Universidade do Minho
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Production of the flavor compound 2-phenylethanol by *Yarrowia lipolytica*

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Thesis submitted in fulfilment of the requirements for Master's degree in biotechnology

Work developed under supervision of

Supervisor: Doctor Adelaide Correia Braga

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DECLARAÇÃO

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ABSTRACT

The flavor compounds produced by biotechnological processes are widely accepted by consumers, since they are considered as "natural" compounds. In addition, they are of great interest due to the high yields and greener processes. 2-Phenylethanol (2-PE) is an aromatic alcohol with a delicate fragrance of rose petals, widely applied in diverse types of products. Although there are many works in the literature about 2-PE production by several yeasts' strains, the non-conventional yeast *Yarrowia lipolytica* was poorly explored in this field, despite appearing to be a competitive producer.

Thus, the main goal of this work is to develop a 2-PE production process, through L-phenylalanine (L-Phe) bioconversion, combining strain selection, medium composition and culture condition optimization and application of *in situ* product removal techniques (ISPR), using *Y. lipolytica* as a cell factory. Based on this, three *Y. lipolytica* strains (W29, NCYC2904 and CBS2075) were selected. Initially, it was studied the inhibitory effect of 2-PE and L-Phe on *Y. lipolytica* growth, and it was observed that all strains were inhibited by 2-PE concentrations equal or above 2 g L^{-1}. Concerning the effect of L-Phe, a remarkable inhibitory effect on cell growth of NCYC2904 strain was detected for all concentrations tested, while W29 and CBS2075 strains were inhibited by 15 g L^{-1} of L-Phe. Thereafter, the ability of W29 and CBS2075 strains to produce 2-PE was assessed at shake flask-scale, with glucose as the carbon source. The W29 strain was selected for further studies since it produced the highest 2-PE titer (1.01 ± 0.07 g L^{-1}). Afterwards, the influence of L-Phe concentration in the 2-PE production was also evaluated, and it was observed that an increase in the precursor concentration enhances the 2-PE concentration (1.57 ± 0.06 g L^{-1}), bioconversion yield (0.28 ± 0.04 *vs* 0.23 ± 0.02 g g^{-1}) and productivity (6.5 ± 0.3 *vs* 6.0 ± 0.4 mg L^{-1} h^{-1}). 2-PE production with W29 strain was also studied at bioreactor level, and the effect of dissolved oxygen (DO) concentration and medium pH were evaluated. It was observed that increasing the DO concentration from 10 % to 30 % lead to a decrease in 2-PE titer (0.88 *vs* 0.71 g L^{-1}), whereas a more acidic environment (pH 5) allow a high 2-PE titer (1.92 g L^{-1}) and bioconversion yield (0.57 g g^{-1}). Crude glycerol was tested as an alternative carbon source for 2-PE production, and a 33 % increase in 2-PE titer was observed with this carbon source. The effect of substrate concentration was also analyzed, and it was demonstrated that an increase in crude glycerol concentration did not enhance the L-Phe consumption efficiency nor the 2-PE concentration. It was also tested a bioconversion strategy coupled with product removal by adsorption, and the resin XAD-4 was chosen as the best adsorbent since it adsorbed most 2-PE and least L-Phe. The addition of 7 % (wet w/v) resin to the bioconversion system allowed a 1.2-fold increase in the 2-PE concentration compared to the bioconversion without addition of the adsorbent resin. These results show that the *Y. lipolytica* W29 strain is a promising microorganism for 2-PE production.

RESUMO

Os aromas e fragrâncias produzidos por processos biotecnológicos são cada vez mais aceites pelos consumidores, uma vez que são considerados compostos "naturais". Além disso, os rendimentos obtidos são mais elevados e este bioprocesso é mais ecológico, tornando-o mais atrativo. O 2-feniletanol (2-FE) é um álcool aromático com uma delicada fragrância a pétalas de rosas, amplamente utilizado em diversos produtos. Embora existam muitos trabalhos na literatura que reportam a produção de 2-FE por várias estirpes de leveduras, a levedura não-convencional *Yarrowia lipolytica* foi pouco explorada nessa área, apesar de parecer um produtor competitivo.

Assim, o principal objetivo deste trabalho consiste no desenvolvimento do processo de produção de 2-FE, por bioconversão da L-fenilalanina (L-Fe), combinando seleção de estirpes, otimização da composição do meio e condições de cultura e aplicação de técnicas de remoção de produto, utilizando a *Y. lipolytica*. Para tal, foram selecionadas três estirpes de *Y. lipolytica* (W29, NCYC2904 e CBS2075). Inicialmente, foi avaliado o efeito inibitório do 2-FE e da L-Fe no crescimento das diferentes estirpes, e verificou-se que concentrações de 2-FE iguais ou superiores a 2 g L^{-1} inibem completamente o crescimento de todas as estirpes. Em relação ao efeito da L-Fe, esta inibiu totalmente o crescimento da estirpe NCYC2904, em todas as concentrações testadas, e o crescimento das estirpes W29 e CBS2075 foi inibido para uma concentração de L-Fe de 15 g L^{-1}. De seguida, a capacidade de produção de 2-FE pelas estirpes W29 e CBS2075 foi avaliada em matraz, com glucose como fonte de carbono. A estirpe W29 foi escolhida como a mais promissora uma vez que produziu a concentração mais elevada de 2-FE (1,01 ± 0,07 g L^{-1}). Posteriormente, foi avaliado o efeito da concentração de L-Fe na produção de 2-FE, tendo-se verificado que o aumento da concentração do precursor favorece a produção (1,57 ± 0,06 g L^{-1}), o rendimento (0,28 ± 0,04 *vs* 0,23 ± 0,02 g g^{-1}) e a produtividade (6,5 ± 0,3 *vs* 6,0 ± 0,4 mg L^{-1} h^{-1}). A produção de 2-FE pela estirpe W29 foi também estudada em biorreator, tendo sido avaliada a influência da concentração de oxigénio dissolvido (OD) e do pH na produção. Observou-se que um aumento na concentração de OD de 10 % para 30 % levou a uma diminuição na concentração de 2-FE (0,88 *vs* 0,71 g L^{-1}), no entanto a acidificação do meio (pH 5) permitiu aumentar a produção de 2-FE (1,92 g L^{-1}) e o rendimento (0,57 g g^{-1}). O glicerol bruto foi também avaliado como fonte de carbono alternativa para a produção de 2-FE, sendo a concentração de 2-FE obtida com glicerol bruto 33 % superior à obtida com glucose. O efeito da concentração de substrato também foi estudado e foi possível observar que o aumento da concentração do glicerol bruto não aumenta a eficiência de consumo da L-Fe nem a concentração de 2-FE. Foi ainda testada uma estratégia de produção com remoção do produto por adsorção, tendo sido selecionada a resina XAD-4 uma vez que permitiu adsorver mais 2-FE e menos L-Fe. A adição de 7 % (húmido p/v) de resina ao meio de bioconversão permitiu aumentar em 1,2 vezes a produção de 2-FE quando comparada com os ensaios sem remoção de produto. Assim, o presente trabalho demonstra o potencial da estirpe *Y. lipolytica* W29 para a produção de 2-FE.

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LIST OF ABBREVIATIONS

2-FE	*2-feniletanol*
2-PE	2-phenylethanol
ADHx	Alcohol dehydrogenase
ALD	Aldehyde dehydrogenase
ALD3	Gene codifying cytosolic aldehyde dehydrogenase
AROx	Gene codifying aromatic/aminoadipate aminotransferase
ATF	Alcohol acetyltransferase
b	Y-intercept
C_a	Concentration of 2-phenylethanol or L-phenylalanine in the aqueous phase after the absorption
C_b	Concentration of 2-phenylethanol or L-phenylalanine in the aqueous phase before the absorption
CM	Chorismate mutase
DAHP	3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase
DO	Dissolved oxygen
E	Adsorption ratio
FDA	Food and Drug Administration
GAP1	Gene codifying permease
GRAS	Generally Regarded As Safe
HPLC	High Performance Liquid Chromatography
ISPA	*In situ* product removal by adsorption
ISPR	*In situ* product removal
KDC	Phenylpyruvate decarboxylase
LD	Detection limit
L-Fe	*L-fenilalanina*
L-Phe	L-phenylalanine
LQ	Quantification limit
m	Slope
OD	*Oxigénio dissolvido*

OD_{600}	Optical density at a wavelength of 600 nm
PDCx	Gene codifying phenylpyruvate decarboxylases
PES	Polyethersulfone
PT	Prephenate dehydrogenase
RI	Refractive index
rpm	Rotation per minute
S_b	Standard error associated to the y-intercept
S_{b1}	Standard error of the independent term in the regression equation
S_m	Standard error associated to the slope
THI3	Gene codifying phenylpyruvate decarboxylases
TYR1	Gene codifying prephenate dehydrogenase
UHPLC	Ultra High Performance Liquid Chromatography
YNB	Yeast Nitrogen Base
YPDA	Yeast extract Peptone Dextrose Agar
YPD	Yeast extract Peptone Dextrose
YPG	Yeast extract Peptone Glycerol
$Y_{2\text{-PE/L-Phe}}$	2-Phenylethanol yield on L-phenylalanine
$Y_{X/S}$	Biomass yield on substrate

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1. RESEARCH AIM

1.1 Introduction

The most common processes to produce flavor compounds are the extraction from natural sources and the chemical synthesis. However, extraction from plants has many disadvantages such as low concentration of the product of interest, seasonal variation, risk of plant diseases and ecological problems. In fact, chemical synthesis still represents the cheaper technology for their production, nevertheless, the compounds are labeled as "artificial" or "nature identical" decreasing their economic value (Longo and Sanromán, 2006; Vandamme and Soetaert, 2002). The consumers demand for flavor compounds produced by natural means has led to a decrease of natural resources, and, in this scenario, the use of microorganisms as biotechnological platforms for its production is becoming a promising alternative. Nevertheless, the industrial feasibility of a bioprocess depends on its productivity and the main constrain for industrial exploitation of these biotechnological processes is its very poor productivity.

Yarrowia lipolytica is a yeast species already explored for the production of some aroma compounds, particularly γ-decalactone (Braga and Belo, 2016). Moreover, this yeast has a Generally Regarded As Safe (GRAS) status and exceptional performance in utilization of different raw biomaterials (Nicaud, 2012). However, *Y. lipolytica* has been poorly explored for the production of other flavor compounds with industrial relevance, such as 2-phenylethanol (2-PE) (Celińska et al., 2013; Celińska et al., 2015).

The aromatic alcohol 2-PE is of great importance for the flavor and fragrances industry, being its production estimated by around 10 000 tons per year in the global market. Its application in several products from cosmetics and perfumery to food, or its usage as a raw material in the production of other important flavor and pharmaceutical compounds, make the 2-PE one of the most commercially important flavors (Carlquist et al., 2015). In eukaryotes, 2-PE can be produced by *de novo* synthesis from glucose via shikimate pathway or by bioconversion of L-phenylalanine (L-Phe) through the Ehrlich pathway (Etschmann et al., 2002). The main challenge for the biotechnological production of 2-PE is its cytotoxicity (Hua et al., 2010), and a lot of efforts has been made in order to improve the process yields and productivity, such as optimization of medium composition (type and concentration of carbon source and L-Phe concentration) (Celińska et al., 2013; Chantasuban et al., 2018; Chreptowicz et al., 2016; Conde-Báez et al., 2017; Cui et al., 2011; Etschmann et al., 2003; Etschmann et al., 2004; Fabre et al., 1998b; Garavaglia et al., 2007; Huang et al., 2000; Martínez-Avila et al., 2018a), operational conditions (pH, temperature, agitation speed, aeration and mode of operation) (Chantasuban et al.,

2018; Garavaglia et al., 2007; Huang et al., 2001; Martínez-Avila et al., 2018a; Stark et al., 2003b) and application of *in situ* product removal (ISPR) techniques (Chantasuban et al., 2018; Chreptowicz and Mierzejewska, 2018; Gao and Daugulis, 2009; Hua et al., 2010; Kim et al., 2014a; Mihal' et al., 2014; Okuniewska et al., 2017; Sendovski et al., 2010) However, most of these works were performed with *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, that are not able to naturally use residues and wastes as substrates and are Crabtree positive microorganisms (ethanol has a synergetic negative effect with 2-PE).

The main objective of this thesis is to contribute to the improvement of the 2-PE production from L-Phe bioconversion, using the yeast *Y. lipolytica*. Thus, the work address strain selection, medium composition and culture condition optimization and the application of ISPR techniques. The present work will offer an excellent opportunity to overcome the current scientific and technological barriers in the field of bio-industry, for the generation of a platform for sustainable and bio-based production of flavor compounds.

1.2 Outputs of this thesis

Oral communications

1) Braga, A.; Oliveira, A.; Freitas, B.; Nagy, E. S.; Quang D. N.; Belo, I. *Yarrowia lipolytica* as a potential producer of 2-phenylethanol from L-phenylalanine biotransformation. 13th International Chemical and Biological Engineering Conference (CHEMPOR 2018), Aveiro, Portugal, 2-4 October, 2018.

2) Braga, A.; Oliveira, A.; Freitas, B.; Nagy, E. S.; Quang D. N.; Belo, I. An adsorptive approach to enhance the 2-phenylethanol (2-PE) production from L-phenylalanine (L-Phe) biotransformation. Third International Conference on Food Science and Technology (FoodConf), Hungry, Budapest, 29 November – 1 December, 2018.

2. LITERATURE REVIEW

2.1 Flavor compounds

Flavor is the result of the presence of many volatile and non-volatile compounds (Longo and Sanromán, 2006), mostly appreciated by the sense of smell, but also of taste (Burdock, 2010). The compounds with flavoring properties represent a wide variety of chemical classes including ketones, aldehydes, alcohols, carboxylic acids, esters, lactones and terpenoids (Carlquist et al., 2015). These compounds are widely applied in the food, feed, cosmetic, chemical and pharmaceutical sectors (Carlquist et al., 2015; Vandamme and Soetaert, 2002). Over a quarter of the world market for food additives is represented by the flavor market (Longo and Sanromán, 2006) and it was estimated to be close to $ 26.3 billion in 2017 (Leffingweel & Associates, 2018).

The most common processes to produce flavor compounds are the chemical synthesis and the extraction from natural sources however, an increase towards its biotechnological production (bioflavors) has been observed (Vandamme and Soetaert, 2002). In view of the development of the bioflavor concept, the term "natural" has been clearly defined: the U.S. Food and Drug Administration (FDA) and European legislations regard compounds obtained by microbial or enzymatic processes as ''natural'', as long as the precursors used for its production are from vegetal or animal origin (E. C. Regulation, 2008; U.S. Food & Drug Administration, 2016). Besides that, the increasing consumer interest for products labeled as "natural", the adaptation of an healthy life style, associated with more health awareness, and the restriction of some chemical synthesized flavors in food, beverages and cosmetics (according to U.S.A and European legislations) promote the development of biotechnological and eco-efficient production processes (Longo and Sanromán, 2006).

Biotechnological approaches to produce flavor compounds can be divided in two classes: whole microbial cell and enzymatic methods (Carlquist et al., 2015; Longo and Sanromán, 2006). The first one can be subdivided into *de novo* synthesis (simple cultivation media without precursor additions) and biotransformation/bioconversion (addition of precursors to the cultivation media) (Carlquist et al., 2015; Longo and Sanromán, 2006).

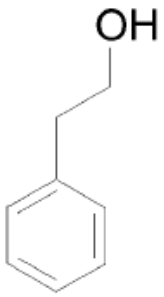
2.2 The flavor compound 2-PE

2-PE is an aromatic alcohol with a delicate fragrance of rose petals (Burdock, 2010) widely applied in diverse types of products, such as perfumes, cosmetics, pharmaceuticals, foods and beverages (Carlquist et al., 2015). Furthermore, 2-PE can also be used as raw material to produce other important flavors compounds, such as 2-phenylethylacetate which is a high-value aromatic ester

(Etschmann et al., 2002; Zhang et al., 2014) and a potential fuel molecule (Guo et al., 2017), phenylacetaldehyde and *p*-hydroxphenylethanol used in pharmaceutical and fine chemical industries (Zhang et al., 2014). 2-PE can also be used as a precursor in the production of biopolymers with flavoring properties, as octane booster ethyl cyclohexane (Chantasuban, 2016).

The economic importance of 2-PE rose-like fragrance is quite significant; in 1990 it was reported that the 2-PE global market was estimated at 7000 tones, with 6000 tons used for fragrances, 10 tons for flavor applications and 990 tons for synthesis of reaction products like esters (Etschmann et al., 2002). With the current available information, the global production of 2-PE is estimated at 10 000 tons per year and is dominated by chemical synthesis (Angelov and Gotcheva, 2012; Hua and Xu, 2011). The information about its natural production is scarce however, in 2002 its market was estimated in 0.5 ton (Etschmann et al., 2002; Schrader et al., 2004). In 2011, 2-PE produced by chemical synthesis was available at around 5 US $ per kg, whereas the same material extracted from flowers was at 1000 US $ per kg (Hua and Xu, 2011). Some physical and chemical properties of this aromatic alcohol are presented in Table 1.

Table 1. Physical and chemical properties of 2-PE (Burdock, 2010; Fabre et al., 1998a).

Physical and chemical properties	Value	Structure
Molecular weight	122.17 g mol^{-1}	 ($C_8H_{10}O$)
Fragrance threshold values	0.015 ppb - 3.5 ppm	
Taste threshold value	20 ppm	
Boiling point	219 - 222 °C	
Density (25 °C)	1.017 - 1.020	
Solubility in water at 20 °C	19 g L^{-1}	

2.3 2-PE production

2.3.1 Natural sources and chemical synthesis

The flavor compound 2-PE is found in the essentials oils of many vegetal sources worldwide, such as narcissus, hyacinth, *Bourbon geranium*, *Alep pine*, rose (*Rose centifolia*), jasmine or lilies, but only 1 - 5 % of 2-PE is present in the essential oils of these plants (Burdock, 2010; Fabre et al., 1998a). An exception to the low concentration of this aromatic alcohol in essentials oils is the rose oil: up to 60 % (v/v) of 2-PE can be obtained from rose floral buds (Burdock, 2010; Etschmann et al., 2002).

However, these extractions have a low yield and its purification is often difficult, making the overall process too expensive (Fabre et al., 1998a; Hua and Xu, 2011; Longo and Sanromán, 2006). Also, seasonal variations and plant diseases lead to a constant shortfall of product supply from natural sources (Longo and Sanromán, 2006).

