique is used to accelerate aging by extracting compounds of oak chips added to vinegar already made. Usually it is used 1-2% (w/v) of oak chips, medium size of 5-10mm, air dried and toasted to imitate the characteristic of wood from barrel. Chips are normally obtained from barrel producers (Tesfaye et al. 2004).

1.5.2 Submerged culture

In this process the acetic acid bacteria are suspended in the acetifying liquid, in which a strong aeration is applied to assure the oxygen demand (Fernández-Pérez et al. 2010b; Tesfaye et al. 2002). The equipment required is a bioreactor that consists on a stainless steel or plastic fermentation tank with air supply system, cooling system, foam controlling system, loading and unloading valves (Garcia-Garcia et al. 2009; Sellmer-Wilsberg 2009; Tesfaye et al. 2002). As oxygen is supplied from air (containing around 23% of oxygen) and only 60-90% of the oxygen supply is used to oxidize ethanol, the volume of air needed is extremely high. For example, an industrial tank holding 25,000 L of culture medium needs 100,000-150,000 L of air per hour. The risk of volatiles being swept (particularly ethanol) by such a massive air supply is very high (Garcia-Garcia et al. 2009). Nowadays, fermenters for wine vinegar production are mostly between 1000 and 110,000 L of working volume (Sellmer-Wilsberg 2009). The productivity of acetification depends on the transfer coefficient of oxygen in the air liquid interface and also driving force. But dissolved oxygen concentration is very low during operation, and the air flow-rate should be low enough to

avoid entrainment losses, but high enough to ensure efficient uptake. (Garcia-Garcia et al. 2009) The advantages of this method are the rapid and forced fermentation (De Ory et al. 2002; Gomez et al. 1994), the strict control of typical parameters of oxidative conversion (temperature, oxygen, alcohol content, and acidity) (Holzapfel 2008) and the short time of production (24–48 h)(Vegas et al. 2010). The disadvantages of this production method are related with relative poorer sensorial characteristics than surface cultured vinegars (Vegas et al. 2010), mainly due to the loss of volatile aromatic compounds (De Ory et al. 2002; Gomez et al. 1994). The submerged culture system is used for the production of most commercial vinegars of higher consumption (Tesfaye et al. 2002).

In order to achieve good performance and efficiency of the bioprocess, bioreactors require advanced regulation procedures. Moreover, to model the arising difficulties it is important to use dynamic biosystems through accurate and fast biochemical sensors. These auxiliary tools are still limited and difficult to handle in practice, which makes optimal bioreactor operation a challenging task (Abdollahi and Dubljevic 2012). To evaluate and control parameters of different bioreactors, the criteria used are specific power consumption, mixing time (the time needed for a defined volume of substance to reach a determined degree of homogeneity), impeller tip speed, and Reynolds number at impeller tip (the ratio of inertial forces to viscous forces) (Barradas et al. 2012)

The bioreactor can be operated in a batch, semi-continuous or continuous way. In Batch it has three main phases: loading of the raw material and inoculation, fermentation and complete unload of biotransformed product (Tesfaye et al. 2002). Semi-continuous, also known as fed-Batch (Schmid 2003), is similar to batch process, but part of the finished product is left in the vessel and it is used to inoculate the next cycle (Nieto et al. 1993). The quantity of discharge product is variable on every single process but commonly 50%of the total volume is removed (De Ory et al. 2004). Usually, fermentation cycle starts with 7-10% of acetic acid and about 5% of ethanol, reaching 8-14% of acetic acid and 0.05%-0,3% of ethanol. Every cycle takes between 18 and 30 hours, depending on the efficiency of the aeration system and the total concentration (Sellmer-Wilsberg 2009). Continuous process consists on extracting a small aliquot of the biotransformed product wile fresh substrate is

continuously supplied (Tesfaye et al. 2002). Maximum concentration obtained through this method is 9-10% acetic acid, because the specific growth rate of the bacteria decreases with decreasing ethanol concentration (Sellmer-Wilsberg 2009) It is possible to use two bioreactors: the first one to degrade alcohol to 2-3%, and the second one to finish fermentation until alcohol is almost depleted (Sellmer-Wilsberg 2009).

1.6 Vinegar post-treatment

Ageing is the time that vinegar is left in the barrel set, after biological transformation (Giudici et al. 2009). During this time, there is an extraction process of phenolic compounds from wood, concentration of solutes due to water loss through wood pores, and several chemical transformations of coexisting compounds, mainly esters (Giudici et al. 2009; Gomez et al. 2006; Tesfaye et al. 2004).

During surface fermentation simultaneous acetification and ageing occur (Tesfaye et al. 2002). Static ageing is made in wooden casks, fill to three quarters of the total volume. It is preferred to use casks that previously had been used to contain wine of the same kind that the one used as raw material for doing the vinegar (Morales et al. 2002). When Sherry wine vinegar is made, it is used a dynamic ageing system, the "Sherry vinegar solera" (Tesfaye et al. 2009b). It consists of a set of casks containing vinegar in different stages of fermentation. The contents of the casks are partially removed from one cask to fill another, following an increasing level of acetification (García-Parrilla et al. 1999). In opposition to the case of TBV, in this case barrels have all the same size and had been previously used for containing wine (García-Parrilla et al. 1999).

Traditionally elaborated vinegars (Sherry, Modena) are subjected to extend their ageing in casks, that can oscillate between 6 months to several years (Gomez et al. 2006). An accelerated ageing with oak chips can be made instead, by adding between 1 and 2% w/v of wooden chips to vinegar. To simulate the conditions of a cask, the chips can be dried and toasted (Gomez et al. 2006). Those vinegars produced by a submerged culture system

can be filtered and bottled for consumption or can be aged in casks to acquire qualitative characteristics (Gomez et al. 2006).
Filtration is the most important post-fermentation treatment to ensure stability and safety in the finished product (Garcia-Garcia et al. 2009). In fact, it is necessary to remove any suspended solids, which might otherwise detract vinegars stability and compromise the quality requirements for human consumption. Upon fermentation, vinegar is a complex suspension that contains microorganisms and parts of cells as solid fraction. Before filtering, the suspension should be decanted in order to reduce the solid concentration as far as possible. In that sense clearing vinegar with clay (e.g. bentonite) often helps to remove colloids and so, as stated above, can accelerate the production of instability by causing precipitation of solids during storage and after bottling (Garcia-Garcia et al. 2009).
After these processes, vinegar is bottled, stored and distributed, in order to be sold (Solieri and Giudici 2009).

1.7 Compounds of wine vinegar

In addition to acetic acid and ethanol, vinegar contains secondary constituents that play an important role regarding its smell, taste and preservation. These constituents have their origin in the raw material, in the added nutrients, and in the water used for dilution. They are also formed by acetic acid bacteria or by chemical reactions between different compounds (Sellmer-Wilsberg 2009). Vinegars volatile profile is clearly influenced by the acetification. Different compounds such as organic acids and phenolics are produced and contribute to the typical flavor of the product (Tesfaye et al. 2002). The aromatic complexity of vinegar is related to the existence of a series of components including carbonyls, ethers, acids, acetals, lactones, alcohols, volatile phenols or esters of different origins. These compounds are either present in the wine substrate (alcohols, ethyl esters), formed during acetification (acetoin, acids), or during the aging process (diacetyl, mainly acetates) (Tesfaye et al. 2009a). Many researches describing the composition of vinegars

were made. Table 3 summarizes studies that have been studied the composition of vinegars from different raw materials and technologies of production.

The level and nature of the organic acids present in any given vinegar can provide information about its origin, processing or ageing to which it has been subjected. Organic acids in wine vinegars comprise volatiles such as acetic and propionic acids and non-volatiles such as tartaric, citric, malic and succinic acids, however the most relevant acid that identifies the vinegar is acetic acid (Castro et al. 2002; Sellmer-Wilsberg 2009).

The phenolic compounds, which are ubiquitous secondary metabolites of plant kingdom, have very important influence on the organoleptic properties of any plant-origin product (color, flavor or astringency) (Andlauer et al. 2000) Further, they draw a great interest due to their postulated health-protecting properties and can be also used as taxonomic markers of botanical origin (Alonso et al. 2004; Davalos et al. 2005). In grapes, phenolic compounds are generally found in the solid parts (Sellmer-Wilsberg 2009; Tesfaye et al. 2009a). In wine vinegars the following phenolics were identified: galic acid, p-OH-benzaldehyde, caffeic acid, vanillic acid, syringic acid, p-coumaric acid, anisaldehyde, epicatechin, sinapic acid and salicylaldehyde (Sellmer-Wilsberg 2009).

1.8 Quality of wine vinegar

The quality of a food item can be evaluated according to different perspectives such as food safety, nutritional value and sensory properties. As far as vinegar is concerned, it is strongly determined by sensory properties once it may modify the overall taste of a given food or meal. (Tesfaye et al. 2002)

Table 3: Components that were studied in different vinegars

substrat	fermentation	aging	Studied compounds	author
Sherry vinegar	surface	traditional aging	Galic acid, 5-(Hydroxymethyl)-2-furaldehyde, Caffeoyltartaric acid, protocatechualdehyde, Cumaroyltartaric acid glycoside, Cumaroyltartaric acid, 2-Furaldehyde, p- Hydroxybenzoic acid, Tyrosol, Hydroxybenzaldehyde, Caffeic acid, Gallic ethyl ester, Vanillin, Syringaldehyde, p-Coumaric acid, Isoquercitrin, Caffeic ethyl ester, p-Coumaric ethyl ester	García-Parrilla et al. (1999)
	submerged	-	Gallic acid, 5HMF, Caffeoyltartaric acid, Protocatechualdehyde, p-Coumaroyltartaric glucosidic ester, Coumaroyltartaric acid, Tyrosol, Caffeic acid, Gallic ethyl ester, p-Coumaric acid, Rutin, Caffeic ethyl ester	Morales et al. (2001)
		traditional aging	Acetaldehyde, ethyl formiate, methyl acetate, ethyl acetate, metanol, etano, diacetyl, 1-propanol, 2-methyl-1-propanol, isoamyl acetate, 2-methyl-1-butanol, 3-methyl-1-butanol, acetoin, hydroxyacetone, ethyl lactate, ç-butyrolactone, diethyl succinate, 2-phenylethanol	Morales et al. (2002)
		Accelerated aging	Galic acid, 5-Hydroxymethyl-2-furaldehyde, Vanillic acid, Syringaldehyde, Coniferaldehyde, Sinapinaldehyde, 2-Furfural, 5-Methylfurfural, Furfury alcohol, trans-β-Methyl-γ-octalactone, Vanillin	Tesfaye et al. (2004)
red wine vinegar	surface	-	Galic acid, Protocatechuic acid, Tyrosol, Caftaric acid, Vanillic acid, (+)-Catechin, Caffeic acid, Syringic acid, Gallic ethyl ester, (-)-Epicatechin, Reseveratrol glucoside, Ellagic acid	Cerezo et al. (2008)
	surface and submerged	-	Asp, Asn, Ser, Glu, His, Gln, Gly, Arg, NH4+, Thr, Ala, Pro, GABA, Cys, Tyr, Val, Met, Orn, Lys, Ileu, Leu, Phe, Trp	Callejon et al. (2008)
		-	Acetaldehydediethylacetal, 2-furaldehyde, benzaldehyde, Methyl acetate, Propyl acetate, Isobutyl acetate, Isoamyl acetate, 2-phenylethyl acetate, ethyl propanoate, ethyl isobutyrate, ethyl butyrate, ethyl isovalerate, ethyl hexanoate, ethyl lactate, ethyl furoate, diethyl succinate, acetoin, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol, Cis-3-hexen-1-ol,furfuryl alcohol, benzyl alcohol, 2-phenylethanol, isovaleric acid, hexanoic acid, octanoic acid, limonene, γ-butyrlactone, guaiacol, eugenol	Callejon et al. (2009)
		-	gallic acid; catechin; chlorogenic acid; caffeic acid; epicatechin; syringic acid; p-coumaric acid; ferulic acid; rutin; resveratrol; hesperidin; apigenin-7-glucoside; rosmarinic acid; eriodictyol; quercetin; naringenin; luteolin; apigenin; acacetin.	Budak and Guzel-Seydim (2010)
white wine vinegar	submerged	-	2-phenylethanol, diethyl succinate, meso 2,3-butanediol, levo 2,3-butanediol, ethyl lactate, acetoin, isoamyl alcohols, 1-propanol, methanol, ethyl acetate, acetaldehyde	Baena-Ruano et al. (2010)
balsamic and red wine vinegar	submerged	traditional aging	Total phenol index, total manometric anthocyanins	Cerezo et al. (2010)
vinegars from white wine, red wine, apple, honey, alcohol, balsamic, and malt	surface and submerged	with and without	n-butyl acetate, ethyl pentanoate, 2-methyl-1-propanol, isoamyl acetate, ethyl hexanoate, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone, Benzaldehyde, 2,3-butanediol, ethyl decanoate, isopentanoic acid, diethyl succinate, benzyl acetate, ethyl-2-phenyl acetate, 2-phenylethyl acetate, R-ionone, benzyl alcohol, 2-phenylethanol, 4-ethylguaiacol, octanoic acid, 4-ethylphenol, decanoic acid	Natera et al. (2003)

Many countries such as Italy and Spain, which are also vinegar producers, are interested in emphasizing the quality of this product, obtained from a raw material of high commercial value. That high quality vinegar can only be successfully manufactured through suitable techniques to obtain sensorial quality parameters according to the raw material used (Antonelli et al. 1997) As vinegar is mostly used as a flavoring agent, it is very important to analyze its sensorial properties.

To appreciate the sensory characteristics of vinegars, it can be presented to the sensorial panel in two distinct ways. The first way encompasses testing the vinegar as it is, using wine glasses. This process is the normal procedure to perform sensory analysis in vinegar cellars. The second one consists of preparing vinegar in the most possibly approximate way as it is normally consumed. So, 25g of lettuce are suspended in 30mL of diluted vinegar. The dilution is made in water, and 1% of acetic acid. Also 1.5 g of sodium chloride in 100 mL are added (GonzalezVinas et al. 1996).

To facilitate the sensory analysis of these sour products, the legislation contemplates other tests such as discriminatory tests that include triangular test (ISO, 1983a, standard 4120) and Paired Comparison tests (ISO, 1983b, standard 5495), among others. The Triangle test in vinegars was developed to evaluate vinegars aged in different types of wood and at different ageing times (Cerezo et al. 2010). However in order to obtain a great insight about the aromatic profile of the samples, it is necessary to use the descriptive analysis (ISO, 1987, standard 6564).(Tesfaye et al. 2002).

Zou et al. (2012) performed a descriptive analysis of Sherry vinegar using the following parameters: Color, Richness in Aroma, Ethyl Acetate, Woody Flavour, Wine Character, Pungent sensation, General Impression. To perform the sensorial analysis of non-aged vinegars, sensorial parameters were divided in four groups: the visual appearance parameter by density, color, and clearness; the aroma parameter was evaluated by deficiency, refinement, intensity and acidity, the texture parameter by fullness, intensity, flavor, harmony and acidity and the global sensation parameter. The Table 4 sums up the amount of attempts made in order to differentiate vinegars according to their quality.

Table 4: Attempts made in order to differentiate vinegars according to their quality

discrimination between:	compound	method	Author
various botanical origins	polyalcohol content	Gas Chromatography-Mass Spectrum (GC-MS)	Antonelli et al. (1994)
		Ion-Exclusion Chromatography, Capillary Electrophoresis,	Antonelli et al. (1997)
	Chiral amino acid	gas chromatography	Erbe and Bruckner (1998)
	EWs (4021, 4058, 4264, 4400, 4853, 5070 and 5273 cm(-1))	Near infrared (NIR) spectroscopy based on effective wavelengths (EWs) and chemometrics	Liu et al. (2008)
	volatile compounds	Pyrolisis mass spectrometry and a sensor technique ('electronic nose')	Anklam et al. (1998)
		stir bar sorptive extraction gas chromatography-mass spectrometry (SBSE-GC-MS)	Marrufo-Curtido et al. (2012)
technology used	Acidity, total extract, ash content, glycerol, alcohol and sulfates		Guerrero et al. (1994)
	eight mineral elements (As, Ca, Cu, Fe, K, Mg, and Zn)		Guerrero et al. (1997)
mature vinegar varieties		Visible_near infrared reflectance (MR) spectroscopy	Zhao et al. (2011)
	transmission spectra in the wavelength range of 800-2500 nm	feasibility of near infrared spectroscopy (NIRS)	Lu et al. (2011)
production area	transmission spectra in the wavelength range of 800-2500 nm	feasibility of near infrared spectroscopy (NIRS)	Lu et al. (2011)
quality, brands, adulterations	volatile compounds		Nieto et al. (1993)
	inorganic elements such as Mg, K, Ph, Zn, Fe, Mn, Ca and Cu	atomic absorption spectrophotometer	Chen et al. (2009)
botanic origin and Technology used	Phenols	High-performance liquid chromatography (HPLC) with diode array detection	García-Parrilla et al. (1997)
Botanic origin, technology used and production area	56 volatile: 15 esters, 10 aldehydes, 5 acids, 12 alcohols, 5 ketones, 4 volatile phenols, 2 pyrazines, and 3 miscellaneous compounds	Headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography mass spectrometry (GC-MS)	Xiao et al. (2011)

Summarizing, vinegar is generally first produced and then analyzed or optimized. Commercial products are too cheap and easy to do to spend resources in investigation for production. Traditional expensive products are traditionally made: many resources are employed for describing, optimizing the production and to certificate authenticity, but not to modify them. Research about quality is mostly focused in describing preexistent products and, sometimes, comparing them; and in authenticity insurance. The only different case is the one of Sherry vinegar: there have been made some studies to optimize production. It has not been found any research that aims to create new kinds of vinegars, nor cheap vinegars neither expensive ones. This panorama in the creation of new types of vinegars reflects both advantages and disadvantages: there is a whole researching field to be explored optimal to produce original works, but no previous experience on which to build.

Chapter 2.

Materials and methods

2.1 Raw material

To perform this work, two different brands of tawny DOC Porto wine, both with around 19% of alcoholic content, were used: wine provided from the Borges winery (wine B) and wine acquired in supermarket from Calem winery (wine C).

In a first stage it was also used the Douro wines in order to adapt the bacteria to the fermentation conditions, namely temperature, aeration and stirring. Two wines were used alternately according to market availability: one from Carm Winery SO_2 free (14% alcohol), and other coming from a private homemade production, in which the addition of antimicrobials was not expected.

