Universidade do Minho
Escola de Engenharia

Carlos Elói Nunes Ferreira Guerreiro

Optimization of γ-decalactones production by genetically modified strains of *Yarrowia lipolytica*

november 2016

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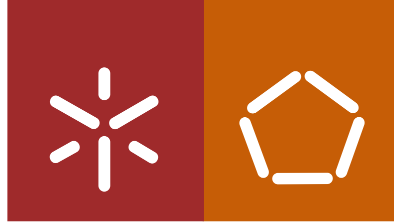

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Carlos Elói Nunes Ferreira Guerreiro

Optimization of γ-decalactones production by genetically modified strains of *Yarrowia lipolytica*

Thesis submitted in fulfilment of the requirements for Master degree in Bioengineering

Work developed under supervision of:
Professor Isabel Maria Pires Belo
Doctor Marlene Alexandra da Silva Lopes

november 2016

“The elevator to success is out of order. You’ll have to use the stairs… one step at a time.”

- Joe Girard

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Findada esta parte crucial do meu percurso académico é importante referir e mostrar o agradecimento àqueles que foram parte integrante e me ajudaram a não perder o ânimo nas alturas mais difíceis.

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RESUMO

No final dos anos 80, os compostos aromáticos produzidos por processos biotecnológicos, tornaram-se muito procurados pelos consumidores. Estes compostos têm uma variedade de aplicações de interesse sensorial (sistema olfativo), como em bebidas, cosméticos e na indústria do papel.

A biotransformação do ácido ricinoleico através de microrganismos, como *Yarrowia lipolytica,* é um processo interessante para produzir compostos aromáticos com cariz "natural". A γ-decalactona é um composto aromático de interesse industrial e possui um aroma cremoso e frutado a pêssego. Este pode ser obtido pela β-oxidação peroxissomal do ácido ricinoleico, usando óleo de rícino como substrato.

A levedura *Y. lipolytica* possui uma família de enzimas, as Acil-CoA oxidases, que são codificadas pelos genes *POX1* até *POX6* e desempenham um papel importante na β-oxidação do ácido ricinoleico. Vários estudos notaram que o gene *POX2* parece degradar os ácidos gordos de cadeia longa (C_{18} to C_{10}) e o *POX3* os ácidos gordos de cadeia curta, sendo que os quatro restantes ainda não têm funções claras.

O objetivo deste trabalho foi estudar o desempenho de estirpes mutantes derivadas da estirpe selvagem W29, MTLY40-2P (sobreexpressa o gene *POX2*) e a JMY3010 (sobreexpressa o gene *LIP2*), sob diferentes condições de operação em bioreatores: concentrações celulares e de óleo de rícino, modo de operação (descontínuo ou descontínuo repetido) e tipo de bioreator (STR ou Air-lift). Além disso, foi estudado pela primeira vez o uso de culturas mistas com ambas as estirpes com o objetivo de melhorar a biotransformação. Concluiu-se que o aumento da densidade celular de 20 g L^{-1} para 40 g L^{-1} não melhorou o processo, mas o aumento de óleo de rícino de 30 g L^{-1} para 60 g L^{-1} teve um efeito positivo na produção de aroma para ambas as estirpes. As culturas mistas em batch (MTLY40-2P e JMY3010) levadas a cabo com 20 g L^{-1} de densidade celular e 60 g L^{-1} de óleo de rícino, obtiveram os melhores resultados para a produção de γ-decalactona (1844 ± 46 mg L^{-1}) e produtividade (80 ± 5 mg L^{-1} h^{-1}). A biotransformação em descontínuo repetido não melhorou o processo, provavelmente por efeitos tóxicos do substrato ou limitação do oxigénio.

A cultura mista descontínua realizada em bioreator air-lift, efetuada com as melhores condições de crescimento celular e óleo de rícino obtidas no STR, conduziu a valores semelhantes de concentração de γ-decalactona, mas o processo foi mais lento.

Por fim, foram realizadas biotransformações descontínuas em frasco de pequena escala e mostraram que a possível acumulação de glicerol no meio, devido à hidrólise do óleo de rícino, tem um pequeno impacto na produção de γ-decalactona e algumas melhorias no processo poderão ser alcançadas através de alterações na composição do meio, tal como o uso de ureia como fonte de azoto.

Palavras-Chave: γ-decalactone, *Y. lipolytica* MTLY40-2P*, Y. lipolytica* JMY3010, Biotransformação, Óleo de Rícino

ABSTRACT

In the final of 80's, aromatic compounds produced by biotechnological processes, have become preferred by consumers, due to natural label. These compounds have a variety of applications of sensorial interest based on the olfactory system, such as in food, drinks, cosmetics and paper industry.

The biotransformation of ricinoleic acid carried out by microorganisms, such as *Yarrowia lipolytica*, is an interesting process to produce aroma compounds with a "natural" label. γ-decalactone is an aromatic compound of industrial interest and possesses a creamy and fruity aroma to peach. It can be obtained from the peroxissomal β-oxidation of ricinoleic acid, using castor oil as natural substrate.

Y. lipolytica possesses a family of enzymes called Acyl-CoA oxidases, Aox, which are encoded by *POX1* to *POX6* genes and play a very important role in β-oxidation of ricinoleic acid. Several studies observed that *POX2* seems to degrade long-chain fatty acids (C_{18} to C_{10}) and *POX3* degrade short-chain fatty acids, and the other four have not so clear functions.

This work aimed to study the performance of mutant strains derived from wild-type W29, MTLY20-2P strain overexpressing *POX2* gene and JMY3010 that overexpresses *LIP2* gene, under different conditions of operation in bioreactors: cellular and castor oil concentration, operation mode (batch or step-wise fed-batch) and bioreactor type STR or air-lift. Moreover, for the first time a co-culture of both strains was used in order to improve biotransformation. It was concluded that cell density increase from 20 g L^{-1} to 40 g L^{-1} did not improve the process, but the increase of castor oil from 30 g L^{-1} to 60 g L^{-1} had positive effects on aroma production for both strains. Batch co-cultures of *Y. lipolytica* mutant strains (MTLY40-2P and JMY3010) obtained with 20 g L^{-1} of total cellular density and 60 g L^{-1} of castor oil concentration led to the best results of γ-decalactone production (1844 ± 46 mg L^{-1}) and productivity (80 ± 5 mg L^{-1} h^{-1}). Step-wise fed-batch biotransformation did not improve the process probably due to toxic effects of castor oil increase or due to oxygen limitation.

Batch co-culture carried out in air-lift bioreactor using the best conditions for cell growth and castor oil biotransformation obtained at STR led to similar values of maximum γ-decalactone concentration, but the process was slower.

Finally, batch biotransformation in small scale flasks were performed and shown that the possible accumulation of glycerol in the medium, due to castor oil hydrolysis, has small impact on γ-decalactone production, and further improvements on the process may be achieved through medium composition changes such as the use of urea as nitrogen source.

KEYWORDS: γ-decalactone, *Y. lipolytica* MTLY40-2P, *Y. lipolytica* JMY3010, Biotransformation, Castor oil

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LIST OF ABBREVIATIONS AND ACRONYMS

AEP	Alkaline extracellular protease
Aox	Acyl-coenzyme A oxidase
ATP	Adenosine triphosphate
C-C bonds	Carbon-Carbon bonds
CoA	Coenzime A
DNS	Dinitrosalicylic acid
FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe
LIP2	Gene of secreted extracellular lipase
NAD^+	oxidized Nicotinamide adenine dinucleotide
NADH	reduced Nicotinamide adenine dinucleotide
OTR	Oxygen transfer rate
***p*-NPB**	*p*-nitrophenyl-butyrate
POX	Acyl-coenzyme A oxidase gene
rpm	Rotations per minute
STR	Stirred Tank Reactor
vvm	volume per volume per minute
XPR2	Gene of alkaline extracellular protease

1 LITERATURE REVIEW

1.1 Aromatic compounds and their biotechnological interest

Aromatic compounds are very sought worldwide for their variety of industrial applications related to their sensorial interest based on the olfactory system, such as in food, drinks, cosmetics and paper industry. The biotechnological production of these compounds is a good alternative to direct extraction from natural products, essentially because it is cheaper to the producers and creates greater incomes. The aroma is a result of a complex mixture of volatile substances from various chemical classes like acids, alcohols, aldehydes, ester, ethers, hydrocarbons, lactones and others. Also, the market that pursuits this kind of products (cosmetic, food and drink industry), represents around 25 % of the whole (Janzantti et al., 2012; Romero-Guido et al., 2011).

Generally, a natural fragrance is defined by natural raw materials which are physically obtained from plants using distillation and extraction. Synthetically reconstituted essential oils, chemically modified natural raw materials (for example by acetylation) and synthetic nature-identical compounds (created by reactive chemistry with same chemical structure of natural aroma) cannot be used in fragrances and labelled as "natural" (Baumann et al., 1988; Surburg & Panten, 2006). A synthetic fragrance may contain natural aroma compounds in combination with synthetic ones, or could be 100% synthetic (Margetts, 2004).

Natural aromas are greener, have greater environmental awareness and value because, when compared to synthetic aroma, they are more sustainable by most of the consumers. They are also safer and more therapeutic and healthful. These consumer perceptions drive to a higher demand for natural aroma. Synthetic aroma creates a bigger repulsive reaction because they can also be produced from petrochemicals, which is not an environmental friendly solution. Synthetic fragrances can last longer and be stronger, are easier to manufacture since their components are more reproducible from lot to lot, but despite of the technical advantages of synthetics, the demand for natural aromatics continues to increase rapidly (Margetts, 2004; Preedy, 2015).

The biotechnological production of aromatic compounds has some advantages compared to the direct extraction from natural products or chemical synthesis, namely higher concentrations of products are obtained and are not dependent of seasonal factors. The greater challenge in this kind of market is to find new viable and economic ways to produce natural aromas or apply them in industry. To be successful

in this area is important to take into consideration an important factor: to obtain high yields with low production costs (Gupta et al., 2013; Soccol et al., 2008).

One important question is the availability of single compounds to formulate aromas and fragrances. Industrially, first compounds were acquired by chemical synthesis in the middle nineteen century, with cinnamic aldehyde, vanillin, methyl salicylate and benzaldehyde. There are about 2000 synthetic compounds available, which show the evolution and work done in this kind of industry (Pometto et al., 2005; Romero-Guido et al., 2011). In the final of 80's, the consumers became more and more interested in natural products. A natural product is the one which comes from an animal, plant or microbial organism using an enzymatic or physical method (Pometto et al., 2005).

1.2 γ-decalactone, an aromatic compound

Lactones are a family of fragrances and flavours that in the past few years gave rise to investigation concerning pathways like β-oxidation (Kunau et al., 1995; Pagot et al., 1998; Waché et al., 2001). These compounds can exhibit oily and fruity properties of peach, coconut and apricot. Among all lactones, the pathway to γ-decalactone production was the first discovered and one of the most produced (Gatfield, 1999; Okui et al., 1963), with market volume of several hundreds of tons per year (Huang & Schwab, 2011). γ-decalactone is commonly used in cosmetics and perfumes and can be easily obtained from hydroxyl fatty acids biotransformation by yeasts (Barth, 2013).

This compound is a cyclic ester and possesses a creamy and fruity aroma with odor to peach which is very strong in concentrations lower than 5 mg L^{-1}. Before the appearance of biotechnological production, this compound was very rare and the costs of production were around 20000 US dollars per kg. With the novel discoveries dropped its price to 1200 US dollars per kg in 1986, 500 US dollars per kg in 1998 and to 300 US dollars per kg, in the last decades (Schrader et al., 2004; Waché et al., 2003).

The interest in biotechnological production of γ-decalactone appeared after the study of Okui in the 60's which consisted in studying the catabolism of hydroxylated fatty acids in numerous organisms (Okui et al., 1963). Industrial processes for γ-decalactone production often use ricinoleic acid as substrate, a fatty acid that constitutes around 90% of castor oil (Puthli et al., 2006).

Castor oil is one of the few commercially available renewable vegetable oil resource that contains one fatty acid in such a high percentage (Hill et al., 1997; Kirk, 1997). The castor oil plant, *Ricinus communis*, is a species of flowering plant in the spurge family, Euphorbiaceae (Scarpa & Guerci, 1982). Seeds of the castor plant are the source of castor oil, which represents approximately 35 % to 55 % of the seeds weight (Akpan et al., 2006). Seeds also contain a toxin called ricin, which is also present in lower concentrations throughout the plant but can be deadly when consumed by humans, since one milligram of ricin is sufficient to kill an adult (Audi et al., 2005; Audi & Belson, 2013). Castor oil is a colorless to very pale yellow liquid with mild or no taste or odor **(Figure 1)**. Its boiling point is 313 °C and its density is approximately 961 kg m^{-3} (Ogunniyi, 2006). Castor oil has many applications namely as bio-based polyol in the polyurethane industry and in food as a mold inhibitor (Wilson et al., 1998).

Figure 1 Extraction of castor oil from *Ricinus communis* plant seeds. Castor oil, a pale yellow colored oil, eco-friendly, non-volatile and non-drying in nature.

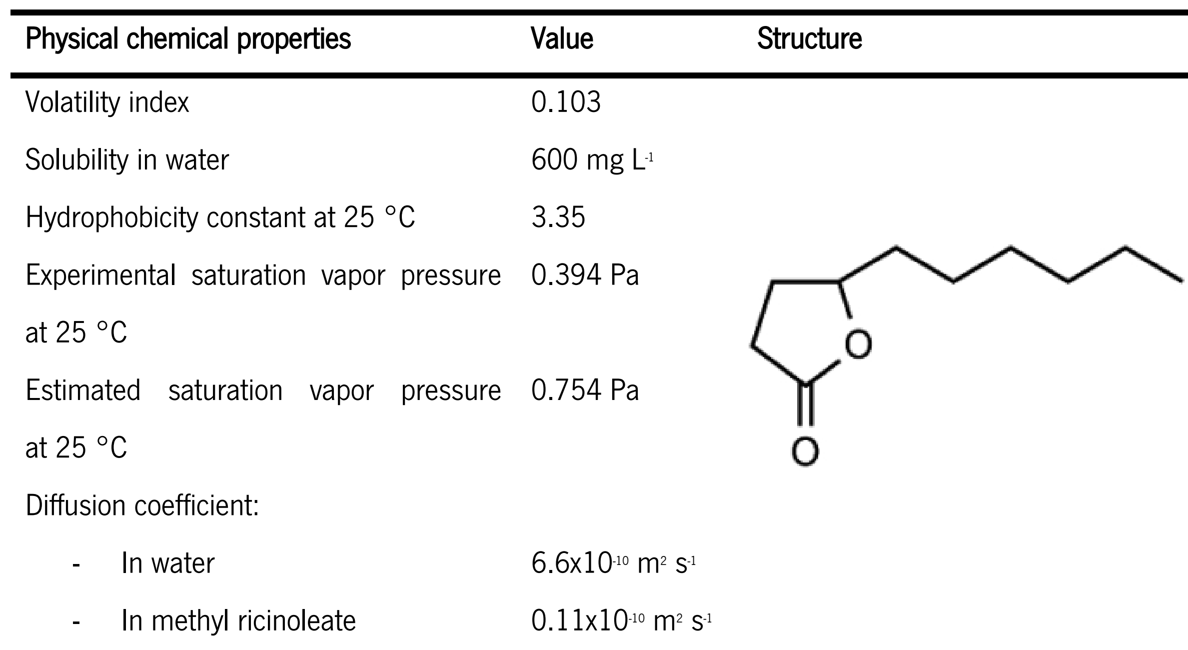
Also, in past century, it was utilized for medicinal purposes as stimulant and lubricating laxative but it can produce fecal incontinence, strong diarrhea, painful cramps and is action can go on for hours, thus this application is not viable nowadays (Siegel & Di Palma, 2005). Ricinoleic acid is the main component of castor oil, and it revealed anti-inflammatory effects (Vieira et al., 2000). More, it is believed that the antibacterial properties of the oil are due to its high ricinoleic acid content (Pereira-Júnior et al., 2007).

