Universidade do Minho
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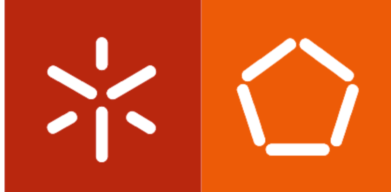

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Ângela Sofia Oliveira Ferreira

Use of n-alkanes as substrate by Yarrowia lipolytica

Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho efetuado sob a orientação da
Doutora Marlene Alexandra da Silva Lopes
e da **Professora Doutora Isabel Maria Pires Belo**

Outubro de 2016

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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ABSTRACT

Hydrocarbons have become one of the most important types of organic pollutants due to splits during the processes of petroleum manipulation and inadequate release of hydrocarbons in several industrial effluents (plastic, cosmetic, solvents and pharmaceutical industries). One approach to treat these residues is their degradation by microorganisms.

Yarrowia lipolytica is a non-conventional yeast, strictly aerobic and is often isolated from oil-polluted environments, dairy products, meat and poultry products. *Y. lipolytica* is able to use n-alkanes, fatty acids and oils as carbon sources, making this yeast a potential agent for remediation of oily wastes.

In this work, the main goal was to evaluate the ability of *Y. lipolytica* W29 to use hexadecane as carbon source and study the effect of several cultivation parameters (nitrogen source, surfactants and oxygenation) that could affect the yeast performance.

Initially, the effect of two hexadecane concentrations on yeast growth was tested and it was observed a significant growth with 5 $g{\cdot}L^{-1}$ of hexadecane, whereas with 10 $g{\cdot}L^{-1}$ of hexadecane no cellular growth was observed. However, the addition of silicone oil to the culture with 10 $g{\cdot}L^{-1}$ of hexadecane eliminated toxic effects and dispersion problems, leading to a considerable yeast growth. It was also observed that the addition of Tween 80 and a nitrogen source (YNB) favored yeast growth in media with hexadecane as carbon source.

The increase of oxygenation conditions did not improved cellular growth and hexadecane consumption. In the experiments carried out with lower agitation and aeration rates, yeast cells accumulated 26 % of their cell dry weight as microbial lipids and shift their morphology from oval form to hyphae form.

The results reported herein prove that *Y. lipolytica* W29 is able to grow in hexadecane medium, producing simultaneously biomass and microbial lipids. Thus, this approach may be adapted to real effluents from refinery industries or other effluents containing hexadecane.

Key words: *Yarrowia lipolytica*, alkanes, hexadecane, biodegradation, dissolved oxygen.

RESUMO

Os hidrocarbonetos tornaram-se um dos poluentes orgânicos mais importantes devido aos derrames que ocorrem durante os processos de manipulação do petróleo e da inadequada libertação de hidrocarbonetos em vários efluentes industriais (indústrias de plásticos, cosméticos, solventes e farmacêuticas). Uma das abordagens para o tratamento destes resíduos é a sua degradação por microrganismos.

Yarrowia lipolytica é uma levedura não-convencional, estritamente aeróbia e que é normalmente isolada a partir de produtos lácteos, carne e produtos de aves e também de ambientes poluídos com petróleo e outros óleos. *Y. lipolytica* é capaz de utilizar n-alcanos, ácidos gordos e óleos como fontes de carbono, o que a torna um potencial agente para a recuperação de resíduos oleosos.

Neste trabalho, o principal objetivo foi avaliar a capacidade de *Y. lipolytica* W29 em usar hexadecano como fonte de carbono e estudar o efeito de vários componentes do meio (fonte de azoto, surfactante e oxigenação) que podem afetar o desempenho da levedura durante o processo.

Inicialmente foi avaliado o efeito de duas concentrações de hexadecano no crescimento da levedura e observou-se um crescimento significativo com 5 $g{\cdot}L^{-1}$ de hexadecano, enquanto com 10 $g{\cdot}L^{-1}$ de hexadecano não foi observado crescimento celular. No entanto, a adição de óleo de silicone à cultura com 10 $g{\cdot}L^{-1}$ de hexadecano eliminou o efeito tóxico e os problemas de dispersão, levando a um crescimento considerável da levedura. Observou-se também que a adição de Tween 80 e uma fonte de azoto (YNB) favoreceu o crescimento de levedura em meios com hexadecano como fonte de carbono.

O aumento das condições de oxigenação não melhorou o crescimento celular nem o consumo de hexadecano. Nas experiências realizadas com taxas de agitação e arejamento inferiores as células de levedura acumularam 26 % do seu peso seco celular em lípidos microbianos e alteraram a morfologia de forma oval para a forma de hifas.

Os resultados aqui expostos provam que a levedura *Y. lipolytica* W29 é capaz de crescer em meio com hexadecano, produzindo simultaneamente biomassa e lípidos microbianos. Assim, esta abordagem pode ser adaptada aos efluentes das indústrias de refinarias de petróleo ou outros efluentes que contenham hexadecano.

PALAVRAS CHAVE: *Yarrowia lipolytica*, alcanos, hexadecano, biodegradação, oxigénio dissolvido.

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LIST OF ABBREVIATIONS

ACS	fatty-acyl-CoA synthetase
CA	citric acid
FADH	fatty alcohol dehydrogenase
FALDH	fatty aldehyde dehydrogenase
FAO	fatty alcohol oxidase
GC	gas chromatography
HPLC	high-performance liquid chromatography
STE	steryl esters
TAG	Trigacylglycerols
TCA	tricarboxylic acid
TPH	total petroleum hydrocarbons
YNB	Yeast nitrogen base
YPD	yeast extract, peptone, glucose medium
YPDA	yeast extract, peptone, glucose and agar medium
μ	Specific growth rate (h^{-1})

1. State of the art

1.1 Pollution by alkane compounds

Since World War II and Industrial Revolution, the increase of industrial and human activities led to a discharge increase of exhausts, particles, and various pollutants into the air, water and soil. As one of the essential energy sources, petroleum is an important material basis for development of the world economy.

Crude oil or petroleum is a complex mixture of different hydrocarbons (Liang et al. 2012). Hydrocarbons are the simplest organic compounds, only with carbon and hydrogen atoms. They are classified into several groups according with their characteristics. Alkanes, also named n-paraffins, have a linear structure without ramifications and only covalent bonds. They are saturated hydrocarbons, with a general formula C_nH_{n+2}. Alkanes are non-polar molecules, which mean that they are insoluble in water, but are soluble in organic solvents. Usually, alkanes are considered to be non-reactive substances, however under suitable conditions they react. At higher temperatures and in the presence of oxygen, saturated hydrocarbons are attacked by oxygen and form carbon dioxide and water in a complete combustion reaction. Hydrocarbons may also react with halogen (fluorine, chlorine, bromine, iodine), in a reaction named halogenation, in which a hydrogen atom is substituted by a halogen atom (Chang 2011).

However, hydrocarbons have become one of the most important types of organic pollutants due to splits during the processes of exploration, extraction, refining and transporting. Moreover, the marketing of petroleum products (Margesin & Schinner 1997) and the inadequate release of hydrocarbons on effluents of various industries that use petroleum or its derivatives, such as plastic, cosmetic, solvents and pharmaceutical industries, have contributed to the increase of pollution by hydrocarbons and n-alkanes.

There are several physical and chemical processes to treat the effluents polluted with alkanes. However, these techniques have some disadvantages such as high energy consumption and secondary pollution from other compounds produced during the process. Thus, biological methods are an attractive alternative because are environmentally-friendly, less expensive and less hazardous. Biological methods can be applied *in situ* (bioremediation) and *ex situ* (in bioreactors) (Pal et al. 2016).

The traditional biological process used is an activated sludge system. This method consists in degradation of pollutants by microorganisms in aerated tanks, producing carbon dioxide, water, and other inorganic compounds. Oily sludge is a hazardous solid waste of oil refinery composed by water, sediments, asphaltenes, wax, heavy metals and hydrocarbons (23.19 %), namely hexadecane. The removal of total petroleum hydrocarbons (TPH) contained in sludge from the oil industry has already reported (Admon et al. 2001; Kuyukina et al. 2003). Marín et al. (2006) obtained a remediation efficiency of 60 % after a 3-month treatment of oil sludge with high concentrations of TPH. Also Roldán-Carrillo et al. (2012) reported degradation rates of 3.6 g TPH kg^{-1} dm d^{-1} - 5.7 g TPH kg^{-1} dm d^{-1} by native microorganisms of oil sludge with alkanes.

Bioreactors technology is also a possible method to *ex situ* alkane treatment. Gargouri et al. (2011) studied the application of bioreactor fermentation for treatment of hydrocarbon-rich industrial effluents. The authors used naturally occurring microorganisms from effluents of petroleum refinery industry in Tunisia to assimilate hydrocarbons and reached a hydrocarbon removal efficiency of 94 %. They also identified six aerobic bacteria from the medium, which were closely related to *Aeromonas punctata (Aeromonas caviae*), *Bacillus cereus*, *Ochrobactrum intermedium*, *Stenotrophomonas maltophilia and Rhodococcus sp*.