2.2 Acetic acid bacteria (AAB) obtainment

Two “vinegar mothers” were tested: a wine vinegar mother, donated by a homemade producer from Douro region, and a cider vinegar mother purchased from a homemade producer. Both of them were adapted to Douro wine in 250 mL flasks with deflectors.

2.3 Analytical methods for Porto wine acetification

Section A. *To evaluate the acetic fermentation of Porto wine by selecting proper inoculums and to obtain fermentation data*

To produce vinegar, several process parameters need optimization. Experiments were carried out to adjust inoculum volume and to set up the method to monitor the fermentation process.

The three experiments performed were carried out on a lab-scale bioreactor equipped with water jacket, maintained at 30 ºC by water circulation, with magnetic agitation (1000

rpm) and aeration (1,4 L/min). Before entering the bioreactor, air passed through a 0.22 µm filter of cellulose acetate (Whatman, Kent, UK) and was introduced in the bioreactor trough a glass air diffuser. The scheme of the arrangement is displayed in Figure 1, and the equipment used is shown in Figure 2. Fermentation pH was measured with a pH electrode (Mettler Toledo pH sensor type InPro 3030/3100. Switzerland) placed inside the reactor.

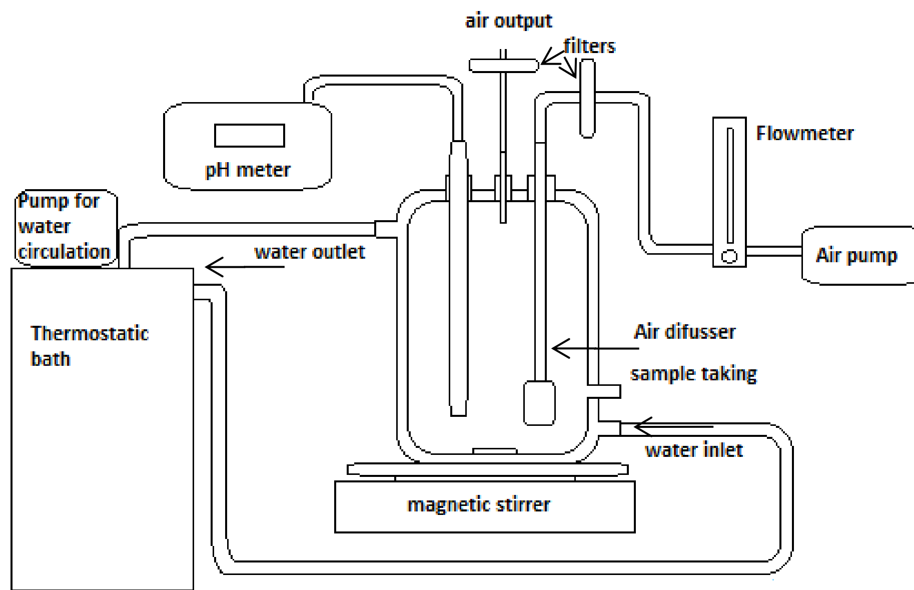


Figure 1: Arrangement of bioreactor

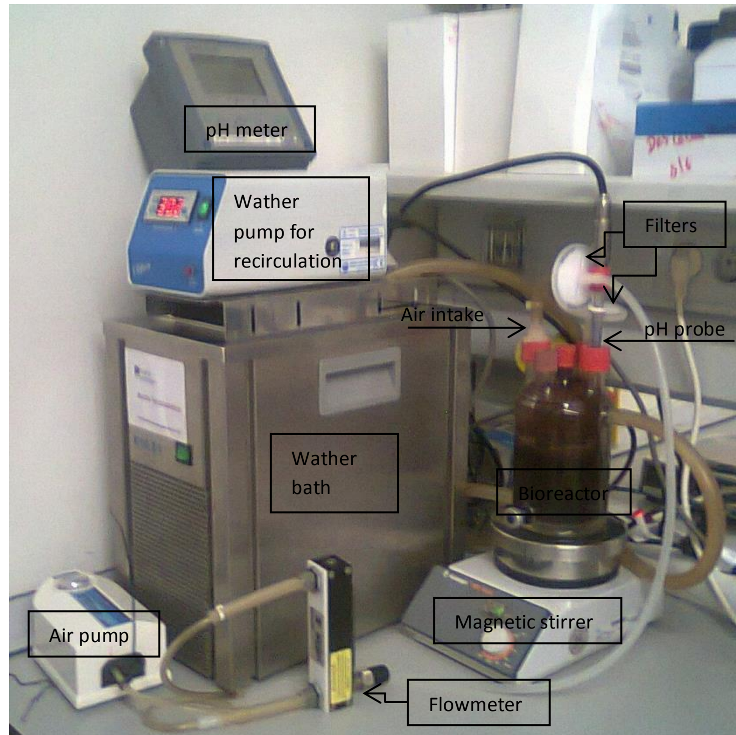


Figure 2: Bioreactor

The bioreactor was operated on feed batch mode. Fermentation started with 5 g of inoculums and 300 mL of wine. It occurred until pH reached a pH value at which the end of fermentation was considered. This pH value was empirically determined by observing the pH behavior during the fermentation process. At that point, 50% of the bioreactor content was unloaded as finished product. After that, fresh wine was feeded to the bioreactor and a new fermentation cylce started. The volume of added wine was determined by pH, using the pH of the inicial mixture as reference. Because of the volume losses during acetification, the fresh wine volume was slightly superior to the unloaded vinegar volume.

2.3.1 First fermentation experience

Wine vinegar fermentation was performed using wine seed vinegar and cider seed vinegar. In both cases, 90 mL of seed vinegar and 160 mL of Douro wine were filtrated through cellulose acetate filters with a porosity of 0,22 µm (Whatman, Kent, UK); 50 µL of silicone-based antifoam were added. During fermentation, fresh medium (Douro wine)

was added when fermentation showed the first increase of pH after an exponential decreasing phase. The volumes of added wine were small in order to not excessively slow down the bacterial proliferation due to the modification of environment. In average, aeration meaning volume of air in volume of medium and in time (minutes) was 4.7 v/v/m Four cycles of fermentation were accomplished with each vinegar seed. In the first case, the second cycle was left to pursue with the aim of discovering whether inoculums hyperoxidates acetic acid to carbon dioxide or not. In fermentation with wine seed vinegar, total acidity was measured by titration with NaOH 0.1 N to assess the results obtained with the pH electrode.

2.3.2 Second fermentation experience

The fermentation was initially started with 75 mL of wine vinegar seed from the first fermentation and 75 mL of Douro wine. A control bioreactor (abiotic assay) was set with 150 mL of Douro wine. The scheme of the disposition is displayed in Figure 3, and the equipment used is shown in Figure 4. Both bioreactors were set in the conditions described before except aeration that was incremented to to 2 L/min. Since, during the fermentation the volume was maintained close to 600 mL and then the aeration can be calculated as 3.33 vvm.

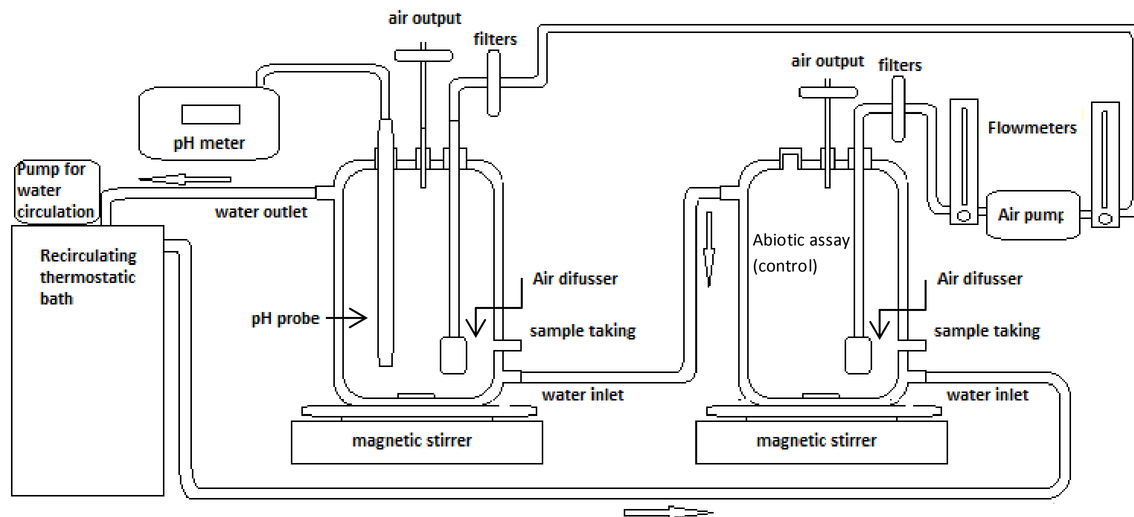


Figure 3: Display of two bioreactors

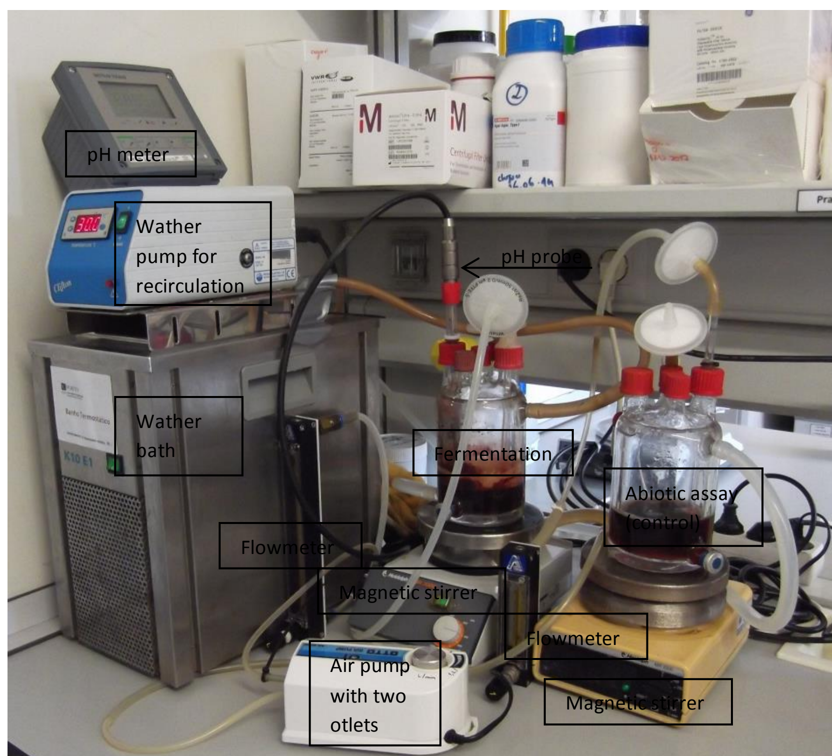


Figure 4: Display of two bioreactors.

After some acetification cycles, 6 aliquots of 50 mL of Porto wine (wine B) were added to each bioreactor. Afterwards, cycles were performed extracting around 50 % of the produced vinegar and adding a similar quantity of wine B, as previously described. When a percentage superior to 98% of Porto wine (wine B) in the bioreactor was reached as well as cycles of fermentation were produced in a repetitive and stable way, samples were taken for analysis. Samples (~12 mL) were taken at the beginning, in the middle and at the end of every five fed-batch cycles. An aliquot of each one (~2 mL) was immediately used for microbiological and chemical analysis by enzymatic test. The remaining was stored at -18ºC for chemical analysis by HPLC and GC-MS. Later on, cycles of acetic acid fermentation were performed using wine C as raw material, and samples were taken and processed as previously described.

2.4Analytical methods for characterize the chemical profile

Section B. *To characterize the obtained product and to find suitable parameters for monitoring the process:*

The concentrations of acetic acid, ethanol glucose and fructose were determined in three points (beginning, middle and end) during three cycles of fermentation. Quantification was made using enzymatic tests and by HPLC.

2.4.1 Determination of chemical composition of product by enzymatic tests

Acetic acid, ethanol glucose and fructose were quantified using the enzymatic kits K-ACETRM, K-ETHO and K-FRUGL (Megazime International Ireland Limited, Wicklow, Ireland), respectively, according to manufacturer's instructions. Absorbances were measured using the microplate reader Synergy HT (BioTek Instruments, Inc., Vermont, USA).

2.4.2 Determination of chemical composition of product by HPLC

Analysis was performed by HPLCusing, a Jasco device equipped with a refractive index detector. A Varian Metacarb 87H column (300 x 6.5 mm) from Agilent was used. The mobile phase consisted of sulphuric acid (Sigma Aldrich) aqueous solution at a concentration of 0,01 N. Elution, with a flow rate of 0,07 mL/min, was conducted at 60 ºC. A Star Chromatography Workstation software (Varian) was used to record and integrate the refractive index detector response. Samples were diluted ten times in distilled water and filtered with a 0,2 µm porosity filter (Whatman, Kent, UK). Standards used to determine the calibration curves were D-glucose anhydrous (Fisher Scientific UK Ltd., Licesterine, UK), D(-)-Fructose (Fisher Scientific UK Ltd., Licesterine, UK), acetic acid glacial (Panreac, Barcelona, Spain) and Ethanol Absolute PA (Panreac, Barcelona, Spain), analytical grade.

2.4.3. Analysis of volatile compounds

2.4.3.1. Chemicals and reagents

The volatile compounds that were studied were (CAS number in brackets): ethyl hexanoate (123-66, Sigma, Madrid, Spain), phenylethyl acetate (103-45-7,Merck), ethyl octanoate (106-32-2, Merck), hexyl acetate (142-92-7, Merck, Darmstadt, Germany), diethyl succinate (123-25-1, Merck), phenylethyl alcohol (60-12-8, Sigma), linalool (78-70-6, Sigma), α-ionone (6901-97-9, Aldrich), β-ionone (6901-97-9, Aldrich). C6–C20 was obtained from Fluka. NaCl and NaOH were purchased from Merck. SPME fibers were purchased from Supelco (Madrid, Spain).

2.4.3.2. Samples description

Volatile compounds were analyzed during fermentation with both raw materials: wines B and C. In each case, three cycles were analyzed as triplicates. Volatile compounds in raw material, were obtained from the product of the fermentation (vinegar) (T2) and at a point in the middle of fermentation (T1) and therefore were analyzed. The point at the beginning of fermentation was put aside because it is only the mixture of vinegar (T2) and raw material in proportion 1/1.

2.4.3.3. Headspace-solid phase microextraction (HS-SPME) conditions

The analysis of the volatile fraction of samples was performed according to the method of Barros et al. (2012) with some changes. The compounds were extracted using a DVB/CAR/PDMS fiber (50/30 μm). SPME fibers were conditioned according to the manufacturer's recommendations. All the extractions were performed in 10 mL vials containing 1.00 mL of sample, 0.5 g of NaCl and internal standard at 1 mg/L. Initially, the samples were continuously stirred at 250 rpm for 5 min at 45 ºC and extracted during 20 min at 45 ºC. HS-SPME procedures were performed using a Combi-PAL autosampler (Varian Pal Autosampler, Switzerland) and the Cycle Composer software (CTC Analytics

System Software, Switzerland). Desorption time into GC injector was 2 min at 230 ºC in splitless mode.

2.4.3.4. Chromatographic conditions

GC-IT/MS analysis was performed on a Varian CP-3800 Gas Chromatograph (USA) equipped with a Varian Saturn 4000 ion trap mass detector (USA) and a Saturn GC-IT/MS workstation software version 6.8. Chromatographic separation was achieved using a capillary column VF-5ms (30 m x 0.25 mm x 0.25 µm) from Varian and a high purity helium C-60 (Gasin, Portugal) as carrier gas at a constant flow of 1.0 mL/min, in splitless injection mode. An initial oven temperature of 40 ºC was held for 1 min, then increasing 5 ºC/min to 250 ºC (45 min) followed by an increase of 5 ºC/min to 300 ºC (10 min). The ion trap detector temperatures were set as follow: 280 ºC, 50 ºC and 180 ºC for the transfer line, manifold and trap, respectively. All mass spectra were acquired in the electron impact (EI). The mass range was 35–600 m/z, with a scan rate of 6 scan/s. The emission current was 50 µA, and the electron multiplier was set in relative mode to auto-tune procedure. The maximum ionization time was 25,000 µs, with an ionization storage level of 35 m/z. The analysis was performed in full scan mode according to Oliveira et al. (2010), Guedes de Pinho et al. (2009a) and Guedes de Pinho et al. (2009b).

2.4.3.5. Qualitative and semi-quantitative analyses

The compounds identification was achieved by comparing the retention time and mass spectra obtained from sample and then once again by comparison with the standard compounds present in the model of synthetic solution injected at the same conditions; by comparing retention times for each reference compound analyzed, the retention index (RI) of each compound, by using a commercial hydrocarbon mixture (C6–C20), in comparison with the retention time index described in the literature and also by comparing the MS fragmentation present with the mass spectra present in the National Institute of Standards and Technology (NIST) MS 14 spectral database. The amounts of each compound were expressed as peak areas x 10 / internal standard peak area.

2.5 Analytical methods to characterize the inoculums used for fermentation

Section C. *To characterize the inoculums used for acetic fermentation of Porto wine*

2.5.1 Enumeration of AAB

The number of microorganisms was determined in samples taken during all stages of fermentation (beginning, middle and end) by culture-dependent and -independent methods. Total cells were enumerated using the 4'-6- diamidino-2-phenylindole (DAPI) staining method, as described before (Manuel et al. 2007). Briefly, one milliliter of diluted sample was filtered through a 0.2 µm-pore-size black polycarbonate membrane (Whatman, Kent, UK) and stained with 100 µg/mL DAPI (Sigma-Aldrich, Steinheim, Germany). The total number of cells was determined by direct counting using an epifluorescence microscope (Nikon, Tokio, Japan). One hundred microscopic fields were counted per preparation, corresponding to a minimum number of 300 cells. YTGD medium was used to isolate and enumerate the cultivable cells, as described by Kanchanarach et al. (2010), modified by preparation in two layers plates (15 mL/plate of medium containing 0.5 % agar, followed by 5 mL/plate of the same media but with double agar concentration). Constituents used were D-Glucose anhydrous (Fisher Scientific UK Ltd., Licesterine, UK), yeast extract (Fisher Scientific UK Ltd., Licesterine, UK), tryptone (Liofilchem S.R.L. Italy), Agar Agar Type I (HiMedia Laboratories Pvt. Ltd., India) and Gycerol 87 % PA (Panreac, Barcelona, Spain). Briefly, the vinegar sample (1 mL) was serially diluted and 100 µL of the dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were superficially plated, and incubated seven days at 30ºC, in triplicate.

