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Vera Mónica Brito Salgado

Dissertação
Mestrado em Microbiologia Aplicada



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This thesis was fully performed at the Department of Bioenergy of Laboratório Nacional de Energia e Geologia (LNEG) under the direct supervision of Dr. Ana Eusébio in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon.

Prof. Dr. Maria Manuela Carolino was the internal designated supervisor in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon

I like nonsense, it wakes up the brain cells.

Fantasy is a necessary ingredient in living,

it's a way of looking at life through the wrong end of a telescope.

Which is what I do, and that enables you to laugh at life's realities.

- Dr. Seuss

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Resumo

Nas regiões da bacia mediterrânica, o processo de extração de azeite por três fases produz aproximadamente 7 a 30 milhões de metros cúbicos de efluente por ano. O descarte deste efluente, denominado por águas ruças devido à cor escura que apresenta, é um sério problema ambiental. Várias soluções têm sido apresentadas através de propostas para tecnologias de tratamento, no entanto nenhuma delas obteve uma eficácia completa. A maioria dos problemas associados com a poluição das águas ruças é atribuída à sua elevada fração orgânica, composta essencialmente por polissacáridos, açúcares simples, compostos fenólicos, taninos, poliálcoois, proteínas, ácidos orgânicos e lípidos. Parte da toxicidade das águas ruças provém dos seus compostos fenólicos, sendo estes responsáveis por sérios danos biológicos, incluindo a fitotoxicidade. Ragazzi e Veronese (1989) descrevem que os compostos fenólicos tirosol e hidroxitirosol são os principais contribuidores para a atividade antimicrobiana deste efluente. Estas águas têm sido usadas como fertilizante para o crescimento vegetal através de sistemas de espalhamento no solo, contudo o elevado teor em sais minerais e em matéria orgânica (ácidos gordos e polifenóis) é um fator desfavorável para a fertilidade do solo.

Apesar das características antimicrobianas apresentadas por este efluente, várias espécies de microrganismos são capazes de sobreviver neste meio, apresentando até uma microbiota moderadamente diversa. Esta microbiota apresenta alguma tolerância, e por vezes resistência, à toxicidade fenólica presente nas águas ruças. Certas espécies, tais como *Yarrowia lipolytica*, *Candida rugosa* e *Candida cylindracea*, conseguem crescer em meios de cultura que contêm águas ruças e produzir compostos com interesse comercial como enzimas e ácidos orgânicos.

Recentemente, devido ao seu conteúdo lipídico, as águas ruças têm sido propostas como uma fonte alternativa de triacilgliceróis para reduzir o custo da produção de éster metílico de ácido gordo (biodiesel, designado pelo acrónimo inglês FAME - fatty acid methyl ester). Tem sido dedicada especial atenção ao uso de lipases como biocatalizadores no processo de produção de biodiesel, sendo considerado um método mais ecológico. As lipases (triacilglicerol hidrolases, EC 3.1.1.3) são um grupo importante de enzimas com aplicações nas indústrias alimentar, de laticínios, dos detergentes e farmacêuticas. Podem ser produzidas por animais, plantas ou por microrganismos. As lipases microbianas apresentam certas características, tais como a estabilidade e a seletividade, que fazem com que sejam bastante procuradas e utilizadas pela área industrial. Os microrganismos que as produzem encontram-se

geralmente em ambientes lipídicos. As águas ruças demonstram potencialidade, não só como fonte de microrganismos produtores de lipases, mas também como meio complexo de crescimento indicado para a produção de lipases que, devido ao seu conteúdo residual lipídico, funciona como um indutor destas enzimas.

O objetivo deste estudo foi isolar, a partir de águas ruças, novas estirpes de leveduras com capacidade de produzir lipases extracelulares e avaliar a adequabilidade das águas ruças para a produção de lipases.

Para tal propósito, trinta e duas estirpes de leveduras foram isoladas de amostras de águas ruças, provenientes de lagares de azeite de Tavira, Portugal e da Jordânia, através dos meios Yeast Malt Agar (YMA), Potato Dextrose Agar (PDA) e Cooke-Rose Bengal Agar (CRBA) e a sua capacidade de produção de lipases foi investigada. Através do cultivo em meios de Tributirina, Tween 20 e Vermelho de Fenol Agar, usando três métodos de deteção rápida em caixas de Petri. Os microrganismos *Yarrowia lipolytica* (LNEG 263F, NRRL Y-323) e *Candida sp.* (LNEG 356F, INETI), duas estirpes de leveduras lipolíticas, foram utilizados como controlos positivos. Após 72 a 96 horas de incubação a 30°C, selecionaram-se seis estirpes de leveduras que conseguiram produzir halos lipolíticos em, pelo menos, 2 métodos de deteção. A atividade lipolítica extracelular foi determinada através do crescimento em meio líquido sintético: 0,5% (m/v) extrato de levedura, 0,1% (m/v) sulfato de magnésio, 0,1% (m/v) cloreto de potássio e 0,5% (v/v) azeite. Os seis isolados e o controlo *Yarrowia lipolytica* foram incubados a 30°C com uma agitação de 180 rpm durante 144 horas e posteriormente avaliados pelo método Gomes *et al.* (2011). O melhor produtor de lipase extracelular foi o isolado designado por “JOR TR 5”, tendo-se obtido uma produção de 0,8 U mL⁻¹ às 96h de incubação. Para a sua identificação, o ADN foi extraído e as regiões D1/D2 do rDNA 28S foram amplificadas por PCR, usando os primers NL1 e NL4. Os fragmentos genómicos amplificados foram enviados para sequenciação (StabVida®). O isolado foi identificado como *Magnusiomyces capitatus* (gene parcial 28S rRNA, estirpe Kw-230) com 99% de similaridade, usando o programa BLAST. *M. capitatus* é uma levedura filamentosa com uma distribuição cosmopolita. Tem uma maior incidência em zonas geográficas com elevada humidade e temperatura. Pode ser encontrada no solo, em frutas e em produtos lácteos (queijos, por exemplo). Também pode ser encontrada no trato digestivo e respiratório de animais e humanos.

O efeito da toxicidade dos compostos fenólicos sobre o crescimento de *M. capitatus* foi avaliado pelo crescimento desta levedura em diferentes diluições do efluente de águas ruças (10, 25, 50 e 100%). Ao meio de cultura natural foi adicionado extrato de levedura

com e sem azeite como suplementos nutricionais. Para avaliar o aumento da biomassa nas experiências com suplemento nutricional, foi elaborada uma reta de correlação entre os valores da densidade ótica a 640 nm que o meio de cultura apresentava e o peso seco da biomassa (g.L^{-1}). Desta forma, foi possível converter nos restantes ensaios os valores de densidade ótica em unidades de peso seco. No ensaio da toxicidade, as várias diluições com réplicas foram incubadas a 30°C com uma agitação de 180 rpm durante 36 horas. Foram retiradas amostras de 1 mL às 0, 12, 22 horas e a todas as horas até às 36 horas. No ensaio de suplemento nutricional, os meios e as suas respetivas réplicas foram postos a incubar a 30°C com uma agitação de 180 rpm durante 120 horas com recolha de amostras de 24 em 24 horas.

Relativamente à influência da toxicidade fenólica no crescimento da biomassa, concluiu-se que o crescimento de *M. capitatus* apresenta alguma inibição devido à toxicidade pelo teor em fenóis presente nas águas ruças. Não houve produção de lipase em nenhuma das diluições nem no efluente bruto. No entanto, com o suplemento nutricional, observou-se que 2 g.L^{-1} de extrato de levedura e 1 g.L^{-1} de azeite obteve os melhores resultados, tanto na produção de lipase (0,33 U.mL^{-1}) como na quantidade da biomassa produzida (13,24 g.L^{-1}).

Com o objetivo de melhorar a produção de lipase da estirpe *M. capitatus*, foi estudada a influência da variação de NH_4Cl e da oxigenação. Com esse objetivo foi delineado um ensaio de acordo com um planeamento experimental que segue a distribuição de Doehlert. Sete ensaios com réplicas foram incubados a 30°C com uma agitação de 200 rpm durante 48h. A concentração de NH_4Cl variou entre 0,2 e 2,8 g.L^{-1} num meio de cultura constituído por águas ruças não diluídas e suplementadas com 2 g.L^{-1} de extrato de levedura e com 1 g.L^{-1} de azeite. A disponibilidade de oxigénio foi estudada através da variação do volume de meio (desde 100 até 400 mL) em erlenmeyers de 1L com anteparas. Estes valores de oxigenação foram convertidos para k_{La} (coeficiente volumétrico de transferência de oxigénio em min^{-1}), variando desde 0,2 até 1,88 min^{-1} . Em todos os ensaios testados, o crescimento da levedura atinge um valor médio de concentração de biomassa de 4,56 g.L^{-1} . A integridade da membrana celular da levedura foi analisada por citometria de fluxo, e observou-se que, na sua grande maioria, em todos os testes, as membranas celulares estavam intactas, indicando que as células não sofreram stress fisiológico. A atividade máxima de lipase foi de 1,16 U.mL^{-1} , com uma suplemento de NH_4Cl de 1,5 g.L^{-1} e um valor de k_{La} de 1,880, em meio de água ruça não diluída.

Com a realização deste trabalho foi possível isolar uma estirpe de levedura com características lipolíticas a partir de um efluente recalcitrante. Esta estirpe foi identificada como *Magnusiomyces capitatus* e, através de um método de otimização das condições de cultura em água ruça não diluída e suplementada com NH_4Cl e oxigénio, foi possível aumentar a produção de lipase por esta estirpe. Estes resultados vêm contribuir com uma nova estirpe produtora de lipases e reforçar o potencial da valorização das águas ruças como meio de fermentação para a indução da produção de lipases e fonte de isolamento de estirpes de leveduras produtoras de lipases.

Palavras-chave: Águas ruças; Lipases; Leveduras lipolíticas; *Magnusiomyces capitatus*; Valorização de efluentes.

Abstract

Olive mill wastewaters (OMW) are effluents originated from olive oil extraction. It consists of a great variety of compounds, including polysaccharides, sugars, phenolic compounds, tannins, polyalcohols, proteins, organic acids and lipids. Due to its olive oil residue (an inducer of lipase) OMW is a potential source of lipase-producing microorganisms and a complex medium potentially suitable for lipase production.

The aim of the present study is to isolate lipolytic yeasts with the ability to produce extracellular lipases from OMW samples, test their phenolic resistance and optimize their lipase production.

For this purpose, thirty-two yeast strains were isolated from OMW samples. Screening for lipase activity using rapid plate detection methods allowed the selection of six isolates. Subsequently, extracellular lipolytic activity was determined in shake-flasks, and the best producer was the isolate "JOR TR 5" with an activity of 0.8 U.mL^{-1} . This strain was identified as *Magnusiomyces capitatus* through DNA sequencing. The growth of *M. capitatus* with OMW as culture medium was assessed using several OMW dilutions and supplementation with yeast extract and olive oil. The culture growth did show some inhibition due to the phenol toxicity present in OMW. Nutrient supplementation improved both lipase production and biomass growth. The influence of ammonium chloride (NH_4Cl) and oxygen availability was shown to improve lipase production, according to a statistical design following the Doehlert distribution. The highest lipase activity obtained was 1.16 U.mL^{-1} , with 1.5 g.L^{-1} of NH_4Cl supplementation and with K_{La} value of 1.880 min^{-1} . The integrity of the yeast cell membrane was detected by flow cytometry and did not showed any signs of physiological stress.

With this work, it was possible to obtain one yeast strain with lipolytic activity isolated from OMW, tolerant to this type of recalcitrant medium and able to produce lipase in undiluted OMW with reduced nutrient supplementation. These results confirm the valorisation of OMW as fermentation medium to induce the production of lipases and as source for isolation of lipase-producing yeasts strains.

Key words: Olive Mill Wastewater; Lipases; Lipase-producing yeasts; *Magnusiomyces capitatus*; Effluent valorisation.

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Abbreviation List

OMW – Olive Mill Wastewater

FAME – Fatty Acid Methyl Ester

COD – Chemical Oxygen Demand

BOD – Biological Oxygen Demand

TSS – Total Suspended Solids

VSS – Volatile Suspended Solids

TKN – Total Kjeldhal Nitrogen

DCW – Dry Cell Weight

OD₆₄₀ – Optical density at 640 nm

YMA – Yeast Malt Agar

CRBA – Cooke-Rose Bengal Agar

PDA – Potato Dextrose Agar

YEPD – Yeast Extract Peptone Dextrose

1. Introduction

The use of fossil fuels to produce energy causes significant damage to the environment and human well-being. This general recognition has led to the research of other more “clean” energy sources. Energy demand is a key concern in this context. Our societies require energy to function and this need has tremendous consequences in our resource use and broader impacts on ecosystems. Renewable energy technologies offer an alternative to increase resource efficiency significantly — allowing society to satisfy its energy requirements at much lower environmental costs (www.eea.europa.eu).

Biodiesel (FAME - fatty acid methyl ester) is a biodegradable and non-toxic fuel that can be obtained from renewable sources, such as vegetable oils, grease or animal fats (second-generation biodiesel). In recent years, cooking oils have been used in biodiesel production. Using low cost feedstock, such as non-edible oils, is essential to reduce production costs and make biodiesel production competitive with petroleum diesel (www.hempcar.org).

Recently, due to their residual lipidic content, olive mill wastewater has been proposed to be used as an alternative source of triacylglycerols to reduce the cost of biodiesel production. Particular attention has been dedicated to the use of lipases as biocatalysts for esterification of vegetable oil wastes, being an environmentally friendly process to produce biodiesel (Yousuf *et al.*, 2010; Gog *et al.*, 2012).

1.1 Olive Mill Wastewater

Olive oil is a product obtained from the olive, the fruit of *Olea europaea* commonly known as olive tree, with an extensive range of therapeutic and culinary applications. Olive oil consumption has considerable health benefits due to its main components, oleic acid, squalene and phenolic compounds, and it is a key ingredient of the “Mediterranean diet” adopted in countries surrounding the Mediterranean Sea (Waterman and Lockwood, 2007).

In this context, around 97% of the total olive oil production worldwide derives from countries in the Mediterranean basin (*Figure 1.1*), of which 80-84% are originated from European Union countries. Average olive oil production in the EU in recent years has been 2.2 million tonnes, representing around 73% of world production. According to the data from 2013/2014, Spain, Italy and Greece account for about 97% of EU olive oil production, with Spain producing approximately 62% of this amount. Outside of this

major production area, olive trees are also grown in the USA, Argentina, the Middle East and Australia (http://ec.europa.eu/agriculture/olive-oil/economic-analysis_en.pdf).



Figure 1.1: Growth of olive trees around the world (<http://www.internationaloliveoil.org>).

In the olive oil production, the wastewater generated by olive processing is one of the strongest industrial effluents, with COD values up to 220 g.L^{-1} and equivalent BOD values up to 100 g.L^{-1} . The amount of wastewater originated from the milling process vary between $0.5\text{-}1.5 \text{ m}^3$ per ton of olives, depending on the process that is used. This wastewater is usually acidic, with a black-brownish colour and is commonly denominated as olive mill wastewater (OMW) (Paixão *et al.*, 1999; Rinaldi *et al.*, 2003). An example of a discharge into the environment can be seen in *Figure 1.2*.



Figure 1.2: Dumping of wastewater into the Hasbani River by a local olive mill in Lebanon (Hashwa and Mhanna, 2010).

The OMW composition varies according to several factors, such as: olive variety, climate conditions, cultivation practices, olive storage time and extraction process used. It is mainly composed by water (83-92%), organic acids, sugars and phenolic compounds. It

also has a high amount of recalcitrant compounds (lignins and tannins), which gives OMW its characteristic dark colour. Additionally, the phenolic compounds and the long-chain fatty acids existent in these wastewaters are very toxic to microorganisms and plants, making the disposal of OMW a very serious environmental issue (Niaounakis and Halvadakis, 2004). Up until now, there is no regulation of olive mill discharges in the European legislation and standards are defined by each individual country. OMW contain an incredibly high organic content, higher than the European legislation permits in effluents, which denies OMW a regular treatment in wastewater treatment plants. The practices that are currently applied involve land disposal, discharge into nearby lakes, rivers or seas and evaporation in lagoons. Soil contamination, underground leakages, water body pollution and foul odour are environmental problems that arise from those practices. (Paraskeva and Diamadopolus, 2006).

Despite their toxicity, several authors describe OMW as a resource for the simple and complex sugars that are required for fermentation processes (Federici *et al.*, 1986; Montedoro *et al.*, 1993; Crognale *et al.*, 2003, Fenice *et al.*, 2003) OMW also contains variable quantities of residual oil, depending on the extraction process efficiency. This contributes to the valorisation of OMW through microbial processes.

