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Orotic acid production from crude glycerol by engineered *Ashbya gossypii*

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Orotic acid is an intermediate of the *de novo* pyrimidine biosynthesis that plays a crucial role in several nutraceutical supplements. In some microorganisms, blockage of this pathway downstream the dihydroorotate dehydrogenase (DHOD) leads to orotic acid accumulation. Here, the *Ashbya gossypii Agura3* pyrimidine auxotroph was shown to accumulate and excrete orotic acid (~1.5 g/L), and this trait was further explored for the production of orotic acid from raw substrates. Metabolic engineering of this strain combined with culture conditions optimization led to a 3.6-fold increase in orotic acid production from crude glycerol. The mitochondrial DHOD encoded by *AgURA9* was shown to be determinant for this phenotype. Heterologous expression of *AgURA9* in engineered *S. cerevisiae* enabled the accumulation and excretion of orotic acid (~0.5 g/L). This study demonstrates the potential of *A. gossypii* for the valorization of crude glycerol to orotic acid and discloses the molecular determinants for its biosynthesis in fungi.

1. Introduction

Orotic acid is an intermediate of the *de novo* biosynthetic pathway of pyrimidine ribonucleotides that plays an important role as a nutraceutical. It was firstly isolated from cow's milk at the beginning of the last century (1905) (Takayama and Furuya, 1989) and designated at the time as vitamin B_{13}. However, as its synthesis occurs not only in microorganisms but also in humans, this classification was abandoned (Takayama and Furuya, 1989; Wishart et al., 2018b). Orotic acid is a minor constituent of the diet that is mainly found in dairy products and some vegetables (such as carrots and beets). The commercial interest in this chemical relies on the use of its salt, orotate. Orotate is the conjugated base of orotic acid and, for a matter of simplicity, independently of its state, it will be mentioned as orotic acid (Carvalho et al., 2016). Orotic acid is used in combination with minerals such as magnesium, calcium, and lithium, acting as a carrier to improve their transport into cells (Wishart et al., 2018a). In general, these formulations are widely appreciated as supplements for athletes and common people but are also valuable in clinical contexts. For instance, magnesium orotate has been successfully used as adjuvant therapy in the treatment of severe heart failure (Stepura and Martynow, 2009), while lithium orotate has beneficial effects for the mental health, being clinically used to reduce the symptoms of bipolar disorder (Lakhan and Vieira, 2008). In addition, as it is a key intermediate of the *de novo* pyrimidine biosynthesis, it is used as a precursor of nucleotides and pyrimidine-derived compounds (Carvalho et al., 2016).

Several bacteria have been reported to accumulate orotic acid and its biotechnological production has been explored with pyrimidine auxotrophs of the bacterium *Corynebacterium glutamicum* (Takayama and Furuya, 1989; Takayama and Matsunaga, 1991). In these prokaryotes, blockage of the pyrimidine biosynthesis downstream the DHOD, generally at the orotidine 5′-phosphate decarboxylase level, is a requiring factor for their orotic acid-producing phenotype. However, when biotechnological important fungi are inspected, additional unknown factors appear to be involved in this phenotype. For instance, *Scura3Δ* uracil-auxotrophs of the model yeast *Saccharomyces cerevisiae* (Fig. 1) do not display orotic acid accumulation and excretion (Nezu and Shimokawa, 2004). On the other hand, the equivalent mutant of the pre-whole genome duplication (pre-WGD) yeast *Kluyveromyces lactis* (*Klura3Δ*) produces orotic acid in standard yeast extract-peptone-glucose (YPD) media (Carvalho et al., 2016). In this work, the deletion of the mitochondrial DHOD (EC 1.3.5.2) encoded by *KlURA9*, which is lacking in *S. cerevisiae*, was shown to be detrimental to orotic acid production. Conversely, deletion of the *KlURA1* gene encoding the cytosolic DHOD (EC 1.3.98.1), which is the only DHOD present in *S. cerevisiae*, did not affect orotic acid production (Carvalho et al., 2016).

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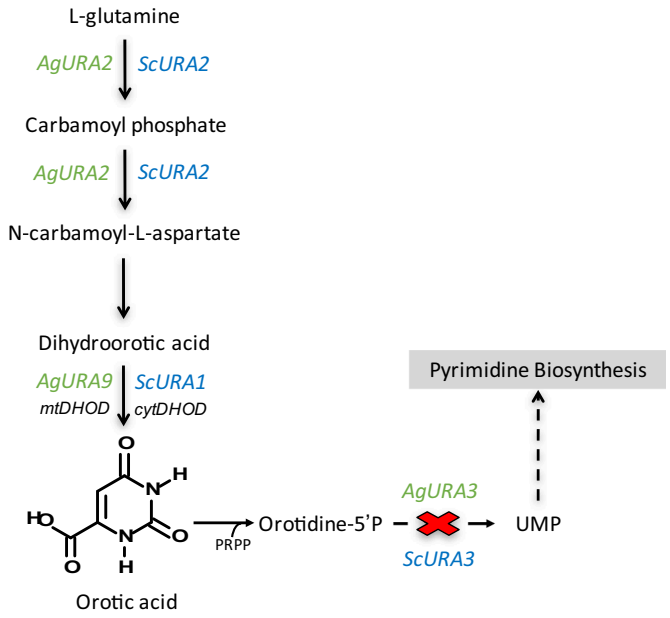

Fig. 1. Schematic representation of the *de novo* biosynthetic pathway of pyrimidines in *A. gossypii* and *S. cerevisiae*. Red × marks where the *de novo* pyrimidine biosynthetic pathway is blocked in the *A. gossypii Agura3* auxotroph (orotidine 5′-phosphate decarboxylase level). Dashed arrows indicate a multistep pathway. Relevant gene names are represented in green and with the *Ag* prefix for *A. gossypii* and in blue and with the *Sc* prefix for *S. cerevisiae*. Acronyms near the gene names represent the corresponding enzyme: mtDHOD, mitochondrial dihydroorotate dehydrogenase; cytDHOD, cytosolic dihydroorotate dehydrogenase; PRPP: phosphoribosyl pyrophosphate; UMP: uridine monophosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Pyrimidine-requiring mutants from *Neurospora crassa* also display an orotic acid-producing phenotype (Mitchell et al., 1948). The genome of this fungus encodes both a cytosolic (NCBI: XP_960964.2) and a mitochondrial (NCBI: XP_961804.1) DHOD. Uracil auxotrophs from *Candida albicans* and *Yarrowia lipolytica*, whose genomes encode only a mitochondrial DHOD, CaUra9p (NCBI: XP_723522.1) and YALI0D18920p (NCBI: XP_503008.1), have been also reported to accumulate and excrete orotic acid (Gojković et al., 2004; Nezu and Shimokawa, 2004; Swietalski et al., 2021). However, *C. albicans* only displayed this phenotype when YPD was supplemented with acetate (Nezu and Shimokawa, 2004). The presence of different DHODs in fungi is linked to evolutionary steps towards anaerobiosis. The cytosolic DHOD (encoded by *URA1*) was acquired by an ancestor of *Saccharomyces* by horizontal gene transfer from bacteria, which permitted these yeasts to start propagating in anaerobic conditions and to lose the mitochondrial/eukaryotic DHOD encoding gene (*URA9*) (Gojković et al., 2004).

