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Research review paper

## *Ashbya gossypii* beyond industrial riboflavin production: A historical perspective and emerging biotechnological applications

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### ARTICLE INFO

#### Article history:

Received 28 May 2015

Received in revised form 28 September 2015

Accepted 4 October 2015

Available online xxxxx

#### Keywords:

*Ashbya gossypii*

Physiology

Metabolism

Genetic engineering

Riboflavin

Recombinant proteins

Single cell oil

Flavour compounds

### ABSTRACT

The filamentous fungus *Ashbya gossypii* has been safely and successfully used for more than two decades in the commercial production of riboflavin (vitamin B<sub>2</sub>). Its industrial relevance combined with its high genetic similarity with *Saccharomyces cerevisiae* together promoted the accumulation of fundamental knowledge that has been efficiently converted into a significant molecular and *in silico* toolbox for its genetic engineering. This synergy has enabled a directed and sustained exploitation of *A. gossypii* as an industrial riboflavin producer. Although there is still room for optimizing riboflavin production, the recent years have seen an abundant advance in the exploration of *A. gossypii* for other biotechnological applications, such as the production of recombinant proteins, single cell oil and flavour compounds. Here, we will address the biotechnological potential of *A. gossypii* beyond riboflavin production by presenting (a) a physiological and metabolic perspective over this fungus; (b) the molecular toolbox available for its manipulation; and (c) commercial and emerging biotechnological applications for this industrially important fungus, together with the approaches adopted for its engineering.

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### 1. Introduction

The filamentous hemiascomycete *Ashbya gossypii* (syn. *Eremothecium gossypii*) has recently emerged as an attractive cell factory to produce yet unexplored high-value products. Industrially exploited for more than 20 years due to its natural ability to overproduce riboflavin, *A. gossypii* is considered a remarkable example of the sustainable White Biotechnology

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business model (Kato and Park, 2012; Lim et al., 2001; Stahmann et al., 2000; zu Berstenhorst et al., 2009). Nevertheless, the scientific prominence of *A. gossypii* is not limited to its biotechnological potential, since it is also extensively used as a model organism in fungal developmental and evolutionary biology studies (Perez-Nadales et al., 2014; Schmitz and Philippsen, 2011; Wendland and Walther, 2005). Its industrial relevance and the basic knowledge accumulated along the years promoted the development of molecular and *in silico* tools that, in turn, have allowed the continuous engineering of *A. gossypii* strains with improved riboflavin production traits, either through random or rational approaches.

The overwhelming capacity of *A. gossypii* to produce riboflavin will continue to be targeted for optimization, even more with the advent of systems biology. However, since the beginning of this decade, we have witnessed the increasingly stronger shift of the *A. gossypii* biotechnological paradigm from riboflavin to other commercially interesting high-value compounds, such as recombinant proteins (Ribeiro et al., 2010), single cell oil (SCO) (Ledesma-Amaro et al., 2014a) and flavour compounds (Ravasio et al., 2014; Ledesma-Amaro et al., 2015). In this review, we will focus on the emerging biotechnological potential of this promising cell factory that nowadays goes far beyond riboflavin production (Fig. 1). This review will first provide a historical perspective over this fungus, highlighting its relevant metabolic and physiological traits (Section 2). Subsequently, the molecular toolbox established and available for *A. gossypii* manipulation will be presented (Section 3) and the random and rational genetic engineering approaches that have been used in the context of riboflavin, recombinant protein, SCO and flavour compounds' production will be described (Section 4). We will conclude by presenting future challenges and directions that should be taken in order to guide these emerging biotechnological applications through the successful track of riboflavin production.

## 2. *A. gossypii*

### 2.1. Habitat

*A. gossypii* was originally isolated from infected cotton bolls (*Gossypium* sp.) harvested in the British West Indies (Caribbean region) and identified as one of the causative agents of stigmatomycosis (Ashby and Nowell, 1926). Subsequently, *A. gossypii* strains were also isolated from other crops widespread along the tropic and sub-tropic regions

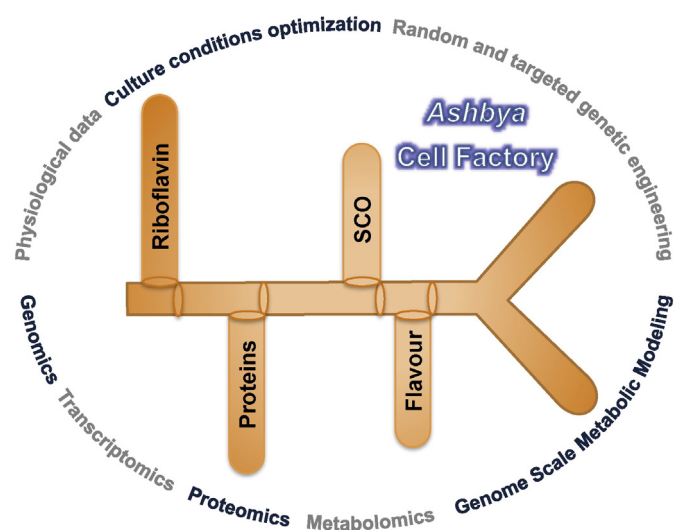


Fig. 1. *A. gossypii*'s biotechnological applications. Following its established industrial use in riboflavin production, emerging biotechnological applications for this fungus are: recombinant protein, single-cell oil (SCO) and flavour compounds' production. The technological approaches used for the development of these biotechnological applications are presented in the closing circle.

of Northern America, Southern America and South Africa, such as coffee, citrus fruits and tomatoes (Batra, 1973; Pridham and Raper, 1950). More recently, *A. gossypii* was isolated from large milkweed bugs found feeding on oleander in Florida, USA (Dietrich et al., 2013). Insect vectors, predominantly those with pierce-sucking mouthparts such as the *Heteroptera*, are essential for the transmission of this fungus, as the fungus itself is unable to penetrate the outer cells of healthy fruits and seeds unless a mechanical injury occurs (Batra, 1973; Pridham and Raper, 1950). Since the most important mode of infection requires insects to transport and inject spores or mycelial fragments into the tissue of the plant (Batra, 1973; Pridham and Raper, 1950), the control of the insect population by insecticides has proven efficient in preventing the dissemination of *A. gossypii* (Dammer and Ravelo, 1990).

The characteristics of the plant tissues where *A. gossypii* has been found (rich in readily usable sugars and/or oils; Caskey and Gallup, 1931) and its insect-dependent infection mode justify modest secretion of extracellular enzymes by *A. gossypii* (Aguiar et al., 2014a; Ribeiro et al., 2010), as its proliferation does not depend on the degradation of complex substrates. On the other hand, the *A. gossypii*'s need to attract insects for spreading benefits from its capacity to produce large amounts of strong volatile aromas (Ravasio et al., 2014; Wendland et al., 2011). Interestingly, this close relationship between *A. gossypii* and certain plant-feeding insects has also provided some hints regarding its riboflavin overproduction capacity (Dietrich et al., 2013; Walther and Wendland, 2012). It has been hypothesised that the overproduction of riboflavin constitutes an ecological advantage for both the fungus and insects in their natural niches, by conferring them protection against some plant defences (Walther and Wendland, 2012; Dietrich et al., 2013). Therefore, a better understanding of the existing biological relationship between *A. gossypii* and its insect vectors holds the potential to unravel new perspectives and opportunities for biotechnology, such as the development of environmentally-friendly insecticides.

