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Evaluation of the resistance of *Acetobacter aceti* to the major chemicals found in winery wastewater

(versão final após defesa)

Nuno Ramos

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Orientadora: Doutora Annabel Dias Barrocas Fernandes
Co-orientadora: Doutora Maria da Conceição Fernandes

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“Quando corre mal. Levanta-te. Recompõe-te. Volta a tentar”.

Resumo

A indústria vinícola gera entre 0,2 e 4 L de águas residuais por litro de vinho produzido, embora esse valor possa ultrapassar os 14 L, dependendo de diversos fatores. O efluente vinícola apresenta, geralmente, elevada salinidade e carga orgânica, contendo uma variedade de compostos orgânicos, como ácidos carboxílicos, açúcares, álcoois e compostos fenólicos. Esses compostos podem inibir o crescimento microbiano, comprometendo os processos de tratamento biológico. Encontrando-se naturalmente presente em todas as fases da vinificação, a *Acetobacter aceti* possui a capacidade de oxidar o etanol a ácido acético e utilizar o acetato produzido no seu metabolismo. O objetivo do presente estudo foi avaliar a tolerância da *A. aceti* a duas concentrações (LC e HC) de misturas de: álcoois-A (etanol, glicerol e metanol), ácidos carboxílicos-CA (acético, sucínico e tartárico) e compostos fenólicos-Ph (tirosol e catecol) presentes num meio modelo, e investigar a possibilidade da sua utilização na biodegradação deste efluente através do crescimento da bactéria, da degradação dos diferentes compostos e da avaliação da carência química de oxigénio (CQO) e carbono orgânico total (COT). Na presença de álcoois, a taxa de crescimento (μ) e rendimento da biomassa ($Y\ ^{x}/_{M}$) da *A. aceti* reduziu para 80% e 77% e para 73% e 88%, em relação ao controlo, para LC e HC, respetivamente. A taxa de consumo do manitol (Q_M) reduziu 59% e 44% em relação ao controlo para LC e HC, respetivamente. A CQO e COT não apresentaram diferenças. Na presença de CA, a μ e $Y\ ^{x}/_{M}$ da *A. aceti* reduziu para 61% e 43% e para 84% e 13% em relação ao controlo para LC e HC, respetivamente. A Q_M reduziu 98% e 93% em relação ao controlo para LC e HC, respetivamente. O CQO e COT não apresentaram diferenças. A *A. aceti* não conseguiu metabolizar o ácido tartárico. A presença de Ph não afetou o crescimento, a Q_M, a CQO ou COT. Os ensaios com o efluente vinícola mostraram que o crescimento da *A. aceti* sofreu uma grande redução, necessitando muito mais tempo para se adaptar e consumir os ácidos orgânicos, com exceção do tartárico. A remoção de CQO e COT foi de 62 e 70%, para as condições sem e com suplementação, respetivamente.

Palavras-chave

Acetobacter aceti;Biodegradação;Crescimento microbiano;Efluente vinícola

Resumo alargado

A indústria vinícola desempenha um papel importante na sociedade, quer a nível económico, quer cultural. Em termos económicos, é uma fonte significativa de emprego em toda a cadeia de produção, desde o cultivo das uvas, até à comercialização do vinho, para além de ser também uma grande impulsionadora do turismo em várias regiões. A nível cultural, o vinho está presente em variadas tradições e identidades nacionais, especialmente em países europeus como a França, a Itália, Portugal e Espanha, sendo uma componente essencial da sua gastronomia.

O efluente vinícola é um resíduo líquido resultante da indústria vinícola, sendo formado durante a maioria dos processos da produção de vinho. Este efluente apresenta normalmente uma elevada carga orgânica, que inclui açúcares, ácidos carboxílicos, álcoois e compostos fenólicos. O seu tratamento inadequado ou a sua descarga direta no meio ambiente pode causar sérios impactos ambientais, entre os quais a contaminação dos recursos hídricos, com consequente desequilíbrio ecológico. Embora o efluente vinícola seja um grande desafio ambiental, é também uma fonte de produtos de valor acrescentado, devido à sua rica composição em compostos orgânicos que podem ser recuperados e aproveitados, promovendo assim a sustentabilidade do setor e a economia circular. Neste contexto, surge a célula de combustível microbiana (MFC), uma tecnologia inovadora que pode ser aplicada no tratamento de efluentes, apresentando o duplo benefício da depuração do efluente e da geração de eletricidade a partir da biodegradação da matéria orgânica. No entanto, o desempenho deste processo, tanto no tratamento do efluente como na produção de eletricidade, é determinado pelo tipo de microrganismos utilizados, sendo que a seleção adequada do inóculo desempenha um papel fundamental na melhoria do desempenho da MFC.

A bactéria *Acetobacter aceti* (*A. aceti*) é um microrganismo que possui a capacidade de oxidar o etanol a ácido acético e utilizar o acetato produzido no seu metabolismo, encontrando-se naturalmente presente em todas as fases da vinificação. Esta presença natural no vinho, combinada com as suas capacidades metabólicas, faz da *A. aceti* uma bactéria muito promissora para o tratamento de efluente vinícola e utilização numa MFC. Assim, o presente estudo teve como objetivo avaliar a tolerância da bactéria *A. aceti* aos compostos presentes no efluente vinícola (alguns dos quais identificados como inibidores do crescimento microbiano, como os fenóis e alguns álcoois) e a viabilidade de utilizá-la para a biodegradação deste efluente.

Numa primeira etapa do trabalho, foi avaliada a ação inibitória no crescimento e metabolismo da *A. aceti* de compostos maioritariamente presentes no efluente vinícola, nomeadamente, álcoois, ácidos carboxílicos e fenóis, e a influência da concentração destes compostos na ação inibidora. Estes ensaios foram realizados utilizando um meio de crescimento modelo, ao qual se adicionaram os diferentes compostos (álcoois, ácidos carboxílicos ou fenóis). Posteriormente, numa segunda etapa, a *A. aceti* foi diretamente exposta ao efluente vinícola real, para avaliar o efeito inibidor da matriz real e complexa. Os ensaios de fermentação foram monitorizados através de determinações de densidade ótica a 600 nm, pH, carência química de oxigénio (CQO), carbono

orgânico total (COT), e quantificação de compostos por cromatografia líquida de alta eficiência (HPLC).

Nos ensaios realizados com soluções modelo contendo os álcoois etanol, glicerol e metanol, foi observado que estes inibem ligeiramente o crescimento bacteriano, em ambas as concentrações estudadas, visto que a taxa de crescimento (μ) e rendimento da biomassa (Y $^{x}/_{M}$) da *A. aceti* reduziu para 80% e 77% e para 73% e 88% em relação ao controlo para a concentração mais baixa e mais alta, respetivamente. A taxa de consumo do manitol (Q_M) também reduziu 59% e 44% em relação ao controlo para a concentração mais baixa e mais alta, respetivamente. Ainda assim, a bactéria foi capaz de degradar a maioria dos compostos orgânicos presentes (remoções de CQO e COT de, aproximadamente, 70%). A conversão do etanol em ácido acético foi observada, bem como a posterior degradação deste produto do metabolismo. Nos ensaios em que a bactéria foi exposta a soluções modelo contendo os ácidos acético, tartárico, lático e succínico, verificou-se uma maior inibição nas primeiras horas de ensaio, principalmente na maior concentração estudada, visto que μ e Y $^{x}/_{M}$ da *A. aceti* reduziu para 61% e 43% e para 84% e 13% em relação ao controlo para a concentração mais baixa e mais alta, respetivamente. Porém, esta inibição foi atenuada com a continuação do ensaio, tendo sido atingidos valores de crescimento significativos. A Q_M também reduziu 98% e 93% em relação ao controlo para a concentração mais baixa e mais alta, respetivamente. Relativamente à remoção de carga orgânica, observou-se que, mesmo inibida, a bactéria conseguiu mineralizar a maioria dos compostos orgânicos presentes (remoções de CQO e COT de, aproximadamente, 70%), apesar de não ter conseguido degradar o ácido tartárico. Por fim, na presença dos compostos fenólicos catecol e tirosol, não foi observada inibição do crescimento bacteriano, tendo a *A. aceti* demonstrado ser capaz de degradar estes compostos. Em suma, em termos de inibição da bactéria, observou-se, nas condições experimentais estudadas, que os compostos que mais inibiram a bactéria foram os ácidos carboxílicos, seguidos dos álcoois e, por último, dos compostos fenólicos (sem inibição observada). A concentração dos compostos influenciou o efeito inibitório, sendo mais acentuado com os ácidos carboxílicos, embora a maior concentração de álcoois também tenha apresentado maior inibição em relação à menor concentração. Independentemente do efeito inibitório observado, a *A. aceti* teve facilidade em degradar quase todos os compostos a que foi exposta.

Os ensaios de fermentação com o efluente vinícola real ($CQO_0 \approx 200$ g L^{-1}) foram realizados em duas condições distintas, com e sem suplementação dos nutrientes essenciais (manitol, extrato de levedura e peptona). Em qualquer uma das situações, foi observada uma forte inibição do crescimento microbiano, tendo, no entanto, esta inibição sido atenuada nas últimas horas dos ensaios com suplementação. Nas condições em que não houve suplementação dos nutrientes essenciais, a bactéria foi incapaz de degradar o glicerol, o ácido lático e o ácido succínico, indicando que a presença destes nutrientes essenciais pode desempenhar um papel no mecanismo de degradação da *A. aceti*, especialmente quando a bactéria é exposta a condições extremas. Ao longo dos ensaios, em ambas condições estudadas, foi observada a formação de produtos metabólicos bacterianos, nomeadamente ácido acético, proveniente da degradação do etanol, e xilose. Ainda assim, a *A. aceti* foi capaz de os degradar também, transformando os seus produtos metabólicos em substratos. Após 2016 horas de ensaio, a CQO foi reduzida para valores

inferiores a 70 g L^{-1} nos ensaios sem suplementação e 40 g L^{-1} nos ensaios com suplementação, sendo a sua percentagem de remoção de 62% e 70%, respetivamente.

Concluiu-se assim que, apesar do metabolismo da bactéria *A. aceti* poder ser inibido pela presença de alguns dos constituintes do efluente vinícola (em maior ou menor grau, dependendo do tipo e da concentração do composto inibidor), para as condições experimentais estudadas, a *A. aceti* consegue degradar a maioria dos compostos orgânicos, apresentando um elevado potencial para ser utilizada no tratamento de efluente vinícola.

Abstract

The wine industry generates between 0.2 and 4 L of wastewater per liter of wine produced, although this figure can exceed 14 L, depending on several factors. Wine effluent typically has high salinity and organic load, containing various organic compounds such as carboxylic acids, sugars, alcohols, and phenolic compounds. These compounds can inhibit microbial growth, compromising biological treatment processes. Naturally present in all stages of winemaking, *Acetobacter aceti* has the ability to oxidize ethanol into acetic acid and use the acetate produced in its metabolism. The present study aimed to evaluate the tolerance of *A. aceti* to two concentrations (LC and HC) of mixtures of: A-alcohols (ethanol, glycerol, and methanol), CA-carboxylic acids (acetic, succinic, and tartaric) and phenolic-Ph compounds (tyrosol and catechol) present in basal media, and to investigate the possibility of its use in the biodegradation of this effluent, through bacterial growth evaluation, degradation of the different compounds and assessment of chemical oxygen demand (COD) and total organic carbon (TOC). In the presence of alcohols, the growth rate (μ) and biomass yield ($Y_{x/M}$) of *A. aceti* decreased to 80% and 77% and to 73% and 88%, compared to the control, for LC and HC, respectively. The mannitol consumption rate (Q_M) decreased by 59% and 44%, compared to the control, for LC and HC, respectively. COD and TOC did not show differences. In the presence of AC, μ and $Y_{x/M}$ of *A. aceti* decreased to 61% and 43% and to 84% and 13%, compared to the control, for LC and HC, respectively. Q_M was reduced by 98% and 93%, compared to the control, for CL and HC, respectively. COD and TOC did not show differences. *A. aceti*, was unable to metabolize the tartaric acid. The presence of Ph did not affect growth, Q_M, COD, or TOC. The tests with real winery wastewater showed that the growth of *A. aceti* suffered a great reduction, requiring much more time to adapt and consume organic acids, except for tartaric. COD and TOC removals were 62% and 70%, for conditions without and with supplementation, respectively.

Keywords

Acetobacter aceti;Biodegradation;Microbial growth;Winery wastewater

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List of Acronyms

A	Alcohols
A. aceti	*Acetobacter aceti*
ATLS	Acetic acid, tartaric acid, lactic acid, and succinic acid
CA	Carboxylic acids
COD	Chemical oxygen demand
COT	Carbono orgânico total
CQO	Carência química de oxigénio
E	Ethanol
EG	Ethanol and glycerol
EGM	Ethanol, glycerol, and methanol
HPLC	High-performance liquid chromatography
IC	Inorganic carbon
m	mass
M	Mannitol
MFC	Microbial fuel cell
OD	Optical density
OD600	Optical density at 600 nm
PEM	Proton exchange membrane
Ph	Phenols
rpm	Rotations per minute
TC	Total carbon
TOC	Total organic and inorganic carbon
TN	Total nitrogen
V	Volume
WW	Winery wastewater
YP	Yeast extract and peptone
YPM	Yeast extract, peptone, and mannitol

Chapter 1
Introduction

Under the scope of the project Wine4H2 – Sustainable wine industry: Green hydrogen production from winery wastewater (2022.02566.PTDC), funded by the Fundação para a Ciência e Tecnologia, the work developed during the master aimed to evaluate the feasibility of using the microorganism *Acetobacter aceti* (*A. aceti*) to degrade winery wastewater (WW) in a microbial fuel cell (MFC). This Introduction section presents a brief description of the main WW characteristics and the technologies applied for its treatment. Additionally, the fundamentals of MFC operation are described, as well as the features of *A. aceti* bacteria for WW biodegradation and simultaneous electricity production in MFC.

1.1. Winery wastewater

The wine industry is one of the most important agro-industrial activities worldwide, particularly in Mediterranean countries like Italy, France, Spain, and Portugal, acting as an economic, social, and cultural pillar, as well as an important driver of tourism [1]. Portugal is the fifth largest wine producer; in 2023, 7.3 million hectoliters were produced [2].

Aside from the unquestionable significance of the wine sector, like any agro-industrial activity, the wine industry has a significant environmental impact, mainly caused by the high consumption of freshwater and generation of solid wastes and wastewater, as approximately 70% of the freshwater consumed is discharged as wastewater [3]. Winery wastewater, resulting from a variety of processes, is a highly pollutant waste stream, with chemical oxygen demand (COD) and total solids concentrations that can reach up to 49 g L^{-1} and 18 g L^{-1}, respectively [1]. The number and type of chemical compounds that can be found in WW are wide, including sugars, alcohols, organic acids, esters, phenolic compounds, and minerals [4,5]. Table 1.1 summarizes the main WW physicochemical characteristics retrieved from the literature.

Grapes are a natural source of organic compounds and, during the winemaking process, several sugars, organic acids, and phenolic compounds are released into the WW, coming from the skin, flesh, and seeds of the grapes. Additionally, other contaminants appear throughout the wine production process, namely from washing water, cooling water, cleaning chemicals, and leaching of solid byproducts [6]. Although water is not an ingredient in wine production, generally, for every liter of wine produced, 10 liters of water are used [7]. The high WW organic load, as well as the toxicity and resistance to biodegradation of some of its constituents, unqualifies untreated WW for discharge into natural water resources. The uncontrolled release of WW leads to the eutrophication of water bodies, due to the fast consumption of dissolved oxygen, which causes the

lack of oxygen for aquatic and amphibious life, causing significant environmental damage [2]. Therefore, WW must be treated before discharge or disposed of by some alternative method [8].

Table 1.1: Physicochemical composition of winery wastewater.

