Universidade do Minho
Escola de Engenharia

Cristiana da Mota Martins Gonçalves

Biotechnological valorization of olive mill wastewaters

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Doctoral Dissertation for PhD degree in Chemical and Biological Engineering

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This is perhaps the easiest and hardest chapter that I have to write. It will be simple to name all the people that who helped to get this done, but it will be though to thank them enough.

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In the beginning there was yeast, and it raised bread, brewed beer, and made wine. After many not days but centuries and even millennia later, it was named Saccharomyces cerevisiae. After more years and centuries there was another yeast, and it was named Schizosaccharomyces pombe; now there were two stars in the yeast heaven. In only a few more years there were other yeasts, and then more, and more, and more. The era of the non-conventional yeasts had begun.

Spencer *et al.* (2002)

LIST OF PUBLICATIONS

This thesis is based on the following original articles and full paper*:

Gonçalves C., Pereira C., Alves M., Belo I., (2010), Olive Mill Wastewater as a renewable resource, *Environmental Engineering and Management Journal*, **9** (3) 319-325;

Gonçalves C., Lopes M., Ferreira J.P., Belo I., (2009), Biological treatment of olive mill wastewater by non- conventional yeasts, *Bioresource Technology*, **100** (15) 3759-3763;

Lopes M., Araújo C., Aguedo M., Gomes N., **Gonçalves C.**, Teixeira J. A., Belo I., (2009), The use of olive mill wastewater by wild type *Yarrowia lipolytica* strains: medium supplementation and surfactant presence effect, *Journal of Chemical Technology and Biotecnhology*, **84**(4), 533-537;

Gonçalves C., Alves M., Belo I., A perspective for OMW valorization through integration of lipase production fermentation and anaerobic digestion, (2010), *Proceedings of the 3rd International Symposium on Energy from Biomass and Waste - Venice 2010 Symposium,* Italy.

The following **submitted** articles were also used:

Gonçalves C., Oliveira F., Belo I., Fed-batch fermentation of olive mill wastewaters for lipase production (May 2011);

Gonçalves C., Oliveira F., Ferreira J. P., Belo I., Influence of phenolic compounds from OMW on the *Yarrowia lipolytica*, *Candida cylindracea* and *Candida rugosa* growth (June 2011).

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* *According to article 8 paragraph 2 of the Portuguese Decree-Law No. 388/70, I certify that were used in this study results published in the publications discriminated.*

Some **methods** of analysis were adapted, due to the complex media used, which resulted in the following publications:

Gomes N., **Gonçalves C**., García-Román M., Teixeira J. A., Belo I., (2011), Optimization of a colorimetric assay for yeast lipase activity in complex systems, *Analytical Methods*, DOI:10.1039/C0AY00680G;

Gonçalves C., Rodriguez-Jasso R. M., Gomes N., Teixeira J. A., Belo I., (2010), Adaptation of dinitrosalicylic acid method to microtiter plates, *Analytical Methods*, **2** (12), 2046-2048.

SUMMARY

Mediterranean countries are known to have favorable conditions for olive oil production. The three-phase extraction technology demands the addition of hot water to the process, and olive oil, olive cake and olive mill wastewater (OMW) are produced. An approach for using this waste as a renewable resource is of greater interest. Accordingly, the present investigation aims the OMW valorization, by producing high-value compounds (lipase and methane) while degrading this waste. Thus, the research work presented in this thesis essentially describes a study about an integrated process, where the effluents are firstly submitted to a lipase producing aerobic fermentation, followed by an anaerobic degradation process, to produce methane. This work is of great interest, since Portugal is one of the world leading producers of olive oil, with crescent production values from campaign to campaign, in the last years.

This work was started with a study about the major problem attributed to the olive mill wastewaters (OMW), the phenolic compounds toxicity. These experiments showed that the non-conventional yeasts *Y. lipolytica*, *C. rugosa* and *C. cylindracea* are able to grow in different phenolic compounds, usually found in OMW. This was later confirmed in bioreactor batch experiments with OMW-based media, where the studied yeasts, not only were capable of achieving similar cell growth, to glucose synthetic media, but also to highly consume the existing and analyzed phenolic compounds. The experiments on batch fermentations, with OMW-based media, were then performed in Erlenmeyer baffled flasks, in order to study the effect of ammonium, cell and surfactant addition as well as to investigate the use of different yeast strains; *Candida rugosa* (PYCC 3238 and CBS 2275), *Candida cylindracea* CBS 7869 and *Yarrowia lipolytica* (CBS 2073, W29 ATCC 20460 and IMUFRJ 50682); and different non-diluted OMW. *C. cylindracea* was the best strain concerning the effluent degradation. After preliminary tests, a study of optimal batch and fed-batch conditions was performed in bioreactor, using *Candida rugosa* CBS 2275, *Candida cylindracea* CBS 7869 and *Yarrowia lipolytica* W29 ATCC 20460. It was confirmed that *C. cylindracea* CBS 7869 was the best lipase-producing yeast (6 U mL^{-1}). However, using a fed-batch strategy, with cell pre-growth directly on the bioreactor, *C. rugosa* CBS 2275 was the strain that obtained the best values of lipase activity (17 U mL^{-1});

achieving at the same time, a significant effluent degradation (64 % of COD, 27 % of phenolics and 77 % of total lipids). Anaerobic biodegradability tests showed that the aerobic treatment had a positive effect on the anaerobic degradation of the OMW. Best results were achieved for the initial concentration of 5 g COD-treated OMW L^{-1}, where 78 % of the COD added was recovered as methane. Furthermore, when the COD degradation in the aerobic step was higher, even better results were possible to achieve, with a faster conversion of COD to methane.

The obtained results demonstrates that the olive mill wastewaters (OMW) are becoming a competitive and valuable growth medium in fermentation processes and also the potential application of non-conventional lipolytic yeasts for OMW valorization, for biomass and enzymes production. This treatment was successful to detoxify the effluent, having a very positive effect in the anaerobic digestion. The utilization of this valorization process will possibly have a positive impact on the environmental problem of OMW management.

RESUMO

Os países que envolvem o Mediterrâneo são conhecidos pelas suas condições favoráveis para a produção de azeite. A tecnologia de extracção de azeite com o sistema em contínuo de 3 fases exige a adição de água quente ao processo, o que dá origem à produção de azeite, águas ruças (OMW) e bagaço. A utilização deste efluente (águas ruças) como um recurso renovável apresenta-se como uma abordagem de elevado interesse, a este problema. Deste modo, o presente trabalho de investigação tem como finalidade a valorização das águas ruças, com produção de produtos de elevado interesse industrial (lipase e metano) enquanto se diminui a carga poluente do resíduo. Assim, o trabalho de investigação apresentado nesta tese descreve essencialmente o estudo de um processo integrado, onde os efluentes são primeiramente submetidos a uma fermentação aeróbia com produção de lipase, à qual se segue um processo de degradação anaeróbia com produção de metano. Este trabalho é de elevado interesse, dado que Portugal está entre os líderes mundiais de produção de azeite, com crescentes valores de produção de campanha para campanha, nos últimos anos.

Assim, este trabalho foi iniciado com um estudo focado no problema mais importante associado às águas ruças, a toxicidade dos compostos fenólicos. Estes ensaios mostraram que as leveduras não-convencionais *Y. lipolytica*, *C. rugosa* e *C. cylindracea* são capazes de crescer nos diferentes compostos fenólicos, que frequentemente constituem as águas ruças. Estes resultados foram mais tarde confirmados em bioreactor, onde as referidas leveduras não só foram capazes de crescer, tanto ou até melhor do que crescem em meio de glucose, como também foram capazes de consumir uma parte considerável de cada composto fenólico, acompanhado ao longo das fermentações. Foram então iniciados os ensaios em descontínuo, em matrazes utilizando meio de águas ruças, de modo a fazer um estudo preliminar sobre o efeito da adição de amónio, surfactante e a da concentração de células, bem como o efeito do uso de diferentes leveduras, nomeadamente *Candida rugosa* (PYCC 3238 e CBS 2275), *Candida cylindracea* CBS 7869 e *Yarrowia lipolytica* (CBS 2073, W29 ATCC 20460 e IMUFRJ 50682) e diferentes amostras de águas ruças não diluídas. Foram seleccionadas algumas condições e a *C. cylindracea* foi a melhor levedura no que diz respeito à degradação do efluente. Depois destes

testes preliminares, estudos sobre as condições óptimas a utilizar em descontínuo e semi-contínuo foram efectuados em bioreactor, utilizando *Candida rugosa* CBS 2275, *Candida cylindracea* CBS 7869 e *Yarrowia lipolytica* W29 ATCC 20460. Concluiu-se que a *C. cylindracea* CBS 7869 é a melhor produtora de lipase em fermentações em descontínuo (6 U mL^{-1}), no entanto, utilizando o modo semi-contínuo, com pré-crescimento das células directamente no reactor a *C. rugosa* CBS 2275 surge como a melhor levedura produtora de lipase, com 17 U mL^{-1}, e com óptimos valores de degradação do efluente (64 % de CQO, 27 % de compostos fenólicos totais e 77 % dos lípidos totais). Os testes de biodegradabilidade anaeróbia demonstraram que a etapa de tratamento aeróbio, que os antecede, tem um efeito positivo na produção de metano. Em ensaios posteriores, foram obtidos melhores resultados para a concentração inicial de 5 g L^{-1} CQO de águas ruças tratadas, com 78 % de conversão do CQO em metano. Mais, verificou-se que quanto melhor é a degradação de CQO na etapa aeróbia do processo integrado, melhores foram os resultados na etapa seguinte, com uma conversão de CQO em metano mais rápida.

Os resultados obtidos demonstram que as águas ruças se estão a tornar num meio de crescimento competitivo e importante, mas também o potencial das leveduras lipolíticas não-convencionais nos processos fermentativos, para a valorização de águas ruças, com produção de enzimas e biomassa. O sucesso destes processos, na posterior etapa de digestão anaeróbia teve um efeito bastante positivo. A utilização deste processo integrado de valorização de águas ruças poderá assim ter um impacto benéfico para a resolução do problema relacionado com a gestão ambiental das águas ruças.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

OMW	Olive Mill Wastewaters
COD	Chemical oxygen demand
pH	Power of hydrogen
TS	Total solids
TVS	Total volatile solids
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
RS	Reducing Sugars
TP	Total Protein
YPD	Yeast Peptone Dextrose media
YNB	Yeast Nitrogen Base media
YPD+P	Yeast Peptone Dextrose media supplemented with a phenolic compound
YNB+P	Yeast Nitrogen Base media supplemented with a phenolic compound
C/N	Carbon-to-nitrogen ratio
YPD+G	Yeast Peptone Dextrose media with extra glucose
LCFA	Long chain fatty acids
Log N/N_0	Decimal logarithm of growth ration
N	Final cell concentration
N_0	Initial cell concentration

CHAPTER 1. INTRODUCTION, RESEARCH AIM AND THESIS OUTLINE

1.1. INTRODUCTION

The olive oil is one of the most important elements in the traditional Mediterranean diet. Olive mill production is a traditional agricultural industry in Mediterranean basin countries, which are known to have favorable conditions for olive oil production. Spain is the most important producer, followed by Italy, Greece, Syria, Tunisia, Turkey, Morocco and Portugal (International Olive Oil Council – IOOC, November 2010).

The olive oil is obtained by two main extraction methods: press (traditional) and continuous (solid-liquid centrifuge) processes. The continuous system can be operated by three- and two-phase extraction technologies, diverging in the water supplies. The three-phase extraction process has a slightly better yield, leading to less amount of olive cake but a significant production of olive mill wastewater, OMW (Roig *et al.*, 2006).

The olive mill wastewater is a stable emulsion composed of ''vegetation waters'' of the olives, water from the processing, olive pulp and oil (Lanciotti *et al.,* 2005). This emulsion contains fats, sugars, phosphates, phenols and metals (Scioli and Vollaro, 1997) and it is also characterized by high values of acidity, organic load, solids content (Eusebio *et al.*, 2002) and a lack of nitrogen. The quality and quantity of olive mill wastewater (OMW) constituents depends on many factors, such as, type of olives, type of soil and production process (D'Annibale *et al.*, 2006; Roig *et al.*, 2006).

The large variety of components found in OMW difficult their treatment (Niaounakis and Halvadakis, 2004) and its incorrect disposal, causes serious environmental problems, such as coloring natural waters, threat to the aquatic life (decrease of fish population), surface and ground water pollution, soil quality, plant growth and odors (Akdemir and Ozer, 2008).

Regarding the seasonality of olive oil production and also that the olive mills are usually small enterprises, scattered around the olive production areas (Paraskeva and Diamadopoulos, 2006), the OMW treatment process should be adjusted to this reality. Several disposal methods have been proposed and mainly include physical-chemical treatments but the most common method applied has been the storage of OMW in lagoons, followed by evaporation during summer season (Azbar *et al.*, 2004). Biological treatment by aerobic microorganisms (fungi and yeasts) has also been

proposed (Eusébio *et al.*, 2002). Yeast species such as *Yarrowia lipolytica* and *Candida cylindracea* can grow well in OMW media, consume the organic material and, at the same time, produce biomass and other valuable products (Scioli and Vollaro, 1997; D'Annibale *et al.*, 2006), like enzymes and organic acids. The anaerobic biological degradation of OMW can lead to methane production and has been the subject of several studies (Boubaker and Ridha, 2010; El-Gohary *et al.*, 2009a; Hamdi, 1996; Ubay and Ozturk, 1997; Beccari *et al.*, 1996; Ergüder *et al.*, 2000).

1.2. RESEARCH AIM

Conventional wastewater treatment methods are relatively ineffective to remove OMW pollutants and, instead of disposal solutions, an approach of using this waste as a renewable resource is of great interest (D'Annibale *et al.*, 2006; Scioli and Vollaro, 1997). For that reason, the research work presented in this thesis aims the OMW valorization, by producing high-value compounds (lipase) while degrading this waste, in a two-step process, where the effluents are submitted to a lipase producing aerobic fermentation that contributes for a partial OMW degradation, followed by an anaerobic methanogenic degradation process to produce methane.

1.3. THESIS SYNOPSIS

This thesis is structured in 9 chapters.

The present section (**CHAPTER 1**) is a general introduction with background information, in which the olive mill wastewater (OMW) problematic is summarily described as well as some treatment methods already proposed. The global research aim is also presented.

The **CHAPTER 2** refers to the **literature review**, where is given a general overview on the current knowledge about the olive mill wastewater, starting with the olive oil production and its by-

products; followed by the OMW characteristics description, some legislation concerning the effluents and the existing valorization methods to be applied to OMW.

In **CHAPTER 3** the main **Materials and Methods** are described. Starting with the microorganisms, their growth and preserving methods. Afterwards, a description of the collection, preservation and storage procedures of the used OMW, as well as its characteristics is presented. The analytical methods are closing this chapter, where methods of the parameters employed to characterize the OMW samples and to monitor fermentation performances are presented. Further specific methods are presented on its proper chapter, in the "Materials and methods" sub-sections.

The different sections of the **Experimental Work** performed, are presented **from CHAPTER 4 to CHAPTER 7**. In these chapters a brief *introduction*, specific *materials and methods*, *results and discussion* and *conclusions* for each subject are given.

In CHAPTER 4, microscale experiments, for the study of the phenolic compounds effect on yeasts growth, are reported. CHAPTER 5 embraces the preliminary batch cultures of six yeast strains, using OMW based media in flasks. The study of batch and fed-batch conditions, in bioreactor, is reported on CHAPTER 6. In the CHAPTER 7 a preliminary integration of the aerobic optimized process and the anaerobic process is described.

Final conclusions as well as suggestions for future work in this field of research are given in **CHAPTER 8**.

Finally, the **CHAPTER 9** gathers all the references used in the elaboration of this work.

CHAPTER 2. LITERATURE REVIEW

Mediterranean countries are known to have favorable conditions for olive oil production. The three-phase extraction technology demands the addition of hot water to the decanter, and olive oil, olive cake and olive mill wastewater (OMW) are produced. An approach for using this waste as a renewable resource is of greater interest. Several authors have been studding physicochemical treatment methods. However, the biological treatments allow not only the treatment but also the effluent valorization, by producing several valuable products, such as methane, biohydrogen, enzymes and organic acids. This effluent is also a source of natural antioxidants (such as hydroxytyrosol) and its extraction is economically attractive. The ideal OMW valorization process could be achieved by the combination of methods, for instance the use of physical-chemical methods as pre-treatment can highly reduce the pollutants concentrations and allows better production efficiency by microorganisms.

This Chapter contains information published in:

Gonçalves C., Pereira C., Alves M., Belo I., (2010), Olive Mill Wastewater as a renewable resource, *Environmental Engineering and Management Journal*, **9** (3) 319-325.

2.1. OLIVE OIL PRODUCTION

Olive oil has been used for centuries in the countries surrounding the Mediterranean Sea. The olive tree (*Olea europaea*), Figure 2.1 – A, grows almost exclusively in these countries where it is a familiar element of the landscape. The olive tree is a small evergreen tree that averages 3 to 5 m in height. The olive tree requires a mild winter and a warm and dry summer with frequent rainfalls from autumn to early spring, the typical climate in Mediterranean area but also in California, parts of Western and South Australia, southwestern South Africa, in isolated sections of Central Asia, and parts of central Chile. The olive tree yield is greatly affected by a biennial cycle: one year it grows and the other year gives more fruits. Therefore more olive oil and wastes are generated every other year (Azbar *et al.*, 2004; Boskou, 2006).

The olive fruit (Figure 2.1 - B) contains water (up to 70 %), which is called "vegetable" water. The average chemical composition of the olive fruit is: water, 50 %; protein, 1.6 %; oil 22 %; carbohydrates, 19 %; cellulose, 5.8 %; minerals (ash), 1.5 % (Boskou, 2006). Other important constituents are pectins, organic acids, pigments, and glycosides of phenols. In the Mediterranean countries, for example, the fruit weight increases in various phases until October or mid-November. Then, it begins to decrease, basically through loss of moisture. As a result, a rise in oil content is observed, usually from October to December. The oil accumulation starts in the period from late July to beginning of August. Through the autumn and winter, the fruit becomes black and the oil content reaches its maximum. Oil is mainly concentrated in the pericarp (96-98 %) (Boskou, 2006).

Figure 2.1 A. Olive tree and **B.** olive fruit.

Thus, it is not surprising that the world's olive oil is mainly produced in Mediterranean countries (Figure 2.2).

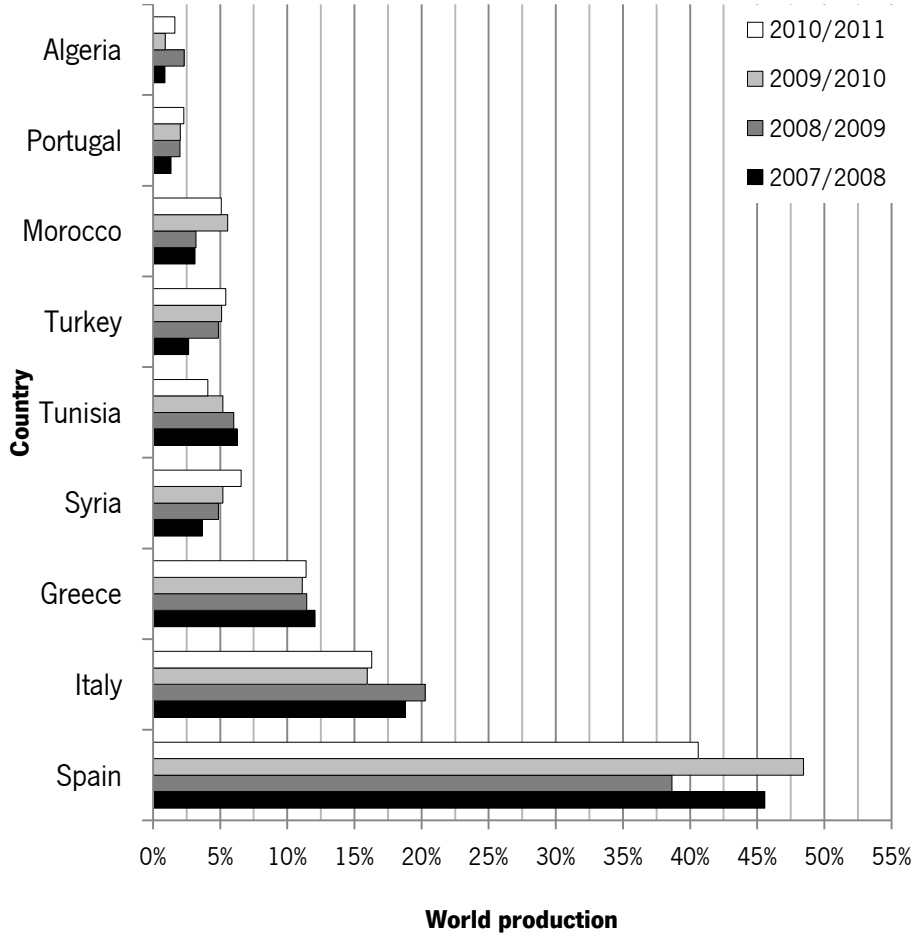

Figure 2.2 World olive oil production (Based on data from the International Olive Oil Council – IOOC, November 2010). The 2010/2011 values are predicted and 2009/2010 provisory.

Spain, Italy and Greece are the most significant olive oil producers with 48 %, 16 % and 11 % of the 2009/2010 world's production, respectively. Portugal was responsible for 2 % (58700 tons) of the world's production in 2009/2010, and 67500 tons were expected for 2010/2011 (International Olive Oil Council statistical series, November 2010). In fact, the Portuguese Olive Oil Association "Casa do Azeite" announced (February 2011) that the Portuguese production in the 2010/2011 campaign amounted 70 000 tons, being the fifth world's leading producer of olive oil. In 2010 it was reported, in several news sources (such as "*Exame* n° 315", a Portuguese magazine), that the

largest olive grove in the world, with 9 700 hectare (or 9.7 x 10^7 m^2) is from *Sovena*, a Portuguese company. The group's headquarters is in Alentejo (Portugal) and their most famous oils are **Andorinha** and **Oliveira da Serra**, in Portugal; **Soleada**, in Spain; and **Olivari**, in Tunísia.

The olive is an environmentally friendly fruit; high quantities of chemicals are unnecessary for its growth and less energy is required for its processing. Olive oil production is expected to be environmentally friendly due to the low-energy and chemicals-free manufacturing process, too. However, generation of large quantities of highly polluted wastes during processing of olives is inevitable (Azbar *et al.*, 2004). Olive oil is produced from olives in olive mills either by the discontinuous press method (traditional) or by the continuous centrifugation (solid-liquid) method (Figure 2.3).