The total biomass of the cultivable microorganisms grown on YPTD medium was further recovered from each plate, by re-suspension with saline solution (NaCl 0.85 %) and centrifugation at 20,800 g during 10 minutes. The obtained pellet was cryopreserved at -20 ºC until further DNA extraction.

2.5.2 Isolation and identification of AAB

Two distinct morphotypes grown on YTGD plates were isolated and purified in the same medium. These isolates, herein named A and B, were further characterized. Their ability to grow on GYC medium culture was tested at 30 ºC during a maximum of 20 days. Colony and cellular morphology, Gram-staining and catalase reaction were performed as described by Smibert & Krieg (1994).

DNA extraction was performed by the thermal shock method. A loopful of biomass was suspended in 50 µL of ultrapure sterile water, heated 10 minutes at 95 ºC in a water bath, cooled down to 4 ºC, on ice, for 5 minutes, and centrifuged 2 minutes at 16000 g. The supernatant containing DNA was transferred into a new sterile micro tube and preserved at -20 ºC.

The 16S rRNA gene of each isolate was amplified through polymerase chain reaction (PCR) and further sequenced, as described elsewhere (Ferreira da Silva et al., 2007). Briefly, the PCR reaction mixture (50 µL) was composed of 1x Buffer KCl and 2.5 mM $MgCl_2$ (Fermentas), 0.2 µM dNTP's, 1 µM Primer 27F (5'-GAG TTT GAT CCT GGC TCA G-3'), 1 µM Primer 1492R (5'-TAC CTT GTT ACG ACT T-3'), 1.25 U Taq polymerase (Fermentas) and 2 µL of target DNA (sample). The PCR program was the following: 5 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C, and a final extension of 20 min at 72 °C (Ferreira da Silva et al. 2007). Nucleotide sequences were determined using a model ABI 3700 DNA Analyser (Applied Biosystems, CA, USA). After checking their quality using the BioEdit software (Hall 1999), the nucleotide sequences (ca. 730 bp) were compared to others available in public databases using the BLAST search tool (http://www.ncbi.nlm.nih.gov) (08/2014).

To confirm the similarity between the two isolates, a Random Amplified Polymorphic DNA PCR (RAPD PCR) was performed, as described by (Ferreira da Silva et al. 2006). The volume of reaction was 25 µL, consisting of 1x Buffer KCl, 1.5 mM $MgCl_2$, 0.2 µM dNTP's, 1.0 µM of primer M13 (5′ GAGGGTGGCGGTTCT3′), 4 U Taq polymerase (Fermentas) and 0.5 µL target DNA (sample). The PCR program was the following: 5 min at 94 °C, followed by 45

cycles of 1 min at 94 °C, 1 min at 34 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C. A positive control was carried out simultaneously. Electrophoresis was performed in 1.5 % agarose gel electrophoresis (90 V, 400 mA, 120 min) with a low DNA mass marker (GeneRuler DNA Ladder thermo Fisher Scientific, USA), and 3 µL per sample of loading buffer (Bromophenol blue 25 % and glycerol 30 %). The gel was stained for 15 min with ethidium bromide and the image was acquired with the Transiluminator UV + Digi Doc (Bio-Rad, Chemidoc XRS, United States).

2.5.3 PCR-DGGE analysis

For vinegar total genomic DNA analysis, 40 mL of sample of vinegar were filtrated through 0.2 µm-pore-size sterile polycarbonate membrane (Whatman, Kent, UK). Filters were preserved at -20 ºC until DNA extraction. Total DNA from vinegar (herein designated as total cell DNA) and from the total biomass of the cultivable microorganisms (herein designated as cultivable cell DNA) was extracted with the Power Soil DNA Isolation Kit (Mo-Bio Laboratories, Inc.) according to manufacturer's protocol. In addition, the DNA from each sample was extracted using the Genomic DNA kit from NucleoSpin Food (Macherey-Nagel) according to the manufacturer's recommendations, with a pre-treatment with 10 mg of glass beeds, cell lysis solution and 5 minutes agitation at 3,000 rpm. The DNA concentration of each product was determined as described by Lopes et al. (2011). All determinations were made in duplicate.

Bacterial community composition of vinegar was assessed by Gradient Gel Electrophoresis (DGGE), as described below. A 200-bp 16S rRNA gene fragment, corresponding to the region V3, was amplified with the 16S rRNA gene primers forward 338F_GC, containing a GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3'), and reverse 518R (5'-ATT ACC GCG GCT GCT GG-3') {Muyzer, 1993 #379}. The mixture for amplification was performed as described by Bondoso et al. (2014), in a reaction volume of 50 µL, and consisted in 0.5x Buffer KCl, 0.5x Buffer.$(NH_4)_2SO_4$-$MgCl_2$, 3mM $MgCl_2$, 0.4mM dNTP's, 2.5µL BSA 3 %, 0,5mM primer 338FGC, 0.5mM primer 518R, 1.5U Taq polymerase (Stabvida, Lisbon, Portugal) and 4 µL

target DNA (sample). The PCR reaction was performed in a thermocycler (MyCycler, Bio-Rad, USA), and the conditions were 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, and a final extension of 20 min at 72 °C. About 800 ng of the PCR products from total cell DNA, 650 ng of cultivable cell DNA and 500 ng of isolate's DNA were loaded in the DGGE gel and the electrophoresis run at 60 °C with DCodeTM universal mutation detection system (Bio-Rad Laboratories, Hercules, CA), using a 25–60% denaturant gradient (where 100% denaturant contained 7 M urea and 40% formamide) in a 8% polyacrylamide gel,, running initially at 20 V for 20 min, and then at 200 V for 5.5 h (Barreiros et al. 2008). The gel was stained for 15 min with ethidium bromide and the image was acquired with the Transiluminator UV + Digi Doc (Bio-Rad, USA). To normalize the DGGE gels, a ruler composed of a set of reference cultures, profile of which covered the whole denaturing gradient in use, was introduced in the extremities of each gel. Gels were performed in duplicate.

2.6 Statistical analysis

Statistical analysis of results was performed using the programs IBM SPSS Statistics and Microsoft Excel. Data from fermentation analysis were correlated using Pearson's correlation. An analysis of variance (ANOVA) and Bonferroni tests were used to estimate the significance or not in the difference between results. Results were considered significant if the associated *p*-value was below 0.05.

Chapter 3.

Results and Discussions

3.1 *Section A. To evaluate the acetic fermentation of Porto wine by selecting proper inoculums and obtainment of fermentation data*

The aggressiveness of wine as substrata for bacteria takes too long to complete scheduled tasks. A total of 347 days of fermentation were performed in total, corresponding 161 days to the first fermentation experience and 186 days to the second one.

3.1.1 First fermentation experience

The disposition of the bioreactor used is displayed in Figure 3. First the fermentation was performed using as inoculums wine vinegar seed and, after that, the one with cider vinegar seed was used.

3.1.1.1 Fermentation with wine seed vinegar

To complete the four cycles of fermentation around 102 days (approximately three months and a half) were necessary. The first cycle of fermentation ends when the pH starts to increase after the first acetification. At that point, an aliquot of around 50 mL is collected and 50 mL of fresh medium (Douro wine) are added. The addition of culture medium marks the beginning of a new fermentation cycle. Four cycles occurred during the first experience. Three aliquots of 50 mL of fresh medium were added on day 15th, 48th and 65th. The evolution in pH and titratable acidity, expressed as grams of acetic acid (AA) in 100 mL, is shown in Figure 5. At the end of 2nd cycle, the ability of bacteria to oxidize acetic acid to carbon dioxide and water was proved: pH increases while acidity concentration decreases. The last cycle was let to continue until pH stopped falling. It is important that it occurs at pH=2.97 (72.7 g AA/L), when that same state as consider to be reached after 2º cycle in pH=3.02 (85.1 g AA/L).

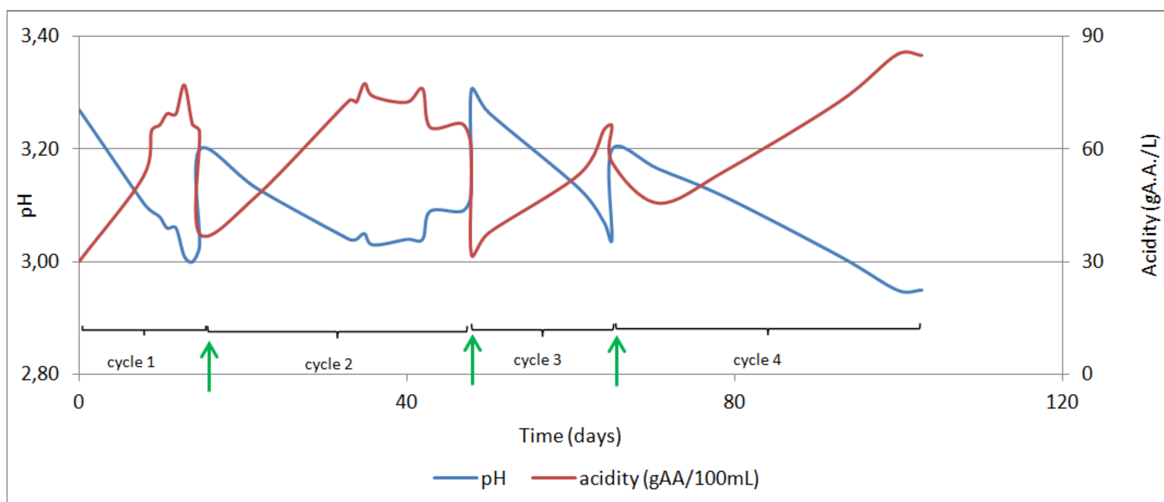


Figure 5: Evolution of pH and acidity (grams of acetic acid by liter) in time (days). Green arrows indicate the points were 50 mL of Douro wine were added, the parenthesis in the bottom of the graphic indicates the extension of every cycle, the number under them marks of which cycle is the case.

The most important parameters of the four cycles are summarized in Table 5. The exploratory character of the research at this stage and the objective of this task do not justify the analysis of further cycles. In this assay, the average length of a cycle is 17 days, with a decrease of pH from 3.29 to 3.05; falling 0.015 units of pH per day. Acidity increases from 32.2 g AA/100mL to 71.2 gAA/mL; what means that 2.3 g AA/100 mL are produced per day.

Table 5: Parameters of the four cycles of fermentation with wine vinegar seed

	time (days)	**pH (internal electrode)**		**(initial pH - final pH)/time**	**acidity (g A.A./L)**		**(initial pH - final pH)/time**
		initial	final		initial	final	
1º cycle	13.8	3.32	3.06	0.02	30.21	66.61	2.64
2º cycle	20.1	3.24	3.03	0.01	37.44	77.5	1.99
3º cycle	16.9	3.32	3.06	0.01	37.34	66.34	1.72
4º cycle	34.8	3.22	2.97	0.01	56.48	85.26	0.83
Average	19.71±5.97	3.28±0.05	3.05±0.02	0.013±0.00	38.28±12.69	70.80±5.36	1.85±0.88

The correlation between titratable acidity and pH was calculated, resulting in -0.933, $p<0.001$ (Figure 6). This result was expected because of the close relationship between both parameters, and support the uselessness of measuring them.

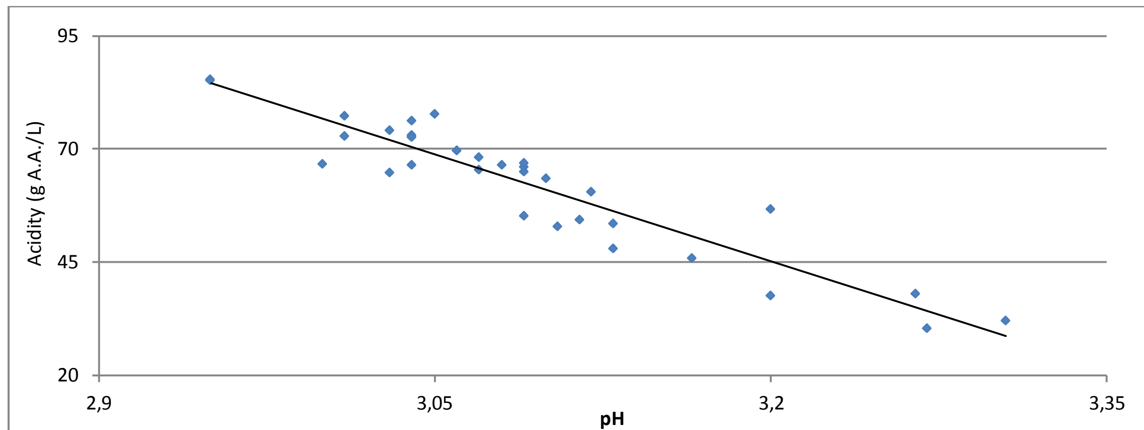


Figure 6: Relation between acidity and pH in fermentation with wine seed vinegar

3.1.1.2 Fermentation with cider seed vinegar

To perform the four cycles of fermentation 43 days were necessary (approximately one and a half month). As in the previous case, the first cycle of fermentation starts with the beginning of assay and ends with the first moment that pH starts increasing . An aliquot of around 50 mL is discharged and 50 mL of fresh medium (Douro wine) are added to start the second cycle of fermentation. The experience goes on repeating that sequence until completing the four cycles. The three aliquots of fresh medium (wine) were added on the day 10th, the 17th and the 32nd. For measuring the length of all cycles, the hours of lag phase and pH decrease were considered, but not the ones after that. The evolution of pH shows a coherent response to the addition of medium, although there is a delay in response after the first and the third cycles (lag phase). That phenomenon has not been seen neither in the other cycles nor in the assay with wine seed vinegar (Figure 7).

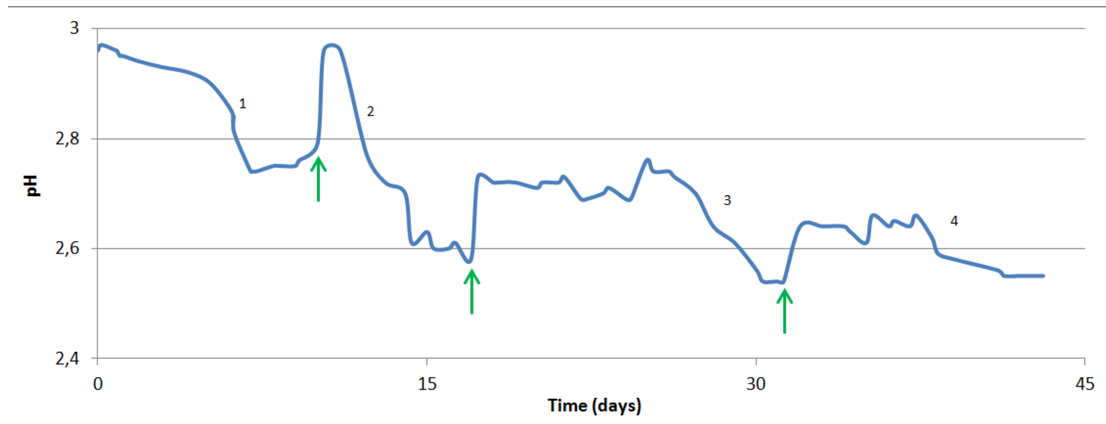


Figure 7: Evolution of pH in time (days). Green arrows indicate the points of addition of medium.

There is a decreasing trend in pH, possibly because the quantity of fresh medium (wine) added at the end of each cycle was not enough to reach the composition at the beginning of the fermentation cycle. It is clear that bacteria do not oxidize acetic acid to carbon dioxide and water, as seen at the end of cycle 2. At the final part of the cycle 1, a very small increase in pH was observed but it might be due to acetic acid loss through evaporation. The cycles obtained by this experience were not regular, and the resulting data is not representative. The duration, and final and initial pH for all cycles are shown in Table 6.

Table 6: Summary of the parameters measured during the cycles of fermentation with cider seed vinegar.

	cycle length (days)	pH		ΔpH/day
		initial	final	
cycle 1	7.0	3.21	2.99	0.03
cycle 2	2.3	3.19	2.96	0.10
cycle 3	13.0	3.1	2.94	0.01
cycle 4	9.3	3.03	2.55	0.05
Average	7.9±4.5	3.1±0.1	2.9±0.2	0.05±0.04

3.1.1.3 Comparison of both fermentations

The evolution of both fermentations is displayed in Figure 8 and the parameters of cycles are summarized in Table 7. At first sight, two facts are noticeable: the pH gap between both fermentations and the difference in time needed for accomplishing the four cycles.

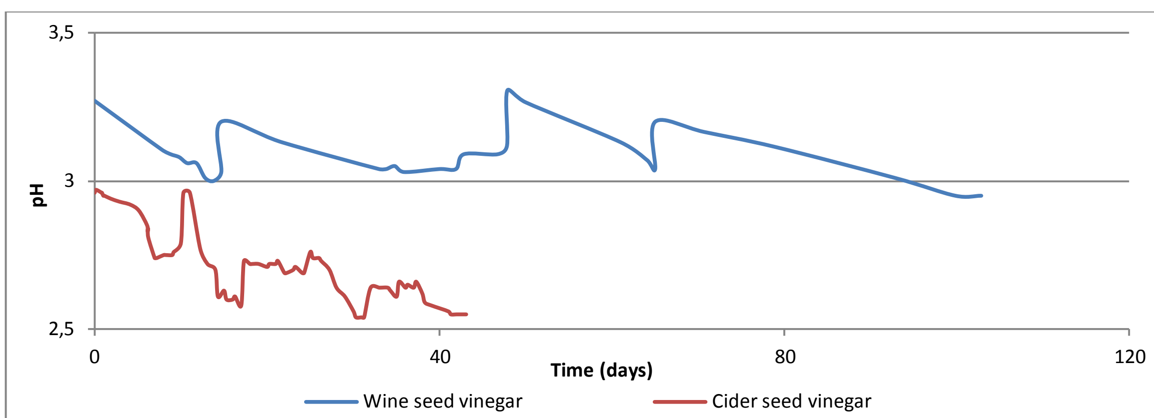


Figure 8: Evolution of pH during fermentation of Douro wine with wine and cider seeds vinegar

Table 7: Comparison of parameters obtained by fermentation with cider seed vinegar and wine seed vinegar

$\bar{X}$ Cycles	cycle length (days)	pH			acidity (gAA/100mL)		
		initial	final	ΔpH/day	initial	final	Δacidity /day
wine seed vinegar	19.71±5.97	3.28±0.05	3.05±0.02	0.013±0.005	38.28±12.69	70.80±5.36	1.85±0.88
cider seed vinegar	7.87±4.49	3.13±0.08	2.86±0.21	0.049±0.039			

The pH gap is of 0.15 and 0.19 points in the pH scale for the average of beginning and end respectively. It is possibly a consequence of a lower initial pH in the case of cider vinegar seed, and from the fact that process controlling was made attending to pH variation, not pH value. The difference in velocity of acetification is very difficult to evaluate because none of the fermentations reached a repetitive and stable working regime. Also, it was not possible to determinate the initial quantity of bacteria in inoculums, due to their exopolisacarid matrix. Thus, the obtained data would indicate that in fermentation with wine seed vinegar pH falls 0.013 points in the pH scale per day, while the fall in the other one is of 0.049 points. It should mean that fermentation with cider seed vinegar is around three and a half times faster than the one with wine seed vinegar. However, the process of fermentation appears to be more regular in the case of fermentation with wine seed vinegar, and for that reason data of velocity might be very variable, therefore not reproducible. The fact of wine seed vinegar is the only one capable of oxidizing acetic acid

is also important: in the future, the excess of acid produced could be reduced by consumption, if it would be necessary. Finally, as wine seed vinegar has origins in a product similar to the one that is aimed to use as raw material, its adaptation is more probable. For these reasons, cider seed vinegar is put aside and the following assay is performed with wine seed vinegar.