Parameter	Unit	Minimum value	Maximum value	Reference
Chemical oxygen demand	mg L^{-1}	150	296119	[1, 2, 5, 6]
Biochemical oxygen demand	mg L^{-1}	120	130000	[1, 2, 5, 6]
Total organic carbon	mg L^{-1}	41	7363	[1, 5]
pH	–	2.5	12.9	[1, 2, 5, 6]
Electrical conductivity	mS cm^{-1}	0.8	5.6	[1, 5]
Total solids	mg L^{-1}	0	82000	[1, 5, 6]
Volatile solids	mg L^{-1}	0	69000	[1, 5, 6]
Suspended solids	mg L^{-1}	0	30300	[1, 5, 6]
Total phosphorous	mg L^{-1}	0.29	280	[1, 6]
Total nitrogen	mg L^{-1}	1.4	415	[1, 6]
Total Kjeldahl nitrogen	mg L^{-1}	3.2	374	[6]
Ammonia-N	mg L^{-1}	0.22	24	[6]
Sodium	mg L^{-1}	0.2	396	[6]
Potassium	mg L^{-1}	20.5	1445	[6]
Total sugars	mg L^{-1}	0	13000	[5]
Maltose	mg L^{-1}	0	13200	[5]
Glucose	mg L^{-1}	0	2700	[2, 5]
Fructose	mg L^{-1}	0	1600	[2, 5]
n-Valeric acid	mg L^{-1}	0	8	[5]
Hexanoic acid	mg L^{-1}	0	5	[5]
Octanoic acid	mg L^{-1}	0	2	[5]
Decanoic acid	mg L^{-1}	1	7	[5]
Citric acid	mg L^{-1}	0	2	[5]
Tartaric acid	mg L^{-1}	0	690	[2, 5]
Malic acid	mg L^{-1}	0	70	[5]
Lactic acid	mg L^{-1}	0	350	[2, 5]
Succinic acid	mg L^{-1}	40	80	[5]
Acetic acid	mg L^{-1}	0	663	[2, 5]
Propionic acid	mg L^{-1}	0	67	[5]
i-Butyric acid	mg L^{-1}	0	2	[5]
n-Butyric acid	mg L^{-1}	0	67	[5]
Ethanol	mg L^{-1}	400	10200	[2, 5]
Methanol	mg L^{-1}	0	15	[5]
Glycerol	mg L^{-1}	140	390	[2, 5]
Total phenolic compounds	mg L^{-1}	0	1450	[1, 2, 5, 6]
2-Phenylethanol	mg L^{-1}	0	5	[5]
Ethyl acetate	mg L^{-1}	2	43	[5]

Winery wastewater treatment technologies are typically divided into physicochemical, biological, advanced oxidation, and membrane processes. Table 1.2 summarizes the main treatment processes applied to WW and the respective average COD removal attained, according to the literature [9]. The most widely used process for treating WW is activated sludge, due to its high efficiency and versatility [4]. Nevertheless, activated sludge treatment has some disadvantages, like the large implantation areas required, the disagreeable odor, and the limited efficiency for some types of pollutants [10].

Table 1.2: Main treatment processes applied to winery wastewater and respective average COD removal (adapted from [9]).

Technology	Process	COD removal
Physicochemical	– Precipitation	9%
	– Sedimentation	20-40%
	– Coagulation	35%
	– Electrocoagulation	47%
Biological	– Membrane bioreactor	94-97 %
	– Activated sludge	98 %
	– Anaerobic sequencing batch reactor	80-97 %
Advanced oxidation	– TiO_2-based	84 %
	– Sulfate radical-based	79 %
	– Fe-based	93 %
	– Ozone-based	30 %
Membrane	– Reverse osmosis	97 %

The biggest challenge to the efficient and cost-effective WW treatment lies in shifting towards resource recovery, viewing WW as a source of nutrients in a circular economy system rather than a waste stream. Although WW is typically seen as a waste that requires treatment, it possesses certain compounds with added value that may be worth recovering from the perspective of biorefinery and circular economy [4]. Rich in phenolic compounds, flavonoids, and anthocyanins with antioxidant properties, WW can be used to extract bioactive substances with posterior use of the remaining stream for energy generation, fertilizer production, or irrigation [11,12]. According to the literature, the use of pretreated WW for vineyard irrigation can have potential benefits for the wine industry, as the high levels of potassium in WW are beneficial to soil fertility [12].

1.2. Microbial fuel cells

Over the last hundred years, the utilization of fossil fuels has had a brutal impact on the economic growth of the most industrially developed countries. According to a projection reported in the literature, the global energy consumption rate will double from 13.5 TW (1 TW = 10^{12} W) in 2001 to 27 TW in 2050, and triple to 43 TW in 2100 [13]. Despite the anticipated effect of the increase

of liquid fuels and natural gas prices on energy demands, it is forecasted that global energy consumption will rise, as a result of population expansion and sustained economic growth, especially in developing countries [14]. Fossil fuels are predicted to be the dominant source of primary energy by 2030, but politicians are becoming increasingly aware of the need to reduce greenhouse gas emissions and promote the development and use of renewable energy sources, which poses a tremendous challenge to fossil fuel-based economies [14].

Among the several renewable energy technologies that have been developed in the past years, microbial fuel cell offers an eco-friendly approach to generating bio-electricity, based on the release of electrons from biochemical reactions facilitated by microbes. Usually, an MFC is comprised of an anodic and a cathodic chamber, separated by a proton exchange membrane (PEM), as illustrated in Figure 1.1 [15]. The biocatalyst at the anode catalyzes the oxidation of organic substrates, generating electrons and protons. The protons migrate to the cathodic chamber via the PEM, while the electrons travel through the electrical external circuit. In the cathodic chamber, the protons and electrons combine, leading to the simultaneous reduction of oxygen to water [16].

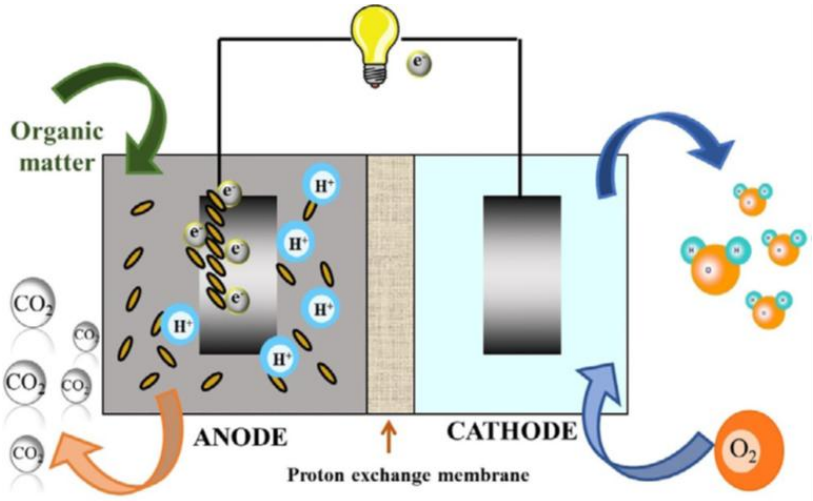


Figure 1.1: Scheme of a typical MFC (retrieved from [15]).

Concurrently with bioelectricity production, MFC can promote wastewater treatment, when this is used as the substrate in the cathodic chamber [17]. As with all technologies, there are advantages and disadvantages to the use of MFC. Figure 1.2 summarizes the main MFC advantages and disadvantages [18]. As can be seen, the disadvantages represent a greater 'weight on the scale' than the advantages. Still, due to the great potential that it is believed can be retrieved from these cells, studies have been carried out to mitigate the disadvantages and, thus, counterbalance the scale.

MFC emerges as a technology for treating wastewater with simultaneous energy production through electroactive bacteria. MFC application for wastewater treatment was initially suggested at the end of the 20th century, being that the advancements made from then significantly

improved the efficiency of this technology [19,20]. Several types of wastewater have been studied as substrate in MFC, namely domestic wastewater, dyeing wastewater, hydrocarbon-containing wastewater, dairy wastewater, brewery wastewater, winery wastewater, rice milling wastewater, molasses-based wastewater, palm oil mill wastewater, food processing wastewater, and pharmaceutical wastewater. It has been found that variations in energy density are directly related to the type of wastewater used and its characteristics [21]. Electricity production in MFC is favored by the use of wastewater rich in sugars, proteins, and starch, which can be easily used by microorganisms.

WW treatment with simultaneous electricity production through MFC is an attractive strategy, in a circular economy approach, for the wine industry [22]. Penteado et al. [23] evaluated the performance of a double-chamber MFC for WW treatment, concluding that, although MFC efficiently generated electricity from WW, the treatment efficiency was low (COD removal of approximately 17%).

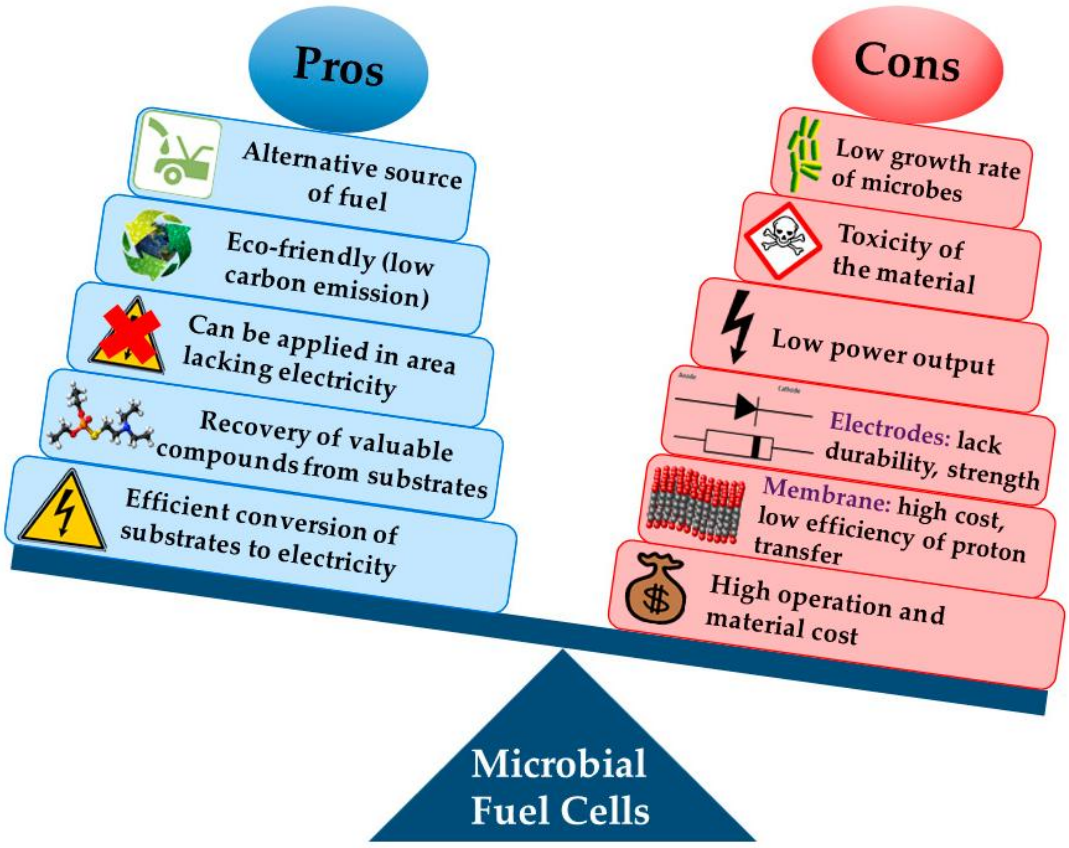


Figure 1.2: MFC main advantages and disadvantages (retrieved from [18]).

The microorganisms that grow in the anodic chamber are fed by the organic substrate supplied by the wastewater, releasing electrons in the process, which are used to produce electricity. The type of microorganisms used determines the process performance in both wastewater treatment and electricity generation [24]. Thus, proper inoculum selection plays a key role in improving MFC performance.

1.3. *Acetobacter aceti*

Acetic acid bacteria, also referred to as Acetobacter sp., are obligate aerobe Gram-negative bacteria, with oxygen serving as the terminal electron acceptor, belonging to the *Alphaproteobacteria* class, *Rhodospirillales* order, and *Acetobacteraceae* family. These bacteria are typically present in tropical and humid areas, inhabiting fruits, flowers, the digestive systems of fruit flies, and certain types of fermented food products [25-27].

Since ancient Babylonian times, acetic acid bacteria have been employed in the production of vinegar. Throughout much of this historical period, vinegar was obtained from the fermentation of natural alcoholic solutions containing approximately 10-15% v/v ethanol, with no extensive comprehension of the underlying natural process. During the early 1800's, several scientists made significant contributions that showed the microbial role in this process. Their findings and succeeding investigation led to an enhanced comprehension of vinegar production and the development of novel production techniques [28].

Acetobacter aceti is a remarkable organism that possesses the ability to oxidize alcohol into acetic acid [29]. This metabolic ability leads to the rapid oxidation of alcohols and sugars, resulting in the production of organic acids, which makes these bacteria particularly suitable for several biotechnological applications, including the synthesis of ascorbic acid (vitamin C) and cellulose [30]. In the food industry, acetic acid bacteria play a crucial role in the production of numerous food and beverage products, including vinegar, cocoa, kombucha, and other fermented beverages. However, it should be noted that the presence and activity of acetic acid bacteria can also lead to the spoilage of other food items and beverages, such as wine, beer, sweet drinks, and fruits [31].

A. aceti can survive in high concentrations of acetic acid, possessing a unique feature in the form of an efflux pump, which aids in the transportation of toxic particles across the cell membrane and cytoplasm, thereby contributing to the maintenance of cellular homeostasis. Moreover, *A. aceti* metabolism exhibits adaptableness to different environments, allowing acetate metabolism whenever needed [32]. In the presence of ethanol, *A. aceti* accumulates acetate, as a result of incomplete oxidation [29]. However, once ethanol is depleted, acetate serves as a carbon and energy source for the tricarboxylic acid cycle [33].

A. aceti has not been documented as a causing agent of human diseases. Its growth is not favored by the conditions found on human skin, thus ensuring its safe handling. Still, certain evidence suggests that, despite its natural occurrence in the environment, *A. aceti* may pose a threat to plants and other flora [34]. Low levels of *A. aceti* can be found in wine. However, when the wine is exposed to air, even briefly, exponential bacteria growth is observed, leading to a notable rise in acetic acid levels. For this reason, *Acetobacter aceti* is known for spoiling wine [35]. Elevated storage temperatures and high wine pH also enhance the growth and activity of *A. aceti* [36]. These bacteria are present at all stages of winemaking, from the mature grape, through

vinification and wine conservation [37]. This natural presence in wine, combined with its metabolic capabilities, makes *A. aceti* a very promising bacteria for WW treatment, with several advantages reported [38]:

- High efficiency in degrading organic compounds;
- Production of acetic acid;
- Low cost and simplicity;
- Sustainability and positive environmental impact;
- Flexibility and adaptability.

Bioelectrocatalysis studies were carried out using *A. aceti*, with promising results in promoting direct electron transfer, with applicability for a microbial fuel cell [39, 40]. Rengasamy and Berchmans [37] evaluated the performance of an MFC with *A. aceti* for bad wine biodegradation and current generation. The authors found a COD removal and an MFC power output of, respectively, 59% and 4.06 A m^{-3}, for a period of 72 h, concluding that MFC technology can be used in wineries for electric current generation.

Chapter 2
Aim of this Work

This chapter describes the objectives of the work developed and the strategy followed to attain those objectives. A description of the content of this dissertation is also presented.

2.1. Aim and strategy

The present study aimed to assess the ability of the microorganism *Acetobacter aceti* to resist winery wastewater constituents and to investigate the feasibility of using this microorganism, known for its capability to oxidize a variety of alcohols and sugars to organic acids, for WW biodegradation.

Using a model growth medium enriched with WW constituents, at different combinations and concentrations, the growth of *A. aceti* and the degradation of the compounds were evaluated, to determine the bacterial degradation capacity and its resistance to the presence of inhibitors in the culture medium. Finally, the degradation capacity and resistance of *A. aceti* were evaluated in a real WW sample.

2.2. Dissertation overview

This dissertation is structured in five chapters. The first chapter consists of a brief literature review of the main WW characteristics, technologies applied for its treatment, MFC fundamentals, and *A. aceti* features for WW biodegradation. Subsequently, in the second chapter, the global aim of the work is identified, as well as the strategy adopted.