1.2 Lipases

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. They catalyse the hydrolysis of triglycerides to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface (Martinelle *et al.*, 1995) and do not hydrolyse dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases, displaying little activity in aqueous solutions containing soluble substrates (Sharma *et al.*, 2001).

The chemical transesterification of vegetable oil for biodiesel production has several disadvantages, such as soap formation, due to the presence of free fatty oils and high reaction temperature. Therefore, enzymatic processes using lipases have been developed to overcome such problems (*Figure 1.3*). These enzymes require minimum amount of water and are very thermostable, even at high temperatures. Despite having low catalytic efficiency, processes such as immobilization, chemical modification and

protein engineering can be used to surpass the overall conversion process (Aires-Barros *et al.*, 1991; Tyagi and Gupta, 1998).

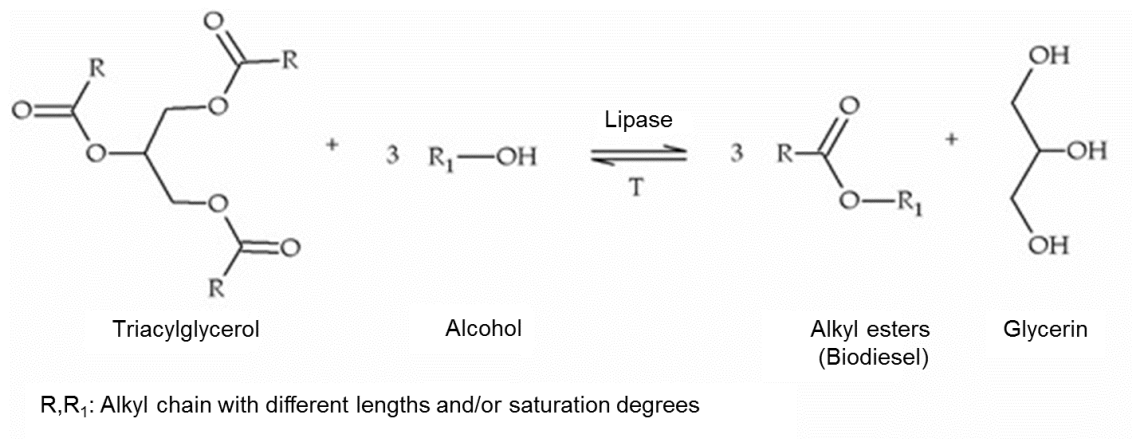


Figure 1.3: Enzymatic transesterification of fatty acid methyl esters, also known as biodiesel.

The ability to perform specific chemical transformations has made them progressively more popular in several industries, such as: food, detergent, cosmetic, organic synthesis and pharmaceutical (Ryu *et al.*, 2006; Gupta *et al.*, 2007; Franken *et al.*, 2009). The use of lipases in organic chemical synthesis is becoming increasingly important. These enzymes catalyse the hydrolysis of water-immiscible triglycerides at water-liquid interface, which will be determined by the amount of water present. When there is little to none water, only esterification and transesterification are favoured. This aspect is very important in the context of enzymatic production of biodiesel (Klibanov *et al.*, 1997).

Lipases perform essential roles in the digestion, transport, and processing of dietary lipids (e.g., triglycerides, fats, and oils) in most living organisms. They are considered as a major group of biotechnologically valuable enzymes, mainly due to the versatility of their applied properties and easy mass production (Bornscheuer *et al.*, 2002; Menoncin *et al.*, 2010).

1.2.1 Microbial lipases

Being more stable than animal or plant lipases, microbial lipases have earned special industrial attention due to their stability, selectivity and broad substrate specificity. The energy consumption required to conduct reactions at elevated temperature and pressure is eliminated as lipases are active under room temperature, reducing the denaturation of labile reactants and products (Dutra *et al.*, 2008; Griebeler *et al.*, 2011).

Lipase-producing microorganisms include actinomycetes and other bacteria, filamentous fungi and yeasts. They can be found in diverse habitats such as industrial wastes,

vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food (Sztajer and Zboinska, 1988). Several methods have been developed to successfully identify these organisms and they usually involve agar plates with a lipidic substrate, in order to identify lipolytic activity. A simple agar medium with lipidic substrates has a turbid appearance and the detection of enzymes is done by the observation of clear-through halos around the colonies (Sierra, 1957; Cardenas *et al.*, 2001). Several substrates (basically, any oil or grease) can be used in this method. The use of chromogenic substrates, such as pH indicator Victoria blue and Phenol red, has also been described (Yeoh *et al.*, 1986; Kouker *et al.*, 1987, Wang *et al.*, 1995, Hou, 1994). Chromogenic dyes can be used to assure a more visible detection, as they induce a change in the medium color around the enzyme-producing colonies. This color change occurs due to the formation of free fatty acids, which causes a change in the medium pH (Bornscheuer and Kazlauskas, 1999). There are other ways to detect lipase production without having to use chromogenic dyes. The precipitation of salts is also used for the detection of lipase-producing microorganisms. The fatty acids resulting from hydrolysis of tween binds with the calcium salts present in the media, forming insoluble crystals, visible around the colonies (Gopinath *et al.*, 2005). Most of the industrial microbial lipases are derived from fungi and bacteria (*Table 1.1*).

Table 1.1: Examples of some commercially available microbial lipases, their most regular applications and producing microorganisms (Jaeger and Reetz, 1998; Sharma *et al.*, 2001; Kademi *et al.*, 2004).

Microorganism	Trade name	Applications	Industry
Bacteria			
<i>Alcaligenes sp</i>	Lipase Lp	Modification of oils and fats	Food processing
<i>Chromobacterium viscosum</i>	Lipase CV	Diagnostics/analytical	Health care and diagnostics
<i>Pseudomonas cepacia</i>	Lipase SL	Chiral synthesis	Food processing
<i>Pseudomonas menodocina</i>	Lumafast	Hydrolysis of oils and fats	Detergent
Filamentous Fungi			
<i>Aspergillus niger</i>	Lipase DS	Dietary supplement	Biopharmaceuticals
	Lipase	Organic synthesis/ analytical	Food processing
<i>Rhizopus oryzae</i>	Lipopan®F	Dough strengthening	Baking and food processing
<i>Rhizomucor miehei</i>	Palatase®	Cheese flavour enhancement	Dairy
Yeast			
<i>Candida cylindracea</i>	Lipase MY	Dietetics	Biopharmaceuticals
	Resinase®	Pitch control	Forest product

<i>Pseudozyma antarctica</i>	Novozym® 435	Oil based specialties	Food processing
<i>Candida rugosa</i>	Lipase AY "Amano" 30	Organic synthesis	Food processing
<i>Geotrichum candidum</i>	Chirazyme® L-8	Oleochemistry	Food processing
	Lipolase®	Oleochemistry	Food processing

These lipases are produced frequently by submerged cultures (Ito *et al.*, 2001), although solid-state fermentation methods can also be used, namely for filamentous fungi (Chisti, 1999). For bacteria and yeasts, it is obligatory to use a submerged culture due to their minimum water requirement. Lipase production is influenced by both the nature and concentration of carbon and nitrogen sources, culture pH, dissolved oxygen concentration and growth temperature. It is reported that lipidic carbon sources appear to be largely crucial in order to obtain a high lipase yield (Elibol and Ozer, 2001).

1.3 Lipase-producing yeasts

In literature, several yeasts have been described to be capable of producing the enzyme lipase. Species belonging to the genus *Candida* are among the most used commercial producers (Larios *et al.*, 2004).

1.3.1 Genus *Candida*

The genus *Candida* is extremely heterogenous and covers a wide diversity of yeasts of ascomycetous affinity with unknown sexual states (*Figure 1.4*).

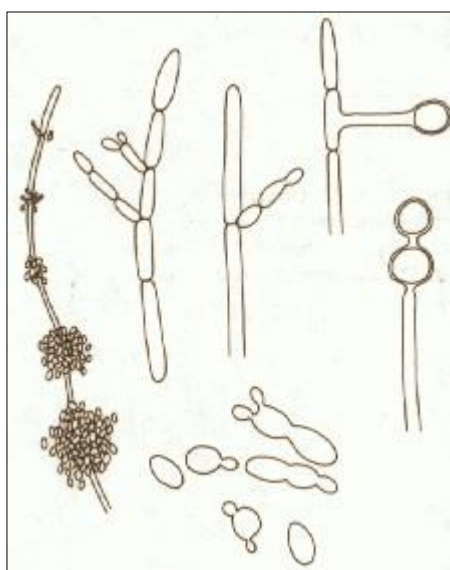


Figure 1.4: Drawings of the microscopic aspect of genus *Candida* (www.botany.utoronto.ca).

Two well-known species that belong to this genus are *Candida cylindracea* and *Pseudozyma Antarctica*. Both species are extensively described in literature due to their capability to produce valuable industrial biocatalysts, such as lipases. Because of their high production yield, they have been studied for the production of biodiesel using several lipidic substrates (Kurtzman *et al.*, 2011). Additionally, lipase produced by *Candida rugosa* is rapidly becoming one of the most used industrial enzymes due to its high activity, both in hydrolysis as well in synthesis (Vakhlu and Kour, 2006). The referred lipases have several isoforms, which greatly contributes to their diverse use in biotechnology. This is due to the broad range specificity (substrate, positional, fatty acid and stereopreference), usually not found in other lipases (de María *et al.*, 2006).

Over the past years, there has been a great demand for the use of non-conventional microorganisms, in this case, non-conventional yeasts (non-*saccharomyces* yeasts) that could be better suited for biotechnological purposes. One of these cases is *Yarrowia lipolytica*, since its finding in 1980, has been made a model of study and research in industrial microbiology (Spencer *et al.*, 2002).

1.3.2 *Yarrowia lipolytica*

Yarrowia lipolytica is an aerobic, nonconventional ascomycetous yeast with multiple biotechnological applications (Figure 1.5). Usually it can be found in hydrophobic substrates rich in fatty acids, such as dairy products (Spencer *et al.*, 2002).

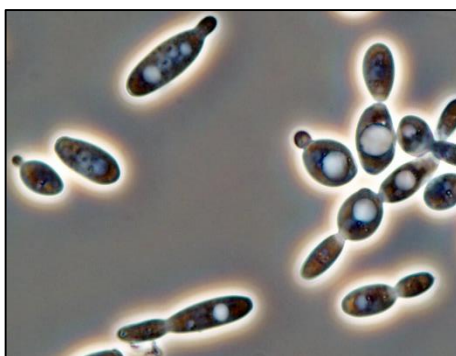


Figure 1.5: Microscopic image of *Yarrowia lipolytica* cells (Taken by Dr^o. Andreas Aurich, UFZ, www.ufz.de).

It can be used for bioremediation, production of biosurfactants and other assorted substances such as citric and isocitric acids and enzymes (proteases, lipases, RNase) (Madzak *et al.*, 2004). The most important of these metabolites is lipase due to its broad technological applications in several different areas. There are many studies related to lipase production by *Y. lipolytica*, in both wild and naturally occurring strains. Some

strains of this species are cultivable in effluents, making them a possible organism for a valorisation process in the case of OMW, for example (Federici *et al.*, 2009; Dermeche *et al.*, 2013).

1.4 Valorisation of Olive Mill Wastewater

In recent years, increasing consideration has been dedicated to the possibility of valorising the olive oil extraction residues. The successful recovery of bioactive chemicals with commercial importance, as well the production of added-value products such as ethanol, organic acids and enzymes appear to be the new frontier in OMW valorisation (Dermeche *et al.*, 2013).

Despite their toxicity, the high level of polyphenols present in OMW can act as a vantage point in their valorisation. Although to different extents, phenolic compounds are known to have antioxidant activity, a characteristic which is very sought out by cosmetic and pharmaceuticals industries. Hydrotyrosol, the main polyphenol in OMW, has the same antioxidant activity comparable to that one detected in other synthetic antioxidants has. Their synthesis is very costly, which means that the acquisition of hydrotyrosol from OMW could constitute an easier and inexpensive alternative for the acquirement of antioxidants (Federici *et al.*, 2009).

The valorisation of OMW can also have a biotechnological approach, using this effluent in the production of added-value products, such as enzymes. As suggested in section 1.1, this effluent can be used as source for the simple and complex sugars required for fermentation processes.

Industrial enzymes can be produced by yeasts and filamentous fungi using OMW as a substrate. D'Annibale *et al.* (2006) assessed the suitability of OMW as growth medium for the production of microbial lipase using several yeasts and filamentous fungi strains from the NRRL collection (Culture Collections of the North Regional Research Laboratory, Peoria, Illinois). All strains were able to grow in undiluted OMW and produce extracellular lipase. The yeast *Candida cylindracea* (NRRL Y-17506) had the highest lipase production (9.23 U.mL^{-1}), but only after supplementation with NH_4Cl (2.4 g.L^{-1}) and olive oil (3 g.L^{-1}). Another case study was presented by Lanciotti *et al.* (2005), in which was used different strains of *Yarrowia lipolytica* to assess their ability to grow in undiluted OMW and compared it to the production of lipase in semi-synthetic medium. The majority of the strains tested did have a higher production of lipase on OMW than in the synthetic medium. They were also able to reduce COD values and polyphenols content.

Another approach to OMW valorisation was made by Yousuf *et al.* (2010), using the oleaginous yeast *Lipomyces starkeyi*. This author used undiluted OMW with the purpose of accumulating lipids as a feedstock for biodiesel production.

Based on the consulted literature, it is safe to assume that OMW has potential to be used in the growth of lipolytic strains and subsequent induction of the production of enzymes by those strains. OMW also has an associated microbiota, which are microorganisms that can live and growth in this effluent, withstanding its phenol toxicity. Isolation and selection of yeasts strains from OMW samples has already been described by Bleve *et al.* (2011). The authors were able to isolate 300 yeasts isolates, showing that OMW has a high quantity of yeasts in its microbiota, probably due to their resistance to high phenolic concentrations.

Due to the oil residue in OMW, it is very likely that most of the associated microbiota has lipolytic properties. Consequently this effluent can be used, not only as a growth medium (due to the presence of complex sugars and other carbon sources), but as well as a source of lipolytic yeasts. Taking these propositions into consideration, this thesis will focus on the isolation of lipolytic yeasts that are able to produce extracellular lipases from OMW samples, test their phenolic tolerance and optimize their lipase production.

2. Materials and Methods

2.1 Olive mill wastewater

2.1.1 OMW origin and processing

Raw OMW samples used in this work were collected from two different locations: a three-phase olive oil mill located in the south region of Portugal (Tavira, Algarve, during the last month of olive oil production campaign of 2013/2014) and several samples from Jordan (Irbid) with two origins: i) enriched OMW in shake-flask; ii) treated OMW in an aerobic bioreactor. All samples were kept at 4°C until further use. Due to high suspended solids in the raw OMW content, a preliminary step of filtration and centrifugation (8600g, 15 min) for the removal of solid was performed before using as culture medium. This filtration is performed with normal gauze or a large strainer.

2.2.2 OMW characterization

The OMW characterization was performed in the subsequent 48 h after its collection. The effluent was characterized for pH, Chemical Oxygen Demand, Total Suspended Solids, Volatile Suspended Solids, Total Kjeldhal Nitrogen, total phenols, nitrates, total lipids and reducing sugars (see section 2.9 Analytical methods).

2.2. Isolation and screening of lipase-producing yeasts strains

The microbial characterization of used OMW samples was performed in Nutrient Agar in order to quantify total heterotrophic bacteria, filamentous fungi and yeasts. Serial dilutions (10^{-1} to 10^{-5}) of the OMW samples were prepared and spread on agar plates, incubated at 30°C during 48 hours, and quantification made in terms of Colony Forming Units (CFU). Yeast colonies were selected and isolated from the obtained CFU counts plates. Selective media, such as YMA (Difco®), CRBA (Difco®) and PDA (Difco®), containing final concentration of 35 µg/mL of chlortetracycline to prevent bacterial growth, were used. The plates were incubated at 30°C during 48 hours. The isolates were first differentiated for their colony morphology and then analysed under optical microscopy (microscope Olympus BX51) in order to identify yeasts strains colonies.

2.2.1 Screening of yeasts strains with lipolytic activity

The yeast isolates were screened for lipolytic activity through rapid plate detection experiments. Three different media were used for that purpose: Tween 20, Phenol-red with lipidic substrates and tributyrin agar (TBA). The lipolytic activity was registered after 48 h of incubation at 30°C.