Gargouri et al. (2015) studied the ability of yeast strains, isolated from effluents of Tunisian petroleum refinery, to grow and degrade crude oil. They found that *Candida tropicalis* and *Trichosporon asahii* degraded n-alkanes and aliphatic hydrocarbons and developed an adaptation mechanism to survive in the harsh environments. These results prove that yeast can be employed to improve the hydrocarbon removal from industrial effluents.

Scholz & Fuchs (2000) studied the applicability of a membrane bioreactor in oily wastewater treatment and observed a 99.9 % of pollutant removal from fuel oil (constituted of 45 % - 50 % of alkanes) and 98 % of pollutant removal from surfactant-oil Akropal (constituted of 50 % of alkanes). Also Soltani et al. (2010) proved that a consortium of microorganisms can degrade hexadecane in a membrane bioreactor. However, only the microorganisms isolated from sea waters seem to be capable to degrade wastewaters with high salinity. Friha et al. (2014) also tested membrane bioreactor efficiency in cosmetic wastewaters with alkanes and achieved good degradation results. These results prove that membrane bioreactor could be an efficient approach to pollutant removal and alkane degradation.

Several microorganisms possess the ability to secrete enzymes that degrade hydrocarbons (Atlas 1992). The rate of alkanes degradation by microorganisms is highest than for other hydrocarbons

(Leahy & Colwell 1990). Therefore, these microorganisms has been successfully applied for the treatment of effluents and sludge with hydrocarbons in controlled systems (Atlas 1995).

Hydrocarbons could be degraded by bacteria, yeast, and fungi species. Mixed populations with enzymatic capacity are required to degrade complex mixtures of hydrocarbons such as crude oil in soil, fresh water and marine environments (Das & Chandran 2011). Using diverse techniques for microbial isolation, different bacterial genera have been characterized from hydrocarbon polluted soils in different geographical and ecological contexts (Chikere et al. 2009). Important hydrocarbon-degrading bacteria in both marine and soil environments are *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter, Bacillus*, *Flavobacterium*, *Nocardia*, *Pseudomonas* spp. and coryneforms (Leahy & Colwell 1990).

There are several factors that affect the degradation of hydrocarbons, such as temperature, pH, nutrients and oxygen available for aerobic microorganisms (Das & Chandran 2011; Atlas 1992). Although biodegradation may occur over a wide range of temperatures, the rate of degradation generally decreases for lower values of temperature (Das & Chandran, 2011). The highest degradation rates generally occur in the range of 30 ^{o}C – 40 ^{o}C in soil environments, 20 ^{o}C – 30 ^{o}C in some water environments, and 15 ^{o}C – 20 ^{o}C in marine environments (Atlas, 1992). Another factor that affects hexadecane degradation by microorganism is the concentration of nutrients. In aquatic ambient, nitrogen and phosphorus becomes the limiting factor for the biodegradation. Additionally, pH is a very important parameter for hydrocarbon biodegradation, and values in the range of 6 - 7.5 are reported as suitable for this biological process (Atlas, 1995).

One of the main problems that limit the cellular growth is the low solubility and high hydrophobicity of alkanes. One solution to this problem is the use of biosurfactants, which are a group of surface-active substances produced by microorganisms that exhibit the ability to reduce surface and interfacial tension. The use of biosurfactants increases the availability of hydrophobic substrate to the microorganisms by decreasing surface tension of the medium (Pacwa-Płociniczak et al. 2011). Pacwa-Płociniczak et al. (2014) observed that *Pseudomonas* sp. P-1 had the ability to produce bioemulsifiers, which increases the degradation of various hydrocarbons.

Several groups studied the ability of various microorganisms to degrade n-alkanes and the ideal conditions to this purpose. In Table 1 are summarized several microorganisms' genera capable of n-alkanes bioremediation studied over the years.

Table 1- Microbial genera often used in studies for alkane biodegradation (Adapted from Ollivier & Magot 2005)

Bacteria	Yeast	Fungi
Achoromobacter	*Candida*	*Aspergillus*
Acinetobacter	*Cryptococcus*	*Cladosporium*
Alcaligenes	*Pichia*	*Dendryphiella*
Bacillus	*Rhodotorula*	*Fusarium*
Brevibacterium	*Saccharomyces*	*Lulworthia*
Corynebacterium	*Torulopsis*	*Penicillium*
Geobacillus	*Yarrowia*	*Virticillium*
Gordonia		
Mycobacterium		
Norcadia		
Pseudomonas		
Rhodococcus		
Sphingomonas		
Streptomyces		
Thermophilium		

1.2 *Yarrowia lipolytica*

Y. lipolytica was discovered by David Yarrow from the Delft Microbiology Laboratory in 1972, and it was firstly referred as *Candida lipolytica*, *Endomycopsis lipolytica* and *Saccharomycopsis lipolytica* (Yarrow 1972). This non-conventional yeast is strictly aerobic, is considered a GRAS organism (generally regarded as safe) and its natural habitats are oil-polluted environments, dairy products (cheese, yogurt, kefir), shoyu, meat and poultry products (Bankar et al. 2009; Coelho et al. 2010; Gonçalves et al. 2014; Sabirova et al. 2011).

Y. lipolytica, a dimorphic fungi, is a suitable model for studies on yeast dimorphism, since it forms yeast cells, pseudo-hyphae or septate hyphae, depending on yeast strain genetics and growth

conditions (Coelho et al., 2010). This morphological change is reversible and is a consequence of cell adaptation to adverse condition and, for this reason, cellular morphology of *Y. lipolytica* is influenced by pH, dissolved oxygen concentration in the medium, carbon and nitrogen sources, some minerals, etc. (Barth & Gaillardin 1997). Ruiz-Herrera & Sentandreu (2002) found that *Y. lipolytica* W29 switch to mycelium morphology form at pH values near neutrality and at pH 3 or 4 the yeast remained in the oval morphology. In the presence of casein, olive oil, N-acetylglucosamine, citrate and serum, *Y. lipolytica* strains switched to mycelium form (Kawasse et al. 2003; Pérez-Campo & Domínguez 2001; Ruiz-Herrera & Sentandreu 2002; Zinjarde et al. 1998). Independently of carbon or nitrogen sources, dissolved oxygen concentration plays an important role in *Y. lipolytica* morphology. Bellou et al. (2014) found that in highly aerated conditions *Y. lipolytica* cells were in oval form, whereas in low aeration conditions hyphae form was induced.

Y. lipolytica strains have been used for studies related to yeast physiology, genetics, gene manipulation and protein expression (Barth & Gaillardin, 1997; Beckerich et al., 1998; Beopoulos et al., 2009; Domínguez et al., 2000; Fickers et al., 2005; Madzak et al., 2004). This microorganism is known as an industrial workhorse because is capable of producing important industrial metabolites, such as lipase, citric acid (CA) , biosurfactants, aroma and microbial lipids (Coelho et al., 2010) and can grow in agro-industrial by-products or wastes such as crude glycerol (Ferreira et al. 2016; Kawasse et al. 2003), oil refinery residues (Rufino et al. 2008), waste cooking oils (Domínguez et al. 2010) and olive mill wastewater (Gonçalves et al. 2009; Lopes et al. 2009).

Y. lipolytica secretes organic acids, intermediates of tricarboxylic acid cycle (TCA), such as citric, isocitric, α-ketoglutaric and pyruvic acids, from a wide range of substrates (glycerol, alkanes, glucose, ethanol and plant oil) (Rymowicz et al., 2008). Microbial lipids, also designed as single cell oil (SCO), are a new category of oil source, and are produced by oleaginous microorganisms. The yeast *Y. lipolytica* are able to accumulate more than 20 % (w/w) of free fatty acids, generally as triacylglycerol and steryl esters in lipid bodies (LP) from agro-industrial residues (Papanikolaou et al. 2003; Papanikolaou & Aggelis 2011; Rakicka et al. 2015) Lipids produced by *Y. lipolytica* represent an attractive source of edible oils and have been considered as an alternative source for the production of polyunsaturated fatty acids and cocoa butter substitutes (Papanikolaou et al. 2002). Moreover, microbial lipids produced by *Y. lipolytica* can be directly converted into biodiesel, as its fatty acid composition is similar to the one from common vegetable oils. Several authors have demonstrated the ability of *Y. lipolytica* to produce biosurfactants and/or bioemulsifiers from a wide range of substrates,

such as soybean oil refinery residue (Rufino et al. 2008), babassu oil (Sarubbo et al. 1999; Vance-Harrop et al. 2003) and alkanes (Cirigliano & Carman 1985; Amaral et al. 2006).