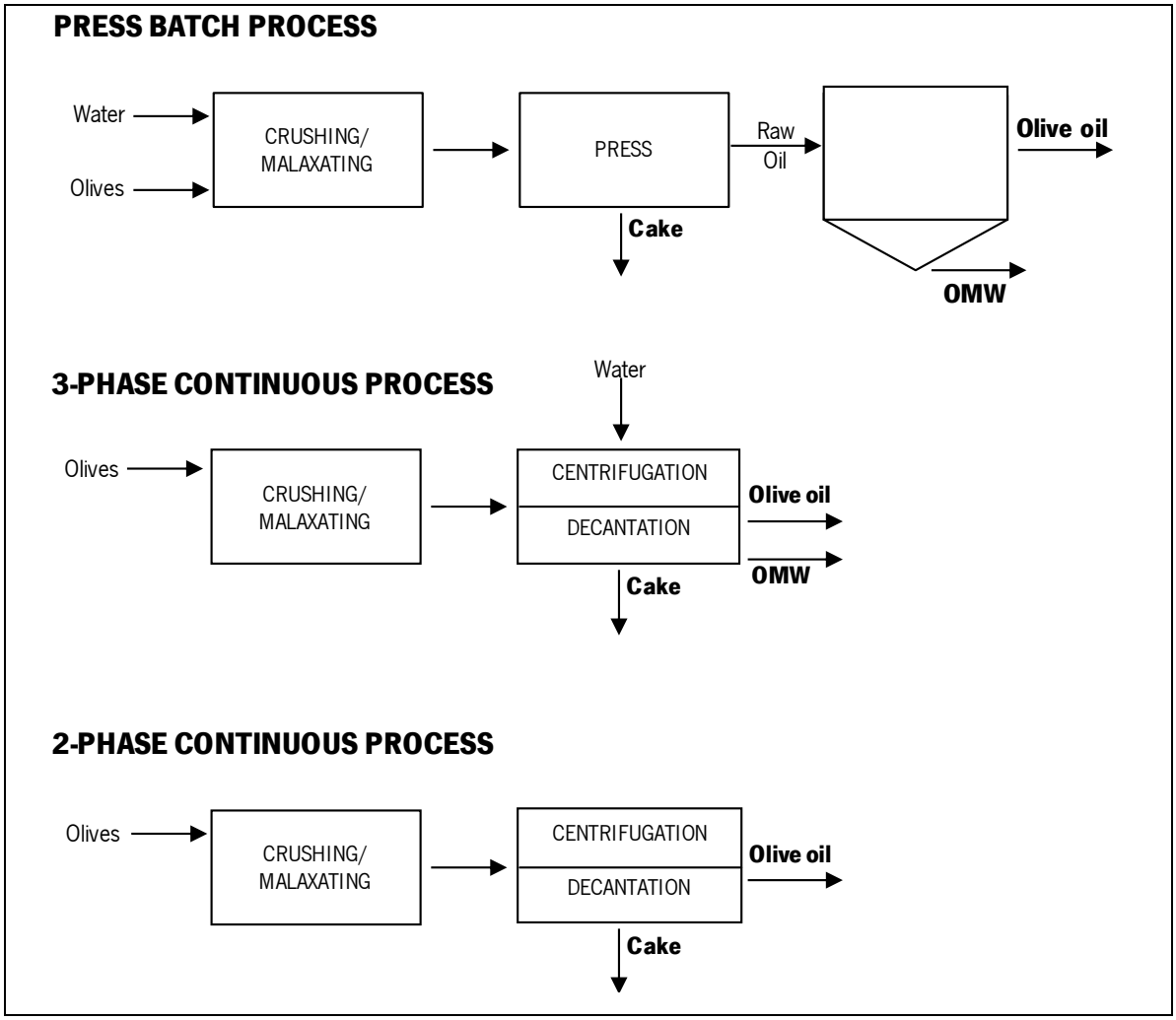

Figure 2.3 Olive oil extraction processes (Azbar *et al.*, 2004).

In the traditional press process, the olives are washed, crushed, and kneaded with the addition of hot water. The resulting paste is then pressed to drain the oil and the liquid waste originating from presses consists of a mixture of olive juice and added water and contains residual oil. Finally, olive oil is separated from the water by vertical centrifugation or decanting (Azbar *et al.*, 2004). The use of the traditional process has decreased and nowadays it is almost only employed in small olive mills.

The continuous system can be operated by three and two-phase extraction technologies, diverging in the water supplies. While the two-phase system does not require the addition of water, producing olive oil and olive cake; the three-phase demands the addition of hot water to the decanter, producing olive oil, olive mill wastewater (OMW) and olive cake (residual solids), Figure 2.4. As a result of these differences, the three-phase extraction process has a slightly better yield, leading to less amount of olive cake but a significant production of olive mill wastewater (Roig *et al.*, 2006). The traditional cold press method typically generates about 50 % of OMW, relative to the initial weight of the olives, while the continuous centrifugation process generates (80 – 110) % of OMW (Mantzavinos *et al.*, 2005). In spite of the seasonal olive oil production, it is estimated that the annual OMW production in the Mediterranean Sea area exceeds 30 x 10^6 m^3 (Casa *et al.*, 2003; Quarantino *et al.*, 2007; Mantzavinos *et al.*, 2005).

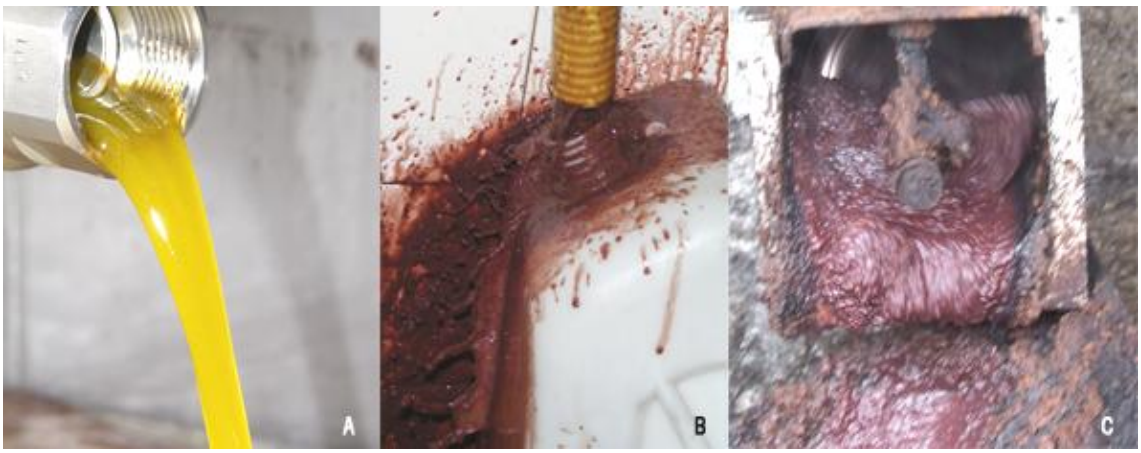

Figure 2.4 A. olive oil, **B.** olive mill wastewater (OMW) and **C.** olive cake (residual solids). Pictures were taken in the olive mill of Amarante ("Lagar de Azeite das Carvalhinhas").

In 2003, Portugal mostly produced olive oil by the traditional press process (about 85 %) followed by the 3-phase process, with approximately 10 % of the production (Roig *et al.*, 2006; IMPEL, 2003). However, considering current statistics about olive oil production in Portugal (INE, 2009), about 70 % of the total olive oil is produced in 2-phase olive mills, 20 % and 9 % are produced in 3-phase and traditional olive mills, respectively. Moreover, most of the olive oil is produced in Alentejo (52 %) where the olive oil is mainly produced by the 2-phase extraction process (91 %).

In the present work, the olive mill wastewaters were collected in the north region of Portugal, responsible for 17 % of the olive oil production, where 17 % of the olive oil is produced in 3-phase olive mills; and 72 % and 11 % on 2-phase and traditional olive mills (INE, 2009).

2.2. OLIVE MILL WASTEWATER CHARACTERISTICS

The olive mill wastewaters are stable emulsions composed of water, olive pulp and residual oil (Lanciotti *et al.*, 2005). The quality and quantity of OMW constituents are determined by many factors, such as, the olives type and maturity, the climacteric conditions and region of origin, the cultivation methods and the technology used for oil extraction (Roig *et al.*, 2006). This dark-coloured wastewater contains fats, sugars, phosphates, phenols and metals (Scioli and Vollaro, 1997). It is also characterised by high values of acidity, organic load, solids content (Eusebio *et al.*, 2002) and the lack of nitrogen.

The most important organic compounds found in the OMW are sugars, tannins, polyphenols, polyalcohols, pectins and lipids (Asses *et al.*, 2009a; Papanikolaou *et al.*, 2008).

The fatty acids commonly found in OMW are acetic acid (C2) and propionic acid (C3), iso-butiric (i-C4), butyric (C4), isovaleric (i-C5), valeric (C5), hexanoic/caproic (C6), octanoic/caprylic (C8), palmitic (C16), palmitoleic (C16:1), stearic (C18), oleic (C18:1) and linoleic (C18:2), (Eusebio *et al.,* 2002; Papanikolaou *et al.,* 2008; Procida and Ceccon, 2006). The free fatty C2–C8 acids come from microbial metabolism, whereas the C16–C18, showing both phytotoxicity and toxic effects towards microorganisms, come from the oil originally present in the olives (Procida and Ceccon, 2006).

The olive pulp is very rich in phenolic compounds but only 2 % of the total phenolic content of the olive fruit remains in the oil phase, while the remaining is lost in the OMW (approximately 53 %) and in the pomace (approximately 45 %) (Rodis *et al.*, 2002). In fact, the phenolic compounds presence are one of the major problems of this effluent since they are difficult to biodegrade (Mantzavinos, *et al.*, 2005; Papanikolaou *et al.*, 2008) due to the phytotoxic effects and anti-microbial activity (Hamdi, 1996; Eusebio *et al.*, 2002; Lanciotti *et al.*, 2005; Quaratino *et al.*, 2007). The phenolic compounds usually found in OMW are benzoic acid; salicylic acid; 2,4 dihydroxybenzoic acid; tyrosol; 2,4 dihydroxybenzaldehyde; syringic acid; 4-hydroxybenzoic acid; caffeic acid (3,4-dihydroxycinnamic acid); syringaldehyde; p-Coumaric acid; ferulic acid, hydroxytyrosol; 3,4 dihydroxyphenyl acetic acid; vanillic acid (El-Gohary *et al.*, 2009b; Asses *et al.*, 2009b).

Table 2.1 shows a summary of OMW characterization obtained by several authors (Hajjouji *et al.*, 2008; Brozzoli *et al.*, 2009; Asses *et al.*, 2009a and 2009b; Aouidi *et al.*, 2009; Tziotzios *et al.*, 2007; Quaratino *et al.*, 2007; Martinez-Garcia *et al.*, 2007; Khoufi *et al.*, 2009; El-Gohary *et al.,* 2009a; Fezzani and Cheikh, 2007; Dhaouadi and Marrot, 2008; Ahmadi *et al.*, 2006; D'Annibale *et al.*, 2006; Chatzisymeon *et al.*, 2008), including the characterizations obtained during this work.

Table 2.1 The OMW characterization: average and boundary-values of the considered literature

Parameter/units	Average	Maximum	Minimum
pH	5.1	5.6	4.6
TS/(g L^{-1})	93.1	190.5	10.5
TSS/(g L^{-1})	24.3	59.7	3.0
TVS/(g L^{-1})	75.4	117.0	7.3
TOC/(g L^{-1})	29.2	65.0	5.8
TN kj/(g L^{-1})	0.8	2.5	0.1
COD/(g L^{-1})	92.9	234.0	19.2
BOD/(g L^{-1})	28.9	60.0	10.6
Total phenols/(g L^{-1})	4.3	12.1	0.7
Total Lipids/(g L^{-1})	4.9	20.0	0.1
Color ($A_{390\ nm}$)	109.0	150.5	72.0
Total sugars/(g L^{-1})	26.0	52.4	3.4
Total proteins/(g L^{-1})	1.0	1.3	0.5

The incorrect disposal of this effluent causes serious environmental problems, such as, coloring natural waters, threat to the aquatic life (decrease of fish population), surface and ground water pollution, soil quality and plant growth and odor (Akdemir and Ozer, 2008).

2.3. RELEVANT LEGISLATION

The agro-industries activity exerts a negative effect on the environment. The sector of the olive oil production is one of the most worrying since it is responsible for the discharge of the olive mill wastewaters, an effluent with high concentrations of organic load (Table 2.1), to water courses. The **Directive 2000/60/EC**, establishing a framework for Community action in the field of water policy, says that member states shall establish standards for the release of certain pollutants, such as biocides and substances that have an unfavorable influence on oxygen balance. The aim of the directive is to make the waters of the European Union safe and clean by 2015. The OMW is directly under the scope of this Directive, together, with other national regulations. The Portuguese

Decree Law No. 74/90 of March 7, repealed by Decree Law 236/98 of August 1 (Water-Law) establishes quality standards, criteria and objectives in order to protect the water environment and improve the water quality considering its main uses.

Some of the legal limits for the discharge of wastewater, presented in Decree-law 236/98 are summarized in Table 2.2.

Table 2.2 Summary of emission limit values for the discharge of wastewater (ELV)

Parameter	ELV
pH	6 - 9
TSS/(mg L^{-1})	60
COD/(g L^{-1})	0,15
Color	*Not visible with a dilution of 1:20*
Fats/(mg L^{-1})	15
TN/(mg L^{-1})	15
Phenolics/(mg L^{-1} of phenol)	0.5

Comparing the ELV (Table 2.2) with the usual parameters of OMW characterization (Table 2.1) it is possible to state that the OMW characteristics far exceeded the limits established by law for discharge, being vital the treatment of this effluent.

The **Council Directive 96/61/EC** on Integrated Pollution Prevention and Control (IPPC), which aims to achieve a high degree of environmental protection for the environment, states that the companies working on certain polluting activities must take all appropriate preventative measures against pollution. Thus, the IPPC directive forces the companies to request and obtain an authorization (which certifies that the proper environmental protection measures are undertaken) from the authorities in order to continue their activity (Panagopoulos and Malliaros, 2002). Concerning this feature, the Portuguese legislation include the **Joint Order Number 626/2000**

(of June 6). This joint order authorizes the use of OMW to irrigate the cultivation soils. The *point* 5 refers that the licensing of the use of OMW for agricultural land irrigation should take into consideration the following aspects:

i). The existence of a tank or pond for storage of all OMW produced during the campaign;

ii). The need to carry out a proper pre-treatment, namely by adjusting the pH;

iii). The use of OMW for irrigation should preferably be made between the months of March to November of each year and must take into account the weather conditions verified in each year;

iv). The OMW should be applied only in shrub or tree crops, while no further studies are performed;

v). The OMW volumes to be used for irrigation should not exceed in any case, 80 m^3 ha^{-1} $year^{-1}$; until there is no further study, to substantiate and justify the change in volume to be applied.

Moreover, the program for the environmental measures implementation to modernize the sector of oil mills by the **Joint Order Number 118/2000 (of July 31)** should also be considered. This document establishes that in the 2000 campaign only can labor the olive mills that gather the following pre-requisites:

i). Have completed the environmental adaptation measures;

ii). Have in physical implementation the appropriate of environmental adaptation measures;

iii). Have submitted, until July 31, 2000, a licensing process to the competent authorities.

2.4. OLIVE MILL WASTEWATER TREATMENT METHODS

The olive oil sector requires improvements in environmental and quality profiles of the whole production chain for economic competitiveness (Azbar *et al.*, 2004). Conventional wastewater treatment methods are relatively ineffective for removing OMW pollutants. Instead of disposal solutions, an approach of using this waste as a renewable resource is of greater interest (Scioli and Vollaro, 1997; D'Annibale *et al.*, 2006). Research efforts have been made in order to extract, or reconvert, fine chemicals for instance: fatty acids, sterols and polyphenols (such as antioxidants), carbohydrates, polysaccharides, sugars and nitrogen compounds. Several studies have been carried out in order to degrade and use this effluent. They can be divided into physico-chemical and biological methods. The most common method applied has been the *lagooning*, the OMW storage in lagoons followed by evaporation during summer season (Azbar *et al.*, 2004; Angelidaki *et al.*, 2002). However, this method is not satisfactory since it only reduces the volume of waste without treating the pollutants and a black foul-smelling sludge, difficult to remove, is produced.

2.4.1. Non-biological processes

Several authors have been studding a numerous of physicochemical methods which, isolated or combined, are able to treat OMW, such as centrifugation, flocculation, coagulation, filtration, oxidation, incineration, ultrafiltration, (reverse) osmosis, ozonation and photolysis. These methods are normally expensive and usually they do not generate valuable products, however they are especially efficient on pollutants removal and consequently very useful as pre- and post-treatment.

Paraskeva *et al.* (2007) showed that is possible to reach high levels of OMW purification, using membrane technology, applying reverse osmosis after ultrafiltration. Akdemir and Ozer (2008) studied the ultrafiltration and concluded that it is possible to reach 80 % of COD removal, without any pretreatment. Drouiche *et al.* (2004), by combining an ultrafiltration with a UV/H_2O_2 oxidation process, accomplish an OMW treatment with an excellent efficiency, despite the dilution needed. In only 1.5 h, COD, COT, TSS degradation and decolourization was obtained.

The electrochemical oxidation OMW compounds over Ti/IrO_2 dimensionally stable anodes, was also investigated (Chatzisymeon *et al.*, 2008), and the decrease of COD and TOC, to low or moderate levels, is accompanied by colour and phenols removal.

Fenton oxidation is a very effective method in several organic pollutants removal. Kallel *et al.* (2009), using hydroxyl radicals generated from zero-valent iron and hydrogen peroxide, has shown that colour disappeared and the phenolic compounds concentration decreased to 50 % only after 3 h of reaction. Rizzo *et al.* (2008) found that coagulation by chitosan is very effective in the removal of TSS (81 %) but, on chitosan coagulated samples, the photo-Fenton oxidation achieved a better performance (e.g., 93 % COD removal) compared to the Fenton one (81 % COD removal).

The performance of platinium and rutherium in the catalytic wet air oxidation (CWAO), on OMW organic load reduction, was studied (Minh *et al.,* 2008) and at the conditions employed, a high amount of OMW pollutants mineralization was achieved. This author also demonstrated that using CWAO as pre-treatment enhanced the yield of methane production in anaerobic digestion.

Supercritical water oxidation (SCWO) can rapidly and efficiently destroy organic substances into H_2O and CO_2, in a significant short residence time. Erkonak *et al.* (2008) shown that SCWO is an effective treatment technology for OMW, increasing the treatment efficiency up to 99.96 % (TOC decomposition) in very short reaction times (10.02 s), using hydrogen peroxide as oxygen source. The most important parameter in the process is the reaction temperature, followed by the system pressure.

Najjar et al. (2009) reported on the catalytic wet hydrogen peroxide oxidation of crude OMW using Fe-BEA catalyst and reached reasonable degradation values of TOC (28 %), OMW phenolic molecules (40 %) and COD (30 %).

Andreozzi *et al.* (2008) tested four combined physicochemical processes: centrifugation-ozonation, centrifugation-solar photolysis, centrifugation-solar modified Fenton, centrifugation-solar Fenton-ozonation; but none was able to achieve COD removal higher than 74.7 %, even for prolonged treatment times.

The preliminary study of Santi *et al.* (2008) provided some results showing that a treatment using a mineral adsorbent (zeolite) could reduce the problems related with the OMW toxic organic loads (COD and polyphenols), easily recoverable after treatment by zeolite regeneration.

The extraction and purification of biologically active compounds (namely biophenols) turns OMW into a source of natural antioxidants. These compounds are object of growing interest in pharmaceutical and food industries since reactive oxygen species are involved in the onset of several human diseases and in the oxidative degradation of food (De Marco *et al.*, 2007).

2.4.2. Biological anaerobic processes

Anaerobic digestion is usually the basic biological process for OMW treatment, however it does not deal with the high organic load and toxicity of this effluent requiring high dilutions prior to treatment, and therefore, introducing serious cost and environmental implications (Khoufi *et al.*, 2009; El-Gohary *et al.*, 2009a). The anaerobic digestion advantages are related with the energy production (El-Gohary *et al.*, 2009a) and the possibility to start the digester after more than 8 months under non-feeding conditions (Martinez-Garcia *et al.*, 2007). In fact, by treating one cubic meter of OMW, 60–80 kWh of energy could be obtained (Schmidt and Knobloch, 2000).

Methane production

Khoufi *et al.* (2009) developed a new process for OMW treatment, which combines electro-Fenton (EF), anaerobic digestion and ultrafiltration (UF). EF-sedimentation procedure achieves high removal of COD (52.6 %), TSS (83.8 %), polyphenolic compounds (78 %) and lipids (93 %), which enhance the anaerobic activity, without any inhibition problems, and a high yield of methane (CH_4) production. Valorisation of biogas to energy is possible with the installation of an electricity generator and, after methanization, approximately 73.5 kWh m^{-3} can be recovered. El-Gohari *et al.* (2009a) recommended the use of a two stage anaerobic system consisting of a classical UASB reactor followed by a hybrid UASB as a post-treatment step for catalytically oxidized OMW, obtaining 59.4 % of methanogenesis. The same author (El-Gohary *et al.,* 2009b) found that the use

of Fenton's reaction as a primary treatment of OMW enhances the efficiency of anaerobic digestion. Azbar *et al.* (2008) obtained an increase of 80 % in biogas production when an OMW chemical pre-treatment is added before anaerobic digestion. The highest biogas production was achieved with the coagulation agent Al_2SO_4, 170 mL of total gas production, in 50 mL of working volume. Fountoulakis *et al.* (2008) found that methane can be produced very efficiently when co-digesting OMW with slaughterhouse water (volume of produced methane per mass of COD added was 0.170 m^3 kg^{-1}) and wine-grape residues (volume of produced methane per mass of COD added was 0.163 m^3 kg^{-1}). Moreover, Martinez-Garcia *et al.* (2007) achieved a biogas production rate of 1.25 L L^{-1} day^{-1} (volume of biogas per volume of reactor) and with OMW previously pre-treated aerobically with cheese whey, by *Candida tropicalis,* achieved 62 % of COD removal. Fezzani and Cheikh (2007) concluded that OMW could be degraded successfully in co-digestion with olive mill solid wastes under thermophilic conditions without previous dilution and without addition of chemical nitrogen substances. The best performance obtained by these authors in methane productivity was 46 L per liter of OMW added per day and 69 % of soluble COD removal efficiency. Dhouib *et al.* (2006) studied the OMW fungal pre-treatment with white rot fungi *Phanerochaete chrysosporium* DSM 6909, followed by anaerobic digestion and ultrafiltration treatment. *P. chrysosporium* decreased the relative toxicity (inhibition to *Vibrio fisheri*) from 100 % to 74 % and the anaerobic filter reactor worked without any apparent toxicity, being the OMW well converted into methane, with methanization yield, of methane per COD, of 0.3 L g^{-1}. Angelidaki *et al.* (2002) found that the combined digestion of OMW with swine manure reaches higher methane production rates than with these effluents alone, and, when using upflow anaerobic sludge blanket (UASB), a COD reduction up to 65 % was obtained. This authors also found that OMW alone needs the addition of nitrogen, achieving better degradation and methane production rates with COD:N ratio in a range of 42 to 61.