Giving the limitations of 2-PE production by natural sources, the chemical synthesis is an attempt to liberate the aroma industry from a dependence upon these factors, producing most of the today's product in the market (Angelov and Gotcheva, 2012; Hua and Xu, 2011). This flavor compound can be chemically synthesized via a Friedel–Craft reaction of benzene and ethylene oxide in the presence of molar quantities of aluminum chloride (Bauer et al., 2001), by hydrogenation of styrene oxide at low temperature using Raney nickel as a catalyst and a small amount of sodium hydroxide (Bauer et al., 2001) or as a by-product of the co-production of propylene oxide and styrene (Oyague et al., 2000).

Even though the chemical synthesis is reliable on an industrial scale and with a very competitive price, there are several problems related with this production process: is an environmentally unfriendly process due to the toxic reagents used and harsh conditions, has a low selectivity for the desired product and accumulation of toxic by-products (Angelov and Gotcheva, 2012; Hua and Xu, 2011; Vandamme and Soetaert, 2002).

2.3.2 Biotechnological production

Considering the disadvantages of chemical production, regarding the quality of the product, health and environmental issues and the inability of natural production at industrial scale, the need to address an alternative platform for 2-PE production through low-cost and environmentally friendly processes became crucial. As previously referred, legislations consider that compounds obtained by microbial or enzymatic processes as "natural", along with the consumers preference for natural products, making the biotechnological processes an interesting alternative for flavors production (Longo and Sanromán, 2006). Moreover, its biotechnological production has several advantages in detriment of chemical and vegetal synthesis, such as causing less damaging conditions, yielding the desirable enantiomeric flavor compound and producing specific single flavor molecules (Vandamme and Soetaert, 2002).

In fact, microorganisms are able to produce 2-PE and other alcohols by normal metabolism as the result of aminoacid catabolism, but the final concentration of the alcohol is very low (Carlquist et al., 2015). However, Chantasuban et al. (2018) reported recently the 2-PE production by *de novo* synthesis with the yeast *Metschnikowia pulcherrima* NCYC373. The concentrations attained ranged from 0.3 to

1.0 g L^{-1} at shake-flask scale in synthetic grape juice media (Chantasuban et al., 2018). The biotechnological approach used to obtain 2-PE by bioconversion of L-Phe, as the sole nitrogen source via the Ehrlich pathway, is the best and simple way to enhance its production (Äyräpää, 1965; Etschmann et al., 2002). Nevertheless, product inhibition is the major problem of this alcohol biosynthesis (Fabre et al., 1998a), along with the synergetic inhibitory effect caused by ethanol formation (Stark et al., 2002).

The 2-PE biosynthesis can be performed by fungi (Lomascolo et al., 2001; Masuo et al., 2015) and bacteria (Zhang et al., 2014). However, yeasts are the most promising producers due to their high catalytic activity, robustness and cell resistance to the inhibitory product (Carlquist et al., 2015). Crabtree negative yeasts are preferable in order to avoid the production of ethanol (Carlquist et al., 2015; Stark et al., 2002).

There are several yeasts described in the literature with the potential to produce 2-PE, being the most studied and reported *K. marxianus* and *S. cerevisiae*, with 2-PE concentrations ranging from 0.4 to 4.0 g L^{-1} (Fabre et al., 1995; Mihal' et al., 2013; Mihal' et al., 2014; Mihal' et al., 2012). Whereas the application of ISPR techniques to the process can allow to achieve a 2-PE concentration of 20.4 g L^{-1} (Gao and Daugulis, 2009). However, recently a novel stress-tolerant yeast *Candida glycerinogenes* WL2002-5 was able to produce 5 g L^{-1} of 2-PE in a simple batch fermentation supplemented with L-Phe (Lu et al., 2016).

Table 2 presents a short summary of the microorganisms described in the literature able to produce 2-PE, the operation conditions and the product concentrations attained.

Table 2. General overview of microorganisms able to produce 2-PE and respective operation conditions.

Strain	Operation conditions	2-PE (g L^{-1})	Reference
C. glycerinogenes WL2002-5	Batch with L-Phe supplementation	5.0	(Lu et al., 2016)
S. cerevisiae JM2014	Batch with L-Phe supplementation	3.6	(Chreptowicz et al., 2016)
	Shake-flask with L-Phe supplementation in whey media supplemented with thick juice	3.3	(Chreptowicz et al., 2017)
K. lactis 6.10	Shake-flask with L-Phe supplementation	3.11	(Celińska et al., 2018)
Pichia cactophila 7.20		3.07	
P. fermentans WUT36	Shake-flask with L-Phe supplementation in whey media supplemented with glucose	2.35	(Chreptowicz et al., 2017)
Y. lipolytica NCYC3825	Shake-flask with L-Phe supplementation	1.98	(Celińska et al., 2013)
Hansenula anomala CBS110	Batch with L-Phe supplementation	1.73	(Albertazzi et al., 1994)
M. pulcherrima NCYC373	Shake-flask with L-Phe supplementation in synthetic grape juice media	1.71	(Chantasuban et al., 2018)
S. cerevisiae	Shake-flask without L-Phe supplementation in tobacco waste and glucose media	1.55	(Wang et al., 2013)
M. pulcherrima NCYC373	Shake-flask without L-Phe supplementation in synthetic grape juice media	1.0	(Chantasuban et al., 2018)
K. marxianus CBS600	Batch with L-Phe supplementation	1.0	(Adler et al., 2011)
K. marxianus ITD00262	Shake-flask without L-Phe supplementation in whey media	0.96	(Conde-Báez et al., 2017)
K. marxianus (Hansen) van der Walt	Shake-flask with L-Phe supplementation	0.92	(Fabre et al., 1998b)
K. marxianus CBS600	Shake-flask with L-Phe supplementation in beet molasses media	0.89	(Etschmann et al., 2003)
S. cerevisiae Ye9-612	Shake-flask with L-Phe supplementation	0.85	(Eshkol et al., 2009)
P. fermentans L-5	Shake-flask with L-Phe supplementation	0.52	(Huang et al., 2001)
K. marxianus CBS6556	Shake-flask with L-Phe supplementation in grape must media	0.47	(Garavaglia et al., 2007)

Table 2. General overview of microorganisms able to produce 2-PE and respective operation conditions (continuation).

Strain	Operation conditions	2-PE (g L^{-1})	Reference
K. marxianus (Hansen) van der Walt	Fed-batch process without L-Phe supplementation	0.40	(Fabre et al., 1995)
K. marxianus ATCC10022	Fed-batch process with L-Phe supplementation in solid-state fermentation of sugarcane bagasse media	0.010 (mass of product per mass of solid)	(Martínez-Avila et al., 2018a)

2.4 Biochemical pathways

As referred above, 2-PE and other alcohols are naturally produced by microorganisms as part of their aminoacid catabolism. In yeasts, 2-PE biosynthesis is connected to the shikimate, Ehrlich and cinnamate pathways (Figure 1) (Albertazzi et al., 1994; Carlquist et al., 2015; Etschmann et al., 2002; Tzin et al., 2012). Through the shikimate pathway, 2-PE is obtained by *de novo* synthesis from carbohydrate precursors (Albertazzi et al., 1994; Carlquist et al., 2015; Etschmann et al., 2002; Tzin et al., 2012), the Ehrlich pathway give 2-PE from L-Phe bioconversion (Albertazzi et al., 1994; Carlquist et al., 2015; Etschmann et al., 2002), and the cinnamate pathway degrade L-Phe, but does not produce 2-PE (Etschmann et al., 2002).

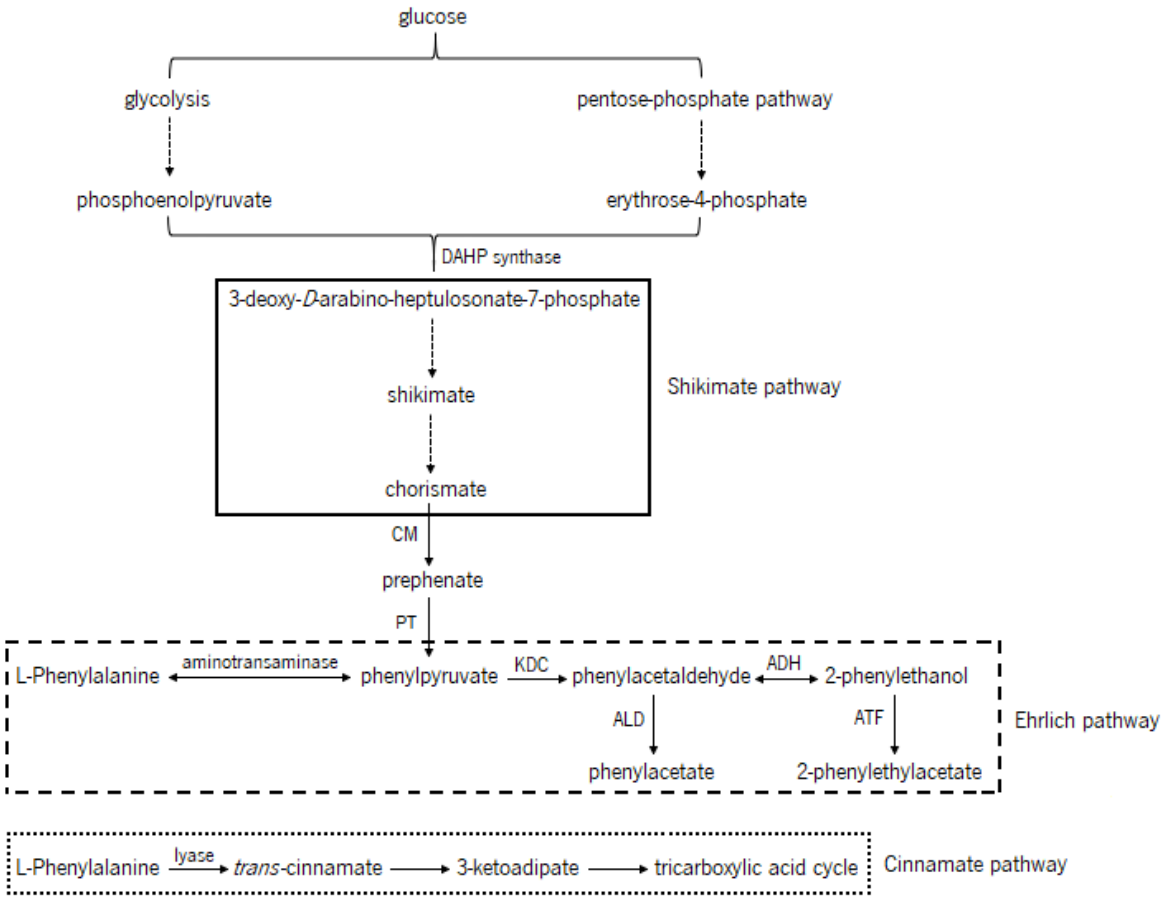


Figure 1. Schematic representation of the pathways related to the 2-PE biosynthesis in yeasts. DAHP synthase, 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase; CM, chorismate mutase; PT, prephenate dehydrogenase; KDC, phenylpyruvate decarboxylase; ADH, alcohol dehydrogenase; ALD, aldehyde dehydrogenase; ATF, alcohol acetyltransferase (adapted from Etschmann et al., 2002; Kang et al., 2014; Tzin et al., 2012).

The shikimate pathway is an important natural pathway in organisms to synthesize aromatic aminoacids such as L-Phe, tryptophan and tyrosine from carbohydrate precursors, found in bacteria, yeasts, fungi and plants (Tzin et al., 2012). In the shikimate pathway, the intermediates from glycolysis and pentose-phosphate pathway (phosphoenolpyruvate and erythrose-4-phosphate) are converted to chorismate. The synthesis of the aminoacid L-Phe begins with chorismate, the terminal metabolite of the shikimate pathway, and ends with the formation of phenylpyruvate (Tzin et al., 2012). Phenylpyruvate is then decarboxylated to phenylacetaldehyde followed by a dehydrogenation to 2-PE (Carlquist et al., 2015; Etschmann et al., 2002). Nevertheless, *de novo* synthesis is inefficient and is not economically viable or applicable to a bioprocess, since glycolysis and the pentose-phosphate pathway are mainly used for cell growth, producing very low concentrations of 2-PE (Carlquist et al., 2015; Etschmann et al., 2002).

Through the Ehrlich pathway, L-Phe is firstly converted to phenylpyruvate by transamination, which is then transformed to phenylacetaldehyde by decarboxylation (Carlquist et al., 2015; Etschmann et al., 2002). Then, the derivative aldehyde may be reduced to 2-PE by an alcohol dehydrogenase or oxidize in phenylacetate (Hazelwood et al., 2008; Kim et al., 2014a). Furthermore, alcohol acetyltransferase can further convert 2-PE to its ester derivative, 2-phenylethylacetate (Guo et al., 2018; Huang et al., 2001). The Ehrlich pathway is a convenient and fast pathway to synthesize 2-PE (ÄYräpää, 1965; Etschmann et al., 2002) however, cheaper precursors than L-Phe should be used to achieve a more economic production (Kim et al., 2014b; Zhang et al., 2014).

In the cinnamate pathway, the first step in the L-Phe degradation is the removal of the amino group by phenylalanine ammonia lyase to yield *trans*-cinnamate; then, if the aminoacid is the sole nitrogen source, it is degraded via protocatechuate to 3-ketoadipate and fed into the tricarboxylic acid cycle (Etschmann et al., 2002). However, this alternative pathway of L-Phe utilization does not lead to 2-PE formation, being undesirable. The aminoacid degradation can be shifted to the Ehrlich pathway, yielding 2-PE, when L-Phe is the only nitrogen source available in culture media (ÄYräpää, 1965). However, even with high L-Phe concentrations, the cinnamate pathway is not completely repressed (Etschmann et al., 2002).

2.5 *Yarrowia lipolytica*

Yarrowia lipolytica is an eukaryotic microorganism from the Fungi kingdom (class Ascomycetes and sub-class Hemiascomycetes) (Barth and Gaillardin, 1997; Nicaud, 2012; van Der Walt and von Arx, 1980). Despite its classification as a non-conventional yeast (term to differentiate the frequently used and studied conventional yeasts from the not well known), it became one of the most studied yeast, such as *S. cerevisiae* (Barth and Gaillardin, 1997; Beopoulos et al., 2010; Nicaud, 2012). Due to its peculiar characteristics, phenotypes, unique metabolic properties and knowledge of its complete genome sequence, *Y. lipolytica* has emerged as a good model organism (Nicaud, 2012). Studies, such as hydrophobic substrates utilization, peroxisome biogenesis, identification of genes involved in the yeast-to-hyphae transition, protein secretion and development of efficient tools for heterologous protein production, represent the main achievements about the application of *Y. lipolytica* (Nicaud, 2012).

This strictly aerobic yeast has a maximal growth temperature at 32 - 34 °C and is considered as non-pathogenic and classified as GRAS by the FDA (Barth and Gaillardin, 1997; Beopoulos et al., 2010). *Y. lipolytica* is also a dimorphic microorganism, capable to grow reversibly in two distinct morphological forms, usually as single oval cells or as filamentous hyphae (Beopoulos et al., 2010;

Nicaud, 2012). Wild type strains can exhibit various colony shapes, smooth and glistening to heavily convoluted and mate, determined by growth conditions and genetic background of the strain (Beopoulos et al., 2010; Nicaud, 2012).

In nature, *Y. lipolytica* can be found in dairy products, such as cheese and yoghurts, but also in sausages, kefir, shoyu and salads containing meat or shrimps (Barth and Gaillardin, 1997; Beopoulos et al., 2010). Some *Y. lipolytica* strains have also been isolated from lipid-rich media, such as soil, sewage or oil-polluted environments and also from hypersaline environments indicating its versatile metabolism and ability to survive under extreme conditions (Beopoulos et al., 2010).

Carbon sources, such as glucose, galactose, mannitol, organic acids, aminoacids, glycerol and ethanol can be used by *Y. lipolytica*. Besides those, this specie can utilize hydrophobic substrates, such as alkanes, *n*-paraffins, lipids and triglycerides (Barth and Gaillardin, 1997; Ledesma-Amaro and Nicaud, 2016). Nowadays, its capability for the bioconversion of different substrates into high-value compounds, like lipases and proteases and flavor compounds, intracellular accumulation of oil and proteins, production of dicarboxylic acids, organic acids and sweeteners, make this yeast industrially attractive (Beopoulos et al., 2010; Nicaud, 2012; Thevenieau et al., 2009).

2.5.1 2-PE production with *Y. lipolytica*

Yarrowia lipolytica has been very poorly explored for the production of 2-PE, despite its interesting characteristics for this process, such as the Crabtree negative trait and GRAS status (Spencer et al., 2002).

Celińska et al. (2013) firstly reported the ability of different *Y. lipolytica* strains to produce 2-PE. In this study, six *Y. lipolytica* strains were cultured without supplementation of L-Phe in order to access their ability to produce 2-PE via *de novo* synthesis. It was observed that the production is not uniform among all strains tested, being the process efficiency strain dependent, as already reported for other species (Albertazzi et al., 1994; Eshkol et al., 2009; Etschmann et al., 2003; Lomascolo et al., 2001). Further experiments were also performed with the best strain, NCYC3825, at shake-flask scale under non-optimized conditions, with glucose as the carbon source and L-Phe addition in the late stationary phase. Under those conditions, it was reported a 2-PE production of 1.98 g L^{-1}, with a productivity and a process yield of 20 mg L^{-1} h^{-1} and 0.31 g g^{-1}, respectively. The use of glycerol as an alternative carbon source to produce 2-PE was also evaluated at bioreactor scale (L-Phe was also added to the culture medium, but at the beginning of the experiment), attaining a 2-PE production of 0.77 g L^{-1}, productivity of 14.52 mg L^{-1} h^{-1} and yield of 0.09 g g^{-1}.

In fact, the maximum bioconversion yield reported for *Y. lipolytica* NCYC3825 (0.31 g g^{-1}) is lower than the maximum theoretical value of 0.75 g g^{-1} (Etschmann et al., 2002). Nevertheless, comparing the 2-PE titers obtained in this study with others described in the literature indicates the potential of *Y. lipolytica* for the production of this flavor compound.

2.6 New insights into 2-PE production

To improve the 2-PE concentration and process yield, different strategies were investigated, begin the most common strain selection, genetic manipulation or mutagenesis and medium composition or culture conditions optimization (Hua and Xu, 2011).