Regarding aroma production, γ-decalactone can be obtained using castor oil, a cheap natural raw-material, from the biotransformation of ricinoleic acid. The catalization of the process is accomplished by enzymes present in microorganisms with GRAS status.

The process to obtain γ-decalactone is based in the condensation of the alcohol group –OH and a carboxylic acid group –COOH of the same molecule. This compound has a single endocyclic oxygen atom and a closed ring consisting of four carbon atoms which are coupled with an adjacent ketone. It has a

molecular weight of 170 g mol^{-1} (Aguedo, 2002a). Some physical chemical properties of γ-decalactone are presented in **Table 1**.

Table 1 Physical chemical properties of γ-decalactone (Souchon, 1994).



Physical chemical properties	Value	Structure
Volatility index	0.103	
Solubility in water	600 mg L^{-1}	
Hydrophobicity constant at 25 °C	3.35	
Experimental saturation vapor pressure at 25 °C	0.394 Pa	
Estimated saturation vapor pressure at 25 °C	0.754 Pa	
Diffusion coefficient:		
- In water	6.6×10^{-10} m^2 s^{-1}	
- In methyl ricinoleate	0.11×10^{-10} m^2 s^{-1}	

There are several studies concerning the optimization of γ-decalactone production by a larger number of microorganisms, such as *Pseudomonas*, *Pichia*, *Candida*, *Rhodoturula* and *Sporobolomyces*. However, the most relevant studies are the ones which use the yeast *Yarrowia lipolytica* as the biological machinery, in which concentrations up to 12 g L^{-1} were achieved (Alchihab et al., 2009; Alchihab et al., 2010; Waché et al., 2002).

Table 2 presents a summary of microorganisms described in the literature, capable of producing γ-decalactone and respective aroma concentrations attained.

Table 2 Microorganisms capable to produce γ-decalactone and aroma concentration obtained.

Microorganism		[γ-decalactone] (mg L^{-1})	Reference
Bacteria	*Acetobacter, Bacillus, Pseudomonas, Xanthomonas*	c.a 6600	(Gocho et al., 1998)
Filamentous fungi	*Mucor sp.*	10500	(Kumin & Munch, 1998)
	Trichoderma harzianum	260	(Serrano-Carreon et al., 1997)
Yeast	*Candida albicans, C. krusei, C. parakrusei, C. guilliermondii, C. pseudotropicalis, C.rugosa, C.tropicalis, C. stellatoidea*	200 to 860	(Farbood et al., 1990; Farbood & Willis, 1983)
	Hansenula saturnus	200 to 860	(Farbood & Willis, 1983; Meyer, 1993)
	Pichia guiliermondi	100 to 500	(Endrizzi et al., 1993; Iacazio et al., 2002)
	Sporidiobolus odorus	80 to 200	(Cheetham et al., 1993; Lee & Chou, 1994; Lee et al., 1995)
	Sporidiobolus johnsonii, Sp. ruinenii, Sp. salmonicolor, Sp. pararoseus	12 to 5500	(Dufossé et al., 1998; Feron et al., 1996a; Lee et al., 1998; Lee et al. 1999)
	Rhodotorula aurantiaca	4500 to 6500	(Alchihab et al., 2009; Alchihab et al., 2010)
	Yarrowia lipolytica	200 to 12000	(Aguedo, 2002a; Ambid et al,, 1999; Braga et al., 2015b; Braga et al., 2015c; Farbood & Willis, 1983; Farbood & Willis, 1985; Escamilla-García et al., 2009; Gomes et al., 2007; Gomes et al., 2010; Gomes et al., 2012; Gomes et al., 2013; Groguenin et al., 2004; Meyer, 1993; Pagot et al., 1998; Rabenhorst & Gatfield, 2001; Reis et al., 2006; Waché et al., 1998; Waché et al., 2000a; Waché et al., 2000b; Wang et al., 1999)

Y. lipolytica has demonstrated a high capability to degrade various lipids and proteins but also revealed its importance in other fields such as lactone production (Aguedo et al., 2005a; Gomes et al., 2012; Moradi et al., 2013; Pagot et al., 1998; Waché et al., 2001), biosynthesis and degradation of peroxisomes (Gunkel et al., 1999), secretion of proteins and metabolites (Beckerich et al., 1998; Madzak, et al., 2004) and dimorphism (Chang et al., 2007; Titorenko et al., 2000).

The fact that *Y. lipolyica* is considered non-pathogenic and it has GRAS statute, is a major motivation to choose this yeast for the development of a process capable to produce γ-decalactone (Nicaud, 2012). Being considered as GRAS, also all the products obtained from this yeast will have the same label, therefore, are safe for human.

1.3 *Yarrowia lipolytica*

In the past few years the use of microorganisms and enzymes for the production of natural flavor compounds became a natural way to obtain nontoxic products with interest to industry, as an alternative to the traditional ways.

Y. lipolytica is one of the most studied species in biotechnology and in industry (Barth & Gaillardin, 1997) and it has the ability to use a wide range of carbon sources, including hydrophobic substrates such as alkanes and triglycerides, also has a strong secretory activity of several metabolites of industrial interest and a high resistance to inhibitory agents. Therefore, the large scale production of this biocatalyst can be achieved through the utilization of a low cost diversity of substrates, like agro-industrial sub-products or residues, contributing to their valorization and minimize the environmental impact created.

The ascomycetous yeast *Y. lipolytica*, is a strictly aerobic microorganism, non-pathogenic and an eukaryotic belonging to the Fungi kingdom (Holzschu et al., 1979). It was first classified as *Endomycopsis lipolytica* (Wickerham et al., 1970), then as *Saccharomycopsis lipolytica* and finally as *Yarrowia lipolytica* (Yarrow, 1972). This yeast was named *lipolytica* because of its high ability to hydrolyze lipids and it can be easily isolated from several habitats such as soil and manure, as well from food rich in protein and fat like meats, shrimps, milk, cheese, yogurt and olive oil (Barth & Gaillardin, 1997). Moreover, *Y. lipolytica* strains have been isolated from lipid-rich media (sewage, oil polluted media) or marine and hypersaline environments (Mafakher et al., 2010; Smyth et al., 2010; Thevenieau et al., 2009). It has and inability to survive in anaerobic conditions, which facilitates its elimination from dairy products (Barth & Gaillardin, 1997; McKay, 1992).

Y. lipolytica is a dimorphic yeast, which means it has the ability to grow in two distinct morphological forms, single oval cells or filamentous hyphae, and can switch between these forms, depending on environmental and growth conditions like aeration, carbon and nitrogen source, pH, as well on the genetic background of the strain (Kawasse et al., 2003; Sugiyama, 2001; Szabo, 2001; van der Walt & von Arx, 1980). True mycelium consists of septate hyphae with 3 μm to 5 μm in width and up to several milimeters in length. Apical cells can exceed 100 μm, and segments are 50 μm to 70 μm long. There is a single

nucleous per segment and they show an ascomycete-type central pore, unusual for other filamentous yeasts, with endoplasmic reticulum extending through it from one segment to the next (Kreger-van Rij & Veenhuis, 1973).

This yeast exhibits multiple colony shapes ranging from smooth and glistening to heavily convoluted and opaque. Most strains are unable to grow above 32 °C and its susceptibility to genetic manipulation and efficient transformation systems make this host an appropriate model for the study in dimorphism in yeasts (Cruz et al., 2000; Holzschu et al., 1979; Szabo, 2001).

This species revealed itself a good producer of a great number of important metabolites, like citric and isocitric acids and other organic acids (Finogenova et al., 2002). Since 1960, *Y. lipolytica* started to be known because of its ability to use hydrophobic substrates such as *n*-alkanes, oils, fats and fatty acids. Its properties in secretion of a large quantity of organic acids, intracellular accumulation of oil, production of dicarboxylic and hydroxyl acids, and most importantly the ease of protease and lipase secretion leaded it to the attention has a novel producer of fine chemicals and basic commodities (Barth, 2013). This attention leads to the development of molecular biology and genetic tools that improves the robustness of this organism and has guaranteed a spot as a profitable industrial organism. Some carbon sources such as oils (castor oil), alkanes (decane and hexadecane), fatty acids (ricinoleic acid, plamitic acid, lauric acid) and fats (Fickers et al., 2005), glucose, mannitol and galactose (Bankar et al., 2009), aminoacids (methionine), organic acids (citrate and lactate), glycerol (Papanikolaou & Aggelis, 2003) and ethanol (Barth & Gaillardin, 1997) are susceptible to be used by *Yarrowia lipolytica*. In lipid-rich media this species secretes an extracellular lipase encoded by *LIP2* gene (Pignède et al., 2000).

One of the advantages of using *Yarrowia lipolytica* is because of its metabolic machinery. This yeast is a current model in terms of studies that focus in catabolic processes like peptide degradation or peroxisomal oxidation of fatty acids. This last metabolic process is very well known and it's called β-oxidation (Nelson & Cox, 2008).

Due to the great sensorial interest in aroma compounds, namely γ-decalactone, several efforts have been made in with the aim of optimizing γ-decalactone production. Therefore, some modified strains of *Y. lipolytica* have been utilized in order to canalize the metabolism of carbon sources like castor oil or more specifically its derivate, ricinoleic acid, to aroma production. In the past few years this aim has been accomplished through gene deletions and/or increase of copy numbers that play key functions in the degradation of fatty acids in β-oxidation, such as *POX* gene family. There are three quite known strains, or inbred lines, designed by different groups that can be used in this type of investigation, the German (H222), the French (W29) and the American (CBS6124-2) (Beopoulos et al., 2009).

1.4 γ-decalactone production through β-oxidation

The conversion of fatty acids to acetyl-CoA by a process known as β-oxidation is a central pathway in many organisms. The electrons removed from fatty acids by this process pass through the respiratory chain, resulting in ATP synthesis, and molecules of acetyl-CoA produced from fatty acids may be completely oxidized to CO_2 in citric acid cycle, resulting in further energy conservation. The target of acetyl-CoA is different depending on the organism but the mechanism of oxidation is essentially a repetitive four-step process (Nelson & Cox, 2008). This four-step process consists basically in two steps of oxidation, one hydration and one cleavage reaction which are catalyzed by three enzymes. At each cycle, the compound gives rise to a two-carbon shorter metabolite and an acetyl. This pathway depends on the pools of acyl-CoA and free CoA, the presence of NAD and the presence of molecular oxygen, because the first step in peroxisomal oxidation is catalyzed by an oxidase. The β-oxidation cycle is theoretically repeated until the complete degradation of the substrate.

Over the centuries, naturally organisms have evolved to adapt and optimize their own processes of obtaining energy trough many ways. Scientists have also played a part in evolution using techniques of metabolic engineering, in which they alter metabolic pathways to optimize the obtaining of certain products of interest as they like, many of them destined to industry. This is not different with β-oxidation cycle, in fact, there are many published works (Aguedo et al., 2005a; Bankar et al., 2009; Beopoulos et al., 2009; Fickers et al., 2011; Nicaud, 2012) where, yeasts like *Y. lipolytica*, are genetically modified in this specific pathway to obtain aroma compounds, like the already described γ-decalactone (Feron et al., 1996b; Pagot et al., 1997; Pagot et al., 1998; Waché et al., 2000b; Waché et al., 2001).

In the yeast *Y. lipolytica*, β-oxidation can be used to obtain aromatic compounds, with sensorial interest, namely decalactones, decenolides and dodecalactones from ricinoleic acid **(Figure 2)**. The biotransformation of ricinoleic acid in γ-decalactone requires the lactonisation at C_{10} level (when hydroxyl group is in γ-position).

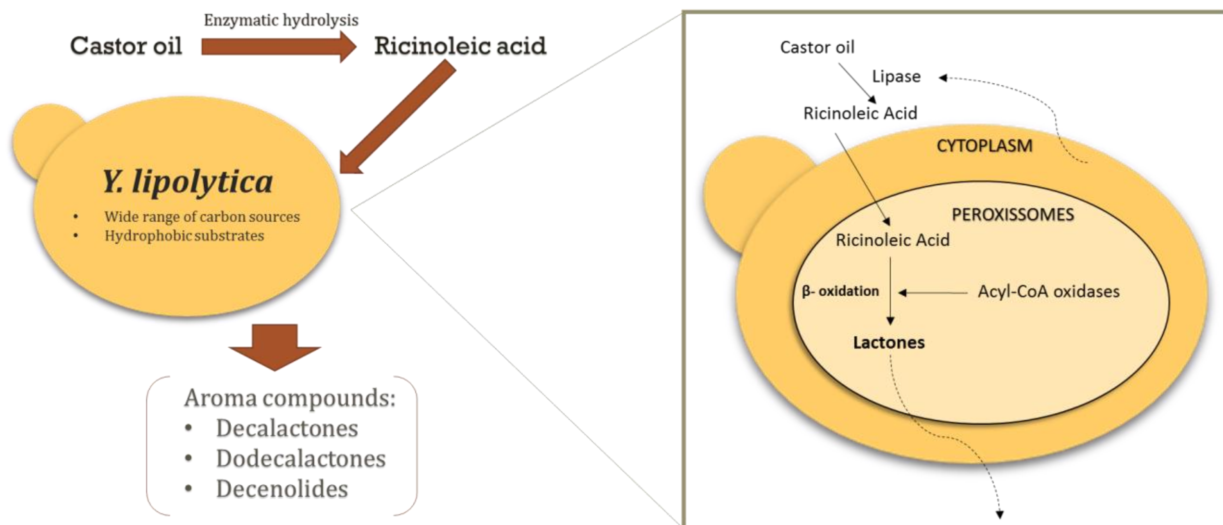


Figure 2 Representation of castor oil conversion into aroma compounds using *Yarrowia lipolytica* as biocatalyst. Using castor oil as a natural carbon source it is possible to obtain various aromas such as decalactone, dodecalactones and decenolides, taking advantage of the peroxissomal activity.