Biohydrogen production

Hydrogen is an ideal alternative energy source to substitute fossil fuels and can be obtained by changing the pH value and the hydraulic retention time (HRT) in order to obtain the appropriate microflora in an anaerobic reactor (Fountoulakis and Manios, 2009). Eroglu *et al.* (2008)

considered the effect of OMW clay pre-treatment process before photofermentative hydrogen production (with *Rhodobacter sphaeroides* O.U.001) and doubled the hydrogen production comparing with the isolated photofermentative hydrogen production. In a recently study, Eroglu *et al.* (2009) found that ozone and Fenton's reagent had a powerful effect on the color removal (90 %) but the resulting effluent was unsuitable for both hydrogen production and bacterial grow.

Ntaikou *et al.* (2009) studied the feasibility of using OMW as initial substrate, diluted 1:4 (v/v) with tap water, for combined biohydrogen and biopolymers production in a two stage system: anaerobic reactor operated at continuous mode and an aerobic sequential batch reactor. Hydrogen and volatile fatty acids (VFAs) were produced via anaerobic fermentation and subsequently, the acidified OMW was used as substrate for aerobic biodegradable polymer production. The main VFAs produced were acetate, butyrate and propionate, at different ratios depending on the hydraulic retention time (HRT) and it was possible to achieve biogas and hydrogen production rates of 633.6 mL day^{-1} and 201.6 mL day^{-1}, respectively. The aerobic reactor was inoculated with an enriched culture of *Pseudomonas putida*, and was operated in sequential cycles of nitrogen offer (growth phase) and nitrogen limitation (PHAs accumulation phase), achieving of 8.94 % PHA's per dry biomass weight, without optimization.

The use of glycerol as a co-substrate was evaluated with the intention of improving biogas and hydrogen production during the anaerobic treatment of a mixture (1:4) of OMW and slaughterhouse wastewater (Fountoulakis and Manios, 2009), obtaining 0.7 mmol of H_2 per gram of glycerol added. The supplementation with crude glycerol (1 % v/v) increased the methane production rate from 479 mL day^{-1} to 1210 mL day^{-1}.

2.4.3. Biological aerobic processes

The residual oil contained on OMW makes this effluent potentially suitable as a liquid growth medium for lipolytic microorganisms. Some lipolytic yeast species can grow well in OMW media, consume the organic material and, at the same time, produce biomass and other valuable products (Scioli and Vollaro, 1997; D'Annibale *et al.*, 2006), such as enzymes (lipases) and organic acids.

Lipases (triacylglycerol hydrolases EC 3.1.1.3) constitute the most important group of biocatalysts for biotechnological applications (Jaeger and Eggert, 2002). There are immense applications of lipase in food, dairy, detergent and pharmaceutical industries. They are also defined as glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. Lipase is a water-soluble enzyme that catalyzes esterification, interesterification, acidolysis, alcoholysis and aminolysis in addition to the hydrolytic activity on triglycerides (Joseph *et al.*, 2008). Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agro-chemicals, and flavour compounds (Jaeger and Eggert, 2002). Lipases are produced by a widespread number of microorganisms, bacteria (Jaeger *et al.*, 1994), yeast and fungi (Rapp and Backhaus, 1992). In particular, lipases produced by bacteria such as Pseudomonas sp. and fungi belonging to the genera *Penicillium*, *Rhizopus*, *Rhizomucor*, *Geotrichum* and *Candida* sp. are well-known industrial lipase producers. Microbial commercial lipases are mainly produced from *Pseudomonas*, *Mucor*, *Geotrichum*, *Rhizopus* and *Candida* spc (Gordillo *et al.*, 1998). A common characteristic in lipase production is the use of a lipid or related substance (triglycerides, fatty acids, detergents...), sole or jointly with sugars as carbon source, as inducer of the production under aerobic conditions. (Gordillo *et al.*, 1998)

Several works on OMW treatment and valorization, with lipase production, have been performed, using bacteria, fungi and yeasts.

Bacteria

Aouidi *et al.* (2009) investigated the improvement of OMW decolourization by *Lactobacillus paracasei* with cheese whey (CW) addition, and concluded that the proportion of OMW/CW that permits the highest colour (47 %) and phenolic compounds (22.7 %) removal was 10/90. An enhanced decolourisation (up to 93 %) and a total phenolic reduction (50 %) of the mixture was obtained when co-fermentated sequentially and pH corrected by lime addition. Hajjouji *et al.* (2008) investigated the conditions (pH, C/N ratio, aeration and temperature) for the optimization

of OMW aerobic treatment and found that optimized conditions (pH=5.7 and C/N=57) led to a 94 % drop in polyphenols.

Fungi and yeasts

D'Annibale *et al.* (2004) found that throughout the OMW treatment with *Panus tigrinus* CBS 577.79 the production of extracellular laccase and manganese peroxidase (MnP) occurred. Quaratino *et al.* (2007) employed the sequential use of laccase and *Panus tigrinus* and also achieved good degradation of phenolic compounds (81 %).

D'Annibale *et al.* (2006) investigated the valorization of OMW by its use as a possible growth medium for the microbial production of extra-cellular lipase. Strains of *Geotrichum candidum* (NRRLY552 and Y553), *Rhizopus arrhizus* (NRRL2286 and ISRIM 383), *Rhizopus oryzae* (NRRL6431), *Aspergillus oryzae* (NRRL1988 and 495), *Aspergillus niger* (NRRL334), *Candida cylindracea* (NRRLY17506) and *Penicillium citrinum* (NRRL1841 and 3754, ISRIM118) were screened, and were all able to grow on the undiluted OMW and to generate extracellular lipase activity. *C. cylindracea* showed the highest lipase activity, obtained with OMW supplemented with NH_4Cl (2.4 g L^{-1}) and olive oil (3.0 g L^{-1}).

Yeasts

In contrast to the conventional yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, widely used in biotechnological processes in the last decades, all the other yeasts are termed non-conventional. The interest in non-conventional yeasts has growing in recent years (Spencer *et al.*, 2002).

Yarrowia lipolytica (originally classified as *Candida lipolytica*) is one of the most extensively studied ''non-conventional'' species. It has been studied for many years for its aptitude to grow on hydrophobic substrates like oil, fatty acids and thus for its capacity to produce lipid-degrading enzymes (Fickers *et al.*, 2004). In fact, this specie is capable of producing important metabolites and having an intense secretory activity, which justifies efforts to use it in industry (as a

biocatalyst), in molecular biology and in genetics studies (Coelho *et al.*, 2010). The ascomycetous *Yarrowia lipolytica* is used as a model for the study of protein secretion, dimorphism, hydrophobic substrate degradation, biolipid production and other related fields. It is strictly aerobic microorganism, and it is because of this inability to survive under anaerobic conditions that permits their easy elimination (Barth and Gaillardin, 1997). Is often found in environments rich in hydrophobic substrates, such as alkanes or lipids, and has developed sophisticated mechanisms for the efficient use of hydrophobic substrates (HS) as the sole carbon source (Barth and Gaillardin, 1997; Fickers *et al.*, 2005). *Yarrowia lipolytica* presents the ability to grow on Olive Mill Wastewater (Papanikolaou *et al.*, 2008) as well as to consume organic compounds, including aliphatic and aromatic hydrocarbons, often accompanied by biosurfactants production. One of the most important products secreted by this microorganism is lipase which can be exploited for several applications in the detergent, food, pharmaceutical, and environmental industries. In addition, *Y. lipolytica* is able to produce citric acid and aroma from a variety of carbon sources, including sugars, alkanes, plant oils, starch hydrolysates, ethanol, and glycerol (Coelho *et al.*, 2010).

Candida rugosa and ***Candida cylindracea*** are not so well-known and studied as *Yarrowia lipolytica*, but they are closely connected with the industrial lipase production. *Candida cylindracea* was regarded as a synonym of *Candida rugosa*. However, Kurtzman and Robnett (1998) showed from ribosomal DNA sequence analysis that *C. cylindracea* is a distinct species from *C. rugosa* and that the two species occur in separate clades among the ascomycetous yeasts. *Candida rugosa,* a spherical-shaped unicellular microfungi, is a renowned lipase producing yeast, *Candida rugosa* lipase is becoming one of the most widely used enzymes in industry (Mancheno *et al.*, 2003). Genetic studies have identified up to seven genes coding for different lipases in *Candida rugosa* (Lotti *et al.*, 1993). *Candida cylindracea* is a well-known industrial lipase producing yeast. It is non-ascosporic, often unicellular, non-pathogenic and recognized as GRAS (generally regarded as safe). Lipase produced by *C. cylindracea* has been one of the most widely used enzymes in research due to its high activity in hydrolytic reactions as well as synthetic chemistry (Kim and Hou, 2006).

Candida rugosa and *Candida cylindracea* are being studied and used for lipase production from OMW (Gordillo *et al.*, 1998; Brozzoli *et al.*,2009).

Several works were performed using yeasts to treat OMW. The lipase production by *Candida cylindracea* NRRL Y17506 was studied by Brozzoli *et al.* (2009) and the best results were obtained with OMW characterized by low COD and low total sugars content. Experiments in shake flasks led to enzyme activity of approximately 10 U mL^{-1} (with 2.4 g L^{-1} of NH_4Cl and 3 g L^{-1} of olive oil as supplements) and in a stirred tank reactor it was obtained lipase activities of 1.8 U mL^{-1} (pH controlled to 6.5), 18.7 U mL^{-1} (uncontrolled pH) and 20.4 U mL^{-1} (pH let free to oscillate bellow 6.5). Avoiding the harmful anoxic conditions for the yeast during exponential growth phase, through a variable stirring regime, these authors achieved good lipase production, 123.3 U L^{-1} h^{-1}. Asses *et al.* (2009a) have used *Geotrichum candidum* to produce lignin peroxidase (LiP), manganese peroxidase (MnP) and lipases from OMW and obtained 60 % and 50 % of COD and color removal, respectively, in settler or bubble column bioreactors. Lipolytic activity was superior in batch bubble reactor however production of LiP and MnP was increased in the settler. The decrease of hydraulic retention time has reduced the process performance. Asses *et al.* (2009b), using *Geotrichum candidum* to OMW biodegradation, found that COD and color removal is higher for fresh than for stored OMW (diluted), achieving COD and color reduction of 75 % and 65 %, respectively. These authors observed that the sugars consumption provides a pH decrease from 6 to 4.1 and also that the high OMW decolorization was correlated with the production of extracellular peroxydases. Furthermore, the results obtained, with *G. candidum,* suggests that culture conditions that increased the yield of lignin and manganese peroxidases activity lead to higher decolorization, due to the depolymerisation of high molecular-mass phenolic compounds, which are the most recalcitrant. Lipase production is improved by olive oil addition (Brozzoli *et al.*, 2009; Asses *et al.*, 2009a; D'Annibale *et al.*, 2006) and agitation (Brozzoli *et al.*, 2009; Asses *et al.*, 2009a) as well as by the type of nitrogen source (D'Annibale *et al.*, 2006).

Papanikolaou *et al.* (2008) used *Yarrowia lipolytica* ACA-DC 50109 to produce citric acid, on OMW enriched with glucose, and reached a notable quantity of total citric acid produced (28.9 g L^{-1}). It was also observed that the adaptation of the strain in OMW-based media favored the biosynthesis

of cellular unsaturated fatty acids (principally of oleic and palmitoleic acids).

Some problems occur under aerobic conditions, such as the OMW color intensification due to the auto-oxidation of the phenolic compounds (Hamdi *et al.*, 1992), which is enhanced by the addition of NaOH. The application of the fungi on a larger scale is limited by the difficulty of achieving continuous culture because of the formation of filamentous pellets and mycelia. Moreover, COD reduction and color removal obtained after OMW biotreatment varied with the same microorganism and operation conditions, since the variable polymerization of phenolic compounds in the effluent during storage is not taken into consideration (Assas *et al.*, 2002). OMW became darker during storage because of the auto-oxidation and subsequent polymerization of tannins, giving dark coloured phenolic compounds (Assas *et al.*, 2002).

CHAPTER 3. MATERIALS AND METHODS

Some methods presented in this Chapter were published in:

Gomes N., **Gonçalves C.**, García-Román M., Teixeira J. A., Belo I., (2011), Optimization of a colorimetric assay for yeast lipase activity in complex systems, *Analytical Methods*, DOI:10.1039/C0AY00680G;

Gonçalves C., Rodriguez-Jasso R. M., Gomes N., Teixeira J. A., Belo I., (2010), Adaptation of dinitrosalicylic acid method to microtiter plates, *Analytical Methods*, 2 (12), 2046-2048.

3.1. MICRORGANISMS, MEDIA AND CULTURE CONDITIONS

3.1.1. Used microorganisms

Strains of *Candida rugosa* (PYCC 3238 and CBS 2275), *Candida cylindracea* CBS 7869 and *Yarrowia lipolytica* (CBS 2073, W29 ATCC 20460 and IMUFRJ 50682) were used in the present work.

3.1.2. Microorganisms preservation

These microorganisms were stored at - 80 °C in cryogenic tubes (Microbank, Pro-Lab Diagnostics, Canada). After thawing, each strain was cultured for 48 h on YPD agar (YPDA) medium (Table 3.1) at 27°C. The colonies were maintained in YPD agar at 4 °C, to be used later.

3.1.3. Microorganisms growth medium

Unless otherwise stated, the cells were pre-grown in 500-mL baffled Erlenmeyer flask containing 200 mL of YPD medium (Table 3.1) and then harvested (12225 g, 5 min) from the pre-culture and re-suspended in the OMW-based media. The medium was sterilized using an autoclave, at 121 °C, during 20 minutes.

Table 3.1 YPD and YPDA composition used in the cellular growth

Compound	Concentration (g L^{-1})
Glucose	20
Peptone	20
Yeast Extract	10
Agar*	30

* Only in YPDA

3.2. OLIVE MILL WASTEWATERS

3.2.1. OMW collection and storage

The OMW samples were collected from different three phases'olive oil mills, from the north region of Portugal. They were chosen among the olive mills recognized by the *Ministry of Agriculture, Rural Development and Fisheries* ("Ministério da Agricultura, do Desenvolvimento Rural e das Pescas").

Directly in the output of the process, the OMW were collected to clean 20 L or 30 L containers that were closed and transported to the laboratory (Figure 3.1). The OMW samples usually arrived to the laboratory still warm. The storage, the preserving methods and the sample characterization were performed in the subsequent 72 hours.

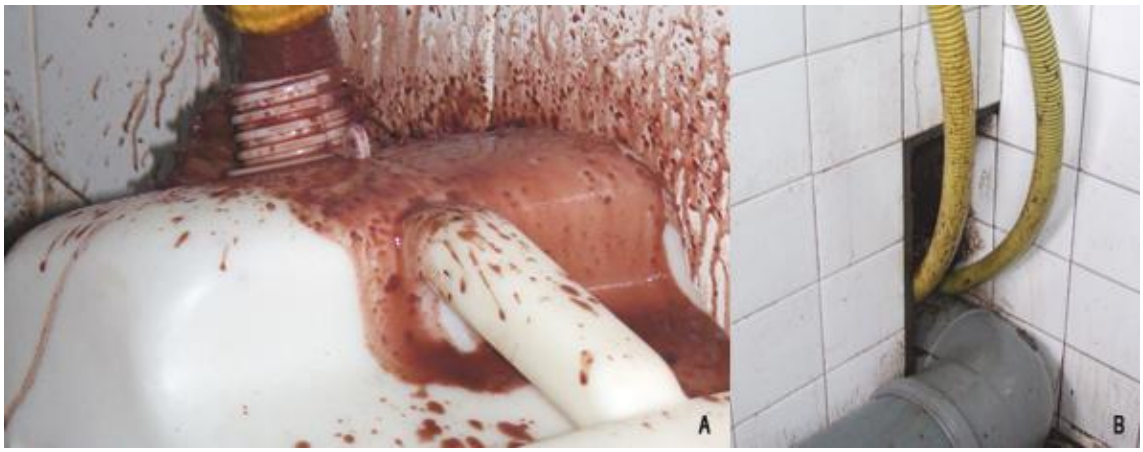

Figure 3.1 A. Olive mill wastewater collection, directly in the containers **B.** output of the process.

These OMW samples were obtained during consecutive campaigns throughout the present research work:

OMW-A. Vila Flor (2005/2006)

OMW-B. Guarda (2006/2007)

OMW-C. Amarante – *Lagar de Azeite das Carvalhinhas* (2006/2007)

OMW-D. Amarante – *Lagar de Azeite das Carvalhinhas* (2007/2008)

OMW-E. Bragança (2007/2008)

OMW-F. Figueira de Castelo Rodrigo (2009/2010)

OMW-G. Amarante – *Lagar de Azeite das Carvalhinhas* (2010/2011)

The olive mill wastewaters collection was performed once a year, per campaign. Therefore, to allow the OMW use along the year, until a new campaign starts, the sample storage and preservation was seen as a key step. In fact, inhibition of biological activity in a sample is often particularly important since microorganisms can consume, partially or completely, a number of substances required for their growth, thus modifying the sample. Methods of preservation include cooling, freezing, pH control, and chemical addition. The applied methods were:

a). Storage at 4° C

b). Sterilization and storage at 4°C

c). Acidification with HCl (pH 2) and storage at 4°C

d). Freezing (- 20 °C)

The methods a) and b) were ineffective to prevent microorganisms proliferation and c) changed chemically and visually the OMW samples. The freezing method was the most adequate to the samples but, even with this, additional proceedings were required. For instance, the sample was only thawed in the day it was needed; moreover it was thawed slowly, well mixed and immediately used, either to be characterized or to prepare OMW-based media. It is important to note that, previously to the application of preservation methods to the samples; they were distributed to smaller containers from 500 mL to 5 L.

3.2.2. OMW characterization

The OMW characterization was performed during the 72 h after their collection. These effluents were characterized for pH, chemical oxygen demand (COD), total solids (TS), total volatile solids (TVS), total suspended solids (TSS), total nitrogen Kjeldhal (TKN) or total nitrogen (TN), total organic carbon (TOC), phenols, reducing sugars (RS), total protein (TP) and lipids (Table 3.2). Note that the description of the used methods is positioned in the *Analytical Methods* of CHAPTER 3.

Table 3.2 OMW characterization

Parameter/unit	OMW-A	OMW-B	OMW-C	OMW-D	OMW-E	OMW-F	OMW-G
pH	4.8	4.8	4.9	4.7	5.5	4.5	4.9
COD/(g L^{-1})	19.6	184 ± 2	191 ± 2	115 ± 1	179 ± 2	261 ± 25	97± 2
TS/(g L^{-1})	10.5	115 ± 3	120 ± 0.2	148 ± 3	143 ± 0.2	155 ± 1	58 ± 1
TVS/(g L^{-1})	7.3	84 ± 12	84 ± 42	117 ± 6	114 ± 2	116 ± 6	46 ± 3
TSS/(g L^{-1})	-[a]	-[a]	-[a]	-[a]	-[a]	55.4 ± 8.8	31.0 ± 0.4
TKN/(mg L^{-1})	50	95 ± 17	60 ± 12	192 ±17	-[a]	-[a]	-[a]
TN/(mg L^{-1})	-[a]	-[a]	-[a]	-[a]	-[a]	198 ± 10	3.4 ± 0.1
TOC/(g L^{-1})	-[a]	-[a]	-[a]	-[a]	-[a]	45.6 ± 0.0	15.3 ± 3.4
Phenols/(g L^{-1})	0.8	9.7 ± 0.2	12.1 ± 0.2	5.5 ± 0.1	5.7 ± 0.1	7.9 ± 1.9	2.7 ± 0.2
RS/(g L^{-1})	3.4	45.5 ± 0.5	34.4 ± 0.9	12.9 ± 0.7	52 ± 0.4	68.5 ± 1.2	16.5 ± 5.1
Lipids/(g L^{-1})	-[a]	-[a]	-[a]	-[a]	-[a]	31.9 ± 15.1	3.5 ± 0.6

Data are mean values ± standard deviation (n = 20). -[a] Not determined.

Long chain fatty acids and phenolic compounds were also determined for the OMW of 2009/2010 and 2010/2011 campaigns (Table 3.3). A description of these methods is located on *Analytical Methods* of CHAPTER 3. The long chain fatty acids found were: palmitic acid (C16:0), palmitoleic acid (C16:1), Stearic Acid (C18:0) and Oleic Acid (C18:1).

Table 3.3 LCFA and phenols found in OMW-F and OMW-G

		OMW-F	OMW-G
LCFA (mg L^{-1})	C16:0	2446 ± 120	660 ± 82
	C16:1	*N*[a]	127 ± 5
	C18:0	733 ± 71	106 ± 4
	C18:1	2390 ± 76	2166 ± 96
Phenols (g L^{-1})	hydroxytyrosol	2.0 ± 0.4	-[b]
	tyrosol	1.2 ± 0.7	-[b]
	oleuropein	0.1 ± 0.0	-[b]
	caffeic acid	0.3 ± 0.2	-[b]

N[a] negligible; -[b] Not determined.

3.3. ANALYTICAL METHODS

3.3.1. pH

The power of Hydrogen (pH) was obtained using an immersion electrode (Microprocessor pH-Meter pH 320, WTW).

3.3.2. Chemical oxygen demand (COD)

Chemical oxygen demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. COD often is used as a measurement of pollutants in wastewater and natural waters (APHA, 1998).

In the Chapter 5 experiments and for OMW-A, B, C and E characterization, COD was determined according to the closed reflux colorimetric method, described in the 20th edition of the *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998) as follows:

> "Most types of organic matter are oxidized by a boiling mixture of chromic and sulfuric acids. When a sample is digested, the dichromate ion oxidizes COD material in the sample. This results in the change of chromium from the hexavalent (VI) state to the trivalent (III) state. Both of these chromium species are colored and absorb in the visible region of the spectrum. The dichromate ion ($Cr_2O_7^{2-}$) absorbs strongly in the 400-nm region, where the chromic ion (Cr^{3+}) absorption is much less. The chromic ion absorbs strongly in the 600-nm region, where the dichromate has nearly zero absorption."

For the COD determinations in the remaining chapters and OMW characterization, were used the test kits from Hach Lange, LCK 114 (150 – 1000 mg L^{-1}), whose the principle of operation, according to the brochure of the COD LCK 114 method, is described as follows:

> "Oxidizable substances react with sulphuric acid – potassium dichromate solution, in the presence of silver sulphate as a catalyst. Chloride is masked by mercury sulphate. The green coloration of Cr^{3+} is evaluated at 620 nm."

3.3.3. Solids

Solids refer to matter suspended or dissolved in water or wastewater. They can affect water or effluent quality adversely in a number of ways (APHA, 1998).

According to Standard Methods for the Examination of Water and Wastewater (APHA, 1998) "**Total solids**" (TS) corresponds to the residue left in the vessel after evaporation of a well-mixed sample and its subsequent drying in an oven at a defined temperature (105 °C). Moreover, the weight loss on ignition (550 ° C for 1 h in a muffle furnace) is called "volatile solids" (**Total Volatile Solids**, TVS) and the "**Total Suspended Solids**" (TSS) are the dry portion of total solids retained by a filter after drying in an oven at 105 °C.

