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# Benchmarking recombinant *Pichia pastoris* for 3-hydroxypropionic acid production from glycerol

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## Summary

**The use of the methylotrophic yeast *Pichia pastoris* (*Komagataella phaffi*) to produce heterologous proteins has been largely reported. However, investigations addressing the potential of this yeast to produce bulk chemicals are still scarce. In this study, we have studied the use of *P. pastoris* as a cell factory to produce the commodity chemical 3-hydroxypropionic acid (3-HP) from glycerol. 3-HP is a chemical platform which can be converted into acrylic acid and to other alternatives to petroleum-based products. To this end, the *mcr* gene from *Chloroflexus aurantiacus* was introduced into *P. pastoris*. This single modification allowed the production of 3-HP from glycerol through the malonyl-CoA pathway. Further enzyme and metabolic engineering modifications aimed at increasing cofactor and metabolic precursors availability allowed a 14-fold increase in the production of 3-HP compared to the initial strain. The best strain (PpHP6) was tested in a fed-batch culture, achieving a final concentration of 3-HP of 24.75 g $l^{-1}$, a product yield of 0.13 g $g^{-1}$ and a volumetric productivity of 0.54 g $l^{-1}$ $h^{-1}$, which, to our knowledge, is the highest volumetric productivity reported in yeast. These results benchmark *P. pastoris* as a promising platform to produce bulk chemicals for the revalorization of crude glycerol and, in particular, to produce 3-HP.**

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## Introduction

In 2004, the US Department of Energy (DOE) published the list of the top value-added bio-based chemicals to be produced from biomass (Werpy and Petersen, 2004). The production of such bio-based products in a biorefinery is necessary to complement biofuel production, which is a low value-added product with a high price volatility. Co-producing biofuels and chemicals in an integrated biorefinery would allow the design of an economically robust and sustainable process, making it an attractive investment option. The DOE report ranked 3-hydroxypropionic acid (3-HP) among one of these top value-added chemicals to be produced in a biorefinery. The largest application of 3-HP is its conversion into acrylic acid and other products typically derived from petroleum (Della Pina *et al*., 2011; Kumar *et al*., 2013). The global market size of acrylic acid is estimated to reach 22 550 M$ in 2022 (Grand View Research, 2016). Due to such promising market forecast, Novozymes and Cargill announced a joint agreement to develop a platform to produce acrylic acid from biologically produced 3-HP in 2008 (Novozymes, 2008). Nevertheless, up to date, the process has not been implemented at a large scale, as further development is still required.

While the revalorization of lignocellulosic products consisting of glucose and xylose has been largely investigated, there are other waste products that can be used as substrates to produce bulk chemicals. This is the case of crude glycerol, a side-product obtained during the enzymatic production of biodiesel. It is a mixture made of 60–80% glycerol, 10–20% methanol and 10–20% of soap or other undefined organic matter compounds (Luo *et al*., 2016). The use of this mixture as

substrate is limited by the fact that methanol is toxic to many microorganisms. The methylotrophic yeast *Pichia pastoris* is a promising microorganism for the revalorization of crude glycerol as it can efficiently grow using both glycerol and methanol as carbon sources. Moreover, *P. pastoris* can grow at a low pH, and it is reported that the cost of the downstream process is reduced if the fermentation is performed at a pH below the pKa value of the acidic product (van Maris *et al*., 2004). For all these reasons, in this study, we investigate the production of 3-HP from glycerol in the yeast *P. pastoris*.

*Pichia pastoris* is widely used in industrial biotechnology as an efficient host for recombinant protein production, and it has received increasing interest as a platform to produce fine and bulk chemicals (Schwarzhans *et al*., 2017; Peña *et al*., 2018). Moreover, the efforts of the *P. pastoris* community have allowed the implementation of the necessary tools allowing its use in metabolic engineering research, including up-to-date genetic engineering tools like the GoldenMOCS (Prielhofer *et al*., 2017) and CRISPR-Cas9 (Weninger *et al*., 2016), metabolic genome-scale models (GSM; Tomàs-Gamisans *et al*., 2016), metabolomics and fluxomics protocols (Carnicer *et al*., 2012; Ferrer and Albiol, 2014), and a wide knowledge of its behaviour at the bioreactor scale (Looser *et al*., 2014; Yang and Zhang, 2018). Another interesting trait of *P. pastoris* is that it is a Crabtree-negative yeast. This is an interesting feature in metabolic engineering because overflow metabolism to undesired by-products, such as ethanol or glycerol, can be minimized. The full oxidation of the carbon source leads to a higher energetic yield than in Crabtree-positive yeasts, like *Saccharomyces cerevisiae*, thus leading to potentially higher product yields (Dai *et al*., 2018; Peña *et al*., 2018). Moreover, the extracellular concentration of some intermediate metabolites of the TCA cycle (i.e. malate or citrate) is lower in *P. pastoris* than in *S. cerevisiae* under similar conditions (Carnicer *et al*., 2012). This trait simplifies the downstream processing of the products of interest.

The biological production of 3-HP has been largely investigated (de Fouchécour *et al*., 2018). Several pathways have been tested in a number of microorganisms, including the industrial workhorses *Escherichia coli* and *S. cerevisiae*. Each pathway to produce 3-HP is named according to its precursor. The route that has obtained the highest yields and productivities is the coenzyme B12-dependant glycerol pathway (Raj *et al*., 2008; Rathnasingh *et al*., 2009). However, the implementation of this route in *P. pastoris* is currently unfeasible at an industrial scale due to the high production costs caused by the requirement of coenzyme B12 addition, which is an expensive compound (Chen *et al*., 2014). The 3-HP route through β-alanine has been investigated in *S. cerevisiae* (Borodina *et al*., 2015) and *E. coli* (Song *et al*., 2016) and the route which uses malonyl-CoA as a precursor has been implemented in several microorganisms, including *E. coli* (Rathnasingh *et al*., 2012; Liu *et al*., 2013, 2016), *S. cerevisiae* (Chen *et al*., 2014; Kildegaard *et al*., 2016) and *Shizosaccharomyces pombe* (Suyama *et al*., 2017).