2.2.1.1 Tween 20 plate assay

Tween 20 agar plates were prepared according to Gopinath *et al.* (2005). The culture media contained peptone (10 g.L⁻¹), NaCl (5 g.L⁻¹), CaCl₂ · 2H₂O (0.1 g.L⁻¹), agar (20 g.L⁻¹) and tween 20 (10 mL, v/v). About 20 mL were distributed in Petri dishes and the isolates were inoculated. Lipolytic activity was indicated by the appearance of visible precipitate, as a result of deposition of calcium crystal salts formed by the fatty acid liberated by the extracellular lipase.

2.2.1.2 Phenol-red plate assay

Chromogenic substrate plates were prepared according to Singh *et al.* (2006) by using phenol red (0.01%) along with 1% olive oil, 0.1% CaCl₂ and 2% agar. pH was adjusted to 7.3-7.4 by using NaOH 0.1 N. Cell suspensions were made using 1 mL of sterile distilled water, having a final concentration of approximately 10⁷ CFU/mL (optical

turbidity, using a McFarland scale). A 3-5 mm diameter sterile paper discs were immersed in each cell suspension and placed in the phenol-red medium. The change in color of phenol from red to bright yellow, forming a halo, was used as an indicator of lipolytic activity.

2.2.1.3 TBA plate assay

The lipase production was examined in a yeast malt agar media supplemented with 0.5% (v/v) tributyrin, after autoclaving. Subcultures of each isolate were inoculated in the tributyrin plates and incubated at 30°C during 72h. In the positive responses, a translucent halo around the colonies was formed.

2.2.2 List of yeast strains used for comparative tests

Three yeasts strains were obtained from Private Collection of Microorganisms of Bioenergy Unit of LNEG as described in *Table 2.1*. These strains were tested for lipolytic activity and compared with yeast strains isolated from OMW.

Table 2.1: List of yeasts cultures from Private Collection of Microorganisms of Bioenergy Unit of LNEG used for comparative tests.

Microorganism/Isolate	LNEG strain ID	Origin and reference	Lipase production (Literature)
<i>Yarrowia lipolytica</i>	263F	NRRL Y-323	Positive (Ogrydziak, 1988)
<i>Candida sp.</i>	356F	INETI	Positive (van Uden & H.R. Buckley, 1970)
11-T AR	----	INETI	Unknown

NRRL – Recently changed to ARS (Agriculture Research Service) Culture collection; INETI – Instituto Nacional de Engenharia, Tecnologia e Inovação.

2.3 Selection of yeasts for extracellular lipase production

Yeasts isolates were grown in 50 mL of YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) overnight. 300 µL of freshly prepared cultures were used as inoculum. Experiments using 100 mL of control medium (1 g.L⁻¹ yeast extract, 1 g.L⁻¹ chloride potassium and 1 g.L⁻¹ magnesium sulfate) with an inductor (0.5 g.L⁻¹ olive oil) were performed in 500 mL-shake-flasks. Cultures were incubated at 30°C under orbital shaking (180 rpm) during 120 hours. 1 mL samples were collected every 24 hours and absorbance was monitored at 600 nm. The samples were then centrifuged (7500 g, 15 min) at 4°C and the supernatant was assayed for the enzyme activity.

2.3.1 Assay of lipase activity

The measurement of lipase activity was adapted from Gomes *et al.* (2011). A reaction mixture composed by 195 μL of substrate (2.63 mM p-nitrophenyl butyrate in sodium acetate buffer, 0.05 M, pH 6.8, with 4% (v/v) Triton X-100) and 5 μL of sample supernatant was incubated in a microplate at 37°C for 15 min. In blanks, fresh medium was used. The absorbance was measured at 405 nm in a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Fischer Scientific). One unit (U) of lipase activity was defined as the amount of enzyme that produces 1 μmol of p-nitrophenol per minute under assay conditions.

2.4 Yeast strain identification: DNA extraction and sequencing

DNA from selected yeast isolates was extracted according to a standard phenol-chloroform-extraction protocol (Cheng & Jiang, 2006): 1000 μL of cell suspension and a volume corresponding to 200 μL of sterile glass beads (\varnothing 0.43-0.6 mm) were centrifuged at 8600 g for 5 min and the supernatant was removed. Samples were frozen (-20°C) for at least 60 min. After defrosting, 500 μL of lysis buffer (10 mM TrisHCl pH 8, 100 mM NaCl, 1 mM Na₂-EDTA, 1 % SDS, 2 % (v/v) Triton x-100) and 500 μL of TE-phenol-chloroform were added to each sample, which was then vortexed (full speed, 20 min) and centrifuged, at 8600 g and for 25 min. 400 μL of the upper phase and 1000 μL of ethanol (100 %) were mixed and placed at -20°C during 30 min, for precipitation purposes. The mix was then centrifuged at 8600 g for 15 min. The supernatant was removed and 50 μL of TE-RNase (pH 8.0; 50 $\mu\text{g}\cdot\text{mL}^{-1}$) were added. Samples were then placed in a thermoplate at 55 °C with occasional stirring for 15 min to solve the pellet. The DNA purity and quantification was measured via NanoDrop (NanoDrop 2000 Spectrophotometer, Thermo Scientific), in which the DNA absorption peak was observed at 260 nm.

The D1/D2 variable domains of the larger rDNA subunit (26S) were amplified by polymerase chain reaction (PCR) using NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG) and NL4 (5'-GGT CCG TGT TTC AAG ACG G) primers (O'Donnell 1993). PCR was conducted using a 3 min initial denaturation at 94°C, followed by 40 cycles of 30 s denaturation at 94°C, 30 seconds primer annealing at 50°C and 90 seconds extension at 72°C, with a final 4 min extension at 72°C. The amplified fragments were submitted to DNA sequencing (STAB Vida, Portugal). The sequences were analyzed through a BLAST search against the nonredundant nucleotide (nt) database (BLAST). Yeast species were identified on the basis of phylogenetic analysis.

2.5 Evaluation of lipase production by *Magnusiomyces capitatus* in OMW

Selected yeast strain was grown in 500-mL shaking flasks containing 100 mL centrifuged OMW (8600 g, 15 min) and the pH was adjusted to 6.8 before sterilization (121°C for 20 min). The cell growth was monitored by analysis of the absorbance at 640 nm (Thermo Electron Corporation Spectrophotometer, model Genesys 6, Madison, USA).

The DCW values were obtained by filtrating 1 mL of every sample (three replicas of each sample were made) using membrane discs (0.45 µm, MicronSep™ membrane filter) and then dried overnight (18-24h) at 100°C. Before use, the membrane discs were dried at the same conditions, to obtain their dry weight. The same samples were also used to measure the biomass by optical density (Thermo Electron Corporation Spectrophotometer, model Genesys 6, Madison, USA) at 640 nm. A correlation between the biomass dry weight values and absorbance at 640 nm was found.

2.5.1 Toxicity tests

The centrifuged OMW medium was diluted 10%, 25% and 50% using distilled water in order to assess the influence of the phenol concentration presented in the medium. The non-diluted medium was also tested (100% OMW). 300 µL of cell suspension, previously grown in YEPD (1% yeast extract, 2% peptone, 2% dextrose) medium overnight at 30°C, served as inoculum. The flasks were placed in orbital incubator (180 rpm) at 30°C during 36 hours. 1 mL samples were taken after 12 hours and every hour after 22 hours.

2.5.2 Influence of yeast extract and olive oil supplementation on lipase activity

Non-diluted OMW with adjusted pH (6.8) was supplemented with yeast extract (2.0 g.L⁻¹) or olive oil (1.0 g.L⁻¹) or both. 300 µL of cell suspension, previously grown in YEPD medium overnight at 30°C, served as inoculum. The flasks were then incubated at 30°C, 180 rpm, for 96 hours. Crude cell-free extract was obtained by recovering the supernatant after spinning down cell debris (7500 g, 15 min). This preparation was used to estimate lipase activity.

2.6 Optimization of lipase production by experimental design methodology

Experimental distribution for two factors, according to the Doehlert uniform design (Doehlert, 1970), was used to produce response analysis. Seven experiments (and respective replicates) were carried out within an experimental domain with 1000 mL-shake flasks containing working volume ranging from 100 to 400 mL, corresponding to a specific oxygen transfer coefficient k_La between 0.24 and 1.88 min⁻¹. The nitrogen source tested was NH₄Cl, according to D'Annibale *et al.* (2006), varying between 0.2 and

2.8 g.L⁻¹. Cultures were incubated on undiluted OMW supplemented with yeast extract (2 g.L⁻¹) and olive oil (1 g.L⁻¹), at 30°C and 180 rpm for 48 hours.

2.6.1 Statistical analysis

Coded representation of the factors and interaction terms was used for calculation purposes. The response studied in this design was the biomass and lipase activity. The model used to express the responses was a second order polynomial model provided by the Doehlert uniform design (equation 1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_{12} + \beta_{11} X_{12} + \beta_{22} X_{22} \quad (Eq. 1)$$

where Y was the response from each experiment; β were the parameters of the polynomial model and X was the experimental factor level (coded units).

2.7 Analytical methods

COD (g.L⁻¹), TSS (g.L⁻¹), VSS (g.L⁻¹), and TKN (g.L⁻¹) were measured according to Standard Methods (APHA, 2001). Total phenols content was determined by a modified Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965) and expressed as caffeic acid (g.L⁻¹). The nitrates determination (NO³-N, g.L⁻¹), was performed by the method "Nitrate Cell Test 1.14542" (Spectroquant Merck) in a HACH DR/2010 spectrophotometer. Total lipids (g.L⁻¹) content was determined gravimetrically after petroleum ether extraction. Reducing sugars were measured by the DNS method (Miller, 1959) and were expressed as glucose (g.L⁻¹).

A flow cytometer (FACScalibur®, Becton-Dickinson, Franklin Lakes, NJ, USA) was used to evaluate the integrity of yeast cellular membrane of the cultured OMW after the experimental design methodology. The cytometer is equipped with a 488 nm argon laser and with photomultipliers FL1 (530 ± 30 nm), FL2 (585 ± 42 nm), FL3 (>670 nm) and FL4 (600 ± 16 nm). Cellular membrane integrity was assessed by fluorescence of stained cells using fluorochrome propidium iodide (IP, Invitrogen® EUA). The concentration of the IP stock solution was 1 mg.mL⁻¹ (in filtered mili-Q water) and the final concentration in the cellular suspension was 0.5 µg.mL⁻¹. IP fluorescence can be detected in channel FL3, due to this fluorochrome being excited at 536 nm and transmitting at 623 nm.

Samples were pre-treated before being used in the flow cytometer, in order to remove the excess of particles normally presented in the OMW. Cells were centrifuged (5000 g, 10min). Supernatant was discarded and pellet was re-suspended in a buffered saline solution (PBS, pH 7.0, Oxoid® England). This procedure was repeated two more times. Before each analysis, the samples were sonicated during 10 seconds, in order to remove cellular aggregates and guarantee the analysis of single cells. This suspension was then

diluted using the same PBS in order to obtain a cellular concentration of 500 to 1000 events per second. 0.5 μL of IP was added to 499 μL of each sample and the mixture was analysed in the flow cytometer. The data obtained were treated in the program *Windows Multiple Document Interface flow cytometry* (WinMDI 9.0).

3. Results and Discussion

3.1 Isolation of yeasts from OMW

According to several authors, OMW usually contain a diverse microbiota that is able to grow and colonize this effluent despite its high phenolic content. This microbiota consists in several species of yeasts, filamentous fungi and bacteria. Yeasts are especially well adapted to this particular environment, since they have a better tolerance to phenolic compounds when compared to bacteria (Rincón *et al.*, 2006; Morillo *et al.*, 2006; Ben Sassi *et al.*, 2006). Samples of raw OMW of three different origins were analysed in terms of their physical-chemical properties (*Table 3.1.1*). The microbial content of these samples used in this study is shown in *Table 3.1.2*.

Table 3.1.1: Physical-chemical characterization of OMW samples.

Parameter	Raw OMW (Portugal)	Enriched OMW (Jordan)	Treated OMW (Jordan)
pH	4.99	8.44	7.90
COD (g.L^{-1})	55.20	13.00	10.45
Conductivity (mS.cm^{-1})	11.80	4.73	4.45
PO_4^{3-} (g.L^{-1})	0.57	0.25	0.35
NO_3^- (g.L^{-1})	1.42	0.33	0.24
NH_4 (g.L^{-1})	1.70	0.60	0.70
TSS (g.L^{-1})	21.9	1.12	4.83
VSS (g.L^{-1})	21.10	1.06	4.35
Total phenols (g.L^{-1})	3.97	0.07	0.05
Lipids (g.L^{-1})	3.20	n.d.	n.d.

n.d. – not determined

Table 3.1.2: Microbial counts in OMW.

OMW sample	Microorganisms	Counts (CFU.mL ⁻¹)
Enriched OMW, Jordan	Bacteria	2.32x10 ⁷
	Yeasts	1.54x10 ⁷
	Filamentous Fungi	1x10
Raw OMW, Portugal	Bacteria	---
	Yeasts	3.14x10 ⁷
	Filamentous Fungi	---

(---): absence of growth

The total number of viable yeasts was very high and similar in both samples. The counts of viable bacteria in the Jordanian samples show a clear dominance of this microbial group over remaining groups. This is not observed in the samples originated in Portugal. This is probably due to the Jordanian samples having an alkaline pH, which permits the existence of bacteria. The samples originated from Portugal show a more acidic pH, which is more favorable to the occurrence of fungi (yeasts and filamentous fungi) in general. Another explanation could arise from the phenol content in the samples, since most bacteria have a low tolerance to phenols, while yeasts and filamentous fungi can withstand a higher phenolic content (Ben Sassi *et al.*, 2006). *Figure 3.1.1* shows the morphological diversity of microorganisms obtained in Nutrient Agar (NA) plates. It was observed that colony morphology is very heterogeneous, with a clear dominance of bacteria.

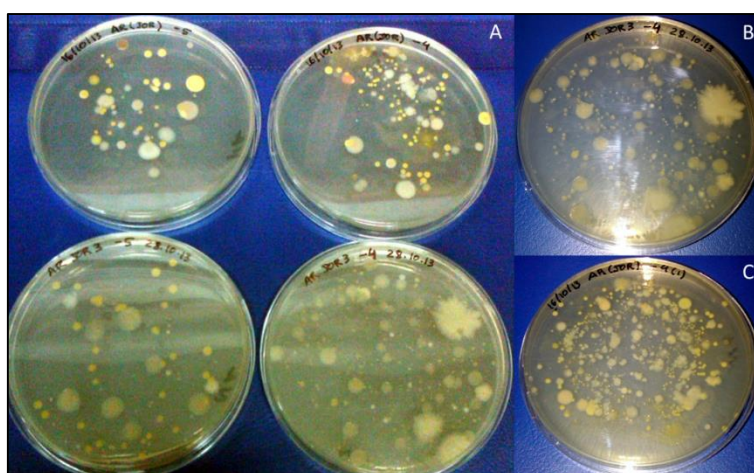


Figure 3.1.1: Nutrient Agar Plates showing an example of the high microbial diversity observed in samples of enriched OMW from Jordan [A], dilutions at 10⁻⁵ (on the left) and 10⁻⁴ (on the right). [B] and [C] are replicas of the dilution at 10⁻⁴.

Yeast colonies were successfully replicated using selective media (PDA, CRBA-supplemented with 35 µg/mL of chlortetracycline and YMA), in order to obtain isolated pure strains. Colonies morphology was very similar in most isolates, forming circular white colonies. Under optical microscopy, the yeast cells were observed with a 40x ocular, making a total amplification of 400x (*Figure 3.1.2*). The 30 isolates obtained were divided into three major groups, based on their microscopic morphology.

In group A, the cells were small sized and round, very similar to the genus *Saccharomyces*, sometimes forming aggregates. In group B, cells had a larger, more rectangular shape, with frequent formation of pseudo mycelia. Group C showed cells with an elliptical shape, without aggregates or filaments. *Table 3.1.2* shows both the origin and the culture medium used to isolate the 32 yeasts strains from OMW samples in this work and the morphological groups in which they were divided.

Table 3.1.2: Yeast strains obtained from OMW with different origins.

