Valorization of crude glycerol as carbon source for the bioconversion of L-phenylamine to 2-phenylethanol by *Yarrowia* species

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Short Title: 2-Phenylethanol production by *Yarrowia* species

Abstract

BACKGROUND: 2-Phenylethanol (2-PE) is an aromatic alcohol with delicate fragrance of rose petals, widely used in perfumes, cosmetics, foods, beverages, and pharmaceutical industries. The yeast *Yarrowia* can produce 2-PE through L-Phenylalanine (L-Phe) biotransformation. However, it is poorly explored in this field, despite its interesting characteristics for this process, such as the Crabtree negative trait.

RESULTS: This study explored the potential of different *Yarrowia* species (*Y. lipolytica* W29, CH 1/5 and CH 3/4 and *Y. divulgata* M 445/4) for 2-PE production. It was observed that all strains were inhibited by 2-PE concentrations above 2 g L⁻¹, but only *Y. lipolytica* W29 was inhibited by 15 g L⁻¹ L-Phe. It was also confirmed that all strains were able to produce 2-PE, with concentrations ranging from 1 to 2.2 g L⁻¹. Afterwards, crude glycerol was used as an alternative carbon source for 2-PE production. All strains grew and produce 2-PE on this carbon source, but CH 1/5 strain was selected for further studies since it allowed the highest 2-PE throughput. The production of 2-PE with CH 1/5 was further studied in bioreactor batch cultures, where a low accumulation of 2-PE was observed. However, a step-wise fed-batch culture allowed to increase the production of 2-PE to 3.2 g L⁻¹.

CONCLUSION: To the best of the author's knowledge, this value corresponds to the highest titer of 2-PE obtained to date with *Y. lipolytica*. In this study, *Y. lipolytica* CH 1/5 is for the first time described as a good natural producer for 2-PE.

Keywords: 2-Phenylethanol (2-PE); Biotransformation; Crude glycerol; L-Phenylalanine (L-Phe); *Yarrowia* species

Introduction

2-Phenylethanol (2-PE) is an aromatic alcohol considered to be an important fragrance ingredient due to its mild, warm, and rose-honey like aroma ^[1-4]. Alongside its derivatives it is widely used in the perfume, cosmetics ^[3], pharmaceutical, food and beverage industries ^[5-7]. The economic importance of 2-PE is quite significant and in 2019 it was reported a market value of \$ 700 million ^[8] with a global 2-PE annual output of nearly 10 000 t.

The flavor compound 2-PE is naturally found in the essential oils of many plants and flowers ^[1,3,9]. However, the process of extraction from these sources is expensive, complex and presents low productivity due to the necessity of multiple separation steps ^[1,10,11]. In addition, seasonal variations, diseases and pests that can attack plants lead to a constant deficit in the supply of these flavors obtained from natural sources ^[12]. Thus, given these limitations, most of commercial 2-PE available nowadays is produced by chemical synthesis ^[13,14]. Although the synthetic 2-PE results in a less expensive process than natural extraction, the compounds produced are classified as "artificial", lowering their market value and limiting the range of applications ^[1,14,15]. Hence, great interest has been arising for the biotechnological production of 2-PE, since these processes are considered healthier and "green" by the consumers ^[2,5,16]. However, its current price is around US\$ 220 kg⁻¹ (at 99 %) ^[17]. Indeed, several microorganisms have been described with the ability to naturally synthesize 2-PE ^[18,19], being the most relevant yeasts due to their high productivity, robustness and tolerance to low pH ^[5]. 2-PE can be synthesized in yeast as a result of the amino acid catabolism by two pathways. In the shikimate pathway, simple sugars can be transformed into 2-PE via *de novo* synthesis ^[1,5,6,20,21]. However, the main bottleneck is that glycolysis and the pentose-phosphate pathways are mainly used for cell growth, instead of 2-PE production, limiting the production of the desired product ^[1,5]. Also, the *de novo* pathway presents a tightly complex feedback

regulation and lengthy reaction steps ^[22]. Therefore, 2-PE production can be significantly improved when L-Phenylalanine (L-Phe) is available in the media. Through the Ehrlich pathway, L-Phe is firstly converted to phenylpyruvate by transamination, which is then transformed to phenylacetaldehyde by decarboxylation; then, the derivative aldehyde is finally reduced to 2-PE by dehydrogenation ^[5,6,22]. The efficiency of this pathway depends on the availability of other nitrogen sources in the media, since in the presence of more readily digestible sources, L-Phe is more susceptible to be metabolized through the cinnamate pathway, limiting 2-PE production ^[6]. Moreover, the main challenge for yeast fermentation of 2-PE is its cytotoxicity ^[23] due to the fact that concentrations between 2 and 3 g L⁻¹ inhibit the cellular growth ^[24].

Yarrowia lipolytica is a nonconventional yeast that presents high potential to produce 2-PE due to several advantageous characteristics, such as Generally Regarded As Safe (GRAS) status, exceptional performance in utilization of different raw materials and its bioconversion into high-value added bioproducts, and the fact that presents Crabtree negative trait and absence of ethanol production ^[25], which is very important in the production of 2-PE. The ability to produce 2-PE by *Y. lipolytica* strains has not yet been extensively described, and Celińska et al. ^[26] first reported the ability of *Y. lipolytica* NCYC3825 strain to produce 2-PE, with an aroma production of 1.98 g L⁻¹ after 168 h cultivation, in shake flasks with glucose as carbon source ^[26]. Furthermore, the use of pure glycerol as an alternative carbon source to produce 2-PE was also evaluated at bioreactor scale, with a 2-PE production of 0.77 g L⁻¹. Even though these results showed a great potential of *Y. lipolytica* for 2-PE production, more comprehensive investigations are still needed. Moreover, the use of crude glycerol as a carbon source is a very interesting alternative since not only solves the environmental problem from waste stream of biodiesel production, but also makes this process more attractive from an economical point of view, considering the use of residues and by-products that can be turned into added value compounds, which meets the development of the circular economy ^[27]. However, crude glycerol is not pure, and the purification is not a cost-effective process, which limits its traditional applications ^[28]. Even so, crude glycerol can be directly used with high efficiency by *Y. lipolytica* ^[26,29].

Furthermore, the choice of mode of operation is especially important in the case of 2-PE production, considering its toxicity to producing cells. In this way, it is possible to select an operation mode capable of removing or diluting the toxic product from the medium, reducing its inhibitory effect and maximizing productivity. The step-wise fed-batch mode

with L-Phe presents advantages in terms of precursor inhibition and consumption [30,31]. Nevertheless, and even though increases in the production of 2-PE have been achieved through the aforementioned strategy, there is still room for improvement [11,32–34].

The main goal of this work was the study of 2-PE production from L-Phe biotransformation, using cheap feedstock as carbon source and *Yarrowia* as cell factory. Thus, the work addresses strains selection, culture conditions optimization and scale-up of the process to bioreactor level. The present work is thus expected to contribute to the development of a sustainable process for the valorization of industrial by-products, contributing for the generation of a platform for sustainable and bio-based production of flavour compounds.