Using glucose as substrate, the maximum theoretical yield of the β-alanine to 3-HP pathway is higher than the route starting from malonyl-CoA, as more ATP is required for the latter. However, when glycerol is used as a substrate, the maximum theoretical yield for both pathways equals 1, as there is net ATP production in both cases (see Supplementary materials Section Data S1). While the β-alanine pathway would require the expression of 3 heterologous genes (Borodina *et al*., 2015) to achieve 3-HP production in *P. pastoris*, it is reported that the single expression of the bifunctional enzyme malonyl-CoA reductase from *Chloroflexus aurantiacus* ($MCR_{Ca}$) triggers 3-HP production in yeast through the malonyl-CoA route (Chen *et al*., 2014). This enzyme performs two NADPH-consuming consecutive reactions sequentially converting malonyl-CoA to malonate semialdehyde (MSA), and then, MSA is converted to 3-HP (Fig. 1).

In this study, the production of 3-HP using glycerol as substrate has been implemented in *P. pastoris* through the malonyl-CoA pathway. The expression of $mcr_{Ca}$ leads to 3-HP production in *P. pastoris*. This base strain has been further modified using two different strategies: protein engineering and metabolic engineering. The independent expression of the two subunits of the malonyl-CoA reductase has shown higher 3-HP production in other microorganisms (Liu *et al*., 2013). This strategy has been further tested in *P. pastoris,* yielding a substantial improvement compared to the initial strain. Further modifications have been implemented to increase the fluxes producing the substrates of the malonyl-CoA pathway to 3-HP (NADPH and malonyl-CoA). The strain producing the highest 3-HP titre has been characterized in a fed-batch culture using glycerol as a substrate. Overall, the potential of *P. pastoris* as a promising host for 3-HP production from this renewable feedstock was demonstrated for the first time.

## Results and discussion

### *Expression of* $mcr_{Ca}$ *in* P. pastoris *leads to 3-HP production*

In the present work, we expressed $mcr_{Ca}$ gene in *P. pastoris* under the control of the constitutive and strong GAP promoter (Waterham *et al*., 1997). Comparison of the resulting strain, PpHP1, to the reference strain (X-33) in triplicate shake flasks on buffered minimal glycerol (BMG) medium showed that the sole expression of $mcr_{Ca}$ resulted in 3-HP production, while it did not affect cell growth (the $\mu_{max}$ of the control and

 

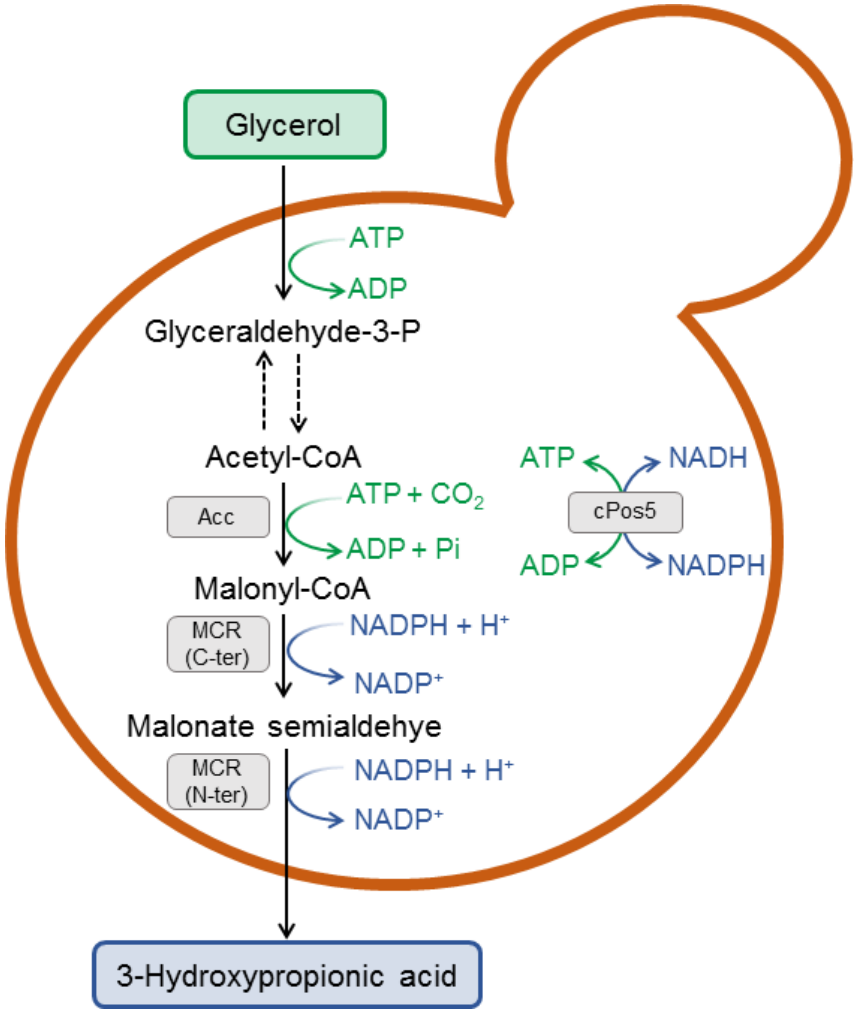


**Fig. 1.** Simplified representation of the conversion of glycerol to 3-HP through the malonyl-CoA route. The metabolic engineering targets to increase the availability of the precursors of the malonyl-CoA to 3-HP pathway are included. Acc, acetyl-CoA carboxylase; cPos5, cytosolic NADH kinase; MCR (C-ter), C-terminal domain of malonyl-CoA reductase; MCR (N-ter), N-terminal domain of malonyl-CoA reductase.

PpHP1 strains was the same within the precision range, $0.24 \pm 0.01\ h^{-1}$ and $0.24 \pm 0.01\ h^{-1}$ respectively). Moreover, no by-products were detected in any of the two strains (Fig. S2).