The third chapter describes all the materials and methods used in the experimental work, including a description of the inoculum and solutions preparation, the characterization of the winery wastewater sample used, and a description of the fermentation experiments performed and of the analytical methods used to follow those experiments.

Chapter four presents a description of the experimental work developed and the results obtained in the different studies performed as described in the third chapter. The results are here discussed and the main conclusions are presented.

Finally, the fifth chapter summarizes the concluding remarks attained during this research work, regarding *A. aceti* ability to resist and degrade WW constituents. Future perspectives are also discussed.

Chapter 3
Materials and Methods

This chapter describes all the materials and methods used in the experimental work. It covers the description of stock solutions, inoculum, and the experiments preparation, as well as winery wastewater used, the analytical determinations, and the fermentation experiments performed.

3.1 Microorganism and media

3.1.1 Stock culture

The bacterium used in this study, *Acetobacter aceti ATCC 15973*, was purchased from Ambifirst (Setúbal, Portugal), which sent the active culture in two Petri dishes. Upon receiving it, each plate was repotted onto two new plates and kept at 25-30 ºC until colony-forming units grew. The bacterium growth was then started in liquid YPM medium (Yeast extract, Peptone, and Mannitol), prepared as described in Table 3.1, according to the supplier guidelines. To preserve the bacteria, just before reaching the stationary phase, 750 µL of fermentation broth was collected in microtubes containing 750 µL of 0.9% NaCl and 58.6% glycerol. After being gently inverted to involve the cells in the glycerol solution, a freezing procedure was carried out, by putting microtubes at -80 ºC.

3.1.2 Inoculum preparation

Before its use in the fermentation experiments, the frozen cells were taken from the -80 ºC freezer, thawed, and placed into 250 mL erlenmeyers containing 100 mL of YPM medium, ensuring that the required amount of oxygen for the bacterium growth was available. The bacterium growth was carried out for 48 hours (time required to reach its exponential phase) at (27 ± 1) ºC, under dark and stirring (200 rpm) conditions, using an RSLAB-7PRO orbital shaker (NOVEDUC, Massamá, Portugal). The bacterium growth was monitored through the visual aspect (turbidity) and optical density measurements at 600 nm (OD600). For the OD600 measurements, the samples were diluted with 0.9% NaCl solution, to prevent the bacterial membrane from destabilizing.

3.1.3 *Acetobacter aceti* characterization and dry weight determination in YPM media

To understand the time needed to accomplish the study a growth curve was established with *A. aceti* in YPM media along the time. YPM is composed of yeast extract, peptone, and mannitol at 5, 3, and 25 g L^{-1}, respectively. After inoculating the media with 10 mL of inoculum, the samples collected were used to measure the absorbance at 600 nm (OD600). The growth curve profile was obtained by representing the ratio of OD600 at time t (OD_t) by OD600 at the beginning of the

experiment (OD_0), for each collected sample. Whenever necessary a dilution of sample was made with 0.9% NaCl to attain an OD lower than 0.7, to follow the Beer-Lambert law. Periodically, samples were collected from the erlenmeyer flasks and ideal dilutions were performed for subsequent OD600 determinations. Each sample was analyzed in duplicate.

To obtain the yield of biomass, dry weight determination of *A. aceti* was performed with the following procedure:

a) At the 48th hour of growth (exponential phase), a sample was collected and several dilutions from it were prepared (1:2; 1:5; 1:10; 1:20; 1:40; 1:50), using 50 mL volumetric flasks.
b) For each diluted sample, the OD600 measurement was performed.
c) Each diluted sample was vacuum filtered through glass microfiber filters with a pore size of 0.45 μm, previously dried and weighed, being the filters (containing the retained bacterium) dried in an oven at 105 °C overnight.
d) After cooling to room temperature in a desiccator, the filters were weighed, and the value of the dry weight, for each diluted sample, was calculated through Equation (1), where the mass of the filters is in g and V is the volume of the sample filtered in mL.
e) The plot of OD600 versus dry weight was established.

$$\text{Dry Weight} = \frac{m_{\text{filter+bacterium}} - m_{\text{filter}}}{V} \quad (1)$$

The slope obtained in the graphical representation was used to calculate the biomass obtained at the specific time chosen to calculate yields. To determine the biomass yield ($Y_{X/M}$) of the fermentation, Equation (2) was used, where X_t is the concentration of cells at time t, X_0 is the initial concentration of cells, S_0 is the initial concentration of mannitol, and S_t the concentration of mannitol at time t.

$$Y_{X/M} = \frac{\text{grams of produced cells}}{\text{grams } of\ consumed\ substrate} = \frac{Xf - Xo}{So - Sf} \quad (2)$$

The substrate consumption rate (Q_M) was obtained from Equation (3), where CS_t corresponds to the measured substrate concentration (g L^{-1}) at time t (h), CS_0 corresponds to the substrate concentration (g L^{-1}) at time 0 h, and t corresponds to the time measured in h.

$$Q_M = \frac{CS_0 - CS_t}{t} \quad (3)$$

3.1.4 Stock solutions

To prepare the growth media (control) and testing media in the presence of an inhibitor it was necessary to prepare stock solutions, to have all media prepared in the same way. The composition of the various stock solutions utilized in the study, as well as the characteristics of the reagents

used, are presented in Table 3.1. All the solutions utilized in the study were prepared with pure water, obtained from a Thermo Scientific Pacific TII 7 UV water purification system (Thermo Fisher Scientific, Waltham, Massachusetts, USA). To maintain sterility in the solutions containing alcohols and phenols, the pure water was autoclaved before its use, for 20 minutes at 121 ºC, using a Tuttnauer 2540ML autoclave, and the inhibitors were added in the flow chamber. All manipulations to prepare these solutions were made inside a Telstar Aeolus H laminar airflow cabinet. Solutions prepared outside the laminar airflow cabinet were autoclaved after preparation.

Table 3.1: Composition of the different stock solutions and components utilized for the preparation of media to be studied.

Solution	Reagent	Chemical formula	Supplier	Purity	Concentration ($g\ L^{-1}$)
Mannitol (M)	Mannitol	$C_6H_{14}O_6$	Scharlau	> 97%	100
YP 10x	Yeast extract	–	Scharlau	–	50
	Peptone	–	Biolife	–	30
Salts 10x	Sodium sulfate	Na_2SO_4	Carlo Erba	≥ 99.6%	18.5
	Calcium chloride	$CaCl_2$	Chemlab	> 95%	0.94
	Potassium sulfate	K_2SO_4	Chemlab	> 99%	22.6
Ethanol (E)	Ethanol	C_2H_6O	AGA	99.5%	50
EG 10x	Ethanol	C_2H_6O	AGA	99.5%	50
	Glycerol	$C_3H_8O_3$	Himedia	> 99%	3.2
EGM 10x	Ethanol	C_2H_6O	AGA	99.5%	50
	Glycerol	$C_3H_8O_3$	Himedia	> 99%	3.2
	Methanol	CH_4O	Sigma	99.9%	0.15
ATLS 10x	Acetic acid	$C_2H_4O_2$	Fluka	> 99.8%	6.6
	Tartaric acid	$C_4H_6O_6$	Merck	≥ 99%	5.3
	Lactic acid	$C_3H_6O_3$	Sigma	85-90%	3.5
	Succinic acid	$C_4H_6O_4$	Merck	> 99.5%	0.8
Phenols (Ph) 10x	Tyrosol	$C_8H_{10}O_2$	TCI	> 98%	1.5
	Catechol	$C_6H_6O_2$	Alfa Aesar	99%	1.5

3.2 Fermentation experimental design

Table 3.2 presents the concentration of the inhibitors present for each test, and the volume used of the stock solution to prepare the media for the fermentation experiments performed in this study are shown in Table 3.3. Considering that *A. aceti* is known for its ability to degrade ethanol into acetic acid, the study was started by the evaluation of the resistance of *A. aceti* to the major alcohols found in winery wastewater (ethanol and glycerol) and to the toxic alcohol methanol, also identified in WW.

Due to equipment limitations *A. aceti* bacterium was exposed to two different concentrations of alcohols (ethanol, glycerol, and methanol), carboxylic acids (acetic acid, lactic acid, tartaric acid, and succinic acid), and phenols (catechol and tyrosol), to assess whether the studied concentrations inhibited the activity of *A. aceti*. The concentrations studied, shown in Table 3.2, were chosen to simulate a WW at average and extreme conditions, according to the compound's concentration reported by Mosse et al. [5]. All assays were done in duplicates.

Table 3.2: Composition of basal media and inhibitor concentration present in the experiments performed.

Assay	**#**	**Compounds concentration (g L^{-1})**			
		Ethanol	**Glycerol**	**Methanol**	
A-I*	A1	37.5	—	—	
	A2	37.5	2.4	—	
	A3	37.5	2.4	0.1	
A-II	A4	—	—	—	
	A5	2.5	0.16	0.0075	
	A6	5.0	0.32	0.015	
A-III	A7	—	—	—	
	A8	2.5	0.16	0.0075	
	A9	5.0	0.32	0.015	
		Acetic acid	**Tartaric acid**	**Lactic acid**	**Succinic acid**
CA	CA1	—	—	—	—
	CA2	0.66	0.53	0.35	0.08
	CA3	1.32	1.06	0.70	0.16
		Tyrosol		**Catechol**	
Ph	Ph1	—		—	
	Ph2	0.15		0.15	
	Ph3	0.30		0.30	
Basal media		**Yeast extract**	**Peptone**	**Mannitol**	**Salts****
		5	3	5.5	1.85, 0.094, 2.26

*- Control without basal media; ** Salt concentrations: sodium sulfate, calcium chloride, and potassium sulfate, respectively

Table 3.3: Description of the fermentation experiments performed.

Experiment	Solution	M	YP	Salts	E	EG	EGM	ATLS	Ph	Inoculum	Water	WW
		mL										
A-I	A1	—	—	—	150	—	—	—	—	50	—	—
	A2	—	—	—	—	150	—	—	—	50	—	—
	A3	—	—	—	—	—	150	—	—	50	—	—
A-II	A4	11	20	20	—	—	—	—	—	10	140	—
	A5	5	20	20	—	—	10	—	—	10	135	—
	A6	5	20	20	—	—	20	—	—	10	125	—
A-III	A7	11	20	20	—	—	—	—	—	10	140	—
	A8	11	20	20	—	—	10	—	—	10	130	—
	A9	11	20	20	—	—	20	—	—	10	120	—
CA	CA1	11	20	20	—	—	—	—	—	10	140	—
	CA2	11	20	20	—	—	—	20	—	10	120	—
	CA3	11	20	20	—	—	—	40	—	10	100	—
Ph	Ph1	11	20	20	—	—	—	—	—	10	140	—
	Ph2	11	20	20	—	—	—	—	20	10	120	—
	Ph3	11	20	20	—	—	—	—	40	10	100	—
WW	WW1	11	20	20	—	—	—	—	—	10	140	—
	WW2	—	—	—	—	—	—	—	—	10	—	191
	WW3	11	20	—	—	—	—	—	—	10	—	160

Since inorganic ions are the main WW constituents, they were also added to the fermentation experiments, at the average concentration of that reported by Mosse et al. [5]. To complement the study with model solutions, fermentation experiments were performed with real WW, and the influence of the presence of yeast extract, peptone, and mannitol was assessed.

The aerobic fermentation experiments were conducted with six 500 mL erlenmeyer flasks, two for each condition assayed (Figure 3.1). The total volume in each erlenmeyer was set to be 200 mL. The experiments were run at dark conditions, continuous orbital stirring (200 rpm), and room temperature (± 27 °C). Periodically, 5 mL samples were withdrawn from each erlenmeyer for analytical determinations to experiment monitoring. When required, the samples were diluted with 0.9% NaCl solution for OD600 determination. For other physicochemical analyses, samples were centrifuged for 20 minutes at 4000 rpm and 4 °C in a HERMLE Z 400 K centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany), discarding the formed bacterial pellet and preserving the supernatant for analysis.

Figure 3.1: Experimental setup. Created in BioRender.com

The fermentation experiments were monitored through OD600, pH, COD, total organic carbon (TOC), total nitrogen (TN), and high-performance liquid chromatography (HPLC) determinations. Utilizing the OD600 values, the *A. aceti* growth curve was plotted as $ln(OD600_t/OD600_o) = f(t)$, where $OD600_t$ is the OD600 at time t and $OD600_o$ is the initial OD600. The *A. aceti* growth rate was determined from the growth curve, corresponding to the growth curve slope, utilizing the data interval leading to the highest curve slope, between the initial point of fermentation and the point closest to 24 hours of testing.

3.3 Analytical determinations

3.3.1 Optical density at 600 nm and pH

Optical density at 600 nm, known as OD600, provides a straightforward method to assess the growth stage of a bacterial culture. The optical density quantifies the extent of light scattering

induced by the bacteria present in the culture; as the bacterial population increases, the light scattering also increases. The selection of the 600 nm wavelength for bacterial OD assessments is deliberate, as this wavelength is non-detrimental to the culture, unlike UV wavelengths. By tracking the progression of OD600 over time, it becomes possible to pinpoint the lag, log, and stationary phases within a bacterial culture.

When taking a culture sample, it needs to be well mixed and the measurement should be taken immediately, as the cells can start to settle, leading to inaccurate results. For this reason, in this work, the samples were always vortexed before being read on the spectrophotometer.

An OD reading higher than 1 is not accurate, since OD readings this high are beyond the dynamic range of most spectrophotometers, which means that the readings do not increase linearly as the concentration of cells increases. Therefore, when required, prior dilutions were made with 0.9% NaCl solution to ensure that the OD was always below 1. This NaCl solution also acted as a blank before taking the readings.

The pH measurements were conducted immediately after sample collection, in falcon tubes, using a Metrohm 827 pH Lab pH meter, after being calibrated.

3.3.2 Chemical oxygen demand

The chemical oxygen demand is commonly utilized to indirectly assess the quantity of organic material present in samples. COD determination methods rely on the principle that the carbon content present in organic compounds can be completely oxidized to carbon dioxide by a strong oxidizing agent, in an acidic environment. Thus, COD quantifies the amount of oxygen required to oxidize the organic material within the sample using a strong oxidizing agent. Its results are typically reported in units of oxygen concentration.

In this work, COD was determined by the closed reflux titrimetric method, according to Section 5220C of the Standard Methods [41]. This method uses dichromate ion as an oxidant, being subsequently reduced to chromic ion. The samples are digested in a strongly acidic solution with a predetermined amount of potassium dichromate in excess. Additionally, a silver catalyst is included to oxidize persistent organic compounds, while mercury sulfate is added to mitigate interferences from the oxidation of chloride ions. The closed reflux digestion of the samples was carried out in a Titronic 500 thermoreactor, for 2 hours at 150 ºC. After digestion, the $K_2Cr_2O_7$ that was not reduced and remained in the solution was titrated with ferrous ammonium sulfate, using a ferroine solution as the indicator. This titration procedure was carried out in a TM 235 automatic titrator. The amount of dichromate consumed by the sample is directly proportional to the amount of oxygen required for the oxidation of the organic compounds.

3.3.3 Total, organic and inorganic carbon

The total organic carbon gives the organic carbon concentration present in the samples. Unlike COD, TOC remains unaffected by the oxidation state of the organic compounds.

TOC determination was conducted through the high-temperature combustion technique, as described in Section 5310B of the Standard Methods [41], using a Shimadzu TOC-L CSH analyzer that integrates combustion catalytic oxidation at 680 °C with a non-dispersive infrared (NDIR) detection technique. Through an automated process, the samples were introduced into the combustion furnace and subjected to combustion at 680 ºC with a platinum catalyst in an oxygen-rich environment. During this process, both organic and inorganic carbon are oxidized to CO_2 and H_2O, being the CO_2 concentration subsequently measured using a non-dispersive infrared detector. This CO_2 concentration corresponds to the total carbon (TC) of the sample. The inorganic carbon (IC) is measured separately, by introducing the sample into a reaction chamber, acidifying it, and converting all inorganic carbon into CO_2. By subtracting IC from TC, TOC concentration is determined.

