Yarrowia lipolytica as a potential producer of 2-phenylethanol from L-phenylalanine biotransformation

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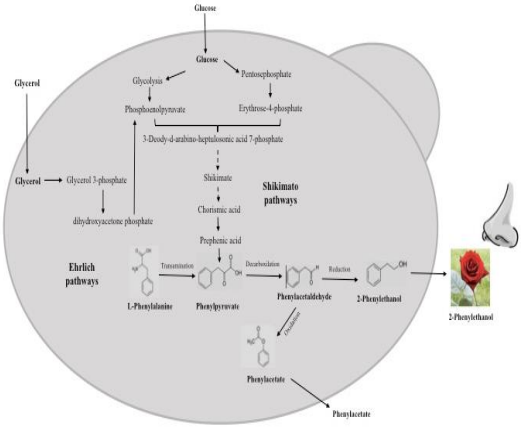

2-Phenylethanol (2-PE) is an aromatic alcohol with a delicate fragrance of rose petals. The non-conventional yeast *Yarrowia lipolytica* is extensively explored for flavor compounds production, but the production of 2-PE has been very poorly described. This study investigated the potential of different *Y. lipolytica* strains (W29, CBS2075, CH 1/5 and CH 3/4) for 2-PE production. It was confirmed that all strains were able to produce 2-PE by L-phenylalanine (L-Phe) bioconversion, but were inhibited by 2-PE concentrations above 2 g L^{-1}. The strain *Y. lipolytica* CH 1/5 was selected for further studies since it produced the highest 2-PE titer (2.2 g L^{-1}). Afterwards, the effect of L-Phe concentration and carbon source (glucose and crude glycerol) on 2-PE production was studied, and it was observed that increasing L-Phe concentration decreases the aroma production, and that the highest titer was obtained with glycerol. This study demonstrates the promising production of 2-PE using *Y. lipolytica* as biotechnological platform for flavors production.

Introduction

2-Phenylethanol (2-PE) is an aromatic alcohol with a fresh rose scent, and is the second most widely used flavor after vanillin [1]. It is commonly used in the cosmetics and perfumes industries [1], with a world market volume of nearly 10 000 t, mainly produced by chemical synthesis. Nevertheless, the use of the chemically synthesised flavor compounds is restricted to some applications and natural flavor compounds are preferred by consumers [2]. However, its natural production includes the extraction from plants and this process involves several steps of down-stream operations, which makes the market price of natural 2-PE more expensive ($1000/kg) than the chemically synthesised ($5/kg) [3]. Hence, great interest has been arising for the biotechnological production of 2-PE. Several microorganisms have been described as possessing the ability to synthesise 2-PE [1]. 2-PE can be synthesized in yeast through shikimate and Ehrlich pathways (Graphical abstract). Either way, the main bottleneck for yeast fermentation of 2-PE is its toxicity [1] due to the fact that concentrations between 2 and 3 g L^{-1} inhibit the cellular growth [1] and the results obtained so far do not seem viable for industrial scale-up. Among several microorganisms able to produce 2-PE, the yeast *Y. lipolytica* appears to be promising due to its interesting characteristics, such as the Crabtree negative trait and absence of ethanol production, however this process has been fairly described.

Material and Methods

The strains used in this work were *Y. lipolytica* W29 (ATCC 20460), CBS2075, CH 1/5 and CH 3/4 (isolated from chesee). *Y. lipolytica* strains were cultivated for 16-17 hours in of YPD medium (glucose 20 g L^{-1}, peptone 20 g L^{-1}, yeast extract 10 g L^{-1}) at 200 rpm and 27 ºC, and further used to inoculate the bioconversion experiments with an initial OD_{600} of 0.5. Bioconversion of L-Phe to 2-PE was carried out in cultivation medium containing per liter of deionized water: glucose or glycerol 40 g, KH_2PO_4 15 g, $MgSO_4.7H_2O$ 0.5 g, YNB without amino acids 0.02 g, thiamine 3 mg, pH 6.5, supplemented with L-Phe 4 g or 6 g incubated at 27 ºC and 200 rpm. Glucose and glycerol were quantified by high-performance liquid chromatography. The 2-PE and L-Phe quantification was obtained using a SHIMADZU UHPLC system equipped with a diode array detector (SPD-M20A) at a fixed wavelength of 215 nm. LC separation was carried out with a YMC ODS-Aq (250 mm × 4.6 mm) reverse phase column at 25 °C. For elution, water (solvent A) and acetonitrile (solvent B) were applied as the mobile phases at a flow rate of 1 mL min^{-1}. A gradient was used, where the amount of solvent A was increased stepwise: 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.

Results and Discussion

The main challenge for microbial production of 2-PE is its cytotoxicity [1]. To study the tolerance of different *Y. lipolytica* strains toward 2-PE, cells were cultivated in solid medium containing various concentrations of 2-PE. As shown in Fig. 1 the growth of all strains was completely repressed at a 2-PE concentration of 2 g L^{-1}. Comparing the performance of the four *Y. lipolytica* strains herein studied it was possible to observe that W29 and CBS2075 strains were more tolerant to 2-PE.