Notably, the PpHP1 strain produced $0.19 \pm 0.03\ g\ l^{-1}$ of 3-HP after 24 h of cultivation. Such 3-HP titre is considerably higher than those achieved in *S. pombe* (Takayama *et al*., 2018) and *S. cerevisiae* (Chen *et al*., 2014) harbouring a similar genetic construction and using glucose as a substrate ($0.016\ g\ l^{-1}$ and $0.093\ g\ l^{-1}$ respectively). Furthermore, the C-yield (Cmol of 3-HP per Cmol of substrate, i.e. glycerol or glucose) for the PpHP1 strain ($0.015 \pm 0.002$ Cmol $Cmol^{-1}$) was remarkably higher than the one observed in other yeasts (0.0003 Cmol $Cmol^{-1}$ in *S. pombe*, and 0.0048 Cmol $Cmol^{-1}$ in *S. cerevisiae*). Remarkably, the specific activity of MCR in PpHP1 was $0.30 \pm 0.06\ U\ mg^{-1}$ of protein, which is remarkably higher than the specific activity reported in *S. cerevisiae* ($0.008\ U\ mg^{-1}$; Chen *et al*., 2014). The combined effect of the strength of the *P. pastoris* GAP promoter and the fact that glycerol is more reduced than glucose, which leads to net production of ATP from the substrate, instead of net ATP consumption (Fig. S1), points at *P. pastoris* as a promising cell factory for the bioproduction of 3-HP from glycerol.

### *Setting up the screening conditions for 3-HP producing* P. pastoris *clones*

We established two independent analytical methods based on NMR and HPLC-MS for 3-HP quantification, which were cross-validated. The results using the HPLC-MS method diverge from those obtained using NMR when glycerol is still present in the medium (Fig. S3). This is explained by the matrix effect caused by the glycerol co-eluting with 3-HP from the column, which affects

 

the ionization efficiency at the ionization source. However, once the glycerol is fully consumed, the HPLC-MS and the NMR methods yielded statistically identical results when measuring the final 3-HP concentrations in the PpHP1 shake flask cultures described in Section 3.1 (i.e. 3-HP final concentrations of 0.18 ± 0.02 g $l^{-1}$ of 3-HP and 0.19 ± 0.02 g $l^{-1}$ of 3-HP respectively).

Consumption of 3-HP has been reported in several microorganisms (Zhou *et al.*, 2014; Yang *et al.*, 2017). In order to evaluate whether the PpHP1 strain was able to assimilate this compound, this strain was grown in triplicate shake flasks using BMG, BMG medium supplemented with 2.5 g $l^{-1}$ of 3-HP (BMG3HP) or buffered minimal medium supplemented with 2.5 g $l^{-1}$ of 3-HP as a sole carbon source (BM3HP). Samples were collected after 24, 48 and 72 h. The 3-HP concentration remained constant throughout the 72 h in the cultures grown on BM3HP (no growth was observed). After 24 h, glycerol was exhausted from the BMG and BMG3HP, while the concentration of 3-HP remained unaltered between the 24 and the 72 h (data not shown), thereby indicating that *P. pastoris* does not assimilate 3-HP as C-source within the experimental time frame tested.

Considering these results, screening of the 3-HP-producing strains was performed taking end-point samples after 48 h of incubation to ensure full consumption of glycerol, followed by 3-HP quantification using the HPLC-MS method.

### *Improvement of 3-HP production by MCR enzyme engineering*

Recent studies have shown the positive effect of dissecting the MCR enzyme in the two subunits catalysing each of the two reactions converting malonyl-CoA into 3-HP (Liu *et al.*, 2013). The presence of three point mutations causing three amino acidic changes (N940V, K1106R and S1114R) had an additional positive impact on 3-HP production in *E. coli* (Liu *et al.*, 2016). The same outcome has been demonstrated in *S. pombe*, where the expression of the N-terminal and the improved version of the C-terminal domain of MCR using two independent expression cassettes led to a 30-fold improvement in the final 3-HP titre, compared to the starting strain expressing the original sequence of $MCR_{Ca}$ (Takayama *et al.*, 2018). Therefore, we introduced two expression cassettes into *P. pastoris* allowing the independent expression of the coding DNA sequences for the N-terminal and the C-terminal domains of MCR (including the wild-type and the mutated version of the latter) under the control of the GAP promoter.

As shown in Fig. 2, the dissection of MCR (strain PpHP2) had a positive impact on 3-HP production, triggering a 12.5-fold increase in 3-HP production (1.63 ± 0.09 g $l^{-1}$ 3-HP). Nevertheless, the introduction of the 3 point mutations in the C-terminal domain (strain PpHP3) resulted in a non-producing strain. Similarly, the introduction of these 3 point mutations to a non-dissected version of MCR resulted in a non-producing *P. pastoris* strain (data not shown). These point mutations are far from the reactive site and the NADPH binding site of the enzyme. It remains poorly understood how distant mutations may affect catalytic properties. Moreover, as the structure of the C-terminal domain of MCR from *C. aurantiacus* is not available, the exact effect of the point mutations on the protein conformational stability/quality is hard to predict, particularly when the protein is synthetized at high rates (i.e. using a strong

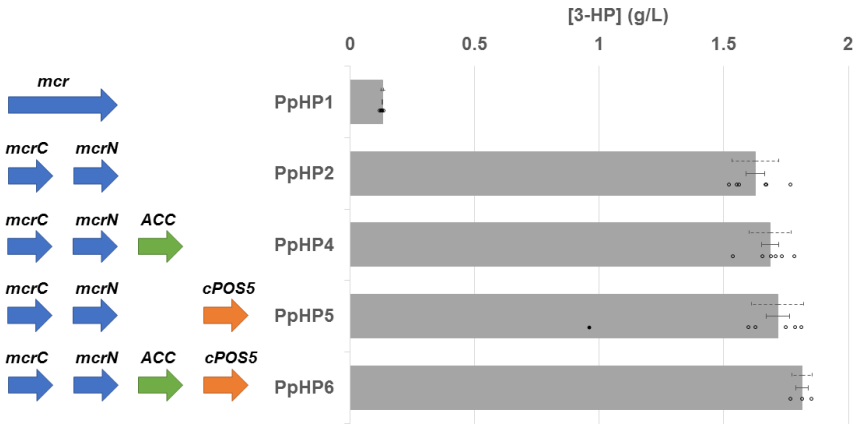


**Fig. 2.** Production of 3-HP for each strain in the screening experiments. The genes heterologously expressed in each strain are depicted in the left side of the graph. The grey bars show the average 3-HP concentration at the end of the culture, the discontinuous line shows the standard deviation, the solid line indicates the SE and the circles show the average result for each clone. The solid circle of a PpHP5 clone shows the result of a clone which was discarded for the calculations, as it had a different behaviour from the rest of the clones of that strain. For PpHP6, only 3 clones could be screened. Despite that more than 50 transformants of PpHP6 from 2 independent transformations were checked using colony PCR, only 3 clones resulted positive.