3.3.4. Nitrogen

Total nitrogen Kjeldhal (TKN) is the sum of organic nitrogen and ammonia nitrogen and was determined as described by to Standard Methods for the Examination of Water and Wastewater (APHA, 1998):

> "In the presence of H_2SO_4, potassium sulfate (K_2SO_4), and cupric sulfate ($CuSO_4$) catalyst, amino nitrogen of many organic materials is converted to ammonium. Free ammonia also is converted to ammonium. After addition of base, the ammonia is distilled from an alkaline medium and absorbed in boric or sulfuric acid. The ammonia may be determined colorimetrically, by ammonia-selective electrode, or by titration with a standard mineral acid."

The catalyst used was selenium (Se) and not cupric sulfate ($CuSO_4$).

Total nitrogen (TN), inorganically and organically bonded nitrogen, was quantified by the test kits from Hach Lange, LCK 338 (20 – 100 mg L^{-1}), whose the principle of operation, according to the pamphlet of the kit LCK 338, is described as follows:

> "Inorganically and organically bonded nitrogen is oxidized to nitrate by digestion with peroxodisulphate. The nitrate ions react with 2.6-dimethylphenol in a solution of sulphuric and phosphoric acid to form a nitrophenol."

3.3.5. Total Organic Carbon (TOC)

The organic carbon in water and wastewater is composed of a variety of organic compounds in various oxidation states. Total organic carbon (TOC) is a more convenient and direct expression of total organic content than biochemical oxygen demand (BOD), assimilable organic carbon (AOC) and chemical oxygen demand (COD), but does not provide the same kind of information. (APHA, 1998). TOC was quantified by the test kits from Hach Lange, LCK 387 (300 – 3000 mg L^{-1}),

whose the principle of operation, according to the brochure for the kit LCK 338, is described as follows:

> "In a two-stage process, the total inorganic carbon (TIC) is first expelled with the help of the TOC-X5 shaker, then the total organic carbon (TOC) is oxidized to carbon dioxide (CO_2). The CO_2 passes through a membrane into the indicator cuvette, where it causes a colour change to occur, which is evaluated with a photometer."

3.3.6. Total Lipids

The lipids (total fat) content was extracted with diethyl ether, in a Soxtec System HT2 1045-extraction unit, after samples lyophilisation (Official Methods of Analysis, 2007).

The sample to be analyzed is weighed into thimbles and inserted in the Extraction Unit. The solvent is added in a closed system. The cups, containing boiling stones and 50 mL of diethyl ether, are heated by the plates were they are placed. The temperature of the circulating heating fluid was 90°C and the condensers were connected to a recirculation cold bath. The 4-step extraction procedure consists of boiling, rinsing, recovery and pre-drying. Afterwards, the samples were weighed and dried until a constant weight was reached.

3.3.7. Long Chain Fatty Acids (LCFA)

Long chain fatty acids were determined as described by Neves *et al.* (2009), where, 2 mL of the sample, once homogenized were transferred into glass vials, afterwards, 1.5 mL of the Internal Standard solution (1000 mg L^{-1}) and 1.5 mL of HCl:1-Propanol (25 % v/v) and 2 mL of DCM were subsequently added. The mixture was vortex-mixed, to promote good contact between the two phases, and was digested at 100 °C for 3.5 h. After digestion, the content of the vial was transferred with 2 mL of ultra-pure water to a different vial, rubber covered, and the contact between the two phases was further promoted. These new vials were kept in inverted position for

30 min, after which 1 mL of the organic phase was collected. 1 mL of this sub sample was analysed by GC.

3.3.8. Reducing sugars

Reducing sugars (sugars with an aldehyde group or that are capable of forming one, in solution through isomerisation) were measured by the well-known dinitrosalicylic acid (DNS) method.

In the presence of reducing sugars, 3,5-dinitrosalicylic acid, of the DNS reagent, is reduced into 3-amino-5-nitrosalicylic acid, a brownish compound that strongly absorbs light at 540 nm, allowing a quantitative spectrophotometric measurement of the amount of reducing sugars present in a given sample.

In the initial experiments (Chapter 5) and for OMW-A, B, C, D and E characterization, the traditional DNS method was used; where 500 μL of dinitrosalicylic acid reagent and 500 μL of sample are added to test tubes. The tubes are dived in a water bath (100 °C), for 5 minutes and then cooled in frozen water, while 5 mL of distilled water are added to each tube, resulting on the final reaction mixture. The addition of water while the tubes are dived in cold water is performed to stop immediately the reaction.

In the remaining chapters and OMW characterization the newly adapted method was used. The reaction was carried out in wells of 340 mL, adding 25 mL of DNS reagent to 25 mL of sample (previously diluted). Subsequently, in order to perform the reaction, the microtiter plate, with cap, was placed in the oven (105 °C) by 10 min, then it was placed on ice and 250 mL of distilled water were immediately added to each well. Finally, when the room temperature was reached the absorbance was read, on an ELISA espectrophotometer (Sunrise, Tecan - Switzerland).

3.3.9. Phenolic compounds

Phenols, defined as hydroxyl derivatives of benzene and its condensed nuclei, may occur in domestic and industrial wastewaters, natural waters, and potable water supplies. Chlorination of such waters may produce odorous and objectionable-tasting chlorophenols (APHA, 1998).

The quantification of phenolic compounds by using Folin–Ciocalteu reactive is a well-known method (Folin–Ciocalteu Index). It involves oxidation, in alkaline solution, of phenols by the yellow Folin–Ciocalteu reagent (a mixture of phosphomolybdate and phosphotungstate) and colorimetric measurement of the resultant mixture of blue oxides of molybdenum and tungsten (Singleton and Rossi, 1965; Slinkard and Singleton, 1977). This blue coloration has a maximum absorption in the region of 750 nm, which is proportional to the total phenolic compounds.

In the first experiments (Chapter 5) and for OMW-A, B, C, D and E characterization, the conventional method of Folin–Ciocalteu Index in assay tubes was used (Compendium of International Methods of Wine and Must Analysis from *International Organisation of Vine and Wine*, 2009) and it worked perfectly. However it is very time-consuming when it is necessary to analyze a large number of samples, thus, the volumes were proportionally reduced so that the reaction could occur directly on the microtiter plate-well, instead of occurring in the assay tube.

The microplate adapted method of Folin–Ciocalteu Index was then tested and after that used routinely (in the remaining chapters and OMW characterization). Therefore, for this strictly order, 5 µL of sample (previously diluted), 15 µL of of Folin–Ciocalteu reagent (from Panreac), 60 µL sodium carbonate solution (15 % w/v) and 200 µL of distilled water were added to test tubes; the microplate is immediately placed in a 60 ° C oven and removed after 10 min; after reaching the room temperature the absorbance was read at 740 nm. Caffeic acid is often used as standard but since it has serious problems to dissolve in cool water, tyrosol was used instead.

Phenolic compounds extraction, detection and quantification

The phenolic compounds were extracted from the OWM by a liquid-liquid extraction by acidified ethyl acetate, according to the procedure of De Marco *et al.* (2007). The presence and amount of phenolic compounds in the OMW extracts were assessed by HPLC analysis. The analysis was performed using a Jasco chromatograph 2080-PU intelligent pump equipped with a Jasco 2070-UV intelligent UV-VIS detector at 280 nm and a Jasco AS-2057 Plus intelligent auto sampler (Jasco, Tokyo, Japan) with a Nucleosil 120-5 C 18 (5 µm particle size, Macherey-Nagel, Düren, Germany) column. The following conditions were applied: column at room temperature; 25 % of methanol and 0.1 % of formic acid in water as mobile phase; a flow rate of 0.9 mL min^{-1} and injection volume of 20 µL. The phenolic compounds were identified by comparison with relative retention times of pure compounds.

3.3.10. Cell counting

Cell concentration was determined by direct counting in the microscope, using a Neubauer-improved counting chamber (Paul Marienfeld GmbH & Co, Lauda-Königshofen, Germany) (Mather and Roberts, 1998).

3.3.11. Lipase

Extracellular lipase activity was measured in the samples supernatant by a spectrophotometric method developed and validated by our research group (Gomes *et al.*, 2011), using *p*-nitrophenyl-butyrate (*p*-NPB) in sodium acetate buffer 50 mM (pH 5.6) as substrate at 37 °C for 15 min. One unit of activity was defined as the amount of enzyme that produces 1 µmol of *p*-nitrophenol per minute, under essay conditions.

The reaction mixture was composed of 980 µL of substrate (2.63 mM *p*-nitrophenyl butyrate in 50 mM sodium acetate buffer, pH 5.6, with 4 % (v/v) Triton X-100) and 20 µL of broth sample. It was

incubated for 15 minutes at 37 °C and the reaction was stopped by the addition of 2 mL of acetone. The absorbance was measured at a wavelength of 405 nm.

The substrate p-NPP was also evaluated, but no significant differences in the activities of the samples, from all the strains, were found.

3.3.12. Protease

Protease activity was quantified at 37 °C for 40 min, as described elsewhere (Pinto, 1998), using 0.5 % (w⁄v) azocasein in acetate buffer (50 mM, pH 5.0) as substrate. The reaction mixture was composed of 500 µL of substrate and 500 µL of broth sample. After incubation, 10 % (w/v) trichloroacetic acid was added to precipitate residual protein not hydrolyzed by the proteolytic enzymes. The sample was then centrifuged (3000 rpm, 5 min) and 1 mL of 5 N potassium hydroxide, was added to the supernatant, producing a pinky-orange color, characteristic of the azo groups in alkaline pH. The intensity of this coloration was measured at a wavelength of 428 nm. One unit of activity was defined as the amount of enzyme that caused an increase of 0.01 of absorbance relatively to the blank per minute, under assay conditions.

3.3.13. Methane content

Methane content of the biogas was measured by gas chromatography (GC) using a Porapack Q (80/200 Mesh) column, with Argon as the carrier gas, at 16 mL min^{-1}, and a thermal conductivity detector. Temperatures of the detector, injector and oven were 110 °C, 110 °C and 35 °C, respectively.

CHAPTER 4. INFLUENCE OF PHENOLIC COMPOUNDS FROM OMW ON YEASTS GROWTH

The olive mill wastewaters (OMW) represent a serious environmental problem, mainly due to their composition in phenolic compounds. A microscale study on the phenolic compounds' effect, from OMW, was carried out by the growth of yeast strains (*Yarrowia lipolytica*, *Candida cylindracea* and *Candida rugosa*).

Although this study proved that *Y. lipolytica*, *C. rugosa* and *C. cylindracea* are able to grow in different media containing phenolic compounds, the strains mostly use other available carbohydrate sources. The presence of catechol exhibited an inhibitory effect in the growth of all the used strains. This conclusion was confirmed by respirometry experiments where a strong respiratory activity inhibition was caused by catechol to *Y. lipolytica*, in contrast to the OMW samples effect.

These results confirmed that *Y. lipolytica*, *C. rugosa* and *C. cylindracea* are potential microorganisms to be applied in OMW treatment.

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And published in:

Gonçalves C., Lopes M., Ferreira J.P., Belo I., (2009), Biological treatment of olive mill wastewater by non-conventional yeasts, *Bioresource Technology*, **100** (15) 3759-3763;

4.1. INTRODUCTION

The olive mill wastewaters (OMW) causes serious environmental problems, such as, coloring natural waters, threat to the aquatic life (decrease of fish population), surface and ground water pollution, soil quality, plant growth and odours (Akdemir and Ozer, 2008). In fact, the foremost problem attributed to the olive mills effluent (OMW) is the phenolic compounds toxicity. The olive pulp is very rich in phenolic compounds and approximately 53 % are lost in the OMW (Rodis *et al.*, 2002). The phenolic compounds usually found in OMW are benzoic acid; salicylic acid; 2,4 dihydroxybenzoic acid; tyrosol; 2,4 dihydroxybenzaldehyde; syringic acid; 4-hydroxybenzoic acid; caffeic acid (3,4-dihydroxycinnamic acid); syringaldehyde; p-Coumaric acid; ferulic acid, hydroxytyrosol; 3,4 dihydroxyphenyl acetic acid; vanillic acid (El-Gohary *et al.*, 2009b; Asses *et al.*, 2009b). These compounds are commonly described as responsible by the OMW toxicity, antimicrobial activity and biodegradation (Bisignano *et al.*, 1999).

A possible recycling option of OMW is to, once phytotoxic effects are neutralized, spread it on agricultural land. Piotrowska *et al.*, 2011, have shown that dephenolized OMW has smaller impact onto soil performance than crude OMW. In fact, the large variability in phenolic compounds of olive mill wastewaters composition constitutes a major limiting factor for their re-use in agricultural systems (Hanifi and Hadrami, 2008). In spite of the fact that some low-molecular-weight phenolics of OMW are degraded during evaporation in ponds, thus reducing the antibacterial effect (Saez *et al.,* 1992), this traditional OMW management process is very slow, which reveals the important need of alternative options for OMW treatment. The research on OMW treatment and valorization is presently focused on the phenolic compounds degradation, responsible for the OMW black color, as their breakdown is considered to be the limiting step in the bio-treatment of OMW.

Fungi cultures have been proposed for the OMW treatment due to their ability in phenolic degrading enzymes production. For instance, a decrease of the phytotoxicity, as described by the parameter Germination Index, was noticed in the OMW treated with some *Pleurotus* spp strains, although this decrease was not proportional to the phenolic removal (Tsioulpas *et al.*, 2002). Also, yeast species such as *Trichosporon cutaneum* have shown to be able to degrade phenolic compounds, particularly the low molecular weight ones (Chtourou *et al.*, 2004).

The present work **aimed** to study the effect of phenolic compounds, from Olive Mill Wastewaters (OMW), on the growth of the yeast species: *Candida cylindracea* CBS 7869, *Candida rugosa* CBS 2275 and *Yarrowia lipolytica* W29 (ATCC 20460). This study was performed at microscale using microplate wells as bioreactor systems. To confirm the microplate trials, additional respirometry trials were also performed.

4.2. MATERIALS AND METHODS

4.2.1. Phenolic Compounds Media

YPD (10-fold diluted) and YNB (13.4 g L^{-1} of Yeast Nitrogen Base, from Fluka, USA) media were used as blank and in the phenolic media composition. Each phenolic medium (YPD+P or YNB+P) was prepared with 50 % of YPD (or YNB) and 50 % of a phenolic compound solution (2 g L^{-1} of a phenolic compound in phosphate buffer 0.5 M pH 7). The phenolic compounds used are usually found in OMW: catechol (99 %) from Sigma-Aldrich; caffeic acid (95 %), oleuropein (pure), hydroxytyrosol (pure) and tyrosol (pure), all from Extrasynthèse.

4.2.2. Microplate trials

The phenolic compounds toxicity tests were carried out in sterilized 96-cells microplates, with wells of 340 μL. Firstly, 250 μL of every prepared medium was transferred to each well. Afterwards, each medium was inoculated with 20 μL of cells suspension with cellular concentration (dry weight) of 0.4 ± 0.1 g L^{-1} that corresponds to (1.5 ± 0.5)×10^7 cells mL^{-1}.

The experiments were performed during 4 days at 27 °C and 160 rpm. Through the experiment, the absorbance (optical density at 650 nm) of the 96-wells was read, on an ELISA espectrophotometer (Sunrise, Tecan - Switzerland), to analyse cell growth. To avoid contamination, the absorbance was read with the microplate transparent cap placed. Throughout the experiments, the microplate was also covered with an opaque cap. This protection from external light was performed in order to prevent the phenolic compounds auto-oxidation. The cell growth in these

trials was monitored by the absorbance of the suspension at 620 nm and converted to grams of cell dry weight per liter dividing by the previously obtained calibration factor of 0.9.

4.2.3. Respirometry trials

Respiratory activity trials were carried on a Biological Oxygen Monitor System (YSI 5300A) with a stirred thermostatic bath. *Y. lipolytica* W29 culture with approximately 22 h of growth was harvested, washed and re-suspended in sodium phosphate buffer 50 mM pH 7.0 in order to obtain a suspension with a final cellular concentration of 9 x 10^6 cell mL^{-1}. This suspension was aerated for 30 min to ensure the oxygen saturation and after this time was placed in the temperature-controlled vessel at 27 °C. Each vessel contains an oxygen probe connected to an oxygen meter. The vessels were closed and the decrease in dissolved oxygen tension (DOT) was monitored over time. The linear decrease observed between time zero and carbon source addition corresponds to the endogenous respiration rate. To determine the oxygen uptake rate (OUR), due to substrate oxidation, solutions of glucose (0.48 g L^{-1}), catechol (0.32 g L^{-1}) or OMW (3.8 g COD L^{-1}) were injected into the vessel. An essay without carbon source addition was performed simultaneously and used as control for endogenous OUR determination.

4.2.4. Statistical Analysis

All the experiments were replicated twice. The results were evaluated by variance analysis (ANOVA) and the significant difference in ANOVA ($p < 0.05$) was detected by the Tukey's HSD test, which was applied to compare the differences among samples. The "Paquete de diseños experimentales. FAUANL, version 2.5" (Olivares, 1994) was the software used for data analysis.

4.3. RESULTS AND DISCUSSION

4.3.1. Microplate growth trials

These experiments were performed, in triplicate, with the 3 yeast strains. As described previously these strains grew in 12 different media, distributed per well: YPD and YNB, used as controls, and the 10 phenolic compounds media, composed of YPD or YNB, with 5 different phenolic compound each (YPD+P and YNB+P).

YPD was chosen since it is a well-known complete medium for yeast growth and YNB due to its composition in all essential nutrients, required for yeast growth, except a source of carbohydrate. These characteristics could turn YNB proper to evaluate the yeast ability to use a compound added to the medium as the only source of carbon. The obtained results are summarized on Figure 4.1. Tukey's HSD test results shown in the figure are only for each strain, in YNB or YPD based media. Tukey's tests between other groups of samples (for example, comparing yeasts or all the media for each yeast) were also conducted, apart from these.

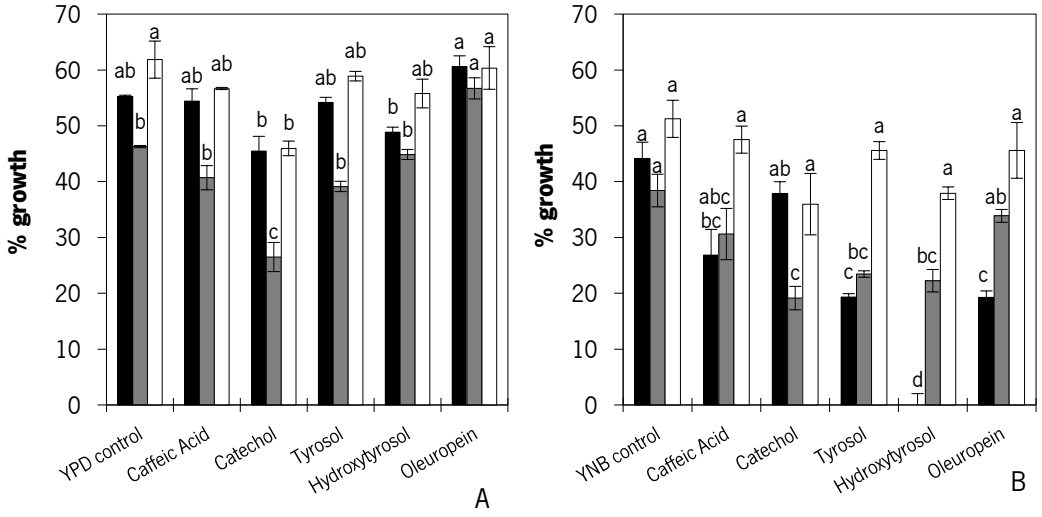

Figure 4.1 Cellular growth, described as percentage of cell mass increase, of the 3 yeast strains: *Candida cylindracea* (black bars), *Candida rugosa* (grey bars) and *Yarrowia lipolytica* (white bars); on different phenolic compound media: **A.** YPD+P and **B.** YNB+P. Each value represents the average of independent two determinations; vertical error bars represent standard deviation of the mean, for each set of

determinations. For each strain, in YNB or YPD based media, the same letter above bars indicates the absence of significant differences ($P < 0.05$; Tukey test).

It is known that phenolics interfere with the integrity of cell membranes or inhibit the germination of spores (Russel and Chopra, 1990). Nevertheless, it was observed that in general the three strains used in this work were able to grow, in the presence of 1 g L^{-1} of each phenolic compound.

Tukey's multiple comparisons were used to separate significant differences among the growth percentage results. In YNB based media the cell growth was globally inferior when compared with the obtained in YPD media, for all strains and phenolic media (significantly different by Tukey test, $p < 0.05$). This result could imply that the used yeasts are able to grow in the presence of phenolic compounds but they are mostly using other available compounds as carbohydrate sources, since YNB essentially differs from YPD by its lack of a carbohydrate source.

In YPD based media, *Y. lipolytica* globally presented the highest cell growth values, followed by *C. cylindracea* (6 % lower than *Y. lipolytica*); but *C. rugosa* had the lowest growth values (25 % lower than *Y. lipolytica*). Thus, for YPD and YPD+P media the growth percentage values, between yeasts, were globally significantly different ($p < 0.05$; by Tukey test). On the other hand for YNB and YNB+P media, the overall cell growth between *C. cylindracea* and *C. rugosa* was not significantly different between both, but significantly different from *Y. lipolytica*, with growth values globally superior (twice). Therefore, *Y. lipolytica* presented higher cell growth in all media. This strain also presents similar cell growth between oleuropein and YPD control (Figure 4.1), but in the presence of catechol the cell growth decreased (24 %). Moreover, all the strains presented an inferior growth percentage in the presence of catechol, than in the other phenolics (Figure 4.1). Mahadevan and Reddy (1968) studied the effect of phenolic compounds (concentrations between 0.003 M and 0.006 M) on the growth of the fungus *Fusarium oxysporum f. vasinfeetum.* They discovered that catechol and particularly phloretin inhibited fungal growth for the concentration of 0.006 M (0.67 g L^{-1} and 1.64 g L^{-1}, respectively) but also, that no relationship between chemical structure of catechol, phloroglucinol and gallic acid and their effect on fungal growth could be demonstrated.

C. cylindracea was also inhibited by hydroxytyrosol, especially on YNB based medium where no growth was observed. This strain could be particularly sensitive to hydroxytyrosol toxicity when no other carbohydrate source is available in the medium. On the other hand, in all YNB based media the *Y. lipolytica* growth is statistical characterized as identical (Figure 4.1), showing that in the absence of other carbohydrate this strain has similar behavior in the presence of each phenolic compound.

Nevertheless it's important to notice that the antimicrobial activity of phenolic compounds could increase because of synergic effects between them or with other antimicrobial compounds (García-Ruiz *et al.*, 2008), which could occur in effluents like olive mill wastewaters.