Group	Isolate	Sample origin	Culture medium
A	JOR ER 1	Enriched OMW (Jordan)	Cooke-Rose Bengal Agar
	JOR ER 2	Enriched OMW (Jordan)	Potato Dextrose Agar
	JOR TR 1	Treated OMW (Jordan)	Cooke-Rose Bengal Agar
	PT OMW 1	Raw OMW (Portugal)	Yeast Malt Agar
	JOR TR 2	Treated OMW (Jordan)	Potato Dextrose Agar
	JOR TR 3	Treated OMW (Jordan)	Yeast Malt Agar
B	PT OMW 2	Raw OMW (Portugal)	Yeast Malt Agar
	PT OMW 3	Raw OMW (Portugal)	Yeast Malt Agar
	JOR ER 3	Enriched OMW (Jordan)	Cooke-Rose Bengal Agar
	5 Fenol	Raw OMW (Portugal)	Yeast Malt Agar
	1 Fenol	Raw OMW (Portugal)	Yeast Malt Agar
	6 Fenol	Raw OMW (Portugal)	Cooke-Rose Bengal Agar
	PT OMW 4	Raw OMW (Portugal)	Potato Dextrose Agar
	PT OMW 5	Raw OMW (Portugal)	Potato Dextrose Agar
	3 Fenol	Raw OMW (Portugal)	Yeast Malt Agar
	PT OMW 6	Raw OMW (Portugal)	Yeast Malt Agar
	JOR TR 4	Treated OMW (Jordan)	Yeast Malt Agar
	JOR TR 5	Treated OMW (Jordan)	Yeast Malt Agar
	JOR TR 6	Treated OMW (Jordan)	Potato Dextrose Agar
	JOR ER 4	Enriched OMW (Jordan)	Yeast Malt Agar
	JOR ER 5	Enriched OMW (Jordan)	Potato Dextrose Agar
	JOR ER 6	Enriched OMW (Jordan)	Cooke-Rose Bengal Agar
C	JOR TR 7	Treated OMW (Jordan)	Cooke-Rose Bengal Agar
	PT OMW 7	Raw OMW (Portugal)	Yeast Malt Agar
	PT OMW 8	Raw OMW (Portugal)	Cooke-Rose Bengal Agar
	PT OMW 9	Raw OMW (Portugal)	Cooke-Rose Bengal Agar
	4 Fenol	Raw OMW (Portugal)	Yeast Malt Agar
	7 Fenol	Raw OMW (Portugal)	Yeast Malt Agar
	PT OMW 10	Raw OMW (Portugal)	Yeast Malt Agar
	PT OMW 11	Raw OMW (Portugal)	Yeast Malt Agar
	PT OMW 12	Raw OMW (Portugal)	Yeast Malt Agar
	PT OMW 13	Raw OMW (Portugal)	Yeast Malt Agar

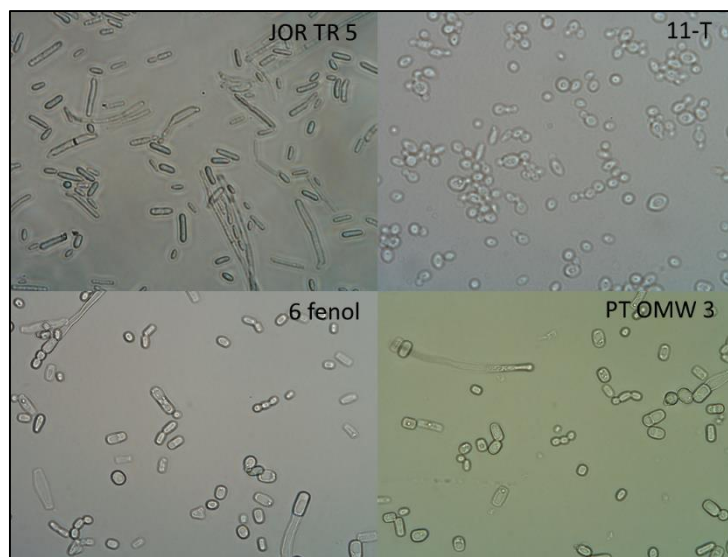


Figure 3.1.2: Isolate 11-T AR belongs to group A (amplification 1000x), while isolates JOR TR5, 6 fenol and PT OMW 3 are part of group B (amplification 400x).

The content of filamentous fungi in the samples is evidently low, when compared to the findings of other authors (Millán *et al.*, 2000). However, this could be due to a short incubation time of the plates (48h) as other authors describe an incubation time of 4-8 days. The bacteria and yeast counts in the Jordanian effluent are very similar, at relatively high amounts. Previous studies show that the microbial populations of olive mill wastewaters are formed by lactic acid bacteria and yeasts, which exist naturally at the surface of olives (Mouncif *et al.*, 1993).

To compare the presumable lipase-producing ability of these isolates with the activity of other yeasts, three strains were used from cultures of the Private Collection of Microorganisms of Bioenergy Unit of LNEG. Namely, *Yarrowia lipolytica* and *Candida sp.* are yeasts described in literature for being capable of producing lipase and were used as positive controls. The isolate 11-T AR was also used due to being previously isolated from OMW, but its lipase production was unknown.

3.2 Screening of yeasts strains with lipolytic activity

Plate assays for rapid detection of hydrolytic enzymes have long been used in microbiology. The base of this method consists in the addition of substrates that can only be hydrolyzed by certain enzymes to the media. Chromogenic dyes, such as pH indicator dye Victoria blue, are specific for lipase detection. However, they are not very sensitive and require a long incubation time. Gupta *et al.* (2006) describes a highly sensitive and rapid pH-based protocol for detection of lipases which consists in using phenol-red as a

chromogenic substrate. When the lipases break the lipids present in the medium, this causes the pH to drop and induces a change of color from pink red to yellow (Gupta *et al.*, 2006).

As previously stated, OMW usually contains oil residue, which allows the growth of yeasts able to produce lipases. The lipolytic activity of the 33 isolates was tested using three different detection media: Tween 20 agar; Phenol-Red agar and Trybutyrin agar. Yeasts that are positive for lipase production form a catalytic halo around the colonies (Figure 3.2.1). The medium Phenol-Red shows a change of color around the colonies (Figure 3.2.1-A), while the medium Tween 20 forms precipitates (Figure 3.2.1-B and Figure 3.2.1-C).

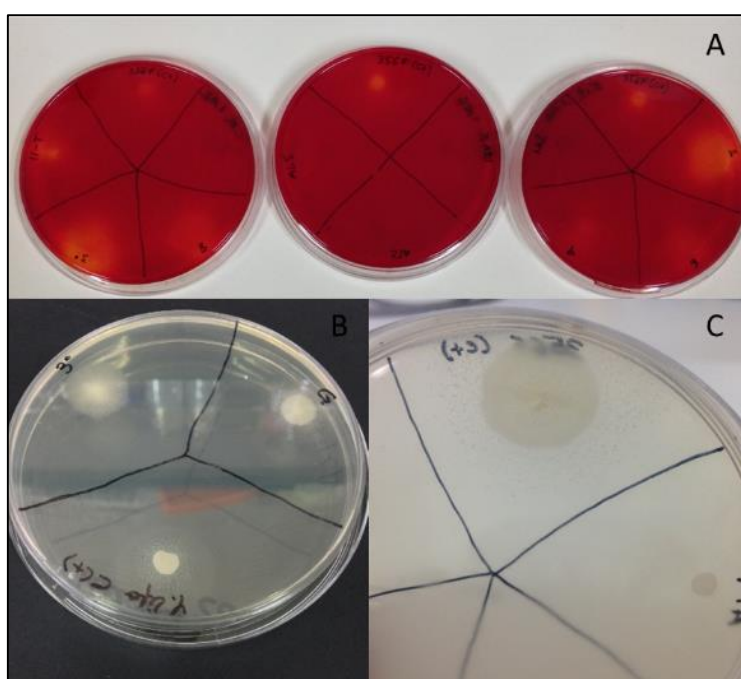


Figure 3.2.1: Detection of lipase production in plate assays. Two strains, *Yarrowia lipolytica* and *Candida sp.*, were used as positive controls. [A] Several isolates in phenol-red agar plates supplemented with 1% olive oil (substrate). [B] Isolates PT OMW3, JOR TR5 and *Yarrowia lipolytica* in a Tween 20 agar plate. [C] Close-up of a *Yarrowia lipolytica* with the precipitation halo in a Tween 20 agar plate.

After 72 to 96 hours at 30°C, six isolates showed lipolytic activity (Table 3.2.1). These strains were referred as 1 Fenol, PT OMW3, JOR TR5, 6 Fenol, PT OMW6 and 11-T AR; showing similar results to the positive controls (*Yarrowia lipolytica* and *Candida sp.*).

Table 3.2.1: Screening of lipase activity in plate assays.

Isolate	Tween 20	Tributyrin	Phenol-red with 1% olive oil
<i>Y. lipolytica</i> NRRL Y-323	+	+	+
<i>Candida sp.</i> 356F	+	+	+
11-T AR	+	+	+
JOR ER 1	-	-	n.d.
JOR ER 2	-	-	n.d.
JOR TR 1	-	-	n.d.
PT OMW 1	+	-	n.d.
JOR TR 2	-	-	n.d.
JOR TR 3	-	-	n.d.
PT OMW 2	-	+	n.d.
1 Fenol	+	-	++
JOR ER 3	-	+	n.d.
PT OMW 3	+	-	++
5 Fenol	-	-	n.d.
6 Fenol	+	-	++
PT OMW 4	-	-	n.d.
PT OMW 5	+	-	n.d.
3 Fenol	-	-	n.d.
PT OMW 6	+	+	n.d.
JOR TR 4	-	-	n.d.
JOR TR 5	++	+	-
JOR TR 6	-	-	n.d.
JOR ER 4	-	-	n.d.
JOR ER 5	-	-	n.d.
JOR ER 6	-	-	n.d.
JOR TR 7	-	-	n.d.
PT OMW 7	-	-	n.d.
PT OMW 8	-	-	n.d.
PT OMW 9	-	-	n.d.
4 Fenol	-	-	n.d.
7 Fenol	-	-	n.d.
PT OMW 10	-	+	n.d.
PT OMW 11	-	+	n.d.
PT OMW 12	+	-	n.d.
PT OMW 13	+	-	n.d.

n.d: not defined; -: negative result; +: positive result; ++: positive result, but halo is visibly larger than the positive control halo.

The medium phenol-red showed poor performance in the evaluation of lipolytic activity of the isolates, most of it showing inconclusive results. However, this method is generally described in literature as a good lipase evaluator (Holmberg and Kielland, 1978). The remaining 27 isolates only revealed a positive result in one method and were not considered for further experiments. These tests are only a qualitative evaluation of lipase production, allowing the detection of producing isolates. However, the quantity of enzymatic production is not discerned. The next assays will evaluate extracellular lipase activity in liquid medium and optimize the lipase production of the selected strain in OMW as growth medium.

3.3 Production of extracellular lipase in shake-flask experiments

The strains 1 Fenol, PT OMW 3, 6 Fenol, PT OMW 6, 11-T and JOR TR 5 were tested for the production of extracellular lipases.

The cultures were grown during 120 hours in a synthetic medium with olive oil as an inductor. The strain *Yarrowia lipolytica* served as a control. It was grown in the same conditions as the remaining strains and had a maximum lipase activity of 0.12 U.mL⁻¹ at 120 hours (Figure 3.3.1).

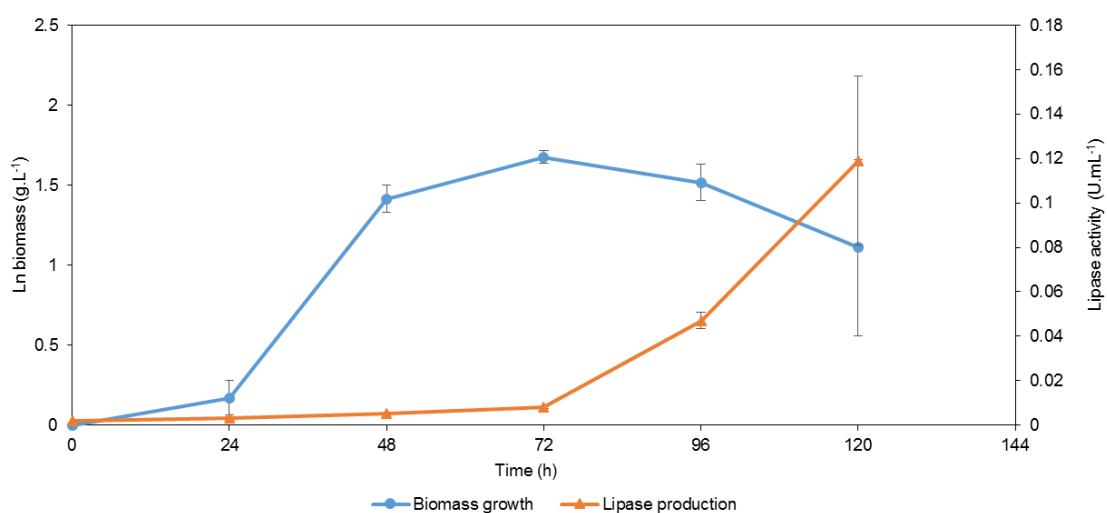


Figure 3.3.1: Time course of biomass growth and lipase production by *Yarrowia lipolytica* NRRL Y-323. All results were obtained in triplicate. Standard deviation is indicated by the error bars.

Figure 3.3.2 shows the biomass growth and enzymatic production of strains 1 Fenol, PT OMW 3 and 6 Fenol. Two strains, 11-T and PT OMW 6, did not exhibit lipase production

(Figure 3.3.3). Figure 3.3.4 shows biomass growth and enzymatic production of strain JOR TR 5. Biomass growth of isolates 11-T, PT OMW 6 and JOR TR 5 was only determined during the first 72 hours, due to the occurrence of flocculation. The flocculation presented itself as small circular aggregates, floating in the medium (Figure 3.3.5). However, lipase production in all isolates was measured until 120 hours of culture growth in order to obtain comparable data.

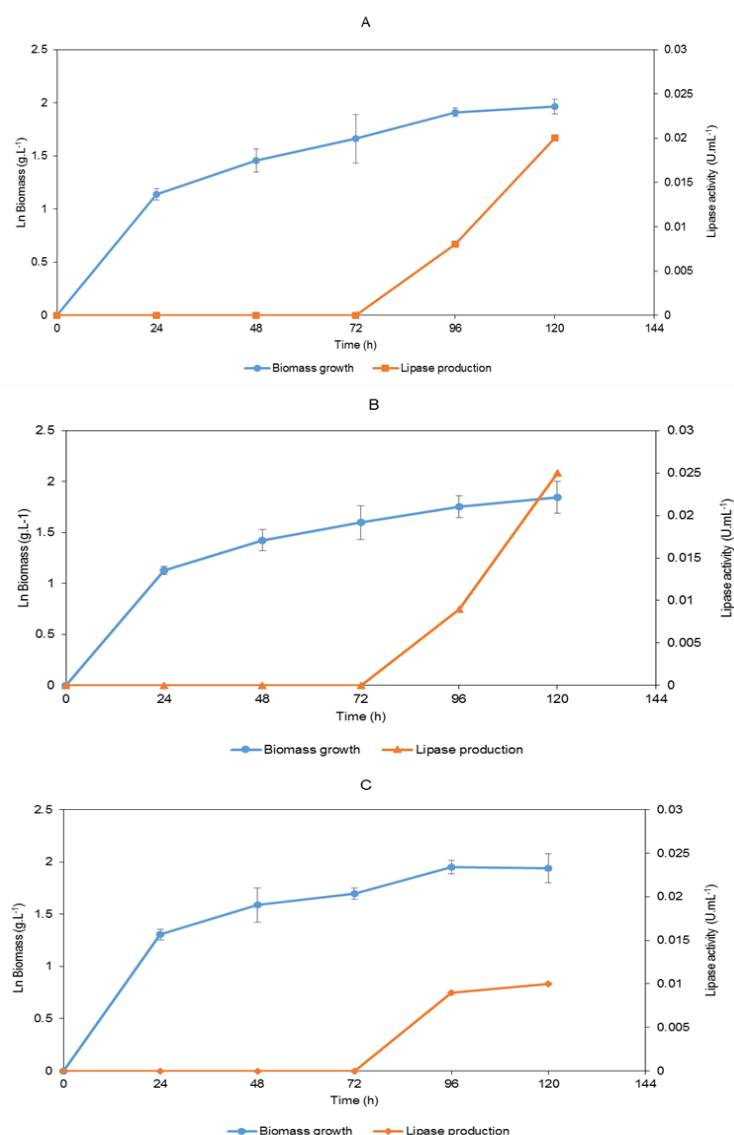


Figure 3.3.2: Biomass growth and lipase production of: **A** - 1 Fenol, **B** - PT OMW 3 and **C** – 6 Fenol, in synthetic medium with 0.5 g.L⁻¹ olive oil. All results were obtained in triplicate. Standard deviation is indicated by the error bars.