### 2.2. Taxonomic history

*A. gossypii* was first characterized in 1926 by Ashby and Nowell (Ashby and Nowell, 1926), who at the time named it *Nematospora gossypii*, based on the close resemblance of its sporangia and spores to those of other *Nematospora* species. In 1928, based on cytological and phylogenetic studies, Guilliermond (1928) placed this species in the class Hemiascomycetes and assigned to it a new genus name, *Ashbya*, because in contrast to other *Nematospora* species it grew exclusively in a mycelial form and polynucleated cells were in the origin of its sporangia. Fragoso and Ciferri (1928) considered this species to belong to the order Saccharomycetales (Endomycetales) and also suggested separating it from the *Nematospora* genus, presenting a Latin description for it under the generic name *Ashbya*.

As this species appears to possess characteristics of several families, further classification attempts differed along the years. Ascospore morphology (needle-shaped with a whiplike filament at one end) and the presence of hyphal growth were considered to separate the genus *Ashbya*, at least at the family level, from yeast genera belonging to the Saccharomycetales (von Arx and van der Walt, 1987). However, the close relationship between *A. gossypii* and *Saccharomyces cerevisiae* started to become obvious when the sequence analysis of the first set of clones of *A. gossypii* genomic DNA (gDNA) revealed an unpredictably high degree of synteny to the gene order of their homologues in *S. cerevisiae* (Altmann-Jöhl and Philippsen, 1996; Steiner and Philippsen, 1994). Based on phylogenetic analysis of ribosomal DNA (rDNA) sequence divergence, Kurtzman (1995) placed the genera *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora* in a single genus, *Eremothecium*, and introduced the family Eremotheciaceae for this genus. Prillinger et al.'s (1997) data on cell wall sugar composition, dihydroxytyrosine content in ascospores, ubiquinone side chains and sequence analysis of the genes coding for the 18S rDNA and ITS region supported

the inclusion of these genera within a single genus, as proposed by Kurtzman (1995), but within the Saccharomycetaceae family.

The sequencing and annotation of the *A. gossypii* genome finally elucidated the close relation between *A. gossypii* and *S. cerevisiae*, revealing homologues in *S. cerevisiae* for 94% of the 4776 annotated protein-coding genes of *A. gossypii*, 90% of which at syntenic positions (Brachat et al., 2003; Dietrich et al., 2004, 2013). Only 6% of the *A. gossypii*'s protein-coding genes have no homologue in *S. cerevisiae*, but most of these genes have a syntenic homologue in other closely related species (Dietrich et al., 2004, 2013; Wendland et al., 2011; Wendland and Walther, 2014). The small size (9.12 Mb) and organization of the *A. gossypii*'s genome into only seven chromosomes also provided compelling evidence for an ancient whole-genome duplication (WGD) of the *Saccharomyces* lineage after its split from the *Ashbya* lineage (Dietrich et al., 2004). With the genome sequence of several fungal species available, fungal phylogenomic studies have been conducted (Fitzpatrick et al., 2006; Wang et al., 2009), congruently placing *A. gossypii* in the order Saccharomycetales, close to other pre-WGD species, like *Kluyveromyces lactis*, *Kluyveromyces waltii* and *Saccharomyces kluyveri*. However, the monophyly of these four species is still unsettled (Wang et al., 2009). Kurtzman and Robnett (2003) suggested that these species are paraphyletic, assigning the genus *Eremothecium* to the clade 12 of the *Saccharomyces* complex, *K. lactis* to the clade 11 and *K. waltii* and *S. kluyveri* to the clade 10. In contrast, other studies suggest that the four species are monophyletic, constituting themselves a clade (Fitzpatrick et al., 2006; Wang et al., 2009).

### 2.3. Life cycle

*A. gossypii* is one of the simplest filamentous fungal species studied, which due to its close relationship to yeast, small genome size and easy genetic manipulation arose as an attractive model to study fungal developmental biology (reviewed in Perez-Nadales et al., 2014; Schmitz and Philippson, 2011; Wendland and Walther, 2005). Its life cycle (Fig. 2) starts with a short period of isotropic growth that initiates the germination of its uninucleate haploid spores and leads to the formation of a spherical germ bubble at the centre of the spores, which is where the nucleus is positioned (Alberti-Segui et al., 2001; Wendland and Philippson, 2000). Then, actin patches start to accumulate in a region of the germ bubble cortex perpendicular to the axis defined by the spore needle, marking the emergence of the first germ tube and the switch to polarized growth (Knechtle et al., 2003). The tip of the germ

tube continues extending and an actin ring at the neck between the germ bubble and the first germ tube initiates the formation of the first septum (Knechtle et al., 2003). Septation and nuclear division are uncoupled events in *A. gossypii*, and thus each hyphal compartment encloses multiple nuclei (Kaufmann and Philippson, 2009; Wendland and Philippson, 2000). These exhibit asynchronous division cycles and variable ploidy, despite sharing a common cytoplasm (Anderson et al., 2015; Gladfelter et al., 2006). At the opposite side of the germ bubble a second germ tube is formed, giving rise to a bipolar germling (Wendland and Philippson, 2000). From these initial hyphae a young mycelium is generated by lateral branching. Hyphal tip growth speed increases during maturation and concomitantly hyphal tips start to undergo dichotomous branching, leading to the formation of Y-shaped hyphal filaments (Ayad-Durieux et al., 2000). To deal with the high demand for novel cell surface at the growing tip, secretory vesicles containing new membrane and cell wall components are transported along the actin cytoskeleton to the tip of fast-growing hyphae, where they accumulate in Spitzenkörper-like spherical structures and fuse with the plasma membrane, releasing their content to the extracellular space (Köhli et al., 2008). The secretion of proteins required for cell wall synthesis and lysis of substrates is thus thought to occur preferentially (although not exclusively) at the hyphal tip (Read, 2011).

The vegetative growth of *A. gossypii* ceases upon nutrient limitation and old mycelia eventually enter the sporulation phase, event that is linked with the production of riboflavin (vitamin B<sub>2</sub>), which dramatically increases during this phase (Stahmann et al., 2001). However, riboflavin production is not exclusively connected to sporulation, as non-sporulating mycelia also accumulate riboflavin (Walther and Wendland, 2012). Old hyphae fragment at septal sites to form sporangia that usually contain eight endospores bound together by filaments, which are set free by lysis of the sporangia cell walls (Wendland and Walther, 2005). On solid media, the sporulation zone is rich in strongly aggregated hyphae that rise above the agar and form synnemata-like structures (Grünler et al., 2010). In liquid media, old mycelia undergo autolysis during the latest phase of its life cycle (Pridham and Raper, 1950).

