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**Use of yeasts as bioremediation agents:
Application to hydrocarbons and
phenolic compounds degradation**

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ABSTRACT

Liquid effluents of petrochemical industry and olive oil processing (olive mill wastewater, OMW) are two examples of effluents contaminated with pollutant compounds, respectively hydrocarbons and phenolic compounds. Biodegradation strategies involving microorganisms to simultaneously degrade these pollutants and obtain high added-value products have become an interesting and environment friendly approach.

This work address the study of the ability of ten yeast species to grow on hydrocarbons (hexadecane) and phenolic compounds (tyrosol, phenol and catechol) as sole carbon and energy source. After a preliminary screening in Petri plates and 96-well microplate, two yeast species demonstrated an extraordinary ability to grow on hexadecane $1 \text{ g}\cdot\text{L}^{-1}$ (*Yarrowia lipolytica* W29) and phenolic compounds $1 \text{ g}\cdot\text{L}^{-1}$ (*Candida tropicalis* ATCC 250).

In Erlenmeyer flask experiments, it was observed that *Y. lipolytica* W29 was able to grow on hexadecane $10 \text{ g}\cdot\text{L}^{-1}$, hexadecene $7.5 \text{ g}\cdot\text{L}^{-1}$ and in a mixture with both hydrocarbons ($5 \text{ g}\cdot\text{L}^{-1}$ of each one).

The increase of oxygen transfer, by raising the ratio of the volumes of headspace and liquid medium in flasks, had a positive effect on cellular growth and metabolites production. In hexadecane-based medium, for example, yeast cells produced lipase (up to $1260 \text{ U}\cdot\text{L}^{-1}$) and accumulated microbial lipids (up to 15 %, w/w), demonstrating its ability to valorize effluents contaminated with hydrocarbons. As *C. tropicalis* shown high potential to grow in phenolic compounds, its ability to grow on undiluted OMW (supplemented and non-supplemented with YNB) and produce added-value compounds was tested in Erlenmeyer flasks. The increase of flask headspace clearly enhanced the cellular growth, but did not favor the intracellular lipids accumulation. By contrast, it was observed that nitrogen limitation led to an enhancement of microbial lipids content: 39 % (w/w) and 24 % (w/w) of lipids were accumulated, respectively, in non-supplemented OMW medium and in repeated batch culture (pulses of OMW without YNB).

Microbial lipids accumulated both by *Y. lipolytica* and *C. tropicalis* were mainly composed by oleic and linoleic acids and the unsaturated fraction exceeded the saturated one. The composition of these lipids, similar to that of common vegetable oils, makes them a potential feedstock for biodiesel production.

Keywords: Hydrocarbons, olive mill wastewater, *Yarrowia lipolytica*, *Candida tropicalis*, microbial lipids, lipase.

RESUMO

Os efluentes líquidos da indústria petroquímica e do processamento do azeite (águas ruças) são dois exemplos de efluentes contaminados com compostos poluentes, respetivamente hidrocarbonetos e compostos fenólicos. A biodegradação destes por microrganismos, obtendo simultaneamente compostos de alto valor acrescentado, tem-se tornado uma abordagem interessante e amiga do ambiente.

Este trabalho aborda o estudo da capacidade de 10 espécies de leveduras para crescerem em hidrocarbonetos (hexadecano) e compostos fenólicos (tirosol, fenol e catecol) como única fonte de carbono e energia. Os ensaios preliminares em placa de Petri e microplaca demonstraram que *Yarrowia lipolytica* W29 crescia bem em 1 g·L⁻¹ de hexadecano e *Candida tropicalis* ATCC 250 era capaz de crescer em 1 g·L⁻¹ de cada composto fenólico.

Nos ensaios realizados em frascos Erlenmeyer, *Y. lipolytica* W29 conseguiu crescer na presença de 10 g·L⁻¹ de hexadecano, 7.5 g·L⁻¹ de hexadeceno e numa mistura dos dois (5 g·L⁻¹ de cada hidrocarboneto). O aumento da transferência de oxigénio, por aumento da razão do volume de *headspace* e do meio líquido dos frascos, teve um efeito positivo no crescimento e na produção de metabolitos. No meio com hexadecano, as células produziram lipase (1260 U·L⁻¹) e acumularam lípidos intracelularmente (15 %, p/p), demonstrando o seu potencial para valorizar efluentes contaminados com hidrocarbonetos. Como a levedura *C. tropicalis* demonstrou potencial para crescer em compostos fenólicos, foi testada em frascos Erlenmeyer a sua capacidade para crescer em águas ruças não diluídas (com e sem suplementação com YNB) e produzir metabolitos de interesse. O aumento do volume do frasco levou ao aumento do crescimento celular, mas não favoreceu a acumulação de lípidos. Foi também observado que a limitação de azoto favorecia a acumulação de lípidos: no meio não suplementado com YNB obteve-se um conteúdo de lípidos igual a 39 % (p/p) e nos ensaios realizados em culturas descontínuas repetidas (pulsos de águas ruças sem YNB) obteve-se 24 % (p/p) de lípidos microbianos.

Os lípidos microbianos acumulados por *Y. lipolytica* e *C. tropicalis* eram compostos maioritariamente por ácidos oleico e linoleico, e a fração insaturada era maior que a saturada. A composição destes lípidos microbianos, semelhante à dos óleos vegetais comuns, torna-os uma potencial matéria-prima para a produção de biodiesel.

Palavras-chave: Hidrocarbonetos, águas ruças, *Yarrowia lipolytica*, *Candida tropicalis*, lípidos microbianos, lipase.

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LIST OF ABBREVIATIONS

ACS	Acetyl-CoA synthetase
BOD	Biological oxygen demand
C/N	Carbon/nitrogen ratio
CoA	Coenzyme A
COD	Chemical oxygen demand
DNS	Acid 3,5-dinitrosalicylic
ER	Endoplasmic reticulum
FA	Fatty acids
FADH	Fatty alcohol dehydrogenase
FALDH	Fatty aldehyde dehydrogenase
FAME	Fatty acid methyl ester
FAO	Fatty alcohol oxidase
FDA	Food and Drug Administration
FFA	Free fatty acids
GC	Gas chromatography
GRAS	Generally recognized as safe
HS	Hydrophobic substrate
k_{La}	Oxygen volumetric mass transfer coefficient (h^{-1})
LB	Lipid body
LCFA	Long chain fatty acid
<i>LIP2</i>	Gene of secreted extracellular lipase
N	Nucleus
P	Peroxisome
<i>p</i> -NPB	<i>p</i> -nitrophenyl-butyrate
PAH's	Polycyclic aromatic hydrocarbons
PRE	Petroleum refinery effluents
PUFA	Polyunsaturated fatty acid
rpm	Rotation per minute
SCO	Single cell oil
SE	Steryl ester

SmF	Solid-state fermentation
SSF	Submerged fermentation
STR	Stirred tank reactor
OD	Optical density
OMW	Olive mill wastewater
PRW	Petroleum refinery wastewater
TAG	Triacylglycerols
TCA	Tricarboxylic-acid
TPH	Total petroleum Hydrocarbons
vm	Volume of air per volume of medium per minute
$X_{\max}$	Maximum biomass
YNB	Yeast nitrogen base
YPD	Yeast extract, peptone, dextrose medium
YPDA	Yeast extract, peptone, dextrose and agar medium
$Y_{x/s}$	Biomass yield

1. LITERATURE REVIEW

Large amounts of pollutant compounds are present in industrial effluents and, often, they are not totally degraded by physical and/or chemical methods before the discharge into the environment. Biodegradation strategies involving microorganisms to simultaneously degrade these wastes and obtain high added-value products become an interesting approach, since the abundance of these compounds ensures the economic viability of bioprocesses while prevents major environmental problems.

Hydrocarbons and phenolic compounds are two examples of pollutants present in agro-industrial effluents, respectively in petroleum refinery effluents and olive mill wastewater (OMW). Oftentimes these pollutants are not degraded before the discharge of effluents, causing damage to the environment.

In this Chapter, the microbial degradation and transformation of hydrocarbons and OMW is addressed describing the state of the art on this topic. Moreover, a brief overview on high added-value metabolites production by microorganisms from hydrocarbons and OMW is presented. Finally, the main objectives of this work are also highlighted.

1.1 Pollutant compounds in the environment

A large variety of chemicals are synthesized and commercially produced every year. During their manufacture and handling, these chemicals are often discharged into the environment without pre-treatments. Many of them degrade slowly and exert toxic effects on plants and animals, thus causing environmental problems in a large scale (Annachhatre and Gheewala, 1996). Although the rate and extent of degradation of a chemical compound largely depends upon its structure and the environment in its vicinity, in case of organic compounds, in particular, are found to be biochemically resistant (Grady, 1985).

Currently, petroleum is considered one of the major pollutants and its heterogeneous composition includes compounds very different in terms of solubility, molecular weight, toxicity, stereochemistry and biodegradability. The main constituents of petroleum are alkanes, iso-alkanes, cycloalkanes, alkenes, aromatic hydrocarbons, condensed aromatic hydrocarbons, phenol and its derivatives (Csutak *et al.*, 2010). Petroleum refinery effluents (PRE) are wastes generated by the industries of refining crude oil and manufacturing fuels, lubricants and petrochemical intermediates. As the process of refining crude oil consumes large amounts of water, significant volumes of wastewater contaminated with hydrocarbons are generated (Diya'uddeen *et al.*, 2010; Varjani, 2016).

Other example of a problematic effluent that is a source of local water pollution, due to its phenolic fraction, organic load and dark color, is the liquid waste of olive oil industry. Olive mill wastewater (OMW) is a stable emulsion, composed by water from the olive processing, olive pulp and oil. This emulsion represents a serious pollution problem as the waste is a dark acidic liquid with a strong oily smell containing fats, sugars, phosphate, phenol and metals (Fickers *et al.*, 2005). The large diversity of components found in OMW makes its treatment difficult, and their disposal becomes a critical environmental problem (Lopes *et al.*, 2009). However, most of the problems associated with OMW pollution can be attributed to the phenolic fraction, because olive pulp is rich in phenolic compounds and approximately 53 % are lost in the OMW. In fact, phenolic compounds are responsible for OMW black coloring and for several biological effects in microorganisms, including antibiosis and phytotoxicity. Moreover, the extremely high organic content of OMW leads to oxygen depletion from the water causing adverse effects on the aquatic life (Lanciotti *et al.*, 2005; Rharrabti and Yamani, 2018).

Bioremediation is defined as the process by which various biological agents (microorganisms, enzymes) degrade the pollutant compounds into less toxic forms. The first patent for a biological remediation agent was registered in 1974, using a strain of *Pseudomonas putida* to degrade petroleum

(Bhatnagar and Kumari, 2013). The main strategy of bioremediation exploits the metabolic pathways of living organisms (mainly microorganisms) for biodegradation of organic pollutants, leading to their partial or complete mineralization into carbon dioxide, water, and inorganic compounds (Kothari *et al.*, 1986). Based on removal and transportation of wastes contaminated with pollutant compounds, bioremediation technology can be classified as *in situ* and *ex situ*. *In situ* bioremediation involves treatment of contaminated material at the same site, while *ex situ* involves complete removal of contaminated material from one site and its transfer to another site (for example laboratory scale), where is treated using biological agents (Bhatnagar and Kumari, 2013).

Bioremediation technology has many advantages compared to the conventional decontamination techniques, namely: maintains the ecological equilibrium; the contaminants are eliminated through microbial metabolic processes; biological systems use less energy and the cost are relatively low; bioremediation may be used in combination with other treatment technologies (Csutak *et al.*, 2010).

Aerobic and anaerobic biological processes are used to treat OMW, reducing the content of organic matter and phenolic compounds, thus facilitating further management before its disposal (Salgado *et al.*, 2016). Beyond be used as substrate for the cultivation of microorganisms, resulting in the remediation of the waste, also high added-value compounds could be produced (Sarris *et al.*, 2017). Some published works also report the biodegradation of effluents contaminated with hydrocarbons by microbial species, but the production of added-value compounds from these pollutants are almost unexplored (Dixit *et al.*, 2017; Varma *et al.*, 2017).

1.2 Hydrocarbons

Hydrocarbons are organic molecules consisting entirely of carbon and hydrogen. Hydrocarbons of crude petroleum can be classified as alkanes, cycloalkanes, aromatics, polycyclic aromatics, asphaltines and resins (Kothari *et al.*, 1986; Varjani and Upasani, 2017). Among the petroleum hydrocarbons, *n*-alkanes such as hexadecane, are the most amenable to biodegradation (Das and Chandran, 2011; Kothari *et al.*, 1986). Hydrocarbon pollution is a serious problem in the environment and represents 70 % of environmental pollutants (Mahmoud *et al.*, 2015). Some hydrocarbons are carcinogenic and neurotoxic to different life forms and the accumulation of these pollutants in marine animal and plant tissues may cause death or mutation (Kothari *et al.*, 1986).

Due to the use of crude petroleum for fuel and chemical production, the number of petrochemical industries is increasing worldwide. For this reason, a great amount of effluents polluted with hydrocarbons

are introduced into the environment, becoming one of the main environmental problems faced at a global level. Moreover, the leaks and accidental spills that can occur during exploration, production, refining, transport and storage of crude petroleum and petroleum products increase the hydrocarbons concentration in soils and water systems (Kothari *et al.*, 1986; Singh *et al.*, 2011).

As the pollution by crude oil/oil spill is one of the main causes of environmental damage and can occur in both terrestrial and marine environments or in freshwater (Gonçalves *et al.*, 2014), a contaminated site may require a combination of procedures to allow the optimum remediation (Khan *et al.*, 2004). Physical, chemical and biological technologies may be used individually or simultaneously to reduce the contamination to a safe and acceptable level (Khan *et al.*, 2004). For example, the technologies commonly used for the remediation of soil contaminated with petroleum hydrocarbons include mechanical burying, evaporation, dispersion and washing (Singh *et al.*, 2011). Soil washing is a chemical method that uses liquids (usually water and occasionally combined with solvents) for cleaning the soil. These solvents are selected on the basis of their ability to solubilize specific contaminants, and on their environmental and health effects. However, while soil washing is cost-effective because it reduces the quantity of material that would require further treatment by another technology, this method has also some drawbacks such as: organic compounds adsorbed onto clay particles are difficult to remove and when soil washing does not destroy or immobilize the contaminants, the resulting soil must be disposed of carefully (Khan *et al.*, 2004).

Physical methods are commonly used to control oil spills in a water environment. They are mainly used as a barrier to control the spreading oil spill without changing its physical and chemical characteristics and a variety of barriers are used to control oil spills (Khan *et al.*, 2004). In the treatment of petroleum refinery effluents two basic treatments are applied: the first phase consists in a series of pre-treatment steps, in which suspended matter, oil and grease are reduced by mechanical and physicochemical treatments. In the second phase, wastewater contaminants are deposited by gravity in separation tanks, to certain acceptable discharge limits. Beside the high cost and time consuming, chemical and mechanical methods are not very effective and are often not environment friendly (Diya'uddeen *et al.*, 2010).

1.2.1 Biodegradation of hydrocarbons by microorganisms

Biodegradation of hydrocarbons is a promising approach for the treatment of contaminated sites and effluents as it is cost effective and can lead to complete mineralization of these pollutant compounds.

However, several environmental factors influence biodegradation of hydrocarbons such as temperature, pH and nutrient and oxygen availability. Regarding pH value, highest rates of hydrocarbons degradation are generally observed at neutral pH (Atlas, 1995; Kothari *et al.*, 1986). Sathishkumar *et al.* (2008) reported highest percentage degradation of crude oil by *Bacillus* sp. IOS1-7 at pH 7. By contrast, Raju *et al.* (2017) concluded that optimal growth of *Bacillus thuringiensis* B3 and *Bacillus cereus* B6 in crude oil medium was to a pH of 6.0. Varjani and Upasani (2017) reported that optimum growth of *Pseudomonas aeruginosa* NCIM 5514, when use crude oil as carbon source, was observed at pH 7.2. Since in these studies the only carbon source used was crude oil, the pH values described correspond to the optimum pH for the degradation of this compound.

Although biodegradation of hydrocarbons may occur over a wide range of temperatures, degradation rates generally decrease at low temperatures (Das and Chandran, 2011). Once the increase in temperature increases solubility of hydrophobic pollutants and consequently improves its degradation. By contrast, at low temperatures their water solubility is decreased which delay onset of biodegradation (Leahy and Colwell, 1990). Ferreira *et al.* (2012) studied the effect of different temperatures in degradation of crude oil by *Y. lipolytica* and concluded that the maximum biodegradation rate (90 %) was attained at 28 °C. By contrast, at lower temperatures (20 °C – 24 °C) the maximum biodegradation rate ranged 21 to 62 %. Varjani and Upasani (2017) reported that *Pseudomonas aeruginosa* NCIM 551 was capable to degrade crude oil at 37 °C. Antizar-Ladislao *et al.* (2004) studied the biodegradation of polycyclic aromatic hydrocarbons (PHAs) by a consortium of bacteria at different temperatures and concluded that the maximum biodegradation rate was attained at 38 °C.

Nitrogen and phosphorus has been mentioned as important ingredients for hydrocarbons biodegradation and their limitation could affects the biodegradation process (Das and Chandran, 2011; Leys *et al.*, 2005). Liebega and Cutright (1999) observed that biodegradation of diesel fuel by *Achromobacter* sp. (ATCC 21910) and *Mycobacterium* sp. (ATCC 21676) was higher when culture medium was supplemented with phosphorus. According to Atlas and Bartha (1972), when nitrate and phosphate were simultaneously added to the medium, petroleum biodegradation was improved. However, has been also reported that the addition of inorganic nutrients has a negative effect on different aspects of pollutant degradation kinetics, such as lag time, degradation rate and degradation extent (Leys *et al.*, 2005). According to Liebega and Cutright (1999), the degradation rate of PHAs by some bacteria strains increased with the addition of micronutrients (calcium and magnesium) and decreased in cultures supplemented with macronutrients (nitrogen and phosphorus). Leys *et al.* (2005) reported that the

addition of high concentrations of nitrogen or phosphorus could totally block the biodegradation of PAHs by *Sphingomonas* and *Mycobacterium* strains due to the high increments in the salinity.

Oxygen is a crucial factor for hydrocarbons degradation, since the initial hydrocarbons degradation occurs by the action of oxygenase enzymes (Kothari *et al.*, 1986). Also peroxidases, reductases, hydroxylases and dehydrogenases play an important role in aerobic and anaerobic pathways of microbial degradation of petroleum hydrocarbon (Varjani and Upasani, 2017). Volke-Sepúlveda *et al.* (2002) reported that one reason for the incomplete biodegradation of hexadecane by fungi in liquid submerged fermentation may be the limitation of oxygen. Ferreira *et al.* (2012) tested a wide range of stirring rates (70 rpm – 250 rpm) in crude oil biodegradation by *Y. lipolytica* and concluded higher stirrings lead to an increase of hydrocarbons consumption.

A wide variety of organisms are known to degrade hydrocarbon compounds under different environmental conditions (Table 1.1). However, the extent to which bacteria, yeasts and filamentous fungi participate in the biodegradation of hydrocarbons has been the subject of only limited studies. Bacteria are the most active agents in petroleum degradation and work as primary degraders of spilled oil in environment. Some bacteria are even known to use exclusively hydrocarbons as carbon source. *Pseudomonas putida* is considered proper for bioremediation of hydrocarbons, since it can efficiently degrade benzene and toluene (Das and Chandran, 2011; Kothari *et al.*, 1986). *Acinetobacter* sp. was found to be capable of utilizing n-alkanes (C₁₀–C₄₀) as a sole carbon source. In addition, many bacteria possess emulsifying activity, producing biosurfactants from hydrocarbons, which increase the availability of hydrocarbons to microbial strains, enhancing the degradation rate of these compounds (Obayori *et al.*, 2009).

Some fungi have ability to degrade hydrocarbons of complex structure (Kobayashi and Rittmann, 1982). According to Al-Jawhari (2014), *A. niger* and *A. fumigates* have the ability to remove hydrocarbons from petroleum-contaminated environments. Ali *et al.* (2012) reported that *A. terreus* can completely degrade PAHs, including naphthalene and anthracene. Thamer *et al.* (2013), have reported 80% biodegradation of crude oil by *B. thuringiensis* in 27 days.

Studies on yeasts able to use various petroleum components as sole carbon source, showed that the biodegradability of hydrocarbons decreases from *n*-alkanes > branched alkanes > low molecular weight aromatic hydrocarbons > cycloalkanes > high molecular weight aromatic and polar compounds (Csutak *et al.*, 2010). *Y. lipolytica* and *C. maltosa* are able to use mono branched alkanes as sole carbon and energy source (Csutak *et al.*, 2010), however the increase in the complexity of the *n*-alkanes structure leads to less degradation by the yeasts. Zinjarde *et al.* (1998) observed that *Y. lipolytica* NCIM 3589

degraded 60 % of hexadecane. By contrast, only 58 % of aliphatic fraction of crude oil was degraded. Farag and Soliman (2011) studied the utilization of different *n*-alkanes as carbon source by *C. tropicalis*. A decrease of 3.2 % in consumption was observed with *n*-heptane as carbon source compared to *n*-hexane.

Table 1.1 – Examples of studies focused on hydrocarbons degradation by microbial species (yeasts, fungi and bacteria).

Effluents/ Hydrocarbons	Concentration range	Microbial species	Principal Results	References
Acidic oily sludge	10 g·L ⁻¹ of total petroleum hydrocarbons	<i>Candida digboiensis</i>	73 % degradation after 7 days	(Sood and Lal, 2009)
Hydrocarbon-rich wastewater	10 g·L ⁻¹ of total petroleum hydrocarbons	<i>Candida tropicalis</i>	97 % degradation after 20 days	(Gargouri <i>et al.</i> , 2015)
		<i>Trichosporon asahii</i>	95 % degradation after 20 days	
Diesel oil	20 mL·L ⁻¹	<i>Trichosporon asahii</i>	95 % degradation after 10 days	(Chandran and Das, 2010)
Mixture of dodecane and hexadecane	37.5 g·L ⁻¹	<i>Candida maltosa</i>	56 % degradation after 7 days	(Chrzanowski <i>et al.</i> , 2006)
Crude oil	10 mL·L ⁻¹	<i>Yarrowia lipolytica</i>	68 % degradation after 7 days	(Hassanshahia <i>et al.</i> , 2012)
Crude oil	0.1 % – 4.0 % (v/v)	<i>Yarrowia lipolytica</i>	90 % degradation of 0.1 % (v/v) petroleum	(Ferreira <i>et al.</i> , 2012)
Diesel oil	4 g·kg ⁻¹ soil	<i>Yarrowia lipolytica</i>	95 % degradation after 155 days	(Margesin and Schinner, 1997)
Hexadecane	10 g·L ⁻¹	<i>Yarrowia lipolytica</i>	60 % degradation after 2 days	(Zinjarde <i>et al.</i> , 1998)

Table 1.1 – Examples of studies focused on hydrocarbons degradation by microbial species (yeasts, fungi and bacteria) (continuation).