In this species, at least four lactones are accumulated during the catabolism of ricinoleic acid, through peroxisomal β-oxidation. γ-decalactone, 3-hidroxi-γ-decalactone (a functionalized lactone with no sensorial interest) and its dehydration products which are of great sensorial interest, dec-3-en-4-olide and dec-2-en-4-olide (Escamilla-García et al., 2007). After concluded the principal path of β-oxidation cycle, lactones keep accumulating in two metabolic crossed steps, which take place right before the two oxidation steps **(Figure 3)**. The accumulation of dec-3-en-4-olide and dec-2-en-4-olide was associated to a low activity of a 3-hidroxyacyl-CoA dehydrogenase (Escamilla-García et al., 2007). This activity depends on the NAD^+/NADH ratio, because NAD^+ is a co-factor for this enzyme.

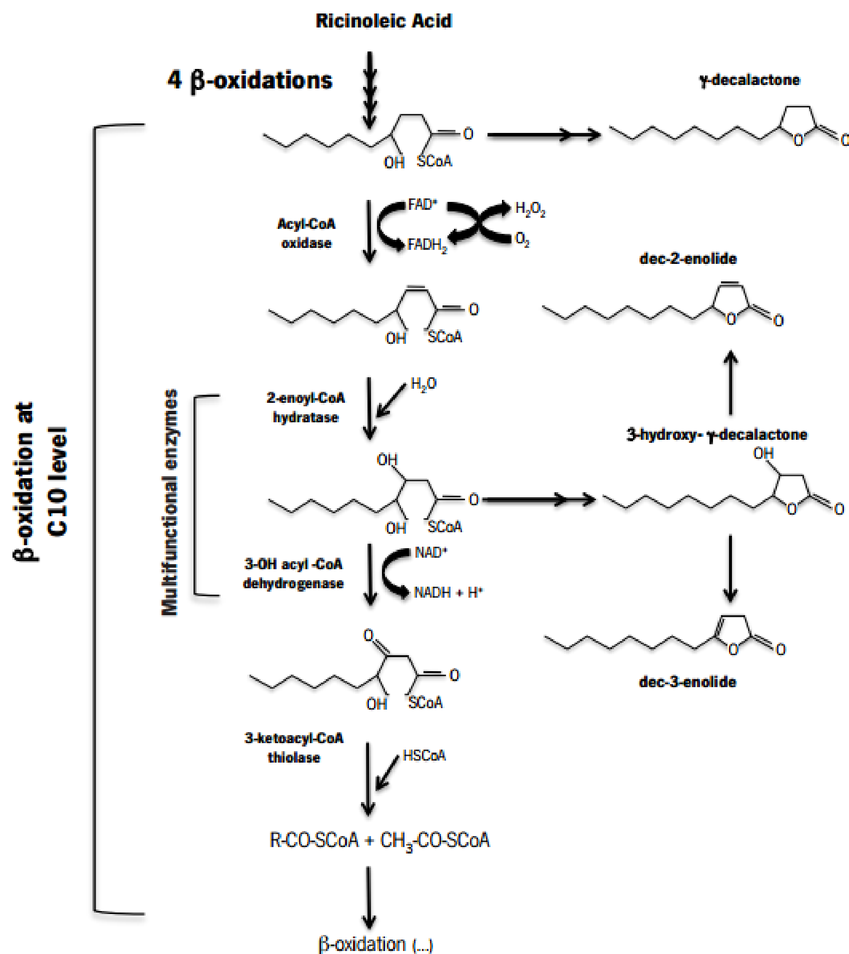


Figure 3 Accumulation of lactones from the β-oxidation cycle, using ricinoleic acid as precursor. Adapted from (Blin-Perrin et al., 2000)

As some studies have already shown, this mechanism is also dependent on the mitochondrial respiration (Waché et al., 2001; Waché et al., 2002). As shown in **Figure 3**, depending on the stage or the cycle that the process is occurring different enzymes will act. 4-hidroxy-decanoyl-CoA is the γ-decalactone precursor and it is degraded by an acyl-CoA oxidase. The other three principal lactones (3-hidroxy-γ-decalactone and decenolides) have the same precursor, 3,4-dihidroxydecanoyl-CoA, which is degraded by a 3-hidroxiacyl-CoA dehydrogenase. The yeast *Y. lipolytica* possesses a family of six acyl-CoA oxidases, Aox1-6, which are respectively encoded by *POX1* to *POX6* genes. Generally, the first enzyme of the whole pathway is considered as the limiting step of the catalysis and the role of each acyl-CoA oxidases started be understood with the mutations in *POX* genes (Groguenin et al., 2004; Waché et al., 2000b; Waché et al., 2001; Waché et al., 2002). The deletion or disruption of *POX1* gene resulted in an increased β-oxidation activity, but inconveniently, this decreases the production of γ-decalactone (Pagot et al., 1998). The most relevant discoveries concerned chain specificity and activity, being Aox2 long-chain specific (Luo et al., 2002) and Aox3 short-chain specific (Luo et al., 2000). Aox1 did not exhibit any

detectable activity and Aox4, Aox5 and Aox6 have shown weak activity in the whole spectrum of straight-chain acyl-CoA, from C_{18} to C_4 (Groguenin et al., 2004) **(Figure 4)**. As consequence, in some *POX* mutants, β-oxidation of C_{10} and smaller acyl-CoA lead to a lower yield, derivate to γ-decalactone consumption (Groguenin et al., 2004; Waché et al., 2002).

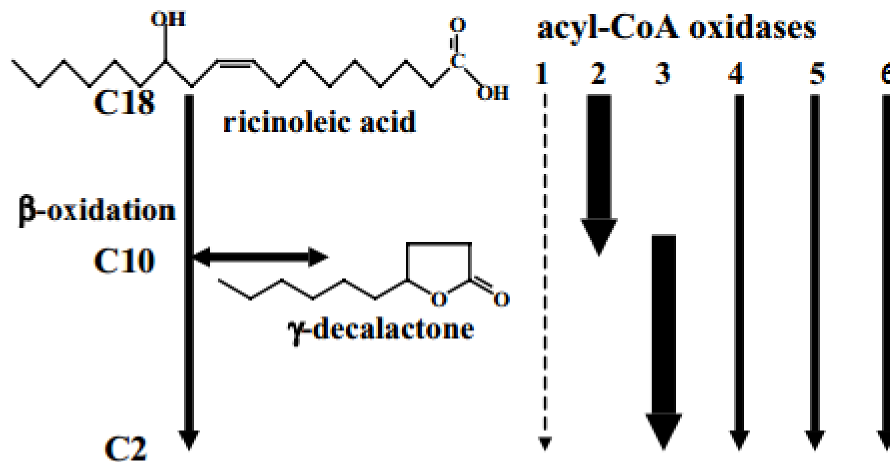


Figure 4 Representation of the ricinoleic acid conversion into γ-decalactone and activities of the acyl-CoA oxidase family in Yarrowia lipolytica. Here is shown that the acyl-CoA oxidades with bigger impact are Aox 2 and 3, followed by 4,5 and 6 and in last Aox 1, which has almost no impact. Adapted from (Groguenin et al., 2004).

Y. lipolytica possesses a family of six acyl-CoA oxidases which are the responsible enzymes for the catalysis of the first reaction of β-oxidation cycle. Therefore, is important to understand the role of every Aox in order to build a strain that won't affect cellular growth and still has the ability to produce γ-decalactone without degrading it.

Developments have been made for lactone production using *Y. lipolytica* wild-type strains W29. As an attempt to increase γ-decalactone concentration and yields, serial knockouts for each enzymes, Aox1 to Aox6, were made (Waché et al., 2000b; Waché et al., 2001; Waché et al., 2002). Studies aiming to understand the involvement of these enzymes in the biotransformation of γ-decalactone by *Y. lipolytica* and build strains that have deletions in one or several *POX* genes have been developed. It was observed that the strain disrupted for *POX2*, *POX3* and *POX5* genes (which still possess *POX4*, one of the weakly active Aox) and with *POX2* reincorporated in a multicopy plasmid, produced more γ-decalactone and that was not consumed further (Waché et al., 2001; Waché et al., 2002).

Groguenin and collaborators also used *Y. lipolytica* W29 as wild-type, and observed that the deletion of *POX1* gene did not seem to modify the enzymatic activity as if Aox1 was inactive towards straight-

chained fatty acids or acyl-CoA (Groguenin et al., 2004). Moreover, the deletion of *POX5* gene decreased activity on the entire chain-length spectrum and the deletion of *POX4* gene exhibited no significant effect. Several strains were built with the goal of investigating the effect of Aox4 and Aox5 on the γ-decalactone degradation, and construct a strain unable to degrade γ-decalactone produced and still grow with the wild-type rate **(Table 3)**. Authors constructed a mutant, named MTLY40-2P (from parental strain *Y. lipolytica* W29), which was disrupted in *POX2*, *POX3*, *POX4* and *POX5* genes and with a *POX2* single copy added to restore is character. This mutant was able to grow almost at the same rate as wild-type and was capable to produce about 10 times more γ-decalactone in only 48 h, maintaining approximately the amount during 250 h.

Table 3 POX genotype and growth characteristics of Yarrowia lipolytica strains. The growth media in wild-type and in the other mutants has the same carbon source (methyl ricinoleate). (Groguenin et al., 2004)

Strains	Genotype	Aox expressed	Growth
W29	Wild type	None	Normal
MTLY36	Δ*pox2,pox3,pox5*	None	Reduced
MTLY37	Δ*pox2,pox3,pox4,pox5*	None	No growth
MTLY40	Δ*pox2,pox3,pox4,pox5*	None	No growth
MTLY36-2P	Δ*pox3,pox5*	Aox2p	Normal
MTLY40-2P	Δ*pox3,pox4,pox5*	Aox2p	Normal

1.5 Biotransformation in biphasic media

In addition to the advances in the construction of modified strains which allowed to achieve higher concentration of γ-decalactone in the culture medium, the biotransformation of castor oil or ricinoleic acid with *Y. lipolytica* has gained attention from researchers in many different features.

Numerous studies were published aiming the toxic effect of these substrates and the lactone on the producer organism, production of aroma at large-scale level and improvement in the process productivity. In these studies, castor oil was the consensual substrate utilized due to its large availability compared to the other sources and lower price.

The γ-decalactone production is possible using its main precursor, ricinoleic acid. In some cases hydrophobic substrates such as castor oil or other esters (Page & Eilerman, 1996), as methyl ricinoleate, are also used as substrates.

Castor oil is a natural oil obtained from seeds of castor plant and chemically it is a triglyceride of fatty acids. From the triglycerides present in castor oil, glycerol is esterified at a level of approximately 90% by ricinoleic acid, an hydroxylated, monounsaturated fatty acid with 18 carbons having a double bond at position C_9-C_{10} and a hydroxyl group at C_{12} (Kirk, 1979). This characteristic combined with unsaturation occurs in castor oil and it is responsible for this oil and ricinoleic acid to be unusually polar, also allowing chemical derivatization that is not practical with other biological oils. The other most relevant fatty acids in castor oil are eicosanoic acid (0.3%), linolenic acid (0.3%), dihydroxystearic acid (0.7%), plamitic acid (0.1%), stearic acid (1%), oleic acid (2%) and linoleic acid (4%) (Naughton, 2000; Omari et al,, 2015). This substrate has the peculiarity of being practically insoluble in water and therefore forms a second phase. To facilitate contact between the yeast present in the aqueous phase and the substrate, an emulsion is formed by applying a surfactant.

Emulsifying agents or surfactants play an important role in various industrial sectors, being one of the most important class of chemical compounds (Nitschke & Pastore, 2002) and can be produced by a synthetic or biological approach (Amaral et al., 2006).

Surfactants are amphipathic molecules consisting of a non-polar hydrophobic group and a polar hydrophilic group. The non-polar group is often a hydrocarbon chain, while the polar group can be either cationic or anionic, non-ionic or amphoteric (Nitschke & Pastore, 2002). They tend to be distributed at the interface between liquid phases with different degrees of polarity (water-in-oil and oil-in-water). The formation of a molecular film, placed at the interface, reduces the interfacial and surface tensions, being responsible for the unique properties of surfactants (Nitschke & Pastore, 2002).

Several surfactants like SDS (anionic character), Saponin (neutral character), CTAB (cationic character), Tween 80 and Triton X-100, were tested in the bioprocess of γ-decalactone production (Aguedo, 2002a). In this study the objective was to determine the effects of the various surfactants in viability of yeast cells, emulsion, hydrophobicity of yeasts surface, membrane interaction and in biotransformations. They concluded that Tween 80, a non-ionic surfactant derived from olyethoxylated sorbitan and oleic acid, was the ideal surfactant. This group observed that this compound permitted to obtain greater γ-decalactone concentrations. Furthermore, among all compounds tested, this emulsifier didn't affect cell viability and had no interaction with their membranes, promoting a greater interfacial surface to the medium and the largest relative surface hydrophobicity to the cells. This way, the interfacial

area between the organic and aqueous phases becomes important in favoring the access of substrate to the cells. The lipid phase is dispersed as droplets in the aqueous phase (oil-in-water emulsion), providing a large interface between lipids and yeasts.

Y. lipolytica cells are hydrophilic with a good attraction to hydrophobic surfaces or molecules, when previously immersed in water. Waché *et al.* (2000a) observed that the transfer between lipid phase and microorganisms does not depend on the size of the fat globules but on the size of the cells (Waché et al., 2000a).

The growth rate depends on the ratio of the droplets size and cells size. When both are about the same size the growth rate is minimal, and maximal when cells are smaller or larger than the droplets. During the biotransformation of castor oil by *Y. lipolytica*, there is direct contact between the surface of the cells and the small substrate droplets and it is possible to increase this interaction (mediated by proteins or glycoproteins of cell wall (Amaral et al., 2006)) by choosing an appropriate surfactant with affinity for the yeast (cationic surfactant) (Aguedo et al., 2004). Also, the cell-surface adhesion can be improved by intentionally modify the cell surface properties. The surface charges may be altered by modifying the medium pH or ionic force. Pre-culturing *Y. lipolytica* on a hydrophobic carbon source instead a classical glucose medium increases the subsequent adsorption of droplets on the cell surface (Aguedo et al., 2003b). The addition of a charged surfactant in the medium enables to obtain lipid droplets with a controlled charge and a consequent optimized adhesion (Ravaine et al., 2002).

For adhesion to be possible, the cell surface must present an affinity for the organic phase having, for example, globally or partially a hydrophobic character, or the medium must contain emulsifiers which will be located at the oil/water interface, as it is the case of the emulsifier Tween 80. Thus, the role of emulsifying agents is very important for an optimal degradation of lipids. The hydrophobicity of the cell surface is considered one of the most important factors in the assimilation of hydrophobic compounds (Kim et al., 2000). In case of diffusion, several mechanisms may be involved including Lewis acid-base, electrostatic interactions, Van der Waals and hydrophobic (Aguedo et al., 2003b).

In γ-decalactone production, high concentrations of aroma compounds reached during the biotransformation may lead to toxic effects towards the producing microorganism (Dufossé et al., 1999). Yeast cells are able to degrade γ-decalactone, however, it becomes toxic at a certain concentration level. Studies concerning the mechanisms leading to lactone toxicity showed that the hydrophobic lactone could take part in the cell membranes and increase its fluidity. Therefore, γ-decalactone concentrations higher than 150 mg L^{-1} lead to a dissipation of cell membrane potential (Aguedo et al., 2002b; Aguedo et al.,

2003b). Retention of aroma compounds within cell membranes and a consequent toxic effect, thus needs to be considered for those compounds with hydrophobic properties (Aguedo et al., 2002b).