A. gossypii is a pre-WGD member of the Saccharomycetaceae family, more closely related to *S. cerevisiae* and *K. lactis* than to other filamentous fungi (Gomes et al., 2014). This fungus is known for its native capacity to overproduce riboflavin (vitamin B_2), for which it is industrially exploited for more than 25 years (Aguiar et al., 2015), and also for its ability to *de novo* biosynthesize other important chemicals such as flavours and fragrances (Birk et al., 2019; Silva et al., 2019a, 2021). In this filamentous fungus, unlike the purine biosynthetic pathway, which is strongly connected with the overproduction of riboflavin (Aguiar et al., 2015), investigation on pyrimidine biosynthesis is limited. However, *A. gossypii* presents some interesting singularities regarding the regulation of pyrimidine biosynthesis that deserve attention (Silva et al., 2019b). For instance, the *de novo* pyrimidine biosynthesis pathway of this fungus, in contrast with *S. cerevisiae* and similarly to *K. lactis*, *N. crassa*, *C. albicans*, and *Y. lipolytica*, owns a putative mitochondrial DHOD, encoded by *AgURA9* (Fig. 1). This fact suggests that an orotic acid-producing phenotype can be displayed by *A. gossypii*. This could lead to the biotechnological exploration of the *Agura3* pyrimidine auxotroph of this filamentous fungus (Aguiar et al., 2014) for orotic acid production and give experimental evidence of the factors involved in orotic acid production in Saccharomycetales. Moreover, *A. gossypii* presents a good native ability to grow in glycerol (Ribeiro et al., 2012), a capacity that was recently explored for the production of lipids (Díaz-Fernández et al., 2019).

Glycerol is a by-product accumulated in several industrial processes, namely in biodiesel and bioethanol production processes (Klein et al., 2016; Luo et al., 2016). Biodiesel production had its golden period between 2005 and 2015, where it grew at an annual rate of 23% (Naylor and Higgins, 2017). The latest data indicates that despite a slowdown, biodiesel production will stay steady, with an annual production until 2025 around 40,000–46,000 million L *per annum* even in an era of low oil price (Severo et al., 2019; IEA - International Energy Agency, 2020). For 10 kg of biodiesel produced, 1 kg (~10%) of crude glycerol is accumulated (Luo et al., 2016). Thus, it is imperative to convert crude glycerol into higher-value products to improve the economic sustainability of the biodiesel industry and mitigate the environmental impacts of crude glycerol waste disposal (Luo et al., 2016; Naylor and Higgins, 2017).

With this in mind, the production of orotic acid by the pyrimidine auxotroph *A. gossypii Agura3* (Aguiar et al., 2014; Silva et al., 2015) was here first disclosed. Subsequently, the study pursued two confluent objectives: i) development of the potential of *A. gossypii* for the production of orotic acid from glycerol, by improving the innate capacities of this fungus through metabolic engineering and culture conditions optimization; ii) understanding the genetic factors for orotic acid accumulation and excretion in fungi, which are for long unknown (Nezu and Shimokawa, 2004) and may have impaired the development of biotechnological processes for the production of this commercially valuable chemical. For this, the pyrimidine biosynthesis pathway of *S. cerevisiae* was engineered at the DHOD level.

2. Materials and methods

2.1. Strains

The *A. gossypii* strains used and generated during this study are listed in Table 1. The pyrimidine auxotroph studied *A. gossypii Agura3* (Aguiar et al., 2014) was the core genetic background used. Stock cultures of these strains were kept as spores suspended in spore buffer containing 200 g/L glycerol, 8 g/L NaCl, and 0.25% (v/v) Tween 20 and stored at −80 °C. Spores were prepared as previously described in Silva et al. (2019b). Yeast strains were maintained at 4 °C on agar plates.

2.2. Media and culture conditions for A. gossypii

Ashbya full medium (AFM; 10 g/L yeast extract, 10 g/L tryptone, 1 g/L myo-inositol, and 20 g/L glucose) was the base medium formulation used for investigating orotic acid production in *A. gossypii*. In alternative to glucose, synthetic or crude glycerol was also used at different concentrations (AFM-glycerol). Additionally, some low-cost medium formulations were also tested, which contained complex nutrients and/or industrial wastes such as crude glycerol (CG), raw yeast extract (RYE; Kelbert et al., 2015), corn steep liquor (CSL; Pereira et al., 2010) and sugarcane molasses (SM). CG was kindly provided by CVR-Centre for Waste Valorisation (Guimarães, Portugal), and before use, it was pretreated to eliminate soap and methanol, essentially as described elsewhere (Ruhal et al., 2011). CG was first diluted in distilled water (1:4 v/v) to reduce the viscosity of the fluid. The pH of the mixture was then adjusted to 3 with HCl (6 M) to convert soap into free fatty acids. The precipitate formed was separated from the mixture by centrifugation at 7000 rpm for 10 min. Next, the pH was adjusted to 12 with KOH (5 M)

Table 1
The strains of *A. gossypii* and *S. cerevisiae* used in this work.

Strains	Relevant genotype	Parental strain	Reference
A. gossypii ATCC 10895	NCBI: txid284811	–	Prof. P. Philippsen, (University of Basel)
A. gossypii Agura3	*Agura3Δ*	ATCC 10895	Aguiar et al., 2014
S. cerevisiae CENPK113-7D	NCBI: txid889517	–	Nijkamp et al., 2012
S. cerevisiae CENPK113-5D	*MATa, Scura3-52*	*S. cerevisiae* CENPK113-7D	
A. gossypii ATCC_AFL067W	*AFL067WpΔ::loxP-KanMX4-loxP-AgGPDp*	ATCC 10895	This work
*A. gossypii Agura3*_AFL067W (uG)	*Agura3Δ AFL067WpΔ::loxP-KanMX4-loxP-AgGPDp*	*Agura3*	
A. gossypii uGU2	*Agura3Δ AFL067WpΔ::loxP-AgGPDp AgURA2pΔ::loxP-KanMX4- loxP-AgGPDp*	uG (*kanmx*-)	
A. gossypii uGU2.9	*Agura3Δ AFL067WpΔ::loxP-AgGPDp AgURA2pΔ::loxP-AgGPDp AgURA9pΔ::loxP-KanMX4-loxP-AgGPDp*	uGU2 (*kanmx*-)	
S. cerevisiae AgURA9	*CENPK113-7D Scura3Δ::loxP-KanMX4-loxP-AgGPDp-AgURA9*	*S. cerevisiae* CENPK113-7D	
S. cerevisiae AgURA9_Δura1	*CENPK113-7D Scura3Δ::loxP-KanMX4-loxP-AgGPDp-AgURA9 Scura1Δ::loxP-NatMX4-loxP*	*S. cerevisiae AgURA9*	
S. cerevisiae 5D_Δura1	*CEN.PK113-5D Scura1Δ::loxP-NatMX4-loxP*	*S. cerevisiae* CENPK113-5D	

and the suspension was filtrated through qualitative filter paper (Advantec) to remove suspended debris. Then, the pH of the filtrate was adjusted to 6.8–7.0. Finally, the methanol contained in the solution was removed by evaporation during autoclaving and the concentration of glycerol was quantified by high-performance liquid chromatography (HPLC). GLY_{CR} was then added to medium formulations at a final concentration equivalent to approximately 25 g/L glycerol. The origin and preparation mode of the RYE and CSL are reported elsewhere (Kelbert et al., 2015; Pereira et al., 2010), respectively. SM was kindly provided by RAR: Refinarias de Açúcar Reunidas, S.A. (Porto, Portugal) and used at a final concentration of approximately 25 g/L sucrose. Whenever indicated, $CaCO_3$ (2 g/L) was used to buffer the medium. All the constituents of each media were sterilized together by autoclaving. As standard procedure, cultivations were performed in 250 mL shake-flasks with a working volume of 50 mL and carried out at 28 °C in an orbital shaker at 200 rpm. The inoculum was set to approximately 10^7 spores/L. However, since different growth conditions and medium formulations were tested, they are described in the "Results and discussion" section and figures captions. Cell dry weight (CDW) was determined by filtration, collection of the mycelium into a pre-weighed dried tube and drying at 105 °C until constant weight (~24 h).

