**Universidade do Minho**
Escola de Engenharia

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# Exploitation of *Ashbya gossypii* for the production of high-value products from glycerol feedstocks

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Mestrado em Bioengenharia

Trabalho efetuado sob a orientação da
**Professora Doutora Lucília Domingues**
e co-orientação da
**Doutora Tatiana Aguiar**

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Declaração

**Autor**
Rui Miguel Correia da Silva

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# Abstract

The implementation of biorefineries has been proposed as a mean to increase the economic viability of the biobased industries. In its "conventional" form, a biorefinery makes use of by-products (*e.g.* crude glycerol) generated during the production of biodiesel to co-produce high-value products. For this, it is necessary to develop robust microorganisms, easily metabolically and genetically manipulated in order to develop tailor made cell factories. *Ashbya gossypii* is considered an example of the sustainable white biotechnology business model in what concerns the industrial production of riboflavin. Its biotechnological relevance has allowed the development of directed and based-knowledge methodologies for strain optimization for riboflavin production.

The metabolic engineering strategies tested to create overproducing strains have focused on pathways strongly connected with riboflavin production. However, the *A. gossypii* pyrimidine pathway and its connection with the riboflavin biosynthetic pathway had not yet been assessed. We report that the blockage of the *de novo* pyrimidine biosynthetic pathway in the recently generated *A. gossypii Agura3* uridine/uracil auxotrophic strain led to improved riboflavin production. Considering that the riboflavin and the pyrimidine pathways share the same precursors and that riboflavin overproduction may be triggered by stress, we suggest that overproduction of riboflavin by *A. gossypii Agura3* may occur as an outcome of a nutritional stress response and/or of an increased availability in precursors for riboflavin biosynthesis, due to their reduced consumption by the pyrimidine pathway.

Despite the recognized capabilities of *A. gossypii* to use industrial wastes as substrate for the production of riboflavin, glycerol had not yet been exploited. In this study we explore riboflavin production by *A. gossypii* from glycerol. Additionally, we designed a strategy to improve its glycerol consumption profile by overexpressing glycerol uptake proteins (*GUP1*) genes. *A. gossypii* strains overexpressing the native *GUP1* gene (*pRSAG*) or *GUP1* gene from *Saccharomyces cerevisiae* (*pRSSG*) under the control of the *AgTEF* promoter displayed significantly improved glycerol-dependent hyperosmotic stress tolerance and glycerol consumption profiles, indicating that our strategy successfully led to the improvement of glycerol utilization.

These results contribute to the further development of *A. gossypii* as an environmental-friendly cell factory organism, contributing to its establishment in the biorefinery concept.

# Resumo

A implementação de biorrefinarias tem sido proposta como um meio para aumentar a viabilidade económica das bioindústrias. Na sua forma "convencional", uma biorrefinaria faz uso de sub-produtos (*e.g.* glicerol em bruto) gerados durante a produção de biodiesel para co-produzir produtos de valor acrescentado. Para isto, é necessário o desenvolvimento de microrganismos robustos, facilmente manipuláveis metabólica e geneticamente, de modo a desenvolver fábricas celulares feitas à medida. *Ashbya gossypii* é considerado um exemplo do modelo de negócio sustentável da biotecnologia branca no que diz respeito à produção industrial de riboflavina. A sua relevância biotecnológica tem permitido o desenvolvimento de metodologias baseadas no conhecimento direcionado para a otimização de estirpes para produção de riboflavina.

As estratégias de engenharia metabólica testadas têm-se focado em vias metabólicas fortemente relacionadas com a produção de riboflavina. No entanto, a via das pirimidinas de *A. gossypii* e a sua relação com a via biossintética da riboflavina ainda não tinham sido avaliadas. Nós reportamos que o bloqueio da via de biossíntese *de novo* das pirimidinas na recentemente gerada estirpe auxotrófica para uridina/uracilo *A. gossypii Agura3* levou a uma melhorada produção de riboflavina. Considerando que as vias da riboflavina e das pirimidinas partilham os mesmos precursores e que a sobre-produção de riboflavina pode ser desencadeada por stress, nós sugerimos que a sobre-produção de riboflavina por *A. gossypii Agura3* pode ocorrer como resultado de uma resposta a stress nutricional e/ou de uma aumentada disponibilidade de precursores para a biossíntese de riboflavina, devido ao seu reduzido consumo pela via das pirimidinas.

Apesar das reconhecidas capacidades de *A. gossypii* para utilizar resíduos industriais para a produção de riboflavina, o glicerol ainda não havia sido explorado. Neste estudo, exploramos a produção de riboflavina por *A. gossypii* a partir de glicerol. Adicionalmente, desenhamos uma estratégia para melhorar o seu perfil de consumo de glicerol a partir da sobre-expressão de genes que codificam proteínas envolvidas no transporte de glicerol para dentro da célula (*GUP1*). Estirpes de *A. gossypii* a sobre-expressar o gene nativo *GUP1* (pRSAG) ou gene de *Saccharomyces cerevisiae GUP1* (pRSSG) sob o controlo do promotor *AgTEF* apresentaram uma tolerância ao stress híper-osmótico dependente de glicerol e um perfil de consumo de glicerol significativamente melhorados, indicando que a nossa estratégia levou com sucesso a uma utilização melhorada de glicerol.

Estes resultados contribuem para o desenvolvimento de *A. gossypii* como uma fábrica celular amiga do ambiente, contribuindo para o seu estabelecimento no conceito de biorrefinaria.

# Publications

The Chapter 2 of this thesis is based on the following original article:

Silva, R., Aguiar, T.Q., Domingues, L., 2015. Blockage of the pyrimidine biosynthetic pathway affects riboflavin production in *Ashbya gossypii*. Journal of Biotechnology, 193, 37–40.

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Data represent average ± standard error of the mean (SEM) from two biological replicates. Where not seen, error bars were smaller than the symbol.

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KCl addition) for that strain. Data for maximum colony radial growth after 13 days represent average ± standard error of the mean (SEM) from three biological replicates.

Maximum Colony Radial Growth (0.8 M KCl): a,c – differ significantly ($p < 0.01$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3). Maximum Colony Radial Growth (1 M KCl): b,d – differ significantly ($p < 0.05$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3).

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X (g $L^{-1}$) of column 40 g $L^{-1}$ glycerol: a,c – differ significantly ($p < 0.01$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3). Y ($g_{mycelium}$ $g_{glycerol}^{-1}$) of column 40 g $L^{-1}$ glycerol: b,d – differ significantly ($p < 0.01$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3).

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# List of abbreviations

| | |
|---|---|
| **a.a.** | Amino acid |
| **ACL** | ATP-citrate lyase |
| **ADP** | Adenosine-5'- pyrophosphate |
| **AFM** | *Ashbya* Full Medium |
| **Amp** | Ampicillin |
| ***Ag*** | *Ashbya gossypii* |
| **AGD** | Ashbya Genome Database |
| **AGT** | Alanine:glyoxylate aminotransferase |
| **AICAR** | 5-Amino-4-imidazolecarboxamide ribotide; |
| **ARS** | Autonomously replicating sequences |
| **BASF** | BASF -The Chemical Company |
| **cAMP** | cyclic Adenosine monophosphate |
| **CBHI** | Cellobiohydrolase I |
| **CTP** | Cytidine-5'-triphosphate |
| **DHA** | Dihydroxyacetone |
| **DHAP** | Dihydroxy-acetone-phosphate |
| **DNS** | 3,5-Dinitrosalicylic acid |
| **DTT** | Dithiothreitol |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **EU** | European Union |
| **EGI** | Endoglucanase I |
| **GlyOH** | Glycerol |
| **GSMM** | Genome-scale metabolic model |
| **GPI** | Glycosylphosphatidylinositol |
| **GTP** | Guanosine-5-triphosphate |
| **G3P** | Glycerol-3-phosphate |
| **G418** | Geneticin |
| **HPLC** | High performance liquid chromatography |
| **ICL** | Isocitrate lyase |
| **IGP** | D-erythro-imidazole-glycerol-phosphate |
| **IMP** | 5'-Inosine monophosphate |
| **LB** | Luria-Bertani medium |
| ***MATa*** | a-Factor pheromone |
| ***MATα*** | $\alpha$-Factor pheromone |
| **OD** | Optical density |
| **ORF** | Open reading frame |
| **PCR** | Polymerase chain reaction |
| **PRA** | Phosphoribosylamine |
| **PRPP** | Phosphoribosyl pyrophosphate |

| | |
|---|---|
| **PUFA** | Polyunsaturated fatty acids |
| **Ribulose-5P** | Ribulose 5-phosohate |
| **RPM** | Revolutions per minute |
| **SAP** | Shrimp Alkaline Phosphatase |
| ***Sc*** | *Saccharomyces cerevisiae* |
| **SC** | Synthetic Complete medium |
| **SCO** | Single cell oil |
| **SCP** | Single cell protein |
| **SEM** | Standard error of the mean |
| **SHMT** | Serine hydroxymethyltransferase |
| **TCA** | Tricarboxilic acid cycle |
| **TEF** | Translation elongation factor |
| **TM** | Transmembrane domains |
| **UMP** | Uridine monophosphate |
| **UP** | Ultrapure |
| **US$** | United States dollar |
| **UV** | Ultraviolet |
| **YNB** | Yeast Nitrogen Base |

# AIMS

The implementation of biorefineries has been proposed as a mean to increase the economic viability of the biofuels industry. Biodiesel production generates large amounts of crude glycerol that urgently need to be exploited in order to assure the cost effectiveness of the biodiesel industry. For this, it is necessary to develop robust microorganisms able to convert this by-product into value-added commodities. Metabolic and genetic engineering advances endow researchers with the tools necessary to create tailor made cell factories able to contribute to the economic and environmental sustainability envisioned by the white biotechnology.

*Ashbya gossypii* is a filamentous fungus that has long been exploited for the industrial production of riboflavin from plant oils due to its natural ability to overproduce this vitamin. Owing to its industrial relevance, it has received much attention in the field of biorefinery technology. The molecular toolbox available together with the sequencing and annotation of its genome has effectively helped to delineate metabolic engineering strategies for strain optimization not only for riboflavin production but also for the development of novel biotechnological applications, such as recombinant proteins, single cell oil and aroma production.

Within the scope of the Fundação para a Ciência e Tecnologia project AshByofactory, our research group obtained some interesting outcomes that needed to be further investigated. Particularly, during the development of the Cre-l*ox*P-based system for *A. gossypii* (Aguiar et al., 2014b) an uridine/uracil auxotrophic strain was generated, *A. gossypii Agura3*, which unexpectedly displayed superior riboflavin production. On the other hand, the physiological characterization of this fungus revealed promising capacities to produce ethanol and recombinant proteins from glycerol (Ribeiro et al., 2012; Aguiar et al., 2014a; Magalhães et al., 2014). Taking into account these findings, the overall goal of this work was to expand the exploitation of *A. gossypii* as an environmental-friendly cell factory organism, contributing to its establishment in the biorefinery concept. Therefore, we specifically aimed to:

1. explore and understand the effect that genetic manipulations on the pyrimidine pathway have on growth and riboflavin production by *A. gossypii*;
2. assess the production of riboflavin from glycerol;
3. create *A. gossypii* strains with improved glycerol uptake, through the overexpression of a native or a heterologous glycerol transport protein (*GUP1*) gene from *Saccharomyces cerevisiae*.

# CHAPTER 1

## General Introduction

## 1.1. *Ashbya gossypii*

*Ashbya gossypii* (syn. *Eremothecium gossypii*) is a filamentous hemiascomycete fungus that was isolated from cotton plants and characterized for the first time in 1916 (Nowell, 1916). A complete characterization performed by Ashby and Nowell (1926) documented this fungus as the cause of the "stigmatomycosis" disease on cotton plant. In the first half of the XX century, *A. gossypii* was considered an important crop pathogen that caused significant economical concerns owing to its wide distribution through various types of crops. Its widespread distribution was due to the action of certain insects, which transferred spores or parts of mycelia during their feeding process (Pridham and Raper, 1950). The distinctive symptom of cotton plants infected by this disease was the yellow staining acquired by its lint fibers. This yellow pigment was later identified as riboflavin (vitamin $B_2$). Beyond the economical concern of *A. gossypii* as a plant pathogen, its ability to overproduce a value-added product (riboflavin) arouse the interest of researchers in exploring *A. gossypii* to develop an industrial process for riboflavin production (Pridham and Raper, 1950; Stahmann et al., 2000).

The scientific prominence of *A. gossypii* is far from being just restricted to its role in crop pathogenicity and riboflavin production. The close relationship between *A. gossypii* and *Saccharomyces cerevisiae* is a well-explored subject. Referred to as an "yeast like fungus" (Pridham and Raper, 1950) or a "filamentous yeast" due to similar shared habitat (Wendland and Walther, 2005), *A. gossypii* was proposed to be a member of the Saccharomycetaceae family by Prillinger et al. (1997) based on comparisons of cell wall sugars, ubiquinone side chains, dityrosine and ribosomal DNA sequences. Regarding molecular genetics, *A. gossypii* is also more similar to yeast than to filamentous fungi (Wendland and Walther, 2005). Dietrich et al. (2004) published the complete genome sequencing and annotation of *A. gossypii*. This has helped to settle the basis of comparison between these two closely related species and allowed a better investigation into other issues related to the *S. cerevisiae* genome, *e.g.* provided new insights supporting the idea that the evolution of *S. cerevisiae* comprised a whole genome duplication (Brachat et al., 2003). *A. gossypii* has the smallest known genome of a free-living eukaryote (9.2 Mb organized on seven chromosomes) and haploid nuclei. 95% of the *A. gossypii* genes show homology with *S. cerevisiae*'s genes. From these, only 4% do not have a shared synteny pattern. The *A. gossypii* genome shows higher GC content than that of *S.*

*cerevisiae* (about 52% against 38%, respectively), providing it more stability (Dietrich et al., 2004).

The availability of the *A. gossypii* genome sequence led to the development of the Ashbya Genome Database (AGD), which enables researchers to freely explore genomic and transcriptomic data and compare information with other databases of other organisms (*e.g.*, searching for homology and synteny networks between *A. gossypii* and *S. cerevisiae*; Gattiker et al., 2007). Already considered a paradigm of the white biotechnology due to its capacity to overproduce riboflavin, *A. gossypii* is also a great example regarding the utilization of bioinformatics tools to fully exploit and understand all the potential of this fungus. Recently, the *A. gossypii* genome was re-annotated (Gomes et al., 2014) and its reaction set compiled to create an *in-house* genome scale metabolic model (GSMM) able to simulate the cell behavior, iDG1082 (Gomes et al., 2013). This tool can potentiate fundamental research in the pursuit of understanding metabolic networks in *A. gossypii* and also help to find new targets to further improve the biotechnological application of this fungus. Ledesma-Amaro et al. (2014a) have also published another GSMM for *A. gossypii* (iRL766), which was validated by predictions and comparisons with experimental data of three parameters: biomass growth, riboflavin production and substrate utilization.

Even before its genome sequencing, and driven by the attractiveness of riboflavin production, *A. gossypii* had become an attractive microorganism for genetic manipulation, leading then to the development of several molecular tools. In 1991, replicative transformation of *A. gossypii* with autonomously replicating sequences (ARS) from *S. cerevisiae* were successfully applied, thus becoming *A. gossypii* the first fungus that could bear a transformation system using ARS from *S. cerevisiae* for free replication of plasmids (Wright and Philippsen, 1991). Latter, its efficient mechanism of homologous recombination was described, being DNA integrated without detectable non-homologous recombination events (Steiner et al., 1995). Meanwhile, a high degree of similarity between the *A. gossypii* and *S. cerevisiae* translation elongation factor 1 (*TEF*) genes was discovered, as well as the existence of conserved short DNA segments responsible for high levels of expression in the promoter region of the *AgTEF* gene (Steiner and Philippsen, 1994). In 1996, construction of a selection marker (*AgTHR4*) for *A. gossypii* showed that these open reading frames (ORF) were conserved in order and orientation with *S. cerevisiae* genome; this was the first report of genome synteny with the baker's yeast (Altmann-Jöhl and Philippsen, 1996).

Taking advantage of the knowledge acquired in the studies cited in the last paragraph, along with the molecular tools already established in *S. cerevisiae*, Wendland et al. (2000) developed and successfully implemented a polymerase chain reaction (PCR) based gene targeting methodology for *A. gossypii*, allowing gene disruption. This was the first molecular tool of this type to be applied in a filamentous fungus. The ORF of *Escherichia coli* $kan^R$ gene under the control of the *S. cerevisiae TEF* promoter and terminator was used as selection marker and allowed a one-step protocol for functional genomics in *A. gossypii* (Wendland et al. 2000). Since then, some new selection markers have been developed (Alberti-Segui et al., 2001; Kaufmann, 2009; Schlüpen, 2003; Jiménez et al., 2008). Recently, the widely used Cre-*lox*P system, also applied in *S. cerevisiae* (Sauer, 1988; Güldener et al., 1996), was adapted for use in *A. gossypii*. The great outcome of this system consists on the removal and further possibility of reuse of selection markers, opening new perspectives for multiple gene deletion and allowing an integrative study of function of more than one gene (Aguiar et al., 2014b).

*A. gossypii* presents two main life structures: spores, which only possess one nucleus, and hyphae, which contain multiple nuclei (Wendland and Walther, 2005). In a recent study, it was estimated that the average nuclei per hypha was about 4 (Nieland and Stahmann, 2013). The filamentous growth is predominant in *A. gossypii*, being the yeast-like growth confined to two moments: the germination of the spores and the moment of fragmentation of the mycelium preceding sporulation (Wendland and Walther, 2005). The *A. gossypii* life cycle begins with the germination of the spores that result in spherical germ cells (isotropic growth phase). The development of a germ tube that grows in one direction is the first phase of the filamentous growth; the next step is similar, *i.e.*, a second germ tube begins to lengthen in the opposite direction to the first. After this, there is a continuous formation of lateral hyphae along the germ tubes. As the maturation of mycelium progresses, hyphae will branch in a dichotomous way, resulting in Y-shapped hypha. Since the beginning, the spherical germ cells as well as all the hypha are separated from each other by a septum. It is in parts near to this septum that the mycelium breaks, yielding single-cell sporangia containing spores inside. Sporulation is the final stage of the *A. gossypii* life cycle; herein, lysis of sporangia results in release of spores (Wendland and Walther, 2005).

The transition from vegetative growth to sporulation is critical for fungi to survive to unfavorable conditions. *A. gossypii* is regarded as a homothallic hemiascomycete. Current efforts are being made in order to clarify if *A. gossypii* possesses or not a sexual life cycle and

how sporulation is regulated in this fungus. The pheromone-signaling transduction pathway has been characterized (Wendland et al., 2011; Wasserstrom et al., 2013). The sequenced strain *A. gossypii* ATCC 10895 only harbors genes encoding functional a-factor pheromone (*MAT**a***). The lack of identified $\alpha$-factor pheromone (*MAT*$\alpha$) strains precludes *A. gossypii* for sexual cycle testing (Wendland et al., 2011). Conversely, a new *A.* gossypii strain isolated from an insect of the *Heteroptera* subfamily recently described and sequenced, unlike the ATCC 10895 strain, harbors two *MAT*$\alpha$ genes (*MAT*$\alpha$*1* and *MAT*$\alpha$*2*). This finding can leverage the search for a sexual life cycle in *A. gossypii* (Dietrich et al., 2013).

The physiological characterization of *A. gossypii* was made mainly in the subsequent years after the fungus being isolated. An extensive analysis of nitrogen and carbon sources was engaged and auxotrophies for myo-inositol, biotin and thiamine were described (Pridham and Raper, 1950). In 2011, a physiological characterization of 4 *A. gossypii* strains (3 related and 1 unrelated strains) was performed. Summarily, *A. gossypii* strains could utilize D-glucose, glycerol and starch as carbon sources, contrasting with xylose or arabinose that could not be used for growth; as nitrogen sources, yeast extract and ammonium were suitable, whereas nitrate was not (Ribeiro et al., 2012).

In comparison with previous reports, the work of Ribeiro et al. (2012) showed that: *A. gossypii* can grow on ammonium when the pH was 6.5, contradicting some authors that reported an absence of or a poorly growth on this nitrogen source (Farries and Bell, 1930; Buston et al., 1938; Wright and Philippsen, 1991); the same happened regarding the utilization of starch as carbon source, where others reported inability to grow or degrade this compound (Marsh, 1926; Tanner et al., 1949). Other interesting findings were reported. First, *A. gossypii* was found to display more sensitiveness to lower pH than most fungi. Second, *A. gossypii* could grow and produce ethanol from glycerol (Ribeiro et al., 2012). Regarding glycerol utilization, these traits are largely attractive for biotechnological purposes.

*A. gossypii* is a microorganism that early captured the attention of the industry owing to its flavinogenic profile. However, as described in this section, *A. gossypii* is also a noteworthy fungus for more fundamental research fields (*e.g.*, evolutionary and fungal development biology). All these characteristics make *A. gossypii* a prominent microorganism, playing a central role either in fundamental and applied research. For all these reasons, *A. gossypii* is one of the best microorganisms that systemically combine both sides of scientific research in order to create value for society.

## 1.2. Biotechnological applications of *A. gossypii*

Riboflavin production is the industrially established biotechnological application that makes *A. gossypii* a proof of concept of the white biotechnology business model. Years of strain improvement mainly by random mutagenesis (*e.g.*, isolation of mutants by anti-metabolites) culminated in an economically competitive process of riboflavin production employing *A. gossypii*. In 1996, chemical synthesis of this vitamin was overcome and the chemical company BASF has preferred this bioprocess instead of the chemical process (Stahmann et al., 2000). This takeover from biotechnological production by *A. gossypii* was the outcome of approximately 30 years of process amelioration since the first commercial fermentation for riboflavin production with this fungus was announced (Wickerham et al., 1946; Stahmann et al., 2000).

The easy genetic manipulation and small genome of *A. gossypii* discovered in the following years made this fungus a perfect synergy model for the development of bioinformatics and molecular tools, as mentioned in the previous section. These tools allowed acquiring a directed and based-knowledge methodology for manipulating strains with improved riboflavin production rather than through random mutagenesis. In the subsection *1.2.1.,* an extensive summary of strategies undertaken over the years, either by random mutagenesis or metabolic engineering by gene manipulation, will be provided. In the same subsection, the ecological advantages and the molecular and regulatory mechanism behind riboflavin overproduction by *A. gossypii* will be briefly reviewed.