Experimental Procedures

Microorganism, media and culture conditions

The strains used in this work were *Y. lipolytica* W29 (ATCC 20460) (widely used for the production of flavor compounds, such as γ -decalactone [35]), *Y. lipolytica* CH1/5 and CH3/4 isolated from cheeses, and *Yarrowia divulgata* M445/4, isolated from raw ground beef. Strains *Y. lipolytica* CH1/5 and CH3/4 and *Y. divulgata* M 445/4 were supplied by Szent István University, Budapest [36,37]. The strains were stored at -80 °C in cryogenic tubes. Cells were reactivated in YPDA medium (10 g L⁻¹ of yeast extract, 20 g L⁻¹ of peptone, 20 g L⁻¹ of glucose and 10 g L⁻¹ of agar), during 24 h at 27 °C. Cells were cultivated for 16-17 hours in a 500 mL baffled Erlenmeyer flask containing 200 mL of YPD medium (10 g L⁻¹ of yeast extract, 20 g L⁻¹ of peptone and 20 g L⁻¹ of glucose) or YPG (10 g L⁻¹ of yeast extract, 20 g L⁻¹ of peptone and 20 g L⁻¹ of glycerol), according to the carbon source used in the biotransformation experiments. Pre-cultures were incubated in an orbital shaker, at 27 °C and 200 rpm. After growing overnight, the cells were centrifuged (8000 rpm, 10 min), washed with a NaCl solution (9 g L⁻¹) and later used to inoculate the biotransformation medium with 0.5 of optical density at 600 nm (OD_{600nm}) (equivalent to a cell concentration of approximately 0.7 g L⁻¹).

Inhibitory Effect of 2-Phenylethanol and L-Phenylalanine

In order to evaluate the inhibitory effect of 2-PE and L-Phe towards the different strains of *Y. lipolytica* and *Y. divulgata*, cells were prepared as described in the previous section and used to inoculate 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), during 14 h, at 27 °C and 200 rpm

in an orbital shaker. After that, cells were diluted to a final OD_{600nm} of 0.6 in a 9 g L⁻¹ NaCl solution. Then, successive dilutions (10⁰, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴) were performed in NaCl 9 g L⁻¹ for each strain, and 3 µL of each solution were plated in solid medium (20 g L⁻¹ of glucose, 2 g L⁻¹ of (NH₄)₂SO₄ and 20 g L⁻¹ of agar) containing different concentrations of 2-PE (0 (control), 1, 2, and 3 g L⁻¹) or L-Phe (0 (control), 5, 8 and 15 g L⁻¹)) [32]. The plates were incubated at 27 °C for 48 h, being subsequently observed and photographed using the ChemiDoc TMXRS software.

Bioconversion Experiments in Shake Flasks

The L-Phe bioconversion experiments were carried out in 500 mL flasks with 200 mL of culture medium containing, per liter of water: glucose or crude glycerol 40 g, KH₂PO₄ 15 g, MgSO₄·7H₂O 0.5 g, Yeast Nitrogen Base (YNB) without amino acids 0.02 g, thiamine 3 mg, supplemented with L-Phe 4 g (adapted from Celińska et al. [38], incubated at 27 °C and 200 rpm, in an orbital shaker. YNB without amino acids, thiamine and L-Phe solutions were sterilized by filtration through a sterile syringe filter. All remaining solutions were sterilized in an autoclave at 110 °C for 30 min. The raw glycerol was supplied by Prio Energy - Prio Biocombustíveis, SA, and has the following composition (w w-1), according to the supplier: 82 % glycerol, 11.8 % water, 4.7 % NaCl, less than 0.01 % methanol, 0.5 % organic matter (which is not glycerol) and less than 1 % of other impurities.

Comparison of the 2-PE *de novo* synthesis was carried out in 500 mL shake flasks with 200 mL of a medium containing 10 g L⁻¹ of yeast extract and 40 g L⁻¹ of glucose, incubated at 27 °C and 200 rpm [38].

Bioconversion Experiments in Bioreactor

The bioreactor experiments were carried out in a 3.7 L bioreactor (RALF PLUS SOLO, Bioengineering) with a diameter of 17 cm and 31 cm height. Air was supplied with a sparger located at the base of the agitator, with a flow-rate automatically controlled. Stirring rate was promoted by an agitator with two Rushton impellers, 6-blade and 6 cm outside diameter. The medium pH was automatically controlled by the addition of potassium hydroxide (2 M) or hydrochloric acid (4 M), using a Peripex peristaltic pump (Bioengineering).

The pre-culture, prepared as described in the previous section, was used to inoculate the bioreactor with 1.1 L of the bioconversion medium previously described, with an OD₆₀₀

nm of 0.5 using crude glycerol (final concentration of glycerol - 40 g L⁻¹) and supplemented with an initial concentration of L-Phe of 4 and 8 g L⁻¹. After defining the optimal operating conditions for 2-PE production, different step-wise fed-batch strategies were tested. In the first strategy, after 80 h of the batch stage with 8 g L⁻¹ of L-Phe, a pulse of 4 g L⁻¹ L-Phe was added. In the second strategy, the batch stage was performed with 8 g L⁻¹ of L-Phe and pulses of 4 g L⁻¹ L-Phe were added after 80 h and 142 h of the beginning of the experiment.

Analytical Methods

Samples were taken at regular intervals for the analysis of the concentration of biomass, glucose, L-Phe and 2-PE. Biomass concentration was determined by measuring the optical density (OD) using a microplate reader at a wavelength of 600 nm. The OD values obtained were converted into dry cell weight (g L⁻¹) using a calibration procedure.

High-performance liquid chromatography was applied for glucose and glycerol quantification using a JASCO system associated with an RI detector (RI-2031). The samples were analyzed using an Aminex HPX-87H column from Bio-Rad, which was kept at 60 °C; 5 mM H₂SO₄ was used as mobile phase with a flow rate of 0.5 mL min⁻¹. Identification of 2-PE was performed by gas chromatography-mass spectrometry (GC-MS). The culture supernatant was collected by centrifugation at 10 000 g for 10 min. 2-PE was extracted from the supernatant with chloroform (1:1, v:v) and dried with Na₂SO₄. The extract was analyzed by GC-MS (Varian 4000) using a pulsed pressure injection of 1 µL onto a Restek RXI-5Sil MS column (30 m, 0.25 mm, 0.25 µm). Helium was used as the carrier gas. Injector and detector temperatures were 270 °C and 250 °C, respectively. The following temperature program was applied: 100 °C for 3 min, followed by a 12 °C/min increase ramp to 200 °C. Mass detector parameters were as follows: transfer line D 250 °C and scan mode range D 38-750 m/z [39].

The concentrations of 2-PE and L-Phe were determined by UHPLC (Nexera X2, SHIMADZU), with a diode array detector (SPD-M20A) at 215 nm, using a YMC ODS-Aq reverse phase column (250x4.6 mm) at 30 °C (CTO-30A). The samples were injected automatically (SIL-30AC Autosampler, SHIMADZU) at a flow rate of 1 mL min⁻¹, using water (solvent A) and acetonitrile (solvent B) as eluents. The separation was obtained using a gradient, in which the concentration of eluent A increased: 0 min - 100% A, 10 min - 100% A, 16.7 min - 70% A, 26.7 min - 70% A, 33.3 min - 100% A; 41.7 min - 100% A. The data were interpreted with the LabSolutions software.