 

promoter). Notably, the 12.5-fold improvement obtained by dissecting MCR is in the same range as those reported in *S. pombe* and *E. coli*.

### *Metabolic engineering of* P. pastoris *to improve 3-HP production*

Metabolic engineering for 3-HP production through the malonyl-CoA pathway was aimed at increasing the availability of the two precursors of this route, namely NADPH and malonyl-CoA. To do so, the $ACC_{Yl}$ and $cPOS5_{Sc}$ genes have been heterologously expressed in the PpHP2 strain. The reactions catalysed by the enzymes encoded by these two genes are depicted in Fig. 1. Acc produces malonyl-CoA from acetyl-CoA, which is the precursor of the central carbon metabolism for 3-HP production. The overexpression of such enzyme has already been performed in *E. coli* (Rathnasingh *et al*., 2012), *S. cerevisiae* (Chen *et al*., 2014; Kildegaard *et al*., 2016) and *S. pombe* (Takayama *et al*., 2018) to increase the production of 3-HP. The enzyme cPos5 produces NADPH by means of NADH and ATP consumption, and the overexpression of its gene leads to an increase in the NADPH/NADP ratio (Tomàs-Gamisans *et al*., 2020).

As shown in Fig. 2, the overexpression of Acc or cPos5 in the strain PpHP2 – resulting in the strains PpHP4 and PpHP5, respectively – led to a small increase in 3-HP production. Still, such increases were not statistically significant compared to the parental strain PpHP2 (*P*-values of 0.27 and 0.15 respectively). However, when both genes were overexpressed at the same time (PpHP6), a significant increase in the final 3-HP concentration was observed ($P = 0.015$).

The PpHP6 strain produced $1.81 \pm 0.04$ g $l^{-1}$ of 3-HP, which represents a 14-fold increase compared to the starting strain (PpHP1), and a 12% increase compared to PpHP2. The C-yield for PpHP6 was $0.146 \pm 0.003$ Cmol $Cmol^{-1}$.

Similar strategies in other microorganisms resulted in similar outcomes. For example, in *E. coli*, the overexpression of either ACC or PntAB (a transhydrogenase encoding gene) led to a twofold increase, while the co-expression of both genes led to a threefold increase (Rathnasingh *et al*., 2012). It is also worth noticing that in *S. pombe*, enzyme engineering and expression level adjustment of MCR led to a 30-fold increase in 3-HP titres, while increasing the availability of acetyl-CoA and CoA in this strain resulted in a twofold increase. Altogether, these results point at the flux from malonyl-CoA to 3-HP as the main limiting factor for 3-HP production. Further metabolic studies should corroborate this hypothesis. Indeed, the increase of the number of copies of the *mcr* gene has already been demonstrated to have a positive effect in 3-HP production in both *S. cerevisiae* (Kildegaard *et al*., 2016) and *S. pombe* (Takayama *et al*., 2018).

Overall, the product yield obtained by the strain PpHP6 during the screening phase ($0.146 \pm 0.003$ Cmol $Cmol^{-1}$) is significantly higher (around 1.8-fold) to the highest yield observed in yeast under comparable conditions (deep-well plate culture using defined medium), i.e. using the *S. cerevisiae* strain 3HP-M11 producing 3-HP through the malonyl-CoA pathway, with a yield on glucose of $0.080 \pm 0.008$ Cmol $Cmol^{-1}$ (calculated from data given by Kildegaard *et al*., 2016).

### *Production of 3-HP in a fed-batch culture*

The strain PpHP6 was further cultivated in a controlled fed-batch culture. First, a batch experiment was performed in order to determine the $\mu_{max}$ of PpHP6, and also the initial biomass concentration ($X_0$) and the biomass to substrate yield ($Y_{X/S}$), which are the parameters required to set the exponential feeding rate (see Section 2.4). The $\mu_{max}$ was $0.19 \pm 0.1$ $h^{-1}$, the $X_0$ was $18.6 \pm 0.2$ g $l^{-1}$ and the $Y_{X/S}$ $0.47 \pm 0.01$ g $g^{-1}$.

After the initial batch phase (22 h), the exponential feeding rate was set to maintain a growth rate equal to 0.1 $h^{-1}$ (approximately 50% of the $\mu_{max}$; Fig. 3A). After 44 h of cultivation (22 h of feeding phase), glycerol accumulation was observed, and the feeding pump was stopped. Thereafter, the fermentation was terminated after 45.5 h of cultivation, when the $pO_2$ increased, indicating that all the glycerol had been consumed. Overall, 195 g $l^{-1}$ of glycerol was added into the reactor, and $24.75 \pm 0.54$ g $l^{-1}$ of 3-HP was produced. The only by-product detected by NMR was arabitol in the late stages of the batch and the fed-batch phases.