1.6 Bioreactors employed for γ-decalactone production

During biotransformation of castor oil in γ-decalactone by *Y. lipolytica*, an emulsion is formed where the fat droplets and cells are suspended. It is, therefore, necessary to disperse the cells evenly by the medium, with air and/or agitation. Some bioreactors configurations have been used for lactone production as an attempt to prevent the problems with medium emulsification.

There are no studies that make strictly use of normal or baffled Erlenmeyer flasks to produce γ-decalactone by *Y. lipolytica*. The main reason is because these systems have scale limitations and do not allow to control the aeration or agitation separately. Nevertheless, when using baffled Erlenmeyer flasks it is possible to improve process performance since its design increases the turbulence of the culture medium and improves the efficiency of oxygen transfer (Gatfield, 1988; Okui et al., 1963; Serrano-Carreón et al., 1997).

In the literature, most of the studies that use Erlenmeyer flasks are mainly about aroma production taking advantage from agro-industrial wastes. Some groups achieved fruity aroma production from waste-based substrates like coffee husk supplemented with glucose, wheat bran, cassava bagasse and sugarcane in 250 mL Erlenmeyer flasks, using as biocatalyst a microorganism called *Ceratocystis fimbriata* (Christen et al., 1997; Rossi et al., 2009; Soares et al., 2000). Also, a group produced aroma from citric pulp and soya bran using *Kluyveromyces marxianus* as biocatalyst in 250 mL Erlenmeyer flasks (Medeiros et al., 2000).

Stirred tank bioreactors (STR) are the most common bioreactor configuration used for bioprocesses development. Normally, the air is supplied at the bottom of the tank with a Rushton turbine immediately above to reduce the bubbles size and increase the oxygen transfer rate from gaseous phase to liquid phase. With this kind of systems, it is possible to control agitation and aeration rates to obtain a desired dissolved oxygen in the medium. Stirred tank bioreactors are widely used for γ-decalactone production. Feron and collaborators used a 7 L stirred bioreactor to produce γ-decalactone (1.6 g L^{-1}) from methyl ricinoleate (41 g L^{-1}) by *Sporidiobolus ruinenii* and *Sporidiobolus salmonicolor* (Feron et al., 1996b). Braga *et al.* (2015) reached a maximum concentration of 1.5 g L^{-1} of γ-decalactone by biotransformation of castor oil (30 g L^{-1}) by *Y. lipolytica* W29 in a 3.7 L STR (Braga et al., 2015c). Most of the works regarding γ-decalactone production are performed in batch mode using STR bioreactors, although the studies

carried out by Gomes *et al.* (2012) and Braga *et al.* (2015b), make use of a STR bioreactor, operating in fed-batch mode.

Despite STR bioreactors being the most common bioreactors used for aerobic fermentations, some disadvantages are recognized to this type of design, namely high agitation rates are needed to achieve the high values of oxygen mass transfer, which can cause damage to the cells. Moreover, the mechanical energy input is high, leading to greater costs and requires special attention to overheat problems. Due to their complexity, they are more expensive and less robust than several other types of bioreactors (Chisti, 1989a).

Due to the design disadvantages shown by stirred tank reactors, efforts have been made to investigate other approaches (Chisti & Moo-Young, 1989b). Air-lift bioreactors are starting to gain reputation and because of being pneumatically agitated they are often employed in bioprocesses where gas-liquid transference is important. In this bioreactor, usually a sparger is placed at the bottom and the ascending of gas bubbles causes mixing, which facilitates mass transfer process. The liquid recirculation is possible because of the riser, downcomer, gas separator and bottom. This type of bioreactors overcome the problem energy consumption because of the simplicity of its construction with no moving parts. Also, they have other features like uniform distribution of shear (Chisti & Moo-Young, 1989b; Merchuk et al., 1994).

Air-lift type bioreactors have some limitations also, such as its application for high viscosity systems. Nevertheless this is an unexplored area and there are still few studies concerning lactone production into air-lift bioreactors (Braga et al., 2015c; Escamilla-García et al., 2014; Gomes, 2011).

2 General Methodology

2.1 Microorganisms, Media and Culture Conditions

Yeast species used during this work are listed in **Table 4**.

Table 4 *Yarrowia lipolytica* strains used in the present work.

Strain	Genotype	Reference
MTLY40-2P	Deletions in *POX3*, *POX4* and *POX5*; Overexpression in pPOX2-*POX2*	Groguenin et al. (2004)
JMY3010	Additional copy of *LIP2*, pTEF-*LIP2*	Supplied by Prof. Nicaud, MICALIS (INRA-AgroParisTech)

Y. lipolytica strains were stored at -80 °C in cryogenic tubes (Microbank, Pro-Lab Diagnostics, Canada). After thawing, each strain was cultured for 24 h on YPDA medium (30 g L^{-1} agar, 20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract) at 27 °C and stored at 4 °C, up to a maximum of two weeks.

2.2 Bioreactor experiments

2.2.1 Stirred Tank Reactor (STR)

Unless otherwise noted along the dissertation, the cell colonies previously prepared (section 2.1) were used to inoculate (cell density of 0.5 g L^{-1}) a 500 mL Erlenmeyer flask containing 200 mL of YDP medium (20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract). The medium was sterilized in an autoclave, at 121 °C during 20 minutes.

Flasks incubation was carried out at 140 rpm and 27 °C for 19 h until the total consumption of glucose, equivalent to cell cultures reaching the late logarithmic phase, with a final optical density at 600 nm (OD_{600}) of 5 (corresponding to cell density of 5.1 g L^{-1}). This subculture was used to inoculate 1.7 L of YPD medium in bioreactor.

Cellular growth was carried at 27 °C, 500 rpm and 3 L min^{-1} of aeration rate for 24 h until reaches the stationary phase of growth.

When the exponential growth phase finished, biotransformation phase was started. Biotransformation phase was carried out at the same conditions of growth phase except the aeration rate (5 L min^{-1}, corresponding to 2.9 vvm) and controlled pH (6.0 ± 0.5). The components of biotransformation medium were directly added, as an emulsion, to YPD medium containing the cells, in order to start the biotransformation.

According to Gomes et al., 2010 and Gomes et al., 2012, a medium composition of 30 g L^{-1} of castor oil and 3 g L^{-1} of Tween 80 was adopted to use in the experiments. In some experiments different oil concentrations were used and therefore the Tween 80 concentration were modified, but the ratio between the two compounds was kept constant.

The base biotransformation medium composition is presented on **Table 5**.

Table 5 Biotransformation medium composition.

Compound	Concentration (g L^{-1})
Castor oil	30 or 60
Tween 80	3 or 6
NH_4Cl	2.5
Yeast Nitrogen Base (YNB) with amino acids	6.7

After establishing the optimal batch operating conditions for γ-decalactone production, we aimed to perform a step-wise strategy using a co-culture, based on one addition of substrate.

A STR bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) working with a maximum volume of 3.7 L, with 31 cm height and 17 cm diameter was used as a STR bioreactor. In this bioreactor, besides temperature and pH, also oxygen dissolved concentration can be controlled **(Figure 5)**.

The desired air supply was delivered to the vessel with a sparger located at the base of the agitator, with an automated flow-rate. Medium pH was kept constant by addition of 21 % (v/v) orthophosphoric acid or potassium hydroxide (2 M), through Peripex W1 peristaltic pumps (Bioengineering, Switzerland) and pH probe (405-DPAS-SC-K8s/325, Mettler Toledo, USA). The measurement of dissolved oxygen concentration was performed with a polarographic-membrane probe (InPro 6000, Mettler Toledo, USA) using the BioScadaLab software.

Figure 5 RALF PLUS SOLO bioreactor working with biotransformation medium.

2.2.2 Air-Lift

Unless otherwise noted along the dissertation, the cell colonies previously prepared (section 2.1) were used to inoculate (cell density of 0.5 g L^{-1}) a 500 mL Erlenmeyer flask containing 200 mL of YDP medium (20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract). The medium was sterilized in an autoclave, at 121 °C during 20 minutes.

The flasks incubation was carried out at 140 rpm and 27 °C for 19 h until the total consumption of glucose, equivalent to cell cultures reaching the late logarithmic phase, with a final optical density at 600

nm (OD_{600}) of 5 (corresponding to cell density of 5.1 g L^{-1}). This subculture was used to inoculate 4.5 L of YPD medium in air-lift bioreactor.

Cellular growth occurred at 27 °C, 7 L min^{-1} (1.6 vvm) of aeration rate for 24 h until a final cell density of 20 g L^{-1}.

When the exponential growth phase finished, biotransformation phase was started. Biotransformation phase was carried out at the same conditions of growth phase but with an aeration rate of 7 L min^{-1} and a controlled pH varying from 5.5 to 6.5. The components of biotransformation medium were directly added, as an emulsion, to the YPD medium containing the cells, in order to start the biotransformation. The biotransformation components were the same as described in **Table 5**, using 60 g L^{-1} of castor oil as substrate.

Air-lift bioreactor is made in glass and has a working volume of 4.5 L. The reactor has an inside diameter of 7 cm and a riser-tube of 37 cm height with an inside diameter of 3.2 cm **(Figure 6)**. Air was used as gas stream in the gas-liquid contactor and it was fed at the bottom of the bioreactor using a five holes sparger. The inlet gas flow-rate was measured and controlled with a mass flow controller (Alicat Scientific, USA). The dissolved oxygen concentration was measured by a polarographic-membrane probe (12/220 T-type, Metler Toledo, USA) connected to a O_2 amplifier (type 170 ppm, Ingold, USA).

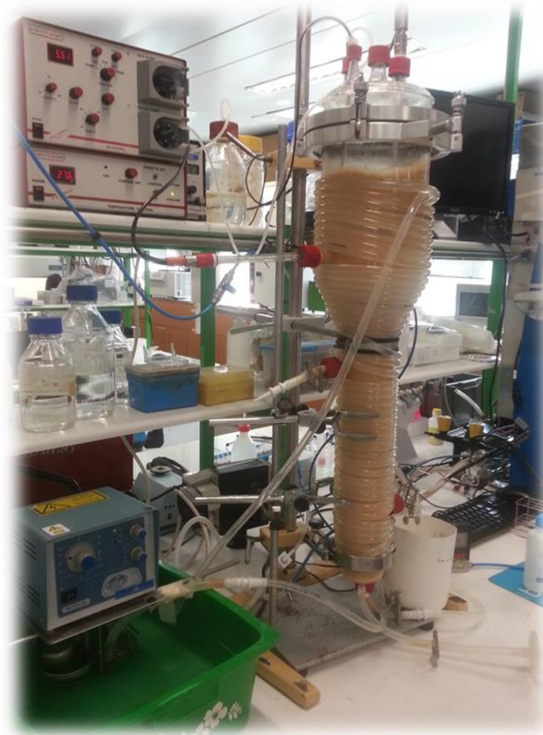


Figure 6 Air-lift bioreactor working with biotransformation medium.

2.3 Erlenmeyer flask experiments

Unless otherwise noted along the dissertation, the cell colonies previously prepared (section 2.1) were used to inoculate (cell density of 0.5 g L^{-1}) a 500 mL baffled Erlenmeyer flask containing 200 mL of YDP medium (20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract). The medium was sterilized in an autoclave, at 121 °C during 20 minutes.

The flasks incubation was carried out at 140 rpm and 27 °C for 19 h until the total consumption of glucose, equivalent to cell cultures reaching the late logarithmic phase, with a final optical density at 600 nm (OD_{600}) of 5 (corresponding to cell density of 5.1 g L^{-1}). That subculture was then used to inoculate the biotransformation medium.

After growth phase, biotransformations components were directly added, as an emulsion, in order to start the biotransformation. As described in **Table 5**, all biotransformation components were added according to an initial castor oil concentration of 30 g L^{-1}.

Two different experiments were performed using erlenmeyer flasks. Firstly, two different conditions were tested in order to simulate total hydrolysis of 30 g L^{-1} and 60 g L^{-1} of castor oil, adding respectively 3 g L^{-1} and 6 g L^{-1} of glycerol into the biotransformation medium. Secondly, different nitrogen sources (urea and ammonium sulfate) and YNB concentrations were tested in biotransformation phase. YNB was used with normal concentration (6.7 g L^{-1}) and half concentration (3.35 g L^{-1}). Urea and ammonium sulfate concentrations were calculated in order to have the same nitrogen mass as in 6.7 g L^{-1} YNB with amino acids (**Table 6**). Moreover, a control experiment was carried out, in which no nitrogen source was added.

Table 6 Different nitrogen sources used in Erlenmeyer flask experiments and respective concentrations.

Nitrogen source	Concentration (g L^{-1})
YNB with amino acids	6.7
	3.35
Urea	2.27
Ammonium Sulfate	5

2.4 Analytical Methods

Samples were collected at appropriate intervals for analysis of cell concentration, glucose and lactones quantification. In some experiments, lipase activity, and glycerol concentration were also determined.

2.4.1 Biomass quantification

For biomass quantification, two methods were applied depending on culture medium composition. In YPD medium, density of cultures was measured at 600 nm and converted to cell dry weight (g L^{-1}) by a calibration curve that relates optical density and cell dry weight. In biotransformation medium, cellular concentration was determined by cell counting in the 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 L^{-1}).

2.4.2 Quantification of reducing sugars

Reducing sugars were measured by an adaptation of the dinitrosalicylic acid (DNS) method. In the presence of reducing sugars, 3,5-dinitrosalicylic acid is reduced into 3-amino-5-nitrosalicylic acid, a brownish compound that strongly absorbs light at 540 nm, allowing a quantitative spectrophotometric measurement of reducing sugars concentration present in the sample (Miller, 1959).

The reaction was carried out in glass test tubes, adding DNS reagent to the sample (1:1, v/v), previously filtered (pore size 0.20 µm, Orange Scientific, Belgium), or to the distilled water (blank), in a bath at 100 °C during 5 minutes. After this time, 1 mL of distilled water was immediately added to each test tube (with 200 mL of reaction mixture), in order to stop the reaction.

The values of absorbance (read in a microtiter plate reader (Sunrise Basic, Tecan, Switzerland) were converted to reducing sugar concentration expressed g L^{-1}, using a calibration curve previously prepared. The calibration curve was obtained by preparing six solutions of glucose with concentrations between 0.5 g L^{-1} and 4 g L^{-1} and representing graphically OD as a function of the known concentrations of each solution.

2.4.3 Measurement of Lipase activity

Extracellular lipase activity was measured in the samples supernatant according to an adaptation of a previously developed and validated spectrophometric method for complex systems described by Morín (Morín et al., 2003). This method was performed in glass test tubes and uses a mixture of *p*-nitrophenyl-butyrate 0.42 mM (*p*-NPB) in acetone 4 % (v/v) dissolved in PBS 50 mM (pH 7.3) as substrate. The reaction (sample and substrate in a ratio of 1:24 v/v) was carried out at 37 °C during 15 min and stopped with an ice and cold water bath. Blank experiments were performed replacing sample by distilled water.