Results and discussion

Inhibitory Effect of 2-Phenylethanol and L-Phenylalanine on *Yarrowia* spp

The major hurdle in the biotechnological production of 2-PE is its strong inhibitory effect on microbial growth. In fact, superior alcohols are well known for their antimicrobial properties ^[32,40] being responsible for the increase in the membrane fluidity ^[41] and consequent reduction of sugars and amino acids uptake, ^[42,43] respiratory capacities and protein and RNA synthesis ^[24,41].

Concentrations between 2 and 3 g L⁻¹ of 2-PE inhibit the growth of various bacteria and fungi ^[24]. Consequently, and assuming that 2-PE can diffuse the cell membrane without any major resistance, it can be expected that if exogenously added, this compound has a similar impact on the cell as if it was produced endogenously^[41]. Taking into consideration that the tolerance to 2-PE and L-Phe by *Yarrowia* cells has not yet been extensively described, the response of different *Yarrowia* strains to the increase of 2-PE and L-Phe concentrations was firstly assessed. Cells were cultured in solid medium with different concentrations of these compounds, and cell growth was subsequently evaluated (Figure 1).

Figure 1

Through the analysis of the results presented in Figure 1, it was possible to verify an inhibitory effect of 2-PE on the growth for all strains under study, at concentrations equal to or above 2 g L⁻¹. This behaviour is similar to that described by Etschmann et al. ^[19] for *K. marxianus* CBS 600 where 2.6 g L⁻¹ of 2-PE inhibited yeast growth completely. However, comparing the performance of the four strains, it was observed that *Y. lipolytica* W29 was the one that presented high resistance to 2-PE. In fact, for this strain there was no difference on the growth in the presence of 1 g L⁻¹ of 2-PE compared with control. However, for the strain *Y. divulgata* M 445/4, the growth was significantly inhibited by 1 g L⁻¹ of 2-PE compared to the control, even for cultures with high inoculum concentration (without dilution, 10⁰). Thus, this strain presented the lowest resistance to the product. For *K. marxianus*, a 2-PE concentration of 1.4 g L⁻¹ was determined by Fabre et al. ^[30] 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 ^[30,44]. For *S.*

cerevisiae, several studies reported the critical value of 4 g L⁻¹ 2-PE, and above this value 2-PE was toxic for yeast proliferation [11,32,41]. More recently, Yan et al.^[45] reported that the growth of the new isolated *Meyerozyma* sp. strain YLG18 was also completely inhibited by a 2-PE concentration of 4 g L⁻¹.

Regarding the L-Phe tolerance, the cell growth of *Y. lipolytica* W29 strain was not affected in culture media containing 5 and 8 g L⁻¹ of L-Phe. However, in the experiments with 15 g L⁻¹ of L-Phe the cellular growth was completely repressed, contrary to what was observed for the remaining strains. The strain *Y. lipolytica* CH 1/5 showed the highest resistance to L-Phe, as it grew for all L-Phe concentrations tested, even at higher inoculum dilutions. A similar result was also reported by Lu et al. [32] 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 [30,46,47]. Thus, it is possible to conclude that the inhibitory effect of 2-PE and L-Phe is not uniform for all strains under study and these differences may be related with the fact that these strains have different origins. In fact, 2-PE and L-Phe are often found in diverse food products, and the strains isolated from different environments or products with 2-PE or L-Phe, even in small amounts, may presented some resistant [36].

Bioconversion Experiments in Shake Flask

The production of 2-PE by L-Phe biotransformation is one of the simplest and mostly used strategies to produce this compound. Thus, considering that the production of 2-PE by *Yarrowia* cells is poorly studied, the ability of *Y. lipolytica* W29, CH 1/5, CH 3/4 and *Y. divulgata* M 445/4 to produce this aroma by bioconversion of L-Phe was evaluated (Figure 2). Therefore, the media were supplemented with 4 g L⁻¹ of L-Phe as the only nitrogen source.

Figure 2

Analysing the cell growth profile for the four strains under study, it was observed that for the strain *Y. lipolytica* W29 the maximum biomass concentrations (11.2 ± 0.7 g L⁻¹) were obtained 110 h after the beginning of the experiment, while *Y. lipolytica* CH 1/5 reached 9.9 ± 0.6 g L⁻¹ after 71 h. However, *Y. lipolytica* CH 3/4 and *Y. divulgata* M 445/4 only

reached the stationary phase after 143 h, with maximum biomass concentrations of $6.7 \pm 0.1 \text{ g L}^{-1}$ and $11.1 \pm 1.1 \text{ g L}^{-1}$, respectively.

Regarding the glucose consumption, for *Y. lipolytica* CH 3/4 and W29 strains, glucose was completely consumed after 168 h. Nevertheless, for strains *Y. lipolytica* CH 1/5 and *Y. divulgata* M 445/4, glucose was not completely consumed, and at the end of the experiment there were still about 7.0 ± 2.4 and $2.3 \pm 0.8 \text{ g L}^{-1}$, respectively. The carbon source was still being consumed when the biomass concentration remained constant, which leads to assume that glucose was used for maintenance purposes [48].

Concerning the L-Phe consumption profile, the precursor was almost totally consumed for the strains *Y. lipolytica* W29 and CH 1/5, remaining only 0.3 g L^{-1} after about 200 h. However, the same was not observed for the other strains (*Y. lipolytica* CH 3/4 and *Y. divulgata* M 445/4), with approximately 1.5 g L^{-1} of L-Phe being detected at the end of the experiment. In fact, during the exponential growth phase, part of the supplemented L-Phe was being used by the cells for growth. The highest rates of L-Phe consumption ($44.6 \text{ mg L}^{-1} \text{ h}^{-1}$ and $50.35 \text{ mg L}^{-1} \text{ h}^{-1}$ during the first 71 h) were observed for strains *Y. lipolytica* W29 and CH 1/5, respectively, since after 71 h only 0.54 g L^{-1} of L-Phe (on average for both strains) were detected in the medium. However, for the other two strains, the consumption was slowest ($37.11 \text{ mg L}^{-1} \text{ h}^{-1}$ and $28.02 \text{ mg L}^{-1} \text{ h}^{-1}$ during the first 71 h, for *Y. lipolytica* CH 3/4 and *Y. divulgata* M 445/4, respectively).

The exponential cell growth phase was accompanied by a low production of 2-PE, being subsequently observed a significant increase of its production in the stationary phase, remaining constant until the end of the experiment (Figure 2B). These results indicate that the bioconversion of L-Phe into 2-PE continued to occur after the exponential growth phase, indicating that the Ehrlich pathway was more active in the stationary phase. This behaviour was also previously reported for *Y. lipolytica* NCYC3825 [38], *K. marxianus* CBS5670 [48] and *K. marxianus* CBS6556 [49], where a high production rate of 2-PE was observed during the stationary phase and at the end of the experiments.