4.3.2. Respiratory activity trials

In order to confirm the results obtained in the microplate trials, in which the presence of catechol inhibits the cell growth, respirometric short-term trials were made with this phenolic compound, glucose and OMW, as carbon sources. Oxygen uptake rate (OUR), due to carbon source oxidation, was assessed by the slope of linear decrease in the dissolved oxygen tension with time and compared with the endogenous one obtained without any carbon source present.

Figure 4.2 shows an example of the respiratory profiles of a cellular suspension, with and without injection of carbon source (OMW 3.8 g COD L^{-1} and catechol 0.32 g L^{-1}).

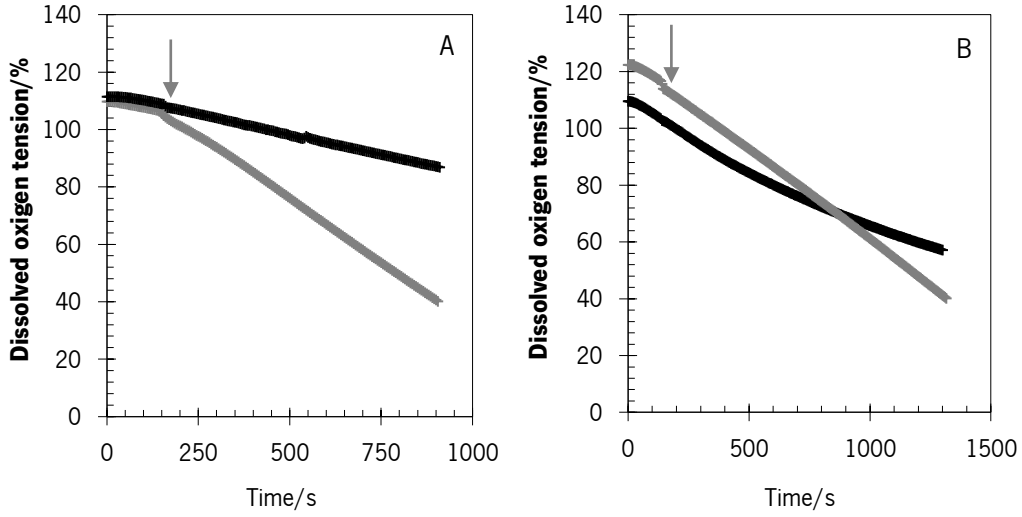

Figure 4.2 Comparison of the oxygen consumption by *Y. lipolytica* suspension in phosphate buffer without carbon source (black) and with **A.** the addition of OMW or **B.** catechol (grey). The arrow represents the injection of carbon source.

The injection of OMW in cell suspension increase threefold the OUR comparatively to that found in the essay without this addition. A twofold OUR increase was found in the essay with addition of 0.5 g L^{-1} glucose solution (data not shown). The addition of catechol to *Y. lipolytica* suspension leads to an inhibitory effect in the respiratory activity of this strain, since the OUR value obtained with catechol in the medium was 93 % of the endogenous value, that is in accordance with the results obtained in the trials on microplates and other studies (Obied *et al.*, 2005). In spite of some authors (Obied *et al.*, 2005; El Hadrami *et al.*, 2004) reported catechol as one of the most abundant compounds in traditional OMW, the results presented in herein work suggested that the OMW used has a lower concentration of this phenolic compound, once OMW was not inhibitory to growth and respiratory activity of *Y. lipolytica* W29.

4.4. CONCLUSION

The present investigation describes a study for the study of the foremost problem attributed to the olive mills effluent (OMW), the phenolic compounds toxicity. Although numerous authors have referred it, few have focused on studying this problem thoroughly.

In conclusion, it has been shown that *Y. lipolytica*, *C. rugosa* and *C. cylindracea* are able to grow in different phenolic synthetic media (1 g L^{-1} of each phenolic compound). Moreover, the used yeasts mostly use other available compounds, than phenolics, as carbohydrate sources. *Y. lipolytica* globally presented the highest cell growth values, but all the used strains presented an inhibition of cellular growth in the presence of catechol. This conclusion was confirmed by respirometry experiments where a strong respiratory activity inhibition was caused by catechol to *Y. lipolytica*, in contrast to the OMW samples effect.

These results confirmed that *Y. lipolytica*, *C. rugosa* and *C. cylindracea* are potential microorganisms to be applied in OMW treatment.

CHAPTER 5. USE OF OMW BY NON-CONVENTIONAL YEASTS: BATCH TRIALS

The ability of lipolytic yeasts to grow on olive mill wastewater (OMW) degrade this waste was studied in several batch experiments (in Erlenmeyer baffled flasks). Factors that affect cellular growth and OMW degradation, such as nitrogen supplementation, cells concentration and surfactant addition, were tested.

Studying *Y. lipolytica* strain W29 and strain IMUFRJ 50682, W29 presented the highest potential for extracellular lipase production in OMW medium. Lipase productivity was improved by the medium supplementation with ammonium sulphate up to 6 g L^{-1}, leading to 80 % of COD degradation and 70 % of total phenols reduction. The surfactant Tween 80 enhanced cell growth and COD degradation, but had a negative effect on lipase activity.

Using OMW, with COD ranging from 100 g L^{-1} to 200 g L^{-1}, all strains, of *Candida rugosa*, *Candida cylindracea* and *Yarrowia lipolytica*, were able to grow, to consume reducing sugars and to reduce COD. *C. cylindracea* was the best strain concerning the effluent degradation.

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5.1. INTRODUCTION

The large diversity of components found in OMW (carbohydrates, polysaccharides, sugars, lipids and phenolic compounds) makes their treatment difficult, and their disposal becomes a critical environmental problem (Niaounakis and Halvadakis, 2004). Thus, these effluents have great importance from an environmental and economical point of view and can be considered not only as a waste to be treated but also a resource to be recovered. Although many methods have been proposed for the treatment of OMW, including physicochemical (Kotta *et al.*, 2007) as evaporation (natural or forced), biological (aerobic and anaerobic) (D'Annibale *et al.*, 2004) and also land application (Cabrera *et al.*, 1996), the most commonly used has been the storage of OMW in lagoons, followed by liquid evaporation during summer season (D'Annibale *et al.*, 2004). The OMW treatment in traditional biological plants is limited by the inhibitory effects of phenolic and lipidic compounds on biomass activity. Some proposals have been reported to reduce this problem, including the use of aerobic microorganisms isolated in OMW but no valorization of the OMW was attempted (Eusébio *et al.*, 2002). The use of fungi can lead to OMW valorization through the enzymes production (Crognale *et al.*, 2006). The anaerobic treatment of OMW can also represent an effluent recovery due to methane production. OMW detoxification by polyphenols reduction has been tested by chemical (Fenton's reagent) and enzymatic (laccase) methods. Yeasts can also be used to degrade phenolic compounds in OMW (Ettayebbi *et al.*, 2003).

Specifically, *Yarrowia lipolytica* strains are good candidates for the OMW treatment and recovery since they can grow well on OMW; degrade lipids and polyphenols; possibly consume the organic material; and produce, at the same time, biomass and other valuable products (Scioli and Vollaro, 1997, Papanikolaou *et al.*, 2008). Nevertheless, the efficiency of organic and polyphenolic content reduction, as well as, the organic acids or lipases production from OMW are strongly dependent on the yeast strain (Lanciotti *et al.*, 2005).

Thus, the **aim** of this chapter was to perform some preliminary tests to evaluate the ammonium, cell and surfactant addition as well as the use of different yeasts and OMW, on the OMW valorization, in batch conditions.

5.2. MATERIALS AND METHODS

5.2.1. OMW-based medium

For the experiments testing the effect of ammonium, cell and surfactant concentration, non-diluted OMW-A was used. OMW were supplemented with 6 g L^{-1} of ammonium sulphate, $(NH_4)_2SO_4$, and 1 g L^{-1} of yeast extract, in order to counteract the lack of nitrogen and vitamins (Scioli and Vollaro, 1997). The pH of the medium was adjusted to 5.6, prior to sterilization. To investigate the possible nitrogen limitation on the OMW use by the yeast strains, a two-fold increase of ammonium concentration was also performed.

The OMW, used to the trials testing the effect of using different OMW (OMW-B, OMW-C, OMW-D and OMW-E; Table 3.2), were enriched with a different source of nitrogen, ammonium chloride and yeast extract and once again, after sterilization, its pH was adjusted to 5.6. The supplementation was made proportionally to its organic composition, in order to counteract the lack of nitrogen. The ammonium chloride (NH_4Cl) concentration added was about 10 % (w/w) to 15 % (w/w) of the OMW content in COD and reducing sugars, respectively. Yeast extract concentration used was approximately 40 % (w/w) of the NH_4Cl added.

5.2.2. Culture conditions

Effect of ammonium, cell and surfactant concentration trials

Batch cultures were carried out with both *Y. lipolytica*, W29 and IMUFRJ 50 682, strains in 500 mL Erlenmeyer flasks with 200 mL of initial medium. The cultures, with an initial concentration of 2 x 10^6 cells mL^{-1}, were incubated at 27 °C and 240 rpm of stirring rate. According to the results of preliminary experiments and to further improve OMW utilization by *Y. lipolytica* W29, trials with this strain were conducted in baffled conical flasks, with an increased initial cell concentration (10^8 cells mL^{-1}). OMW medium was supplemented with 6 g L^{-1} or 12 g L^{-1} of ammonium sulphate and 1 g L^{-1} of yeast extract, with and without the addition of 1 g L^{-1} of Tween 80. An experiment with no OMW medium supplementation of yeast extract and ammonium sulphate was also performed. All trials were repeated at least twice. Cultures were incubated for approximately 100 h and samples

were taken along time to monitor and correct pH values. The final culture volume was around half the value of the initial one. Cell density was immediately determined by cell counting and samples were stored at - 20 °C for further analysis. Cells were observed in an Olympus BX51 microscope immediately after sampling (48 h of growth) for the morphology changes detection, under light microscopy coupled to a DP71 digital camera (Olympus) for acquisition of images (original magnification×400).

Effect of using different OMW

The batch cultures, with different OMW base media, were carried out in Erlenmeyer baffled flasks, with 1000 mL of total volume and 400 mL of initial medium. The batch cultures, with an initial concentration of approximately 10^6 cells mL^{-1}, were incubated at 27 °C with a stirring rate of 240 rpm.

Throughout the process time, culture samples were collected for several analyses. Cell density, assessed by cell counting in the microscope, and pH adjustment were made at each sampling time. Cells were observed in an Olympus BX51 microscope immediately after sampling (48 hours of growth) for the morphology changes detection.

The samples were stored at -20 °C for further analyses.

5.3. RESULTS AND DISCUSSION

5.3.1. Effect of ammonium concentration to *Y. lipolytica* (W29 and IMUFRJ 50682)

This experiments were performed with *Y. lipolytica* W29 and IMUFRJ 50 682 in OMW-A (only 20 g COD L^{-1}), supplemented with 6 g L^{-1} and 12 g L^{-1} of ammonium sulphate (Figure 5.1).

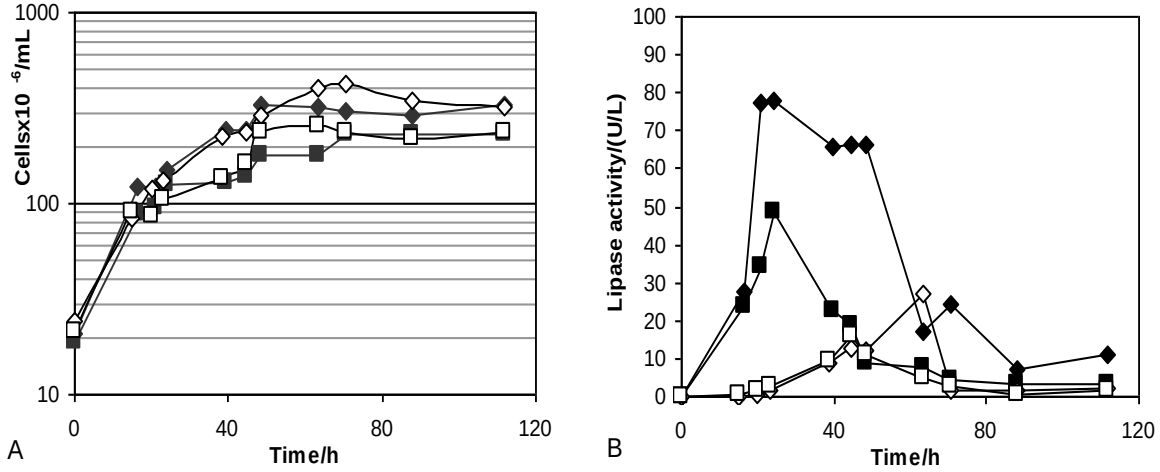

Figure 5.1 A. *Y. lipolytica* strain W29 (closed symbols) and strain IMUFRJ 50682 (open symbols) growth in OMW media with 6 g L^{-1} (♦) and 12 g L^{-1} (■) of ammonium sulphate at 240 rpm and 27 °C. **B.** Time course of extracellular lipase activity.

The effluent degradation (sugars, phenols and COD) and the lipase production for the cultures of strains W29 and IMUFRJ 50 682, supplemented with 6 g L^{-1} (N6) and 12 g L^{-1} (N12) of ammonium sulphate, is presented in Table 5.1.

Table 5.1 Total degradation percentage of sugars, phenols and COD; maximum values of lipase activity and productivity for the cultures of strains W29 and IMUFRJ 50 682, supplemented with 6 g L^{-1} (N6) and 12 g L^{-1} (N12) of ammonium sulphate.

	Reduction sugars (%)	Phenols (%)	COD (%)	Lipase activity (U L^{-1})	Lipase Productivity (U L^{-1} h^{-1})
W29-N6	90 ± 8	72 ± 8	61 ± 5	78 ± 6	3.7 ± 0.4
W29-N12	63 ± 5	57 ± 7	79 ± 8	49 ± 3	2.3 ± 0.3
IMUFRJ-N6	90 ± 9	68 ± 8	75 ± 6	27 ± 3	0.43 ± 0.12
IMUFRJ-N12	76 ± 6	39 ± 5	80 ± 7	16 ± 2	0.36 ± 0.08

*The values are replicate mean ± standard deviation

Both *Y. lipolytica* strains were able to grow in OMW supplemented with yeast extract and ammonium sulphate (Figure 5.1 - A). The growth curves of both strains were identical for the same culture conditions and a 15-fold cell number increase was obtained after 110 h of cultivation, for the lowest value of ammonium sulphate concentration used. Increasing ammonium supplementation did not improve cell growth; on the contrary, a reduction of 30 % in final cell density was obtained with 12 g L^{-1} of ammonium added. The increase in ammonium concentration also had a negative effect on extracellular lipase production for both strains (Figure 5.1 - B). The strain IMUFRJ 50682 has been reported to be an efficient lipase producer (Amaral *et al.*, 2006), but the strain W29 showed a higher potential for lipase production in OMW based medium. In fact, W29 strain has been reported to be a good candidate for the OMW use in lipase production (Lanciotti *et al.*, 2005). The work presented in this chapter, and already published (Lopes *et al.,* 2009), is the first report on the behavior of IMUFRJ 50682 strain in OMW based medium. The low lipase activity level found for this strain in OMW medium can be explained by the use of the medium nutrients to produce other biomolecules, such as biosurfactants (Amaral *et al.*, 2006).

Both strains were able to consume the reducing sugars present in OMW (Table 3.2), but highest consumption was obtained for the lowest amount of ammonium supplied, which is in accordance with the cell growth profile (Figure 5.1 - A). This was also observed for phenolic compounds degradation (Table 5.1). However, the increase in ammonium supply slightly improved the COD degradation. The maximum values of COD degradation obtained (up to 80 %) were similar to the maximum values previously reported for OMW aerobic treatment with fungi (Paraskeva *et al.*, 2006).

5.3.2. Effect of cell concentration and surfactant to *Y. lipolytica* W29

The main goal of these experiments was the OMW use as a substrate for cell growth and lipase production. Therefore, the 6 g L^{-1} ammonium concentration and the strain with highest values of lipase activity (W29) were selected for further studies concerning the influence of culture conditions on the process: no supplementation, with 1 g L^{-1} yeast extract and 6 g L^{-1} $(NH_4)_2SO_4$ and with 1 g L^{-1} yeast extract, 6 g L^{-1} $(NH_4)_2SO_4$ and 1 g L^{-1} of Tween 80 (Figure 5.2).

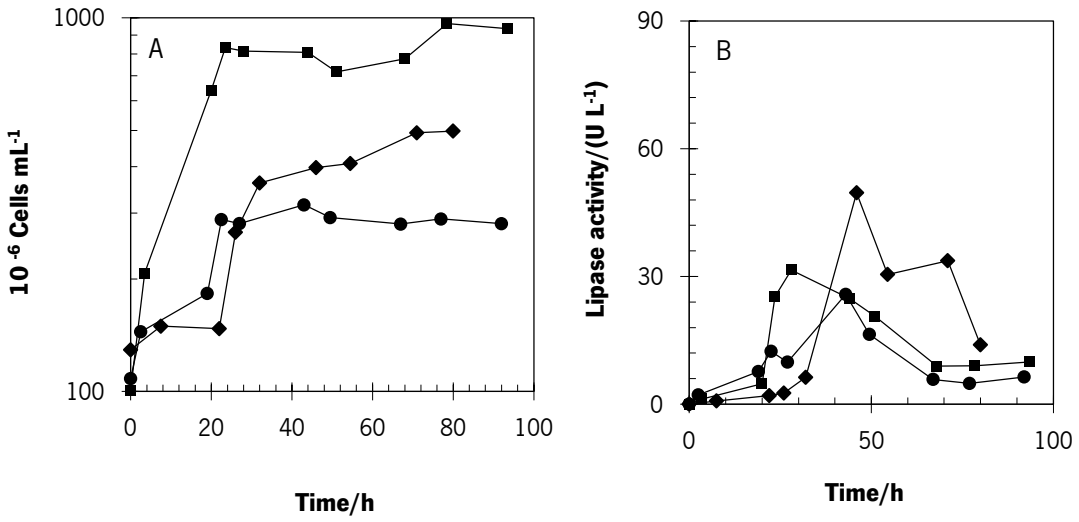

Figure 5.2 A. *Y. lipolytica* W29 growth in OMW media in baffled conical flasks with an initial cell concentration of 10^8 cells mL^{-1} at 240 rpm and 27 °C. **B.** Time course of extracellular lipase activity: (●) OMW without supplementation, (◆) OMW supplemented with 1 g L^{-1} yeast extract and 6 g L^{-1} $(NH_4)_2SO_4$ and (■) OMW supplemented as previous with the addition of 1 g L^{-1} of Tween 80.

In these batch trials, the initial cell concentration was increased (10^8 cells mL^{-1}) and baffled flasks were used to ensure strong mixing in this increased cell density culture. For these culture conditions, in comparison with the experiments with a lower cell density inoculum, an increase in final cell density was reached (around 1.6-fold, for the trial with ammonium sulphate and no surfactant) (Figure 5.2). The 1 g L^{-1} of Tween 80 addition to the medium strongly improved cell growth and a final cell concentration rise of 1.9-fold was observed, which indicates that the surfactant enhanced mass transfer between substrates in the liquid phases, thus improving cell growth. On the other hand, the use of a cell culture with higher cell density in the OMW-based medium did not favor the kinetics of lipase production (Figure 5.2-B). In fact, a 36 % reduction in the maximum value of extracellular lipase activity was found (Table 5.1 and 5.2) compared with values with lower cell density. The reduction in the lipase production by Tween 80 in synthetic media with *Y. lipolytica* cultures was also observed by other authors (Domínguez *et al.*, 2003).

Cell growth on OMW with no supplementation was also studied. The results obtained show the ability of *Y. lipolytica* W29 to grow in OMW, as well as the induction of lipase secretion in OMW without modification (Table 5.2).

Table 5.2 Total degradation percentage of sugars, phenols and COD; maximum values of lipase activity and productivity for W29 cultures, supplemented with 6 g L^{-1} (N6) of ammonium sulphate, with 1 g L^{-1} of Tween 80 (N6 – T) and without supplementation (N0).

	Reducing sugars (%)	Phenols (%)	COD (%)	Lipase Activity (U L^{-1})	Productivity (U L^{-1} h^{-1})
N6	86 ± 7	70 ± 6	54 ± 5	49.7 ± 4	1.1 ± 0.1
N6-T	88 ± 8	47 ± 4	74 ± 7	31.5 ± 3	1.1 ± 0.2
N0	75 ± 6	43 ± 4	63 ± 6	25.8 ± 3	0.6 ± 0.1

*The values are replicate mean ± standard deviation

According to the Table 5.2 data, *Y. lipolytica* W29 is also able to degrade COD and phenols without the need for OMW supplementation. The low nitrogen content of the medium (Table 3.2) and the lack of some vitamins may, however, limit the lipase production. Surfactant addition to the supplemented OMW medium improved organic load degradation, but had a different effect on the total phenols reduction.

Figure 5.3 shows the variation of sugars, phenols and COD during cultivation time in the presence of Tween 80.

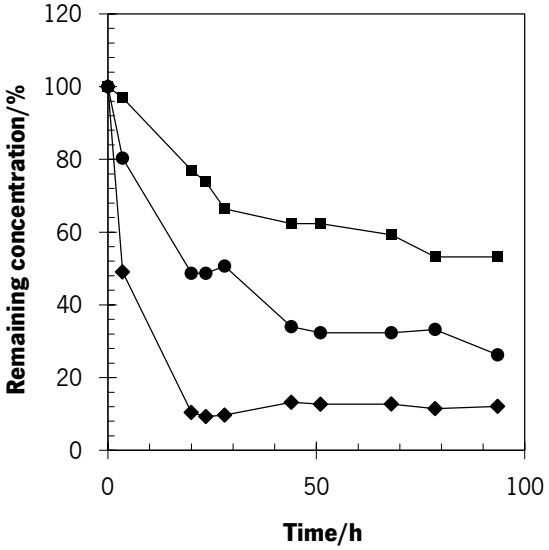

Figure 5.3 Degradation of reducing sugars (◆), COD (●) and phenols (■), obtained with *Y. lipolytica* W29 cultures in OMW supplemented with 6 g L^{-1} of ammonium sulphate, 1 g L^{-1} of yeast extract and 1 g L^{-1} of Tween 80.

Identical profiles were obtained for other conditions, showing in all cases that the first substrates consumed were sugars and the most difficult to consume were the phenolic compounds. The high percentage (around 70 %) of total phenols degradation achieved, at the same conditions that also favored the lipase production, indicates that the OMW use by *Yarrowia lipolytica* has a high potential of OMW valorization and degradation, since the effluent detoxification through the phenols content reduction is a crucial step for further treatment in biological plants.

5.3.3. Effect of using different OMW and yeasts

Experiments with *Y. lipolytica* W29 and *C. rugosa* PYCC 3238, in OMW-B and OMW-C (with almost 200 g COD L^{-1}), were performed using 1000 mL Erlenmeyer baffled flasks, with 400 mL of OMW-based media. In Figure 5.4, the cell growth of both strains in both OMW is depicted.

In previous experiments (5.3.1 and 5.3.2), was used $(NH_4)_2SO_4$ as nitrogen source however, ammonium chloride (NH_4Cl) will be used is these experiments due to its positive effect on lipase production, as already shown by other authors (D'Annibale *et al.*, 2006).