In the last years, the suitability and potential of *A. gossypii* as a host for recombinant protein production has been assessed. Filamentous fungi are excellent models with recognized capacities either for fungal origin proteins or non-fungal origin proteins. However, the protein secretion potential of *A. gossypii* was poorly investigated. Indeed, very little was known about this subject in this fungus (Stahmann et al., 1997; Althöefer et al., 2001; Ribeiro et al., 2010). To counteract this trend and to explore this possible biotechnological application, some works were performed in order to evaluate the level of expression and secretion of homologous and heterologous proteins in *A. gossypii* (Ribeiro et al., 2010; Magalhães et al. 2014; Aguiar et al., 2014a). Strategies of random and directed mutagenesis to improve the secretory ability of this fungus were also explored (Ribeiro et al., 2013). Moreover, another recent study performed a first characterization of the *A. gossypii* secreted N- glycome, providing new insights on the

genomic determinants of the N-glycosylation pathway in this fungus (Aguiar et al., 2013). Subsection *1.2.2.* will address in more detail each work here cited.

Besides these two biotechnological applications, which will be the main focus of the work performed throughout this thesis, it has been possible to identify and explore novel biotechnological applications. One of these applications is the adaptation of *A. gossypii* for single-cell oil (SCO) production, an alternative source of oil for the oleochemical/biofuel industries, which have seen their principal main sources (crude oil, and animal fat and plant oil) linked to negative environmental and economical effects (Ledesma-Amaro et al., 2014b; Ledesma-Amaro et al., 2014c). The lipidogenic profile of *A. gossypii* had been studied already 20 years ago (Stahmann et al., 1994). In that longstanding report, it was found that *A. gossypii* could store considerable amounts of lipids as a reserve of energy, depending on the carbon source (10 to 20% of mycelial dry weight). The fatty acid composition of lipids stored revealed a dominance of unsaturated fatty acids in both works, particularly oleic acid and palmitoleic acid (approximately 50% and 20%, respectively; Stahmann et al., 1994; Ledesma-Amaro et al., 2014b).

By definition, to be considered an oleaginous microorganism, *A. gossypii* has to be able to retain more than 20 – 25% of lipids in relation to its cell dry weight. In two works by Ledesma-Amaro et al. (2014b; 2014c), *A. gossypii* was metabolically engineered to fit the essential requisites in order to become a reliable candidate for SCO production. First, two strategies were adopted: deletion of gene(s) involved in the beta-oxidation pathway to block the metabolization of fatty acids; and the expression of a heterologous ATP-citrate lyase (ACL) from *Yarrowia lipolytica* that is lacking in *A. gossypii*, with the purpose of rising the pool of cytosolic acetyl-CoA that can be used for fatty acid formation and thus increasing the total fatty acids accumulated. These strategies implemented in separate or together led to an enhancement in lipids accumulation, which reached the maximum of *ca.* 70% fatty acid of mycelial dry weight (Ledesma-Amaro et al., 2014b). The other study was based on the characterization of the elongation and desaturation system of *A. gossypii* and consequent deletion and overexpression of the genes codifying these enzymes, which are responsible for the length and level of unsaturation of fatty acids, respectively (Ledesma-Amaro et al., 2014c). This work has enabled the creation of a repertoire of strains that can be applied in different industries according to their fatty acid profile. For instance, a strain lacking the gene encoding an elongase responsible for the elongation of fatty acid 18:0 to 22:0 (*A. gossypii elo624*Δ), had a fatty acid profile that

fulfill the requirements established by an European quality standard for biodiesel production intended for vehicle use (EN14214; Ledesma-Amaro et al., 2014c).

The strong fruity aroma displayed by *A. gossypii* led Ravasio et al. (2014) to explore the aromatic compounds produced by *A. gossypii*. The predominant volatile compounds identified were isoamyl alcohol and 2-phenylethanol (banana/fruity and rose/flower aromas, respectively). Only high levels of isoamyl alcohol were also identified in the relative *E. cymbalariae*. However, the main difference between these two species resided on the genetic information related to the aromatic amino acids catabolism (Ehrlich pathway). While *E. cymbalariae* has only one gene related to this pathway (*ARO8a*), *A. gossypii* encodes four genes (*ARO8a*, *ARO8b*, *ARO10* and *ARO80*) (Ravasio et al., 2014). Exploration of the function of each gene and its influence on aromatic compounds production have allowed concluding that mutants lacking the genes described suffered alterations on aromatics production, suggesting that the Ehrlich pathway is engaged with in the production of aromatic compounds in *A. gossypii*, particularly 2-phenylethanol. Additionally, overexpression of *ARO80* led to an increase of 50% in volatile compounds, specifically in isoamyl alcohol (Ravasio et al., 2014).

Interest has been raised on non-conventional yeast as natural flavour producers, opening the outlook of exploring these non-*Saccharomyces* species in fermented beverages, for example in co-fermentation with *S. cerevisiae* in order to improve the aromatic/flavour profile of the product (Domizio et al., 2010). The Saccharomycetaceae filamentous fungus *A. gossypii* (and also *E. cymbalariae*) demonstrated interesting potential for flavour production, supporting and reinforcing the idea that exploring these strains may result in a new wide line of applications for food and beverage industries (Ravasio et al., 2014).

The physiological plasticity and robustness of *A. gossypii* combined with its easy genetic manipulation and with the considerable amount of bioinformatics resources available for this fungus, have endorsed a directed and sustained exploration of all its capacities. All the biotechnological applications succinctly described here, covering different areas and industries, proclaim this “filamentous yeast” as a real cell factory organism and a remarkable example of white biotechnology.

*1.2.1. Riboflavin production by A. gossypii*

Riboflavin is a water-soluble vitamin (vitamin $B_2$) possessing a strong yellow color. Many plants and microorganisms can produce the essential amount of riboflavin needed for their own growth, whereas humans and animals cannot. Consequently, its exogenous supply is essential for the latter. Riboflavin deficiency is related to many malady conditions, among which lips and tongue inflammation, hyperemia, vision deterioration and growth failure are some examples. At the molecular level, riboflavin is a precursor of cofactors of dehydrogenases and oxidoreductases, as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Therefore, its principal applications are as food/feed additives and can also be applied in cosmetics. Furthermore, due to its intense yellow color, it is used as a coloring agent denominated E-101 (Stahmann et al., 2000; Survase et al., 2006; Kato and Park, 2012).

Nowadays, industrial production of this vitamin has completely turned to microbial production rather than chemical synthesis. *A. gossypii* is one of the largest (if not the largest) industrial producers of riboflavin. In 2009, the industrial production of riboflavin by microorganisms was predicted to reach the 6000 tons per year and the prices of its related products were estimated to be between US$35–50 per kg for food market or US$15 per kg for feed market (Tajima et al., 2009; Kato and Park, 2012). Usually, biosynthesis of this type of pseudo-secondary metabolites (*e.g.*, riboflavin) are matched by a strong regulatory machinery that does not let their production exceed the levels required for growth. However, *A. gossypii* has the capability to overcome this regulation and to produce more than 20 g $L^{-1}$ of riboflavin (Stahmann et al., 2000; Survase et al., 2006).

The industrial production of riboflavin by *A. gossypii* was the first biotechnological process of this vitamin to be preferred over chemical synthesis, an accomplishment performed by BASF in 1996 (Stahmann et al., 2000). Riboflavin is synthesized from stored fatty acids after the growth stops, with the contribution of five pathways: glyoxylate cycle, gluconeogenesis, pentose phosphate pathway, purine biosynthetic pathway and riboflavin biosynthetic pathway (Park et al., 2011) (Fig. 1.1). The riboflavin biosynthetic pathway comprises six genes: *RIB1*, *7*, *2*, *3*, *4* and 5 that encode GTP cyclohydrolase II, 2,5-diamino-6-(ribitylamino)-4(3H)-pyrimidinone-5'-phosphate reductase, 2,5-diamino-6-(5-phospho-D-ribitylamino)-pyrimidin-4(3H)-one-deaminase, 3,4-dihydroxy-2-butanone-4-phosphate (DHBP) synthase, 6,7-dimethyl-8-ribityllumazine synthase and riboflavin synthase, respectively (Kato

and Park, 2012). Some of the genes belonging to the riboflavin biosynthetic pathway (*RIB* genes) are strongly up-regulated during the stationary phase, *i.e.*, during the production phase of riboflavin (Schlösser et al. 2001; 2007).

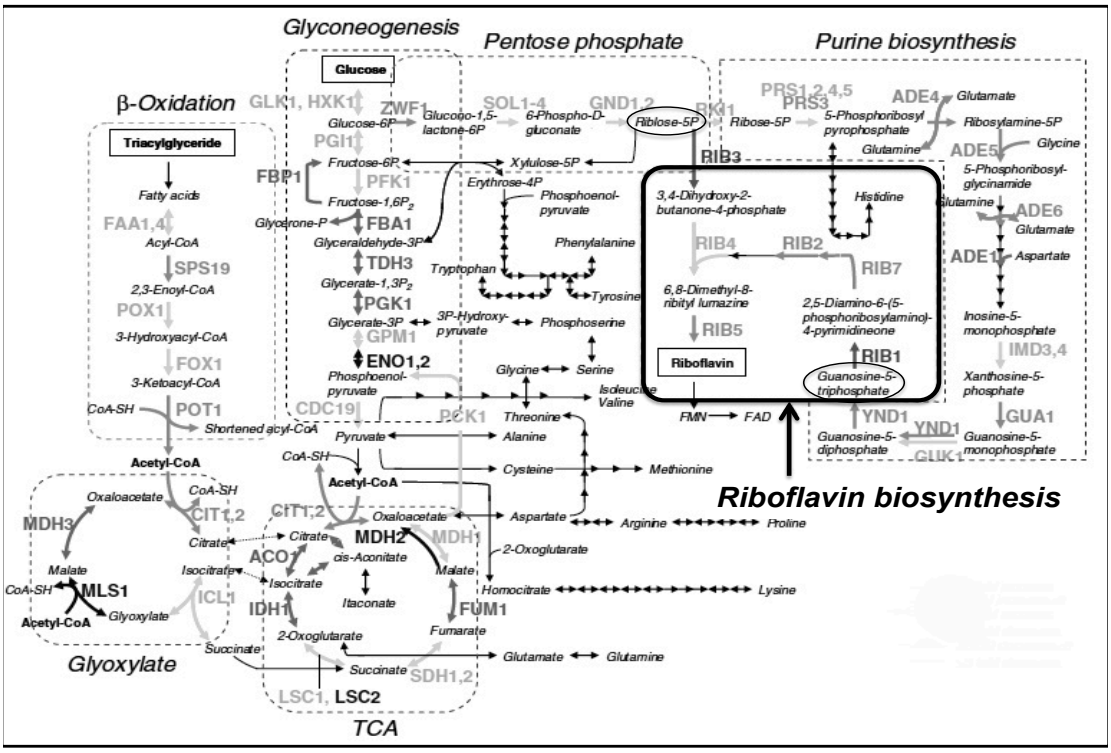


**Figure 1.1 –** Metabolic networks and corresponding pathways and genes involved in riboflavin production by *A. gosyypii*. TCA: tricarboxilic acid cycle. Adapted from Park et al. (2011).

Guanosine triphosphate (GTP) and ribulose 5-phospate (R5P) are the main precursors of the riboflavin biosynthetic pathway, each synthesized from the purine biosynthetic pathway and pentose phosphate pathway, respectively. The importance of GTP in riboflavin production was very recently highlighted throughout a comparative metabolic flux analysis of the central carbon pathway of a wild type and a riboflavin-overproducing mutant (Jeong et al. 2014). On the other hand, there are several unspecific precursors of riboflavin that enhance its production when they are present in the medium. Glycine is one of the most documented, being involved in purine biosynthetic pathway and consequently being important for GTP formation (Stahmann et al., 2000; Lim et al., 2001).

The industrial applicability and establishment of riboflavin production by *A. gossypii* has become a landmark of white biotechnology, arising the interest of the scientific community. From then on, researchers have been engaging efforts to further improve the process by

several approaches, such as: culture conditions and medium optimization; generation of improved strains by random mutagenesis followed by anti-metabolite screening; genetic and metabolic engineering; identification and characterization of regulatory mechanisms (genetic and molecular) responsible for riboflavin production in *A. gossypii*.

Vegetal oils are the preferred carbon source to enhance the yield of riboflavin production in *A. gossypii*. The medium composition for riboflavin production, regarding the carbon and nitrogen source, as well as supplementation, is a well-studied subject (Lim et al., 2001). However, foreseeing a more environmental friendly and economical production process some other culture conditions have been attempted. Lim et al. (2003) increased riboflavin production in *A. gossypii* by introducing a mineral support to the culture. This mineral support, in addition to adsorb the vegetal oil, was suitable for the filamentous growth of the fungus, enhancing the oil consumption and riboflavin production. Waste activated bleaching earth (wABE) is a discarded material from crude oil refining industries, which is used to adsorb impurities of the process. Since it also adsorbs large quantities of vegetable oil (*e.g.*, palm oil and rapeseed oil), it was used as feedstock for testing riboflavin production in *A. gossypii* (Ming et al., 2003; Park et al., 2004a; Park et al., 2004b; Park et al., 2007; Tajima et al., 2009). These findings, reporting growth and riboflavin production in industrial wastes have inferred about the physiological robustness of *A. gossypii*. Likewise, the usage of this type of waste feedstocks makes possible reducing the production cost of riboflavin and leaves open the possibility of incorporating *A. gossypii* in a biorefinery scenario.

The search and modification of *A. gossypii* strains for enhanced riboflavin production capabilities has followed two tracks: random mutagenesis or rational directed genetic manipulation. The identification and characterization of the *A. gossypii* isocitrate lyase (ICL), an enzyme belonging to the glyoxylate cycle that is strongly inhibited by itaconate and oxalate, disclosed a positive correlation between the enzyme activity and riboflavin production when soybean oil was used as carbon source (Schmidt et al, 1996a; Schmidt et al., 1996b). Using itaconate as an anti-metabolite it was possible to isolate an overproducing strain in soybean oil, denominated *ItaGS01* (Schmidt et al., 1996b). This correlation was confirmed later, when mutants lacking the gene encoding this enzyme (*AgICL1*) were impaired in riboflavin production compared to the parent strain (Kanamasa et al., 2007). This enzyme has been the principal target for screening overproducing strains by antimetabolite (mainly itaconate) in random mutagenesis studies.

Randomly mutated strains have been obtained by different means: ultraviolet (UV) light (Park et al. 2007), chemical mutagens (Tajima et al. 2009) and more recently by disparity mutagenesis (Park et al., 2011). This last approach was attempted with the intention of creating more stable mutants than those obtained by the other two approaches. This methodology consisted in the transformation of *A. gossypii* with a plasmid carrying a mutated DNA polymerase *∂*, which has a defective repair function, suitable for generating errors in DNA during cell division (Park et al., 2011). On the other hand, without the application of any mutagenic agent, Sugimoto et al. (2010) could isolate a strain with high riboflavin production through inoculation of spores of an *A. gossypii* wild type strain on a medium containing oxalate. More recently, during an analysis of itaconate metabolism and genetics in *A. gossypii*, the related compounds *cis*-aconitate and *trans*-aconitate were detected in the production phase of riboflavin (Sugimoto et al., 2014). It is known that the latter (*trans*-aconitate) has an inhibitory effect on aconitase (*AgACO1*) in the TCA. Therefore, *trans*-aconitate was suggested as a novel and more available anti-metabolite towards riboflavin overproduction in *A. gossypii* (Sugimoto et al., 2014).

The traits of the *A. gossypii* genome and posteriorly the bioinformatics tools developed for it, have made this fungus a microorganism appropriate for genetic modifications. Therefore, there is a wide range of studies describing rational modification of strains in order to improve its flavinogenic profile. These works have focused on identifying the function of certain genes, which made them possible targets for genetic/metabolic engineering manipulations. These metabolic engineering studies focused on pathways/genes beyond the riboflavin biosynthetic pathway, *i.e.*, on pathways interfering with the formation of precursors for riboflavin production.

The availability of glycine is a limiting step for riboflavin production, therefore some metabolic engineering works focused on that subject. Monschau et al. (1998) overexpressed the *AgGLY1* gene, which encodes the enzyme responsible for the conversion of threonine to glycine, in order to increase the cellular pool of glycine. This genetic alteration was capable of increasing 9-fold the production of riboflavin by *A. gossypii*. However, this was only displayed to when threonine was available in the medium, suggesting a limiting step at threonine biosynthesis (Monschau et al., 1998). For the same purpose, Schlüpen et al. (2003) isolated the *AgSHM1* and *AgSHM2* genes, encoding a mitochondrial and a cytosolic serine hydroxymethyltransferase (SHMT), respectively. These enzymes catalyze the reverse reaction of glycine to serine. The disruption of *AgSHM2* resulted in an improvement of riboflavin

production by these mutants, possibly because the flux of glycine to serine was reduced and more glycine became available (Schlüpen et al., 2003). Some years later, Kato and Park (2006) constructed and expressed a plasmid carrying a heterologous *S. cerevisiae AGX1* gene in *A. gossypii*, aiming also to increase glycine. This gene, which is not present in the *A. gossypii* genome, encodes the enzyme alanine:glyoxylate aminotransferase (AGT) that converts glyoxylate into glycine. However, the increase in riboflavin concentration was not as substantial as the authors expected, only 2-fold higher than the strain carrying the plasmid without the *ScAGX1* gene (Kato and Park, 2006).

The glyoxylate cycle is an important pathway for riboflavin production and for growth in non-fermentable carbon source, as vegetable oils. Consequently, the genes involved in it may play an important role as evidenced by the importance of the *AgICL1* already mentioned. Sugimoto et al. (2009) investigated the function of *AgMLS1* (which encodes the enzyme malate synthase) in riboflavin production and oil consumption in *A. gossypii*. A mutant strain lacking this gene was impaired in riboflavin production and oil consumption. However, a strain overexpressing *AgMLS1* was only able to produce 1.7-fold more riboflavin than the control strain (Sugimoto et al., 2009)

One of the industrial advantages of *A. gossypii* is the autolysis of the mycelium in late stationary phase, which therefore prevents expensive recovery processes (Park et al., 2004a; Ledesma-Amaro et al., 2014a). Before that moment, the riboflavin produced by *A. gossypii* is either transported to the medium or accumulated in the vacuole, approximately in equal amounts (Förster et al., 1999). The deletion of the *A. gossypii AgVMA1* gene (encoding a vacuolar ATPase subunit A) led to a redistribution of the flux of riboflavin, directing it almost completely to the medium (Förster et al., 1999). In addition, this study has shown the first *vma1* mutant that was not completely or conditionally impaired in growth as reported for other fungi (*e.g.*, *S. cerevisiae* and *Neurospora crassa*). In the same line of this work, Förster et al. (2001) performed a biochemical analysis of the transport systems of riboflavin in *A. gossypii*. These authors concluded that the uptake system of riboflavin had low activity and high affinity. Conversely, the efflux of riboflavin is carried by an independent system with high activity, *i.e.*, through a specific riboflavin export carrier (Förster et al., 2001). Until date, no gene encoding an export carrier of riboflavin was identified.

The metabolic flux and the availability of phosphoribosyl pyrophosphate (PRPP), a central intermediate of carbon and nitrogen metabolism, was also a target for metabolic

engineering in *A. gossypii*. PRPP is the first committed metabolite of the purine biosynthetic pathway, being essential for GTP formation. In the first step of this pathway occurs the conversion of the PRPP to phosphoribosylamine (PRA) by the action of PRPP amidotransferase encrypted by the gene *AgADE4*. Jiménez et al. (2005) followed two approaches in order to increase the production of riboflavin through the engineering of *AgADE4*: overexpression of this gene with the strong and constitutive promoter in order to overcome transcriptional inhibition and replacement of amino acid (a.a.) residues connected to feedback inhibition through site-directed mutagenesis of *AgADE4*. These two strategies combined resulted in the construction of a strain capable of producing 10-fold more riboflavin in comparison with the wild type strain (Jiménez et al., 2005). Another report followed a similar strategy, namely, overexpression and site-directed mutagenesis to overcome transcriptional and post-translational feedback inhibition, respectively. However, this time the targets chosen by Jiménez et al. (2008) were genes encoding the PRPP synthetase, *AgPRS2,4* and *AgPRS3*. The maximum riboflavin production levels achieved by the metabolically engineered strains generated in this study were approximately 2-fold higher than the wild type strain (Jiménez et al., 2008).

Mateos et al. (2006) investigated the role of a gene encoding a transcription factor of the Myb family, *AgBAS1*. These authors discovered that this transcription factor was directly involved in the regulation of *AgADE4* and *AgSHM2* expression. Afterwards, a strain harboring an *AgBAS1* gene with a deletion in a C-terminal regulatory domain was constructed. This modification enhanced riboflavin production approximately 9-fold in comparison to the wild type after 96 hours on rich medium (Mateos et al., 2006).

It is clear that the biotechnological production of riboflavin by *A. gossypii* is a process well harnessed by the industry. Moreover, the scientific community has been continuously studying it so that it can become even more profitable. However, intriguing questions remain to be answered: Why does *A. gossypii* naturally overproduce riboflavin? Is it an ecological benefit for the fungus? Although the molecular and regulatory mechanisms of this *A. gossypii* trait are not fully understood, there are some works that have addressed the issue.

Stahmann et al. (2001) showed that riboflavin might play a photoprotectant role for the ascospores of *A. gossypii* against UV light. This was shown by exposing the spores of *A. gossypii* and *Aspergillus nidulans* to UV light in the presence or absence of riboflavin. In the presence of vitamin $B_2$ both spores were more resistant to UV light. Conversely, when riboflavin was not present, *A. gossypii* hyaline spores revealed to be more sensitive than *A. nidulans*

spores. Moreover, addition of cyclic adenosine monophosphate (cAMP), a known stress signal that negatively affects sporulation, also abolished riboflavin production in *A. gossypii* (Stahmann et al., 2001). Supported by this evidence and by the fact that sporulation and riboflavin production are elevated in the productive phase (after the growth ceases), these authors established a connection between sporulation and riboflavin production. However, riboflavin production is not exclusively connected to sporulation, because it is possible to see non-sporulating mycelia accumulating riboflavin (Walther and Wendland, 2012).

Later, other kind of stress stimuli was connected to riboflavin production. For instance, Schlösser et al. (2007) concluded that constant and unaltered conditions were not suitable for riboflavin overproduction in *A. gossypii*. During a chemostatic culture, only after a strong downshift of the dilution rate was it possible to note riboflavin overproduction. Even if the dilution rate was low from the beginning, it was still not possible to observe riboflavin overproduction. Therefore, these authors concluded that riboflavin overproduction is not linked to low growth rates *per se*, but some type of stress stimulus is needed, such as the declining nutrition observed in the transition between the growth and stationary phase in batch cultivations (Schlösser et al. 2007).