These experiences were useful not only for selecting one of the seed vinegar but also for obtaining some fermentation data about pH and time needed for fermentation.

3.1.2 Second fermentation experience

Bioreactors settings are shown in Figure 3 and in Figure 4. The pH progression during 186 days (approximately 6 months) of this experience is shown in Figure 9. It started with 3 cycles of fermentation of Douro wine (20 days) to readapt the inoculated bacteria to fermentation conditions. After that, a progressive addition of Porto wine (wine B) was performed to promote the adaptation to the new media (x days). During successive cycles, the percentage of Douro wine was reduced, being gradually replaced by Porto wine. After X days, fermentation cycles become more reproducible and samples were collected for further analysis. After 16 cycles (83 days), Porto wine B used as substrate was changed to Porto wine C, to obtain data of fermentation with different substrata and compare the results. Technical problems occurred with the air pump. When fermentation cycles become again more reproducible new samples were collected for further analysis. Every stage is describeb in detail in the following sections.

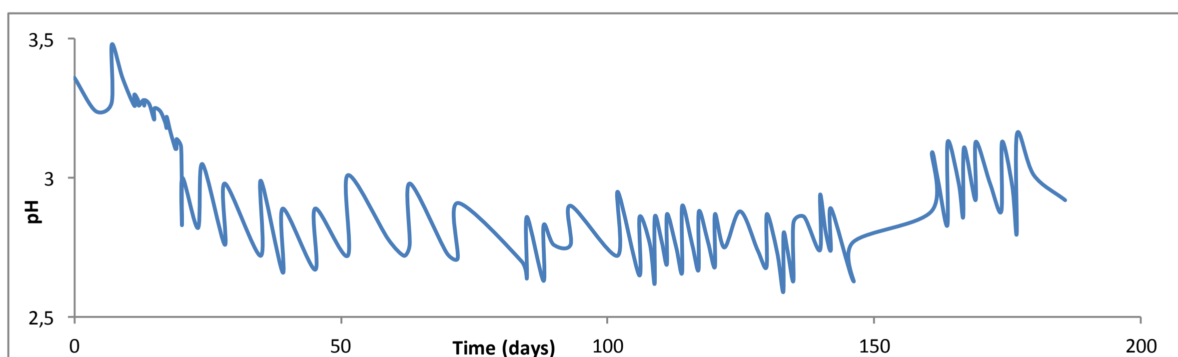


Figure 9: pH in function of time (days) during second fermentation

3.1.2.1 Adaptation of inoculums to Porto wine

It was successfully accomplished in the initial 70 days. The evolution of pH in both reactors is shown in Figure 10. The difference in initial pH between both assays is due to the addition of seed vinegar to the "biotic" assay. In the graphic, the arrows mark the occasions when addition of raw material occur: green arrows mark the addition of Douro wine, red arrows mark the addition of 50 mL of Porto wine without extracting content of the previous cycle and orange arrows mark the addition of Porto wine, extracting an aliquot of vinegar before adding wine. The addition of Porto wine led to a decrease in pH, both at the beginning and end of each cycle, attributable to lower pH of Porto wine in comparison with the Douro wine (pH 3,16 and 3,51 respectively). When the addition is made without extracting fermented product, this growth is more marked because the formed acid is never withdrawn from bioreactor. Formed acid of one cycle is added to formed acid in the next cycle. When fermented product is withdrawn (after cycle 9), part of the acid sintetized is withdrawn and the effect is a rise of pH.

Abiotic reactor (control): during the adaptation, showed a slightly decrease of pH, mainly caused by the progressive addition of Porto wine. Small (centesimal) and random variances were observed and can be attributted to pH measurement errors. There is no evidence of acetic acid production in the control reactor during a period longer than two months.

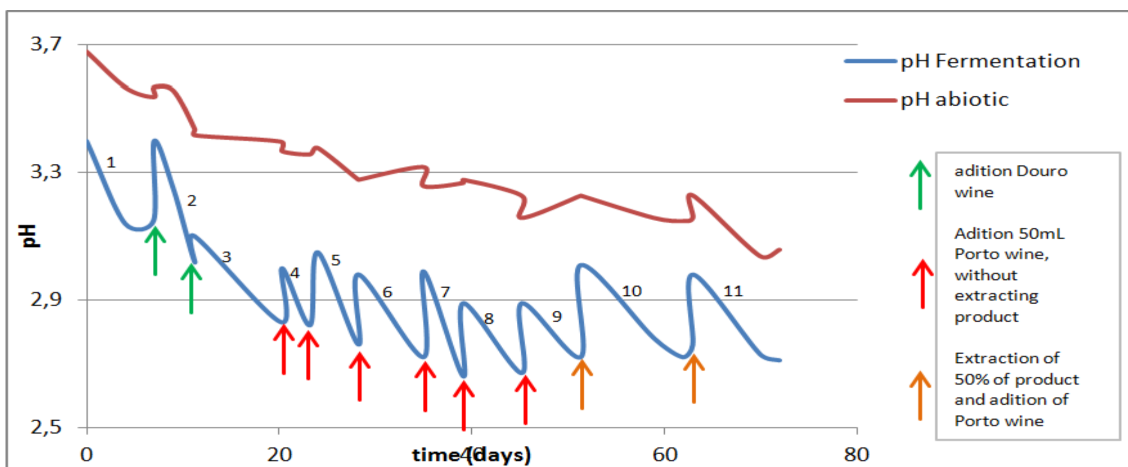


Figure 10: pH of acetic acid fermentation and abiotic assay in time (days). The numbers (1 to 11) near the pH of fermentation curve enumerates de cycles of fermentation

3.1.2.2 Replacement of Douro wine with Porto wine

The replacement of Douro wine with Porto wine (wine B) was made by carrying out sequential fermentation cycles by discharging around the 50% of the bioreactor volume adding an equivalent quantity of Porto wine. This procedure started after the cycle 9, when bioreactor content was 50% of Porto wine. Progressively, the percentage of Porto wine increases, reaching to 98% after the cycle 16 (102 days). After that, it is considered that the adaptation of bacteria to the new media is completed and bioreactor is in working regime. Some cycles were done in working regime. Similar characteristics of initial and final pH and fermentation rate were observed (Figure 11).

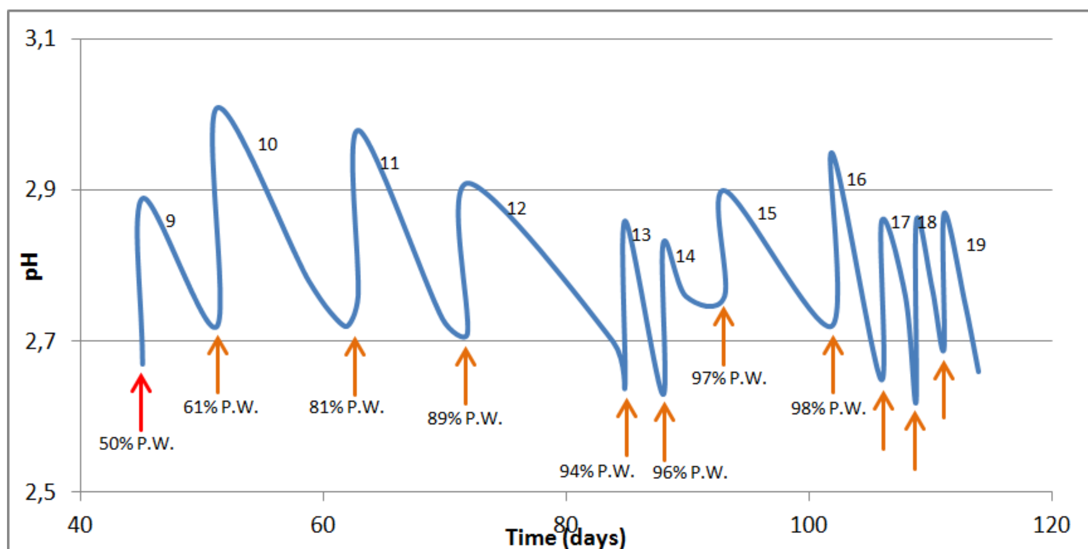


Figure 11: Evolution of pH in time during fermentation in a mix of Porto and Douro wine. Porto wine: P.W. Red arrow shows an addition of Porto wine without previous extraction of produced vinegar. Orange arrows indicates the point were 50% of volume in bioreactor was extracted and a similar quantity of fresh Porto wine was added. Numbers from 9 to 19 indicates the number of cycle of fermentation.

3.1.2.3 Working regime

Figure 12 shows the evolution of pH during cycles 16 to 36. Once the bioreactor system entered working regime, five cycles were produced (17 to 21). This regime was interrupted during some periods due to technical problems in the aeration pumps. In the middle of the cycle 22, altered aeration flow interrupted the working scheme (last part of cycle 22 and 23). It was expected to regain predictability in the scheme (cycles 24 and 25),

to start assays with the second raw material (Porto wine C), starting at the cycle 26. Adaptation of bacteria was not necessary because substrates had similar characteristics. Technical problems occurred again during cycles 28 to 30. In that period it was also observed that, as wine C is not as acid as wine B, the values of pH used as parameters for beginning and end of cycles must be corrected. For this reason, both parameters become slightly higher after cycle 30. Between cycles 31 to 35, predictable and stable working scheme was achieved again. At that moment, fermentation was considered to be in working regime. After that, an extra cycle was made before ending the assay.

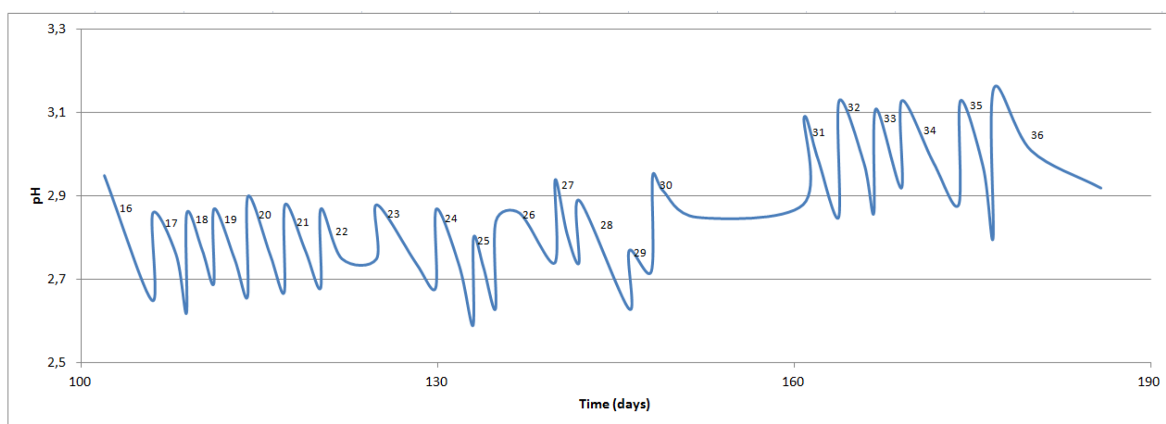


Figure 12: **Variation** of pH during 21 cycles of fermentation. Cycles 16 to 25 corresponds to wine B, cycles 26 to 30 for transition between wines B and C, and cycles 31 to wine C.

With these data, is possible to make a comparison between the data obtained in the 1st fermentation (Douro vinegar) with wine seed vinegar, and the two different Porto wines used in the 2nd fermentation (cycles 17 to 21 for wine B and 31 to 35 for wine C). Data are displayed in Table 8.

Table 8: Comparison of parameters of fermentation of vinegar made from Douro wine (1st fermentation) and two Porto wines (2nd fermentation)

	cycle length (days)	pH		
		initial	final	ΔpH/day
Douro wine	17.0±3.2	3.29±0.05	3.05±0.02	0.01±0.00
wine B	2.8±0.3	2.87±0.02	2.66±0.03	0.07±0.01
wine C	3.1±1.0	3.12±0.02	2.86±0.04	0.09±0.02

Although the initial and final pHs are different in all cases, the pH variation (ΔpH) during fermentation cycles is similar, around 0,24 in the pH scale in all the cases. The difference in pH values is consequence of the differences in pH in the raw material (wines). From this results it is evident that fermentation rate of Porto wine is higher than the one with Douro wine, possibly due to slight differences in aeration, or even by differences in quantity of biomass. Additionally, since wine seed vinegar comes from Douro valley, the vinegar made with it may has been added Porto wine, enriching the alcohol tolerant bacteria at the expense of those that were not. By the application of ANOVA test with Bonferroni, the difference between the fermentation of both Porto (B and C) vinegars is non significant, and the difference between both Porto wines and Douro wine is significant ($p < 0.05$). Porto wine is more similar to the substrata from witch this particular vinegar mother was extracted.

The comparison with other published results about fermentations is not simple due to differences in fermentation parameters and techniques for acetification monitorization. Moreover, the aeration cannot be simply described by volume of air injected per time unit, volume of medium and agitation, it also depends on the ability of the aerator to introduce air with fine bubbles into the fermentation liquid.

The acetification of sherry wine (120 g/L of alcohol, 0.6 g A.A./L of titratable acidity, 29 g/L of glucose, 62 g/L of fructose, pH 3.4, Variety: 100% Grenache) performed by the submerged method (air flow 150 L/h, temperature 30 °C, stirring speed 450 rpm working volume 3.4 L) was considerably faster. The acidity increased from >3% (w/v) to 7% (w/v) during 33 h (Hidalgo et al. 2010). Lower alcoholic content on sherry wine may allow more abundant growth of acetifying bacterias.

The relationship between pH of raw material and pH in different stages of fermentation was analysed (Table 9). The information of only two cases is not enough to make generalizations but the similarity between the results with both wines was clear and this may be an useful information for future research. It also exposes the similarity between pH modifications in both fermentations.

Table 9: Relationship between pH of raw material and the pH of different stages of fermentation

	pH raw material	PH in stages of fermentation			Ratio $pH_{stage}/pH_{raw\ material}$			Ratio $pH_{end}/pH_{init.}$
		initial	middle	end	initialtion	middle	end	
Wine B	3.16	2.88	2.76	2.67	0.91	0.87	0.84	0.93
Wine C	3.47	3.12	2.98	2.86	0.90	0.86	0.82	0.92

According to the performed experiments, the predicted pH of vinegars made with this seed is around 0.83 pH of wine, and the initial pH of the mixture wine/seed vinegar is around 0.90 pH of wine. These data are important for planning and controlling fermentation in future works.

3.2 *Section B. To characterize the obtained product and finding suitable parameters for monitoring the process*

Changes on concentrations of glucose, fructose, ethanol and acetic acid during acetification were assessed using enzymatic methods and HPLC. Poor precision was observed when applying enzymatic methods. Only HPLC results will be discussed.

The analysis of wines used as raw material is summarized in Table 10. The ratios glucose/fructose for both wines were similar: 0.71 for wine B and 0.73 for wine C. Acetic acid was in a very low quantity in both cases. The content of ethanol expressed as percentage was 20.2% m/m for wine B and 18.8% m/m for wine C.

Table 10: Characterization of Porto wines used as raw material

	pH	Glucose (g/L)	Fructose (g/L)	Acetic Acid (g/L)	Ethanol (g/L)
Wine B	3.16	51.2±5.8	71.9±8.0	0.6±0.3	159.3±7.3
Wine C	3.47	41.1±1.7	56.1±2.3	0.4±0.0	148.2±5.0

3.2.1 Chemical composition changes during acetification

Samples were collected during successive fermentation cycles. Three cycles of working regimes with both Porto wines were selected. In the case of wine B, cycles 19, 20 and 21

were chosen; and 31, 32 and 34 in the case of wine C. Cycle 33 was discharged because the cycle midpoint sample was missed.

Results are summarized in Table 11. As observed, ethanol is the main substrate, and glucose and fructose are not being used significantly as substrate during the fermentation process. Acetic acid is being produced during the process, growing from 13.4 to 28.5 g/L in the case of Wine B and from 18.1 to 34.5 g/L in the case of wine C.

