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Biotechnological approach for waste cooking oils valorization based on yeast *Yarrowia lipolytica*

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“If I cannot do great things, I can do small things in a great way”

- Martin Luther King Jr.

ABSTRACT

Daily, huge quantities of waste cooking oils (WCO) are produced worldwide. These residues rich in lipids can be used as substrate in several biotechnological processes, such as those based in *Yarrowia lipolytica* cultures. This yeast species has the special ability to degrade lipid-rich substrates while producing value-added metabolites, such as lipase and microbial lipids of interesting TAGs composition. The effective use of WCO by *Y. lipolytica* depends on the WCO concentration, the presence of surfactants and pH, among other parameters, thus its optimization is crucial for the development of an industrial process of WCO valorization.

Initially, it was intended to study the effect of medium composition (pH, WCO concentration and arabic gum concentration) on lipase and microbial lipids production in experiments carried out in Erlenmeyer flasks using an experimental design based on Taguchi method. The initial pH value of the production medium was the most influential parameter on production of lipase and microbial lipids. The increase of pH value from 5.6 to 7.2 improved lipase production by *Y. lipolytica*, since lipase activity was 4-fold higher at pH value of 7.2. By contrast, highest microbial lipids accumulation was attained in the experiments performed at pH 5.6. The remaining parameters did not show any influence on lipase production; however, it was observed that gum arabic concentration was an important parameter for the production of microbial lipids by *Y. lipolytica* W29. The interaction between the parameters WCO concentration and arabic gum concentration was the interaction with most influence on both production of lipase and microbial lipids by yeast. Taguchi also established optimum conditions for the production of lipase and microbial lipids (pH 7.2, WCO 10 $g \cdot L^{-1}$ and pH 5.6, WCO 30 $g \cdot L^{-1}$ and arabic gum 5 $g \cdot L^{-1}$ for lipase and microbial lipids production, respectively). In both cases, the experimental results obtained in the optimum conditions were identical to the expected values.

The influence of oxygen mass transfer on lipase and microbial lipids production by *Y. lipolytica* W29 was also studied. Increasing k_La from 9 h^{-1} to 93 h^{-1} improved cell growth as well as protease production. By contrast, values of k_La above 16 h^{-1} led to a decrease in lipase production. The accumulation of microbial lipids by yeast was also favored at lower k_La values. The fatty acids composition of microbial lipids accumulated by *Y. lipolytica* cells was mainly linoleic (≥60 %) and oleic (≥30 %) acids, demonstrating the potential of these lipids to be used as food supplements.

Keywords: Waste cooking oils, *Yarrowia lipolytica*, lipase, microbial lipids, biorefinery approach.

RESUMO

Diariamente, são produzidas grandes quantidades de óleos alimentares usados (OAU) em todo mundo. Estes resíduos, ricos em lípidos, podem ser utilizados como substrato em vários processos biotecnológicos e a levedura *Yarrowia lipolytica* é capaz de degradar compostos ricos em lípidos enquanto produz metabolitos de valor acrescentado, como lipase e lípidos microbianos. A concentração da fonte de carbono, a presença de surfactantes bem como o pH do meio são parâmetros que podem influenciar produção de lipase e lípidos microbianos por *Y. lipolytica*. A otimização destes parâmetros é essencial para uma máxima produção de lipase e lípidos microbianos.

Inicialmente, foi aplicado um desenho experimental baseado no método de Taguchi e estudou-se o efeito dos parâmetros pH, concentração de WCO e de goma arábica na produção de lipase e lípidos microbianos por *Y. lipolytica* em experiências realizadas em frascos de Erlenmeyer. O valor de pH inicial do meio de produção foi o parâmetro com maior influência na produção de lipase e lípidos microbianos. O aumento do pH favoreceu a produção de lipase, uma vez que 4 vezes mais atividade lipolítica foi observada a pH 7.2. Pelo contrário, uma maior acumulação de lípidos foi conseguida nos meios de produção a pH 5.6. Os restantes parâmetros não demonstraram qualquer influência na produção de lipase; contudo, observou-se que a concentração de goma arábica foi um parâmetro importante para a produção de lípidos microbianos por *Y. lipolytica* W29. A interação entre os parâmetros concentração de OAU e concentração de goma arábica foi a interação com maior influência na produção de lipase e lípidos microbianos. Taguchi também estabeleceu as condições ótimas para a produção de lipase e lípidos microbianos (pH 7.2, 10 $g \cdot L^{-1}$ de OAU e pH 5.6, 30 $g \cdot L^{-1}$ de OAU e 5 $g \cdot L^{-1}$ de goma arábica para a produção de lipase e lípidos microbianos, respetivamente). Em ambos os casos, os resultados experimentais obtidos nas condições ótimas foram idênticos aos valores esperados.

A influência da transferência de oxigénio na produção de lipase e lípidos microbianos em culturas descontínuas de *Y. lipolytica* W29 foi também estudada. O aumento do k_La de 9 h^{-1} até 93 h^{-1} favoreceu o crescimento celular bem como a produção de protease. Por outro lado, valores de k_La acima de 16 h^{-1} levaram a um decréscimo na produção de lipase. A acumulação de lípidos microbianos pela levedura foi também favorecida a valores k_La mais baixos. A composição, em ácidos gordos, dos lípidos microbianos acumulados pela levedura era, maioritariamente, ácido linoleico (≥60 %) e ácido oleico (≥30 %), demonstrando o potencial destes lípidos para serem utilizados como suplementos alimentares.

Palavras-chave: óleos alimentares usados, *Yarrowia lipolytica*, lipase, lípidos microbianos, biorefinaria.

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACL	Acetyl-CoA lyase
ACS	Acetyl-CoA synthetase
ARA	Arachidonic acid
Aox	Acyl-coenzyme A oxidase
C	Dissolved oxygen concentration in the liquid ($mg \cdot L^{-1}$)
C_i	Dissolved oxygen concentration in the beginning ($mg \cdot L^{-1}$)
C_0	Dissolved oxygen concentration when aeration is restarted ($mg \cdot L^{-1}$)
C*	Solubility of oxygen in the liquid ($mg \cdot L^{-1}$)
C/N	Carbon/nitrogen ratio
CLA	Conjugated linoleic acid
CoA	Coenzyme A
DAG	Diacylglycerol
DO	Dissolved oxygen
ER	Endoplasmic reticulum
FAME	Fatty acid methyl esther
FDA	Food and Drug Administration
GLA	γ-linoleic acid
GC	Gas chromatography
GRAS	Generally recognized as safe
LA	Linoleic acid
LB	Lipid body
LCFA	Long chain fatty acid
LIP2	Gene of secreted extracellular lipase
μ	Maximum specific growth rate (h^{-1})
$k_L a$	Oxygen volumetric mass transfer coefficient (h^{-1})
p-NPB	*p*-nitrophenyl-butyrate
PUFA	Polyunsaturated fatty acid

rpm	Rotation per minute
SCO	Single cell oil
SCP	Single cell protein
SE	Steryl ester
SI	Severity index (%)
STR	Stirred tank reactor
T	Time (h)
t_0	Time when aeration is restarted (h)
TAG	Triacylglycerol
TCA	Trichloroacetic acid
V_a	Volume of sample (mL)
V_t	Total volume (mL)
Vvm	Volume of air per volume of medium per minute
WCO	Waste cooking oil
YNB	Yeast nitrogen base
YPD	Yeast extract, peptone, dextrose medium
YPDA	Yeast extract, peptone, dextrose and agar medium

1 LITERATURE REVIEW

Waste cooking oils (WCO) are lipid-rich residues produced in great quantities worldwide. The design of strategies involving microorganisms to simultaneously degrade these wastes and obtain high added-value products become an interesting approach, since the huge abundance and low cost of WCO ensures the economic viability of the bioprocesses while prevents major environmental problems.

Yarrowia lipolytica is capable to produce lipase and microbial lipids using WCO and that can be an interesting and alternative approach for valorization of WCO.

In this Chapter, the microbial valorization of waste cooking oil is addressed describing state-of –the art on this topic. The utilization of hydrophobic substrates by *Y. lipolytica* is also discussed. Moreover, a brief overview on lipase and microbial lipids production by *Y. lipolytica* from WCO is presented.

The main objectives of this work are also highlighted.

1.1 Waste cooking oils (WCO)

Waste cooking oils are generated from vegetable oils (coconut, sunflower, soybean, palm tree, cottonseed, rapeseed and olive) used at high temperature for food frying (Azócar *et al.*, 2010; Tsoutsos *et al.*, 2016). During the frying process, vegetable oils undergo many physical and chemical modifications and several toxic compounds are formed through several thermolytic, hydrolysis and oxidation reactions. Generally, vegetable oils are used at temperatures between 160 °C and 200 °C for long periods of time. During food frying, due to the low levels of oxygen, the hydrolysis of saturated fatty acids originates some by-products such as alkanes, symmetric ketones, propene and propane diesters and diacylglycerols. Acrolein, CO and CO_2 also result from non-oxidative decomposition of saturated fatty acids. In other hand, unsaturated fatty acids originate mostly dimeric compounds, such as dehydrodimers and polycyclic compounds (Nawar, 1984). Furthermore, these fatty acids could react with oxygen molecules through free radical mechanisms, generating hydroperoxides as the primary products. The scission of O-O bond of hydroperoxides results in alkoxy radicals, which further produces some chemicals such as aldehydes, hydrocarbons, semi-aldehydes and acids. These chemicals, resulting from cleavage of the alkoxy radicals, are carcinogenic (Kulkarni & Dalai, 2006).The molecules of water in the food is also a problem, since these molecules can enter into the oil and cause the hydrolysis of triglycerides into free fatty acids (FFA), glycerol, monoglycerides and diglycerides (Nawar, 1984; Mittelbach & Enzelsberger, 1999; Hanisah *et al.*, 2013).

Although WCO have carcinogenic compounds, oil-frying method is increasingly popular due to the good taste that this method confers to food. As vegetable oils on food frying method should not be used for a long period, an accumulation of WCO is inevitable (Hanisah *et al.*, 2013), especially in developed countries (Chhetri *et al.*, 2008). It is estimated that approximately 29 million tons of WCO are generated per year worldwide (Maddikeri *et al.*, 2012) and 700 thousand ton to 1 million tons are produced in European Union (Hanisah *et al.*, 2013). In 2015, the production of WCO in Portugal was approximately 77 thousand tons, from which about 25 % were generated in the domestic sector, 69 % in the hotel and catering sector and a small fraction (6 %) was from the food industry sector. It is expected that approximately 58 thousand ton of WCO can be recovered,

but only 23 thousand ton of WCO were sent for recycling or valorization, which means that 35 thousand ton of WCO were wasted and released through public sewerage system[1].

In wastewater treatment plants, these oily wastes are considered another residue which should be removed, increasing the water treatment costs[2]. Moreover, these wastes rich in lipids can hinder the sewage treatment at wastewater treatment plants owing to: (i) foam formation due to the accumulation of non-degraded fatty acids, (ii) adsorption of lipids and long chain fatty acids (LCFA) onto the biomass, causing sludge flotation and mass transfer problems and (iii) inhibition of anaerobic microbial communities due to an increase of LCFA (Hanaki *et al.*, 1981; Angelidaki & Ahring, 1992; Alves *et al.*, 2009; Appels *et al.*, 2011). In order to avoid the illegal practice of discharge WCO through public sewerage system, many developed countries have set policies that penalize this action. In Portugal, Decree-Law No. 267/2009 establishes the legal regime of waste cooking oils management produced by the industrial, hotel and catering and domestic sectors. This directive created a set of rules aimed to the implementation of selective collection circuits, its proper transport, treatment and recovery by properly licensed operators for this purpose. Moreover, the industries identified as generating WCO are obligated to report information about the WCO management to Portuguese Environment Agency (APA)[3].

Traditionally, WCO have been used as additives in domestic animals feedstock or in the production of soaps (Domínguez *et al.*, 2010). However, to avoid the transfer of carcinogenic compounds to humans through food chain, in the European Union is strictly prohibited the recycling of WCO for animal feed since 2002 (Lam *et al.*, 2016). WCO are as well an inexpensive raw material used for biodiesel production, once WCO have a fatty acid composition similar to that of pure vegetable oils: oleic, palmitic, linoleic, stearic and palmitoleic acids (Leung & Guo, 2006; Papanikolaou *et al.*, 2011). WCO, which price in 2016 was around 0.65 €/L are less expensive than pure vegetable oils, which price was approximately 0.73 €/L. WCO used as raw material for biodiesel production replacing pure vegetable oils, enhances the economic viability of biodiesel production (raw material represents 70 % - 95 % of the overall cost of biodiesel production) (Fischer & Connemann, 1998). However, the conditions used in frying methods, as well the fried material

[1] Portuguese environment agency, https://www.apambiente.pt/_zdata/Politicas/Residuos/FluxosEspecificosResiduos/OAU/relatorio_Gesto%20OAU%202015.pdf, accessed in October 2017.

[2] Portuguese site of wastes management, http://www.reciclimpa.pt/index-2.html, accessed in May 2017.

[3] Portuguese environment agency, https://www.apambiente.pt/index.php?ref=16&subref=84&sub2ref=197&sub3ref=282, accessed in October 2017.

(vegetables, meat, fish), cause physical and chemical changes that affects the biodiesel production: (a) the high content of FFA and water may difficult the separation of esters from glycerol and form soap, affecting the final biodiesel conversion efficiency; (b) the presence of dimeric and polymeric acids and glycerides in WCO, which increase its viscosity, will also affect negatively the alkaline-catalyzed transesterification reaction (method normally adopted for biodiesel production); (c) low values of molecular mass and iodine value and high values of density and saponification are also some of the difficulties that must be overcome to produce biodiesel from WCO (Enweremadu & Mbarawa, 2009; Ho *et al.*, 2014). Furthermore, an increase of foam formation and modifications in the surface tension and color of the vegetable oils after frying process also interfere in the biodiesel conversion (Cvengroš & Cvengrošová, 2004).

1.2 Microbial valorization of WCO

The use of WCO for biodiesel production can be a promising alternative for its efficient reutilization. However, as stated before, all chemical and physical changes that occur during the frying method affects biodiesel final yield and increase the processing costs of biodiesel, which makes the product costly. Furthermore, though great quantities of WCO are generated per year, this amount is not enough to produce all biodiesel needed to satisfy the requests of all countries in the world. Thus, the recycling of WCO through alternative cost-efficient processes in order to obtain added-value products should be studied.

The design of strategies involving microorganisms to simultaneously degrade hydrophobic substrates (such as oily wastes) and obtain high added-value products have been developed and can become an interesting approach, since the huge abundance and low cost of WCO are attractive for ensuring the economic viability of the bioprocesses as well as preventing major environmental problems. A large number of microorganisms, such as yeasts, fungi and bacteria, have been studied for that purpose, since they can use WCO as carbon source and convert that substrate into added-value products (Table 1.1).

Table 1.1 Added-value products obtained by microbial conversion of waste cooking oils.

Added-value product	Microorganism	Mode of operation	Initial WCO concentration	Reference
Carotene	*Blakeslea trispora* ATCC 14271 and ATCC 14272	Batch (flasks)	50 g·L^{-1}	Nanou & Roukas (2016)
Citric acid	*Y. lipolytica* SWJ-1b	Batch	80 g·L^{-1}	Liu *et al.* (2015)
Erythritol	*Y. lipolytica* M53	Batch	30 g·L^{-1}	Liu *et al.* (2017)
Lipase	*Aspergillus niger* NRRL 363	Batch (flasks)	15 g·L^{-1}	Papanikolaou *et al.* (2011)
	Bacillus cereus ASSCRC-P1	Batch (flaks)	Without information	Awad *et al.* (2015)
	Pseudomonas sp 3AT	Batch (flasks)	20 g·L^{-1}	Haba *et al.* (2000)
	Y. lipolytica CECT 1240	Batch	30 g·L^{-1}	Domínguez *et al.* (2010)
	Y. lipolytica M53	Batch	30 g·L^{-1}	Liu *et al.* (2017)
Microbial lipids	*Aspergillus niger* sp. ATHUM 3482	Batch (flasks)	15 g·L^{-1}	Papanikolaou *et al.* (2011)
	Yarrowia lipolytica	Batch	5 g·L^{-1}	El Bialy *et al.* (2011)
	Yarrowia lipolytica NCIM 3589	Batch (flasks)	100 g·L^{-1}	Katre *et al.* (2012)
Polyhydroxyalkanoates (PHAs)	*Cupriavidus necator* H16	Batch (flasks)	20 g·L^{-1}	Verlinden *et al.* (2011)
	Cupriavidus necator DSM 28	Batch	20 g·L^{-1}	Cruz *et al.* (2016)
	Pseudomonas aeruginosa 7a	Batch (flasks)	2 g·L^{-1}	Costa *et al.* (2009)

Table 1.1 Added-value products obtained by microbial conversion of waste cooking oils (continuation).

Added-value product	Microorganism	Mode of operation	Initial WCO concentration	Reference
Rhamnolipids	*Pseudomonas aeruginosa* 47T2 NCIB 40044	Batch (flasks)	40 g·L^{-1}	Haba *et al*. (2000)
	Pseudomonas aeruginosa ATCC 10145	Batch (flasks)	25 g·L^{-1}	Wadekar *et al*. (2012)
Riboflavin	Mutant of *Ashbya gossypii* ATCC 1089532	Batch (flasks)	40 g·L^{-1}	Wei *et al*. (2013)

Concerning the valorization of WCO by bacteria species, several strains of *Pseudomonas* sp. have been described as having the ability to utilize WCO oils to produce metabolites of interest. Haba *et al*. (2000) concluded that waste cooking oils are suitable for rhamnolipids (bacterial surfactants) production by *Pseudomonas aeruginosa* 47T2 NCIB. Costa *et al.* (2009) also studied different strains of *P. aeruginosa* for the production of rhamnolipids and polyhydroxyalkanoates (PHA's) and concluded that, among the substrates studied (glycerol, cassava wastewater and WCO), WCO was the best substrate for the production of both metabolites. Wadekar *et al*. 2012 also studied the production of rhamnolipids by *P. aeruginosa* ATCC 10145 and observed that waste cooking oil was more suitable for rhamnolipids production than other waste substrates like sweet water and waste glycerol. It was reported that *Cupriavidus necator* DSM 428 is capable of use WCO as sole carbon source to produce polyhydroxyalkanoates. The successfully production of lipase from WCO by bacterial species, such as *Pseudomonas* sp 3AT, *P. aeruginosa* ATCC 111 and *Bacillus cereus* ASSCRC-P1 was already reported (Haba *et al.,* 2000; Awad *et al*., 2015).