Other report that reinforced the idea that riboflavin overproduction in *A. gossypii* is a stress-induced phenomenon was presented by Kavitha and Chandra (2009), when they showed an increase in riboflavin concentration when vitamin E and menadione were added to the medium. This increase was accompanied by an augment in the levels of enzymes related to oxidative stress, suggesting that these oxidants acted as stressors (Kavitha and Chandra, 2009). The inherent fungal plasticity of *A. gossypii* also affects riboflavin production. For instance, it was shown that hyphal cells of *A. gossypii* in the same culture differed in terms of riboflavin production/accumulation, being some overproducing/accumulating riboflavin and other not (Nieland and Stahmann, 2013).

Notwithstanding these findings that have shown interesting links between riboflavin, sporulation and stress phenomena, it is crucial to identify the molecular determinants and explain the regulatory network of riboflavin production in *A. gossypii*. In this regard, some efforts have been engaged. First, genes involved in the riboflavin biosynthetic pathway (*AgRIB3, AgRIB4, AgRIB5*) were found to be transcriptionally regulated by an induction of its promoter during the production phase (Schlösser et al., 2001; Schlösser et al., 2007). This transcriptional regulation of some *RIB* genes and the studies linking riboflavin overproduction to

stress stimuli were complemented by a report of Walther and Wendland (2012). In this paper, the authors investigated the role of a transcription factor of the Yap-family encoded by the gene *AgYAP1*. The recognized function of Yap1p in oxidative stress response was verified to have in *A. gossypii* a strong connection with riboflavin production. A mutant lacking the *AgYAP1* gene was more sensitive to oxidative agents and displayed diminished riboflavin production. Moreover, Yap1p directly induced the *AgRIB4* expression in the presence of an oxidative agent, having the promoter region of this gene 3 binding motifs for Yap1p binding (Walther and Wendland, 2012). Table 1.1 lists the genes described in this subsection and summarizes its regulatory or metabolic contribution to riboflavin production.

**Table 1.1 –** Regulatory or metabolic role of the genes studied to improve riboflavin production in *A. gossypii*.

| Gene | Contribution to riboflavin production | Reference |
|---|---|---|
| *AgICL1* | Anti-metabolite identification for strain isolation (itaconate and oxalate) | Schmidt et al., 1996b |
| *AgGLY1* | Overexpression to increase glycine | Monschau et al., 1998 |
| *AgVMA1* | Disruption to direct riboflavin to the medium | Förster et al., 1999 |
| *AgRIB3-5* | Transcriptionally regulated by promoter induction | Schlösser et al., 2001; 2007 |
| *AgSHM1* & *AgSHM2* | Disruption to increase glycine | Schlüpen et al., 2003 |
| *AgADE4* | Overexpression and engineering to increase GTP | Jiménez et al., 2005 |
| *ScAGX1* | Overexpression to increase glycine | Kato and Park, 2006 |
| *AgBAS1* | Transcription factor regulating expression of *AgADE4* and *AgSHM2* | Mateos et al., 2006 |
| *AgPRS2,4* & *AgPRS3* | Overexpression and engineering to increase PRPP | Jiménez et al., 2008 |
| *AgMLS1* | Overexpression to increase oil consumption | Sugimoto et al., 2009 |
| *AgYAP1* | Induction of *RIB4* promoter under oxidative stress | Walther and Wendland, 2012 |
| *AgACO1* & *AgTMT1* | Anti-metabolite identification for strain isolation (*trans*-aconitate) | Sugimoto et al., 2014 |

The outline presented here on the research employed in riboflavin production by *A. gossypii* after its industrial establishment allows us to understand the extensive and varied work performed. Additionally, it shows what is still missing to fully exploit the paradigmatic flavinogenic trait of this industrially relevant fungus.

### *1.2.2. Production of recombinant proteins by A. gossypii*

As mentioned above, in the last few years the recognized fungal traits for native or recombinant protein secretion began to be exploited in *A. gossypii*. Ribeiro et al. (2010) successfully expressed two cellulases from *Trichoderma reesei* in *A. gossypii*, endoglucanase I (EGI) and cellobiohydrolase I (CBHI). The native signal sequences of these heterologous proteins directed them in *A. gossypii* to the extracellular medium. However, the presence of extracellular CHBI was hard to detect. Indeed, no extracellular CBHI activity was detected, although its presence was confirmed by Western blot analysis. These results suggested that more active EGI was produced/excreted than CBHI. The production levels and secretion of both proteins in *A. gossypii* were similar to those achieved in *S. cerevisiae*. A partial glycosylation characterization of both proteins showed that they were more glycosylated than the *T. reesei* native proteins, but less that those produced by *S. cerevisiae*. Thereby, these results demonstrated that filamentous growth *per se* does not guarantee high levels of secretion (Ribeiro et al., 2010).

Two other works further explored the potential of *A. gossypii* to produce and secrete proteins (Aguiar et al., 2014a; Magalhães et al. 2014). Aguiar et al. (2014a) characterized the genetic information exclusively responsible for the expression of an active extracellular invertase in *A. gossypii* (*AgSUC2*). The expression of this gene, which encodes the invertase, was found active in the *A. gossypii* supernatant and in cell-associated fractions, is repressed by glucose at the transcription level. *AgSUC2* has a high degree of homology with *ScSUC2*, and the production and secretion of the heterologous ScSuc2p in *A. gossypii* was able to complement the AgSuc2p function (Aguiar et al., 2014a). Magalhães et al. (2014) successfully expressed and secreted a β-galactosidase from *A. niger* in *A. gossypii*. After testing different native and heterologous promoters for high expression, transformants with considerably high levels of secretion were obtained: about 37 times higher levels of secretion than a *S. cerevisiae* laboratory strain and even *ca.* 2 times superior than a high β-galactosidase producing *S.*

*cerevisiae* strain (Magalhães et al., 2014). A converging topic relating these two papers were the improved yields of protein secretion (30-fold for invertase and 1.5-fold for β-galactosidase) when glycerol was used as carbon source instead of glucose (Aguiar et al., 2014a; Magalhães et al. 2014).

The first steps into the development of *A. gossypii* as a host for recombinant protein production have already been taken. Beyond the evaluation of its natural production/secretion capacities of heterologous and native proteins, a characterization of its secreted N-glycome/N-glycosylation pathway was already done (Aguiar et al., 2013). Moreover, different approaches were undertaken in order to improve its secretory ability, such as random mutagenesis and targeted engineering by gene disruption (*AgGAS1A* and *AgGAS1B*) (Ribeiro et al., 2013). However, to fully explore this biotechnological application of *A. gossypii* it is required a deeper understanding of the global apparatus involved in protein secretion and delineate genetic/metabolic strategies to efficiently create new strains with improved protein production and secretion.

## 1.3. Glycerol feedstocks

Glycerol is a sugar alcohol abundantly distributed in nature. Normally, it is found naturally linked to other compounds (*e.g.,* triglycerides), serving as a structural backbone. Glycerol can be used as a carbon and energy source by microorganisms. Also, it is one of the preferred osmolytes used for osmoregulation. Industrially, glycerol has an effective presence in several industries, such as food, cosmetics, plastic, pharmaceutical and many more. For instance, it can be used as a stabilizer or cryoprotectant (da Silva et al., 2009; Abad and Turon, 2012; Dobson et al., 2012; Nicol et al., 2012). According to some projections, the current world consumption of glycerol is over 1 million tones per year (Nicol et al., 2012).

The industrial sources of glycerol are based on purification, microbial or chemical synthesis processes from oleochemical industrial by-products (da Silva et al., 2009; Nicol et al., 2012). Biodiesel production has taken the top of the suppliers' chain of glycerol. During the production process of biodiesel it is generated approximately 10% of crude glycerol from the feedstock, *i.e.* from 10 liters of biodiesel produced, 1 liter of crude glycerol is generated (da Silva et al., 2009; Abad and Turon, 2012; Dobson et al., 2012; Nicol et al., 2012). Nonetheless, it is important to note that crude glycerol (industrial waste material) has not the

same characteristics of refined glycerol (industrially applicable). Crude glycerol contains methanol and soap as main contaminants. Other contaminants may be also present (*e.g.*, unused reactants, phosphorous, zinc, silicon, potassium, sodium) and physico-chemical properties can be altered (such as pH), depending on the type of feedstock used for biodiesel production (Abad and Turon, 2012; Dobson et al., 2012; Nicol et al., 2012).

The intensification of biodiesel production in the 15 years was astonishing. For instance, between 2000 and 2005 an augment of 295% was reported (Nicol et al., 2012). Due to this situation and despite its industrial significance, the huge accumulation of glycerol stocks has affected its commercial value. The upshot of a rising negative pressure was the collapse of glycerol price (Dobson et al., 2012). A recent paper, reported that glycerol with 75-80% purity only costs about US$0.03–US$0.05 per kg (Nicol et al., 2012). The offer clearly exceeded the demand. Consequently, the usage of these enormous stocks of glycerol by the industries mentioned above could be compromised for economical reasons, since purification of crude glycerol into refined glycerol that presents the desired degree of purity could become an unviable process (Abad and Turon, 2012; Chatzifragkou and Papanikolaou, 2012).

Data collected from the European Biodiesel Board demonstrate that despite a slowdown in the levels of effective production, the production capacity has been steadily increasing in the European Union (EU; Fig. 1.2). The conjuncture of the last years caused by the financial-economic crisis all around the world may justify the freezing of biodiesel production. On the other hand, the solid investment in the construction of production plants, together with legislation benefits associated with the renewable energies are evidences suggesting a continued strong biodiesel production.

For all the reasons aforementioned it is urgent to develop industrial processes capable of valorizing crude glycerol (Nicol et al., 2012). The production of high-value products from biotechnological processes is one of the strongest and more sustainable applications considered. As mentioned above, many microorganisms can use glycerol as carbon and energy source. Thus, it has the potential to become a new, and more plentiful and inexpensive raw material for fermentation processes with a better degree of reduction than sugars (da Silva et al., 2009; Abad and Turon, 2012). The genes, enzymes and pathways for the uptake and bioconversion of glycerol have been described for some microorganisms. The high-value products produced from glycerol employing microorganisms have been increasing considerably. This already extensive list includes: 1,3-propanediol, dihydroxyacetone,

polyhydroxyalcanoate, acetic acid, citric acid, succinic acid, pyruvic acid, propionic acid, $\alpha$-ketoglutaric acid, butanol, ethanol, pigments (*e.g.*, $\beta$-carotene and astaxanthin), biosurfactants (*e.g.*, sophorolipids) and polyunsaturated fatty acids (PUFA; Abad and Turon, 2012; Chatzifragkou and Papanikolaou, 2012; da Silva et al., 2009; Dobson et al., 2012; Nicol et al., 2012; Rywińska et al., 2013). Other applications such as SCO and single cell protein (SCP) production were also reported (Chatzifragkou and Papanikolaou, 2012; Nicol et al., 2012; Rywińska et al., 2013).

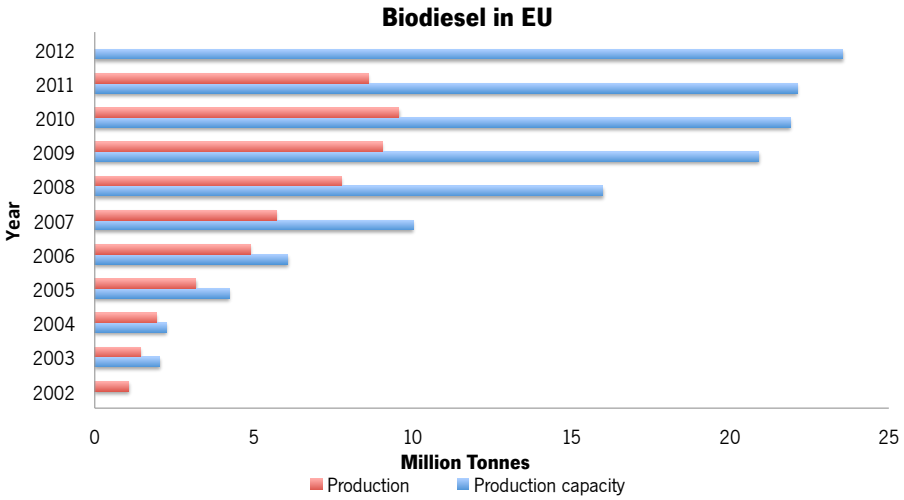


**Figure 1.2 –** Evolution of the total biodiesel production and nominal installed capacities (translated as production capacity) in the European Union. Data collected from the European Biodiesel Board, accessed at http://www.ebb-eu.org/stats.php in 2014.

Metabolic and genetic engineering have been becoming a common practice in order to broaden and improve glycerol-based fermentations for production of high value commodities. For instance, some reports can be found for metabolically engineered *S. cerevisiae* strains (Yu et al., 2010a; Yu et al., 2010b; Jung et al., 2011). Valorization of crude glycerol is both a need and a tremendous opportunity, even for improving the profitableness of biodiesel production if applied in a biorefinery context (Abad and Turon, 2012).

# CHAPTER 2

## Blockage of the pyrimidine biosynthetic pathway affects riboflavin production in *Ashbya gossypii*

**Abstract**

The *Ashbya gossypii* riboflavin biosynthetic pathway and its connection with the purine pathway have been well studied. However, the outcome of genetic alterations in the pyrimidine pathway on riboflavin production by *A. gossypii* had not yet been assessed. Here, we report that the blockage of the *de novo* pyrimidine biosynthetic pathway in the recently generated *A. gossypii Agura3* uridine/uracil auxotrophic strain led to improved riboflavin production on standard agar-solidified complex medium. When extra uridine/uracil was supplied, the production of riboflavin by this auxotroph was repressed. High concentrations of uracil hampered this (and the parent) strain growth, whereas excess uridine favored the *A. gossypii Agura3* growth. Considering that the riboflavin and the pyrimidine pathways share the same precursors and that riboflavin overproduction may be triggered by nutritional stress, we suggest that overproduction of riboflavin by the *A. gossypii Agura3* may occur as an outcome of a nutritional stress response and/or of an increased availability in precursors for riboflavin biosynthesis, due to their reduced consumption by the pyrimidine pathway.

Keywords: *Ashbya gossypii*; pyrimidine pathway; riboflavin production; uridine/uracil auxotrophy; nutritional stress

## 2.1. Introduction

The filamentous hemiascomycete *Ashbya gossypii* (syn. *Eremothecium gossypii*) is a remarkable riboflavin (vitamin B2) overproducer that has long been exploited for the industrial production of this vitamin (Stahmann et al., 2000; Kato and Park, 2012). Years of strain optimization lead to the establishment of a cost-efficient industrial riboflavin production process using *A. gossypii* (by BASF in 1996), which sent chemical synthesis of this vitamin for second plan (Stahmann et al., 2000). Since then, the scientific community has been mainly focused on the development of new strategies to further improve riboflavin production yields and, more recently, there has been a growing interest in unraveling the molecular and regulatory mechanisms behind riboflavin production: to discover under what conditions is the overproduction of this vitamin activated and weather it may offer a major ecological advantage to the fungus. In this regard, there are some reports supporting that riboflavin overproduction by *A. gossypii* is an environmental stress-induced phenomenon (Stahmann et al., 2001; Shlösser et al. 2007; Kavitha and Chandra, 2009; Walther and Wendland, 2012).

Riboflavin is synthesized from ribulose 5-phosohate (ribulose-5P) and/or guanosine-5-triphosphate (GTP) through a pathway controlled by six genes (Shlösser et al., 2007). Riboflavin overproduction starts when the *A. gossypii* growth rate declines and is characterized by a strong increase in the expression of three of these genes (*AgRIB3*, *AgRIB4* and *AgRIB5*), which are regulated at the transcription level (Shlösser et al., 2001; Shlösser et al., 2007; Walther and Wendland, 2012). Stahmann et al. (2001) reported that riboflavin could act as a photoprotectant agent for *A. gossypii* hyaline spores against ultraviolet light, being riboflavin overproduction by *A. gossypii* strongly reduced when sporulation is inhibited. Beyond this association between riboflavin production and sporulation, the developmental program that results in spore formation has been reported to also be induced by some kind of environmental stress (Wendland et al., 2011; Nieland and Stahmann, 2013; Wassersrtom et al., 2013). Schlösser et al. (2007) demonstrated that stable cultivation conditions like continuous culture at constant dilution rate, even under unfavorable conditions for high growth rates (*e.g.* reduced oxygen supply, different temperature and salt stress), were unsuitable for riboflavin overproduction in *A. gossypii*. Nonetheless, sudden decreases of the dilution rate in continuous culture resulted in immediate riboflavin overproduction by *A. gossypii*, suggesting that stress stimuli are required to trigger riboflavin overproduction (Schlosser et al., 2007). On the other

hand, Kavitha and Chandra (2009) showed that *A. gossypii* growing on culture medium supplemented with vitamin E (a pro-oxidant) and menadione (a well-known oxidant) presented increased riboflavin production, which was associated to increased intracellular activity of enzymes related to oxidative stress (Kavitha and Chandra, 2009). AgYap1p (encoded by *AgYAP1*), a well-known oxidative stress response regulator, was recently shown to mediate an AgYap1p-dependent increase in riboflavin production during oxidative stress (Walther and Wendland 2012). AgYap1p was determined to play a particularly important role in the modulation of the *AgRIB4* expression, but it is probably involved in the regulation of the *AgRIB3* and *AgRIB5* expression as well (Walther and Wendland 2012).

As described above, environmental stress, such as nutritional and oxidative stress, can be the stimulus that triggers the molecular mechanisms for riboflavin overproduction in *A. gossypii*. However, since the riboflavin biosynthetic pathway shares its precursors with other metabolic pathways (Fig. 2.1), riboflavin production is also influenced by alterations in these pathways (Park et al., 2011). Increased purine/GTP biosynthesis, either through the overexpression of genes encoding transcription factors (*AgBAS1*; Mateos et al., 2006) or enzymes directly involved in this pathway (*AgPRS2-4*, *AgPRS3* or *AgADE4*; Jimenez et al. 2005; Jimenez et al. 2008), or by increasing the intracellular availability of glycine (Monschau et al., 1998; Schlüpen et al., 2003; Kato and Park, 2006; Mateos et al., 2006), an important precursor for the *de novo* purine biosynthesis, leads in *A. gossypii* to increased riboflavin production. Alterations in the pyrimidine pathway are also expected to affect riboflavin production, as high concentrations of uracil in the medium inhibit riboflavin production by *Eremothecium ahsbyii* (Goodwin and Pendlington, 1954). However, to our knowledge, the effect of manipulations in this pathway on riboflavin production by *A. gossypii* has not yet been addressed.

In the scope of the development of Cre-*lox*P-based tools for genetic manipulation of *A. gossypii*, we recently deleted the entire coding region of the *A. gossypii* ATCC 10895 *AgURA3* gene with a dominant marker cassette that was subsequently removed using the Cre-recombinase, thus generating an *A. gossypii* uridine/uracil auxotrophic strain free of exogenous marker genes (Aguiar et al., 2014). In this study, we explored the growth and flavinogenic phenotypes of this strain (*A. gossypii Agura3*), which has the de novo pyrimidine biosynthetic pathway blocked at the orotidine-5'-phosphate decarboxylase (AgUra3p) level (Fig. 2.1).

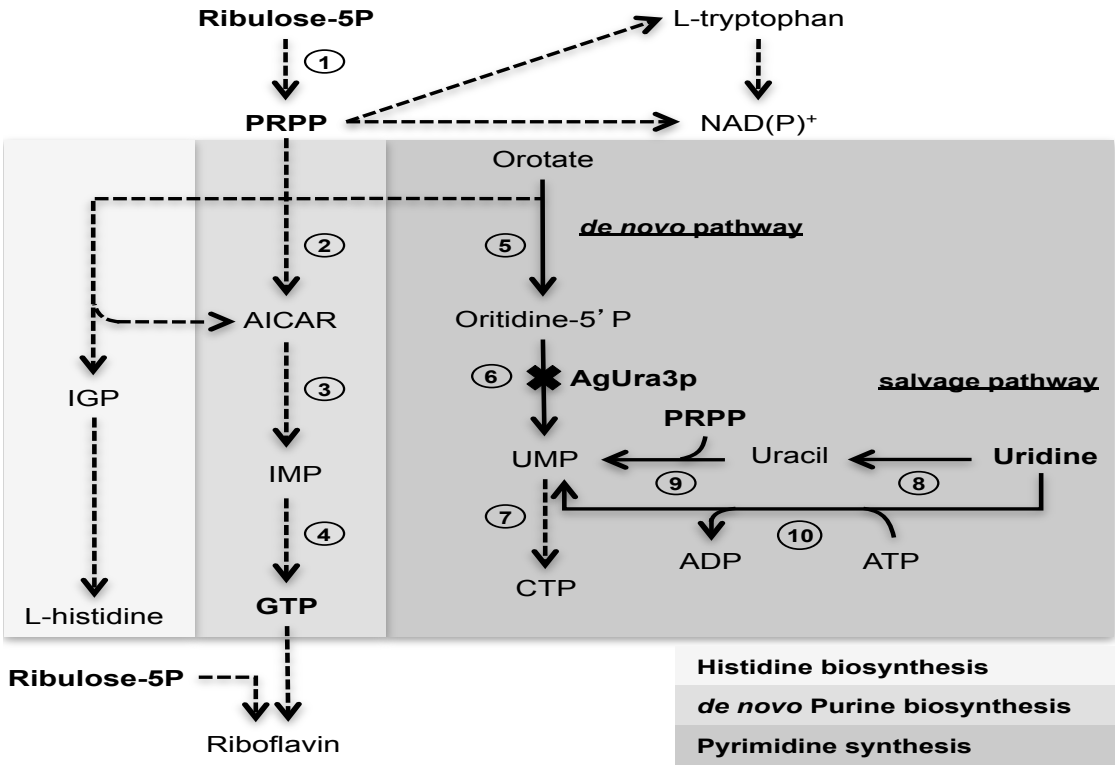


**Figure 2.1 –** Schematic representation indicating the contribution of ribulose-5P, PRPP and GTP to riboflavin biosynthesis and to other metabolic pathways. The ✖ marks where the pyrimidine biosynthetic pathway is blocked in the *A. gossypii Agura3* uridine/uracil auxotrophic strain. Dashed arrows indicate a multi-step pathway. IGP: D-erythro-imidazole-glycerol-phosphate; AICAR: 5-amino-4-imidazolecarboxamide ribotide; IMP: 5'-inosine monophosphate; NAD(P): nicotinamide adenine dinucleotide (phosphate); CTP: cytidine-5'-triphosphate; ADP: adenosine-5'-pyrophosphate; ATP: adenosine-5'-triphosphate. Numbers indicate the enzymes involved in each step of the purine and pyrimidine pathways. 1: ribose-5-phosphate ketol-isomerase and PRPP synthetase; 2: PRPP amidotransferase, phosphoribosylglycinamidine synthetase, glycinamide ribotide transformylase, 5'-phosphoribosylformyl glycinamidine synthetase, phopshoribosylaminoimidazole synthetase, phosphoribosylamino-imidazole carboxylase, phosphoribosyl amino imidazolesuccinocarbozamide synthetase and adenylosuccinate lyase; 3: AICAR transformylase and IMP cyclohydrolase; 4: IMP dehydrogenase, guanosine-monophosphate synthase, guanylate kinase and nucleoside diphosphate kinase; 5: orotate phosphoribosyltransferase; 6: orotidine-5'-phosphate decarboxylase; 7: uridylate kinase and CTP synthase; 8: uridine nucleosidase; 9: uracil phosphoribosyltransferase; 10: uridine kinase.