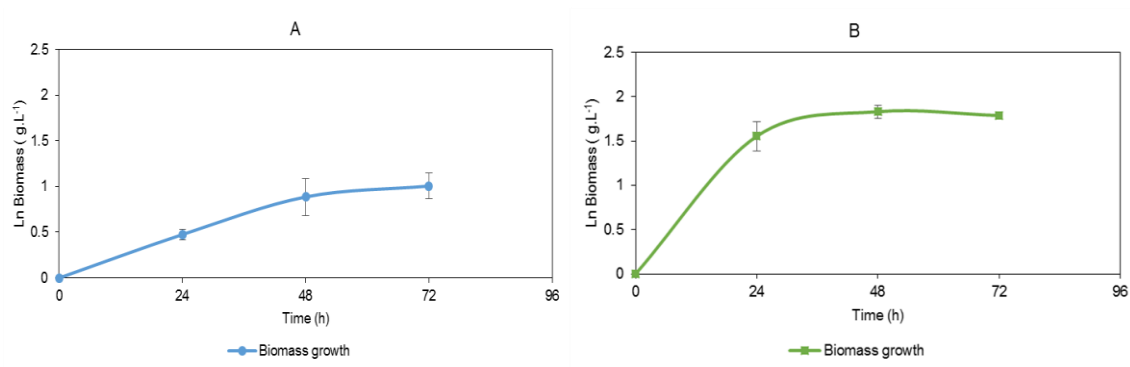


Figure 3.3.3: Biomass growth of: **A** – 11-T and **B** – PT OMW 3, in synthetic medium with 0.5 g.L⁻¹ olive oil. All results were obtained in triplicate. Standard deviation is indicated by the error bars.

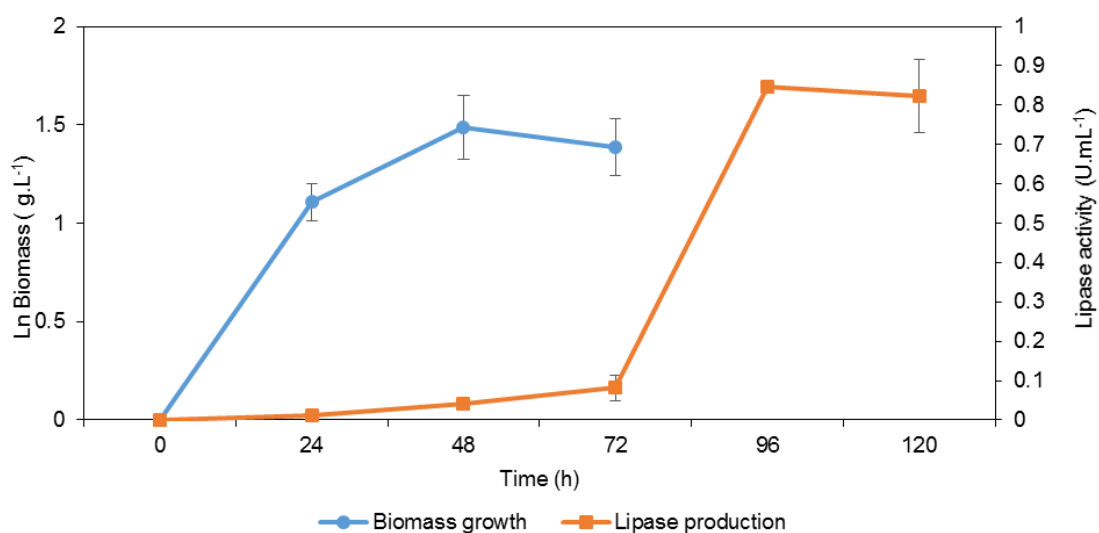


Figure 3.3.4: Time course of biomass growth and lipase production by strain JOR TR 5 in synthetic medium with 0.5 g.L⁻¹ olive oil. All results were obtained in triplicate. Standard deviation is indicated by the error bars.

With these assays, strains 1 Fenol, PT OMW 3 and JOR TR 5 were selected as the most prominent lipase producers, with 0.02 U.mL⁻¹, 0.03 U.mL⁻¹ and 0.85 U.mL⁻¹, respectively. Strain JOR TR 5 showed similar flocculation profile to the strains 11-T and PT OMW 6.



Figure 3.3.5: Flocculation of strain 11-T at 96 hours of incubation in synthetic medium with 0.5 g.L⁻¹ olive oil.

Some of the strains presented a flocculating behavior. Flocculation can be triggered by environmental factors, such as nitrogen starvation, or pH changes, so it might protect the cells in the middle of the flocs from the environment, or by genetic factors. Certain authors (Holmberg and Kielland-Brandt, 1978; Straver *et al.*, 1993) believe that the flocculation of microbial cultures may be related to the levels of oxygen provided during the culture growth. Powell *et al.* (2003) also suggests that the flocculation potential of a cell may alter throughout the lifespan, older cells being more flocculent. This could provide an explanation as to why the cultures 11-T, PT OMW 6 and JOR TR 5 only exhibited flocculation at 72 hours, in a late stationary phase.

Strains PT OMW 3 and JOR TR 5 achieved the best results (0.03 U.mL⁻¹ and 0.85 U.mL⁻¹) under the experimental tested conditions used. Strains 11-T and PT OMW 6 did not produce extracellular lipase. However, the existence of intracellular production is not excluded (this production was not studied) since they revealed lipase activity in previous solid media screening. The production of extracellular enzymes is more advantageous in an industrial production point of view due to the reduced cost of the downstream processing. In an extracellular enzyme production, only the separation and purification of enzymes is necessary. In an intracellular production, the enzyme must be extracted from the inside of the cell, meaning that the cells have to be lysed, adding an extra step to the downstream processing and raising the costs. Therefore at this point these strains were excluded from further assays.

Olive oil was chosen as the lipidic inductor, due to having the best results demonstrated in the literature (D'Annibale *et al.*, 2006) and being similar to the residual oil present in OMW, which will be used in the following assays. Strains PT OMW 3 and JOR TR 5 were further identified and strain JOR TR 5 was chosen to be used in the forthcoming experiments.

3.4 Identification of yeast strains PT OMW 3 and JOR TR 5 by DNA sequencing

To successfully identify isolates PT OMW 3 and JOR TR 5, the primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG) and NL4 (5'-GGT CCG TGT TTC AAG ACG G) were used to amplify the D1/D2 variable domains of the larger- rDNA subunit (26S), after which the amplified fragments were sequenced (O'Donnell, 1993). A similarity search was performed using the BLAST tool (BLAST). The results obtained are shown in *Table 3.4.1*.

Table 3.4.1: Phylogenetic affiliations of the nucleotide sequences obtained from amplified DNA fragments of 26S and 18S rDNA gene.

Isolate	Similar organism	Access Code	Similarity	Class
PT OMW 3	<i>Galactomyces geotrichum</i>			
	partial 18S rRNA gene, strain LMA-70	JQ668740	99%	Saccharomycetes
JOR TR 5	<i>Magnusiomyces capitatus</i> partial 26S rRNA gene, strain Kw-230	HG313854	99%	Saccharomycetes

Galactomyces geotrichum, previously known as *Endomyces geotrichum* or *Dipodascus geotrichum*, is a filamentous yeast and was first described by E.E. Butler and L.J. Peterson in 1972, isolated from soil samples originated from Puerto Rico. It has a cosmopolite distribution, being usually found in soil (Kurtzman *et al.*, 2011). Phillips and Pretorius (1991) reported extracellular lipase production by *G. geotrichum* strain CBS 772.71.

Magnusiomyces capitatus, previously known as *Geotrichum capitatum*, *Blastoschizomyces capitatus* or *Dipodascus capitatus*, is also a filamentous yeast with a cosmopolite distribution, being prevalent in geographic areas of high humidity and temperature. This species was first described by Diddens & Lodder in 1942. It can be found in diverse natural substrata including soil, fruits and dairy products. *M. capitatus* also appears in the digestive and respiratory tract of animals and humans (Kurtzman *et al.*, 2011). This species was reported, for biotechnological purposes, by Yamada-Onodera *et al.* (2007) as a producer of N-benzyl-3-pyrrolidinol dehydrogenase. To current knowledge, *M. capitatus* has yet to be reported on the production of lipases.

Petterson and Kurtzman (1991) used the variable D2 domain near the region of the 5' end of the large subunit (26S) rDNA from two yeast sister species to determine if closely related species could be separated from substitutions in that region. They concluded that different species had more than 1% substitutions, thus providing an empirical means to

recognize species. The great majority of yeast species can be successfully identified from the sequence divergence in the D1/D2 domain.

The isolates PT OMW 3 and JOR TR 5 were successfully identified as being closely related with *Galactomyces geotrichum* strain LMA-70 and *Magnusiomyces capitatus* strain Kw-230 respectively, both with a similarity of 99%. Since *M. capitatus* was the strain with the highest lipase production observed, it was chosen for further experiments.

3.5 Influence of phenol toxicity of OMW in *Magnusiomyces capitatus* growth

In order to evaluate the effect of OMW phenolic content in the growth rate of *M. capitatus*, the culture was grown in three different dilutions (10%, 25% and 50% OMW) and undiluted OMW during 36 hours. Two different conditions of undiluted OMW were also tested on growth rate of *M. capitatus*: one with adjusted pH to 6.8 and another supplemented with ammonium chloride (NH_4Cl , 0.63 g.L^{-1}). Figure 3.5.1 shows the biomass growth in each condition tested, while Table 3.5.1 shows the growth rate obtained for each culture.

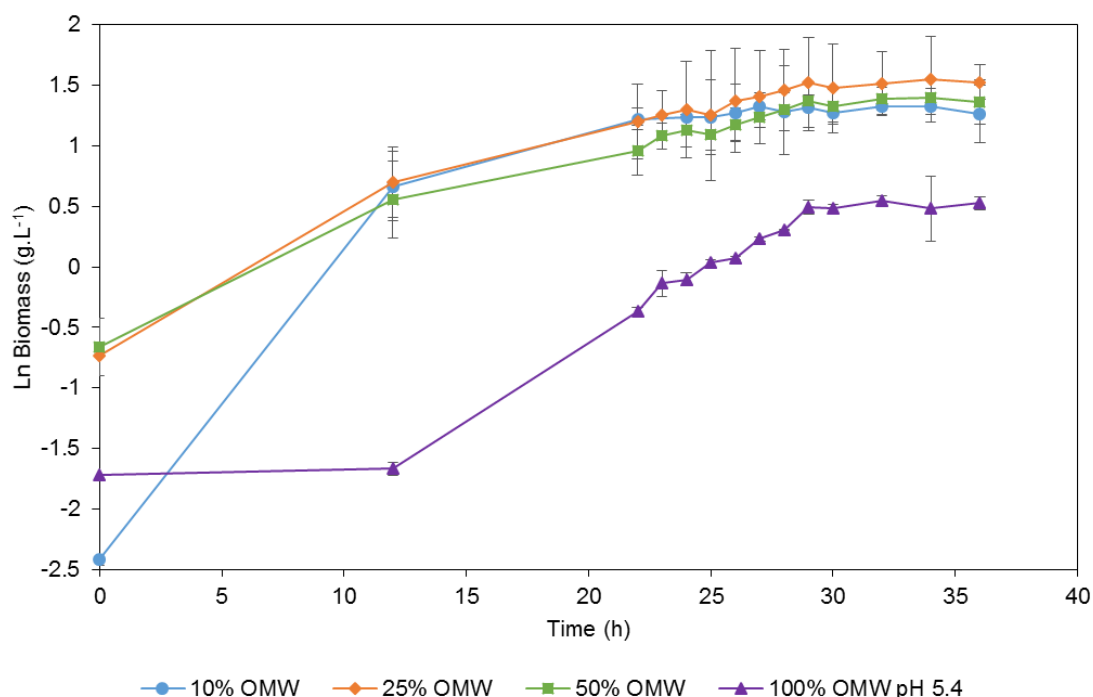


Figure 3.5.1: Biomass growth of *M. capitatus* in different dilutions of medium. The legend indicates the amount of OMW used in each dilution (100% is undiluted OMW). All results were obtained in duplicate. Standard deviation is indicated by the error bars.

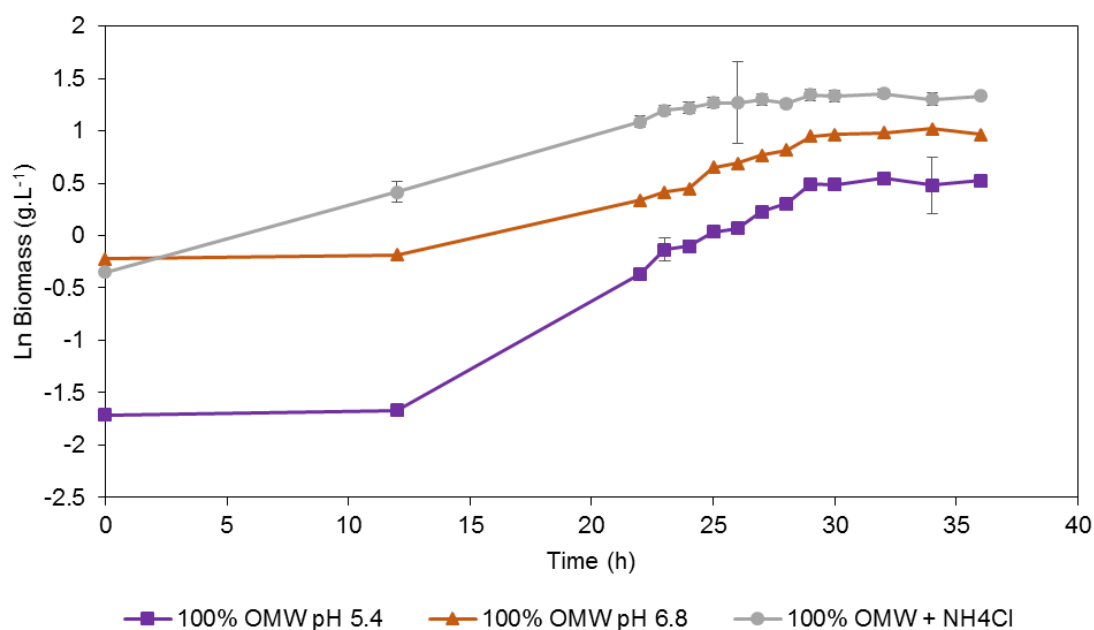


Figure 3.5.2: Biomass growth of *M. capitatus* in undiluted OMW media. All results were obtained in duplicate. Standard deviation is indicated by the error bars.

Table 3.5.1: Growth rate (μ , h^{-1}) of *M. capitatus* in OMW diluted at 10%, 25%, 50% and in undiluted OMW (100% 5.6, 100% 6.8 and 100% with NH_4Cl).

Experiment condition	Growth rate (μ , h^{-1})
10% OMW	0.28
25% OMW	0.12
50% OMW	0.05
100% OMW at pH 5.4	0.13
100% OMW at pH 6.8	0.07
100% OMW suppl. with NH_4Cl	0.21

The growth rate of the yeast strain was also compared against the phenolic content of each OMW dilution to provide a faster way to observe if the growth was indeed affected by the presence and concentration of phenolic compounds in the growth media (Figure 3.5.3). The phenolic content of the OMW media in each experiment was quantified, in order to observe if *M. capitatus* was able to degrade these phenolic compounds.