1.2.1 Biodegradation of alkanes by *Y. lipolytica*

Y. lipolytica is able to use n-alkanes, fatty acids and oils as carbon sources, which makes this yeast a potential agent in remediation of environments contaminated with vegetable and mineral oil wastes. This ability could be used for biodegradation, in which yeast is inoculated in polluted places to degrade hydrocarbons *in situ*, or to treat industrial effluents contaminated with hydrocarbons at the same time that produce biomass or added-value products in bioreactors. Since the studies regarding the treatment of effluents contaminated by alkanes are scarce, the degradation of petroleum could give an idea and solution that could be adapted to effluents treatment. Margesin & Schinner (1997) observed that *Y. lipolytica* degraded 68 % of diesel oil after 10 days of culture, at 15 °C – 20 °C. Zinjarde et al. (1998) reported that *Y. lipolytica* NCIM 3589, isolated from contaminated sea water, was able to degrade pure alkanes, namely hexadecane (60 %), n-tetradecane (50 %), n-octadecane (45 %), n-decane (40 %) and n-dodecane (40 %) after 24 h of culture at 30 °C under aerobic conditions, being the degradation of alkanes correlated to yeast morphology. Zogała et al. (2005) observed that was possible to achieve *in situ* soil decontamination using *Y. lipolytica* A-101 strain. Schmitz et al. (2000) tested the ability of six yeast species to degrade alkanes and found that *Y. lipolytica* and *Candida maltosa* are capable to degrade 96 % of n-tetradecane.

Several cultivation parameters can affect the percentage of hydrocarbons degradation 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 $g \cdot L^{-1}$ – 1.9 $g \cdot L^{-1}$), and observed a higher degradation (98 %) at 28 °C, 160 rpm, 0.1 % (v/v) of petroleum and 1 $g \cdot L^{-1}$ of initial cellular concentration. Also cell morphology appears to affect alkanes degradation by *Y. lipolytica* strains. In some studies, it was established that yeast morphology plays a role in hydrocarbon degradation. When a yeast culture, predominantly in mycelium form, was inoculated into a medium containing alkanes, it was observed a rapid transition to the yeast form, indicating that this morphology is more suitable for the use of alkanes (Zinjarde et al. 1998; Zinjarde et al. 2008).

It was reported that *Y. lipolytica* cells displayed an oval form during hexadecane degradation and surfactant production (Rodriguez & Domínguez 1984; Gutierrez & Erickson 1977; Zinjarde et al. 1997; Zinjarde et al. 1998). In aerobic conditions, *Y. lipolytica* cells growing in mycelium form rapidly

switched to yeast form when n-dodecane was added to the culture (Zinjarde et al. 2008). Palande et al. (2014) observed that hexadecane affected negatively the mycelial development of *Y. lipolytica* var. *indica*.

It was reported that *Y. lipolytica* had the ability to form biofilms in the presence of alkane as carbon source. This result is important because cells in biofilms are more protected by the matrix formed and can survive during periods of stress (Bankar et al. 2009).

The uptake of alkanes by *Y. lipolytica* is not completely explained, but there are findings that support two possible ways of substrate transportation. The metabolism of alkanes is illustrated in Figure 1.

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 biosurfactant. Substrate droplets enter the cell by passive transport or by transport/export mechanisms of the cell (hydrophobic outgrowths) (Barth & 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).

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 & 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). After that fatty aldehydes suffer the same transformation described previously. Here in peroxisome, the activation of fatty acids is made by fatty-acyl-CoA synthetase II (ACS II) also generating fatty-acyl-CoA.

Fatty-acyl-CoA formed in cytosol and in peroxisome enters 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 trigacylglycerols (TAG) or steryl esters (STE) (Fickers et al. 2005; Fukuda 2013).

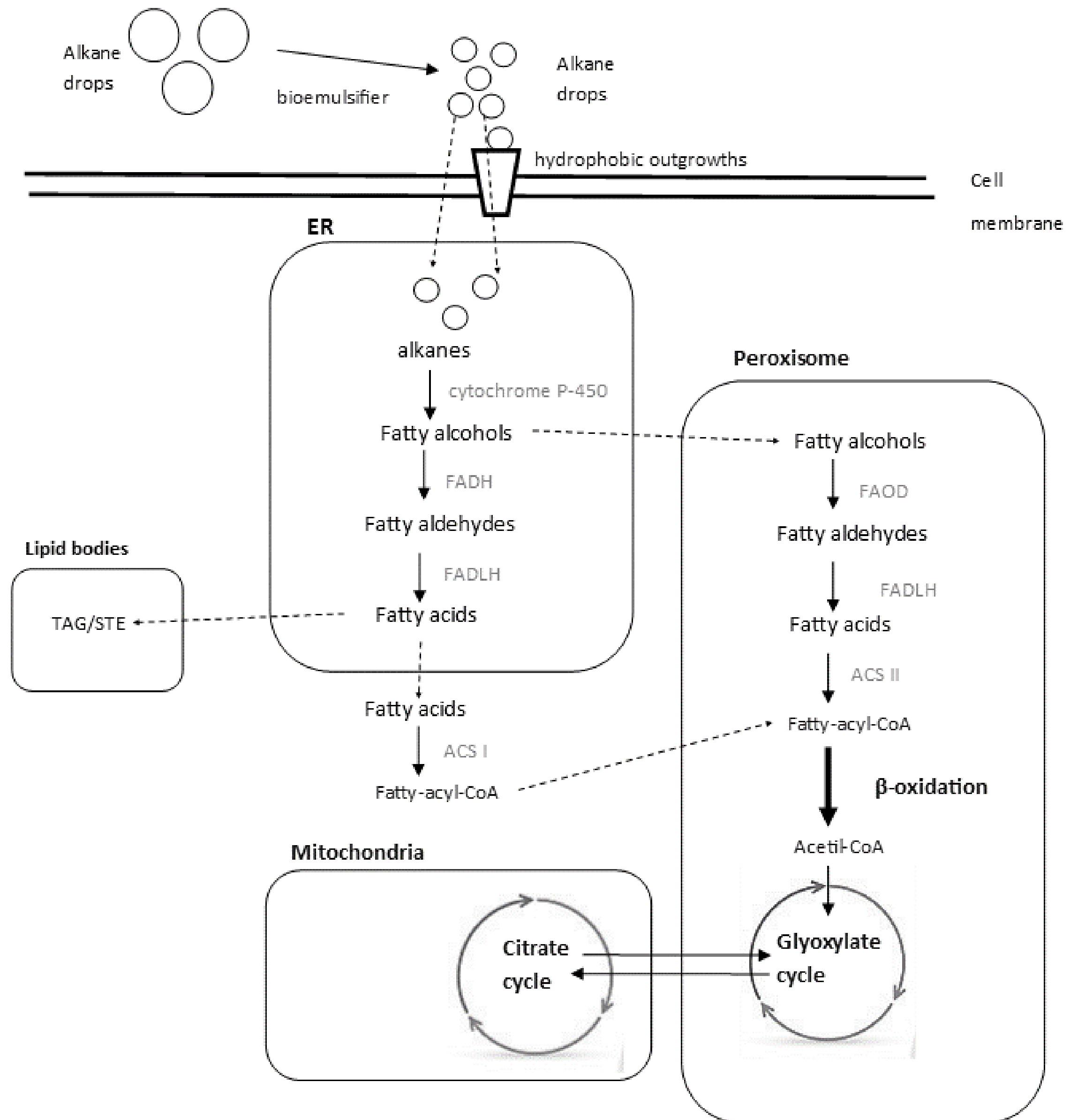

Figure 1- Main metabolic pathways and cellular compartments involved in alkane degradation. Main metabolic flux during alkane oxidation is shown with black arrows (enzymatic steps), enzymes are written in grey next to the arrows and dashed arrows represent metabolites transportation.

Y. lipolytica produces a wide range of metabolites from several substrates. When alkanes are used as carbon source, this yeast has the ability to produce biosurfactants and organic acids, such as citric and isocitric acids (Zinjarde et al. 1998; Crolla & Kennedy 2001; A. Crolla & Kennedy 2004; Wojtatowicz et al. 1993).

Biosurfactants production has the function of decrease the surface tension and enable the cellular uptake of hydrophobic substrates such as alkanes (Cirigliano & Carman 1984; Nakahara et al. 1977; Pareilleux 1979). Cirigliano & Carman (1985) characterized an emulsifier, called Liposan, which was produced in culture medium with hexadecane and was constituted by carbohydrate (83 %) and protein (17 %). Zinjarde et al. (1998) also showed that *Y. lipolytica* NCIM 3589 produced an emulsifier in a medium with alkanes as carbon source in conditions of excess of substrate.

Nakahara et al. (1977) reported a production of biosurfactant by *Y. lipolytica* ATCC 8662 with hexadecane as carbon source and observed a small increase in the maximum growth rate and a longer exponential phase with the increase of hexadecane concentration from 1 % to 10 % (v/v) in the culture medium.