Sporulation in *A. gossypii* is affected by the mitogen-activated protein (MAP)-kinase pathway that governs the mating pheromone response and the starvation/filamentous growth response, and by the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway that governs all aspects of cell physiology, particularly morphogenesis, growth and sporulation (Wasserstrom et al., 2013, 2015; Wendland et al., 2011). However, the specific mechanisms of spore formation in *A. gossypii* are still poorly understood, not being clear whether there is a meiotic component to sporulation (Wasserstrom et al., 2013, 2015). The *A. gossypii* reference strain ATCC10895 was found to harbour four identical mating-type cassettes in its genome containing *MATa* information, but no *MATα* orthologs were found (Dietrich et al., 2013; Wendland et al., 2011), which led to question the existence of a sexual cycle in this fungus (Wendland et al., 2011). However, the recent genome sequencing of a new *A. gossypii* strain (*FDAG1*) isolated from large milkweed bugs revealed that this wild isolate encodes both *MATa* and *MATα* sequences, suggesting that some *A. gossypii* strains likely have a sexual cycle (Dietrich et al., 2013).

### 2.4. Metabolism and physiology

*A. gossypii* is a natural overproducer of riboflavin (vitamin B<sub>2</sub>), which confers to its mycelia a characteristic yellow colour (Wickerham et al., 1946). Overproduction of this vitamin in *A. gossypii* is known to start at the stationary phase, when the growth rate declines, concomitantly with sporulation (Karos et al., 2004; Stahmann et al., 2001). As a rule, similar kinetics of production have been seen during microbial synthesis of secondary metabolites, but not primary metabolites. Therefore,

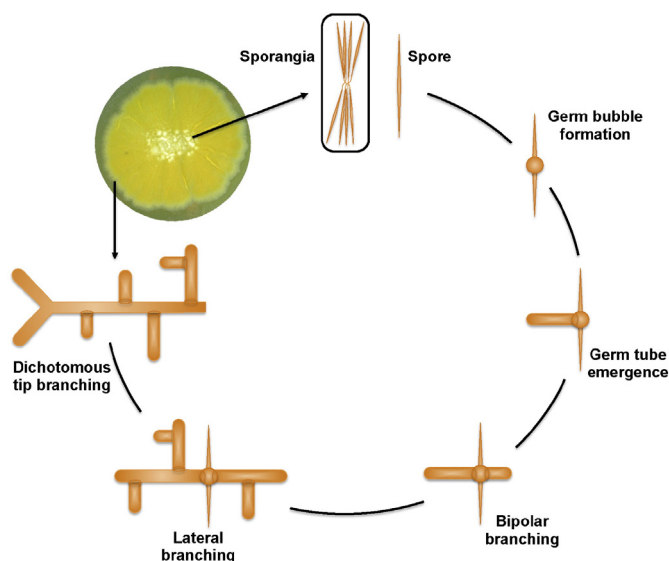


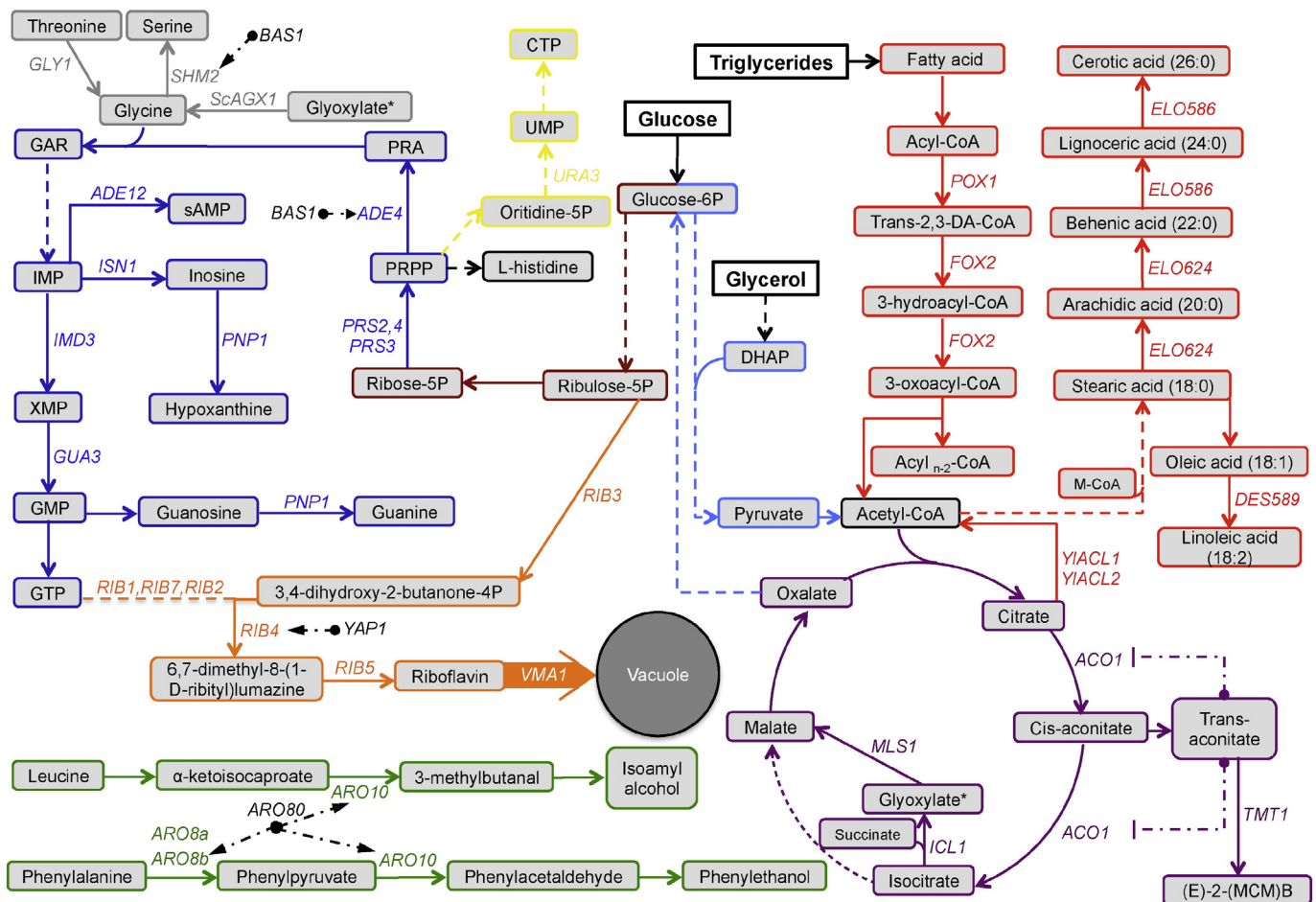
Fig. 2. Schematic representation of the *A. gossypii* life cycle. The colony photo represents growth for eight days on agar-solidified complex medium.

riboflavin is considered in *A. gossypii* as a pseudo-secondary metabolite (Schlösser et al., 2001).