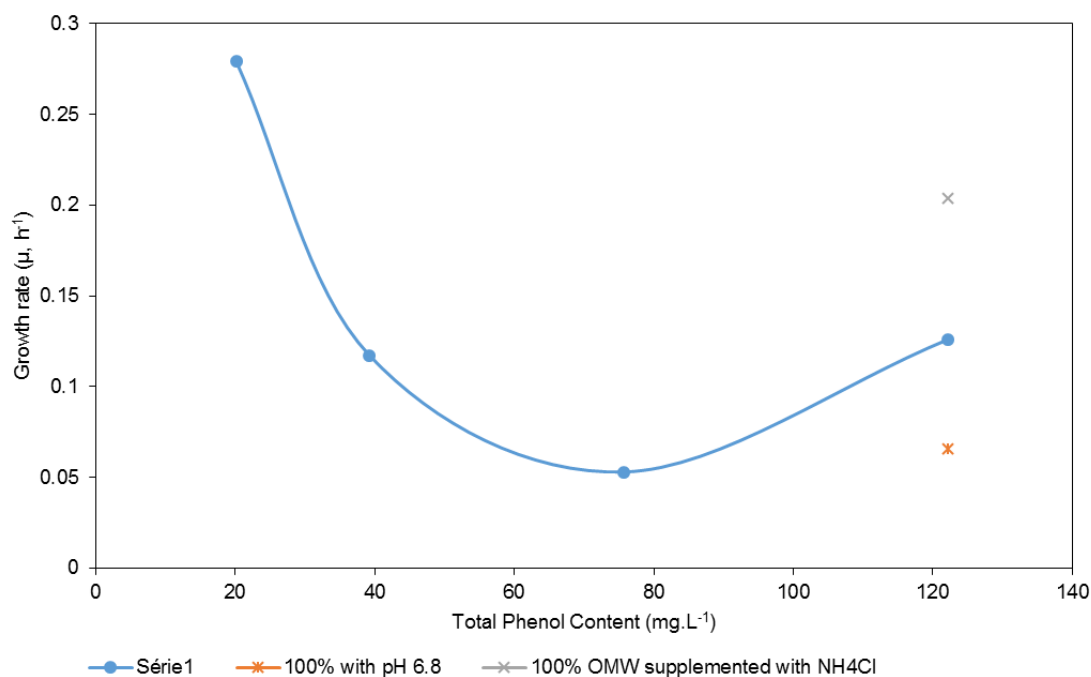


Figure 3.5.3: Effect of phenol content on *M. capitatus* growth rate in OMW media.

As the phenolic content increases in the growth medium, *M. capitatus* growth rate declines. This phenomenon is more evident between the dilution 10% and 25% (represented in Figure 3.5.3 by the total phenol content values of 20.17 and 39.28 mg.L⁻¹, respectively), where the growth rate decreases from 0.28 h⁻¹ to 0.12 h⁻¹. The lowest growth rate observed was of 0.05 h⁻¹ in 50% OMW (Figure 3.5.3, 75.75 mg.L⁻¹). However, it was observed that, at the highest phenolic value (Figure 3.5.3, 122.18 mg.L⁻¹), *M. capitatus* growth rate increases from 0.05 h⁻¹ to 0.13 h⁻¹, a value slightly higher than the one observed with dilution 25%. Increasing the OMW pH to 6.8 resulted in a decrease of the growth rate from 0.13 h⁻¹ to 0.07 h⁻¹. It was observed that supplementing 100% OMW with NH₄Cl (0.63 g.L⁻¹) resulted in a high increase of the growth rate, reaching 0.21 h⁻¹. This growth rate is the second highest observed, with the first being obtained in dilution 10%. This suggests that phenolic inhibition is not the only factor influencing *M. capitatus* growth in OMW medium.

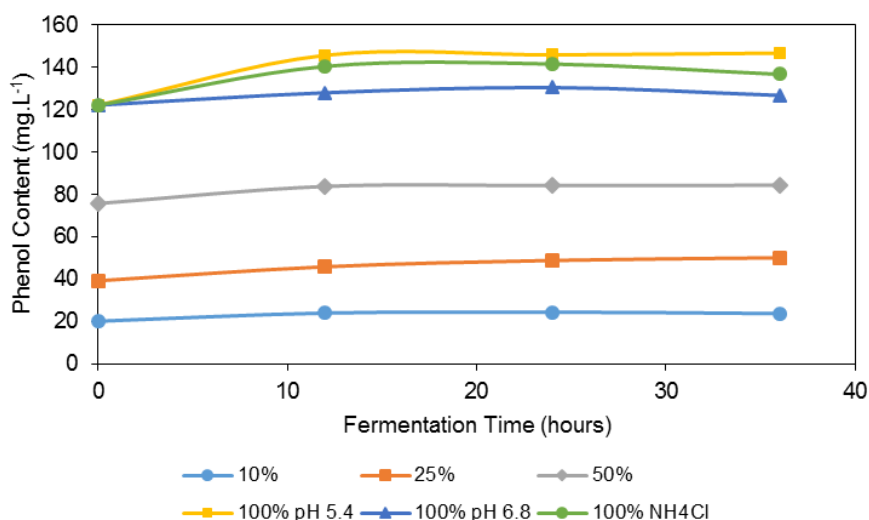


Figure 3.5.4: Variation of phenol content along time in different OMW culture medium concentrations.

It was observed that there was no phenolic degradation throughout the 36 hours of microbial growth (Figure 3.5.4), which suggests that this strain is not able to degrade phenolic compounds and probably uses alternative carbon sources.

The high concentrations of polyphenols (tannins, catechins and anthocyanins) are the primary responsible for the toxic properties of the OMW. More specifically, monomeric phenols have been directly associated with the phytotoxic and antimicrobial characteristics of this effluent (D'Annibale *et al.*, 1998). In the total phenol content, the lower value corresponds to the more diluted medium (10%). As the percentage of OMW increases (25%, 50% and 100%), so does the phenolic content. *M. capitatus* growth rate had its highest value in the more diluted medium (10%) and experienced a decline with the increase of concentration of OMW in the media. Sassi *et al.* (2006) describes a similar situation. The authors isolated 120 yeasts strains from three different OMW and only 20 were able to grow in OMW plate media. The authors suggest that the strains that were unable to grow could be not well adapted to OMW and suggested such could be related to phenolic inhibition. Some authors demonstrated that monomeric phenols indeed affect the microbial growth during the biological treatment of OMW, diminishing microbial growth rate or completely inhibiting its growth (Robles *et al.*, 2000; Brozzoli *et al.*, 2009). However, the growth rate obtained in undiluted OMW pH 5.6 was not the lowest value obtained. It was observed that supplementation of undiluted OMW with NH_4Cl resulted in an improvement in *M. capitatus* growth rate. This could indicate that, only to a certain point, does phenolic inhibition affects *M. capitatus* growth in OMW.

3.6 Evaluation of lipase production by *Magnusiomyces capitatus* in OMW

In figures 3.6.1 and 3.6.2, we can observe the effect of yeast extract and olive oil supplementation on *M. capitatus* growth in Figure 3.6.1 and its lipase production in Figure 3.6.2.

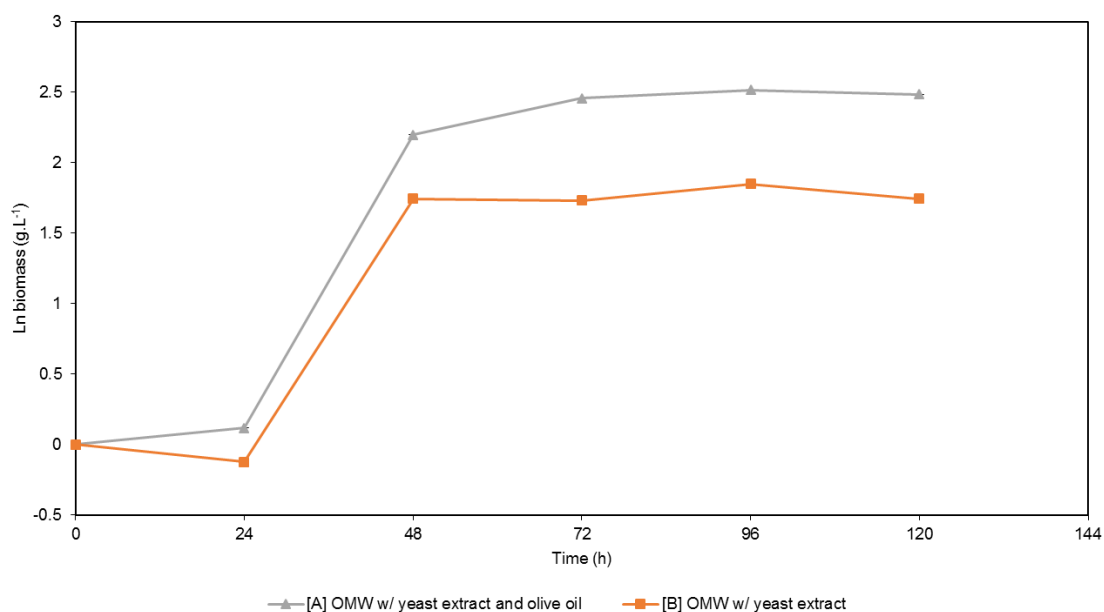


Figure 3.6.1: Biomass growth of *M. capitatus* in the two OMW-based media. All results were obtained in duplicate. Standard deviation is indicated by the error bars.

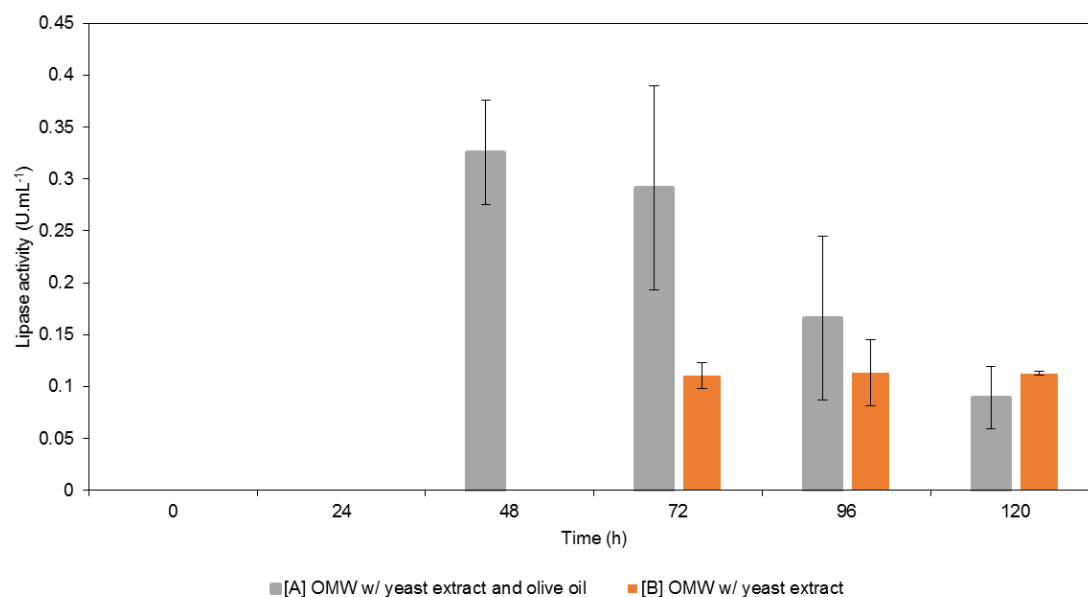


Figure 3.6.2: Lipase production of *M. capitatus* in the OMW-based media. All results were obtained in duplicate. Standard deviation is indicated by the error bars.

The OMW-based medium supplemented with both olive oil and yeast extract (medium [A]) achieved the best results, when compared to the other medium. Medium [A]

maximum lipase production value of 0.33 U.mL⁻¹ was first achieved at 48 hours, while medium [B] only produced 0.11 U.mL⁻¹ at 96 hours. As to the biomass values with medium [A], *M. capitatus* had the highest value of 12.34 g.L⁻¹ at 96 hours. With medium [B], *M. capitatus* was only able to attain 6.34 g.L⁻¹ of biomass at 96 hours, almost half of the value medium [A] has able to achieve. In both media, *M. capitatus* reached the stationary state at 48 hours.

Previous studies using other lipase-producing yeast assumed that *M. capitatus* needed nutritional supplementation in order to successfully produce lipase (Gonçalves *et al.*, 2007; Fadil *et al.*, 2003). Since the lipid content OMW samples were relatively low, olive oil supplementation could aid production. Yeast extract can also help with the nutritional requirements. Fadil *et al.* (2003) referred the need of supplementation with nitrogen or phosphorous in aerobic biological treatment process of OMW as nutritional requirements for microorganisms to degrade the organic and phenolic fraction. Moreover, D'Annibale *et al.* (2006) studied the addition of several oils in OMW-based media (to induce lipase production) and concluded that olive oil was the most effective inducer when used at a concentration higher than 0.3 g.L⁻¹. Gonçalves *et al.* (2011) described the supplementation of OMW with yeast extract to assure a minimum amount of vitamins necessary to the microbial growth.

It was observed that flocculation did not occur in any of the OMW media, unlike previous experiments in the synthetic medium growth. This is probably due to the lack of certain nutrients necessary to culture growth in synthetic medium, since growth conditions were not altered.

The supplementation with both a nutrient source (yeast extract) and lipidic inducer (olive oil) benefits the growth and lipase production of *M. capitatus* in an OMW-based medium.

3.7 Optimization of lipase production by *M. capitatus* on OMW medium

Nitrogen content and oxygen availability are two key factors that must be used for improving lipase production by *M. capitatus*. The combined effect of those factors is unknown. The surface response methodology according to the Doehlert distribution for two factors was used to find optimum conditions for lipase production and highlight possible interactions between these two factors. In addition, flow cytometry was used to assess the cell membrane integrity in all tested conditions. *Table 3.7.1* shows the results obtained from the experimental design. The two factors taken into consideration were:

shaken flask working volume, as a means to control oxygen availability in terms of the specific oxygen transfer coefficient, (k_{La}) and nitrogen effect (NH_4Cl).

Table 3.7.1: Tested conditions and the obtained results of each experiment according to a Doehrlert distribution for two factors. Tests were made in duplicate.

Test	Experimental conditions			Response	
	Volume (mL)	k_{La} (min^{-1})	NH_4Cl ($g.L^{-1}$)	Biomass ($g.L^{-1}$)	Lipase activity ($U.mL^{-1}$)
A	170	1.06	1.5	6.59 ± 0.18	0.43 ± 0.16
B	100	1.88	1.5	6.73 ± 0.39	1.12 ± 0.05
C	400	0.24	1.5	5.06 ± 2.43	0.19 ± 0.13
D	130	1.47	2.8	6.25 ± 0.55	0.99 ± 0.12
E	190	0.65	0.2	6.97 ± 0.46	0.05 ± 0.00
F	130	1.47	0.2	6.96 ± 0.13	0.26 ± 0.00
G*	190	0.65	2.8	6.21	0.98

*data referent to the replica was not used due to bacterial contamination.

It was observed that when the concentration of NH_4Cl was kept constant ($1.5 g.L^{-1}$) and k_{La} values varied (tests A, B and C), it induced changes in the biomass and in lipase concentration values. When k_{La} was $0.24 min^{-1}$, the biomass value was $5.06 g.L^{-1}$, being the lowest value obtained in the experimental design. However, as the k_{La} value increased to $1.06 min^{-1}$, the biomass obtained was substantially higher ($6.6 g.L^{-1}$). This increase was also observed in lipase production, varying from 0.19 to $0.43 U.mL^{-1}$, a value 2.29-fold higher than the former. Further increase in the k_{La} value to $1.88 min^{-1}$ did not induce major changes ($6.7 g.L^{-1}$). This could mean that the oxygen is the limiting nutrient in the interval $0.26-1.06 min^{-1}$. In the lipase production, the k_{La} increase continued to induce higher production, reaching $1.12 U.mL^{-1}$, resulting in a production increase of 2.6-fold (comparing with the previous value).

Maintaining the k_{La} values constant and varying the NH_4Cl concentration also induced changes, more visible in the lipase production than in the biomass growth. With a k_{La} value of $1.47 min^{-1}$ (tests D and F), the increase of NH_4Cl from 0.2 to $2.8 g.L^{-1}$ increased the lipase production by 3.8-fold (0.26 to $0.99 U.mL^{-1}$), while decreasing biomass growth from 6.97 to $6.25 g.L^{-1}$. Similar observation was denoted when the design used the constant k_{La} of $0.65 min^{-1}$, (tests E and G) changing from 0.05 to $0.98 U.mL^{-1}$ in lipase production. This 19.6 fold production increase is the highest observed. Similarly, a slight decrease in the biomass growth when the NH_4Cl concentration is increased (tests E and G) is observed (6.97 to $6.2 g.L^{-1}$).

The results obtained for biomass growth under different tested conditions are consistent with the corresponding consumption of reducing sugars (*Figure 3.7.1*). Test C, carried out at the lowest k_{La} (0.24 min^{-1}), has shown the lowest sugar consumption (about 9%). As for lipase production, the best conditions found for sugar consumption (around 43%) were tests B and D, at highest oxygen availability (k_{La} 1.88 and 1.47 min^{-1} , respectively) and at highest concentration of NH_4Cl (1.5 and 2.8 g.L^{-1} , respectively).