## 2.2. Materials and methods

### *2.2.1. Sterilization procedures*

All thermo-resistant material, culture media and solutions were sterilized by autoclaving at 121 °C and 1 bar of pressure during 20 min. Thermo-labile culture media and

solutions, as stock solution of Yeast Nitrogen Base (YNB) without amino acids, Amino Acid drop out mix and Lysing Enzymes from *Trichoderma harzianum* (Sigma- Aldrich) were sterilized by filtration with 0.2 $\mu$m filters.

### *2.2.2. Strains*

The strains used in this chapter were *A. gossypii* ATCC 10895 and *A. gossypii Agura3*. *A. gossypii* ATCC 10895, here referred as the parent strain, was kindly provided by Prof. P. Philippsen (Biozentrum, University of Basel, Switzerland). Its uridine/uracil auxotrophic derivate *A. gossypii Agura3* (*Agura*3Δ::*loxP*) was obtained in another study (Aguiar et al., 2014b). Stock cultures were maintained as spores in sterile spore buffer, containing 0.8% (w/v) NaCl, 0.025% (v/v) tween 20 and 20% (v/v) glycerol, that were obtained using the following protocol: *A. gossypii* strains were inoculated in agar-solidified *Ashbya* Full Medium (AFM; Altmann-Jöhl and Philippsen, 1996) containing 1% (w/v) tryptone, 1% (w/v) yeast extract, 2% glucose (w/v) and 0.1% (w/v) myo-inositol. After 8 days of incubation at 30 °C, mycelium was collected from the Petri dish with a sterile loop into a 15 mL falcon tube containing 5 mL of sterile water. This suspension was incubated about 4 h at 30 °C with 4 mg $mL^{-1}$ of Lysing Enzymes from *T. harzianum* (Sigma- Aldrich), to allow total digestion of hyphal cells and releasing of spores. The suspension was centrifuged at 3000 rpm for 5 minutes and the supernatant discarded. The spores were washed twice with 5 mL of sterile spore buffer. Spores were quantified in an improved Neubauer chamber under a microscope and stored at -80 °C in aliquots of 1 mL.

### *2.2.3. Media and culture conditions*

Agar-solidified AFM was used in order to evaluate the effect of uridine and uracil on riboflavin production. When indicated, AFM was supplemented with 0.05, 0.1, 0.2, 0.5 and 1 mM uridine or 0.2, 0.5 and 1 mM uracil. Agar-solidified AFM and agar-solidified Synthetic Complete (SC) medium (Ribeiro et al., 2012), buffered with 0.1% (w/v) calcium carbonate and containing 2% (w/v) glucose, 10% (v/v) of a 6,8% (w/v) stock solution of YNB without amino acids and 3% (v/v) Amino Acid drop out mix (Table 2.1.) were used in order to evaluate the effect of uridine and uracil on colony radial growth. When indicated, AFM was supplemented

with 5 or 10 mM uridine/uracil and SC medium without uracil/uridine was supplemented with 2 mM uridine/uracil. In all culture conditions, $10^5$ spores of *A. gossypii* strains were inoculated on the agar plates and incubated at 30 °C. All the photographs presented here are representative of 3 biological replicas.

**Table 2.1 –** Amino acid composition of the drop out mix used in SC medium.

| Amino acid | Concentration (g $L^{-1}$) |
|---|---|
| L-Aspartic acid | 8.87 |
| L-Isoleucine | 17.5 |
| L-Lysine | 3.03 |
| L-Phenylalanine | 2.77 |
| L-Serine | 3.50 |
| L-Tyrosine | 1.00 |
| L-Valine | 3.90 |
| L-Arginine | 11.6 |
| L-Methionine | 4.90 |
| L-Tryptophan | 2.73 |
| Adenine hemisulfate | 0.450 |
| L-Leucine | 8.73 |
| L-Threonine | 3.96 |
| L-Histidine | 1.93 |

*2.2.4. Analytical procedures*

Cell dry weight was determined by collecting the mycelium from each Petri dish into a pre-weighed dried tube, drying overnight at 105 °C and weighing. Colony radial growth was determined by measuring the diameter of colonies grown on agar-solidified medium in 90 mm diameter Petri dishes. For total riboflavin estimation, the mycelium from each Petri dish was collected after 3 days of growth and digested with 4 mg $mL^{-1}$ Lysing Enzymes from *T. harzianum* (Sigma- Aldrich) for 1 h. Cell debris was removed by centrifugation and the supernatant's optical density was measured at 445 nm wavelength, and converted into riboflavin concentration using a standard curve ($R^2$ = 0.996) constructed with pure riboflavin standards (Sigma-Aldrich) of 15, 30, 45, 60 and 90 mg $L^{-1}$. Riboflavin concentration (mg $L^{-1}$) was calculated by the following conversion factor: 0.0134 mg (optical density units $L^{-1}$) $^{-1}$. Specific riboflavin production was calculated as $mg_{riboflavin}\ g_{mycelium}^{-1}$.

*2.2.5.Statistical analysis*

The data from comparative analyses of specific riboflavin production and colony radial growth between *A. gossypii* ATCC 10895 and *A. gossypii Agura3* on agar solidified AFM without supplementation and with 0.5 mM uridine were analyzed in GraphPad Prism® (GraphPad Software, Inc.). One-way ANOVA and Tukey's multiple comparisons tests were used to find means significantly different from each other.

## 2.3. Results and discussion

The *A. gossypii Agura3* auxotrophic strain requires the supply of exogenous uridine/uracil to compensate its nutritional deficiency (Aguiar et al., 2014b). However, in this work uracil was detrimental for the *A. gossypii* growth (Fig. 2.2A and B). In AFM medium, the growth inhibitory effect of high concentrations of uracil was more evident in the *Agura3* strain than in its parent strain (Fig. 2.2B). Nevertheless, in SC medium, clearly showed to negatively affect the parent strain growth as well (Fig. 2.2A). Similar concentrations of uracil were reported not to affect the growth of the *A. gossypii* close relative *E. ahsbyii* (Goodwin and Pendlington, 1954). By contrast, uridine did not cause growth inhibition (Fig. 2.2A and B). On the contrary, it significantly favored the *Agura3* strain growth, which only reached the level of the parent strain growth under extra uridine supplementation conditions (Fig. 2.3; Table 2.2).

Excess of exogenous uracil (but not uridine) was recently reported to have a strong inhibitory effect on the *Aspergillus nidulans* growth, being an auxotrophic strain deleted in *pyrG* (encoding orotidine-5'-phosphate decarboxylase) also more sensitive to uracil than the wild type strain (Sun et al., 2013). This uracil toxicity is believed to be related with the misincorporation of uracil into DNA when uracil is accumulated in excess (Sun et al., 2013). Uridine cannot be directly incorporated into DNA, which may explain why excess uridine did not cause growth inhibition in *A. nidulans* (Sun et al., 2013) and *A. gossypii*.

The *A. gossypii Agura3* strain presented a riboflavin overproducing phenotype on standard agar-solidified AFM, detected by the yellow color of the colonies, which was not displayed by the parent strain under the same conditions (Fig. 2.2C). However, when increasing concentrations of uridine/uracil were added to AFM, the production of riboflavin by *A. gossypii Agura3* gradually diminished (Fig. 2.2D and E), whereas the flavinogenic phenotype

of the parent strain remained unaffected (*cf.* Fig. 2B–D). High concentrations of uracil in AFM inhibited riboflavin production by *A. gossypii Agura3*, but lead to decreased growth (Fig. 2.2E; Dinis, 2012). Hence, the riboflavin overproducing phenotype of this uridine/uracil auxotroph is inversely correlated with the accessibility to these nutrients.

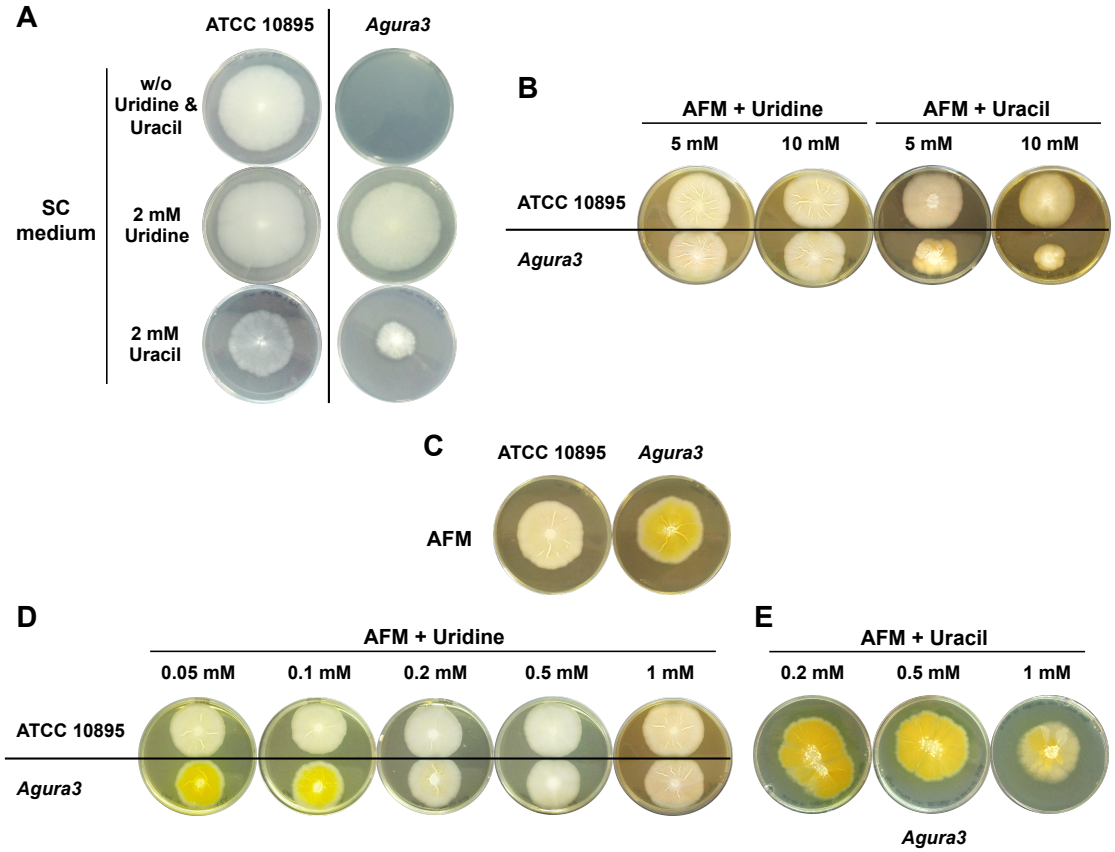


**Figure 2.2 –** Effect of uridine/uracil supplementation on growth and riboflavin production by the *A. gossypii Agura3* and ATCC 10895 strains. (A) *A. gossypii* ATCC 10895 and *Agura3* grown for 5 days on agar-solidified SC medium lacking uridine and uracil or supplemented with 2 mM of either uridine or uracil. (B) *A. gossypii* ATCC 10895 and *Agura3* grown for 5 days on agar-solidified AFM supplemented with either uridine or uracil at 5 or 10 mM. (C) *A. gossypii* ATCC 10895 and *Agura3* grown for 5 days on standard agar-solidified AFM. (D) *A. gossypii* ATCC 10895 and *Agura3* grown for 5 days on standard agar-solidified AFM supplemented with 0.05, 0.1, 0.2, 0.5 or 1 mM uridine. (E) *A. gossypii Agura3* grown for 8 days on agar-solidified AFM containing 0.2, 0.5 or 1 mM uracil. All images are representative of three biological replicas. Photographs corresponding to Petri dishes of AFM supplemented with uracil were adapted from Dinis (2012).

These results clearly showed that addition of 0.5 mM uridine to AFM resulted in an approximately 9-fold decrease in riboflavin production by *A. gossypii Agura3* and, as mentioned above, in significant improved colony radial growth (Fig. 2.3; Table 2.2).

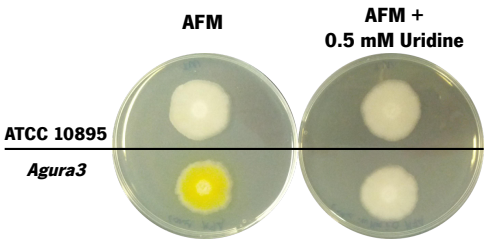


**Figure 2.3 –** *A. gossypii Agura3* and ATCC 10895 strains after 3 days of growth on standard agar-solidified AFM and AFM supplemented with 0.5 mM uridine. In this figure are depicted representative photographs of the three biological replicas used for riboflavin specific production determination and colony radial growth measurements.

**Table 2.2 –** Riboflavin specific production and colony radial growth by *A. gossypii* ATCC 10895 and *A. gossypii Agura3* after 3 days of incubation in AFM and AFM supplemented with 0.5 mM uridine. Data represent average ± standard error of the mean (SEM) from three biological replicas.

| | AFM | AFM + 0.5 mM uridine |
|---|---|---|
| *A. gossypii* ATCC 10895 | | |
| Riboflavin (mg $g_{biomass}^{-1}$) | $1.00 \pm 0.12^{a}$ | $0.923 \pm 0.098^{a}$ |
| Colony radial growth (mm) | $27.4 \pm 0.2^{b}$ | $27.3 \pm 0.2^{b}$ |
| *A. gossypii Agura3* | | |
| Riboflavin (mg $g_{biomass}^{-1}$) | $7.51 \pm 0.23^{c}$ | $0.789 \pm 0.104^{a}$ |
| Colony radial growth (mm) | $25.3 \pm 0.2^{d}$ | $26.8 \pm 0.1^{b}$ |

Riboflavin: a,c – significantly different ($p < 0.0001$) as determined by one-way ANOVA and Tukey's test (n = 3); Colony radial growth: b,d – significantly different ($p < 0.0001$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3).

Nutritional stress can trigger riboflavin overproduction in *A. gossypii* (Schlösser et al., 2007). The cascade of events involved in this stress response has not been fully elucidated, but it is known to induce the expression of genes of the riboflavin biosynthetic pathway, thus increasing the metabolic flux to riboflavin synthesis (Schlösser et al., 2007; Walther and Wendland, 2012). A very recent genome-wide metabolic re-annotation of *A. gossypii* made *in-house* by Gomes et al. (2014) showed that comparatively with *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, this fungus does not possess exclusive enzymes directly involved in the riboflavin biosynthetic pathway that could explain its riboflavin overproducing trait. These findings suggest that riboflavin overproduction by *A. gossypii* should be related with regulatory mechanisms instead of metabolic capabilities. On the other hand, a set of enzymes associated with the metabolism of GTP and glyoxylate (both strongly connected to riboflavin production,

even though the latter only when oils are used as a carbon source) was found exclusively in *A. gossypii*, thus reinforcing the importance of these pathways that are responsible for the synthesis of riboflavin's precursors (Gomes et al., 2014).

The metabolic flux to riboflavin synthesis is determined by the amount of precursors available (Kato and Park, 2012). Ribulose-5P is obtained from the carbohydrate metabolism through the oxidative branch of the pentose phosphate pathway and can be directly used as a precursor for riboflavin synthesis or isomerized to ribose-5-phosphate (Park et al., 2011). Ribose-5-phosphate is in its turn required for the synthesis of phosphoribosyl pyrophosphate (PRPP), an intermediate metabolite necessary for the synthesis of other riboflavin precursor (GTP), purine, pyrimidine and pyridine nucleotides, histidine and tryptophan (Fig. 2.1) (Jimenez et al., 2008). Increased PRPP synthetase activity led in *A. gossypii* to increased riboflavin production (Jimenez et al., 2008). Moreover, a riboflavin overproducing *A. gossypii* mutant isolated by disparity mutagenesis displayed threefold higher expression of PRPP synthetase encoding genes during the riboflavin production phase than its parent strain (Park et al., 2011). Therefore, alterations in the intracellular PRPP pool affect riboflavin production in *A. gossypii*.

Given that the purine and pyrimidine synthesis was estimated to consume most of the synthesized PRPP (Hove-Jensen, 1988) and that *A. gossypii Agura3* has the *de novo* pyrimidine biosynthetic pathway blocked (Fig. 2.1), an increase in the PRPP available for riboflavin synthesis through the *de novo* purine/GTP biosynthetic pathway and/or a reduction in PRPP synthesis driven by its intracellular accumulation, with consequent increased availability of ribulose-5P for riboflavin synthesis, might have occurred when no extra uridine/uracil was supplied. Any of these events could explain the overproduction of riboflavin by this strain under these conditions. Moreover, increased flux in the riboflavin pathway triggered by a nutritional stress response may have also contributed to this phenotype. Conversely, when extra uridine/uracil was supplied, the nutritional requirements of this auxotrophic strain were gradually compensated, thus preventing nutritional stress. Additionally, the synthesis of uridine monophosphate (UMP) from uridine/uracil through the pyrimidine *salvage* pathway must have increased, therefore reducing the PRPP availability for GTP/riboflavin synthesis. Increased demand for PRPP may have also led to the redirection of ribulose-5P for its synthesis.

The findings reported here show that blockage of the *A. gossypii* pyrimidine biosynthetic pathway led to increased riboflavin production, which was inversely correlated with the accessibility to the nutrients required to compensate the auxotrophy caused by this blockage. Although changes in the purine pathway have been described to affect riboflavin production, these are the first experimental results documenting alterations in riboflavin production caused by metabolic engineering of the pyrimidine pathway, thus broadening the knowledge about rational metabolic engineering strategies that improve riboflavin production in *A. gossypii*. Beyond the direct impact on riboflavin production by *A. gossypii* and on the knowledge about its physiology, a broader scientific interest of this work resides in the description of a wider effect of mutations in the *URA3* gene on fungal physiology, beyond uridine/uracil auxotrophy.

# CHAPTER 3

## Exploration of glycerol as substrate for riboflavin production by *Ashbya gossypii* and of strategies to improve its consumption


**Abstract**

Glycerol is one of the most promising substrates for biotechnological applications given the worldwide scenario of strong investment in biodiesel production, which consequently has led to the accumulation of large stocks of this compound. The filamentous fungus *Ashbya gossypii* has recognized biotechnological capabilities. The production process of its most remarkable trait (riboflavin production) is well established on plant oils and some industrial wastes were already studied for this purpose in order to increase the process profitability. However, exploitation of glycerol for the production of riboflavin or other biotechnological application had not yet been performed. Here, we report riboflavin production from pure glycerol by *A. gossypii* at the same level as that produced from glucose, confirming the suitability of this carbon source for this purpose. Additionally, we designed a strategy to improve its glycerol consumption profile by overexpressing glycerol uptake protein (*GUP1*) genes. *A. gossypii* strains overexpressing the native *GUP1* gene (*pRSAG*) or the *GUP1* gene from *Saccharomyces cerevisiae* (*pRSSG*) were able to show glycerol-dependent hyperosmotic stress tolerance and glycerol consumption profiles significantly improved. *A. gossypii pRSAG* achieved the best results. This strain was able to grow under hyperosmotic stress caused by 1 M KCl, showing a shorter lag phase and a significantly higher final radial growth than the control strain. Additionally, it translated its faster glycerol consumption profile in medium with 40 g $L^{-1}$ glycerol into 2.5-fold higher yields of biomass production (0.15 ± 0.01 $g_{mycelium}$ $g_{glycerol}^{-1}$) in comparison with the control strain.



Keywords: *Ashbya gossypii*; glycerol; *GUP1*; hyperosmotic stress; glycerol consumption

## 3.1. Introduction

The establishment of the biodiesel industry will bring new questions and challenges that need to be addressed. Among them, the most prominent lies on the valorization of the main by-product of the process, crude glycerol. Owing to its degree of impurity, it is impossible to directly apply this by-product in the manufacturing of commodities derived from refined glycerol (Abad and Turon, 2012; Dobson et al., 2012; Nicol et al., 2012). This matter needs to be solved not only due to environmental concerns but also to guarantee the cost-effectiveness of biodiesel production. In this context, the scientific community has been looking at the accumulation of crude glycerol stocks has an opportunity for developing biotechnological processes from this renewable resource. Glycerol presents itself as a profitable alternative to the conventional sugars for fermentation processes and due to its characteristics it can offer its own range of high-value products (da Silva et al., 2009; Abad and Turon, 2012).

A rising number of microorganisms have been exploited for different biotechnological applications from glycerol, such as the non-conventional yeast *Yarrowia lipolytica* (Rywińska et al., 2013). *Y. lipolytica* is one of the most explored microorganism for the biotechnological valorization of this substrate. The available range of applications studied in this cell factory comprises some organic acids (such as citric, succinic, acetic and $\alpha$-ketoglutaric acids), invertase, sugar alcohols, single cell oil (SCO) and single cell protein (SCP) (Rywińska et al., 2013). Among these, citric acid production from glycerol has been studied for over 10 years, whereas SCO and SCP have become suitable processes for commercialization in the last 5 years (Rywińska et al., 2013). Although little is known about the glycerol metabolism in *A. gossypii*, the existing studies have demonstrated interesting outcomes. Förster et al. (1998) identified a highly active glycerol uptake system in *Ashbya gossypii* under hyperosmotic stress conditions and demonstrated that glycerol is the principal osmoprotectant of this fungus. On the other hand, Ribeiro et al. (2012) reported high specific growth rates of *A. gossypii* on glycerol, comparing to those obtained by its closely related yeast *Saccharomyces cerevisiae*, and a noteworthy ability of *A. gossypii* to convert glycerol into ethanol. Moreover, exploration of recombinant protein production in this fungus demonstrated that the productivities obtained for some proteins are higher on glycerol than on glucose (Aguiar et al., 2014a; Magalhães et al., 2014).