Lipase activity (U L^{-1}) was quantified using Eq.2.1.

$$Activity = \frac{1}{\varepsilon} \times \frac{A}{t} \times \frac{Vt}{Va} \times 1000 \qquad \text{(Eq.2.1)}$$

where ε represents the molar extinction coefficient of *p*-nitrophenol (1.711 mM^{-1}); A represents the absorbance of the reactional mixture at a wavelength of 410 nm; V_t represents the total volume (mL) of the reactional mixture in the test tube; Va represents the volume of sample (mL) used in reactional mixture and t (min) represents the time during which the enzymatic reaction occurred.

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

2.4.4 Lactone Extraction and Quantification

To determine the production of γ-decalactone in all experiments, 2 mL of biotransformation medium were removed and their pH was lowered to 2 with HCl (37 % (v/v)) to promote the total lactonization of 4-hydroxydecanoic acid. As internal standard, γ-undecalactone (5 g L^{-1}) was added and the extraction of γ-decalactone was performed with diethyl ether 1:1 (v/v), mixing gently during 1 minute.

When the separation of phases was completed, the ether phase (organic) was collected and analyzed by gas chromatography (Varian 3800 instrument, Varian, Inc., USA) with TR-WAX capillary column (30 nm x 0.32 nm x 0.25 µm, Teknokroma, Spain). The temperature of split injector and detector was respectively 250 °C and 300 °C. The separation of lactones was achieved using a temperature gradient from 60 °C to 145 °C (at a rate of 5 °C min^{-1}) and from 145 °C to 215 °C (at a rate of 2 °C min^{-1}). Helium was used as a carrier gas.

Data were analyzed using the acquisition and integration software Star Chromatography Workstation v. 6.30 (Varian, Inc., USA) and the calibration curves for γ-decalactone and γ-undecalactone.

The determination of the response coefficient (K) was made by the average of various concentration/area ratios, for both compounds. It was obtained from the ratio between the average peak areas corresponding to γ-decalactone and γ-undecalactone.

The determination of K allows quantifying γ-decalactone using Eq. 2.2.

$$[\gamma\text{-decalactone }] = [\gamma\text{-undecalactone}]\times \frac{1}{K}\times\frac{A\gamma\text{-decalactone}}{A\gamma\text{-undecalactone}} \qquad \text{(Eq.2.2)}$$

[γ-decalactone] represents the concentration of γ-decalactone (mg L^{-1}); [γ-undecalactone] represents the concentration of γ-undecalactone (mg L^{-1}); $A_{\gamma\text{-decalactone}}$ corresponds to the area of γ-decalactone and $A_{\gamma\text{-undecalactone}}$ refers to the area of γ-undecalactone.

The calibration curve was obtained by preparing five solutions of equal concentrations of γ-decalactone and internal standard varying between 50 mg L^{-1} to 3000 mg L^{-1} (Annex I).

2.4.5 Glycerol Quantification

Glycerol concentration was determined by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H (BIORAD) column (300x7.8) coupled to an infrared detector (RI-2031, JASCO inc., USA). The column was eluted with H_2SO_4 0.005 M at 0.7 mL min^{-1} and the column temperature was 60 ◦C.

Prior injection in HPLC, a pre-treatment of the samples was performed in order to remove oil and Tween 80: 2 mL of biotransformation medium was added to 2 mL of diethyl ether, mixed and the aqueous phase was collected and filtered (pore size 0.20 µm, Orange Scientific, Belgium) to HPLC vials.

Data were analyzed using the acquisition and integration software CHROMPASS v1.8.6.1 (LCNetII/ADC JASCO inc., USA) and the calibration curves for glycerol. The calibration curve was achieved by preparing eight glycerol solutions with concentrations between 0.05 g L^{-1} and 10 g L^{-1} and representing graphically the peak Area (mV.min) as a function of the known concentrations of each solution.

3 RESULTS AND DISCUSSION

3.1 Stirred Tank Reactor

3.1.1 Batch mode operation

The influence of castor oil (in biotransformation phase) and glucose (in growth phase) concentrations on γ-decalactone production was investigated, using genetically modified strains of *Y. lipolytica*: MTLY40-2P and JMY3010. An increase of aroma production using the strain *Y. lipolytica* MTLY40-2P compared to JMY3010 is expected, due to its modification in β-oxidation pathway, which leads to a decrease on short-chain fatty acid degradation and enhances long-chain fatty-acid β-oxidation (Waché et al., 2002). Moreover, extracellular lipase expressed by *Y. lipolytica* JMY3010 in γ-decalactone production may contribute to faster hydrolysis of castor oil, releasing ricinoleic acid and glycerol as it has been described (Braga et al., 2015b). For these reasons, in this study the production of γ-decalactone was evaluated using pure cultures of *Y. lipolytica* MTLY40-2P or *Y. lipolytica* JMY3010, as well as co-cultures of these two strains.

Figure 7 shows the profiles of γ-decalactone production in batch cultures of both mutant strains after a 24 h growth phase in YPD medium with 20 g L^{-1} of glucose. As described in section 2.2.1, biotransformation phase started when a total cellular concentration of 20 g L^{-1} was reached and was carried out at 27 °C, 500 rpm of stirring rate and 3 L min^{-1} of aeration rate. It was observed that in all experiments, glucose concentration was low (below 2 g L^{-1}) at the beginning of biotransformation, indicating that yeast cells consumed almost all the glucose during growth. For both strains, the increase of castor oil concentration from 30 g L^{-1} to 60 g L^{-1} led to an improvement of aroma production. Similar results are obtained by Braga *et al*. (2015b) in batch cultures of wild and engineered strains of *Y. lipolytica*. However, Alchihab *et al*. (2010) observed that γ-decalactone concentration obtained in *Rhodotorula aurantiaca* cultures, decreased with the raise of initial castor oil concentration. Also, Endrizzi *et al*. (1993) reported that high concentrations of methyl ricinoleate reduced the yield of aroma produced by *Pichia guilliermondii* (Endrizzi et al., 1993).

The effect of castor oil concentration on γ-decalactone production was more pronounced for *Y. lipolytica* JMY3010 strain. When the concentration of substrate varied from 30 g L^{-1} to 60 g L^{-1}, the

maximum γ-decalactone concentration increased from 710 mg L^{-1} to 1699 mg L^{-1}, which represents a 58 % improvement in γ-decalactone production. In cultures with *Y. lipolytica* MTLY40-2P, aroma production increased, from 1424 mg L^{-1} to 1714 mg L^{-1}, which represents only a 17 % improvement in γ-decalactone production. Lower improvement in aroma production by *Y. lipolytica* MTLY40-2P could be explained by lower lipase activity detected in the extracellular medium, compared to *Y. lipolytica* JMY3010 that overexpresses *LIP2* gene. In spite of the fact that at the beginning of the biotransformation, similar values of lipase (around 400 U L^{-1}) in the medium were observed for both strains the lipase secretion during biotransformation was higher for JMY3010 than for MTLY40-2P strains reaching the maximum values of 2759 U L^{-1} and 1045 U L^{-1}, respectively for 60 g L^{-1} of castor oil. The increment of castor oil concentration could be exerting inhibitory effects which are not so accentuated in the lipase overexpressing strain, since oil is rapidly hydrolyzed into its fatty acids (mostly ricinoleic acid) and glycerol.

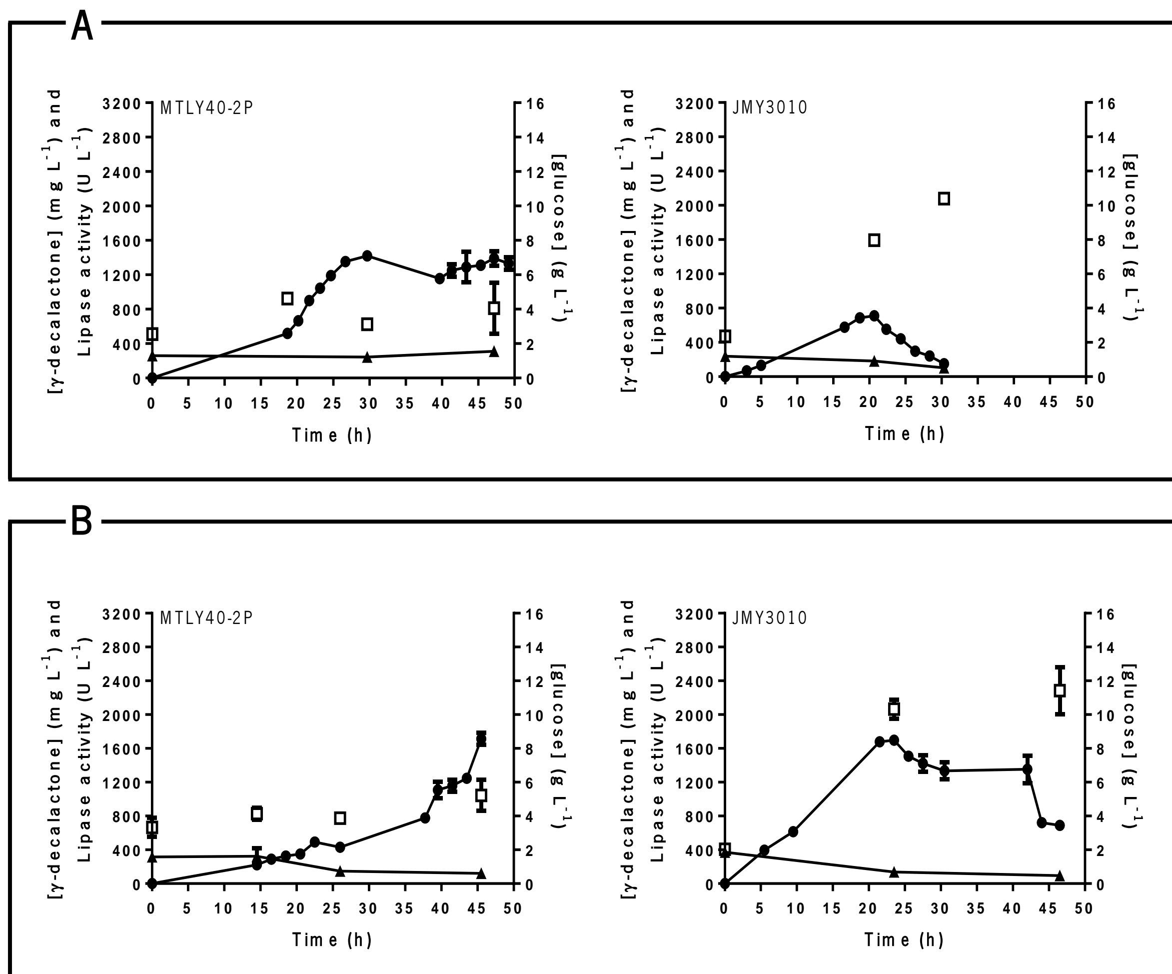


Figure 7 Effect of castor oil concentration (A: 30 g L^{-1}; B: 60 g L^{-1}) on γ-decalactone production (●), lipase activity (□) and glucose concentration (▲) during the biotransformation phase after a 24 h growth phase in YPD medium (glucose concentration of 20 g L^{-1}). Data are presented as mean and standard deviation of two independent experiments.

For MTLY40-2P strain, the increase in substrate concentration led to a higher maximum γ-decalactone concentration but, contrarily to JMY3010, more time was needed to reach maximum aroma concentration compared to the experiment with initial concentration of 30 g L^{-1} **(Figure 7 A)**. Braga *et al.* (2015b), also reported an increase of maximum γ-decalactone concentration by increasing castor oil concentration from 30 g L^{-1} to 60 g L^{-1}, for *Y. lipolytica* MTLY40-2P and JMY3010 strains.

Some research groups observed that after reaching the maximum concentration, γ-decalactone started to disappear due to the ability of *Y. lipolytica* wild-type to consume aroma as a carbon source (Aguedo, 2002a; Braga et al., 2015c; Braga & Belo, 2015a). Same result was observed in this study in the experiments with *Y. lipolytica* JMY3010. It was observed that, after reaching the maximum, γ-decalactone concentration started to decrease gradually until the end of biotransformation. Probably, γ-decalactone is consumed due to the short-chain fatty acids degradation (C_{10} or lower) which comes from *Y. lipolytica* JMY3010 Aox3 activity (Waché et al., 2000b). However, and regardless the initial castor oil concentration, in *Y. lipolytica* MTLY40-2P cultures no aroma consumption was observed. These results were expected since *Y. lipolytica* MTLY40-2P has a disruption in *POX3* and maintains an Aox2 activity (responsible for converting ricinoleic acid into γ-decalactone), resulting in a reduced or almost null degradation of short-chain fatty acids, or in this case, γ-decalactone (Waché et al., 2000b; Waché et al., 2002). In fact, once strain *Y. lipolytica* MTLY40-2P lack this gene, it can still consume γ-decalactone due to the residual activity of Aox1 and Aox6, but in this study that was not observed.

The use of an extracellular enzyme in the biotransformation medium to reduce lag phase observed in γ-decalactone production was proven by Gomes *et al.* (2013). It was claimed that the reduction occurred because the oil was faster hydrolyzed into ricinoleic acid. Since this is a lengthy and time consuming process, increasing lipase activity is of great importance for industrial production (Braga et al., 2012; Gomes et al., 2013). Therefore, the use of an engineered strain overexpressing *LIP2* gene (express extracellular lipase) would bridge the gap of this problem by improving extracellular lipase secretion and, consequently, γ-decalactone production with no extra costs with commercial enzymes. In the present study lipase activity was measured and higher values were obtained with *Y. lipolytica* JMY3010 (**Figure 7 A** and **Figure 7 B**). In *Y. lipolytica* JMY3010 cultures, lipase activity reached a maximum value of 2758 U L^{-1}, while in *Y. lipolytica* MTLY40-2P cultures reached only 1045 U L^{-1} (experiments with 60 g L^{-1} of castor oil). These differences in extracellular lipase were expected, since *Y. lipolytica* MTLY40-2P has no additional copy of *LIP2* gene and could explain the delay observed in γ-decalactone production observed for this strain. The existence of a lag phase in γ-decalactone production

(particularly in *Y. lipolytica* MTLY40-2P cultures), can be attributed to the time required for the synthesis of lipases involved in the hydrolysis of castor oil, in order to enable the availability of ricinoleic acid (direct precursor of γ-decalactone production through β-oxidation oxidation) to the cells.

In this experiment it was observed that, in cultures with *Y. lipolytica* MTLY40-2P, the maximum productivity decreased from 51 mg L^{-1} h^{-1} to 38 mg L^{-1} h^{-1}, when the castor oil concentration varied from 30 g L^{-1} to 60 g L^{-1}. However, this effect was not observed for *Y. lipolytica* JMY3010, contrariwise, an increase of maximum productivity from 37 mg L^{-1} h^{-1} to 78 mg L^{-1} h^{-1} was attained **(Table 7)**.