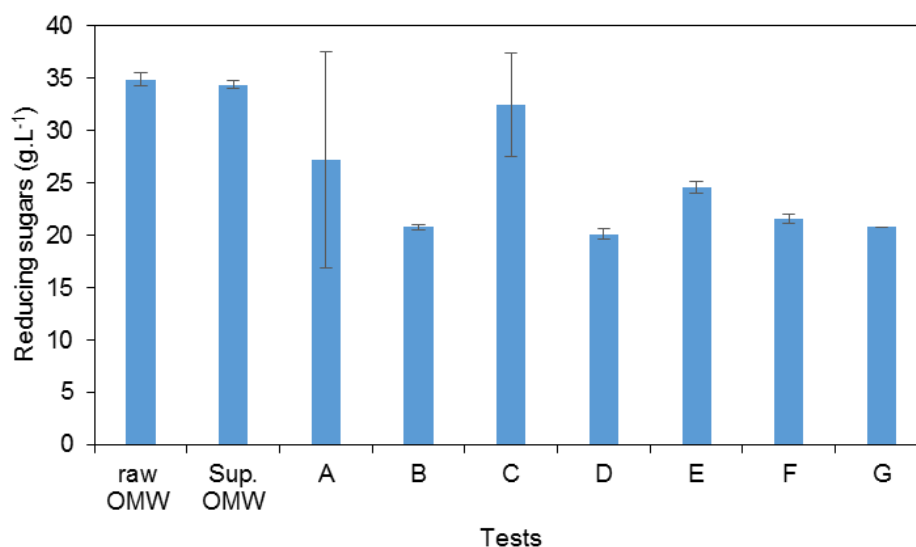


Figure 3.7.1: Reducing sugars quantification in each experiment after the 48 hours of microbial growth. The samples raw OMW and supplemented OMW represent the concentration of total sugars in the beginning of the experiment. All results were obtained in duplicate. Standard deviation is indicated by the error bars.

In order to observe if the different culture conditions induced physiological stress to the cells, flow cytometry was used. *Figure 3.7.2* shows several plots concerning the flow cytometry data. Using propidium iodide (PI), the cells with permeabilized membranes are stained, since PI can successfully enter the cell and bind to its DNA. However, PI cannot enter cells with a viable membrane. The samples were analyzed, first to differentiate the cellular population from other particles, and then to detect PI fluorescence. *Table 3.7.2* presents the flow cytometry analysis, showing the percentage of viable or permeabilized cells.

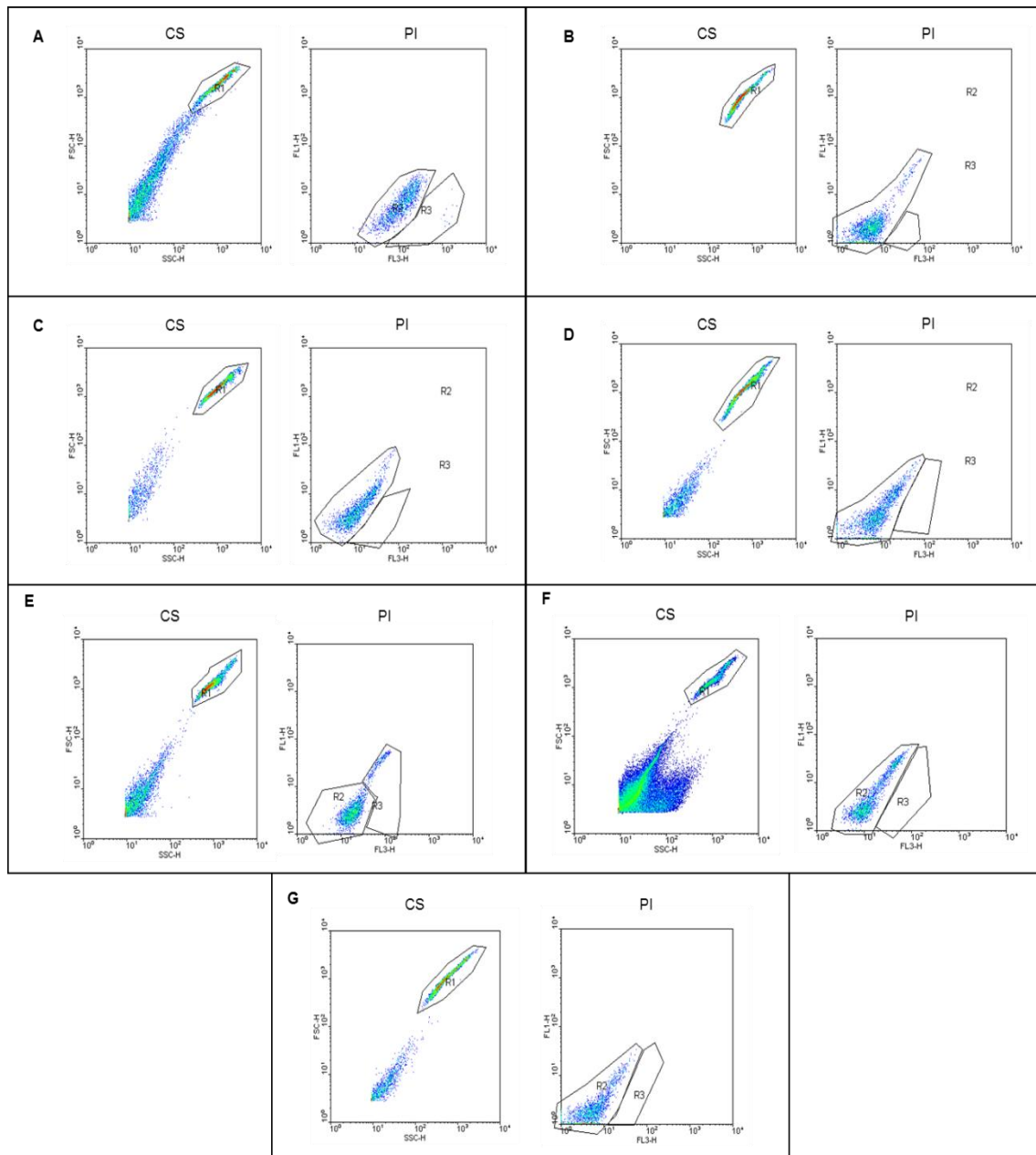


Figure 3.7.2: Flow cytometry results of each experiment. CS – Cellular sample. Detection of cells using forward and side scatter scan. The marked region (R1) represents the total population of cells. PI – Detection of propidium iodide (PI) staining using the FL3 channel. R2 is the region of non-stained cells and R3 is the region with stained cells. The figures of the replicas can be found in annex (Figures S1-S6).

Table 3.7.2: Flow cytometry results (in percentage) using PI staining, taken from the analysis of the graphs on Figure 3.7.2.

Test	Experimental conditions			Response	
	Volume (mL)	K _L a (min ⁻¹)	NH ₄ Cl (g.L ⁻¹)	Intact cytoplasmic membrane (%)	Permeabilized cytoplasmic membrane (%)
A	170	1.06	1.5	93.7 ± 4.5	5.8 ± 5.1
B	100	1.88	1.5	99.7 ± 0.1	0.1 ± 0.1
C	400	0.24	1.5	97.9	0.4
D	130	1.47	2.8	99.5 ± 0.1	0.2 ± 0.2
E	190	0.65	0.2	90.9 ± 10.6	7.5 ± 10.2
F	130	1.47	0.2	97.2 ± 1.1	1.4 ± 0.5
G	190	0.65	2.8	98.6	0.4

From the analysis of Figure 3.7.2 and Table 3.7.2, it could be concluded that the studied factors did not influence the integrity of the cytoplasmic membrane since values higher than 90% were obtained for all tested conditions, which could indicate that the cells were not under any physiological stress and were able to endure the several conditions of the experiments. The lowest percentages of permeabilized cells (0.09 – 0.2%) correspond to the experimental conditions where lipase activity achieved was higher (tests B and D: maximal oxygen availability tested and maximal nitrogen concentration tested, respectively).

The data obtained from the experimental design were further used for regression analysis and polynomial model-derived parameters (b₀ to b₂₂) shown in Table 3.7.3. These parameters describe the relative influence of both factors individually on the responses and how they interact within the experimental domain. b₀ represents the analyzed response at the center of the experimental domain. The magnitude of b₁ and b₂ indicates the importance of each factor (oxygen availability and nitrogen concentration) on the responses. b₁₂ is an interaction parameter and express how the effect of one factor depends on the level of the other factor.

Table 3.7.3: Parameters of the polynomial models representing the studied responses.

	Model	Biomass (g.L ⁻¹)	Lipase (U.mL ⁻¹)	IC	PC
Model parameters	β_0	6.59	0.54	93.71	2.23
	β_1	1.14	0.31	0.53	0.02
	β_2	-0.42	0.48	0.71	-0.37
	β_{12}	0.03	-0.11	1.20	-0.61
	β_{11}	-1.56	0.16	5.10	-1.98
	β_{22}	0.53	-0.02	4.54	-1.52
Model validation (Fischer test)	Effectiveness of the parameters	5.68	22.16	3.37	16.58
	Significance level (%)	$\alpha = 0.01$	$\alpha = 0.001$	$\alpha = 0.06$	$\alpha = 0.001$
	Lack of fit	36.32	51.10	0.52	9.09
	Significance level (%)	$\alpha = 0.001$	$\alpha = 0.0001$	$\alpha = 0.001$	$\alpha = 0.001$
R²	(coefficient of multiple determination)	0.78	0.93	0.67	0.91

β_0 , response at the center of the experimental domain; β_1 and β_2 , parameters of the factors; β_{12} , parameter of the interaction of the factors; β_{11} and β_{22} , self-interaction parameters of the factors. Critical and calculated values in both F-tests were used to test the effectiveness of the parameters and the lack of fit.

For the statistical analysis, the observations were according with the model representation of the experimental domain (*Table 3.7.3*). The relative effect of the two studied factors, oxygen availability (k_{La}) and nitrogen concentration, in biomass growth, lipase production, intact (IC) and permeabilized cells (PC) was given by the values of β parameters. The influence of the oxygen availability ($\beta_1 = 0.31$) is lower than nitrogen concentration ($\beta_2 = 0.48$) in lipase production. The interaction of both factors ($\beta_{12} = -0.11$) shows that these two factors act independently, since their joint action does not exhibit an improvement in lipase activity, except when these factors are at their highest value. Concerning biomass, there is a positive interaction of the two factors for biomass growth ($\beta_{12} = 0.03$) but the influence of oxygen availability ($\beta_1 = 1.14$) has more effect on yeast growth than nitrogen concentration ($\beta_2 = -0.42$). The interaction of the two factors also has a positive response for intact membrane cells ($\beta_{12} = 1.20$).

Data regression transformed the statistical data into contour plots. The profiles shown by the isoresponse contours of Lipase (A), Biomass (B), percentage of Intact cells (C) and Permeabilized cells (D) with growing concentrations of nitrogen and oxygen are shown in *Figure 3.7.3*.

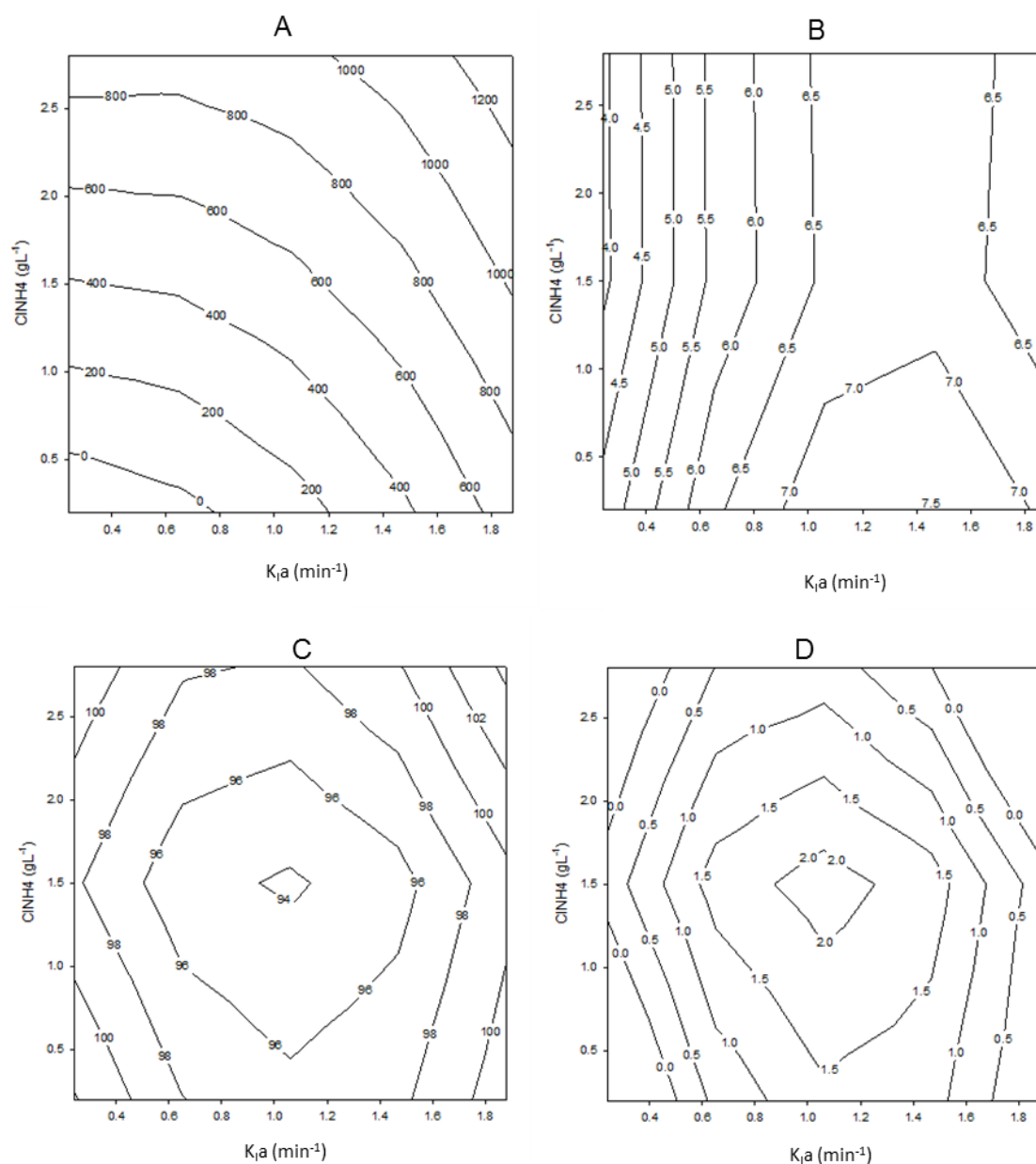


Figure 3.7.3: Response surface for factors nitrogen concentration (0.2 - 2.8 g.L⁻¹ NH₄Cl) and oxygen availability (K_{La} 0.24 – 1.88 min⁻¹) in: **(A)** lipase production (U.mL⁻¹); **(B)** biomass growth (g.L⁻¹); **(C)** FC, Intact cell membranes (%); **(D)** CC, Compromised cell membranes (%).

The response surface in *Figure 3.7.3 - A* shows the variation of lipase production within the experimental domain. The increase on NH₄Cl concentration and k_{La} increased the production of lipase. However, the enzyme activity seems to be more affected by nitrogen concentration due to the horizontal lines observed on the upper left response surface. The importance of oxygen availability on biomass growth is expressed by the vertical lines on the response surface in *Figure 3.7.2 - B*, with the nitrogen concentration having little influence. It becomes clear that the optimum k_{La} values vary between 1.0 and 1.7 min⁻¹. In this range, the limiting nutrient is in excess and the nitrogen concentration becomes the most influent factor. The increase to highest k_{La} values

(achieved with low volumes of culture media), did not improve biomass production, meaning that a good stability for the growth of *M. capitatus* under the experimental domain was achieved. This stability can be observed in the response surface in *Figures 3.7.2 - C and D*, since there is no variation in the response of intact and permeabilized cell membranes resultant from the interaction of both factors, oxygen transfer rate (k_{La}) and nitrogen concentration (NH_4Cl).

It is generally known that OMW have a low content of nitrogen, sometimes being necessary to supplement these effluents. For example, Fadil *et al.* (2003) report a study on the low content of nitrogen and phosphate of OMW and how these nutrients are necessary for a successful degradation of that effluent using *Geotrichum* sp., *Aspergillus* sp. and *Candida tropicalis*. This was also observed in section 3.5, in which supplementation of undiluted OMW with NH_4Cl (0.63 g.L^{-1}) resulted in an increase of *M. capitatus* growth rate.