2.3. Construction of integration cassettes and transformation of *A. gossypii*

DNA manipulations were made using standard molecular biology procedures (Domingues, 2017). For the overexpression of the genes *AgAFL067W* (AFL067W; NCBI Reference Sequence: NM_210835.2), *AgURA2* (ACR263C; NCBI Reference Sequence: NM_209018.2) and *AgURA9* (ACL035C; NCBI Reference Sequence: NM_208722.1), the transformation cassettes used to substitute the native promoter of the genes were constructed as follows: the *loxP-KanMX-loxP* selectable marker, conferring resistance to geneticin (G418), and the promoter sequence of the *AgGPD* gene was amplified by PCR using specific primers (Section 1 of Supplementary data) from a vector described previously (Silva et al., 2019a). These primers were used to introduce recombinogenic flanks for the locus corresponding to regions starting upstream 50 bp from the start codon and downstream the ATG initiation codon.

The overexpression modules were used to transform spores of *A. gossypii* using the Cre-*lox*P system (Aguiar et al., 2014). The primary heterokaryotic transformants were selected on agar-solidified AFM containing 250–300 μg/mL geneticin (G418). The selection of *A. gossypii* homokaryotic clones was performed through the germination of uninucleated haploid spores obtained from the primary heterokaryotic transformants on agar-solidified AFM-G418. The correct genomic integration of the cassette, as well as the homokaryotic genotype of each clone, were confirmed by analytical PCR. For this, a pin-head of mycelium from single colonies was suspended in 30 μL of extraction buffer (0.05 M carbonate buffer pH 6.9, 2% (w/v) PVP 40, 0.2% (w/v) BSA and 0.05% (v/v) Tween 20) and incubated for 10 min at 95 °C. Afterwards, the tubes were centrifuged at maximum speed. The supernatant was used as a template in the PCR reactions using the specific primers listed in Section 1 of Supplementary data.

2.4. Construction of integration cassettes, transformation and characterization of engineered *S. cerevisiae*

For the overexpression of the gene *AgURA9* (ACL035C; NCBI Reference Sequence: NM_208722.1) in the *ScURA3* locus (YEL021W; GenBank: CM001526.1), the transformation cassette containing the *loxP-KanMX-loxP* selectable marker, conferring resistance to geneticin (G418), the promoter sequence of the *AgGPD* gene and the entire open reading frame (ORF) of the gene *AgURA9* was used to substitute the entire ORF of *ScURA3* plus 82 bp upstream the ATG initiation codon. This sequence was amplified by PCR using specific primers (Section 1 of Supplementary data), containing recombinogenic flanks for the *ScURA3* locus, from gDNA of the strain *A. gossypii* uGU2.9. For the deletion of the gene *ScURA1* (SCEN_K00200; NCBI Reference Sequence: CP046091.1), the transformation cassette containing the *loxP-NatMX-loxP* selectable marker, conferring resistance to nourseothricin (clonNAT), was used to substitute the entire ORF of *ScURA1* plus 80 bp upstream the ATG initiation codon. This sequence was amplified by PCR using specific primers (Section 1 of Supplementary data), containing recombinogenic flanks for the *ScURA1* locus, from a vector of our lab stock containing the corresponding selection marker (gRNA_pCfB2310-CAN1).

The protocol used for the transformation of yeast was the LiAC/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). Transformants were selected on agar-solidified YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) containing 200 μg/mL geneticin (G418) or 100 μg/mL nourseothricin (clonNAT). The correct genomic integrations of the cassettes were confirmed by analytical PCR. For this, a small amount of biomass was suspended in 15 μL of 20 mM NaOH and incubated for 15 min at 95 °C. Afterwards, the tubes were centrifuged at maximum speed and the supernatant was used as a template in the PCR reactions using the specific primers listed in Section 1 of Supplementary data.

YPD was the base medium formulation used for accessing orotic acid production in *S. cerevisiae*. Cultivations were performed in 250 mL shake-flasks with a working volume of 25 mL and carried out at 30 °C in an orbital shaker at 200 rpm. The inoculum was set to an initial OD_{600}/biomass of 0.1, made by an overnight grown pre-inoculum in the same conditions.

2.5. Radial growth of A. gossypii strains in hyperosmotic stress conditions

Agar-solidified AFM containing 1 M KCl and 30 mM glycerol were inoculated with 10 μL of a suspension of spores (10^7 spores/mL) and incubated at 30 °C for 7 days. The control condition consisted of agar-solidified AFM. Colony radial growth was determined daily by measuring the diameter of colonies in 90 mm diameter Petri dishes in two perpendicular directions, through two guidelines previously drawn on the lower outer face of the plates (Brancato and Golding, 1953). Radial growth measurements were performed in biological duplicates.

2.6. Analytical methods

The concentration of glucose, glycerol and sucrose in the supernatant of the culture samples was determined by HPLC using a BioRad Aminex HPX-87H (300 × 7.8 mm) column at 60 °C and 0.005 M sulfuric acid as eluent in a flow rate of 0.6 mL/min. The corresponding peaks were detected using a Knauer-IR intelligent refractive index detector. Orotic acid was the chemical compound studied (PubChem CID: 967). Orotic acid anhydrous (98%) from Acros Organics was used as a standard for the Ultra High Performance Liquid Chromatography (UHPLC).

The orotic acid present in the supernatant of culture samples was quantified with SHIMADZU Nexera X2 UHPLC chromatography. The column used for separation was a BRISA LC2 C18 (250 mm × 4.6 mm, 5 μm particle size; from Teknokroma, Spain) maintained at room temperature. The column effluent was monitored at 280 nm using a SHIMADZU SPD-M20A diode array detector. The eluent used was 25 mM K_2HPO_4/KH_2PO_4 (pH 7.0) at a flow rate of 0.5 mL/min during a total run of 40 min (Carvalho et al., 2016). Samples for orotic acid quantification were diluted with 500 mM NaOH when appropriate.

2.7. Statistical analyses

GraphPad Prism for IOS version 6.0 was used to carry out the statistical analyses. Differences to a control strain were analysed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. Unless indicated otherwise, statistical significance was established at $p < 0.05$ for the comparisons.

3. Results and discussion

3.1. A. gossypii Agura3 accumulates and excretes orotic acid to the culture medium

An initial assessment of orotic acid production by *A. gossypii Agura3* in shake-flask cultivations revealed that this strain can produce orotic acid in the range of g/L from both glucose and glycerol (Section 2 of Supplementary data). This orotic acid is virtually all excreted to the extracellular medium since no relevant differences were found between its concentration in the supernatant and total culture broth (lysed cells plus supernatant). Similar to what was previously observed with the yeast *K. lactis* (Carvalho et al., 2016), time-course analysis of orotic acid production by *A. gossypii Agura3* showed production kinetics associated with growth, stabilizing at the stationary phase (Section 2 of Supplementary data).