Table 11: Summary of contents in glucose, fructose, acetic acid and ethanol during three stages of fermentation

		Wine B			Wine C		
		1st cycle	2nd cycle	3rd cycle	1st cycle	2nd cycle	3rd cycle
glucose (g/L)	initiation	45.7 ± 3.0^{a}	52.8 ± 0.9^{ab}	56.3 ± 0.7^{b}	60.0 ± 2.1^{a}	51.8 ± 1.3^{b}	46.9 ± 1.1^{c}
	middle	59.8 ± 6.0^{a}	58.7 ± 0.3^{a}	66.1 ± 2.4^{a}	56.5 ± 1.2^{a}	53.1 ± 1.6^{a}	49.3 ± 6.7^{a}
	end	58.3 ± 1.5^{a}	62.8 ± 8.4^{a}	59.0 ± 5.9^{a}	59.2 ± 1.5^{a}	54.6 ± 1.7^{b}	52.7 ± 0.9^{b}
fructose (g/L)	initiation	66.7 ± 4.8^{a}	76.4 ± 1.0^{ab}	81.8 ± 0.2^{b}	83.3 ± 3.0^{a}	69.0 ± 1.1^{b}	61.8 ± 1.9^{c}
	middle	86.9 ± 8.5^{a}	85.5 ± 1.3^{a}	95.3 ± 4.3^{a}	77.6 ± 1.2^{a}	71.3 ± 2.0^{a}	65.3 ± 8.7^{a}
	end	79.6 ± 1.6^{a}	90.2 ± 11.6^{a}	86.2 ± 9.1^{a}	80.8 ± 1.5^{a}	73.2 ± 1.6^{b}	69.9 ± 1.5^{b}
acetic acid (g/L)	initiation	13.7 ± 3.4^{a}	11.7 ± 2.1^{a}	14.9 ± 0.7^{a}	22.6 ± 2.3^{a}	14.8 ± 0.9^{b}	16.8 ± 1.1^{b}
	middle	24.3 ± 2.6^{a}	22.5 ± 0.8^{a}	31.1 ± 5.9^{a}	20.2 ± 0.6^{a}	23.6 ± 0.4^{a}	22.00 ± 2.2^{a}
	end	21.4 ± 0.5^{a}	32.1 ± 0.2^{a}	32.1 ± 1.4^{a}	42.4 ± 0.3^{a}	37.9 ± 1.2^{b}	32.2 ± 1.7^{c}
ethanol (g/L)	initiation	93.7 ± 6.5^{a}	113.0 ± 1.3^{b}	115.3 ± 2.2^{b}	76.8 ± 0.6^{a}	105.5 ± 2.5^{b}	107.6 ± 3.6^{b}
	middle	61.1 ± 5.5^{a}	64.6 ± 4.4^{a}	64.5 ± 18.2^{a}	40.8 ± 0.1^{a}	52.7 ± 1.2^{a}	43.2 ± 5.0^{a}
	end	$32,7\pm1.1^{a}$	38.8 ± 1.3^{ab}	46.6 ± 4.4^{b}	12.2 ± 1.0^{a}	29.4 ± 1.1^{b}	11.6 ± 0.2^{a}

For each wine, values not sharing the same superscript letter (a, b) within the horizontal line are different according to ANOVA and Tukey test.

The existence of statistically significant differences between the content of each compound in successive cycles reflects some flaws in controlling the fermentation. For wine B, statistically significant differences were observed for the initial glucose, fructose and ethanol concentrations, and for ethanol concentration at the end of the cycles. For wine C, statistically significant differences for glucose, fructose, acetic acid and ethanol concentrations were not observed in the middle of fermentation only. These observations point to some imprecision in setting the final time for each cycle and the volume of raw material to begin a new one.

For a better analysis, results are graphically presented in Figure 13. To elaborate the figure in a proper way, cycle 34 (with wine C) is represented immediately after the end of cycle 32, as if they were consecutive.

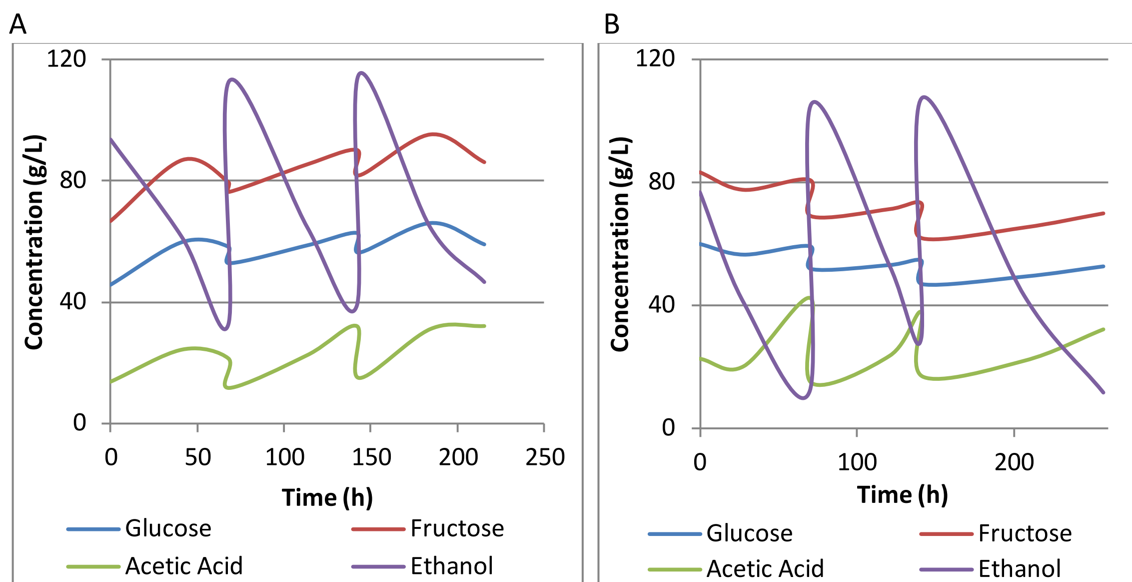


Figure 13: Evolution of glucose, fructose, acetic acid and ethanol in time. A): in wine B and B): in Wine C.

Thereafter, the evolution of the four compounds during the fermentation process is discussed in depth. Due to the interrelationship between the analytes studied, rather than interpret the evolution of each of them separately, they are grouped into two groups of two compounds. First group comprises glucose and fructose, because both are sugars and have a similar behavior during fermentation. Second group includes acetic acid and ethanol, because acetic acid is generated when ethanol is consumed.

3.2.1.1 First group: Glucose and Fructose

Concentration of glucose and fructose varies during the fermentation. It is very important to understand the cause of this variability to interpret what is happening inside the bioreactor.

3.2.1.1.1 Variations during the fermentation cycle

Concentrations of glucose and fructose slightly increased along every cycle of fermentation with both substrates. Despite this general trend, the increase is only

statistically significant for glucose in fermentation with wine B. An increase of sugar concentration during individual cycles can be observed graphically (Figure 13). Additionally, in the case of wine C a decreasing trend between cycles was also observed. The intracycle concentration effect may result from the evaporation of fermentation media due to the high aeration.

The mean glucose/fructose ratios during the fermentation cycles were 0.694±0.008 in samples collected during wine B fermentation and 0.742±0.001 on samples collected during wine C fermentation. Similar ratios were observed in the raw materials: 0.601±0.000 for wine B and 0.732±0.000 for wine C. The most striking is how stable this ration is during fermentation. A Pearson correlation between the concentrations of both sugars allowed to obtain r=0.968 and $p<0.001$ (Figure 14).

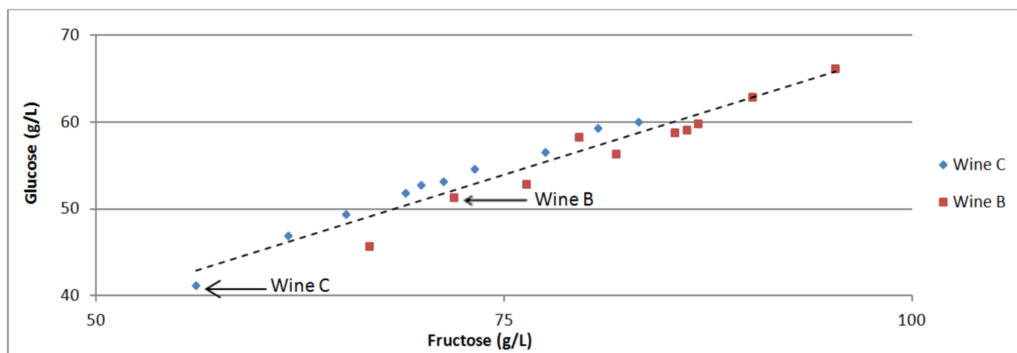


Figure 14: Correlation between the concentrations in g/L of sugars (r = 0.968, $p<0.001$). The arrows indicate the points for raw materials, wines B and C

The points corresponding to fermentation of wine B are under the trendline while the ones from wine C are over it. However, the point corresponding to wine C itself is under the trendline. Other interesting experimental points are the one obtainded during wine B fermentation that has less of both sugars than the raw material and the one that is over the trendline. The first case corresponds to a point in the initiation of first cycle, and it is possible that there was still influence of the raw material previously used, Douro wine, that has a lower quantity of sugars. In the second case, the point corresponds to the end of first cycle, and it can be seen in the graphic that the relationship glucose/fructose is different to the one in the previous and following points.

As the ratio between both sugars remained constant, it is noteworthy that there was no evidence of glucose or fructose consumption. The occurrence of bacterial growth with glucose or fructose as substrate can be discarded.

Taken into account what was said before it can be inferred some data about bacteria metabolism. They consume ethanol before glucose and fructose. Additionnaly, in each fermentation cycle, after a decrease in pH, pH begins to increase, probably because the produced acetic acid is being consumed. It is known that oxidation of sugars to water and carbon dioxide will not affect noticeably the pH.

Assuming no consumption of sugars, changes in their concentration can be used to estimate evaporation rate from the reactor. The evaporation rate is related to the increase in glucose ou fructose concentration in the bioreactor. Volume loss was calculated for the cycles in study assuming an initial volume of 600 mL. Results are shown in Table 12.

Table 12: Percentage of losses of volume during cycles of fermentation, in total and per day of acetification

% of volume losses	Wine B				Wine C			
	according to glucose concentration		according to fructose concentration		according to glucose concentration		according to fructose concentration	
	total	per day	total	per day	total	per day	total	per day
1º cycle	21.5	7.6	16.1	5.7	4.6	1.6	4.0	1.4
2º cycle	15.9	5.2	15.3	5.0	5.1	1.7	5.8	2.0
3º cycle	4.5	1.5	5.0	1.7	11.0	2.3	11.7	2.4

The similarity between volume loss calculated from the rise of both sugar concentrations may confirm the inicial assumption of no or insignificant consumption of glucose and fructose. Although data are consistent in every single cycle, differences between cycles can be observed. In the case of the 3rd cycle of C wine, the acetification became slower in the second half of the cycle; the cycle was longer than the others. When the two stages of fermentation are separated, losses of volume are: 1,87% and 2,02% per day in the first part of fermentation and 3,01% and 3,14% per day in the second part. The reason for extending this cycle is not clear, but it is believed that in normal situations the overall performance of the fermentation would be more similar to the first part of the cycle that

to the second part. On the other hand, the volume loss on 1st and 2nd cycle with wine B is approximately three times higher when compared with other fermentation cycles, but their length is similar. These differences might be explained by the effect of colligative properties of the fermentation solution. There is an inverse relationship between the total quantity of solutes and the ability to the evaporation of the solvent. It was calculated the correlation between the total content of sugars (glucose and fructose) at the beginning of every cycle and the percentage of volume loss (according both sugars). Although the quantity of results is too small to generalize, results would indicate a very strong correlation between both values ($r_s = 0.879$ for samples with wine B and $r_s = 0.863$ for wine C), and are shown in Figure 15.

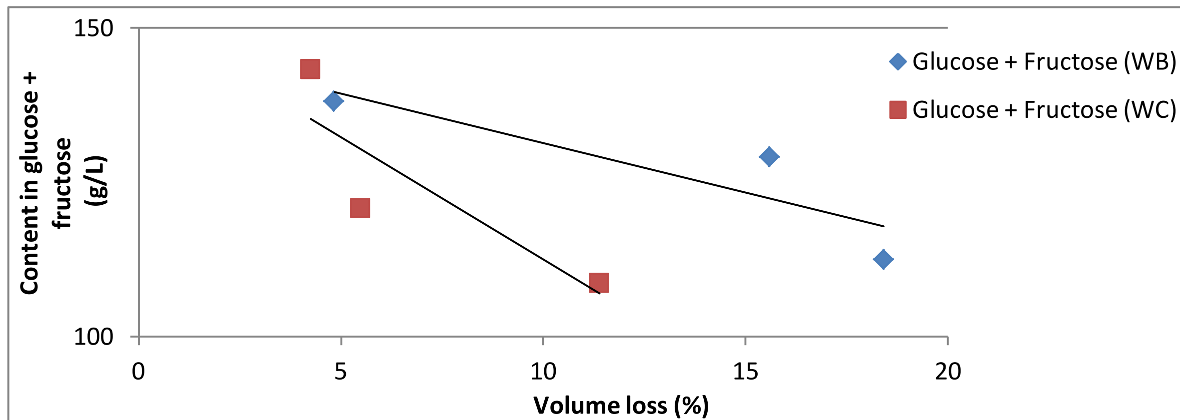


Figure 15: Correlation between de the volume loss and the content in sugars at the beginning of every cycle

Is interesting that, in figure 15, fermentations with different wines had different slopes, probably due the effect of other compounds present in the raw material. This fact highlights the effect of colligative properties and the importance of controlling sugar concentration as a determinant of evaporation rate during the fermentation process.

3.2.1.1.2 Variations during successive cycle

To study the modification of concentration of glucose and fructose during consecutive cycles is important because it gives a hint about the effectiveness of process control. The comparison between the values obtained with both wines is presented in Figure 16.

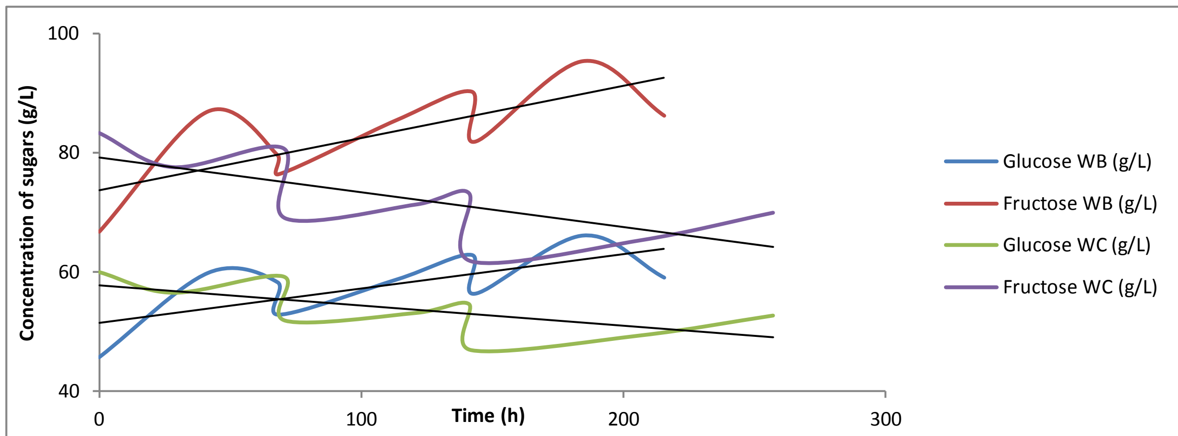


Figure 16: Concentration of glucose and fructose in time for fermentations with wine B (WB) and wine C (WC)

There was a growing trend for both compounds during wine B fermentation, while the same parameters decreased in wine C fermentation. For wine B, the smallest values both for glucose and fructose concentrations were observed at the beginning of first cycle (45.70±3.03 g/L and 66.74±4.79 g/L respectively) while the higher values occured at the middle of the last cycle (66.09±2.44 g/L and 95.33±4.34 g/L respectively). On the other hand, for wine C the smallest glucose and fructose concentrations occured at the beginning of last cycle (46.87±1.08 g/L and 61.76±1.94 g/L respectively), while the higher values (59.96±2.06 g/L for glucose and 83.27±3.01 g/L for fructose) were observed at the beginning of the first cycle. In all cases, the slopes are small and can be measured in centesimals (g/L/h). The intercepts are similar to the values obtained for glucose and fructose concentration in raw material for wine B, but not in the case of wine C (Table 13). If glucose and fructose are not consumed during fermentation, it is expected a high correlation between intercepts and glucose and fructose concentration in wine used as raw material. The correlation will increase with the number of fermentation cycles with the same raw material because the final product will tend to have a sugar concentration close to raw material. With wine B, 6 fermentation cycles were performed before the analyzed ones (from 13º to 18º) successively increasing the proportion of wine B or wine B products in the biorreactor up to 97% (with a 0,5 ratio between the inoculum volume and

the volume of the bioreactor). With wine C, the analysed cycles were performed after 5 initial cycles, necessary to reach to quantity of wine C up to 94% (with a 0,5 ratio between the inoculum volume and the volume of the bioreactor).

Small negative or positive slopes will be associated with the transition among the previous initial raw material used, the actual one and the ratio between inoculums and raw material added. Wine C has lower concentrations of both sugars in comparison to wine B, and that would be the reason why there was a decreasing trend in sugar concentration during cycles. Before the use of wine B, Douro wine was used, because it has lower concentration of sugars than any Porto wine. The previously discussed volume losses by evaporation from the bioreactor contributed to a positive slope and will be the main determinant after some cycles.

Table 13: Comparison between concentration of glucose and fructose in wines B and C and intercepts of the tendency lines of concentration of those compounds during fermentation

	Glucose		Fructose	
	Concentration (g/L)	intercept	Concentration (g/L)	intercept
Wine B	51.2±5.8	51.515	71.9±8.0	73.753
Wine C	41.1±1.7	57.662	56.1±2.3	79.185

3.2.1.1.3 Glucose and fructose in relation with pH

The correlations for both glucose and fructose with pH are presented in Figure 17. As the pH of raw materials is different, the pH of the product in fermentation is also different. Even the pH that indicates the end of fermentation cycle with one wine may be the pH of the middle or even the initial pH with other one. For that reason, they must be analyzed separately. The average pH for beginning, middle and end were 2.88, 2.76 and 2.67 for wine B, and 3.12, 2.98 and 2.86 for wine C. Initial samples from fermentation cycles with wine B and samples at the end of cycle with wine C not only had a very similar pH but also similar values of concentration of glucose and fructose (Glucose: 51.25±5.42 g/L for wine B and 55.52±3.38 g/L for wine C; Fructose: 74.99±7.65 g/L for wine B and 74.63±5.56 g/L for wine C). Although this similarity seems random, it is only due to the composition of raw materials.