Effluents/ Hydrocarbons	Concentration range	Microbial species	Principal Results	References
Hexadecane	20 g·L ⁻¹ - 80 g·L ⁻¹ (SmF)	<i>Aspergillus niger</i>	62 % consumption of hexadecane 80 g·L ⁻¹	(Volke- Sepúlveda <i>et al.</i> , 2003)
	45 g·L ⁻¹ - 180 g·L ⁻¹ (SSF)		100 % consumption of hexadecane 45 g·L ⁻¹	
Anthracene oil	0.5 g·L ⁻¹	<i>Phanerochaete chrysosporium</i>	70 % to 100 % degradation of PHAs of anthracene oil after 27 days	(Bumpus, 1989)
Crude oil	5 µL·mL ⁻¹	<i>Aspergillus niger</i>	8 % degradation	(Elshafie <i>et al.</i> , 2007)
		<i>Aspergillus terreus</i>	15 % degradation	
		<i>Penicillium chrysogenum</i> .	10 % degradation	
Crude oil	30 mL·L ⁻¹	<i>Rhodococcus erythropolis</i>	71 % degradation after 30 days	(Pi <i>et al.</i> , 2017)
Diesel oil	10 mL·L ⁻¹	<i>Bacillus coagulans</i>	60 % degradation after 15 days	(Dixit <i>et al.</i> , 2018)
		<i>Bacillus cereus</i>	67 % degradation after 15 days	
		<i>Bacillus subtilis</i>	82 % degradation after 15 days	
		<i>Bacillus megaterium</i>	49 % degradation after 15 days	
Soil contaminated with petroleum sludge	0.01 g·g ⁻¹ soil	<i>Bacillus pumilus</i>	97 % removal of total petroleum hydrocarbons after 122 days	(Varma <i>et al.</i> , 2017)

The yeasts *Yarrowia lipolytica* and *Candida maltosa* are described as able to use mono-branched alkanes as sole carbon and energy source, which can be incorporated in lipids, converted into soluble cellular compounds (proteins, amino acids), intermediate metabolites (dicarboxylic acids with β-methyl group) and partially oxidized to CO₂ (Csutak *et al.*, 2010). According to Chrzanowski *et al.* (2005), *C. maltosa* EH 15 degraded 56.5 % of hydrocarbons after 7 days in a culture with 37.5 g·L⁻¹ of hexadecane

and dodecane (1:1 w/w). Schmitz et al. (2000) tested the ability of six yeast species to degrade alkanes and found that a co-culture with *Y. lipolytica* and *C. maltosa* are able to degrade 96 % of tetradecane.

Yarrowia lipolytica is a strictly aerobic yeast capable of producing important metabolites with industrial interest (enzymes, organic acids, aromas, microbial lipids). It is considered as nonpathogenic and several processes based on this organism were classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA) (Coelho *et al.*, 2010). Usually can be found in environments containing hydrophobic substrates, rich in alkanes and oily substances. Because of its ability to use alkanes, fatty acids and oils, *Y. lipolytica* is regarded as a potential agent in bioremediation of environments for the degradation of vegetal and mineral oil wastes (Gonçalves *et al.*, 2014). However, few authors have studied the degradation of hydrocarbon compounds by *Y. lipolytica* strains. And since 1954 that no author studied the degradation of alkenes by yeasts. Margesin and Schinner (1997) observed that *Y. lipolytica* degraded 68 % of diesel oil after 10 days. Zinjarde et al. (1998) reported that *Y. lipolytica* NCIM 3589, isolated from contaminated sea water, was able to degrade 78 % of the aliphatic fraction in 5 days. Moreover, this strain was also able to degrade pure alkanes, namely hexadecane (60 %), tetradecane (50 %), octadecane (45 %), decane (40 %) and dodecane (40%) after 24 h of culture (Zinjarde *et al.*, 1998). Bruyn (1954) reported *Candida lipolytica* was able to degrade double-stranded hydrocarbons (hexadecene), producing 1,2-hexadecanediol. According to Hassanshahian et al. (2012), *Y. lipolytica* PG-20 and *Y. lipolytica* PG-32 degraded 68 % and 58 % of crude oil, respectively, after 7 days.

Several cultivation parameters can affect the degradation of hydrocarbons by *Y. lipolytica* strains. Ferreira et al. (2012) tested a wide range of temperatures (20 °C – 34 °C), stirring rates (70 rpm – 250 rpm), petroleum concentration (0.1 % - 4 %, v/v) and inoculum density (0.1 g·L⁻¹ – 1.9 g·L⁻¹), and observed a higher degradation (98 %) at 28 °C, 160 rpm, 0.1 % (v/v) of petroleum and 1 g·L⁻¹ of initial cellular concentration. Hassanshahian et al. (2012) reported that the optimal *Y. lipolytica* growth and simultaneous biodegradation of hydrocarbons occurred in ONR medium (an artificial seawater mineral salts medium based on the ionic composition of seawater) with an acidic pH (pH 5).

In order to assimilate hydrocarbons/oils substrates, microorganisms have developed specific mechanisms (Thevenieau *et al.*, 2018). The uptake of alkanes by *Y. lipolytica* is not completely explained, but there are findings that support two possible ways of substrate transportation. *Y. lipolytica* has the ability to produce biosurfactants when is in contact with hydrophobic substrates in order to enhance the contact area between the substrate and the cell, favoring their absorption. For assimilation of alkanes, an emulsion is formed and their droplets diameter is reduced by the secreted biosurfactants. Substrate droplets enter the cell by passive transport or by transport/export mechanisms of the cell (hydrophobic

outgrowths) (Barth and Gaillardin, 1997). Additionally, it is known that cellular membrane increases hydrophobicity when it is in contact with hydrophobic substrates to favor absorption (Chrzanowski *et al.*, 2008). The metabolism of alkanes assimilation by *Y. lipolytica* is illustrated in Figure 1.1.

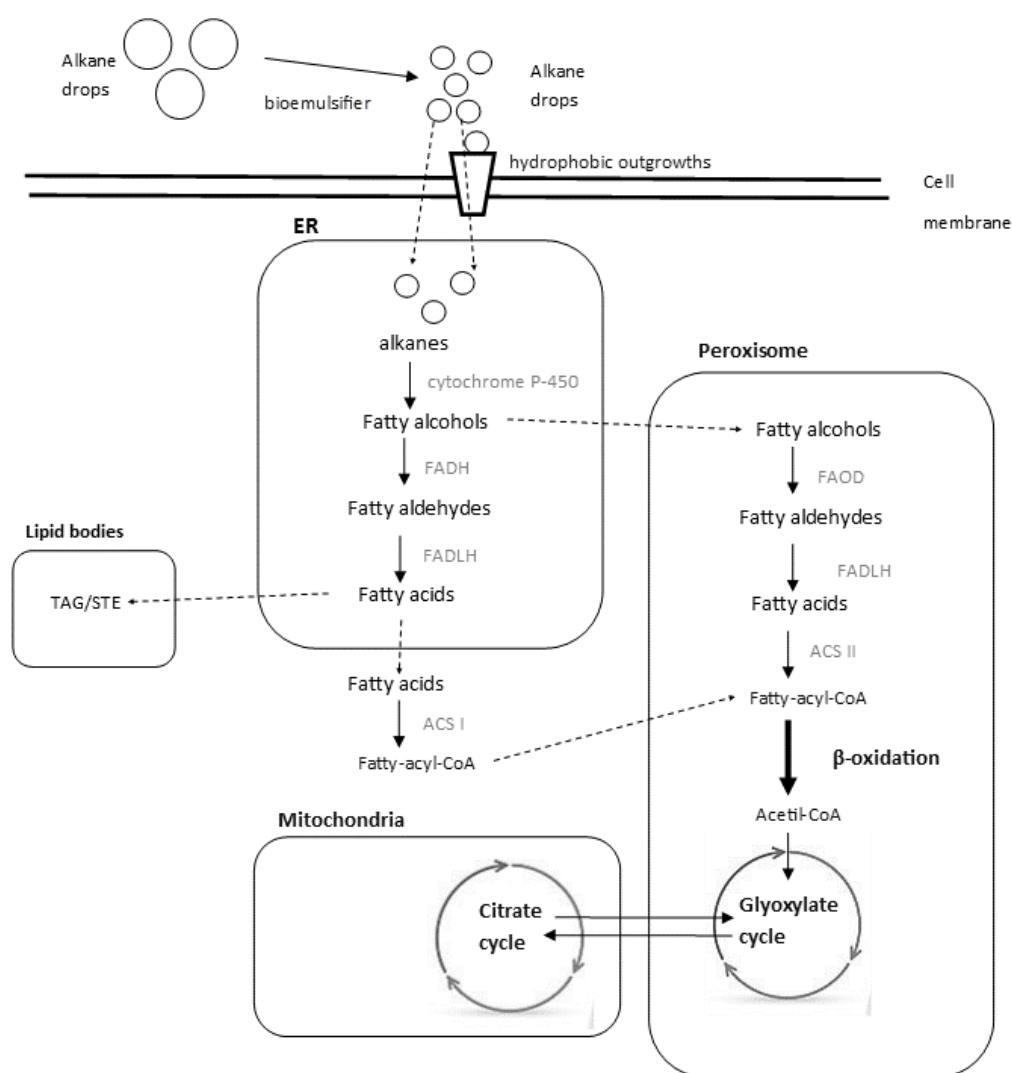


Figure 1.1 – Main metabolic pathways and cellular compartments involved in alkane degradation by *Y. lipolytica*. Main metabolic flux during alkane oxidation is shown with black arrows (enzymatic steps), enzymes are written in grey next to the arrows and dashed arrows represent metabolites transportation (Ferreira, 2016).

Once in cytoplasm, hydrocarbons are transported to endoplasmic reticulum (ER), suffering hydroxylation by cytochrome P-450 monooxygenase. This enzyme is activated in yeast when it grows in alkanes, and genetic studies have shown that different mutations in gene encoding the protein affect the use of alkanes by *Y. lipolytica* (Barth and Gaillardin, 1997). In this first step, fatty alcohol-alkane is formed, which may remain in the endoplasmic reticulum or be transported into the peroxisome. In the ER, the

fatty alcohol is oxidized by the enzyme fatty alcohol dehydrogenase (FADH) generating a fatty aldehyde. The next step is the hydrolysis of fatty aldehyde to fatty acids by fatty aldehyde dehydrogenase (FALDH), which are then transported to the cytosol. In the cytosol, fatty acids are activated by fatty-acyl-CoA synthetase I (ACS I) forming fatty-acyl-CoA which is conducted to the peroxisome. Alternatively, in peroxisome fatty alcohol is also oxidized to fatty aldehydes by fatty alcohol oxidase (FAO). Once in peroxisome, the activation of fatty acids is made by fatty-acyl-CoA synthetase II (ACS II) also generating fatty-acyl-CoA (Ferreira, 2016). Fatty-acyl-CoA formed in cytosol and in peroxisome enter in the β -oxidation pathway. From this path results Acetyl CoA that will be used in the glyoxylate cycle which communicates directly with the citrate cycle that takes place in the mitochondria. In the case of excess of substrate, cell can accumulate acids from the citrate cycle or storage directly the fatty acids from the ER into lipid bodies as triacylglycerols (TAG) or steryl esters (STE) (Fickers *et al.*, 2005; Fukuda, 2013).

1.3 Olive mill wastewater (OMW)

Olive oil production is a traditional industry in Mediterranean countries, that accounts for about 97 % of the world production of olive oil and among which Portugal is one of the ten major producers (Rharrabti and Yamani, 2018). In 2016, Spain was the main olive oil-producing country, followed by Italy, Greece, Turkey, Tunisia and Morocco. In the 2016/2017 season, 2.3 million tons of olive oil were produced in European Union countries (Rharrabti and Yamani, 2018) and Portugal contributed with 94 thousand tons (Portuguese Olive Oil Association “Casa do Azeite”). Despite the economic importance of olive oil extraction industry, great amounts of wastewaters are generated by olive oil mills, causing high pollution.

Three systems are used worldwide for the industrial-scale extraction of oil from olives, namely (i) the traditional press-cake system, (ii) the three-phase decanter system and (iii) the modern two-phase centrifugation system. Nowadays, two-phase and three-phase centrifugation systems are most commonly used (Morillo *et al.*, 2009). The two-phase system separates the olive mixture into oil and wet pomace. In the other hand, the manufacturing process of olive oil in three-phase centrifugation system separates the olive mixture into oil and pomace, generated a black liquid effluent called olive mill wastewater (OMW) (Rharrabti and Yamani, 2018). This black liquid is composed by the water naturally present in olives, as well as the water used in the process and soft tissues from the olive pulp, forming a very stable emulsion (García *et al.*, 2000). Additionally, OMW is composed of residual oil (Lanciotti *et al.*, 2005) and the organic fraction of OMW includes sugars, polyalcohols, tannins, polyphenols, pectins, organic acids and lipids

that results in high values of chemical oxygen demand (COD) (Rharrabti and Yamani, 2018). The phenolic fraction, known by its phytotoxic and antimicrobial effects, is considered the most pollutant part of OMW (Ettayebi *et al.*, 2003). The phenolic compounds are derived from phenol and they are composed of one or more aromatic benzene rings, one or more hydroxyl groups and one or more functional side chains. At present, more than 50 different phenolic compounds have been identified in OMW (Torrecilla, 2010). Because OMW degradation rate, in particular the phenolic compounds, is much slower than for other substances (sugars or volatile acids with short chains), OMW is not easily biodegradable. Due to their high content of phenolic compounds, organic matter and long-chain fatty acids, OMW may be toxic to microorganisms and plants, and cannot be directly disposed into the environment (Salgado *et al.*, 2016). However, OMW is often poured into the soil or disposed of in sewage, causing soil and water pollution. The dark coloration, high biological and chemical oxygen demand (BOD/COD), and the presence of phytotoxic substances do not allow the direct discharge of OMW in the water or land. In fact, untreated OMW is able to change the microbial composition of the soil through their antibacterial activity. Moreover, the discharge of OMW directly onto soil may have impact in physical and chemical properties of soil, such as porosity and pH (Bevilacqua *et al.*, 2009; Mcnamara *et al.*, 2008).

The fearsome ecological impact of this wastewater has incited researchers to develop various technological processes in order to decrease the OMW toxicity. Some chemical, physical and biological processes have been proposed, but many difficulties have been encountered during these processes: technical-economic difficulties, weak efficiency, cell death during bioprocess and the requirement of a large installation space (Ettayebi *et al.*, 2003). Several systems for OMW purification have been reported, including evaporation basins and physical-chemical treatments (Flouri *et al.*, 1997; Martinez-Garcia *et al.*, 2007). Simple physical processes such as dilution, evaporation, sedimentation, filtration and centrifugation have been carried out to treat OMW, however none of these processes alone is able to reduce the organic load and toxicity of OMW to acceptable limits (Paraskeva and Diamadopoulos, 2006). Techniques such as adsorption or ion exchange are methods that could be used to eliminate phenols and polyphenols, but these methods are usually applied in combination with others (Torrecilla, 2010). The dilution of OMW can be used prior to biological treatment to reduce toxicity to the microorganisms responsible for organic matter decomposition. Centrifugation and filtration increase the pH and conductivity of the effluents and remove the organic matter through, respectively, phase separation and exclusion (Paraskeva and Diamadopoulos, 2006).

Electrocoagulation is a chemical method that has recently attracted attention for the treatment of olive mill and other industrial effluents (Paraskeva and Diamadopoulos, 2006). It is a technique for

destabilizing suspended, emulsified, or dissolved pollutants in an electrocoagulation cell by introducing an electric current without adding a chemical coagulant. Electrocoagulation process allowed removal of total solids and COD of about 82.5 % and 47.5 %, respectively at 45 mA/cm² after 70 min by using coupled iron-aluminum electrodes (Rharrabti and Yamani, 2018). Adsorption is another chemical process used to remove hazardous inorganics and organic compounds, especially phenols from OMW.

Fenton oxidation is a very effective oxidation method in several organic pollutants removal. Fenton reaction is based on chemical oxidation and coagulation of organic compounds present in OMW by means of hydrogen peroxide and ferrous sulfate (Torrecilla, 2010). Apart from the COD reduction, also the phenolic content is notably reduced by Fenton reaction (Azabou *et al.*, 2007). Kallel et al. (2009), using hydroxyl radicals generated from zero-valent iron and hydrogen peroxide, has shown that color disappeared and the phenolic compounds concentration decreased to 50 % only after 3 h of reaction.

The total removal of phenolic compounds and/or COD from OMW could be reached using a combination of physicochemical processes. Amor et al. (2015) applied Fenton's reaction followed by anaerobic digestion for OMW treatment. This combined process resulted in a significant improvement on organic load removal, reaching COD degradations up to 88 %.

Although these non-biological methods are normally expensive and do not generate valuable products, they are especially efficient on pollutants removal and can be useful as pre- and post-treatment of OMW.

1.3.1 Biodegradation of OMW by microorganisms

Biological processes for the treatment of wastewaters have worldwide applications. They are considered environmentally friendly, reliable and, in most cases, cost-effective. Beyond that, this treatment is able to remove organic matter and inorganic nutrients (Paraskeva and Diamadopoulos, 2006). OMW represents a possible resource for simple and complex sugars that might be a basis for fermentation processes (Asses *et al.*, 2009). The biological treatments are considered most favorable because beyond they are friendly to the environment compared to other procedures, in addition, the biological treatment of OMW opens the possibility for its conversion into products of industrial interest. Diverse approaches using different microorganisms have been designed to convert OMW into added-value products, such as organic acids, biopolymers, enzymes, ethanol, single cell proteins or microbial lipids, among others (Herrero *et al.*, 2018).

Comparing anaerobic to aerobic biodegradation, the former process requires generally higher capital investment, expert labor, and transport of waste from generation point to treatment point, resulting in higher fuel costs and higher emissions (Mansour *et al.*, 2011). In addition, the anaerobic biological degradation of OMW can lead to methane production but large periods of biomass adaptation and low phenolic compounds degradation have been reported as a disadvantage of the process (Araujo *et al.*, 2004). Likewise, some problems occur under aerobic conditions, such as the OMW color intensification due to the auto-oxidation of the phenolic compounds (Gonçalves, 2011).

Several investigations have been carried out using microorganisms capable of growing aerobically on diluted OMW in order to reduce the initial organic load, dark color, toxicity of the effluents, chemical oxygen demand (COD) and phenolic content (Fadil *et al.*, 2003; Mansour *et al.*, 2011). OMW can be biodegraded by bacteria, yeasts and fungi (Ettayebi *et al.*, 2003; Fadil *et al.*, 2003; Paraskeva and Diamadopoulos, 2017) as shown in Table 1.2. A number of different bacteria species have been tested in aerobic processes to treat OMW. The effectiveness of bacteria in reducing the phytotoxicity of OMW varies greatly. Bacteria appear to be very effective against some phenolic compounds and relatively ineffective against others, such as the more complex polyphenolics responsible for the dark coloration of OMWs (Mcnamara *et al.*, 2008). For example, *Bacillus pumilus* was able to completely degrade protocatechuic acid and caffeic acid, but had much less effect on tyrosol degradation (Ramos-Cormenzana *et al.*, 1997).

In general, literature shows that fungi (filamentous fungi and yeasts) are more effective than bacteria to degrade both simple and more complex phenolic compounds present in olive-mill wastes (Morillo *et al.*, 2009). The reason for this lies in the structure of the aromatic compounds present in OMWs since they are analogous to that of many lignin monomers (Mansour *et al.*, 2011). In addition to reduction of COD and removal of simple phenolic compounds, fungi are also effective at reducing coloration of OMW (Mcnamara *et al.*, 2008).

A variety of white rot fungi have been used for remediation of OMW. Fountoulakis *et al.* (2002) reported that *Pleurotus ostreatus* can grow in OMW without the addition of any nutrients, removing phenols from the culture medium, but decolorization of OMW was observed only when sterilized medium was used. Nevertheless, the use of filamentous fungi (compared with bacteria) for OMW treatment in large-scale processes is considered problematic due to the difficulty of achieving a continuous culture because of the formation of fungal pellets and other aggregations (Morillo *et al.*, 2009). Moreover, bio-treatments based on cultivation of filamentous fungi require long-lasting fermentation cycles of no less than 2 weeks, as well as diluted OMW to minimize the waste toxicity.

An interesting approach is the utilization of some yeast species that have shown a great tolerance to the OMW antimicrobial effect (Ettayebi *et al.*, 2003). In fact, yeasts are the more adapted and resistant to high concentrations of phenolic compounds and low pH found in OMW (Bevilacqua *et al.*, 2009). Particularly, yeasts are good candidates for the OMW treatment and recovery because they can grow well on lipids; they can consume the organic material, degrade lipids and polyphenols; at the same time, they can produce biomass and other valuable products. Nevertheless, the efficiency of organic and polyphenolic content reduction, as well as metabolites production from OMW is strongly dependent of the yeast strain (Araujo *et al.*, 2004; Lanciotti *et al.*, 2005). Yeasts such as *Candida oleophila* and *Candida tropicalis* have been proposed to detoxify and/or decolorize OMW (Martínez-García *et al.*, 2007).

Table 1.2 – Examples of studies focused on OMW degradation by microbial species (yeasts, fungi and bacteria).

OMW	Microbial species	Principal Results	References
Undiluted; Supplemented with ammonium chloride and yeast extract	<i>Candida cylindracea</i>	84 % reduction of reducing sugars 27 % reduction of phenolic compounds 70 % reduction of COD	(Gonçalves <i>et al.</i> , 2009)
	<i>Candida rugosa</i>	83 % reduction of reducing sugars 20 % reduction of phenolic compounds 59 % reduction of COD	
	<i>Yarrowia lipolytica</i>	85 % reduction of reducing sugars 25 % reduction of phenolic compounds 51 % reduction of COD	
Diluted; Supplemented with yeast extract, potassium di-hydrogenophosphate and ammonium sulfate	<i>Candida tropicalis</i>	12 % reduction of phenolic compounds 39 % reduction of COD	(Ettayebi <i>et al.</i> , 2003)
Diluted	<i>Yarrowia lipolytica</i>	51 % reduction of phenolic compounds 58 % reduction of color	(Sarris <i>et al.</i> , 2017)

Table 1.2 – Examples of studies focused on OMW degradation by microbial species (yeasts, fungi and bacteria) (continuation).