Y. lipolytica can also produce organic acids from n-alkanes (Akiyama et al. 1973; Wojtatowicz et al. 1993). Akiyama et al. (1973) obtained higher citric and isocitric acids production with *Y. lipolyitica* mutant strains from alkanes as carbon source. Wojtatowicz et al. (1993) produced citric and isocitric acids from hexadecane with *Y. lipolytica* A- 101 and attained a yield of 1.7 g total acid/g hexadecane.

Crolla & Kennedy (2004) studied the effect of stirring rate in citric acid production by *Y. lipolytica* NRRL-Y-1095 from alkanes and concluded that higher cellular growth and citric acid production were attained in the range of 800 rpm – 1000 rpm. Crolla & Kennedy (2001) concluded that temperature, inoculum density and alkane concentration are factors that affect citric acid production by *Y. lipolytica* NRRL-Y-1095.

Also, acid α-ketoglutaric can be produced with hexadecane (and other alkanes) as carbon source by *Y. lipolytica*. With addition of thiamine (a vitamin not synthesized by *Y. lipolytica* but necessary for α-ketoglutarate dehydrogenase activity) and the studied of best conditions it was possible increase the concentration of this acid to 108.7 $g \cdot L^{-1}$ (yield, 120% petrolatum, w/w) (Finogenova et al. 2005). And Kamzolova et al. (2012) produced succinic acid from alkanes with *Y. lipolytica* VKM Y-2412 and reached maximum acid concentration of 38.8 $g \cdot L^{-1}$ and 82.45 % of the alkanes consumed.

2. AIMS

The non-conventional yeast *Y. lipolytica*, a strictly aerobic yeast, is capable to use n-alkanes as carbon source to grow and to produce products of industrial interest.

Our objective was to study the growth of *Y. lipolytica* W29 in batch cultures using hexadecane as substrate. Cultivation parameters that could affect the yeast performance, such as nitrogen source, surfactants and oxygenation, were also intended to study.

Therefore, the specific goals of this work were:

- Evaluate the effect of several parameters (n-hexadecane concentration, surfactant and nitrogen source) on cellular growth of *Y. lipolytica* W29 cultures in Erlenmeyer flask cultures.

- Assess the effect of dissolved oxygen concentration in use of n-hexadecane as a carbon source in bioreactors experiments.

- Characterization of metabolites produced by *Y. lipolytica* W29 from n-hexadecane as sole carbon and energy source.

- Characterization of yeast morphology during cellular growth in hexadecane and evaluation of possible impact on the culture behavior.

3. MATERIALS AND METHODS

3.1 Yeast strain and culture media

Y. lipolytica W29 (ATCC20460:CLIB89) was 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.

Yeasts cells were pre-grown overnight (pre-inoculum) in 500 mL Erlenmeyer flasks with 200 mL of YPD (glucose 20 g·L^{-1}, peptone 20 g·L^{-1} and yeast extract 10 g·L^{-1}) at 27 °C in an incubator shaker at 140 rpm. Pre-inoculum medium was used to inoculate media with hexadecane.

3.2 Erlenmeyer flask experiments

In order to evaluate the ability of *Y. lipolytica* W29 cells to use hexadecane as carbon source, several factors were tested, namely: (a) initial concentration of hexadecane - 5 g L^{-1} and 10 g·L^{-1} ; (b) presence of a surfactant - Tween 80 (equal to 10 % of carbon source concentration, in order to maintain the concentration of Tween 80 above the critical micelle concentration); (c) presence of a nitrogen source – Yeast nitrogen base (YNB) without amino acids (6.7 g L^{-1}); (d) presence of amino acids in the medium - YNB with amino acids (6.7 g·L $^{-1}$); and (e) influence of low viscosity Silicone Oil (10 %, v/v). All media were prepared in potassium phosphate buffer 0.1 M, pH 6. Taking into account these factors, 7 experiments were planned (Table 2). All experiments were carried out in 500 mL Erlenmeyer flasks filled with 200 mL of each medium described in Table 2, with an initial cell density of 1 g·L $^{-1}$, in an orbital incubator at 140 rpm and 27 °C.

Table 2- Medium composition of each experiment carried out in Erlenmeyer flasks

Experiment	Composition
1	Hexadecane (5 g·L^{-1})
2	Hexadecane (10 g·L $^{-1}$)
3	Hexadecane (10 g·L $^{-1}$) and silicone oil 10 % (v/v)
4	Hexadecane (5 g·L $^{-1}$) and Tween 80 10 % (v/v)
5	Hexadecane (5 g·L $^{-1}$) and YNB without amino acids (6.7 g·L $^{-1}$)
6	Hexadecane (5 g·L $^{-1}$) and YNB with amino acids (6.7 g·L $^{-1}$)
7	Hexadecane (5 g·L $^{-1}$), Tween 80 10 % (v/v), YNB with amino acids (6.7 g·L $^{-1}$)

3.3 Bioreactor experiments

After the preliminary experiments carried out in Erlenmeyer flasks, several batch cultures were performed in a lab-scale bioreactor, in order to evaluate the effect of dissolved oxygen concentration on hexadecane utilization by *Y. lipolytica* W29 cultures.

Batch experiments were carried out in a 2 L bioreactor (Biolab, B. Braun, Germany), filled with 1 L of medium, at 27 °C and pH was maintained at 6 by addition of NaOH 4 M. To monitor dissolved oxygen concentration, bioreactor is equipped with a polarographic oxygen probe (12/220 T-type, Metler Toledo, USA) and the respective meter (type 170).

Medium composition and operational conditions used in each experiment in bioreactor are described in Table 3. All media were prepared in potassium phosphate buffer 0.1 M, pH 6.

Table 3- Medium composition and operational conditions used in the experiments carried out in bioreactor

Experiment	Medium composition	Operational conditions
1 (control)	YNB without amino acids (6.7 g·L^{-1}); Tween 80 (5 g·L^{-1})	200 rpm and 0.5 vvm
2	Hexadecane (5 g·L^{-1}); YNB without amino acids (6.7 g·L^{-1}); Tween 80 (5 g·L^{-1})	200 rpm and 0.5 vvm
3	Hexadecane (5 g·L $^{-1}$); YNB without amino acids (6.7 g·L^{-1}); Tween 80 (5 g·L^{-1})	400 rpm and 1 vvm

Culture medium (without YNB and hexadecane) was sterilized in bioreactor vessel by autoclaving at 121 °C for 15 min. YNB and hexadecane was sterilized by filtration through 0.2 μm filter and added to the medium in sterile conditions.

3.3 Analytical methods

Samples were collected at appropriate intervals for measurement of cell concentration and hexadecane concentration.

For cell concentration, density of cultures was measured at 600 nm and converted to cell dry weight ($g{\cdot}L^{-1}$) by a calibration curve. A blank test without cells (supernatant of culture) was read at 600 nm in order to assess the influence of hexadecane on optical density measurement and no effect was observed.

Cell viability was estimated by the methylene blue staining method (Jones, 1987), in which viable cells are colorless and non-viable cells are stained of blue. To perform this procedure, 500 μL of cell culture was added to 500 μL of methylene blue (0.1 $g{\cdot}L^{-1}$), stirred by vortexing and incubated for 5 min at room temperature and protected from light. After the incubation period, the mixture was stirred again and 10 μL was transferred into a Neubaeur chamber (Blaubrand, Germany) in order to count the viable and non-viable cells in the optical microscope (Leica ATC 2000).

The viability of the cultures (%) was determined by the equation:

$$Viability\ (\%) = \frac{number\ of\ viable\ cells}{total\ number\ of\ cells} \times 100$$

Hexadecane concentration was quantified by gas chromatography (GC) after extraction with diethyl ether (purity 99.9 %). One milliliter of culture (cells + supernatant) was taken every 24 h and 0.1 mL of undecane 15 $g{\cdot}L^{-1}$ (internal standard - IS) was added. For liquid/liquid extraction, 2 mL of diethyl ether was added to the sample (culture + undecane), mixed (by vortex) for sixty seconds and stand for 2 hours. 200 μL of organic phase were collected, diluted with 800 μL of diethyl ether and injected in GC chromatograph.

Chromatograph (Bruker Scion chromatograph GC-436) was equipped with a capillary column Bruker BR1-MS (15 m of length, 0.25mm of diameter and 0.25 mm of thick film). Analyses were carried out with constant flow mode, using hydrogen (99.999 % purity) as carrier gas. The hydrogen

pressure in column was 5 bar. Injector was at 250 °C. The oven temperature was programmed from 60 °C (1 min) to 170 °C at a temperature gradient of 4 °C/min.