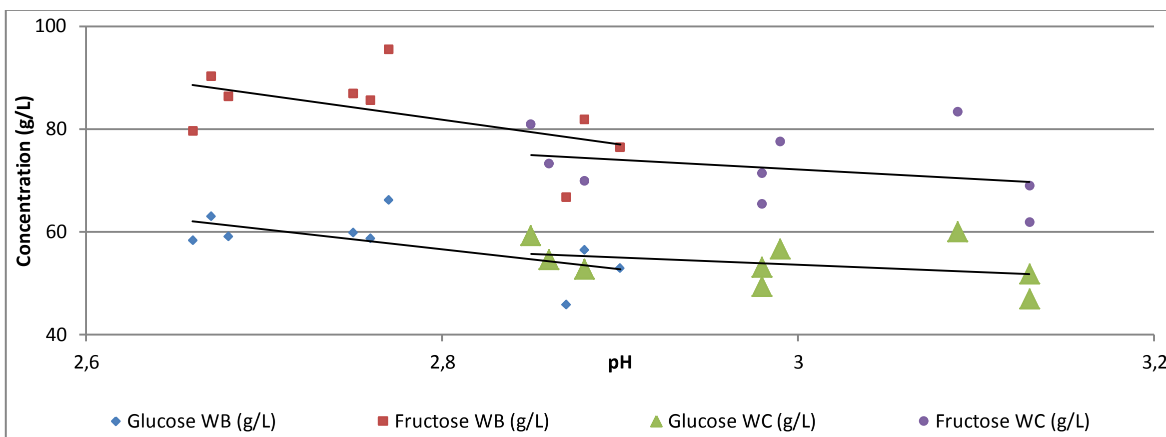


Figure 17: concentration of glucose and fructose in function of pH. Wine B: WB, Wine C: WC.

In the case of wine B, strong correlations between pH and sugars were observed being -0.525 (*p*= 0.072) for glucose and 0.610 (*p*= 0.081) for fructose. In the case of wine C, correlations were very week: -0.162 (*p*= 0.702) for glucose and -0.104 (*p*= 0.807) for fructose. These differences may reflect differences in the pH record and evolution across fermentation cycles. Sugar concentration trough fermentation cycles with wine C still reflect two different effects: a decreasing trend due to previous raw material and an increasing trend due to sugar concentration and due to evaporation losses. Instead, in the case of wine B, sugar concentration increases both through each cycle of fermentation and through successive cycles, resulting in higher correlations with pH.

From previous analysis it is possible to infer that the concentrations of glucose and fructose are not suitable parameters for monitoring the fermentation process. However, monitoring sugar concentration during the fermentation can help the production of homogenous batches of vinegars.

3.2.1.2 Ethanol and acetic acid

The evolution of acetic acid and ethanol concentrations during fermentation with both wines are shown in Figure 18. It is clear that fermentation rate was very similar with both substrates, even though their pH and amount of acid produced were different. Cycles of fermentation started with an average concentration of ethanol around 102 g/L. Nevertheless, at the end of cycles, average ethanol concentration was different, being

39.4 g/L for wine B and 17.7 g/L for wine C. Acetic acid content at the beginning of fermentation cycles was relatively similar for all cases and is around 15.8 g/L. Although, there were some differences between fermentations: the concentration of acetic acid was smaller in the end of cycles with wine B in comparison with the ones with wine C: on average, 28.5 g/L and 37.5 g/L, respectively. Also, the concentration of ethanol at the end of 1st and 3rd cycles with wine C was very similar (12.2 g/L and 11.6 g/L) but the concentration of acetic acid at the same points was different (42.4 g/L and 32.2 g/L respectively).

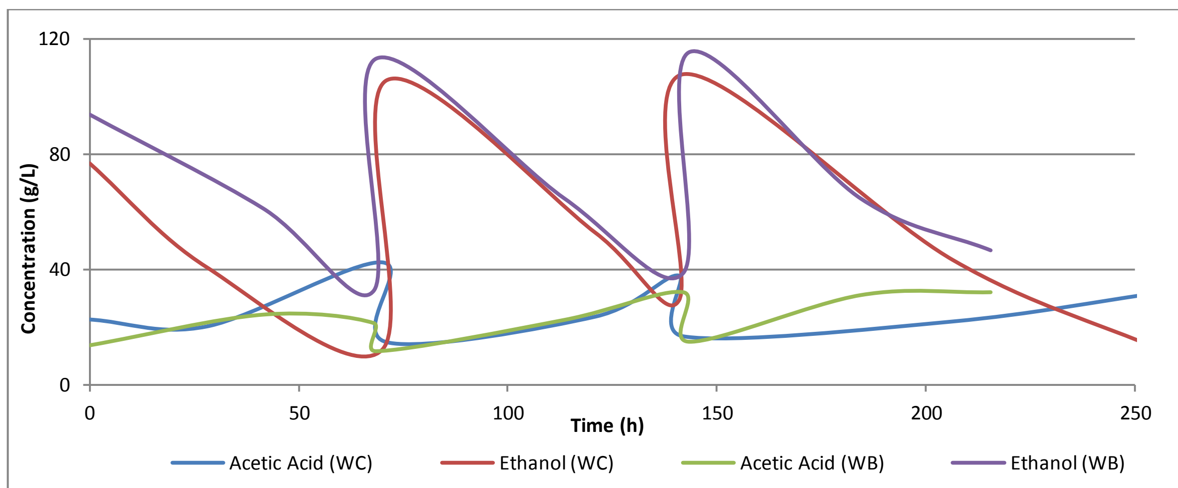


Figure 18: Comparison of the evolution of acetic acid and ethanol between the fermentations of wine B (WB) and wine C (WC)

The correlations between acetic acid and ethanol were very strong and significant: 0.936 ($p < 0.001$) for wine B and 0.881 ($p = 0.002$) for wine C. Slope and intercept for both linear regressions were remarkably similar (Figure 19).

According to Garcia-Garcia et al. (2009), the conversion of ethanol to acetic acid by AAB has around 95-98% of the stoichiometric value so even stronger correlation were expected.

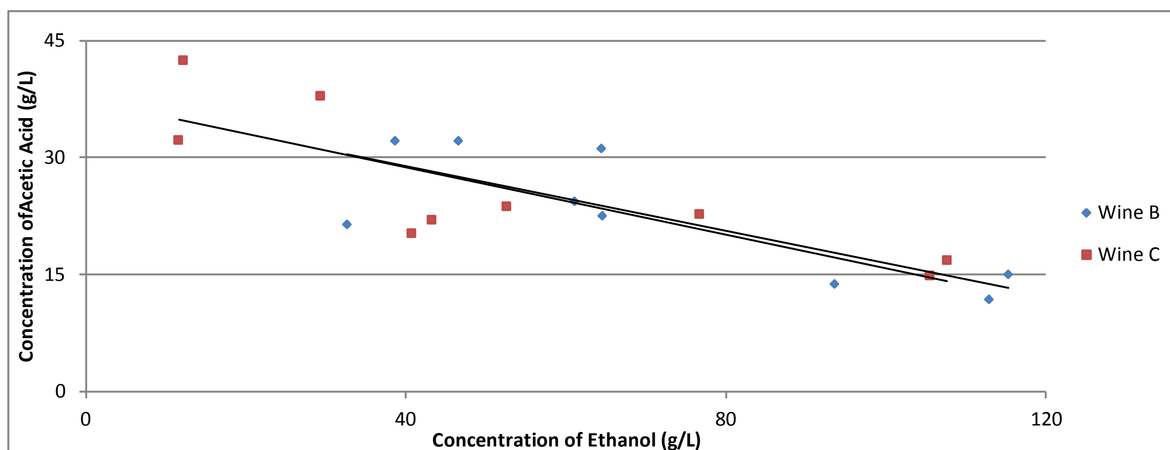


Figure 19: relationship between concentration of acetic acid (g/L) and ethanol (g/L)

To compare the results obtained from other studies, with other vinegars, it was necessary to measure titratable acidity, expressed as grams of acetic acid by liter, because that method was the most commonly used. The values obtained for ethanol content and acidity are compared in Table 14 with the ones obtained for 49 samples of wine vinegar, 12 samples of cider, 4 samples of alcohol (Gerbi et al. 1998) and 6 samples of Sherry wine vinegar (Castro et al. 2002; Morales et al. 2002; Tesfaye et al. 2004).

Table 14. Comparison of values of ethanol and acidity in different vinegars

	wine vinegar	Alcohol vinegar	cider vinegar	Sherry wine vinegar	fermented wine B	fermented wine C
Ethanol (g/L)	4.19±7.98	3.47±3.63	1.65±1.02	1.0±0.2	39.3±6.9	17.8±10.0
Acidity (gA.A./L)	66.0±8.1	73.5±10.8	54.0±4.5	82.4±6.0	30.5±1.7	41.5±4.0

It is clear that the product of fermentation with both wines B and C has more ethanol and less acetic acid than vinegar of wine, alcohol, cider and Sherry wine. Probably, the oxidation of ethanol was being limited by the oxygen transfer rate in the bioreactor. It is reported that under oxygen limitation and low ethanol concentration AAB oxidize preferably acetic acid instead of ethanol (Schmid 2003).

The theoretical and real quantities of acetic acid produced were calculated and results are presented in Table 15. The total quantities of acetic acid and ethanol compounds were corrected considering volume losses. In table 15, the topic “lost ethanol” is the difference

in the ethanol amount between the beginning and the end of every cycle. That loss is a sum of the alcohol both consumed and lost by volatilization, being not possible to differentiate them. The "calculated acetic acid production" is the calculus made considering that all the loss ethanol is biotransformed into acetic acid stechiometrically with an efficiency of 95%. The "acetic acid measured" is the difference between the acid quantity at the beginning and end of every cycle. That is the produced acetic acid minus the losses by volatilization and by bacterial consumption. The "gap" is the diference between "calculated acetic acid production" and "measured acetic acid".

Table 15: Differences between calculated acetic acid according to stechiometry and empiric results

	Lost etanol (g)	calculated acetic acid production (g)	acetic acid measured (g)	gap(g)
1º cycle wine B	43.1	53.4	0.3	53.1
2º cycle wine B	51.2	63.4	6.7	56.7
3º cycle wine B	43.9	54.3	8.5	45.8
1º cycle wine C	39.3	48.7	9.7	39.0
2º cycle wine C	47.6	58.9	11.4	47.5
3º cycle wine C	58.3	72.2	7.3	64.9

This results clearly expose a loss of compounds, probably, acetic acid, during fermentation. There is no information on bibliography about the amont of mass losses during acetic fermentations, possibly because those values depend on the equipment used, and the work conditions. Acetic acid was lost even when there was still ethanol available for consumption. The lack of oxygen due to low rates of oxygen transfer in the interface air-liquid may be the cause of ethanol not being totally turned into acetic acid, even when there is a high volume of air entering to the system. Acetic acid loss may be produced by evaporation or oxidized because of bacteria not being able of consuming ethanol.

3.2.1.2.1 Acetic acid and ethanol in relation with pH

Fermentation was monitored measuring pH and assuming a strong correlation of this parameter with ethanol and acetic acid. According to the data obtained, this assumption is true for both wines. Correlations between pH and ethanol were positive, very strong and

significant, 0.983 (p<0.001) and 0.953 (p<0.001) for wine B and C, respectively. Correlations between pH and acetic acid were negative, very strong and significant, -0.946 (p<0.001) and -0.921 (p<0.001) for wine B and C, respectively.

Linear regression analysis showed slight differences between wines, which can be attributed to differences between initial pH (Figure 20 and Table 16). These results validate the usefulness of measuring pH to follow the fermentation.

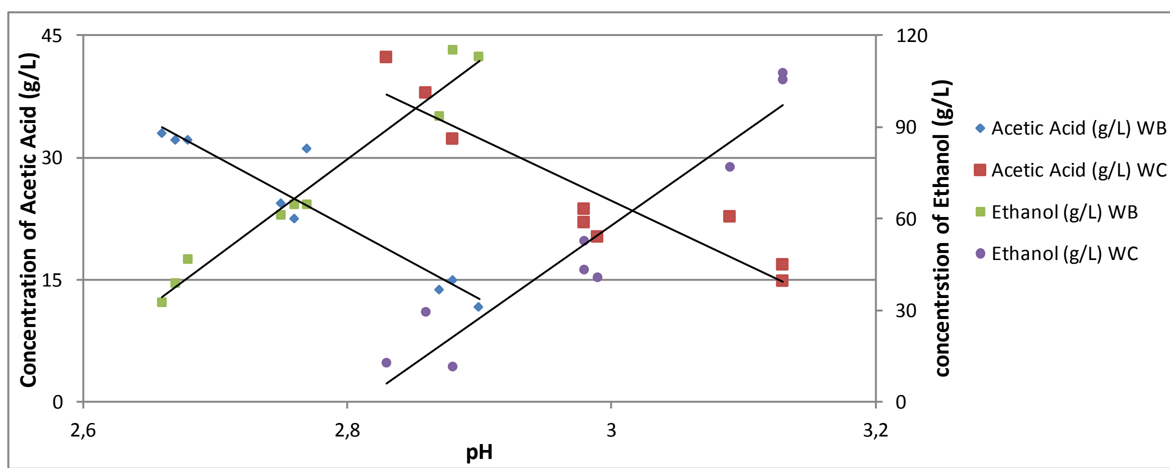


Figure 20: Relationship between pH and ethanol and acetic acid

Table 16: Parameters of the relationship pH / acetic acid and pH /ethanol, with both wines in study

	Slope		Intercept		Correlation	
	Acetic Acid	Ethanol	Acetic Acid	Ethanol	Acetic Acid	Ethanol
Wine B	-88.122	322.92	268.13	-824.81	-0.946	0.983
Wine C	-76.701	303.8	254.83	-853.65	-0.921	0.958

Comparing the slopes of trendlines and intercepts for wines B and C, it is noticed that both acetic acid and ethanol trendlines and intercepts are bigger in the case of wine B. However, the differences are small.

3.2.2 Comparative study between the result of enzymatic tests and HPLC

HPLC is generally considered an accurate and precise methodology. However, HPLC requires high initial investment costs and specialized training of human resources. On the other hand, determinations using enzymatic tests can reduce initial investment costs and

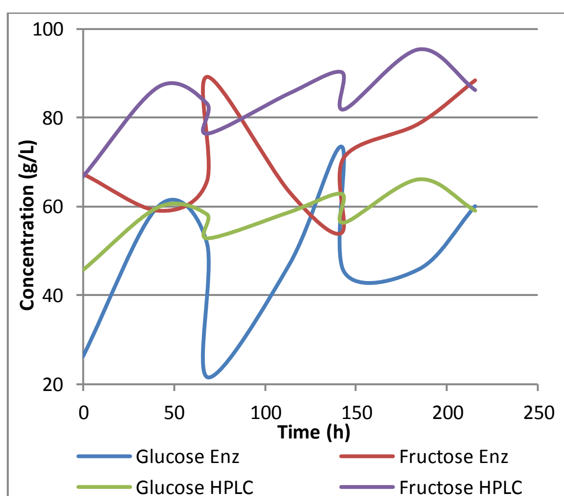
require relatively less analytical skills. Therefore, enzymatic tests can be useful tools for monitoring acetic fermentations.

The results obtained by both methodologies in samples from cycles 19, 20 and 21 (with wine B) are summarized in Table 17. For a better comparison, glucose and fructose concentrations (Figure 21A) and acetic acid and ethanol concentrations (Figure 21B) are graphically displayed.

Table 17: Concentrations of glucose, fructose, acetic acid and ethanol according to two different methods (HPLC and enzymatic assays) for three cycles of fermentation with wine B.

		1st cycle		2nd cycle		3rd cycle	
		HPLC	Enzimatic d.	HPLC	Enzimatic d.	HPLC	Enzimatic d.
glucose (g/L)	beginning	45.7±3.0	26.3±4.2	52.8±0.9	21.5±2.5	56.3±0.7	45.2±4.2
	middle	59.8±6.1	60.1±8.3	58.7±0.3	47.0±0.0	66.1±2.4	45.8±10.3
	end	58.3±1.5	51.9±2.7	62.8±8.4	73.5±14.0	59.0±5.9	60.1±0.0
fructose (g/L)	beginning	66.7±4.8	67.3±10.7	76.4±1.0	89.1±2.0	81.8±0.2	71.1±5.5
	middle	86.9±8.5	59.0±2.8	85.5±1.3	63.2±2.7	95.3±4.3	78.6±3.8
	end	79.6±1.6	65.5±4.1	90.2±11.6	54.0±1.5	86.2±9.1	88.4±10.9
acetic acid (g/L)	beginning	13.7±3.4	14.2±0.4	11.7±2.1	12.3±0.6	14.9±0.7	12.3±0.1
	middle	24.3±2.6	28.8±11.4	22.5±0.8	17.5±0.5	31.1±5.9	20.0±0.7
	end	21.3±0.5	34.5±4.3	32.1±0.2	27.7±1.8	32.1±1.4	30.7±1.2
ethanol (g/L)	beginning	93.7±6.5	90.5±4.0	113.0±1.3	67.5±7.5	115.3±2.2	67.3±5.6
	middle	61.1±5.5	61.3±7.4	64.6±4.4	42.4±7.6	64.5±18.2	68.7±4.0
	end	32.7±1.1	45.4±5.7	38.8±1.3	53.9±1.4	46.6±4.4	52.3±10.3

A



B

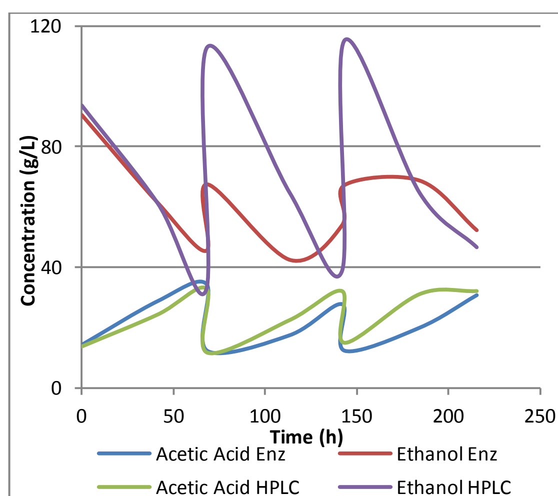


Figure 21: Concentration in time of glucose and fructose (A) and acetic acid and ethanol (B) according to two different methods -HPLC and enzymatic (Enz) assays- for three cycles of fermentation with wine B

If two analytical methods measure the same quantity, the relationship between the results provided by both methods should be linear with intercept = 0 and the slope = 1. Applied linear regression analysis between the data provided by HPLC (xx axis) and enzymatic tests (yy axis) showed an intercept far from 0 and a slope far form 1 (Table 18). The correlation coefficients between HPLC and enzymatic methods results are not significantly different from 0 in case of fructose and ethanol results. For glucose the results obtained by both methods are strongly correlated but the linear regression slope is close to 2.0 and intercept is -67.6. Acetic acid results showed the best association between both methods. The results are also strongly correlated (r = 0,67), linear regression slope is close to 0.7 and intercept is 5.9.