OMW	Microbial species	Principal Results	References
Undiluted; without supplementation	<i>Pichia fermentans</i>	18 % reduction of phenolic compounds 31 % reduction of COD	(Taccari and Ciani, 2011)
	<i>Candida</i> sp.	15 % reduction of phenolic compounds 22.4 % reduction of COD	
Diluted; Supplemented with ammonium nitrate and yeast extract	<i>Geotrichum</i> sp.	47 % reduction of phenolic compounds 55 % reduction of COD	(Fadil <i>et al.</i> , 2003)
	<i>Aspergillus</i> sp.	44 % reduction of phenolic compounds 53 % reduction of COD	
	<i>Candida tropicalis</i>	52 % reduction of phenolic compounds 63 % reduction of COD	
Diluted; Supplemented with glucose	<i>Candida tropicalis</i>	17 % reduction of phenolic compounds	(Dourou <i>et al.</i> , 2016)
	<i>Saccharomyces cerevisiae</i>	13 % reduction of phenolic compounds	
Diluted	<i>Paecilomyces variotii</i>	68 % reduction of phenolic compounds 59 % reduction of COD	(El-aziz <i>et al.</i> , 2016)
Diluted	<i>Geotrichum candidum</i>	64 % COD reduction in the bubble column and 57% in the settler bioreactors	(Asses <i>et al.</i> , 2009)
Diluted; Supplemented with ammonium nitrate and ammonium sulfate	<i>Phanerochaete chrysosporium</i>	92 % reduction of phenolic compounds	(García <i>et al.</i> , 2000)
	<i>Aspergillus niger</i>	76 % reduction of phenolic compounds	
	<i>Aspergillus terreus</i>	64 % reduction of phenolic compounds	
	<i>Geotrichum candidum</i>	20 % reduction of phenolic compounds	
Diluted	<i>Pseudomonas putida</i>	66 % reduction of phenolic compounds 85 % reduction of COD 93 % reduction of BOD 75 % reduction of color	(Mansour <i>et al.</i> , 2011)

The yeasts *Yarrowia lipolytica* and *Candida* sp. have been reported as good candidates for OMW detoxification (Gonçalves *et al.*, 2009; Lanciotti *et al.*, 2005). Lanciotti *et al.* (2005) evaluated the ability of different *Y. lipolytica* strains to grow in OMW and concluded that these yeast strains are able to reduce COD (1.5 % and 41 % of the initial value) and total polyphenols concentration (2 % - 23 %). Gonçalves *et al.* (2009) studied the ability of three *Y. lipolytica* strains to grow on undiluted OMW and observed a reduction on COD (37 % - 51 %), total phenols (20 % - 31 %) and reducing sugars (76 % - 85 %).

Candida sp. has been one of the most yeast studied (Ruiz-Ordaz *et al.*, 2001), particularly the genus *tropicalis*, because can metabolize phenolic compounds as the only carbon and energy source (Tuah *et al.*, 2009). Fialová *et al.* (2004) reported that the yeast *Candida maltosa* can degrade phenol up to 1700 mg·L⁻¹, but only *Candida tropicalis*, isolated from acclimated activated sludge, could degrade phenol concentrations beyond 1700 mg·L⁻¹ (Yan *et al.*, 2005). Krug *et al.* (1985) concluded that *Candida tropicalis* HP 15 degraded phenol at concentrations up to 2.5 g·L⁻¹ and resorcinol could be degraded up to a concentration of 7.5 g·L⁻¹. *Candida tropicalis* is one of the most versatile yeast species and exhibits three main morphologies: yeast, pseudohyphae and hyphae isolated. This yeast is isolated from various environments, from normal human mucocutaneous flora and immunocompromised patients, to natural environments such as polluted soil or water and industrial effluents (Csutak *et al.*, 2017; Jiang *et al.*, 2016). Varma and Gaikwad (2008) identified *C. tropicalis* NCIM 3556 as a high phenol-degrading strain, which was further investigated for its potential to degrade other derivatives of phenol such as o-cresol, m-cresol, 2,6-dimethyl phenol, α -naphthol, o-chlorophenol and p-nitrophenol. Bevilacqua *et al.* (2009) reported that *Candida tropicalis* can reduce up to 52 % of phenol present in OMW. Douro *et al.* (2016) observed a reduction on color (56 %) and phenolic compounds (17 %) as well as consumption of reducing sugars (58.9 g·L⁻¹) of OMW by *C. tropicalis*. Fadil *et al.* (2003) also reported that *C. tropicalis* was able to reduce COD (54 %), total phenols (58 %) and reducing sugars (85 %) of diluted OMW.

1.4 Metabolites produced from hydrocarbons and OMW

Processes that add value to effluents contaminated with pollutant compounds, by producing valuable metabolites, have been developed. A wide range of high added-value products, such as enzymes, biofuels and organic acids can be obtained upgrading the sub-products of agro-industrial activity (Liguori *et al.*, 2013). Several microorganisms are able to degrade pollutant compounds present in petroleum refinery effluents or wastes contaminated with *n*-alkanes/hydrocarbons, producing added-value metabolites, as shown in Table 1.3.

Table 1.3 – Examples of high added-value products obtained from microbial valorization of hydrocarbons or effluents contaminated with these compounds.

Metabolite	Microbial species	Hydrocarbons/Effluents	References
Microbial lipids	<i>Candida tropicalis</i>	<i>n</i> -hexadecane Petroleum	(Csutak <i>et al.</i> , 2017)
	<i>Rhodococcus opacus</i>	<i>n</i> -hexadecane	(Castro <i>et al.</i> , 2017)
	<i>Candida tropicalis</i>	<i>n</i> -alkanes	(Mishina <i>et al.</i> , 1977)
	<i>Candida lipolytica</i>		
Succinic acid	<i>Yarrowia lipolytica</i>	<i>n</i> -alkanes	(Kamzolova <i>et al.</i> , 2012)
Lipase	<i>Burkholderia glumae</i>	Hexadecane	(Boekema <i>et al.</i> , 2007)
	<i>Acinetobacter lwoffii</i>	<i>n</i> -alkanes	(Breuil <i>et al.</i> , 1978)
	<i>Aspergillus terreus</i>	Crude oil	(Mahmoud <i>et al.</i> , 2015)
Biosurfactants	<i>Yarrowia lipolytica</i>	Petroleum	(Csutak <i>et al.</i> , 2015)
	<i>Rhodococcus erythropolis</i>	Crude oil	(Pi <i>et al.</i> , 2017)
	<i>Pseudomonas</i> sp.	Engine oil	(Obayori <i>et al.</i> , 2009)
	<i>Candida tropicalis</i>	<i>n</i> -hexadecane Petroleum	(Csutak <i>et al.</i> , 2017)

OMW contains various carbon sources, organic compounds and minerals, and from a biotechnological point of view, could an exploitable substrate for various bioprocesses (Lanciotti *et al.*, 2005). The residual oil contained on OMW makes this effluent suitable as growth medium for lipolytic

microorganisms. Actually, several microorganisms have been proved to be able to grow on OMW and produce various high added-value compounds as shown in Table 1.4.

Table 1.4 – Examples of high added-value products obtained from microbial valorization of OMW.

Metabolite	Microbial species	OMW medium	References
Microbial lipids	<i>Yarrowia lipolytica</i>	Diluted; Supplemented with glycerol and glucose	(Dourou <i>et al.</i> , 2016)
	<i>Lipomyces starkeyi</i>		
	<i>Yarrowia lipolytica</i>	Diluted; Supplemented with glucose	(Sarris <i>et al.</i> , 2017)
Citric acid	<i>Yarrowia lipolytica</i>	Diluted; Supplemented with glycerol	(Dourou <i>et al.</i> , 2016)
	<i>Yarrowia lipolytica</i>	Diluted; Supplemented with glucose	(Sarris <i>et al.</i> , 2017)
Biosurfactants	<i>Aureobasidium thailandense</i>	Diluted; Supplemented with glucose and yeast extract	(Meneses <i>et al.</i> , 2017)
Mannitol	<i>Yarrowia lipolytica</i>	Diluted; Supplemented with glycerol	(Dourou <i>et al.</i> , 2016)
Ethanol	<i>Candida tropicalis</i>	Diluted; Supplemented with glucose	(Dourou <i>et al.</i> , 2016)
	<i>Saccharomyces cerevisiae</i>		
Lipase	<i>Candida rugosa</i>	Undiluted; Supplemented with ammonium chloride and yeast extract	(Gonçalves <i>et al.</i> , 2009)
	<i>Yarrowia lipolytica</i>		
	<i>Candida cylindracea</i>		
	<i>Aspergillus</i> strains	Undiluted; Diluted with vinasses	(Salgado <i>et al.</i> , 2016)
Flavor compounds	<i>Rhizopus oryzae</i> and <i>Candida tropicalis</i>	Diluted without supplementation	(Guneser <i>et al.</i> , 2016)

1.4.1 Lipase

Lipases (E.C.3.1.1.3) constitute a versatile group of enzymes which catalyze different sorts of reactions such as hydrolysis, esterification, transesterification, aminolysis, acidlysis and alcoholysis (Joseph *et al.*, 2008). These enzymes are a class of hydrolases, which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over an oil–water interface. Generally, lipases are secreted in the presence of lipids, such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, bile salts and glycerol (Peng *et al.*, 2016; Thakur, 2012; Treichel *et al.*, 2010). The multifaceted features have enabled the use of lipases in medicine, food, detergent, leather, paper industries and waste treatment (Peng *et al.*, 2016).

Lipase is produced by animals, plants, and microorganisms. Microbial lipases have gained special industrial attention due to their stability, selectivity, and broad substrate specificity. Many microorganisms are known as potential producers of extracellular lipases, including bacteria, yeast, and fungi (Thakur, 2012). Ertugrul *et al.* (2007) isolated 17 bacterial strains that could grow on media based on OMW and selected the most promising strain for lipase production. After the screening in tributyrin agar medium, a strain of *Bacillus* sp. was identified as the best lipase producer.

In case of lipase production by fungi, it differs according to the species, the composition of the growth medium, cultivation conditions, pH, temperature, and the nature of carbon and nitrogen sources (Thakur, 2012). Some of the most commercially important lipase-producing fungi are recognized as belonging to the genera *Rhizopus* sp. and *Aspergillus* sp. Salgado *et al.* (2016) observed that the maximum lipase activity ($1253.7 \text{ U}\cdot\text{L}^{-1}$) was obtained by *A. ibericus* in fermentations with undiluted OMW. Also Abrunhosa *et al.* (2012) observed that *A. ibericus* have ability to produce lipases in OMW supplemented with mineral nutrients. The lipase activity achieved in bioreactor batch cultures was $2927.0 \text{ U}\cdot\text{L}^{-1}$. D'Annibale *et al.* (2006) observed that, in *Rhizopus* sp. cultures with OMW-based medium, lipase production was 0.30 to $0.36 \text{ U}\cdot\text{mL}^{-1}$. Li *et al.* (2006) studied the effect of different carbon sources on lipase production. *R. arrhizus* was able to produce lipase with olive oil, peanut oil and glycerol in concentrations of 157.0 , 163.0 and $62.5 \text{ U}\cdot\text{mL}^{-1}$ respectively.

Several species of yeasts were found as producers of lipase, such as those belonging to *Candida* and *Yarrowia* genera (Vakhlu and Kour, 2006; Thakur, 2012). *Candida tropicalis* is able to lipase extracellularly from polar and non-polar organic compounds as substrate (Peng *et al.*, 2016; Vakhlu and Kour, 2006). *Yarrowia lipolytica* is known by its ability to produce intra and extracellular lipase from a wide range of substrates, including agro-industrial residues (OMW, animal fat, waste cooking oils, etc.) (Lopes *et al.*, 2009; Lopes *et al.*, 2018b; Liu *et al.*, 2017).

Lipase production is significantly improved by the presence of lipidic substrates, including oily-wastes such as olive mill wastewaters (Lopes *et al.*, 2009; Salgado *et al.*, 2016; Ertugrul *et al.*, 2007), waste cooking oils (Lopes *et al.*, 2018b) or palm oil mill wastewater (Theerachai *et al.*, 2017). Other carbon sources, such as olive oil (Lopes *et al.*, 2008), hexadecane, Tween 80 (Boekema *et al.*, 2007) and organic solvents (Peng *et al.*, 2016) are also known as inducers of lipase production.

Microbial lipases are mostly extracellular and their production is greatly influenced by physicochemical and operational factors such as temperature, pH and dissolved oxygen (Thakur, 2012). Extracellular lipase production by *Staphylococcus aureus* was higher at optimized culture conditions: pH 7.5, 37 °C and olive oil concentration of 30 mg·L⁻¹ (Khatape *et al.*, 2015). Corzo and Revah (1999) concluded that pH affects significantly lipase production by *Y. lipolytica* 681 and reported that a maximum lipase production was achieved in pH range of 5.8 – 7. In relation to the temperature, Corzo and Revah (1999) observed that temperatures around 30 °C were the optimal for lipase production by *Y. lipolytica*. On the other hand, Tan *et al.* (2003) reported that temperatures above 28 °C led to a decrease on lipase production by *Candida* sp. 99-125.

In lipase producing cultures, which can occur in water-immiscible substrates such as oils, fatty acids and hydrocarbons, oxygen availability is a crucial parameter because fatty substrates coat the gas-liquid interface with a layer of oil, reducing the oxygen mass transfer (Lopes *et al.*, 2018a). In fact, it has been demonstrated that dissolved oxygen is the main chemical engineering parameter that influences the physiological mechanisms involved during lipase synthesis. Oxygen limitation tends to decrease the activity of the *Lip2* promoter, which is responsible for the production of the main extracellular lipase by *Y. lipolytica* (Brigida *et al.*, 2014). According to Lopes *et al.* (2008) and Tan *et al.* (2003) the increase of oxygen solubility by raising total air pressure led to an enhancement of lipase production by *Y. lipolytica* W29. Furthermore, dissolved oxygen is also affected by operations conditions in a stirred-tank bioreactor, such as agitation and aeration rates. Lipase biosynthesis by *Y. lipolytica* was favored at low aeration rates for the same agitation speed, being the maximum obtained in cultures performed at 400 rpm and 0.2 vvm (Deive *et al.*, 2010). Elibol & Ozer (2000) observed that, in *Rhizopus arrhizus* cultures, a 2-fold improvement on lipase production was attained by the increase of dissolved oxygen level from 20 % to 70 %.

Lipase productivity is affected by different physical and chemical factors, however several works in the literature have demonstrated the influence of media composition on lipase biosynthesis, being the carbon and nitrogen sources two of the most important (Brigida *et al.*, 2014). Lipase production is significantly increased in the presence of lipidic substrates, such as olive oil (Lopes *et al.*, 2008), but the

use of glucose and glycerol as carbon sources can represses lipase induction (Fickers *et al.*, 2011). In addition to carbon sources, nitrogen sources should also be carefully considered. A review by Brigida *et al.* (2014) emphasized that organic nitrogen sources such as peptone, casein, yeast extract and tryptone are suitable for high extracellular lipase production.

1.4.2 Microbial lipids

Microbial lipids, also called microbial oils, single cell oils or intracellular lipids, are hydrophobic bodies mainly composed of triacylglycerols and sterol esters (Murphy, 2001). Oleaginous microorganisms are able to accumulate more than 20 % of their cell dry weight as intracellular lipids. Its use as source of oils and fats (microbial lipids), in large-scale operations, may have a great industrial interest (Papanikolaou and Aggelis, 2011). Once microbial lipids enriched with essential fatty acids, are of considerable importance for food industry and production of biodiesel (Bélignon *et al.*, 2016; Csutak *et al.*, 2017; Papanikolaou *et al.*, 2002).

In the past years, several oleaginous species of bacteria, yeast and fungi have been investigated for their ability to accumulate storage lipids and for the diversity of fatty acids inherent with these lipids. Compared with other species, yeast possesses certain advantages for both basic and applied researches as a microbial cell factory (Dey and Maiti, 2013). However, because most oily yeasts show low growth rates, the success of lipid accumulation relies on the rate of the exponential phase of growth (Ageitos *et al.*, 2011). The extraction of microbial lipids from yeasts has also some disadvantages, for including the presence of a thick cell wall that renders the yeast cells resistant to many solvents. For this reason, most of the lipid extraction methods involve cellular breakage, either by cold or heat, or by chemical or enzymatic hydrolysis, and an extraction with organic solvents, such as petroleum ether, methanol, or chloroform (Ageitos *et al.*, 2011).

Lipids may be accumulated by yeasts via two different pathways: *de novo* synthesis, involving the production, in defined conditions, of fatty acid precursors, such as acetyl and malonyl-CoA and their integration into the storage lipid biosynthetic pathway (the Kennedy pathway); and the *ex novo* accumulation pathway, involving the uptake of fatty acids, oils and triacylglycerols (TAG) from the culture medium and their accumulation in an unchanged or modified form within the cell (Beopoulos *et al.*, 2009). The *de novo* pathway of fatty acids (FA) biosynthesis requires precursors such as acetyl-CoA and malonyl-CoA and this is the first dedicated step to biosynthesizing FA. This is followed by the elongation process through FA synthetase. In the *ex-novo* pathway of lipid accumulation, the FA enters into the cell

and becomes activated. Then, it is absorbed as CoA-thioesters into the storage lipid. The synthesized or imported lipid may also be degraded to other lipid-derived metabolites (Abghari and Chen, 2014). The storage lipids are accumulated in a specialized compartment known as lipid body (LB), which consists of a lipid core encased in a phospholipid monolayer, within which many proteins with diverse biochemical activities are embedded. These lipid bodies contain neutral lipid, essentially composed by triacylglycerols (TAG), free fatty acids (FFA) and small amounts of steryl ester (SE) (Ferreyra *et al.*, 2006; Beopoulos *et al.*, 2009; Dey and Maiti, 2013). The most important metabolic processes and genes involved in lipid synthesis by yeasts is illustrated in Figure 1.2.

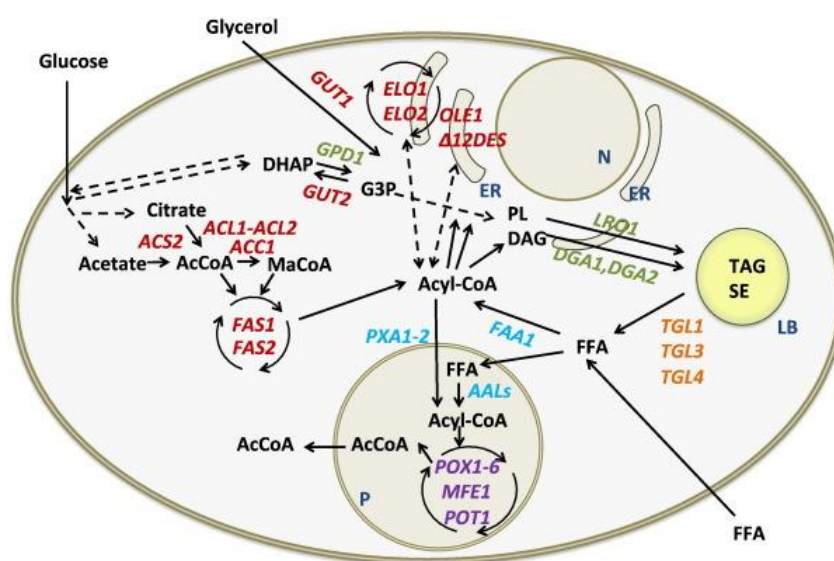


Figure 1.2 – Schematic representation of the metabolic pathways leading to the production of neutral lipids (triacylglycerol (TAG) and steryl esters (SE)) from either glucose or free fatty acids (FFA). Dashed lines indicate multiple steps. Gene colors of the genes indicate different metabolic pathways: in red fatty acid synthesis and elongation and desaturation system, in green triacylglycerol synthesis, in orange lipid remobilization, in light blue fatty acid activation and transport, and in purple fatty acid degradation. The different organelles are indicated by the dark blue letters where N is the nucleus, ER the endoplasmic reticulum, LB the lipid body and P the peroxisome. DHAP (dihydroxyacetone phosphate), G3P (glycerol-3-phosphate), AcCoA (acetyl-CoA), MaCoA (malonyl- CoA), PL (phospholipid), DAG (diacylglycerol) (Ledesma-Amaro and Nicaud, 2016).

New intracellular fatty acids previously absent in the substrate, can be produced by oleaginous yeast fermentation under certain conditions. An important application of new fats with polyunsaturated fatty acids (PUFA) is as food or nutritional supplements (Gonçalves *et al.*, 2014). Beyond that, microbial fatty acids became of large interest for biofuel production, an ecological response to the growing world crisis due to depletion of conventional fuel resources (Csutak *et al.*, 2017). Therefore, isolation of naturally

existing yeast strains having suitable fatty acid profiles is foremost important for their potential applications (Dey and Maiti, 2013).

The yeasts able to produce and accumulate high levels of lipids and fatty acids are denominated as oleaginous yeasts and belong mainly to *Candida*, *Rhodotorula*, *Yarrowia*, *Cryptococcus* and *Trichosporon* genera (Csutak *et al.*, 2017). Generally, oleaginous microorganisms begin to accumulate lipids when an element in the medium becomes limiting and the carbon source is present in excess (Beopoulos *et al.*, 2009). Several carbon sources have been reported as suitable for microbial lipids production, such as glucose, xylose, glycerol, starch, cellulose hydrolysates, alkanes, vegetable oils, animal fat and agro-industrial wastes (Bélignon *et al.*, 2016; Lopes *et al.*, 2018a; Papanikolaou and Aggelis, 2011). The predominant fatty acids produced by oleaginous yeasts are myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids (Ageitos *et al.*, 2011; Dey and Maiti, 2013).

Lipid accumulation depends primarily on microorganism physiology, nutrient limitation and environmental conditions, such as temperature and pH (Beopoulos *et al.*, 2009). It is also affected by the production of secondary metabolites, such as citrate and ethanol. In case of nutrient limitation, the excess of carbon and nitrogen limitation are essential factors for the induction of *de novo* synthesis in oleaginous microorganisms. The use of culture media with proper carbon/nitrogen (C/N) ratios is essential to maximize the production of microbial lipids. Generally, it is considered that a molar ratio C/N above 20 is the optimum for microbial lipids accumulation. By contrast, very high C/N ratios, above 70, influence negatively the process of intracellular lipids accumulation (Lopes *et al.*, 2018a; Papanikolaou and Aggelis, 2011). Besides that, lipid accumulation is influenced by the concentration of some microelements (Mg^{+2} , Zn^{+2} , Mn^{+2} , Cu^{+2} , and Ca^{+2}) and inorganic salts (Ageitos *et al.*, 2011). In the other hand, in *ex novo* lipid accumulation process (as occurs in hydrophobic substrates), the C/N ratio is not a limiting parameter and microbial oils production is independent of C/N ratio level.