Riboflavin is synthesized *de novo* from guanosine-5-triphosphate (GTP) and ribulose 5-phosphate (ribulose 5-P) through a multi-step pathway controlled by six *RIB* genes (Fig. 3) (Karas et al., 2004). The riboflavin production phase 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 (Schlösser et al., 2001, 2007; Walther and Wendland, 2012). Overproduction of riboflavin by *A. gossypii* has been shown to be triggered by environmental stresses, such as nutritional and oxidative stress (Kavitha and Chandra, 2009, 2014; Silva et al., 2015; Schlösser et al., 2007; Walther and Wendland, 2012). Alongside with the induction of riboflavin overproduction and secretion, oxidative stress has been shown to also induce the activity of enzymes that are involved in the detoxification of reactive oxygen species (ROS), e.g. catalase and superoxide dismutase (Kavitha and Chandra, 2009, 2014). Hence, the secretion of large amounts of riboflavin by *A. gossypii* was suggested to function as a possible stress defence mechanism against the ROS produced by plant defences (Walther and Wendland, 2012). The *AgYap1p*, a transcription factor that plays a major role in directing the cellular responses to various stresses, was shown to regulate the expression of the *AgRIB4* gene and to mediate an *AgYap1p*-dependent increase in riboflavin production during oxidative stress (Walther and Wendland, 2012). *AgYap1p* also seems to

regulate the expression of *AgRIB3* and *AgGSH1* (gene encoding  $\gamma$ -glutamyl cysteinyl synthase, a rate-limiting enzyme in the biosynthesis of glutathione – molecule involved in the *A. gossypii* antioxidant defence), both of which have been shown to be up-regulated in response to oxidative stress (Kavitha and Chandra, 2014). These evidences show that riboflavin overproduction by *A. gossypii* is related with regulatory mechanisms rather than with metabolic capabilities. Indeed, comparatively with its close relatives *S. cerevisiae* and *K. lactis* that do not overproduce riboflavin, *A. gossypii* does not possess exclusive enzymes directly involved in the riboflavin biosynthetic pathway that could explain its overproducing trait (Gomes et al., 2014). On the other hand, significant differences exist at the intergenic level between *A. gossypii* and its non-flavinogenic relatives (Brachet et al., 2003; Wendland et al., 2011), which hints at the existence of regulatory differences between them.

The first physiological studies focusing on the influence of some common environmental factors on the *A. gossypii*'s growth started to appear in the literature as a reflection of the eventual interest in using this fungus for industrial applications. As early as 1930, Faries and Bell (1930) noted the production of a yellow pigment (riboflavin) by certain *A. gossypii* strains when investigating the nitrogen requirements of this species. In their study, potassium nitrate and ammonium salts were not utilized as nitrogen sources, observations later confirmed by Buston et al. (1938). However, Ribeiro et al. (2012) recently verified



**Fig. 3.** Pathways and genes involved in the *A. gossypii* metabolism that have been targeted for biotechnological purposes. The pathways are identified according to the following colour scheme: glycolysis and gluconeogenesis (light blue),  $\beta$ -oxidation and fatty acid biosynthesis (red), TCA cycle and glyoxylate cycle (purple), pentose phosphate pathway (brown), *de novo* purine biosynthetic pathway (dark blue), glycine pathways (grey), *de novo* pyrimidine biosynthetic pathway (yellow), riboflavin biosynthetic pathway (orange), leucine and phenylalanine degradation (Ehrlich pathway) (green). Dashed arrows indicate a multi-step pathway, dotted arrows indicate regulatory roles and filled arrows indicate a transport reaction. (E)-2-(MCM)B, (E)-2-8(methoxycarbonylmethyl)butenedioate; DHAP, dihydroxy-acetone-phosphate; GAR, 5'-phosphoribosylglycinamide; GMP, guanosine-5'-phosphate; M-CoA, malonyl-CoA; PRA, 5-P- $\beta$ -D-ribosyl-amine; Trans-2,3-DA-CoA, Trans-2,3-dehydroacyl-CoA; XMP, xanthosine-5-phosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that several *A. gossypii* strains actually grow well on chemically defined medium containing ammonium as sole nitrogen source when the medium pH is 6.5, but poorly at pH 4.5, indicating that the lack or limited growth previously reported probably reflected low medium pH. Supporting their observations is the fact that *A. gossypii* is genetically equipped for ammonium (but not for nitrate) assimilation (Ribeiro et al., 2012).

The sensitivity of *A. gossypii* to low medium pH had been previously observed by Tanner et al. (1949), whom verified that media initially adjusted to pH 4.0 gave little growth and riboflavin production. Between pH 4.5 and 5.5 there was a good and rapid multiplication, but the best riboflavin yields were obtained when the initial pH of the medium was above pH 5.5, preferably in the range of pH 6.0 to 7.0 (Tanner et al., 1949). The growth temperature also has an impact on riboflavin production. Although *A. gossypii* grows over a rather wide temperature range (20 to 40 °C), the greatest riboflavin accumulation occurs when cultures are incubated at 26 to 28 °C, which is below the optimum for growth (30 to 35 °C) (Özbas and Kutsal, 1986; Pfeifer et al., 1950; Tanner et al., 1949). The presence of low concentrations of NaCl and Na<sub>3</sub>PO<sub>4</sub>, or slightly higher concentrations of mannitol, sorbitol and KCl, highly inhibit the *A. gossypii*'s growth, displaying this fungus acute sensitivity to hyperosmotic stress (Förster et al., 1998; Nikolaou et al., 2009).

Myo-inositol and biotin have early been found to be required for *A. gossypii*'s growth (Buston and Pramanik, 1931; Farries and Bell, 1930; Kögl and Fries, 1937), as this fungus lacks the genes necessary for their biosynthesis (Gomes et al., 2014). In fact, the *A. gossypii*'s requirement for biotin played an important role in the discovery of this vitamin (Kögl and Fries, 1937). Thiamine is also necessary for adequate growth, as *A. gossypii* synthesizes it in sub-optimal amounts (Pridham and Raper, 1950). When these “accessory factors” are added to the medium, *A. gossypii* is capable of utilizing simple mixtures of amino acids, ammonium aspartate or asparagine as nitrogen sources (Buston and Kasinathan, 1933), the latter supporting better growth in defined media (Demain, 1972). Peptone and casein constitute, however, better nitrogen sources for *A. gossypii* (Farries and Bell, 1930). Yeast extract is also a good source of several nutrients necessary for *A. gossypii*'s growth (Wickerham et al., 1946) and we have observed that it alone can support residual growth of *A. gossypii* in liquid medium without addition of carbon source (Aguiar et al., personal communication). Crude nitrogen and vitamin sources, like animal steep liquor, corn steep liquor and distillers' solubles have, however, been preferably employed in large-scale riboflavin production (Pfeifer et al., 1950; Smiley et al., 1951; Tanner et al., 1949).