The titre of orotic acid produced by *A. gossypii Agura3* in glucose (1.51 ± 0.04 g/L) was slightly higher than that obtained in glycerol (1.23 ± 0.04 g/L). The concentration of orotic acid stabilized after approximately 72 h of growth when glucose was already depleted from the medium and 5.83 ± 0.58 g/L of glycerol remained in the culture. Biomass production was similar in glucose (5.52 ± 0.33 g_{CDW}/L) and glycerol (5.73 ± 0.07 g_{CDW}/L). As expected, orotic acid was not detected in cultures of the wild strain *A. gossypii* ATCC 10895 (data not shown). On the other hand, the addition of 5 mM uridine to AFM, which has been shown to rescue the growth of the *A. gossypii Agura3* auxotroph (Silva et al., 2015, 2019b), abolished the production of orotic acid by this strain (data not shown). Thus, these results show that: (i) blockage of the *de novo* pyrimidine biosynthesis downstream the formation of orotic acid (*e.g.*, deletion of *AgURA3*) leads to the accumulation and subsequent excretion of this compound by *A. gossypii*; (ii) when high amounts of intermediates of the pyrimidine salvage pathway (such as uridine) are present in the medium, this pathway is recruited and the metabolic flux through the *de novo* biosynthesis is reduced or null (Silva et al., 2019b).

3.2. A. gossypii AgURA9 is a determinant for orotic acid production

The biotechnological exploration of orotic acid production in fungi has been limited due to two main factors: (i) only a few species are known to be able to accumulate and excrete orotic acid (Carvalho et al., 2016; Nezu and Shimokawa, 2004; Takayama and Furuya, 1989); (ii) the genetic determinants for this phenotype are poorly understood. In addition to *K. lactis* (Carvalho et al., 2016), *N. crassa* (Mitchell et al., 1948), *C. albicans* (Nezu and Shimokawa, 2004) and *Y. lipolytica* (Swietalski et al., 2021), *A. gossypii* was here shown to be one of the fungal species whose pyrimidine auxotrophs display this phenotype. *K. lactis* owns two putative DHODs: the cytosolic KlUra1p (EC 1.3.98.1; NCBI: XP_453064.1) and the mitochondrial KlUra9p (EC 1.3.5.2; NCBI: XP_452611.1) (Carvalho et al., 2016; Gomes et al., 2014) (Fig. 1). The *N. crassa* genome also encodes a cytosolic (NCBI: XP_960964.2) and a mitochondrial (NCBI: XP_961804.1) DHOD. In turn, *A. gossypii*, *C. albicans* and *Y. lipolytica* only have the mitochondrial DHOD (EC 1.3.5.2): AgUra9p (NCBI: NP_983369.1) (Gomes et al., 2014), CaUra9p (NCBI: XP_723522.1) (Gojković et al., 2004) and YALI0D18920p (NCBI: XP_503008.1) (Swietalski et al., 2021), respectively. *S. cerevisiae*, whose uracil auxotrophs do not accumulate orotic acid, only owns the cytosolic DHOD (EC 1.3.98.1), ScUra1p (NCBI: NP_012706.1) (Nezu and Shimokawa, 2004). Carvalho et al. (2016) advanced the importance of the presence of the mitochondrial DHOD for the *K. lactis* orotic acid-producing phenotype. In their study, the deletion of the *KlURA9* gene in the *K. lactis Klura3Δ* strain reduced orotic acid production by 3-fold, whereas the deletion of the *KlURA1* gene did not affect orotic acid production (Carvalho et al., 2016). Here, to assess if the presence of a mitochondrial DHOD would turn *S. cerevisiae* into an orotic acid producer and thus demonstrate that, beyond the pyrimidine auxotrophy, the presence of this enzyme is determinant for orotic acid accumulation and excretion, the pyrimidine biosynthesis pathway of this yeast was engineered with the *A. gossypii AgURA9* gene.

From the prototrophic strain *S. cerevisiae* CENPK113-7D, an auxotrophic strain for pyrimidines was constructed, strain *S. cerevisiae AgURA9*, where the *ScURA3* locus was substituted by an *AgURA9* expression cassette (Table 1; for details see Section 2.4). However, no orotic acid was detected in the cultures of this new strain. Despite the presence of *AgURA9*, *S. cerevisiae* still maintained intact the native cytosolic DHOD encoded by *ScURA1*. Therefore, it was hypothesized that the metabolic flux of the *de novo* pyrimidine biosynthesis would continue to be made preferentially by the native pathway (*i.e.*, *via* ScUra1p). Consequently, strain *S. cerevisiae AgURA9_Δura1* was subsequently constructed by further deleting *ScURA1* (Table 1). The rationale followed was that this modification would block the formation of orotic acid by any native enzyme and would thus funnel its biosynthesis through the action of *AgURA9*. With this modification, this new strain accumulated and excreted orotic acid to the medium unlike any other *S. cerevisiae* strain tested (Table 2).

S. cerevisiae AgURA9_Δura1 was able to excrete 0.53 ± 0.17 g/L orotic acid, corresponding to a specific production of 0.21 ± 0.07 g/g_{CDW} at 24 h. The absorption spectrum of the peak identified in this *S. cerevisiae* strain matches the spectrum of a pure orotic acid standard chromatogram (Section 3 of Supplementary data). This is the experimental evidence that the presence of a mitochondrial DHOD, encoded by *URA9* genes, is a major factor for orotic acid accumulation and excretion in fungi. With this in mind, other species encoding a native mitochondrial DHOD (Gojković et al., 2004) will likely be candidates for

Table 2
Orotic acid production by engineered *S. cerevisiae* strains. Cultivations were carried out in YPD at 30 °C and 200 rpm using 250 mL shake-flasks with 10% working volume. Data represent average values ± standard deviations of biological duplicates at 24 h.

S. cerevisiae strain	*AgURA9_Δura1*	*AgURA9*	5D_*Δura1* (*ura-*)	CENPK113-5D (*ura-*)	CENPK113-7D (WT)
Orotic acid (g/L)	0.53 ± 0.17	n.d.	n.d.	n.d.	n.d.
Final CDW (g/L)	2.47 ± 0.00	2.15 + 0.33	2.97 + 0.26	2.86 + 0.57	2.80 + 1.16

n.d. – not detected.

orotic acid production as well. Beyond those, other species may be engineered using as a basis the rationale used in this study to confer *S. cerevisiae* an orotic acid-producing phenotype: i) blockage of the pyrimidine *de novo* biosynthesis downstream the formation of orotic acid (deletion of *ScURA3*); ii) expression of a gene encoding a mitochondrial DHOD (*AgURA9*); and iii) deletion of the gene encoding the native cytosolic DHOD (*ScURA1*).