Temperature and pH are parameters that must be controlled for a maximum microbial lipids production. Lipid accumulation is favored by a slightly acidic pH (5 to 6.5) (Kuttiraja *et al.*, 2017) and pH values in the range of 5 - 7 have been reported as the optimum for microbial lipids production by *Y. lipolytica* strains (Papanikolaou *et al.*, 2002; Lopes *et al.*, 2018b). According to Kuttiraja *et al.* (2017), culture of *Y. lipolytica* SKY7 in bioreactor with controlled pH at 6.5 produced $7.78\text{ g}\cdot\text{L}^{-1}$ of lipids, whereas with uncontrolled pH only $6.58\text{ g}\cdot\text{L}^{-1}$ of microbial lipids were produced. In the other hand Dey and Maiti (2013) observed that lipids produced by *C. tropicalis* cells from glucose were not significantly affected by the changes of pH values of the cultured media. Temperature is other factor that critically influences the

lipid accumulation by *Y. lipolytica* cultures, being the range between 28 °C and 30 °C the most commonly used (Sarris *et al.*, 2011).

Several authors recognized the importance of oxygen on lipid biosynthesis and also consider that during the anabolic fatty acid biosynthesis pathways, high dissolved oxygen concentration drives the microbial metabolism toward the synthesis of lipid-free biomass alternatively to the accumulation of lipids. Moreover, for strictly aerobic microorganisms, the amount of oxygen available in the medium is a factor with an important impact on yeast growth and microbial lipids production (Coelho *et al.*, 2010). Rakicka *et al.* (2015) suggest that an oxygen limitation could lead to an inhibition in carbon metabolism, resulting in citric acid secretion instead of lipids accumulation. However, these authors also reported that *Y. lipolytica* JMY4086 accumulated higher lipid contents in conditions of lower dissolved oxygen concentration in culture medium. Moreover, it has been reported that high stirring and aeration rates do not improve the *ex novo* lipids accumulation and may enhance their degradation by increasing the acyl-CoA oxidase activity (Papanikolaou *et al.*, 2007; Rakicka *et al.*, 2015). Papanikolaou *et al.* (2011) also concluded that the highest quantities of lipids accumulated by *A. niger* coincided with the lower values of dissolved oxygen in the medium. By contrast Bellou *et al.* (2014) observed that *Y. lipolytica* ACA-DC 50109 accumulated high lipid content in medium with high dissolved oxygen concentrations. Lopes *et al.* (2018a) also reported that increasing the oxygen transfer rate from 96 mg·L⁻¹·h⁻¹ to 192 mg·L⁻¹·h⁻¹ clearly enhanced lipid accumulation by *Y. lipolytica* W29.

2. GOALS

Large quantities of industrial effluents (e.g. olive mill wastewater and effluents contaminated with hydrocarbons) are produced daily around the world and discharged into the environment without any treatment. The treatment of these effluents is an obligatory condition to decontaminate and add value to them. Biological treatment by microorganisms of contaminated-effluents may represent an economic and ecological advantage over traditional physical and chemical methods. Moreover, besides the decontamination of pollutant load of effluents, the microbial degradation has the advantage to produce added-value compounds, valorizing these wastes.

The main objective of this thesis was to study the biodegradation of pollutant compounds (hydrocarbons and olive mill wastewater) by yeast species, producing added-value metabolites. Therefore, the specific objectives were:

- Screening of yeast species with the ability to degrade pollutant compounds (hydrocarbons and phenols) and use them as sole carbon and energy source in 96-well microplate experiments;
- Evaluate the maximum hydrocarbons concentration in which *Yarrowia lipolytica* W29 is able to grow in 96-well microplate experiments;
- Assess the ability of *Candida tropicalis* ATCC 250 to grow on diluted and undiluted OMW in 96-well microplate experiments;
- Scale-up the hydrocarbons and OMW degradation by *Y. lipolytica* W29 and *C. tropicalis* ATCC 250, respectively, to Erlenmeyer flasks;
- Evaluate the metabolites production from hydrocarbons and OMW;
- Study the effect of oxygen mass transfer, by raising headspace volume, on yeasts growth and metabolites production.

3. MATERIALS AND METHODS

In this Chapter, the general methods, operation conditions and equipment used in this work are presented.

3.1 Yeast species and maintenance

Candida tropicalis ATCC 250, *Candida utilis* CBS 621, *Candida rugosa* PYCC 3238, *Candida cylindracea* CBS 7869, *Yarrowia lipolytica* IMUFRJ, *Yarrowia lipolytica* CBS 2073, *Yarrowia lipolytica* NYCC 1026, *Yarrowia lipolytica* CBS 2075, *Yarrowia lipolytica* W29 and *Pichia pastoris* CBS 2612 were maintained on YPDA medium (glucose 20 g·L⁻¹, peptone 20 g·L⁻¹, yeast extract 10 g·L⁻¹ and agar 20 g·L⁻¹) at 4 °C for a maximum of two weeks.

3.2 Screening of yeast species

3.2.1 Petri plate experiments

The ability of ten yeast species to grow on solid medium with some pollutant compounds (catechol, tyrosol, phenol and hexadecane) as carbon source was initially evaluated in Petri plate experiments. For each yeast species, cells were pre-grown overnight in 100 mL-Erlenmeyer flasks filled with 20 mL of YPD medium. This pre-inoculum was diluted to obtain a yeast culture with an optical density of 0.6 and successive dilutions (up to 10⁻⁵) from this culture were prepared in NaCl 0.9 %. 30 µL of each dilution were inoculated on agar plate containing 0.5 g·L⁻¹ of each pollutant compound as carbon source, 6.7 g·L⁻¹ of yeast nitrogen base (YNB) without amino acids and 20 g·L⁻¹ of agar. Two control experiments were also carried out: (1) glucose as carbon source and (2) no addition of any carbon source (YNB medium). Petri plates, inoculated with different yeast species were placed inverted in a static incubator oven at 30 °C during 48 hours. Cellular growth on each carbon source was evaluated visually and photos were taken in a Chemi Doc XR[®] (Bio-Rad, USA).

3.2.2 96-well microplate experiments

After the first screening on Petri plate experiments, the ability of six yeast species to grow on liquid medium with the same pollutant compounds as carbon source was evaluated in 96-well microplate experiments. For each yeast species, cells were pre-grown overnight in 100 mL-Erlenmeyer flasks filled with 10 mL of YPD medium. Each well of microplate was inoculated with 30 µL of pre-inoculum, 240 µL of YNB without aminoacids (final concentration on the well 6.7 g·L⁻¹) and 30 µL of pollutant compounds (final concentration on the well 1 g·L⁻¹). Two control experiments were also carried out: (1) no inoculation of medium to reduce the interference of color development on absorbance due to the oxidation of pollutant

compounds and (2) no addition of any pollutant compound. The 96-well microplates, inoculated with different yeast species, were placed in a static incubator oven at 30 °C during 48 hours. Cellular growth on each carbon source was assessed by optical density ($\lambda = 600$ nm) at the beginning and the final of the experiments.

3.3 *Yarrowia lipolytica* growth in hydrocarbons-based media

3.3.1 96-well microplate experiments

The ability of *Yarrowia lipolytica* W29 to grow on different concentrations of hydrocarbons as sole carbon and energy source was first evaluated in batch cultures, in 96-wells microplates during 48 hours. Yeast cells were pre-grown overnight in YPD medium. Then, 30 μ L of cells suspension were transferred to each microplate well with 270 μ L of sterilized hydrocarbon-based medium, composed by hexadecane or hexadecene (1 g·L⁻¹ - 10 g·L⁻¹), YNB without amino acids 6.7 g·L⁻¹ and Tween 80 1 % (w/v). Additionally, two control experiments were carried out: (1) no addition of hydrocarbon and inoculated with *Y. lipolytica* W29 and (2) no inoculation of hydrocarbon-based medium to reduce the interference of color development due to the oxidation of hydrocarbons. The 96-well microplates were placed in a static incubator for 48 hours at 30 °C and cellular growth was assessed by optical density ($\lambda = 600$ nm) at the beginning and the final of the experiments.

3.3.2 Erlenmeyer flask experiments

Batch experiments with hexadecane (10 g·L⁻¹), hexadecene (7.5 g·L⁻¹) and a mixture of both hydrocarbons (5 g·L⁻¹ of each hydrocarbon) as carbon source were carried out in 250-mL Erlenmeyer flasks filled with 95 mL of each culture medium (hydrocarbons, YNB without amino acids 6.7 g·L⁻¹ and Tween 80 1 % (w/v)) and inoculated with 5 mL of *Y. lipolytica* W29 pre-culture that grew overnight. The experiments were carried out at 27 °C and 200 rpm in an orbital incubator.

To evaluate the effect of oxygen mass transfer on yeast growth and metabolites production, experiments with a higher ratio between the volume of headspace and the volume of culture medium were performed. Yeast growth was carried out in 500-mL Erlenmeyer flasks filled with 100 mL of culture medium (hexadecane or mixture of hydrocarbons).

An additional experiment, in repeated batch mode, was carried out in 500-mL Erlenmeyer flasks filled with 100 mL of hexadecane 10 g·L⁻¹, YNB without amino acids 6.7 g·L⁻¹ and Tween 80 1 % (w/v). After 72 hours and 144 hours of yeast growth, freshly medium (hexadecane 10 g·L⁻¹ and YNB 6.7 g·L⁻¹) was added to the Erlenmeyer flasks.

In all experiments, at appropriate intervals, samples were taken to quantify cellular concentration, pH, chemical oxygen demand (COD), extracellular lipase activity, microbial lipids and long chain fatty acids (LCFA).

3.4 *Candida tropicalis* growth in olive mill wastewater-based media

3.4.1 96-well microplate experiments

The ability of *Candida tropicalis* ATCC 250 to grow on different concentrations of olive mill wastewater was first evaluated in batch cultures, in 96-wells microplates during 48 hours. Yeast cells were pre-grown overnight in YPD medium. Then, 30 µL of cell suspension were transferred to each microplate well with sterilized OMW-based medium, composed by OMW (5 % – 50 %, v/v) and YNB without amino acids 6.7 g·L⁻¹. Additionally, two control experiments were carried out: (1) no addition of OMW and inoculated with *C.tropicalis* ATCC 250 and (2) no inoculation of OMW-based medium to reduce the interference of color development due to the oxidation of phenolic compounds of OMW. The 96-well microplates were placed in a static incubator for 48 hours at 30 °C and cellular growth was assessed by optical density (λ = 600 nm) at the beginning and the final of the experiments.

Olive mill wastewater used in this work was collected from “Lagar de Oliveira das Carvalhinhas” located at Amarante, Portugal. The OMW characterization for pH, chemical oxygen demand (COD), total nitrogen, total phenols and reducing sugars is shown in Table 3.1.

Table 3.1 – Main characteristics of OMW characterization. Data are average and standard deviation of two independent replicates.

Parameter/unit	OMW
pH	4.44 ± 0.01
COD (g·L ⁻¹)	87.2 ± 2.8
Total Nitrogen (mg·L ⁻¹)	949 ± 28.5
Total Phenols (g·L ⁻¹)	2.1 ± 0.2
Reducing sugars (g·L ⁻¹)	11.4 ± 0.8

3.4.2 Erlenmeyer flask experiments

Batch experiments with OMW 50 % (v/v), OMW 100 % (v/v) and OMW 100 % (v/v) supplemented with YNB without amino acids $6.7 \text{ g}\cdot\text{L}^{-1}$, were carried out in 250-mL Erlenmeyer flasks. Five mL of *C. tropicalis* ATCC 250 pre-culture that grew overnight on YPD medium was used to inoculate each Erlenmeyer flask. The experiments were carried out at 30 °C and 200 rpm in an orbital incubator.

To evaluate the effect of oxygen mass transfer on yeast growth and metabolites production, experiments with a higher ratio between the volume of headspace and the volume of culture medium were performed. Yeast growth was carried out in 500-mL Erlenmeyer flasks filled with 100 mL of culture medium (OMW 100 % (v/v) supplemented with YNB without aminoacids $6.7 \text{ g}\cdot\text{L}^{-1}$).

An additional experiment, in repeated batch mode, was carried out in 500-mL Erlenmeyer flasks filled with 50 mL of OMW 100 % (v/v) supplemented with YNB without aminoacids $6.7 \text{ g}\cdot\text{L}^{-1}$. After 72 hours and 144 hours of yeast growth, 50 mL of freshly medium was added to the Erlenmeyer flasks.

In all experiments, at appropriate intervals, samples were taken to quantify cellular concentration, pH, chemical oxygen demand (COD), microbial lipids and long chain fatty acids (LCFA).

3.5 Analytical methods

3.5.1 Cellular growth

To evaluate the yeast growth, cellular concentration was quantified by cell counting in a binocular bright-field microscope (Leica DM 750, Wetzlar, Germany), using a Neubauer counting chamber (Paul Marienfeld GmbH & Co, Lauda-Königshofen, Germany) and converted to cell dry weight ($\text{g}\cdot\text{L}^{-1}$) by a calibration curve.

3.5.2 Total phenols: Folin-Ciocalteu method

The quantification of total phenols was measured by method of Folin-Ciocalteu (Commission Regulation - EEC - N° 2676/90). In this method, 100 μL of sample supernatant were added to 2 mL of sodium carbonate 15 % (w/v), 500 μL of Folin-Ciocalteu reagent and 7.5 mL of distilled water. Blank assays were performed replacing supernatant of culture medium by distilled water. The test tubes were placed in a bath at 50 °C during 5 minutes. After the tubes cool down to room temperature, 300 μL

were transferred into a 96-well microplate to read the absorbance at 700 nm in a microtiter plate reader (Synergy HT, BioTek, USA).

The absorbance was converted to total phenols concentration ($\text{g}\cdot\text{L}^{-1}$) by a calibration curve (tyrosol was used as standard). To construct the calibration curve, tyrosol solutions with several concentrations ($0.2 \text{ g}\cdot\text{L}^{-1} - 1 \text{ g}\cdot\text{L}^{-1}$) dissolved in absolute ethanol were prepared, following the same procedure as the samples.

3.5.3 Reducing sugars: DNS method

Reducing sugars concentration was quantified by the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959): 0.5 mL of culture supernatant was added to 0.5 mL of 3,5-dinitrosalicylic acid and the test tubes were immersed in a bath at 100 °C during 5 minutes. Blank assays were performed replacing culture supernatant by distilled water. After cooling the mixture in a cold water bath, 5 mL of distilled water were added to each test tube, vortex-mixed and 300 μL were transferred into a 96-well microplate to read the absorbance at 540 nm in a microtiter plate reader (Synergy HT, BioTek, USA).

The absorbance was converted to reducing sugars concentration ($\text{g}\cdot\text{L}^{-1}$) by a calibration curve (glucose was used as standard). To construct the calibration curve, glucose solutions with several concentrations ($0.25 \text{ g}\cdot\text{L}^{-1} - 2 \text{ g}\cdot\text{L}^{-1}$) were prepared and these solutions were submitted to the same procedure as the samples.

3.5.4 Lipase activity

Extracellular lipase activity was measured in culture supernatants (50 μL) using *p*-nitrophenyl-butyrate 1 mM as substrate in 250 μL of the reaction medium composed by acetone 4 % (v/v) and Triton-X (4 %, v/v) dissolved in phosphate buffer 50 mM (pH 7.3). The enzymatic reaction was followed by the absorbance measurement at 410 nm in a microtiter plate (Cytation 3, BioTek, USA) at 37 °C, every 30 seconds during 10 minutes.

Lipase activity ($\text{U}\cdot\text{L}^{-1}$) was quantified by Eq. 1.1:

$$\text{Activity } U \cdot L^{-1} = D \times \frac{1}{\varepsilon} \times \frac{V_t}{V_a} \times 1000 \quad (\text{Eq.1.1})$$

where D was calculated by linear regression of absorbance (nm) *versus* time (minutes); ϵ represents the molar extinction coefficient of *p*-nitrophenol (7.821 mM^{-1}); V_t represents the total volume of reaction mixture (0.29 mL) and V_s represents the volume of sample used in reactional mixture (0.05 mL).

One unit of activity was defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol per minute under the assay conditions.

3.5.5 Chemical oxygen demand (COD)

Chemical oxygen demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The chemical oxygen demand was measured by a test kit (LCK 914 (Lange, Hach, Germany)).

3.5.6 Microbial lipids: Phospho-vanillin method

Intracellular lipids were quantified in lyophilized cells (10 mg) by phospho-vanillin colorimetric method after extraction with methanol and chloroform (1:1, v/v) (Inouye & Lotufo, 2006). After vortex-mixing the mixture (cells + methanol + chloroform) for 3 minutes, 250 μL of the extraction mixture was transferred to test tubes and heated at 100°C to allow the evaporation of solvents. After the evaporation, 100 μL of pure sulfuric acid was added to each tube, vortex-mixed and heated at 100°C for 10 minutes. After the solution cool down to room temperature, 2.4 mL of phospho-vanillin reagent (0.24 g of vanillin dissolved in 40 mL and 160 mL of orthophosphoric acid 85 %) was added. All samples rested for 15 minutes at room temperature and 300 μL were transferred into a 96-well microplate to read the absorbance at 490 nm in a microtiter plate reader (Synergy HT, BioTek, USA). The absorbance was converted to lipids concentration ($\text{g}\cdot\text{L}^{-1}$) by a calibration curve (olive oil was used as standard). To construct the calibration curve, several concentrations of olive oil ($0 \text{ mg}\cdot\text{L}^{-1}$ – $2500 \text{ mg}\cdot\text{L}^{-1}$) dissolved in acetone were submitted to the same procedure of samples, except the extraction with methanol and chloroform.

3.5.7 Long chain fatty acids (LCFA)

Intracellular lipids were extracted with chloroform and transformed into their corresponding methyl esters with a mixture of methanol: H_2SO_4 (15:85, v/v) (adapted from Castro *et al.*, 2017). A defined amount of lyophilized cells was transferred to glass vials, adding 1.5 mL of pentadecanoic acid (internal standard), 3 mL of methanol: H_2SO_4 and 1.5 mL of chloroform. The mixture was vortex-mixed and digested

at 100 °C for 3 hours. After digestion, the mixture was vortex-mixed and 1.5 mL of ultra-pure water was added. The content of glass vial was transferred into a 10 mL-vial. These new vials were closed with a rubber stopper and aluminum crimp cap and kept in inverted position for around 30 minutes, after which 2 mL of organic phase was collected and transferred into a 2 mL-vial. In order to prevent any water from entering the gas chromatography (GC) column, a small amount of sodium thiosulfate was added to all 2 mL-vials. After sodium thiosulfate settled, the supernatant was transferred to a new 2 mL-vial, closed with an aluminum crimp cap, and stored at - 20 °C until the moment of injection.

To construct the calibration curve, several dilutions with chloroform from the stock solution with all standards of fatty acid methyl esters (lauric acid, C12:0; myristic acid, C14:0; palmitic acid, C16:0; palmitoleic acid, C16:1; margaric acid, C17:0; stearic acid, C18:0; oleic acid, C18:1; linoleic acid, C18:2) were made in order to attain a range of concentrations between 25 mg·L⁻¹ and 1000 mg·L⁻¹. These different concentrations of standards were submitted to the same procedure of samples (1.5 mL of each standard solutions + 1.5 mL of internal standard + 3 mL of methanol: H₂SO₄ solution).

The fatty acid methyl esters in organic phase (FAME) was quantified by GC (CP-3800 gas chromatograph (Varian Inc., USA)) fitted with FID detector and TRACSIL TR-WAX capillary column (30 m x 0.25 mm x 0.25 µm, Teknokroma, Spain). The injector and detector temperatures were 250 °C and helium was used as carrier gas at 1 mL·min⁻¹. The initial oven temperature was 50 °C, maintained for 2 minutes, followed by a 10 °C·min⁻¹ ramp up to 225 °C, and the final isothermal conditions were maintained for 10 minutes.

Data were analyzed using the acquisition and integration software Star Chromatography Workstation v. 6.30 (Varian, Inc., USA).

3.5.8 Statistical analysis

The analysis of variance (ANOVA) provides information about the relative importance of each parameter and allows identify the most significant parameters. The ANOVA was done in GraphPad Prism, version 6. Additionally, Tukey 's test was carried out to evaluate the statistical differences between the results of the experiments.

4. RESULTS AND DISCUSSION

In this chapter it is described the results regarding the screening of yeast species able to degrade and use as carbon source several pollutant compounds, usually found in industrial effluents. Two yeast species, *Yarrowia lipolytica* W29 and *Candida tropicalis* ATCC 250, stand out as promising to grow, respectively, in hydrocarbons and olive mill wastewater-based media. Moreover, both species were able to produce added-value compounds from these low-cost residues. *Yarrowia lipolytica* produced lipase and microbial lipids from hydrocarbons and *Candida tropicalis* accumulated unsaturated fatty acids from olive mill wastewaters.

4.1 Screening of yeast species

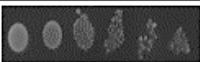
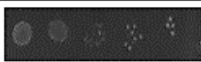
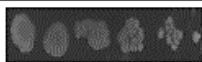
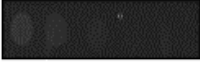
4.1.1 Petri plate experiments

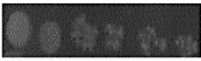
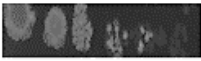
Over the last few years, several attempts have been made to degrade the pollutant compounds present in agro-industrial effluents. Although several authors have studied the ability of various yeasts to degrade some pollutants present in agro-industrial effluents, many studies still fall short, particularly for pollutant compounds such as hydrocarbons or phenols. Therefore, the main objective of this work was to evaluate the ability of several yeast species to degrade some pollutant compounds belonging to the classes of hydrocarbons and phenols.

Firstly, a broader screening on agar plates was performed to distinguish, among the ten yeast species tested, which had the ability to grow on hydrocarbons (hexadecane) and phenolic compounds (catechol, tyrosol and phenol) as sole carbon and energy source. Additionally, two control experiments for each yeast species were carried out: (1) glucose as carbon source and (2) no addition of carbon source (YNB medium). The yeast growth after 48 hours was evaluated qualitatively by observation of colonies on agar plates (Table 4.1). As expected, all yeast species grew well on glucose-based medium, appearing large colonies on the agar, even for the highest dilutions. Contrariwise, lower growth was observed in the medium without any carbon source (YNB medium).

Among the phenolic compounds tested, it was observed that growth of all yeasts tested was lower on catechol and phenol as carbon source, compared with tyrosol. In fact, 6 yeast species (*Yarrowia lipolytica* strains and *Candida cylindracea*) did not grow on catechol-based medium, even when the agar plate was inoculated with a non-diluted cellular suspension. Gonçalves et al. (2009) had already demonstrated that, among several phenolic compounds, catechol was the most inhibitory for the growth of *C. rugosa* CBS 2275 and *Y. lipolytica* W29. However, *C. cylindracea* CBS 7869 and *Y. lipolytica* W29 were able to degrade total phenols of olive mill wastewater (Gonçalves et al., 2009). The yeast *Y. lipolytica* is described in the literature as a good species to degrade phenolic compounds. Lee et al. (2000) reported that *Y. lipolytica* Y103 was able to degrade catechol, phenol and 4-chlorophenol. Other authors reported that *Y. lipolytica* strains can be considered a candidate for OMWs bioremediation, since they are able to degrade the phenolic compounds present in these effluents (Araujo et al., 2004; Lopes et al., 2009; Sarris et al., 2017).