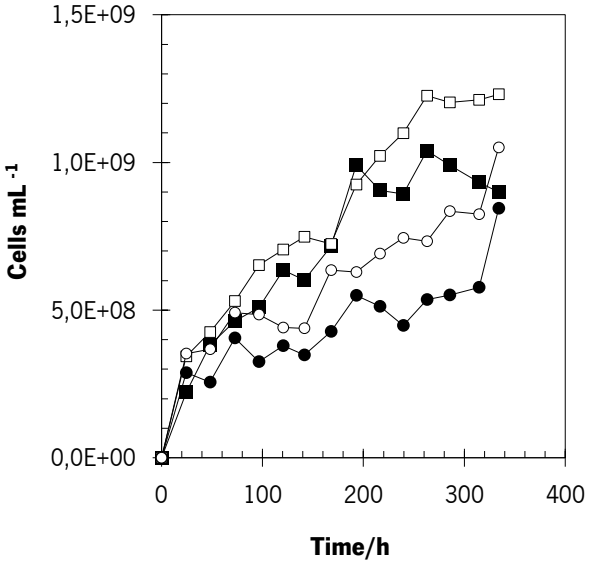

Figure 5.4 Growth of *Y. lipolytica* W29 ATCC 20460, in OMW-B (■) and OMW-C (●); and *C. rugosa* PYCC 3238, in OMW-B (□) and OMW-C (○).

The OMW used were OMW-B and OMW-C. Both strains were able to grow on both OMW, without dilution (Figure 5.4), increasing about 1.7 log the cell number. Cell mass production was higher for OMW-B than for OMW-C, for both strains, probably due to the higher content of sugars and lower content of phenolic compounds in this medium (Table 3.2).

Table 5.3 summarizes the effluent degradation obtained in these experiments.

Table 5.3 OMW organic matter degradation, for different conditions of OMW and strains

	Reducing Sugars reduction/%		Phenolic compounds reduction/%		COD reduction/%	
Yeast strain	OMW-B	OMW-C	OMW-B	OMW-C	OMW-B	OMW-C
Y. lipolytica W29 ATCC 20460	90.5	71.8	19.2	20.6	52.6	29.5
C. rugosa PYCC 3238	80.2	64.2	12.2	*N*[a]	62.2	35.8

[a] Negligible

Both strains were able to consume almost all of the sugars present in the media and to significantly reduce COD (Table 5.3). In spite of the low degradation of phenolic compounds, no cell growth inhibition was noticed. The COD, reducing sugars and phenolic compounds reductions were greater in OMW-B, which has slightly lower COD and phenolic compounds concentration, than OMW-C. The strain of *C. rugosa* seemed to be more efficient than *Y. lipolytica* reducing the OMW COD, but *Y. lipolytica* degraded better the phenolic compounds (Table 5.3).

The effluent degradation was more difficult with both OMW-B and C, than with OMW-A, due to a higher COD and phenolic compounds concentration. As a result, further improvements should be performed to permit a better degradation when high polluted OMW are used.

Both yeast cells were observed by optical microscopy (Figure 5.5 and 5.6), in YPD control media (synthetic media) and in OMW-based media. Cells displayed a typical oval form in all the trials, demonstrating that cell growth in OMW medium did not induce hyphae formation for both strains but the cells seems to be more aggregated than in YPD medium.

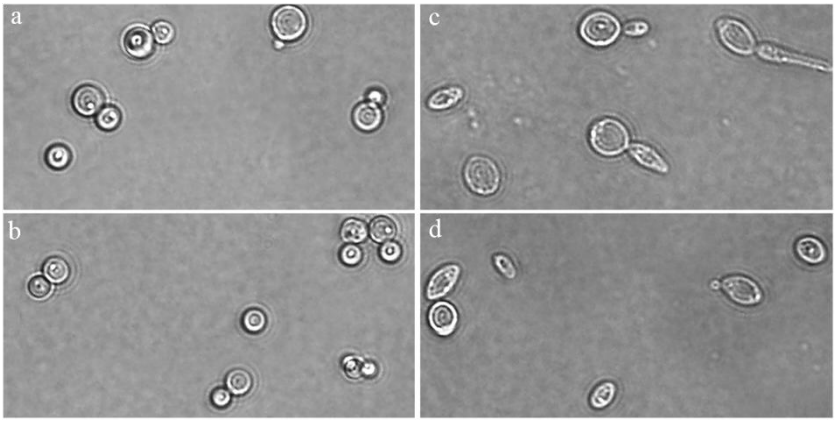

Figure 5.5 Microscopic morphological characteristics of *Candida rugosa* **(a, b)** and *Yarrowia lipolytica* **(c, d)** image, in synthetic medium (magnification 400x).

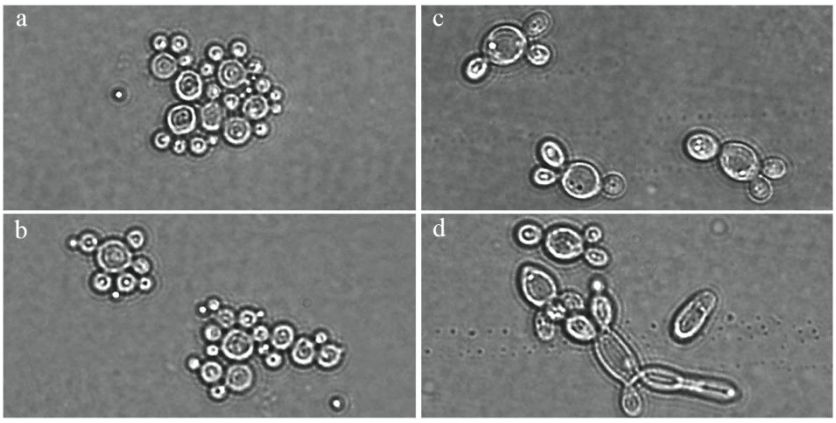

Figure 5.6 Microscopic morphological characteristics of *Candida rugosa* **(a, b)** and *Yarrowia lipolytica* **(c, d)** image, in OMW medium (magnification 400x).

Yeasts are capable of forming aggregates, as a survival strategy in adverse conditions (Calleja *et al.*, 1987). Moreover, the presence of lipids in the OMW can induce the cell aggregation around oil droplets, particularly for strains with hydrophobic cell surfaces, as *Y. lipolytica* (Aguedo *et al.*, 2005). A greater cell aggregation occurred for *C. rugosa* strains, which could possibly cause substrates availability limitations, to the cells. These limitations could explain the weak cell growth observed, for this strain.

Batch trials in 1000 mL Erlenmeyer baffled flasks, with 400 mL of working volume, were then performed with the six strains of *C. rugosa*, *C. cylindracea* and *Y. lipolytica*, using the OMW samples OMW-D and OMW-E (Table 3.2). These results are shown on Table 5.4.

Table 5.4 OMW degradation, using different OMW and strains

Yeast strain	Reducing Sugars reduction/%		Phenolic compounds reduction/%		COD reduction/%	
	OMW-D	OMW-E	OMW-D	OMW-E	OMW-D	OMW-E
Y. lipolytica W29 ATCC 20460	55.5	85.1	N[a]	31.3	21.6	36.9
Y. lipolytica CBS 2073	56.5	85.3	N[a]	25.3	23.5	51.3
Y. lipolytica IMUFRJ 50682	58.8	76.0	N[a]	20.0	23.1	50.9
C. rugosa PYCC 3238	56.5	82.6	N[a]	20.4	20.4	58.7
C. rugosa CBS 2275	55.6	68.7	N[a]	15.3	31.1	40.9
C. cylindracea CBS 7869	54.8	84.3	N[a]	27.0	45.8	70.2

[a] Negligible

All strains were able to grow on both OMW, without dilution and with similar growing profiles to those previously presented. OMW-E seems to be more easily degradable, since higher reduction values of sugars; phenolic compounds and COD were obtained for this sample than for OMW-D. The phenolic compounds degradation in OMW-D was negligible for all strains. The strains of *Y. lipolytica* W29 and *C. cylindracea* CBS 7869, presented the better performance degrading phenolic compounds of OMW-E. Moreover, *C. cylindracea* was the most efficient strain on COD degradation in both OMW samples. Concerning the reducing sugars consumption, no significant differences were found among the cultures but the strains of *Y. lipolytica* and *C. cylindracea* presented a slight better performance.

Lipase production by *Y. lipolytica* W29 grown on OMW-based medium was already demonstrated in the first experiments herein presented with OMW-A. Using undiluted OMW-D and OMW-E and the 6 yeast strains was also possible to detect lipase activity (Table 5.5).

Table 5.5 Maximum of lipase activity produced for the different strains and OMW, in Erlenmeyer baffled flasks

	Lipase Activity ($U \cdot L^{-1}$)	
Yeast strain	OMW-D	OMW-E
Y. lipolytica W29 ATCC 20460	29.9 ± 16.8	21.2 ± 12.9
Y. lipolytica CBS 2073	68.9 ± 12.1	54.8 ± 9.3
Y. lipolytica IMUFRJ 50682	35.3 ± 12.3	24.6 ± 8.5
C. rugosa PYCC 3238	28.7 ± 12.9	32.7 ± 13.7
C. rugosa CBS 2275	55.1 ± 5.8	24.7 ± 2.6
C. cylindracea CBS 7869	145.6 ± 55.0	58.1 ± 21.8

Data are mean values ± standard deviation (n=2)

The kinetic profile demonstrates that the activity increases up to a maximum, after what it decays, which is in accordance with the ones obtained in the first experiments (Figure 5.2 – B). In both OMW, *C. cylindracea* was the strain that expressed the highest value of lipolytic activity. This result agree with the one obtained by D'Annibale *et al.* (2006), in which the authors demonstrated the high potential of lipase production with a strain of *C. cylindracea* NRRL Y-17506. An increase of the lipase activity in OMW-D with *C. cylindracea* was obtained, compared with the experiment with *Y. lipolytica* W29. Except for *C. rugosa* PYCC 3238, all strains reached the highest value of lipolytic activity in OMW-D, probably due to lower content in sugars that can repress lipase production (Fickers *et al.*, 2003; Dalmau *et al.*, 2000).

5.4. CONCLUSIONS

The potential application of the yeast *Y. lipolytica* for OMW valorization by its use as culture medium for biomass and enzymes production is confirmed. The strain W29 showed better

performance in this hostile medium than the wild type strain IMUFRJ 50682. This result confirms the wide range of W29 application in bioprocesses development, particularly in media with lipidic components (Aguedo *et al.*, 2004). The utilization of olive mill wastewaters for biological production of high value products may have a positive impact on the environmental problem of OMW management, since it can act also as a first step in effluent treatment.

The results of this study also confirmed the potential application of other non-conventional lipolytic yeasts for OMW valorization, by its use as culture medium for biomass and enzymes production. The ability of all strains used, to produce lipase from undiluted OMW was shown. Moreover, *C. cylindracea* was the best strain concerning the effluent degradation.

CHAPTER 6. BATCH AND FED-BATCH FERMENTATION OF OMW FOR LIPASE PRODUCTION

The aim of this chapter was to study the lipase production and OMW degradation by *Candida cylindracea* CBS 7869, *Candida rugosa* CBS 2275 and *Yarrowia lipolytica* W29 (ATCC 20460), in batch and fed-batch experiments using a lab-scale bioreactor with OMW based media. Additionally, the effect of the OMW medium on the yeasts growth was performed in the bioreactor.

Comparing cellular growth in OMW and in YPD media, the studied yeasts not only were capable of achieving similar or even better cell growth, than in glucose synthetic media, but also to highly consume the existing phenolic compounds. In the batch operation mode, *Candida cylindracea* was the best lipase-producing yeast (30 U L^{-1} h^{-1}), confirming the Erlenmeyer batch experiments previously performed (**CHAPTER 5**), while *Candida rugosa* was the most efficient in fed-batch culture, concerning lipase production (130 U L^{-1} h^{-1}) and effluent degradation (reduction of 64 % of COD, 27 % of phenolics and 77 % of total lipids).

Therefore, these results at bioreactor scale suggest that *Candida rugosa* CBS 2275 has a great potential to be applied in OMW treatment, as a significant lipase producer while removing oil and COD from this effluent.

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6.1. INTRODUCTION

The OMW content in simple and complex sugars, residual oil (lipids), proteins, mineral elements and phenols, turns this effluent in a renewable resource, as it can be extracted and purified or used for fermentative production process (Brozzoli *et al.*, 2009; Crognale *et al.*, 2006; Ertugrul *et al.*, 2007; Lanciotti *et al.*, 2005). In addition, the residual oil of OMW turns this waste in a potential growth medium to lipolytic microorganisms (Brozzoli *et al.*; 2009, Asses *et al.*; 2009a). Although the use of OMW, as a growth medium to produce lipase, has been studied by several authors (Brozzoli *et al.*, 2009; Lanciotti *et al.*, 2005; D'Annibale *et al.*, 2006), it has been mostly conducted using batch operation, even though many reports revealed that, with synthetic media, the highest lipase production was achieved with fed-batch strategy (Gordillo *et al.*, 1998; Gordillo *et al.*, 1995; Montesinos *et al.*, 1996).

The **aim** of this study was to compare the performance of three yeast species in batch and fed-batch cultures in OMW-based medium, with the purpose of optimizing the lipase production as well as the OMW degradation.

6.2. MATERIALS AND METHODS

6.2.1. Media

The OMW-based media used is composed by OMW-F or OMW-G (Table 3.2), without dilution. The medium was supplemented with a source of nitrogen (NH_4Cl), previously chosen by its positive effect on lipase production, and yeast extract (in a reason of 2:5 relatively to the NH_4Cl added). Before sterilization, the pH was adjusted with NaOH and 6 drops of silicon antifoam agent were added.

For the study of the OMW effect of cells growth, the growth in OMW based medium (OMW-F) was compared with the culture growth in YPD (Table 3.1), supplemented with extra glucose (YPD+G).

This control medium (YPD+G) diverge from YPD in the increased amount on glucose concentration (68.5 g L^{-1} of glucose) that equals the one present in the used OMW.

6.2.2. Culture conditions

Batch trials for OMW effect on cell growth

Each strain, with an initial concentration of approximately 10^6 cells mL^{-1}, was grown in two different media, non-diluted OMW and YPD+G (the control) in a 2-L bioreactor (Biolab, B. BRAUN; h_t =21 cm, D_t = 11 cm). The cultures were incubated at 27 °C, pH 5.6, 2 vvm of aeration and 500 rpm of agitation rate. Throughout the fermentation time, culture samples were collected and cell density was assessed by cell counting.

Batch and fed-batch trials

Designing the growth medium or physico-chemical factors, may greatly influence the production of lipase. Batch and fed-batch trials were performed in a 2-L bioreactor (Biolab, B. BRAUN). A study of the batch conditions, for lipase production, was firstly carried out, concerning C/N ratio, pH and agitation. The pH was automatically adjusted by means of a pH-meter and delivery pumps loaded with NaOH 1N and HCl 1N. An aeration rate of 1.5 L min^{-1} was used. Experiments were carried out, in duplicate, during 9 days.

To the batch experiments, the reactor was filled with 1.3 L of OMW-based medium and, after sterilization; it was inoculated with yeast cells previously grown in YPD medium, as described above.

The fed-batch experiments were initiated with a batch phase of 24 h, after which the OMW-based medium began to be fed. The feeding was performed with two pulses per day, of 10 % (v/v) of the medium inside the reactor, each. Thus, the fed-mode of operation was approximated by a

repeated-batch operation. The dilution rate was maintained between 0.02 h^{-1} and 0.01 h^{-1}. The fed-batch was stopped, when the reactor was filled up to 1.5 L, with OMW-based medium, achieving 9 days of fermentation. In these experiments, two different methodologies were studied for OMW-based media inoculation: cells pre-grown outside and inside the bioreactor. In the first case, cells were grown in a 1-L Erlenmeyer baffled flask (with 400 mL of YPD) and then harvested and re-suspended in the OMW-based medium within the bioreactor with an initial volume of 400 mL. In the second case, the cells were grown within the bioreactor (with 400 mL of YPD) and the OMW-based medium was fed afterward.

The time course of cell density, total phenols, reducing sugars and lipase activity was followed throughout the experiments. However, the chemical oxygen demand (COD), total nitrogen (TN) and total organic carbon (TOC) were only assessed at the beginning and at the end point of the experiments.

6.3. RESULTS AND DISCUSSION

6.3.1. Effect of OMW based medium toxicity on cell growth

Experiments in 2 L-bioreactor were performed in order to verify the results previously obtained in microplates (CHAPTER 4). Results obtained of cell growth and reducing sugars concentration through time are depicted in Figure 6.1.

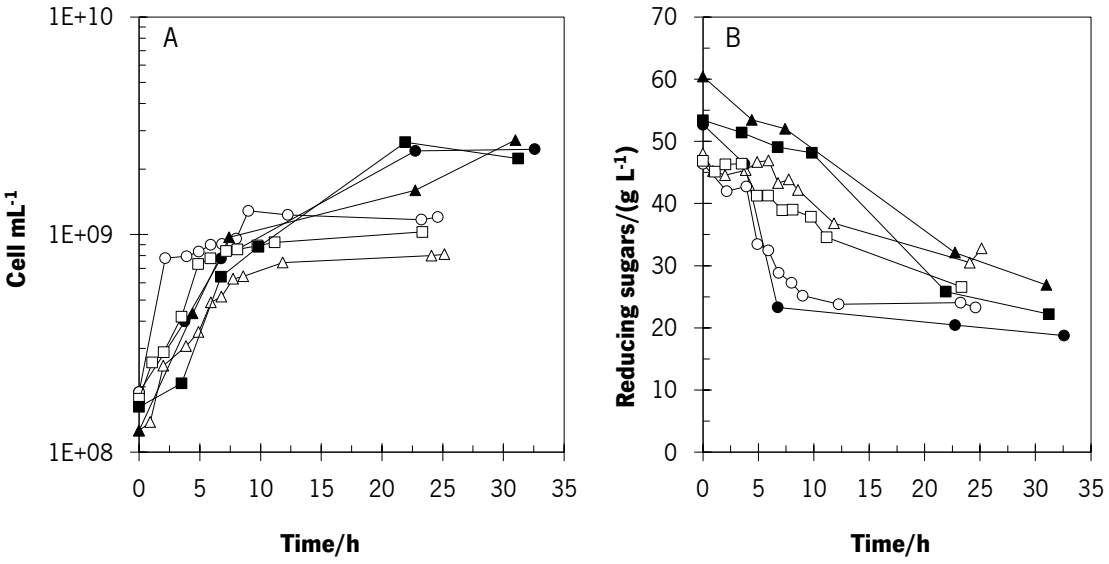

Figure 6.1. Time courses of **A.** Cell growth and **B.** Reducing sugars consumption of the 3 yeast strains: *Candida cylindracea* (●), *Candida rugosa* (▲) and *Yarrowia lipolytica* (■) on different media: YPD+G (open symbols) and OMW (closed symbols). Each point represents the average of two replicates (standard deviation <10 % of the mean).

The results on a lab-scale bioreactor indicate initial cellular growth retardation for *Candida cylindracea* and *Yarrowia lipolytica* (Figure 6.1) when grown in OMW. Nevertheless, in the end of the fermentation the cell density in YPD+G media has stabilized while in the OMW media was still increasing. Moreover, the specific growth rates of *Candida cylindracea*, *Candida rugosa* and *Yarrowia lipolytica* grown in YPD+G were 0.66, 0.21 and 0.28; while in OMW these values were 0.21, 0.28 and 0.17, respectively. In fact, phenolic extracts containing mainly phenolic acids, have been described as being more active against bacteria than against yeasts, suggesting that yeasts have a stronger resistance to the action of these compounds (García-Ruiz *et al.*, 2008), but *Candida cylindracea* suffers a strong initial inhibition. The hydroxytyrosol contained in the OMW-based medium is possibly inhibitory to this yeast strain, since in microplate trials *C. cylindracea* was also inhibited by hydroxytyrosol media.

The COD degradation in these experiments was negligible, but, similarly for all strains, a degradation of (88.0 ± 6.0) %, (66.0 ± 2.0) %, (65.6 ± 1.4) % and (71.5 ± 1.5) % was obtained for hydroxytyrosol, tysosol, oleuropein and caffeic acid, respectively. Among these, hydroxytyrosol was the most degraded, statistically different from the others, at a 95 % confidence level by the Tukey's

HSD test. It seems that, in the presence of a great amount of a carbohydrate easier to degrade (glucose), cells were also able to degrade each of these phenolic compounds. The ability of olive fruit bacteria to remove phenolic compounds from OMW was studied by Tziotzios *et al.* (2007), which reported a maximum phenolic removal of 60 % with a bench-scale reactor; on the other hand, approximately 60 % of the initial glucose was consumed. Glucose was consumed by (42 ± 5) % and (59 ± 3) % in YPD+G and OMW, respectively (Figure 6.1 – B). These results might confirm that the studied yeasts, in the presence of other carbohydrates besides the phenolic compounds, not only are capable of achieving similar or better cell growth, but also to consume the existing phenolic compounds.

6.3.2. Batch cultures

The aim of this study was to investigate the performance of three yeast species in batch and fed-batch cultures in OMW-based medium, with the purpose of optimizing the lipase production as well as the OMW degradation. Firstly, the C/N ratio effect was evaluated, as well as the pH and stirring rate. Table 6.1 shows a summary of the results, in terms of maximum lipase activity achieved and cells growth (Log N/N_0), obtained in different experiments, testing changes on the operational conditions.

Table 6.1 Results of the batch conditions study for *Y. lipolytica*

pH	Agitation/rpm	C/N	Lipase activity/(U L^{-1})	Log N/N_0
5.6	400	5	0.50 ± 0.06	1.07 ± 1.04
5.6	400	15	0.56 ± 0.15	1.24 ± 0.73
7.2	400	15	1.09 ± 0.03	1.08 ± 1.42
7.2	500	15	2.92 ± 1.40	1.41 ± 1.19

Data are the replicates (n=3) mean value ± 95 % confidence interval.

As previously referred, to counteract the characteristic lack of nitrogen of the raw OMW (D'Annibale *et al.*, 2006; Eusébio *et al.*, 2002), the medium was supplemented with a source of nitrogen, NH_4Cl. Yeast extract was also added to assure a minimum amount of vitamins. In the present study, the C/N ratios of 5 and 15 were tested.

Figure 6.2 shows the time courses of cell-growth, reducing sugars consumption and lipase activity of the culture broth, for *Y. lipolytica* experiments when C/N ratios of 5 and 15 are used. *C. cylindracea* and *C. rugosa* presented similar behavior.