Comparing the performance of the four strains under study, it was shown that all strains were capable to produce the desired compound, however with different efficiencies, being the strain *Y. lipolytica* CH 1/5 the one that produced the highest 2-PE concentration. Thus, the maximum concentration of 2-PE, $2.2 \pm 0.3 \text{ g L}^{-1}$, was obtained with strain *Y. lipolytica* CH 1/5, with a productivity of $9.5 \pm 1.8 \text{ mg L}^{-1} \text{ h}^{-1}$ (Table 1), followed by $1.7 \pm 0.1 \text{ g L}^{-1}$ with strain *Y. lipolytica* CH 3/4, $1.06 \pm 0.01 \text{ g L}^{-1}$ and $1.0 \pm 0.1 \text{ g L}^{-1}$ by strains *Y. divulgata* M 445/4 and *Y. lipolytica* W29, respectively, that were the strains were lowest

productivities values were observed. The comparison of the concentrations obtained in this work with the data described in the literature indicates the potential of these strains for the production of 2-PE, particularly the strain CH 1/5 that produced a 2-PE titer that is 11% higher than the reported value by Celińska et al. [26] for the strain NCYC 3825. Gut et al [22] reported a 2-PE concentration that is 21 % higher than the value attained in this study, however in this case a genetically modified strain was used. When comparing to other species, Huang et al. [50] reported the production of 0.5 g L⁻¹ of 2-PE from 1 g L⁻¹ of L-Phe in 16 h with *Pichia fermentans* L-5 and Lomascolo et al. [51] obtained 1.4 g L⁻¹ of 2-PE from 6 g L⁻¹ of L-Phe in 9 days, using *Aspergillus niger*. More recently, Adame-Soto et al. [52] obtained 1.02 g L⁻¹ from 9 g L⁻¹ of L-Phe, using yeast *K. marxianus* ITD0090.

Therefore, taking into account the bioconversion yields of 2-PE with respect to L-Phe, *Y. lipolytica* CH 3/4, CH 1/5 and *Y. divulgata* M 445/4 were best producers since the obtained yields were around 40 % higher than the values obtained with W29 strain (Table 1). Additionally, they represent an improvement of 57 % when compared with the yield of 0.31 g g⁻¹ obtained by Celińska et al. [26], with strain *Y. lipolytica* NCYC3825. However, the obtained yields were 1.6 times lower (on average) than the theoretical maximum yield of 0.75 g g⁻¹ [6]. Taking into consideration that Stark et al. [41] also achieved an incomplete bioconversion in 2-PE production with *S. cerevisiae* GIV2009 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 [41]. As previous described, a possible explanation 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 cells [6,14].

Taking into consideration that the strains CH 1/5 and CH 3/4 produced the highest 2-PE titers, these strains were selected as the best candidates for the following experiments.

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, is not pure, and the purification is not a cost-effective process, which limits its traditional applications [28]. Nonetheless, it can be naturally consumed with high efficiency by *Y. lipolytica* [26]. Thus, the use of crude glycerol as a carbon source not only solves the environmental problem from waste stream of biodiesel production, but also makes this process more attractive from an economic point of view, considering the use

of residues and by-products that can be turned into added value compounds, which meets the development of the circular economy^[27]. Based on this, the ability of both strains to produce 2-PE was also studied using crude glycerol as an alternative carbon source (Figure 3).

Figure 3

Both strains were able to consume glycerol at similar rate. For *Y. lipolytica* CH 3/4 the maximum biomass concentration was very similar to the one obtained when glucose was used as carbon source ($6.3 \pm 0.1 \text{ g L}^{-1}$). However, for strain CH 1/5, glucose was the preferred carbon source for the production of biomass, when compared to glycerol, since the maximum biomass concentration was 51 % lower with glycerol. This preference for glucose over glycerol has already been reported by other authors for other strains^[53]. Moreover, glycerol was not completely consumed, and at the end of the experiment around 9 g L^{-1} were still present in the culture medium. The L-Phe consumption profile was also similar to both strains, since approximately 0.9 g L^{-1} of L-Phe, an average for both strains, still remained at the end of the experiment. Furthermore, the strain *Y. lipolytica* CH 1/5 was the one that presented the highest productivity (Table 1) and 2-PE concentration ($1.72 \pm 0.28 \text{ g L}^{-1}$) followed by $0.87 \pm 0.01 \text{ g L}^{-1}$ with strain *Y. lipolytica* CH 3/4. The concentrations obtained with *Y. lipolytica* CH 1/5 and CH 3/4 are higher than the concentration of 2-PE attained with *Y. lipolytica* NCYC3825 (0.8 g L^{-1}), using pure glycerol as a carbon source^[26], which highlights the potential of these strains. It is also worth highlighting the behaviour of *Y. lipolytica* CH 1/5 strain that clearly shifted its metabolism to produce 2-PE, which also justifies the low biomass mentioned previously. Despite the preference of glucose as a carbon source for cell growth, the production of 2-PE with *Y. lipolytica* CH 1/5 was little affected by the carbon source (Table 1). In fact, Huang et al.^[50] obtained a conversion of 0.41 g g^{-1} in the production of 2-PE with glycerol by *P. fermentans* L-5, greater than that obtained with glucose utilization (0.23 g g^{-1}). Hence, it is possible to conclude that the performance of *Y. lipolytica* CH 1/5 was not significantly impacted by the use of crude glycerol, without statistically significant differences ($p > 0.05$) in comparison with the values obtained with glucose. Consequently, since the use of glycerol makes this process more attractive from an economic and environmental point of view, it was chosen as the carbon source for all the following experiments, in addition to the use of the strain *Y. lipolytica* CH 1/5.

Table 1

Bioconversion Experiments at bioreactor scale

Batch experiments

Most of the studies describe the 2-PE production in agitated Erlenmeyer flasks [11,19,26,54] 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. The production and accumulation of 2-PE is highly dependent on the environmental conditions such as pH [46,55], aeration and agitation rates [49,55]. Since *Y. lipolytica* is a strictly aerobic microorganism, the oxygenation of the culture is also a key factor. Studies with other microorganisms have shown that high 2-PE productions were obtained when high oxygen concentrations were available in the culture medium [32,44,46,55]. Additionally, pH can affect enzymatic activities, and consequently the production and composition of flavor compounds [56] and also influence cell growth. In order to analyze the effect of aeration and agitation rates on the 2-PE production by *Y. lipolytica* CH 1/5, experiments were carried at bioreactor scale, using glycerol as the carbon source, with 4 g L⁻¹ of L-Phe, and without pH control (during the experiments the medium pH range from 6.5 to 5.5 (*data not shown*)) (Table 2).

Table 2

No statistically significant differences were found in the 2-PE concentration, productivity and yield obtained in bioreactor experiments at the different conditions analyzed. However, a 6-fold decrease in the concentration of 2-PE and a 2.4-fold decrease in the 2-PE productivity was obtained in bioreactor experiments when compared with the values obtained in batch cultures in shake flasks (Table 1). A possible explanation for the differences observed in both conditions is the impact of mechanical mixing in the cell morphology and physiology. Alonso et al. [57] observed that, in lipase production with *Y. lipolytica* IMUFRJ 50682, high agitations rates could lead to a decrease in the cellular growth due to shear stress caused by the impellers [57]. Braga et al. [58] also reported the negative effect of high agitation and aeration rates in *Y. lipolytica* W29 cells morphology in biphasic culture medium for aroma production from castor oil. Under these conditions, a morphological change was observed and the number of cells in pseudo-hyphae form

increased, as a cell mechanism response to stress conditions ^[58,59]. Under the conditions here studied, an aqueous homogenous medium, *Y. lipolytica* cells seemed to be more sensitive to mechanical agitation, leading to a lower biomass formation. In Figure 4, images of *Y. lipolytica* CH 1/5 cells collected during bioconversion experiments are presented. It was possible to observe that an increase in the agitation and aeration rates leads to an increase in the pseudo-hyphae morphotype (filamentous form) of the cells (see supplementary Figure S1B and S1C), reinforcing a dimorphic transition probably caused by an increased stress in the cells by agitation increase. On the other hand, it was clearly observed that lower agitation and aeration rates (see supplementary Figure S1A) promoted the growth in yeast morphotype (oval form).