3.3. Improvement of glycerol consumption and hyperosmotic stress tolerance through AFL067W overexpression

The development of a microorganism as a cell factory requires the optimization of its substrate consumption range, together with the improvement of its robustness towards industrial conditions (Czajka et al., 2017; Nielsen and Keasling, 2016; Yadav et al., 2012). After confirming the production of orotic acid by *A. gossypii Agura3*, it was interesting to note that the production levels from glycerol were comparable to those obtained in glucose, even though glycerol is consumed at slower consumption rates. This demonstrates that this polyol, the main by-product of the biodiesel industry, could be a suitable carbon source for exploring orotic acid production with this filamentous fungus. Glycerol is an important industrial by-product (Klein et al., 2016; Luo et al., 2016) and *A. gossypii* can utilize this carbon source for biotechnological purposes (Magalhães et al., 2014; Díaz-Fernández et al., 2019). Therefore, to further explore orotic acid production from glycerol feedstock, a chassis strain with improved utilization of glycerol was firstly sought.

In *S. cerevisiae*, ScGup1p was firstly identified as a glycerol transporter by Holst et al. (2000), who reported its role in the growth of this yeast from glycerol, as well as in cell recovery from hyperosmotic stress when glycerol was present in the medium. Although other functions have been associated with *ScGUP1* beyond glycerol metabolism, overexpression of this gene was revealed to be an effective strategy to increase glycerol consumption by *S. cerevisiae* (Jung et al., 2011; Yu et al., 2010a). *A. gossypii* owns a syntenic homolog of *ScGUP1* – *AFL067W*. In previous work, *AFL067W* and *ScGUP1* were overexpressed in *A. gossypii* ATCC 10895 strain using an episomal multi-copy vector (Silva, 2014). The resulting strains, in particular the strain overexpressing the native *AFL067W*, presented enhanced glycerol consumption and improved hyperosmotic tolerance when glycerol was present in the medium (Silva, 2014). However, the use of episomal vectors in *A. gossypii* raises stability issues that are a concern for industrial applications. Therefore, the overexpression of the native *AFL067W* in the *Agura3* strain was here tested through the replacement of its native promoter by the strong and constitutive *AgGPD* promoter (for details see Section 2.4). Subsequently, the glycerol consumption profile of the strain overexpressing *AFL067W* through a stable integrative overexpression cassette (*Agura3_AFL067W*) was compared with the parental *Agura3* strain (Fig. 2).

Agura3_AFL067W demonstrated a slight improvement in glycerol utilization, being able to consume almost completely all the glycerol present in AFM-glycerol (~45 g/L) after 8 days (192 h) (Fig. 2). At this time point, approximately 5 g/L of glycerol were still present in the cultivation of the *Agura3* strain. Moreover, overexpression of *AFL067W* did not affect orotic acid production.

Besides functioning as a carbon source, glycerol is a major osmoprotectant for *A. gossypii* cells (Förster et al., 1998). In turn, ScGup1p was shown to have a complementary role in counteracting hyperosmotic

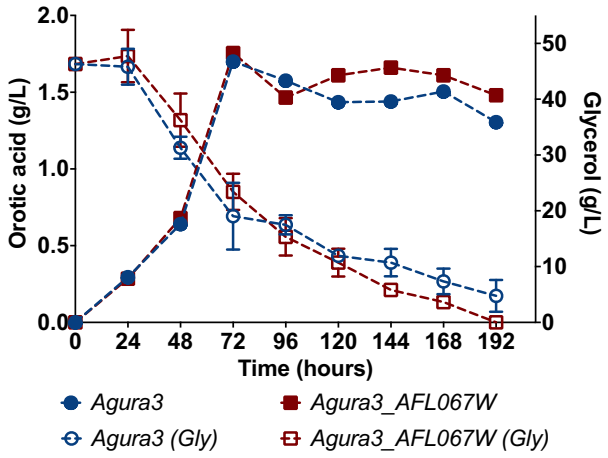

Fig. 2. Orotic acid production and glycerol consumption by *A. gossypii Agura3* and *A. gossypii Agura3_AFL067W* in AFM-glycerol. *A. gossypii Agura3* is represented by circles (●) and *A. gossypii Agura3_AFL067W* by squares (■). Filled symbols represent orotic acid production and empty symbols glycerol consumption. Strains were grown at 28 °C and 200 rpm. Data represent average values and standard deviations of biological duplicates. Where not seen, error bars were smaller than the symbols. The final CDW was 9.7 ± 1.5 g_{CDW}/L for the strain *Agura3* and 11.2 ± 1.3 g_{CDW}/L for the strain *Agura3_AFL067W*.

stress conditions in *S. cerevisiae* (Holst et al., 2000; Yu et al., 2010b). Therefore, to assess if the overexpression of *AFL067W* could improve the hyperosmotic stress tolerance, the growth of *Agura3_AFL067W* strain was monitored on agar-solidified AFM containing an osmotic stress agent (1 M KCl) and 30 mM glycerol (Fig. 3). To determine if the effects on hyperosmotic stress tolerance were strain-dependent, *AFL067W* was also overexpressed in the wild strain ATCC 10895 (ATCC_ *AFL067W*).

Fig. 3A shows that the overexpression of *AFL067W* improved the radial growth of the strains at hyperosmotic stress conditions that completely abolished the growth of the parental strains (*Agura3* and ATCC 10895). Interestingly, both overexpressing strains presented similar improvements in response to the hyperosmotic conditions (Fig. 3B). Glycerol is the major osmoprotectant used by *A. gossypii* under hyperosmotic stress (Förster et al., 1998). Under these conditions, the preferential mechanism adopted by *A. gossypii* cells is to accumulate glycerol through its biosynthesis. This is also observed in *S. cerevisiae* (Blomberg and Adler, 1989; Förster et al., 1998; Holst et al., 2000). The improvement observed here suggests that the overexpression of *AFL067W* complement the main mechanism of the fungus to hyperosmotic stress conditions (glycerol biosynthesis) by being involved in the uptake of exogenous glycerol when this is available.

In sum, the overexpression of *AFL067W* was revealed to be a flexible engineering strategy that allowed enhancing the consumption rate of glycerol, but also improving the robustness of the fungus towards hyperosmotic stress. During the neutralization of crude glycerol salts as potassium or sodium chloride are formed, which may consequently increase the osmotic stress for microorganisms (Ruhal et al., 2011). Since *A. gossypii* is among the most sensitive fungi to osmotic stress (Nikolaou et al., 2009), these results are relevant for biotechnological purposes in which industrial wastes as crude glycerol are intended to be utilized. For

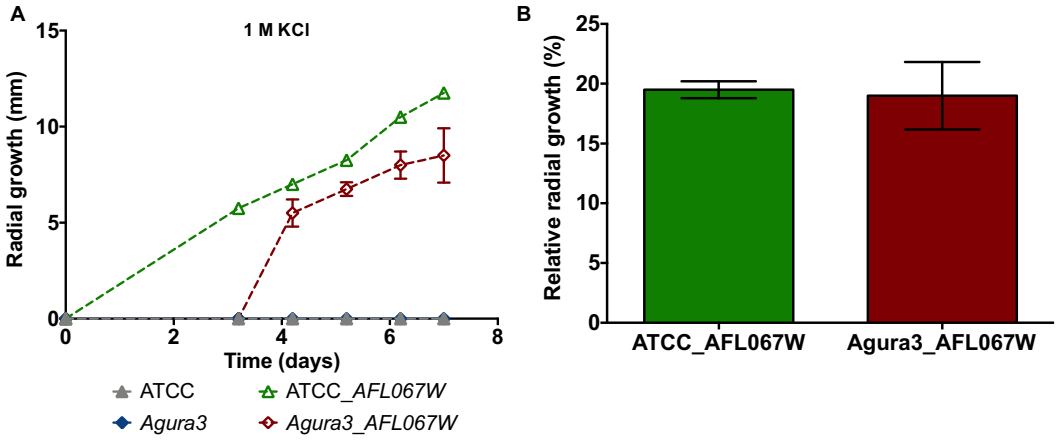

Fig. 3. (A) Radial growth of *A. gossypii* strains on agar-solidified AFM with 1 M KCl and 30 mM of glycerol. Strains were grown at 30 °C. (B) Relative radial growth (%) of strains overexpressing *AFL067W* (ATCC_*AFL067W* and *Agura3*_*AFL067W*). Relative growth was obtained by calculating the ratio between the final radial growth of each strain in AFM with and without 1 M KCl. Data represent average values and standard deviations of biological duplicates. Where not seen, error bars were smaller than the symbols.