3.3.4 Total nitrogen

The total nitrogen determination was conducted using a TN measuring unit TNM-L ROHS coupled to the TOC-L CSH analyzer. In this process, all nitrogen compounds present in the sample are initially transformed into nitrogen monoxide and nitrogen dioxide through catalytic combustion in a furnace. Subsequently, the nitrogen species react with ozone, resulting in the formation of an excited state of nitrogen dioxide. The emitted light energy generated when the excited state returns to the ground state is then quantified using a chemiluminescence detector.

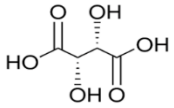
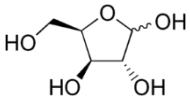
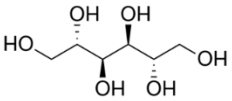
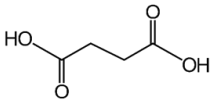
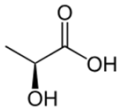
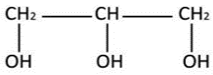
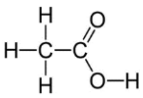
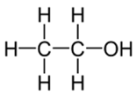
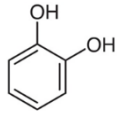
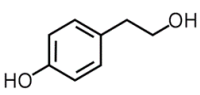
3.3.5 Quantification of substrates and metabolic products

High-Performance Liquid Chromatography (HPLC) is a method used for separating chemical compounds in solution, employed in analytical chemistry to identify and quantify components in a mixture. A basic HPLC process can be described as follows: 1) the solvent, known as the mobile phase, is stored in a reservoir; 2) to ensure a specific flow rate of the mobile phase, typically measured in milliliters per minute, a high-pressure pump, also referred to as a solvent delivery system, is utilized; 3) the sample is injected into the continuously circulating mobile phase stream by an injector, which transports the sample to the HPLC column; 4) the column contains the necessary chromatographic retention material for the separation process, known as the stationary phase; 5) a detector is used to observe the separated compounds as they elute from the HPLC column.

In this work, HPLC was performed using a Shimadzu HPLC(Shimadzu, Kyoto, Japan) system equipped with an SCL-40 System Controller SCL-40, a degassing unit DGU-405, a solvent delivery module LC-40D XR, an Auto Sampler SIL-40C XR, a column oven CTO-40C, a

photodiode array detector SPD-M40, and a refractive index detector RID-20A. A cationic Biorad Aminex HPX-87H column was used, and the elution was performed isocratically with a sulfuric acid aqueous solution (5 mM) at a flow rate of 0.6 mL min^{-1} and 50 ºC. For the analysis, the sample volume injected was 5 µL. The different compounds identified, as well as their retention times and the range of the calibration curves used for subsequent quantification, are presented in Table 3.4.

Table 3.4: Chemical compounds detected and quantified by HPLC.

Chemical compound	Chemical Structure	Retention time (min)	Calibration curve range (mg L^{-1})
Tartaric acid		8.642	0.5–250
Xylose		9.779	0.5–250
Mannitol		10.098	5–250
Succinic acid		11.745	0.5–250
Lactic acid		12.772	0.4–210
Glycerol		13.512	0.1–50
Acetic acid		15.221	0.4–210
Ethanol		22.188	0.4–198
Catechol		33.701	6–300
Tyrosol		58.540	6–300

Chapter 4
Results and Discussion

This chapter describes the experimental work developed and presents and discusses the results obtained in the different studies performed. The first step was to characterize the growth profile of *Acetobacter aceti* and calculate its dry weight. Next, its behavior in the presence of various inhibitors was evaluated, and finally, its behavior in the presence of winery wastewater as well as the characterization of this wastewater.

4.1 Growth profile of *Acetobacter aceti* in YPM media

The growth curve of the bacterium *Acetobacter aceti* was determined following the procedure described in section 3.1.3 The data from this experiment (e.g., sampling periodicity, sample dilution, absorbance, and OD600 values) are presented in Appendix I—Supplementary Material, Table S1. Utilizing the data from Table S1, the *A. aceti* growth curve was plotted as $ln(OD600_t/OD600_0) = f(t)$ (Figure 4.1).

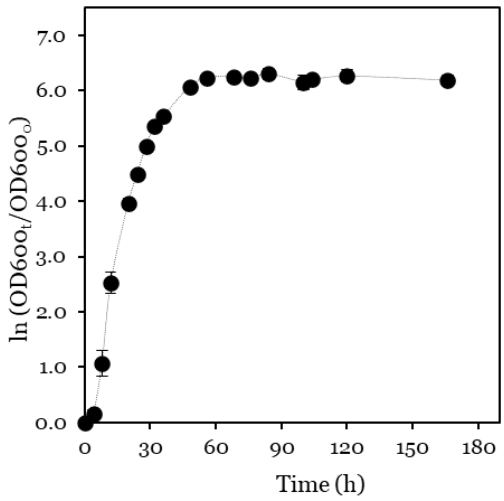


Figure 4.1: *Acetobacter aceti* growth curve in YPM media.

According to Figure 4.1, three phases can be identified in the growth curve:

1. Lag phase – observed in the initial hours (between 0 and 8 h) and corresponding to the bacteria adaptation period to its new conditions;
2. Exponential or Log phase – between the 8th and the 48th hour, where the cells have already accumulated everything they need for growth and, thus, they proceed to cell division. The healthiest and most uniform cells are found in this phase;
3. Stationary phase – between the 48th and the 166th hour, where the bacterial population runs out of essential nutrients or is inhibited by its waste products, ceasing growth.

4. Death or Decline phase—Although it is not identified in this growth curve, this stage is usually characterized by the decrease in the number of viable cells as the culture conditions deteriorate to a point where the cells are irreparably damaged.

The *A. aceti* growth presented a "classic" growth curve, allowing to access essential information for the subsequent experiments, such as understanding the bacterium life cycle, determining growth parameters, evaluating cultivation conditions, and monitoring possible contaminations. From the growth curve obtained in the *A. aceti* characterization experiment (Figure 4.1), the *A. aceti* growth rate was determined to be (0.205 ± 0.001) h^{-1} (R^2 = 0.975).

The calculation of dry weight and OD600 was performed as indicated in Section 3.1. for *A. aceti*. The plot of OD600 versus dry weight (calibration curve) obtained is presented in Figure 4.2 The experimental data utilized for this plot, obtained from the dry weight determination procedure is given in Table S2 (Appendix I—Supplementary Material).

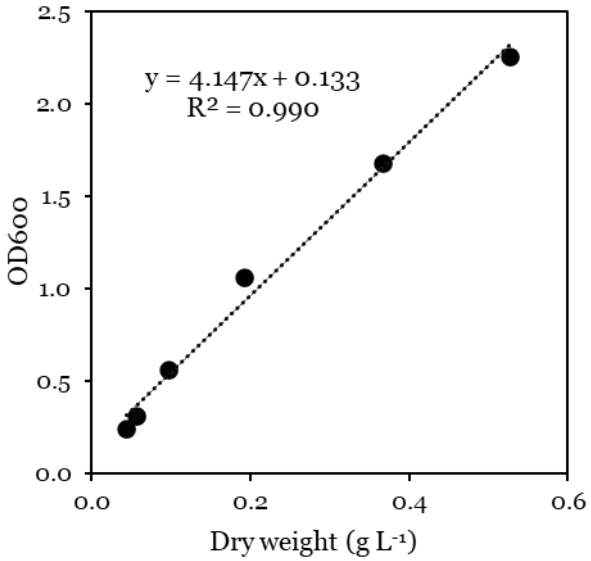


Figure 4.2: Plot of OD600 versus dry weight for *Acetobacter aceti*.

A linear relation between *A. aceti* OD600 and dry weight was found, with a coefficient of determination (R^2) of 0.990, which is quite acceptable in this context. In this study, the growth curve method was chosen for monitoring the growth of *A. aceti* during the tests performed. Still, it should be noted that there are some disadvantages related to growth control through absorbance measurements, such as the fact that it is not possible to distinguish between live and dead cells.

4.2 *Acetobacter aceti* tolerance to alcohols

The set of fermentation experiments A-I, (Table 3.3), was aimed to evaluate the *A. aceti* growth in the presence of alcohol compounds, namely ethanol, glycerol, and methanol, assays were run

with a mixture of the alcohol's aqueous solution with the inoculum. The obtained results, presented in Table S3 (Appendix I—Supplementary Material), showed that no growth occurred. This could be due to the inhibitory effect of the alcohols at the studied concentration, the lack of suitable nutrients, or both. In light of these results, a second set of experiments was conducted A-II (Table 3.3), in which the concentration of the alcohols was reduced and the media was supplemented with yeast extract, peptone, mannitol, and salts. The yeast extract and peptone were added at the same concentration as that used in the YPM medium, but as for mannitol, its concentration was reduced, aiming to force the bacterium to degrade the inhibitory compounds (Table 3.2).

Figure 4.3 shows the *A. aceti* growth curves from experiments A-II (A4-A6), obtained from the experimental data presented in Appendix I—Supplementary Material, Table S4, as well as pH, COD variation, and all HPLC determinations of concentrations of mannitol, ethanol, glycerol, acetic acid, and xylose.

From the figure below, it can be seen that the control assay (A4), without alcohols, presented a growth profile similar to Figure 4.1, but the final OD was half of the first assay (3 instead of 6). This could be explained by the difference in the concentration of mannitol present in both media. When the growth was carried out in YPM medium the concentration of mannitol present was 25 g L^{-1}, while in the control test (A4) the concentration of mannitol present was only 5.5 g L^{-1}. This difference can explain the results obtained in assay A4.

Growth profile in the presence of alcohol presents a diauxic behavior at the time near 50 h, indicating possible use of two sources of carbon. In general, the growth of bacteria in the control assay was higher than in the assays with alcohols. Moreover, the higher the alcohol concentration, the lower the growth at the end of the exponential phase. These results indicate that, although not to a great extent, the presence of alcohol seems to have decreased *A. aceti* growth. This could also be justified by the lower concentration of mannitol, leaving the doubt of which factor had the major effect. The initial growth rate determined for assays LC (A5) and HC (A6) (Table 4.1), confirms the slight *A. aceti* growth decrease in the presence of the alcohols. The fact that there were no differences between the two studied alcohol concentrations may indicate that mannitol concentration could be the factor that affected *A. aceti* growth. The results indicated that new conditions should be used when performing the tests guaranteeing the same conditions of mannitol for the control and in the presence of inhibitors. Relatively to biomass yield (Table 4.1), the higher values obtained in the presence of ethanol and glycerol are explained because the calculation was made with the basis of mannitol as substrate, but ethanol and other alcohol were also used to grow.

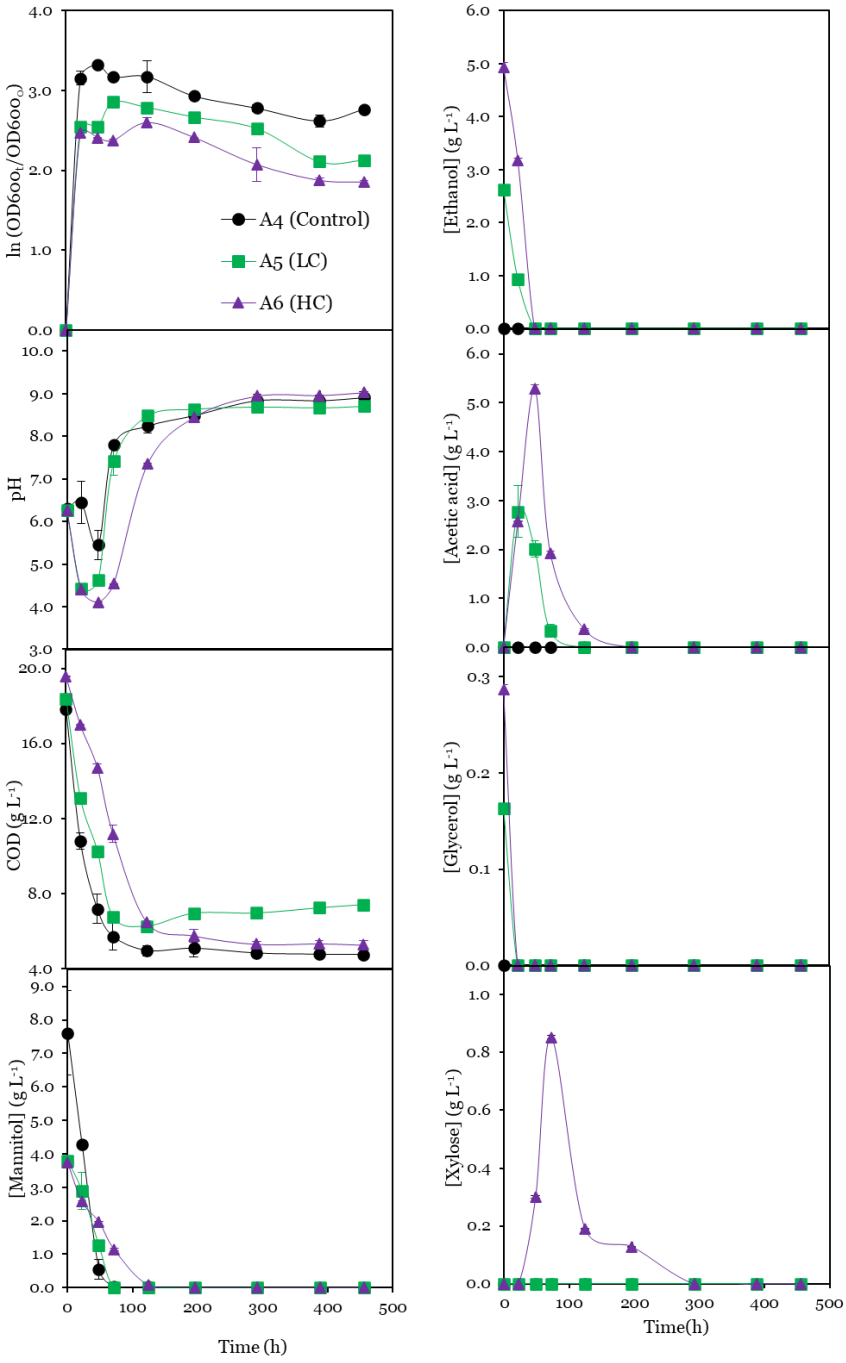


Figure 4.3: Profile of growth (ln ($OD600_t/OD600_0$), pH, COD, [Mannitol], [Ethanol], [Acetic acid], [Glycerol] and [Xylose] of fermentation of *A. aceti* in basal media (Control), low concentration LC (2.5, 0.16, and 0.0075 g L^{-1}) and high concentration HC (5.0, 0.32, and 0.015 g L^{-1}) of ethanol, glycerol and methanol, respectively.

About pH variation along assays A4-A6 (experiment A-II), a decrease can be seen during the first hours of assays is observed, being this decrease more pronounced with the increase in alcohol concentration. This more pronounced pH decrease is probably due to acetic acid formation from ethanol biodegradation, a metabolism already known to be characteristic of *A. aceti*. This variation was observed in a previous study with this bacterium [42] where a conversion of alcohol

to acetate (acetic acid) was found. The proportion of pH decrease is higher in experiment A6 (HC) with higher alcohol concentration.

Table 4.1: Initial growth rate, biomass yield, and COD removal for fermentation of *A. aceti* in basal media (Control), with low concentration LC (2.5, 0.16, and 0.0075 g L^{-1}) and high concentration HC (5.0, 0.32, and 0.015 g L^{-1}) of ethanol, glycerol and methanol in basal media, respectively.

Assay	Control (A4)	LC (A5)	HC (A6)
Initial μ (h^{-1})	0.143 ± 0.004	0.116 ± 0.003	0.112 ± 0.002
R^2	1	1	1
$Y_{X/M}$	0.563 ± 0.081	0.712 ± 0.004	0.885 ± 0.026
COD removal (%)	73 ± 0.034	60 ± 0.176	73 ± 1.151

COD is one of the most utilized parameters in wastewater characterization, the ability of *A. aceti* to reduce the organic load was evaluated through COD determinations along the assays. For all the conditions assessed (A4-A6), the COD was reduced by 83, 60, and 73% for control, LC (A5), and HC (A6), respectively (Table 4.1), in just over 100 hours. A slight decrease in the COD removal efficiency was observed for assay A5, which could be justified by the difficulty of the bacteria to cope with a lower concentration of mannitol. However, at the HC (A6) assay, the bacteria could cope better with this situation, perhaps because of the higher value of ethanol. The results show that *A. aceti* is capable of degrading alcohol compounds.