2.6.1 Metabolic engineering

Since yeasts are good candidates for 2-PE production from L-Phe bioconversion, considerable progresses have been achieved by applying different metabolic engineering strategies of the related pathways. As previously referred, the Ehrlich pathway is a convenient and fast pathway to synthesize 2-PE thus, most of the works explore the main genes and corresponding proteins involved in this pathway (Kim et al., 2014a; Wang et al., 2017; Yin et al., 2015). In yeasts, several genes are involved in the catabolism of L-Phe to 2-PE via Ehrlich pathway. Aro8p and Aro9p enzymes encoded by *ARO8* and *ARO9* genes were both characterized as aminotransferases that catalyze the transamination from L-Phe to phenylpyruvate and the decarboxylation reaction was described with five genes (*ARO10*, *THI3*, *PDC1*, *PDC5* and *PDC6*) encoding phenylpyruvate decarboxylases (KDC) (Hazelwood et al., 2008). Kim et al. (2014a) reported the first metabolic engineering approach for 2-PE production in *S. cerevisiae*, overexpressing *ARO9* and *ARO10* genes. This study allowed genetic engineered strains to produce 0.45 g L^{-1} of 2-PE after 96 h. The deletion of *ALD3* gene, that encodes for cytosolic aldehyde dehydrogenase (ALD) who catalyze the phenylacetaldehyde oxidation to phenylacetate, was also tested. Being phenylacetate a competitor to 2-PE formation, the deletion of this gene increased the 2-PE production to 4.8 g L^{-1} (Kim et al., 2014a). The studies conducted by Wang et al. (2017) with *S. cerevisiae* YS58, that overexpress *ARO8* and *ARO10* genes, leads to a 2-PE production of 3.2 g L^{-1} after 60 h, increasing in 42 % its production when compared with parental strain. More recently, the over-expressing of GATA transcriptions factors Gln3p and Gat1p was reported as a novel approach to enhanced 2-PE production (Chen et al., 2017; Wang et al., 2018). These transcription factors regulate the transcription of genes encoding permeases and catabolic enzymes for nitrogen sources and aminoacid metabolism. Their overexpression increases the expression of *ARO9* and *ARO10* genes, as well as the *GAP1* gene

(encodes a permease in the aminoacid transportation), resulting in a more efficiently transportation of L-Phe into cells (Chen et al., 2017; Wang et al., 2018). These studies demonstrated the improvement of 2-PE production by overexpression of key enzymes in the Ehrlich pathway, providing new perspectives to enhance ''natural'' 2-PE production.

Nevertheless, the Ehrlich pathway uses L-Phe as a precursor for 2-PE production and precursors cheaper than L-Phe should be considered to achieve an economic production process (Kim et al., 2014b; Zhang et al., 2014). Thus, different strains were genetically engineered for overproduction of 2-PE from monosaccharide (Guo et al., 2018; Kang et al., 2014; Kim et al., 2014b; Shen et al., 2016; Zhang et al., 2014). Most studies focus on over-expressing the KDC and alcohol dehydrogenase (ADH) enzymes but also the 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase (DAHP), the chorismate mutase (CM) and the prephenate dehydrogenase (PT), which are the rate-limiting enzymes in *de novo* synthesis pathway. Kim et al. (2014b) overexpressed the *ARO10* and *ADH2* genes of *S. cerevisiae* in *K. marxianus* BY25569. Then, serial subcultures with an L-Phe analog, *p*-fluorophenylalanine, were conducted in order to obtain an evolved strain resistant to the L-Phe analog. Finally, the overexpression of *ARO*G gene from *Klebsiella pneumoniae*, that encodes a feedback resistant mutant of DAHP synthase, was also performed. This genetically modified strain was able to produce 1.3 g L^{-1} of 2-PE from glucose without addition of L-Phe (Kim et al., 2014b). Cordente et al. (2018) also studied the 2-PE and 2-phenylethylacetate production by natural and chemically mutagenised populations of *S. cerevisiae* wine strain, AWRI796. With these new strains it was possible to increase in 20-fold the 2-PE and 2-phenylethylacetate concentrations. The resistant colonies presented mutations in the *ARO4* and *TYR1* genes, which encodes the DAHP and PT, the rate-limiting enzymes of *de novo* synthesis pathway, being responsible for the product overproduction (Cordente et al., 2018).

As previously referred, yeasts are the most promising producers of 2-PE, unlike bacteria whose production do not seem viable for industrial scale (Jollivet et al., 1992). However, the microbial production of 2-PE by engineered *Escherichia coli* and *Enterobacter* sp. was recently reported (Guo et al., 2017; Guo et al., 2018; Kang et al., 2014; Liu et al., 2018; Zhang et al., 2014). Zhang et al. (2014) isolated and identified a new *E.* sp. CGMCC 5087 strain, which is able to produce 2-PE by *de novo* synthesis. By the overexpression of the DAHP synthase and CM-PT enzymes, the engineered strain produces 0.33 g L^{-1} of 2-PE in 12 h. Guo et al. (2018) constructed a heterologous pathway to produce 2-PE directly from glucose in *E. coli*. The coordinated overexpression of DAHP synthase and CM-PT to increase phenylpyruvate production, followed by the heterologous expression of KDC and overexpression of ADH, and finally the heterologous expression of aminotransferase to redirect the

metabolic flux to phenylpyruvate, allowed a 2-PE concentration of 1.0 g L^{-1}. It was discovered that *Proteus mirabilis* JN458 bacteria contained a novel pathway to produce 2-PE, which is different from those find in plants and yeasts (Stumpf and Green, 1944) . More recently, Liu et al. (2018) imitated this pathway from *P. mirabilis* in *E. coli* and the recombinant strain was able to produce 3.21 g L^{-1} of 2-PE with L-Phe supplementation.

2.6.2 Culture media

2-PE production is highly dependent on media composition and culture conditions (Garavaglia et al., 2007). Several studies described different types and concentrations of carbon sources that are able to increase 2-PE production (Celińska et al., 2013; Chantasuban et al., 2018; Chreptowicz et al., 2016; Conde-Báez et al., 2017; Cui et al., 2011; Etschmann et al., 2003; Etschmann et al., 2004; Fabre et al., 1998b; Garavaglia et al., 2007; Huang et al., 2000; Martínez-Avila et al., 2018a).

Huang et al. (2000) studied the effect of different carbon sources (glucose, fructose, galactose, xylose, maltose, lactose, sucrose, soluble starch, sorbitol, glycerol and ethanol) and their concentration, as well as the influence of yeast extract and L-Phe concentrations in 2-PE production by *P. fermentans* L-5. Their results showed that glycerol and sucrose were the most suitable carbon sources to produce 2-PE, and L-Phe concentrations up to 0.1 % increases its production. Celińska et al. (2013) also reported the use of glycerol as carbon source in bioconversion of L-Phe to 2-PE by *Y. lipolytica* NCYC3825, reaching a 2-PE production of 0.77 g L^{-1} after 54 h. In *de novo* production of 2-PE by *M. pulcherrima* NCYC373, Chantasuban et al. (2018) reported a less efficient production in glycerol and xylose when compared with glucose.

Current agricultural and industrial practices have led to the generation of large amounts of agro-industrial residues that can also be used in the production of added value compounds, such as 2-PE. An interesting alternative carbon source for 2-PE production from L-Phe bioconversion with *K. marxianus* CBS6556 is grape must, that allowed a final titer of 0.77 g L^{-1} in a batch process (Garavaglia et al., 2007). Etschmann et al. (2003) also reported the use of molasses-based medium to produce 2-PE by *K. marxianus* CBS600, being observed a 4-fold increase in the production levels in comparison to synthetic medium. Tobacco was also reported as an alternative waste media for 2-PE production via *de novo* synthesis by *S. cerevisiae*, reaching a maximum titer of 1.55 g L^{-1} (Wang et al., 2013). The use of whey, a by-product from milk and cheese processing, is another interesting residue recently applied in the 2-PE production. *K. marxianus* ITD00262 was able to produce a maximum 2-PE concentration of 0.96 g L^{-1} using sweet whey as a substrate (Conde-Báez et al., 2017). Chreptowicz et al. (2017) also

described the application of whey for 2-PE production. However, the culture media was supplemented with glucose or by-products from sugar beet processing, as a fermentable carbon source. The authors were able to identify new strains capable to produce over 2 g L^{-1} of 2-PE through the L-Phe bioconversion in a batch mode (Chreptowicz et al., 2017) . Another recent alternative approach for this flavor production was reported by Martínez-Avila et al. (2018a). In the proposed system, *K. marxianus* ATCC10022 used the available nutrients from a residue-substrate (sugarcane bagasse) supplemented with L-Phe, achieving a 2-PE production of 10.21 mg g^{-1} (mass of product per mass of solid) in a fed-batch system.

The aminoacid L-Phe is another important media component. As already referred, high L-Phe concentrations are needed to shift the cell metabolism to the Ehrlich pathway and enhancing the 2-PE production (Etschmann and Schrader, 2006).

2.6.3 Mode of operation

In microbial processes, three different modes of operation have been extensively applied: batch, fed-batch and continuous mode. In batch mode, all nutrients required are added at the beginning of the fermentation and remain in the bioreactor until the process ends. With a fed-batch process, the addition of components occurs gradually during the cycle until the reactor volume reaches its maximum and, as batch mode, the components remain in the bioreactor until the process ends. Continuous mode allows the continuous addition of nutrients to the bioreactor and fractions of the medium are removed at the same flow rate as the supplied nutrients are added so, the volume of the culture is kept constant. This feature is especially interesting for 2-PE production, considering its toxicity to the cells. With that approach, it is possible to remove or dilute 2-PE in the medium reducing the inhibitory effect and maximizing the productivity.

Several studies described the L-Phe bioconversion in batch mode to produce 2-PE (Albertazzi et al., 1994; Celińska et al., 2013; Chantasuban et al., 2018; Chreptowicz et al., 2016; Cui et al., 2011; Gao and Daugulis, 2009; Garavaglia et al., 2007; Lu et al., 2016; Stark et al., 2003b). Garavaglia et al. (2007) obtained a 2-PE titer of 0.77 g L^{-1} with *K. marxianus* CBS6556, and a bioconversion yield of 0.62 g g^{-1}. With *S. cerevisiae* CWY132 and Giv 2009 strains, a 2-PE production of 3.52 g L^{-1} and 2.35 g L^{-1} was obtained (Cui et al., 2011; Stark et al., 2003b). More recently, L-Phe bioconversion to 2-PE by *C. glycerinogenes* WL2002-5 and *S. cerevisiae* JM2014 achieved a 2-PE concentration of 5.0 and 3.6 g L^{-1}, respectively (Chreptowicz et al., 2016; Lu et al., 2016). In a batch process, the accumulation of 2-PE coupled with ethanol formation have synergetic inhibitory effects, stopping the fermentation at low 2-

PE concentrations (Stark et al., 2003b).

Fed-batch mode on glucose under oxidative conditions is very useful to avoid ethanol formation and, consequently, increasing the 2-PE production (Etschmann and Schrader, 2006; Stark et al., 2002). Gao and Daugulis (2009) described an increase in the product concentration from 13.7 g L^{-1} in batch to 20.4 g L^{-1} with fed-batch (with ISPR). Stark et al. (2002) reported the 2-PE concentration level of 12.6 g L^{-1} (with ISPR) and a bioconversion yield of 0.3 g g^{-1} after 93 h, with *S. cerevisiae* Giv 2009. In comparison, Etschmann and Schrader (2006) obtained 10.2 g L^{-1} of 2-PE with *K. marxianus* CBS600 (with ISPR), but with a higher bioconversion yield (0.74 g g^{-1}). Martínez-Avila et al. (2018a) also described an enhancement in 2-PE and 2-phenylethylacetate production by *K. marxianus* ATCC10022, in solid-state fermentation of sugarcane bagasse, from 16 to 18 mg g^{-1} (mass of product per mass of solid) when feeding strategies were applied. On the contrary, *de novo* production of 2-PE by *M. pulcherrima* NCYC373 decreased when a fed-batch strategy was applied (Chantasuban et al., 2018). Although the batch fermentation allowed a 2-PE concentration of 0.70 g L^{-1}, the production level decreases to approximately 0.35 g L^{-1} in a fed-batch strategy (Chantasuban et al., 2018).

In a continuous process, the ethanol toxicity is avoided, but the L-Phe bioconversion yields are low since most of the precursor is lost in the outflow of the continuous culture (Stark et al., 2003b). Thus, in order to achieve high bioconversion yields, the continuous culture most be performed at a high L-Phe concentration in order to maintain a residual precursor concentration in culture media (Stark et al., 2003b). Wang et al. (2011) compared the 2-PE bioconversion by *S. cerevisiae* R-UV3 in fed-batch and continuous process with product removal. They reported that the fed-batch and continuous mode gave the same molar yield of 2-PE (0.80 mol mol^{-1}), but the continuous culture allowed a productivity of 0.90 g L^{-1} h^{-1}, being the highest reported for 2-PE production (Wang et al., 2011). More recently, *de novo* production of 2-PE by *M. pulcherrima* NCYC373 reached higher titers with continuous mode rather than batch and fed-batch cultures (Chantasuban et al., 2018). In continuous fermentation, 2-PE concentration levels reached 1.5 g L^{-1}, before it became to toxic and caused the flush out (Chantasuban et al., 2018).

2.6.4 ISPR strategies

Even with the efforts to optimize the culture medium and cultivation conditions, and choose the most producing microorganism, product inhibition is still the major problem of 2-PE biosynthesis (Carlquist et al., 2015). Some strategies, such as ISPR techniques, which are the fast removal of product from a producing cell preventing its subsequent interference with cellular components, have

been developed to reduce the 2-PE toxicity in the fermentation medium increasing its production (Carlquist et al., 2015). The potential benefits of the ISPR techniques are the avoidance or reduction of growth inhibition or toxicity by the product, product stabilization and the facilitation of downstream processing (Pérez, 2001). These techniques act by maintaining the product concentration around the cells below an inhibitory level, and the strains are able to continue the 2-PE production (Hua and Xu, 2011). According to the different separation tools employed, the methods can be divided into: two-phase extraction, which could use organic solvents or immiscible ionic liquids; adsorption, where resins or other adsorption media capture the product; solvent immobilization; organophilic pervaporation unit coupled to a bioreactor and membranes coupled with solvent extraction.

The two-phase extraction with organic solvents is the most efficient and commonly method used for 2-PE bioconversion (Gao and Daugulis, 2009; Martínez-Avila et al., 2018b; Mei et al., 2009). This two-phase system involves an aqueous phase where the bioconversion occurs and an organic phase with high 2-PE selectivity who continuously collects the product (Martínez-Avila et al., 2018b). However, the use of organic solvents has several drawbacks: its viscosity leads to the origin of emulsions between the two phases which difficult the recovery process (Gao and Daugulis, 2009; Mei et al., 2009); these compounds usually present high boiling points meaning that they evaporate slowly, being the removal from the aqueous phase more complicated (Hua and Xu, 2011); additionally, the application of organic solvents in a bioprocess could also modify the product quality by affecting its organoleptic properties due to residual odors as well as the cell viability due to the toxicity of some organic solvents (Gao and Daugulis, 2009; Hua and Xu, 2011). There are several organic solvents reported for the two-phase extraction, such as oleic acid (Stark et al., 2002), oleyl alcohol (Chantasuban et al., 2018; Etschmann et al., 2003) or polypropylene glycol 1200 (Etschmann and Schrader, 2006; Kim et al., 2014a). Recently, Chantasuban et al. (2018) reported the application of oleyl alcohol as an extraction phase in the 2-PE production by *M. pulcherrima* NCYC373. The production levels were enhanced with the application of this ISPR technique, achieving a 2-PE concentration of 1.96 g L^{-1} in the aqueous phase and an overall production of 3.13 g L^{-1}. Another two-phase extraction system was reported by Chreptowicz and Mierzejewska (2018) where rapeseed oil was used as the extractant in the bioconversion of L-Phe to 2-PE by *S. cerevisiae* JM2014 achieving a final titer of 9.8 g L^{-1} and an overall productivity of 135.97 mg L^{-1} h^{-1}. Furthermore, the use of immiscible ionic liquids, such as [HMPyr][NTf_2] in two-phase extraction system, allows a suitable extraction capacity and an enhanced biocompatibility (Hua and Xu, 2011; Martínez-Avila et al., 2018b). Okuniewska et al. (2017) reported

the 2-PE production by *S. cerevisiae* AM1-d in the presence of the ionic liquid [HMPyr] [NTf_2], attaining a 2-PE concentration of 3.7 g L^{-1}.

In situ product removal by adsorption (ISPA) uses adsorbent resins or other adsorption media to enable the product recovery (Hua and Xu, 2011). This technique is widely used in the production of high-valued aromatic compounds however, is very poorly applied in the 2-PE production (Gao and Daugulis, 2009; Hua et al., 2010; Mei et al., 2009). With this approach, it is possible to reduce the product toxicity and perform its simultaneously purification, making the downstream process more practical and feasible (Gao and Daugulis, 2009; Hua et al., 2010; Mei et al., 2009). Moreover, contrarily to the organic solvents, the adsorbent resins can be more biocompatible and less inexpensive, non-volatile and do not compromise the product quality (Gao and Daugulis, 2009; Hua et al., 2010). The addition of the macroporous resin D101 and the resin HZ818 to the bioconversion broth, allowed an increase in the production level, being reached a 2-PE concentration of 6.2 and 6.6 g L^{-1}, respectively (Hua et al., 2010; Mei et al., 2009). Gao and Daugulis (2009) reported a highly significant enhancement in the 2-PE production using a solid-liquid two-phase partition bioreactor with polymer beads as the sequestering immiscible phase. The batch mode system reached a final 2-PE concentration of 13.7 g L^{-1} (88.74 g L^{-1} in the polymer phase and 1.2 g L^{-1} in the aqueous phase), whereas the fed-batch achieved an overall titer of 20.4 g L^{-1} (97.0 g L^{-1} in the polymer phase and 1.4 g L^{-1} in the aqueous phase) (Gao and Daugulis, 2009). More recently, Chantasuban et al. (2018) reported a 2-PE production of 14 g L^{-1} with *M. pulcherrima* NCYC373, in a batch fermentation using powdered activated carbon as the adsorbent phase.

Table 3 summarizes some of the works that reported the 2-PE production with ISPR techniques.

Table 3. Overview of 2-PE production with ISPR techniques.