Hexadecane concentration was quantified according to equation:

$$[Hexadecane] = [Undecane] \times \frac{1}{K} \times \frac{A_{Hexadecane}}{A_{Undecane}}$$

where [Hexadecane] is hexadecane concentration (g/L); [Undecane] is undecane concentration (g/L); K is the response coefficient; $A_{hexadecane}$ is the area of hexadecane peak; and $A_{undecane}$ is the area of undecane peak. Coefficient K was obtained by a calibration curve previous prepared. Coefficient K was determined by the slope of graphical representation of hexadecane peak area/undecane peak area *versus* hexadecane concentration/undecane concentration.

For image analysis, samples was taken every 48 hours and used without preparation. Images were acquired at 1360 × 1024 pixels and 8-bit format with 100× total magnification, through the commercial software CellBˆ (Olympus, Tokyo, Japan) by a Olympus BX51 optical microscope (Olympus, Tokyo, Japan) coupled with an Olympus DP72 camera (Olympus, Tokyo, Japan). Images were analyzed by image processing and analysis programs (program comprise the image pre-processing, segmentation, hyphae and yeast cells recognition, and image post processing steps) developed in Matlab 7.3 (The Mathworks, Inc., Natick) language to determine yeast and hyphae morphology (Braga et al. 2016).

Yeast cells were observed in an Olympus BX51 microscope immediately after sampling (240 h of growth) for the intracellular lipids visualization.

For lipid quantification, a sample was taken in the final of cultivation, centrifuged, washed tree times with PBS 50 mM pH 7.3 and lyophilized. 10 mg of lyophilized biomass was used to extract lipids with methanol and chloroform (1:1, v/v). 250 µL of the extraction mixture were evaporated at 100 °C and 100 µL of pure sulfuric acid were added to the sediment and heated at 100 °C during 15 min. After the solution cool down to room temperature, 2.4 mL of phospho-vanillin (vanillin dissolved in ortophosphoric acid 85 %) was added and left 15 min at room temperature. The absorbance of the mixture was read at 490 nm. The absorbance was converted to lipids concentration ($g \cdot L^{-1}$) by a calibration curve (olive oil was used as standard) (Inouye & Lotufo 2006).

Citric acid concentration was measured by high-performance liquid chromatography (HPLC) using an YMC ODS-Aq (250 x 4.6 mm) reverse phase column coupled to a diode array detector at 214

nm. The mobile phase was KH_2PO_4 20 mM, pH 2.8 at room temperature and a rate flow of 0.7 $mL \cdot min^{-1}$.

4. Results and discussion

4.1 Preliminary experiments in Erlenmeyer flask

4.1.1 Effect of hexadecane concentration

Y. lipolytica has the ability to use several substrates as carbon source, namely hydrophobic compounds such as alkanes. Nowadays, this ability could be of great interest to simultaneously degrade alkanes in waste streams and use it to grow and produce added-value compounds, such single cell protein. Therefore, the effect of substrate (hexadecane) concentration on *Y. lipolytica* growth was evaluated. Two concentrations of hexadecane (5 $g{\cdot}L^{-1}$ and 10 $g{\cdot}L^{-1}$) were tested and yeast cellular growth profiles are presented in Figure 2.

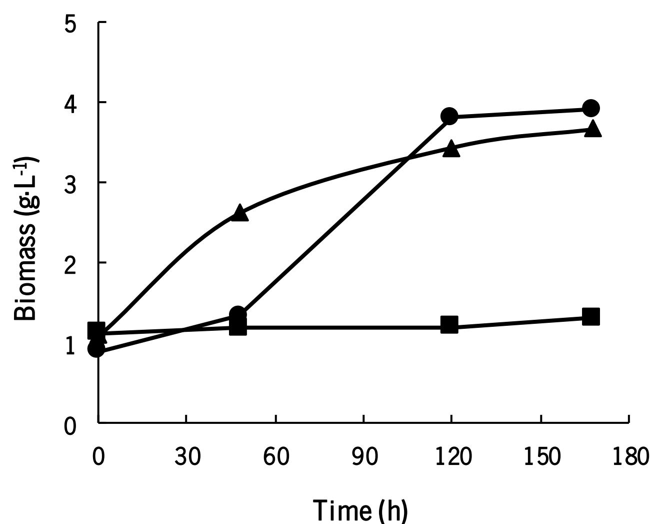

Figure 2- Cellular growth profiles of *Y. lipolytica* W29 obtained in experiments carried out with 5 $g{\cdot}L^{-1}$ of hexadecane (●), 10 $g{\cdot}L^{-1}$ of hexadecane (■) and 10 $g{\cdot}L^{-1}$ of hexadecane with silicone oil 10 % (v/v) (▲).

In the experiment performed with 5 $g{\cdot}L^{-1}$ of hexadecane, yeast growth had a lag phase of approximately 48 hours, followed by an exponential phase. In the last hours of fermentation, the stationary phase seems to be reached. However, when *Y. lipolytica* W29 cells were grown in other carbon sources, such as glucose and glycerol, does not showed such a long lag phase (Braga & Belo 2015; Ferreira et al. 2013). The time needed to reach the exponential phase with alkane as a carbon source indicates that yeast cells need more time to adapt and assimilate alkane when compared to

other carbon sources. Because hexadecane is not immiscible with water, it uptake requires morphological and physiological modifications, notably in cell adhesion properties (surface hydrophobicity) or in the production of emulsifiers (biosurfactants) (Sinigaglia et al. 1994). However, after this adaptation phase, yeast is able to grow on hexadecane and reached a final biomass concentration of 3.9 $g{\cdot}L^{-1}$.

Hassanshahian et al. (2012) demonstrated the ability of two *Y. lipolytica* strains, isolated from an oil-polluted area in the Persian Gulf, to grow and degrade crude oil. However, authors observed that *Y. lipolytica* PG-20 strain was more capable to degrade long chain length alkanes (C_{16}-C_{22}) than *Y. lipolytica* PG-32 strains. Probably, the strain used in the present work degrades more easily medium chain length alkanes than long chain ones.

In the experiment performed with 10 $g{\cdot}L^{-1}$ of hexadecane no significant cellular growth was observed, which may show that this hexadecane concentration has an inhibitory effect on yeast metabolism. Contrariwise, this yeast strain grown well and no inhibitory effects of substrate concentration was observed with 10 $g{\cdot}L^{-1}$ of glucose (Braga et al. 2016) or glycerol (Ferreira et al. 2016).

Silicone oil is often mentioned as an effective absorbent of hydrophobic compounds and has been used by other authors as a pollutant reservoir (Darracq et al. 2010; Castro et al. 2014; Ascon-Cabrera & Lebeault 1995). Considering that silicone oil has the ability to capture and continuously release substrate to the medium, it was decided to study the addition of silicone oil (10 %, v/v) to the medium with 10 $g{\cdot}L^{-1}$ of hexadecane and evaluate the effect on yeast growth. In these conditions, cellular growth not exhibited a lag phase and the exponential phase started in the first hours of fermentation, which shows that silicone oil addition eliminated the inhibitory effect observed in the experiments carried out only with 10 $g{\cdot}L^{-1}$ of hexadecane.

Despite the increase of initial growth rate in the experiment performed with 10 $g{\cdot}L^{-1}$ of hexadecane and silicone oil compared to the experiment carried out with 5 $g{\cdot}L^{-1}$ of hexadecane, similar final biomass concentration was reached. Thus, it is possible to suggest that the presence of silicone oil favored the specific growth rate in the first hours of growth, instead of final biomass concentration.

Cellular viability of *Y. lipolytica* cells remained above 90 % for all experiments, even for that carried out with 10 $g{\cdot}L^{-1}$ of hexadecane. This indicates that hexadecane was not toxic to cells in the range of concentrations tested. Probably, the low cellular growth observed was due to limited substrate dispersion in the culture medium and consequently weaker contact between droplets and cells that may occur at 10 $g{\cdot}L^{-1}$ of hexadecane. When the silicone oil was added to the medium, hexadecane was

distributed into both phases (organic and aqueous), decreasing its concentration in aqueous culture medium and reducing possible toxic effects or dispersion problems.

As the higher biomass yield was obtained in the experiment with 5 g·L^{-1} of hexadecane as carbon source the following experiments were carried out with this concentration of substrate.

4.1.2 Effect of surfactant

One major constraint of use hydrophobic substrates (such as hexadecane) in microbial cultures is the requirement of an efficient dispersion of organic phase (containing the hydrophobic substrate) into the aqueous medium. One approach to minimize this problem is the use of an emulsifier, a compound that comprises both hydrophobic and hydrophilic components. Emulsifiers are dispersed at the oil–water or water–oil interface, decreasing the interfacial tension and increasing the hexadecane availability to the cells (Bos & Van Vliet 2001). Moreover, the beneficial effect of surfactants (produced *in situ* by microorganisms or added to the medium) was already proved in the treatment of sludge oil (Hu et al. 2013) .Thus, the effect of surfactant addition (Tween 80) on *Y. lipolytica* W29 growth was studied and the cellular growth profiles are shown in Figure 3.