In the literature, there are some researches about the use of WCO as carbon source by fungi and yeasts strains to achieve high added-value products. Some strains of *Aspergillus sp.* cultivated in WCO accumulated up to 64 % (w/w) of dry fungal mass as intracellular lipids (Papanikolaou *et al*., 2011). Other important metabolites, such as lipase, riboflavin and carotenes were successfully produced from WCO by *A. niger* NRRL 363, *Ashbya gossypii* and *Blakeslea trispora*, respectively (Papanikolaou *et al*., 2011; Wei *et al*., 2013; Nanou & Roukas, 2016). Among

yeast species, *Y. lipolytica* is one of the most studied yeasts for utilization of WCO as carbon source, due its special ability to degrade WCO while produces several added-value products. Katre *et al.* (2012) and El Bialy *et al.* (2011) reported that *Y. lipolytica*, growing in a WCO based-medium, accumulated great amount of microbial lipids with high content of essential fatty acids known as polyunsaturated fatty acids (PUFA). Domínguez *et al.* (2010) studied the biodegradation of WCO by *Y. lipolytica* CECT 1240 and concluded that the addition of WCO to medium led to a significant increase in extracellular lipase production, compared to oil-free cultures. It was also reported that *Y. lipolytica* SWJ-1b is capable to produce citric acid and accumulate intracellular lipids from WCO as carbon source (Liu *et al.*, 2015).

1.3 The non-conventional yeast *Yarrowia lipolytica*

Y. lipolytica is a non-conventional and strictly aerobic yeast that can be found in environments containing hydrophobic substrates, such as *n*-alkanes and lipids (Spencer *et al.*, 2002) or marine and hypersaline environments (Katre *et al.*, 2012). This microorganism can also be isolated from lipid containing food, such as dairy products (Corbo *et al.*, 2001; Vasdinyei & Deák, 2003) and cheeses (Roostita & Fleet, 1996; Suzzi *et al.*, 2001).

Firstly, this yeast was denominated *Candida lipolytica* and was included in the deuteromycetous group. Over the years, it was reclassified and renamed as *Endomycopsis lipolytica*, *Saccharomycopsis lipolytica* and, finally, *Yarrowia lipolytica* (Yarrow, 1972; van der Walt & von Arx, 1980). The species name, "*lipolytica*", refers to the ability of this yeast to degrade lipidic substrates. Now, *Y. lipolytica* is considered a eukaryotic organism (Fungi kingdom), a hemiascomycetous dimorphic fungus that belongs to the class of *Ascomycetes*, subclass *Hemiascomycetes* and order *Saccharomycetales* (Kurtzman *et al.*, 2011).

Y. lipolytica is one of the most widely studied organisms from the group "non-conventional" yeasts. This term is used to differentiate this group from yeasts more commonly used as study models, called "conventional yeasts", such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Barth & Gaillardin, 1997). Since its maximum growth temperature is below 34 °C, this yeast is not considered pathogenic and the processes based in this microorganism are classified as "generally recognized as safe" (GRAS) by American Food and Drug

Administration (Spencer *et al.*, 2002; Coelho *et al.*, 2010). Furthermore, the inability of *Y. lipolytica* to grow under anaerobic conditions allows its effortless elimination from dairy products, in contrast to *Kluyveromyces marxianus,* for example (McKay, 1992).

Y. lipolytica has been considered an adequate model for dimorphism studies, since it is easy to distinguish between two morphological forms, oval yeast and pseudo-hyphae (van der Walt & von Arx, 1980) (Figure 1.1). Ruiz-Herrera & Sentandreu (2002) reported that various environmental factors such as pH, carbon and nitrogen sources affect the formation of mycelium in different *Y. lipolytica* strains. The authors also observed that mycelium formation was maximal at pH near neutrality and decreased as pH was lowered and become almost null at pH 3 for *Y. lipolytica* W29 and *Y. lipolytica* Po1a. Several authors reported that morphology transition of *Y. lipolytica* to a true mycelium occurs when hydrophobic substrates, such as tallow, crude oils and palm-oil mill effluent, was used as carbon source (Oswal *et al.*, 2002; Papanikolaou *et al.*, 2007; Zinjarde *et al.*, 2008). By contrast, Bellou *et al.* (2014) and Papanikolaou *et al.* (2009) verified that *Y. lipolytica* strains growing at low dissolved oxygen concentration, the mycelial or pseudo-mycelial forms predominated, independently of the carbon and nitrogen sources used. Anaerobic stress also affects dimorphism in *Y. lipolytica* and semi-anoxic conditions induce the formation of extremely long hyphae (Ruiz-Herrera & Sentandreu, 2002).

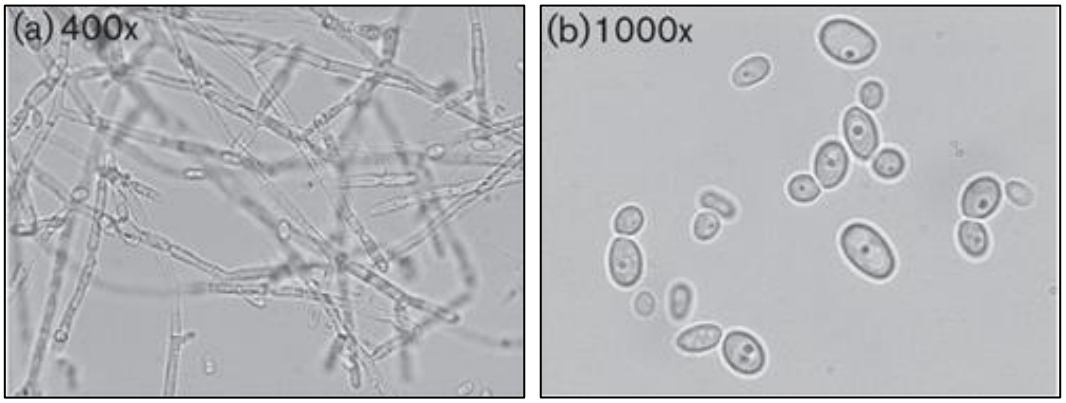

Figure 1.1 Two distinct morphological forms of *Y. lipolytica* growth as (**a**) pseudo-hyphae and as (**b**) oval cells (Bellou *et al.*, 2014).

Y. lipolytica has the ability to assimilate a wide range of carbon sources. This yeast can degrade hexoses, such as glucose (Papanikolaou *et al.*, 2006; Moeller *et al.*, 2007; Papanikolaou *et al.*, 2009) and fructose (Lazar *et al.*, 2014). However, the cellular membrane of *Y. lipolytica* is almost impermeable to sucrose, since the yeast does not have the sucrose-cleaving enzyme invertase (Pereira-Meirelles *et al.*, 1997). *Y. lipolytica* is also capable to use organic acids such as acetic, lactic, propionic, malic, succinic and citric acids as sole carbon and energy sources (Rodrigues & Pais, 2000; Fontanille *et al.*, 2012; Kolouchová *et al.*, 2015). Currently, there is a great interest in the ability of *Y. lipolytica* to degrade efficiently hydrophobic substrates such as mixtures rich in saturated free fatty acids (Papanikolaou *et al.*, 2002; Papanikolaou & Aggelis, 2003; Papanikolaou *et al.*, 2007) and *n*-alkanes (Fukuda, 2013), and residues such as olive mill wastewater (Lopes *et al.*, 2009; Wu *et al.*, 2009; Sarris *et al.*, 2011; Moftah *et al.*, 2013) and waste cooking oils (Domínguez *et al.*, 2010; Liu *et al.*, 2017). Some authors reported that *Y. lipolytica* also can grow in pure glycerol (Papanikolaou & Aggelis, 2002; Papanikolaou *et al.*, 2002; Tomaszewska *et al.*, 2012) and crude glycerol (Papanikolaou & Aggelis, 2009; Dobrowolski *et al.*, 2016), while it produces several added-value products such as lipase, citric acid, erythritol, mannitol and accumulates intracellular lipids.

1.3.1 Hydrophobic-substrates metabolism in *Y. lipolytica*

Y. lipolytica growth on hydrophobic substrates requires the contact between the hydrophobic substrate and the cell surface. Two mechanisms have been formulated to explain this process: (i) surfactant-mediated transport, in which substrates can be solubilized or pseudo-solubilized by biosurfactants produced by *Y. lipolytica* during growth on hydrophobic substrates (Cirigliano & Carman, 1984; Zinjarde & Pant, 2002); or (ii) direct interfacial transport, in which droplets of substrate can adhere directly to the cell wall (Gutierrez & Erickson, 1977) through several mechanisms such as electrostatic, hydrophobic, Lewis (acid and base) and van der Waals interactions (Rosenberg, 1991; Bellon-Fontaine *et al.*, 1996).

The assimilation of hydrophobic substrates (HS), such as triglycerides, fatty acids and *n*-alkanes, is a complex metabolism which involves several metabolic pathways that occur in different

subcellular components, such as endoplasmic reticulum (ER), peroxisomes and mitochondria (Fickers *et al.*, 2005).

When the HS enter into the cell, several metabolic pathways are involved in their degradation such as monoterminal alkane oxidation, β-oxidation and the interaction between the glyoxylate and the citrate cycles. HS degradation is then finalized trough the β-oxidation pathway in the peroxisomes (Fickers *et al.*, 2005). To assimilate HS from the medium, *Y. lipolytica* can produce biosurfactants that reduce the size of the HS droplets, facilitating the surface-mediated transport. When *Y. lipolytica* utilizes triglycerides as carbon source, the first step of their catabolism involves the hydrolysis into free fatty acids (FFA) and glycerol by the extracellular enzyme lipase. When FFA are in the cell their degradation is finalized through the β-oxidation pathway in the peroxisomes. However, when HS are in excess, substrates are stored in lipid bodies in the form of triglyceride (TAG) and steryl esters (SE). After hydrolysis, lipids accumulated intracellularly could be then mobilized by intracellular lipases, encoded by the *TGL* genes, to the peroxisome to carry out the β-oxidation. Alternatively, *n*-alkanes can be attached to protrusions formed on the yeast surface and then migrate to the ER via plasma membrane, by direct interfacial transport (Beopoulos *et al.*, 2009). A simplified scheme of the HS catabolism is depicted in Figure 1.2.

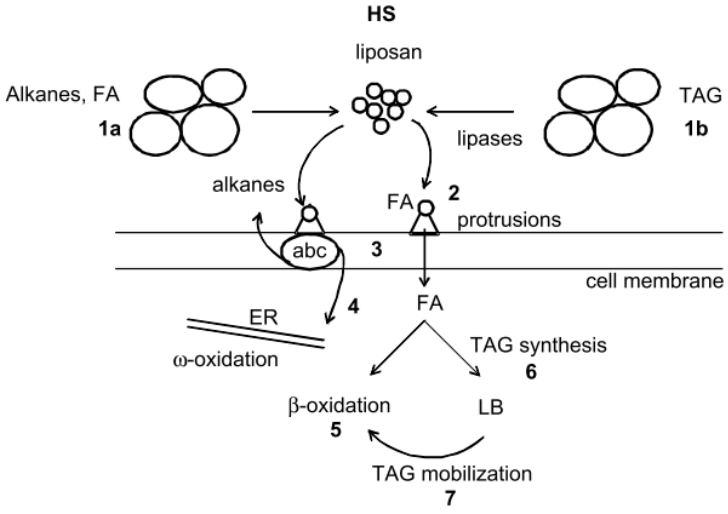

Figure 1.2 Schematic representation of the of hydrophobic substrates (HS) assimilation by *Y. lipolytica.* (**1b**, **1a**) HS droplets diameters is reduced by secreted liposan (biosurfactant); (**1b**) Extracellular lipase cleave TAGs and originate fatty acids (FAs); (**2**) FAs droplets bind onto the protrusions on the cell surface; (**3**) n-Alkanes enter into the cell via transport mechanisms (as ABC transporters); (**4**) ω-Oxidation of n-alkanes in the ER by the P450 monooxygenase systems; (**5**) Fatty acids are degraded by β-oxidation or (6) storage into lipid bodies as TAGs; (**7**) Mobilization of TAGs after hydrolysis by intracellular lipases. Circle sizes represent schematically the size of HS droplets (Beopoulos *et al.*, 2009).

1.4 Metabolites produced by *Y. lipolytica*

Y. lipolytica is a microorganism with multiple biotechnological applications. This yeast has high potential for the production of organic acids, not only citric acid (Kamzolova *et al.*, 2003; Sarris *et al.*, 2011), but also isocitric, α-ketoglutaric, succinic and acetic acids (Finogenova *et al.*, 2002; Kamzolova *et al.*, 2009; Papanikolaou *et al.*, 2009; Holz *et al.*, 2011). *Y. lipolytica* has also been used for the production of mannitol and erythritol, organic compounds used commonly in the food industry as thickeners and sweeteners (Tomaszewska *et al.*, 2012). This yeast is also capable of producing γ-decalactone, a peach like aroma compound with great industrial interest, obtained during the biotransformation of methyl ricinoleate (Aguedo *et al.*, 2005; Braga & Belo, 2015). Additionally, an important industrial application of *Y. lipolytica* strains has been the production of biomass for use as single cell protein (SCP) (Barth & Gaillardin, 1997; Nicaud, 2012).

Biosurfactants are also important compounds produced by *Y. lipolytica*. These molecules, composed predominantly of glycolipids, are essential for the processes of hydrophobic compounds assimilation. Generally, the growth of *Y. lipolytica* in hydrophobic substrates is accompanied by the production of bio-surfactants, which increase the contact surface between the hydrophobic substrate and the cell surface and facilitate the interaction (Christofi & Ivshina, 2002; Fickers *et al.*, 2005).

Among the metabolites produced by *Y. lipolytica*, one of the most important is lipase, an enzyme that has gained considerable interest due to its extensive technological applications in food, pharmaceutical and detergent industries (Talon *et al.*, 1996; Keller & Layer, 2003; Saisubramanian *et al.*, 2006; Nerurkar *et al.*, 2013; Li *et al.*, 2014). Moreover, currently there is a great interest in the ability of this yeast to store lipids intracellularly that can be used in the production of microbial lipids enriched with essential fatty acids, which are of considerable importance for food industry (Papanikolaou *et al.*, 2003; Béligon *et al.*, 2016).

1.4.1 Lipase

Lipases are serine hydrolases, referred as triacylglycerol acylhydrolases (E.C. 3.1.1.3) and catalyze the hydrolysis of ester bond of tri-, di- and mono-glycerides of long chain fatty acids into free fatty acids and glycerol (Sarda & Desnuelle, 1958), at the interface between an insoluble phase and an aqueous one (Macrae & Hammond, 1985). In addition to hydrolysis, they can catalyze several reactions of industrial interest, like esterification, inter-esterification and transesterification.

Lipases are an important group of enzymes which are exploited for several industrial applications, such as detergents (Saisubramanian *et al.*, 2006; Nerurkar *et al.*, 2013; Li *et al.*, 2014), for the treatment of oily wastewaters (Gonçalves *et al.*, 2009; Lopes *et al.*, 2009; Celson *et al.*, 2016) and in food industry (mostly for flavor development) (Talon *et al.*, 1996; Braga & Belo, 2015).

There are some microbial species capable of producing extracellular lipase: fungi (*Aspergillus oryzae* (Toida *et al.*, 1998) and *Penicillium aurantiogriseum* (Lima *et al.*, 2003)), bacteria (*Bacillus subtilis* (Sánchez *et al.*, 2002) and *Bacillus pumilus* (Joyce & Kurup, 1999)) and yeasts (*Candida rugosa* (Benjamin & Pandey, 1996) and *Y. lipolytica* (Lopes *et al.*, 2008; Lopes *et*

al., 2009; Domínguez *et al.*, 2010; Liu *et al.*, 2017)). *Y. lipolytica* is capable to produce several lipases (extracellular, membrane-bound and intracellular). Pignède *et al.* (2000) described the *LIP2* gene as the responsible for all extracellular lipolytic activity of *Y. lipolytica* W29. This extracellular lipase Lip2p is responsible for hydrolysis of long-chain triglycerides, with preference for oleic substrates (Darvishi, 2012). Besides this, other five lipases (*LIP7*, *LIP8*, *LIP12* and *LIP14*) from *Y. lipolytica* strains were also purified and characterized (Fickers *et al.*, 2005; Zhao *et al.*, 2011; Kumari & Gupta, 2012). In table 1.2 are summarized several studies regarding lipase production by *Y. lipolytica* strains from different carbon sources.

Table 1.2 Examples of studies developed for lipase production by *Y. lipolytica* strains from different carbon sources.

Strain	Carbon source(s)	Mode of operation	Reference
Y. lipolytica YB 432-12	Hazelnut oil cake + glucose	Solid-state fermentation	Saygün *et al.* (2014)
Y. lipolytica NCIM 3589	Mustard oil cake	Solid-state fermentation	Imandi *et al.* (2013)
Y. lipolytica CBS 2073, W29 and IMUFRJ 50682 *Y. lipolytica* W29 and IMUFRL 50682 *Y. lipolytica* NRRL Y-1095	Olive mill wastewater	Batch (flasks)	Gonçalves *et al.* (2009) Lopes *et al.* (2009) Moftah *et al.* (2013)
Y. lipolytica W29 *Y. lipolytica* NCIM 3639 *Y. lipolytica* LMI 91	Olive oil	Batch Batch Batch	Lopes *et al.* (2009) Sathish Yadav *et al.* (2011) Gonçalves *et al.* (2013)
Y. lipolytica ACA-DC 50109	Stearin	Batch	Papanikolaou *et al.* (2007)

Table 1.2 Examples of studies developed for lipase production by *Y. lipolytica* strains from different carbon sources (continuation).