It was previously shown that, using batch cultures of wild-type *Y. lipolytica* W29, an increase of γ-decalactone productivity during biotransformation phase can be attained by increasing cell concentration from 30 g L^{-1} to 60 g L^{-1} (Braga & Belo, 2015a). When the authors increased cell concentration, γ-decalactone productivity increased 2.6 times in cultures with an initial castor oil concentration of 30 g L^{-1}. However, in cultures with a substrate concentration of 60 g L^{-1}, no significant effect on aroma productivity was observed. In the present study, it was intended to assess the influence of cell concentration of culture in aroma production, thus biotransformation phase was carried out with a cell concentration of 40 g L^{-1} of *Y. lipolytica* MTLY40-2P. Therefore, cells were firstly grown in batch cultures for 24 h in YPD medium with 40 g L^{-1} of glucose. **Figure 8** shows the γ-decalactone production for those cultures during biotransformation at two different castor oil concentrations: 30 g L^{-1} **(Figure 8 A)**, and 60 g L^{-1} **(Figure 8 B)**. Briefly, when both biotransformations started, glucose concentration was still very high, 11.5 g L^{-1} and 14.3 g L^{-1}, respectively in experiments carried out with 30 g L^{-1} and 60 g L^{-1} of castor oil. In both cultures a higher delay in aroma production at the beginning of the process, comparatively to the experiments described above, was noticed. Moreover, γ-decalactone production only started when glucose reached low concentrations in the medium. This can be explained by the preference of *Y. lipolytica* for carbon sources more easily assimilable (such as glucose) instead of castor oil, which must be firstly hydrolyzed by lipase into ricinoleic acid to be consumed by yeast. Thus, only after the depletion of glucose from culture medium, castor oil hydrolysis begun and was assimilated by yeast to induce aroma production. These results show that growth phase time should be adjusted in order to allow total glucose consumption.

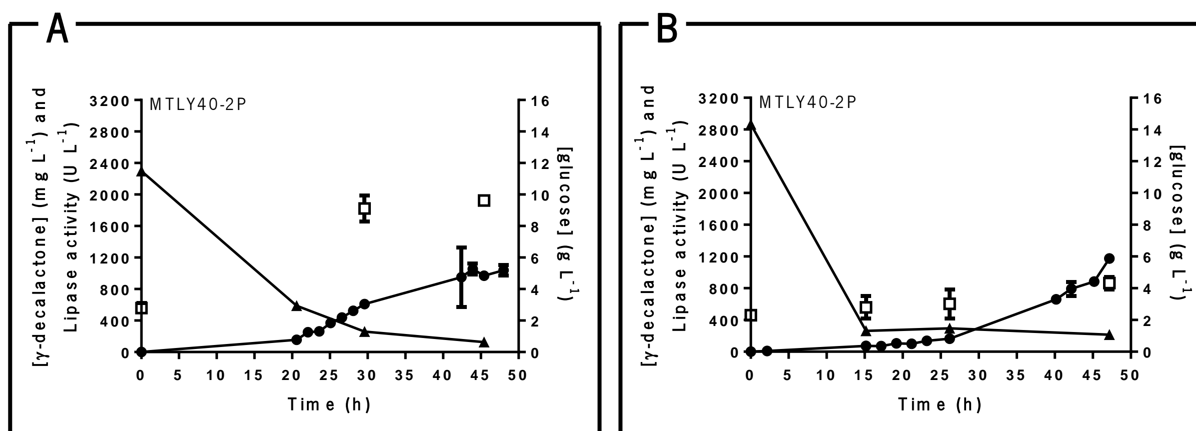


Figure 8 Effect of castor oil concentration (A: 30 g L-1; B: 60 g L-1) on γ-decalactone production (●), lipase activity (□) and glucose concentration (▲) during the biotransformation phase after a 24 h growth phase in YPD medium (glucose concentration of 40 g L-1). Data are presented as mean and standard deviation of two independent experiments.

Y. lipolytica MTLY40-2P achieved higher γ-decalactone concentrations and productivities. With twice the cell concentration than the previous experiments, in 30 g L^{-1} and 60 g L^{-1} castor oil concentrations, γ-decalactone concentrations of 1055 mg L^{-1} and 1175 mg L^{-1} were obtained, respectively. Also, similar productivities were achieved in both cultures, 24 mg L^{-1} h^{-1} and 25 mg L^{-1} h^{-1}, respectively. These results showed that *Y. lipolytica* MTLY40-2P achieved better aroma production with a cell concentration of 20 g L^{-1} and 30 g L^{-1} of castor oil **(Figure 7 A)**.

Taking into account the results obtained and described above with pure cultures of *Y. lipolytica* MTLY40-2P and *Y. lipolytica* JMY3010 **(Figure 7** and **Figure 8)**, the use of co-cultures of both yeast strains was attempted. The aim was to evaluate if co-culture could overcome the problem of the delay in aroma production observed in batch cultures of *Y. lipolytica* MTLY40-2P and avoid aroma consumption noticed in the case of *Y. lipolytica* JMY3010 cultures. Moreover, in this case, co-culturing these strains has the advantage of both being derived from the same yeast species, so there is no need to worry about an optimization of growth and/or operating conditions. It is worth to notice that is the first time that this approach is described, since studies reported in the literature are still focused in optimizing operational conditions for maximal γ-decalactone production using pure culture of each mutant strain or even with wild-type W29 strain. **Figure 9** shows γ-decalactone production (biotransformation phase) and dissolved oxygen concentration (growth and biotransformation phases) in batch co-cultures after a 24 h growth phase in YPD medium with 20 g L^{-1} of glucose **(Figure 9 A)**, and 40 g L^{-1} of glucose **(Figure 9 B)**, being both experiments performed with an initial castor oil concentration of 60 g L^{-1}. In these experiments, growth medium was inoculated with same cellular concentration of both yeast strains. It was firstly

checked that *Y. lipolytica* MTLY40-2P and *Y. lipolytica* JMY3010, have the same specific growth rate in glucose medium, thus no competition was expected in the growth phase of both strains in co-culture.

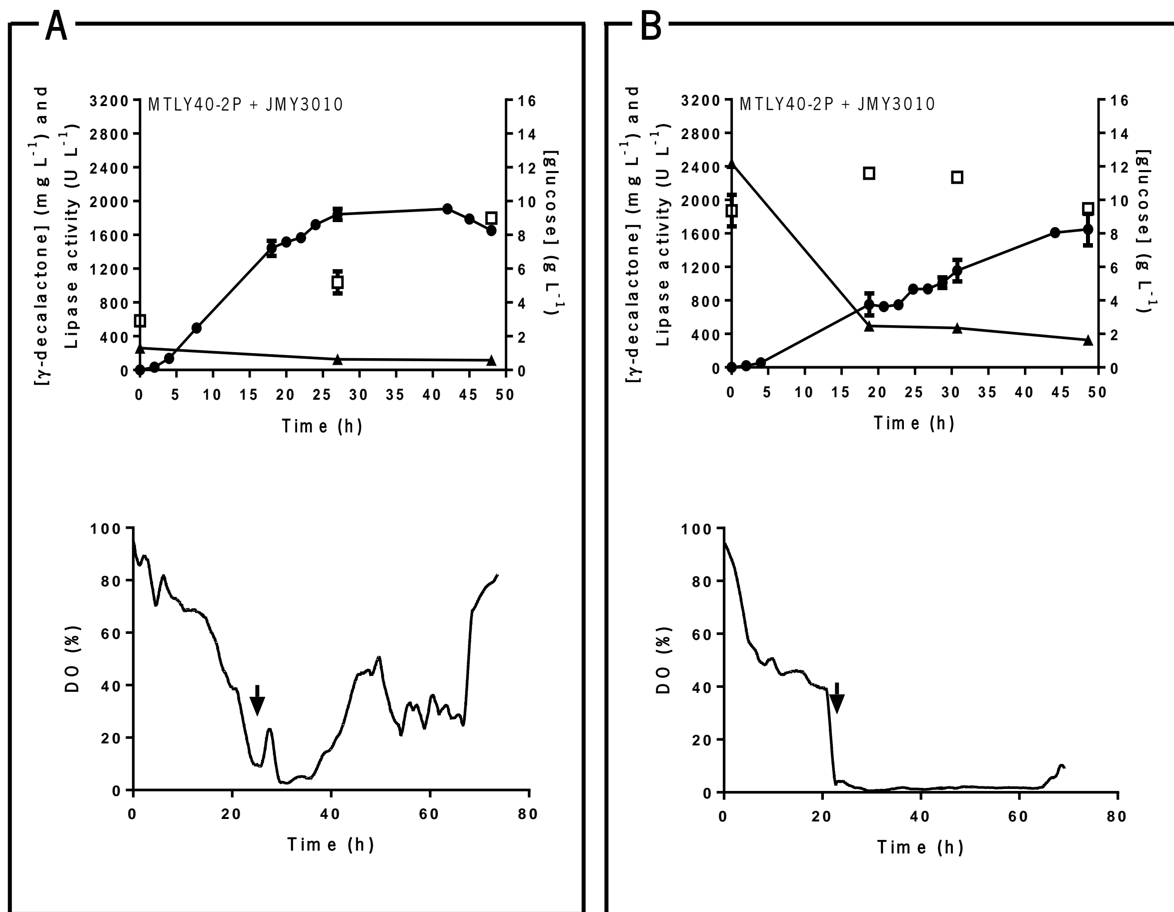


Figure 9 Profiles of γ-decalactone production (●), lipase activity (□) and glucose concentration (▲) and dissolved oxygen concentration (% of saturation), obtained in co-cultures of *Y. lipolytica* MTLY40-2P and *Y. lipolytica* JMY3010 with 60 g L^{-1} of castor oil, after a 24 h growth phase in YPD medium with 20 g L^{-1} (A) and 40 g L^{-1} (B) of glucose. Data are presented as mean and standard deviation of two independent experiments. Arrows indicate the beginning of biotransformation phase.

Comparing the results of co-cultures with pure ones, it was observed the disappearance of the delay in γ-decalactone production, previously observed in *Y. lipolytica* MTLY40-2P experiments **(Figure 9 A)**. Furthermore, a maximum γ-decalactone concentration of 1844 mg L^{-1} was obtained (which was higher than the maximum values obtained in pure cultures with glucose concentration of 20 g L^{-1}, **Figure 7**). Moreover, an aroma productivity of 80 mg h^{-1} L^{-1} was obtained, which almost matched the highest productivity observed in all batch experiments, when using pure cultures of *Y. lipolytica* JMY3010 **(Table 7)**. The simultaneous action of both strains resulted in a faster castor oil hydrolysis into ricinoleic acid by *Y. lipolytica* JMY3010, which was further converted in γ-decalactone by *Y. lipolytica* MTLY40-2P.

From the experiment with 20 g L^{-1} of glucose **(Figure 9 A)**, it can be concluded that the balance of both effects of having cells overproducing lipase but consuming the aroma and cells that only produces

aroma without consuming it, lead to final results with interesting values for maximum aroma concentration and productivity. Co-culture with a cell concentration of 40 g L^{-1} **(Figure 9 B)** revealed the same problem observed when pure cultures of *Y. lipolytica* MTLY40-2P **(Figure 8)** were used. When biotransformation phase started, 12 g L^{-1} of glucose was still in the medium, which resulted in a delay in γ-decalactone production, since *Y. lipolytica* is not able to begin castor oil hydrolysis until total glucose consumption. Using a co-culture **(Figure 9 B)** resulted in an increment in maximum γ-decalactone concentration (27 %) and productivity (38 %), when compared to pure *Y. lipolytica* MTLY40-2P culture **(Figure 8 B)**, when using a cell concentration of 40 g L^{-1}. These operating conditions avoided γ-decalactone consumption during all the process. Moreover, lipase activity was higher in the experiment with a cell concentration of 40 g L^{-1} **(Figure 9 B)**, compared to the experiment carried out with a cell concentration of 20 g L^{-1} **(Figure 9 A)**, 2319 U L^{-1} and 1799 U L^{-1}, respectively.

From the dissolved oxygen percentage profiles of the co-culture, carried out with 20 g L^{-1} of glucose **(Figure 9 A)** and 40 g L^{-1} of glucose **(Figure 9 B)**, no significant differences were observed between the experiments during the growth phase in spite of the higher final cellular biomass obtained using 40 g L^{-1} of glucose. However, differences among the dissolved oxygen profiles during biotransformation phase were observed. Dissolved oxygen was near to 0 % (between 23 h and 40 h), which corresponds to the interval time of higher γ-decalactone production rate in the experiment with lower cell density **(Figure 9 A)**. When the peak of γ-decalactone was obtained and the production stabilized, the dissolved oxygen started to increase and stabilized in values above 20 %. This indicates that, in order to reach γ-decalactone production peak, cells had to consume all the supplied oxygen. However, in the experiment with higher cell density **(Figure 9 B)**, the percentage of dissolved oxygen (in biotransformation phase) was always near to 0 % during the monitored period of the biotransformation (between 21 h and 70h). In this experiment γ-decalactone productivity was 50 % lower when compared to the one of lower cell concentration and did not seem to reach a plateau of γ-decalactone production. Therefore, this indicates that there was a limitation of the process by oxygen.

Escamilla-García *et al.* (2007) reported that, under very low aeration conditions a high accumulation of γ-decalactone was observed, due to the inhibition of acyl-CoA oxidase. Also, Escamilla-García *et al.* (2009) showed that, with slightly higher aeration conditions, 3-hydroxyacyl-CoA dehydrogenase was improved due to NAD regeneration (respiration dependent), resulting in 3-hydroxy-γ-decalactone production (did not affect acyl-CoA oxidase activity). Braga and Belo (2015a) also showed that increasing

oxygenation rates led to higher γ-decalactone concentration, but more time was needed to reach the maximum aroma concentration at higher OTR.

All the results from batch cultures are summarized in **Table 7**. Regarding γ-decalactone production by pure cultures, it is clear that the highest aroma productivity was obtained in *Y. lipolytica* JMY3010 cultures but, due to its Aox3 activity, γ-decalactone consumption was observed after approximately 20 h of biotransformation. Overall, the best results were obtained when a co-culture was used with 20 g L^{-1} cell concentration and 60 g L^{-1} of castor oil. With these conditions, the highest a γ-decalactone concentration and productivity were obtained, overcoming the lag phase observed in all MTLY40-2P experiments. Moreover, it is important to stress out that the use of the co-culture in this biotransformation process using castor oil as substrate suits well in the definition of biorefinery, since, it is possible to obtain two added-value products: γ-decalactone and lipase.

Table 7 Maximum γ-decalactone concentration and productivity obtained in batch pure cultures and co-cultures. Data are presented as the mean and standard deviation of two independent experiments.

Strain	[Castor Oil] (g L^{-1})	Cell concentration (g L^{-1})	[γ-decalactone]$_{maximum}$ (mg L^{-1})	γ-decalactone productivity $_{maximum}$ (mg L^{-1} h^{-1})
MTLY40-2P	30	20	1424 ± 4	51 ± 2
		40	1055 ± 48	24 ± 1
	60	20	1714 ± 4	38 ± 2
		40	1175 ± 4	25 ± 0
JMY3010	30	20	710 ± 15	37 ± 1
	60	20	1699 ± 42	78 ± 3
Co-culture	60	20	1844 ± 46	80 ± 5
		40	1610 ± 26	40 ± 7

3.1.2 Step-wise Fed-batch operation

Cultures in fed-batch mode are frequently used for the production of several bioproducts including, proteins, primary and secondary metabolites and biopolymers. This mode of operation allows to achieve a higher cell density than normal batch cultures and is often applied to obtain high yields and productivities of the desired product, by controlling nutrients or substrates (Lee et al., 1999). In this work, this is a really interesting feature, considering the potential toxicity of substrate to yeast cells (Feron et al., 1997; Lee et al., 1998; Lee et al., 1999; Lee et al., 1995; Lin et al., 1996).