Taking into consideration that the differences between the 2-PE concentration obtained in the previous experiments are not statistically significant, 600 rpm and 1 L min⁻¹ were selected for further bioreactor experiments, since these conditions causes less hydrodynamic stress to the cells ^[58].

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 ^[60]. 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. To study the effect of medium pH on the 2-PE production, bioconversion experiments were performed at controlled pH 5.5, 6.5 and 7.5, with an agitation rate of 600 rpm and an air flow rate of 1 L min⁻¹.

Table 3

Looking to the obtained results, it was possible to observe that the 2-PE concentration increased as the initial pH of the culture medium decreases from 7.5 to 5.5. The decrease in the concentration of 2-PE obtained with an increase in pH also leads to a decrease in the same ratio for process yield and productivity (Table 3). In the bioreactor experiments at pH 5.5, a significant decrease in the production of 2-PE concentration was observed comparing with the shake-flask experiments (9.5-fold decrease). This difference is probably due to the more marked cell growth seen in the bioreactor experiments, which led to faster consumption of L-Phe and, consequently, to a lower production of 2-PE (see supplementary Figure S2) ^[5,6]. Comparing the results obtained at pH 5.5 with the ones attained without pH control (Table 2), it was possible to conclude that the 2-PE

production with *Y. lipolytica* CH 1/5 was not significantly affected by the pH control. Huang et al. [50] studied 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. [56] observed that a low pH is more favorable for cell growth but is unfavorable for 2-PE production. The highest 2-PE titer was obtained at a pH 8 [56]. 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. [46] reported a high 2-PE production with *M. pulcherrima* at pH 4 than at pH 5. We also observed a similar behavior with *Y. lipolytica* CH 15, since a high 2-PE production was attained with a low medium pH. In this case, the acidic environment clearly led to an enhancement in the activity of the Ehrlich pathway enzymes. A maximum 2-PE concentration of 0.31 g L⁻¹ was obtained at pH 5.5, with an increase in the bioconversion yield and productivity. Chreptowicz et al. [11] 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* CH 1/5, the productivity values of the shaking flask experiments with *S. cerevisiae* JM2014 were lower than the ones attained at bioreactor scale [11].

Previous reports [30,41,61] 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 in bioreactor, L-Phe was completely consumed (see supplementary Figure S2). Based on this, new experiments were carried out to analyze the behavior of the *Y. lipolytica* CH 1/5 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 2). Therefore, a L-Phe concentration of 8 g L⁻¹ was selected for the following studies (Figure 4).

Figure 4

The maximum biomass concentration obtained was 19.15 g L⁻¹, at 79.5 h, when the cells had already reached the stationary phase of growth. It was also observed that glycerol was completely consumed after 55.5 h and biomass concentration was much higher than

the values obtained in flask scale experiments. Also, L-Phe, was completely consumed after 31.5 h, but after 24 h, 94 % of the precursor had already been consumed. Under these conditions, and contrary to the shake-flask experiments, all L-Phe was consumed. When comparing the 2-PE concentration and yield obtained in bioreactor experiments with 8 g L⁻¹ of L-Phe (0.76 g L⁻¹ of 2-PE and a yield of 0.092 g g⁻¹) with the ones obtained with 4 g L⁻¹ of L-Phe (Table 3), it was observed a 40 % and 18 % increase in both parameters, respectively. Nevertheless, the concentrations of 2-PE obtained in the bioreactor experiments were still reduced when compared to the flask scale.

Step-wise Fed-batch Cultures

Despite the simple control over culture conditions, batch mode in bioreactors present disadvantages, as previously mentioned, such as long lag time and accumulation of toxic compounds [62]. Furthermore, as previously mentioned, this difference in the 2-PE concentrations obtained in flask scale and bioreactor experiments is probably due to the more marked cell growth seen in the bioreactor experiments, which led to faster consumption of L-Phe and, consequently, to a lower production of 2-PE [5,6]. Therefore, it was decided to test the production of 2-PE in step-wise fed-batch mode with pulses of L-Phe. Thus, it was possible to provide the precursor again so that it could integrate the Ehrlich pathway, and avoid inhibition of cell growth by the precursor. In fact, high concentrations of L-Phe can inhibit not only the cell growth, but also transporters of this amino acid [10,49,54]. Consequently, for the step-wise fed-batch experiments, a pulse of 4 g L⁻¹ was added 80 hours after the beginning of the experiment (in the stationary phase of growth) (Figure 5).

Figure 5

Considering the cellular growth profile, a maximum concentration of 22.7 ± 3.1 g L⁻¹ was obtained, 79.5 h after the beginning of the experiment, and glycerol was completely consumed after 55.5 h. In comparison with the batch experiments, the biomass concentration was similar. After the addition of the L-Phe pulse it was possible to verify a decrease in biomass concentration, which can be explained by the addition of a considerable volume of L-Phe solution, which led to the dilution of the constituents of the medium, including the biomass.

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Regarding the consumption of L-Phe, before the addition of L-Phe, the precursor was already below the detection limit. After the addition of the precursor, the concentration of L-Phe was $4.19 \pm 0.98 \text{ g L}^{-1}$, and L-Phe was rapidly consumed, with a 99 % decrease in just 15.5 h. It should be noted that when the precursor was added to the medium, as expected, the stationary phase of cellular growth had already been reached, which indicates a greater availability of L-Phe to integrate the Ehrlich pathway and produce 2-PE. Consequently, and considering the production of 2-PE, at the end of the experiment a concentration of 3.2 g L^{-1} was obtained, allowing a 4.64-fold increase in comparison with the batch experiment at bioreactor scale. Thus, a productivity and conversion of $14.3 \text{ mg L}^{-1} \text{ h}^{-1}$ and 0.29 g g^{-1} , respectively, were achieved. Thereafter, and considering that L-Phe was completely consumed, the addition of a second pulse was tested. Consequently, pulses of 4 g L^{-1} were added 80 and 142 hours after the beginning of the experiment. However, after the addition of the second pulse, the 2-PE concentration remained constant until the end of the experiment (around $1.69 \pm 0.10 \text{ g L}^{-1}$). In this case, the strategy with only one pulse allowed more promising results, and it can be concluded that the addition of the second pulse did not produced the expected results, as there was no consumption of the precursor provided (*data not show*).