HPLC determinations were performed to monitor the variation of the most significant compounds in experiment A-II. As described above, the initial mannitol concentration was reduced to half in the assays containing alcohol. Although the mannitol was completely degraded at all the conditions assayed, which was expected since it is a preferred substrate of *A. aceti*, a decrease in the mannitol consumption rate with the increase in alcohol concentration was observed.

Ethanol and acetic acid variations during the experiment were evaluated. It is possible to see that acetic acid was obtained from the consumption of ethanol, in which after 48-h experiment, ethanol was completely degraded, for both assayed concentrations. The decrease in ethanol concentration was accompanied by an increase in acid acetic concentration, showing the unique *A. aceti* ability to convert ethanol into acetic acid, as described above. It can also be observed that, after ethanol was completely degraded, acetic acid degradation occurred, explaining the increase in pH after 48 h of fermentation. This behavior was reported by Jucker and Ettlinger (1985) [42].

Regarding glycerol and xylose variations during the experiment it can be seen that the bacterium had no difficulty in degrading glycerol since, after 22 hours of experiment, glycerol was completely degraded. As for xylose, its formation was observed only in the assay with higher alcohol

concentration (A6), reaching a maximum value after 72 h of fermentation, with a posterior decrease until its complete degradation until 292 h.

Although the results obtained in experiments A-II were very promising, the fact that the control assay (A4) had a higher mannitol concentration than that of A5 and A6 raised questions about its influence on the A5 and A6 performance. The reason why it was chosen to use a lower mannitol concentration in A5 and A6 was to promote the consumption of the inhibitors by the bacterium, by avoiding it to opt for its preferred substrate. However, given the difference observed in the *A. aceti* growth, a question arose whether the lower growth in A5 and A6 was due to the action of the inhibitors or due to the lack of mannitol. To clarify this question, a third experiment with alcohols was performed (experiment A-III), where the mannitol concentration was kept the same between the different assays (Table 3.3).

Figure 4.4 shows the *A. aceti* growth curves from experiments where basal media was applied to all conditions supplemented with the inhibitors for assay A-III (A7-A9) (Table 3.2), obtained from the experimental data presented in Appendix I—Supplementary Material, Table S5, as well as pH, COD and TOC variation and all HPLC determinations of concentrations of mannitol, ethanol, acetic acid, and xylose. Table 4.2 shows the initial growth rate, the biomass yield for all conditions (A7-A9) as well as the substrate consumption rate for mannitol and ethanol, and COD and TOC removal percentage, for the studied conditions.

Growth curves from experiment A-III, where the concentration of mannitol is equivalent in all assays (basal media), are very similar to the ones obtained from experiment A-II. Again, a diauxic behavior is observed, the first growth when ethanol and mannitol are consumed and the second growth when acetate is consumed, for LC (A8), 27 and 167 hours respectively, and for HC (A9), 33 and 167 hours respectively. The initial growth rates (Table 4.2) are similar to those of experiment A-II (Table 4.1). However, the biomass yield obtained in the presence of inhibitors in assays A-III is smaller than in the control assay. These results indicate that the presence of alcohol inhibits *A. aceti* growth. Jucker and Ettlinger (1985) found similar behavior in their studies, including the diauxic profile.

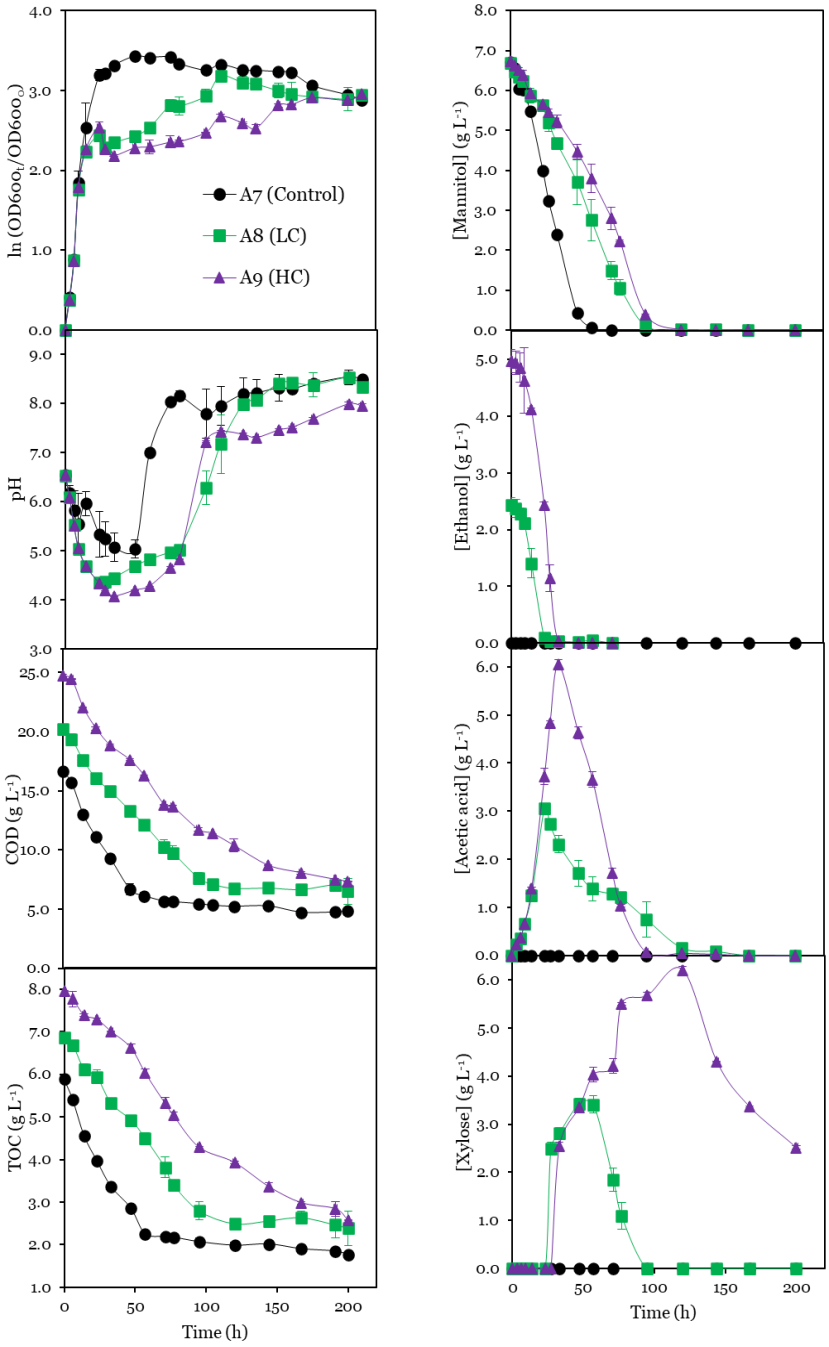


Figure 4.4: Profile of growth (ln (OD600t/OD6000), pH, COD, TOC, [Mannitol], [Ethanol], [Acetic acid], and [Xylose] of fermentation of *A. aceti* in basal media (Control) and with low concentration LC (2.5, 0.16, and 0.0075 g L^{-1}) and high concentration HC (5.0, 0.32, and 0.015 g L^{-1}) of ethanol, glycerol and methanol, respectively.

The pH variation along this third trial (assays A7-A9) presented a similar trend to the one observed in experiment A-II. As explained before, this variation is due to ethanol conversion to acetate, followed by its use as a carbon source.

Table 4.2: Initial growth rates, biomass yield, and substrate consumption rate (mannitol and ethanol), COD and TOC removal for *A. aceti* of fermentation in basal media (Control) and with low concentration LC (2.5, 0.16, and 0.0075 g L^{-1}) and high concentration HC (5.0, 0.32, and 0.015 g L^{-1}) of ethanol, glycerol and methanol, respectively.

Parameter	Control(A7)	LC (A8)	HC (A9)
Initial μ (h^{-1})	0.147 ± 0.006	0.1170 ± 0.0004	0.113 ± 0.003
R^2	0.945	0.866	0.878
$Y_{X/M}$	0.562 ± 0.009	0.410 ± 0.078	0.458 ± 0.073
Q_M Mannitol (g L^{-1} h^{-1})	0.117 ± 0.000	0.069 ± 0.010	0.051 ± 0.006
Q_M Ethanol (g L^{-1} h^{-1})	-	0.089 ± 0.004	0.142 ± 0.001
COD removal (%)	73 ± 0.034	60 ± 0.176	73 ± 1.151
TOC removal (%)	70 ± 0.347	65 ± 5.440	68 ± 0.120

Figure 4.4 also shows the COD decays in experiment A-III. Due to the increase in mannitol concentration in the assays with alcohol, compared to the A-II experiment, the initial COD of A8 and A9 (experiment A-III) is higher (20.25 and 24.73 g L^{-1} respectively) than the homologous A5 and A6 from experiment A-II (18.38 and 19.56 g L^{-1} respectively). Still, COD removals of approximately 70-75% were achieved, as observed for experiment A-II, although the decrease in COD removal rate with the increase in alcohol concentration is more evident in experiment A-III results. Alongside COD, TOC analyses were performed to evaluate the degree of mineralization of the organic compounds' biodegradation, as removal in TOC indicates that the organic compounds were mineralized to inorganic carbon species. Figure 4.4 also shows the TOC decays in experiment A-III. TOC removal followed the same trend as that of COD (Table 4.2), achieving removals of 60-73%, which indicates a high mineralization degree of the organic compounds.

Contrarywise to the observed in experiment A-II, in experiment A-III the initial concentration of mannitol was the same in the three assays (A7-A9). For all the conditions studied, complete mannitol biodegradation was attained, although the consumption rate decreased with the increase in alcohol concentration since the COD/TOC removal decreased slightly from control to LC (A7) but presented the same removal percentage in HC (A8) assay, as previously observed in experiment A-II.

Ethanol and acetic acid variations during experiment A-III were studied. Once again, the present data are similar to the results found in experiment A-II. In this set of experiments (A-III) samples

were taken more often, which allowed the establishment of the metabolization trend more accurately. The ethanol consumption rate (Table 4.2) obtained for HC(A9) was double the obtained for LC (A8), which was expected.

After 9 hours of fermentation, glycerol was no longer present in the assays with LC (A8) and HC (A9) concentrations of alcohols. This result indicates that its degradation occurred earlier than that perceived from Figure 4.3 (AII), since in A-II tests there were no data before 22 h of fermentation. Xylose variation during experiment A-III (Figure 4.4) is different from the observed in experiment A-II. In the A-III test, xylose formation was observed for both LC and HC alcohol concentrations, being their value dependent majorly on ethanol concentration. This increased xylose concentration can be due to the higher mannitol concentration in solutions A8 and A9, compared to experiments A-II, as it was the only parameter changed between A-II and A-III experiments. However, it should be noted that no xylose formation was observed in the control assays (A4 and A7), suggesting that its production was due to the presence of other alcohols, besides mannitol.

Although the xylose variation profile was similar in both experiments, being initially formed as a product of *A. aceti* metabolism and then consumed by it, for HC (A9) the complete degradation of xylose was not observed, due to the short length of the fermentation time. If the experiment had been prolonged above 200 hours, most likely a total degradation of xylose in HC (A9) could have been observed. On the other hand, the column used to quantify the alcohols and sugar by HPLC has the possibility of co-eluting xylose and mannose simultaneously. To clarify which sugar was formed, further characterization of this sample should be performed. This possibility arises from the consumption of mannitol and its possible metabolization to mannose.

The comparison between experiments A-II and A-III allows us to conclude that the lowest growth rates observed in the presence of alcohol were not due to the lack of mannitol in experiment A-II but to a slight inhibition of *A. aceti* by the alcohols. Still, *A. aceti* was able to mineralize most of the organic compounds present.

4.3 *Acetobacter aceti* tolerance to carboxylic acids

Since the wine wastewater presents several acids, *A. aceti* was grown in the presence of the major carboxylic acids found in it. In the present work, the studied acids were acetic acid, tartaric acid, lactic acid, and succinic acid. A mixture of the indicated acid was evaluated in experiment CA (Table 3.3.). Results obtained from the experimental data presented in Appendix I— Supplementary Material, Table S6, as well as pH, COD, and TOC variation and all HPLC determinations of concentrations of mannitol, acetic, tartaric, lactic and succinic acids, as well as xylose, are represented in Figure 4.5. It shows the *A. aceti* growth curves from the experiment with carboxylic acids (CA1-CA3). The control assay presented the same profile as the one observed with equivalent conditions in the study with alcohols. In the presence of a lower concentration of carboxylic acids LC (CA2), the growth profile was similar to the control assay, although with a

minor growth. In the presence of higher concentration, a diauxic growth is observed at 100h. This diauxic behavior is due to the consumption of xylose. Table 4.3 presents the initial growth rates determined for assays CA1-CA3, biomass yield, substrate consumption rate for mannitol and acetic acid, COD, and TOC removal for the CA assays.

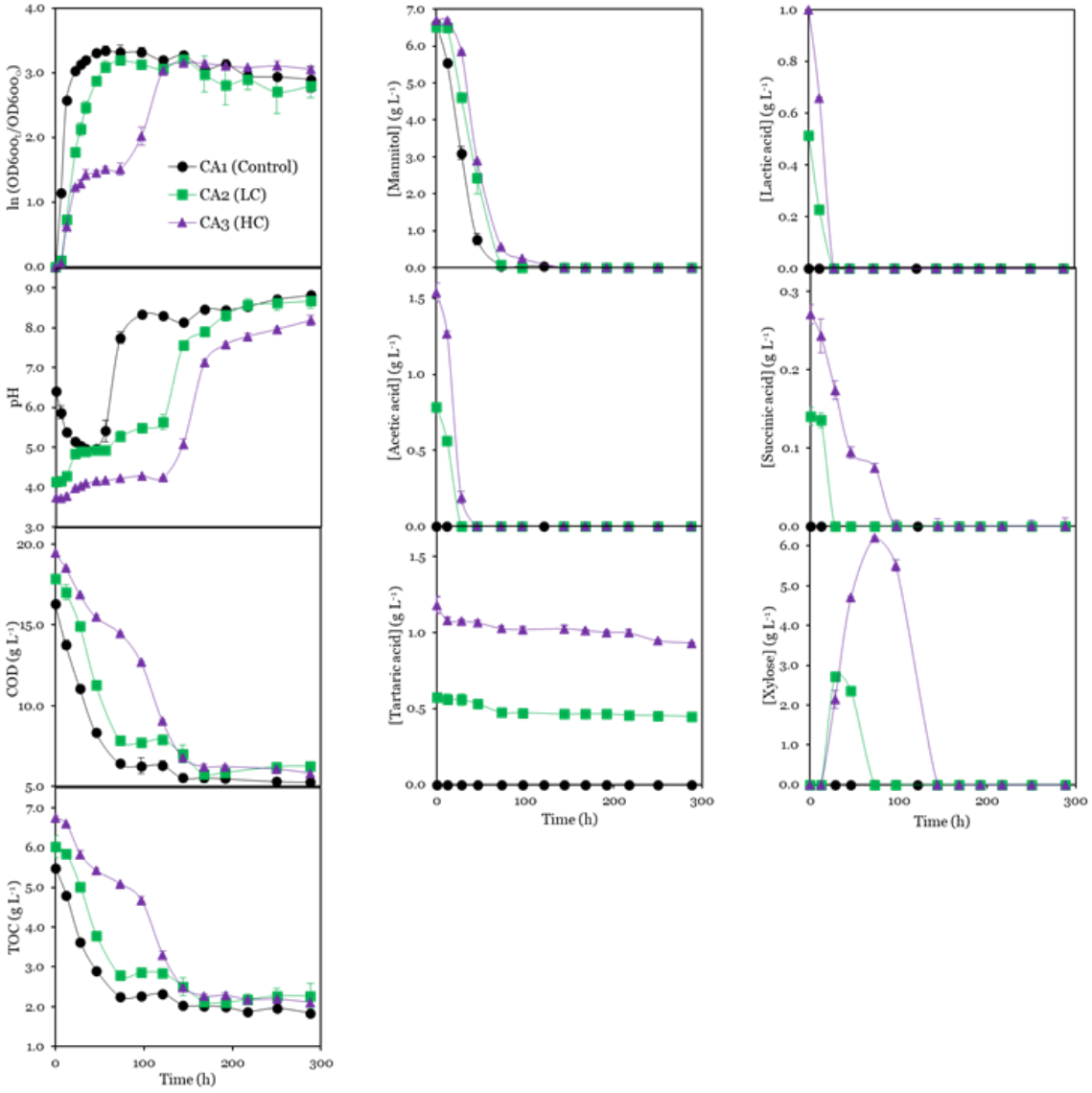


Figure 4.5: Profile of growth (ln (OD600t/OD6000), pH, COD, TOC, [Mannitol], [Acetic acid], [Tartaric acid], [Lactic acid], [Succinic acid] and [Xylose] of *A. aceti* fermentation in basal media and with low concentration LC (0.66, 0.53, 0.35, 0.08 g L^{-1}) and high concentration HC(1.32, 1.06, 0.70, 0.16 g L^{-1}) of acetic, tartaric, lactic and succinic acid, respectively.