Several studies on the *A. gossypii*'s carbon requirements indicated that glucose, fructose, sucrose, starch, maltose and glycerol support good growth, but plant oils (such as corn oil and soybean oil) are superior and favour riboflavin production and lipid accumulation (Kutsal and Özbas, 1989; Ledesma-Amaro et al., 2014a; Pridham and Raper, 1950; Ribeiro et al., 2012; Tanner et al., 1949). Ethanol supports more limited growth rates (Pridham and Raper, 1950). Cellulose, pentoses (arabinose, xylose), galactose and lactose are not utilized as carbon sources (Pridham and Raper, 1950; Ribeiro et al., 2012), which may be explained by the absence of enzymes and/or pathways necessary for the metabolism of these substrates in the *A. gossypii*'s genome (Gomes et al., 2014; Ribeiro et al., 2012). Correlated with the limited range of carbon sources that *A. gossypii* utilizes, the variety and amount of hydrolytic enzymes secreted by *A. gossypii* to the extracellular medium is rather low (Aguiar et al., 2014a; Ribeiro et al., 2010, 2013). Of these, only a lipase and an invertase have been experimentally characterized thus far (Aguiar et al., 2014b; Stahmann et al., 1997).

Under aerobic conditions, glucose is oxidized by *A. gossypii* more or less completely to carbon dioxide and water (Mickelson, 1950; Mickelson and Schuler, 1953). However, considerable amounts of ethanol and traces of pyruvic, acetic and citric acid are formed during the course of the fermentation (Mickelson, 1950; Mickelson and Schuler,

1953). Volatile fusel alcohols and esters derived from the catabolism of amino acids are also formed via the Ehrlich pathway (Fig. 3), with particularly high level production of isoamyl alcohol and 2-phenylethanol, which confer to *A. gossypii*'s cultures a characteristic fruity/floral aroma (Ravasio et al., 2014). *A. gossypii* is also able to ferment glucose anaerobically, yielding 2 moles of carbon dioxide per mole of sugar consumed, which approaches the theoretical yield of a typical yeast alcoholic fermentation (Mickelson, 1950). On the other hand, aerobiosis is required for riboflavin overproduction (Demain, 1972; Pfeifer et al., 1950; Tanner et al., 1949).

*A. gossypii* has been shown to accumulate high amounts of lipid bodies as carbon and energy storage pools (Stahmann et al., 1994; Ledesma-Amaro et al., 2014a). Although the carbon sources utilized play a determinant role in its lipidogenic profile, *A. gossypii* predominantly accumulates unsaturated fatty acids (FA) (Stahmann et al., 1994; Ledesma-Amaro et al., 2014a). When lipids are used as carbon source, the glyoxylate cycle plays a fundamental role with respect to *A. gossypii*'s growth and riboflavin synthesis, as the precursors of riboflavin, GTP and ribulose 5-P, originate from the carbohydrate metabolism (Fig. 3) (Schmidt et al., 1996a, 1996b). The first enzyme of this pathway, isocitrate lyase (ICL), is essential for catalysing the cleavage of isocitrate to succinate and glyoxylate, thus diverting the carbon flux from the tricarboxylic acid (TCA) cycle into a carbon-conserving pathway. The final result of this anaplerotic activity is a net conversion of fats to carbohydrates through gluconeogenesis (Maeting et al., 1999; Schmidt et al., 1996a, 1996b). An extracellular lipase secreted by *A. gossypii* plays an important role in the degradation of plant oils to fatty acids and glycerol before their uptake into intracellular organelles (where  $\beta$ -oxidation takes place) (Maeting et al., 2000; Stahmann et al., 1997). Addition of non-ionic surface active agents to the culture medium (like Tween 80) stabilizes the activity of this enzyme (Stahmann et al., 1997) and enhances riboflavin production (Demain, 1972; Goodman and Ferrera, 1954).

Riboflavin biosynthesis is closely related to other biosynthetic pathways, such as the purine synthesis pathway (Fig. 3), all of which compete for the same substrates. In addition to being directly used in the riboflavin biosynthesis pathway, ribulose 5-P can alternatively be isomerized to ribose 5-P (Kruger and von Schaeuwen, 2003). GTP, the other riboflavin precursor, is synthesized through the purine pathway, which starts with the formation of phosphoribosyl pyrophosphate (PRPP) from ribose 5-P and ATP. Interestingly, *A. gossypii* was recently shown to secrete high amounts of inosine and guanosine, which are purine nucleosides produced via this same pathway (Ledesma-Amaro et al., 2015). PRPP is not only required for the *de novo* and salvage pathways of purine, pyrimidine and pyridine (NAD<sup>+</sup>, NADP<sup>+</sup>) nucleotides, but also as a precursor of histidine (Fig. 3) and tryptophan biosynthesis (Jiménez et al., 2008). The addition of purines (like hypoxanthine) and, most importantly, glycine to the culture medium has yield-enhancing effects on riboflavin production (Demain, 1972; Monschau et al., 1998). Glycine is an important precursor during the *de novo* purine biosynthesis and is endogenously obtained through the conversion of threonine by the AgGly1p threonine aldolase (Monschau et al., 1998).

One important aspect of the *A. gossypii* physiology that needs to be emphasized is the fact that significantly different sporulation efficiencies and growth parameters, such as colonial radial growth rate, have been reported not only for different strains within the species but also for the same strain deposited in different culture collections (Ribeiro et al., 2012). This inherent plasticity of *A. gossypii* also affects riboflavin production/accumulation, as Nieland and Stahmann (2013) recently showed that *A. gossypii* hyphal cells growing in the same culture exhibit phenotypic differences in what concerns overproduction and accumulation of riboflavin. Therefore, one must be cautious when analysing the available data on the metabolism and physiology of this fungus.

Recent findings reporting the existence of differences between the mating type of different *A. gossypii* strains (Dietrich et al., 2013) and of variable degrees of ploidy within the same cell at different cell cycle

stages (Anderson et al., 2015) may help explain why physiological and metabolic differences are observed between and within strains, and thus warrant further investigation. On the other hand, as the production of industrially interesting metabolites varies significantly among wild strains (Ledesma-Amaro et al., 2015), the systematic exploration of this fungus natural biodiversity may unravel new biotechnological potential for *A. gossypii*, and should therefore be considered as a standard procedure in the development of novel applications (see Section 4).