Wang et al. ^[63] reported the addition of pulses of 2.5 g L^{-1} of L-Phe 17 hours after the beginning of the batch culture with *S. cerevisiae* R-UV3 strain, that led to 2-PE concentration of 4.38 g L^{-1} and a productivity of $0.31 \text{ g L}^{-1} \text{ h}^{-1}$. In comparison with a batch system, where a concentration of 2-PE of 2.8 g L^{-1} and a productivity of $0.12 \text{ g L}^{-1} \text{ h}^{-1}$ were obtained, the addition of pulses represented an improvement of 1.6 and 2.6-fold, respectively. More recently, Tian et al. ^[33] also reported an increase from 3.95 g L^{-1} to 4.73 g L^{-1} (20 % increase), with a single-dose fed-batch strategy, with *Wickerhamomyces anomalus* 1D6, where L-Phe was added one time at 24 h and 600 g L^{-1} glucose storage liquor were continuously added from 18 to 72 h.

Conclusions

Y. lipolytica strain CH 1/5 was for the first time reported as a 2-PE producer with better performance than other strains. Despite some problems identified in the scale-up of the process from batch cultures in small Erlenmeyer flasks to bioreactor, mainly attributed to hydrodynamic stress and metabolic shift to cell growth, the step-wise fed-batch cultures in the bioreactor allowed to improved 2-PE production. To the best of the author's knowledge, the 2-PE concentrations obtained in this study correspond to the highest titer

of 2-PE obtained to date with non-genetically modified *Y. lipolytica*. In addition, the obtained 2-PE titer is competitive with the ones achieved by other wild-type microorganisms. Nevertheless, and even though a considerable increase in the production of 2-PE has been achieved through the aforementioned strategy, this concentration still leaves room for improvement when compared to the concentrations reported for other genetically modified microorganisms and using other operating strategies [11,32–34].

Acknowledgements

The authors thank the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UIDB/ BIO/04469/2020 unit and BioTecNorte operation (NORTE-01-0145- FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte. The authors thank Dr. D.Q. Nguyen and Dr. E.Nagy from Szent István University, Budapest, Hungary, for kindly providing *Y. lipolytica* CH 1/5, CH 3/4 and *Y. divulgata* M445/4 strains. The authors also want to express their gratitude to Prio Energy – Prio Biocombustíveis, SA for providing the glycerol used in this experimental work.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

References

1. Martínez-Avila O, Sánchez A, Font X, Barrena R. Bioprocesses for 2-phenylethanol and 2-phenylethyl acetate production: current state and perspectives. *Appl Microbiol Biotechnol* 2018;102(23):9991–10004.
2. Qian X, Yan W, Zhang W, Dong W, Ma J, Ochsenreither K, et al. Current status and perspectives of 2-phenylethanol production through biological processes. *Crit Rev Biotechnol* 2019;39(2):235–48.
3. Fabre CE, Blanc PJ, Goma. 2-Phenylethyl Alcohol: An Aroma Profile. *Perfum Flavorist* 1998;23(3):43–5.
4. Šimko I, Roriz E, Gramblička M, Illeová V, Polakovič M. Adsorption separation of 2-phenylethanol and L-phenylalanine on polymeric resins: Adsorbent screening, single-component and binary equilibria. *Food Bioprod Process* 2015;95:254–63.
5. Carlquist M, Gibson B, Yuceer K, Paraskevopoulou A, Sandell M, Angelov A, et

- al. Process engineering for bioflavour production with metabolically active yeasts - a mini-review. *Yeast* 2015;32:123–43.
6. Etschmann M, Bluemke W, Sell D, Schrader J. Biotechnological production of 2-phenylethanol. *Appl Microbiol Biotechnol* 2002;59(1):1–8.
 7. Quintelas C, Braga A, Mesquita D, Amaral A, Ferreira E, Belo I. NIR spectroscopy applied to the determination of 2-Phenylethanol and L-phenylalanine concentrations in culture medium of *Yarrowia lipolytica*. *J Chem Technol Biotechnol* 2019;94(3):812–8.
 8. Pandal N. Global Markets for Flavours and Fragrances. BCC Res Wellesley, MA 2014;
 9. Burdock GA. Fenaroli's handbook of flavour ingredients. Boca Raton: CRC Press/Taylor & Francis Group; 1976.
 10. Mei J, Min H, Lü Z. Enhanced biotransformation of L-phenylalanine to 2-Phenylethanol using an *in situ* product adsorption technique. *Process Biochem* 2009;44(8):886–90.
 11. Chreptowicz K, Wielechowska M, Głowczyk-Zubek J, Rybak E, Mierzejewska J. Production of natural 2-phenylethanol: From biotransformation to purified product. *Food Bioprod Process* 2016;100:275–81.
 12. Longo MA, Sanromán MA. Production of food aroma compounds: Microbial and enzymatic methodologies. *Food Technol Biotechnol* 2006;44(3):335–53.
 13. Angelov AD, Gotcheva V. Biosynthesis of 2-phenylethanol by yeast fermentation. *Sci Work UFT* 2012;59:490–5.
 14. Hua D, Xu P. Recent advances in biotechnological production of 2-phenylethanol. *Biotechnol Adv* 2011;29(6):654–60.
 15. Vandamme EJ, Soetaert W. Bioflavours and fragrances via fermentation and biocatalysis. *J Chem Technol Biotechnol* 2002;77(12):1323–32.
 16. Conde-Báez L, López-Molina A, Gómez-Aldapa C, Pineda-Muñoz C, Conde-Mejía C. Economic projection of 2-phenylethanol production from whey. *Food Bioprod Process* 2019;115:10–6.
 17. Martínez-Avila O, Sánchez A, Font X, Barrena. Fed-Batch and Sequential-Batch Approaches to Enhance the Bioproduction of 2-Phenylethanol and 2-Phenethyl Acetate in Solid-State Fermentation Residue-Based Systems. *J Agric Food Chem* 2019;67(12):3389–99.
 18. Forti L, Mauro S, Cramarossa MR, Filippucci S, Turchetti B, Buzzini P. Non-

- conventional yeasts whole cells as efficient biocatalysts for the production of flavours and fragrances. *Molecules* 2015;20(6):10377–98.
19. Etschmann MMW, Sell D, Schrader J. Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. *Biotechnol Lett* 2003;25(7):531–6.
 20. Albertazzi E, Cardillo R, Servi S, Zucchi G. Biogenesis of 2-phenylethanol and 2-phenylethylacetate important aroma components. *Biotechnol Lett* 1994;16(5):491–6.
 21. Tzin V, Galili G, Aharoni A. Shikimate Pathway and Aromatic Amino Acid Biosynthesis. *eLS* 2012;(December 2017):1–10.
 22. Gu Y, Ma J, Zhu Y, Xu P. Refactoring Ehrlich pathway for high-yield 2-phenylethanol production in *Yarrowia lipolytica*. *ACS Synth Biol* 2020;
 23. Hua D, Lin S, Li Y, Chen H, Zhang Z, Du Y, et al. Enhanced 2-phenylethanol production from L-phenylalanine via *in situ* product adsorption. *Biocatal Biotransformation* 2010;28(4):259–66.
 24. Lester G. Inhibition of Growth, Synthesis, and Permeability in *Neurospora crassa* by Phenethyl Alcohol. *J Bacteriol* 1965;90(1):29–37.
 25. Spencer J, Ragout de Spencer A, Lalue C. Non-conventional yeasts. *Appl Microbiol Biotechnol* 2002;58(2):147–56.
 26. Celińska E, Kubiak P, Białas W, Dziadas M, Grajek W. *Yarrowia lipolytica*: The novel and promising 2-phenylethanol producer. *J Ind Microbiol Biotechnol* 2013;40(3–4):389–92.
 27. Li C, Gao S, Yang X, Lin C. Green and sustainable succinic acid production from crude glycerol by engineered *Yarrowia lipolytica* via agricultural residue based in situ fibrous bed bioreactor. *Bioresour Technol* 2018;249:612–9.
 28. Amaral P, Ferreira T, Fontes G, Coelho M. Glycerol valorization: new biotechnological routes. *Food Bioprod Process* 2009;87(3):179–86.
 29. Pereira AS, Miranda SM, Lopes M, Belo I. Factors affecting microbial lipids production by *Yarrowia lipolytica* strains from volatile fatty acids: Effect of co-substrates, operation mode and oxygen. *J Biotechnol* 2021;331(February):37–47.
 30. Fabre CE, Blanc PJ, Goma G. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. *Biotechnol Prog* 1998;14(2):270–4.
 31. Sáenz D, Chianelli M, Stella C. L-Phenylalanine Transport in *Saccharomyces cerevisiae*: Participation of GAP1, BAP2, and AGP1. *J Amino Acids*