Characteristic of ISPR technique		Strain	2-PE_{total} (g L^{-1})	Reference
Two-phase extraction	Oleyl alcohol	*K marxianus* CBS 600	3.0	(Etschmann et al., 2003)
		M. pulcherrima	3.1	(Chantasuban et al., 2018)
	Ionic liquid BMIM[Tf_2N]	*S. cerevisiae* Ye9-612	2.4	(Sendovski et al., 2010)
	Ionic liquid [HMPyr] [NTf_2]	*S. cerevisiae* AM1-d	3.7	(Okuniewska et al., 2017)
	Rapeseed oil	*S. cerevisiae* JM2014	9.8	(Chreptowicz and Mierzejewska, 2018)
	Polypropylene glycol (PPG) 1200	*K. marxianus* CBS 600	10.2	(Etschmann and Schrader, 2006)
		S. cerevisiae JHY315	6.1 (in the organic phase)	(Kim et al., 2014a)
	Oleic acid	*S. cerevisiae* Giv 2009	12.6	(Stark et al., 2002)
Adsorption	Solid-liquid Two-Phase Partitioning Bioreactor (TPPB) with polymer Hytrel ® 8206	*K. marxianus* CBS 600	13.7 (batch) 20.4 (fed-batch)	(Gao and Daugulis, 2009)
	Activated carbon	*M. pulcherrima*	14.0	(Chantasuban et al., 2018)
	Macroporous resin D101	*S. cerevisiae* BD	6.2	(Mei et al., 2009)
	Resin HZ818	*S. cerevisiae* P-3	6.6	(Hua et al., 2010)
Solvent immobilization	Microcapsules with a hydrophobic core of dibutyl sebacate and an alginate-based wall	*S. cerevisiae* Giv 2009	5.6	(Stark et al., 2003a)
	Diputylsebacate entrapped into a polyethylene matrix		3.8	(Serp et al., 2003)
Organophilic pervaporation	Organophilic pervaporation unit, equipped with a polyoctylmethylsiloxane (POMS) membrane at 40 °C	*K. marxianus* CBS 600	5.9 (2-PE and 2-phenylethylacetate)	(Etschmann et al., 2005)

Table 3. Overview of 2-PE production with ISPR techniques (continuation).

Characteristic of ISPR technique		Strain	2-PE_{total} (g L^{-1})	Reference
Membrane extraction	Hybrid system combined of STR and immersed extraction membrane using pentane as the organic phase	*S. cerevisiae* (baker's yeast)	21.22 (volumetric productivity)	(Mihal' et al., 2014)
	Integrated batch fermentation with membrane extraction using Miglyol as the organic phase	*K. marxianus* CBS600	4.0 (2-PE and 2-phenylethylacetate)	(Adler et al., 2011)

3. MATERIALS AND METHODS

3.1 Microorganisms

The strains used in this work were *Y. lipolytica* W29 (ATCC20460), *Y. lipolytica* CBS2075 (isolated from rancid margarine and used in cheese flavor) and *Y. lipolytica* NCYC2904 (isolated from a maize-processing plant).

3.2 Yeast preservation

Yarrowia lipolytica strains were stored at – 80 °C in cryo-stocks tubes. After thawing, each strain was cultured on YPDA medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 20 g L^{-1} agar, sterilized in an autoclave at 110 °C for 30 min) and incubated at 27 °C during 24 h. The colonies were stored at 4 °C, to be posteriorly used to inoculate the culture media.

3.3 Pre-culture conditions

Cells were prepared as described in section 3.2 and used to inoculate a 500 mL Erlenmeyer flask with 200 mL of YPD medium or YPG medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} pure glycerol, sterilized in an autoclave at 121 °C for 20 min), according to the carbon source used in the bioconversion experiments.

Pre-cultures were incubated in an orbital shaker (Shel Lab, Sheldon Manufacturing, Cornelius, Oregon, USA) at 27 °C, 200 rpm during 16 to 17 h. After this time, cells were washed with NaCl solution (0.9 % (w/v)), centrifuged (8000 rpm during 5 min) and used to inoculate the bioconversion medium with an initial optical density at a wavelength of 600 nm (OD_{600}) of 0.5 (equivalent to a cell density of 0.46 g L^{-1}).

3.4 Inhibitory effect of 2-PE and L-Phe on *Y. lipolytica* cells

To study the tolerance of the different *Y. lipolytica* strains towards 2-PE and L-Phe, cells were prepared as described in section 3.2 and used to inoculate a 100 mL Erlenmeyer flasks with 30 mL of YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose, sterilized in an autoclave at 110 °C for 30 min), during 14 h, at 27 °C and 200 rpm in an orbital shaker (Shel Lab, Sheldon Manufacturing, Cornelius, Oregon, USA). After that, cells were diluted to a final OD_{600} of 0.6 (equivalent to a cell density of 0.58 g L^{-1}) in a 0.9 % (w/v) NaCl solution.

Cells (3 µL) at a dilution of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were spotted on solid media (20 g L^{-1} glucose, 2 g L^{-1} $(NH_4)_2SO_4$ and 20 g L^{-1} agar, sterilized in an autoclave at 110 °C for 30 min) containing

different concentrations of 2-PE (0, 1, 2 and 3 g L^{-1}) and L-Phe (0, 5, 8 and 15 g L^{-1}), that was sterilised by filtration through a 0.2 µm sterile syringe filter (PES, Whatman) (based on Lu et al., 2016).

Plates were incubated at 27 °C during 48 h being posteriorly observed and photographed using ChemiDoc™XRS software.

3.5 Bioconversion experiments at shake-flask scale

The bioconversion of L-Phe to 2-PE were carried out in 500 mL Erlenmeyer flasks with 200 mL of a modified cultivation medium (based on Celińska et al., 2013) containing per liter of deionized water: 15 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 40 g glucose or 50 g or 70 g crude glycerol, 20 mg YNB without aminoacids, 3 mg thiamine, supplemented with 5 g or 8 g L-Phe, incubated in an orbital shaker (Shel Lab, Sheldon Manufacturing, Cornelius, Oregon, USA) at 27 °C and 200 rpm. YNB without aminoacids, thiamine and L-Phe solutions were sterilised by filtration through a 0.2 µm sterile syringe filter (PES, Whatman). All remaining solutions were sterilized in an autoclave at 110 °C for 30 min.

Crude glycerol, which was provided by Prio Energy – Prio *Biocombustíveis*, SA., as the following composition by mass: 52.9 % glycerol, 9 % water, 4.9 % NaCl and less than 0.001 % methanol and 0.5 % organic matter (non-glycerol), according to the supplier.

3.6 Bioconversion experiments at bioreactor scale

The bioreactor experiments were carried out in a 3.7 L bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) with 31 cm height and 17 cm diameter (Figure 2). Air was supplied with a sparger located at the base of the agitator, with a flow-rate automatically controlled. Agitation was carried out with two Rushton impellers, 6-blade and 6 cm outside diameter.

Different experiments were carried out in order to access the effect of pH and DO concentration in 2-PE production. The pH values tested were 5, 7 and without pH control (initially adjusted to 6.5). The DO concentrations studied were 10 and 30 %. The set point values for pH and DO were automatically controlled by a control unit coupled to the bioreactor. The medium pH was controlled by addition of potassium hydroxide (4 M) or hydrochloric acid (2 M), using a Peripex peristaltic pump (Bioengineering, Switzerland). The DO concentration in the culture medium was controlled by feedback control of the stirring speed from 350 until a maximum of 900 rpm and an air-flow rate from 1 to 5 mL min^{-1}.

Cells were prepared as described in section 3.2 and used to inoculate a 1000 mL Erlenmeyer flask containing 300 mL of YPD, incubated at 27 °C, 200 rpm during 16 to 17 h. This pre-culture was

further used to inoculate the bioreactor with 1.7 L of bioconversion medium previously described, using 40 g L^{-1} of glucose as carbon source and 8 g L^{-1} L-Phe, with an initial OD_{600} of 0.5 (equivalent to a cell density of 0.46 g L^{-1}).

Figure 2. RALF PLUS SOLO bioreactor with bioconversion medium.

3.7 ISPR experiments

3.7.1 Selection of adsorbent resins

To evaluate the affinity of 2-PE and L-Phe for the adsorbent resins, three different resins were used: Amberlit XAD-7HP, XAD-4 and XAD-16 (Table 4). Prior to use, the resins were agitated in methanol to remove impurities and then washed with distilled water to remove the solvent.

Table 4. Physical characteristics of the adsorbent resins used, according to the supplier, Sigma-Aldrich.

	Adsorbent resins		
	XAD-7HP	**XAD-16**	**XAD-4**
Characteristics	Acrylic ester used to adsorb molecules up to MW 60 000	Hydrophobic polyaromatic used to remove hydrophobic compounds up to 40 000 MW and separation of large organic molecules	Hydrophobic polyaromatic used to remove small hydrophobic compounds
Matrix	Acrylic	Sytrene-divinylbenzene	Sytrene-divinylbenzene
Dipole moment	1.8	0.3	0.3
Diameter (Angstroms)	90	100	50
Surface area ($m^2\ g_{dry}^{-1}$)	450	900	750
Wet density ($g_{wet}\ mL^{-1}$)	1.05	1.02	1.02
Skeletal density ($g_{dry}\ mL^{-1}$)	1.24	1.08	1.08
Porosity ($mL\ g_{dry}^{-1}$)	1.14	1.82	0.98

To study the affinity of 2-PE and L-Phe for the adsorbent resins, experiments were performed in 250 mL Erlenmeyer flasks with 50 mL of bioconversion medium containing 15 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4.7H_2O$, 40 g L^{-1} glucose, 20 mg L^{-1} YNB without aminoacids, 3 mg L^{-1} thiamine, supplemented with 10 g L^{-1} of 2-PE and L-Phe (adapted from Hua et al., 2010). The resin (5 %, wet w/v) was added to the flasks and incubated in an orbital shaker (Shel Lab, Sheldon Manufacturing, Cornelius, Oregon, USA) at 27 °C, 200 rpm for 2 h (Hua et al., 2010). The concentrations of 2-PE and L-Phe in the aqueous phase after adsorption were further analysed by UHPLC. The adsorption ratios (1) of the resins were calculated as follows:

$$E = \frac{100\ (C_b - C_a)}{C_b} \quad (1)$$

where E (%) represents the adsorption ratio, and C_b and C_a (g L^{-1}) the concentrations of 2-PE or L-Phe in the aqueous phase before and after the absorption, respectively.

To establish the optimum amount of the resin in the bioconversion experiments, 10 g L^{-1} of 2-PE and L-Phe were dissolved in the bioconversion medium (previously described), and different amounts of resin were added (1, 5, 7, 9 and 11 % (wet w/v). Concentrations of 2-PE and L-Phe in the aqueous phase after adsorption were posteriorly analysed by UHPLC.

3.7.2 Desorption of 2-PE and L-Phe and reuse of the resin

The resin was separated from the bioconversion broth by sedimentation. Then, it was first washed with distilled water (volume equivalent to 5-fold of wet bead weight). After, the resin was eluted with absolute ethanol (1:2 (w/v)). Desorption was performed in an orbital shaker (Shel Lab, Sheldon Manufacturing, Cornelius, Oregon, USA) at 27 °C, 200 rpm, during 2h and repeated three times (Hua et al., 2010). The ethanol phases collected were then combined and analysed by UHPLC to determine the concentrations of 2-PE and L-Phe after desorption.

After desorption, the resin was soaked in methanol and washed with distilled water to remove the solvent, allowing it to be reused.

3.7.3 Bioconversion experiments coupled with ISPR

In the bioconversion experiments with ISPR, the adsorbent resin was prepared according to the section 3.7, weighed, sterilized with ethanol, and washed with sterile distilled water before being added to the bioconversion medium, priori to inoculation. Experiments were performed in 500 mL Erlenmeyer flasks, using crude glycerol (50 g L^{-1}) as carbon source and with 8 g L^{-1} of L-Phe.

3.8 Analytical methods

Samples from the bioconversion medium were taken at regular intervals for the analysis of biomass concentration, glucose, glycerol, L-Phe consumption and 2-PE production.

Cell concentration was determined by measuring the OD_{600} of the cell cultures in a microplate reader (Sunrise, Tecna, Männedorf, Switzerland). The OD_{600} was converted to cell dry weight (g L^{-1}), using a calibration curve previously obtained (Annex I).

Samples from the bioconversion broth were centrifuged at 8000 rpm during 5 minutes. Culture supernatants were then filtered through 0.2 µm syringe filter (PES, Whatman) and stored in vials at - 20 °C to further analysis.

Glucose and crude glycerol concentrations were quantified by HPLC using a JASCO system equipped with a RI detector (RI-2013). Samples were analysed using an ion exchange column (Aminex HPX-87H, 300 x 7.8 mm, Bio-Rad) which was kept at 60 °C. They were automatically injected at a flow rate of 0.5 mL min^{-1} using 5 mM H_2SO_4 as mobile phase during 25 min. Identification of glucose and crude glycerol was made with the Jasco ChromPass software and the concentrations obtained using a calibration curve (Annex II).

For the quantification of 2-PE and L-Phe, samples were analysed using a SHIMADZU UHPLC system (Nexera X2, Shimadzu) equipped with a diode array detector (SPD-M20A) at 215 nm. Liquid chromatography separation was carried out with a reversed phase column (YMC ODS-Aq, 250 x 4.6 mm) kept at 30 °C (CTO-30A). Samples were automatically injected (SIL-30AC Autosampler, Shimadzu) at a flow rate of 1 mL min^{-1} using water (solvent A) and acetonitrile (solvent B) as mobile phase components. A gradient was performed during 42 min as follows: 0 min – 100 % component A, 10 min – 100 % component A, 16.7 min – 70 % component A, 26.7 min – 70 % component A, 33.3 min – 100 % component A, 41.7 min – 100 % component A. The identification of 2-PE and L-Phe was performed with the LabSolutions software and the concentrations obtained using a calibration curve (Annex III).

4. RESULTS AND DISCUSSION

4.1 Inhibitory effect of 2-PE and L-Phe on *Y. lipolytica* cells

The major hurdle in the biotechnological production of 2-PE is the strong inhibitory effect that this flavor has on microorganism's growth. In fact, superior alcohols are well known for their antimicrobial properties (Lester, 1965; Lu et al., 2016; Naz et al., 2013; Zhu et al., 2011) being responsible for the increase of the membrane fluidity (Stark et al., 2003b), reduction of sugars and aminoacids uptake, (Lester, 1965; Naz et al., 2013; Stark et al., 2003b; Zhang et al., 2013), respiratory capacities and protein and RNA synthesis (Lester, 1965; Naz et al., 2013; Stark et al., 2003b).

Consequently, the main bottleneck for microbial production of 2-PE is its cytotoxicity (Etschmann et al., 2002). It should be kept in mind that this effect is strain dependent (Etschmann et al., 2003). Mihal' et al. (2012) reported that *S. cerevisiae* (baker's yeasts) can tolerate up to 4 g L^{-1} of 2-PE, however 2 g L^{-1} of 2-PE could completely inhibit the cell growth of *K. marxianus* (Hansen) van der Walt (Fabre et al., 1998b). On the other hand, it is also known that the precursor concentration, in this case L-Phe, is a highly important component in the bioconversion process that can greatly affect its productivity (Tseng and Wayman, 1975).

The tolerance to 2-PE and L-Phe by *Y. lipolytica* strains has not yet been extensively described, thus the strains response to the increase of 2-PE and L-Phe concentrations was firstly assessed (Figure 3). The strains *Y. lipolytica* W29, CBS2075 and NCYC2904 were cultivated in solid medium with different concentrations of 2-PE and L-Phe to evaluate its cellular growth (Figure 3).

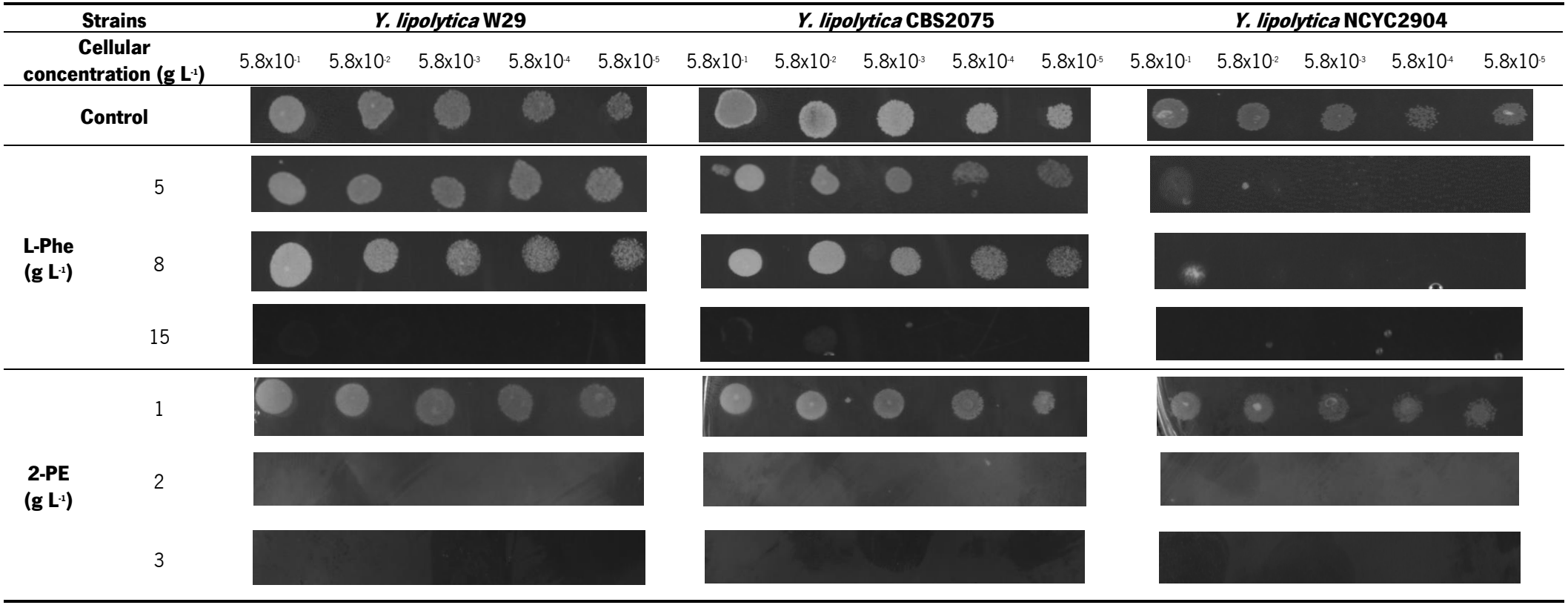


Figure 3. Inhibitory effect of 2-PE and L-Phe on cell growth of *Y. lipolytica* W29, CBS2075 and NCYC2904 strains. After the cellular growth in liquid media, 3 µL of the cellular suspension (cell density of $5.8x10^{-1}$ g L^{-1}) was spotted at different dilutions on solid medium containing different concentrations of 2-PE (0, 1, 2 and 3 g L^{-1}) and L-Phe (0, 5, 8 and 15 g L^{-1}). Cell cultures were incubated at 27 °C for 48 h.