Riboflavin production is the overwhelming trait of *A. gossypii* that makes it a paradigm of white biotechnology (Stahmann et al., 2000; Kato and Park, 2012). This process is well exploited on hydrophobic substrates, being industrially implemented from plant oils. Since the industrial establishment of process with *A. gossypii* there has been a constant search to further increase its profitability. For instance, some industrial wastes containing oils have been explored for the production of riboflavin (Ming et al., 2003; Park et al., 2004a; Park et al., 2004b; Park et al., 2007; Tajima et al., 2009). Given the increasing accumulation of crude glycerol stocks, it would be interesting to explore this industrial waste for riboflavin production by *A. gossypii*. However, to the extent of our knowledge, riboflavin production by *A. gossypii* has not even been tested from pure glycerol.

The genes, enzymes and pathways for the biosynthesis, utilization and uptake of glycerol have been described for some microorganisms (da Silva et al., 2009). In *S. cerevisiae*, identification of the genes involved in the glycerol metabolism was obtained mainly by the study of the osmotic stress response and/or the glycerol utilization as carbon source. In this yeast, glycerol biosynthesis starts from dihydroxyacetone-phosphate (DHAP) that is converted to glycerol-3-phosphate (G3P) by the action of a G3P dehydrogenase encoded by *GPD1*/*GPD2* (Albertyn et al., 1994; Eriksson et al. 1995). Conversion to glycerol is performed through the action of a G3P phosphatase encoded by *GPP1*/*GPP2* (Norbeck et al., 1996). For the utilization of glycerol there are two pathways: the aerobic and anaerobic routes. The aerobic utilization of glycerol is the best-characterized route and is formed by two enzymes: a glycerol kinase (encoded by *GUT1*; Pavlik et al., 1993) and a G3P dehydrogenase (encoded by *GUT2*; Rønnow et al., 1993). Gut1p is responsible for converting glycerol into the intermediate G3P, which is finally converted to DHAP by Gut2p. DHAP can then be directed to the glycolysis and/or gluconeogenesis pathways. The anaerobic route is not well described in *S. cerevisiae*, but consists also in a two reactions pathway. First, glycerol is converted to dihydroxyacetone (DHA) by a glycerol dehydrogenase encoded by *GCY1*, and subsequently a DHA kinase (encoded by *DAK1*/*DAK2*) catalyzes the conversion of DHA into DHAP (Norbeck and Blomberg, 1997).

The transport of glycerol in *S. cerevisiae* has been associated mainly to three different genes: *FPS1*, *GUP1* and *STL1*. *FPS1* encodes an aquaglyceroporin associated with the uptake of glycerol when glucose was present in the medium (Sutherland et al., 1997) and with different responses to osmotic stress conditions, allowing the efflux of glycerol during hypo-

osmotic stress and preventing the release of glycerol during hyperosmotic stress conditions (Luyten et al., 1995; Tamas et al., 1999; Oliveira et al., 2003; Geijer et al., 2012). Furthermore, Jung et al. (2011) reported that its overexpression did not lead to increased glycerol consumption in engineered *S. cerevisiae* strains.

*GUP1* encodes a member of the membrane-bound O-acyltransferases family (Hofmann, 2000). Holst et al. (2000) identified the *GUP1* gene in *S. cerevisiae* and reported its involvement in growth when glycerol was used as energy and carbon source, and with glycerol-dependent cell recovery from hyperosmotic stress (Holst et al., 2000). Further studies suggested other biological roles and cellular functions for this gene beyond its role on glycerol metabolism (Neves et al., 2004; Oliveira and Lucas, 2004): glycosylphosphatidylinositol (GPI) anchor biosynthesis (Bosson et al., 2006), cell wall composition (Ferreira et al., 2006) and lipid metabolism (Ferreira and Lucas, 2008), among others. Moreover, in engineered *S. cerevisiae* strains generated for ethanol and 1,2-propanediol production from glycerol, overexpression of *GUP1* led to faster glycerol consumption and improved hyperosmotic stress tolerance (Yu et al., 2010a; 2010b; Jung et al., 2011).

*STL1* encodes a member of the sugar transporter family and it was reported to be the glycerol proton symporter of the plasma membrane in *S. cerevisiae*, being strongly but transiently induced when cells are under osmotic stress. Moreover, strains deleted for *STL1* grew poorly on glycerol (Ferreira et al., 2005).

*A. gossypii* possesses a homolog gene for each one of the aforementioned *S. cerevisiae* genes involved in the metabolism of glycerol, but only the *AgFPS1* gene has been experimentally characterized so far (Geijer et al., 2012). Among these homologs, only *AgSTL1* does not share the syntenic pattern of its homolog, *ScSTL1*.

In this study, we aimed at exploring the production of riboflavin by *A. gossypii* from glycerol. Furthermore, taking into account the slower growth and substrate consumption by *A. gossypii* in glycerol cultivations compared to glucose cultivations (Ribeiro et al., 2012), we proposed to increase the uptake of glycerol in *A. gossypii* by overexpressing the native *AgGUP1* or the heterologous *ScGUP1* from *S. cerevisiae*. Here, we performed the first metabolic engineering manipulation to improve the glycerol consumption profile in *A. gossypii*.

## 3.2. Materials and methods

### *3.2.1. Sterilization procedures*

All thermo-resistant material, culture media and solutions were sterilized by autoclaving at 121 °C and 1 bar of pressure during 20 min. Thermo-labile culture media and solutions, as Lysing Enzymes from *Trichoderma harzianum* (Sigma-Aldrich), antibiotics and solutions for *A. gossypii* transformation, were sterilized by filtration with 0.2 μm filters.

### *3.2.2. Strains*

Microbial strains used in this chapter are listed in Table 3.1.

**Table 3.1 –** Microbial strains used in this chapter.

| Strain | Genotype | Source |
|---|---|---|
| *Escherichia coli NZ5α* | fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | NzyTech |
| *S. cerevisiae* CEN.PK113-7D | *MATα*, *MAL2-8*c,*SUC2* | INSA, Toulouse, France |
| *A. gossypii* ATCC 10895 | Sequenced strain, here referred as parent strain | Prof. P. Philippsen (Biozentrum, University of Basel, Switzerland) |
| *A. gossypii Agura3* | Uridine/uracil auxotrophe derived from the parent strain (*Agura3*Δ::*lox*P) | Aguiar et al., 2014b |
| *A. gossypii pFMT_EV* | *A. gossypii* carrying the empty vector pFMT | Magalhães et al., 2014 |
| *A. gossypii pRSAG* | *A. gossypii* overexpressing the *AgGUP1* from *A. gossypii* | This study |
| *A. gossypii pRSSG* | *A. gossypii* overexpressing *ScGUP1* from *S. cerevisiae* | This study |

### *3.2.3. Vector*

The vector used as backbone for the overexpression of the *GUP1* genes from *A. gossypii* and *S. cerevisiae* was pFMT (Magalhães et al., 2014). This vector contains the following relevant features: CoLE1 origin of replication; 2-micron origin of replication; gene that

confers resistance to ampicillin; GEN3 cassette conferring resistance to geneticin (G418); *AgTEF* promoter and *ScPGK1* terminator with a multiple cloning sequence (MCS) in between.

### *3.2.4. Primers*

Primers used in this chapter are listed in Table 3.2.

**Table 3.2 –** Primers used in this chapter. Underlined characters represent the recognition sites of the restriction enzymes used in the cloning procedures.

| Primer name | Sequence (5'->3') | Tm (°C) | Use |
|---|---|---|---|
| AgGUP1_Fw | TCTATCCGGCCGATGCTCCATTCGCTCTGG | 55 | Amplification of *AgGUP1* from *A. gossypii* and verification of insertion into pFMT |
| AgGUP1_Rv | GGGGTACCCACAATGAGGTAGTATGTGGCG | 55 | |
| ScGUP1_Fw | CGGAATTCTGCAAGAAATGTCGCTGATC | 53 | Amplification of *ScGUP1* from *S. cerevisiae* and verification of insertion into pFMT |
| ScGUP1_Rv | CCGCTCGAGTCAGCATTTTAGGTAAATTCCGTG | 53 | |
| PGK_Rv | GGCATTAAAAGAGGAGCG | 54 | Verification of insertion into pFMT |
| pMI_MCS_Fw | CCAGTGAATTGGCCGATGC | 57 | |

### *3.2.5. Media*

*Escherichia coli* strains were grown in Luria-Bertani (LB) medium containing 1% (w/v) sodium chloride (NaCl), 1% (w/v) yeast extract, 0.5% (w/v) tryptone (pH 7.5). This medium was added with ampicillin to a final concentration of 100 μg/mL (LB-amp) for transformants selection. *A. gossypii* strains were grown in *Ashbya* Full Medium (AFM; Altmann-Jöhl et al., 1996) containing 1% (w/v) tryptone, 1% (w/v) yeast extract, 2% glucose (w/v) and 0,1% (w/v) myo-inositol. This medium was added with geneticin (G418) to a final concentration of 200

μg/mL (AFM-G418) for transformants selection. When indicated, glucose was replaced by 2.5% or 4% (w/v) pure glycerol (AppliChem, 99%) (AFMGlyOH). AFM and AFMGlyOH used in submerged cultures were supplemented with 0.15% (w/v) glycine. Agar-solidified AFM and LB media were obtained by adding 1.5% (w/v) agar.

### *3.2.6. Storage conditions for bacteria and spores*

Bacteria cultures were maintained for up to 2 weeks at 4 °C in appropriate selective medium, on inverted agar plates sealed with parafilm. For long time storage, permanent stocks were prepared using the following protocol: an overnight culture in liquid LB-amp medium was diluted 10 times in the same but fresh medium and grown for 5 h more. Subsequently, a solution of 0.3 mL of sterile glycerol was added to 1 mL of the culture, mixed by vortexing and incubated on ice for 10 min. The tubes were stored at -80 °C. For culture recovery, the frozen cells were scrapped and spread on agar-solidified LB-amp. The permanent stock was stored and re-used.

*A. gossypii* was maintained as spores in sterile spore buffer, containing 0.8% (w/v) sodium chloride, 0.025% (v/v) tween 20 and 20% (v/v) glycerol, that were obtained using the following protocol: *A. gossypii* strains were center inoculated on appropriate agar-solidified AFM. After 8 days of incubation at 30 °C, mycelium was collected from the Petri dish with a sterile loop into a 15 mL falcon tube containing 5 mL of sterile water. This suspension was incubated about 4 h at 30 °C with 4 mg $mL^{-1}$ of Lysing Enzymes from *T. harzianum* (Sigma-Aldrich), to allow total digestion of hyphal cells and releasing of spores. The suspension was centrifuged at 3000 rpm for 5 min and the supernatant discarded. The spores were washed twice with 5 mL of sterile spore buffer. Spores were quantified in an improved Neubauer chamber under a microscope and stored at -80 °C in aliquots of 1 mL.

### *3.2.7. Molecular biology procedures*

#### *3.2.7.1. Plasmid DNA preparation from E. coli strains*

For rapid plasmid DNA extraction, the following protocol was used: *E. coli* cells were collected from a fresh culture on agar-solidified LB-amp, resuspended in 200 μL of ultrapure

(UP) $H_2O$ and mixed by vortexing. 200 μL of Solution I containing 1% (w/v) SDS and 0.2 M NaOH were used for cells lysis, followed by 4 times inversion to mix. Afterwards, 200 μL of Solution II, containing 3 M Potassium acetate pH 5.5, were used to neutralize and precipitate proteins. Again the tube was inverted 4 times to mix and incubated for 5 min on ice to enhance precipitation. The suspension was centrifuged for 2 min at 13200 rpm. Then, the supernatant was mixed with 500 μL of 100% isopropanol and centrifuged for 2 min. In the end, the supernatant was removed and the pellet was air dried and dissolved in 30 μL of UP $H_2O$.

To obtain higher quantities and purity of plasmid DNA, the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) was used according to the manufacturer's protocol.

*3.2.7.2. Genomic DNA extraction*

*A. gossypii* genomic DNA was extracted according to the following protocol (Aguiar et al., 2014b). A piece of mycelium was collected from the periphery of a fresh colony grown on agar-solidified AFM with a sterile toothpick to a collection tube containing 200 μL of DNA Extraction Buffer (50 mM NaCl, 1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0 and 0.5% (v/v) Triton X-100) and mixed by vortexing. Next, in a chemical fume hood 100 μL of Phenol:Chloroform:Isoamyl alcohol (25:24:1) were added to this solution that was mixed for 10 seconds on vortex followed by incubation on ice for 10 s. This step was repeated 4 times. After this, the mixture was centrifuged at room temperature for 15 min at 13000 rpm in order to form a system with two phases clearly separated. In the end, 20 μL of the upper phase were diluted in 80 μL of sterile UP $H_2O$.

The AxyPrepTM Multisource Genomic DNA Miniprep Kit (Axygen Biosciences) was used for extraction of genomic DNA from *S. cerevisiae* CEN.PK113-7D. All procedures were performed according to the manufacturer's instructions.

*3.2.7.3. DNA storage*

DNA solutions were stored at -20 or 4 °C in TE buffer containing 10 mM Tris/HCl pH 8.0 and 1 mM EDTA, EB buffer containing 10 mM Tris/HCl pH 8.5, or alternatively in UP $H_2O$.

*3.2.7.4. DNA quantification*

Nucleic acid concentration and purity was determined in a NanoDrop 1000 Spectrophotometer (Thermo Scientific) by loading 2 μl of sample. The concentration in ng $\mu L^{-1}$ was calculated from the absorbance at 260 nm and the sample purity was assessed by the ratio of absorbance at 260 nm and 280 nm. A value of approximately 1.8 for DNA, and 2.0 for RNA, was accepted as indicative of pure nucleic acid solution. Lower values may indicate the presence of protein, phenol or other contaminants. The ratio of absorbance at 260 and 230 nm was used as another measure of nucleic acid purity, which should be in the range of 1.8-2.2 for pure nucleic acid solutions. An appreciably lower ratio may indicate the presence of other contaminants.

*3.2.7.5. Amplification of DNA fragments by Polymerase Chain Reaction (PCR)*

DNA amplification by PCR for subcloning procedures in *E. coli* was performed with the Phusion High-Fidelity DNA polymerase (Finnzymes). The *GUP1* genes from *A. gossypii* ATCC 10895 (*AgGUP1*) and *S. cerevisiae* CEN.PK113-7D (*ScGUP1*) were amplified using the primers listed in Table 3.2 The reaction mixture consisted of 20 μL of 5x Phusion HF buffer, 2 μL of 10 mM dNTPs, 1.5 μL of 20 μM of each corresponding primer, 5 μL of *A. gossypii* ATCC 10895 genomic DNA/0.5 μL of *S. cerevisiae* CEN.PK113-7D genomic DNA, 1 μL of Phusion High-Fidelity DNA Polymerase (2U $\mu L^{-1}$) and UP $H_2O$ to a final volume of 100 μL. The *AgGUP1* and *ScGUP1* amplification was performed concomitantly using the following protocol: initial denaturation at 98 °C during 1 min; 30 cycles of 10 s denaturation at 98 °C, 30 s annealing at 60 °C and 45 s extension at 72 °C; final extension step of 10 min at 72 °C.

DNA amplification by PCR for verification of correct insertion of *AgGUP* and *ScGUP1* into pFMT was performed with Taq DNA polymerase (NZYTech). The reaction mixture consisted of 2 μl of NzyTaq buffer 10x, 0.4 μL of 10 mM dNTPs, 0.6 μL of 50 mM $MgCl_2$, 0.6 μL of 20 μM of each corresponding primer, 2 μL of plasmid DNA extracted through a rapid plasmid DNA extraction method (*3.2.7.*), 0.3 μL of NZYTaq DNA Polymerase (5U $\mu L^{-1}$) and UP $H_2O$ to a final volume of 20 μL. The *AgGUP1* and *ScGUP1* amplification was performed concomitantly using the following protocol: initial denaturation at 95 °C during 5 min; 30

cycles of 30 s denaturation at 95 °C, 30 s annealing at 53 °C and 2 min extension at 72 °C; final extension step of 10 min at 72 °C.

*3.2.7.6. DNA Electrophoresis*

Analysis of DNA fragments was performed by electrophoresis in 1% (w/v) agarose gels dissolved in 1x TAE buffer (prepared from 50x TAE Buffer containing 2 M Tris-base, 50 mM EDTA and pH 8.0 adjusted with acetic acid). 0.006% (v/v) Green Safe Premium (NZYtech) was added to gels for nucleic acid staining. Each DNA sample was mixed with 1x Loading Dye (diluted from a 6x solution containing 25% (w/v) glycerol, 20 mM EDTA and 0.25% (w/v) Bromophenol blue), which was used to allow the visualization of the running velocity and to increase the samples density. Electrophoretic runs were performed at 70-100 V, in 1x TAE buffer. Gels were visualized and photographed in a Molecular Imager ChemiDoc$^{TM}$ XRS + Imaging System (Bio-Rad) and analyzed using the Image Lab 4.0 software.

The DNA molecular weight marker used in agarose gels was NZYDNA Ladder III (NZYTech) (Table 3.3).

**Table 3.3 –** Molecular weight of the bands produced by NZYDNA Ladder III.

| Band | Size (bp) |
|---|---|
| 1 | 10000 |
| 2 | 7500 |
| 3 | 6000 |
| 4 | 5000 |
| 5 | 4000 |
| 6 | 3000 |
| 7 | 2500 |
| 8 | 2000 |
| 9 | 1400 |
| 10 | 1000 |
| 11 | 800 |
| 12 | 600 |
| 13 | 400 |
| 14 | 200 |

*3.2.7.7. DNA purification of PCR products*

PCR products purification was performed using the QIAquick PCR purification Kit (Qiagen) according to the manufacturer's instructions.

*3.2.7.8. DNA purification from agarose gel*

DNA purification from agarose gels was performed using the QIAquick Extraction Gel Kit (Qiagen), according to the manufacturer's instructions.

*3.2.7.9. Enzymatic modification of DNA*

The restriction endonucleases used for plasmid DNA and PCR fragments digestion were purchased from New England Biolabs. All the digestions were performed at 37 °C according to the manufacturer's instructions. For cloning procedures, the digested vectors were dephosphorylated with Fermentas™ Shrimp Alkaline Phosphatase (SAP) to prevent their recircularization. The appropriate amount of SAP (1 unit per pmol of plasmid *termini*) was added to the tube one hour before the end of the restriction reaction. Restriction analyses were also performed for verifying the correct insertion of *AgGUP* and *ScGUP1* into pFMT.

*3.2.7.10. Ligation reactions*

Ligation of DNA fragments to linearized dephosphorylated vectors was performed overnight at 4 °C with T4 DNA Ligase (Promega). The quantity of insert to use in ligation reactions was calculated using the following formula:

ng of insert = ((ng of vector × Kb of insert) ÷ Kb of vector) × insert:vector molar ratio (1)

For all ligations, the amount of vector used was 100 ng and an insert:vector molar ratio of 3.5:1 was used. The DNA ligation mix was completed with 1 μL of the provided 10x Ligase Buffer, 1 U of T4 DNA Ligase and UP $H_2O$ to a final volume of 10 μL.

*3.2.8. Transformation of E. coli cells*

NZY5α Competent Cells (NZYtech) were transformed with the constructs resulting from the ligation reactions through the heat-shock method. This method was performed accordingly the manufacturer's instructions. Briefly, competent cells were thawed on ice and mixed gently. To 100 μL of competent cells, the total volume of the ligation reaction (*3.2.16.*) was added and then the tube was gently tapped to mix. The cells were incubated on ice for 30 min and then heat-shocked for 40 s in a 42 °C water bath. The tubes were placed on ice for 2 min before adding 900 μL of room temperature SOC medium containing 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4.7H_2O$, 10 mM $MgCl_2.6H_2O$ and 20 mM glucose. The cells were incubated at 225 rpm and 37 °C for 1 h, to allow recovering, and then the cell suspension was spread on agar solidified LB-amp plates in appropriate dilutions and incubated overnight at 37 °C.

*3.2.9. Transformation of A. gossypii*

Transformation of *A. gossypii* with the constructs was performed by electroporation. The protocol here used was adapted from Wendland et al. (2000). Two pre-inocula of approximately $5x10^6$ spores were grown for 10-12 h at 30 °C and 200 rpm in 500 mL Erlenmeyer flasks with 100 mL of AFM. The mycelium from both flasks was harvested by vacuum filtration through filter paper in sterile conditions. The mycelium was washed twice with 200 mL of sterile cold water, collected to a 50 mL sterile falcon with 40 mL of AT Buffer containing 25 mM Dithiothreitol (DTT) and 50 mM potassium phosphate buffer (pH 7.5). The resuspended mycelium was incubated for 30 min at 30 °C and 100 rpm. Afterwards, this suspension was filtered again in with the same type of filter paper and washed twice with 40 mL of chilled STM Buffer containing 270 mM sucrose, 10 mM Tris-HCl (pH 7.5) and 1 mM $MgCl_2.6H_2O$. The electrocompetent mycelium was then resuspended in STM buffer at a ratio of 1 mL per 0.3 g of mycelium and preserved on ice until electroporation.

Electroporation was made using Gene Pulser Xcell™ Electroporation System (Bio-Rad) at a voltage of 1500 V, a capacitance of 25 μF and a resistance of 100 Ω, resulting in time constants between 2.1 for both constructs. For this, the following procedures were undertaken: 150 μL of electrocompetent mycelium and 50 μL of DNA solution (5 μg of plasmid DNA) were added to a chilled 0.2 cm electroporation cuvette (Bio-Rad). The cuvette was incubated for 5

min on ice and then placed into the electroporation system for the pulse. Immediately after the pulse, 1 mL of AFM at room temperature was added to the cuvette. Finally, the mycelium suspension was spread over agar-solidified AFM-G418 plates, in different dilutions (20, 50, 200 e *ca.* 900 μL). The plates were incubated at 30 °C for 24-36 h to allow the growth of transformants.

### *3.2.10. Shake-flask cultivations*

All cultivations were performed in an orbital incubator at 30 °C and 200 rpm in 250 mL Erlenmeyer flasks containing 50 mL of medium. Cultivations presented in section *3.3.1.* were performed in AFM or AFMGlyOH supplemented with 0.15% (w/v) glycine and were inoculated with $10^6$ spores of *A. gossypii* ATCC 10895 or *A. gossypii Agura3*. Cultivations presented in section *3.3.4.* were performed in AFMGlyOH supplemented with 0.15% (w/v) and inoculated with $10^6$ or $10^7$ spores of *A. gossypii pFMT_EV*, *pRSAG* and *pRSSG*. Samples were taken to monitor growth by measuring the optical density at 600 nm ($OD_{600nm}$) and to collect the supernatant by centrifugation (10 min at 13000 rpm and 4°C) for further analyses (riboflavin estimation, quantification of extracellular invertase activity and quantification of glycerol by high performance liquid chromatography (HPLC). Cell dry weight was determined at the end of the cultivations.