Some studies reported an increase of γ-decalactone production in fed-batch cultures when compared to batch mode (Ambid et al., 2003; Lee et al., 1995). Lee *et al.* (1995) reported an increase of γ-decalactone production by *Sporobolomyces odorus* from castor oil, using a three-step feeding of carbon source oil. Also, Ambid *et al.* (2003) observed a considerably improvement of γ-decalactone production by *Sporobolomyces odorus* using additions of methyl ricinoleate every 24 h. These were in fact modes of operation that approximate to fed-batch mode since substrate feeding was not continuous but occurred by pulses (step-wise), that is more adequate for viscous substrates such as lipidic ones.

Thus, in this work a step-wise fed-batch strategy was attempted, since although γ-decalactone consumption has been avoided using a co-culture **(Figure 9)**, higher γ-decalactone concentrations and productivities are sought. **Figure 10** shows γ-decalactone production and dissolved oxygen concentration (growth and biotransformation phases) in a step-wise fed-batch co-culture after 24 h growth phase in YPD medium with 20 g L^{-1} of glucose and 60 g L^{-1} of castor oil, in which a pulse of 60 g L^{-1} of castor oil was added to the bioreactor.

Dissolved oxygen profile **(Figure 10 B)** was similar to the batch co-culture experiment with higher cell density **(Figure 9 B)**, since the percentage of dissolved oxygen (in biotransformation phase) was always near to 0 % during the monitored period of the biotransformation (between 24 h and 79h). In this experiment γ-decalactone productivity decreased 43 % compared to batch co-culture of lower cell concentration **(Figure 9 A)** and did not seem to reach a plateau of γ-decalactone production. Therefore, this indicates that there was a limitation of the process by oxygen.

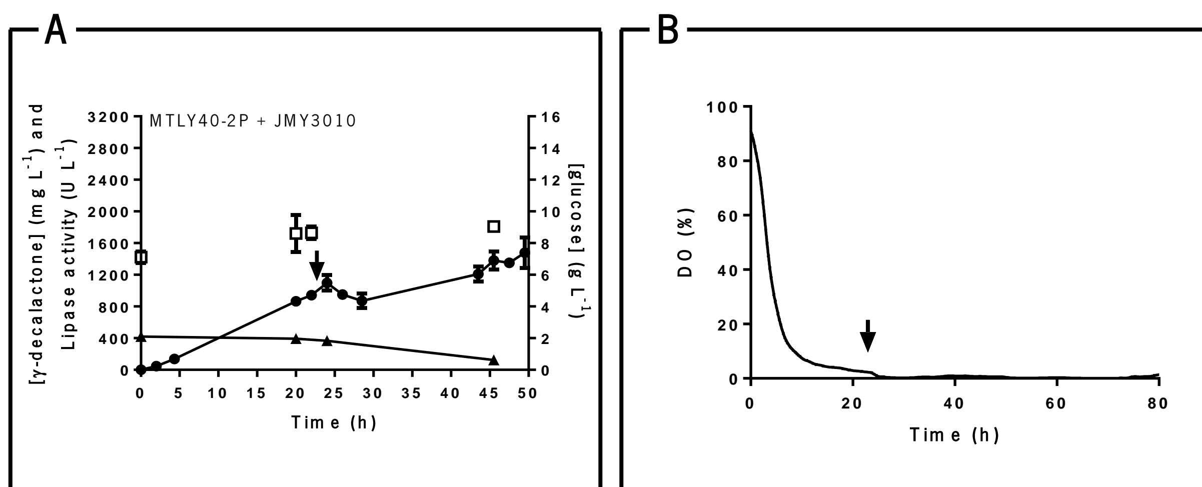


Figure 10 A: Profile of γ-decalactone production (●), lipase activity (□) and glucose concentration (▲); B: dissolved oxygen concentration (% of saturation); obtained in step-wise fed-batch co-cultures of *Y. lipolytica* MTLY40-2P and *Y. lipolytica* JMY3010 with 60 g L^{-1} of castor oil, after a 24 h growth phase in YPD medium (20 g L^{-1} of glucose). In A: solid arrow represents a 60 g L^{-1} pulse of castor oil, which was applied after 23 h of biotransformation phase. In B: solid arrow indicates the beginning of biotransformation phase. Data are presented as mean and standard deviation of two independent experiments.

A maximum γ-decalactone concentration of 1478 mg L^{-1} was attained at the end of the experiment (48 h) and a productivity of 46 mg L^{-1} h-^{1} obtained at a 24 h of biotransformation immediately after the pulse addition of substrate to the culture. Lipase activity and glucose concentration were similar to those values obtained previously in one step batch biotransformation **(Figure 9 A).** Thus, opposed to what was expected the substrate addition did not lead to further increase of γ-decalactone in the medium. The initial castor oil concentration combined with the pulse addition (both 60 g L^{-1}) may have led to toxic levels of substrate concentration, which resulted in lower aroma production. Further experiments with lower concentration of initial castor oil and different pulse additions could be performed. An approach with multiple pulses, with lower concentrations, could also be tested.

Braga *et al.* (2015b) also tried a similar approach, using *Y. lipolytica* W29, *Y. lipolytica* MTLY40-2P and *Y. lipolytica* JMY3010 (pure cultures), where two pulses of 60 g L^{-1} of castor oil were added during a biotransformation of 80 h, using the identical operation conditions but higher stirring speed as used in the herein reported work. It was observed that no greater improvement in γ-decalactone production was achieved when using *Y. lipolytica* W29 and *Y. lipolytica* JMY3010. However, for *Y. lipolytica* MTLY40-2P, maximum γ-decalactone concentration was increased from batch to step-wise fed-batch. In general, it was concluded that the productivity values of γ-decalactone obtained with step-wise fed-batch and batch where quite similar, and slightly lower in the fed-batch cultures. Moreover, they concluded that higher

initial castor oil concentrations (60 g L^{-1}) can be used in batch cultures instead of lower substrate step-wise feeds.

Braga and Belo *et al*. (2015a) studied γ-decalactone production by *Y. lipolytica* wild-type W29 using a step-wise fed-batch approach with two 60 g L^{-1} pulses of castor oil (maximum pulse concentration, so far, described in the literature). In experiments performed with 60 g L^{-1} of castor oil in biotransformation phase, substrate additions did not enhance aroma production (similar to batch mode). Moreover, a consumption of γ-decalactone was observed, which was supposed to be minimized by this operation mode. It was concluded that γ-decalactone measurement resulted from an equilibrium between production and consumption of the aroma (Braga & Belo, 2015a).

The productivity of 46 mg L^{-1} h^{-1} obtained **(Figure 10)**, was similar to the values reported by Braga and Belo *et al*. (2015a) when using 30 g L^{-1} of initial castor oil and two pulses of 60 g L^{-1} (41 mg L^{-1} h^{-1}). In this particular case, results revealed that it was possible to achieve a similar productivity using a lower cell density and substrate amount.

Results obtained using a co-culture were firstly reported here and also indicates that a pulse addition of castor oil (step-wise fed-batch strategy) was not beneficial for the biotransformation process, compared to batch process, under the same experimental conditions used.

3.2 Air-lift: integration of the best results obtained in STR batch experiments

Traditionally, STR bioreactors are the commonly vessels used in laboratory and industrial production of added- value products in fermentations. However, STR have some disadvantages: the power input due to mechanical stirring is high (meaning high costs); problems of overheating that have to be controlled; and the stirring rates required to achieve sufficient oxygen mass transfer may cause damage to the microorganisms (Braga et al., 2015c). Therefore, air-lift bioreactors are being increasingly considered in the scientific community because of their advantages when compared to conventional STR, such as minimal cellular hydrodynamic stress, higher OTR promoted by air bubbles and lower power consumption. Nowadays, there are few studies concerning γ-decalactone production in this type of bioreactor. In order to assess the applicability of air-lift bioreactors on γ-decalactone production, a batch co-culture was performed in an airlift bioreactor under the best biotransformation conditions obtained in section 3.1.1.

Figure 11 shows γ-decalactone production in a batch co-culture of *Y. lipolytica* MTLY40-2P and *Y. lipolytica* JMY3010 after a 24 h growth phase in YPD with 20 g L^{-1} of glucose. As described in section 2.2.2, biotransformation phase was carried out with a castor oil concentration of 60 g L^{-1}, at 27 °C and 7 L min^{-1} of aeration rate. Briefly, when biotransformation phase started, glucose was almost totally consumed, remaining in the medium 5.9 g L^{-1}. It seems that this glucose still present in the medium did not lead to a delay in aroma production at the beginning of the process. In this operating condition, a maximum γ-decalactone concentration of 1847 mg L^{-1} was obtained, which is similar to the results attained in co-cultures batch experiments performed in stirred tank bioreactor **(Figure 9 A)**. However, aroma productivity was much lower than the previous co-culture (20 mg h^{-1} L^{-1} vs 80 mg h^{-1} L^{-1}).

Lipase activity (ranging from 2087 U L^{-1} to 3010 U L^{-1}) was relatively higher in this experiment when compared to the STR experiment carried out with 20 g L^{-1} of glucose and 60 g L^{-1} of castor oil (increased 40 %) **(Figure 9 A)**.

STR experiments were performed with a higher specific aeration rate than the airlift experiment during biotransformation phase (2.9 vvm vs 1.6 vvm) due to limitation of bioreactor instability and foaming caused by high aeration rates in the airlift reactor. During biotransformation phase the dissolved oxygen was always near to 0 %, which indicates that probably the process was limited by oxygen. *Y. lipolytica* is aerobic, therefore the deficit in oxygen supply have important impact on cell activity and on aroma production (decreased γ-decalactone productivity). The concentration of dissolved oxygen and the oxidative state of the medium are important factors influencing the formation of γ-decalactone in *Y.*

lipolytica, due to the oxygen role on the β-oxidation (Aguedo et al., 2005b; Fickers et al., 2005; Kamzolova et al., 2003). Thus, the OTR from the gas to the liquid is a key factor in the biotransformation process optimization. Therefore, in an air-lift bioreactor higher aeration rates should be employed in order to match STR experiments and increase dissolved oxygen percentage in the medium.

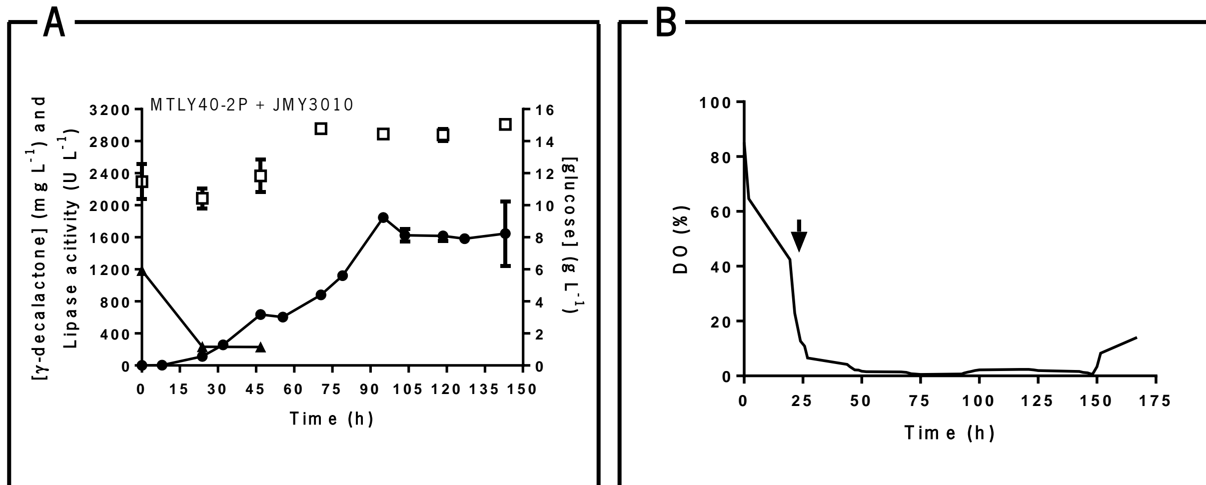


Figure 11 Profiles γ-decalactone production (●), lipase activity (□) and glucose concentration (▲) and dissolved oxygen concentration (% of saturation), obtained in air-lift co-cultures of *Y. lipolytica* MTLY40-2P and *Y. lipolytica* JMY3010 with 60 g L⁻¹ of castor oil, after a 24 h growth phase in YPD medium (20 g L⁻¹ of glucose). Data are presented as mean and standard deviation of two independent experiments. Arrow indicates the beginning of biotransformation phase.

Few studies have been already reported of γ-decalactone production in an airlift bioreactor. Gomes (2011) studied γ-decalactone production by *Y. lipolytica* W29 wild-type strain in an air-lift bioreactor and was concluded that, when compared to STR experiments, air-lift experiments resulted in higher aroma concentrations, although larger times to achieve the maximum γ-decalactone concentrations were needed. In this study, similar results were observed, since higher productivities were obtained using STR bioreactor.

Braga *et al.* (2015c) also studied γ-decalactone production by *Y. lipolytica* W29 wild-type strain in an air-lift bioreactor. It was observed that the reduction of the oxygen transfer rate decreased the time needed to reach the peak of γ-decalactone production, resulting in higher productivities but lower final aroma concentrations were attained.

In this experiment, it was observed that, four times higher productivities were attained with STR bioreactor co-cultures **(Figure 9 A)** when compared to air-lift (same biotransformation conditions), while maximum γ-decalactone concentrations were similar.

3.3 Effect of medium components

3.3.1 Effect of glycerol on γ-decalactone production

The hydrolysis of castor oil into ricinoleic acid by extracellular lipase secreted by *Y. lipolytica* results in a mixture of other compounds that affect the cells, such as oleic acid and glycerol (Gomes et al., 2013). Glycerol, contrarily to fatty acids and alkanes, may repress *POX2* promoters (responsible for the long-chain acyl-CoA degradation), inhibiting γ-decalactone production by *Y. lipolytica* (Barth, 2013).

It has been reported that *POX2* promoter is induced in the presence of hydrophobic substrates such as fatty acids or triglycerides, and repressed or not induced in the presence of glycerol (Juretzek et al., 2000; Sassi et al., 2016).

In this subchapter it was intended to evaluate the effect of glycerol on γ-decalactone production, taking into account the maximum theoretical glycerol concentration that could be obtained from total castor oil hydrolysis. In all previous experiments (section 3.1 and 3.2), concentrations of 30 g L^{-1} or 60 g L^{-1} of castor oil was employed, which correspond to a maximum theoretical glycerol concentration of 3 g L^{-1} and 6 g L^{-1}, respectively. **Figure 12** shows γ-decalactone production by *Y. lipolytica* MTLY40-2P in batch cultures in small scale Erlenmeyer's flasks, with and without addition of glycerol to the biotransformation medium.