Table 4.1 – Photos of yeast colonies after growth on agar plates with different carbon sources (glucose, catechol, tyrosol, hexadecane and phenol) and without carbon source (YNB).

	<i>Pichia pastoris</i> CBS 2612	<i>Candida utilis</i> CBS 621	<i>Candida rugosa</i> PYCC	<i>Candida</i> <i>cylindracea</i> CBS 7869	<i>Candida tropicalis</i> ATCC 250
Glucose					
Catechol					
Tyrosol					
Hexadecane					
YNB					
Phenol			NCO		

	<i>Yarrowia</i> <i>lipolytica</i> IMUFRJ	<i>Yarrowia lipolytica</i> W29	<i>Yarrowia lipolytica</i> CBS 2073	<i>Yarrowia lipolytica</i> NCYC 1026	<i>Yarrowia lipolytica</i> CBS 2075
Glucose					
Catechol					
Tyrosol					
Hexadecane					
YNB					
Phenol	NCO	NCO	NCO	NCO	

NCO: Not carried out

Candida tropicalis growth was not inhibited by any phenolic compound and the size of colonies obtained on catechol and phenol were quite similar to that obtained on glucose. As demonstrated by other authors, *C. tropicalis* is able to degrade several phenolic compounds, including the total phenols of OMW (Dourou *et al.*, 2016; Ettayebi *et al.*, 2003; Krug *et al.*, 1985; Varma and Gaikwad, 2008; Yan *et al.*, 2005). The results of Yan *et al.* (2005) showed that pure cultures of *C. tropicalis* has high potential for phenol degradation (up to 2 g·L⁻¹) and high inoculum volume lessened phenol toxicity for the cells, increasing cellular growth and phenol biodegradation velocity. According to Ettayebi *et al.* (2003), *C. tropicalis* YMEC14 was able to grow on diluted OMW supplemented with yeast extract, potassium dihydrogenophosphate and ammonium sulfate at a specific growth rate of 0.25 h⁻¹. In these conditions, 12 % of phenol was biodegraded by the yeast after 24 h of cultivation. Dourou *et al.* (2016) reported that

C. tropicalis LFMB 16 was able to grow on diluted OMW, but cellular growth was improved when glucose was used as co-substrate.

All yeast species tested demonstrated the ability to grow on hexadecane as carbon source, although *C. tropicalis* was the yeast that grew better. Gargouri et al. (2015) reported that *C. tropicalis*, isolated from industrial refinery wastewater, grew well on decane, hexadecane, diesel oil and crude oil but failed to grow on aromatic hydrocarbons. Hassanshahian et al. (2012) observed that two *Y. lipolytica* strains, isolated from an oil-polluted area in the Persian Gulf, were able to grow on *n*-alkanes (undecane, tridecane, tetradecane, pentadecane and hexadecane) and diesel oil as sole carbon sources. However, cellular growth of yeast strains was inhibited on aromatic hydrocarbons (naphtalene, phenantere and pyren).

4.1.2 96-well microplate experiments

After the Petri plate screening, 6 yeast species were selected to a new screening in liquid medium, performed in 96-well microplates (Figure 4.1). The carbon source concentration was the same used on agar plates ($1\text{ g}\cdot\text{L}^{-1}$) and, once again, YNB without amino acids was used as control.

In general, yeast growth on media with hexadecane and phenolic compounds as carbon source was lower than that on YNB medium. *Candida utilis* CBS 621 growth on catechol and hexadecane was statistically equal to the growth on YNB (control, no carbon source). Tyrosol was inhibitory to yeast growth, but a considerable growth comparatively to the control was observed on phenol-based medium. According to Mikulášová et al. (1990), the inhibition dose of phenol for 100 % *C. utilis* growth inhibition was higher than $1.8\text{ g}\cdot\text{L}^{-1}$ after 24 hours. Hofmann et al. (1988) observed that *C. utilis* was not able to use phenol ($1\text{ g}\cdot\text{L}^{-1}$) and *n*-alkanes (octane and hexadecane) as substrate to grow.

All phenolic compounds had an inhibitory effect on growth of *P. pastoris* CBS 2612 and the yeast growth on hexadecane was statistically equal to that on YNB medium. In our best knowledge, there are no reports in the literature about the effect of hydrocarbons and phenolic compounds on cellular growth of non-genetically modified *P. pastoris* strains.

Candida cylindracea CBS 7869 growth was inhibited by the presence of catechol and phenol as sole carbon source. The addition of tyrosol and hexadecane to the medium slightly decreased the yeast growth comparatively to the control, but no statistical differences were found. According to Gonçalves et al. (2009), *C. cylindracea* CBS 7869 was able to grow on undiluted OMW, using 27 % of phenolic

compounds as carbon source. Until now, no works focused on *C. cylindracea* growth in hydrocarbons were published.

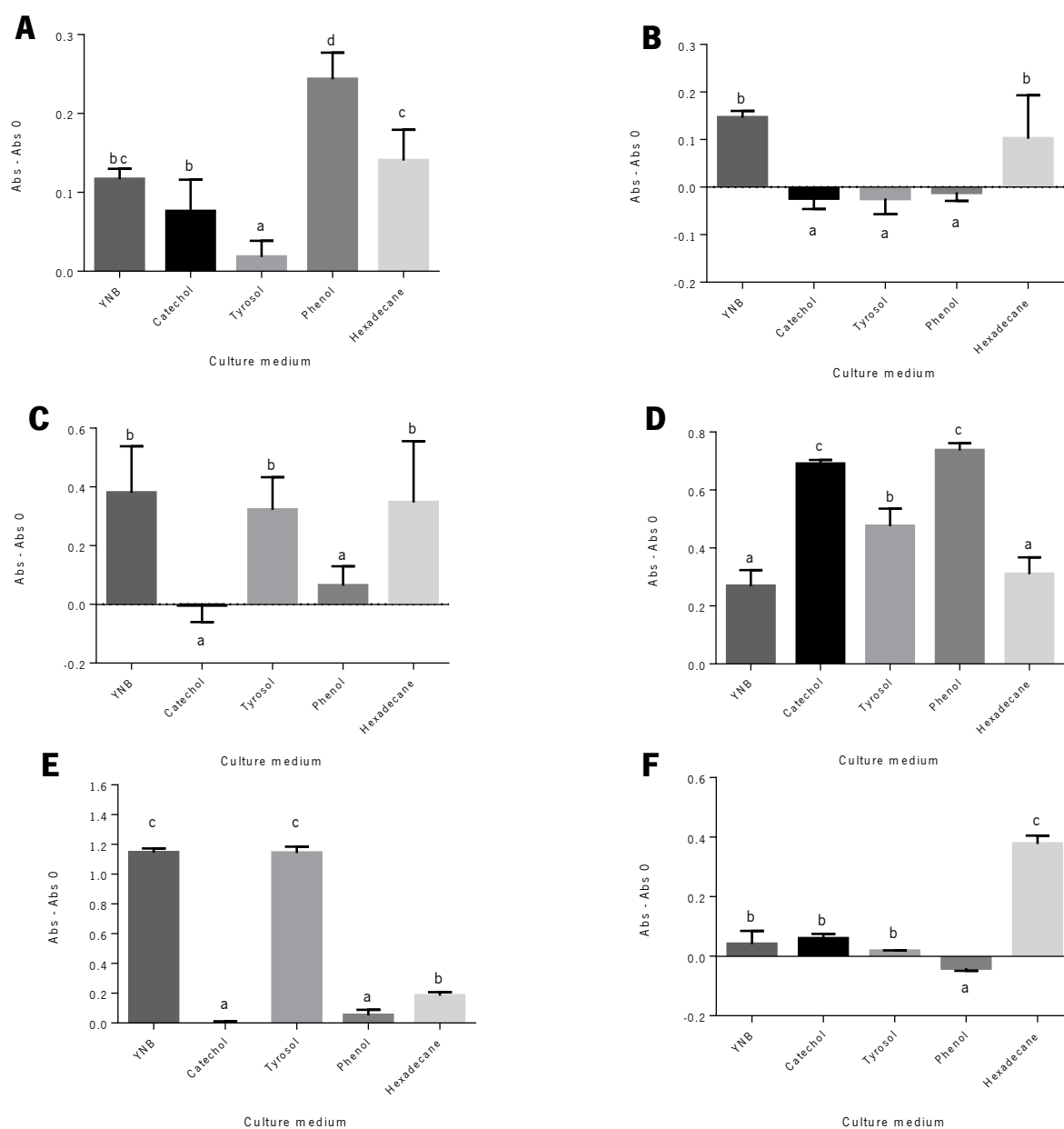


Figure 4.1 – Cellular growth of *Candida utilis* CBS 621 (**A**), *Pichia pastoris* CBS 2612 (**B**), *Candida cylindracea* CBS 7869 (**C**), *Candida tropicalis* ATCC 250 (**D**) *Yarrowia lipolytica* CBS 2075 (**E**) and *Yarrowia lipolytica* W29 (**F**) on YNB, hexadecane- and phenolic compounds-based media. The error bars represent the standard deviation of two independent replicates. Letters above each bar indicate the results of Tukey's test. Bars with the same letter do not present statistically significant differences ($p \leq 0.05$).

Candida tropicalis ATCC 250 demonstrated the ability to grow significantly on phenolic compounds-based medium, being the final biomass obtained in catechol, tyrosol and phenol statistically higher than that in YNB medium. Although yeast growth was not inhibited by hexadecane, no significant differences were found comparatively to the control. Rui-Ordaz et al. (2001) and Kumar et al. (2017) also reported that two *C. tropicalis* strains were able to grow on phenol as carbon source. Wang et al. (2012) concluded that *C. tropicalis* W1 cannot grow on 4-chlorophenol as the sole carbon source, but when cultivated in the mixture of phenol and 4-chlorophenol, the strain grew well. Schmitz et al. (2000) and Farag and Soliman (2011) observed that *C. tropicalis* strains were able to grow on tetradecane and crude petroleum as sole carbon and energy source.

The growth of *Y. lipolytica* CBS 2075 was strongly inhibited by the presence of catechol, phenol and hexadecane as carbon source, although the yeast growth on tyrosol had been statistically equal to that obtained in YNB medium. Contrariwise, *Y. lipolytica* W29 growth was inhibited by all phenolic compounds, but a significant growth on hexadecane was attained comparatively to the control (YNB medium). Despite *Y. lipolytica* W29 growth on hexadecane-agar plates had not been significant, this yeast was selected for liquid screening since it is reported by several authors its ability of degrading hydrocarbons (Fickers *et al.*, 2005; Lee *et al.*, 2000). In fact, the yeast growth on liquid medium was statistically higher than that obtained on YNB medium. Probably, the emulsion created by hexadecane and Tween 80 was more effective on liquid medium than on agar plates, being the carbon source more accessible to be used by the yeast. *Yarrowia lipolytica* is one of the most extensively studied “non-conventional” yeasts for degradation of hydrophobic substrates such as *n*-alkanes (Fickers *et al.*, 2005). According to the literature, this yeast is able to degrade crude oil and metabolize the hydrocarbons present on it (Hassanshahian *et al.*, 2012; Zinjarde and Pant, 2000). Zinjarde and Pant (2000) studied the crude oil degradation by *Y. lipolytica* NCIM 3589 and concluded that this strain degraded 78 % of the aliphatic fraction of crude oil in a period of 5 days. Hassanshahian et al. (2012) reported that two *Y. lipolytica* strains degraded 68 % and 58 % of crude oil, respectively, after 7 days.

In opposition to what was observed in these experiments, other authors have reported the ability of *Y. lipolytica* strains to use phenolic compounds as carbon sources and grow well on phenolic-rich effluents, such as OMW (Lopes *et al.*, 2009; Gonçalves *et al.*, 2009). Lopes et al. (2009) concluded that *Y. lipolytica* W29 and *Y. lipolytica* IMUFRJ 50682 were able to grow in OMW with 0.8 g·L⁻¹ of total phenols concentration. Gonçalves et al. (2009) observed that *Y. lipolytica* W29 growth was inhibited by catechol and caffeic acid, but not by tyrosol, hydroxytyrosol and oleuropein. Moreover, the yeast had the ability to grow significantly in OMW with approximately 10 g·L⁻¹ of total phenols concentration. Sarris et al. (2017)

reported that *Y. lipolytica* ACA-YC 5033 had the ability to grow significantly in OMW-based medium with an initial total phenols concentration up to 5.5 g·L⁻¹. Dourou et al. (2016) also observed the ability of *Y. lipolytica* A6 to grow in OMW-based medium with an initial phenolic compounds concentrations of 1.9 g·L⁻¹.

After the screening on liquid medium, carried out on 96-well microplates, it was concluded that three species have the potential to be used as degradation agents of effluents contaminated with phenolic compounds (*C. tropicalis* ATCC 250), phenol (*C. utilis* CBS 621) and hexadecane (*Y. lipolytica* W29), since they can grow on these pollutant compounds as sole carbon and energy source.

4.2 *Yarrowia lipolytica* growth in hydrocarbons-based media

4.2.1 96-well microplate experiments

Effluents of petrochemical industries contain several toxic contaminants such as aromatic hydroxyl compounds, hydrocarbons, grease, heavy metals, among others. Alkanes and alkenes, one example of hydrocarbons present in petroleum, are a high-energy compound and a variety of microorganisms have acquired the ability to assimilate these pollutants as sole carbon and energy source (Fukuda, 2013; Hassanshahian *et al.*, 2012).

In this work, it was evaluated the ability of *Y. lipolytica* W29 to grow on several concentrations of hexadecane and hexadecene as sole carbon source (Figure 4.2).

It was observed that *Y. lipolytica* W29 was able to grow on media with hexadecane and hexadecene up to 10 g·L⁻¹ as sole carbon source. However, cellular growth on hexadecene was substantially lower than on hexadecane. Aliphatic hydrocarbons (alkanes and alkenes) are more rapidly and easily degraded by microorganisms than aromatic hydrocarbons. Yet, alkenes (hexadecene) have a double bond between the carbon atoms, which makes its biodegradation by microorganisms more difficult (Leahy and Colwell, 1990).

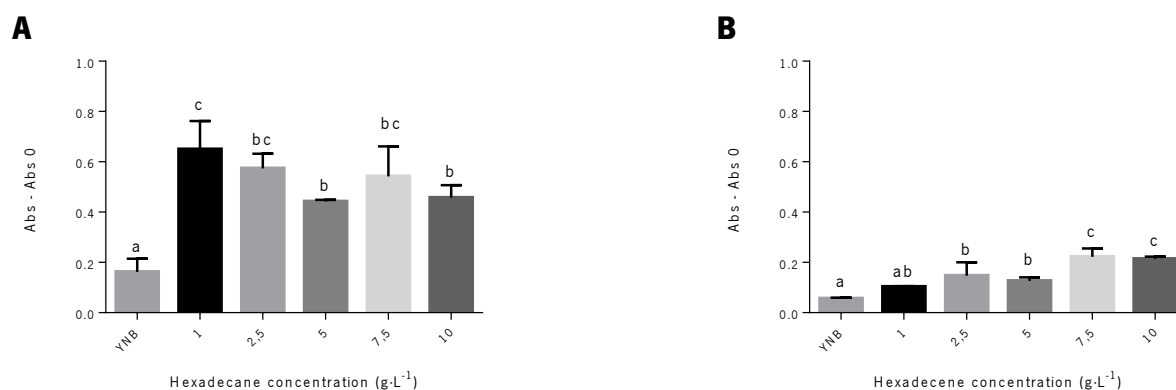


Figure 4.2 – *Yarrowia lipolytica* W29 growth on several concentrations of hexadecane (A) and hexadecene (B). YNB medium was used as control. The error bars represent the standard deviation of two independent replicates. Letters above each bar indicate the results of Tukey's test. Bars with the same letter do not present statistically significant differences ($p \leq 0.05$).

In literature, some authors reported that yeasts are good degraders of hydrocarbons. Nevertheless, there are still few studies that correlate yeasts and hydrocarbons assimilation, being the majority focused on bacteria and filamentous fungi. Hassanshahian et al. (2012) observed that two strains of *Y. lipolytica* were able to grow in crude oil-based medium, which is composed by several hydrocarbons. Bruyn (1954) reported *Candida lipolytica* was able to degrade double-stranded hydrocarbons (hexadecene), producing 1,2-hexadecanediol. However, only this study is found in the literature regarding the utilization of hexadecene by *Y. lipolytica* strains.

4.2.2 Erlenmeyer flask experiments

After the 96-well microplate experiments, in which several concentrations of hexadecane and hexadecene were tested, a scale-up was performed in 250-mL Erlenmeyer flasks. In addition to the experiments with hexadecane (10 g·L⁻¹) or hexadecene (7.5 g·L⁻¹) as sole carbon source, an experiment with a mixture of both hydrocarbons (5 g·L⁻¹ of each) was carried out (Figure 4.3A).

Yarrowia lipolytica is a strictly aerobic yeast, which means that dissolved oxygen concentration in the culture medium is an important factor for yeast growth and consequently for substrate consumption (Coelho *et al.*, 2010). Also, it is known that hydrocarbon biodegradation in wastewater can be limited by many factors, including oxygen, since this parameter may influence the assimilation of carbon sources by the microorganisms (Gargouri *et al.*, 2011). In order to study the effect of oxygen mass transfer on *Y. lipolytica* W29 growth and metabolites production from hydrocarbons, experiments raising the volume of

headspace were performed in 500-mL Erlenmeyer flasks (Figure 4.3B). In order to study the effect of the addition of carbon source (hexadecane) during the process, a new 500-mL Erlenmeyer flask experiment was performed, in which pulses of fresh medium (hexadecane 10 g·L⁻¹ and YNB 6.7 g·L⁻¹) were added after 72 hours and 144 hours (repeated batch, Table 4.2).

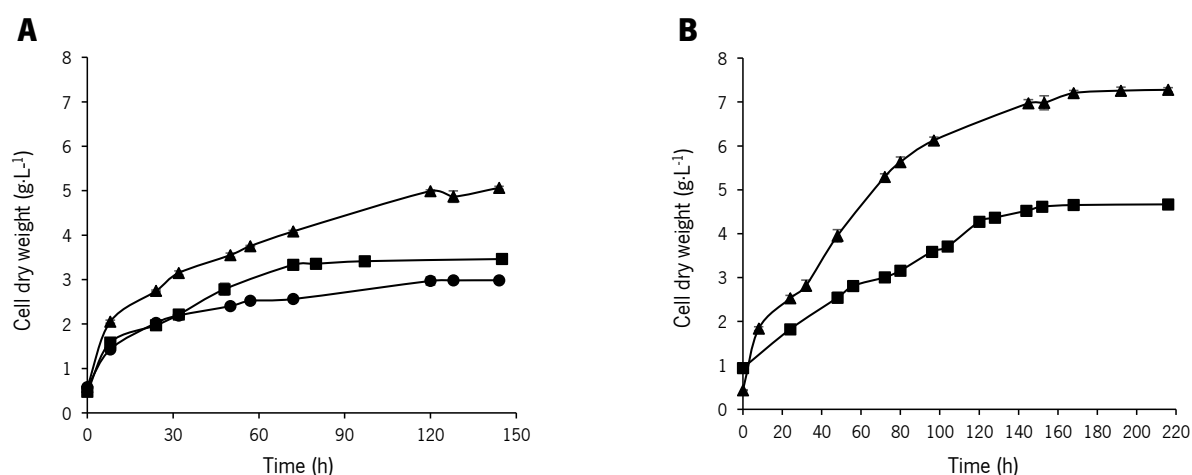


Figure 4.3 – Growth profile of *Y. lipolytica* W29 obtained in experiments carried out in 250-mL Erlenmeyer flasks (A) and 500-mL Erlenmeyer flasks (B) with different hydrocarbons: hexadecane 10 g·L⁻¹ (■), hexadecene 7.5 g·L⁻¹ (●) and mixture of hexadecane 5 g·L⁻¹ and hexadecene 5 g·L⁻¹ (▲). The error bars represent the standard deviation for two independent replicates.

As observed in microplate experiments, also in Erlenmeyer flasks *Y. lipolytica* W29 was able to grow with 10 g·L⁻¹ of hexadecane and 7.5 g·L⁻¹ of hexadecene. Moreover, the use of both hydrocarbons as carbon source led to an increase of final biomass concentration (Figure 4.3A). A 1.5- and 1.7-fold improvement on cellular growth was obtained in the mixture of hydrocarbons comparatively to the experiments performed with hexadecane and hexadecene, respectively. By contrast, Csutak et al. (2017) observed that *C. tropicalis* growth in a complex and real mixture of hydrocarbons (petroleum) was lower than that obtained with pure hydrocarbons. A cellular concentration of 1×10⁸ cells·mL⁻¹ and 0.5×10⁶ cells·mL⁻¹ was attained with hexadecane and petroleum, respectively.

The increase of oxygen mass transfer, by raising the volume of Erlenmeyer headspace, had a clearly positive effect on cellular growth, regardless of carbon source used (Figure 4.3B). The final biomass concentration attained in these experiments was 30 % and 26 % higher than that obtained in 250-mL Erlenmeyer experiments, respectively for the mixture of hydrocarbons and hexadecane.

In 250-mL Erlenmeyer flask experiments, the highest biomass yield was obtained in cultures with a mixture of hydrocarbons, followed by hexadecene and hexadecane-based media (Table 4.2). The

increase of oxygen mass transfer, by raising the volume of flask headspace, led to an enhancement of biomass yield, particularly in hexadecane experiments. A 1.7- and 1.2-fold improvement on biomass yield was attained increasing headspace volume, respectively in cultures with hexadecane and mixture of hydrocarbons.

Table 4.2 – Values of maximum biomass ($X_{\max}$), reduction of chemical oxygen demand (COD) and biomass per mass of substrate ($Y_{x/s}$) obtained in *Y. lipolytica* W29 cultures carried out in 250- and 500-mL Erlenmeyer flasks with different hydrocarbons as carbon source. Data are average and standard deviation of two independent replicates.

Hydrocarbon	Flask capacity (mL)	$X_{\max}$ (g·L ⁻¹)	Reduction of total COD (%)	$Y_{x/s}$ (g·g ⁻¹)
Hexadecene	250	2.99 ± 0.01	79.0 ± 1.6	0.40 ± 0.04
Hexadecane	250	3.53 ± 0.05	93.6 ± 2.1	0.33 ± 0.05
	500	4.66 ± 0.03	93.9 ± 1.8	0.45 ± 0.05
Mixture	250	5.07 ± 0.04	71.0 ± 1.5	0.63 ± 0.03
	500	7.27 ± 0.05	93.9 ± 2.6	0.73 ± 0.02
Hexadecane Repeated batch	500	8.14 ± 0.33	85.1 ± 1.9	0.91 ± 0.04

Although some articles report that *Y. lipolytica* grows on hydrocarbon-based media, the maximum biomass concentration is lower than that found in this work. According to Hassanshahian et al. (2012), two strains of *Y. lipolytica* grown in crude oil-based medium at an optical density up to 1.06 and 1.04.