As already inferred from the evolution of physiological growth parameters over time, growth rate was not maintained during the whole feeding phase. Furthermore, the decrease in the growth rate was accompanied with the accumulation of glycerol and the production of arabitol towards the end of the cultivation. These results differ from previous observations of *P. pastoris* producing heterologous proteins in fed-batch cultures operated under analogous conditions (i.e. using a pre-programmed exponential substrate feeding strategy for controlled specific growth rate at 0.1 $h^{-1}$), where the $\mu$ remained constant throughout the feeding phase and no arabitol accumulation was detected, achieving up to 100 g $l^{-1}$ of dry cell biomass (Garcia-Ortega *et al*., 2013). The spline curves fitted to the evolution of the growth rate ($\mu$), product yield ($Y_{P/S}$), $qP_{3\text{-HP}}$ and $qP_{Arabitol}$ throughout the cultivation further reveal that the decrease in the growth rate coincides with the onset of glycerol accumulation and arabitol by-product excretion (Fig. 3B). Conversely, product yield and $qP_{3\text{-HP}}$

 

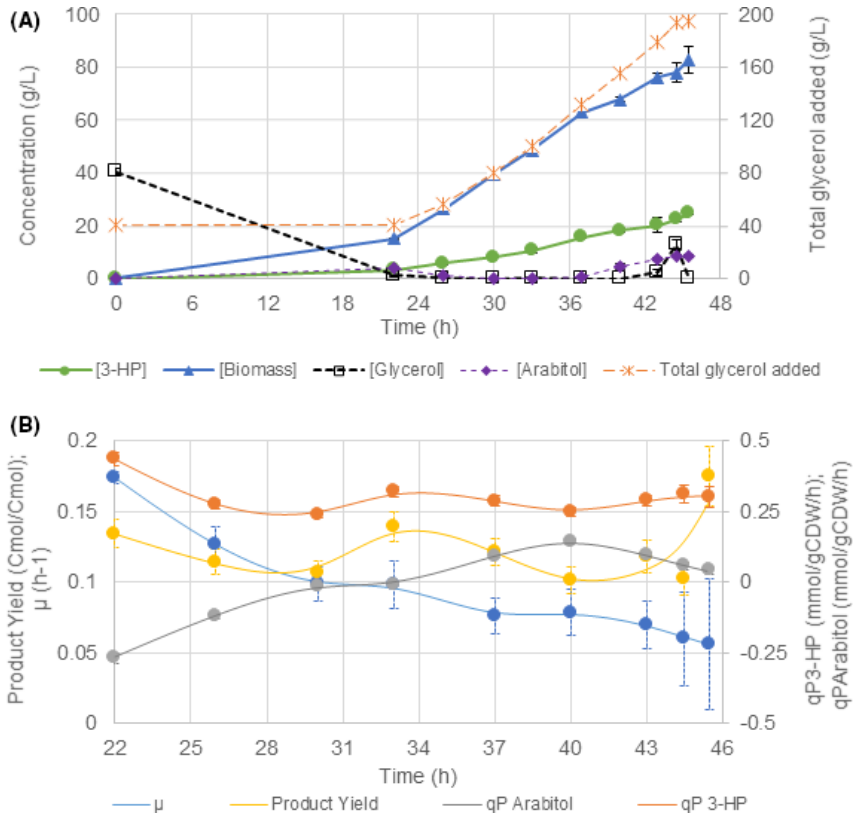


**Fig. 3.** Fed-batch culture of PpHP6.
A. Biomass and metabolites concentration during cultivation and total glycerol added (per litre) into the reactor is shown. It was calculated considering the volume of feeding added to the reactor and the actual culture volume. Error bars denote SE.
B. Growth rate (μ), product yield ($Yield_{3\text{-}HP/Glyc}$), q-rate of 3-HP (qP 3-HP), and q-rate of arabitol (qP Arabitol) of the strain PpHP6 throughout the feeding phase of the fed-batch culture at a pre-set μ of 0.1 $h^{-1}$. Error bars show the SE.

followed the opposite trend, i.e. the $Y_{P/S}$ is reduced from 0.134 ± 0.010 Cmol $Cmol^{-1}$ at the first part of the feeding phase to yields close to 0.1 Cmol $Cmol^{-1}$ coinciding with the highest $qP_{Arabitol}$ values. These results show how during the beginning of the feeding phase the C-yield was close to the one obtained in the screening phase (0.146 ± 0.003 Cmol $Cmol^{-1}$), even though the average yield of the overall fed-batch culture was lower (0.130 ± 0.003 Cmol $Cmol^{-1}$ and 0.127 ± 0.003 g $g^{-1}$).

A possible explanation for the observed trends is the increase in the 3-HP concentration. Accumulation of 3-HP in the fermentation broth has been reported to be toxic for other microorganisms like *E. coli* and *S. cerevisiae*. Such toxicity may be triggered by the conversion of 3-HP into 3-hydroxypropanaldehyde (reuterin), which causes oxidative stress to the cells through its interaction with reduced glutathione (Schaefer *et al.*, 2010; Kildegaard *et al.*, 2014). The sequestration of glutathione leads to a decrease in the maximal growth rate of *S. cerevisiae* at high 3-HP concentrations (Kildegaard *et al.*, 2014).

Another plausible explanation of the decrease in the μ at the final stage of the culture may be the increase in the ATP expenditure for maintenance at high extracellular concentrations of weak organic acid, i.e. 3-HP. Protonated acid molecules may enter the cytoplasm by diffusion and later on dissociate, leading to an ATP cost for the re-secretion of the organic acid and the restoration of the intracellular pH. The expression of a 3-HP exporter in *E. coli* has proven to be beneficial, as it reduces stress caused by the intracellular dissociation of 3-HP (Nguyen-Vo *et al.*, 2020). Moreover, higher extracellular concentrations of 3-HP have a direct impact on the thermodynamics of its secretion, leading to an increase in ATP expenses for 3-HP export (van Maris *et al.*, 2004). All these phenomena would ultimately impact on the $Y_{X/S}$ in *P. pastoris*, explaining the decrease in the growth rate and the accumulation of glycerol at the later stage of the fed-batch culture.

Moreover, we observe the production of arabitol at the end of the batch and the fed-batch phases (Fig. 3A). Arabitol production in *P. pastoris* has been related to stress conditions caused by the unfolded protein response (UPR; Tredwell *et al.*, 2017), high osmolarity (Dragosits *et al.*, 2010) or hypoxic conditions, where

 

arabitol production has been proposed to be used as a redox sink (Baumann *et al*., 2010). Additionally, arabitol by-product formation has been observed in lactic acid-producing recombinant *P. pastoris* growing in batch cultures using glycerol as carbon source (Melo *et al*., 2020). Therefore, the presence of arabitol points to a redox imbalance, either caused by 3-HP-derived toxicity effects, or by overexpression of cPos5. Further metabolomic and fluoxmic studies will help elucidating the cause.