The effect of nutrients requirement and oxygen supply has been studied on biomass growth and for improvement of lipase production by filamentous fungi and yeast strains. D'Annibale *et al.* (2006) described the influence of different nitrogen sources (NH_4Cl , $(NH_4)_2SO_4$, $NaNO_3$ and urea) on lipase production in *Candida cylindracea* and the study showed that highest lipase activity of 1.45 U.mL^{-1} was achieved with the use of ammonium salts mainly NH_4Cl . Brozzoli *et al.* (2009) also concluded that lipase production can be enhanced on nitrogen-supplemented OMW based media by *Candida cylindracea*. This is consistent with the results obtained, since the lowest lipase values (0.05 and 0.26 U.mL^{-1}) corresponded to the lowest nitrogen concentration (0.2 g.L^{-1}) in the medium.

Alonso *et al.* (2005) concluded that oxygen availability influenced lipase production, both in quantity as well the phase of secretion^[77]. Pereira-Meirelles (1997) had described that maximum lipase activity was detected in the late stationary phase, when the lipids had been fully consumed. However, when oxygen availability is enhanced (higher stirring speeds or higher air flow rates) an early lipase release into the culture medium (related to a faster lipid uptake) was observed. Results show that oxygen did show some influence in lipase production, as the highest lipase value (1.12 U.mL^{-1}) was obtained at the highest k_{La} (1.88 min^{-1}). In the biomass production, oxygen was the factor with the larger influence (*Figure 3.7.3 – B*).

4. Conclusions

The purpose of this study was to isolate novel yeast strains with extracellular lipolytic capacity from OMW and to assess the suitability of the same effluent as growth medium for lipase production.

Using OMW samples from three phase system olive mills in Irbid (Jordan) and Tavira (Portugal), it was possible to isolate 32 yeasts strains, eight of them with lipolytic activity. This selection was made using qualitative parameters (presence or absence of catalytic halo in plate tests) through the rapid detection method. The determination of extracellular lipase production in a synthetic medium with olive oil as inducer, allowed to select isolate JOR TR 5 as the best producer. This isolate was further affiliated with *Magnusiomyces capitatus* (partial 28S rRNA gene, strain Kw-230), with 99% similarity through BLAST search. This species had not yet been described in literature as having lipolytic properties.

For *M. capitatus* growth, the use of OMW as medium showed that there was a slight inhibition, likely due to phenol toxicity. The growth in undiluted OMW was evidently slower than in the medium with the highest dilution of OMW (10%) and there was no lipase production. However, supplementation with yeast extract and olive oil to the undiluted OMW did improve both the biomass growth and the production of lipase. This could mean that undiluted OMW did not have the necessary nutrients for the *M. capitatus* growth.

Optimization of lipase production by *M. capitatus* in selected growth conditions, undiluted OMW supplemented with yeast extract (2 g.L⁻¹) and olive oil (1 g.L⁻¹) as inducer, was done using two variables, nitrogen source (NH₄Cl) concentration and oxygen level (k_La). The statistical design following the Doehlert distribution for these two factors allowed to conclude that maximal lipase production (1.12 U.mL⁻¹) was achieved at NH₄Cl concentration and K_La of 1.5 g.L⁻¹ and 1.88 min⁻¹, respectively. Under tested conditions, biomass growth was stable as shown through by flow cell cytometry analysis in which membrane cells presented an average of 99% integrity.

In conclusion, this study shows that it was possible to isolate lipolytic yeast strains from OMW and to produce lipase in undiluted OMW, although some nutrient supplementation is necessary. The valorisation of OMW is confirmed as fermentation medium to induce the production of lipases and as source for isolation of new lipase-producing yeasts strains.

5. Future work

The optimization results in shake-flasks by *M. capitatus* have shown that the influence of oxygenation level and its correlation with nitrogen source concentration could be improved and lipase production could be increased. However, as the volume of shake-flasks is a limiting factor, future studies will have to be performed in larger scale, preferably in a bioreactor which allows increasing oxygenation. Future work should exploit the use of cheaper oils instead of olive oil as inducer sources to be supplemented to OMW.

For evaluation of biodiesel production by lipase transesterification during growth of *M. capitatus* in OMW medium, further studies should be done to assess methanol tolerance of this strain. Moreover, the transesterification activity of the lipase produced by *M. capitatus* should be tested and characterized.

6. References

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Annex

Galactomyces geotrichum strain LMA-70 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence
Sequence ID: [gb|JQ668740.1](#) Length: 5126 Number of Matches: 1
Range 1: 2133 to 2671

Score	Expect	Identities	Gaps	Strand	Frame
977 bits(529)	0.0()	536/539(99%)	1/539(0%)	Plus/Plus	
Features:					
Query 16	ACGGCGAGTG-AGCGGCAAAAGCTCAAATTTGAAATCGGCCTCCAGGTCGAGTTGTAATT	74			
Sbjct 2133	ACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCGGCCACCAGGTCGAGTTGTAATT	2192			
Query 75	TGTAGATTGATTTTGTAGAGCGGATTAAAGTCTGTTGGAACACAGCGCCTTAGAGGGTGA	134			
Sbjct 2193	TGTAGATTGATCTTTGTAGAGCGGATTAAAGTCTGTTGGAACACAGCGCCTTAGAGGGTGA	2252			
Query 135	CAGCCCCGTAAAATCTATTCTCATTGTAAGATACTTTGGAAGAGTCGAGTTGTTGGGAA	194			
Sbjct 2253	CAGCCCCGTAAAATCTATTCTCATTGTAAGATACTTTGGAAGAGTCGAGTTGTTGGGAA	2312			
Query 195	TGCAGCTCTAAGTGGGAGGTAAATTCCTTCTAAAGCTAAATATTGACGAGAGACCGATAG	254			
Sbjct 2313	TGCAGCTCTAAGTGGGAGGTAAATTCCTTCTAAAGCTAAATATTGACGAGAGACCGATAG	2372			
Query 255	CGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAAAGTACGT	314			
Sbjct 2373	CGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAAAGTACGT	2432			
Query 315	GAAATTGTTAAAGGGAAGGGTATTGAATCAGACTTGGTGCTGTTGTTCAACTGTGTTTC	374			
Sbjct 2433	GAAATTGTTAAAGGGAAGGGTATTGAATCAGACTTGGTGCTGTTGTTCAACTGTGTTTC	2492			
Query 375	GGCATACTGTACTCAGCAGTACTAGGCCAAGTGGGGTGTGTTGGGAGTGAAAAAGAGTA	434			
Sbjct 2493	GGCATACTGTACTCAGCAGTACTAGGCCAAGTGGGGTGTGTTGGGAGTGAAAAAGAGTA	2552			
Query 435	GGAACGTAACCTTCGAGTGTTATAGCCTACTTTTCATAGCTCCTCAGCGCCTCAGGAC	494			
Sbjct 2553	GGAACGTAACCTTCGAGTGTTATAGCCTACTTTTCATAGCTCCTCAGCGCCTCAGGAC	2612			
Query 495	TGCGCTTCGGCAAGGACCTTGGCATAATGATTCTATACCGCCCGCTTTGAAACACGGAC	553			
Sbjct 2613	TGCGCTTCGGCAAGGACCTTGGCATAATGATTCTATACCGCCCGCTTTGAAACACGGAC	2671			

Figure S1: Results of the Blast search for isolate PT OMW 3, using the DNA fragments from the domain D1/D2 of the 26S rDNA. The sequences were analyzed using a Blast search against the nonredundant nucleotide (nt) database.

Magnusiomyces capitatus partial 28S rRNA gene, strain Kw-230
Sequence ID: [emb|HG313854.1](#) Length: 450 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
715 bits(387)	0.0	397/401(99%)	3/401(0%)	Plus/Plus
Query 20	ACGGCGAGTG-AGCGGC-AAAGCTCAATTTGAAAACGCTTCGGCGTGTGTAAGTAAAGT	77		
Sbjct 49	ACGGCGAGTGAAGCGGCAAAAGCTCAATTTGAAAACGCTTCGGCGTGTGTAAGTAAAGT	108		
Query 78	GGTTAGACGCTTTTTAATAAGTCCCTTGAACAGGCGCCATAGAGGGTGATAGCCCCGT	137		
Sbjct 109	GGTTAGACGCTTTTTAATAAGTCCCTTGAACAGGCGCCATAGAGGGTGATAGCCCCGT	168		
Query 138	ATTAAATTGTCTAGTGTGCTACTTTAGAGCGAGTCGAGTTGTTGGGAATGCAGTCAAA	197		
Sbjct 169	ATTAAATTGTCTAGTGTGCTACTTTAGAGCGAGTCGAGTTGTTGGGAATGCAGTCAAA	228		
Query 198	TAGGTGGTAACTCCATCTAAAGCTAAATATTGCTGGGAGACCGATAGCGAACAGTACA	257		
Sbjct 229	TAGGTGGTAACTCCATCTAAAGCTAAATATTGCTGGGAGACCGATAGCGAACAGTACA	288		
Query 258	GTGATGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAATAGCGTGTGAAATTGTGGA	317		
Sbjct 289	GTGATGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAATAGCGTGTGAAATTGTGGA	348		
Query 318	GGGGAAGGCGATGGTAGGAATAAGAGGCTGCGGTTTGAAATAATTATTTTCGGGCCACG	377		
Sbjct 349	GGGGAAGGCGATGGTAGGAATAAGAGGCTGCGGTTTGAAATAATTATTTTCGGGCCACG	408		
Query 378	GTCTCCTGAGCCTGCTTTTCGACCCGCTTTGAAAACACGGA	418		
Sbjct 409	GTCTCCTGAGCCTGCTTTTCGACCCGCTTTGAAA-CACGGA	448		

Figure S2: Results of the Blast search for isolate JOR TR 5, using the DNA fragments from the domain D1/D2 of the 26S rDNA. The sequences were analyzed using a Blast search against the nonredundant nucleotide (nt) database.

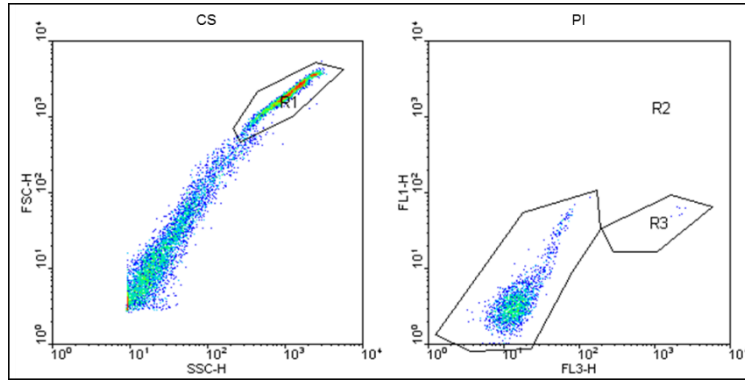


Figure S3: Flow cytometry results of replica of experiment A. CS – Cellular sample. The marked region (R1) represents the total population of cells. PI – Detection of propidium iodide (PI) staining using the FL3 channel. R2 is the region of non-stained cells and R3 is the region with stained cells.

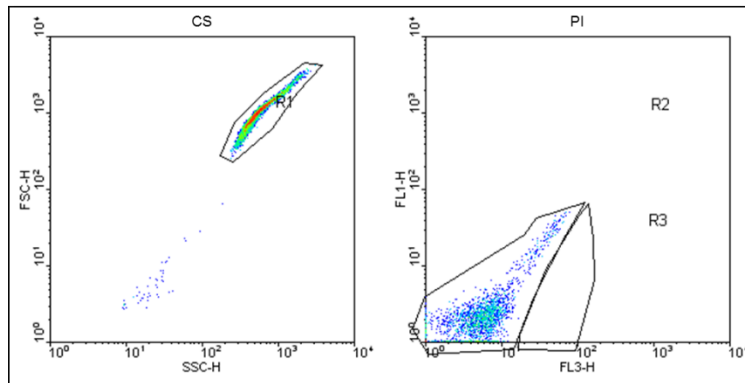


Figure S4: Replica of experiment B. CS – Cellular sample. The marked region (R1) represents the total population of cells. PI – Detection of propidium iodide (PI) staining using the FL3 channel. R2 is the region of non-stained cells and R3 is the region with stained cells.

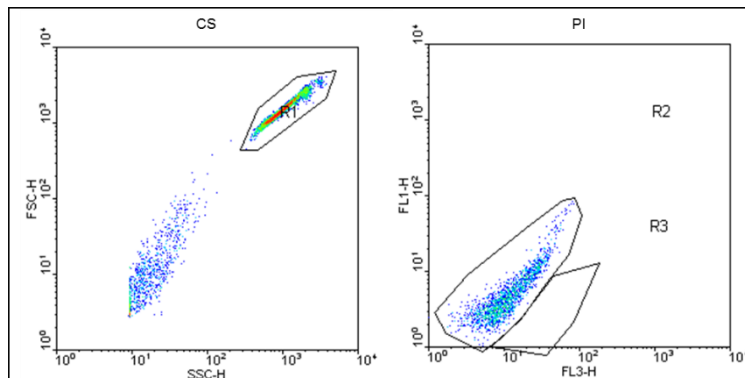


Figure S5: Replica of experiment C. CS – Cellular sample. The marked region (R1) represents the total population of cells. PI – Detection of propidium iodide (PI) staining using the FL3 channel. R2 is the region of non-stained cells and R3 is the region with stained cells.

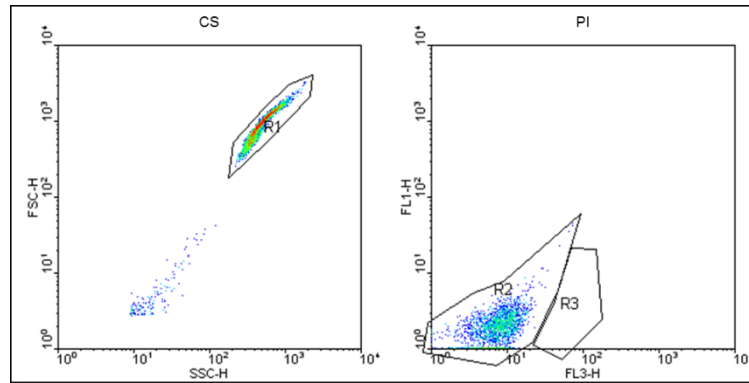


Figure S6: Replica of experiment D. CS – Cellular sample. The marked region (R1) represents the total population of cells. PI – Detection of propidium iodide (PI) staining using the FL3 channel. R2 is the region of non-stained cells and R3 is the region with stained cells.

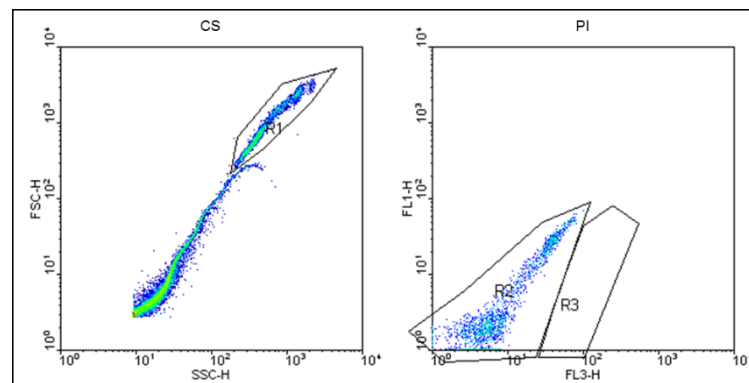


Figure S7: Replica of experiment E. CS – Cellular sample. The marked region (R1) represents the total population of cells. PI – Detection of propidium iodide (PI) staining using the FL3 channel. R2 is the region of non-stained cells and R3 is the region with stained cells.

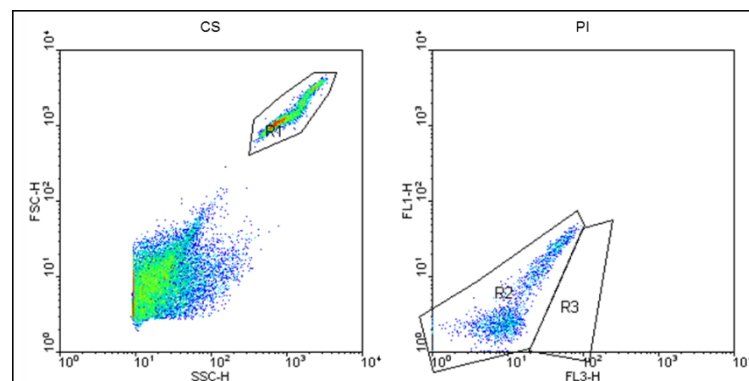


Figure S8: Replica of experiment F. CS – Cellular sample. The marked region (R1) represents the total population of cells. PI – Detection of propidium iodide (PI) staining using the FL3 channel. R2 is the region of non-stained cells and R3 is the region with stained cells.