In this work, biomass yield was determined by grams of biomass produced per mass of substrate consumed ($Y_{x/s}$). The substrate (hexadecane and hexadecene) consumption was measured by the chemical oxygen demand (COD). It should be stressed out that the initial COD value is mainly due to hydrocarbons, since the other component of medium (YNB without aminoacids) has a very low COD value (140 mg·L⁻¹). In all experiments, more than 70 % of hydrocarbons were consumed by *Y. lipolytica* W29 (Table 4.2). It is worth to notice that hexadecane consumption was significantly higher than hexadecene consumption, corroborating the results of cellular growth obtained in 96-well microplate experiments (Figure 4.2). The raise of headspace volume, which enhances oxygen transfer from the gas phase to the liquid medium, had a clear positive effect on hydrocarbons consumption when hexadecane and hexadecene were mixed in the medium. A 23 % increase of COD reduction was attained in this experiment, compared to that obtained in 250-mL Erlenmeyer flask experiments. It should be noted that

the values of hydrocarbons consumption obtained herein were comparable or even higher than others found in the literature. Zinjarde and Pant (2000) reported that *Y. lipolytica* NCIM 3589, isolated from contaminated sea water, was able to degrade 78 % of the aliphatic fraction of crude oil in 5 days. Moreover, this strain was also able to degrade pure alkanes, namely hexadecane (60 %), tetradecane (50 %), octadecane (45 %), decane (40 %) and dodecane (40%) after 24 h of culture.

Since *Y. lipolytica* is a strictly aerobic yeast, the amount of oxygen available in culture medium is an important parameter that affects cellular growth and substrates consumption. According to Lopes et al. (2009), the increase of oxygen transfer rate, by raising the total air pressure, led to an enhancement of *Y. lipolytica* growth and glucose consumption. Papanikolaou et al. (2007) also observed that higher oxygenation, owing to higher agitation rate in bioreactor, significantly enhanced the biomass concentration of *Y. lipolytica* ACA-DC 50109. Hydrocarbons biodegradation under aerobic conditions is strongly related to the oxygen concentration in the culture medium and/or in the gas phase. Volke-Sepúlveda et al. (2002) reported that one reason for the incomplete biodegradation of hexadecane by fungi in liquid submerged fermentation may be the limitation of oxygen. Ferreira et al. (2012) tested a wide range of stirring rates (70 rpm – 250 rpm) in crude oil biodegradation by *Y. lipolytica* and concluded higher stirrings lead to an increase of hydrocarbons consumption. Ferreira et al. (2016) observed that higher volumetric oxygen mass transfer coefficient ($k_L a$) values promote higher maximum growth and crude glycerol consumption rates by *Y. lipolytica*.

One factor that may affect cellular growth and metabolites production from hydrocarbons, as well the hydrocarbons degradation, is the carbon source concentration in the culture medium. It was observed in the previous experiments that hexadecane was totally consumed before the end of the process. Thus, yeast growth and metabolites production may be limited by carbon source availability. Highest biomass concentration ($8.1 \text{ g}\cdot\text{L}^{-1}$) was achieved in repeated batch experiment, probably due the addition of carbon source two times, avoiding substrate limitation. A 1.7- and 1.8-fold improvement in final biomass concentration and biomass yield, respectively, were obtained in repeated batch mode comparatively to the batch experiment. It should also be noted that, despite higher hexadecane concentration was added to the medium ($30 \text{ g}\cdot\text{L}^{-1}$ in the total), yeast cells were able to degrade up to 85 % of COD. Thus, repeated batch mode, could be a strategy to improve *Y. lipolytica* W29 growth from hexadecane without an inhibitory effect of excess of carbon source.

The non-conventional dimorphic yeast *Y. lipolytica* has been efficiently cultivated on various hydrophobic substances producing many extra- or intracellular metabolites of industrial significance

(Papanikolaou *et al.*, 2007). The metabolites produced during these experiments (lipase and microbial lipids) were also quantified, as showed in Table 4.3.

Table 4.3 – Values of maximum lipase, microbial lipids content and microbial lipids concentration obtained by *Y. lipolytica* W29 in experiments carried out in 250- and 500-mL Erlenmeyer flasks. Data are average and standard deviation of two independent replicates.

Hydrocarbon	Flask capacity (mL)	Lipase (U·L ⁻¹)	Microbial lipids (% w/w)	Lipids concentration (g·L ⁻¹)
Hexadecene	250	610 ± 112	9.9 ± 1.4	0.30 ± 0.04
Hexadecane	250	1260 ± 125	8.7 ± 0.7	0.31 ± 0.03
	500	2730 ± 304	15.1 ± 0.1	0.707 ± 0.001
Mixture	250	567 ± 134	8.5 ± 0.1	0.431 ± 0.003
	500	868 ± 270	6.6 ± 0.1	0.48 ± 0.01
Repeated batch	500	1602 ± 97	14.1 ± 1.2	1.20 ± 0.20

Yarrowia lipolytica demonstrated that has the ability to produce lipase and accumulate lipids intracellularly from hexadecene and hexadecane as sole carbon and energy source. In these experiments the maximum lipase activity was obtained at 32 h and 24 h in the experiments carried out in 250-mL and 500-mL Erlenmeyer flasks, respectively. Regardless of hydrocarbon used and flask volume, the profile of lipase production was always the same, reaching a peak after which the production decayed (Figure 4.4). Profile of lipase production by *Y. lipolytica* is dependent on yeast strain and culture conditions, especially on the carbon sources used (Lopes *et al.*, 2018a). Lopes *et al.* (2018b) observed that at pH 5.6 and 6.5, lipase activity reached a maximum value after 9 h of cultivation, diminishing after that time. Also others authors reported that when *Y. lipolytica* strains grew on stearin (Papanikolaou *et al.*, 2007) or pure olive oil (Lopes *et al.*, 2008), lipase production reached a maximum value and decreased during the stationary growth phase.

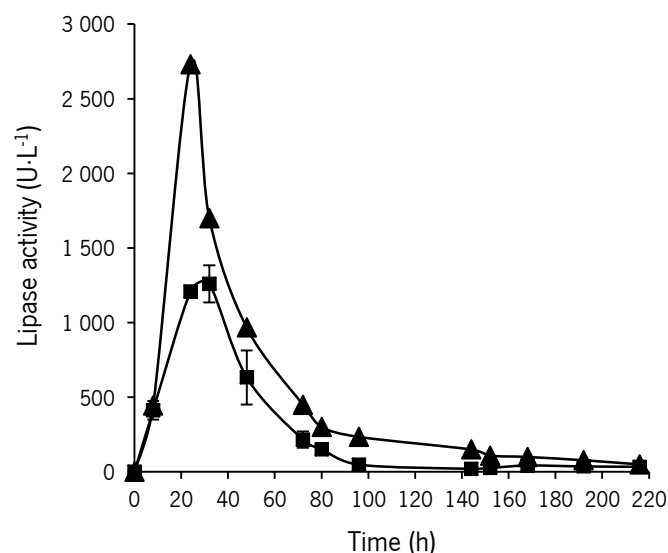


Figure 4.4 – Example of lipase activity profiles obtained in *Y. lipolytica* cultures growing in hexadecane and carried out in 250-mL Erlenmeyer flasks (■) and 500-mL Erlenmeyer flasks (▲). The error bars represent the standard deviation for two independent replicates.

Regardless of Erlenmeyer flask volume, the maximum value of lipase activity was obtained with hexadecane as sole carbon source (2 times more than that attained with hexadecene and mixture of both hydrocarbons). Furthermore, lipase activity was significantly enhanced with the raise of oxygen transfer from the gas phase to the culture medium, as demonstrated by the results obtained in the 500-mL Erlenmeyer flasks. A 2.2- and 1.5-fold improvement in lipase activity was obtained in hexadecane and mixture experiments, respectively, compared to 250-mL flask experiments. Tan et al. (2003) concluded that the raise of dissolved oxygen concentration in the medium significantly enhanced the lipolytic activity by *Candida* sp. 90-17. Lopes et al. (2007) also demonstrated that the increase of oxygen solubility by raising total air pressure led to an enhancement of lipase production by *Y. lipolytica* W29. Fickers et al. (2009) observed that lipase production by mutant *Y. lipolytica* LgX64.81 was enhanced by the increase of dissolved oxygen concentration.

Although in repeated batch mode higher biomass concentration was obtained compared to batch mode experiment, lipase production was not favored by this mode of operation. In this experiment, the maximum lipase activity was 1.7 times lower than that obtained in hexadecane batch culture. According to Espinosa et al. (1990), lipase production decreased as carbon source concentration increased in the culture medium. This could be the reason why lower lipase activity was obtained in repeated batch experiments, since higher hexadecane was added to the medium (30 g·L⁻¹ in the total).

According to literature, there are other microbial species able to produce lipase from hydrocarbons. Mahmoud et al. (2015) reported that fungus *Aspergillus terreus* can produce lipase when it grows on crude oil polluted soils. Boekema et al. (2007) studied the lipase production by *Burkholderia glumae* from hexadecane and concluded that when 10 (% v/v) of hexadecane was included in the growth medium, lipolytic activity increased 7-fold after 24 h of growth.

Beyond to the hydrocarbons as carbon source, these media also contained Tween 80 with the aim to create a stable emulsion, allowing a better dispersion of the carbon source in the medium. According to some authors, the use of Tween 80 in hydrocarbon media favors the production of lipase. Boekema et al. (2007) related that lipase production could be improved by including additives, such as Tween 80, in the growth medium.

Microscopic observations of *Y. lipolytica* W29 cells cultivated in medium with hydrocarbons revealed that the presence of these hydrophobic substrates and/or increase of oxygen transfer (by raising headspace volume) did not induced hyphae formation and oval form was observed in all experiments (Figure 4.5A). In addition, the presence of lipid bodies inside the yeast cells can be observed after Nile Red staining (Figure 4.5B).

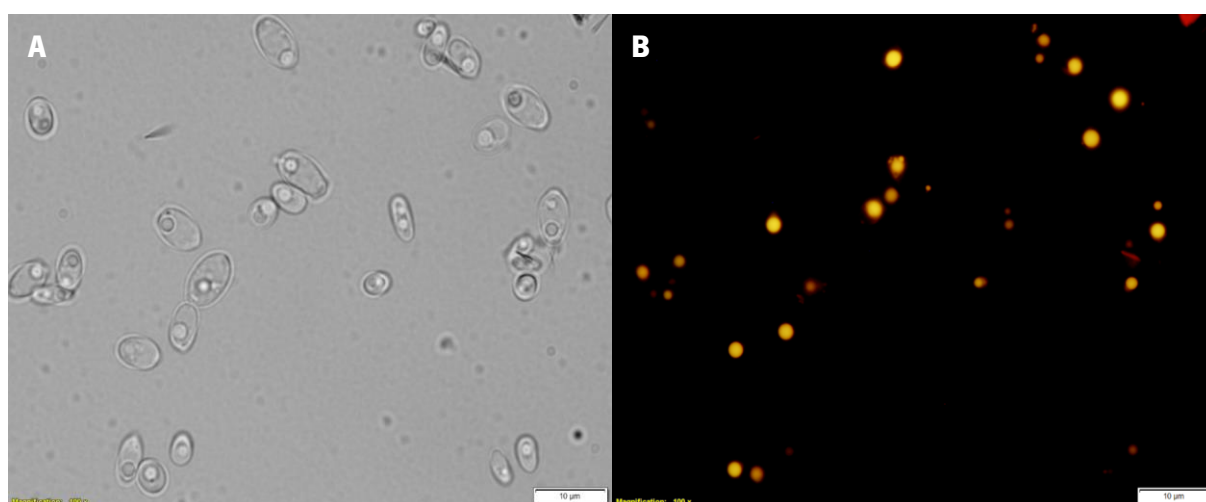


Figure 4.5 – Light microscopy image (A) and fluorescence microscopy image after staining with Nile Red (B) of *Y. lipolytica* W29 cells with intracellular lipid bodies in the cytoplasm after 216 h of growth in 10 g·L⁻¹ of hexadecane in 500-mL Erlenmeyer flask (magnification 1000×).

In 250-mL Erlenmeyer flask experiments, there are no statistically significant differences in cultures with hexadecene and hexadecane as sole carbon source, as well in mixture of hydrocarbons. However, as the final biomass concentration in hexadecene experiments was lower than that of hexadecane cultures, the intracellular lipids concentration (gram of lipids per liter of culture medium)

obtained in this condition was lower. This can be a factor to take into account and to be optimized in the future in order to favor the production of intracellular lipids from hexadecene as sole carbon source. By contrast, as the biomass concentration was higher in mixture experiments, the concentration of intracellular lipids was higher in this condition, comparatively to the experiments with sole hexadecane and hexadecene. A 1.4-fold improvement on lipids concentration was obtained using both hydrocarbons simultaneously.

The raise of headspace volume clearly favored the microbial lipids accumulation by yeast cells growing in hexadecane-based medium. Approximately 2-fold improvement in lipid content and lipid concentration was obtained comparatively with the 250-mL flask experiments. However, in the case of both hydrocarbons were used as carbon source (mixture experiments), the increase of headspace led to a decrease of lipid content. Yet, as the final biomass concentration in 500-mL flask experiments was significantly higher, the intracellular lipids concentration was also enhanced by the raise of headspace volume. According to Tai and Stephanopoulos (2013), fermentations highly aerated favored the lipid accumulation by *Y. lipolytica* mutants. Bellou et al. (2014) also observed that *Y. lipolytica* ACA- DC 50109 accumulated high lipid content in medium with high dissolved oxygen concentrations.

Regarding to repeated batch experiment, the lipid content was statistically equal to that obtained in 500-mL batch experiment. However, the intracellular lipids concentration in repeated batch experiment was 1.7 times higher, due to the higher biomass concentration attained. Although some authors report that lipid accumulation is favored by excess of carbon and limitation of nitrogen (Ageitos *et al.*, 2011; Papanikolaou and Aggelis, 2011), in this experiment the nitrogen was not totally limited since the fresh medium add was composed by hexadecane and YNB. According to Papanikolaou and Aggelis (2011), oleaginous microorganisms consume their own storage lipids when their metabolic abilities cannot be saturated by the extracellular carbon source. However in this experiment, due to high hexadecane concentration added to the medium (30 g·L⁻¹ in the total), the cells did not have to consume the intracellularly accumulated lipids.

The lipid accumulation by yeasts grown in hydrocarbons is already described in literature. To our best knowledge, only one recent study described the formation of lipid droplets. Csutak et al. (2017) reported that numerous lipid droplets were observed inside *C. tropicalis* CMGB114 cells growing in hexadecane and petroleum. However, contrary to this, only in older studies lipid content was quantified. The lipid content observed in this present work is comparable and even higher than these studies. Mishina et al. (1976) reported that *C. lipolytica* cells accumulated 4 % (w/w) of intracellular lipids from a mixture of *n*-alkanes (decane, undecane, dodecane and tridecane). Thorpe et al. (1972) observed that *C. tropicalis*

growing in individual *n*-alkanes (dodecane, tridecane, tetradecane, pentadecane and hexadecane) as carbon source, accumulated up to 17.9 % (w/w) as intracellular lipids.

In lipid production by oleaginous microorganisms, two distinct metabolic pathways are involved: *ex novo* lipid accumulation (from hydrophobic substrates) and *de novo* lipid synthesis (from hydrophilic substrates) (Lopes *et al.*, 2018a). The oleaginous yeasts growing on various hydrophobic materials (e.g. oils, fats, free-fatty acids), uptake TAGs and fatty acids from the culture medium and accumulate it in an unchanged or modified form. In the other hand, the *de novo* synthesis from hydrophilic substrates (e.g. glucose or glycerol) involves the production of fatty acid precursors, such as acetyl and malonyl-CoA and their integration in lipid storage biosynthesis (Aggelis *et al.*, 1997; Gonçalves *et al.*, 2013; Papanikolaou and Aggelis, 2011). Long chain fatty acids composition of microbial lipids accumulated by *Y. lipolytica* cells from hexadecane and hexadecene is show in Table 4.4.

Table 4.4 – Fatty acids composition of microbial lipids accumulated by *Y. lipolytica* W29 cells from hydrocarbons. Data are average and standard deviation of two independent replicates.

Hydrocarbon	Flask (mL)	Long chain fatty acids (% w/w)				
		Palmitic (C16:0)	Palmitoleic (C16:1)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)
Hexadecene	250	14.0 ± 1.1 ^f	—	19.1 ± 2.3 ^g	32.4 ± 2.7 ^h	34.4 ± 0.7 ^h
Hexadecane	250	12.1 ± 1.4 ^{ef}	3.8 ± 0.8 ^{ab}	6.4 ± 0.1 ^{bcd}	48.3 ± 2.9 ^a	29.3 ± 0.7 ⁱ
	500	10.9 ± 0.2 ^{ef}	3.1 ± 0.2 ^a	5.1 ± 1.8 ^{abc}	47.8 ± 0.8 ^a	33.1 ± 1.1 ⁱ
Mixture	250	12.3 ± 0.7 ^f	—	12.3 ± 3.4 ^f	37.2 ± 3.6 ^{lmn}	38.3 ± 0.8 ^{mn}
	500	11.6 ± 0.5 ^{ef}	—	11.9 ± 0.1 ^{ef}	36.5 ± 0.7 ^{lm}	40.1 ± 0.1 ⁿ
Repeated batch	500	23.6 ± 2.6 ⁿ	7.2 ± 0.1 ^{cd}	14.0 ± 0.1 ^f	46.3 ± 1.1 ^o	9.0 ± 1.4 ^{de}

^{a-o} Values followed by the same letter do not present statistically significant differences ($p \leq 0.05$).

— Not detected

In this work, when hexadecane were used as carbon source, lipids accumulated by *Y. lipolytica* W29 were composed by negligible amounts of stearic acid (5 % - 6 %) and palmitoleic acid (3 % - 4 %) and considerable percentages of linoleic (25 % - 35 %) and oleic (45 % - 50 %) acids. It should be stressed out

that, only with hexadecane, yeast cells were able to accumulate palmitoleic acid. The saturated fraction of lipids accumulated from hexadecane was, respectively, 18.5 % and 16 % in 250-mL and 500-mL Erlenmeyer flask experiments. The unsaturated fraction largely exceeded the saturated one, and was 81.5 % and 84 % in 250-mL and 500-mL Erlenmeyer flask experiments, respectively. Interestingly, in repeated batch experiment, which was carried out in 500-mL Erlenmeyer flasks, the saturated fraction (37.6 %) was 2.4 times higher than that obtained with hexadecane batch experiments in the same flasks capacity. Probably due to a higher concentration of carbon and nitrogen sources in the culture medium.

This enhancement of saturated fraction was due to the significant increase of palmitic and stearic acids content and the remarkable decrease of linoleic acids. In fact, linoleic acid in repeated batch experiments was approximately 4 times lower than in batch mode. In the experiments with hexadecene as sole carbon source, lipids accumulated by yeast cells were considerably more saturated (33 % of saturation) than those accumulated from hexadecane. Yet, these lipids were also mainly composed by oleic and linoleic acids. In the experiments with the mixture of both hydrocarbons, oleic and linoleic acids were also the main fatty acids accumulated. The saturated fraction varied between 24.6 % (250-mL flask experiments) and 23.5 % (500-mL flask experiments) and the unsaturated one ranged from 75.4 % (250-mL flask experiments) and 76.5 % (500-mL flask experiments). From these results, it was observed that unsaturated fraction of microbial lipids accumulated by *Y. lipolytica* W29 cells largely exceeded the saturated one, independently of hydrocarbon used as carbon source. Additionally, the increase of oxygen transfer by raising the headspace volume, led to an enhancement of unsaturated fraction, particularly when hexadecane is used as sole carbon source.

Yarrowia lipolytica accumulates lipids with a different fatty acids composition, depending on medium conditions, i.e. it is possible to manipulate the operational conditions and culture medium composition to obtain the desired lipids. In the literature, there are few works demonstrating that different *Y. lipolytica* strains growing in hydrocarbons can accumulate distinctly fatty acids. Klug and Markovetz (1967) reported that *C. lipolytica* cells growing in hexadecane, accumulated lipids rich in palmitic acid (30 %), followed by oleic acid (25 %), palmitoleic acid (20 %) and stearic acid (10 %). However, with 1-hexadecene as carbon source, the highest percentage of fatty acids belonged to linoleic acid (36.1 %), followed by oleic acid (9.6 %), and the remaining percentages of others fatty acids were less than 9 %. Thorpe and Ratledge (1972) also studied the fatty acid distribution in triglycerides of *Candida* 107 grown on hexadecane and concluded that these lipids were mainly unsaturated (74 %).

The results reported herein demonstrated long chain fatty acids profile of lipids accumulated by *Y. lipolytica* W29 cells could be manipulated by changing the hydrocarbon used as carbons source, as well

the headspace volume of Erlenmeyer flask. Since the microbial lipids obtained in this work have a high content of unsaturated fatty acids, allow them to be used as an excellent source of food supplement. Particular those lipids rich in linoleic acid (omega-6 family), which is considered an essential fatty acid and cannot be synthesized by mammals and must be ingested through diet (Bélignon *et al.*, 2016). In addition, is it noteworthy the valorization of pollutant compounds presents in environment by producing microbial lipids that can be used for animal feedstock and biodiesel production. Once that hydrocarbons have undesired substances that could be transfer to the human body, will be more sustainable the production of biodiesel from lipids produced with this pollutant compounds. Biodiesel is mainly produced by a transesterification reaction with vegetable oils (edible or non-edible), waste cooking oils and animal fats. However, due to global food securities, oil derived from food sources cannot fulfill the requirement for biodiesel production in large scale (Patel *et al.*, 2016). So, researchers are looking for novel oil resources to produce biodiesel. Among various resources, lipid produced by microorganisms, involving yeasts, known as single cell oil (SCOs) are considered as promising feed-stock for biodiesel production due to its similarity with vegetable oils in fatty acid compositions. The advantages of oleaginous yeast culture is the independence of weather conditions and arable land that is more prone in the case of plants, faster growth rate, shorter life cycle and easier scale-up (Leiva-Candia *et al.*, 2014; Patel *et al.*, 2016; Rakicka *et al.*, 2015).

4.3 *Candida tropicalis* growth in olive mil wastewater-based media

4.3.1 96-well microplate experiments

Olive oil production is a traditional agricultural industry in Mediterranean countries, that accounts for about 95 % of the world production and among which Portugal is one of the ten major producers (Araujo *et al.*, 2004). The manufacturing process of olive oil in three-phase centrifugation system generates a black liquid effluent called olive mill wastewater.