### 3. Molecular toolbox

Various genetic tools and molecular methods have been developed for use in *A. gossypii*, among which random mutagenesis with physical (ultraviolet (UV) radiation) or chemical (nitrogen mustard, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and ethyl methane sulfonate (EMS)) mutagens followed by screening of colonies with the desired phenotype has been the methodology used for the longest time to generate and isolate *A. gossypii* mutants with improved traits (Park et al., 2007; Pridham and Raper, 1952; Perlman, 1979; Ribeiro et al., 2013; Schmidt et al., 1996a; Tajima et al., 2009). In fact, random mutagenesis has been the main classical genetic approach used to manipulate *A. gossypii* strains, although adaptive evolution strategies under selective conditions also allowed isolating spontaneous mutants with improved phenotypes (Pfeifer et al., 1950; Sugimoto et al., 2010). Other random mutagenesis techniques that do not involve the use of chemical or physical mutagens have also been used to generate genetic diversity in *A. gossypii*, such as insertional mutagenesis by in vitro transposition (Santos et al., 2005) and disparity mutagenesis using an error-prone DNA polymerase (Park et al., 2011). The absence of a known sexual cycle in this fungus has impeded the use of other classical genetic approaches (Dietrich et al., 2013; Wendland et al., 2011). A significant technical difficulty associated with the *A. gossypii* mutagenic screens that needs to be highlighted is the inability to easily isolate single spores, as they clump together via terminal filaments (Wendland et al., 2011).

With the introduction of recombinant DNA technology in *A. gossypii*, reverse genetic approaches have been added to mutational techniques as a means to further improve strains in a rational way. Transformation methods for inserting foreign DNA into *A. gossypii* were initially developed by Wright and Philippsen (1991). The surprising result of their study was that freely replicating plasmids are propagated in *A. gossypii* based on *S. cerevisiae* autonomously replicating sequences (ARS) (*ARS1* and 2-micron). However, an ARS element and centromere of the *A. gossypii* chromosome V, which function in *Holleya sincauda* (a close relative of *A. gossypii*; Kurtzman, 1995), were found not to be functional in *S. cerevisiae* (Schade et al., 2003). *S. cerevisiae* centromeres do not function in *A. gossypii* as well (Wendland and Walther, 2005). Plasmids containing *S. cerevisiae* ARS elements have been widely used since to express homologous and heterologous genes in *A. gossypii* (Aguiar et al., 2014b; Altmann-Jöhl and Philippsen, 1996; Ayad-Durieux et al., 2000; Kato and Park, 2006; Magalhães et al., 2014; Monschau et al., 1998; Ribeiro et al., 2010).

Strong, constitutive promoters are available for driving homologous and heterologous gene expression in *A. gossypii*, such as those of the *A. gossypii* translation elongation factor 1 $\alpha$  (*AgTEF*) or glyceraldehyde-3-phosphate dehydrogenase (*AgGPD*) (Revuelta et al., 1999; Steiner and Philippsen, 1994). The *S. cerevisiae*'s *TEF2*, *PDC1*, *PGK1*, *LEU2* and *ADH1* constitutive promoters have also been used to drive the expression of genes in *A. gossypii* (Kaufmann, 2009; Magalhães et al., 2014; Ribeiro et al., 2010; Wendland et al., 2000). Regulatable promoters, such as the *A. gossypii*'s and *S. cerevisiae*'s *MET3*, the *S. cerevisiae*'s *THI3* and the *A. gossypii*'s *SUC2* promoters have been described to regulate the expression of genes in *A. gossypii* as well (Aguiar et al., 2014b; Dünkler and Wendland, 2007; Kaufmann, 2009).

Another interesting characteristic of *A. gossypii* is that, in contrast to other filamentous fungi and similarly to *S. cerevisiae*, it has a highly

efficient homologous recombination system (Steiner et al., 1995). This has facilitated the establishment of simple PCR-based gene targeting techniques in *A. gossypii* (Wendland et al., 2000). Integrative transformation of *A. gossypii* results in primary heterokaryotic transformants, which contain both wild and mutant nuclei. These heterokaryotic transformants generate uninucleate spores that when grown under selective conditions allow the isolation of homokaryotic mutants (Steiner et al., 1995). Thus, compared with other filamentous fungi, gene analysis in *A. gossypii* is particularly straightforward.

*A. gossypii* strains auxotrophic for threonine (*Agthr4 $\Delta$* ; Altmann-Jöhl and Philippsen, 1996) and leucine (*Agleu2 $\Delta$* ; Ayad-Durieux et al., 2000) have been developed for use in molecular genetic studies. These auxotrophies are complemented by the corresponding *S. cerevisiae* homologue genes (Altmann-Jöhl and Philippsen, 1996; Ayad-Durieux et al., 2000). Several heterologous selectable marker cassettes for dominant selection have also been developed, such as the *GEN3*, *NATPS* and *BLE3* marker cassettes, which confer resistance to G418/geneticin (Wendland et al., 2000), clonNAT/nourseothricin (Hoepfner in Kaufmann, 2009) and phleomycin (Ribeiro et al., 2013), respectively. A hygromycin resistance (*Hyg<sup>r</sup>*) marker gene has also been used to select *A. gossypii* transformants (Jiménez et al., 2008; Mateos et al., 2006).

Reporter proteins, like the *Escherichia coli* and *Aspergillus niger*  $\beta$ -galactosidase and the green fluorescent protein (GFP), have been widely used in *A. gossypii* to study gene expression and protein localization (Ayad-Durieux et al., 2000; Dünkler and Wendland, 2007; Gladfelter et al., 2006; Magalhães et al., 2014; Monschau et al., 1998; Walther and Wendland, 2012). In this context, Kaufmann (2009) developed a plasmid collection for PCR-based gene targeting in *A. gossypii* that covers a wide selection of modules for fluorescent protein tagging combined with different heterologous selection markers. Additionally, several fluorescent dyes can be used in *A. gossypii* to stain various cellular organelles and cytoskeleton structures (Gladfelter et al., 2006; Walther and Wendland, 2004; Wendland and Philippsen, 2001).

Important molecular tools for genetic manipulation of *A. gossypii* have been established, but optimal strain design for industrial applications requires flexible transformation systems that allow the removal and recycling of exogenous marker genes, especially those conferring resistance to antibiotics. With this in mind, the Cre-*loxP* recombination system of the bacteriophage P1 was adapted for generating *A. gossypii* strains free of exogenous marker genes, opening new perspectives for the deletion and/or integration of multiple genes (Aguiar et al., 2014c). In the scope of this work, three heterologous recyclable drug marker cassettes and two Cre recombinase expression vectors were created, being suitable for use in both laboratorial and industrial *A. gossypii* strains, as they do not require any predetermined genetic background. These tools also allowed the generation of stable auxotrophic strains for uridine/uracil (*Agura3 $\Delta$* ), adenine (*Agade1 $\Delta$* ) and both (*Agura3 $\Delta$ , Agade1 $\Delta$* ) (Aguiar et al., 2014c). Unlike the others auxotrophies already mentioned (*A. gossypii* *Agleu2 $\Delta$*  and *Agthr4 $\Delta$*  strains), the corresponding *S. cerevisiae*'s homologue could not complement the deletion of *AgURA3* (Pompejus et al., 1999). Therefore, these auxotrophic strains will be important for future engineering projects as soon as a selection system based on the complementation of these auxotrophies becomes available.