Strain	Carbon source(s)	Mode of operation	Reference
Y. lipolytica IMUFRJ 50682	Soybean bran + soybean bran sludge	Solid-state fermentation	Farias *et al.* (2014)
Y. lipolytica CECT 1240	Vegetable oils	Batch (flasks)	Domínguez *et al.* (2003)
Y. lipolytica 704		Batch	Kamzolova *et al.* (2005)
Y. lipolytica KKP 379		Batch (flasks)	Fabiszewska & Białecka-Florjańczyk (2015)
Y. lipolytica YB 432-12		Batch (flasks)	Saygün *et al.* (2014)
Y. lipolytica CECT 1240	Waste cooking oil	Batch	Domínguez *et al.* (2010)
Y. lipolytica SWJ-1b			Liu *et al.* (2015)
Y. lipolytica M53			Liu et al. (2017)

Lipase productivity is affected by different physical and chemical factors, being the carbon and nitrogen sources two of the most important. Lipase production is significantly increased in the presence of lipidic substrates, such as olive oil (Lopes *et al.*, 2008), soybean oil (Maldonado *et al.*, 2012) and rapeseed oil (Dobrev *et al.*, 2015), and the use of glucose and glycerol as carbon sources can represses lipase induction (Fickers *et al.*, 2011). Additionally, several oily-wastes has been used as carbon and inductor source for lipase production: olive mill wastewaters (Lopes *et al.*, 2009; Moftah *et al.*, 2013) and waste cooking oils (Domínguez *et al.*, 2010; Liu *et al.*, 2017). Lopes *et al.* (2009) and Moftah *et al.* (2013) studied the use of olive mill wastewater (OMW) by several *Y. lipolytica* strains and concluded that all strains had a great potential for OMW valorization by its use as culture medium for lipase production. Domínguez *et al.* (2010) studied the biodegradation and utilization of WCO by *Y. lipolytica* CECT 1240 and concluded that the addition of WCO to the culture medium led to a significant improvement in extracellular lipase production.

In addition to carbon sources, nitrogen sources should also be carefully considered. A review by Brígida *et al.* (2014) emphasized that organic nitrogen sources such as peptone, casein, yeast extract and tryptone are suitable for high extracellular lipase production. By contrast, inorganic (or mineral) nitrogen sources, such as ammonium chloride, ammonium sulfate and

ammonium citrate do not trigger lipase synthesis (Fickers *et al.*, 2004; Darvishi *et al.*, 2009; Liu *et al.*, 2017). However, Almeida *et al.* (2012) reported that the use of ammonium nitrate as nitrogen source influenced positively the production of lipase by *Candida viswanathii*.

Lipase production by *Y. lipolytica* is also affected by pH and temperature. An optimal pH value between 6 and 7.5 was found, depending on the substrates (Fickers *et al.*, 2011). Corzo & Revah (1999) also concluded that pH affects significantly lipase production and reported that a maximum lipase production was achieved in pH range of 5.8 – 7. Several authors agree about the remarkable influence of temperature on lipase production by *Y. lipolytica*. The environment from which *Y. lipolytica* strains were obtained seems to play an important role in determining the optimal temperature (Brígida *et al.*, 2014). Some authors reported that temperatures range from 25 °C to 30 °C improved lipase production by *Y. lipolytica* W29 (Wu *et al.*, 2009). Corzo & Revah (1999) and Domínguez *et al.* (2010) observed that temperatures around 30 ° C were the optimal for lipase production by *Y. lipolytica* 681 and *Y. lipolytica* CECT 1240, respectively. By contrast, Tan *et al.*, (2003) reported that temperatures above 28 °C led to a decrease on lipase production.

The amount of oxygen available in *Y. lipolytica* cultures is also an important parameter to consider in lipase production, since this yeast is strictly aerobic. There are several researches regarding the effect of agitation and aeration rates and their importance for maintaining the adequate dissolved oxygen concentration in the culture medium. However, the information found in the literature about the optimum dissolved oxygen concentration or the suitable agitation and aeration rates for maximization of lipase production is contradictory. Tan *et al.* (2003) concluded that a higher stirring rate increased the dissolution of oxygen in the medium and significantly enhanced the lipolytic activity. 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. Elibol & Ozer (2000) improved lipase productivity by increasing simultaneously the agitation rate from 200 rpm to 300 rpm and aeration rate from 0.5 vvm to 1.5 vvm. By contrast, Alonso *et al.* (2005) and Corzo & Revah (1999) concluded that low levels of aeration increased lipase production since an increase of stirring rate above 300 rpm seemed to reduce lipase activity levels.

1.4.2 Microbial lipids

Microbial lipids, also called microbial oils, single cell oils and intracellular lipids, are hydrophobic bodies mainly composed of triacylglycerols and steryl esters (Murphy, 2001).

Several microorganisms are known as capable of accumulate intracellular lipids, though only the oleaginous microorganisms can accumulate significant quantities of lipids, corresponding to more than 20 % (w/w) of their dry cell mass (Papanikolaou & Aggelis, 2011a). Microalgae have the ability to fixe CO_2 and convert it into carbohydrate and later into intracellular lipids. Intracellular lipids accumulated by microalgae are very often rich in polyunsaturated fatty acids, belonging to omega-3 and omega-6 families, which have wide applications as food supplements (Ratledge & Cohen, 2008). Oleaginous yeasts and filamentous fungi are able to accumulate significant amounts of intracellular lipids, ranging from 25 % to 40 % (w/w), which are composed mostly by (TAGs). Monoglycerides, diglycerides, steryl-esters and sterol are also present in these microbial lipids. Commonly, TAGs are rich in unsaturated fatty acids (44 %), such as linoleic and oleic acids (Donot *et al.*, 2014). In conditions of nutrient limitation, oleaginous microorganisms can accumulate lipids to levels exceeding 70 % of their cell dry weight (Beopoulos *et al.*, 2009).

An alternative to synthesize intracellular TAGs is the production of lipoids (reserve polymers) by bacteria. Their structure is similar to that was found in TAGs. Generally, lipoids are anchored in the membrane in inclusions, being difficult to extract these compounds (Alvarez & Steinbüchel, 2002; Meng *et al.*, 2009).

Intracellular lipids in oleaginous yeasts and filamentous fungi can be stored by two different pathways: (1) "*de novo"* synthesis and (2) "*ex novo*" synthesis. The synthesis "*de novo*" involves the production, in defined conditions, of fatty acid precursors, such as acetyl and malonyl-CoA, and their integration into the storage lipid biosynthetic pathway. Hydrophilic substrates are the carbon sources used in "*de novo*" lipid accumulation. The key step for "*de novo*" synthesis in the oleaginous microorganisms is the exhaustion of an essential nutrient (usually nitrogen) from the culture medium (Beopoulos *et al.*, 2009). During the nutrient limitation, the synthesis of proteins and nucleic acids, essential to cell growth, represses some metabolic pathways while the synthesis of fatty acids and triglycerides are induced and further accumulated into lipid bodies (Gonçalves *et al.*, 2014). The excess of carbon source and nitrogen depletion are important factors for the induction of lipogenesis in *de novo* process and the use of the suitable molar ratio C/N

(carbon/nitrogen ratio) is essential to maximize the accumulation 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 (Papanikolaou & Aggelis, 2011b). Nevertheless, some authors reported that excess of carbon source (e.g. glycerol) in the medium could lead to the production of organic acids such as citric acid, in the detriment of microbial lipids accumulation (Papanikolaou 2008; André *et al.*, 2009; Papanikolaou & Aggelis, 2009). Karatay & Dönmez (2010) and Tsigie *et al.* (2012) observed an intracellular lipid content around 60 % (w/w) and 48 % (w/w) in *Y. lipolytica* strains when molasses and rice bran hydrolysate were used as carbon sources, respectively. Papanikolaou & Aggelis (2002) reported that *Y. lipolytica* LGAM S(7)1 achieved a microbial lipids content up to 43 % (w/w) when is cultivated on crude glycerol as sole carbon source.

The process of "*ex novo*" synthesis involves the uptake of fatty acids, oils and triacylglycerols from the culture medium, and their accumulation in an unchanged or modified form inside the cell (Beopoulos *et al.*, 2009). Generally, this synthesis of "*ex-novo*" lipids involves the bio-modification of fats and oils by the microorganisms. The degradation of hydrophobic substrates involves the secretion of lipases that catalyze the hydrolysis of substrate into free fatty acids. These compounds are incorporated into the cell and can be used both for growth or transformed to produce different fatty acids (Aggelis *et al.*, 1997; Fickers *et al.*, 2005). The use of hydrophobic substrates as carbon sources for lipid accumulation is, generally, a growth-associated process (Papanikolaou *et al.*, 2001, 2007; Papanikolaou *et al.*, 2002; Martínez *et al.*, 2015). Papanikolaou *et al.* (2011) reported that when waste cooking olive oil was added to the growth medium, several strains of *Aspergillus* sp. and *Penicillium expansum* accumulated remarkable quantities of intracellular lipids, up to 64 % (w/w). El Bialy *et al.* (2011) reported that *Y. lipolytica* grown on media containing WCO as carbon sources accumulated a lipid content between 38 % (w/w) and 58 % (w/w).

Degradation of hydrophobic materials in *Y. lipolytica* strains is a multi-step process that requires different enzymes, acyl-coenzyme A oxidases (*Aox*), with different enzymatic activities: *Aox*3p is specific for short-chain acy-CoAs, *Aox2*p is specific for long-chain acyl-CoAs and *Aox*1p, *Aox*4p and *Aox*5p do not present specificity for any chain length. At higher aeration and agitation rates and, consequently, high dissolved oxygen levels, short-chain acyl-CoA oxidases, such as *Aox*3p, demonstrate increased activity, which direct the carbon towards the synthesis of acetyl-

CoA, for lipid-free biomass production instead of storage lipids (Luo *et al.*, 2000; Mlícková *et al.*, 2004; Fickers *et al.*, 2005).

Currently, *Y. lipolytica* is considered an outstanding biolipid producer and, therefore, it is used as unicellular model microorganism to study fatty acids metabolism and lipid accumulation. The most important metabolic processes and genes involved in lipid synthesis are summarized in Figure 1.3.

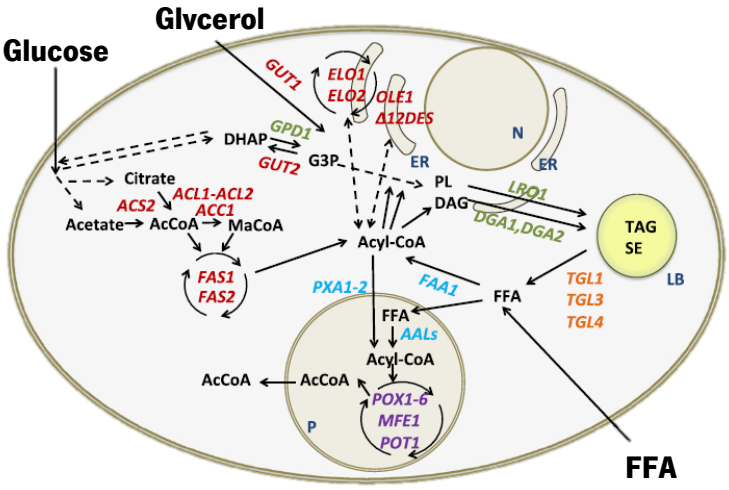

Figure 1.3 Schematic illustration of the metabolic pathways for production of neutral lipids (triacylglycerol (TAG) and steryl esters (SE)) from glucose, glycerol or free fatty acids (FFA). Dashed lines indicate multiple steps. The colors of the genes indicate different metabolic pathways: in red, fatty 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 & Nicaud 2016).

Several metabolic pathways to accumulate lipids are possible in *Y. lipolytica* cells depending on the carbon source (glucose, glycerol or oily substrates). In the cytosol, the fatty acids are produced from acetyl-CoA, which can be derived from *ACS* genes (acetyl-CoA synthetase), a

pyruvate dehydrogenase complex, an amino acid degradation pathway or *ACL* genes (ATP citrate lyase) (present only in the genome of oleaginous yeasts).

When glucose is used as carbon source, the citrate in the cytosol is converted to acetyl-CoA by *ACL* genes and, then, acetyl-CoA can be transformed into malonyl-CoA by acetyl-CoA carboxylase (*ACC1*), which overexpression has been associated with lipid overproduction. The fatty acid synthase (FAS) produce acyl-CoA that can be released with a chain length of 16 or 18 carbons. These C16:0 and C18:0 molecules can be substrates for elongases (which produce long chain fatty acids or very long chain fatty acids) and desaturases (located in endoplasmic reticulum (ER) and that produce palmitoleic (C16:1) or oleic (C18:1) acids through *OLE1* gene (Δ9 desaturase) and linoleic acid (C18:2) through *FAD2* gene (Δ12 desaturase)).

When *Y. lipolytica* grows in an oily environment, a large battery of extracellular lipases secreted by the yeast hydrolyse fats and oils, producing free fatty acids (FFA), which will be rapidly incorporated into the cell. In the cytosol, these free fatty acids can be activated by fatty acyl-CoA synthetase (*FAA1*) to produce acyl-CoA. *Y. lipolytica* accumulates lipids in an intracellular particle called lipid body (LB), mainly formed of neutral lipids, particularly triacylglycerols (TAGs, 85 %) and some steryl esters (SEs, 8 %). TAGs are formed through the Kennedy pathway, where preformed diacylglycerol (DAG) is converted to TAG from acyl-CoA by *DAG1* and *DAG2*. SEs are formed from acyl-coA and sterol is formed by *ARE1*. The pathways to form TAGs and SEs may take place between the endoplasmic reticulum (ER) and the lipid body surface (LB), where the responsible enzymes are found. The hydrolysis of TAGs from lipid bodies by intracellular lipases release free fatty acids at the surface of the LB. In *Y. lipolytica* there are two genes for that purpose: *TGL4* and *TGL3*. Free fatty acids, from medium or resulting from the action of *TGL* genes, must be activated and transported to the peroxisome to carry out the β-oxidation. FFAs can enter the peroxisome directly by unknown transporters or be activated by *FFA1* gene to acyl-CoA and stored in lipid body, or enter the peroxisome in an activated form through specific transporters *Pxa1*/ *Pxa2*. In the peroxisome, the FFAs must be activated by *AAL* genes to be able for use by β-oxidation enzymes (Ledesma-Amaro & Nicaud, 2016).

The promising production of microbial lipids rich in high added-value unsaturated fatty acids by oleaginous yeasts has been considered as a process economically viable, particularly if the carbon sources used were low-cost substrates, as olive mil waste waters or waste cooking oils.

Despite to accumulate fewer lipids than other oleaginous yeast species, *Y. lipolytica* is the only one able to accumulate large amounts of linoleic acid, representing more than 50 % of the fatty acids accumulated by the yeast (Beopoulos *et al.*, 2009). Other fatty acids accumulated by *Y. lipolytica* are oleic, stearic, palmitoleic and palmitic acids (Athenstaedt *et al.*, 2006; Bialy *et al.*, 2011; Cheirsilp & Louhasakul, 2013). An important characteristic of microbial lipids produced by *Y. lipolytica* is the significant presence of linoleic acid, an essential fatty acid known by polyunsaturated fatty acid or PUFA. These PUFAs cannot be synthesized by mammals and must be ingested in diet. Linoleic acid (LA) are a precursor for the synthesis of more highly unsaturated and longer-chained omega-6 family fatty acids, which gathers γ-linoleic acid (GLA), arachidonic acid (ARA) and conjugated linoleic acid (CLA). ARA is the most abundant PUFA in humans and has a major role as structural lipid associated predominantly with phospholipids. Moreover, is the principal omega-6 fatty acid present in the brain, being the direct precursor of eicosanoids which regulate lipoprotein metabolism, blood rheology, leucocyte function and platelet activation (Béligon *et al.*, 2016). In other hand, one of the most common applications for intracellular lipids accumulated by yeasts is the production of microbial substitutes for cocoa butter, commonly used in the food technology for chocolate fabrication process. However, oleaginous yeasts accumulate lipids rich in unsaturated fatty acids and cocoa butter contains 55 % to 67 % (w/w) of saturated fatty acids, such as palmitic and stearic. In order to overcome this problem, several approaches have been used to increase the level of this fatty acids, such as growth of yeasts in medium rich in stearic acid, the use of desaturase inhibitors, low oxygenation of the growth medium and genetic manipulation of strains (Papanikolaou & Aggelis, 2011b). In table 1.3 are summarized several studies regarding microbial lipids production by *Y. lipolytica* strains from different carbon sources.

Table 1.3 Examples of studies developed for microbial lipids production by *Y. lipolytica* strains from different carbon sources.