### *3.2.11. Osmotic stress assays*

Agar-solidifed AFM-G418 plates containing 30 mM glycerol and different osmotic stress agents were centrally inoculated with $10^5$ spores of *A. gossypii pFMT_EV*, *pRSAG* and *pRSSG*. The stocks of spores for the inoculum, which are maintained in sterile spore buffer, were centrifuged 5 min at 3000 rpm and at room temperature, the supernatant was discarded and the spores washed 2 times in sterile spore buffer without glycerol. Finally, the spores were resuspended in sterile spore buffer without glycerol. Osmotic stress was applied using KCl (0, 0.4, 0.6, 0.8 and 1 M), NaCl (1M) and sorbitol (1 and 1.5 M). For testing osmotic stress response in the absence of glycerol, glycerol was not added to the medium. The colony radial growth of each transformant was measured over time. Each assay was performed in triplicate for each strain.

*3.2.12. Analytical procedures*

Cell dry weight was determined by collecting the mycelium by centrifugation (10 min at 13000 rpm and 4°C) or filtration trough filter paper into a pre-weighed dried tube, drying overnight at 105 °C and weighing.

Colony radial growth was determined by measuring over time the diameter of colonies grown on agar-solidified medium in 90 mm diameter Petri dishes.

For riboflavin estimation, the collected supernatant's optical density was measured at 445 nm wavelength, and converted into riboflavin concentration using a standard curve ($R^2$ = 0.996) constructed with pure riboflavin standards (Sigma-Aldrich) of 15, 30, 45, 60 and 90 mg $L^{-1}$. Riboflavin concentration (mg $L^{-1}$) was calculated by the following conversion factor: 0.0134 mg (optical density units $L^{-1}$)$^{-1}$. Specific riboflavin production was calculated as $mg_{riboflavin}\ g_{mycelium}^{-1}$.

Glycerol was quantified by HPLC using a Varian MetaCarb 87H column, eluted with 0.005 M sulfuric acid at 60 °C and at a flow rate of 0.7 mL $min^{-1}$. The peaks corresponding to glycerol were detected using a refractive index detector.

*3.2.12.1. Extracellular invertase activity*

To quantify extracellular invertase activity 50 μL of culture supernatant were incubated with 50 μL of 0.2 M Sucrose (Applichem) and 100 μL of 0.1 M Na-Phosphate-citrate Buffer pH 6.0 for 90 min at 40 °C. The reaction was stopped by boiling the samples at 100 °C for 10 min. As a blank an extra assay was made in which the culture supernatant was only added to the mixture immediately before the inactivation at 100 °C. After inactivation, the released reducing sugars were measured by 3,5-Dinitrosalicylic acid (DNS) assay. Reducing sugars quantification by DNS was performed by addition of 90 μL of each sample to 90 μL of DNS, containing 1% (w/v) DNS, 0.4 N NaOH and 30% (w/v) potassium sodium tartrate tetrahydrate, in a 2 mL collection tube. Then, this mixture was incubated for 5 min at 100 °C. After this, 900 μL of distilled water was added to the mixture and the optical density was measured at 540 nm. Reducing sugars concentration was obtained using a standard curve ($R^2$ = 0.991) constructed with pure glucose standards of 0.5, 1, 2, 3 and 5 g $L^{-1}$. Reducing sugars concentration (g $L^{-1}$) was calculated by the following conversion factor: 0.2286 g (optical

density units $L^{-1})^{-1}$. One enzymatic unit (U) was defined as the amount of enzyme that hydrolyzed sucrose to liberate 1 µmol of glucose per minute at 40 °C and pH 6.

*3.2.12.2. Bioinformatics analysis*

PlasmaDNA software (Angers-Loustau et al., 2007) was used for *in silico* designing of cloning procedures performed in this chapter, including plasmid constructions. The deduced amino acid sequence encoded by the putative *GUP1* gene from *A. gossypii* (AFL067W; 1737 bp; GenBank accession no. AAS53305.2) and the *GUP1* gene from *S. cerevisiae* CEN.PK113-7D (YGL084C; 1683 bp; GenBank accession no. EIW10273.1) were aligned in the Clustal Omega server (Sievers et al., 2011; http://www.ebi.ac.uk/Tools/msa/clustalo/) using the default parameters. Predictive transmembrane domains (TMs) for AgGup1p and ScGup1p were obtained using TMHMM Server v.2.0 (Krogh et al., 2001) (http://www.cbs.dtu.dk/services/TMHMM/).

*3.2.13. Statistical analysis*

The data from comparative analyses of riboflavin production (*3.3.1.*), colony radial growth under osmotic stress conditions (*3.3.3.*) and glycerol consumption (*3.3.4.*) by *A. gossypii pFMT_EV*, *pRSAG* and *pRSSG* were analyzed in GraphPad Prism® (GraphPad Software, Inc.). One-way ANOVA, two-way ANOVA and Tukey's multiple comparisons test were used to find means significantly different from each other.

## 3.3. Results and discussion

*3.3.1. Riboflavin production by different A. gossypii strains using glycerol as carbon source*

The production of riboflavin by *A. gossypii* has been extensively studied in different carbon sources. Glucose is the most commonly used carbon source at laboratory scale for testing overproducing strains, but the industrial process of riboflavin production by *A. gossypii* is established from plant oils, as they support higher production levels (Stahmann et al., 2000; Lim et al., 2001). Some industrial wastes containing plant oils have also been tested, to further improve the profitability of this biotechnological process (Ming et al., 2003; Park et al., 2004a;

Park et al., 2004b; Park et al., 2007; Tajima et al., 2009). Glycerol is the principal by-product of the continuously growing industry of biodiesel. However, in *A. gossypii*, glycerol is a very poorly explored substrate that was only tested for ethanol and recombinant protein production (Ribeiro et al., 2012; Aguiar et al., 2014a; Magalhães et al. 2014). This abundant and cheap carbon source had not yet been explored for riboflavin production. Therefore, to evaluate the production of riboflavin by *A. gossypii* from glycerol, some preliminary shake-flask cultivations were performed with two different *A. gossypii* strains (ATCC 10895 and *Agura3* (Fig. 3.1)) in AFMGlyOH (pure glycerol as carbon source) and also in AFM (glucose as carbon source), for comparison. For these experiments both media were supplemented with glycine.

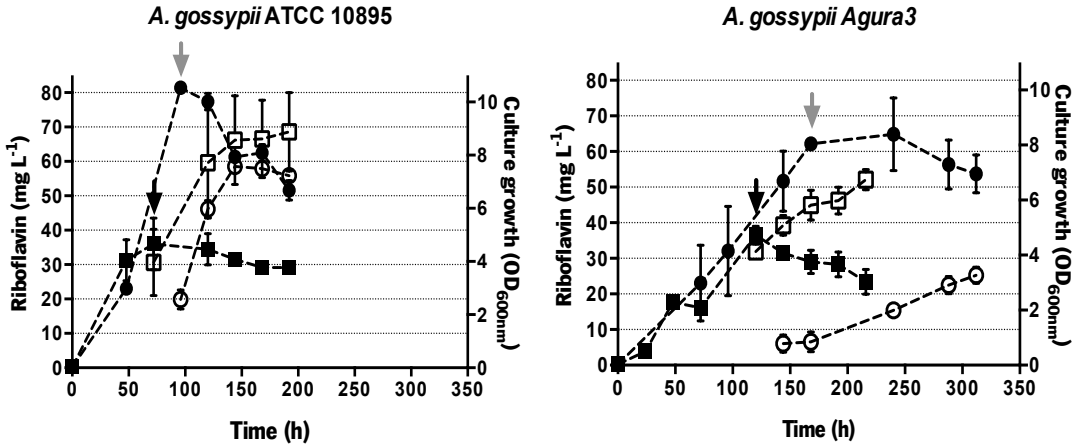


**Figure 3.1 –** Culture growth and riboflavin production by *A. gossypii* ATCC 10895 and *Agura3*. Squares (■) correspond to AFM and circles (●) correspond to AFMGlyOH. Both media were supplemented with glycine. Filled symbols represent culture growth ($OD_{600nm}$) and empty symbols represent riboflavin production (mg $L^{-1}$). The beginning of the stationary phase is indicated by black arrow in AFM cultivations and grey arrow in AFMGlyOH cultivations. Data represent average ± standard error of the mean (SEM) from three biological replicates. Where not seen, error bars were smaller than the symbol.

Riboflavin is considered a pseudo-secondary metabolite in *A. gossypii*, as its overproduction starts when growth stops (*i.e.* stationary phase) (Stahmann et al, 2000; Kato and Park, 2012). In the shake-flask cultivations performed here, at the beginning of the stationary phase the amount of riboflavin produced by *A. gossypii* ATCC 10895 in AFM was about 1.5-fold higher than in AFMGlyOH, whereas in *A. gossypii Agura3* it was approximately 4.9-fold higher than in AFMGlyOH (Fig. 3.1). Nevertheless, the differences observed in volumetric riboflavin production, as well as in the maximum volumetric concentration achieved in AFM and AFMGlyOH at this time point (Table 3.4), were only statistically significant ($p < 0.05$) for *A. gossypii Agura3*. By safeguarding the growth pattern divergences of these strains

in the conditions tested through the normalization of the volumetric riboflavin production with the produced biomass (Table 3.4), the same tendency for higher levels of riboflavin production in AFM than in AFMGlyOH were still observed, but no longer statistically significant ($p > 0.05$).

**Table 3.4 –** Riboflavin production parameters of *A. gossyppi* ATCC 10895 and *A. gossyppi Agura3* in AFM and AFMGlyOH cultivations. *A. gossypii* ATCC 10895 values for maximum production correspond to 120 and 144 h in AFM and AFMGlyOH, respectively. *A. gossypii Agura3* values for maximum production correspond to 144 and 312 h in AFM and AFMGlyOH, respectively. These values are matched with the corresponding values of volumetric and specific riboflavin concentration. Data represent average ± standard error of the mean (SEM) from three biological replicates.

| | **AFM** | **AFMGlyOH** |
|---|---|---|
| *A. gossypii* ATCC 10895 | | |
| Specific riboflavin concentration at the beginning of the stationary phase (mg $g_{mycelium}^{-1}$) (Time) | 7.4 ± 2.2 (72 h) | 4.5 ± 0.6 (96 h) |
| Volumetric riboflavin concentration (mg $L^{-1}$) (Time) | 59 ± 16 (120 h) | 58 ± 1.3 (144 h) |
| Specific riboflavin concentration (mg $g_{mycelium}^{-1}$) (Time) | 14 ± 4 (120 h) | 13 ± 0 (144 h) |
| Specific riboflavin productivity (mg $g_{mycelium}^{-1}$ $h^{-1}$) | 0.12 ± 0.03 | 0.092 ± 0.002 |
| *A. gossypii Agura3* | | |
| Specific riboflavin concentration at the beginning of the stationary phase (mg $g_{mycelium}^{-1}$) (Time) | 8.9 ± 0.7 (120 h) | 2.3 ± 1.3 (168 h) |
| Volumetric riboflavin concentration (mg $L^{-1}$) (Time) | 39 ± 3 (144 h) | 25 ± 2 (312 h) |
| Specific riboflavin concentration (mg $g_{mycelium}^{-1}$) (Time) | 11 ± 0 (144 h) | 7.8 ± 1.6 (312 h) |
| Specific riboflavin productivity (mg $g_{mycelium}^{-1}$ $h^{-1}$) | 0.076 ± 0.003 | 0.025 ± 0.005 |

Concerning the differences observed in riboflavin production between strains, the volumetric production values obtained were generally higher in *A. gossypii* ATCC 10895 than in *A. gossypii Agura3* (Fig. 3.1). However, by comparing the production levels of the two strains in the same condition, *i.e.* by matching production levels of these strains in AFM and AFMGlyOH separately, this tendency for higher volumetric riboflavin production by *A. gossypii* ATCC 10895

was not statistically significant ($p > 0.05$) according to the ordinary one-way ANOVA and Tukey's multiple comparison tests. In fact, *A. gossypii Agura3* was the strain that reached the highest specific riboflavin production levels at the beginning of the stationary phase (8.91 ± 0.73 $mg_{riboflavin}\ g_{mycelium}^{-1}$ in AFM), a tendency that is consistent with the results discussed in Chapter 2. Nonetheless, the specific riboflavin production obtained by both *A. gossypii* strains in AFM was not very different from that reported for an overproducing itaconate resistant strain (*ITAGS01*) in a medium containing soybean oil as carbon source and also supplemented with glycine (10.5 $mg_{riboflavin}\ g_{mycelium}^{-1}$; Förster et al., 1999). It is worth noting that the specific riboflavin production obtained with *A. gossypii* ATCC 10895 in AFM at the beginning of the stationary phase was also very consistent with previously reported for the same strain in similar medium (7.5 $mg_{riboflavin}\ g_{mycelium}^{-1}$ after 72 h of cultivation; Monschau et al., 1998).

A drawback of using glycerol for riboflavin production by *A. gossypii* is that the stationary phase on this carbon source is reached later than on glucose, which affects riboflavin productivity (Fig. 3.1, Table 3.4). *A. gossypii* ATCC 10895 reached the stationary phase around 72 h (3 days) in AFM and around 96 h (4 days) in AFMGlyOH. In turn, *A. gossypii Agura3* took more time than its parent strain, only reaching the stationary phase after 120 h (5 days) in AFM and 168 h (7 days) in AFMGlyOH. Nonetheless, the maximum cell densities reached by both *A. gossypii* strains were higher in AFMGlyOH than in AFM (Fig. 3.1). The maximum riboflavin production levels (Table 3.4) followed the same trend. For *A. gossypii* ATCC 10895, the maximum levels of productivity were obtained 24 h later in AFMGlyOH than in AFM (144 and 120 h, respectively), whereas for *A. gossypii Agura3* the difference was more noteworthy, being the maximum levels of productivity detected in AFMGlyOH only 168 h after they were obtained in AFM (Table 3.4).

Ribeiro et al. (2012) had already reported lower colony radial growth rate for *A. gossypii* ATCC 10895 (and three other *A. gossypii* strains) on defined Verduyn medium containing glycerol as sole carbon source than in the same medium containing glucose as sole carbon source. Nonetheless, the specific growth rates achieved by *A. gossypii* strains in complex medium with glycerol were higher than those obtained for *S. cerevisiae*, indicating that *A. gossypii* is more efficient in utilizing glycerol as carbon source than its closely related yeast *S. cerevisiae* (Ribeiro et al., 2012).

Comparison between strains showed that *A. gossypii Agura3* grew slower than *A. gossypii* ATCC 10895 in both carbon sources. However, *A. gossypii Agura3* reached maximum

cell densities ($OD_{600nm}$) similar to those of *A. gossypii* ATCC 10895 in AFM, although in AFMGlyOH the maximum cell densities achieved by *A. gossypii Agura3* were lower (Figs. 3.1.). These growth limitations of *A. gossypii Agura3* may be explained by its uridine/uracil auxotrophy, which may be overcome by increasing the exogenous availability of uridine (as explored in Chapter 2). Given that uridine/uracil supplementation was also shown to significantly affect riboflavin production by this strain (Chapter 2), further investigation of riboflavin production by *A. gossypii Agura3* in submerged cultures is required in order to assess if higher production levels can be achieved.

These results provide important insights regarding the potential of using glycerol for riboflavin production as they settle a basis of comparison with a commonly used carbon source for riboflavin production (glucose). Detection of riboflavin production in glycerol cultivations, at production levels comparable to those obtained in glucose, indicates that glycerol is a suitable carbon source for riboflavin production in *A. gossypii*. However, we identified the slower growth on glycerol as the main bottleneck of the process, which could influence riboflavin production, specifically the productivity of the process. Therefore, the next step for glycerol exploration by *A. gossypii* underwent the genetic manipulation of this fungus in order to increase its glycerol uptake.

### *3.3.2. Construction of recombinant A. gossypii strains overexpressing the native or the S. cerevisiae GUP1 genes*

The *S. cerevisiae GUP1* is a well-studied gene that is reported to play important roles in several cellular functions. Among these, its role in the active uptake of glycerol was the first to be described (Holst et al., 2000) and overexpression of this gene in engineered *S. cerevisiae* strains successfully improved their glycerol consumption (Yu et al. 2010a; 2010b; Jung et al., 2011). In *A. gossypii*, the glycerol uptake system has been studied under hyperosmotic stress conditions (Förster et al., 1998). However, the glycerol metabolism in *A. gossypii* has been poorly explored, with one gene associated with glycerol transport (*AgFPS1*; Geijer et al., 2012) experimentally characterized in *A. gossypii* so far.

*AgGUP1* is a syntenic homolog of *ScGUP1*. The a.a. sequence of AgGup1p (578 a.a.) and ScGup1p (560 a.a.) share a percent identity matrix of 63% and a similarity of 76% (Fig. 3.2). Moreover, AgGup1p and ScGup1p are predicted to be transmembrane proteins with 11

and 10 putative transmembrane domains (TMs), respectively (Fig. 3.2) (Krogh et al., 2001). TMs correspond to a set of hydrophobic residues that are connected to the cell membrane and are an integral part of the protein function, namely two-way membrane transport (Ramasarma et al., 2012). These predictive results have already been reported for ScGup1p (Holst et al., 2000).

```
AgGUP1      MLHSLWDATLTCVSRLFSLEALDARLAPSNDPKIRQRITMQATPSRWKTAEFKFYYAVFA        60
ScGUP1      ------MSLISILSPLITSEGLDSRIKPSP----KKDASTTTKPSLWKTTEFKFYYIAFL        50
                   : :: :* *:: *.**:*: **     ::  :  :.** ***:****** .*

AgGUP1      IAVPLMFKAAVDATNAWNPNYKKIEPLLDQGWLFGRKVDNSDSQYGFFRGNFALLFALAA        120
ScGUP1      VVVPLMFYAGLQASSPENPNYARYERLLSQGWLFGRKVDNSDSQYRFFRDNFALLSVLML        110
            :.***** *.::*:.  **** : * **.*************** *** ***** .*

AgGUP1      GHLLTKRVALMLFPIQKLTFDLYFGLFFIVVAHGVNTFRVLIHVVIMFAIARILRNQRKA        180
ScGUP1      VHTSIKRIVLYSTNITKLRFDLIFGLIFLVAAHGVNSIRILAHMLILYAIAHVLKNFRRI        170
             *   **:.*    * ** *** ***:*:*.*****::*:* *::*::***::*:* *:

AgGUP1      ATILCWVYGIGTLFFNSKYDTYPFGDVLPFLSFLDTAYKGLIKRWDVFYNFTLLRILSHN        240
ScGUP1      ATISIWIYGISTLFINDNFRAYPFGNICSFLSPLDHWYRGIIPRWDVFFNFTLLRVLSYN        230
            ***  *:***.***:*.:: :****::  *** **  *:*:* *****:******:**:*

AgGUP1      LDYLEKWSDEPQQEASRSVSPSQEFDTFKDGKCPELLDERSRLTAPHPISDYNMANYLAY        300
ScGUP1      LDFLERWENLQKKKSP-----SY---ESKEAKSAILLNERARLTAAHPIQDYSLMNYIAY        282
            **:**:*.:  ::::      *      *:.*.  **:**:**** ***.**.: **:**

AgGUP1      VAYTPLYIAGPILTFNDYLYQTRRTLPSITPSRTFYYALNFLCCIMTMEVVLHFIYVVAA        360
ScGUP1      VTYTPLFIAGPIITFNDYVYQSKHTLPSINFKFIFYYAVRFVIALLSMEFILHFLYVVAI        342
            *:****:*****:*****:**:::*****. .  ****:.*: .:::**.:***:****

AgGUP1      AKAKAWEGDTPFQLSMIGLFNLNIIWLKLLIPWRFFRLWALIDGIDPPENMIRCVDNNYS        420
ScGUP1      SKTKAWENDTPFQISMIGLFNLNIIWLKLLIPWRLFRLWALLDGIDTPENMIRCVDNNYS        402
            :*:**** *****:*******************:******:**** *************

AgGUP1      TLAFWRAWHRSYNKWVVRYIYVPLGGSTNRILTSLAVFSFVAIWHDIELKLLVWGWLIVL        480
ScGUP1      SLAFWRAWHRSYNKWVVRYIYIPLGGSKNRVLTSLAVFSFVAIWHDIELKLLLWGWLIVL        462
            :********************:*****.**:**********************:*******

AgGUP1      FLLPEIVASSVLSAYSNRVWFRFLCSVGAIVNIWMMMIANLVGFCLGTDGTITLLKDMFS        540
ScGUP1      FLLPEIFATQIFSHYTDAVWYRHVCAVGAVFNIWVMMIANLFGFCLGSDGTKKLLSDMFC        522
            ******.*:.::* *:: **:*.:*:***:.***:******.*****:*** .**.***.

AgGUP1      TVKGISFFIVANGALFVGTQTMFEQREHEKRQGIYVKC*     578
ScGUP1      TVSGFKFVSLASVSLFIAVQIMFEIREEEKRHGIYLKC*     560
            **.*:.*. :*. :**:..* *** **.***:***:***
```

**Figure 3.2 –** Bioinformatics analyses and comparison of putative AgGup1p and ScGup1p sequences. Amino acid sequence alignment of AgGup1p and ScGup1p using Clustal Omega server (Sievers et al., 2011; http://www.ebi.ac.uk/Tools/msa/clustalo/; default parameters). Asterisk (*) indicates positions that have a single, fully conserved residue. Colon (:) indicates conservation between groups of strongly similar properties. Period (.) indicates conservation between groups of weakly similar properties. Predictive transmembrane domains are underlined and were obtained using TMHMM Server v.2.0 (Krogh et al., 2001; http://www.cbs.dtu.dk/services/TMHMM/).

Although the similarities found between AgGup1p and ScGup1p indicate related functions, other homolog proteins involved in glycerol transport (AgFps1p and ScFps1p) have been demonstrated to exhibit considerable functional differences related to their different transmembrane core regions (Geijer et al., 2012). Given that AgGup1p is predicted to have an

extra TM compared to ScGup1p, one should expect functional differences between these homologs as well. Thus, to evaluate the effect of *GUP1* overexpression in *A. gossypii* and determine its efficiency in improving the uptake of glycerol by this fungus, both native (*AgGUP1*) and heterologous (*ScGUP1*) genes were separately overexpressed using the multi-copy vector pFMT under the regulation of the constitutive *A. gossypii* translation elongation factor (*AgTEF*) promoter, which has been reported to drive high gene expression in *A. gossypii* (Steiner and Philippsen, 1994; Magalhães et al., 2014).