All these molecular tools have been important for the development of stable *A. gossypii* strains for biotechnological purposes (as it will be discussed in the following section), but the improvement of existing tools and development of new ones will be crucial to meet the demands of the biotechnology industry and to develop *A. gossypii* as a new cell factory. In this regard, the establishment of flexible genome editing methods (e.g., CRISPR-Cas system; DiCarlo et al., 2013) for engineering this fungus is of great interest. Moreover, as the number of characterized promoters currently available for use in *A. gossypii* is limited, screening and identification of other promoters with desired characteristics will settle the basis for the development of new and more flexible gene

expression tools. Similar screening strategies should be followed for designing new reporter proteins and secretion signal sequences for use in the scope of recombinant protein production. Beyond these pressing needs, a major outcome of the upcoming years may be related with the arising of forward genetic tools for *A. gossypii*, fading away the dependency on reverse genetics. However, significant work is still needed in order to elucidate the conditions needed for sexual reproduction. The first step has already been taken with the isolation of wild *A. gossypii* strains possessing mating type regions distinct from those presented by the first sequenced strain, ATCC10895 (Dietrich et al., 2013).

#### 4. Commercial and emerging biotechnological applications

The filamentous fungus *A. gossypii* presents several industrially attractive features, such as good performance in large-scale fermentation processes and ability to grow in inexpensive waste-derived substrates to high cell densities (Park et al., 2007; Tajima et al., 2009; Wei et al., 2013). Its capacity to secrete proteins and metabolites to the culture medium and to undergo autolysis during the late stationary phase of growth or at low temperature is also industrially appealing, as they facilitate downstream product recovery (Aguiar et al., 2014a; zu Berstenhorst et al., 2009). These features, combined with the successful industrial process of riboflavin production by *A. gossypii* and with the availability of several tools for its easy genetic manipulation, have raised attention to this fungus for the production of other metabolites and/or proteins. In the last year, systems biology provided essential *in silico* predictive tools to optimize existing and develop new biotechnological applications for *A. gossypii*. In this context, the *A. gossypii* genome was re-annotated (Gomes et al., 2014) and its reaction set compiled to create a genome scale metabolic model (GSMM) able to simulate the cell behaviour, being now this model in its experimental validation phase (Gomes et al., personal communication). Other GSMM for *A. gossypii* was already published (iRL766) and successfully validated by predictions and comparisons for three experimental parameters: biomass growth, riboflavin production and substrate utilization (Ledesma-Amaro et al., 2014b). Together, these new tools are leveraging the *A. gossypii* potential as a cell factory organism.

##### 4.1. Riboflavin production

*A. gossypii* constitutes a paradigm of the sustainable White Biotechnology with regard to industrial riboflavin production. Riboflavin is a precursor of the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are essential co-factors for numerous enzymes (including dehydrogenases, oxidases, oxidoreductases) that participate in a range of redox reactions critical for major biological processes. This vitamin is commercially used as a yellow colourant and animal food additive, and for many years it was mainly obtained by chemical synthesis, until its industrial production shifted to microbial fermentation (Stahmann et al., 2000; zu Berstenhorst et al., 2009). As an outcome of

classical improvement of naturally overproducing strains by random mutagenesis and optimization of culture conditions, the industrial fermentation process using *A. gossypii* for riboflavin production is among the first biotechnological processes that replaced the chemical process in use due to its higher economical competitiveness (Kato and Park, 2012; Lim et al., 2001; Stahmann et al., 2000; zu Berstenhorst et al., 2009).

Wild *A. gossypii* strains have been reported to produce up to 5 g/L of riboflavin in optimized culture media, but randomly improved strains can produce from 14 to 20 g/L of riboflavin (Demain, 1972; Lim et al., 2001; Park et al., 2011). Many randomly improved *A. gossypii* strains for riboflavin production were obtained by several rounds of mutagenesis followed by screening of colonies with increased yellow pigmentation (Perlman, 1979). Since the industrial establishment of this process, different mutagenic agents and screening procedures were adopted and combined in different manners (Table 1). UV radiation (Park et al., 2007; Schmidt et al., 1996b; Wei et al., 2012), chemical mutagens (Lizama et al., 2007; Tajima et al., 2009) and disparity mutagenesis (Park et al., 2011) have been applied to create mutations. For the screening procedures, antimetabolites (mainly itaconate and oxalate) have been the most common way to isolate overproducing strains, essentially due to the correlation between the *A. gossypii* isocitrate lyase (Icl1p; glyoxylate cycle) activity and riboflavin production (Schmidt et al., 1996a, 1996b). In fact, Sugimoto et al. (2010) were able to isolate a riboflavin overproducing strain only by growing *A. gossypii* spores in a medium containing oxalate, without the application of any mutagenic agent. More recently, through the analysis of the itaconate metabolism and genetics, its related compounds *cis*-aconitate and *trans*-aconitate were detected in the riboflavin production phase and, as the latter has an inhibitory effect on aconitase in the TCA, the authors proposed *trans*-aconitate as a novel and more available antimetabolite for screening of riboflavin overproduction (Sugimoto et al., 2014).

Since the 1990s, when BASF (Germany) launched a riboflavin production plant with *A. gossypii*, the performance of *A. gossypii* strains could be much improved by rational design as well. The constantly increasing molecular toolbox for this fungus (see Section 3) and the deep understanding acquired along the last two decades about the *A. gossypii* riboflavin biosynthetic pathway (reviewed in Kato and Park, 2012) supported this. Among the rational strategies tested (Table 2), the majority focused on increasing the flux of the riboflavin biosynthetic pathway and of the purine/glycine biosynthetic pathways by (1) overexpressing the *AgRIBs* genes (Althöfer et al., 1999; Althöfer and Revuelta, 2003); (2) overexpressing the *AgGLY1* gene, involved in the conversion of threonine to glycine (Monschau et al., 1998); (3) disrupting the *AgSHM2* gene, which encodes a cytosolic serine hydroxymethyltransferase that converts glycine into serine (Schlüpen et al., 2003); (4) overexpressing the *S. cerevisiae*'s *AGX1* gene, which is not present in *A. gossypii* and encodes an alanine:glyoxylate aminotransferase that converts glyoxylate into glycine (Kato and Park, 2006); (5) overexpressing

**Table 1**

Random engineering approaches applied in *A. gossypii* for biotechnological purposes.