Strain	Carbon source(s)	Mode of operation	Reference
Y. lipolytica LGAM S(7)1	Crude glycerol	Batch (flasks)	Papanikolaou & Aggelis, (2002)
Y. lipolytica LFMB 19, LFMB 20 and ACA-YC 5033		Batch (flasks)	André *et al.* (2009)
Y. lipolytica QU21		Batch (flasks)	Poli *et al.* (2014)
Y. lipolytica JMY4086		Fed-batch	Rakicka *et al.* (2015)
Y. lipolytica A101		Batch	Dobrowolski *et al.* (2016)
Y. lipolytica SKY7		Batch	Mathiazhakan *et al.* (2016)
Y. lipolytica LGAM S(7)1	Crude glycerol + stearin	Batch (flasks)	Papanikolaou *et al.* (2003)
Y. lipolytica LGAM S(7)1	Glucose + stearin	Batch (flasks)	Papanikolaou *et al.* (2006)
Candida lipolytica	Molasses	Batch (flasks)	Karatay & Dönmez (2010)
Y. lipolytica ACA-YC 50109	Olive oil mill wastewater	Batch (flasks)	Papanikolaou *et al.* (2008)
Y. lipolytica ACA-YC 5028, W29 and ACA-YC 5033			Sarris *et al.* (2011)
Y. lipolytica TISTR 5054, TISTR 5151, TISTR 5212, and TISTR 5621	Palm oil mill effluent	Batch (flasks)	Cheirsilp & Louhasakul (2013)
Y. lipolytica Po1g	Rice bran hydrolysate	Batch (flasks)	Tsigie *et al.* (2012)
Y. lipolytica ACA-YC 50109	Stearin	Batch	Papanikolaou *et al.*, 2002) Papanikolaou *et al.* (2007)
Y. lipolytica	Waste cooking oil	Batch (flasks)	El Bialy *et al.* (2011)
Y. lipolytica NCIM 3589		Batch	Katre *et al.* (2012)
Y. lipolytica SWJ-1b			Liu *et al.* (2015)

Temperature and pH are parameters that must be controlled for a maximum microbial lipids production. 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; Najjar *et al.*, 2011; Sarris *et al.*, 2011; Fontanille *et al.*, 2012). Also, temperature critically influences lipid accumulation by *Y.*

lipolytica and the range between 28 °C and 30 °C has been used for microbial lipids production by *Y. lipolytica* cultures (Bialy *et al.*, 2011; Sarris *et al.*, 2011; Cheirsilp & Louhasakul, 2013; Nambou *et al.*, 2014).

The amount of dissolved oxygen concentration in *Y. lipolytica* cultures is also a key parameter to take into account in microbial lipids production. Rakicka *et al.* (2015) and Yen & Zhang (2011) reported that *Y. lipolytica* JMY4086 and *Rhodotorula glutinis* BCRC 22360, respectively, accumulated higher lipid contents in conditions of lower dissolved oxygen concentration in culture medium. Papanikolaou *et al.* (2011) also concluded that the highest quantities of lipids accumulated by *Aspergillus niger* coincided with the lower values of dissolved oxygen saturation in the medium. By contrast, Tai & Stephanopoulos (2013) studied the lipid accumulation by mutant *Y. lipolytica* in glucose medium and observed highest lipid content in fermentations highly aerated. Bellou *et al.* (2014) also observed that *Y. lipolytica* ACA-DC 50109 accumulated high lipid content in medium with high dissolved oxygen concentrations.

2 GOALS

Great amounts of waste cooking oils are daily produced throughout the world. From both economic and ecological standpoint, the development of feasible ways to valorize these residues is very important. Valorization of waste cooking oils based on microorganisms can be a promising biotechnological approach, since there are microorganisms able to degrade these residues in order to obtain several added-value products.

The non-conventional yeast *Y. lipolytica* is capable to use residual oils from WCO for lipase production and microbial lipids accumulation. However, several culture parameters can affect both lipase and microbial lipids production. Moreover, due to the extensive application of lipases and microbial lipids in industry, the scale-up of bioprocess is a crucial issue for industrial application. Thus, the optimization strategies for maximization of lipase and microbial lipids production must consider some key operational parameters, such as oxygen mass transfer.

The main goal of this thesis was the valorization of WCO by *Y. lipolytica* for co-production of lipase and microbial lipids. Therefore, the specific goals were:

- Study the effect of medium composition (pH, WCO concentration and arabic gum concentration) on lipase and microbial lipids production in Erlenmeyer flasks using an experimental design based on Taguchi method;

- Scale-up the bioprocess to a stirred tank bioreactor (STR), studying the effect of oxygen mass transfer rate on 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 strain preservation

Yarrowia lipolytica W29 (ATCC 20460) were pre-grown in YPD medium (glucose 20 $g \cdot L^{-1}$, peptone 20 $g \cdot L^{-1}$ and yeast extract 10 $g \cdot L^{-1}$) overnight at 27 °C. For the preparation of crio-stocks, 800 μL of yeast culture and 200 μL of glycerol (99.5% of purity) were added to a sterile 1.5 mL microtube. Stocks were stored at - 80 °C and 1 microtube was subsequently used to inoculate each pre-inoculum.

3.2 Optimization of lipase and microbial lipids production – experimental design

Lipase and microbial lipids production was optimized using the Taguchi method, a fractional factorial experimental design. This method uses orthogonal arrays for the optimization of different parameters studying a few pairs of parameters combinations instead of all the possible combinations, which reduces time and resources. Orthogonal arrays selection is decided according to the number of parameters (P) and the variation of levels (L) of each parameter. The number of experiments (N) is calculated by the relation N-(L-1)P+1.

The experimental design was performed using a L9 orthogonal array with Qualiteck-4 software (Nutek, Bloomfield Hills, USA). Three factors (WCO concentration, pH and arabic gum concentration) were combined and varied in three levels (Table 3.1). From Qualiteck-4 software a total of 9 experiments were planned. The experiments were carried out in 500 mL Erlenmeyer flasks with baffles filled with 200 mL of production medium. The medium was sterilized in an autoclave at 121 °C during 15 minutes. Yeast cells were pre-grown in YPD medium (this pre-inoculum was inoculated with 1 crio-stock described in section 2.1), centrifuged and resuspended in the production medium, in order to achieve a cell density of 0.5 $g \cdot L^{-1}$: WCO as carbon source, yeast nitrogen base (YNB) without amino acids with ammonium sulfate (6.7 $g \cdot L^{-1}$) as nitrogen source and arabic gum dissolved in Tris-HCl buffer 400mM. The bioprocess was performed at 170 rpm and 27 °C for 24 hours in orbital incubator.

The responses of interest (lipase activity and microbial lipids production) obtained in the experimental design was processed in the Qualiteck-4 software with "bigger is better" quality

characteristics to evaluate the optimal culture conditions to maximize the lipase and microbial lipids production.

Table 3.1 Parameters and levels used to optimize the growth conditions for lipase production *by Y. lipolytica* W29.

Experiment	pH	WCO concentration	arabic gum concentration
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2
Level	**pH**	**WCO ($g \cdot L^{-1}$)**	**arabic gum ($g \cdot L^{-1}$)**
1	5.6	10	0
2	6.5	30	5
3	7.2	50	10

WCO, collected in the public canteen of University of Minho, were previously filtered to remove the major solid impurities. The content of lipids of these WCO was (0.92 ± 0.02) g of lipids·g^{-1} of oil. The olive oil (Gallo Reserva virgem extra®) used in comparative experiments has a maximum acidity of 0.4 % and the content of lipids was (0.76 ± 0.01) g of lipids·g^{-1} of oil. The fatty acids composition of each lipidic substrate used in this work (WCO and olive oil) is shown in Table 3.2.

Table 3.2 Fatty acids composition (%) of WCO and olive oil used in this study. Data are the average and standard deviation of two independent replicates.

Fatty acid (%)	WCO	Olive oil
Palmitic (C16:0)	7.5 ± 0.2	11.9 ± 0.0
Stearic (C18:0)	3.2 ± 0.0	3.3 ± 0.0
Oleic (C18:1)	24.9 ± 0.1	72.9 ± 0.2
Linoleic (C18:2)	64.3 ± 0.1	11.9 ± 0.2
Saturated fraction (%)	11	15
Unsaturated fraction (%)	89	85

3.3 Bioreactor experiments – effect of oxygen mass transfer

In order to evaluate the effect of oxygen mass transfer (namely volumetric oxygen transfer coefficient, $k_L a$) on lipase production, several batch experiments were carried out in a 2-L bioreactor (BIOLAB, B. Braun, Germany) (Figure 3.1) at 27 °C for 48 hours, varying the specific aeration rate (0.3 vvm - 1 vvm) and stirring rate (200 rpm - 500 rpm).

Yeast cells were pre-grown overnight in 500 mL Erlenmeyer flasks filled with 200 mL of YPD medium (this pre-inoculum was inoculated with 1 crio-stock described in section 2.1) at 27 °C and 170 rpm, centrifuged and resuspended (at an initial cell density of 0.5 $g \cdot L^{-1}$) in the production medium (1.6 L) composed by: WCO 10 $g \cdot L^{-1}$, YNB without amino acids and with ammonium sulfate 6.7 $g \cdot L^{-1}$ and Tween 80 1 $g \cdot L^{-1}$ dissolved in Tris-HCl buffer 400 mM, pH 7.2. The medium pH was kept at 7.2 by the addition of KOH 2 M. Dissolved oxygen concentration was measured with an optical probe (InPro 6000, Mettler Toledo, USA).

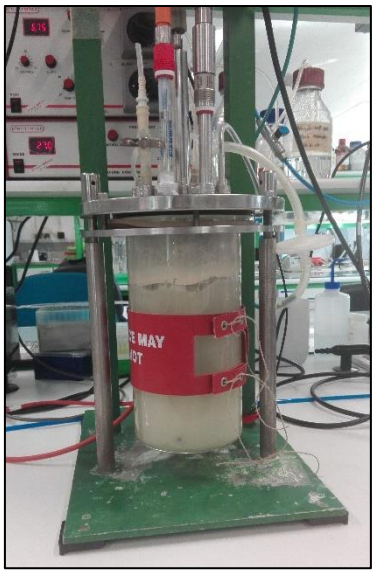

Figure 3.1 Stirred tank bioreactor (BIOLAB, B. Braun, Germany) with optimized medium for lipase production.

3.3.1 k_La measurement: dynamic gassing-out technique

k_La was determined using the dynamic gassing-out technique, in the presence of active cells, during the lipase production by *Y. lipolytica* W29. This method is based on following the dissolved oxygen concentration in cultivation medium during a short interruption of the air supply (Bandyopadhyay *et al.*, 1967). In the presence of active cells and in the absence of aeration, the respiratory activity of yeast cells leads to the depletion of oxygen of the liquid medium. The procedure involves two steps: the first one is to stop the aeration and the second one is to restart aeration at the operating conditions. Thus, in the first step, monitoring the decrease of dissolved oxygen concentration will allow to determine the specific oxygen uptake rate (OUR) (Eq.2.1):

$$\frac{dC}{dt} = -OUR \qquad \text{(Eq. 2.1)}$$

Air supply is restarted before reaching the critical dissolved oxygen concentration value (0.1 $mgO_2 \cdot L^{-1}$) (Tribe *et al.*, 1995). After the restart of aeration in the operating conditions (second step), the oxygen mass balance in the liquid phase is expressed by Eq.2.2:

$$\frac{dC}{dt} = k_{\mathrm{L}}a\ (C^* - C) - OUR \qquad (\mathrm{Eq.\ 2.2})$$

Considering the pseudo-steady state immediately before the determination, OUR can be replaced by Eq.2.3:

$$k_{\mathrm{L}}a\ (C^*\ -\ C_i) =\ OUR \qquad (\mathrm{Eq.\ 2.3})$$

C_i is the dissolved oxygen concentration at the beginning of the determination, C* is the dissolved oxygen concentration for each time during the gas-out period and $k_{\mathrm{L}}a$ is the oxygen volumetric mas transfer coefficient. The combination of two previous equations and integration of Eq.2.3 result in the Eq.2.4:

$$\ln\left(\frac{C_i - C}{C_i - C_0}\right) =\ -k_{\mathrm{L}}a\ (t - t_0) \qquad (\mathrm{Eq.\ 2.4})$$

Where C_0 and t_0 are the dissolved oxygen concentration and the time when aeration was restarted and C and t are the dissolved oxygen concentration for each time during the gas-out period and the time, respectively. By the graphical representation of $\ln\left(\frac{C_i - C}{C_i - C_0}\right)$ as a function of time a line was obtained whose slope corresponds to the (- k_{L}a) value.

3.4 Analytical methods

3.4.1 Total lipids

Lipid content of WCO and olive oil (g lipids·g^{-1} sample) was quantified after the extraction with chloroform and methanol, based on the method for total lipid extraction and purification developed by Bligh & Dyer (1959). A mass of 0.4 g of sample was mixed with water (1.6 mL). Then, 4 mL of chloroform and 2 mL of methanol was added to homogenate, filtered (0.45 μm)

and transferred to a separatory funnel. The lower phase (chloroform and lipids) was collected in a beaker previously weighted and after the evaporation of chloroform the beaker was weighted again. The amount of lipids was calculated by the Eq. 2.5:

$$\frac{g_{lipids}}{g_{sample}} = \frac{w_3 - w_2}{w_1} \qquad \text{(Eq. 2.5)}$$

where w_1 is the exact mass of sample; w_2 is the mass of beaker + lower phase, after the evaporation of chloroform and w_3 is the mass of beaker.

3.4.2 Cellular 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 ($g \cdot L^{-1}$) by a calibration curve.

3.4.3 Lipase activity

In a 96-well microplate, 240 µL of a mixture of p-nitrophenyl-butyrate 1 mM (p-NPB) in acetone 4 % (v/v) dissolved in PBS 50 mM (pH 7.3) (substrate) was added to a volume of 50 µL of culture supernatant. Immediately, the microplate was placed in a microtiter plate reader (Cytation 3, BioTek, USA) and an enzymatic run was performed at 37 °C during 10 minutes, with record of the absorbances (read at wavelength of 410 nm) every 30 seconds. Blank assays were performed replacing sample by distilled water or PBS buffer.

Lipase activity ($U \cdot L^{-1}$) was quantified using Eq.2.6:

$$Activity\ (U \cdot L^{-1}) = D \times \frac{1}{\varepsilon} \times \frac{V_t}{V_a} \times 1000 \qquad \text{(Eq. 2.6)}$$

where D represents the slope of the linear zone of the graphical representation 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 (mL) of the reactional mixture (0.29 mL) and V_a represents the volume of sample (mL) 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 assay conditions.

3.4.4 Protease activity

Protease activity was determined according to Pinto (1998). In microtubes, 0.5 mL of azocasein 0.5 % (w/v) dissolved in sodium acetate 50 mM (pH 5) buffer was added to 0.5 mL of sample supernatant. Blank assays were performed replacing sample by distilled water. The microtubes were incubated at 37 °C during 40 minutes. Then, 1 mL of trichloroacetic acid (TCA) 10 % (w/v) was added. The mixtures were centrifuged at 3000 rpm during 10 minutes, the supernatant (approximately 2 mL) was recovered to test tubes and 1 mL of KOH 5 M was added. The absorbance was read at a wavelength of 428 nm (microtiter plate reader (Sunrise Basic, Tecan, Switzerland)).

Protease activity ($U \cdot L^{-1}$) was determined using the Eq.2.7:

$$Activity\ (U \cdot L^{-1}) = \frac{A}{t \times V_a} \qquad \text{(Eq. 2.7)}$$

where A represents the absorbance of the reactional mixture at the wavelength of 428 nm; t (minutes) represents the time during mixture were incubated and V_a represents the volume of sample used in reactional mixture (0.5 mL).

One unit of activity was defined as the amount of enzyme that causes an increase of 0.01 of absorbance relative to the blank per minute under assay conditions.

3.4.5 Microbial lipids (phospho-vanillin method)

Intracellular lipids were extracted from dry cells with methanol and chloroform (1:1, v/v) and quantified by phospho-vanillin colorimetric method (Inouye & Lotufo, 2006) After vortex-mixing the mixture (cells + methanol + chloroform) for 5 minutes, a volume of 250 µL of supernatant was transferred to test tubes and heated at 100 ºC to allow the evaporation of solvent. After the evaporation, 100 µL of pure sulfuric acid was added to each tube, vortex-mixed and heated at 100 ºC for 10 minutes. After this time, samples were cooled to room temperature, 2.4 mL of vanillin reagent (0.24 g of vanillin dissolved in 40 mL of hot or warm water and 160 mL of 85 % orthophosphoric acid) was added and the mixture was vortex-mixed. 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 (microtiter plate reader (Sunrise Basic, Tecan, Switzerland)). The absorbance was converted to lipids concentration ($mg \cdot L^{-1}$) by a calibration curve (olive oil was used as standard). To construct the calibration curve, several concentrations of olive oil (0 $mg \cdot L^{-1}$ – 2500 $mg \cdot L^{-1}$) dissolved in acetone were submitted to the same procedure of samples, except the extraction with methanol and chloroform.

3.4.6 Long chain fatty acids (LCFA)

Intracellular lipids were transformed into their corresponding methyl esters with methanol:H_2SO_4 (15:85, v/v), followed by extraction with chloroform (adapted from Castro *et al.* (2017)). A defined amount of dry cells was transferred to glass vials where 1.5 mL of pentadecanoic acid, C15:0 (internal standard), 3 mL of methanol:H_2SO_4 solution and 1.5 mL of chloroform were added. The mixture was vortex-mixed and digested at 100 °C for 3.5 hours. After digestion, the mixture was votex-mixed and 1.5 mL of ultra-pure water was added. The content of glass vial was transferred to 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 to 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 (caproic acid, C6:0; caprylic acid, C8:0; capric acid, C10:0; 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 $mg \cdot L^{-1}$ and 1000 $mg \cdot L^{-1}$. 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:H_2SO_4 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) using heptadecanoic acid (C17:0) as internal standard. The injector and detector temperatures were 220 °C and 250 °C, respectively, 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).

4 RESULTS AND DISCUSSON

In this work, an experimental design based on Taguchi method was applied to evaluate the effect of the parameters pH, WCO concentration and arabic gum concentration on lipase and microbial lipids production by *Y. lipolytica* W29. pH was the parameter with more influence on both lipase and microbial lipids production by yeast. Besides the individual effect, an important influence of the interaction between WCO concentration and arabic gum concentration was observed. Different values of parameters levels were found as the optimal for lipase (pH 7.2; WCO concentration 10 $g \cdot L^{-1}$; arabic gum concentration 0 $g \cdot L^{-1}$) and microbial lipids production (pH 5.6; WCO concentration 30 $g \cdot L^{-1}$; arabic gum concentration 5 $g \cdot L^{-1}$).