The vectors pRSAG (*AgGUP1* under the control of the *AgTEF* promoter) and pRSSG (*ScGUP1* under the control of the *AgTEF* promoter) were constructed as follows. The coding region of *AgGUP1* was amplified from *A. gossypii* ATCC 10895 genomic DNA (*3.2.7.5*) with the primers AgGUP1_Fw and AgGUP1_Rv and the coding region of *ScGUP1* was amplified from *S. cerevisiae* CEN.PK113-7D genomic DNA (*3.2.7.5*) with the primers ScGUP1_Fw and ScGUP1_Rv (Table 3.2, Fig. 3.3). To clone the *AgGUP1* into pFMT (*3.2.3.*), restriction sites for *Eag*I and *Kpn*I were added through the AgGUP1_Fw and AgGUP1_Rv primers, respectively. For *ScGUP1* cloning, restriction sites for *EcoR*I and *Xho*I were introduced through the ScGUP1_Fw and ScGUP1_Rv primers, respectively.

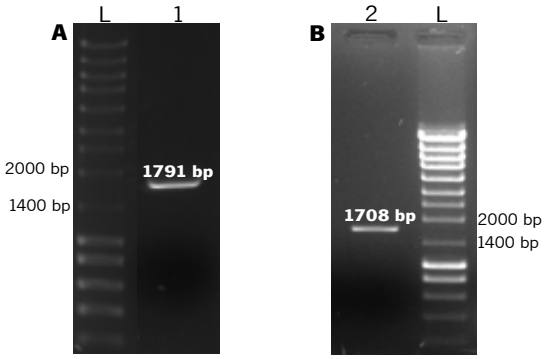


**Figure 3.3 –** PCR fragments of *AgGUP1* amplified from *A. gossypii* ATCC 10895 with primers AgGUP1_Fw and AgGUP1_Rv (A) and of *ScGUP1* amplified from *S. cerevisiae* CEN.PK113-7D with primers ScGUP1_Fw and ScGUP1_Rv (B). Lane L: molecular weight marker; lane 1: *AgGUP1* PCR product; lane 2: *ScGUP1* PCR product.

After digestion of the purified PCR products (*3.2.7.7.*) and pFMT with the restriction enzymes indicated above (*3.2.7.9.*) and ligation of the fragments (*3.2.7.10.*), the constructs were cloned into *E. coli* (*3.2.8.*) and the transformants were screened using two different procedures. First, plasmid DNA obtained from different colonies by the rapid extraction method (*3.2.7.1*), was run in agarose gel (*3.2.7.6.*) against a negative control (the empty vector (EV) pFMT) to search for plasmids with higher molecular weight than the negative control (Fig. 3.4).

From this screening, 2 plasmids containing the *AgGUP1* (constructs 1 and 7) and 3 plasmids containing the *ScGUP1* (constructs 5, 9 and 15) were chosen and confirmed by PCR (*3.2.7.5*), using the same primers used for the corresponding genes amplification (Fig. 3.5). Subsequently, correct insertion of *AgGUP1* in construct 7 (named pRSAG vector) was confirmed by digestion with *Eag*I and *Kpn*I (Fig. 3.5) and of *ScGUP1* in construct 9 (named pRSSG vector) was confirmed by digestion with *EcoR*I and *Xho*I (Fig. 3.6). Finally, the orientation and sequence of *AgGUP1* and *ScGUP1* were successfully confirmed by sequencing using the primers AgGUP1_Fw, PGK_Rv, pMI_MCS_Fw for pRSAG and ScGUP1_Fw, PGK_Rv, pMI_MCS_Fw for pRSSG.

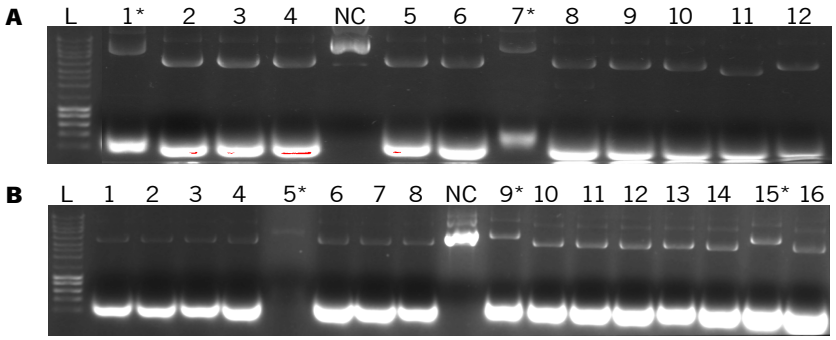


**Figure 3.4 –** Screening of colonies transformed with *AgGUP1* (A) or *ScGUP1* (B) inserted into pFMT. Lane L: molecular weight marker; NC: pFMT_EV (negative control). Numbers with an asterisk (*) represent the constructs chosen for the next confirmation step.

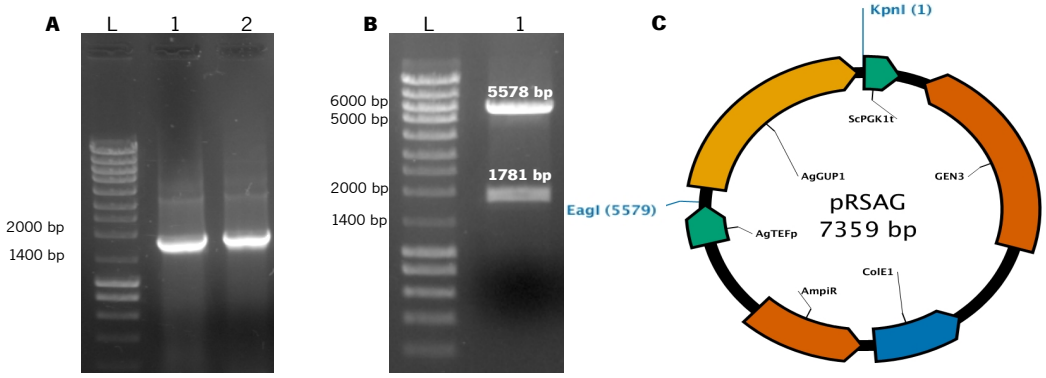


**Figure 3.5 –** Confirmation of correct insertion of *AgGUP1* into pFMT. (A) PCR fragments amplified from the selected constructs 1 (lane 1) and 7 (lane 2) with the primers AgGUP1_Fw and AgGUP1_Rv. Expected amplicon size of 1791 bp. Lane L: molecular weight marker. (B) Restriction analysis of the selected construct 7 with *Eag*I and *Kpn*I (lane 1). Expected fragment sizes indicated in the image. Lane L: molecular weight marker. (C) Representation of the final vector, which was named pRSAG.

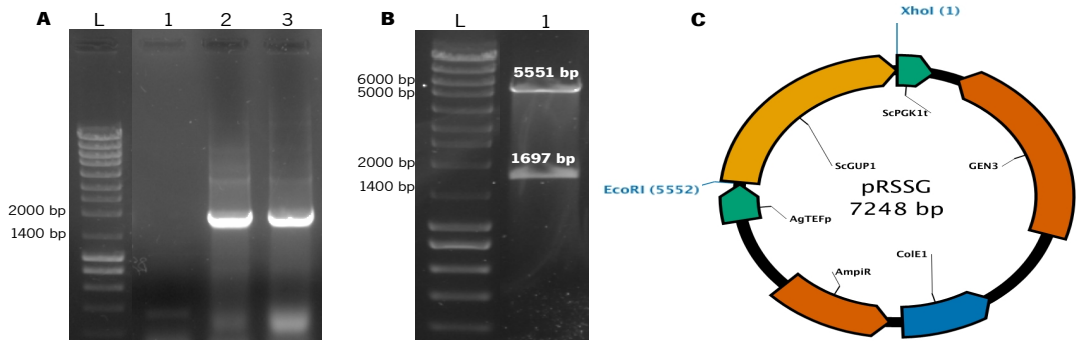


**Figure 3.6 –** Confirmation of correct insertion of *ScGUP1* into pFMT. (A) PCR fragments amplified from the selected constructs 5 (lane 1), 9 (lane 2) and 15 (lane 3) with the primers ScGUP1_Fw and ScGUP1_Rv. Expected amplicon size of 1708 bp. Lane L: molecular weight marker. (B) Restriction analysis of the selected construct 9 with *EcoR*I and *Xho*I (lane 1). Expected fragment sizes indicated in the image. Lane L: molecular weight marker. (C) Representation of the final vector, which was named pRSSG.

*A. gossypii* ATCC 10895 was then transformed with the constructed vectors (*3.2.9.*) with an efficiency of 16 transformants $\mu g^{-1}$ DNA for pRSAG and 61 transformants $\mu g^{-1}$ DNA for pRSSG. Transformants were selected on agar-solidified AFM-G418 and then transferred to agar-solidified AFMGlyOH-G418 for screening of those with faster radial growth. One transformant of each new strain (*A. gossypii pRSAG* and *A. gossypii pRSSG*) was chosen for the experiments performed in the next sections.

### *3.3.3. Growth characterization of A. gossypii pRSAG and A. gossypii pRSSG under hyperosmotic stress conditions*

*ScGUP1* has been determined to be involved in *S. cerevisiae*'s cell recovery from hyperosmotic stress when glycerol is present in the medium (Holst et al., 2000). Moreover, engineered *S. cerevisiae* strains overexpressing *GUP1* present enhanced resistance to hyperosmotic stress (Holst et al., 2000; Yu et al., 2010b). Therefore, to test whether overexpression of *GUP1* leads in *A. gossypii* to a glycerol-dependent improved resistance to hyperosmotic stress, the *A. gossypii pRSAG* and *pRSSG* transformants and corresponding empty vector control strain (*A. gossypii pFMT_EV*) were grown on agar-solidified AFM-G418 supplemented with 30 mM glycerol and different concentrations of KCl (0.4, 0.8 and 1 M) or sorbitol (1 M and 1.5 M) (Fig. 3.7). The duration of the experiment was determined by the time that the transformants growing under the control condition (without addition of KCl or sorbitol) took to reach the stationary phase (*3.2.12.*) in 9 mm plates.

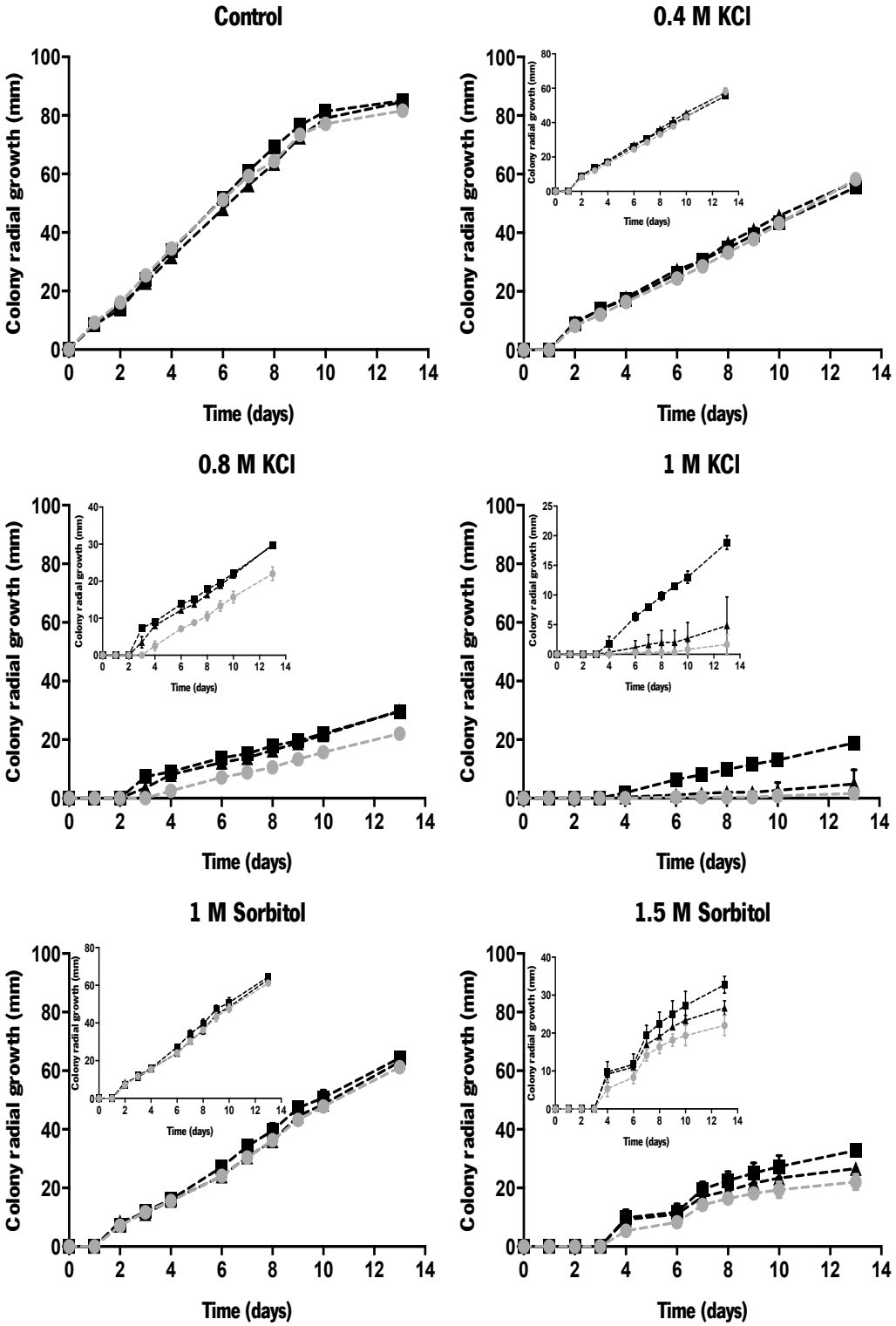


**Figure 3.7 –** Colony radial growth profile of *A. gossypii* transformants grown under different osmotic conditions: *A. gossypii pFMT_EV* (●), *A. gossypii pRSAG* (■) and *A. gossypii pRSSG* (▲) strains. Control indicates assays performed without addition of KCl or sorbitol. The small graphics presented by condition represent the same data but with smaller Y axis scales, to allow a better comparison between strains within each condition tested. Data represent average ± standard error of the mean (SEM) from three biological replicates. Where not seen, error bars were smaller than the symbol.

Increasing concentrations of KCl led to increasingly longer lag phases and to gradual decreasing of the relative growth of all *A. gossypii* transformants tested (Fig. 3.7, Table 3.5).

**Table 3.5 –** Growth parameters of *A. gossypii* transformants grown on media containing various KCl concentrations. Asterisk (*) indicates assays performed without the addition of 30mM glycerol to the media. Relative growth was calculated from the maximum colony radial growth after 13 days under each condition relative to the corresponding control condition (no KCl addition) for that strain. Data for maximum colony radial growth after 13 days represent average ± standard error of the mean (SEM) from three biological replicates.

| | **KCl concentration (M)** | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **0.4** | **0.8** | **1** | **0.8*** | **1*** |
| *A. gossypii pFMT_EV* | | | | | | |
| Lag phase (days) | 0 | 1 | 3 | 5 | – | – |
| Maximum colony radial growth (mm) | 81.7 ± 0.9 | 58.3 ± 2.0 | 22.0 ± 1.8[a] | 1.67 ± 1.67[b] | 0 | 0 |
| Relative Growth (%) | 100 | 71 | 27 | 2 | 0 | 0 |
| *A. gossypii pRSAG* | | | | | | |
| Lag phase (days) | 0 | 1 | 2 | 3 | – | – |
| Maximum colony radial growth (mm) | 85.0 ± 0.0 | 55.5 ± 0.3 | 29.7 ± 0.9[c] | 18.8 ± 2.0[d] | 0 | 0 |
| Relative Growth (%) | 100 | 65 | 35 | 22 | 0 | 0 |
| *A. gossypii pRSSG* | | | | | | |
| Lag phase (days) | 0 | 1 | 2 | 3 | – | – |
| Maximum colony radial growth (mm) | 84.7 ± 0.3 | 57.5 ± 2.5 | 29.8 ± 0.7[c] | 4.83 ± 4.83[b] | 0 | 0 |
| Relative Growth (%) | 100 | 68 | 35 | 6 | 0 | 0 |

Maximum Colony Radial Growth (0.8 M KCl): a,c – differ significantly ($p < 0.01$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3). Maximum Colony Radial Growth (1 M KCl): b,d – differ significantly ($p < 0.05$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3).

Exposure to 0.4 M KCl did not lead to any differences in the growth profile between transformants, which showed similar growth parameters under these conditions (Table 3.5). However, at higher osmotic conditions (0.8 and 1 M KCl), the strains overexpression *GUP1* presented better growth phenotypes than the empty vector control strain. In the presence of 0.8 M KCl both *A. gossypii pRSAG* and *pRSSG* strains showed similar growth phenotypes.

Compared to the control strain, the lag phase of these strains was shorter (2 days for *pRSAG* and *pRSSG* vs 3 days for the control) and their relative growth higher (35% for overexpressing strains, contrasting with 27% for the control strain; Table 3.5). Moreover, the maximum colony radial growth of *A. gossypii pRSAG* (29.7 ± 0.9 mm) and *A. gossypii pRSSG* (29.8 ± 0.7 mm) after 13 days differed significantly ($p < 0.01$) from that of the control strain (22.0 ± 1.8 mm). In the presence of 1 M KCl, the lag phase of both *GUP1* overexpressing strains continued to be shorter than that of the control strain (3 and 5 days, respectively), but only *A. gossypii pRSAG* (18.8 ± 2.0 mm) presented significantly improved growth ($p < 0.05$) in comparison with *A. gossypii pRSSG* (4.83 ± 4.83 mm) and the control strain (1.67 ± 1.67 mm). This hyperosmotic condition led to nearly 100% reduction of growth in *A. gossypii pRSSG* and control strain.

High salinity conditions (0.8 and 1 M NaCl) were already reported to lead to an almost total reduction in *A. gossypii* growth (Förster et al., 1998; Nikolaou et al., 2009). Moreover, the most commonly used salt for osmotic stress experiments, NaCl, was found to be more toxic to *A. gossypii* than KCl, reducing approximately 2-fold more its growth rate and final growth than KCl at similar osmolality (Förster et al., 1998). Nikolaou et al. (2009) later confirmed this marked toxic effect of NaCl, identifying *A. gossypii* together with *Saccharomyces pombe* as the most sensitive fungal species (among 14 species tested) to this ionic stress agent. This particular sensitivity to NaCl was also tested in *A. gossypii pRSAG* and *pRSSG* strains, using NaCl (1 M) instead of KCl in AFM-G418 plates with 30 mM of glycerol, but none of the strains tested were able to grow (data not shown), indicating that *GUP1* overexpression did not produce any favorable effect under this condition.

We also investigated whether *GUP1* overexpression led to an improved phenotype under other osmotic stress conditions. For this, the same plate tests were performed with 1 and 1.5 M sorbitol, which causes non-ionic hyperosmotic stress. The effect of sorbitol on the *A. gossypii* transformants growth was much less pronounced than that caused by KCl (Fig. 3.7). In the presence of 1 M sorbitol, all the transformants presented not very long lag phases (only 1 day) and final relative growth of only about 74-75% in comparison with the corresponding strain in control conditions. In 1.5 M sorbitol, the differences between strains were more noteworthy. Both *A. gossypii pRSAG* and *pRSSG* strains showed slightly better growth profiles than the control strain (Fig. 3.7, Table 3.6). Still, this tendency was not statistically significant ($p > 0.05$).

**Table 3.6 –** Growth parameters of *A. gossypii* transformants grown on media containing various sorbitol concentrations. Relative growth was calculated from the maximum colony radial growth after 13 days under each condition relative to the corresponding control condition (no sorbitol addition) for that strain. Data for maximum colony radial growth after 13 days represent average ± standard error of the mean (SEM) from three biological replicates.

| | **Sorbitol concentration (M)** | | |
|---|---|---|---|
| | **Control** | **1** | **1.5** |
| *A. gossypii pFMT_EV* | | | |
| Lag phase (days) | 0 | 1 | 3 |
| Maximum colony radial growth (mm) | 81.7 ± 0.9 | 61.4 ± 1.2 | 22.0 ± 2.6 |
| Relative Growth (%) | 100 | 75 | 27 |
| *A. gossypii pRSAG* | | | |
| Lag phase (days) | 0 | 1 | 3 |
| Maximum colony radial growth (mm) | 85.0 ± 0.0 | 64.8 ± 1.3 | 32.8 ± 1.8 |
| Relative Growth (%) | 100 | 76 | 39 |
| *A. gossypii pRSSG* | | | |
| Lag phase (days) | 0 | 1 | 3 |
| Maximum colony radial growth (mm) | 84.7 ± 0.3 | 63.0 ± 1.5 | 26.7 ± 1.9 |
| Relative Growth (%) | 100 | 74 | 32 |

The non-ionic agent sorbitol caused lesser osmotic pressure than the ionic agents, being the percentages of relative *A. gossypii* growth obtained for 1.5 M sorbitol similar to those obtained for 0.8 M KCl. This is in line with previous studies where both *S. cerevisiae* and *A. gossypii* were shown to be less sensitive to sorbitol than to NaCl (Nikolaou et al., 2009). Even a *S. cerevisiae* strain defective on *GUP1* was almost not affected by 1.5 M sorbitol, unlike the observed for 1 M NaCl or KCl (Holst et al., 2000). Förster et al. (1998) also reported a stronger effect of ionic than non-ionic osmotic stress in *A. gossypii* growth, using NaCl/KCl or sorbitol/mannitol (an isomer of sorbitol). Additionally, the same authors demonstrated that *A. gossypii* could uptake exogenous mannitol under hyperosmotic conditions. These findings suggested mannitol as a compatible solute that could act as an osmotically active substance, but not at the same extent as the predominant osmoprotectant, glycerol (Förster et al., 1998).

A similar effect of exogenous sorbitol accumulation was reported for *S. cerevisiae* (Meikle et al., 1988). This accumulation of non-fermentable polyols (such as mannitol and sorbitol) as a response to osmotic pressure has also been reported for other yeast and filamentous fungi (Jennings, 1983; Förster et al., 1998).