this specific study, these improvements were added up to a genetic background (*Agura3*) that was already harnessed for orotic acid production. Thus, this engineered strain (*Agura3*_*AFL067W*), hereafter designated as strain uG, was the chassis used to optimize orotic acid production from glycerol in *A. gossypii*.

3.4. Optimization of aeration conditions for orotic acid production in shake-flask

The *de novo* pyrimidine pathway is linked to the respiratory chain in *A. gossypii* through a mitochondrial DHOD. Therefore, good oxygenation of the culture should be important for orotic acid production, as previously demonstrated for *K. lactis* (Carvalho et al., 2016). Before undergoing additional strain engineering, improved aeration conditions for orotic acid production were first sought. To assess this, shake-flask cultivations with strain uG were performed under different aeration conditions in AFM-glycerol (Fig. 4). To provide a superior level of aeration, the ratio between working volume/shake-flask capacity was increased from 1:5 to 1:10. For that, the headspace in shake-flasks was increased by reducing the working volume of the medium (25 mL in shake-flasks of 250 mL; 25/250 mL) or by using a shake-flask with higher capacity (50 mL in shake-flasks of 500 mL; 50/500 mL).

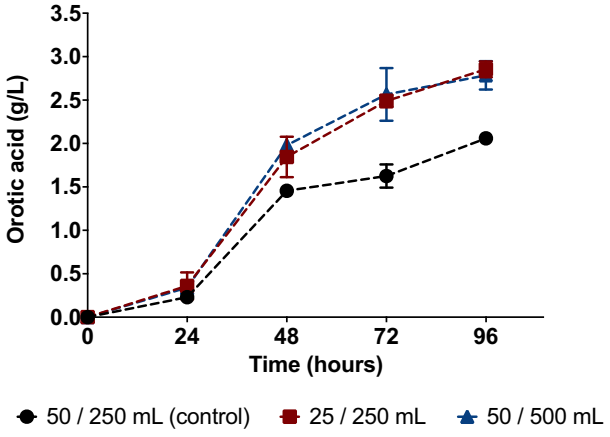

Fig. 4. Effect of aeration on orotic acid production by *A. gossypii* uG during shake-flask cultivations in AFM-glycerol (40 g/L glycerol). The control condition consisted of 50 mL of working volume in shake-flasks of 250 mL (ratio of 1:5). Increased aeration was achieved by increasing the headspace in shake-flasks to a ratio of 1:10 (25/250 mL and 50/500 mL). Strains were grown at 28 °C and 200 rpm. Data represent average values and standard deviations of biological duplicates. Where not seen, error bars were smaller than the symbols.

The production of orotic acid in shake-flask was positively affected by increased aeration, with similar improvements achieved in both conditions tested (25/250 mL and 50/500 mL). After 96 h, a maximum concentration of 2.85 ± 0.09 g/L orotic acid was reached. This represented an increase of 39% in orotic acid production in comparison with the control condition (50/250 mL). Orotic acid production was also evaluated using 50 mL cultures in baffled flasks of 500 mL, but the percentage of improvement remained similar (data not shown). These results confirmed that good culture oxygenation is important for orotic acid production in *A. gossypii*. This indication is in line with the general preference of *A. gossypii* for aerobic cultivations (Aguiar et al., 2015). The production levels obtained in these conditions (in the range of 1–3 g/L) are comparable to those reported for other fungi in shake-flask, such as *N. crassa* (Mitchell et al., 1948) or *K. lactis* (Carvalho et al., 2016).

3.5. Improvement of the A. gossypii metabolic flux towards orotic acid biosynthesis

After the construction of a chassis strain and the definition of better aeration conditions that do not constrain the evaluation of strain performance, the overexpression of key genes of the *de novo* pyrimidine biosynthesis was the strategy chosen to further improve orotic acid production by *A. gossypii*. In *S. cerevisiae*, the first two steps of the *de novo* biosynthesis of pyrimidines are catalysed by an enzyme encoded by *ScURA2* that display bi-functional activity, carbamylphosphate synthetase and aspartate transcarbamylase (Denis-Duphil, 1989; Souciet et al., 1982). These steps convert L-glutamine to carbomoyl phosphate and then the latter into N-carbamoyl-L-aspartate (Fig. 1). There are other carboymylphosphate synthetases (*ScCPA1* and *ScCPA2*) encoded in the genome of *S. cerevisiae* (Lim and Powers-Lee, 1996). However, despite being assigned to steps of the pyrimidine biosynthesis in some databases, such as KEGG (Kanehisa et al., 2021), these enzymes are involved in arginine biosynthesis and not in pyrimidine biosynthesis (Lim and Powers-Lee, 1996). *A. gossypii* owns a syntenic homolog of these *S. cerevisiae* genes, namely of *ScURA2* – *ACR263C*, here designated as *AgURA2*. Therefore, due to the orthogonal importance of Ura2p in the regulation of pyrimidine biosynthesis (Benoist et al., 2000), the overexpression of the native *AgURA2* in *A. gossypii* was also tested (for details see Section 2.4). The enzyme that catalyses the synthesis of orotic acid from dihydroorotic acid is a mitochondrial DHOD encoded by *AgURA9* (*ACL035C*) (Gomes et al., 2014). Thus, *AgURA9* was also overexpressed

(Fig. 1). Two new strains were constructed: strain uGU2, comprising the genetic modifications of strain uG plus the overexpression of *AgURA2*; and strain uGU2.9, comprising the genetic modifications of strain uGU2 plus the overexpression of *AgURA9* (Table 1). To test the production of orotic acid from glycerol by these new strains, shake-flask cultivations using the aeration conditions selected in the last section were performed (Fig. 5).

In comparison with the parental strain (*Agura3*), all the additional genetic modifications increased the final titre of orotic acid production: strain uG (2.99 ± 0.37 g/L), strain uGU2 (3.35 ± 0.18 g/L), strain uGU2.9 (3.72 ± 0.28 g/L) (Fig. 5A). These genetic modifications led to a maximum 51% increase in volumetric production (t = 96 h). From the three engineered strains, only the combination of the overexpression of the three genes (*AFL067W*, *AgURA2* and *AgURA9*) led to statistically significant differences in orotic acid-specific production in comparison with the parental strain (*Agura3*) (Fig. 5B). Strain uGU2.9 (0.49 ± 0.08 g/g_{CDW}) presented 53% more production per biomass than strain *Agura3* (0.32 ± 0.08 g/g_{CDW}). These data confirm the importance of an elevated metabolic flux through the *de novo* pyrimidine biosynthesis for orotic acid production by *A. gossypii*. To the extent of our knowledge, this is the first time that this metabolic engineering strategy is used for orotic acid production in fungi.