Its composition presents a large diversity depending on various parameters such as the variety of olives and their maturity, the region of origin, and especially the technology used for oil extraction (Dourou *et al.*, 2016). From a biotechnological standpoint olive mill wastewater (OMW), containing various carbon sources, organic compounds and minerals, would be considered as an exploitable resource that could be valorized as substrate in various bioprocesses (Lanciotti *et al.*, 2005). Actually, several microorganisms have been proved able to grow on OMW and produce various high-added value products

and several investigations have been carried out using microorganisms capable of growing aerobically on OMW in order to reduce the initial organic load and the phenolic content (Dourou *et al.*, 2016; Fadil *et al.*, 2003).

In this work, the ability of *C. tropicalis* ATCC 250 to grow on several concentrations of olive mill wastewater as sole carbon source was evaluated (Figure 4.6).

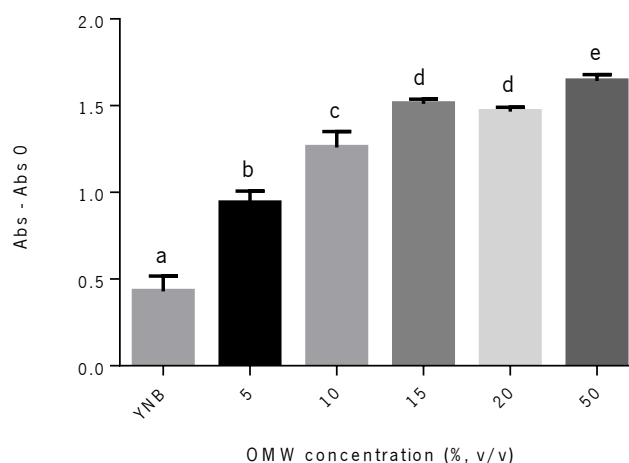


Figure 4.6 – *Candida tropicalis* ATCC 250 growth on several concentrations of olive mill wastewater. YNB medium was used as control. The error bars represent the standard deviation of two independent replicates. Letters above each bar indicate the results of Tukey's test. Bars with the same letter do not present statistically significant differences ($p \leq 0.05$).

It was observed that *C. tropicalis* ATCC 250 was able to grow on diluted OMW up to 50 % (v/v) as substrate. In fact, in this condition, a 3-fold improvement on cellular growth was obtained compared to the control. Until now, there are few studies regarding the growth of *C. tropicalis* strains in OMW. *Candida tropicalis* isolated from Moroccan OMW shown the ability to grow on diluted OMW and use monophenols and polyphenols as carbon source (Ettayebi *et al.*, 2003). Fadil *et al.* (2003) also reported the ability of a strain of *C. tropicalis* isolated from OMW to grow on diluted OMW-based medium and degrade polyphenols. Dourou *et al.* (2016) observed that *C. tropicalis* LFMB 16 grew in diluted OMW supplemented with glucose up to a maximum biomass of $2.6 \text{ g}\cdot\text{L}^{-1}$ in Erlenmeyer flasks.

4.3.2 Erlenmeyer flask experiments

After the 96-well microplate experiments, in which several concentrations of OMW were tested, a preliminary scale-up was performed in 250-mL Erlenmeyer flasks (Figure 4.7). Besides the experiment

with OMW (50 %, v/v), an experiment with 100 % of OMW was carried out, since no growth inhibition was observed in the experiment with 50 % of OMW in microplate experiments. Additionally, it was tested the effect of no with a nitrogen source (YNB). Throughout this experiment was also quantified the consumption of reducing sugars and total phenols.

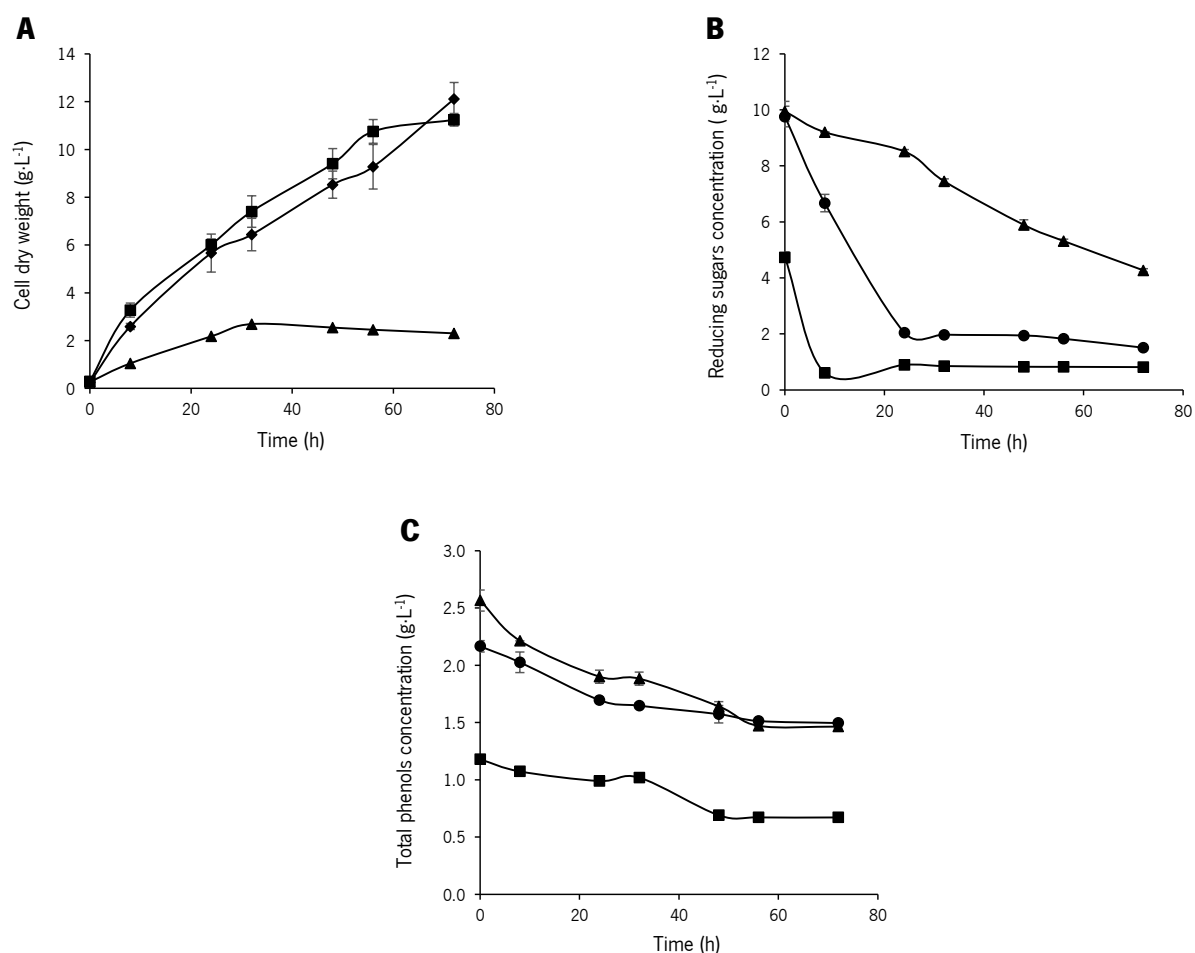


Figure 4.7– Profiles of *C. tropicalis* ATCC 250 growth (**A**), reducing sugars consumption (**B**) and total phenols consumption (**C**) obtained in experiments carried out in 250-mL Erlenmeyer flasks: 50 % OMW supplemented with YNB (■); 100 % OMW supplemented with YNB (●) and 100 % OMW without YNB (▲). The error bars represent the standard deviation for two independent replicates.

As observed in microplate experiments, also in Erlenmeyer flasks *C. tropicalis* ATCC 250 was able to grow with 50 % of OMW. Additionally, the yeast had also the ability to grow on undiluted OMW, demonstrating that there was no growth inhibition by OMW components, particularly phenolic compounds. It was also proved that the supplementation of these OMWs with a nitrogen source is crucial, since the yeast growth on OMW without YNB was very low (Figure 4.7A). In fact, a 5.3-fold improvement on cellular growth was obtained in supplemented OMW 100 % comparatively to the experiments

performed without supplementation (OMW 100 % without YNB). In the medium with diluted OMW (50 %), *C. tropicalis* consumed almost all of reducing sugars and total phenols, corresponding to an 82.8 % and 43 % of reduction (Table 4.5), respectively. In experiments with undiluted OMW, a significant difference in reducing sugars consumption was obtained between the supplemented and non-supplemented experiments. A 84.5 % of reducing sugars consumption was obtained in OMW supplemented medium, while only 57.1 % was attained in non-supplemented medium. Regarding the total phenols consumption, no significant differences were obtained between the experiments with and without supplementation.

Table 4.5 – Values of maximum biomass (X_{max}) and reduction of COD, reducing sugars and total phenols obtained in *C. tropicalis* ATCC 250 preliminary cultures carried out in 250-mL Erlenmeyer flasks. Data are average and standard deviation of two independent replicates.

OMW (% v/v)	X_{max} (g·L ⁻¹)	Reduction of COD (%)	Reduction of reducing sugars (%)	Reduction of total phenols (%)
50 with YNB	11.2 ± 0.3	35.6 ± 1.1	43.0 ± 1.7	82.8 ± 0.5
100 without YNB	2.3 ± 0.3	4.6 ± 0.5	42.9 ± 1.0	57.1 ± 0.8
100 with YNB	12.1 ± 0.7	23.7 ± 1.6	30.9 ± 1.8	84.5 ± 0.6

Some authors have described yeast *C. tropicalis* as a microorganism with high efficiency to remove phenolic compounds from effluents (Abdelhadi *et al.*, 2010; Kumar *et al.*, 2017). According to literature, few authors investigated the ability of *C. tropicalis* to grow on OMW. Moreover, these studies were carried out with diluted OMW and supplemented with other carbon sources (Ettayebi *et al.*, 2003; Fadil *et al.*, 2003). In this work, it was proved that *C. tropicalis* ATCC 250 was able to grow on undiluted OMW and without the addition of other carbon sources.

COD is often used as a measurement of organic load in wastewater and effluents (APHA, 1998). Besides quantifying the consumption of total phenols and reducing sugars, in this work also the reduction of chemical oxygen demand (COD) was measured, since OMW is composed by other organic compounds, such as lipids. Moreover, COD is a parameter that must be taken into account before the discharge of these effluents into the environment. The highest reduction of COD was observed in 50% OMW-based medium, probably because this medium was diluted, and was easily to the yeast degrade the organic load. Comparing the undiluted media, COD reduction was higher in supplemented medium (5.2 times higher than in non-supplemented medium). Ettayebi *et al.* (2003) studied the decontamination of OMW

by *C. tropicalis* YMEC14 cells during 24 h and observed that 12 % and 39 % of total phenols and COD, respectively, were consumed. Likewise, Fadil et al. (2001) studied polyphenol biodegradation by *C. tropicalis* in diluted OMW and concluded that 54 %, 58 % and 85 % of COD, total phenols and reducing sugars, respectively, were degraded. Martinez-Garcia et al. (2007) reported a 62 % of COD removal in OMW pretreated aerobically by *C. tropicalis* and supplemented with cheese whey. Bevilacqua et al. (2009) reported that *C. tropicalis* can reduce up to 52 % of phenol present in OMW.

Normally, OMW is a nitrogen-rich wastewater and, for this reason, many authors state that the addition of an external nitrogen source is not required. Fezzani and Cheikh (2007) concluded that OMW could be degraded successfully in co-digestion with olive mill solid wastes under thermophilic conditions without previous dilution and without addition of chemical nitrogen substances. Herrero et al. (2008) also observed that the highest values of cellular biomass of *R. opacus* PD630 growing on OMW were obtained without the addition of mineral salts and a nitrogen source (NH_4Cl). However, in this work, nitrogen addition proved to be essential to yeast growth and reducing sugars consumption. This is maybe due to the low amount of total nitrogen that this OMW have (Table 3.2, Materials and Methods section). Likewise, Angelidaki et al. (2002) found that OMW-based medium required the addition of nitrogen for the anaerobic treatment.

Candida tropicalis has been studied by its ability to metabolize phenolic compounds as the only carbon and energy source (Tuah *et al.*, 2009). It was observed, in the preliminary experiments described above, that *C. tropicalis* ATCC 250 was not able to consume all total phenols during the 72 hours of the bioprocess. Thus, additional experiments with undiluted OMW supplemented with YNB were carried out during 216 hours in 250-mL Erlenmeyer flasks. Moreover, *Candida tropicalis* is an aerobic yeast, meaning that dissolved oxygen concentration in the culture medium is an important factor for yeast growth and substrate consumption. Besides that, it is known that phenolic compounds biodegradation presents in OMW can be limited by many factors, including oxygen, since this parameter may influence the assimilation of carbon sources by the microorganisms (Krastanov *et al.*, 2013; Rucká *et al.*, 2017). In order to study the effect of oxygen mass transfer on *C. tropicalis* ATCC 250 growth, carbon sources consumption and metabolites production from OMW, experiments with a higher ratio of headspace volume/medium volume were performed in 500-mL Erlenmeyer flasks (Figure 4.8).

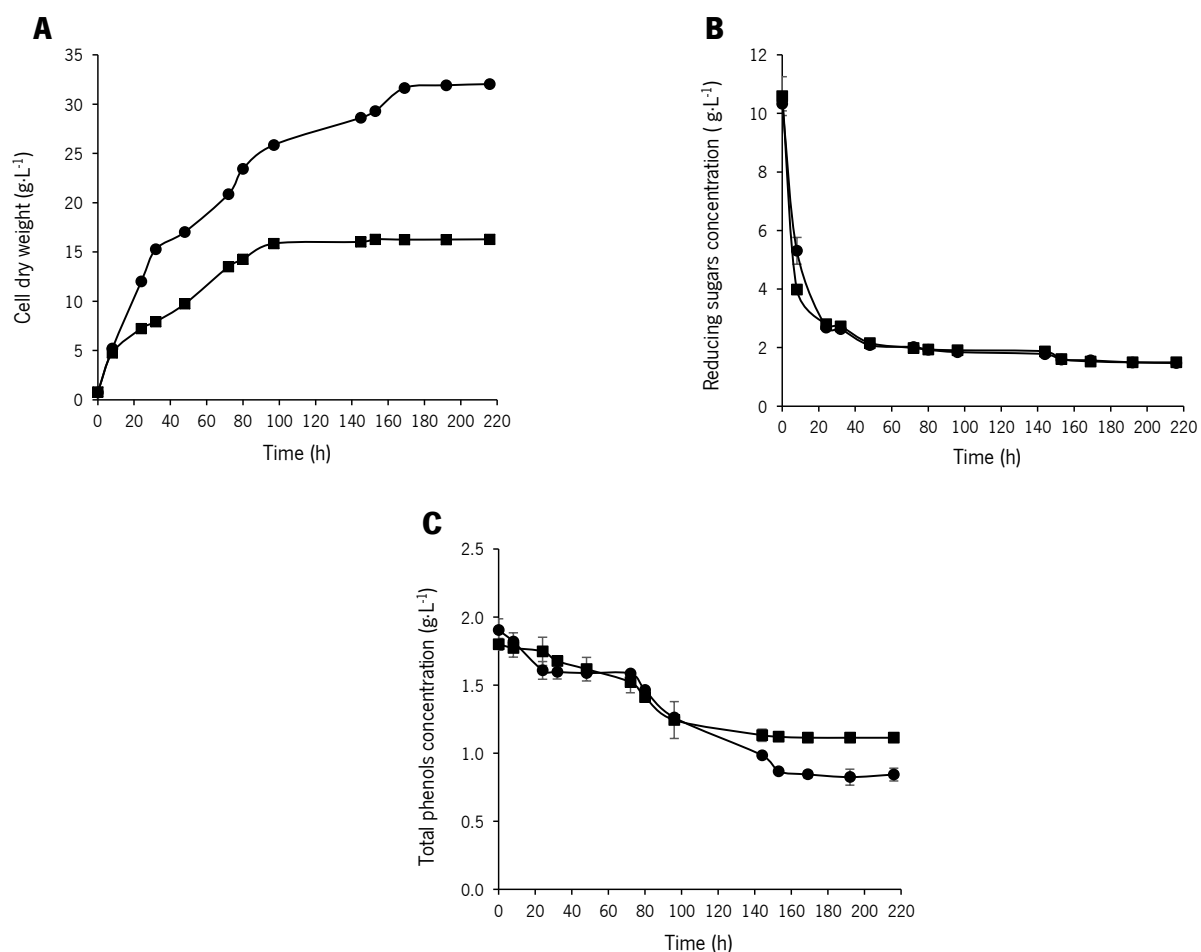


Figure 4.8 – Profiles of *C. tropicalis* ATCC 250 growth (**A**), reducing sugars consumption (**B**) and total phenols consumption (**C**) obtained in experiments with undiluted OMW supplemented with YNB, carried out in 250-mL Erlenmeyer flasks (■) and 500-mL Erlenmeyer flasks (●). The error bars represent the standard deviation for two independent replicates.

Regarding the experiments carried out in 250-mL Erlenmeyer flasks, a 1.3-fold improvement on cellular growth was obtained after 216 hours, comparatively to that found at 72 hours of growth. The consumption of reducing sugars and total phenols obtained in these new experiments were, respectively, 84.2 % and 38.2 % (Table 4.6). This means that extend the culture time had effect on total phenols consumption and had no effect on reducing sugars consumption. The increase of oxygen mass transfer, by raising the volume of Erlenmeyer headspace, had a clearly positive effect on cellular growth. The final biomass concentration attained in this experiment was 2-fold higher comparatively to 250-mL Erlenmeyer flask experiments. The consumption of total phenols was slightly enhanced by the increase of oxygen transfer. A 1.5-fold improvement on total phenols degradation was observed in 500-mL Erlenmeyer flasks. By contrasts, the profiles of reducing sugars consumption were equal for both experiments and no differences were found by raising the headspace volume.

Table 4.6 – Values of maximum biomass (X_{max}) and reduction of COD, reducing sugars and total phenols obtained in *C. tropicalis* ATCC 250 cultures carried out in 250- and 500-mL Erlenmeyer flasks with OMW 100% supplemented with YNB. Data are average and standard deviation of two independent replicates.

Flask capacity (mL)	X_{max} (g·L ⁻¹)	Reduction of COD (%)	Reduction of reducing sugars (%)	Reduction of total phenols (%)
250	16.3 ± 0.03	49.9 ± 2.1	38.2 ± 1.1	84.2 ± 0.9
500	32.0 ± 0.4	47.4 ± 1.8	55.7 ± 1.3	85.2 ± 0.6

As stated above, few studies were performed regarding the growth of *C. tropicalis* on OMW, particularly on undiluted OMW. However, other yeast species have been explored for their ability to grow on OMW-based medium. Taccari and Ciani (2011) screened 105 isolated for growth on plates containing OMW and 5 were selected and identified as *Pichia fermentans* and *Candida* sp. Particularly the strain *Candida* sp. (Y4) was able to grow on undiluted OMW and reduced 18 % and 31 % of total phenols and COD, respectively. Abdelhadi et al. (2009) observed that some strains of *Candida* sp., such as *C. holstii*, *C. diddensiae* and *C. boidinii*, had the ability to grow in OMW and to reduce phenolics concentration, even this reduction was lower than 50 %. Sarris et al. (2017) studied the ability of yeast *Y. lipolytica* ACA-YC 5033 to grow in OMW and concluded that, when the initial phenolics concentration was 5.5 g·L⁻¹, the yeast grown up to 3.0 g·L⁻¹ in 216 h of experiment. In this work, the final biomass concentration of *C. tropicalis* was much higher comparatively to other values found in the literature. Probably, *C. tropicalis* ATCC 250 has special features that enables the consumption of all carbon sources present on OMW, enhancing its growth.

It was observed that COD reduction was enhanced by extend the time of the culture. A 2-fold improvement on COD removal was obtained after 216 hours, comparatively to the experiment carried out during 72 hours. However, the increase of oxygen transfer had no positive effect on COD reduction, since its value was slightly lower than that found in 250-mL flasks experiments. It should be noted that, even without an optimization, the values of COD removal obtained in this work are comparable, or even higher, that others found in the literature. Gonçalves (2011) reported that the COD degradation of OMW-based medium, in bioreactor cultures, was 14 % and 17 %, respectively by *C. cylindracea* and *Y. lipolytica*. Gonçalves et al. (2013) also studied the ability of *C. rugosa* and *C. cylindracea* to grow in undiluted OMW and observed a 70 % and 59 % of COD reduction. Asses et al. (2009) observed that during fermentation

of OMW by *Geotrichum candidum* in bubble column reactor the COD concentration decreased approximately 30 g·L⁻¹.

Dourou et al. (2016) reported a 16.5 % of total phenols reduction by *C. tropicalis* LFMB 16 in bioreactor cultures. Mansour et al. (2010) concluded that the aeration of *Pseudomonas putida* mt-2 cultures favored the total phenols removal from OMW, achieving 66 % of reduction.

In addition to biomass production from OMW, *C. tropicalis* was also able to produce high added-value metabolites, such as microbial lipids (Table 4.7). The content of microbial lipids ranged from 18 % to 39 % depending on culture conditions, proving that this yeast may be considered an oleaginous yeast and it has the potential to accumulate significant amounts of intracellular lipids from OMW.

Table 4.7 – Values of microbial lipids content and microbial lipids concentration obtained in *C. tropicalis* ATCC 250 cultures. Data are average and standard deviation of two independent replicates.

OMW (%)	Flask capacity (mL)	Microbial lipids (% w/w)	Lipids concentration (g·L ⁻¹)
50	250*	21.7 ± 0.3	2.4 ± 0.1
100 without YNB	250*	38.9 ± 1.4	0.9 ± 1.4
	250*	26.3 ± 1.3	3.2 ± 0.2
100 with YNB	250**	18.3 ± 1.5	3.0 ± 0.8
	500**	19.8 ± 1.9	6.3 ± 0.8
Repeated batch	500**	24.3 ± 0.5	8.52 ± 0.04

*72 hours of culture; **216 hours of culture.

In the preliminary experiments, carried out during 72 hours in 250-mL Erlenmeyer flasks, it was observed that the accumulation of lipids was higher with undiluted OMW. This could be due to the fact that, in undiluted OMW, the concentration of carbon sources, including olive oil concentration was higher, favoring the accumulation of microbial lipids. Moreover, in the experiments without the supplementation of nitrogen, a considerable amount of intracellular lipids was attained. A 1.5-fold improvement on microbial lipids production was obtained in this experiment comparatively to that with YNB. It is reported in the literature that excess of carbon source and nitrogen limitation is one parameter that may increase the accumulation of lipids by oleaginous yeasts (Dey and Maiti, 2013; Papanikolaou and Aggelis, 2011; Rakicka *et al.*, 2015). However, as the final biomass was very low in this experiment, the lipid concentration attained was negligible compared to the experiment supplemented with nitrogen source.