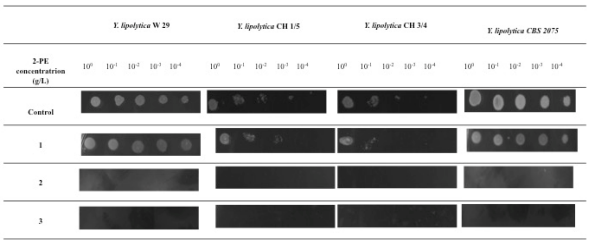

Figure 1. Inhibitory effect of 2-PE for the *Y. lipolytica* W29, CH 1/5, CH 3/4 and CBS2075 strains. Cells were incubated in YPD medium for 16 h and diluted to an OD_{600} of 0.6. Cells (3 µL) at a dilution of 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were spotted on solid media (glucose 40 g L^{-1}, agar 20 g L^{-1} and $(NH_4)_2SO_4$ 2 g L^{-1}) containing different concentrations of 2-PE (0 (control), 1, 2 and 3, g L^{-1}). Cells were incubated at 30 ºC during 48h.

Since the 2-PE production by *Y. lipolytica* was poorly explored, it was studied the ability of the four *Y. lipolytica* strains to produce this aroma through L-Phe biotransformation (Table 1).

Table 1. Maximum concentration, productivity and yield of 2-PE for the *Y. lipolytica* strains with 4 g L^{-1} of L-Phe. (Data are presented as average and standard deviation of two independent experiments).

Strains	**2-PE ($g\ L^{-1}$)**	**Yield ($g\ g^{-1}$)**	**Productivity ($mg\ L^{-1}\ h^{-1}$)**
W29	1.01 ± 0.07	0.24 ± 0.01	5.8 ± 0.4
CH 1/5	2.17 ± 0.27	0.7 ± 0.1	11.4 ± 0.3
CH 3/4	1.71 ± 0.14	0.8 ± 0.2	8.9 ± 0.7
CBS 2075	0.80 ± 0.02	0.19 ± 0.01	4.8 ± 0.1

Comparing the performance of the four strains tested, it was possible to observe that all strains were able to produce 2-PE, although with different strain-dependent production efficiencies, being the CH 1/5 strain the one with the highest production titer (Table 1), 2.17 ± 0.27 g L^{-1}. Taking into consideration the 2-PE titers reported by Huang et al. [4] (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 tested *Y. lipolytica* strains for this metabolite production. Therefore, given that the bioconversion yields of 2-PE with respect to L-Phe, *Y. lipolytica* CH 1/5 and CH 3/4 were the best producers, since the obtained yield was 68% and 75% higher than the values obtained with the strains W29 and CBS2075, respectively. Taking the above into account and considering that the highest 2-PE concentration and productivity were obtained with CH 1/5 strain, this strain was selected as the best candidate for the following experiments. Previous reports [5] have shown that the L-Phe concentration in the media influences 2-PE production. Since that, in the previous experiments L-Phe was completely consumed and new experiments were carried out to analyze the behavior of the *Y. lipolytica* CH 1/5 strain in the presence of 6 g L^{-1} of L-Phe. Alternatively it was also studied the ability of this yeast to produce 2-PE in the presence of crude glycerol, a by-product of the biodiesel industry, that can be naturally consumed by *Y. lipolytica* [6] (Table 2).

Table 2. Maximum concentration, productivity and yield of 2-PE for the *Y. lipolytica* CH 1/5 with 6 g L^{-1} of L-Phe using glucose and glycerol as carbon sources. (Data are presented as average and standard deviation of two independent experiments).

Carbon source	**2-PE ($g\ L^{-1}$)**	**Yield ($g\ g^{-1}$)**	**Productivity ($mg\ L^{-1}\ h^{-1}$)**
Glucose	0.57 ± 0.0005	0.13 ± 0.03	2.6 ± 0.1
Glycerol	1.2 ± 0.1	0.33 ± 0.01	5.0 ± 0.5

Regarding the 2–PE concentrations obtained under these conditions, it was possible to observe that, for glucose, increasing the L-Phe concentration decreases the titer of 2-PE (0.57 *vs* 2.17 g L^{-1}, for the experiments with 4 and 6 g L^{-1} the L-Phe, respectively). However, the 2-PE titer, yield and productivity attained with glycerol are 53 % higher than the ones obtained with glucose, under the same conditions. Despite the preference of *Y. lipolytica* to use glucose as carbon source for cell growth, glycerol is a better substrate for 2-PE production. The same behavior was also reported by Huang et al. [4] with *P. fermentans* L-5. The authors also reported a higher 2-PE yield with glycerol (0.56 mol mol^{-1}) when compared with glucose (0.31 mol mol^{-1}). The low titer of 2-PE obtained with 6 g L^{-1} of L-Phe in the presence of glucose can be probably attributed to the production of other metabolites in the Ehrlich pathway, such as phenylacetate (Graphical abstract). It seems that some enzymes in this pathway can be inhibited in the presence of glycerol.

Comparison of the obtained final titers of 2-PE with the literature data indicates that *Y. lipolytica* shows the potential to efficiently produce this metabolite. For example *P. fermentans* L-5 produced 0.5 g L^{-1} 2-PE from 1 g L^{-1} L-Phe [4], strains of *K. marxianus* produced up to 0.9 g L^{-1} of 2-PE [7] in a non-optimized process. In the scope of the above, the process of 2-PE production with *Y. lipolytica* CH 1/5 appears promising, however it requires further research and optimization.

Acknowledgements

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