3.6. Effect of medium composition on orotic acid production by *A. gossypii*

To establish the grounds of a biotechnological process that contributes to a circular bioeconomy, the potential of the host to produce the desired product from renewable raw materials was analysed. Some preliminary experiences were made with *A. gossypii* (strain uG), in which low-cost industrial by-products were assessed as culture medium components. Crude glycerol (CG), sugarcane molasses, raw yeast extract, and corn steep liquor were the industrial by-products tested. The production of orotic acid by *A. gossypii* from different combinations of these substrates was tested in shake-flask cultivations (Section 4 of Supplementary data). *A. gossypii* uG was able to produce orotic acid from all the medium formulations exclusively made of low-cost substrates. Noteworthy, from a medium constituted by 2% raw yeast extract plus CG, *A. gossypii* uG was able to produce 1.15 ± 0.02 g/L of orotic acid after 96 h. These results demonstrated that *A. gossypii* not only withstands these industrial by-products but also produces orotic acid from a medium completely based on industrial by-products.

These preliminary data were important as they highlighted the most promising raw materials for *A. gossypii* cultivations, namely CG and yeast extract. Therefore, these two media components were used to test the potential of *A. gossypii* uGU2.9 in shake-flask cultivations. Since strain uGU2.9 displayed the highest specific orotic acid production per biomass, the medium composition was modified to increase biomass production. For this, the amount of yeast extract used in the AFM formulation was doubled. The focus on yeast extract (YE) followed two main reasons: i) yeast extract is the main carbon donor for anabolic processes during growth of *A. gossypii* in riboflavin industrial cultivations (Schwechheimer et al., 2018); and ii) *A. gossypii* can use raw yeast extract coming from the brewery industry (Section 4 of Supplementary data), so the results obtained here can be extrapolated for future optimizations. Additionally, both synthetic and crude glycerol were tested to compare the titres obtained (Fig. 6).

The data showed that independently of the source of glycerol used, synthetic (SG) or crude (CG), the doubling of the YE concentration (1% *vs* 2%) had a positive effect on the final volumetric production of orotic acid (Fig. 6A & B), with improvements in the order of 35% in SG (3.30 ± 0.19 *vs* 4.46 ± 0.47 g/L) and 31% in CG (3.10 ± 0.28 *vs* 4.06 ± 0.41 g/L). These improvements in volumetric production resulted from an increase in biomass production, as clearly observed in the SG cultivations, where a significant difference was observed between the CDW in 1% *vs* 2% YE, but specific production ($Y_{P/X}$) remained similar (Fig. 6C & D). However, in CG cultivations, no significant difference was observed in CDW nor in final specific orotic acid production (Fig. 6C & D). These results indicate that higher orotic acid titres can be obtained by increasing biomass production through modification of the medium composition, namely through optimization of YE concentration. In addition, they show that the performance of strain uGU2.9 was similar in media using either SG or CG, reaching final yields of orotic acid produced per amount of glycerol ($Y_{P/S}$) of 0.153 ± 0.016 g/g_{gly} and 0.165 ± 0.017 g/g_{gly} after 96 h using 2% YE, respectively. Indeed, these final orotic acid yields obtained in shake flask from glycerol, namely CG, are similar to those reported for *K. lactis Klura3* cultivations in a bioreactor from commercial glucose (~0.15 g/g_{gluc}; Carvalho et al., 2016). Moreover, an average orotic acid yield of ~0.76 g/g_{CDW} was reached in CG cultivations supplemented with 2% YE (Fig. 6D), which is comparable to the best specific production yields ($Y_{P/X}$) reported in the literature from commercial glucose (Table 3). Thus, these results show that *A. gossypii* uGU2.9 is capable of producing orotic acid using industrial waste as substrate without loss of titre, yield, and production rate in comparison with synthetic substrates (commercial glucose or glycerol), and that the best production yields obtained in shake-flask from CG ($Y_{P/S}$

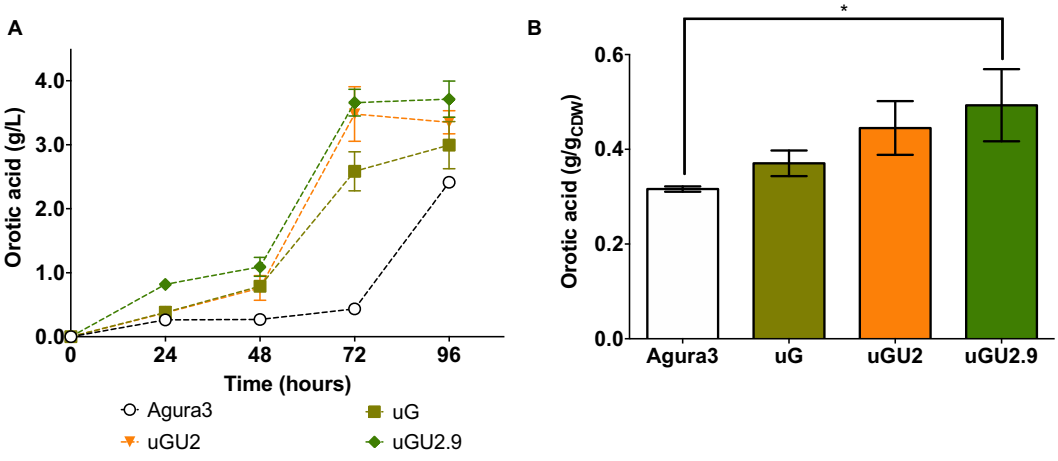

Fig. 5. Orotic acid production by *A. gossypii* engineered strains. (A) Time-course volumetric (g/L) and (B) final specific production (g/g_{CDW}) of orotic acid. Cultivations were carried out in AFM-glycerol (20 g/L) at 28 °C and 200 rpm using 250 mL shake-flasks with 10% working volume. Data represent average values and standard deviations of at least biological triplicates. Where not seen, error bars were smaller than the symbols. Asterisk represents significant differences (* $p < 0.05$) compared to the control strain (*Agura3*).