Altogether, these results suggest that the pre-established bioreactor fed-batch cultivation protocols for heterologous protein production in *P. pastoris* are not necessarily optimal for the production of weak acids such as 3-HP, as its accumulation in the extracellular space and/or the toxic effect at high concentrations may lead to physiological changes negatively impacting on the bioprocess parameters such as the $\mu_{max}$ or $Y_{X/S}$. Moreover, the current cultivation protocol led to the production of arabitol as a by-product at the end of the fed-batch process, which caused a significant decrease in the product yield. Deletion of the arabitol dehydrogenase (ArDH) gene in a lactic acid-producing strain reduced arabitol production, resulting in a 20% increase in product titres (Melo *et al*., 2020).

The tolerance to 3-HP should also be addressed in order to increase further the product yield. To this end, both adaptive laboratory evolution (ALE) experiments and rational engineering strategies have proven successful to increase 3-HP tolerance in other yeast and could be therefore transferred to *P. pastoris*. Specifically, ALE experiments led to the conclusion that overexpression of the S-(hydroxymethyl)glutathione dehydrogenase gene (*SFA1*) in *S. cerevisiae* restored growth at 50 g $l^{-1}$ of 3-HP (Kildegaard *et al*., 2014). Expression of two mutated versions of *SFA1* under the control of the native SFA1 promoter was also able to restore cell growth at 50 g $l^{-1}$ of 3-HP. The *SFA1* residues which were mutated in *S. cerevisiae* are conserved in the homologous *P. pastoris* gene (PAS_chr3_1028). Therefore, a similar approach to avoid 3-HP toxicity could be tested in *P. pastoris*.

The overall productivity of the fed-batch culture was $0.54 \pm 0.01$ g $l^{-1}$ $h^{-1}$. To our knowledge, this is the highest 3-HP productivity reported in yeast (de Fouchécour *et al*., 2018; Lis *et al*., 2019), and it is almost identical to the highest productivity reported using the malonyl-CoA pathway, which was 0.56 g $l^{-1}$ $h^{-1}$ and it was reported in *E. coli* (Liu *et al*., 2016). Such promising results can be attributed to the combination of three main factors: (i) the high specific MCR activity due to the strength of pGAP, extensively proven for heterologous protein production in *P. pastoris*; (ii) the use of glycerol as a substrate, which delivers more ATP than glucose under aerobic conditions, leading to higher biomass and product yields compared to the use of glucose; and (iii) the use of glycerol as substrate allows for higher growth rates during the feeding phase compared to typical fed-batch cultivations of Crabtree-positive yeasts growing on glucose as substrate, thereby supporting higher volumetric productivities, while minimizing by-product formation.

## Conclusions

In this study, we successfully introduced the malonyl-CoA to 3-HP pathway in *P. pastoris* for 3-HP production. The use of pGAP, a strong constitutive promoter, to drive expression of the biosynthetic *mcr* gene, combined with the use of glycerol as carbon source, proved to be key to obtain higher space-time yields than in other yeasts. The subsequent combination of protein and metabolic engineering strategies performed in this study have led to a 14-fold increase in the 3-HP yield. Moreover, a controlled fed-batch strategy has shown the ability of *P. pastoris* to produce up to 24.75 g $l^{-1}$ of 3-HP in 45.5 h, achieving an overall yield of 0.13 Cmol $Cmol^{-1}$, and a productivity of 0.54 g $l^{-1}$ $h^{-1}$. Overall, we benchmarked *P. pastoris* for 3-HP production, demonstrating the potential of this cell factory for platform chemicals bioproduction. In addition, this study serves as a basis for further optimization of this platform through integrated systems metabolic engineering and bioprocess engineering strategies, which are paramount to reach economically attractive metrics for 3-HP production from crude glycerol in *P. pastoris*.

## Experimental procedures

### *Strain construction*

A series of strains derived from the parental strain *P. pastoris* X-33 (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) was generated. Plasmids and strains used during this study are listed in Table 1. Three heterologous genes were expressed in *P. pastoris*, encoding for: a bifunctional malonyl-CoA reductase from *Chloroflexus aurantiacus* ($mcr_{Ca}$, Uniprot: Q6QQP7_CHLAU), an acetyl-CoA carboxylase from *Yarrowia lipolytica* ($ACC_{Yl}$, Uniprot: YALI0_C11407) and a cytosolic NADH kinase from *S. cerevisiae* ($cPOS_{Sc}$; Tomàs-Gamisans *et al*., 2020). The expression of all the heterologous genes was controlled by the strong and constitutive GAP promoter. A detailed description of the molecular cloning protocols used to generate the plasmids for this study is available in the Supplementary Materials Section (Data S1).

Electrocompetent *P. pastoris* cells were prepared as described elsewhere (Sears *et al*., 1998). Plasmids derived from pBIZi_pGAP (Bioingenium SL, Barcelona,

 

**Table 1.** List of plasmids and *P. pastoris* strains used during this study.

| Plasmid name | Expression cassettes in the plasmid | Source |
|---|---|---|
| pBIZi_pGAP | | Bioingenium SL |
| pBIZi_pGAP_MCR | $P_{GAP}$ - $mcr_{Ca}$ - AOX1tt | This study |
| pBIZi_pGAP_MCR_N | $P_{GAP}$-$mcr_{Ca}$(N-ter)-AOX1tt | This study |
| pBIZi_pGAP_MCR_C | $P_{GAP}$-$mcr_{Ca}$(C-ter)-AOX1tt | This study |
| pBIZi_pGAP_MCR_E | $P_{GAP}$-$mcr_{Ca}$($Cter_{N940V/K1106W/S1114R}$)-AOX1tt | This study |
| pBIZi_pGAP_MCR_NC | $P_{GAP}$-$mcr_{Ca}$(N-ter)-AOX1tt<br>$P_{GAP}$-$mcr_{Ca}$(C-ter)-AOX1tt | This study |
| pBIZi_pGAP_MCR_NE | $P_{GAP}$-$mcr_{Ca}$(N-ter)-AOX1tt<br>$P_{GAP}$-$mcr_{Ca}$($Cter_{N940V/K1106W/S1114R}$)-AOX1tt | This study |
| BB1_23 | | Prielhofer *et al.* (2017) |
| BB1_12_pGAP | | Prielhofer *et al.* (2017) |
| BB1_34_ScCYC1tt | | Prielhofer *et al.* (2017) |
| BB1_34_RPS3tt | | Prielhofer *et al.* (2017) |
| BB2_AB | | Prielhofer *et al.* (2017) |
| BB2_BC | | Prielhofer *et al.* (2017) |
| BB3eH_14 | | Prielhofer *et al.* (2017) |
| BB3eH_AC | | Prielhofer *et al.* (2017) |
| BB3eH_ACC | $P_{GAP}$-$ACC_{Yl}$(Δ*Bbs*I)-ScCYC1tt | This study |
| BB3eH_cPOS5 | $P_{GAP}$-$cPOS5_{Sc}$-ScCYC1tt | This study |
| BB3eH_ACC_cPOS5 | $P_{GAP}$-$ACC_{Yl}$(Δ*Bbs*I)-ScCYC1tt<br>$P_{GAP}$-$cPOS5_{Sc}$-ScCYC1tt | This study |