The extent of cultivation time led to a decrease of microbial lipids content. Probably, lipids accumulated in the first hours of cultivation were mobilized to yeast growth or other metabolic functions, due to the depletion of extracellular carbon supply (Beopoulos *et al.*, 2011). According to Beopoulos *et al.* (2009), if the extracellular carbon supply is exhausted, stored lipids may be mobilized, once that lipid accumulation is always dependent on the influx of carbon substrates. This complex regulation makes it difficult to achieve high rates of lipid accumulation in batch culture. The increase of oxygen transfer, by raising headspace volume, did not favor the lipid accumulation by *C. tropicalis* cells. However, as the final biomass concentration obtained in 500-mL flask experiments were the double of that in 250-mL flask experiments, the lipid concentration was 2 times higher in the first case.

Apart from there is little information in the literature regarding the use of OMW by *C. tropicalis*, in our best knowledge there are no studies concerning the production of microbial lipids by this yeast species from OMW. However, some authors reported that the increased of oxygen favored the accumulation of microbial lipids by yeasts (Lopes *et al.*, 2018a; Lopes *et al.*, 2018b). Lopes *et al.* (2018a) observed that increasing the oxygen transfer rate from 96 mg·L⁻¹·h⁻¹ to 192 mg·L⁻¹·h⁻¹ clearly enhanced lipid accumulation by *Y. lipolytica* W29 from pork lard. Also Lopes *et al.* (2018b) observed that the raise of k_{La} from 9 h⁻¹ to 16 h⁻¹ had a clearly positive effect on microbial oils production by *Y. lipolytica* when waste cooking oils was used carbon source.

It is reported in the literature that the excess of carbon and nitrogen deficiency is an essential factor for the induction of lipogenesis in oleaginous microorganisms (Papanikolaou and Aggelis, 2011). Moreover, it was observed in the preliminary experiments, that the non-supplementation with YNB of undiluted OMW favored the accumulation of lipids. Thus, an experiment with the addition of undiluted OMW without YNB after 72 hours and 148 h hours of cultivation (repeated batch) was carried out. A 1.2-fold improvement in microbial lipids content was obtained compared to the experiment carried out in batch mode. Ageitos *et al.* (2011) reported that lipid accumulation normally takes place when the oleaginous yeasts grows in the presence of high amounts of a carbon source and the nitrogen source is kept limited. Dey and Maiti (2013) stated that oleaginous microorganisms require the exhaustion of nutrient, usually nitrogen, to allow that excess carbon be incorporated into lipids. Cultures of *C. tropicalis* SY005, growing in basal medium with xylose and glycerol as carbon source and subjected to nitrogen-limited growth, accumulated 45 % of intracellular lipids after 4 days.

Microscopic observations of *C. tropicalis* ATCC 250 cells cultivated in undiluted OMW supplemented with YNB revealed the presence of intracellular lipid bodies (Figure 4.9). Almost all cells had more than one lipid body, which was proved by staining with Nile Red.

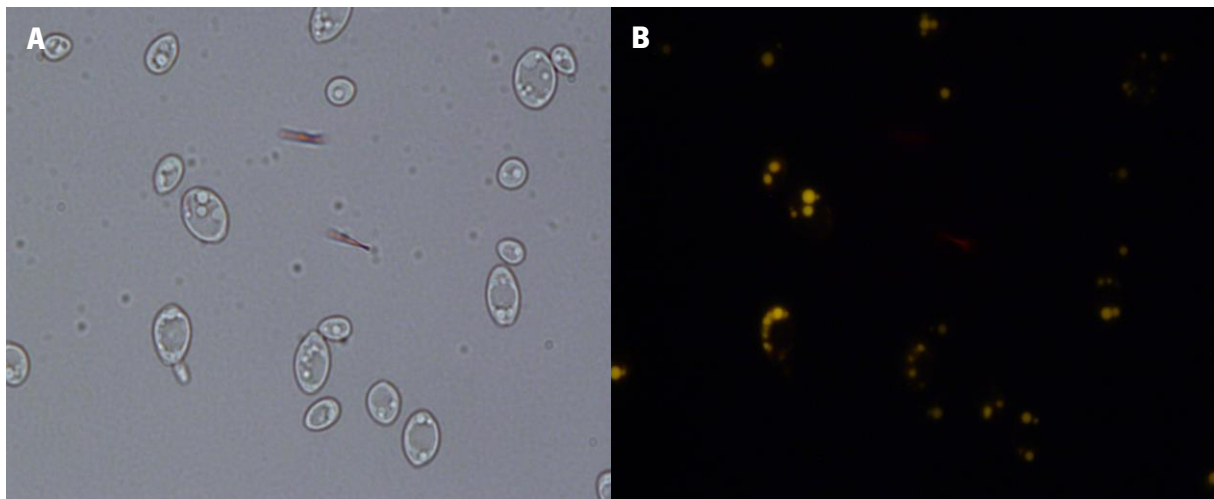


Figure 4.9 – Light microscopy image **(A)** and fluorescence microscopy image after staining with Nile Red **(B)** of *C. tropicalis* ATCC 250 cells with intracellular lipid bodies in the cytoplasm after 216 h of growth in undiluted OMW supplemented with YNB in 500-mL Erlenmeyer flask (magnification 1000×).

Microbial lipids composition is significantly influenced by the fatty acids composition of lipidic substrate and by their specific uptake rate (Papanikolaou *et al.*, 2006; Papanikolaou and Aggelis, 2011). OMW is an effluent with lipidic substrate, olive oil. As the lipid composition of olive oil is known for the high content of oleic acid, it is expected lipids accumulated by the yeast will be rich in oleic acid (Table 4.8).

Regardless of culture conditions and medium composition, microbial lipids accumulated by *C. tropicalis* cells were mainly composed by oleic acid (60 % - 68 %), followed by linoleic (21 % - 25 %), palmitic (7 % - 12 %) and stearic (3 % - 8 %) acids. Moreover, there were no statistical differences in the saturated and unsaturated fractions between the experiments. However, the unsaturated fraction (80 % - 90 %) exceeded by far the saturated one (10 % - 20 %) in all conditions.

Due to the composition of these microbial lipids, rich in oleic and linoleic acids, they can be used as an excellent source of food supplement or used for animal feedstock. Moreover, as the lipids accumulated by *C. tropicalis* cells from OMW are mainly constituted by unsaturated fatty acids, they can be used for biodiesel production.

Table 4.8 – Fatty acids composition of microbial lipids accumulated by *C. tropicalis* ATCC 250 cells from OMW. Data are average and standard deviation of two independent replicates.

OMW (%, v/v)	Experiment flask (mL)	Long chain fatty acids (%)			
		Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)
50 with YNB	250*	12.2 ± 7.1 ^d	7.6 ± 5.8 ^{abcd}	59.5 ± 6.7 ^f	20.7 ± 6.2 ^e
100 without YNB	250*	10.8 ± 0.2 ^{cd}	5.5 ± 0.3 ^{abc}	61.7 ± 0.4 ^{fg}	22.0 ± 0.3 ^e
100 with YNB	250*	8.4 ± 0.1 ^{bcd}	4.2 ± 0.4 ^{ab}	62.7 ± 0.4 ^{gh}	24.7 ± 0.1 ^e
	250**	7.4 ± 0.6 ^{abcd}	4.1 ± 0.3 ^{ab}	67.5 ± 0.7 ^{hi}	21.0 ± 0.3 ^e
	500**	7.0 ± 0.6 ^{abcd}	2.8 ± 0.9 ^a	68.2 ± 0.5 ⁱ	21.9 ± 0.4 ^e
Repeated batch	500**	7.5 ± 0.1 ^{abcd}	4.8 ± 0.3 ^{ab}	65.4 ± 0.6 ^{ghi}	22.3 ± 0.2 ^e

^{a-i} Values followed by the same letter do not present statistically significant differences ($p \leq 0.05$); *72 hours of culture; **216 hours of culture.

The high cost of feedstocks to produce biodiesel is the major obstacle in the commercialization of the product (Leiva-Candia *et al.*, 2014). Moreover, currently biodiesel is being widely produced as alternative to petroleum (Darvishi *et al.*, 2017). Agro-industrial waste could be used as substrate to cultivate oleaginous microorganisms and a number of microorganisms have the ability to accumulate lipids under specific cultivation conditions. Since microbial lipids can be produced using various waste streams (e.g. whey, lignocellulosic hydrolysate) as substrate, it has been considered a potential feedstock to support a sustainable biodiesel industry (Patel *et al.*, 2016; Leiva-Candia *et al.*, 2016). So, from the economic point of view, is to value the use of a waste without great economic value to produce a biofuel.

There are no studies that report the accumulation of microbial lipids by *C. tropicalis*. However, some works regarding the accumulation of microbial lipids by other yeast species from OMW are found in the literature. Papanikolaou *et al.* (2008) observed that *Y. lipolytica* ACA-DC 50109 accumulated unsaturated fatty acids (principally oleic and palmitoleic acids) from OMW. Dourou *et al.* (2016) studied the fatty acid composition of lipids produced during different growth phases of *Lipomyces starkeyi* and *Y. lipolytica* on OMW and concluded that oleic acid was the most abundant fatty acid in both yeasts lipids, representing 40 % – 63 % of total fatty acids. Palmitic and linoleic acids were also found in considerable concentrations, regardless of the growth medium. These results are in accordance with those obtained in this work with *C. tropicalis*, since in both works, the unsaturated fraction largely exceeded the saturated

one. Saris et al. (2017) studied the fatty acid composition of *Y. lipolytica* ACA-YC 5033 cells cultivated in nitrogen limited OMW medium and concluded that intracellular lipids were mainly composed by unsaturated fatty acids.

5. CONCLUSIONS AND FUTURE WORK PERSPECTIVES

In this chapter, the general conclusions of the work are presented as well as suggestions for future work related with this research theme.

5.1 Final Conclusions

Due to the advancement of technology and increased industrial production, thousands of liters of effluents are produced by the industries every day. After the screening of yeasts species for the biodegradation of pollutants, it was concluded that petroleum-derived compounds, e.g. hydrocarbons, can be degraded by *Y. lipolytica* W29. On the other hand, *C. tropicalis* ATCC 250 stands out by its ability to degrade phenolic compounds.

In 96-well microplate experiments, *Y. lipolytica* W29 was able to grow on media with hexadecane 10 g·L⁻¹ and hexadecene 7.5 g·L⁻¹ as sole carbon source. *Candida tropicalis* ATCC 250 growth was observed in OMW-based media, even in concentrations of 50 (% v/v).

After the 96-well microplate experiments, a scale-up was performed in 250- and 500-mL Erlenmeyer flasks. In hydrocarbons-based media the increase of oxygen mass transfer, by raising the volume of Erlenmeyer headspace, had a clearly positive effect on *Y. lipolytica* growth. The final biomass concentration attained in these experiments was approximately 30 % higher than that obtained in 250-mL Erlenmeyer experiments. Lipase and microbial lipids production was also improved by the raise of headspace volume. This is particularly important for the economic production of an important enzyme and lipids enriched with essential fatty acids, which simultaneously have wide application in biodiesel production.

In OMW experiments, a 5-fold improvement on *C. tropicalis* growth was obtained in undiluted OMW supplemented with YNB comparatively to the experiments carried out without supplementation. The raise of oxygen mass transfer led to an increase of final biomass concentration and total phenols consumption.

Independently of carbon source, microbial lipids accumulated by *Y. lipolytica* and *C. tropicalis* were mainly composed by oleic and linoleic acids and the unsaturated fraction exceeded the saturated one. Due to their composition, similar to that of common vegetable oils, these lipids could be used for biodiesel production.

The work described herein could be inserted in the concept of circular economy: hydrocarbons and OMW are recycled for the production of lipids that could be used for biodiesel production, with no generation of more residues.

5.2 Future work perspectives

The present work brings new perspectives on the biodegradation/removal of pollutant compounds from contaminated effluents and a biotechnological production of high added-value metabolites, such as lipase and microbial lipids by *Y. lipolytica* W29 and *C. tropicalis* ATCC 250.

Some authors observed that nitrogen limitation enhances lipid accumulation by yeast but inhibit cell growth, whereas a higher level of dissolved oxygen (DO) enhances biomass accumulation. Thus, the development of a two-step process to improve cellular growth (high dissolved oxygen concentration during exponential growth phase) and microbial lipids (pulses only with carbon source) production could be attempted. This approach is carried out in a lab-scale bioreactor, in which dissolved oxygen can be measured and controlled. Two approaches to study the effect of oxygen mass transfer could be tested: (a) vary the stirring rate and air flow rate to obtain different values of oxygen transfer rate or (b) maintain the dissolved oxygen constant, manipulating the stirring speed and specific air flow rate, through a cascade control mode.

Additionally, other bioreactors configurations (pressurized bioreactor and air-lift bioreactor), which are normally associated to high oxygen mass transfer, could be tested. It was already proved that lipase production by *Y. lipolytica* from olive oil was improved by the raise of total air pressure in a pressurized bioreactor. However, there are no reports regarding the effect of pressurized conditions on microbial lipids production from hydrocarbons or from OMW.

Finally, the degradation of phenol by *C. utilis* CBS 621 is another aspect to explore, since this yeast species demonstrated the ability to grow on phenol up to 1 g·L⁻¹ in Petri plate and 96-well experiments.

6. REFERENCES

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SCIENTIFIC OUTPUTS

Poster communications:

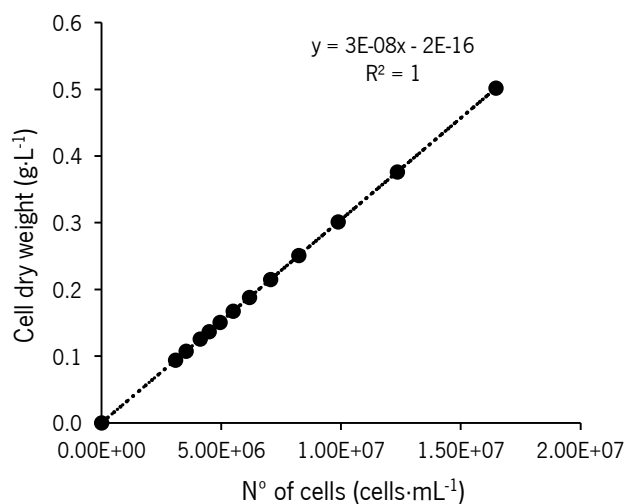
1. Lopes, M., **Ramôa, R.**, Miranda, S., Belo, I. "Simultaneous degradation of hydrocarbons and production of valuable compounds by *Yarrowia lipolytica*." 13th International Chemical and Biological Engineering Conference - CHEMPOR 2018. Aveiro, Portugal, October 2-4, 2018, pp.334–335.

The abstract with the results presented in this conference was published in the book of proceedings: Book of Extended Abstracts of 13th International Chemical and Biological Engineering Conference - CHEMPOR 2018. Aveiro, Portugal.

ANNEX I – CALIBRATION CURVES

Equations of the calibration curves with a confidence interval of 95 %. LOD: Limit of detection and LOQ: Limit of quantification; AA: Area of acid; AIS: Area of internal standard; [A]: Acid concentration; [IS]: Internal standard concentration.

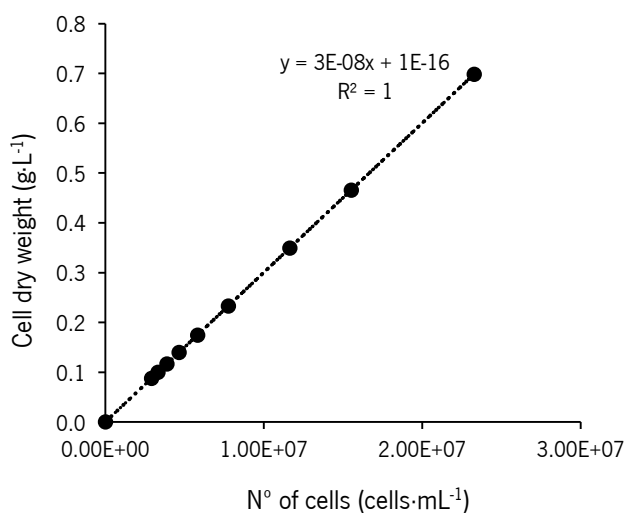
AI.1 Biomass of *Yarrowia lipolytica* W29



$$\text{Cell dry weight (g.L}^{-1}\text{)} = 3 \times 10^{-8} \times \text{N}^{\circ} \text{ of cells (cells.mL}^{-1}\text{)} + (-2 \times 10^{-16} \pm 2.5 \times 10^{-17})$$

LOD (g.L⁻¹): 1.1×10^{-9} ; **LOQ** (g.L⁻¹): 3.7×10^{-9}

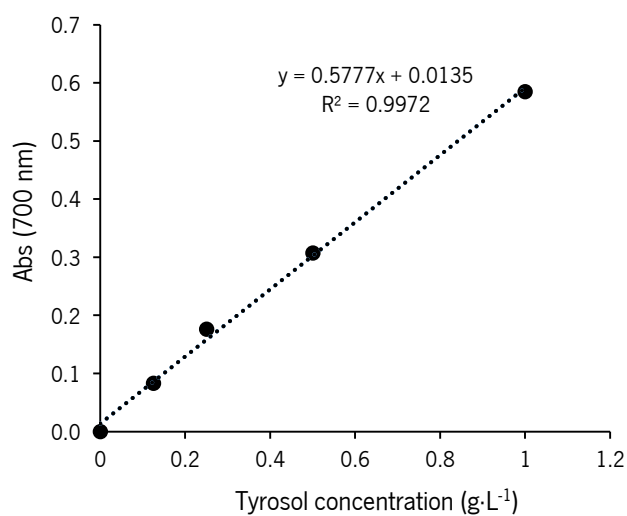
AI.2 Biomass of *Candida tropicalis* ATCC 250



$$\text{Cell dry weight (g.L}^{-1}\text{)} = (3 \times 10^{-8} \pm 3.7 \times 10^{-9}) \times \text{N}^{\circ} \text{ of cells (cells.mL}^{-1}\text{)} + (1 \times 10^{-16} \pm 9.7 \times 10^{-10})$$

LOD (g.L⁻¹): 3.8×10^{-17} ; **LOQ** (g.L⁻¹): 1.3×10^{-16}

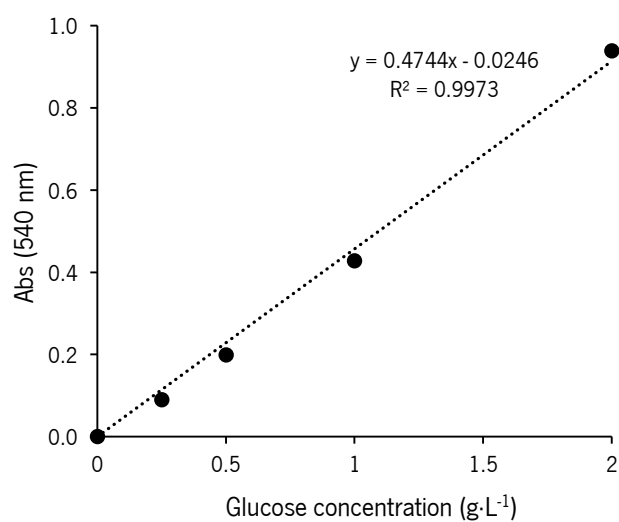
AI.3 Total phenols



$$\text{Abs (700 nm)} = (0.58 \pm 0.06) \times [\text{tyrosol}] \text{ (g·L}^{-1}\text{)} + (0.01 \pm 0.03)$$

LOD (g·L⁻¹): 0.05; **LOQ** (g·L⁻¹): 0.16

AI.4 Reducing sugars

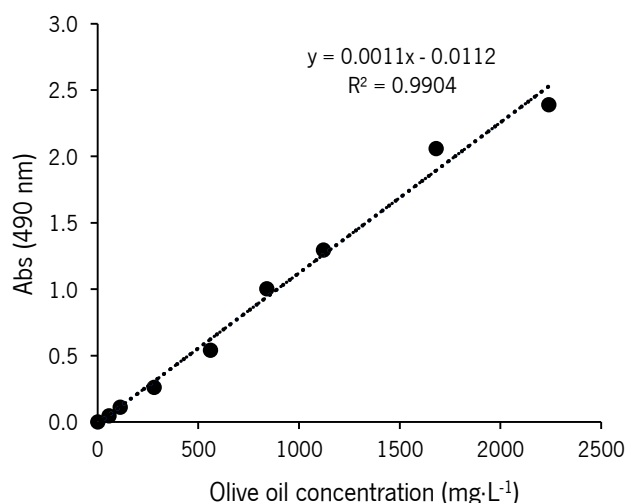


$$\text{Abs (540 nm)} = (0.47 \pm 0.05) \times [\text{glucose}] \text{ (g·L}^{-1}\text{)} + (-0.02 \pm 0.05)$$

LOD (g·L⁻¹): 0.09; **LOQ** (g·L⁻¹): 0.31

AI.5 Microbial lipids

This is an example of a calibration curve obtained in Phospho-vanillin method, because at every time that this method was performed, a new calibration curve was made.



$$\text{Abs (490 nm)} = (0.001 \pm 9.9 \times 10^{-5}) \times [\text{olive oil}] \text{ (mg} \cdot \text{L}^{-1}) + (-0.01 \pm 0.11)$$

LOD (g·L⁻¹): 118.6; **LOQ** (g·L⁻¹): 395.3

AI.6 Long chain fatty acids (LCFA)

Acids		Calibration curves	LOD (g·L ⁻¹)	LOQ (g·L ⁻¹)
Lauric acid	AA / AIS = (0.9670 ± 0.0097) × [A] / [IS] + (-0.0012 ± 0.0046)		0.0067	0.0223
Myristic acid	AA / AIS = (0.9446 ± 0.0118) × [A] / [IS] + (-0.0027 ± 0.0056)		0.0083	0.0276
Palmitic acid	AA / AIS = (1.0490 ± 0.0153) × [A] / [IS] + (0.0125 ± 0.0075)		0.0101	0.0335
Palmitoleic acid	AA / AIS = (0.9831 ± 0.0102) × [A] / [IS] + (-0.0077 ± 0.0048)		0.0069	0.0230
Margaric acid	AA / AIS = (1.0634 ± 0.0108) × [A] / [IS] + (-0.0080 ± 0.0052)		0.0068	0.0226
Stearic acid	AA / AIS = (1.1288 ± 0.0189) × [A] / [IS] + (0.0072 ± 0.0091)		0.0113	0.0376
Oleic acid	AA / AIS = (1.0607 ± 0.0133) × [A] / [IS] + (-0.0084 ± 0.0074)		0.0097	0.0324
Linoleic acid	AA / AIS = (0.4707 ± 0.0069) × [A] / [IS] + (-0.0049 ± 0.0034)		0.0102	0.0341