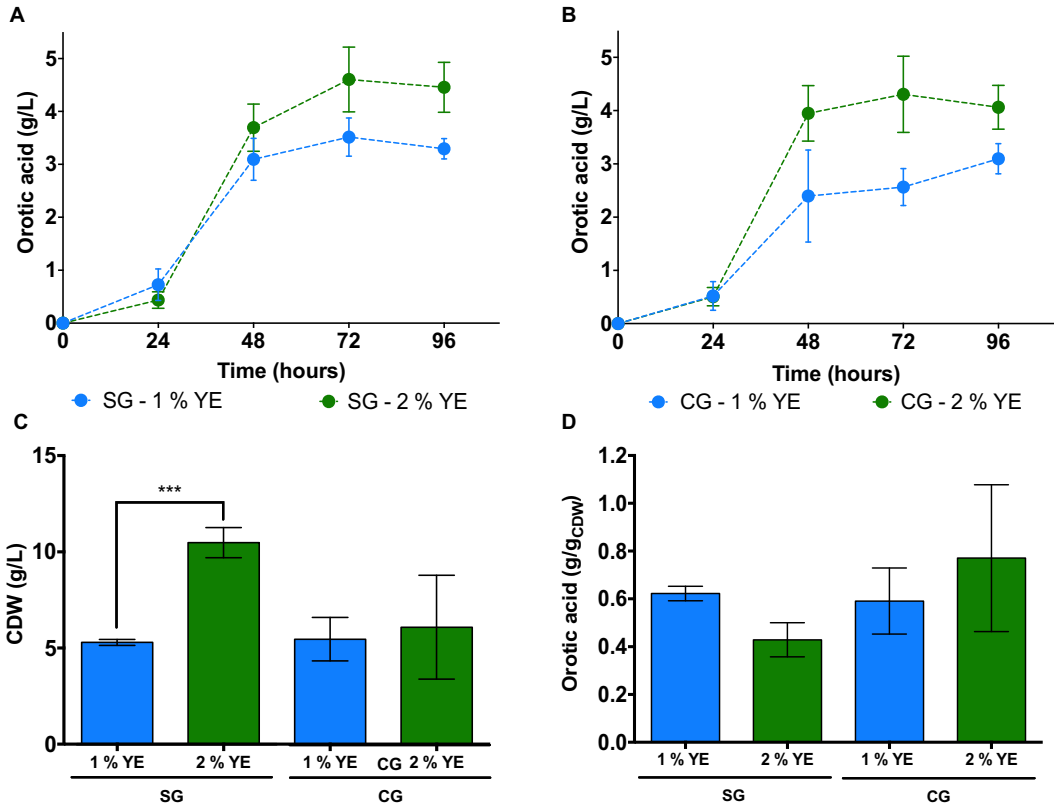

Fig. 6. Orotic acid production by *A. gossypii* uGU2.9 in AFM-YE and using synthetic (SG) or crude glycerol (CG) (~25 g/L). (A) Time-course volumetric production (g/L) using SG. (B) Time-course volumetric production (g/L) using CG. (C) Final cell dry weight (CDW) of the strain in the different conditions. Asterisk represents significant differences (***$p < 0.001$) between conditions. (D) Final specific production (g/g_{CDW}) of orotic acid in the different conditions. Cultivations were carried out at 28 °C and 200 rpm using 250 mL shake-flasks with 10% working volume. Data represent average values and standard deviations of at least biological triplicates. Where not seen, error bars were smaller than the symbols.

and $Y_{P/X}$) are comparable to those reported in the literature from commercial glucose (Table 3), highlighting the potential of *A. gossypii* to valorize CG in a circular economy context for orotic acid production.

Given the need to improve the economic and environmental sustainability of the biodiesel industry, there is a demand for value-added processing solutions for crude glycerol derived from small/medium-sized biodiesel producers, for which the refining of crude to pure glycerol is not economically feasible, and its discard represents a high financial and environmental burden (Luo et al., 2016). On the other hand, important savings can be done in biotechnological processes by substituting commercial substrates with cheaper raw substrates. The use of glycerol as a carbon source in biotechnological processes has several advantages (Klein et al., 2016), the most recognized of which is that it does not exert the so-called Crabtree effect observed in aerated glucose-based cultures, which facilitates the achievement of high yields of biomass or biomass-associated in large-scale bioreactors. For biotechnological processes with *A. gossypii*, glycerol has also advantages as an osmoprotectant, as seen in this work and others (Förster et al., 1998; Silva, 2014). Thus, crude glycerol is a particularly attractive feedstock for the cost-effective production of orotic acid. Here, through metabolic engineering and culture conditions optimization, the orotic acid production titre of *A. gossypii* from crude glycerol could be improved by 3.6 fold (1.23 ± 0.04 *vs* 4.46 ± 0.47 g/L) in shake-flask. Based on the groundwork established in this study, improvements in orotic acid production can be achieved by optimizing the culture aeration and biomass production. Taking as an example the best orotic acid production process reported in the literature, which uses a mutant *C. glutamicum* strain and sugar-based feedstock (Takayama and Matsunaga, 1991), up to 5 fold improvements in volumetric production were obtained with the scaling-up of the process to a bioreactor (Table 3). Thus, further improvements in orotic acid production from crude glycerol can be expected from controlled bioreactor fermentations. Considering the many variables associated with the disposal, valorization and bioconversion of crude glycerol to orotic acid, a comprehensive techno-economic analysis should be done in the future to address the industrial viability of this process.

4. Conclusions

This study discloses the native potential of *A. gossypii Agura3* as a novel host for the production of orotic acid and the importance of the mitochondrial AgUra9p DHOD for this phenotype. This trait was improved through metabolic engineering of the *de novo* pyrimidine biosynthesis (at the AgUra2p and AgUra9p level) in a robust chassis strain engineered with improved glycerol consumption and hyper-osmotic stress tolerance. With this strain (*A. gossypii* uGU2.9), an orotic acid production yield of ~0.17 g/g_{gly} was reached in shake-flask using crude glycerol feedstock, thus establishing the groundwork for a novel valorization route to this residue.

CRediT authorship contribution statement

Rui Silva: Conceptualization, Methodology, Investigation, Writing – original draft. **Tatiana Q. Aguiar:** Conceptualization, Methodology, Writing – review & editing. **Lucília Domingues:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Table 3
Orotic acid production levels reported for different microbial strains and from different substrates. Specific orotic acid production ($Y_{P/X}$ in g/g_{CDW}) is presented between brackets only when the available information allowed its determination. Production levels obtained from bioreactor cultivations are indicated with an *.

Microorganism	Substrate (required additives)	Production levels	Culture time	Reference
Neurospora sp. (*pyr-*)	15 g/L sucrose (17 mg/L cytidine sulfate)	1.3 g/L	96 h	Mitchell et al., 1948
Candida albicans SGY243 (*ura-*)	20 g/L glucose (10 g/L yeast extract + 20 g/L acetate)	3.0 g/L	120 h	Nezu and Shimokawa, 2004
Candida tropicalis (*ade-/hyp-*)	n.i.	7.0 g/L	n.i.	Takayama and Furuya, 1989
Candida lipolytica (*ura-*)	n.i.	7.6 g/L	n.i.	Takayama and Furuya, 1989
Yarrowia lipolytica PO1f (*ura-*)	30 g/L glucose (10 g/L yeast extract)	8.2 g/L* (0.45 g/g_{CDW})	32 h	Swietalski et al., 2021
Kluyveromyces lactis Klura3Δ (*ura-* mutant derived from DSM 70799)	50 g/L glucose (10 g/L yeast extract)	6.7 g/L* (0.74 g/g_{CDW})	96 h	Carvalho et al., 2016
Brevibacterium ammoniagenes 1043 (*ura-* mutant derived from CCEB 364)	50 g/L glucose (5 g/L yeast extract)	5.8 g/L (0.65 g/g_{CDW})	72 h	Škodová et al., 1969
Corynebacterium glutamicum T-30 (mutant derived from the *ura-* ATCC 14275)	200 g/L blackstrap molasses (50 mg/L uracil)	11.2 g/L	72 h	Takayama and Matsunaga, 1991
Corynebacterium glutamicum T-30 (mutant derived from the *ura-* ATCC 14275)	200 g/L blackstrap molasses (60 mg/L uracil)	56 g/L*	84 h	Takayama and Matsunaga, 1991

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2022.100992.

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