2014;2014:283962.

32. Lu X, Wang Y, Zong H, Ji H, Zhuge B, Dong Z. Bioconversion of L-phenylalanine to 2-phenylethanol by the novel stress-tolerant yeast *Candida glycerinogenes* WL2002-5. *Bioengineered* 2016;7(6):418–23.
33. Tian S, Liang X, Chen J, Zeng W, Zhou J, Du G. Enhancement of 2-phenylethanol production by a wild-type *Wickerhamomyces anomalus* strain isolated from rice wine. *Bioresour Technol* 2020;318(October):124257.
34. Červeňanský I, Mihal' M, Markoš. Pertraction-adsorption in situ product removal system: Intensification of 2-phenylethanol bioproduction. *Sep Purif Technol* 2020;251(June).
35. Braga A, Belo I. Biotechnological production of γ -decalactone, a peach like aroma, by *Yarrowia lipolytica*. *World J Microbiol Biotechnol* 2016;32(10).
36. Nagy E, Niss M, Dlačný D, Arneborg N, Nielsen D, Péter G. *Yarrowia divulgata* f.a., sp. nov., a yeast species from animal-related and marine sources. *Int J Syst Evol Microbiol* 2013;63(PART 12):4818–23.
37. Nagy ES. Isolation and diversity of food spoilage *Yarrowia* yeast strains from meat. *Acta Aliment* 2014;43:101–6.
38. Celińska E, Kubiak P, Białas W, Dziadas M, Grajek W. *Yarrowia lipolytica*: The novel and promising 2-phenylethanol producer. *J Ind Microbiol Biotechnol* 2013;40(3–4):389–92.
39. Guo D, Zhang L, Pan H, Li X. Metabolic engineering of *Escherichia coli* for production of 2-Phenylethylacetate from L-phenylalanine. *Microbiologyopen* 2017;4(6):1–5.
40. Zhu YJ, Zhou HT, Hu YH, Tang JY, Su MX, Guo YJ, et al. Antityrosinase and antimicrobial activities of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid. *Food Chem* 2011;124(1):298–302.
41. Stark D, Zala D, Münch T, Sonnleitner B, Marison IW, Von Stockar U. Inhibition aspects of the bioconversion of L-phenylalanine to 2-phenylethanol by *Saccharomyces cerevisiae*. *Enzyme Microb Technol* 2003;32(2):212–23.
42. Naz S, Cretenet M, Vernoux JP. Current knowledge on antimicrobial metabolites produced from aromatic amino acid metabolism in fermented products. *Microb Pathog Strateg Combat them Sci Technol Educ (A Méndez-Vilas, Ed)* 2013;(July 2016):337–46.
43. Zhang H, Fauré R, François JM, Blanc PJ, de Billerbeck GM. Xylosylation as an

effective means for reducing yeast growth inhibition by 2-phenylethanol. J Basic Microbiol 2013;53(9):792–5.

44. Gao F, Daugulis AJ. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by *Kluyveromyces marxianus*. Biotechnol Bioeng 2009;104(2):332–9.
45. Yan W, Zhang X, Qian X, Zhou J, Dong W, Ma J, et al. Comprehensive investigations of 2-phenylethanol production by high 2-phenylethanol tolerating *Meyerozyma* sp. strain YLG18. Enzyme Microb Technol 2020;140(June):109629.
46. Chantasuban T, Santomauro F, Gore-lloyd D, Parsons S, Henk D, Scott J, et al. Elevated production of the aromatic fragrance molecule , 2-phenylethanol , using *Metschnikowia pulcherrima* through both de novo and ex novo conversion in batch and continuous modes. J Chem Technol Biotechnol 2018;93(8):2118–30.
47. Chung H, Lee SL, Chou CC. Production and molar yield of 2-phenylethanol by *Pichia fermentans* L-5 as affected by some medium components. J Biosci Bioeng 2000;90(2):142–7.
48. Wittmann C, Hans M, Bluemke W. Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. Yeast 2002;19(15):1351–63.
49. Garavaglia J-, Flôres S, Pizzolato T, Peralba M, Ayub M. Bioconversion of L-phenylalanine into 2-phenylethanol by *Kluyveromyces marxianus* in grape must cultures. World J Microbiol Biotechnol 2007;23(9):1273–9.
50. Huang C, Lee S, Chou C. Production and molar yield of 2-phenylethanol by *Pichia fermentans* L-5 as affected by some medium components. J Biosci Bioeng 2000;90(2):142–7.
51. Lomascolo A, Lesage-meessen L, Haon M, Navarro D, Antona C, Faulds C, et al. Evaluation of the potential of *Aspergillus niger* species for the bioconversion of L-phenylalanine into 2-phenylethanol. World J Microbiol Biotechnol 2001;17:99–102.
52. Adame-Soto P, Aréchiga-Carvajal E, López M, González-Herrera S, Moreno-Jiménez M, Urtiz-Estrada N, et al. Potential production of 2-phenylethanol and 2-phenylethylacetate by non-Saccharomyces yeasts from *Agave durangensis*. Ann Microbiol 2019;69(9):989–1000.
53. Rakicka M, Kieroń A, Hapeta P, Neuvéglise C, Lazar Z. Sweet and sour potential of yeast from the *Yarrowia* clade. Biomass and Bioenergy 2016;92:48–54.
54. Eshkol N, Sendovski M, Bahalul M, Katz-Ezov T, Kashi Y, Fishman A.