To evaluate the effect of oxygen mass transfer on lipase and microbial lipids production, batch cultures of *Yarrowia lipolytica* W29 was carried out in a lab-scale stirred tank bioreactor. The increase of $k_L a$ from 9 h^{-1} to 93 h^{-1} enhanced cell growth and protease production. By contrast, a $k_L a$ of 16 h^{-1} was found as the best condition for the maximization of lipase and microbial lipids production. The fatty acids composition of lipids accumulated by *Y. lipolytica* W29 was mainly linoleic (≥60 %) and oleic (≥30 %), demonstrating the potential of these lipids to be used as food supplements.

4.1 Optimization of culture conditions for lipase and microbial lipids production – experimental design

The production of lipase and microbial lipids by *Y. lipolytica* strains is dependent on growth conditions such as pH and medium composition (Destain *et al.*, 1997; Domínguez *et al.*, 2003; Fickers *et al.*, 2004; Lopes *et al.*, 2009; Kebabci *et al.*, 2012; Bellou *et al.*, 2016; Liu *et al.*, 2017).

Therefore, in this work an experimental design based on Taguchi method was applied to evaluate the effect of pH, WCO concentration and arabic gum concentration on lipase and microbial lipids production by *Y. lipolytica* W29. Although several authors have studied the individual effect of each parameter on lipase or microbial lipids production, the combined effect between the three factors was not fully explored.

4.1.1 Lipase production

The results of lipase activity ($U \cdot L^{-1}$) obtained from the nine experiments are shown in Table 4.1. Lipase production were dependent on the combination of the various parameters studied and ranged from 64 $U \cdot L^{-1}$ to 523 $U \cdot L^{-1}$. The higher values of lipase activity were obtained in the experiments 7, 8 and 9, which have in common the pH value (7.2). Contrariwise, in the experiments performed with pH 5.6, considerable lower lipase production was achieved. On average, lipase activity obtained at pH 7.2 was 4-fold higher than that reached at pH 5.6.

These results show the potential of WCO to successfully induce lipase production by *Y. lipolytica* W29 cultures. In the literature, some authors also cited that oily substrates are potential inducers of lipase by *Y. lipolytica* cells (Domínguez *et al.*, 2003; Lopes *et al.*, 2009; Moftah *et al.*, 2013).

Table 4.1 Maximum lipase activity ($U \cdot L^{-1}$) obtained in batch cultures of *Y. lipolytica* W29 in the experiments designed by Taguchi L9 orthogonal array. Data are average and standard deviation of two independent replicates.

Experiment	pH	WCO concentration ($g \cdot L^{-1}$)	arabic gum concentration ($g \cdot L^{-1}$)	Lipase activity ($U \cdot L^{-1}$)
1	5.6	10	0	231 ± 79
2	5.6	30	5	79 ± 47
3	5.6	50	10	64 ± 34
4	6.5	10	5	285 ± 11
5	6.5	30	10	372 ± 8
6	6.5	50	0	313 ± 10
7	7.2	10	10	489 ± 54
8	7.2	30	0	466 ± 11
9	7.2	50	5	523 ± 16

Two different profiles on lipase production were observed: (a) in the experiments whose initial pH was 5.6 and 6.5 (experiments 1 to 6), lipase activity reached a maximum value after 9 hours of cultivation, after which it decreases; (b) when initial pH was 7.2 (experiments 7 to 9), the maximum value of lipase activity was also observed after 9 hours, but no decrease of lipase activity was observed until the end of cultivation. An example of these two profiles (experiment 6 performed at pH 6.5 and experiment 9 performed at pH 7.2) are depicted in Figure 4.1.

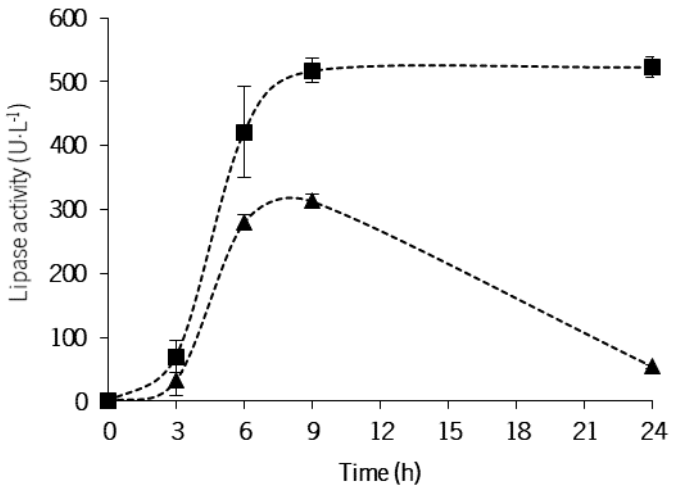

Figure 4.1 Profiles of lipase activity ($U \cdot L^{-1}$) obtained in experiments 6 (▲) and 9 (■). The conditions of each experiment were: experiment 6 - pH 6.5, WCO concentration 50 $g \cdot L^{-1}$ and arabic gum concentration 0 $g \cdot L^{-1}$; experiment 9 - pH 7.2, WCO concentration 50 $g \cdot L^{-1}$ and arabic gum concentration 5 $g \cdot L^{-1}$. The error bars represent the standard deviation for two independent replicates.

The individual effects of each parameter at different levels on lipase production by *Y. lipolytica* W29 are shown in Figure 4.2. The increase of pH from level 1 (5.6) to level 3 (7.2) had a remarkable positive effect on lipase synthesis, since lipase production at pH 7.2 was 4-fold higher than that obtained at pH 5.6. Change WCO and arabic gum concentration had a less relevant effect than pH variation, since the difference in both responses due to changes in the parameters levels were small. It should be stressed out that only 10 $g \cdot L^{-1}$ of WCO was enough to induce the maximum lipase production, but no inhibition effect was observed by increasing WCO concentration up to 50 $g \cdot L^{-1}$.

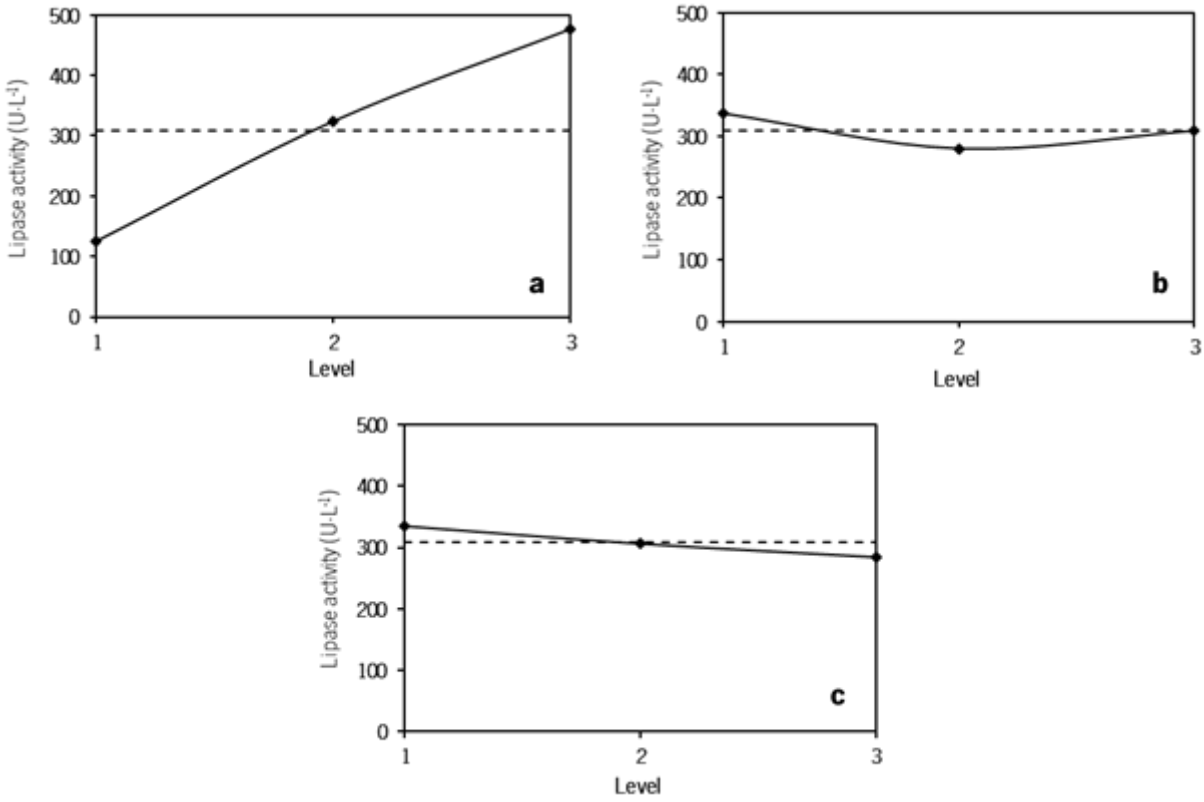

Figure 4.2 Individual effects of pH (**a**), arabic gum concentration (**b**) and WCO concentration (**c**) on lipase production by *Y. lipolytica* W29 obtained from Qualitek-4 software. pH levels: 1 – 5.6, 2 – 6.5, 3 – 7.2; WCO concentration ($g \cdot L^{-1}$) levels: 1 – 10, 2 – 30, 3 – 50; arabic gum concentration ($g \cdot L^{-1}$) levels: 1 – 0, 2 – 5, 3 – 10.

The analysis of variance (ANOVA) provides information about the relative importance of each parameter and allows identify the most significant parameters for lipase production (F-ratio > 9, confidence level of 90 %) and those with less significance. The contribution of each individual parameter (P, %) is calculated by the ratio between pure sum and the total sum of squares, and the higher percentage represents the parameter with more influence on the process (Table 4.2). According to ANOVA analysis, pH was by far the most significant parameter, with 84.8 % contribution toward variation in lipase production. This result agrees with the largest slope for the parameter pH in the graph of individual effects (Figure 3.1a). WCO and arabic gum concentration had no significant influence on lipase production by *Y. lipolytica* W29 in the range of the present study.

Table 4.2 Analysis of variance (ANOVA) of Taguchi L9 orthogonal array for lipase production by *Y. lipolytica* W29 cultures.

Factor	Sum of squares	Variance	F-ratio	P (%)
pH	372531.2	186265.6	50.9	84.8
WCO concentration	7929.2	3964.6	1.1	0.1
arabic gum concentration	9899.6	4949.8	1.35	0.6
Error	40195.9	3654.2	-	14.4

In the present study, the increase of initial pH of culture medium from 5.6 to 7.2 had a remarkable positive effect on lipase production by yeast and the highest values of lipase activity were obtained in experiments 7, 8 and 9 which had the same initial pH (7.2). The influence of medium pH on lipase synthesis by *Y. lipolytica* strains was previously reported, though the conclusions had not been consensual, demonstrating that lipase production depends on yeast strain as well on other culture conditions (Corzo & Revah, 1999; Domínguez *et al.*, 2010; Sathish Yadav *et al.*, 2011; Gonçalves *et al.*, 2013). Corzo & Revah (1999) observed that *Y. lipolytica* 681 produced maximum lipase at pH 4.5, while Gonçalves *et al.* (2013) reported that in cultures of *Y. lipolytica* LMI 91 the maximum lipase activity was obtained at initial pH 5.0. Sathish Yadav *et al.* (2011) concluded that initial pH between 5.0 and 6.0 was the most suitable for maximization of lipase production by *Y. lipolytica* NCIM 3639 cells, decreasing sharply after pH 7.0. By contrast, Destain *et al.* (1997) and Domínguez *et al.* (2010) observed that *Y. lipolytica* CBS 6303 and *Y. lipolytica* CECT 1240 cells, respectively, showed maximum lipase activity at pH values around 7.0.

Throughout the batch cultures, the medium pH in experiments performed at an initial pH equal to 5.6 and 6.5 dropped to approximately 2, while in experiments carried out at an initial pH of 7.2 the final pH was about 5.8. This abrupt decrease of medium pH in experiments 1 to 6 probably explains the lower lipase production obtained in these experiments, since the pH value was very different from the optimum pH values referred in literature. The drop of pH until values so low can be due to the release of free fatty acids (which result from hydrolysis of WCO by lipase) to the culture medium, since no organic acids were detected in the supernatant.

In spite of WCO concentration had no influence on lipase production by *Y. lipolytica* W29 cells in the range of the present study, the influence of different concentrations of lipidic carbon

sources on lipase activity has been explored by some authors. The results obtained in the present work indicate that any WCO concentration within the range of concentrations tested can be used without inhibition of lipase production by *Y. lipolytica* W29. However, the lowest concentration (10 $g \cdot L^{-1}$) was sufficient to induce the maximum lipase activity. Domínguez *et al.* (2010) also concluded that maximum lipase production by *Y. lipolytica* CECT 1240 was achieved with lower concentration of WCO (5 $g \cdot L^{-1}$), but concentrations above 10 $g \cdot L^{-1}$ led to the decrease of lipase activity. Lima *et al.* (2003) also concluded that concentrations of olive oil above 1 % (v/v) had an inhibitory effect on lipase production by *Penicillium aurantiogriseum*. Almeida *et al.* (2012) studied the effect of different concentrations of olive oil on lipase production by *Candida viswanathii* and also concluded that lipase activity decreased with olive oil concentrations above 20 $g \cdot L^{-1}$. Liu *et al.* (2015) cultivated *Y. lipolytica* SWJ-1b in medium with WCO and observed that concentrations of WCO above 40 $g \cdot L^{-1}$ lead to a decrease on lipase production By contrast, Liu *et al.* (2017) studied the lipase production by *Y. lipolytica* M53 and observed a maximum lipase activity with the maximum WCO concentration tested, 80 $g \cdot L^{-1}$. Papanikolaou *et al.* (2007) observed that an increase of stearin (a solid industrial derivate of tallow) concentration from 10 $g \cdot L^{-1}$ to 20 $g \cdot L^{-1}$ improved lipase production by *Y. lipolytica* ACA-DC 50109 and Kamzolova *et al.* (2005) observed that concentration of rapeseed oil was an important parameter to lipase production by *Y. lipolytica* strains and concentrations under 5 $g \cdot L^{-1}$ resulted in decrease on lipase activity.

According to some authors, the amount of lipidic substrate in the culture medium must be controlled, since high oil concentrations can affect the oxygen mass transfer in the liquid phase and the accumulation of great amounts of free fatty acids can inhibit the lipase induction (Rathi *et al.*, 2002; Tamilarasan & Dharmendira Kumar, 2011).

Surfactants or emulsifying compounds, which increase the fatty substrates availability for yeast, have been referred either as enhancers (Corzo & Revah, 1999) and inhibitors (Domínguez *et al.*, 2003; Lopes *et al.*, 2009; Dheeman *et al.*, 2010) of lipase production. The results obtained in this work showed that arabic gum concentration had no influence on lipase production by *Y. lipolytica* W29 and no addition of arabic gum is needed to achieve the maximum lipase activity in flask experiments. The fatty acids in the fermentation medium (obtained by the hydrolysis of TAGs of WCO), which are composed of a hydrophilic carboxylic head and a hydrophobic aliphatic tail, act as surfactants at neutral pH (Alves *et al.*, 2009). This fact could be the reason why, in the present study, no arabic gum is needed for maximum lipase production.

Apart the individual effect of each factor, it is also important assess the interaction between parameters. Estimated interaction severity index (SI) allows understanding the influence of the interaction of two parameters (Table 4.3). It should be notice that highest SI value is not associated with the most significant parameters (individual effect). The interaction between WCO concentration and arabic gum concentration, both parameters with no individual influence on lipase activity, had the higher SI value (52.1 %). The interactions between pH (parameter with more influence individual) and WCO concentration and pH and arabic gum concentration had lower SI values, 28.0 % and 14.6 %, respectively. This bioprocess is developed in a biphasic system, an oil-in-water emulsion with WCO and arabic gum as constituents of the culture medium. In two liquid-phase systems, with organic phase dispersed in the aqueous phase, the access of the cells to the oily substrate depends on the stability of the emulsion. Surfactants or emulsifiers in the medium decrease the lipid-water interfacial tension and promote the dispersion of the lipidic substrate (Delorme *et al.*, 2011). Considering that arabic gum acts as an emulsifying agent, it makes sense that the interaction between WCO concentration and arabic gum concentration has the highest severity index.

Table 4.3 Estimated interactions of studied parameters based on severity index (SI, %).

Interacting factor pairs	SI (%)
WCO concentration *vs* arabic gum concentration	52.1
WCO concentration *vs* pH	28.0
pH *vs* arabic gum concentration	14.6

Taguchi method established the optimum conditions for maximization of lipase production considering the experimental data, and predicted a theoretical value in such conditions (Table 4.4). The results obtained showed that the minimum concentration of WCO (10 $g \cdot L^{-1}$) is sufficient to induce maximum lipase activity by *Y. lipolytica* cells and no arabic gum addition is required for the maximum lipase biosynthesis. To attest the validity of the experimental design, verification experiments with three replicates were performed at optimal conditions predicted by the method for lipase production. Additionally, an experiment with olive oil instead of WCO was carried out, since this vegetable oil is traditionally used for lipase induction (Corzo & Revah, 1999; Lopes *et al.*,

2008; Najjar *et al.*, 2011). Extracellular lipase activity profiles were similar in WCO and olive oil-based media. The maximum value of lipase activity was achieved after 9 hours of growth and no decrease of lipase activity was observed until the end of cultivation. However, higher values of enzyme activity were obtained when WCO was used as both carbon and inducer sources. The maximum lipase activity attained in WCO medium was close to the value predicted by Taguchi method and the double of that obtained with olive oil. These results confirm the suitability of WCO as an inducer of lipase biosynthesis and is particularly promising since WCO is an inexpensive substrate when compared with olive oil, which makes the process for lipase production more attractive both from an economic and environmental point of view.

Table 4.4 Optimum culture conditions predicted by Taguchi method for maximization of lipase production by *Y. lipolytica* W29 from WCO, predicted values of lipase activity ($U \cdot L^{-1}$) in optimal conditions and experimental results obtained in WCO and olive oil-based media.