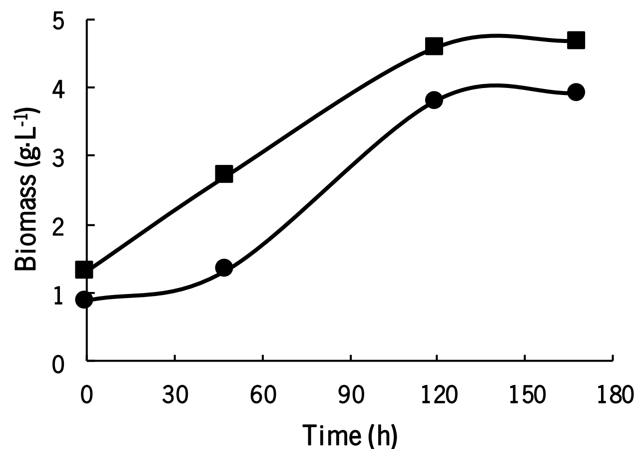

Figure 3- Cellular growth profile of *Y. lipolytica* W29 in the experiments with 5 g·L^{-1} of hexadecane and with (■) and without (●) Tween 80.

In experiment performed with Tween 80, no lag phase was observed in cellular growth, unlike as occurred in the experiments without surfactant. The exponential phase began in the first hours of

fermentation and reached a final cell dry weight of 4.5 $g{\cdot}L^{-1}$. The cellular growth rate for both experiments was similar (Table 4) but the biomass yield obtained for the experiment carried out with Tween 80 was slightly higher. Tween 80 (as well other emulsifiers) is a compound that helps the formation of micelles or microdroplets of hydrophobic substrates, increasing the contact area between cells and substrate, which leads to a higher assimilation of substrate by yeast cells.

Table 4- Specific growth rate (μ) and biomass yield (Yx/s) obtained for experiments carried out without and with Tween 80

	μ (h^{-1})	**Yx/s**
Without Tween 80	0.011	0.60
With Tween 80	0.010	0.67

Whitworth et al. (1973) studied the effect of several emulsifiers in alkanes degradation by *Y. lipolytica* ATCC 8661 and found that in the presence of Tween 80 cellular growth was higher compared to control (no emulsifiers) and to other emulsifiers tested. In *Halomonas sp*. C2SS100 cultures, the addition of Tween 80 significantly decreased the adaptation phase of bacteria to the medium (waters collected from petroleum reservoirs) and increased the initial cellular growth rate (Mnif et al. 2009). In *Pseudomonas aeruginosa* cultures, the addition of a surfactant enhanced hexadecane biodegradation (Noordman et al. 2002).

As the higher biomass yield was obtained in the experiments with Tween 80, as well no lag phase was observed, it was decided to perform the following experiments with Tween 80.

4.1.3 Effect of a nitrogen source

As the presence of a nitrogen source is often associated to an improvement of cellular growth (Cruz et al. 2002), the effect of the addition of YNB (which has ammonium sulfate as nitrogen source) was studied. Additionally, the influence of amino acids in YNB on cellular growth was assessed. The cellular growth profiles of *Y. lipolytica* W29 obtained in experiments with YNB with and without amino acids are presented in Figure 4.

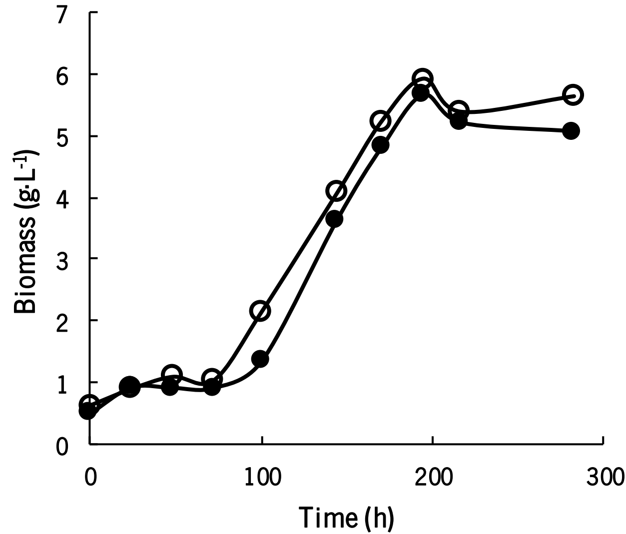

Figure 4- Cellular growth profile of *Y. lipolytica* W29 in the experiments carried out with 5 $g \cdot L^{-1}$ of hexadecane and YNB with amino acids (closed symbols) and without amino acids (open symbols).

In these experiments, a lag phase of approximately 70 h was observed, followed by an exponential phase until 190 h of growth, reaching then the stationary phase. When compared to the experiment without nitrogen source (see chapter 4.1.2), the final biomass was improved from 4.68 $g \cdot L^{-1}$ to 5.67 $g \cdot L^{-1}$ (which corresponds to a 17 % increase) and the specific growth rate increased from 0.011 h^{-1} to 0.016 h^{-1} (which corresponds to a 30 % increase). These results indicate that the addition of a nitrogen source had a positive effect on cellular growth in medium with hexadecane as carbon source.

In both experiments performed with YNB (one with amino acids and the other without amino acids), the specific growth rate was 0.016 h^{-1} and biomass yield of 1. Thus, it can be concluded that the addition of amino acids had no major benefits on *Y. lipolytica* W29 growth in medium with hexadecane.

Taking into account the results of these preliminary experiments carried out in Erlenmeyer flasks, a medium composition for the maximum cellular growth of *Y. lipolytica* W29 on hexadecane was defined: hexadecane 5 $g \cdot L^{-1}$, Tween 80 10 % and YNB without amino acids 6.7 $g \cdot L^{-1}$ dissolved in potassium phosphate buffer 0.1 M, pH 6.

4.2 Bioreactor experiments

Y. lipolytica is a strictly aerobic yeast, which means that dissolved oxygen concentration in the culture medium is an important factor for carbon source assimilation and, consequently, yeast growth.

Thus, the effect of oxygenation on cellular growth was studied in bioreactor batch experiments with different aeration and agitation rates (in order to obtain different oxygen transfer rates and consequently different dissolved oxygen concentrations in the culture medium). The growth profiles, hexadecane degradation and dissolved oxygen concentration profiles obtained in bioreactor experiments are presented in Figure 5.

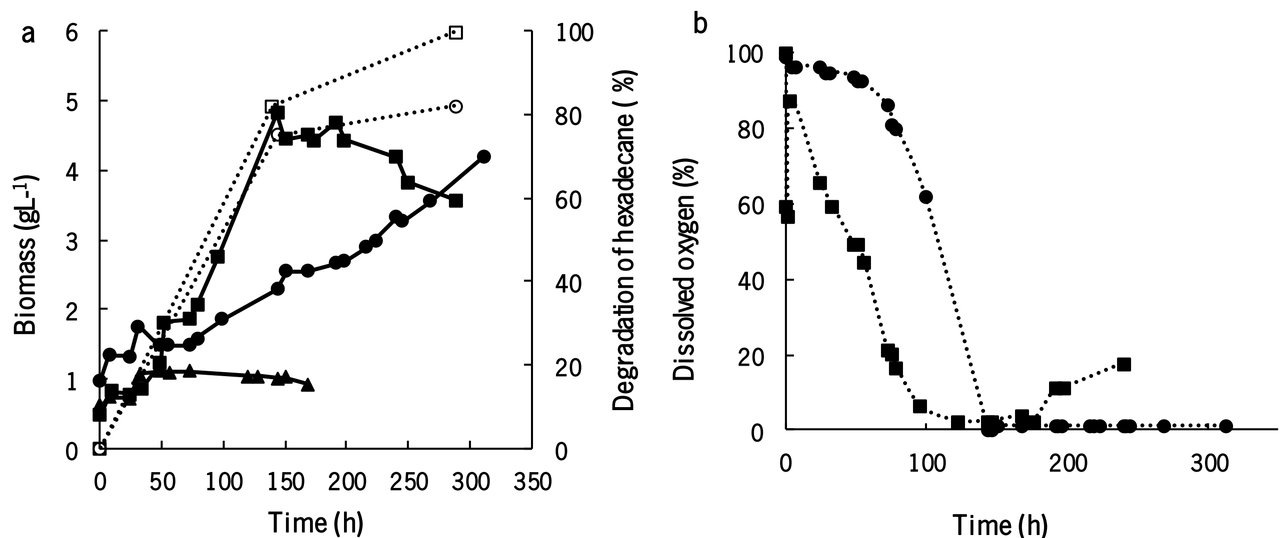

Figure 5- (a) Cellular growth profile of *Y. lipolytica* W29 in batch cultures (closed symbols) and percentage of hexadecane degradation (open symbols) in the experiments carried out without hexadecane (▲) and with 5 g·L-1 of hexadecane at 200 rpm and 0.5 vvm (■) and at 400 rpm and 1 vvm (●); **(b)** dissolved oxygen profiles obtained in the experiments carried out with 5 g·L-1 of hexadecane at 200 rpm and 0.5 vvm (■) and at 400 rpm and 1 vvm (●).