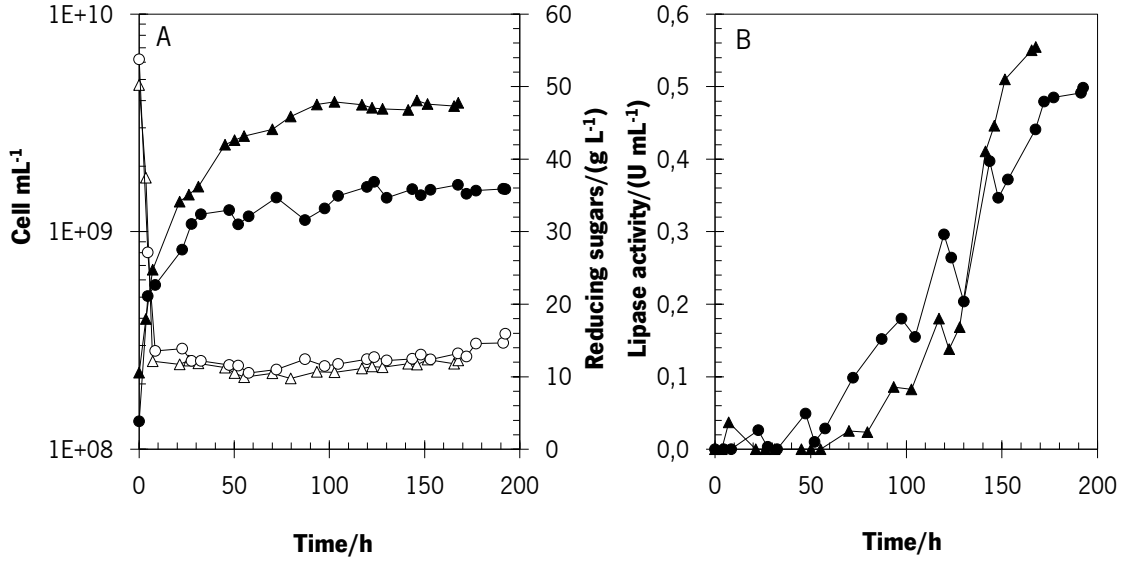

Figure 6.2 A. Time course of cell growth (closed symbols), reducing sugars consumption (open symbols) and **B.** lipase production, by *Y. lipolytica*, with C/N rations of 15 (▲) and 5 (●), in batch experiments performed on the bioreactor, with OMW-F.

The increase of the C/N ratio leads in general to higher cell growth (up to 0.9 log units) but similar glucose consumption and lipase production profiles for both. Although the C/N ratio of 15 did not increase significantly the production of lipase, it will be used in further experiments since less nitrogen is spent in the process.

The enzyme production is also profoundly influenced by initial pH of the medium and by the amount of oxygen available. In previous set of experiments (Chapter 5) OMW based media with initial pH of 5.6 was used but a pH around 7.0 is frequently used for lipase production (Brozzoli *et al.*, 2009; Freire *et al.*, 1997). The results obtained confirmed that neutral pH favors lipase production in OMW and also that the increase of stirring rate lead to a significant improvement of lipase in this emulsion type of medium.

Figure 6.3 depict the results obtained in experiments using the best batch conditions (500 rpm, C/N 15 and pH 7.2) for lipase secretion and for the three yeast species used.

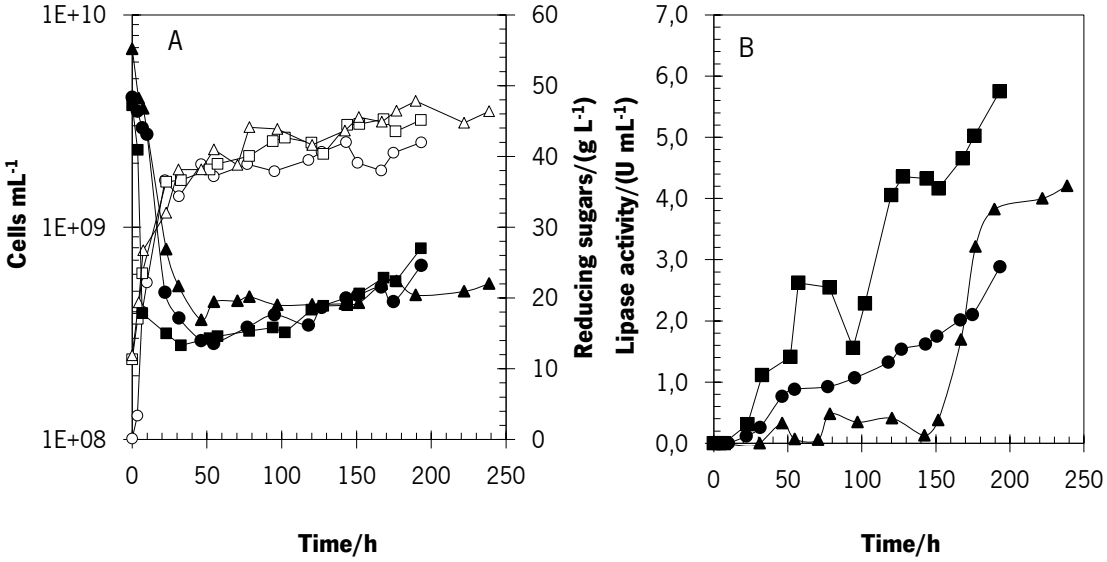

Figure 6.3 A. Time course of cell growth (open symbols), reducing sugars consumption (closed symbols) and **B.** lipase production, by *C. cylindracea* (■), *Y. lipolytica* W29 (●) and *C. rugosa* (▲) in batch experiments with OMW-F.

All strains present a similar cellular growth profile on the OMW medium described above showing a remarkable cell adaptation to the undiluted OMW, since no cellular growth inhibition by OMW was observed, for *C. cylindracea*, *Y. lipolytica* and *C. rugosa*. Reducing sugars consumption profiles were quite similar between strains and overall sugars degradation was 49 %, 43 % and 54 % for *C. cylindracea*, *Y. lipolytica* and *C. rugosa*, respectively. Most of this consumption (about 85 %) was achieved in the early hours, especially for *C. cylindracea* CBS 7869, which showed higher extracellular lipase productivity (30 U L^{-1} h^{-1}), than *C. rugosa* (20 U L^{-1} h^{-1}) and *Y. lipolytica* (7 U L^{-1} h^{-1}). The sugar consumption profile is indicative of a short lag phase for all yeasts under study, which corroborate the good adaptability of these strains to OMW based medium. Brozzoli *et al.* (2009) also obtained highest initial rates of total sugars consumption by *Candida cylindracea* NRRL Y-17506, grown in a 3-L bench-top stirred tank reactor on OMW-based medium. *C. cylindracea*

showed the best lipase productivity, in the optimized batch conditions. Several authors had previously screened some yeast strains and other microorganisms for lipase production; *C. cylindracea* is often pointed out as the best lipase-producer. For instance, in previous experiments (Chapter 5) batch cultures in Erlenmeyer baffled flasks were performed using 6 different strains: *Candida rugosa* (PYCC 3238 and CBS 2275), *Yarrowia lipolytica* (CBS 2073, W29 ATCC 20460 and IMUFRJ 50682) and *Candida cylindracea* CBS 7869. Similarly, to the work herein reported at bioreactor scale, this last strain was the best extracellular lipase producer. Moreover, strains of *Geotrichum candidum* (NRRL Y-552 and Y-553), *Rhizopus arrhizus* (NRRL 2286 and ISRIM 383), *Rhizopus oryzae* (NRRL 6431), *Aspergillus oryzae* (NRRL 1988 and 495), *Aspergillus niger* (NRRL 334), *Candida cylindracea* (NRRL Y-17506) and *Penicillium citrinum* (NRRL 1841 and 3754, ISRIM 118) were also screened by D'Annibale *et al.* (2006). They found as well that a strain of *C. cylindracea*, NRRL Y-17506, have the highest lipase activity.

Other authors reported that the COD concentration gradually decreases throughout the experiments (Crognale *et al.*, 2006; D'Annibale *et al.*; 2006; Tziotzios *et al.*, 2007), however in these experiments a negligible COD degradation was obtained. In fact, the OMW used in these experiments presented an extremely high value of COD (261 g L^{-1}) while other authors generally use OMW with COD values below 100 g L^{-1} (Aouidi *et al.*, 2009; Crognale *et al.*, 2006; D'Annibale *et al.*, 2006; Tziotzios *et al.*, 2007). Although this concentration has not been toxic for the microorganisms, it did not allow the COD degradation.

It is important to refer that undesirably, the color of the OMW-based media becomes more intense throughout each experiment. This was probably a consequence of the alkaline solution addition, to control the pH during the fermentation, inducing the phenolic compounds oxidation but also, as referred by Hamdi *et al.* (1992), because of the auto-oxidation of these compounds in the aerobic conditions.

6.3.3. Fed-batch cultures

Other modes of operation, than batch mode, have been proved to be useful to improve lipase production in synthetic media (Gordillo *et al.*, 1998; Gordillo *et al.*, 1995; Montesinos *et al.*, 1996).

Montesinos *et al.* (1996) studied the interaction between medium components, microorganisms and the operational mode, to produce lipase by *Candida rugosa* ATCC 14830 in synthetic media, and found that the specific productivity in continuous culture was slightly higher than in batch cultures. Furthermore, Gordillo *et al.* (1998) also studied the lipase production from *Candida rugosa* (ATCC 14830) in synthetic media and reported that the best operation mode tested was a fed-batch, with controlled specific growth rate, which increased the productivity 10-fold comparatively to batch operation.

Firstly, fed-batch experiments were performed using OMW-F (Table 3.2) with *Candida cylindracea* and *Yarrowia lipolytica*. Figure 6.4 shows the time courses of cell-growth, reducing sugars consumption and lipase activity of the culture broth for the both yeasts.

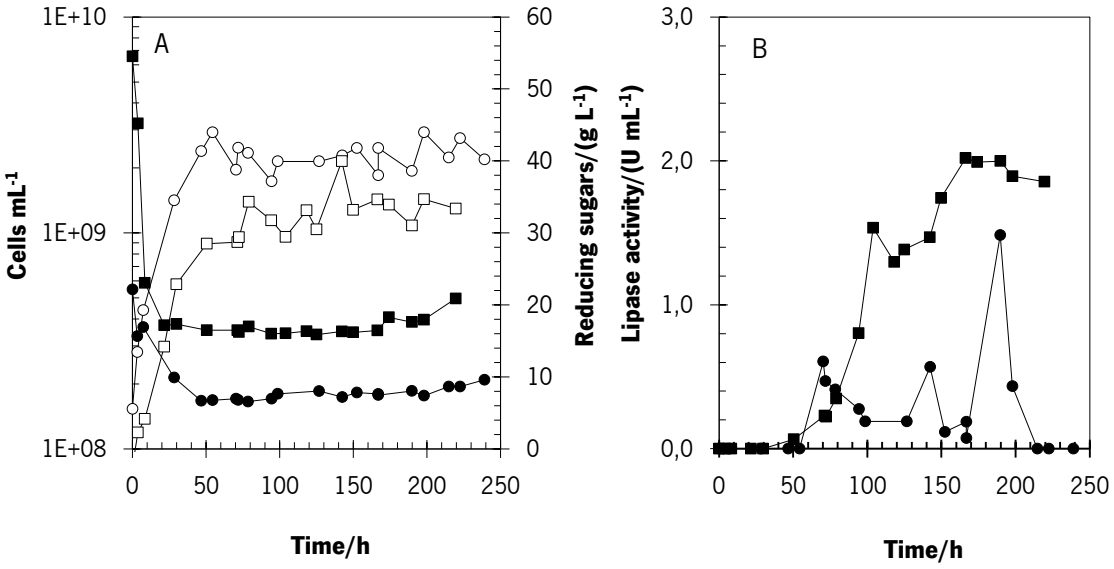

Figure 6.4 A. Time course of cell growth (open symbols), reducing sugars consumption (closed symbols) and **B.** lipase production, by *C. cylindracea* (■) and *Y. lipolytica* (●) for fed-batch cultures, in which the cells were grown in flasks with YPD medium and then harvested and added to OMW-based medium within the bioreactor (OMW-F).

The cell growth for fed-batch cultures (Figure 6.4 - A) was 79 % higher with *Y. lipolytica* than with *C. cylindracea*. The sugar consumption was also superior (7 %) for *Y. lipolytica* than *C. cylindracea*

experiments. On the other hand, in the experiments using *C. cylindracea*, high values of extracellular lipase activity were achieved (Figure 6.4 - B). Thus, the cell growth and sugar consumption were higher for the yeast with lower values of extracellular lipase (*Y. lipolytica*). These results suggest that an inverse relation exists between cell growth and the produced extracellular lipase in the media. Observing Brozzoli *et al.* (2009) work it is also possible to verify this tendency. In fact, in the current work, until the cell growth and glucose consumption stabilize (at the end of the exponential phase), there was no extracellular lipase production. However, after the first 50 hours of fermentation (beginning of the stationary phase), the production of extracellular lipase begins and increases during time. In fact, Pereira-Meirelles *et al.* (1997) reported that the extracellular lipase activity was only observed at high levels, at late stationary phase, whereas intracellular lipase levels were constant and almost undetectable during the cultivation period. This fact suggests that the produced enzyme was attached to the cell wall, mainly at the beginning of cultivation. This behaviour is dependent of species, since, Freire *et al.* (1997) referred that lipase production is growth associated, and found a direct relation between the lipase production and the *Penicillium restrictum* growth.

The COD degradation, by *C. cylindracea* and *Y. lipolytica,* was 14 % and 17 %, respectively. Therefore, even using the OMW-F (261 g L^{-1} of COD), it was possible to obtain some COD degradation, possibly owed to a slight dilution of the OMW, promoted by the fed-batch mode itself. Nevertheless, it must be stressed out that the ratio of reducing sugars per COD values, of the OMW samples used, where about 26 % and 17 %, for OMW-F and OMW-G, respectively. Thus, it basically seems that these yeasts are mostly consuming sugars and the other components, such as phenolic compounds, are only slightly used.

Although the fed-batch feeding conditions slightly improved the COD degradation, relatively to the batch mode, it did not improve lipase production. Thus, as this work aims the improvement of lipase production, while degrading the effluent, a modification in the fermentation conditions was tested, and the cells were pre-grown directly inside the reactor and afterwards the OMW started to be fed. This modification pretends to early adapt the cells to the reactor conditions. These experiments were performed with the three yeast strains (*C. rugosa*, *Y. lipolytica* and *C.*

cylindracea), using OMW-G. Figure 6.5 shows the time courses of cell-growth, reducing sugars consumption and lipase activity of the culture broth for the 3 yeasts.

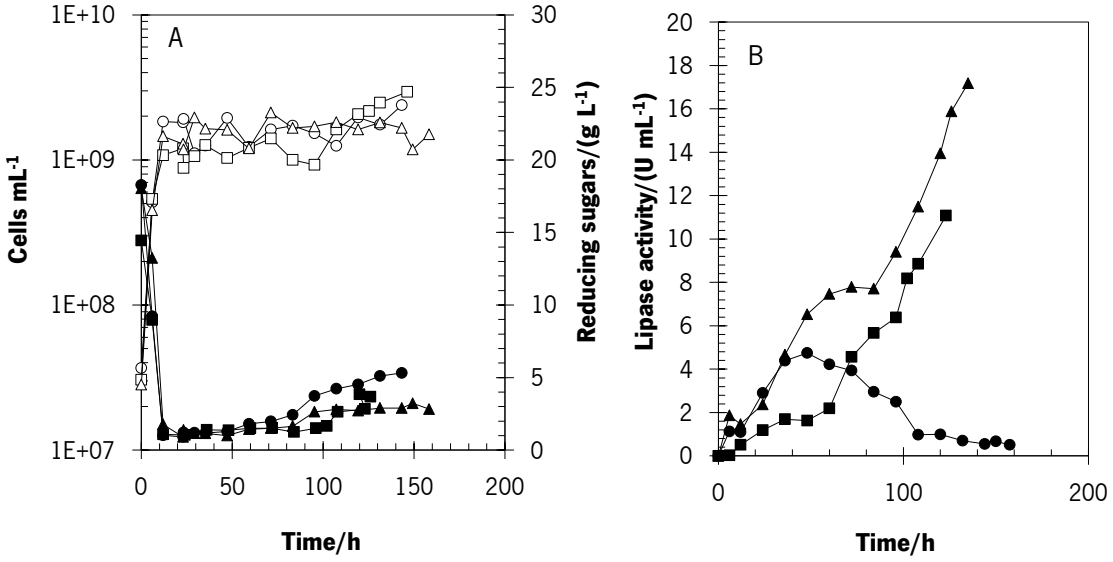

Figure 6.5 A. Time course of cell growth (open symbols), reducing sugars consumption (closed symbols) and **B.** lipase production, by *C. cylindracea* (■), *Y. lipolytica* (●) and *C. rugosa* (▲) for fed-batch cultures, in which the cells were pre-grown in YPD within the bioreactor, using OMW-G.

The results obtained showed, in general, higher cell growth (more than 1 log units), sugar consumption and lipase production (Figure 6.5), comparatively to the previous fed-batch experiments (Figure 6.4). The introduction of this modification, along with the use of OMW-G (with "only" 97 g L^{-1} of COD), led to overall better results, especially for *C. rugosa*.

In previous fed-batch experiments, it was observed that lipase production was higher at the stationary phase. In these experiments, as the exponential phase ends at 12 h, a longer stationary phase occurs and higher values of lipase activity are obtained. In fact, a lipolytic activity of 17 U mL^{-1} was achieved by *C. rugosa*, the best lipase producer in these trials. *C. cylindracea* also reached higher lipase activity values (11 U mL^{-1}) in these conditions than in the previous fed-batch strategy. The obtained results are consistent with those obtained by Gomes *et al.* (2010) who obtained higher productivity values with a start-up procedure of cells grown within the bioreactor in

the biotransformation process of methyl ricinoleate, with the same strain of *Y. lipolytica* used herein.

The typical lipase production kinetics of *Y. lipolytica*, with decay phase after achieving a maximum value of lipase activity (5 U mL^{-1}), was observed (Figure 6.5 - B). This behavior can be observed in several other works using this yeast to produce lipase (Gomes *et al.*, 2010; Pereira-Mairelles *et al.*, 1997). A possible reason for this behavior is the presence of protease (Figure 6.6), which can be responsible for lipase breakdown. In fact, for *Y. lipolytica* was obtained a higher increase of protease than for the others.

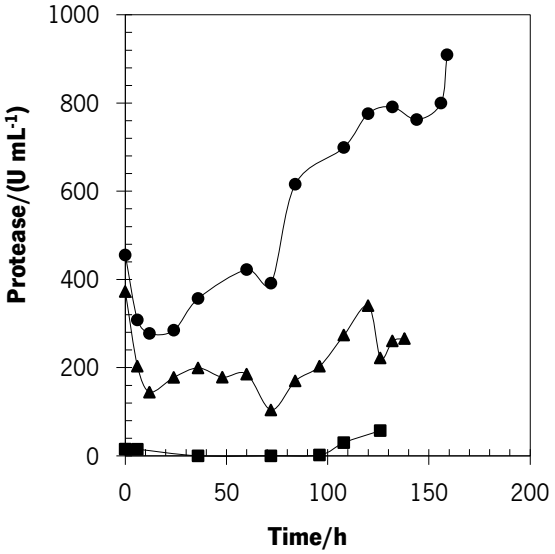

Figure 6.6 Time course of protease activity produced by *C. cylindracea* (■), *Y. lipolytica* (●) and *C. rugosa* (▲).

Comparing the lipase (Figure 6.5 - B) and protease (Figure 6.6) production profiles by *Y. lipolytica* is observed an inverse relation between both. Some authors (Pereira-Meirelles *et al.*; 1997, Freire *et al.*, 1997; Gombert *et al.*, 1999) also reported a sharp decrease on lipolytic activity caused by protease. Furthermore, Freire *et al.* (1997) and Gombert *et al.* (1999) reported that lipase activity decreases; not only due to proteolysis, but also because of media pH increase, which frequently

occurs in the final of the experiments, performed. To verify that, Pereira-Meirelles *et al.* (1997) and Freire *et al.* (1997) used the serine protease inhibitor PMSF (phenylmethylsulphonyl fluoride) in the culture medium to reduce the decrease on lipase activity, and the maximum level of lipase activity was maintained for an additional period of time.

The global results obtained in these experiments are summarized in Table 6.2, in terms of COD, reducing sugars, phenolic compounds and lipids reduction, as well as the obtained maximum extracellular activity and productivity in lipase.

Table 6.2 Overall results of the effluent degradation (COD, reducing sugars, phenolic compounds and lipids consumption) and lipase production (maximum extracellular activity and productivity) obtained for fed-batch cultures, in which the cells were pre-grown in YPD inside the bioreactor

Parameter	*C. cylindracea*	*C. rugosa*	*Y. lipolytica*
COD/%	58	64	50
Reducing sugars/%	52	66	54
Phenolics/%	4	27	1
Lipids/%	56	77	55
Lipase Activity/(U mL^{-1})	11.3	17.2	4.7
Lipase productivity/(U L^{-1} h^{-1})	90	130	100

As referred above, the fed-batch feeding strategy used with cells pre-grown in YPD within the bioreactor improved the lipase production (Figure 5.4 and Table 5.2). Furthermore, the process was also more efficient in terms of effluent degradation, since it leads to good COD and lipids reduction, both higher than 50 %. *C. rugosa* achieved the highest value of lipase yield (130 U L^{-1} h^{-1}), in parallel with highest lipids reduction (77 %).

The phenolic compounds reduction is usually negligible, concerning the performed experiments, except when lower COD concentrations are used (OMW-A, about 20 g COD L^{-1}). However, in these

fed-batch experiments, performed with cell pre-growth inside the bioreactor, 27 % of phenolic compounds removal was reached using *C. rugosa*.

Freire *et al.* (1997) observed the time course for lipase production and lipids consumption, by *Penicillium restrictum*, and found that the maximum lipolytic activity corresponded to the depletion of the carbon source (lipids), which is in accordance to our results.

6.4. CONCLUSION

In conclusion, the results in this chapter demonstrates that the olive mill wastewaters are becoming a competitive and valuable growth medium in fermentation processes, with lipolytic microorganisms, since they allow a significant production of lipase while degrading the effluent.

The studied yeasts were not only capable of achieving similar or even better cell growth in OMW based medium, than in glucose synthetic media, but also to highly consume the existing phenolic compounds. In the batch operation mode, *C. cylindracea* CBS 7869 was the best lipase-producing yeast (30 U L^{-1} h^{-1}). However, using a fed-batch strategy, with cell pre-growth directly on the bioreactor, *C. rugosa* CBS 2275 was the strain that obtained the best values of lipase productivity (130 U L^{-1} h^{-1}) achieving at the same time, a significant effluent degradation (64 % of COD, 27 % of phenolics and 77 % of total lipids). The color of the OMW-based media becomes more intense throughout each experiment, especially on batch fermentations. The results of this work suggest that *C. rugosa* CBS 2275 has a great potential to be applied for the removal of oil and COD from OMW with simultaneous lipase production.