| Strain name | Plasmid integration | Source |
|---|---|---|
| X-33 | | Invitrogen-Thermo Fisher Scientific |
| PpHP1 | pBIZi_pGAP_MCR | This study |
| PpHP2 | pBIZi_pGAP_MCR_NC | This study |
| PpHP3 | pBIZi_pGAP_MCR_NE | This study |
| PpHP4 | pBIZi_pGAP_MCR_NC<br>BB3eH_ACC | This study |
| PpHP5 | pBIZi_pGAP_MCR_NC<br>BB3eH_cPOS5 | This study |
| PpHP6 | pBIZi_pGAP_MCR_NC<br>BB3eH_ACC_cPOS5 | This study |

Spain) were linearized using AvrII (New England Biolabs, Ipswich, MA, USA), while the ones derived from BB3eH were linearized using PmeI (New England Biolabs). Transformation was performed using 100 ng of purified linear DNA according to a previously described protocol (Sears *et al.*, 1998).

Recombinant *P. pastoris* strains were selected on YPD agar plates (1% yeast extract, 2% peptone, 2% dextrose, 15 g $l^{-1}$ agar) supplemented with the appropriate antibiotic (100 μg $ml^{-1}$ zeocine or hygromycin 200 μg $ml^{-1}$; InvivoGen, CA, USA). When the plates contained both zeocine and hygromycin, the concentration of each antibiotic was reduced by half (50 μg $ml^{-1}$ zeocine and hygromycin 100 μg $ml^{-1}$).

### *Shake flask cultures*

Cultures were first grown overnight on 5 ml of YPG medium (1% yeast extract, 2% peptone and 1% v/v glycerol) supplemented with the appropriate antibiotic in 50-ml Falcon tubes, at 30°C, and 180 rpm. The overnight cultures were then diluted to an optical density at 600 nm ($OD_{600}$) of 0.5 on 5 ml of YPG and grown for 8 h at the same cultivation conditions. Afterwards, these cultures were used to inoculate a 500-ml baffled shake flask containing 50 ml of buffered minimal glycerol medium (BMG; 100 mM potassium phosphate buffer pH 6, 1.34% yeast nitrogen base (YNB), 0.4 mg $l^{-1}$ biotin and 1% v/v glycerol) at a starting $OD_{600}$ of 0.05. The shake flask cultures were grown at 30°C and 180 rpm in an incubator shaker Multitron Standard (Infors HT, Bottmingen, Switzerland) with a 2.5 cm orbit.

### *Screening in deep-well plates*

*Pichia pastoris* strains were inoculated from cryovials into a 24 deep-well plates containing YPG medium supplemented with the appropriate antibiotic. Cultures were placed on a platform with a slope of 20° in an incubator shaker Multitron Standard with 25 mm orbit and grown overnight at 28°C and 220 rpm. Afterwards, 50 μl of overnight cultures was used to inoculate a 24 deep-well plate containing 2 ml of BMG medium. If not stated otherwise, six clones of each strain were tested in

 

triplicates. The cultures were grown for 48 h at the same conditions.

### *Enzymatic assay*

*Pichia pastoris* X-33 and *P. pastoris* PpHP1 cells were grown overnight on YPG medium supplemented with zeocine when appropriate. The grown cultures were used to inoculate a 100-ml shake flask containing 25 ml of fresh BMG medium at an initial $OD_{600}$ of 0.1. When the cells reached an $OD_{600}$ of about 2, 5 ml of each culture were centrifuged at 6000 *g*. The pellets were washed twice with ice-cold PBS buffer and resuspended in 1 ml of breaking buffer (50 mM HEPES, 150 mM KCl, 1 mM DTT, 1 mM EDTA, pH 7.5, Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific)). Resuspended cells were then lysed using glass beads applying 5 cycles of 1 min of vortexing followed by 2 min of incubation on ice. Cell lysates were centrifuged at 16 000 *g* and 4°C for 20 min. The supernatant (soluble fraction) was transferred to a new tube, and the amount of protein in the cell extract was quantified using the Bradford method (Pierce™ Coomassie Plus, Thermo Fisher Scientific). The MCR specific activity was quantified as previously reported (Chen *et al*., 2014). As MCR is a bifunctional enzyme consuming 2 NAPDH molecules to convert malonyl-CoA into 3-HP, one unit of enzyme activity was defined as 2 μmol NADPH consumed per minute.