Factor	Level	Values	Predicted results	Experimental results
pH	3	7.2	531 $U \cdot L^{-1}$	532 $U \cdot L^{-1}$ ± 21 $U \cdot L^{-1}$ (WCO)
WCO concentration ($g \cdot L^{-1}$)	1	10		274 $U \cdot L^{-1}$ ± 28 $U \cdot L^{-1}$ (olive oil)
arabic gum concentration ($g \cdot L^{-1}$)	1	0		

4.1.2 Microbial lipids production

The results of microbial lipids content (%, w/w) and long chain fatty acids composition (%) obtained in the nine experiments are shown in Table 4.5. As observed for lipase production, also microbial lipids accumulation was dependent on the combination of the various parameters studied, ranging from 21 % (w/w) to 53 % (w/w). The highest microbial lipids accumulation was attained in the experiments performed at pH 5.6 (experiments 1, 2 and 3). On the other hand, the lowest intracellular lipids accumulation was obtained in the experiments that have in common the pH 7.2. On average, the lipid content obtained at pH 5.6 was 2-fold higher than that obtained at pH 7.2. However, it is important to highlight that, in all experiments, cells of *Y. lipolytica* W29 accumulated more than 20 % of their cell dry weight as intracellular lipids, which is in accordance with the principles of oleaginous yeasts. Particularly for the experiment 2, this value exceeded 50 % (w/w), demonstrating that WCO is suitable and advantageous for bioprocesses which main goal

is to maximize the microbial lipids production. This oil content is similar to others found in the literature for microbial lipids accumulation from lipidic substrates. El Bialy *et al.* (2011) observed a lipid content above 50 % (w/w) for *Y. lipolytica* cultivated in WCO, while Liu *et al.* (2015) reported a lower microbial lipids content (42 % (w/w)) for *Y. lipolytica* SWJ-1b growing on 80 $g \cdot L^{-1}$ of WCO. Papanikolaou *et al.* (2002) observed that, for *Y. lipolytica* ACA-DC 50109 cultivated in stearin medium, the lipid content ranged from 44 % (w/w) to 54 % (w/w). Sarris *et al.* (2011) studied the lipid accumulation of *Y. lipolytica* W29 in medium with olive mill wastewaters and observed a yeast lipid content of 34 % (w/w). Saygün *et al.* (2014) tested different oil sources for lipid accumulation by *Y. lipolytica* YB 423-12 and concluded that highest lipid accumulation was achieved with linseed oil (61.7 %, w/w), trout oil (53.8 %, w/w) and *Echium* oil (56.9 %, w/w). Papanikolaou *et al.* (2011) studied the lipid accumulation by *Aspergillus* sp. ATHUM 3482 from waste cooking olive oil and observed that an intracellular lipid content up to 64 % (w/w) was achieved.

Table 4.5 Microbial lipids content and fatty acids composition obtained in batch cultures of *Y. lipolytica* W29 in the experiments designed by Taguchi L9 orthogonal array. Data are average and standard deviation of two independent replicates.

Experiment	Microbial lipids (%, w/w)	Long chain fatty acids (%)				
		Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	Unsaturated fraction (%)
1	44 ± 5	28.1 ± 0.5	3.4 ± 0.3	39.8 ± 0.4	28.7 ± 0.3	69 ± 0
2	53 ± 1	33.1 ± 0.5	3.6 ± 0.2	38.2 ± 0.3	25.1 ± 0.2	63 ± 0
3	46 ± 3	31.8 ± 0.3	3.6 ± 0.2	35.5 ± 0.3	29.0 ± 0.4	65 ± 0
4	32 ± 2	26.4 ± 0.4	3.1 ± 0.3	31.2 ± 0.4	39.3 ± 0.2	71 ± 0
5	35 ± 6	28.8 ± 0.2	2.8 ± 0.1	30.6 ± 0.2	37.8 ± 0.3	68 ± 0
6	22 ± 1	24.9 ± 0.5	2.7 ± 0.2	28.5 ± 0.4	43.9 ± 0.3	72 ± 0
7	24 ± 1	27.4 ± 0.5	5.1 ± 0.3	30.8 ± 0.5	36.7 ± 0.4	68 ± 1
8	21 ± 1	26.8 ± 0.4	2.3 ± 0.2	26.1 ± 0.4	44.8 ± 0.5	71 ± 1
9	27 ± 1	30.0 ± 0.4	2.5 ± 0.3	27.0 ± 0.4	40.5 ± 0.3	68 ± 0

When hydrophobic substrates are used as carbon sources, lipids accumulation by oleaginous yeasts is a primary anabolic process ("*ex novo*" lipid synthesis), which occurs simultaneously with cell growth and the production of other lipid-free metabolites. Hence, microbial lipids composition is significantly influenced by the fatty acids composition of lipidic substrate and

by their specific uptake rate (Aggelis & Sourdis, 1997; Papanikolaou & Aggelis, 2010). In this work, where WCO were used as carbon source, lipids accumulated by *Y. lipolytica* W29 were composed by negligible amounts of stearic acid (2 % - 5 %) and considerable percentages of linoleic (25 % - 45 %), oleic (26 % - 40 %) and palmitic (25 % - 33 %) acids (Table 4.5). In all experiments, the amount of palmitic and oleic acids accumulated by the yeast was higher than that found in the initial substrate (WCO). It should be noticed that the higher quantities of oleic acid were achieved in the experiments with the lowest initial medium pH (5.6). In other hand, linoleic acid was preferentially accumulated by yeast cells cultivated at initial pH of 7.2. By contrast, the percentages of stearic and palmitic acids were similar in all experiments. The amount of each long chain fatty acid and the percentage of saturated (from 28 % to 37 %) and unsaturated (from 63 % to 72 %) fatty acids were different comparatively to the initial substrate. A bio-modification of WCO was observed and *Y. lipolytica* accumulated lipids with a different fatty acids composition, depending on medium conditions. It was observed that in all experiments the saturated fraction of intracellular lipids was much higher when compared to WCO used. Particularly in experiments performed at pH 5.6, this result was more evident and in experiment 2, intracellular lipids accumulated by yeast were much more saturated (37 %) than the WCO (11 %). These results showed that long chain fatty acids profile of lipids accumulated by *Y. lipolytica* W29 cells could be manipulated through the optimization of medium composition, particularly by the manipulation of medium pH, creating an opportunity to produce tailor-made lipids from WCO (Papanikolaou & Aggelis, 2010).

In the literature, there are some works demonstrating that different *Y. lipolytica* strains growing in WCO can accumulate distinctly fatty acids profiles. Katre *et al.* (2012) reported that microbial lipids accumulated by *Y. lipolytica* NCIM 3589 from WCO were mainly composed by caprylic (25 %), oleic (21 %) and palmitic (21 %) acids and only 12 % of linoleic acid. By contrast, *Y. lipolytica* strain NCIM 3472 accumulated only oleic (72 %), lignoceric (27 %) and behenic (2 %) acids from WCO, and no linoleic or palmitic acids were found. On other hand, cells of *Y. lipolytica* NC-I growing in waste cooking oils from frying vegetables accumulated preferentially oleic acid (50 %), followed by palmitic (20 %) and linoleic (17 %) acids (El Bialy *et al.*, 2011). It is worth to notice that the fatty acid composition of these intracellular lipids is very different from each other, probably due to the different composition of WCO used in each work.

Several authors reported that, through biotechnological valorization, the composition of oily wastes without any value can be upgrade by its bio-modification by oleaginous microorganisms,

obtaining lipids with high added-value. Aggelis *et al.* (1997) reported that *Candida* spp., *Langermania* sp. and *Rhodotorula* sp. accumulated less unsaturated lipids, despite the initial substrate rich in linoleic acid (evening primrose oil). It was reported that *Y. lipolytica* CICC1778 cultivated in mixtures of mutton fat/methyl stearate accumulated lipids enriched with oleic, stearic and palmitic acids, with a fatty acid profile resembled the composition of cocoa butter (Xiong *et al.*, 2015). Saygün *et al.* (2014) observed that the fatty acid profiles of oils accumulated by *Y. lipolytica* YB 423-12 using different oily substrates depend on oil type, though the oleic acid was the most accumulated, independently of the fatty substrate. The main fatty acids accumulated were: oleic and palmitic acids from trout oil, oleic and linoleic acids from borage oil, and α-linolenic and oleic acids from linseed oil. Papanikolaou *et al.* (2011) observed that cells of *Aspergillus niger* LFMB 2 accumulated lipids richer in palmitic acid than the initial oily substrate (waste cooking olive oil). By contrast, *Aspergillus* sp. ATHUM 3482 accumulated lipids enriched with stearic acids but less rich in palmitic and oleic acids comparatively to initial substrate.

The intracellular lipids obtained in this work, with high content of unsaturated fatty acids (oleic and linoleic acids), can be used as an excellent food supplement. Particular those lipids rich in linoleic acid (omega-6 family), which is considered an essential fatty acid or PUFA (polyunsaturated fatty acid) and cannot be synthesized by mammals and must be ingested through diet (Béligon *et al.*, 2016). The traditional source of PUFAs are fish oils, but the decrease of fish populations, their undesirable odor and presence of mutagenic compounds and heavy metals leads to a search for better and sustainable sources. Despite the lipids accumulated by *Y. lipolytica* have less linoleic acid than the initial substrate, in the European Union the recycling of WCO for food supplement and animal feedstock is strictly prohibited (Lam *et al.*, 2016). The biotechnological valorization of WCO by *Y. lipolytica* described herein is a cheaper approach to convert oily wastes without any nutritional value into lipids with high added-value.

The individual effect of each parameter at different levels on microbial lipids production by *Y. lipolytica* W29 from WCO is shown in Figure 4.3. Unlike to the observed for lipase production, the increase of pH from level 1 (5.6) to level 3 (7.2) had a negative influence on microbial lipids accumulation, since the lipid content obtained at pH 5.6 was on average 2-fold higher than that obtained at pH 7.2. As verified for lipase production, WCO and arabic gum concentrations had a less relevant effect than pH variation, since the difference in both responses due to changes in the parameters levels was small. The intermediate levels of WCO and arabic gum concentrations (30

g·L^{-1} and 5 g·L^{-1}, respectively) were found as the optimum for the maximal production of microbial lipids, nevertheless no inhibition effect was observed by increasing WCO concentration up to 50 g·L^{-1} and arabic gum up to 10 g·L^{-1}.

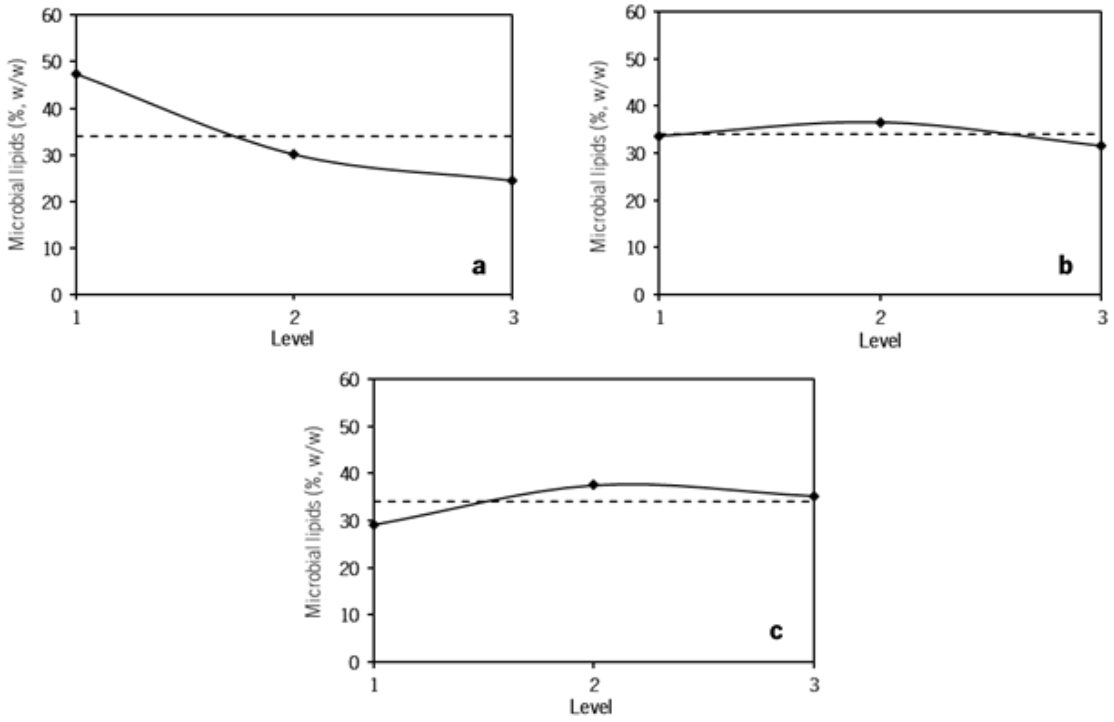

Figure 4.3 Individual effect of pH (**a**), arabic gum concentration (**b**) and WCO concentration (**c**) on microbial lipids production by *Y. lipolytica* W29 obtained from Qualitek-4 software. pH levels: 1 – 5.6, 2 – 6.5, 3 – 7.2; WCO concentration (g·L^{-1}) levels: 1 – 10, 2 – 30, 3 – 50; arabic gum concentration (g·L^{-1}) levels: 1 – 0, 2 – 5, 3 – 10.

The analysis of variance (ANOVA) for the results of L9 orthogonal array is very important to define which parameters have more influence on microbial lipids production by *Y. lipolytica* W29 (Table 4.6). According with these results, pH was by far the most significant parameter, with 80.6 % contribution toward variation in microbial lipids production. This result agrees with the largest slope for the parameter pH in the graph of individual effects (Figure 4.3a). However, arabic gum concentration had a smaller significant effect than pH, and WCO concentration had no significant effect on microbial lipids production in the range of the present study.

Table 4.6 Analysis of variance (ANOVA) of Taguchi L9 orthogonal array for microbial lipids accumulation by *Y. lipolytica* cultures.

Factor	Sum of squares	Variance	F-ratio	P (%)
pH	1740.1	870.1	96.5	80.6
WCO concentration	72.3	36.2	4.0	2.5
arabic gum concentration	226.2	113.1	12.6	9.7
Error	99.2	9.0	-	7.2

As demonstrated by ANOVA analysis, microbial lipids accumulation by *Y. lipolytica* W29 from WCO was strongly affected by initial pH, being this parameter the most influential on the bioprocess. The highest content of microbial lipids was obtained in the experiments carried out at pH 5.6. Papanikolaou *et al.* (2002) also identified the medium pH as a crucial factor for lipids production by *Y. lipolytica* ACA-DC 50109 on a stearin based medium and concluded that lipid accumulation by yeast was favored at pH 6. Generally, values of pH in the range of 4 – 7 are reported for lipids accumulation by *Y. lipolytica* cells from fatty substrates (Papanikolaou *et al.*, 2002; El Bialy *et al.*, 2011; Sarris *et al.*, 2011; Liu *et al.*, 2015).

Despite the WCO concentration had no significant effect on microbial lipids production in the present study, some authors recognized that initial lipidic substrate concentration can affect the final intracellular lipid yield. Depending on the nature and fatty acid composition of carbon source, several concentrations of substrate have been reported as optimal for microbial lipids accumulation by *Y. lipolytica* strains. Katre *et al.* (2012) concluded that 30 $g \cdot L^{-1}$ of WCO is the optimal concentration for lipids accumulation by *Y. lipolytica* NCIM 3472 strain and higher concentrations are inhibitory for both growth and lipid yield. By contrast, for *Y. lipolytica* NCIM 3589 strain no inhibition was noticed for WCO concentrations up to 100 $g \cdot L^{-1}$ (Katre *et al.*, 2012). Liu *et al.* (2015) also reported that no inhibition was observed with WCO concentrations up to 140 $g \cdot L^{-1}$, but 40 $g \cdot L^{-1}$ of WCO was enough to maximize the accumulation of lipids by *Y. lipolytica* SWJ-1b. Papanikolaou *et al.* (2007) observed that an increase of stearin concentration from 10 $g \cdot L^{-1}$ to 20 $g \cdot L^{-1}$ enhanced the lipid content accumulated by *Y. lipolytica* ACA-DC 50109, regardless the variations in the nitrogen concentration in the medium.

In literature it was reported that in *ex novo* lipid synthesis, which involves the uptake of fatty substrates from the culture medium, the accumulation of lipids by microorganisms is independent of molar C/N ratio (Papanikolaou *et al.*, 2002; Papanikolaou *et al.*, 2011; Donot *et al.*, 2014). In this work, different C/N ratios were used, since different WCO concentrations were tested but the same concentration of nitrogen source was maintained for all experiments. The results obtained herein showed that the variation of WCO concentration and, consequently, C/N ratio, had no significant effect on microbial lipids production by *Y. lipolytica* in the range of the present study, which is in accordance with the previous studies.

A slight significant effect of arabic gum on microbial lipids accumulation was observed, and only 5 $g \cdot L^{-1}$ is required to maximize the lipid accumulation by *Y. lipolytica* cells. As mentioned before, in a biphasic system, the access of the cells to the oily substrate is favored by the presence of surfactants/emulsifiers, which decrease the lipid-water interfacial tension and promote the dispersion of the lipidic substrate. In this work, arabic gum concentration had no significant influence on lipase production and a slightly effect on microbial lipids accumulation. Since low amounts of arabic gum are needed for emulsifying this process, this represents an advantage during industrial process because the addition of emulsifying agents increases the production costs.

Estimated interaction severity index (SI) allows understanding the influence of two parameters interaction (Table 4.7). As occurred for lipase production, also for microbial lipids accumulation the interaction between WCO concentration and arabic gum concentration had the higher SI value. On other hand, the interaction between the pH (parameter with more individual influence) and WCO concentration or arabic gum concentration had lower SI values. This result was expected since, as already stated, in two liquid-phase systems, with organic phase dispersed in the aqueous phase, the access of the cells to the oily substrate depends on the stability of the emulsion. Arabic gum acts as an emulsifying agent and, thus, it is not surprising that the interaction between WCO concentration and arabic gum concentration has the highest severity index for both lipase and microbial lipids production.