As shown in Figure 3, a remarkable inhibitory effect of L-Phe on cell growth of *Y. lipolytica* NCYC2904 strain was detected. When compared with the control, the only colony formed with 5 g L^{-1} of L-Phe presents a very low opacity. Regarding the L-Phe tolerance exhibited by the *Y. lipolytica* W29 and CBS2075 strains, a similar behaviour was observed in both strains; in media containing 5 and 8 g L^{-1} of L-Phe, the cell growth was not affected. However, in the experiments with 15 g L^{-1} of L-Phe the cellular growth is completely repressed. A similar result was also reported by Lu et al. (2016) that observed a decrease in cellular growth of the stress-tolerant yeast *C. glycerinogenes* for L-Phe concentrations up to 7 g L^{-1}. On the other hand, for *P. fermentans*, *K. marxianus* and *M. pulcherrima*, an increase in L-Phe concentrations up to 2, 5 and 30 g L^{-1}, respectively, did not affect significantly the cellular growth (Chantasuban et al., 2018; Fabre et al., 1998b; Huang et al., 2000). These results allow to state that L-Phe concentrations equal or above 15 g L^{-1} have a significant inhibitory effect on the cellular growth of *Y. lipolytica* W29 and CBS2075.

Concerning the effect of 2-PE, a remarkable inhibitory effect on cell growth was detected for all the strains tested with 2-PE concentrations equal or above 2 g L^{-1} (Figure 3). However, comparing the performance of the three strains with 1 g L^{-1} of 2-PE, it was possible to observe that the strains W29 and CBS2075 show the highest resistance to the product, since they present more opaque colonies, even at high dilutions. The NCYC2904 strain showed the lowest resistance to the 2-PE since it was observed a low opacity in the colonies, especially at high dilutions. For *K. marxianus*, a 2-PE concentration of 1.4 g L^{-1} was determined by Fabre et al. (1998b) as the critical value for a significant decrease in the viability of the cells, since a 2-PE concentration of 2 g L^{-1} leads to a total inhibition of cellular growth (Fabre et al., 1998b; Gao and Daugulis, 2009). For *S. cerevisiae*, several studies reported the critical value of 4 g L^{-1} 2-PE, being higher 2-PE concentrations toxic for yeast proliferation and production (Chreptowicz et al., 2016; Lu et al., 2016; Stark et al., 2003b; Zhang et al., 2013).

Thus, it was possible to observe that the inhibitory effect of 2-PE and L-Phe is not uniform for all the strains studied, which is in agreement with the results reported by Etschmann et al. (2003). Since the main goal of this work is to develop a bioprocess for 2-PE production through L-Phe bioconversion, it is important to select a strain with high resistance to 2-PE but also to the precursor. Based on this, the W29 and CBS2075 strains were selected for following bioconversion experiments.

4.2 Production of 2-PE by L-Phe bioconversion

4.2.1 Bioconversion experiments at shake-flask scale

Several microorganisms are able to naturally produce 2-PE however, the final titers obtained are still uncompetitive. As previously referred, 2-PE can be produced via *de novo* synthesis, nevertheless the obtained titers are very low since the glycolysis and the pentose-phosphate pathways are mostly used for biomass formation rather than 2-PE production (Angelov and Gotcheva, 2012; Etschmann et al., 2002; Hua and Xu, 2011). Thus, taking into account that the production of 2-PE by *Y. lipolytica* strains is poorly explored (Celińska et al., 2013; Celińska et al., 2015), the ability of *Y. lipolytica* W29 and CBS2075 strains to produce 2-PE was studied. Therefore, the addition of 5 g L^{-1} of L-Phe as the sole nitrogen source was evaluated as an alternative way to increase 2-PE production through the Ehrlich pathway, at shake-flask scale using glucose as carbon source (Figure 4).

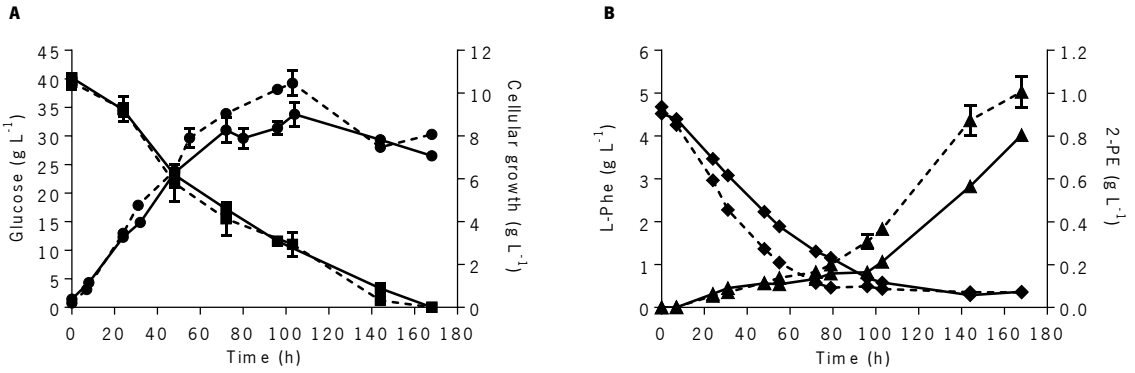


Figure 4. Cellular growth (●), glucose consumption (■) (**A**), 2-PE production (▲) and L-Phe consumption (◆) (**B**) with *Y. lipolytica* W29 (dashed lines) and CBS2075 (solid lines) strains, in shake-flasks cultures, supplemented with 5 g L^{-1} L-Phe. Data are presented as average and standard deviation of two independent experiments (if no error bars are visible, the standard deviations were too small to be displayed).

Analysing the cell growth profile of both strains, it was possible to observe that during the first 104 h of fermentation, cells grew exponentially reaching the maximum biomass concentration of 9.0 ± 0.6 g L^{-1} and 10.5 ± 0.6 g L^{-1} for *Y. lipolytica* CBS2075 and W29 strains, respectively (Figure 4A). Regarding the glucose consumption profile, after the exponential growth phase, about 11 g L^{-1} of the carbon source was still present in the bioconversion broth (Figure 4A). In fact, glucose was only completely depleted after 168 h of fermentation. The carbon source was still being consumed even though the biomass concentration remained constant, which leads to assume the use of glucose for maintenance purposes (Wittmann et al., 2002).

Concerning the L-Phe consumption profile for both cultures, it was possible to observe a precursor consumption of around 4.25 ± 0.08 g L^{-1} and 3.94 ± 0.01 g L^{-1} during the exponential growth phase, for the W29 and CBS2075 strains respectively, with an average 2-PE production of only 0.3 ± 0.1 g L^{-1} (Figure 4B). After 168 h of fermentation, solely 0.37 ± 0.10 g L^{-1} and 0.35 ± 0.07 g L^{-1} of L-Phe remained in the medium, for the CBS2075 and W29 strains, respectively.

Comparing the performance of both strains tested for 2-PE production, it was possible to observe that a maximum 2-PE concentration of 1.01 ± 0.07 g L^{-1} was obtained with the W29 strain, and that the CBS2075 strain was able to produce 0.80 ± 0.02 g L^{-1} of 2-PE (Figure 4B). Taking into consideration the 2-PE titers reported by Garavaglia et al. (2007) (0.77 g L^{-1} of 2-PE from 3 g L^{-1} of L-Phe), Fabre et al. (1998b) (0.70 g L^{-1} of 2-PE from 2 g L^{-1} of L-Phe) and Adler et al. (2011) (1.0 g L^{-1} of 2-PE from 9 g L^{-1} of L-Phe) using *K. marxianus*, Eshkol et al. (2009) (0.85 g L^{-1} of 2-PE from 4 g L^{-1} of L-Phe) for *S. cerevisiae* and Huang et al. (2001) (0.5 g L^{-1} of 2-PE from 1 g L^{-1} of L-Phe) with *P. fermentans* L-5, the obtained results elucidate the potential of the *Y. lipolytica* strains tested for the production of this metabolite.

Analysing the L-Phe consumption and 2-PE production profiles, at least two phases with distinct strain physiological characteristics could be distinguished (Figure 4B). Whereas the exponential growth was accompanied by only a slight accumulation of 2-PE in the cultivation broth, its production rate was significantly enhanced in the stationary growth phase (from 1.7 ± 0.1 mg L^{-1} h^{-1} to 8.9 ± 0.1 mg L^{-1} h^{-1} for the W29 strain and 2.5 ± 0.2 mg L^{-1} h^{-1} to 9.1 ± 1.0 mg L^{-1} h^{-1} for the CBS2075 strain), steadily accumulating until the end of cultivation. At the same time, the precursor consumption rate decreases from 40.0 ± 0.3 mg L^{-1} h^{-1} to 4.5 ± 0.1, for the W29 strain, and from 53.4 ± 2.1 mg L^{-1} h^{-1} to 1.5 ± 1.8 mg L^{-1} h^{-1}, for the CBS2075 strain. This indicates that the bioconversion of L-Phe to 2-PE continued to occur after the exponential growth phase, meaning that the Ehrlich pathway was more active under the stationary phase and at the end of the experiment. The same behaviour was also been described by Celińska et al. (2013), Garavaglia et al. (2007) and Wittmann et al. (2002), with *Y. lipolytica* NCYC3825, *K. marxianus* CBS6556 and *K. marxianus* CBS5670. These authors observed a high production rate of 2-PE during stationary growth phase and at the end of the experiments, indicating that the bioconversion was still active under non-growing conditions. Nevertheless, the 2-PE production in yeasts is usually associated with cellular growth (Angelov and Gotcheva, 2012; Etschmann et al., 2002).

According to Wittmann et al. (2002), L-Phe could be considered the dominating precursor for 2-PE production and the contribution of glucose as a product precursor can be neglected. Therefore,

given the obtained yield of 2-PE with respect to L-Phe, *Y. lipolytica* W29 was the best producer since the attained yield is 21 % higher than the value achieved with the CBS2075 strain, although the 2-PE titers and bioconversion yields did not present statistically significant differences ($p \leq 0.05$) (Table 5). Nevertheless, it was 1.3 times lower than the bioconversion yield reported by Celińska et al. (2013) for *Y. lipolytica* NCYC3825 and far below from the theoretical value of 0.75 g g^{-1} (Etschmann et al., 2002). The same behaviour was also been described by Stark et al. (2003b), that also achieved a bioconversion yield lower than the theoretical one. This result suggests that a fraction of L-Phe is probably degraded through other pathways and complete conversion of L-Phe to 2-PE cannot be attained. The highest productivity was also obtained with the W29 strain, being 1.3 times higher than the value reached by the CBS2075 strain, presenting statistically significant differences ($p \leq 0.05$) (Table 5).

As previously described, a possible explanation for this behavior can be the utilization of L-Phe for cell growth and in the cinnamate pathway, which constitutes one of several possibilities of L-Phe degradation by yeast (Angelov and Gotcheva, 2012; Etschmann et al., 2002; Hua and Xu, 2011). Even though the presence of L-Phe as the only nitrogen source allows the predomination of the Ehrlich pathway, a certain concentration of L-Phe is always degraded through the cinnamate pathway, being impossible to attain a complete conversion of L-Phe to 2-PE (Etschmann et al., 2002). Metabolic network analysis of 2-PE production with *K. marxianus* CBS5670 allowed to determine the metabolic fate of L-Phe in a glucose-grown batch culture (Wittmann et al., 2002). This study reported that 73.3 % of L-Phe was converted to 2-PE or 2-phenylethylacetate, 22.4 % was lost into the cinnamate pathway and 4.3 % was used for protein biosynthesis (Wittmann et al., 2002). We can consider that the same type of catabolic loses of L-Phe may occur in *Y. lipolytica,* however it is important to stressed out that the 2-PE production is strain dependent. Another possible explanation for the low bioconversion yields attained may be the formation of intermediate compounds of the Ehrlich pathway, such as phenylacetate and 2-phenylethylacetate. In a glucose-grown culture of *S. cerevisiae* cells, 90 % of L-Phe is converted to 2-PE, whereas the remaining is directed to the phenylacetate production (Hazelwood et al., 2008). The 2-phenylethylacetate formations can occur through direct esterification (lipase catalysed) of an alcohol and acid, or by transesterification of an alcohol and ester (Białecka-Florjańczyk et al., 2012; Kuo et al., 2014). Hence, 2-PE can be further converted to the 2-phenylethylacetate ester, since *Y. lipolytica* is known for its highly secretory activity of lipases (Białecka-Florjańczyk et al., 2012).

Taking into consideration that the strain W29 produced the highest 2-PE titer, this strain was selected as the best candidate for the following experiments. Previous reports (ÄYräpää, 1965; Fabre et

al., 1998b; Stark et al., 2003b) have shown that the L-Phe concentration in the media influences 2-PE production and that a high L-Phe concentration is necessary to direct the precursor to the Ehrlich pathway. Also, in the previous experiments, L-Phe was completely consumed. Based on this, new experiments were carried out to analyze the behavior of the *Y. lipolytica* W29 strain in the presence of a higher L-Phe concentration. From the cytotoxicity results with L-Phe, it was possible to conclude that L-Phe concentrations equal to 15 g L^{-1} have a significant inhibitory effect on the cellular growth (Figure 3). Therefore, a L-Phe concentration of 8 g L^{-1} was selected for the following studies (Figure 5).

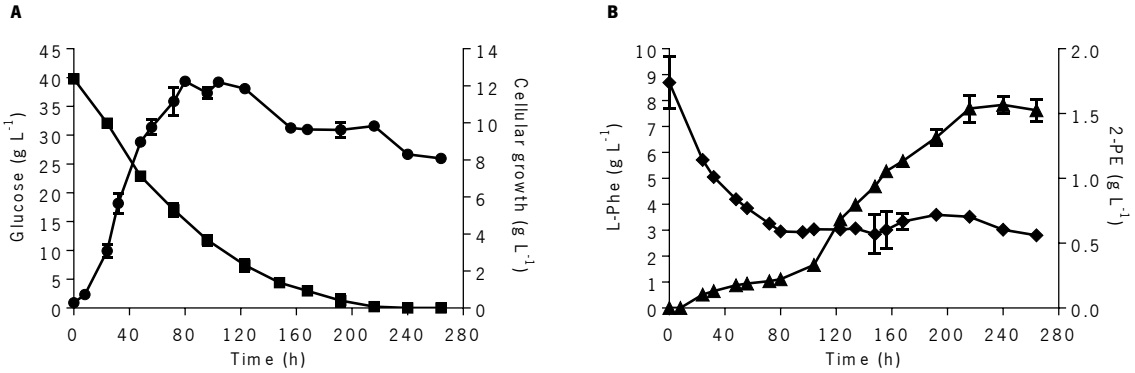


Figure 5. Cellular growth (●), glucose consumption (■) (**A**), 2-PE production (▲) and L-Phe consumption (◆) (**B**) with *Y. lipolytica* W29, in shake-flasks cultures, supplemented with 8 g L^{-1} L-Phe. Data are presented as average and standard deviation of two independent experiments (if no error bars are visible, the standard deviations were too small to be displayed).

During the first 80 h of fermentation, cells grew exponentially reaching a maximum biomass concentration of 12.2 ± 0.1 g L^{-1} (Figure 5A). At the same time, approximately, 17 g L^{-1} of glucose remained in the bioconversion broth, being completely depleted after 216 h of fermentation (Figure 5A). Comparing the growth and glucose consumption profiles under the new conditions with 5 g L^{-1} L-Phe experiments (Figure 4A), a similar trend was observed however, a 1.2-fold increase in the maximum biomass concentration was attained, with a higher growth rate and glucose yield ($Y_{X/S}$ 0.53 g g^{-1} *vs* 0.36 g g^{-1}). In fact, an increase in the precursor concentration led to an enhancement in biomass concentration, with lower carbon source consumption. This reinforces the hypothesis of L-Phe utilization for cell growth, as previously described.

Concerning the precursor consumption profile (Figure 5B), it was possible to observe that after 264 h of cultivation it was not completely converted, and after 80 h only 66 % of the total precursor added was consumed. In fact, during the exponential growth phase it was observed a high L-Phe consumption rate (71.9 ± 12.6 mg L^{-1} h^{-1}) that further decreases in the stationary phase (0.8 ± 0.8 mg L^{-1} h^{-1}). As such, the bioconversion yield and productivity achieved with 8 g L^{-1} of L-Phe were similar with

the values obtained with 5 g L^{-1} of L-Phe (the differences not presenting statistically significant differences at $p \leq 0.05$) (Table 5). Nevertheless, the bioconversion yield was still far below from the theoretical value (Etschmann et al., 2002).

Table 5. Maximum productivity and yield of 2-PE for *Y. lipolytica* CBS2075 and W29 strains, in shake-flasks cultures, supplemented with 5 g L^{-1} and 8 g L^{-1} L-Phe. Data are presented as average and standard deviation of two independent experiments.

Strain	L-Phe (g L^{-1})	$Y_{2\text{-PE/L-Phe}}$ (g g^{-1})	Productivity (mg L^{-1} h^{-1})
Y. lipolytica CBS2075	5	0.19 ± 0.01^{a}	4.8 ± 0.1^{a}
Y. lipolytica W29	5	$0.23 \pm 0.02^{a\,A}$	$6.0 \pm 0.4^{b\,A}$
	8	0.28 ± 0.04^{A}	6.5 ± 0.3^{A}

$^{a\ b\ A}$ Values followed by the same letter do not present statistically significant differences ($p \leq 0.05$) by the t test analysis. Lowercase letters compare the CBS2075 and W29 strains. Uppercase letters are for the cultures supplemented with 5 and 8 g L^{-1} of L-Phe.

Regarding the 2-PE production profile, a maximum 2-PE concentration of 1.57 ± 0.06 g L^{-1} was obtained with 8 g L^{-1} L-Phe, after 240 h (Figure 5B). With a 1.6-fold increase in precursor concentration it was possible to achieve a 1.6-fold improvement in 2-PE concentration (the differences present statistically significant differences at $p \leq 0.05$). In fact, at the beginning of the stationary growth phase it was possible to observe an increase in the production rate from 2.8 ± 0.2 mg L^{-1} h^{-1} to 9.7 ± 0.7 mg L^{-1} h^{-1}. However, in the last 48 h of fermentation, the 2-PE concentration in the cultivation broth remains constant. In fact, this point coincides with glucose depletion (Figure 5A), meaning that the cells cannot stay metabolically active without substrate, and the 2-PE production stabilizes. Also, there was still some L-Phe in the bioconversion medium, and it seems that the precursor consumption may be repressed by the accumulation of 2-PE intermediates, that can be toxic and repress the L-Phe uptake (Fabre et al., 1998b).