Table 18: Linear regression analysis between the data provided by HPLC (xx axis) and enzymatic tests (yy axis) and Pearson correlation

	Linear regression			**Pearson correlation**	
	intercept	slope		Coefficient	P
Glucose	-67.6	2.0		0.72	0.03
Fructose	82.6	-0.1		-0.10	0.81
Acetic Acid	5.9	0.7		0.67	0.05
Ethanol	39.3	0.3		0.65	0.06

By the comparison of Figures 23 A and B, an interference of ethanol, acetic acid, titratable acidity or pH in glucose and fructose determination using enzymatic kits can be hipothesized. Acetic acid was the only compound properly measured with enzymatic kits but, since its concentration is strongly correlated with pH, a parameter easier to measure, the determination of acetic acid with enzymatic kits is nor crucial neither more convenient for controlling the process.

3.2.3 Legislation

According to the Portuguese legislation, the content of acetic acid, ethanol and other substances in vinegar are legislated by Annex of Article 6 of Decree-Law nº 174/2007 of May 8th from the Ministry of Agriculture, Rural Development and Fisheries. It specifies

that the minimum acidity, expressed as acetic acid by 100 mL, is of 6 g/100 mL for wine vinegar and 5 g/ 100 mL for other vinegars; the maximum of residual ethanol, at 20 ºC, is 1.5 % (v/v) and the maximum of other constituents is 0.5 % (m/v).
The average composition of the products obtained at the end of each fermentation cycle is presented in Table 19. It can be noticed that all parameters are out of the legal parameters for wine vinegar.

Table 19: Acidity, ethanol and other compounds (glucose and fructose) at the end of cycles with both wines

	acidity (g a.a./100mL)	Ethanol %(v/v)	glucose (g/100mL)	fructose (g/100mL)
required	≥6.00	≤1.50	≤1.00	≤1.00
Wine B	3.0±0.2	5.0±0.9	6.0±0.5	8.6±0.7
Wine C	4.1±0.4	2.2±1.3	5.5±0.3	7.5±0.5

As already discussed, the end product has low acetic acid and high ethanol content when compared to regular vinegars. This can be a consequence of intrinsic microbiological inhability to extend the acetification and/or a lack of oxygen transfer rate, as discussed previously. Even if a technological change would allow carrying on acetification until levels of acidity and residual ethanol required to vinegars, the contents of glucose and sucrose would not change during the process. It would be necessary an additional treatment for consuming or extracting sugars excess, but it would modify one of the intrinsic sensorial characteristics.
A more efficient aeration control (agitation, air flow, bubble size, etc) and temperature are highly relevant to further research in process optimization and scale-up.

3.2.4. Analysis of volatile compounds

3.2.4.1. Linearity

The calibration curves for eight esters, five alcohols, one aldehyde, three ketones, three phenols and four acids, in a model synthetic solution were constructed using the

optimized HS-SPME method. Linear curves were fitted on to the calibration points. Good linearity for most compounds in large concentration ranges were obtained.

3.2.4.2 Volatile compounds in vinegar

Eight esters (ethyl hexanoate, ethyl octanoate, hexyl acetate, ethyl 2-phenylacetate, phenylethyl acetate, benzyl acetate, diethyl succinate, diethyl malate), one aldehyde (2,4-Dimethylbenzaldehyde), five alcohols (phenylethanol, benzyl alcohol, (E)-linalool oxide B and, (E)-linalool oxide A, alpha-terpineol), three ketones (pulegone, ionone, damascenone), three phenols (eugenol, 4-ethylphenol, 4-ethylguaiacol) and four acids (isovaleric acid, hexanoic acid, octanoic acid, decanoic acid) were analyzed. The results of the analysis of volatile compounds by mass chromatography are summarized in Table 20. Results are semi-quantitative: compounds were identified and the areas corresponding to each compound were integrated and compared with the area of internal standard (expressed as area x 10/area internal standard).

Although it is not possible to say what the total content of each compound is, these data provide valuable information about their modification during fermentation. The first comparison that can be made is the difference between the raw material (RW) and the obtained product of fermentation (T2). The second is the modification during fermentation, given by the comparison between T1 and T2; and eventually with a calculated T0 given by the combination between the results for vinegar (T2) and raw material in the proportion in which they were mixed.

Table 20: Concentration of volatile compounds by mass chromatography in area x 10/area internal standard (mean ± standard deviation)

Compounds	Wine B			Wine C		
	RM	T1	T2	RM	T1	T2
ESTERS						
Ethyl hexanoate	25.9^{b}	1.42±0.16^{a}	nd^{a}	23.9^{b}	0.564±0.108^{a}	nd^{a}
Ethyl octanoate	32.3^{b}	nd^{a}	nd^{a}	56.5^{b}	nd^{a}	nd^{a}
Hexyl acetate	0.730^{a}	2.22±0.19^{b}	0.782±0.099^{a}	3.01^{b}	1.22±0.21^{a}	0.700±0.274^{a}
Ethyl 2-phenylacetate	17.4^{a}	279±28^{b}	587±126^{c}	nd^{a}	17.3±2.4^{c}	12.9±0.8^{b}
2-Phenylethyl acetate	5.67^{a}	103±3^{b}	104±10^{b}	9.23^{a}	436±26^{b}	861±173^{c}
Benzyl acetate	nd^{a}	1717±26^{b}	2142±16^{c}	nd^{a}	1.25±0.07^{a}	4.36±1.41^{b}
Diethyl succinate	1193^{b}	312±45^{b}	304±2^{b}	279^{a}	905±57^{b}	1102±94^{c}
Diethyl malate	72.2^{a}	75.5±62.6^{a}	14.8±4.3^{b}	6.00^{a}	68.0±4.8^{b}	64.2±3.4^{b}
ALDEHYDE						
2,4-Dimethylbenzaldehyde	70.9	61.1±32.8	59.9±18.8	48.3^{a}	323±16^{c}	283±2^{b}
ALCOHOLS						
2-Phenylethanol	479^{a}	510±22^{b}	614±46^{b}	468^{a}	472±1^{a}	542±44^{b}
Benzyl alcohol	nd^{a}	2.85±0.20^{b}	3.09±0.23^{b}	3.73^{a}	3.85±1.93^{a}	5.22±1.84^{b}
(*E*)-Linalool oxide B	0.136^{a}	0.337±0.004^{a}	0.312±0.238^{a}	0.082^{a}	0.208±0.015^{b}	0.308±0.018^{c}
(*E*)-Linalool oxide A	0.119^{a}	0.228±0.014^{b}	0.373±0.014^{c}	0.069^{a}	0.328±0.090^{b}	0.274±0.010^{b}
α– Terpineol	0.534^{b}	nd^{a}	nd^{a}	0.140^{a}	nd^{b}	nd^{b}
KETONES						
Pulegone	18.9^{b}	18.7±0.4^{ab}	13.3±3.7^{a}	nd^{a}	14.9±5.3^{b}	9.51±2.43^{b}
Unidentified Ionone	2.45^{a}	2.20±0.08^{a}	3.39±0.18^{a}	0.732^{a}	2.15±0.03^{ab}	3.43±0.29^{b}
β-Damascenone	0.459^{c}	0.180±0.019^{b}	0.115±0.007^{a}	0.316^{b}	0.060±0.005^{a}	0.075±0.020^{a}
PHENOLS						
Eugenol	nd^{a}	21.6±5.0^{b}	26.1±2.7^{b}	nd^{a}	6.45±2.26^{b}	6.16±0.13^{b}
4-Ethylphenol	nd^{a}	1.53±2.47^{b}	nd^{a}	nd^{a}	1.63±1.63a^{b}	3.07±0.97^{b}
4-Ethylguaiacol	4.00^{a}	39.6±0.8^{b}	37.0±7.2^{b}	nd^{a}	4.04±0.30^{b}	4.76±0.40^{b}
ACIDS						
Isovaleric acid	nd^{a}	4.47±0.27^{b}	6.70±0.02^{c}	nd^{a}	6.59±0.57^{b}	8.68±1.64^{b}
Hexanoic acid	4.86^{a}	16.2±1.0^{b}	22.8±0.8^{c}	5.77^{a}	16.0±0.1^{b}	19.8±0.7^{c}
Octanoic acid	nd^{a}	57.7±0.8^{b}	68.9±7.6^{c}	nd^{a}	39.0±5.4^{b}	48.1±4.2^{b}
Decanoic acid	nd^{a}	4.03±0.14^{c}	2.92±0.06^{b}	nd^{a}	2.72±0.64^{b}	2.15±0.58^{b}

RM: Raw material, nd: not detected. For each wine, values not sharing the same superscript letter (a, b) within the horizontal line are different according to the Tukey test.

Comparing raw material (RM) and "vinegar" (T2) it is seen similar behavior with both substrates for most compounds. Esters have important influence on vinegar aroma (Callejon et al. 2008). In this case, two of them decreased (ethyl hexanoate, ethyl octanoate) and three increased (ethyl 2-phenylacetate, 2-phenylethyl acetate and benzyl acetate), being the differences statistically significant. The formation of acetic esters from

the reaction of alcohols with acetic acid is a well-known phenomena (Morales et al. 2002). This statement was verified in the case on benzyl acetate, but not for hexyl acetate. In red wine vinegar production, ethyl esters are mostly hydrolyzed throughout acetification, although exceptions exists (Callejon et al. 2009). Ethyl 2-phenylacetate behaviour during Wine B acetification was one of those exceptions. According to Callejon et al. (2009), diethyl succinate increases in submerged acetifications but decreases in surface acetification, suggesting that the hydrolysis or synthesis of ethyl esters may depend on the length of acetification or on the acetic acid bacteria strains involved (Callejon et al. 2008). In the present research, diethyl succinate and diethyl malate decreased in the case of wine B and increase with C; while hexyl acetate remained constant for wine B and decreased for wine C. The fermentation technology and inoculums were the same in both fermentations. This variation might result from the differences in the composition of raw materials: enrichment in a given compound may shift the equilibrium of a chemical reaction to the reactants or products.

The aldehyde, 2,4-dimethylbenzaldehyde, showed also differences with both substrates: it decreased slightly in the case of wine B (from 70.9 to 59.9 in area x 10/area internal standard) and markedly increased in wine C fermentation (from 48.3 to 283 in area x 10/area internal standard) being the difference statistically significant.

Four out of five alcohol compounds (phenylethanol, benzyl alcohol, (E)-linalool oxide B and (E)-linalool oxide A) increased significantly during fermentation, while alpha-terpineol significantly decreased. However, the concentrations of (E)-linalool oxide B, (E)-linalool oxide A and alpha-terpineol were low (inferior to 0.4 in area x 10/area internal standard) both in RM and T2. It was postulated that some alcohols are involved in the metabolism of acetic bacteria (2- phenylethanol, within them) because they exhibited random changes in concentration at the end of the fermentation cycle (Baena-Ruano et al. 2010).

In the group of ketones, pulgeone concentration decreased significantly and the unidentified Ionone increased significantly, while β-damascenone concentration decreased in wine B fermentation and increased with wine C.

Phenols and acids concentrations increased significantly during fermentation with both substrates. The only exception was 4-ethylphenol concentration that apparently remained constant. According to Callejon et al. (2009) the increase of acids, such as hexanoic and octanoic acids, results from the metaboism of alcohols by bacteria.

The composition of vinegar depends on raw material and the way alcoholic and acetic fermentation are conducted (Baena-Ruano et al. 2010). The reduction of some compounds during fermentation may be the result of metabolism of acetic acid bacteria. Although acetic acid bacteria uses sugars, alcohols and organic acids as carbon source (Mamlouk and Gullo 2013), the large diversity in the group can provide some strains enzymatic systems suitable for esters and/or other compounds use. Also, it is possible to have losses by volatilization. Submerged acetification have lower increase in volatile compounds than the surface process (Callejon et al. 2009). The increase of other compounds may result from synthesis as by products of fermentation.

When analysing volatile compounds concentrations during booth fermentations, important differences can be observed. Two esters (ethyl hexanoate and ethyl octanoate), one alcohol (alpha-terpineol), two ketones (pulegone and the unidentified ionone) and two phenols (eugenol and 4-ethylguaiacol) concentrations showed no significant differences between T1 and T2 with both substrates; but significant differences between raw materials were detected (phenols and the unidentified ionone increase while the others decrease). This may indicate a synthesis or loss during the first hours of fermentation. Other compounds exhibited a statistically significant constant increase: two esters (ethyl 2-phenylacetate and benzyl acetate) and one alcohol (hexanoic acid). These compounds may be synthesized during the entire fermentative process.

Other compounds exhibit statistical differences with only one of the wines. In fermentation with wine B, diethyl malate, β damascenone and 4-ethylphenol concentrations decreased along fermentation, while (E)-linalool oxide A, isovaleric acid, octanoic acid and decanoic acid concentrations increased. The same compounds in fermentation with wine C remained relatively constant when T1 and T2 results are observed. In wine C fermentation, 2-phenylacetate, phenylethanol, benzyl alcohol and (E)-

linalool oxide B concentrations had a significant increase. In the same period, 2,4-dimethylbenzaldehyde showed a higher concentration in T1, decreasing in T2. This may result from the opposing effect of synthesis and evaporation losses. In fermentation with wine B there are no significant differences between T1 and T2 for these compounds. These differences can be related to initial differences in raw material composition including volatile profile.

It is possible to analyze data in a more global way by summarizing volatile compounds by class (esters, aldehydes, alcohols, ketones, phenols and acids). Bar graphs were plotted to compare the relative concentrations of each class of compounds in different stages of fermentation (Figure 22 A and B)

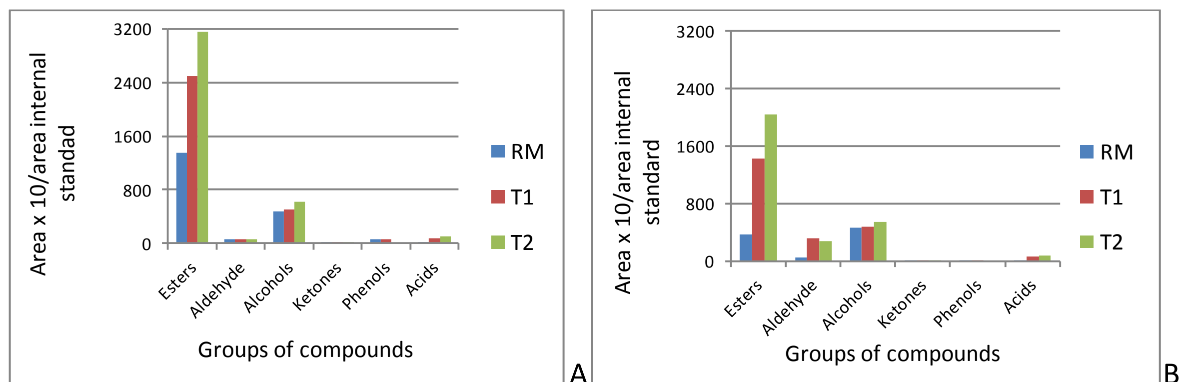


Figure 22: Comparison of the relative concentrations by group of chemical compounds in different stages of fermentation for wine B (A) and wine C (B)

In both cases, esters are in the most conspicuous class of compounds. In T2, esters are around 2.3x higher than in wine B; in the case of wine C, that value rises to 5.4x higher. Higher increments were noticed in volatile acids. Their concentrations in T2 were 21x and 14x higher than in raw material, for wine B and C, respectively. Alcohols exhibited smaller increases: 1.3x in wine B fermentation and 1.2x in wine C fermentation. Although it may be synthesis of alcohols, most of this is the result of concentration by evaporation of solvent. During the fermentations, aldehydes and ketones decreased respectively to 0.84x and 0.77x the wine B concentration but increased respectively 5.9x and 12x the wine C concentration. Wine B is richer in both classes of compounds, and for that reason a higher

amount can be lost by volatilization and/or the chemical reaction of synthesis may be shifted towards the reactants. Phenols disappear during fermentation with both wines.

In all acetification, total volatile compounds are increased (Callejón 2009). In the case of "Porto wine vinegar", total amount of volatile compounds increases 2x and 3x for wine vinegars B and C, respectively. However, Callejon et al. (2009) obtained only a 1.06x increment in vinegar produced by submerged acetification. These differences may result from different analysis of volatile compounds and from the different bacterial strain metabolism (Gluconacetobacter).

Few studies have studied the volatile composition changes during biological acetification. Additionaly, by the fact of not having quantitative data in this study, it is difficult to compare results with what was found for other vinegar making processes. However, relative differences between raw materials and vinegars can be compared. In an acetification process of red wine (Budak 2010), superficial and submerged fermentation technologies were compared. Six acids were measured (gallic, caffeic, chlorogenic, syringic, p-coumaric and ferulic). Mostly, there were no significant differences between the acids contents in raw material and in the resulting vinegars. There was only one exception: chlorogenic acid increased when vinegar was made by submerged method. Other acids were studied during "Porto wine vinegar" production (isovaleric acid, hexanoic acid, octanoic acid, decanoic acid) and they all increased. An increase of acids (isovaleric, hexanoic and octanoic) concentrations between the initiation and the end of red wine vinegar production during submerged fermentation was also reported (Callejón 2009). In the same study it was visible a non-significant decreasing in alcohols and esters. Phenols remain in similar values through acetification in both studies. The differences shown may result from the use of different substrates, but also from different enzymatic pools of the bacteria used as inoculums.

An important conclusion may be extracted from this analysis. Although high aeration rate, high fraction of volatile compounds remain in the product.

3.3 *Section C. To characterize the inoculums used for acetic fermentation of Porto wine*

Three cycles in the period of working regime were analyzed: cycles 17, 18 and 19, corresponding to wine B. As fermentation has very similar parameters with both wines, there is no need of analyzing both them.

3.3.1 Enumeration of AAB

It was expected to observe variations on the total and/or cultivable cells counts over the different stages of fermentation, once at the beginning part the final product was put aside and the reactor was recharged with fresh wine. However, there was not a remarkable difference in the cell counts during the different stages of fermentation (Table 21). In all cases, the number of cultivable cells was lower that the total cells counts. The number of cultivable cells ranged from 3.78x106 UFC/mL and 4.1x107 UFC/mL, while total cells varied between 4.18x107 UFC/mL and 1.38x108 UFC/mL. In general, the cultivable population constituted 8.33% of the total cells. Since the DAPI method does not distinguish viable from non-viable cells, it is not possible to confirm if some of the total cells were in a viable but not cultivable state. The value at the beginning of the second cycle in plate counting is provably an outlier, although it is consistent with the results by triplicate.