Given that glycerol act as a major osmoprotectant for *A. gossypii* cells (Förster et al., 1998), to determine whether the improved osmotic protection phenotypes of the *A. gossypii GUP1* overexpressing strains under hyperosmotic stress caused by KCl were due to a higher capacity to uptake exogenous glycerol (rather than to a higher capacity to retain intracellular glycerol or to biosynthesize it), experiments were performed on AFM-G418 plates supplemented with 0.8 and 1 M KCl but without addition of glycerol (Table 3.5). Under these conditions none of the strains were able to grow, indicating that the osmoprotective action of Gup1p is glycerol-dependent. Moreover, the results obtained for the control strain under exposure to 0.8 M KCl are in agreement with those reported for *S. cerevisiae* by Holst et al. (2000). In this work, the authors referred that under hyperosmotic stress conditions the action of Gup1p was more dramatic in strains defective in glycerol biosynthesis, but in a wild type background (without impairment of glycerol biosynthesis), the uptake of glycerol was also important for appropriate growth under hyperosmotic stress conditions.

In *S. cerevisiae*, the preferred mechanism to overcome hyperosmotic stress lies on the biosynthesis of glycerol (Blomberg and Adler, 1989; Holst et al., 2000). Förster et al. (1998) suggested the same mechanism for *A. gossypii*. The strategy applied here for overexpressing *GUP1* under the strong *AgTEF* promoter might have allowed overcoming this regulation and further improve the hyperosmotic stress response of *A. gossypii* by increasing the uptake of exogenous glycerol. Although overexpression of the *ScGUP1* also led to improved osmotic stress resistance, better hyperosmotic protection was supported by the overexpression of *AgGUP1*. *A. gossypii pRSAG*, overexpressing the native *AgGUP1,* was able to grow even under exposure to 1M KCl, demonstrating the high protection against hyperosmotic stress conferred by the overexpression of this gene. This may be justified by the small differences observed between the native and the *S. cerevisiae* Gup1p (Fig. 3.5), as differences between the transmembrane core regions of AgFps1p and ScFps1p have been identified as responsible for the hyperactive glycerol transport properties of AgFps1p, which are not displayed by ScFps1p (Geijer et al., 2012). Given these results, the better utilization capacities of *A. gossypii* than those of *S. cerevisiae* may be partially justified by the more active glycerol transport properties

of AgFps1p and AgGup1p than those of ScFps1p and ScGup1p. This hypothesis should be experimentally confirmed in future studies.

*3.3.4. Consumption of glycerol as carbon source by A. gossypii pRSAG and A. gossypii pRSSG*

Although the improved osmotic stress resistance demonstrated by *A. gossypii pRSAG* and *pRSSG* strains have confirmed the functionality of Gup1p in glycerol transport in *A. gossypii*, the main goal of this study was focused on improving the glycerol consumption profile of *A. gossypii*. Thus, to evaluate the consumption of glycerol as carbon source by the *GUP1* overexpressing strains, *A. gossypii pRSAG* (*AgGUP1*), *pRSSG* (*ScGUP1*) and *pFMT_EV* (empty vector control) were grown in AFMGlyOH-G418 (Fig. 3.8).

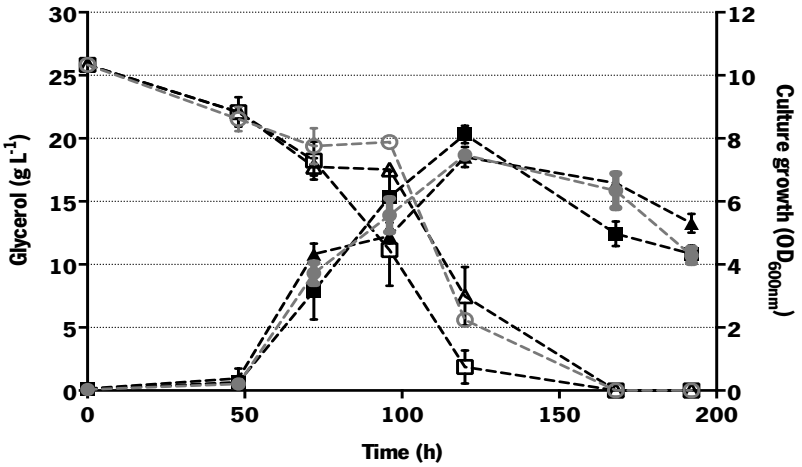


**Figure 3.8 –** Culture growth and glycerol consumption by the *A. gossypii* transformants in AFMGlyOH-G418 (25 g $L^{-1}$): *A. gossypii pFMT_EV* (●), *A. gossypii pRSAG* (■) and *A. gossypii pRSSG* (▲) strains. Filled symbols represent culture growth ($OD_{600nm}$) and empty symbols represent glycerol concentration in the medium (g $L^{-1}$). The initial inoculum was $10^6$ spores. Data represent average ± standard error of the mean (SEM) from two biological replicates. Where not seen, error bars were smaller than the symbol.

Under these conditions, the *AgGUP1* functionality was further confirmed, as the *A. gossypii pRSAG* was able to show faster glycerol consumption than the control strain. At 96 h, while the control strain consumed 6.14 g $L^{-1}$ glycerol, *A. gossypii pRSAG* had already consumed 14.3 g $L^{-1}$. This significant difference ($p < 0.01$) translated into a 2.3-fold increase in glycerol consumption. On the other hand, the glycerol consumption of *A. gossypii pRSSG* was not different from that of the control strain at any time point. Despite the higher glycerol consumption rate of *A. gossypii pRSAG* (0.20 g $L^{-1}$ $h^{-1}$) in comparison with *A. gossypii pRSSG*

(0.15 g $L^{-1}$ $h^{-1}$) and with *A. gossypii pFMT_EV* (0.17 g $L^{-1}$ $h^{-1}$), the final biomass yields were similar for all strains with 25 g $L^{-1}$ glycerol (Table 3.7).

**Table 3.7 –** Kinetic parameters of mycelium production by *A. gossypii* transformants from glycerol. X: final mycelium concentration. Y: yield of mycelium produced per glycerol consumed. Data represent average ± standard error of the mean (SEM) from two (25 g $L^{-1}$ glycerol) and three (40 g $L^{-1}$ glycerol) biological replicates.

| | **Glycerol (g $L^{-1}$)** | |
|---|---|---|
| | **25** | **40** |
| *A. gossypii pFMT_EV* | | |
| X (g $L^{-1}$) | 2.8 ± 0.2 | 2.5 ± 0.5 [a] |
| Y ($g_{mycelium}$ $g_{glycerol}^{-1}$) | 0.11 ± 0.01 | 0.062 ± 0.012[b] |
| *A. gossypii pRSAG* | | |
| X (g $L^{-1}$) | 2.8 ± 0.3 | 5.7 ± 0.6[c] |
| Y ($g_{mycelium}$ $g_{glycerol}^{-1}$) | 0.11 ± 0.01 | 0.15 ± 0.01[d] |
| *A. gossypii pRSSG* | | |
| X (g $L^{-1}$) | 3.2 ± 0.1 | 2.1 ± 0.1[a] |
| Y ($g_{mycelium}$ $g_{glycerol}^{-1}$) | 0.13 ± 0.00 | 0.050 ± 0.006[b] |

X (g $L^{-1}$) of column 40 g $L^{-1}$ glycerol: a,c – differ significantly ($p < 0.01$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3). Y ($g_{mycelium}$ $g_{glycerol}^{-1}$) of column 40 g $L^{-1}$ glycerol: b,d – differ significantly ($p < 0.01$) as determined by one-way ANOVA and Tukey's multiple comparisons test (n = 3).

We further analyzed the glycerol consumption profile of the transformants under other culture conditions: 40 g $L^{-1}$ glycerol and an initial inoculum of $10^7$ spores (Fig. 3.9). Under these culture conditions, the lag phase of all strains tested was approximately 1 day shorter compared with the previously tested conditions, and the glycerol consumption rate of both *GUP1* overexpressing strains was clearly demonstrated to be significantly faster than that of the control strain ($p < 0.0001$), confirming their improved glycerol consumption phenotype.

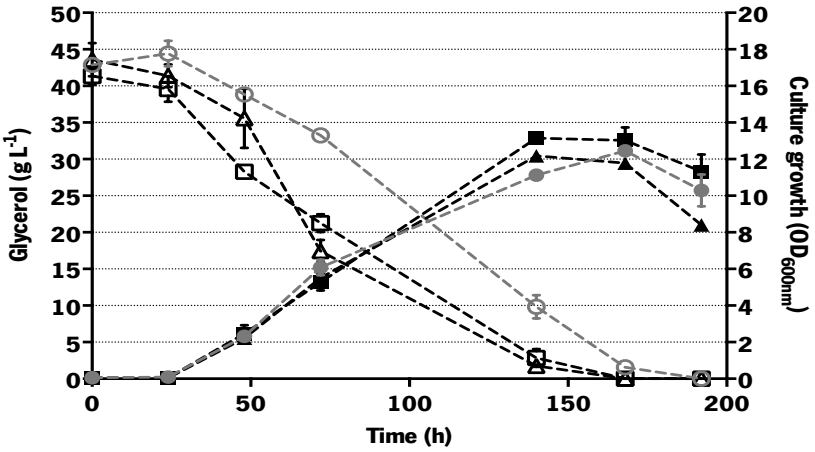


**Figure 3.9 –** Culture growth and glycerol consumption by the *A. gossypii* transformants in AFMGlyOH-G418 (40 g $L^{-1}$): *A. gossypii pFMT_EV* (●), *A. gossypii pRSAG* (■) and *A. gossypii pRSSG* (▲) strains. Filled symbols represent culture growth ($OD_{600nm}$) and empty symbols represent glycerol concentration in the medium (g $L^{-1}$). The initial inoculum was $10^7$ spores. Data represent average ± standard error of the mean (SEM) from three biological replicates. Where not seen, error bars were smaller than the symbol.

*A. gossypii pRSAG* kept showing the fastest glycerol consumption under these conditions. At 48 hours it had already consumed 13.1 g $L^{-1}$ glycerol, which represents a 3.2-fold increase in comparison to the control strain. In turn, although at this time point a slightly higher amount of glycerol had been consumed by *A. gossypii pRSSG* compared to the control strain, this difference was not yet statistically significant ($p > 0.05$). However, significant differences were further found at 72 and 140 h ($p < 0.001$) between the glycerol consumption of both *GUP1* overexpressing strains and the control strain, with approximately 2.4-fold and 1.3-fold higher amount of glycerol consumed by both overexpressing strains at these time points, respectively. Moreover, in the *A. gossypii pRSAG* and *pRSSG* cultivations, after 168 h all glycerol had already been exhausted from the medium, whereas in the control strain the depletion of glycerol was only detected at 192 h.

The results obtained here for *A. gossypii* are very exciting compared to those registered for *S. cerevisiae* overexpressing *GUP1,* where only 1.2-fold increases in glycerol consumption were achieved after 96 h in comparison with the wild type strain (Jung et al., 2011). Moreover, even for a *S. cerevisiae* strain overexpressing not only *GUP1*, but also *GCY* and *DAK*, only 1.6-fold increase in the glycerol consumption rate was obtained at 48 h (Yu et al., 2010a).

Faster glycerol consumption resulted also in slightly faster growth, with *pRSAG* and *pRSSG* transformants reaching the stationary phase earlier than the control strain (Fig. 3.9). Additionally, higher glycerol consumption rates were achieved by the *GUP1* overexpressing

strains (0.27 g $L^{-1}$ $h^{-1}$ for *pRSAG* and 0.30 g $L^{-1}$ $h^{-1}$ for *pRSSG*) than for *A. gossypii pFMT_EV* (0.25 g $L^{-1}$ $h^{-1}$). Under these conditions, and unlike the observed with 25 g $L^{-1}$ glycerol (Fig. 3.9), *A. gossypii pRSAG* was able to translate its improved glycerol consumption profile into a 2.5-fold increase in the yield of mycelium produced per glycerol consumed in comparison with the control strain (Table 3.7). The biomass production yield (0.15 ± 0.01 $g_{mycelium}$ $g_{glycerol}^{-1}$) here achieved by *A. gossypii pRSAG* was not only significantly higher than that of the control strain ($p < 0.01$), but also stands within the range of the biomass yields achieved by *Y. lipolytica* strains in flask cultivations with 30 g $L^{-1}$ pure glycerol (0.14 – 0.27 $g_{biomass}$ $g_{glycerol}^{-1}$; André et al., 2009), which are very efficient in converting glycerol into value-added compounds (Rywińska et al., 2013).

With the confirmation that the approach applied in this study (*GUP1* genes overexpression) enabled to successfully improve glycerol utilization by *A. gossypii*, we further evaluated if this faster glycerol uptake could lead to increased productivity of extracellular proteins (invertase) and riboflavin. The highest production levels of native invertase by *A. gossypii* have been obtained on glycerol (Aguiar et al., 2014a). However, no increases were detected in the secreted invertase activity detected in the *A. gossypii pRSAG* and *pRSSG* compared to the control (Table 3.8) in cultivations with 20 g $L^{-1}$ glycerol and supplemented with glycine (*3.2.5.*).

**Table 3.8 –** Specific invertase activity detected in the culture supernatants of the *A. gossypii* transformants during growth in AFMGlyOH-G418 (20 g $L^{-1}$). Data represents average ± standard error of the mean (SEM) from three biological replicates. The average biomass produced by *A. gossypii* recombinant strains was: 2.29 ± 0.06 g $L^{-1}$ for *pFMT_EV*, 3.52 ± 0.61 g $L^{-1}$ for *pRSAG* and 2.06 ± 0.32 g $L^{-1}$ for *pRSSG*.

| **Time (h)** | *A. gossypii pFMT_EV* | *A. gossypii pRSAG* | *A. gossypii pRSSG* |
|---|---|---|---|
| Extracelular activity (U $mL^{-1}$) | | | |
| 24 | 0.023 ± 0.011 | 0.0053 ± 0.0027 | 0.030 ± 0.007 |
| 48 | 0.11 ± 0.02 | 0.046 ± 0.010 | 0.10 ± 0.02 |
| 72 | 0.069 ± 0.021 | 0.035 ± 0.0090 | 0.011 ± 0.006 |
| 96 | – | 0.013 ± 0.007 | 0.0021 ± 0.0021 |

Moreover, in these shake-flask cultivations, surprisingly no riboflavin production was detected. Even the control strain (*A. gossypii pFMT_EV*) presented a strong impairment in riboflavin production, suggesting that this phenotype was not related with the overexpression of *GUP1*. Other authors (Kato and Park, 2006; Sugimoto et al. 2009) have already reported

riboflavin production impairment by *A. gossypii* transformants carrying plasmids to overexpress genes. The same authors suggested that the plasmid retention and/or the addition of antibiotics to maintain plasmids might be a burden for *A. gossypii*, damaging its secondary metabolism (Kato and Park, 2006; Sugimoto et al. 2009).

These observations suggest that for exploitation of a given product, *GUP1* overexpression should be accompanied by the overexpression of other genes in order to redirect the metabolic carbon flux, as has been done successfully in *S. cerevisiae* by other authors for the production of ethanol (Yu et al., 2010a; 2010b) and 1,2-propanediol (Jung et al., 2011). Moreover, given the evidences observed here and already reported by other authors (Kato and Park, 2006; Sugimoto et al. 2009) regarding riboflavin production, metabolic engineering strategies delineated by gene overexpression should consider genome integration instead of plasmid utilization.

In this chapter we have confirmed that overexpression of *GUP1* led to an improved glycerol consumption profile in *A. gossypii* either through overexpression of the native or the *S. cerevisiae* gene. Both transformants showed faster and improved glycerol consumption rates than the control strain, which resulted in slightly faster growth. Beyond this, *A. gossypii pRSAG* also achieved higher yields of mycelium production per glycerol consumed in comparison with the control strain. As occurred for hyperosmotic stress resistance, overexpression of *AgGUP1* was more effective in generate an *A. gossypii* strain with significant improved rates of glycerol utilization.

The genetic engineering strategy applied in this chapter was able to create *A. gossypii* strains with improved osmotic protection and superior rates of glycerol utilization. Given these results, overexpression of *GUP1* stands as an effective strategy to increase glycerol consumption. For further exploitation of added-value compounds, a combined overexpression of genes related with glycerol metabolism together with the redirection of the carbon flux to produce the metabolites of interest should be considered.

# CHAPTER 4

## Conclusions and future perspectives

The filamentous fungus *A. gossypii* has long been exploited for the industrial production of riboflavin. Driven by its remarkable traits and by its genetic similarity with the eukaryotic model *S. cerevisiae*, over the years *A. gossypii* has been the focus of fundamental and applied investigation, which have culminating in a fruitful synergy for all who explore this fungus. The wealth of molecular and bioinformatics tools available for this microorganism allows a based-knowledge exploitation of the *A. gossypii* full biotechnological potential, which nowadays goes far beyond the production of riboflavin. It is time to take advantage of all the knowledge acquired during these years and to go further in the full exploitation of other biotechnological applications for this fungus, such as the recently reported recombinant protein, single cell oil and aroma production. Nonetheless, it is important to never neglect this overwhelming capacity of *A. gossypii* to produce riboflavin, since new insights are constantly emerging, even more with the advent of the predictive *in silico* tools. This thesis focused on the study of the effect of the blockage of the *de novo* pyrimidine pathway on riboflavin production, exploration of riboflavin production from glycerol and on the investigation of metabolic engineering strategies to improve the glycerol consumption profile of this fungus.

In this study, we reported for the first time that genetic alterations on the *de novo* pyrimidine pathway affected riboflavin production. The uridine/uracil auxotrophic strain *A. gossypii Agura3* was able to show an approximately 7.5-fold increased riboflavin production compared to the parent strain on medium without the addition of uridine/uracil. The addition of uridine and/or uracil to the medium resulted in a gradual and clear reduction of riboflavin production and addition of uridine favored the colony radial growth of this strain, which under these conditions presented a similar phenotype to the parent strain.

The blockage of the *de novo* pyrimidine pathway was suggested to lead to unbalances of the metabolic flux with direct influence on PRPP. PRPP is the first committed intermediate of some important pathways. Among them, the purine and the pyrimidine pathways are those that absorb most of its metabolic flux. Reduction of its flux for the *de novo* pyrimidine pathway could increase the availability of this compound to the purine pathway and increased flux to the purine pathway has been recently shown to be part of the central carbon metabolism of a riboflavin overproducing strain (Jeong et al. 2014). Therefore, these observations suggest that an increase in the PRPP available for riboflavin synthesis through the *de novo* purine/GTP biosynthetic pathway and/or a reduction in PRPP synthesis driven by its intracellular accumulation, with consequent increased availability of ribulose-5P for riboflavin synthesis,

might have occurred when no extra uridine/uracil was supplied. Moreover, nutritional stress caused by the absence of uridine/uracil supplementation may also explain this overproducing phenotype. To confirm these altered metabolic fluxes similar approaches to those described by Jimenez et al. (2005; 2008) and/or Jeong et al. (2014) should be performed in order to monitor the cellular availability of PRPP. Finally, the production of riboflavin by this strain in submerged culture should be optimized in order to take fully advantage of its overproducing phenotype.

Given the results obtained from the preliminary studies on riboflavin production by *A. gossypii* strains from glycerol, we conclude the suitability of this carbon source for this purpose. Further exploitation of this process could bring profitability improvements for riboflavin production by *A. gossypii* due to the actual situation of crude glycerol stocks. However, the slower growth and consumption rates observed on glycerol need to be optimized.

In this thesis, a genetic engineering strategy aimed at improving the yield of value-added products from glycerol in *A. gossypii* was also delineated. For this, the first objective passed by increasing the consumption of the substrate, in this case glycerol. Overexpression of *GUP1* had been already applied with success in *S. cerevisiae* to increase the glycerol consumption. So, we overexpressed not only *AgGUP1* but also *ScGUP1* under the control of the strong *AgTEF* promoter, using the pFMT vector, to check if *GUP1* overexpression resulted in improved glycerol uptake and if there were differences between these two transporters.

Overexpression of both *AgGUP1* (*pRSAG*) and *ScGUP1* (*pRSSG*) genes led to enhanced glycerol consumption. This translated into a significant enhancement of glycerol-dependent hyperosmotic stress tolerance and, as originally proposed, in improved glycerol consumption profiles in shake-flask cultivations. Both overexpressing transformants (*pRSAG* and *pRSSG*) showed improved glycerol consumption, slightly faster growth in comparison with the control strain. Regarding the differences between the overexpressing transformants, *A. gossypii pRSAG* showed the best results. It presented the highest hyperosmotic stress tolerance, growing under exposure to 1 M KCl and the fastest and highest increase in glycerol consumption (3.2-fold more glycerol consumed at 48 h in AFM-G418 with 40 g $L^{-1}$ glycerol than the empty vector control strain). Moreover, in shake-flask cultivations with 40 g $L^{-1}$ glycerol, *A. gossypii pRSAG* presented significant increase in the biomass production yield ($0.15 \pm 0.01$ $g_{mycelium}$ $g_{glycerol}^{-1}$) in comparison with the control strain, which are in the range of those obtained by *Y. lipolytica* strains (André et al., 2009; Rywińska et al., 2013). In future studies, it would be interesting to

confirm whether AgGup1p is actually more efficient than ScGup1p in the uptake of glycerol. For this, the same strategy applied here should be applied in *S. cerevisiae*, as has already been done in a similar way to study differences between AgFps1p and ScFps1p (Geijer et al., 2012).

These findings confirm the effectiveness of our metabolic engineering strategy in increasing the glycerol consumption profile of *A. gossypii*, constituting the first genetic manipulation reported to improve the glycerol metabolism in *A. gossypii*. This strategy may be considered as the upstream module of an integrated strategy accompanied by other gene overexpression for directing the carbon flux of glycerol to a given product, as done with success in *S. cerevisiae* for ethanol (Yu et al., 2010a; 2010b) and 1,2-propanediol production (Jung et al., 2011). Even though *AgSTL1* does not share the syntenic pattern of its homolog (*ScSTL1*), it will be interesting to test the overexpression of both *STL1* genes and check if better results are obtained than those obtained here with *GUP1* overexpression. Regarding riboflavin production, *GUP1* overexpression together with the overexpression of the aerobic route of glycerol utilization (native and heterologous *GUT1* and *GUT2* genes) can be the first multi-gene strategy to be studied. Given the possible burden effects of antibiotic/plasmid retention that may hamper the production of high-value products (*e.g.* riboflavin), a genome integration strategy for gene overexpression should be considered. The availability of the *A. gossypii* GSMM will allow a rational evaluation and design of novel genetic and metabolic engineering strategies to be applied in the future.

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