### *Bioreactor cultivation*

For the bioreactor cultivations, the batch medium consisted of 40 g $l^{-1}$ glycerol, 1.8 g $l^{-1}$ citric acid, 0.02 g $l^{-1}$ $CaCl_2 \cdot 2\ H_2O$, 12.6 g $l^{-1}$ $(NH_4)_2HPO_4$, 0.5 g $l^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 0.9 g $l^{-1}$ KCl, 50 μl antifoam Glanapon 2000 kz (Bussetti and Co GmbH, Vienna, Austria), $4 \cdot 10^{-5}$ g $l^{-1}$ biotin and 4.6 ml $l^{-1}$ of PTM1 trace salts (Maurer *et al*., 2006). The pH of the medium was set to 5 using 5 M HCl. The feeding medium composition was 400 g $l^{-1}$ glycerol, 10 g $l^{-1}$ KCl, 6.45 g $l^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 0.35 g $l^{-1}$ $CaCl_2 \cdot 2\ H_2O$, 0.2 ml $l^{-1}$ antifoam Glanapon 2000 kz, 0.3 mg $l^{-1}$ biotin and 15 ml $l^{-1}$ PTM1 trace salts. For the batch and the feeding media, all components except the biotin and the trace salts were mixed and autoclaved. Biotin and trace salts were filter-sterilized and added to the mixture once the medium had cooled down.

Fed-batch cultures were performed in duplicate in a 5-l Biostat B Bioreactor (Sartorius Stedim, Goettingen, Germany). The pH was maintained at 5 using ammonia 15% (v/v). The temperature was set to 28°C, and air was added into the reactor at an aeration rate of 1 vvm (2 l $min^{-1}$). The agitation was gradually increased from 600 to 1200 rpm to maintain a $pO_2$ above 25%. When increasing the agitation was insufficient to maintain the $pO_2$ above the set point, the agitation was set to 1200 rpm and pure oxygen was mixed with air at the air inlet of the bioreactor to maintain a $pO_2$ above 25%, while maintaining an aeration rate of 2 l $min^{-1}$.

The batch phase was performed with a starting volume equal to 2 l, and the reactor was inoculated at a starting $OD_{600}$ equal to 1. The feeding phase started after a sudden increase in the $pO_2$ indicating that all the glycerol from the batch phase had been consumed. A pre-set exponential feeding rate was used when adding the feeding medium into the reactor. Such exponential feeding rate was calculated according to previous literature (Cos *et al*., 2005), and it aimed to maintain pseudo-steady state conditions at a constant growth rate of 0.1 $h^{-1}$ at C-limiting conditions. The working volume of the reactor was calculated as described elsewhere (Garcia-Ortega *et al*., 2013). To calculate the exponential feeding rate, the biomass concentration at the end of the batch phase ($X_0$) and the biomass to substrate yield ($Y_{X/S}$) were used.

### *Analytical methods and data processing*

The analytical and biological replicates were averaged, and the standard error (SE) for each measurement was calculated considering the number of biological replicates (number of independent samples).

The splines fitting the μ, the q-rates and the product yield during the feeding phase of the fed-batch culture were performed using Matlab 2019 (Mathworks, Natick, MA, USA). The *fit* function was used together with the additional options: *'smoothingspline'* and *'SmoothingParam' = 0.5*.

The $OD_{600}$ measurements of the shake flask and the bioreactor cultures were performed using a Lange DR 3900 spectrophotometer (Hach, Loveland, CO, USA).

To quantify the metabolites, the culture samples were first centrifuged at 12 000 *g* and 4°C and then filtered using a 0.45-μm syringe filter of type HAWP (Millipore, Temecula, CA, USA). Glycerol and arabitol were quantified using an HPLC Dionex Ultimate3000 (Dionex – Thermo Fisher Scientific) coupled to a UV detector at 210 nm and an RI detector (Dionex – Thermo Fisher Scientific). The compounds were separated with an ionic exchange column ICSep ICE-COREGEL 87H3 (Transgenomic, Omaha, NE, USA) using 6 mM sulphuric acid as mobile phase at a flow rate of 0.6 ml $min^{-1}$. As 3-HP and glycerol elute the column at the same time, the 3-HP was quantified using two different methods: HPLC-MS and nuclear magnetic resonance (NMR) spectroscopy. Standard samples for calibration were prepared using 3-hydroxypropionic acid sodium salt from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

 

For the HPLC-MS method, we used a Prominence HPLC (Shimadzu, Kyoto, Japan) coupled to a single quadrupole Shimadzu-2010A mass spectrometry detector using electrospray ionization. The metabolites of the supernatant were separated using an ICSep 87H USP L17 column (Transgenomic, NE, USA) and 16 mM formic acid as mobile phase at a flow rate of 0.15 ml $min^{-1}$. 2 μl samples were injected into the column, and the MS analyser was set to 89 *m*/*z* for negatively charged molecules, which corresponds to the *m*/*z* ratio for unprotonated 3-HP. The detector settings were as follows: curved desolvation line (CDL) temperature at 200°C, heat block temperature at 200°C, voltage of the detector at 1.5 kV, nebulizing gas (nitrogen) flow at 1.5 l $min^{-1}$ and drying gas (nitrogen) flow at 10 l $min^{-1}$. Each sample was analysed in duplicate.

For the NMR method, a Bruker AVANCE 600 spectrometer (600.13 MHz frequency for $^{1}H$) equipped with a 5 mm TBI probehead and an autosampler (Bruker BioSpin, Rheinstetten, Germany) was utilized. The probe temperature was maintained at 300.0 K for all experiments. Once centrifuged and filtered, 300 μl of each culture aliquot was mixed with 300 μl of a $D_2O$ stock solution containing an internal standard (3-(trimethylsilyl)-[2,2,3,3-$^{2}H_4$]-propionic acid sodium salt (TSP), 11.1 mM) and transferred to the NMR tube. All samples were analysed conducting standard quantitative 1D $^{1}H$ NMR experiments with presaturation of the residual water signal. Data were collected into 32k data points during an acquisition time of 1.7 s using a recycle delay of 15 s. Spectra were recorded in the time domain as interferograms (FID) across a spectral width of 9,615 Hz and as the sum of 128 transients. FIDs were automatically Fourier transformed (FT) and the spectra were phased, and baseline corrected. TSP was used as internal reference ($\delta(^{1}H)$ and $\delta(^{13}C)$ at 0.00 ppm).

The glycerol concentration obtained from the IR spectra of the HPLC analysis was corrected accounting for the 3-HP interference. The area at the IR detector corresponding to the amount of 3-HP quantified using the HPLC-MS or the NMR analyses was calculated and subtracted from the total IR area.

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## Conflict of interest

The authors have no conflict of interest to declare.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Data. S1**. Supplementary materials.

 