- Production of 2-phenylethanol from L-phenylalanine by a stress tolerant *Saccharomyces cerevisiae* strain. J Appl Microbiol 2009;106(2):534–42.
55. Huang CJ, Lee SL, Chou CC. Production of 2-phenylethanol, a flavor ingredient, by *Pichia fermentans* L-5 under various culture conditions. Food Res Int 2001;34(4):277–82.
56. Mu L, Hu X, Liu X, Zhao Y, Xu Y. Production of 2-phenylethanol by microbial mixed cultures allows resource recovery of cane molasses wastewater. Fresenius Envir Bull 2014;23:1356–1365.
57. Alonso F, Oliveira E, Dellamora-Ortiz G, Pereira-Meirelles F. Improvement of lipase production at different stirring speeds and oxygen levels. Brazilian J Chem Eng 2005;22:9–18.
58. Braga A, Mesquita DP, Amaral AL, Ferreira EC, Belo I. Aroma production by *Yarrowia lipolytica* in airlift and stirred tank bioreactors: Differences in yeast metabolism and morphology. Biochem Eng J 2015;93:55–62.
59. Kawasse FM, Amaral PF, Rocha-Leão MHM, Amaral AL, Ferreira EC, Coelho MAZ. Morphological analysis of *Yarrowia lipolytica* under stress conditions through image processing. Bioprocess Biosyst Eng 2003;25(6):371–5.
60. Yoshioka K, Hashimoto N. Cellular fatty acid and ester formation by brewers' yeast. Agric Biol Chem 1983;47(10):2287–94.
61. Äyräpää T. The formation of phenethyl alcohol from 14 C-labelled phenylalanine. J Inst Brew 1965;(71):341–347.
62. Lingappa B, Prasad M, Lingappa Y, Hunt D, Biemann K. Phenethyl alcohol and tryptophol: autoantibiotics produced by the fungus *Candida albicans*. Science 1969;163:192–4.
63. Wang H, Dong Q, Guan A, Meng C, Shi X, Guo Y. Synergistic inhibition effect of 2-phenylethanol and ethanol on bioproduction of natural 2-phenylethanol by *Saccharomyces cerevisiae* and process enhancement. J Biosci Bioeng 2011;112(1):26–31.

Tables

Table 1 - 2-PE productivity ($\text{mg L}^{-1} \text{h}^{-1}$) and yields (g g^{-1}) obtained in shake flask, for the different strains and conditions, after 190 h. The values presented correspond to the average of two independent tests $\pm$ standard deviation. Values for each parameter followed by the same letter in columns present statistically significant differences ($p < 0.05$) by the one-way ANOVA analysis.

	Strain	Productivity ($\text{mg L}^{-1} \text{h}^{-1}$)	Yield (g g^{-1})
Glucose	<i>Y. lipolytica</i> CH1/5	$9.5 \pm 1.8^{\text{cd}}$	$0.49 \pm 0.15^{\text{a}}$
	<i>Y. lipolytica</i> CH 3/4	$7.6 \pm 0.6^{\text{a}}$	$0.54 \pm 0.03^{\text{bc}}$
	<i>Y. lipolytica</i> W29	5.8 ± 0.4	$0.24 \pm 0.01^{\text{b}}$
	<i>Y. divulgata</i> M 445/4	$4.68 \pm 0.01^{\text{c}}$	0.40 ± 0.01
Glycerol	<i>Y. lipolytica</i> CH1/5	$7.2 \pm 1.2^{\text{b}}$	0.380 ± 0.004
	<i>Y. lipolytica</i> CH 3/4	$3.2 \pm 0.4^{\text{bad}}$	$0.18 \pm 0.02^{\text{ac}}$

Table 2 - 2-PE concentration, productivity ($\text{mg L}^{-1} \text{h}^{-1}$) and yields (g g^{-1}) obtained under different aeration and agitation rates. The values presented correspond to the average of two independent experiments $\pm$ standard deviation. According with one-way ANOVA analysis, there are no statistically significant differences ($p > 0.05$) for each parameter at different operating conditions.

Aeration and Agitation rates	2-PE Concentration (g L^{-1})	Productivity ($\text{mg L}^{-1} \text{h}^{-1}$)	Yield (g g^{-1})
600 rpm, 1 L min^{-1}	0.314 ± 0.005	3.87 ± 0.07	0.069 ± 0.02
600 rpm, 3 L min^{-1}	0.37 ± 0.06	4.5 ± 0.7	0.08 ± 0.01
900 rpm, 3 L min^{-1}	0.37 ± 0.06	4.5 ± 0.7	0.082 ± 0.004

Table 3 - 2-PE productivity ($\text{mg L}^{-1} \text{h}^{-1}$) and yields (g g^{-1}) obtained under different medium pH. The values presented correspond to the average of two independent tests $\pm$ standard deviation. According with one-way ANOVA analysis, there are no statistically significant differences ($p > 0.05$) for each parameter at different operating conditions.

pH	2-PE Concentration (g L^{-1})	Productivity ($\text{mg L}^{-1} \text{h}^{-1}$)	Yield (g g^{-1})
5.5	0.31 ± 0.01	3.8 ± 0.1	0.07 ± 0.02
6.5	0.185 ± 0.06	2.28 ± 0.05	0.04 ± 0.01
7.5	0.152 ± 0.07	1.8 ± 0.1	0.037 ± 0.004

Figures caption

Figure 1 - Inhibitory effect of 2-PE and L-Phe for strains *Y. lipolytica* W29, *Y. lipolytica* CH 1/5, *Y. lipolytica* CH 3/4, and *Y. divulgata* M 445/4. After growing in liquid medium, 3 μ L of a cell suspension with OD_{600nm} of 0.6 were plated in solid medium with different concentrations of 2-PE (0, 1, 2 and 3 g L⁻¹) and L- Phe (0, 5, 8 and 15 g L⁻¹) in successive dilutions. The cells were incubated at 27 °C for 48 h.

Figure 2 - Cellular growth (○), glucose consumption (◇), L-Phe consumption (△) and 2-PE production (□) for the strains: *Y. lipolytica* CH 1/5 (grey) and *Y. lipolytica* W29 (black) (a and b); *Y. lipolytica* CH 3/4 (grey) and *Y. divulgata* M 445/5 (black) (c and d) at shake flask scale, using glucose as a carbon source and an initial concentration of L-Phe of 4 g L⁻¹. The values presented correspond to the average of two independent tests $\pm$ standard deviation. If no error bars are visible, the standard deviations were too small to be displayed.

Figure 3 - Cellular growth (○), glycerol consumption (◇) (a), L-Phe consumption (△) and 2-PE production (□) (b) for the strains *Y. lipolytica* CH 1/5 (black) and *Y. lipolytica* CH 3/4 (grey), at shake flask scale, using glycerol as a carbon source and an initial concentration of L-Phe of 4 g L⁻¹. The values presented correspond to the average of two independent tests $\pm$ standard deviation. If no error bars are visible, the standard deviations were too small to be displayed.

Figure 4 - Cellular growth (○), glycerol consumption (◇) (a), L-Phe consumption (△) and 2-PE production (□) (b) for the strain *Y. lipolytica* CH 1/5, at bioreactor scale, using glycerol as a carbon source and an initial concentration of L-Phe of 8 g L⁻¹. The values presented correspond to the average of two independent tests $\pm$ standard deviation. If no error bars are visible, the standard deviations were too small to be displayed.

Figure 5 - Cellular growth (○), glycerol consumption (◇) (a), L-Phe consumption (△) and 2-PE production (□) (b) for the strain *Y. lipolytica* CH 1/5, with a pulse of 4 g L⁻¹ at t = 80 h, at bioreactor scale, using glycerol as a carbon source and an initial concentration

of L-Phe of 8 g L^{-1} . The values presented correspond to the average of two independent tests $\pm$ standard deviation. The arrows indicate the moment of the addition of the L-Phe pulse. If no error bars are visible, the standard deviations were too small to be displayed.

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Figure 2

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Figure 3

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Figure 4

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Figure 5

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