With both CA concentrations, it was observed a decrease in the growth rate (0.085 and 0.060, respectively for low and high concentrations, respectively) which confirms *A. aceti* growth inhibition in the presence of carboxylic acids. Compared to that of the alcohols A-III fermentation experiment (Table 4.2), the effect of carboxylic acids on growth rates was more severe.

Table 4.3: Initial growth rate, biomass yield, substrate consumption rate, COD, and TOC of *A. aceti* fermentation in basal media and with low concentration LC (0.66, 0.53, 0.35, 0.08 g L^{-1}) and high concentration HC(1.32, 1.06, 0.70, 0.16 g L^{-1}) of acetic, tartaric, lactic and succinic acid, respectively.

Parameter	Control (CA1)	LC (CA2)	HC (CA3)
Initial μ (h^{-1})	0.140 ± 0.002	0.085 ± 0.001	0.060 ± 0.001
R^2	0.901	0.947	0.950
$Y_{x/M}$	0.520 ± 0.058	0.435 ± 0.002	0.070 ± 0.008
Q_M Mannitol (g L^{-1} h^{-1})	0.090 ± 0.002	0.088 ± 0.000	0.084 ± 0.000
Q_M Acetic (g L^{-1} h^{-1})	-	0.028 ± 0.000	0.048 ± 0.004
COD removal (%)	68 ± 1.104	65 ± 4.337	70 ± 0.797
TOC removal (%)	66 ± 0.244	62 ± 7.007	69 ± 1.843

Compared to the alcohol A-III fermentation experiment, higher growth inhibition is observed during the first hours of the CA experiment, especially for the highest carboxylic acid concentration. Still, both CA2 and CA3 end up achieving the growth level of the control (CA1), taking more time in the presence of a higher concentration of acids (CA3) to achieve the final level of the control.

Related to pH variation along assays in the presence of acids, for control (CA1) a similar behavior is observed compared to the control assays in experiments with alcohols (A4 and A7). In the presence of acid (CA2 and CA3), the initial pH was lower than the control, due to the presence of the carboxylic acids. Since CA3 is more concentrated it is reflected as a lower pH measurement. The increase in pH is related to acetate consumption during the experiment, allowing it to reach a final value similar to control (CA1). This behavior shows how *A. aceti* was resilient to the studied acids concentration.

The ability of *A. aceti* to reduce the organic load can be analyzed from Figure 4.5, which displays the COD decay along the assays CA1-CA3. The highest concentration of carboxylic acids (CA3) required a longer time to reduce the COD, while lower concentrations (CA2) presented a similar removal rate to control (CA1). This longer time required in CA3 agrees with the observed lower growth rate, indicating that the bacterium needed more time to adapt when the concentration of the carboxylic acids was higher. Compared to alcohol assays, COD removals ranging from 70 to 75% were observed, showing that *A. aceti* is capable of degrading the carboxylic acids found in winery wastewater.

TOC decays in experiment CA, followed the same trend as that of COD, achieving removals of 67-71% and indicating a high mineralization degree of the organic compounds.

The concentration of mannitol, carboxylic acids under study, and formed fermentation products were monitored through HPLC. Figure 4.5 also shows the assimilation of mannitol in assays (CA1-

CA3). For all studied conditions, a complete mannitol biodegradation was attained, which was expected since it is the bacteria's preferred substrate. Still, the consumption rate decreased with the increase in carboxylic acid concentration (0.088 and 0.084 for CA2 and CA3 respectively), confirming the increased *A. aceti* inhibition with carboxylic acid concentration.

Acetic and tartaric acids profile during the experiment are presented in Figure 4.5. Acetic acid was easily degraded by *A. aceti*, being completely consumed after 28 hours and 46 hours for experiments with low (CA2) and high (CA3) carboxylic acid concentrations, respectively. This acetic acid consumption by *A. aceti* had already been observed in the alcohol experiments, in which, the acetic acid formed from ethanol metabolization, was higher than in the present tests (CA experiment), being completely consumed. As for tartaric acid, the obtained results indicate that *A. aceti* could not degrade this compound, since its concentration did not vary significantly during the experiment, for both studied concentrations.

The lactic and succinic acids profile during the experiment (Figure 4.5) shows that *A. aceti* degrades both lactic and succinic acids very fast. Lactic acid was consumed completely after 28 hours of the experiment. As for succinic acid, it is all consumed after 28 hours for the lower concentration, in the case of the higher concentration, the profile presents a diauxic consumption, and a residual amount remains in the solution until 100 hours of the experiment.

Once again, xylose formation was observed in the presence of carboxylic acids experiment, except for the control (CA1). As observed for the alcohol experiment, xylose is initially formed as a product of *A. aceti* metabolism and then consumed as a substrate. However, in the presence of the carboxylic acids experiment, both xylose formation and consumption occurred earlier than in the test with alcohols.

A global analysis of the results obtained in the presence of carboxylic acids experiment allows to conclude that, although *A. aceti* growth showed a delay in the first hours of the experiment, the bacterium could reach considerable growth values later. As for the organic load removal, even though the carboxylic acids slightly affected the bacterium growth, *A. aceti* could metabolize most of the studied organic compounds.

4.4 *Acetobacter aceti* tolerance to phenolic compounds

Although generally phenolic compounds are seen as a positive factor for microorganism growth, when present at very high concentrations, like the ones in winery wastewater (Table 1.1) phenolic compounds pose a serious environmental threat that needs to be carefully addressed [2,43,44]. Baía et al (2020) characterized winery wastewater and phenolic compounds such as tyrosol were found in the concentration of 0.079 g L^{-1}. For this study, two phenolic compounds were used to study their effect on the *A. aceti* growth, namely catechol, and tyrosol, which were evaluated in

experiment Ph (Table 3.3). Figure 4.6 shows the *A. aceti* growth profile from the fermentation assays in basal media and in the presence of phenolic compounds Ph (Ph2 and Ph3), obtained from the experimental data presented in Appendix I—Supplementary Material, Table S7, as well as pH and COD variation and all HPLC determinations of concentrations of mannitol, catechol, and tyrosol. Table 4.4 presents the initial growth rates determined for assays Ph1-Ph3, biomass yield, substrate consumption rate for mannitol and phenols as well as COD removal for the phenolic assays.

The control (Ph1) growth curve has a similar profile to the one observed for control with the previous tests. The analysis of the growth profile shows no effects of the presence of tyrosol and catechol. The concentrations used in our study were relatively low, hence the phenols had no repercussions on *A. aceti* growth, as both LC (Ph2) and HC (Ph3) showed similar growth profiles to that of the control assay. This behavior is confirmed by the obtained initial growth rates which was around 0.123 h^{-1} (Table 4.4) for all assays. Biomass yield presented values around 0.759 (Table 4.4.) for the assay with phenolic compounds. No significant changes were also observed for mannitol consumption and COD removal percentage.

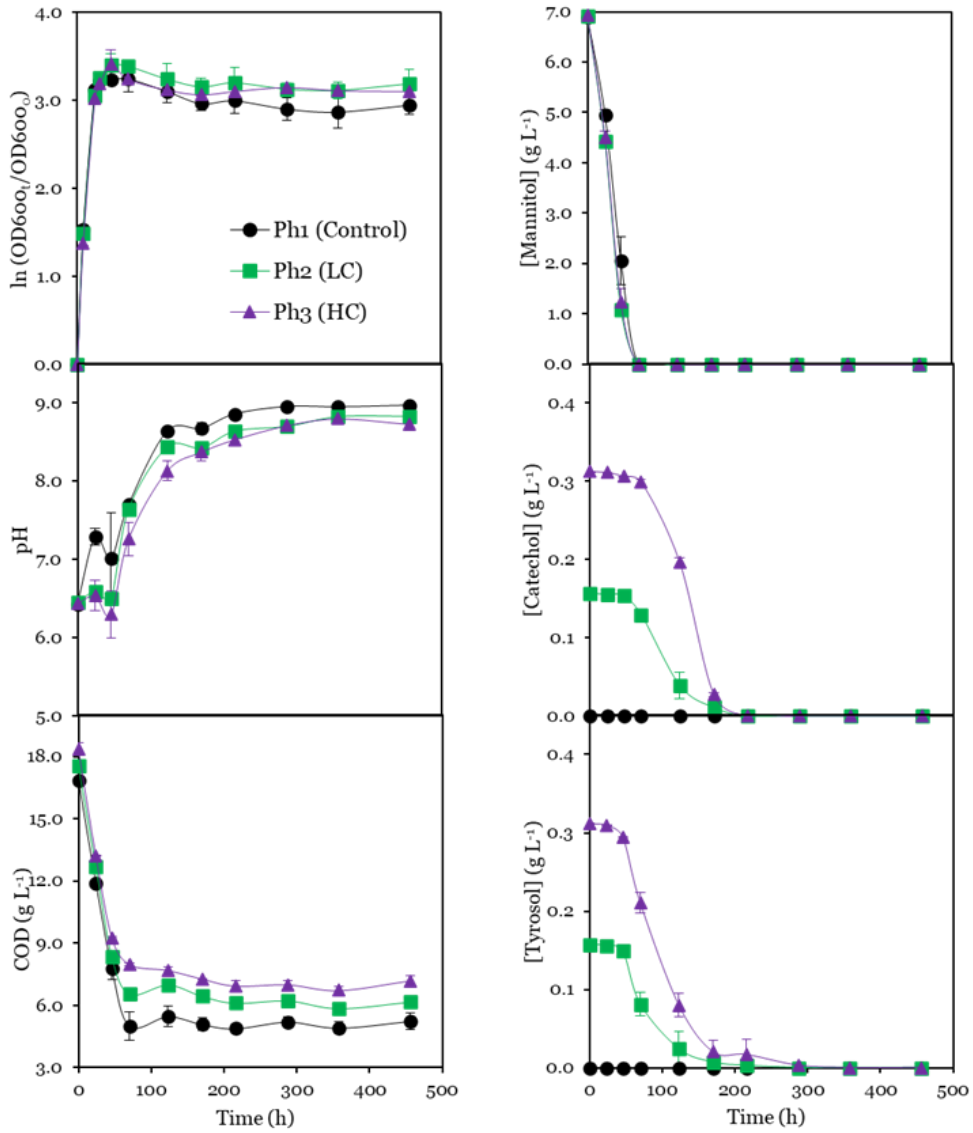


Figure 4.6: Profile of growth (ln (OD600t/OD6000), pH, COD, [Mannitol], [Catechol] and [Tyrosol] of *A. aceti* fermentation in basal media and with low concentration LC (0.15 g L^{-1}) and high concentration HC (0.30 g L^{-1}) of both catechol and tyrosol.

Table 4.4: Initial growth rates, biomass yield, substrate consumption rate, and COD for *A. aceti* fermentation in basal media and with 0.15 and 0.30 g L^{-1} of both catechol and tyrosol for low concentration LC and high concentration HC, respectively.

Parameter	Control (Ph1)	LC (Ph2)	HC (Ph3)
Initial μ (h^{-1})	0.12500 ± 0.00004	0.123 ± 0.002	0.1230 ± 0.0001
R^2	0.967	0.970	0.981
$Y_{X/M}$	0.778 ± 0.099	0.759 ± 0.077	0.813 ± 0.178
Q_M Mannitol (g L^{-1} h^{-1})	0.098 ± 0.000	0.098 ± 0.001	0.099 ± 0.000
Q_M Phenols (g L^{-1} h^{-1})	-	0.001 ± 0.000	0.002 ± 0.000
COD removal (%)	69 ± 2.118	65 ± 0.941	61 ± 1.895

The observed pH variation for all assays presented similar curves. The studied phenols did not affect the pH of the basal media used in the experiments. presenting in this way no difference in pH for control and media containing phenolic compounds. It can be observed for the three assays, as for the growth curves, confirming that the presence of phenols, at the experimental conditions studied, did not influence significantly the *A. aceti* metabolism.

Although phenols had no inhibitory effect on *A. aceti* growth, it was evaluated whether the bacterium was able to degrade these compounds and how their presence affected the other organic compounds' degradation. Figure 4.6 displays the COD decay along the assays Ph1-Ph3. COD removal was effective during the first 70 hours of the experiment, where the removal rate was around 65% for the three assays (Table 4.4), showing that there was no inhibitory effect from phenols. Still, the COD removal followed the sequence control > LC> HC, which can be due to the higher initial COD of Ph2 and Ph3.

Phenolic compounds, as well as mannitol concentration, were followed along the experiment by HPLC analysis. Figure 4.6 shows the profile of mannitol concentration with phenolic compounds. As indicated before, the presence of tyrosol and catechol did not affect mannitol consumption, being mannitol completely consumed until the 70 hours of the experiment, matching the time at which COD removal ceased. Mannitol consumption rates (Table 4.4) were practically the same for all assays.

Catechol and tyrosol decays during the experiment are also presented in Figure 4.6. It can be seen that *A. aceti* can degrade catechol and tyrosol, but this only occurred around 70 h of the experiment, when there was no longer mannitol available. Tyrosol consumption started earlier than catechol, indicating that it was first degraded by *A. aceti*. Still, after 300 h of the experiment, both catechol and tyrosol were completely consumed, independently of their initial concentration. Although metabolism products were expected, they were not detected at the HPLC analysis conditions applied. The phenolic compounds consumption rate (Table 4.4) was very small, 0.001 and 0.002 g L^{-1}, for LC and HC, respectively. HC presented the double value of the LC since the concentration of phenolic compounds was also double. The low values are explained due to *A. aceti* taking much longer to degrade phenolic compounds (300 h) than it takes to degrade mannitol (70 h).

A global analysis of the results obtained in the Ph experiment allows to conclude that, although phenolic compounds represent an environmental concern due to their toxicity, they do not inhibit the *A. aceti* growth and its ability to degrade the organic compounds, including the phenols. The overall results of A, CA, and Ph experiments show that *A. aceti* presents high resistance and degradation ability to the most significant organic compounds in winery wastewater.

4.5 Winery wastewater characterization and studies with *A. aceti*

The WW used in this work was collected in March 2024 from Adega Cooperativa do Fundão C.R.L., before being submitted to any treatment, and was kept refrigerated until its use. WW characterization is presented in Table 4.5. To ensure that *A. aceti* was the only bacterium present during the fermentation experiments, the WW sample was autoclaved, for 20 minutes at 121 ºC, before its use. The characterization of the autoclaved WW is also presented in Table 4.5.

Table 4.5: Characterization of the utilized winery wastewater before and after autoclaving.