Previous studies also reported a positive effect of L-Phe concentration on 2-PE production (Eshkol et al., 2009; Fabre et al., 1998b; Garavaglia et al., 2007; Huang et al., 2000; Lu et al., 2016; Mei et al., 2009). In fact, it was shown that an increase in the L-Phe concentration led to an increase in the 2-PE titers and bioconversion yields (Eshkol et al., 2009; Fabre et al., 1998b; Garavaglia et al., 2007; Huang et al., 2000; Lu et al., 2016; Mei et al., 2009). However, this improvement was only observed up to a certain L-Phe concentration, and this enhancement is strain dependent, suggesting an inhibitory effect of L-Phe on cell activity (as previously observed in Figure 3) or a substrate uptake bottleneck for L-Phe (Fabre et al., 1998b). Despite the need of a high precursor level to obtain a high product yield, an

increase in L-Phe concentration may led to the accumulation of other metabolic intermediates and biomass formation, which decreases the bioconversion yield (Fabre et al., 1998b; Huang et al., 2000).

An interesting behavior can also be observed during the bioconversion experiments, suggesting that in 2-PE production with *Y. lipolytica* W29, the L-Phe consumed via Ehrlich pathway is firstly converted to one or more intermediates of this pathway and further converted to 2-PE. In fact, it was possible to observe that the highest 2-PE production was attained when the L-Phe consumption rate was reduced (Figure 4B and 5B). Unfortunately, it was not possible to measure or identify the metabolic intermediates of this pathway, and this is only a possible explanation for the obtained results.

A literature review revealed that the obtained 2-PE concentrations with 8 g L^{-1} L-Phe were similar with the ones described for L-Phe concentrations higher than 5 g L^{-1}: *Aspergillus niger* produced 1.4 g L^{-1} of 2-PE from 6 g L^{-1} of L-Phe in 9 days (Lomascolo et al., 2001) and *Y. lipolytica* NCYC3825 produced nearly 2 g L^{-1} from 7 g L^{-1} of L-Phe (Celińska et al., 2013).

Considering that an increase in L-Phe concentration enhances the biomass and product concentration, bioconversion yield and productivity, the following experiments were performed with an initial L-Phe concentration of 8 g L^{-1}.

4.2.2 Bioconversion experiments at bioreactor scale – effect of DO concentration

Most of the studies describe the 2-PE production in agitated Erlenmeyer flasks (Celińska et al., 2018; Chreptowicz et al., 2017; Conde-Báez et al., 2017; Eshkol et al., 2009; Etschmann et al., 2003; Fabre et al., 1998b; Huang et al., 2001; Wang et al., 2013) and depending on the agitation, the culture medium may be more or less agitated and aerated. However, these systems do not allow to control the aeration or agitation separately (Gatfield, 1988; Okui et al., 1963; Serrano-Carreón et al., 1997). More recently, the 2-PE production was also described at bioreactor scale with *K. marxianus*, *M. pulcherrima*, *S. cerevisiae* and *C. glycerinogenes* (Adler et al., 2011; Chantasuban et al., 2018; Chreptowicz et al., 2016; Garavaglia et al., 2007; Lu et al., 2016; Martínez-Avila et al., 2018a).

The production and accumulation of 2-PE is highly dependent from the environmental conditions such as pH (Chantasuban et al., 2018; Huang et al., 2001), aeration and agitation rates (Garavaglia et al., 2007; Huang et al., 2001) and DO concentration (Chreptowicz et al., 2016; Etschmann et al., 2005; Stark et al., 2002; Wang et al., 2011). Since *Y. lipolytica* is a strictly aerobic microorganism, the oxygenation of the culture is also a key factor. Studies have shown that high 2-PE productions were obtained when high oxygen concentrations were available in the culture medium (Celińska et al., 2013; Chantasuban et al., 2018; Gao and Daugulis, 2009; Huang et al., 2001; Lu et al., 2016). Thus, some

authors suggest that this variable has to be set around 20 – 40 % of saturation (as DO) (Etschmann et al., 2005; Wang et al., 2011) while others recommend about 100 % of saturation (Chreptowicz et al., 2016; Stark et al., 2002).

In order to analyse oxygen effect on the 2-PE production by *Y. lipolytica* W29, experiments were carried at bioreactor scale, using glucose as the carbon source, with 8 g L^{-1} of L-Phe, and the medium pH initially adjusted to 6.5. The experiments were carried out by varying the oxygen input to the medium through the manipulation of agitation and aeration rates, in order to maintain the levels of the DO concentration at 10 and 30 % of saturation (Figure 6).

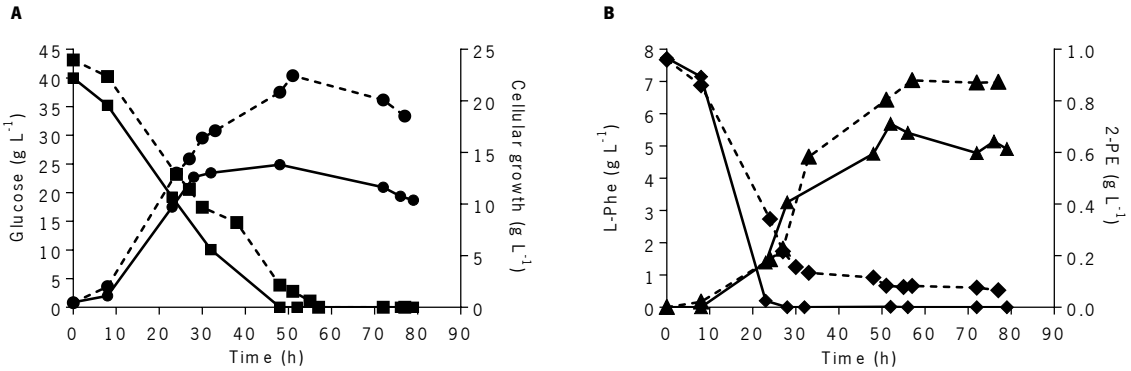


Figure 6. Cellular growth (●), glucose consumption (■) (**A**), 2-PE production (▲) and L-Phe consumption (◆) (**B**) with *Y. lipolytica* W29 with 10 % (dashed lines) and 30 % (solid lines) of DO, in batch bioreactor cultures, supplemented with 8 g L^{-1} L-Phe. The medium pH was initially set to 6.5.

Looking to the cellular growth profile of the experiment with 10 % of DO, it was possible to observe that the cells reached the stationary phase after 51 h, with a maximum biomass concentration of 22.5 g L^{-1} (Figure 6A). Regarding the 30 % DO bioconversion culture, the stationary phase was attained after 32 h, however the maximum biomass concentration achieved was only 13.0 g L^{-1} (Figure 6A). Comparing the obtained results with the ones performed at shake-flask scale under the same conditions (Figure 5A), a 1.8-fold improvement in the cellular growth was observed for the experiments at 10 % DO, whereas only a 1.1-fold increase was observed in the 30 % DO condition. In fact, at bioreactor scale, more oxygen is available in the bioconversion broth due to an increase in the oxygen transfer rate. Thus, *Y. lipolytica* W29 cells grew more and faster than at the shake-flask scale. However, in the bioreactor experiments, the different DO concentrations studied had a clear effect on *Y. lipolytica* W29 growth. The aeration and agitation rates needed to keep the DO at 30 % are higher than the ones needed to maintain the DO at 10 % (Annex IV). A possible explanation for the differences observed in both conditions is the impact of mechanical mixing in the cell morphology and physiology. A similar behaviour was reported by Alonso et al. (2005). They also observed that high agitations rates could lead

to a decrease in the cellular growth due to shear stress caused by the impellers (Alonso et al., 2005). Braga et al. (2015) also reported the negative effect of high agitation and aeration rates in *Y. lipolytica* W29 cells morphology. Under these conditions, a morphological change was observed and the amount of cells in pseudo-hyphae form increases, as a cell mechanism response to stress conditions (Braga et al., 2015; Kawasse et al., 2003). Under the conditions here studied, the *Y. lipolytica* cells can be mechanically more fragile due to the strong mechanical agitation, leading to a lower biomass formation. In Figure 7, images of *Y. lipolytica* W29 cells during bioconversion experiments at 10 and 30 % DO are presented. It was possible to observe that in 30 % DO experiments, pseudo-hyphae form of the cells increased with respect to the 10 % DO bioconversions, reinforcing a dimorphic transition probably caused by an increase stress in the cells with respect to high agitation.

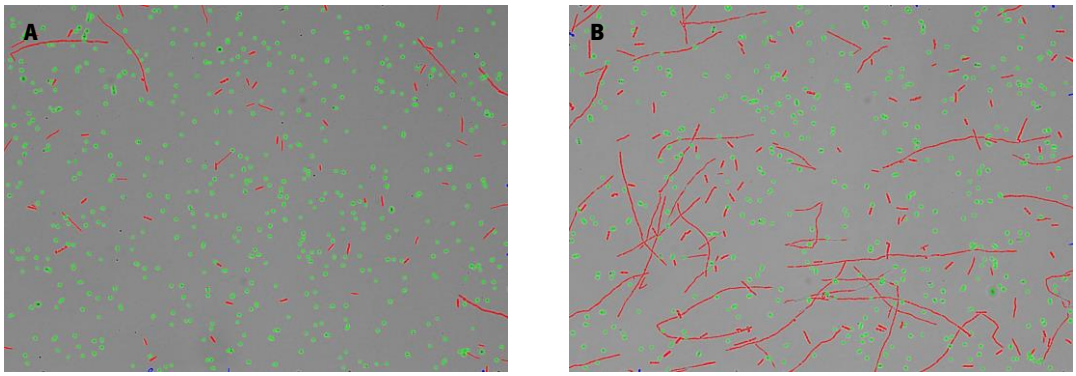


Figure 7. Images of *Y. lipolytica* W29 cells in the experiments at 10 % (**A**) and 30 % (**B**) of DO (hyphae in red and yeasts in green).

Looking at the substrate consumption profile (Figure 6A), it was possible to observe that glucose was completely depleted from the medium after 57 h and 48 h, in the experiments with 10 and 30 % of DO, respectively. Nevertheless, the glucose consumption rate in bioreactor experiments was faster when compared with shake-flask scale experiments (Figure 5A).

For both conditions, the L-Phe was rapidly consumed in the first hours of culture (Figure 6B). In the bioconversion with 30 % DO, the precursor consumption rate decreased from 327.4 mg L^{-1} h^{-1} to 3.6 mg L^{-1} h^{-1} during the first 23 h, whereas at 10 % DO experiments, it decreased from 200.3 mg L^{-1} h^{-1} to 12.3 mg L^{-1} h^{-1} after 33 h. During the exponential growth phase, 7.7 and 7.0 g L^{-1} of L-Phe were consumed to produce only 0.4 and 0.8 g L^{-1} of 2-PE, in the experiments with 30 and 10 % DO, respectively. In fact, under these conditions, and contrary to the shake-flask experiments, all L-Phe was consumed.

Concerning the 2-PE production profiles, it was possible to observe an initial lag phase of around 10 h (Figure 6B). After that, its concentration increases up to a maximum with a production rate of around 17.53 mg L^{-1} h^{-1} (10 % DO) and 16.16 mg L^{-1} h^{-1} (30 % DO), stabilizing after around 57 h with a 2-PE titer of 0.88 and after 52 h with a 2-PE titer of 0.71 g L^{-1}, for the experiments with 10 and 30 % DO, respectively.

It was observed that the growth and 2-PE production decrease as the DO increase. Other studies reported that the alcohol production by microorganisms was affected by shaking speed or aeration rate (Raymond et al., 1990; Yoshizawa, 1966). In fact, oxygen could inhibit the activity of alcohol acetyltransferase that catalyses the 2-PE conversion to its ester, 2-phenylethylacetate. Hence, high shaking speeds are helpful for 2-PE accumulation, avoiding its esterification (Huang et al., 2001). As already referred, some authors suggest that this variable has to be set around 20 – 40 % DO (Etschmann et al., 2005; Wang et al., 2011) or even 100 % DO (Chreptowicz et al., 2016; Stark et al., 2002), in order to increase the 2-PE production. However, a different behaviour was observed in this study, and a high 2-PE production was obtained in the experiment with low DO concentration (10 % DO). This might be related with the high biomass concentration attained under these conditions. As already referred, in the 30 % DO condition cells were more fragile, hence less cellular growth was observed and consequently less product was formed (Chantasuban et al., 2018; Etschmann et al., 2002; Garavaglia et al., 2007; Stark et al., 2003b). The same behaviour was also observed by Garavaglia et al. (2007), with *K. marxianus.* In bioreactor cultures, low aeration and agitation rates were more favourable for 2-PE production, due to high cellular growth (Garavaglia et al., 2007).

However, when comparing the 2-PE concentration and yield obtained in bioreactor experiments with 10 % DO (Table 6) with the ones obtained at shake-flask scale (Figure 5 and Table 5), it was observed a 44 and 54 % decrease in both parameters, respectively. Celińska et al. (2013) also reported a 71 % decrease in the bioconversion yield of *Y. lipolytica* NCYC3825 when the 2-PE production was scale-up to bioreactor scale.

Table 6. Maximum productivity and yield of 2-PE for *Y. lipolytica* W29, in a STR, supplemented with 8 g L^{-1} L-Phe.

Strain	DO (%)	$Y_{2\text{-PE/L-Phe}}$ (g g^{-1})	Productivity (mg L^{-1} h^{-1})
Y. lipolytica W29	10	0.13	15.4
	30	0.09	13.7

Regarding the process productivity, a 2.4-fold increase was observed in comparison with the shake-flask experiments (Table 5). Braga et al. (2015) also reported an increase in γ-decalactone

productivity with *Y. lipolytica* W29 with the process scale-up to bioreactor, since the time needed to reach the maximum γ-decalactone production is reduced.

Taking into consideration that the experiments with 10 % DO lead to a high 2-PE concentration, this condition was selected for further bioreactor experiments.

4.2.3 Bioconversion experiments at bioreactor scale – effect of medium pH

It has been previously reported that alcohol acetyltransferase catalyses the conversion of 2-PE to 2-phenylethylacetate, whereas esterase catalyses the hydrolysis of 2-phenylethylacetate to produce 2-PE and acetic acid (Yoshioka and Hashimoto, 1983). Therefore, it is expected that the pH of the medium would affect the activity of the enzymes involved in the formation of 2-PE, and thus affect its production by *Y. lipolytica* W29.

To study the effect of medium pH on the 2-PE production, bioconversion experiments were performed at pH 5 and 7, with a DO of 10 % (Figure 8).

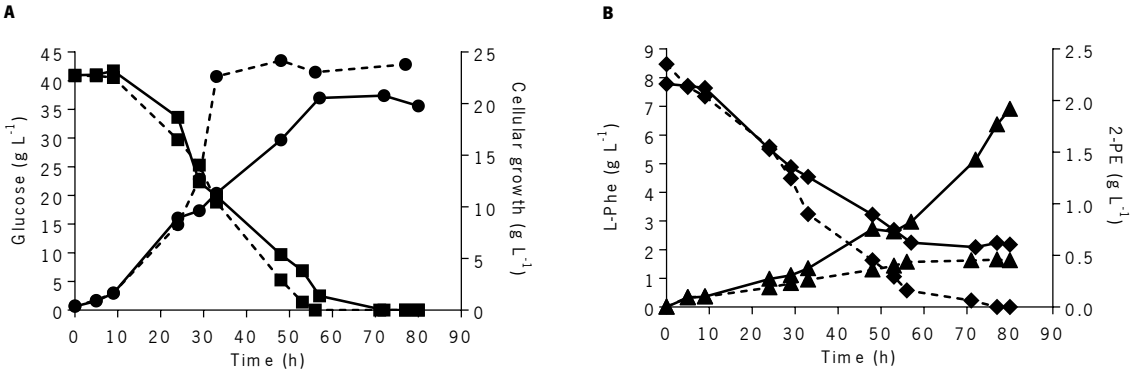


Figure 8. Cellular growth (●), glucose consumption (■) (**A**), 2-PE production (▲) and L-Phe consumption (◆) (**B**) with *Y. lipolytica* W29 at medium pH 5 (solid lines) and 7 (dashed lines), in batch bioreactor cultures, supplemented with 8 g L^{-1} L-Phe.

Figure 8A shows the growth and substrate consumption profiles of *Y. lipolytica* W29 in bioconversion media with pH 5 and 7. In media with an initial pH of 7, cells grew exponentially until 30 h of cultivation, reaching a maximum biomass concentration of 24.2 g L^{-1}. In media with pH 5, the exponential growth phase was attained after 57 h with a maximum biomass concentration of 20.8 g L^{-1}. Comparing the maximum biomass concentration obtained with the one attained in the experiments without pH control (Figure 6A), a 1.1-fold increase in cellular growth was observed with an increase in the medium pH to 7. However, a decrease in pH to 5 led to a 1.1-fold decrease in biomass concentration. The medium pH had a clear effect on the cellular growth, where the neutral pH favoured the biomass formation. Nevertheless, it is important to stress out that *Y. lipolytica* has the ability to grow

even at a low pH. This microorganism is able to adapt to a wide range of pH conditions, shifting the metabolite pattern without impairing growth or the substrate uptake rate (Egermeier et al., 2017).

For both conditions, the carbon source was completely depleted after 72 and 56 h, for the pH 5 and 7 experiments, respectively (Figure 8A).

Looking to the precursor consumption profile, it was possible to observe that during the first 33 h of cultivation its consumption rate is almost the same (158.3 mg L^{-1} h^{-1} for pH 7 and 129.0 mg L^{-1} h^{-1} for pH 5) for both conditions (Figure 8B). Nevertheless, after this time, a high consumption of L-Phe was observed in the experiment with a pH of 7, with an average consumption of L-Phe of 7.9 g L^{-1} after 56 h, and a 2-PE production of 0.44 g L^{-1}. After 77 h of bioconversion, all L-Phe was consumed. In the experiments at pH 5, only 5.5 g L^{-1} of L-Phe was consumed to produce 0.82 g L^{-1} of 2-PE, after 57 h (Figure 8B). In fact, when cells reached the stationary phase, the precursor consumption stabilizes, and around 2.2 g L^{-1} of L-Phe remaining in the bioconversion broth, at the end of the experiment. On the other hand, the precursor consumption rate was negatively affected by the control of medium pH at 5, since only 72 % of the total L-Phe added was consumed in comparison to the total precursor consumption verified with the not controlled pH culture.