As Whitworth et al. (1973) suggested that Tween 80 can be used as carbon source by *Y. lipolytica* when the yeast is grown in glucose and alkanes, it was decided to realize a control experiment without hexadecane in order to understand if cellular growth is due to hexadecane degradation and assimilation by yeast cells or due to another component of the medium. In the absence of hexadecane, no significant cellular growth was observed. In the first 32 hours, a slight increase in yeast growth (from 0.62 g·L-1 to 1.07 g·L-1) was observed, but it stopped after this time. This behavior suggests that, during first hours yeast can use other medium components as carbon source. However, this growth is not significant compared to the cellular growth obtained in experiments with hexadecane, indicating that yeast growth resulted from hexadecane degradation and assimilation as the principal carbon source.

In the experiment with hexadecane carried out at 200 rpm and 0.5 vvm, cellular growth began in the first hours of bioprocess (no considerable lag phase was observed) and remained in exponential phase until 144 hours. At this time, cellular growth stabilized and the stationary phase was reached. In

the experiment conducted at 400 rpm and 1 vvm, also yeast growth began in the first hours of fermentation but the exponential growth phase remained until the final of the experiment.

The specific growth rate obtained in the experiment conducted at 200 rpm and 0.5 vvm was 4-times higher than that obtained in the experiment carried out at 400 rpm and 1 vvm (Table 5). Also maximum productivity in this experiment was 30-times higher than in the experiment with more oxygenation.

Table 5- Specific growth rate (μ) and maximum biomass productivity obtained for bioreactor experiments carried out at different conditions of agitation and aeration

Experiment conditions	μ (h^{-1})	Maximum Productivity ($g{\cdot}L^{-1}{\cdot}h^{-1}$)
200 rpm, 0.5 vvm	0.016	0.030
400 rpm, 1 vvm	0.004	0.010

In the final of process, it was observed a completely degradation of hexadecane in the experiment performed in conditions of 200 rpm and 0.5 vvm, whereas in the experiment at 400 rpm and 1 vvm hexadecane degradation only reached 90 %. In both experiments, same hexadecane degradation profile was observed until 144 h of culture. However, and regarding the same hexadecane consumption, higher biomass (52 % higher) was attained in the experiment with less oxygenation at 144 h. It was expected that the increase of stirring rate would lead to a better dispersion of hexadecane in culture medium, since higher stirring rates break hexadecane into smaller droplets, making it more available to yeast cells. Probably, the increase of dissolved oxygen concentration, associated to possible shear stress induced by higher stirring rate, could lead to metabolic shifts in yeast cell and the final result is a balance between several effects.

Moo-Young et al. (1971) concluded that an increase of agitation rate led to an improvement of biomass yield with alkanes as substrate in *C. lipolytica* ATCC 8661 cultures. However, Crolla & K. J. Kennedy (2004a) observed that ideal agitation rate for alkane degradation by *C. lipolytica* NRRL-Y-1095 was 800 rpm – 1000 rpm and with 1200 rpm of agitation the yeast presented less biomass yields. They suggested that higher agitation lead to negative metabolic effects, or the turbulence and shear may interfere with physical interaction between alkanes and the cells.

It was reported the ability of *Y. lipolytica* to form biofilms in hexadecane as carbon source as a skill for cells counteract possible stress conditions (Dusane et al. 2008). Probably, the mechanical agitation of 400 rpm used in the present work could destroy the biofilm formed by the cells and affect their capacity to grow.

In the experiment without hexadecane, the dissolved oxygen concentration slightly decreased in the first 32 h and remained above 80 % until the final (data not shown). This result was expected since no significant growth was observed. In the other two experiments, the dissolved oxygen concentration decreased from the beginning and reached 0 % after 140 h of culture, corresponding in both cases to the exponential growth phase. However, the oxygen consumption rate was higher in the experiment carried out at 200 rpm and 0.5 vvm, and was in this experiment that a higher specific growth rate was obtained. After 176 h, the oxygen concentration in the culture performed at 200 rpm and 0.5 vvm slightly increased, which corresponded to the stationary growth phase observed in growth profiles. On the other hand, in the experiment at 400 rpm and 1 vvm, oxygen concentration remained equal to 0 % until the end of culture that is in accordance with growth profile, which no stationary phase was reached.

Besides the ability to produce biomass that can be used as single cell protein, *Y. lipolytica* has also the capability to produce organic acids from hexadecane (Zinjarde & Pant 2002; A. Crolla & Kennedy 2004). Taking into account this information, citric acid was measured in the culture supernatants but no production was observed.

The yeast *Y. lipolytica* are able to accumulate free fatty acids, generally as triacylglycerol and steryl esters in lipid bodies (LP). In order to evaluate if *Y. lipolytica* W29 cells was capable to accumulate lipids intracellularly from hexadecane, total microbial lipids were quantified at the end of experiment with higher hexadecane degradation and biomass concentration (5 $g \cdot L^{-1}$ of hexadecane, 200 rpm and 0.5 vvm). It was observed that yeast cells accumulated 26 % of their cell dry weight as microbial lipids, showing that *Y. lipolytica* cells were capable to accumulate a significant amount of lipids from hexadecane. This result is particularly interesting, since simultaneous to hexadecane biodegradation and biomass production, also an added-value product was obtained. Moreover, no studies in the literature regarding the production of microbial lipids from hexadecane are available. From microscopic visualization of *Y. lipolytica* W29 cells grown in hexadecane it was possible to visualize the microbial lipids accumulated as lipid bodies in the cytoplasm (Figure 6).

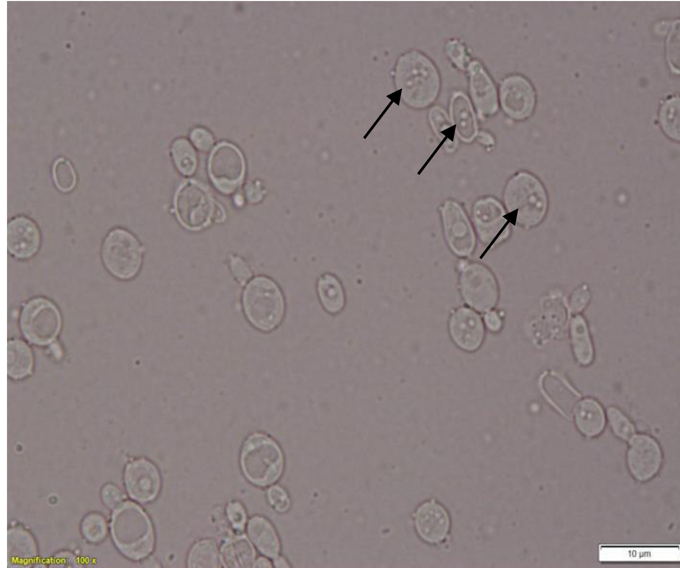

Figure 6 - Light microscopy image of *Y. lipolytica* W29 cells: visualization of lipid bodies in the cytoplasm after 204 h of growth in hexadecane 5 $g \cdot L^{-1}$. The arrows indicate microbial lipids in the cytoplasm.

Papanikolaou et al. (2002) obtained with *Y. lipolytica* ACA-DC 50109 an accumulation of 44 – 0.54 g of lipid per gram of biomass with industrial fat of animal origin, named stearin as carbon source at pH 6 and a temperature of 28 – 33 °C. Bati et al. (1984) reached an accumulation of 55 % (w/w) of lipids with *Y. lipolytica* 1094 growing on corn oil. And Rakicka et al. (2015) already proves that *Y. lipolytica* is capable to produce lipids from industrial residues and obtained with *Y. lipolytica* JMY4086a lipid production of 31 % of of cell dry weight from crude glycerol.

4.2.1 Quantitative image analysis (QIA)

Dimorphic yeast *Y. lipolytica* is capable to grow in two different morphological forms, as single oval form or as filamentous hyphae, being reversible between each other and dependent on yeast strain and growth conditions (Kawasse et al. 2003; Smita et al. 2008; Braga et al. 2016). Since few studies concerning the effect of hexadecane on *Y. lipolytica* morphology are available, a quantitative image analysis of cells growing in bioreactor was performed. The QIA consist in three sub processes: (a) segmentation, where yeast and hyphae morphology was recognized through segmentation by a predefined 0.9 threshold value by a MatLab function; (b) hyphae and yeast cells recognition, where hyphae morphology is determined taking into account a combined width and morphology approach (cells above 3.5 μm of diameter are considered yeast cells or cell aggregates); and (c) image post processing steps, where the final yeasts and hyphae cells binary images are saved after the removal of

image boundaries. Some images were selected to illustrate yeast morphology during the experiments and are presented in Figure 7.