Table 21: Enumeration of total cells and YPGD-cultivable AAB over the three stages of fermentation in the three acetification cycles

Sample	pH	Culturable cells (UFC/mL)	Total cells (cels/mL)
Cycle 17 – initiation	2.86	$6.53x10^{6} \pm 8.39x10^{5}$	$8.65x10^{7} \pm 1.6x10^{6}$
Cycle 17 – middle	2.76	$4.93x10^{6} \pm 1.59x10^{6}$	$8.27x10^{7} \pm 1.77x10^{6}$
Cycle 17 – end	2.65	$1.43x10^{7} \pm 3.53x10^{6}$	$1.38x10^{8} \pm 9.63x10^{5}$
Cycle 18 – initiation	2.86	$4.10x10^{7} \pm 5.59x10^{6}$	$7.06x10^{7} \pm 9.63x10^{5}$
Cycle 18 – middle	2.77	$3.87x10^{6} \pm 7.07x10^{3}$	$6.57x10^{7} \pm 2.73x10^{6}$
Cycle 18 – end	2.69	$1.17x10^{7} \pm 1.27x10^{6}$	$6.38x10^{7} \pm 2.25x10^{6}$
Cycle 19 – initiation	2.87	$4.83x10^{6} \pm 6.51x10^{5}$	$4.18x10^{7} \pm 2.24x10^{6}$
Cycle 19 – middle	2.75	$5.35x10^{6} \pm 1.48x10^{6}$	$1.27x10^{8} \pm 5.14x10^{6}$
Cycle 19 – end	2.66	$7.50x10^{6} \pm 2.83x10^{5}$	$9.19x10^{7} \pm 9.63x10^{6}$

The percentage of culturable cells, in comparison with total cells, was calculated and summarized in Table 22. The proportion is around 9 ± 4.48 %, not showing great variation in the different stages of fermentation. It can be inferred that, if there is a compound affecting the culturavility of cells, it affects all the stages of fermentation in a similar way. This difference cannot be attributed to ethanol since this compound varies widely during fermentation.

Table 22: Percentage of cells that are culturable in the different cycles of fermentation

	% of culturable cells
initiation	9.6 ± 2.8
middle	5.3 ± 1.0
end	12.3 ± 5.4
total	9.0 ± 4.5

Although the percentage of culturable calls does not vary widely, it is in average inferior to 10% of total cells and, for that reason, further analysis were made only with the results of total cells counting. It was verified the relationship between the quantity of microorganisms and the stage of fermentation (hence, pH, concentration of acetic acid and ethanol) by graphing the results in function of time (Figure 23) and in correlation to pH (Figure 24). The beginning of any cycle is supposed to have around the half of cells at the end of the previous cycle, and for this reason the quantity of cells should be related to the fermentation cycle. As bioreactor was in working regime, both the quantity of cells and the variations should be repeatable. Nevertheless, there is no evidence of any tendency; it cannot be confirmed that any of the parameters in study affects the proliferation of bacteria. The variation may be the result.

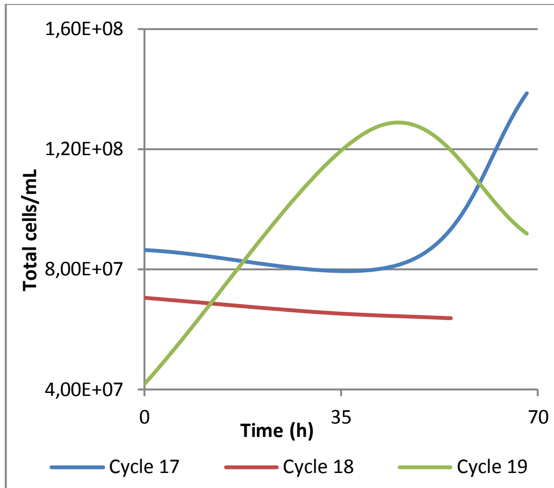


Figure23: Evolution of total cells in time in the three cycles in study

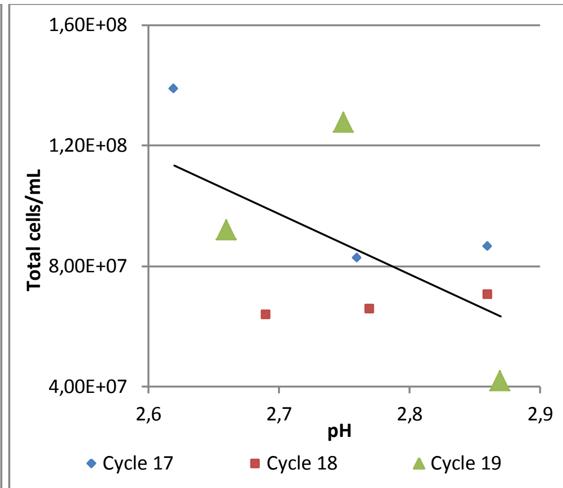


Figure 24: Relationship between total cells and PH

3.3.2 Isolation and identification of AAB

Two isolates, with different colony morphology, were recovered from the YTGD agar plates, and herein, named A and B. In this medium, isolate A formed cream colored colonies, with 2 mm as maximum diameter, slightly translucent and needed 5-6 days to grow at 30ºC. Under the same conditions, colonies of isolate B were bigger (1.5-4 mm Ø), translucent, iridescent, and needed 6-7 days to grow. These differences are shown in Figure 25. Microscopic observation revealed that both isolates have the typic characteristics of AAB: Gram-negative, polymorphous, ellipsoidal to rod-shaped, straight or slightly curved, occurring single, in pairs, or in chains (Sellmer-Wilsberg 2009). Therefore, it was not possible to distinguish both isolates based on their cellular morphology (Figure 26).

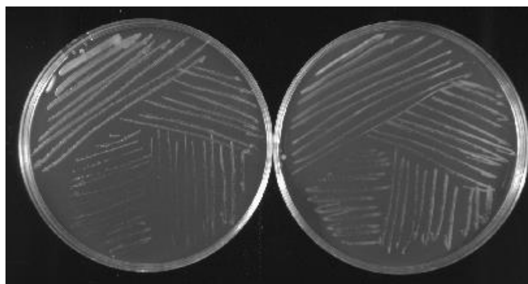


Figure 25: Isolates A (left) and B (right) in YTGD agar with six days of growth.

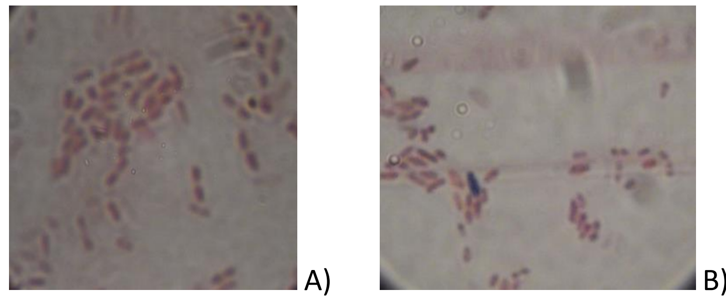


Figure 26: Gram-stained smear from a seven-day-old culture of A) isolate A and B) isolate B on YPGD agar medium at 30°C. Bright field (1000 x) with zoom 10X in photo camera.

The comparison of the 16S rRNA gene sequence of each isolate with the public databases (ezbiocloud.net) showed that they both share 100% similarity with Acetobacter pasteurianus subsp. paradoxus LMG1591 (T). The etymology of the subspecie name “paradoxus” means strange, contrary to all expectation, paradoxical. Contrary to most AAB, they are catalase (-) and unable of growing in GYC agar (Gossele et al. 1983). However, both isolates were catalase (+) and isolate A grows in GYC agar, but isolate B does not. Figure 27 shows a culture of isolate A on GYC agar medium.

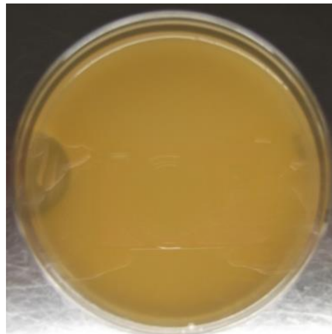


Figure 27: Culture of isolate A on GYC agar. The light zone on the left of the petri plate is caused by solubilization of CO3Ca due to pH drop, caused by the acid produced by bacteria.

Facing these contradictory results, a RAPD-PCR with primer M13 was carried out to compare the two isolates. Results did not allow a clear distinction between isolates. The RAPD-profile is shown in Figure 28. These facts indicate the possibility of novel subspecies. According to Solieri and Giudici (2009), the taxonomy of acetic acid bacteria is nowadays under extensive and continuous revision. As actual taxonomic studies are based on a

polyphasic approach (including morphological, physiological and biochemical aspects, metabolism, ecology, genome characterization and phylogeny) further studies must be developed. In the case of AAB, it has been reported the loss of specific physiological activity as a consequence of spontaneous mutations (Kondo and Horinouchi, 1997). For this reason some phenotypic characters cannot be used for taxonomic purposes (Gullo and Giudici 2009).

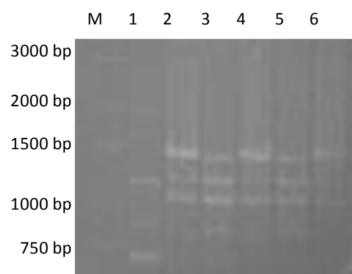


Figure 28: Result of RAPD-PCR. M: marker, line 1: positive control, lines 2, 4 and 6: isolate A, lines 3 and 5 isolated B

3.3.3 PCR-DGGE analysis

In order to assess if vinegar contained more organisms than the two cultivable isolates (A and B), a DGGE analysis was carried out.

From the resulting gels (Figure 29) it is clear that there are no differences between the total genomic DNA and the cultivable bacteria DGGE-profiles, suggesting that vinegar did not contain any other bacteria but those recovered in YTGD medium, irrespective of the fermentation cycle. However, it cannot be excluded the presence of additional uncultivable microorganisms; the primers used may have not been adequate to amplify the gene fragment. In addition, DGGE fails to detect organisms with low abundance (Muyzer et al. 1993)

The DGGE-profiles of the two cultivable isolates were identical. Such results support the conclusions recovered with the RAPD-PCR and the 16S rRNA gene fragment sequence analyses. Such results suggest that the acetic fermentation was carried out by a single AAB species. However, given the fact that the two isolates showed different phenotypic characteristics, further studies are necessary to confirm their taxonomic affiliation.

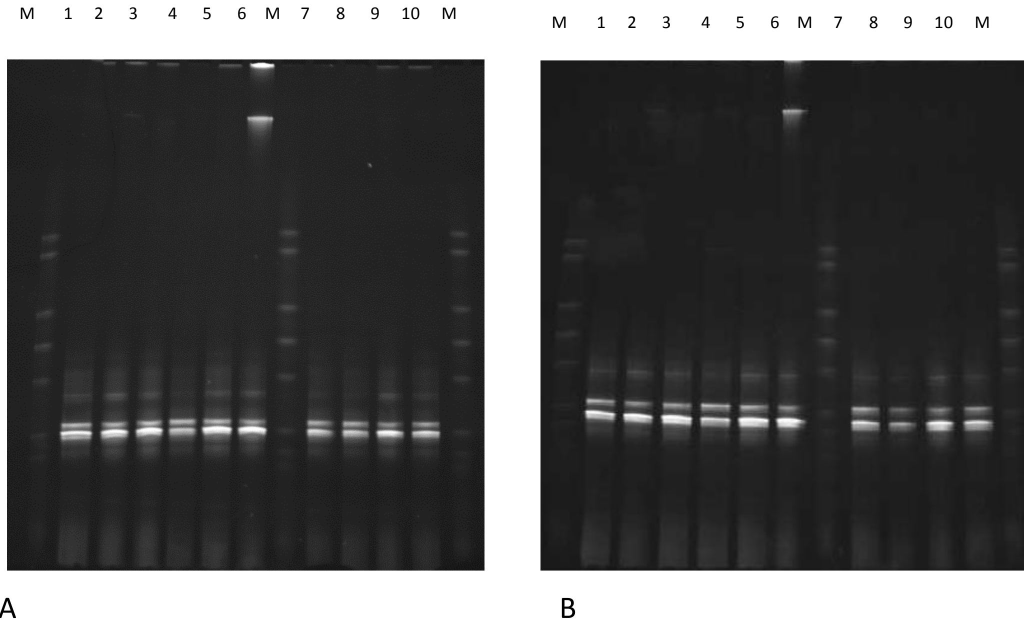


Figure 29: Gels resulting from PCR-DGGE analysis. In both cases, M: marker, line 1: total genomic DNA 1st cycle, Line 2: genomic DNA of culturable bacteria in 1st cycle, line 3: total genomic DNA 2nd cycle, line 4: genomic DNA of culturable bacteria in 2nd cycle, line 5: total genomic DNA 3rd cycle, line 6: genomic DNA of culturable bacteria in 3rd cycle, line 7: genomic DNA isolate A, line 8: genomic DNA isolate B, line 9: genomic DNA isolate A (duplicate), line 10: genomic DNA isolate B (duplicate).

The results obtained in this study are consistent with the conclusions reported by Fernández-Pérez et al. (2010b): single strain “seed vinegar” are found in the vinegars that offer the most stressful conditions.

Chapter 4.

Conclusions

Vinegar has been produced in many places by different ways since ancient times. It is such an easy to do product that very little research about vinegars exists before 1990. To produce commercial mainstream products, established technology is used in high scales making the product relatively cheap, restraining resource allocation to research in product or processing technology improvements. On the other hand, low scale, high quality traditional products are usually expensive. These products and processes are usually widely described to certificate authenticity, but not to modify them or to develop new kinds of vinegar.

Shortly, vinegar production is analyzed and optimized only if vinegar commercial value justifies. Research aiming to create new kinds of vinegars is poorly described. For these reasons, developing this work aiming to produce vinegar from Porto wine has been quite challenging.

Section A.

Two seed vinegars were evaluated: one coming from wine vinegar and other from cider vinegar. Although both were able to produce vinegar from Douro red wine, they showed different characteristics: cider wine vinegar was more active and wine seed vinegar oxidized acetic acid to water and carbon dioxide. No consistent cycles of fermentation were established, but with wine seed vinegar fermentation cycles were more regular. Therefore, and because it was collected in Douro region, wine vinegar seed was selected for Porto wine acetic fermentation.

The abiotic assay showed that no acidification occurs by non-fermentative paths and acid producing bacteria were responsible for pH decrease in the reactor inoculated with “seed vinegar”. Bacteria have successfully adapted to Porto wine during 6 fermentation cycles (32 days) whereas Porto wine fraction in the bioreactor increased from 0% of 50%. Almost complete replacement of Douro wine with Porto wine was performed in 7 cycles and 102 days, until reaching the 98 % of Porto wine. Working regime was achieved with two Porto

wines B and wine C, with similar characteristics but different pH. In the tested conditions (temperature 30 ºC, aeration 2 L/min, stirring 4000 rpm, in 1 L bioreactor filled with 600 mL, with approximately 10^7 bacteria/mL), one cycle of fermentation takes around 3 days with an average rate of acidification of 0.08 pH units per day. Initial and final pH depends on the pH of the wine used as raw material, being the final pH around 0.83x the pH of the raw material and 0.92x the initial fermentation pH. Initial and final pH values were 2.87 and 2.66 respectively for vinegar B, and 3.12 and 2.86 for vinegar C.

Section B.

During fermentation with wine B, glucose increases from 52 g/L to 60 g/L, fructose increases from 75 g/L to 86 g/L, acetic acid increases from 13 to 28 g/L and ethanol decreases from 107 to 39 g/L in wine B. In the case of fermentation with wine C, glucose increases from 53 g/L to 55 g/L, fructose increases from 71 g/L to 75 g/L, acetic acid increases from 18 to 37 g/L and ethanol decreases from 99 to 18 g/L in wine B.

Glucose and fructose are neither consumed nor degraded appreciably during fermentation. Bacteria selectively oxidize ethanol and even acetic acid before sugars. The changes in sugar concentrations trough the fermentation cylces are due to the solvent volume loss by volatilization. These concentration changes allow calculating volume losses in the bioreactor during fermentation between 4% and 18% of initial volume. Changes in the sugars concentrations may affect the flavour of vinegar. Moreover, to accurately control ~~of~~ acetification; total sugar or individual sugars concentration should be measured ideally using a rapid and simple method.

Changes in ethanol and acetic acid concentrations revealed that fermentation cycles are reproductive with both raw materials. Ethanol and acetic acid concentrations were strongly correlated with pH. The final concentration of ethanol for both vinegars was approximately ten times higher than in different commercial vinegars. And the concentration of acetic acid is around twice higher in other vinegars in comparison with Porto wine vinegar. The concentration of acetic acid in the obtained product is too low

while ethanol is excessively high, according to the legislation of the European Union. Low oxygen transfer rates in the air-liquid interface may be impairing further ethanol oxidation and a more efficient air dispersion system may be required. The concentration of both sugars is also higher than legal limit. For sugars, an exception in vinegar legislation is needed, because alternatives to remove sugars in the final product would also eliminate intrinsic characteristics of this singular product. Alternatively, this product may be commercialized under other designation like "Porto wine seasoning sauce".

Fermentation can be successfully monitored using a pH sensor, however control parameters need previous optimization when changes in raw materials occur.

Comparing the volatile profiles of raw materials and final products, a general enrichment of volatiles during acetification was observed: total amount of volatile compounds increases 2x and 3x for wine vinegars B and C, respectively. Comparing the compounds by group, it was observed that esters were the most conspicuous class of compounds. All volatile groups increased or remained constant when comparing the raw materials and the products. This clear enrichment is probably related to synthesis during bacterial metabolism. Although high aeration rate, a high fraction of volatile compounds remain in the product.

Section C.

This work allowed to determine that the number of acetic acid bacteria in vinegar fermentation from Porto wine is around 10^7 total cells/mL. The cultivable cells constituted 9% of the total cells. Only two morphotypes were distinguishable among the cultivable cells. Further studies are necessary to confirm the affiliation of the two cultivable isolates to *Acetobacter pasteurianus subsp paradoxus*. Based on DGGE-profiling, these two isolates seem to be the only microorganisms present in wine vinegar fermentation trough successive acetification cycles.

Summarizing, the production of Porto wine vinegar is possible and the inocculum obtained is adequate. The improvement of the product would be achieved by adding technology to production. Furthermore, legislation adjustments need to be made to facilitate the production and commercialization of this unique product as “vinegar”. Instead, this product may be commercialized under other designation like “Porto wine seasoning sauce“.

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