In the experiments at pH 7, the production of 2-PE was 0.44 g L^{-1} after 56 h of cultivation, coinciding with the glucose depletion and reached the maximum titer of 0.45 g L^{-1} after 80 h (Figure 8B). Its concentration increased as the initial pH of the culture medium decreases (pH 5), with a production rate of 13.8 mg L^{-1} h^{-1}, after 53 h, reaching a maximum titer of 1.92 g L^{-1}, after 80 h (Figure 8B). However, it was not possible to observe a plateau in 2-PE concentration that probably takes more time to achieve under this condition.

The control of media at pH 5, led to a 2.7-fold and a 2.2-fold improvement in 2-PE titer when compared with the concentrations attained in the previous experiments without pH control at 30 % DO and 10 % DO, respectively (Figure 6B). In the bioreactor experiments with 10 % DO and pH 5, a 22 % increase in the 2-PE concentration was observed comparing with the shake-flask experiments (Figure 5B). The same behaviour was also observed for the bioconversion yield and productivity (Table 7) with a 4.4 and 1.8-fold improvement, respectively, when compared with the concentrations attained in the previous experiments without pH control and 10 % DO (Table 6). Comparing with the shake-flask experiments (Table 5), a 104 and 334 % improvement in the bioconversion yield and productivity, respectively, was observed.

Table 7. Maximum productivity and yield of 2-PE for *Y. lipolytica* W29, in a STR, supplemented with 8 g L^{-1} L-Phe.

Strain	pH	$Y_{2\text{-PE/L-Phe}}$ (g g^{-1})	Productivity (mg L^{-1} h^{-1})
Y. lipolytica W29	7	0.05	6.0
	5	0.57	28.2

Several authors reported an enhancement in 2-PE production when the medium pH also favoured the biomass formation (Chantasuban et al., 2018; Garavaglia et al., 2007; Huang et al., 2001). Huang et al. (2001) studied the 2-PE production with *P. fermentans* L-5 at different pH, and they observed that an increase in the initial pH of the culture medium increases the 2-PE production, being reached a maximum 2-PE production of 515.2 mg L^{-1} in the medium with an initial pH of 8.5. Moreover, Mu et al. (2014) observed that a low pH is more favourable for cell growth but is unfavourable for 2-PE production. The highest 2-PE titer was obtained at a pH 8 (Mu et al., 2014). Nevertheless, it is very difficult to clearly identify a trend in the effect of the media pH among the reported systems, and it is probably set in accordance with the particular strain needs. Chantasuban et al. (2018) reported a higher 2-PE production with *M. pulcherrima* at pH 4 than at pH 5. We also observed a similar behaviour with *Y. lipolytica* W29, since a high 2-PE production was attained with a low medium pH. In this case, the more acidic environmental clearly leads to an enhancement in the activity of the Ehrlich pathway enzymes. Additionally, the lipase production in *Y. lipolytica* is highly dependent on medium pH and yeast strain. Therefore, the conclusions of the influence of medium pH over lipase production as not been consensual. Several studies reported the maximum lipase production for *Y. lipolytica* strains at pH 5, for *Y. lipolytica* LMI 91 (Gonçalves et al., 2013), between 5 and 6, for *Y. lipolytica* NCIM3639 (Yadav et al., 2011) or pH 7, with *Y. lipolytica* CBS6303 (Domínguez et al., 2010). It was also possible that the acidic pH in bioconversion experiments was not favourable for lipase production, and the esterification of 2-PE to 2-phenylethlyacetate is reduced, leading to an increase in 2-PE concentration.

A maximum 2-PE concentration of 1.92 g L^{-1} was obtained with a DO of 10 % and at pH 5, with an increase in the bioconversion yield and productivity. In a batch reactor of *Y. lipolytica* NCYC3825 at pH 4, the bioconversion yield and productivity only reached 0.09 g g^{-1} and 14.25 mg L^{-1} h^{-1}, respectively (Celińska et al., 2013). Chreptowicz et al. (2016) reported a bioconversion yield and productivity of 0.68 g g^{-1} and 92.99 mg L^{-1} h^{-1}, respectively, in the first 24 h of batch bioconversion with *S. cerevisiae* JM2014. However, contrary to what was observed with *Y. lipolytica* W29, the productivity values of the

shaking flask experiments with *S. cerevisiae* JM2014 were lower than the ones attained at bioreactor scale (Chreptowicz et al., 2016).

In fact, the obtained 2-PE titer is competitive with the titers achieved by other microorganisms, however the process at bioreactor scale requires further research and optimization.

4.3 Crude glycerol as an alternative carbon source for 2-PE production

Current industrial practices have led to the generation of large amounts of wastes or by-products that can be used in the production of added value compounds, which is in line with the development of circular economy.

Crude glycerol is the main by-product of biodiesel industry, where 10 kg of biodiesel produced generates 1 kg of glycerol. However, crude glycerol is not pure and due to the presence of impurities its use in traditional applications become limited (Johnson and Taconi, 2007) and the purification of crude glycerol is not a cost-effective process (Amaral et al., 2009; Wang et al., 2001). Thus, it becomes necessary to find alternative strategies for the recovery and valorisation of this by-product in its un-purified form (Amaral et al., 2009; Çelik et al., 2008; Johnson and Taconi, 2007) and the biotechnological conversion of crude glycerol by microbial fermentation into value-added products has been proposed as an interesting alternative (André et al., 2010; Koutinas et al., 2007). *Y. lipolytica* is known for its capability to growth on crude glycerol (Celińska and Grajek, 2013) and thus the use of this alternative carbon source to efficiently produce 2-PE could turn this bioprocess economically attractive.

In order to investigate if *Y. lipolytica* W29 exhibits the ability to produce 2-PE in the presence of other carbon source, new experiments were carried out with crude glycerol, in the presence of 8 g L^{-1} of L-Phe (Figure 9). It was also evaluated the impact of carbon source concentration in 2-PE production. Thus, experiments were performed with 40 and 60 g L^{-1} of crude glycerol to assess if an increase in carbon source concentration could promote the complete consumption of L-Phe (Figure 9). Due to the difficulty in access the correct concentration of glycerol in crude glycerol, the initial carbon source concentration in the experiments were higher than expected (about 50.0 g L^{-1} instead of 40 g L^{-1} and 70.0 g L^{-1} instead of 60 g L^{-1}).

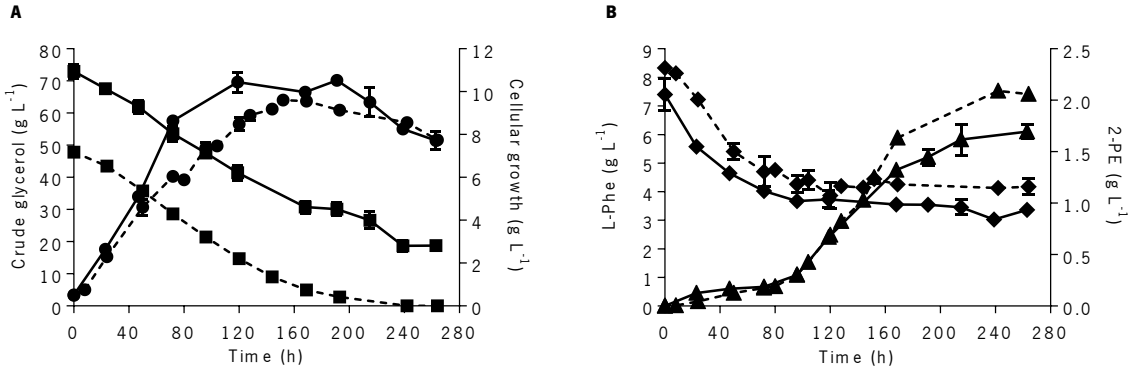


Figure 9. Cellular growth (●), crude glycerol consumption (■) (**A**), 2-PE production (▲) and L-Phe consumption (◆) (**B**) with *Y. lipolytica* W29, in shake-flasks cultures with different crude glycerol concentrations, 50 g L^{-1} (dashed lines) and 70 g L^{-1} (solid lines), supplemented with 8 g L^{-1} L-Phe. Data are presented as average and standard deviation of two independent experiments (if no error bars are visible, the standard deviations were too small to be displayed).

Analysing the growth profile for both conditions, it was possible to observe that a maximum biomass concentration of approximately 10 g L^{-1} was obtained for both cultures after 152 and 119 h of cultivation, for the experiments with 50 and 70 g L^{-1} of crude glycerol, respectively (Figure 9A). These results may be due to the fact that an average of 50 g L^{-1} of crude glycerol was consumed in both conditions, leading to the same biomass formation (Figure 9A). Comparing the obtained results with the ones performed with glucose (Figure 5A) under the same conditions (40 g L^{-1} of initial carbon source), the maximum biomass concentration attained with crude glycerol was about 18 % lower than the values obtained with glucose. The same behaviour was also been observed by Rakicka et al. (2016) with *Y. lipolytica* A-101 reaching lower biomass concentrations with glycerol when compared with glucose..

Regarding the glycerol consumption profile, after 240 h of fermentation, about 20 g L^{-1} of carbon source was still present in the bioconversion broth, in the experiments with 70 g L^{-1} of crude glycerol. Whereas it was completely depleted after 240 h, in the experiments with 50 g L^{-1} of carbon source (Figure 9A).

Looking to the L-Phe consumption profiles for both conditions, it was possible to observe that they were similar, with a consumption rate of 44.7 ± 1.7 mg L^{-1} h^{-1} and 38.9 ± 7.5 mg L^{-1} h^{-1}, during the first 80 h and 96 h, that decreases to 3.2 ± 1.3 mg L^{-1} h^{-1} and 1.8 ± 1.4 mg L^{-1}, for the experiments with 50 and 70 g L^{-1} of crude glycerol, respectively (Figure 9B). A similar behaviour was also been observed in the experiments with glucose (Figure 5B). During the highest L-Phe consumption rate, both cultures consumed an average of 4 g L^{-1} of L-Phe resulting in the average production of 0.25 g L^{-1} of 2-PE. In fact, after 264 h of fermentation only 50 - 55 % of the total L-Phe added was consumed, probably due

to a precursor uptake repression by the accumulation of some intermediates of the Ehrlich pathway, as previously discussed. In fact, the L-Phe uptake in these experiments was lower than the ones obtained with glucose (66 %). It was also clear that an increase in the carbon source concentration did not enhance the L-Phe consumption efficiency and 2-PE concentration. However, a different behaviour was reported by Huang et al. (2000), with *P. fermentans* L-5. The authors reported that an increase in sucrose concentration leads to an enhancement in the L-Phe consumption (Huang et al., 2000).

Regarding the 2-PE production profile, it was possible to observe a maximum 2-PE production of 2.09 ± 0.02 g L^{-1} and 1.69 ± 0.07 g L^{-1}, for the experiments with 50 g L^{-1} and 70 g L^{-1} of crude glycerol, respectively being observed statistically significant differences at $p \leq 0.05$ (Figure 9B). In fact, after 80 h, it was possible to observe an increase in the production rate from 2.4 ± 0.2 mg L^{-1} h^{-1} to 16.2 ± 0.2 mg L^{-1} h^{-1} for the experiments with 50 of crude glycerol, whereas for the 70 g L^{-1} of crude glycerol experiments, the production rate increased from 3.1 ± 0.6 mg L^{-1} h^{-1} to 11.1 ± 0.7 mg L^{-1} after the first 96 h. Moreover, a 1.4-fold increase in substrate concentration led to a 1.2-fold decrease in the 2-PE concentration. Nevertheless, the 2-PE titer attained with 50 g L^{-1} of crude glycerol was 33 % higher than the value obtained with glucose, being observed statistically significant differences at $p \leq 0.05$ (Figure 5B).

In the experiments with 50 g L^{-1} of crude glycerol, the end of the bioconversion was accompanied by stabilization in the 2-PE production, and at the same time the carbon source was depleted, as previously observed (Figure 5). However, for the experiments with 70 g L^{-1} of crude glycerol, the 2-PE production stabilizes at the end of the bioconversion but, in this case, is still observed around 20 g L^{-1} of carbon source in the broth, which reinforce the hypothesis of 2-PE stabilization by accumulation of toxicity intermediates of the Ehrlich pathway. When comparing the process yields of 2-PE obtained in each of the two conditions tested, no significant differences were observed ($p \leq 0.05$) (Table 8). However, the productivities obtained present significant differences at $p \leq 0.05$ (Table 8). Nonetheless, the yield and productivity obtained with 50 g L^{-1} of crude glycerol are 1.8-fold and 1.3-fold higher, respectively, than the ones attained with glucose under the same conditions (Table 5), being observed statistically significant differences ($p \leq 0.05$). However, the bioconversion yields are still lower than the theoretical value (Etschmann et al., 2003). This enhancement in 2-PE production may be related with a lower lipase activity in the glycerol media (Fabiszewska et al., 2015), and therefore a low conversion of 2-PE to 2-phenylethylacetate is observed. It is also suggested a possible inhibition of the alcohol acetyltransferase due to the formation of unsaturated fatty acids from glycerol metabolism (Yoshioka

and Hashimoto, 1983). Huang et al. (2000) studied the 2-PE production with *P. fermentans* L-5 and they also reported high titers and bioconversion yields with glycerol compared to glucose.

Table 8. Maximum productivity and yield of 2-PE for *Y. lipolytica* W29, in shake-flasks cultures with varying crude glycerol concentrations of 50 g L^{-1} and 70 g L^{-1}, supplemented with 8 g L^{-1} L-Phe. Data are presented as average and standard deviation of two independent experiments.

Strain	Crude glycerol (g L^{-1})	$Y_{2\text{-PE/L-Phe}}$ (g g^{-1})	Productivity (mg L^{-1} h^{-1})
Y. lipolytica W29	50	0.50 ± 0.04 [a]	8.6 ± 0.1 [a]
	70	0.42 ± 0.03 [a]	6.5 ± 0.3 [b]

[a] [b] Values followed by the same letter do not present statistically significant differences ($p \leq 0.05$) by the t test analysis.

Taking the above results into consideration, crude glycerol can be considered an interesting and promising alternative carbon source for 2-PE production with *Y. lipolytica* W29. Therefore, the following experiments were performed with 50 g L^{-1} of crude glycerol.

4.4 ISPR experiments

4.4.1 Selection of adsorbent resin

The biotechnological production of 2-PE is a very attractive option to overcome the limitations of chemical synthesis and extraction from natural sources, however its production is still limited due to the cytotoxicity effects of 2-PE towards the production host. Therefore, the recent developments report the 2-PE production by L-Phe bioconversion coupled with ISPR techniques. Thus, the product removal by adsorption, which uses adsorbent resins for the continual product removal, will be the ISPR technique applied in L-Phe bioconversion to 2-PE by *Y. lipolytica* W29 using crude glycerol as carbon source. For this, the affinity of three adsorbent resins (XAD-7HP, XAD-16 and XAD-4) for 2-PE and L-Phe was firstly evaluated, as well as the amount of adsorbent resin used (Figure 10).

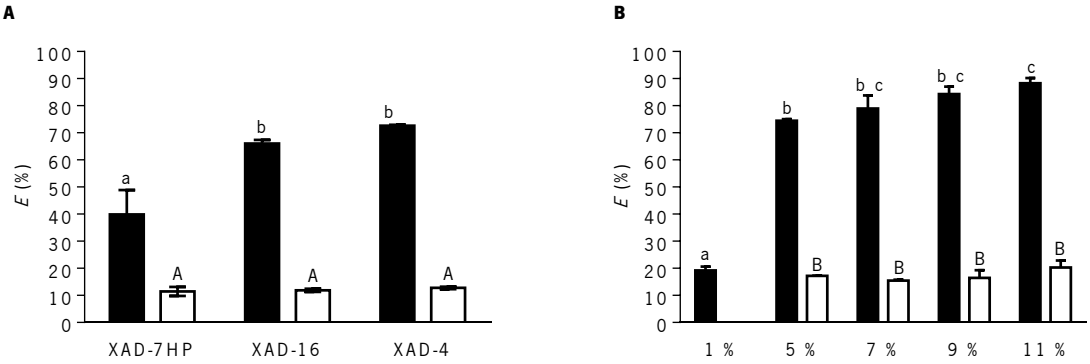


Figure 10. The adsorption capacity (*E*, %) of the resins Amberlit XAD-7HP, XAD-16 and XAD-4 for 2-PE (black) and L-Phe (white) in media containing 10 g L^{-1} of 2-PE and L-Phe, with 5 % (wet w/v) (**A**) and of the adsorption of 2-PE and L-Phe by resin XAD-4 with 1, 5, 7, 9 and 11 % (wet w/v) (**B**). Data are presented as average and standard deviation of two independent experiments (if no error bars are visible, the standard deviations were too small to be displayed). Values followed by the same letter do not present statistically significant differences ($p \leq 0.05$) by the one-way ANOVA test. Lowercase letters are for the 2-PE adsorption values. Uppercase letters are for the L-Phe adsorption values.

The affinities of the selected resins for 2-PE and L-Phe are shown in Figure 10. The choice of the most suitable resin was based on two criteria: its ability to highly adsorb 2-PE, in order to maintain its concentration in the bioconversion broth above the toxic limit, and high specificity to avoid L-Phe sequestration, in order to maintain the precursor available for cells.

After 2 h of incubation, all resins showed the ability to adsorb 2-PE (Figure 10A). The resin XAD-7HP showed the lowest 2-PE affinity (the differences presenting statistically significant differences at $p \leq 0.05$), with no more than 40 % of 2-PE added to the bioconversion broth adsorbed. The adsorption capacities of the resins XAD-16 and XAD-4 were the highest, with 66.1 ± 1.3 % and 72.6 ± 0.3 % of adsorption ratio observed for both resins, respectively, not being observed statistically significant differences at $p \leq 0.05$. Concerning the resins specificity, they presented an average adsorption of 12 % of L-Phe, not being observed statistically significant differences at $p \leq 0.05$ (Figure 10A). The XAD-16 an XAD-4 resins are both hydrophobic cross-linked polystyrene resins, whereas XAD-7HP is a functionalised resin with an acrylic matrix. The hydrophobic cross-linked polystyrene resins preferably adsorb the hydrophobic 2-PE over the hydrophilic L-Phe, due to its non-polarity characteristic and large surface area (Hua et al., 2010; Mei et al., 2009). Contrarily, the functionalised resin, XAD-7HP, has a low adsorption capacity as a consequence of the functionalisation of the base matrix with cationic groups and low surface area (Šimko et al., 2015). Thus, comparing the adsorption performances of the three resins for 2-PE, the polystyrene XAD-4 resin was selected for further studies since it has shown a high specificity for 2-PE adsorption, and the adsorption capacity for L-Phe was equal for all resins tested.