Table 4.7 Estimated interactions of studied parameters based on severity index (SI, %).

Interacting factor pairs	SI (%)
WCO concentration *vs* arabic gum concentration	67.9
WCO concentration *vs* pH	9.8
pH *vs* arabic gum concentration	3.0

Considering the experimental data, Taguchi method established the optimum conditions for maximization of microbial lipids production and predicted a theoretical value in such conditions (Table 4.8). It was observed that the optimal conditions for lipids accumulation are completely different from those for lipase production. While for lipase maximization, pH 7.2 was the optimum value, for microbial lipids pH must be kept at 5.6. A concentration of 10 $g \cdot L^{-1}$ of WCO was sufficient to induce maximum lipase activity, whereas an intermediate concentration (30 $g \cdot L^{-1}$) is required for lipids accumulation. The addition of arabic gum was not essential for lipase secretion, but 5 $g \cdot L^{-1}$ of this emulsifier is needed for the maximum microbial lipids production. As WCO concentration increases in the culture medium (from 10 $g \cdot L^{-1}$ for lipase production to 30 $g \cdot L^{-1}$ for microbial lipids accumulation), a higher amount of arabic gum (none for lipase and 5 $g \cdot L^{-1}$ for lipids) is required to maintain the stability of the oil-in-water emulsion. An important relation between WCO concentration and arabic gum concentration was already shown by severity index (67.9 % and 52.1 % for microbial lipids and lipase production, respectively), demonstrating that the interaction between these two parameters is the most influent interaction in this bioprocess.

Table 4.8 Optimum culture conditions predicted by Taguchi method for maximization of microbial lipids production by *Y. lipolytica* W29 from WCO, predicted and experimental values of microbial lipids content (%, w/w) obtained in optimal conditions.

Factor	Level	Values	Predicted results	Experimental results
pH	1	5.6		
WCO concentration ($g \cdot L^{-1}$)	2	30	53 (%, w/w)	51 % ± 3 % (w/w)
arabic gum concentration ($g \cdot L^{-1}$)	2	5		

The maximum microbial lipids content attained in optimal conditions was close to the value predicted by Taguchi method, which proves the effectiveness of the experimental design and confirms the ability of *Y. lipolytica* W29 to accumulate large amounts of intracellular lipids from WCO. This yeast oil content was one of the higher ever reported for a wild *Y. lipolytica* strain.

It should be noticed that the optimal conditions predicted for maximization of microbial lipids production are the same of experiment 2 (Table 3.1) and, thus, it is not surprising that lipids content was identical for both experiments. Therefore, also the fatty acids composition (oleic 38 %, palmitic 33 %, linoleic 26 % and stearic 3 % acids) was similar to the fatty acids profile obtained in the experiment 2. The microbial lipids were composed by 64 % of unsaturated fatty acids, which makes these lipids a good source to food supplements, since unsaturated lipids are known for their benefits for human health.

Although the optimal conditions for lipase and microbial lipids production were very different, it is possible any time to set the operating conditions for maximum lipase production or maximum microbial lipids production with the same technology and equipment. The bioprocess could be directed for any of these metabolites production considering the market demands, demonstrating that a biorefinery approach may be designed based on WCO. The capacity of a simultaneous production of lipase, which has wide utilizations for industrial applications, and microbial lipids, which currently have a great demand due their application for food supplements, will reduces the production costs of both metabolites.

4.2 Effect of oxygen mass transfer on metabolites production by *Y. lipolytica* in a stirred tank bioreactor (STR)

The several applications of lipases in industry require the development of economic and environmental-friendly large-scale production bioprocesses. A considerable amount of studies focused on bioprocesses for production of lipase from lipidic substrates, however less information is currently available about the optimization of lipase production from WCO in bioreactors.

Y. lipolytica has the special ability to degrade WCO for production of lipase and microbial lipids, under specific growth conditions (Domínguez *et al.*, 2010; El Bialy *et al.*, 2011; Katre *et al.*, 2012; Liu *et al.*, 2017). Moreover, *Y. lipolytica* is a strictly aerobic yeast and some studies have demonstrated the effect of oxygen mass transfer on metabolites production (Alonso *et al.*, 2005; Lopes *et al.*, 2008; Salehmin *et al.*, 2014). In other hand, the use of a lipidic substrate as carbon source implies that mixing conditions, such as agitation and aeration rates, should be adequate for a good mixture, allowing the satisfactory supply of the fatty carbon source to the yeast cells (Deive *et al.*, 2010). Therefore, in this work the effect of oxygen mass transfer on lipase and microbial lipids production by *Y. lipolytica* W29 from WCO was evaluated. Several experiments were carried out in a 2-L stirred tank bioreactor, by changing simultaneously stirring and aeration rates. Additionally, experiments with no addition of WCO into the culture medium (control) were performed.

4.2.1 Lipase production

The results of k_La, maximum biomass concentration and maximum specific growth rate (h^{-1}) obtained for the different conditions of stirring and aeration rate are presented in Table 4.9. As expected for a stirred tank bioreactor, the increment of stirring and aeration rates led to an enhancement of k_La value. A 10-fold improvement in k_La value was achieved by increasing simultaneously the aeration rate from 0.3 vvm to 1 vvm and the stirring rate from 200 rpm to 500 rpm.

Table 4.9 Experimental k_La values under different conditions of stirring and aeration rates, maximum biomass concentration and maximum specific growth rate (h^{-1}) obtained in batch cultures of *Y. lipolytica* W29 carried out in a STR bioreactor with WCO 10 $g \cdot L^{-1}$. Data are average and standard deviation of two independent replicates.

Experimental conditions		k_La (h^{-1})	Maximum biomass concentration ($g \cdot L^{-1}$)	μ (h^{-1})
Aeration rate (vvm)	Stirring rate (rpm)			
0.3*	300	15 ± 1	0.94 ± 0.1	0.008 ± 0.001
0.3	200	9 ± 0	2.8 ± 0.3	0.03 ± 0.01
0.3	300	16 ± 1	6.9 ± 0.4	0.11 ± 0.02
0.3	400	49 ± 0	9.5 ± 0.2	0.09 ± 0.02
1	500	93 ± 4	9.8 ± 1.2	0.18 ± 0.03

*Control (no addition of WCO)

It was observed that the raise of k_La from 9 h^{-1} to 93 h^{-1} had a clearly positive effect on cellular growth. As expected, in the control experiment with no addition of WCO, the maximum biomass concentration observed was very low (0.94 $g \cdot L^{-1}$), demonstrating that the addition of carbon source is crucial for cellular growth. Lowest biomass concentration (2.8 $g \cdot L^{-1}$) was achieved at lowest k_La value, probably due the limitation of dissolved oxygen that occurred in the first hours, impairing the cellular growth. A 4-fold improvement of cellular growth was observed at highest k_La value comparing with the lowest k_La value. No considerable differences were observed for cellular growth profiles in the experiments carried out at highest values of k_La (16 h^{-1}, 49 h^{-1} and 93 h^{-1}) (data not shown). The exception was noticed in the experiment at lower k_La value (9 h^{-1}), in which cells presented a longer lag phase when compared with the cellular growth behavior at higher k_La values. This led to an exponential phase less pronounced, which is in accordance with the lower values of biomass concentration observed at k_La value of 9 h^{-1}.

Different dissolved oxygen profiles were observed in batch cultures of *Y. lipolytica* W29, according with the k_La values (Figure 4.4). During the first hours of yeast fermentation, which corresponds to the exponential growth phase, a decrease on oxygen concentration in the fermentation medium was observed for all experiments, with exception for the experiment at higher k_La value (93 h^{-1}). Indeed, for a k_La value of 9 h^{-1}, a full depletion of oxygen from the medium was observed from the first hour and through all the fermentation, which can justify the lower biomass achieved in these conditions. In the experiments with k_La value equal to 16 h^{-1}, the dissolved oxygen

concentration fell to zero after 8 h of fermentation and stabilized in this value until approximately 40 h of fermentation. For the k_La value of 49 h^{-1}, the oxygen concentration never dropped to zero, although a decline was observed in the first 10 h, and stabilized around 50 % at 25 h. At the highest k_La value (93 h^{-1}), the dissolved oxygen concentration never dropped to zero. The same behavior was observed for control experiments performed with no addition of WCO.

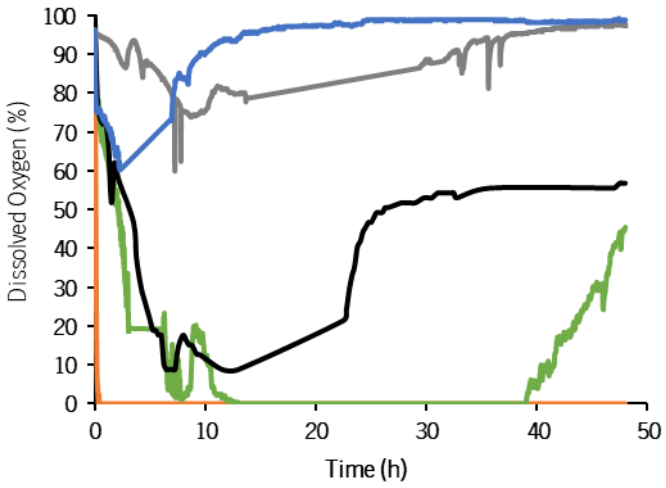

Figure 4.4 Dissolved oxygen concentration profiles during lipase production in batch cultures of *Y. lipolytica* W29 at different k_La values (h^{-1}): 9 (orange line); 16 (green line); 49 (black line); 93 (grey line); control (blue line).

The increase of k_La also improved the specific growth rate from 0.03 h^{-1} to 0.18 h^{-1}, and highest specific growth rate was attained in the experiments performed at k_La equal to 93 h^{-1}. The increase of agitation rate reduces the size of the oil droplets and enhances the rate of extracellular hydrolysis of the oil, which improves the carbon supply to the cells. This phenomenon could be one hypothesis to the enhancement of growth rate and final biomass concentration. Additionally, at higher k_La values the cellular growth was not limited by oxygen concentration.

These results are in accordance with those reported by Lopes *et al.* (2009), since the authors also concluded that cellular growth of *Y. lipolytica* was improved by increasing oxygen transfer rate. Papanikolaou *et al.* (2007) observed that higher oxygenation, owing to higher agitation rate employed in bioreactor, significantly enhanced the biomass concentration of *Y. lipolytica* ACA-DC 50109. By contrast, Alonso *et al.* (2005) and Deive *et al.* (2010) concluded that an increase of

agitation rate led to a decrease in cellular growth of *Y. lipolytica* IMUFRJ 50682 and *Y. lipolytica* CECT 1240, respectively. Both studies suggested that lower cellular growth was due to the shear stress promoted by the impellers (mechanical stress) occurred at high agitation rates and/or oxidative stress resulting from the increase in dissolved oxygen concentration.

The raise of k_La from 9 h^{-1} to 16 h^{-1} had a remarkable positive effect on lipase production, increasing from 1855 $U \cdot L^{-1}$ to 12000 $U \cdot L^{-1}$ (Table 4.10). However, an opposite effect was observed for k_La values above 16 h^{-1}. Indeed, the lowest lipase activity was attained at higher k_La value and a 2.4-fold reduction in lipase production was obtained comparatively with the experiment carried out at a k_La of 16 h^{-1}. The possibility to operate at lower stirring and aeration rates is an important aspect, making this bioprocess economically interesting, since it is possible to reduce the costs with power consumption. In the control experiment with no addition of WCO, the lipase activity observed was much lower when compared with the experiments with addition of WCO, which confirms the requirement of oily substrate for the maximal induction of lipase.

Table 4.10 Experimental k_La values under different experimental conditions, maximum lipase activity ($U \cdot L^{-1}$) and maximum protease activity ($U \cdot L^{-1}$) obtained in batch cultures of *Y. lipolytica* W29 carried out in STR bioreactor. Data are average and standard deviation of two independent replicates.

k_La (h^{-1})	Maximum lipase activity ($U \cdot L^{-1}$)	Maximum protease activity ($U \cdot L^{-1}$)
15 ± 1	66 ± 15[a]	149 ± 46[a]
9 ± 0	1855 ± 408[ab]	603 ± 187[b]
16 ± 1	12000 ± 2869[c]	1453 ± 45[c]
49 ± 0	7438 ± 1636[d]	1959 ± 132[d]
93 ± 4	5050 ± 943[bd]	2111 ± 67[d]

*control (no addition of WCO)

[a,b,c,d]Values followed by the same letter do not present statistically significant differences ($p \leq 0.05$)

Deive *et al.* (2010) and Alonso *et al.* (2005) also reported that lower agitations rates improved lipase production by *Y. lipolytica* strains cultivated in medium with olive oil. Dalmau *et al.* (1998) concluded that, when *C. rugosa* grown in medium with oleic acid, the lipase production was higher at lower stirring rates, once large droplets sizes of oleic acid were found in the fatty

acid/water emulsion. Burkert *et al.* (2005) studied the lipase production by *Geotrichum candidum* in soybean oil medium and concluded that lower values of k_La resulted in 3-fold improvement in lipase activity. Other authors also concluded that agitation rates above 200 rpm led to a decrease on lipase production by *A. niger* cultivated in olive oil medium (El-batal *et al.*, 2016). By contrast, Salehmin *et al.* (2014) studied the production of lipase by *Candida rugosa* using palm oil as carbon source and observed that in experiments carried out at agitation rates below 400 rpm no lipase production was observed and the highest production was attained at 600 rpm. The authors suggested that low agitation rates narrowed lipase production due to the limitations on the supply of carbon source. On the other hand, at agitation rates above 600 rpm, corresponding to a non-limiting supply of carbon situation, cellular growth was favoured (high specific growth rates was observed) instead of lipase production.

In fact, the maximum lipase activity was observed in the experiments carried out at 300 rpm (k_La value of 16 h^{-1}) at 30 h of fermentation, which coincided with a limitation of dissolved oxygen in the medium (oxygen concentration was equal to 0 % between 10 h and 40 h of cultivation). Freire *et al.* (1997) also observed that lipase production by *P. restrictum* cultivated in medium with olive oil was improved by oxygen limitation. By contrast, 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. 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 %. Fickers *et al.* (2009) observed that the raise of lipase production by mutant *Y. lipolytica* LgX64.81 was enhanced with the increased of dissolved oxygen concentration.

It is worth to notice that a 23-fold improvement in lipase activity was obtained in bioreactor experiments comparatively with the Erlenmeyer flasks experiments carried out in optimum conditions. In bioreactor experiments, the pH was controlled at 7.2 contrary to what happened in Erlenmeyer flasks experiments where fluctuations on pH value were observed. The control of pH is an important strategy since Taguchi method has established that pH was the parameter with most influence on lipase production by *Y. lipolytica* W29 from WCO. Domínguez *et al.* (2010) also observed an improvement on lipase activity in bioreactor fermentations when compared with the flaks fermentations. The authors justify this fact with the existence of suitable hydrodynamic

conditions inside the bioreactor such as good aeration and agitation rates, which reduces the resistance to oxygen mass transfer, once the thickness of liquid layer in the gas/liquid interface is weakened.

In the conditions observed as most advantageous for lipase production (k_La 16h^{-1}), the lipase activity sharply decreasing immediately after reaching the maximum activity, as explained above. The depletion of key nutrients and consumption of extracellular lipase for cell maintenance could explain this profile (Deive *et al.*, 2010). On other hand, the decrease of lipase production at the stationary phase has been attributed by some authors to the decrease of lipidic substrate concentration in the medium (Papanikolaou *et al.*, 2007). Moreover, an increase of protease activity was also observed throughout the fermentation and achieved the maximum production at the end of cultivation time. The presence and action of proteases could explain the drastic decrease of lipase activity after reaching the maximum.

Contrary to the results obtained for lipase production, the raise of k_La value from 9 h^{-1} to 93 h^{-1} led to a clearly increase on protease activity. A 3.5-fold enhancement on protease activity was attained at k_La of 93 h^{-1} comparatively to that obtained at k_La of 49 h^{-1}. The presence of protease in the fermentation medium can influence the maximum lipase production because the action of these enzymes could lead to the degradation of lipase. Lopes *et al.* (2009) observed that highest value of protease activity was achieved in the same conditions where it was observed maximum lipase productivity. Alonso *et al.* (2005) concluded that the increase of k_La values from 8.5 h^{-1} to 54.5 h^{-1} do not favored the protease production and maximum protease activity was achieved at lowest agitation rate tested (100 rpm).

4.2.2 Microbial lipids production

Although the experiments in bioreactor were performed under the optimal conditions for lipase production, in the Erlenmeyer flaks experiments it was observed that *Y. lipolytica* W29 cells accumulated great content of lipids from WCO simultaneously with production of lipase. Thus, the lipid content was quantified and the effect of oxygen mass transfer on microbial lipids production was also assessed.

The results of microbial lipids content (%, w/w) are shown in Figure 4.5. As observed for lipase production, the raise of k_La from 9h^{-1} to 16 h^{-1} had a clearly positive effect on microbial lipids production. By contrast, a negative influence on microbial lipids production was observed for k_La values above 16 h^{-1}. Although the conditions were optimal for lipase production, considerable amounts of lipids were accumulated by yeast cells. The lipid content ranged between 10 % (w/w) to 48 % (w/w), which corresponds, in mass of intracellular oil per volume of culture medium, to 0.6 g·L^{-1} and 3.0 g·L^{-1}, respectively. The lipid content accumulated by *Y. lipolytica* in medium with no addition of WCO was only 10 % (w/w), which corresponds to only 0.07 g·L^{-1} due the low biomass achieved in this fermentation.