Parameter	Unit	Collected WW	Autoclaved WW
Chemical oxygen demand	g L^{-1}	208.7 ± 0.8	157.2 ± 0.3
Total organic carbon	g L^{-1}	52.0 ± 0.4	41.3 ± 0.4
Total nitrogen	g L^{-1}	1.23 ± 0.03	1.12 ± 0.04
pH	–	3.82 ± 0.06	3.68 ± 0.05
Ethanol	g L^{-1}	110.5 ± 0.2	66.5 ± 0.1
Glycerol	g L^{-1}	10.4 ± 0.1	9.24 ± 0.03
Acetic acid	g L^{-1}	1.74 ± 0.02	1.58 ± 0.02
Lactic acid	g L^{-1}	2.70 ± 0.02	2.39 ± 0.01
Succinic acid	g L^{-1}	1.89 ± 0.01	1.71 ± 0.01
Tartaric acid	g L^{-1}	1.86 ± 0.01	1.59 ± 0.01

When comparing the wastewater used in the test with Table 1.1, which shows the characteristic composition of wine wastewater, we can see some differences. Firstly, we can see that the wastewater used has very high COD and TOC values, with the TOC value exceeding the range values shown in the table and the COD value is very close to the maximum value, even though it is within the range. Looking at the compounds detected and quantified by HPLC, we can see that all the compounds present in the collected wastewater are in much higher quantities than those shown in Table 1.1., concluding that we are dealing with highly charged wastewater.
Although it is unknown which part of the process the effluent originated, these comparisons and the fact that it is such a highly charged wastewater suggest that it probably came from the first wash of the reactor, justifying the values presented.

It is possible to see that the process of sterilization affected mainly the quantity of ethanol present in wine wastewater, which passed from 110.5 to 66.5 g L^{-1}. As a consequence, the COD also changed after the sterilization process.

Considering that the *A. aceti* fermentation experiments utilizing model solutions of alcohols, carboxylic acids, and phenols revealed promising features for the use of this bacterium in WW

degradation, a fermentation experiment utilizing a real WW was performed, to evaluate the behavior of *A. aceti* with whole WW matrix. Assays (WW1, WW2, and WW3) were prepared as described in Table 3.3, with the control assay (WW1) in basal media, WW containing only the inoculum (WW2), and WW with inoculum plus supplements for basal media (WW3).

Figure 4.7 shows the *A. aceti* growth profiles from the experiments done with WW, obtained from the experimental data presented in Appendix I—Supplementary Material, Table S8. pH, COD, and TOC variation and HPLC determinations of concentrations from mannitol, ethanol, glycerol, acetic, tartaric, lactic, and succinic acid, as well as xylose.

Control assay maintained a growth profile, similar to the ones obtained in the studies with the various groups of inhibitors. The obtained initial growth rate, 0.128 h^{-1} (Table 4.4) is very similar to the obtained in the assay with phenolic compounds. Due to the impossibility of obtaining the correct values of biomass, the dry weight was not calculated, from the OD, as performed for other assays. Only COD and TOC variation was measured, as well as HPLC analysis from the collected samples. It can be seen that the growth of *A. aceti* is strongly inhibited in the WW matrix, being incapable of reaching the level of growth attained by the control experiment (WW1) for both tested conditions. Nonetheless, throughout the experiment, the growth was very slow. The growth profile for WW2 and WW3 is similar at the beginning of fermentation. Yet, the supplementation in WW3 allowed an improvement in bacterial growth after 1128 h. Conversely, WW2 did not present this same behavior, probably due to the lack of components that constricted the growth of *A. aceti*. The initial growth rate was 0.0180 and 0.0160 h^{-1} for WW2 and WW3, respectively (Table 4.6), and was about 1000 smaller than the control assay. This confirms the higher *A. aceti* growth inhibition in the presence of winery wastewater. WW2 could grow with the reminiscent source of carbon present in WW, but after its depletion stabilized. WW3 presented the typical diauxic growth as the one observed in the experiments with alcohols.

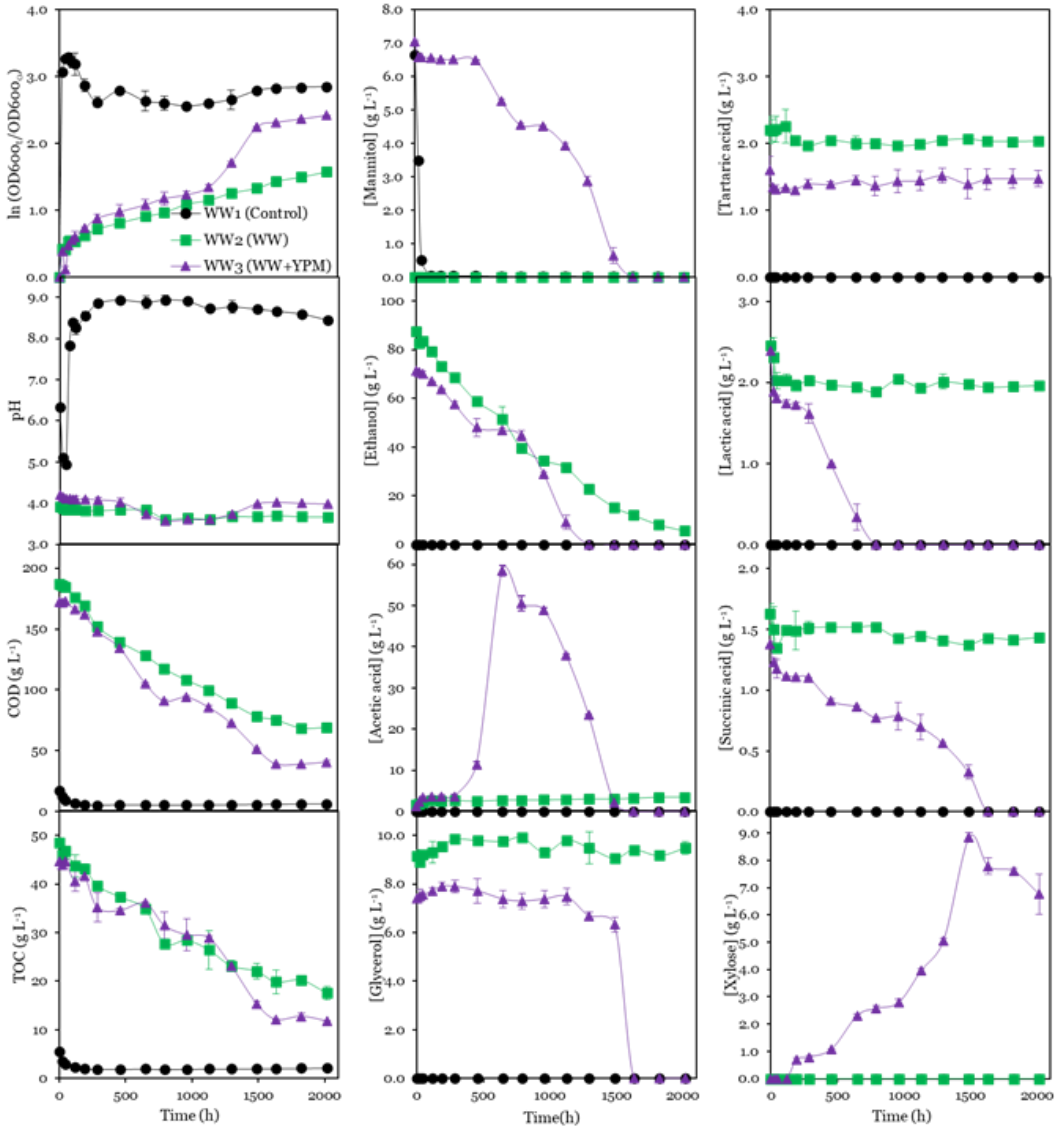


Figure 4.7: Profile of growth (ln (OD600t/OD6000), pH, COD, TOC, [Mannitol], [Ethanol], [Acetic acid], [Glycerol], [Tartaric acid], [Lactic acid], [Succinic acid] and [Xylose] of fermentation of *A. aceti* in basal media, WW (WW2) and WW+basal media supplements (WW3).

Table 4.6: Initial growth rates, COD, and TOC removal for *A. aceti* in basal media, WW (WW2), and WW+basal media supplements (WW3).

Parameter	Control (WW1)	WW2	WW3
Initial μ (h^{-1})	0.128 ± 0.001	0.0180 ± 0.0004	0.0160 ± 0.0001
R^2	1	1	1
COD removal (%)	69 ± 2.118	65 ± 0.941	61 ± 1.895
TOC removal (%)	66 ± 0.244	62 ± 7.007	69 ± 1.843

Related to pH variation along assays WW1-WW3, it was possible to see that pH from the control was very different from the assays with WW. The fact of not having added a buffer to maintain the

pH of the assay could explain the differences observed. This same fact may have helped the limitation of *A. aceti* to grow properly.

The ability of *A. aceti* to reduce the organic load in the real and complex WW matrix was evaluated through COD and TOC determinations along the assays. Figure 4.7 shows that *A. aceti* could reduce the COD from approximately 180 g L^{-1} to values below 70 g L^{-1} in WW2 and 40 g L^{-1} in WW3, respectively. The difference observed between WW2 and WW3 is due on one hand, to the lack of supplementation in assay WW2, where the bacteria could not develop properly to metabolize proportionally, as happened with WW3. On the other hand, the difference in the pH of the WW could also contribute to the difference. The higher removal observed in WW3 suggests that the presence of mannitol, yeast extract, and peptone is beneficial for the biodegradation process.

As for TOC removal, also displayed in Figure 4.7, it can be seen that it followed the same trend as that of COD, indicating a high mineralization degree of the organic compounds, even at such a high concentration. These results promoted the removal of around 65% (Table 4.6) for both COD and TOC in all tested conditions. Keep in mind that the time allowed to ferment in this trial was four times more than the inhibitor's tolerance tests.

The variation of some of the relevant compounds in experiment WW was monitored through HPLC determinations. Mannitol concentration consumption in control and WW3 (as no mannitol was added in WW2), shows that, for WW3, mannitol consumption occurs only after the 456 h of the fermentation probably due to the strong *A. aceti* inhibition caused by the winery wastewater. Still, after 1632 h the bacterium had already consumed all the mannitol available. On the other hand, in the control, mannitol was completely consumed during the first 120 hours.

Figure 4.7 also presents the ethanol and acetic acid variations during experiment WW. Ethanol was progressively consumed by the bacterium during the experiment, being completely consumed after 1296 h in WW3, while in WW2 a residual concentration remained in the solution after the 2016 h of fermentation. Ethanol consumption in WW2 did not result in acetic acid formation, as acetic acid concentration remained practically unchanged during the experiment. However, in assay WW3, acetic acid formation was observed. These results indicate that supplementation plays a key role in the ethanol bioconversion into acetic acid.

Regarding the decay of tartaric and lactic acids during the WW experiment, as observed in the CA experiment, *A. aceti* was not able to degrade tartaric acid under the experimental conditions utilized. As for lactic acid, it was not consumed in WW2, while in WW3, it was completely degraded after 792 h.

Similar behavior to that described for lactic acid was also observed for succinic acid, with insignificant consumption in WW2, and complete degradation in WW3 after 1632 h of fermentation.

Glycerol and xylose concentrations were also monitored during the WW experiment. Along with lactic and succinic acids, glycerol was not consumed in WW2, while in WW3 was completely degraded. In the WW3 assay, it is worth noting that succinic acid consumption became more pronounced when all the lactic acid was degraded (around 1000 h) and glycerol consumption became effective when all the succinic acid was degraded (around 1500 h). As for xylose, its formation was only observed in the WW3 assay, following the profile already described for the previous studies with inhibitors, being initially formed as a product of *A. aceti* metabolism, and then consumed by it.

The overall results obtained in the WW experiment allow to conclude that, although *A. aceti* growth is strongly inhibited by the winery wastewater, this bacterium can degrade most of the organic compounds present in WW, especially if supplemented. The addition of a proper buffer can also have a positive effect.

Chapter 5
Conclusion and Future Perspectives

The main goal of this work was to evaluate the ability of the bacterium *Acetobacter aceti* to resist the compounds present in winery wastewater, as well as to study the performance of this microorganism, known for its ability to oxidize various types of alcohols and sugars, in the biodegradation of winery wastewater.

A. aceti growth can reach the exponential phase of its growth in 48h in YPM or basal media. In the basal media, the initial growth rate was 0.147 ± 0.006 h^{-1} with a biomass yield of 0.562 ± 0.009. The fermentation study of *A. aceti* with basal media with alcohols, carboxylic acids, and phenolic compounds showed in general, at the experimental conditions studied, the bacterium was tolerant to the three groups studied.

The presence of alcohols slightly reduced the initial growth rate, and the biomass yield, even with the highest. The profile growth presented a diauxic growth, resulting from the conversion of ethanol to acetate, this second metabolite used as a carbon source. Mannitol consumption was reduced to 59 and 44% of the control for lower and higher concentration *vs* control assay. COD and TOC removal in the presence of alcohol did not affect its value.

The presence of carboxylic acids had a higher effect on the growth of *A. aceti*. In our study, the pH was not corrected to basal media, but still, the bacteria could grow. The initial growth rate was affected, with increasing carboxylic acid concentration decreasing to 61 and 43% of the one presented by the control. Growth profile at higher concentrations of acids presented a diauxic growth, resulting from the conversion of acetic acid to xylose and its further use as a carbon source. The tartaric could not be metabolized by *A. aceti*, in both tested concentrations. Mannitol consumption was practically the same as the control, reducing the higher concentration to 93% of the control assay.

The studied phenolic compounds did not affect *A. aceti* growth, hence growth rate, biomass yield, and mannitol consumption rate did not change in the presence of tyrosol and catechol. COD also was practically the same as the control.

When *A. aceti* was exposed to real winery wastewater, a differentiated behavior was observed. In the conditions where the essential nutrients (mannitol, yeast extract, and peptone) were not supplemented to the winery wastewater, the bacterium was unable to degrade glycerol, lactic acid, and succinic acid, indicating that the presence of these essential nutrients might play a role in the *A. aceti* degradation mechanism, especially when the bacterium is exposed to extreme conditions. Throughout the experiments, the formation of bacterial metabolic products was observed, namely acetic acid from ethanol degradation and xylose. Still, *A. aceti* was able to degrade them as well,

transforming its metabolic products into substrates. The time needed to metabolize the different substrates/inhibitors present in the WW was 4 times longer than the tests with model solutions. Regardless of the inhibitory effect observed, *A. aceti* found it easy to degrade almost all the compounds to which was exposed. The exception to this was the tartaric acid, which the bacterium was never able to degrade, both for the experiment with the carboxylic acids model solution and the experiment with real winery wastewater.

In conclusion, the work developed in this study revealed that *A. aceti* metabolism can be inhibited by the presence of some of the winery wastewater constituents, to a greater or lesser extent depending on the type and concentration of the inhibitor compound. Still, for all the experimental conditions assayed, including the experiment with real and highly polluted winery wastewater, *A. aceti* was able to grow and degrade most of the organic compounds present, thus presenting a great potential to be applied in winery wastewater treatment.

As for future work perspectives, *A. aceti* association with other bacteria should be studied, aiming for a microbial consortium that leads to the degradation of a wider range of pollutants, thus increasing the efficiency of the treatment process. The combination of this biological process with electrochemical technology, in the form of a microbial fuel cell, to allow the simultaneous winery wastewater treatment and electricity production, should be pursued, in a sustainable and circular economy approach.

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Appendix I

Supplementary Material

Table S1: Experimental data from the *A. aceti* growth curve procedure.

Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
0	1	0.026	0.024	0.025	0.025
4	1	0.032	0.027	0.030	0.030
8	1	0.089	0.060	0.074	0.074
12	1	0.371	0.261	0.316	0.316
20	2	0.732	0.597	0.664	1.329
24	5	0.481	0.406	0.444	2.218
28	5	0.791	0.679	0.735	3.674
32	10	0.564	0.504	0.534	5.336
36	10	0.668	0.609	0.638	6.383
48	10	1.127	1.042	1.085	10.845
56	20	0.659	0.620	0.639	12.780
68	20	0.702	0.603	0.653	13.053
76	20	0.629	0.639	0.634	12.673
84	20	0.662	0.707	0.684	13.688
100	20	0.559	0.620	0.589	11.783
104	20	0.631	0.600	0.615	12.305
120	20	0.632	0.681	0.656	13.128
166	20	0.594	0.627	0.610	12.203

[a] – Mean value of the different absorbance values measured.
[b] – Obtained from the product of dilution factor and mean value.