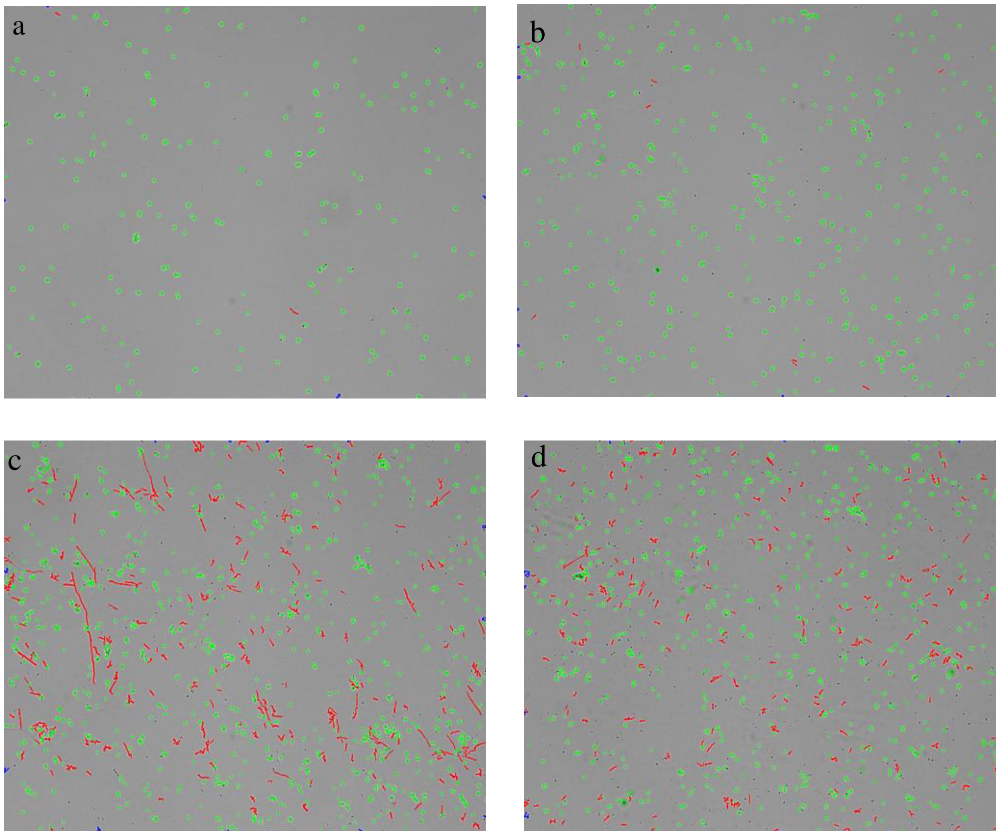

Figure 7- Images obtained in QIA at the beginning of the experiments (a) and at the end of each experiment: (b) 0 $g{\cdot}L^{-1}$ of hexadecane; (c) 5 $g{\cdot}L^{-1}$ of hexadecane carried out at 200 rpm, 0.5 vvm and (d) 5 $g{\cdot}L^{-1}$ of hexadecane carried out at 400 rpm, 1 vvm.

Images were analyzed in order to characterize the most relevant size parameters, such as the ratio of total hyphae length and total yeast area (TH/TY) (Figure 8).

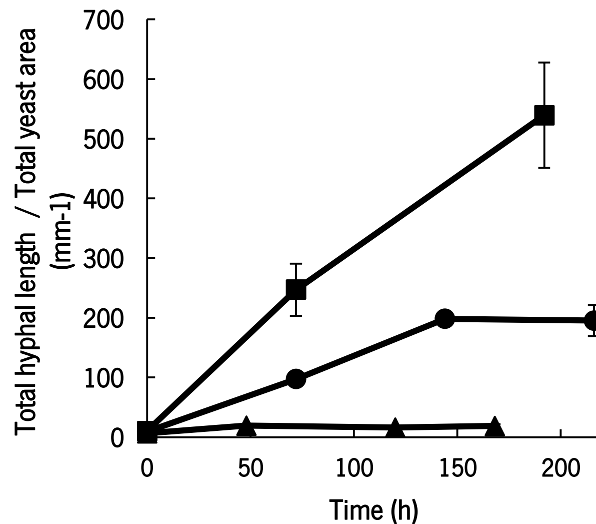

Figure 8- Experimental behavior of total hyphae length per total yeast area ratio for the experiments: without hexadecane (▲), 5 g·L^{-1} of hexadecane, 200 rpm and 0.5 vvm; (■) 5 g·L^{-1} of hexadecane, 400 rpm and 1 vvm (●).

In the experiment performed without hexadecane in the medium yeast cells did not grow and no morphology transition was observed, since TH/TY ratio remained constant, which indicates that cells remained in oval form and the agitation (200 rpm) and aeration (0.5 vvm) solely not promoted a shift in *Y. lipolytica* morphology. However, with the addition of 5 g·L^{-1} of hexadecane to the medium, TH/TY ratio increased to 539.4 mm^{-1} from the beginning until the end of process, indicating a continuous shift from yeast to hyphae form. This result indicates that hexadecane induced hyphae morphology in *Y. lipolytica* W29 cells. However, in the experiment performed with same concentration of hexadecane but with higher dissolved oxygen (400 rpm and 1 vvm), TH/TY ratio increased in the firsts six days and stabilized in 198.3 mm^{-1} until the end of growth. This result indicates that hexadecane can induce hyphae formation but other factors also have influence on yeast morphology transition. However, Braga et al. (2016) reported that, during growth in hydrophobic substrates (castor oil), *Y. lipolytica W29* cells remained in yeast single cell morphology. Also Zinjarde et al. (2008) reported that immediately after the transference of *Y. lipolytica* cells in mycelium form to a dodecane, cells changed to yeast form and Palande et al. (2014) observed that hexadecane affected negatively the mycelial development of *Y. lipolytica* var. *indica*.

5. Conclusions and future work

The possibility to use *Y. lipolytica* W29 to degrade hexadecane (a pollutant compound frequently found in several industrial effluents) and, additionally, produce added-value products may result in an environmentally-friendly and cost-effective process and. Thus, the main goal of this work was to study the growth of *Y. lipolytica* W29 in batch cultures using hexadecane as carbon source and evaluate the effect of several cultivation parameters that could affect the yeast performance.

It was observed that, in the experiment performed with 5 $g{\cdot}L^{-1}$ of hexadecane and no more additional compounds, yeast cells were able to grow, which indicates that yeast growth was related to hexadecane consumption and proves that *Y. lipolytica* W29 can use hexadecane as a carbon source. However, with 10 $g{\cdot}L^{-1}$ of hexadecane, no significant growth was noticed, but cellular viability remained above 90 %. These results mean that the low cellular growth observed was probably due to limited substrate dispersion in the culture medium and consequently weaker contact between droplets and cells, instead of toxic effects. The addition of silicone oil to the cultures with 10 $g{\cdot}L^{-1}$ of hexadecane led to an increase of cellular growth, which indicates that this compound reduced possible toxic effects or dispersion problems. Additionally, it was found that the addition of a surfactant (Tween 80) and a nitrogen source (YNB) increased specific growth rate and biomass production. Thus, a composition of culture medium for *Y. lipolytica* W29 growth on hexadecane was defined: hexadecane 5 $g{\cdot}L^{-1}$, Tween 80 10 %, YNB without amino acids 6.7 $g{\cdot}L^{-1}$ dissolved in potassium phosphate buffer 0.1 M, pH 6.

In order to study the effect of oxygenation on yeast growth and hexadecane consumption, several experiments were carried out in a lab-scale bioreactor, varying simultaneously agitation and aeration rates. It was proved that the increase of dissolved oxygen did not improved cellular growth or hexadecane consumption. In the experiments carried out at 200 rpm and 0.5 vvm, a total consumption of hexadecane was attained and yeast cells accumulated 26 % of their cell dry weight as microbial lipids. This result is important because there are no studies in the literature exploring the production of microbial lipids from hexadecane as carbon source. The morphological characterization of yeast cells by quantitative image analysis techniques revealed that hexadecane caused a dimorphic transition growth towards the hyphae morphotype.

The results reported herein prove that industrial effluents contaminated with hexadecane (e.g. refinery effluents) could be valorized by *Y. lipolytica* W29 cultures to simultaneously degrade

hexadecane and produce biomass (single cell protein) and microbial lipids. Therefore, several suggestions for future work are proposed:

- assess the possibility of using nitrogen sources more economically attractive to industrial application than YNB, such as urea, corn steep liquor or other wastes rich in nitrogen;
- evaluate the effect of mixtures of alkanes on yeast growth;
- optimize the microbial lipids production from hexadecane and characterize the long chain fatty acids profile of lipids;
- assess the yeast growth and metabolites production from refinery effluents.

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