Therefore, it was necessary to determine the most suitable amount of adsorbent resin used in the bioconversion experiments. Experiments were performed with different quantities of the resin XAD-4. As shown in Figure 10B, the 2-PE adsorption capacity increases as the quantity of resin added increases. Less than 20 % of 2-PE was adsorbed when 1 % (wet w/v) of XAD-4 was added to the bioconversion medium. The highest adsorption ratio was obtained with 11 % (wet w/v) of adsorbent resin, with 88.3 ± 1.9 % of the 2-PE added adsorbed. However, it was clear that 7 and 9 % (wet w/v) XAD-4 had almost the same adsorption ability, since the differences in adsorption ration between these experiments were not statistically significant at $p \leq 0.05$). Concerning the L-Phe adsorption, it was null in the experiments with 1 % (wet w/v) and was about 17 % for the other conditions evaluated (without statistically significant differences at $p \leq 0.05$) (Figure 10B). Based on the obtained results, a resin load of 7 % (wet w/v) was chosen for the bioconversion experiments with *Y. lipolytica* W29, since with this amount of resin it is established a balance between the adsorption of 2-PE and L-Phe (high adsorption of 2-PE and low adsorption of L-Phe). Also, considering that the adsorption capacities with 7, 9 and 11 % (wet w/v) are statistically similar and considering the economical aspect of the process, 7 % (wet w/v) of XAD-4 was selected, since it allows the same amount of product extraction using less resin.

4.4.2 Bioconversion experiments with ISPR

Batch bioconversion of L-Phe with the addition of adsorbent resin XAD-4 were performed. The initial concentrations of L-Phe and crude glycerol were 8 g L^{-1} and 50 g L^{-1}, respectively. Resin XAD-4 (7 % (wet w/v) was added to the system at the start of the bioconversion. The obtained results are presented in Figure 11.

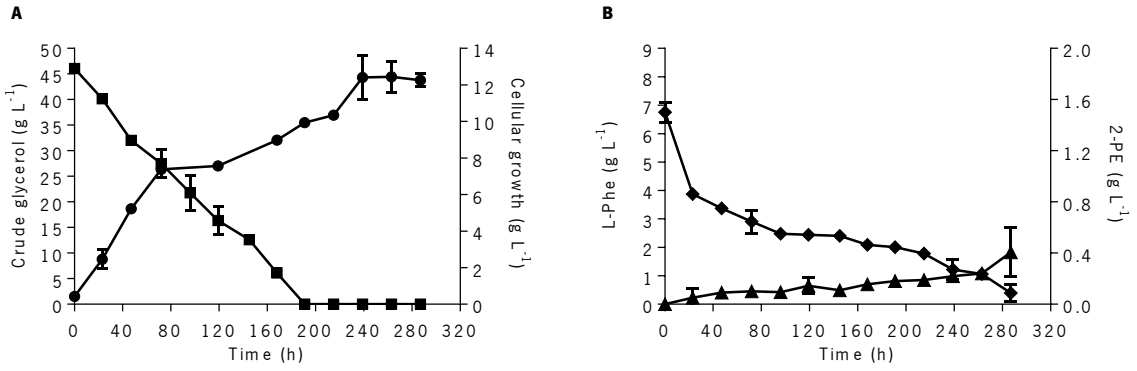


Figure 11. Cellular growth (●), crude glycerol consumption (■) (**A**), 2-PE production (▲) and L-Phe consumption (◆) (**B**) with *Y. lipolytica* W29, in shake-flasks cultures, with crude glycerol as carbon source, supplemented with 8 g L^{-1} L-Phe and 7 % (wet w/v) of adsorbent resin XAD-4. Data are presented as average and standard deviation of two independent experiments (if no error bars are visible, the standard deviations were too small to be displayed).

Comparing the growth profile under this condition with batch without adsorption (Figure 9A), it was clearly confirmed that the viability of the cells is not affected by the presence of the resins, i.e., neither adsorption of essential nutrients to the resin nor counter-ion release into the medium are problematic for the adsorption strategy. The results of Figure 11A evidence this behaviour, since the polymer appeared to have no affinity for crude glycerol, as the substrate consumption profile was not affected. Adler et al. (2011) also observed the same effect in a membrane extraction process.

Analysing the cell growth profile, it was possible to see that the cells grew exponentially for 239 h reaching a maximum biomass concentration of 12.4 ± 1.2 g L^{-1} (Figure 11A). Comparing the obtained results with the conventional bioconversion (Figure 9A), the maximum biomass concentration reached in the scenario with adsorption was 1.2-fold higher than the values obtained without adsorption. As expected, the application of an ISPR technique allowed a high biomass concentration, since the continuous removal of 2-PE from the bioconversion broth avoids the direct contact of the toxic product with cells (Pérez, 2001).

Regarding the L-Phe consumption, during the first 24 h of fermentation, approximately, 43 % of the precursor added was consumed (Figure 11B). During the remaining time of the experiment, the precursor consumption rate was almost constant, and only 0.4 ± 0.29 g L^{-1} of L-Phe was remaining in the bioconversion broth at the end of the experiment. One of the key points also noticing is that the application of the ISPR led to an enhancement in the L-Phe consumption, with a 1.5-fold increase in the precursor consumption rate compared with batch experiments without product removal (Figure 9B). In addition, it was also possible to achieve a L-Phe consumption of about 94 %, which is 44 % higher than the value attained in the culture without product removal. This suggests that the adsorption strategy here tested might also promote the adsorption of the intermediates of the Ehrlich pathway that could inhibit the L-Phe uptake. Therefore, the precursor can be completely consumed, and the 2-PE production increased.

Regarding the production of 2-PE, the results obtained suggest that its kinetic profile is also similar when compared with experiments without product removal (Figure 9B). Nonetheless, lower amounts of 2-PE were detected in the bioconversion broth when the ISPR strategy was used. While for the experiments without product removal 2.09 ± 0.02 g L^{-1} of 2-PE was obtained, the value dropped to 0.41 ± 0.19 g L^{-1} with the product removal strategy (Table 9), being this concentration lower than the toxic limit observed for *Y. lipolytica* W29 (Figure 3).

Table 9. Comparison of the amount of 2-PE produced in the different scenarios with *Y. lipolytica* W29, in shake-flasks cultures with crude glycerol, supplemented with 8 g L^{-1} L-Phe, and with XAD-4 (7 % (wet w/v)). Data are presented as average and standard deviation of two independent experiments.

Strain		2-PE_{resin} (g L^{-1})	2-PE_{broth} (g L^{-1})	2-PE_{total} (g L^{-1})
Y. lipolytica W29	With ISPR	2.04 ± 0.31	0.41 ± 0.19	2.44 ± 0.50 [a]
	Without ISPR	-	2.09 ± 0.02	2.09 ± 0.02 [a]

[a] Values followed by the same letter do not present statistically significant differences ($p \leq 0.05$) by the t test analysis.

In fact, with this strategy a 1.2-fold increase in the 2-PE concentration was achieved, even though it does not present statistically significant differences at $p \leq 0.05$ (Table 9). In addition, the bioconversion yield and productivity obtained were not statistically different from the values reached in the conventional process at $p \leq 0.05$ (Table 10).

Reports by Mei et al. (2009) and Hua et al. (2010), with adsorbent resins added to the bioconversion broth, also shown significant improvements in the 2-PE production. Adler et al. (2011) suggested that the continuous product removal might lead to a disturbance in the enzymatic conversion of phenylacetaldehyde to 2-PE and 2-phenylethylacetate, resulting in an enhancement in L-Phe metabolism via Ehrlich pathway.

Table 10. Maximum productivity and yield of 2-PE for *Y. lipolytica* W29, in shake-flasks cultures with crude glycerol, supplemented with 8 g L^{-1} L-Phe, and with XAD-4 (7 % (wet w/v)). Data are presented as average and standard deviation of two independent experiments.

Strain	$Y_{2\text{-PE/L-Phe}}$ (g g^{-1})	Productivity (mg L^{-1} h^{-1})
Y. lipolytica W29	0.39 ± 0.12	8.5 ± 1.8

5. CONCLUSIONS AND PERSPECTIVES FOR FUTURE WORK

5.1 Conclusions

2-PE production by L-Phe bioconversion using microorganisms is an attractive way to produce this flavor compound. Even more, these compounds are structurally identical with those present in natural sources and possess the "natural" label, which is considered advantageous by the consumer preferences. Although, the scientific community has dedicated time and efforts around the production of "natural" aromas, the overall productivity is still very low.

Thus, this work allowed to develop a 2-PE production process, through L-Phe bioconversion, using *Y. lipolytica* as a cell factory. Firstly, the inhibitory effect of 2-PE and L-Phe on cellular growth of *Y. lipolytica* strains were assessed. It was observed a remarkable inhibitory effect of 2-PE on the cell growth of all the strains tested (*Y. lipolytica* W29, CBS2075 and NCYC2904) in concentrations equal or above 2 g L^{-1}. However, when exposed to 1 g L^{-1} of 2-PE, the W29 and CBS2075 strains showed a higher resistance to the product. Concerning the inhibitory effect of L-Phe, the cellular growth of the NCYC2904 strain was greatly affected by the precursor concentration, even at low L-Phe concentrations. The W29 and CBS2075 strains, presented a slightly high resistance to L-Phe, however the growth of both strains was completely repressed at L-Phe concentrations equal or above 15 g L^{-1}.

Afterwards, the ability to produce 2-PE through L-Phe bioconversion was studied using the W29 and CBS2075 strains, with 5 g L^{-1} of L-Phe and glucose as the carbon source, at shake-flaks scale. It was demonstrated that both strains were able to produce 2-PE, however the production titers attained were strain's dependent. The W29 strain showed a high potential to produce 2-PE, reaching a maximum concentration of 1.01 ± 0.07 g L^{-1} (26 % higher than the value achieved with the CBS2075 strain), with a bioconversion yield of 0.23 ± 0.02 g L^{-1} and a productivity of 6.0 ± 0.04 mg L^{-1} h^{-1}. However, the bioconversion yield attained with both strains was far below from the theoretical value of 0.75 g g^{-1}. Based on this, the W29 strain was selected for the following experiments.

The effect of precursor concentration on 2-PE production by *Y. lipolytica* W29 was also studied. New experiments were performed in the presence of 8 g L^{-1} of L-Phe, and it was possible to conclude that an increase in the precursor concentration led to an enhancement in biomass concentration and also in the maximum concentration of 2-PE (1.57 ± 0.06 g L^{-1}). Nevertheless, an incomplete consumption of the precursor was observed (only 66 %), leading to a bioconversion yield and productivity similar to those obtained in the experiments with 5 g L^{-1} of L-Phe. Taking into consideration that a high L-Phe concentration lead to a high 2-PE production, the next experiments were performed with 8 g L^{-1} of precursor.

The 2-PE production was further studied at bioreactor scale, taking into account the effect of DO concentration and medium pH. It was demonstrated that the high aeration rates needed to maintain a DO at 30 % had a negative effect on the cellular growth of *Y. lipolytica* W29, due to the shear stress caused. Under these conditions, it was possible to observe a dimorphic transition from yeast to hyphae-form. In addition, the 2-PE production decreases as the DO increase, and in the experiments with 10 % DO a maximum 2-PE concentration of 0.88 g L^{-1} was reached. Moreover, it was observed that a more acidic environment (pH 5) had a positive effect in 2-PE production, since a higher 2-PE concentration (1.92 g L^{-1}) was obtained in the experiments at pH 5 when compared with pH 7 (0.45 g L^{-1}). These results revealed that the maximum 2-PE concentration and yield was achieved at pH 5 and with a DO of 10 %. The scale-up of the process also allowed an increase in process yield and productivity.

The ability of *Y. lipolytica* W29 to produce 2-PE using an alternative carbon source, in this case the crude glycerol, was also evaluated. It was demonstrated that this yeast has the ability to produce 2-PE in a culture media where crude glycerol is the carbon source, allowing a higher 2-PE concentration (2.09 ± 0.02 g L^{-1}) when compared to glucose under the same conditions. Furthermore, the bioconversion yield reached with crude glycerol was also enhanced when compared with the experiments with glucose (0.50 ± 0.04 *vs* 0.28 ± 0.04 g g^{-1}). The effect of substrate concentration was also analyzed, and it was demonstrated that an increase in crude glycerol concentration did not enhance the L-Phe consumption efficiency nor the 2-PE concentration.

It was also studied a bioconversion strategy with product removal by adsorption. The affinity of the adsorbent resins XAD-7HP, XAD-16 and XAD-4, for 2-PE and L-Phe was evaluated. The resin XAD-4 was selected since it presented an adsorption capacity of 72.6 ± 0.3 % for 2-PE, with a high specificity, once only an average of 12 % of L-Phe was adsorbed. The results also revealed that a resin load of 7 % (wet w/v) allow to adsorb 79.0 ± 4.8 % of 2-PE. With this amount of resin, it was established a balance between the adsorption of 2-PE and L-Phe (high adsorption of 2-PE and low adsorption of L-Phe). Batch bioconversions of L-Phe with crude glycerol and with 7 % (wet w/v) adsorbent resin XAD-4 were performed, and, as expected, the application of an ISPR technique allowed an increase in biomass production, as well as a 1.2-fold increase in the 2-PE concentration, compared to the bioconversion without addition of the adsorbent resin, with a yield of 0.39 ± 0.12 g g^{-1}. Moreover, it was observed that in the experiments with ISPR, all L-Phe was consumed (a 1.5-fold increase in the precursor consumption rate was observed when compared with batch experiments without ISPR).

5.2 Perspectives for future work

The present work brings new perspectives on the biotechnological production of 2-PE by *Y. lipolytica* W29 contributing with some strategies to increase the flavor production, but there is still room for further studies and developments.

Considering that current agricultural and industrial practices have led to the generation of large amounts of agro-industrial residues that can be used for the production of added value compounds, it would be very interesting to test other alternative substrates such as molasses, grape must and fruit and vegetables wastes, that are available at low cost, all year in Portugal, for 2-PE production.

In this bioprocess it will also be interesting to investigate the fed-batch and continuous mode of operation, since they will decrease the toxicity of the precursor and produced flavor towards the cells. This will be particularly interesting to apply in pneumatically agitated bioreactors, such as airlift bioreactor. This could allow high oxygenation levels without decreasing the biomass formation and, consequently, the 2-PE production levels. Another interesting assignment can pass through the use of immobilized cells that could exhibit higher tolerance to 2-PE. In this context, the selection of the best supports for cell immobilization, optimization of the immobilization methods and culture conditions, and the possibility of reuse the immobilized cells in different cultures, could be assessed. In fact, the use of a continuous mode of operation with immobilized cells will be a very interesting approach for 2-PE production. The integration of the ISPR techniques at bioreactor scale will also be attractive, since it allows the reduction of the product toxicity and its simultaneously purification, leading to a simpler downstream process design.

It will also be important to perform some rational metabolic engineering approaches to increase the 2-PE production, such as inactivation of competing pathways or (over)-expression of genes leading to accumulation of 2-PE (eg. YALI0E20977p, YALI0F19910p).

Finally, in order to increase the viability of the process, it would be important to exploit the by-products accumulated during the 2-PE production, such as lipases and other derivate flavors. Also, it is highly important to elucidate the 2-PE production pathways in *Y. lipolytica*, and it would be interesting to identify and quantify the intermediates of the Ehrlich pathway that could be one of the reasons for lower yields.

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ANNEXES

Annex I – Biomass calibration curves

Table A1. Biomass calibration curve of *Y. lipolytica* W29 and CBS2075 strains.

Strain	Range (g L^{-1})	Biomass (g L^{-1}) = ($m \pm S_m$) x OD_{600} - ($b \pm S_b$)		Detection limit (g L^{-1})	Quantification limit (g L^{-1})
		($m \pm S_m$)	($b \pm S_b$)		
Y. lipolytica W29	0 – 0.50	(0.965 ± 0.063)	- (0.022 ± 0.017)	0.04	0.14
Y. lipolytica CBS2075	0 – 0.51	(1.144 ± 0.097)	- (0.012 ± 0.027)	0.03	0.11

Annex II – Glucose and glycerol calibration curves

Table A2. Calibration curves of glucose and crude glycerol.

Compound (g L^{-1})	Range (g L^{-1})	Area (g L^{-1}) = ($m \pm S_m$) x Concentration (g L^{-1}) - ($b \pm S_b$)		Detection limit (g L^{-1})	Quantification limit (g L^{-1})
		($m \pm S_m$)	($b \pm S_b$)		
Glucose	0 – 0.20	(10.782 ± 0.056)	+ (0.324 ± 0.491)	0.11	0.36
Crude glycerol	0 – 0.21	(8.922 ± 0.017)	+ (0.130 ± 0.152)	0.04	0.13

Annex III – 2-PE and L-Phe calibration curves

Table A3. Calibration curves of 2-PE and L-Phe.

Compound (g L^{-1})	Range (g L^{-1})	Area (g L^{-1}) = ($m \pm S_m$) x Concentration (g L^{-1}) - ($b \pm S_b$)		Detection limit (g L^{-1})	Quantification limit (g L^{-1})
		($m \pm S_m$)	($b \pm S_b$)		
2-PE	0 – 250	(8972.5 ± 121.0)	- (19410.8 ± 1.0)	3.6	11.9
L-Phe	0 – 250	(11711 ± 1289.1)	- (109038 ± 164443.3)	23.0	76.4

Where m represents the slope and S_m its associated standard error and b the y-intercept and S_b its associated standard error.

The detection limit (LD) (2) and quantification limit (LQ) (g L^{-1}) (3) were calculated as follows:

$$LD = \frac{3 \times S_{b1}}{m} \quad (2)$$

$$LQ = \frac{10 \times S_{b1}}{m} \quad (3)$$

Where S_{b1} represents the standard error of the independent term in the regression equation.

Annex IV – Agitation rate profiles

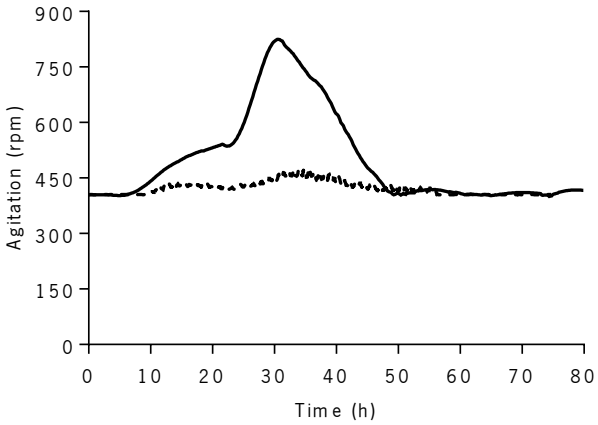

Figure A1. Agitation rate profiles for *Y. lipolytica* W29 at DO of 10 % (dashed lines) and 30 % (solid lines), in batch bioreactor cultures.