CHAPTER 7. A PERSPECTIVE FOR OMW VALORIZATION THROUGH INTEGRATION OF LIPASE PRODUCTION FERMENTATION AND ANAEROBIC DIGESTION

The present chapter aims to study an integrated process for the OMW valorization by producing high-value compounds while degrading the effluent. The OMW is submitted to a lipase-producing aerobic fermentation with a partial OMW degradation, followed by an anaerobic methanogenic degradation process.

The results obtained showed that the aerobic treatment, with production of lipase, had a positive effect on the anaerobic degradation of the OMW. Methane yields were higher in the vials supplemented with treated OMW, relatively to the ones with raw OMW. Best results were achieved for the concentration of 5 g COD OMW L^{-1}, where 78 % of the COD added was recovered as methane.

Using treated OMW from fed-batch trials, even better results were achieved, especially for 5 g COD L^{-1} were a nearly total conversion of the COD in methane was achieved, in almost half of the time needed in the previous set of experiments.

The information presented in this Chapter was submitted in:

Gonçalves C., Alves M., Belo I., A perspective for OMW valorization through integration of lipase production fermentation and anaerobic digestion. *Proceedings of the 3rd International Symposium on Energy from Biomass and Waste - Venice 2010 Symposium*, 2010, (Venice, Italy)

7.1. INTRODUCTION

Conventional wastewater treatment methods are highly ineffective to remove the OMW pollutants. Instead of a disposal solution approach, using this waste as a renewable resource is of great interest (D'Annibale *et al.*, 2006; Scioli and Vollaro, 1997).

The present chapter work **aims** the OMW valorization, by producing high-value compounds while degrading this waste, in a two-step process. The effluent is submitted to a lipase producing aerobic fermentation that contributes for a partial OMW degradation, followed by an anaerobic methanogenic degradation process to produce methane.

7.2. MATERIALS AND METHODS

7.2.1. OMW based media

The OMW-based medium is composed by OMW-G (Table 3.2), without dilution. The medium was supplemented with ammonium chloride (C/N=15) and yeast extract (in a reason of 2:5 relatively to the NH_4Cl added). Before sterilization, the pH was adjusted to 7.2 with NaOH and 6 drops of silicon antifoam agent were added.

A part of the prepared OMW media was aerobically treated in the 2-L bioreactor (Biolab, B. BRAUN), and the remaining medium was stored at -20 °C, to be used as non-treated OMW in the anaerobic digestion trials.

7.2.2. Batch and fed-batch trials

Lipase production by *C. cylindracea* on OMW-based media was performed in a 2 L - bioreactor at 27 °C, 500 rpm and 1.5 L min^{-1} of airflow, during 9 days. The pH was automatically adjusted to 7.2 by means of a pH-meter and delivery pumps loaded with NaOH 1N and HCl 1N.

In the batch experiments, the reactor was filled with 1.3 L of OMW-based medium and, after sterilization; it was inoculated with yeast cells previously grown in YPD medium, as described above. The fed-batch experiments, inoculated in the same way, were initiated with a batch phase of 24 h, after which the OMW-based medium began to be fed. The feeding was performed with two pulses per day, of 10 % (v/v) of the medium inside the reactor, each. Thus, it was approximated by a repeated-batch operation. The dilution rate was maintained between 0.02 h^{-1} and 0.01 h^{-1}. The fed-batch was stopped, when the reactor was filled up to 1.5 L, with OMW-based medium.

7.2.3. Anaerobic digestion experiments

Inoculum

The anaerobic digestion experiments were performed with a granular sludge from a brewery wastewater treatment plant. The activity of this granular sludge in presence of acetate was 50 mL CH_4@STP (g VSS.day)$^{-1}$, approximately. Residual substrate depletion was promoted through overnight incubation at 37 °C, before starting the experiments.

Anaerobic digestion experiments

In order to evaluate the effect of the previous aerobic step in the anaerobic digestion process, biodegradability tests were performed using OMW samples with and without aerobic treatment. In the first set of experiments was used a pre-treated OMW, by *C. cylindracea*, in batch conditions. Then a pre-treated OMW, by *C. cylindracea*, in fed-batch conditions was used. Vials with 12.5 mL of working volume were used. Adequate amount of inoculum was added to each vial in order to obtain 2 - 5 g VS L^{-1}, and preferably around 3 g VS L^{-1}. The basal medium used in these experiments was described elsewhere (Gonçalves *et al.*, 2011). All batch tests were performed in triplicate and incubated at 37 °C and 120 rpm.

Anaerobic biodegradability experiments were performed with OMW-G (with and without aerobic pre-treatment) in concentrations of COD ranging from 5 g L^{-1} to 50 g L^{-1}. The headspace biogas was

sampled periodically to assess the methane content, by gas chromatography. The amount of methane produced at the end of the assay was converted to its COD equivalent. Methane yields were then calculated as the ratio between the methane-COD produced and the OMW-COD added. Experiments with addition of lipase (from *Candida rugosa*, SIGMA), were performed to evaluate the influence of lipase on the anaerobic digestion.

The maximum value of g COD-CH_4 per g COD added achieved by the blanks (vials without substrate) was subtracted to the maximum value of the samples giving the g COD-CH4/ g COD added plateau.

7.3. RESULTS AND DISCUSSION

7.3.1. Lipase production

The treated OMW, used afterward for anaerobic digestion, comes from aerobic fermentation experiments performed during the optimization phase of the process. The results obtained in batch (A) and fed-batch (B) conditions are summarized in Table 7.1, with the final values of COD, reducing sugars, phenolic compounds and lipase activity.

Table 7.1 Overall characterization of the treated OMW in the end of the aerobic fermentation step using batch and fed-batch strategies

Treated OMW	COD ($g\ L^{-1}$)	Reducing Sugars ($g\ L^{-1}$)	Phenolic compounds ($g\ L^{-1}$)	Lipase activity ($U\ mL^{-1}$)
A	58.9 ± 8.1	8.6 ± 4.2	3.1 ± 1.4	3.5 ± 1.2
B	19.4 ± 2.3	2.5 ± 0.1	2.0 ± 0.2	0.9 ± 0.1

No cellular growth inhibition by OMW was observed. The batch conditions did not improve the OMW degradation, but they provide reasonable amounts of lipase. An opposite effect was observed

with the implementation of a fed-bach system that resulted in a 41 % increase of the COD degradation and a decrease on lipase production (about 74 %). These results suggest an inverse relation between cell growth and the produced extracellular lipase in the media. Observing Brozzoli *et al.* (2009) work it is also possible to verify this tendency. The LCFA were assessed during the fed-batch experiments and they all (palmitic acid, palmitoleic acid, stearic acid and oleic acid) increased, about 75 % each. According to Cirne *et al.*, 2006 the lipases enhance the hydrolisys of lipids allowing the accumulation of LCFA's.

7.3.2. Anaerobic Digestion

In the first set of experiments a *Candida cylindracea* pre-treated OMW in batch conditions was used. Biodegradability tests were performed to pre-treated OMW (Figure 7.1 - A) and non-treated OMW (Figure 7.1 - B).

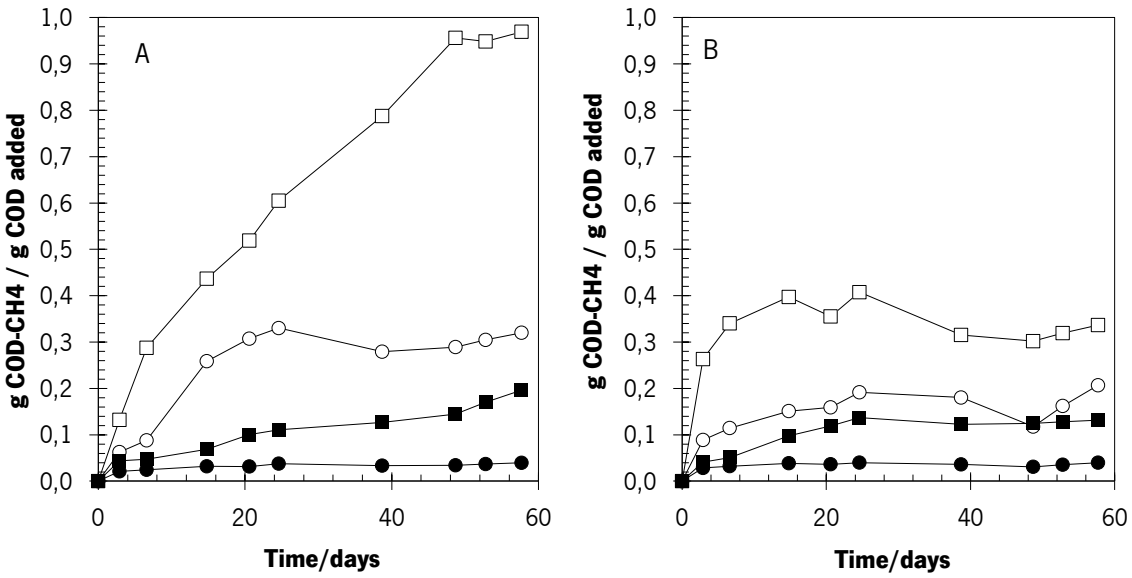

Figure 7.1 Cumulative methane production during biodegradability tests with A. pre-treated and B. raw OMW for different OMW-COD concentrations: 5 g L^{-1} (□) 10 g L^{-1} (○) 20 g L^{-1} (■) and 50 g L^{-1} (●). Each point represents the average of three replicates (standard deviation <10 % of the mean).

Methane yields were higher in the vials supplemented with treated OMW (Figure 7.1 - A), relatively to the ones amended with raw OMW (Figure 7.1 - B). This fact indicates that the aerobic treatment, with production of lipase, has a positive effect on the anaerobic degradation of the OMW. Dhouib *et al.* (2005) worked with fungal (*Phanerochaete chrysosporium*) pretreatment followed by anaerobic digestion and their results show that aerobic treatment has a positive effect on the anaerobic degradation. In the herein work, this effect on the anaerobic biodegradability diminishes with the increase of the OMW-COD concentrations (Figure 7.1). For the concentration of 5 g COD-treated OMW L^{-1} 78 % of the COD added was recovered as methane (g COD-CH_4/ g COD added plateau), while only 15 % is achieved using non-treated OMW. Nevertheless, when higher concentrations of COD were used (50 g L^{-1}) only 2 % of the COD added was recovered as methane (g COD-CH_4/ g COD added plateau), both in the presence of treated and non-treated OMW. Gonçalves *et al.* (2011) studied the anaerobic treatment of olive mill wastewater (OMW) to enhance the biogas production and already concluded that high concentrations of olive mill effluent (50 g COD L^{-1}) may lead to an increase in the toxicity that is induced by the phenolic and lipidic compounds present in this kind of wastewater.

In the aerobic step, LCFAs had increased (about 75 %) while in the anaerobic experiments, oleic acid and palmitic acid were degraded, especially for higher concentrations of OMW COD (99 %). However, palmitoleic and stearic acid had highly increased, until 20 times. This could suggest that these LCFAs were produced, through lipids hydrolysis. The lipase produced in the aerobic experiments was not extracted for these experiments, so it could contribute for lipids hydrolysis on the treated OMW. Lipase activity was assessed before and after this first set of biodegradability tests and it was verified a decay, that was less significant to 5 and 10 g COD L^{-1} (about 60 %), than for higher concentrations (about 95 %).

Lower values of OMW-COD are required for the anaerobic digestion, considering the previous results with 50 g COD L^{-1} of treated OMW and other author's work (Gonçalves *et al.*, 2011). To avoid dilution, and related costs, a higher COD removal efficiency is necessary during the aerobic treatment. Therefore, a new set of experiments of anaerobic biodegradability tests were performed using a *Candida cylindracea* pre-treated OMW in fed-batch conditions (Figure 7.2 - A). In these experiments, as the COD concentration of the treated OMW was only ca. 20 g L^{-1}, the addition of

tap water for OMW COD dilution was much lower than in previous trials and, for the same reason, the concentration of 50 g COD L^{-1} was not possible to use.

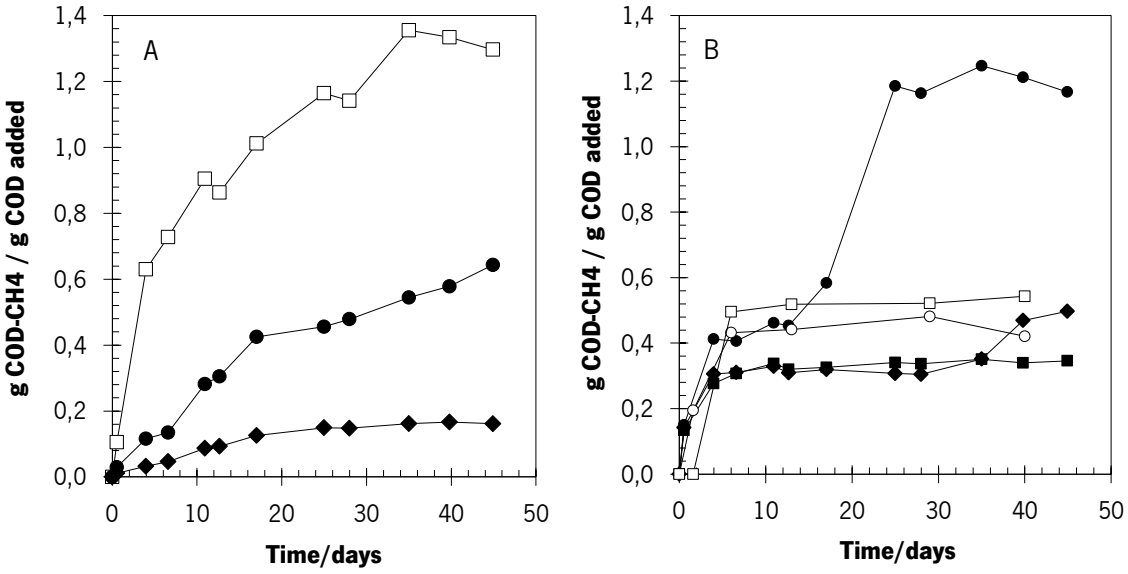

Figure 7.2 Cumulative methane production during biodegradability tests using: **A.** pre-treated OMW for different OMW-COD concentrations, 5 g L^{-1} (□), 10 g L^{-1} (●) and 20 g L^{-1} (◆); and **B.** non-treated OMW with 5 g COD L^{-1} (◆) adding 5 g L^{-1} (●), 2 g L^{-1} (■), 0.5 g L^{-1} (□), and 0.2 g L^{-1} (○), of lipase. Each point represents the average of three replicates (S.D. <10 % of the mean).

In these experiments, using 5 g COD L^{-1} there was a roughly total conversion of the COD in methane, in almost half of the time needed in the previous set of experiments. Even for 10 and 20 g COD L^{-1} the methane production was enhanced 2- and 7-fold, respectively. For the three concentrations used, the degradation of palmitic, palmitoleic and oleic acid, was quite similar, ca. 72 ± 14, 69 ± 20 and 97 ± 6 %, respectively. Nevertheless, it was observed an augmentation in stearic acid of 35 % for 5 g COD/L^{-1} and a triplication was observed for 10 and 20 g COD L^{-1}.

As referred formerly, the lipase produced in the aerobic step was not extracted before anaerobic experiments, thus its influence should be considered. A new set of experiments of anaerobic biodegradability tests were performed using non-treated OMW with 0.0, 0.2, 0.5, 2 and 5 g L^{-1} of lipase, corresponding to the activities of 0.0, 1.6, 4.0, 16 and 40 U mL^{-1}, approximately (Figure 7.2

- B). In order to identify if the added lipase could contribute with both lipolytic activity and COD, to be consumed by the microorganisms, these results were not considered without first measuring the COD concentration of the added lipase. The lipase concentrations used, contributed with an insignificant amount (<0.1 g COD L^{-1}) in the total COD (ca. 100 g COD L^{-1}), thus only the effect of lipase activity will be considered in these experiments. The methane recovery was almost 100 % for non-treated OMW with 5 g L^{-1} of lipase but for non-treated OMW without lipase or 0.2, 0.5 and 2 g L^{-1} of lipase, only slightly deviation was observed. These results reveal that the lipase present in the treated OMW had no significant influence since the lipase quantity was less than 0.5 g L^{-1}. In these experiments the degradation of palmitic, palmitoleic and oleic acid, was quite similar, ca. 83 ± 22, 73 ± 15 and 96 ± 6 %, respectively. Concerning the stearic acid an augmentation (26 %) was observed when no lipase is added to the non-treated OMW but when 5, 2, 0.5 and 0.2 g L^{-1} are added a degradation of this LCFA was observed, ca. 56, 8, 25 and 65 %.

7.4. CONCLUSION

Biodegradability tests showed that the aerobic treatment had a positive effect on the anaerobic degradation of the OMW. This effect diminishes with the increase of the OMW COD. Methane yields were higher in the vials supplemented with treated OMW, relatively to the ones with crude OMW. Best results were achieved for the concentration of 5 g COD-treated OMW L^{-1}, where 78 % of the COD added was recovered as methane. The lipase produced on the aerobic step, when not removed, had loss of activity (60-95 %) throughout the anaerobic process. The treatment of higher concentrations of COD (50 g L^{-1}), even for aerobically pre-treated OMW, was unsuccessful. A new set of experiments showed that using *Candida cylindracea* pre-treated OMW in fed-batch conditions even better results were possible to achieve, since COD degradation in the aerobic step was higher. A nearly total conversion of the COD in methane, in almost half of the time needed in the previous set of experiments was achieved with 5 g COD L^{-1}.

CHAPTER 8. FINAL CONCLUSIONS AND PERSPECTIVES OF FUTURE WORK

8.1. GENERAL CONCLUSIONS

The present investigation essentially describes a study about the potential application of non-conventional yeasts to valorize the olive mill wastewaters, by a lipase producing aerobic fermentation. It is also reported an integration of this process with an anaerobic methanogenic degradation process, to produce methane. This is of great interest since Portugal is one of the world leading producers of olive oil, with crescent production values from campaing to campaign, in the last years.

This work was started with a study about the major problem attributed to the olive mill wastewaters (OMW), the phenolic compounds toxicity. Although numerous authors have referred it, few have focused on studying this problem deeply. These experiments showed that *Y. lipolytica*, *C. rugosa* and *C. cylindracea* are able to grow in different phenolic compounds, usually found in OMW. Only catechol was slightly toxic to these yeasts, which was confirmed by respirometry experiments. Fortunately, catechol is not the most frequent and concentrated phenolic compound in the OMW samples used. This was later confirmed in bioreactor batch experiments with OMW-based media, where the studied yeasts, not only were capable of achieving similar or even better cell growth, than in glucose synthetic media, but also to highly consume the existing phenolic compounds.

The experiments on batch fermentations, with OMW-based media, were then performed in Erlenmeyer baffled flasks, in order to preliminarily study the effect of ammonium, cell and surfactant addition as well as to investigate the use of different yeast strains *Candida rugosa* (PYCC 3238 and CBS 2275), *Candida cylindracea* CBS 7869 and *Yarrowia lipolytica* (CBS 2073, W29 ATCC 20460 and IMUFRJ 50682) and OMW. The results confirmed the potential application of non-conventional lipolytic yeasts for OMW valorization, by its use as culture medium for biomass and enzymes production. The ability of all strains used, to produce lipase from undiluted OMW was shown. Moreover, *C. cylindracea* was the best strain concerning the effluent degradation.

After preliminary tests, a study of optimal batch conditions was performed in bioreactor, using *Candida rugosa* CBS 2275, *Candida cylindracea* CBS 7869 and *Yarrowia lipolytica* W29 ATCC 20460. The fed-batch operation mode was also experienced, with cells pre-growth outside and

directly inside the bioreactor. It was confirmed that *C. cylindracea* CBS 7869 was the best lipase-producing yeast (30 U L^{-1} h^{-1}). However, using a fed-batch strategy, with cell pre-growth directly on the bioreactor, *C. rugosa* CBS 2275 was the strain that obtained the best values of lipase productivity (130 U L^{-1} h^{-1}); achieving at the same time, a significant effluent degradation (64 % of COD, 27 % of phenolics and 77 % of total lipids)

Anaerobic biodegradability tests showed that the aerobic treatment had a positive effect on the anaerobic degradation of the OMW. Best results were achieved for the initial concentration of 5 g COD-treated OMW L^{-1}, where 78 % of the COD added was recovered as methane. Furthermore, the lipase produced on the aerobic step, when not removed, had loss of activity (60-95 %) throughout the anaerobic process. When the COD degradation in the aerobic step was higher, even better results were possible to achieve, with a faster conversion of COD to methane.

In conclusion, the obtained results demonstrates that the Olive Mill Wastewaters are becoming a competitive and valuable growth medium in fermentation processes, with lipolytic microorganisms, since they allow a significant production of lipase while degrading the effluent. The results of this work suggest that *C. rugosa* CBS 2275 has a great potential to be applied for the removal of oil and COD from OMW with simultaneous lipase production in fed-batch cultures. This treatment was successful to detoxify the effluent, having a very positive effect in the anaerobic digestion. The utilization of olive mill wastewaters for biological production of high value products might have a positive impact on the environmental problem of OMW management.

In the context of this dissertation further research should be performed.

8.2. SUGGESTIONS FOR FUTURE WORK

The olive mill wastewaters are a very challenging subject due to its complexity. With this research great strides were made in the understanding of the OMW and how to valorize it, however further research is needed to better comprehend and exploit this rich effluent. For these reasons, future work will concern the use of different yeasts (such as *Candida tropicalis*) and other microorganisms (such as filamentous fungi), or even their combination, in order to achieve even better results on the effluent degradation and on the production of valuable products.

Moreover, some methods have failed when OMW was used, mainly because of its dark color and solids, but also due to a large number of compounds in the effluent composition that could interfere with the principle of operation and equipments of several techniques. Therefore, more methods and procedures should be adapted to reach a better characterization of the effluent and the products obtained from it, such as organic acids, volatile fatty acids, among others.

The extraction, purification and full characterization of the lipases, and other enzymes produced (such as proteases), is also a task to be accomplished in upcoming work. Given the good values of lipase activity achieved, the referred steps will be important for the technical and economical assessment of the product, to me commercialized.

Another interesting assignment is the implementation of a post- or pre-treatment of color removal, since the neutral pH, needed for both aerobic and anaerobic processes, demands the addition of an alkaline solution, which increases, even more, the color intensity of the effluent.

Future work will also comprise the research on the production of phenolic compounds extracts, from olive mill wastewaters. Other authors have already worked in this subject using other olive mill resources, the olive cake. At first glance this may become very economically attractive, given the commercial value of some of these OMW compounds. Nevertheless, the process of extraction, purification and characterization, should be thoroughly studied for that purpose. The effluent treatment, after phenolic compounds extraction, should also be considered since these compounds are not the only cause of pollution.

It is also of huge interest, the complete implementation of the integrated process: aerobic step followed by anaerobic digestion, both in bioreactors. Besides, with the continuous operation of both bioreactors, the process will advance towards the real implementation in olive mills. The implementation of continuous operation of both lab-scale bioreactors (aerobic and anaerobic), will be a considerable challenge, considering the operational differences of each, but with this the real implementation of the integrated process would become nearer, which would bring substantial environmental benefits.

CHAPTER 9. REFERENCES

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