Table S2: Experimental data from the *A. aceti* dry weight procedure.

Dilution	Absorbance	m_{filter} (g)	$m_{filter+bacterium}$ (g)	Dry weight (g L^{-1})
1:2	2.254	0.1038	0.1301	0.526
1:5	1.677	0.1038	0.1221	0.366
1:10	1.064	0.1038	0.1134	0.192
1:20	0.564	0.1038	0.1086	0.096
1:40	0.309	0.1038	0.1066	0.056
1:50	0.240	0.1038	0.1060	0.044

Table S3: Experimental data from the fermentation experiment A-I.

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	0	10	0.330	0.3265	0.328	3.283
	2	10	0.301	0.3155	0.308	3.083
A1	24	10	0.323	0.328	0.325	3.253
	48	10	0.344	0.3525	0.348	3.480
	72	10	0.358	0.3845	0.371	3.710

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	96	10	0.369	0.342	0.355	3.553
	144	10	0.340	0.3555	0.348	3.478
	192	10	0.498	0.358	0.428	4.280
	264	10	0.360	0.414	0.387	3.868
	312	10	0.319	0.3235	0.321	3.210
	360	10	0.389	0.376	0.383	3.825
	432	10	0.352	0.3425	0.347	3.470
	504	10	0.344	0.336	0.340	3.400
	624	10	0.347	0.329	0.338	3.378
A2	0	10	0.332	0.332	0.332	3.318
	2	10	0.302	0.312	0.307	3.068
	24	10	0.325	0.351	0.338	3.375
	48	10	0.329	0.337	0.333	3.325
	72	10	0.337	0.345	0.341	3.408
	96	10	0.352	0.347	0.349	3.490
	144	10	0.347	0.355	0.351	3.510
	192	10	0.366	0.350	0.358	3.575
	264	10	0.356	0.344	0.350	3.495
	312	10	0.318	0.322	0.320	3.198
	360	10	0.365	0.373	0.369	3.685
	432	10	0.346	0.345	0.345	3.450
	504	10	0.339	0.350	0.345	3.445
	624	10	0.332	0.342	0.337	3.365
A3	0	10	0.323	0.326	0.325	3.245
	2	10	0.306	0.319	0.312	3.120
	24	10	0.333	0.464	0.398	3.980
	48	10	0.337	0.337	0.337	3.365
	72	10	0.337	0.367	0.352	3.520
	96	10	0.344	0.370	0.357	3.568
	144	10	0.340	0.346	0.343	3.428
	192	10	0.336	0.351	0.343	3.430
	264	10	0.357	0.365	0.361	3.608
	312	10	0.330	0.330	0.330	3.300
	360	10	0.382	0.384	0.383	3.828
	432	10	0.337	0.315	0.326	3.258
	504	10	0.354	0.358	0.356	3.560
	624	10	0.324	0.344	0.334	3.340

[a] – Mean value of the different absorbance values measured.
[b] – Obtained from the product of dilution factor and mean value.

Table S4: Experimental data from the fermentation experiment A-II.

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	0	2	0.300	0.308	0.304	0.608
	22	20	0.748	0.681	0.714	14.285
	48	20	0.849	0.843	0.846	16.920
	72	20	0.710	0.738	0.724	14.470
A4	124	20	0.625	0.846	0.735	14.705
	196	20	0.552	0.589	0.570	11.400
	292	20	0.469	0.511	0.490	9.800
	388	20	0.390	0.446	0.418	8.355
	456	20	0.476	0.488	0.482	9.640
	0	2	0.313	0.326	0.319	0.639
	22	20	0.417	0.397	0.407	8.140
	48	20	0.401	0.411	0.406	8.120
	72	20	0.531	0.582	0.557	11.130
A5	124	20	0.505	0.535	0.520	10.395
	196	20	0.451	0.471	0.461	9.220
	292	20	0.395	0.404	0.399	7.985
	388	20	0.251	0.277	0.264	5.275
	456	20	0.270	0.267	0.268	5.365
	0	2	0.320	0.314	0.317	0.634
	22	20	0.367	0.382	0.375	7.490
	48	20	0.358	0.350	0.354	7.075
	72	20	0.342	0.341	0.341	6.825
A6	124	20	0.413	0.442	0.427	8.545
	196	20	0.357	0.352	0.354	7.080
	292	20	0.220	0.290	0.255	5.095
	388	20	0.206	0.209	0.207	4.145
	456	20	0.203	0.204	0.203	4.060

[a] – Mean value of the different absorbance values measured.
[b] – Obtained from the product of dilution factor and mean value.

Table S5: Experimental data from the fermentation experiment A-III.

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	0	1	0.496	0.492	0.494	0.494
	3	1	0.764	0.721	0.742	0.742
A7	6	1	1.246	1.153	1.200	1.200
	9	5	0.695	0.563	0.629	3.145
	14	10	0.777	0.501	0.639	6.388

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	23	20	0.635	0.286	0.461	12.065
	27	20	0.618	0.259	0.439	12.320
	33	20	0.688	0.264	0.476	13.610
	47	20	0.756	0.288	0.522	15.220
	57	20	0.750	0.314	0.532	15.000
	71	20	0.774	0.398	0.586	15.120
	77	20	0.690	0.358	0.524	13.900
	95	20	0.642	0.488	0.565	12.785
	105	20	0.689	0.684	0.686	13.725
	120	20	0.637	0.644	0.640	12.805
	129	20	0.635	0.640	0.637	12.745
	144	20	0.627	0.628	0.627	12.540
	153	20	0.617	0.621	0.619	12.375
	167	20	0.526	0.528	0.527	10.530
	191	20	0.467	0.470	0.468	9.365
	200	20	0.443	0.440	0.441	8.820
	0	1	0.475	0.489	0.482	0.482
	3	1	0.696	0.710	0.703	0.703
	6	1	1.135	1.162	1.148	1.148
	9	5	0.550	0.573	0.561	2.806
	14	10	0.446	0.452	0.449	4.490
	23	20	0.274	0.278	0.276	5.520
	27	20	0.239	0.236	0.237	4.745
	33	20	0.238	0.267	0.253	5.050
	47	20	0.272	0.276	0.274	5.480
	57	20	0.296	0.313	0.304	6.085
A8	71	20	0.379	0.429	0.404	8.080
	77	20	0.362	0.438	0.400	7.990
	95	20	0.422	0.486	0.454	9.080
	105	20	0.573	0.590	0.582	11.630
	120	20	0.556	0.516	0.536	10.715
	129	20	0.513	0.536	0.525	10.490
	144	20	0.507	0.461	0.484	9.675
	153	20	0.504	0.422	0.463	9.260
	167	20	0.433	0.464	0.448	8.965
	191	20	0.387	0.489	0.438	8.755
	200	20	0.455	0.462	0.458	9.165
A9	0	1	0.485	0.480	0.483	0.483

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	3	1	0.706	0.700	0.703	0.703
	6	1	1.142	1.134	1.138	1.138
	9	5	0.581	0.573	0.577	2.884
	14	10	0.455	0.474	0.464	4.643
	23	20	0.291	0.317	0.304	6.075
	27	20	0.234	0.229	0.231	4.625
	33	20	0.217	0.212	0.214	4.285
	47	20	0.227	0.246	0.236	4.725
	57	20	0.226	0.253	0.240	4.790
	71	20	0.242	0.266	0.254	5.075
	77	20	0.253	0.259	0.256	5.110
	95	20	0.288	0.285	0.286	5.720
	105	20	0.343	0.356	0.349	6.985
	120	20	0.322	0.324	0.323	6.455
	129	20	0.295	0.310	0.303	6.050
	144	20	0.411	0.393	0.402	8.035
	153	20	0.407	0.407	0.407	8.135
	167	20	0.435	0.450	0.443	8.850
	191	20	0.419	0.441	0.430	8.595
	200	20	0.456	0.468	0.462	9.230

[a] – Mean value of the different absorbance values measured.
[b] – Obtained from the product of dilution factor and mean value.

Table S6: Experimental data from the fermentation experiment CA.

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	0	1	0.524	0.539	0.532	0.532
	6	2	0.831	0.839	0.835	1.670
	12	10	0.697	0.695	0.696	6.958
	22	20	0.555	0.541	0.548	10.955
	28	20	0.614	0.600	0.607	12.130
	34	20	0.643	0.658	0.650	13.005
CA1	46	20	0.721	0.726	0.723	14.465
	56	20	0.780	0.729	0.754	15.080
	73	20	0.666	0.799	0.732	14.645
	97	20	0.685	0.785	0.735	14.700
	121	20	0.663	0.630	0.646	12.925
	144	20	0.687	0.711	0.699	13.975
	168	20	0.565	0.562	0.563	11.265

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	192	20	0.627	0.586	0.606	12.125
	217	20	0.505	0.513	0.509	10.175
	250	20	0.513	0.489	0.501	10.015
	288	20	0.412	0.565	0.488	9.765
CA2	0	1	0.503	0.493	0.498	0.498
	6	2	0.284	0.265	0.274	0.549
	12	10	0.103	0.103	0.103	1.030
	22	20	0.148	0.147	0.147	2.940
	28	20	0.196	0.222	0.209	4.180
	34	20	0.276	0.309	0.292	5.840
	46	20	0.427	0.455	0.441	8.815
	56	20	0.517	0.576	0.546	10.920
	73	20	0.605	0.607	0.606	12.115
	97	20	0.580	0.562	0.571	11.420
	121	20	0.542	0.518	0.530	10.600
	144	20	0.571	0.658	0.614	12.285
	168	20	0.602	0.397	0.499	9.985
	192	20	0.522	0.329	0.425	8.505
	217	20	0.510	0.396	0.453	9.060
	250	20	0.478	0.290	0.384	7.675
	288	20	0.468	0.353	0.410	8.205
CA3	0	1	0.512	0.505	0.508	0.508
	6	2	0.270	0.267	0.268	0.536
	12	10	0.095	0.094	0.094	0.940
	22	20	0.089	0.086	0.087	1.740
	28	20	0.090	0.095	0.092	1.845
	34	20	0.101	0.111	0.106	2.110
	46	20	0.106	0.111	0.108	2.165
	56	20	0.113	0.117	0.115	2.295
	73	20	0.108	0.122	0.115	2.295
	97	20	0.213	0.172	0.192	3.845
	121	20	0.544	0.507	0.525	10.505
	144	20	0.602	0.583	0.592	11.845
	168	20	0.577	0.599	0.588	11.755
	192	20	0.550	0.582	0.566	11.320
	217	20	0.557	0.547	0.552	11.040
	250	20	0.540	0.590	0.565	11.300

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	288	20	0.531	0.543	0.537	10.740

[a] – Mean value of the different absorbance values measured.
[b] – Obtained from the product of dilution factor and mean value.

Table S7: Experimental data from the fermentation experiment Ph.

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
Ph1	0	2	0.314	0.325	0.319	0.639
	8	5	0.603	0.585	0.594	2.969
	24	20	0.712	0.730	0.721	14.415
	31	20	0.797	0.796	0.796	15.925
	46	20	0.820	0.796	0.808	16.155
	70	20	0.891	0.751	0.821	16.415
	123	20	0.762	0.661	0.711	14.220
	170	20	0.643	0.593	0.618	12.355
	216	20	0.694	0.585	0.640	12.790
	288	20	0.625	0.538	0.581	11.625
	358	20	0.629	0.501	0.565	11.290
	456	20	0.640	0.574	0.607	12.140
Ph2	0	2	0.327	0.308	0.318	0.635
	8	5	0.574	0.553	0.563	2.815
	24	20	0.672	0.679	0.676	13.510
	31	20	0.862	0.785	0.823	16.465
	46	20	0.887	1.012	0.949	18.980
	70	20	0.971	0.900	0.936	18.710
	123	20	0.734	0.892	0.813	16.260
	170	20	0.715	0.769	0.742	14.835
	216	20	0.706	0.852	0.779	15.580
	288	20	0.706	0.739	0.722	14.445
	358	20	0.688	0.738	0.713	14.255
	456	20	0.705	0.835	0.770	15.395
Ph3	0	2	0.322	0.327	0.324	0.649
	8	5	0.521	0.509	0.515	2.574
	24	20	0.664	0.666	0.665	13.290
	31	20	0.782	0.788	0.785	15.690
	46	20	0.871	1.110	0.990	19.805
	70	20	0.859	0.797	0.828	16.555
	123	20	0.758	0.709	0.733	14.665
	170	20	0.699	0.690	0.694	13.885

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	216	20	0.744	0.698	0.721	14.415
	288	20	0.758	0.753	0.755	15.100
	358	20	0.752	0.705	0.728	14.565
	456	20	0.725	0.715	0.720	14.400

[a] – Mean value of the different absorbance values measured.
[b] – Obtained from the product of dilution factor and mean value.

Table S8: Experimental data from the fermentation experiment WW.

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
WW1	0	1	0.535	0.554	0.544	0.544
	24	20	0.591	0.586	0.589	11.770
	48	20	0.720	0.714	0.717	14.335
	72	20	0.744	0.725	0.735	14.690
	96	20	0.702	0.646	0.674	13.475
	120	20	0.730	0.598	0.664	13.270
	192	20	0.505	0.456	0.480	9.600
	288	20	0.346	0.402	0.374	7.475
	456	20	0.421	0.464	0.442	8.845
	648	20	0.334	0.428	0.381	7.615
	792	20	0.389	0.349	0.369	7.370
	960	20	0.348	0.353	0.350	7.005
	1128	20	0.360	0.372	0.366	7.315
	1296	20	0.422	0.356	0.389	7.775
	1488	20	0.449	0.436	0.442	8.845
	1632	20	0.456	0.462	0.459	9.180
	1824	20	0.459	0.472	0.465	9.300
	2016	20	0.478	0.460	0.469	9.375
WW2	0	1	1.105	1.128	1.116	1.116
	24	10	0.168	0.174	0.171	1.705
	48	10	0.169	0.168	0.168	1.683
	72	10	0.201	0.182	0.192	1.915
	96	10	0.194	0.193	0.193	1.930
	120	10	0.189	0.194	0.191	1.913
	192	10	0.209	0.208	0.208	2.083
	288	10	0.230	0.230	0.230	2.295
	456	10	0.250	0.254	0.252	2.520
	648	10	0.273	0.281	0.277	2.770
	792	10	0.300	0.289	0.294	2.943

Assay	Time (h)	Dilution factor	Absorbance (600 nm)		Mean value [a]	OD600 [b]
	960	10	0.329	0.339	0.334	3.338
	1128	10	0.355	0.353	0.354	3.535
	1296	10	0.406	0.383	0.394	3.940
	1488	10	0.427	0.419	0.423	4.225
	1632	10	0.468	0.469	0.468	4.683
	1824	10	0.496	0.505	0.501	5.005
	2016	10	0.534	0.548	0.541	5.410
WW3	0	1	1.705	1.698	1.701	1.701
	24	10	0.251	0.250	0.250	2.503
	48	10	0.198	0.183	0.190	1.903
	72	10	0.276	0.273	0.274	2.743
	96	10	0.303	0.295	0.299	2.988
	120	10	0.330	0.296	0.313	3.128
	192	10	0.350	0.357	0.354	3.535
	288	10	0.397	0.427	0.412	4.120
	456	10	0.423	0.486	0.454	4.543
	648	10	0.477	0.530	0.503	5.033
	792	10	0.516	0.592	0.554	5.540
	960	10	0.567	0.604	0.585	5.853
	1128	10	0.674	0.639	0.656	6.563
	1296	10	0.959	0.926	0.942	9.423
	1488	10	1.614	1.601	1.607	16.073
	1632	10	1.729	1.717	1.723	17.230
	1824	10	1.824	1.808	1.816	18.158
	2016	10	1.917	1.912	1.915	19.145

[a] – Mean value of the different absorbance values measured.
[b] – Obtained from the product of dilution factor and mean value.