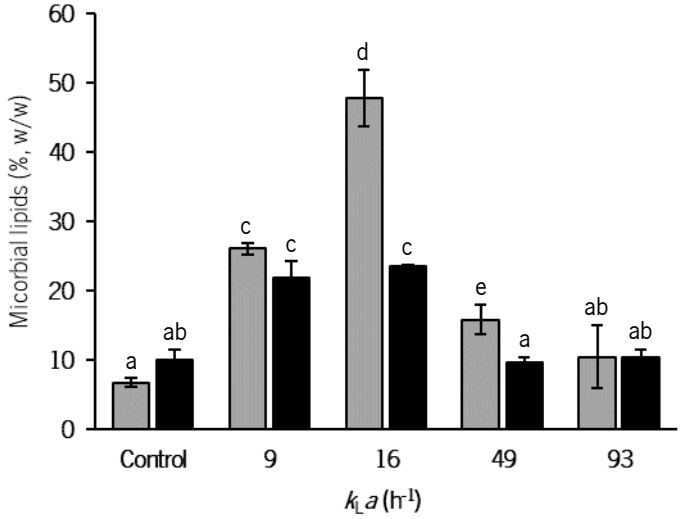

Figure 4.5 Microbial lipids content (%, w/w) of *Y. lipolytica* W29 cells obtained in batch cultures carried out in STR bioreactor after 24 hours of fermentation (grey bars) and 48 hours of fermentation (black bars). The error bars represent the standard deviation of two independent replicates. Bars with the same letter do not present statistically significant differences ($p \leq 0.05$).

The maximum value of lipid content accumulated by *Y. lipolytica* cells (3.0 g·L^{-1}) in this work was similar to other values found in the literature for *Y. lipolytica* strains cultivated in medium with addition of WCO (El Bialy *et al.*, 2011; Liu *et al.*, 2015) and higher than cellular lipid concentration (1.7 g·L^{-1}) observed by Sarris *et al.* (2011) for *Y. lipolytica* W29 cultivated in olive oil mill wastewater medium. By contrast, higher cellular lipids concentrations were obtained by other authors (Papanikolaou *et al.*, 2002; Papanikolaou *et al.*, 2007; Saygün *et al.*, 2014; Bellou *et al.*,

2016). However, in those studies higher biomass concentrations was achieved. Once the lipid content obtained in the bioreactor experiments in this work was 48 % (w/w), which corresponds only to 3.0 $g \cdot L^{-1}$ of lipids, the use of high cellular density cultures could be a strategy to reach higher microbial lipids concentrations.

It is also important to observe the differences in microbial lipids content within each condition. In general, the lipid content (%, w/w) was identical at 24 hours and 48 hours of fermentation ($p > 0.05$). However, in the conditions where the highest content of lipids was attained (k_La 16 h^{-1}), a significant decrease on microbial lipids content from 24 h to 48 h was observed. In batch fermentations, when microorganisms are cultivated in lipidic substrates, the accumulation of intracellular lipids occurs in the first stage of growth. The fast degradation of substrate lead to conditions of carbon starvation that forces the degradation of storage lipids by microorganisms (Aggelis & Sourdis, 1997). Additionally, accumulated lipids could inhibit the uptake of extracellular fatty acids by microorganisms, and the degradation of reserve lipids instead of uptake of extracellular fatty acids is initiated (Aggelis *et al.*, 1997).

In this work, the highest lipid contents were achieved at lower k_La values, which correspond to lower dissolved oxygen concentration in the culture medium. As shown above, the dissolved oxygen concentration in the conditions of lower k_La values remained nearly zero almost all the time (Figure 4.3). The results reported in this work are in accordance with Bati *et al.* (1984), since the authors observed that a dissolved oxygen concentration nearly zero improved microbial lipids accumulation by *Candida lipolytica*. Yen & Zhang (2011) studied the effect of dissolved oxygen on lipid accumulation by *Rhodotorula glutinis* cultivated in glucose and concluded that low dissolved oxygen levels retarded cell growth but had a positive effect in lipid accumulation. Karamerou *et al.*(2015) also observed that low aerated cultures favored lipid accumulation by *R. glutinis* cultivated in biodiesel-derived glycerol. The degradation of hydrophobic substrates by *Y. lipolytica* strains is a multi-step pathway requiring different acyl-CoA oxidases (*Aox*). Apparently, at higher aeration and agitation rates, the activity of short-chain acyl-CoA oxidases (e.g. *Aox*3p) increases, and the carbon source is directed towards the synthesis of acetyl-CoA, indicating that lipid-free biomass formation is favored instead of intracellular lipids accumulation (Mlícková *et al.*, 2004; Fickers *et al.*, 2005; Papanikolaou *et al.*, 2007). By contrast, Tai & Stephanopoulos (2013) studied the lipid accumulation by mutant *Y. lipolytica* in glucose medium and concluded that fermentations highly

aerated favored the lipid accumulation. Bellou *et al.* (2014) also observed that *Y. lipolytica* ACA-DC 50109 accumulated high lipid content in medium with high dissolved oxygen concentrations.

Microscopic observations of *Y. lipolytica* W29 cells cultivated in medium with WCO revealed that the presence of lipidic substrate and/or dissolved oxygen concentration did not induced hyphae formation and oval form was observed in all experiments (Figure 4.6). Moreover, large lipid bodies were observed inside the *Y. lipolytica* cells when fermentation was carried out at k_La of 16 h^{-1} (Figure 4.5a), which is in accordance with the high lipid content described above. By contrast, small lipid bodies inside the yeast cells were observed in the experiments performed at k_La of 93 h^{-1}, which is in accordance with the lower lipid content achieved in these conditions.

Some authors reported that low aeration conditions induce mycelium formation on *Y. lipolytica* cells and in high aeration conditions *Y. lipolytica* cells showed typical oval form, regardless the nature of substrate (hydrophilic, such as glucose and glycerol or hydrophobic, such as olive oil) (Bellou *et al.*, 2014). By contrast, Papanikolaou *et al.* (2007) cultivated *Y. lipolytica* in stearin medium and observed that yeast cells displayed a true mycelium form, independently of substrate concentration and mode of operation.

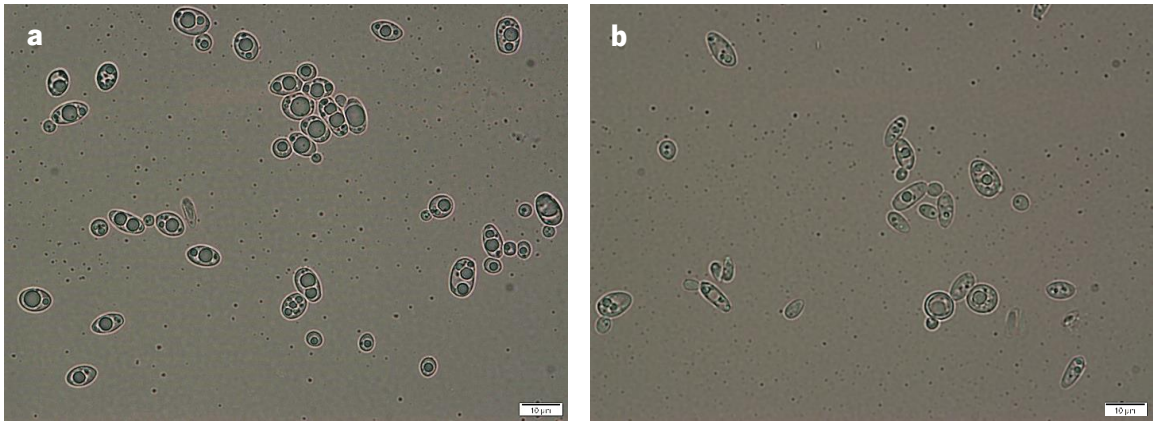

Figure 4.6 Light microscopy image of *Y. lipolytica* W29 cells with intracellular lipid bodies in the cytoplasm after 24 h of growth in WCO: **a**) k_La of 16 h^{-1} and **b**) k_La of 93 h^{-1}.

The fatty acids composition of intracellular lipids accumulated by *Y. lipolytica* W29 cells from WCO is shown in Table 4.11. In all conditions, negligible amounts of stearic acid were detected (1 % - 4 %), while considerable quantities of linoleic (60 % - 71 %), oleic (21 % - 29 %), and

palmitic (6 % - 13 %) acids were accumulated. It should be noticed that in all experiments, the amount of linoleic acid was higher than 60 %. The unsaturated fraction of microbial lipids ranged from 84 % to 93 %.

The content of each fatty acid observed for all conditions between 24 hours and 48 hours of fermentation showed no considerable differences, except for oleic and linoleic acids. Oleic acid content increased between 24 hours and 48 hours in all experiments. By contrast, linoleic acid content decreased between 24 hours and 48 hours for almost all conditions.

Contrary to the results obtained in this work, André *et al.* (2009) observed that lipids accumulated by *Y. lipolytica* ACA-YC 5033 in medium with crude glycerol was more saturated at the exponential growth phase (24 hours of fermentation) due the increase of palmitic and stearic acids concentrations compared with the other phases of growth but in contrast oleic acid content decreased during fermentation. Several studies observed that yeasts cannot accumulated high quantities of oleic acid, since this fatty acid was rapidly incorporated inside cells and was as well rapidly dissimilated for growth requirements (Papanikolaou *et al.*, 2001; Papanikolaou & Aggelis, 2003a, 2003b).

Table 4.11 Long chain fatty acids composition (%) obtained in batch cultures of *Y. lipolytica* W29 carried out in STR bioreactor. Data are average and standard deviation of two independent replicates.

k_La (h^{-1})	Time (h)	Long chain fatty acids (%)				
		Palmitic (C16)	Stearic (C18)	Oleic (C18:1)	Linoleic (C18:2)	Unsaturated fraction (%)
9	24 h	12.5 ± 1.2	3.3 ± 0.6	21.9 ± 0.6	62.2 ± 2.3	84 ± 1
	48 h	10.3 ± 1.0	3.0 ± 0.8	28.7 ± 0.4	58.0 ± 1.7	87 ± 1
16	24 h	6.3 ± 0.1	1.1 ± 0.0	21.2 ± 1.1	71.3 ± 1.0	93 ± 1
	48 h	6.1 ± 0.5	1.6 ± 0.3	26.3 ± 1.4	66.0 ± 0.6	92 ± 0
49	24 h	8.7 ± 1.3	2.8 ± 0.3	27.0 ± 0.0	62.7 ± 0.2	90 ± 1
	48 h	7.3 ± 1.8	2.2 ± 0.5	27.8 ± 0.0	62.7 ± 2.2	91 ± 0
93	24 h	8.7 ± 2.0	2.7 ± 1.2	24.5 ± 0.1	64.0 ± 1.6	89 ± 0
	48 h	10.2 ± 2.6	4.2 ± 1.3	25.3 ± 1.7	60.3 ± 2.2	86 ± 0

Comparatively to the initial substrate, an increase of palmitic acid was observed for all conditions. A slightly increase was also observed in oleic acid content comparatively to the initial

substrates. By contrast, in almost all experiments the stearic acid content observed was slightly lower when compared with the initial substrate as well as linoleic acid content. The exception was the experiments at k_La of 16 h^{-1} once it was observed a higher linoleic acid content than the same fatty acid content found in initial substrate. As observed in Erlenmeyer flasks experiments, the manipulation of conditions such as aeration and agitation rates allowed the production of tailor-made lipids.

Due to the composition of microbial lipids rich in oleic and linoleic acids (mono and polyunsaturated fatty acids, respectively), these intracellular lipids can be used as an excellent food supplement (Béligon *et al.*, 2016). This PUFA belongs to the family of omega-6, acting as a precursor for the synthesis of more highly unsaturated and longer-chained omega-6 family, such as arachidonic acid (ARA), which is the principal omega-6 fatty acid present in the brain. One of the major currently problems are how to provide enough a diet rich in PUFAs to an increasing population. The microbial lipids accumulated by *Y. lipolytica* enriched with linoleic acid could be considered as an alternative source for essential nutrients that are present in diet. The bioprocess described herein is a cheaper and advantageous way to recycle WCO into lipids with high added-value.

The simultaneous production of lipase and microbial lipids by *Y. lipolytica* W29 growing in WCO lead to a sustainable and economical viable production of an important enzyme and lipids enriched with essential fatty acids, which have wide application in pharmaceutical and food industries. Moreover, the operational costs can be reduced since at the same time and with the same equipment and technology, two added-value metabolites with important applications are obtained.

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 field of research.

5.1 Final Conclusions

Y. lipolytica has the special ability to degrade lipid-rich substrates while produces value-added metabolites. WCO can be used as carbon source in the production of lipase and microbial lipids by *Y. lipolytica* under specific growth conditions.

The optimization of medium parameters by experimental design based on the Taguchi method allowed to conclude that pH was the parameter with more influence on lipase and microbial lipids production by *Y. lipolytica* W29 using WCO as substrate. Lipase production ranged from 64.4 $U \cdot L^{-1}$ to 523.1 $U \cdot L^{-1}$ in flask assays and the higher values of lipase activity were obtained in the experiments at pH 7.2. By contrast, highest microbial lipids accumulation was attained in the experiments performed at pH 5.6, ranging from 21 % (w/w) to 53 % (w/w). Arabic gum had a significant influence only for microbial lipids production by yeast. However, the interaction between pH and arabic gum concentration and their influence was found significant for both lipase and microbial lipids production and should be considering for further fermentations. The optimal conditions for maximization of lipase production were: pH 7.2 and WCO 10 $g \cdot L^{-1}$, with no addition of arabic gum. In other hand, optimal conditions for maximization of microbial lipids production were: pH 5.6, WCO 30 $g \cdot L^{-1}$ and arabic gum 5 $g \cdot L^{-1}$. Similar lipase activities were obtained using WCO compared with olive oil, validating the possibility of using WCO as a low-cost carbon source for lipase production by *Y. lipolytica* W29.

The experiments in STR bioreactor showed that increasing k_La up to 93 h^{-1} improve cell growth as well as protease production. On the other hand, values of k_La above 16 h^{-1} led to a decrease in lipase production. The accumulation of microbial lipids was also favored in lower k_La values. The composition, in fatty acids, of microbial lipids accumulated by yeast was mainly linoleic (≥60%) and oleic (≥30%) acids, demonstrating the potential of these lipids to be used as food supplements. The maximum production of microbial lipids was observed for the same k_La conditions (16 h^{-1}), demonstrating that a biorefinery context may be designed to exploit the various metabolites that are simultaneous produce by *Y. lipolytica* W29 from WCO.

5.2 Future work perspectives

The present work brings new perspectives on the biotechnological production of lipase and microbial lipids by *Y. lipolytica* W29, contributing for the optimization of some important parameters that could affect the bioprocess. Nevertheless, there are still some new ideas for future work and developments.

Several authors observed that low levels of dissolved oxygen (DO) enhance lipid accumulation, but inhibit cell growth, whereas a higher level of DO enhances biomass accumulation rather than lipid accumulation. Thus, a two-stage DO strategy to control biomass and improved lipid accumulation by *Y. lipolytica* W29 could be applied.

In order to study the effect of operation mode on lipase and microbial lipids production, different strategies of substrate feeding to the STR bioreactor may be applied: step-wise fed-batch (pulses of WCO at regular intervals) and fed-batch mode (WCO feeding at a constant feeding rate or at an exponential feeding rate). Additionally, studies with constant dissolved oxygen, manipulating the stirring speed and specific air flow rate, through a cascade control mode, could be performed.

Several authors reported high lipid concentration in medium with *Y. lipolytica* strains, but initial cellular concentration used was higher when compared with the initial cellular concentration used in this work. Thus, studies with higher initial cellular concentrations to improved lipid concentration in medium with WCO could be carried out in bioreactor.

Finally, pressurized bioreactors have also great potential of application for lipase production, and it would be interesting to implement in this bioreactor type strategies of gradually increasing pressure to obtain high cell density cultures and to produce lipase and microbial lipids.

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SCIENTIFIC OUTPUTS

The results described herein were already presented in two International Congresses (oral communications) and two manuscripts (one review and one research paper) are being prepared for submission to Peer review journals.

Oral communications

1. Lopes, M., **Miranda, S. M.**, Pereira, A. S., Belo, I. Lipofactory: *Yarrowia lipolytica* as a cell factory to produce microbial oils from hydrophobic substrates. European Biotechnology Congress 2017, May 25 - 27, Dubrovnik, Croatia, 2017.

The abstract of this oral communication is published in Journal of Biotechnology: Lopes, M., Miranda, S. M., Pereira, A. S., Belo, I. (2017) Lipofactory: *Yarrowia lipolytica* as a cell factory to produce microbial oils from hydrophobic substrates. Journal of Biotechnology, 256:S19. DOI:10.1016/jbiotec.2017.06.614.

2. **Miranda, S. M.**, Pereira, A. S., Belo I., Lopes, M. Waste cooking oils: low-cost substrate for co-production of lipase and microbial lipids. 4th International Conference "Wastes – Solutions, treatments and opportunities", September 25 - 26, Porto, Portugal, 2017.

A full paper with the results presented in this conference is published as a chapter in the book: Miranda, S. M. Pereira, A. S., Belo, I., Lopes, M. Waste cooking oils: low-cost substrate for co-production of lipase and microbial lipids. *In* Vilarinho, C., Castro, F., Lopes, M. L. *Wastes – Solutions, treatments and opportunities II*, Taylor & Francis Group, 2017. ISBN: 978-1-138-19669-8.

Poster communications:

1. Lopes, M., Braga, A., Ferreira, P., Pereira, A. S., Guerreiro, C., **Miranda, S. M.**, Belo, I. *Yarrowia lipolytica* as a cell factory to produce valuable compounds. CEB Anual Meeting 2017, July 6, Braga, Portugal, 2017.

The abstract with the results presented in this conference is published in the book of proceedings: Ferreira, E. C. (2017). *Book of Abstracts of CEB Annual Meeting 2017*. Universidade do Minho (Centro de Engenharia Biológica). Braga, Portugal.

Manuscripts in preparation:

1. Co-production of lipase and microbial lipids by *Yarrowia lipolytica* from the low-cost substrate waste cooking oils (research paper).

2. Microbial valorization of waste cooking oils: valuable compounds production (review paper).