All conditions presented similar production profiles **(Figure 12)** with productivities of 6.3 mg L^{-1} h^{-1} (without glycerol), 5.1 mg L^{-1} h^{-1} (3 g L^{-1} of glycerol) and 4.7 mg h^{-1} L^{-1} h^{-1} (6 g L^{-1} of glycerol), that shows a slight decrease (25 %) of productivity in the presence of glycerol. Moreover, same behavior was observed for maximum γ-decalactone concentrations of 1534 mg L^{-1} (without glycerol), 1242 mg L^{-1} (3 g L^{-1} of glycerol) and 1241 mg L^{-1} (6 g L^{-1} of glycerol), corresponding to 19 % decrease. It is interesting to notice that in spite of the final aroma concentration obtained in these low scale experiments were similar to the values obtained in bioreactors, the values of productivities are around 10-fold lower in Erlenmeyer flasks than in bioreactors. The slower rate of the biotransformation in flasks may be explained by limitations in substrates accessibility to the cells due to the weaker mixing as well as lower oxygen transfer rates.

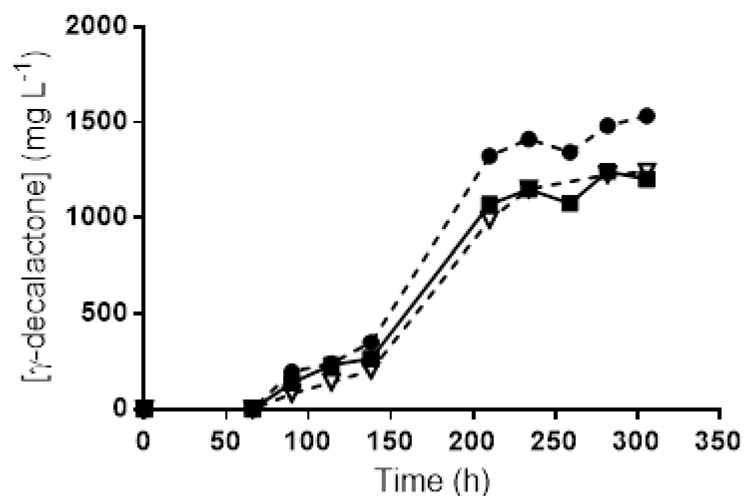


Figure 12 Profiles of γ-decalactone production by *Y. lipolytica* MTLY40-2P in baffled Erlenmeyer flasks, after a 19 h growth phase in YPD (20 g L^{-1} of glucose), with a castor oil concentration of 30 g L^{-1}: without glycerol (●), with 3 g L^{-1} of glycerol (■) and 6 g L^{-1} of glycerol (▽).

As already discussed in section 3.1.1, *POX2* plays an important role in long-chain fatty acids oxidation, and consequently in γ-decalactone production. According to the results herein reported, and in case of total castor oil hydrolysis, a small effect on the final γ-decalactone concentration, due to a possible inhibition of *POX2* by glycerol, is expected.

Thus, possible inhibitory effects of glycerol may explain some low aroma production observed in the experiments previous reported. In the STR batch experiments (section 3.1) HPLC was used to measure glycerol concentration during biotransformation phase and the values did not exceed 0.7 g L^{-1}, which is below the concentrations previously tested: 3 g L^{-1} and 6 g L^{-1} **(Figure 12)**.

3.3.2 Effect of different nitrogen sources on γ-decalactone production

In these experiments the aim was to assess the effect of different nitrogen sources on γ-decalactone production by *Y. lipolytica* MTLY40-2P during biotransformation phase in order to assess the possibility of using nitrogen sources more economically attractive to industrial application. Two organic nitrogen sources (YNB with amino acids and urea) and one inorganic nitrogen source (ammonium sulfate) were tested in batch cultures at small scale (Erlenmeyer flasks). The nitrogen sources used in all experiments described previously in sections 3.1, 3.2 and 3.3.1 were: peptone (included in YPD medium), NH_4Cl and YNB with amino acids. **Figure 13** shows γ-decalactone production with different nitrogen sources and without the nitrogen source. The concentrations tested of ammonium sulfate and urea were determined to have the same nitrogen concentration as 6.7 g L^{-1} of YNB (1.06 g L^{-1}).

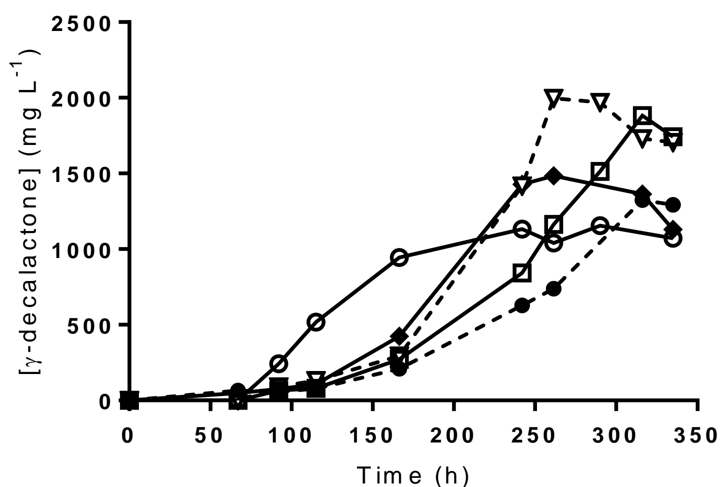


Figure 13 Profiles of γ-decalactone production by *Y. lipolytica* MTLY40-2P in baffled Erlenmeyer flasks, after a 19 h growth phase in YPD medium, with a castor oil concentration of 30 g L^{-1} obtained in the experiments carried out with: 6.7 g L^{-1} of YNB (●), 3.35 g L^{-1} of YNB (□), 2.27 g L^{-1} of Urea (▽), 5 g L^{-1} of ammonium sulfate (◆) and without nitrogen source (○).

Table 8 shows a summary for maximum aroma production and productivities obtained for every condition tested above **(Figure 13)**.

Table 8 Maximum γ-decalactone concentration and productivity by *Y. lipolytica* MTLY40-2P in Erlenmeyer flasks batch cultures using YNB with amino acids, urea and ammonium sulfate as nitrogen sources.

Nitrogen source	Concentration (g L^{-1})	[γ-decalactone] $_{maximum}$ (mg L^{-1})	γ-decalactone productivity $_{maximum}$ (mg L^{-1} h^{-1})
YNB with amino acids	6.7	1324	4.2
	3.35	1882	6.0
Urea	2.27	1996	7.6
Ammonium Sulfate	5	1485	5.9
None	0	1155	6.8

The decrease of YNB concentration led to an increment in γ-decalactone productivity (4.2 mg L^{-1} h^{-1} to 6.0 mg L^{-1} h^{-1}) and production rate (5.3 mg L^{-1} h^{-1} to 19.7 mg L^{-1} h^{-1}). Also, higher γ-decalactone concentration (1882 mg L^{-1}) was obtained by using 3.35 g L^{-1} of YNB. When compared to the experiment without nitrogen source, the use of YNB resulted in lower productivities. However, without nitrogen source, the maximum γ-decalactone concentration decreased 39 % when compared to the experiment in which 3.35 g L^{-1} of YNB were used.

It was expected to obtain similar γ-decalactone concentrations in the experiments with 6.7 g L^{-1} of YNB and 5 g L^{-1} of ammonium sulfate, since ammonium sulfate is the nitrogen source of YNB. Despite YNB being richer in nutrients and amino acids, ammonium sulfate leads to a more directional cellular metabolism in biotransformation phase. Ammonium sulfate seemed to be a better option than YNB, since it is cheaper and did not affect aroma production. Overall, when urea was used, productivity increased and γ-decalactone production was 42 % higher, compared to the experiment without nitrogen source in biotransformation medium **(Table 8)**.

Lipase activity can also be modulated by the nitrogen source (Coelho et al., 2010; Lopes et al., 2016), which could also affect γ-decalactone production. Pereira-Meirelles *et al.* (1997) observed that tryptone and oleic acid led to higher lipase activities by *Y. lipolytica* mutants, when compared to urea or ammonium sulfate (Pereira-Meirelles et al., 1997). Moreover, Fickers *et al*. (2004) stated that lipase activity in *Y. lipolytica* cultures was clearly stimulated in the presence of increased tryptone concentration.

The choice of nitrogen source, organic or inorganic, has proven to be an important parameter for the improvement of lipase production. Playing a different role in enzymes synthesis, organic sources can

supply cells with growth factors and amino acids, while inorganic sources can be quickly used (De Almeida et al., 2013).

Based on Charalambous (1992) findings, the type of nitrogen and carbon sources used in fermentation would have an important bearing on the development of a process for the biotechnological production of natural aroma extracts by *Penicillium italicum*. Different nitrogen sources affected the aroma production both quantitatively and qualitatively (Charalambous, 1992).

In this study similar results were observed since the nitrogen source, being organic or inorganic (YNB vs ammonium sulfate), seemed to have an impact in γ-decalactone production.

Taking into account the studies referred above, it is possible to conclude that nitrogen sources play an important role, both in the quality and the quantity of aroma (or flavor) as well as the lipase activity, which are two of the most important aspects in this bioprocess. All current studies concerning γ-decalactone production by *Y. lipolytica* strains use YNB with amino acids as principal nitrogen source. However, this nitrogen source is more expensive comparatively to other sources, such as urea or ammonium sulfate. Nowadays, production costs are one of the key issues at industrial scale and the results herein reported indicates that further γ-decalactone optimization is possible considering medium composition and strategies of operation at bioreactor scale integrated with the use of genetically improved strains.

4 CONCLUSIONS AND FINAL REMARKS

4.1 Final conclusions

Over the last few decades a lot of studies have been made in order to improve γ-decalactone production by *Y. lipolytica*. The bioprocess studied in this dissertation is attractive from an industrial point of view, since it is possible to obtain two added-value "natural" products in the same biotransformation (γ-decalactone and extracellular lipase). However, despite all the efforts from the scientific community, the overall aroma productivity is still very low.

The studies reported in this dissertation were primarily focused on the optimization of γ-decalactone production in STR bioreactor using two mutant strains derived from *Y. lipolytica* W29 under different operating culture conditions.

STR batch experiments (section 3.1.1) showed that higher γ-decalactone concentration (1844 ± 46 mg L^{-1}) and productivity (80 ± 5 mg L^{-1} h^{-1}) were obtained with a *Y. lipolytica* MTLY40-2P and *Y. lipolytica* JMY3010 co-culture, when compared to pure cultures of each strain. The main reasons were the decrease of the lag phase of the aroma production (observed in MTLY40-2P pure cultures) and the minimization of γ-decalactone consumption (observed in JMY3010 pure cultures).

A step-wise fed-batch co-culture (section 3.1.2) was performed under the same conditions as the best batch experiment (20 g L^{-1} of glucose and 60 g L^{-1} of castor oil). Some authors reported an increase in γ-decalactone production using this approach with MTLY40-2P cultures (Braga & Belo, 2015a; Braga et al., 2015b). However, in this study a single pulse addition of castor oil (60 g L^{-1}) resulted in a decrease of γ-decalactone productivity (43 %). These results suggested that toxic levels of castor oil were achieved by the probable accumulation in the medium.

In air-lift co-culture, similar values of maximum γ-decalactone concentration were attained when compared to batch experiments in STR (20 g L^{-1} of glucose and 60 g L^{-1} of castor oil **(Figure 9 A)**). However, γ-decalactone productivity decreased severely (75 %) since more time was needed to achieve the maximum concentration. Dissolved oxygen profile suggested that the process may be limited by oxygen deficit.

Small scale (Erlenmeyer flasks) experiments were performed to test eventual inhibitory effects of glycerol on γ-decalactone production by *Y. lipolytica* MTLY40-2P and results showed that glycerol

concentrations of 3 g L^{-1} and 6 g L^{-1} did not cause a major effect in the bioprocess. A slight decrease of 19 % in maximum aroma concentration was observed in the presence of glycerol compared to the biotransformation without addition of glycerol at the beginning of the experiment.

Moreover, batch cultures at small scale were also performed to assess the effect of different nitrogen sources in γ-decalactone production by *Y. lipolytica* MTLY40-2P. It was concluded that urea may be used instead of YNB, resulting in higher maximum aroma concentration and productivity compared to standard biotransformation medium with YNB.

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

4.2 Future work perspectives

The present work brings new insights on the biotechnological production of γ-decalactone by *Y. lipolytica* implementing co-cultures of MTLY40-2P and JMY3010 strains, which overcame problems of pure cultures.

However, new operating conditions should be tested in STR and air-lift bioreactors, varying parameters such as agitation or aeration rates in order to optimize oxygen transfer and prevent oxygen limitation. Also, different strategies of substrate feeding to the bioreactor may be applied in order to perform a complete study of step-wise fed-batch operation, such as the use of lower castor oil concentration pulses at higher frequency.

Moreover, future batch biotransformation at STR bioreactor scale should be performed using urea as nitrogen source and assess the effect on γ-decalactone production and lipase activity, compared to normal biotransformation conditions performed in batch experiments (section 3.1.1). At last, it would be very interesting to construct a new strain combining the deletion and expression of genes from the β-oxidation pathway as in MTLY40-2P strain and at the same time overexpressing *LIP2* gene as in JMY3010 strain.

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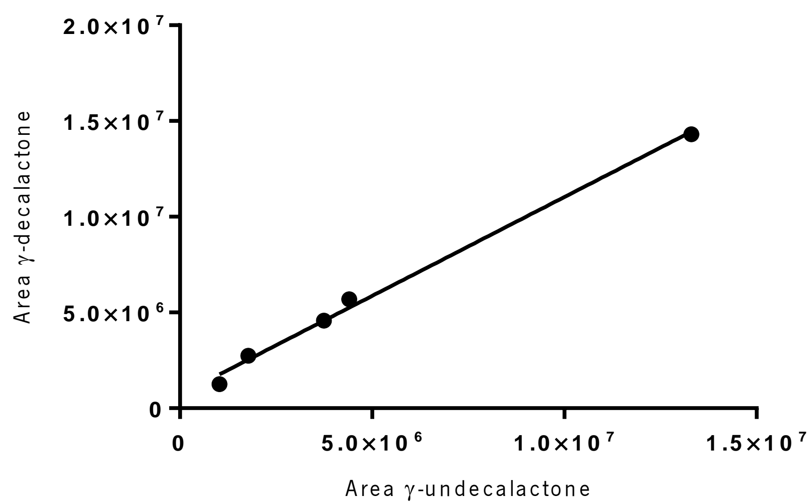
ANNEX I – DECALACTONE CALIBRATION CURVE



Annex I Gas chromatography γ-decalactone calibration curve. The calibration curve was obtained by preparing five solutions of equal concentrations of γ-decalactone and internal standard varying between 50 mg L^{-1} to 3000 mg L^{-1}; y = 1.033 ± 0.042x + 705093 ± 2758; R^2 = 0.9950. The response coefficient (K) corresponds to the slope (1.033).