| Mutagen                         | Screening   | Application                    | Reference(s)                               |
|---------------------------------|---|--------------------------------|--|
| UV light                        | Antimetabolite (itaconate)  | Riboflavin                     | Park et al. (2007), Schmidt et al. (1996b) |
| UV light                        | Yellow colour of the colonies   | Riboflavin                     | Wei et al. (2012)                          |
| MNNG                            | Antimetabolite (itaconate)  | Riboflavin                     | Lizama et al. (2007), Tajima et al. (2009) |
| –                               | Antimetabolite (oxalate)  | Riboflavin                     | Sugimoto et al. (2010)                     |
| Mutated DNA polymerase $\delta$ | Antimetabolite (oxalate and hydrogen peroxide)  | Riboflavin                     | Park et al. (2011)                         |
|                                 | Regeneration of the colonies by serial transfers in selective medium  |                                |  |
|                                 | Stabilization of the colonies in non-selective medium and quantification of riboflavin production                 |                                |  |
| EMS                             | Selective medium containing CMC <sup>a</sup> (for EGI), starch (for $\alpha$ -amylase) or tributyrin (for lipase) | Recombinant protein production | Ribeiro et al. (2013)                      |
|                                 | Determination of specific enzymatic activities  |                                |  |

<sup>a</sup> Carboxymethylcellulose.

**Table 2**  
Target genes of rational genetic engineering strategies in *A. gossypii* for different biotechnological applications.

| Gene                                  | Strategy and outcome  | Reference(s)                               |
|---------------------------------------|---|--|
| <b>Riboflavin production</b>          |   |  |
| <i>AgRIB3-5</i>                       | Overexpression increased the flux of the riboflavin biosynthetic pathway, which resulted in a 2.5-fold increase in riboflavin production  | Althöfer et al. (1999)                     |
| <i>AgRIB1,2,4,7</i>                   | Overexpression increased the flux of the riboflavin biosynthetic pathway, which resulted in an 1.4-fold increase in riboflavin production   | Althöfer and Revuelta (2003)               |
| <i>AgGLY1</i>                         | Overexpression increased glycine formation from threonine, which allowed a 9-fold increase in specific riboflavin production  | Monschau et al. (1998)                     |
| <i>AgSHM2</i>                         | Disruption abolished the conversion of glycine into serine, which led to a 10.6-fold increase in specific riboflavin production   | Schlüpen et al. (2003)                     |
| <i>ScAGX1</i>                         | Heterologous expression allowed glycine biosynthesis from glyoxylate, which resulted in an 1.3-fold increase in specific riboflavin production  | Kato and Park (2006)                       |
| <i>AgADE4</i>                         | Overexpression and elimination of feedback inhibition by site directed mutagenesis increased the flux of the purine biosynthetic pathway, which resulted in a 10-fold increase in riboflavin production | Jiménez et al. (2005)                      |
| <i>AgPRS2,4</i> and <i>AgPRS3</i>     | Overexpression and elimination of feedback inhibition by site directed mutagenesis increased PRPP availability, which resulted in an 1.7- to 1.8-fold increase in riboflavin production                 | Jiménez et al. (2008)                      |
| <i>AgBAS1</i>                         | Deletion of the C-terminal interaction and regulatory domain constitutively activated the purine and glycine pathways, which resulted in a 9.4-fold increase in specific riboflavin production          | Mateos et al. (2006)                       |
| <i>AgURA3</i>                         | Disruption blocked the pyrimidine biosynthetic pathway, thus increasing the availability of riboflavin precursors, which led to a 7.5-fold increase in specific riboflavin production                   | Aguiar et al. (2014c), Silva et al. (2015) |
| <i>AgICL1</i>                         | Overexpression increased riboflavin production from oils (by 2.2-fold)  | Käsler et al. (1997)                       |
| <i>AgMLS1</i>                         | Overexpression increased riboflavin production from oils (by 1.7-fold)  | Sugimoto et al. (2009)                     |
| <i>AgVMA1</i>                         | Disruption reduced the accumulation of riboflavin in the vacuole and increased its excretion to the culture medium (by 2-fold)  | Förster et al. (1999)                      |
| <b>Recombinant protein production</b> |   |  |
| <i>ShcelA1</i>                        | Heterologous expression allowed the recombinant production of <i>S. halstedii</i> endo- $\beta$ -1,4-glucanase  | Althöfer et al. (2001)                     |
| <i>TregII</i> and <i>TrcbII</i>       | Heterologous expression allowed the extracellular production of <i>T. reesei</i> EGI and CBHI   | Ribeiro et al. (2010)                      |
| <i>AnlacA</i>                         | Heterologous expression allowed the extracellular production of <i>A. niger</i> $\beta$ -galactosidase  | Magalhães et al. (2014)                    |
| <i>ScSUC2</i>                         | Heterologous expression allowed the extracellular production of <i>S. cerevisiae</i> invertase  | Aguiar et al. (2014b)                      |
| Single cell oil production            |   |  |
| <i>YIACL1/YIACL2</i>                  | Heterologous expression enabled the production of acetyl-CoA from citrate, which increased lipid accumulation during the trophic phase (by 1.3-fold)  | Ledesma-Amaro et al. (2014a)               |
| <i>AgPOX1</i> and <i>AgFOX2</i>       | Disruption blocked the beta-oxidation pathway, which triggered a 2- to 3.5-fold increase in lipid accumulation  | Ledesma-Amaro et al. (2014a)               |
| <i>AgELO586</i>                       | Overexpression increased cerotic acid (26:0) accumulation and disruption resulted in SCO with properties that meet some biodiesel quality standards   | Ledesma-Amaro et al. (2014c)               |
| <i>AgELO624</i>                       | Overexpression led to nervonic acid (24:1) production and disruption resulted in SCO with properties that meet some biodiesel quality standards   | Ledesma-Amaro et al. (2014c)               |
| <i>AgDES589</i>                       | Overexpression increased linoleic acid (18:2) accumulation  | Ledesma-Amaro et al. (2014c)               |
| <b>Flavour compounds' production</b>  |   |  |
| <i>AgARO80</i>                        | Overexpression increased isoamyl alcohol and isobutanol production (by 2.5-fold)  | Ravasio et al. (2014)                      |
| <i>AgADE12</i>                        | Disruption increased the excretion of inosine (by 11.5-fold) and guanosine (by 1.2-fold)  | Ledesma-Amaro et al. (2015)                |
| <i>AgIMD3</i>                         | Disruption increased inosine excretion (by 1.6-fold) and overexpression increased guanosine excretion (by 1.5-fold)   | Ledesma-Amaro et al. (2015)                |
| <i>AgPNP1</i>                         | Disruption increased the excretion of inosine (by 1.9-fold) and guanosine (by 1.5-fold)   | Ledesma-Amaro et al. (2015)                |
| <i>AgISN1</i>                         | Disruption did not increase inosine excretion   | Ledesma-Amaro et al. (2015)                |
| <i>AgGUA1</i>                         | Overexpression increased riboflavin production but did not increase guanosine excretion   | Ledesma-Amaro et al. (2015)                |
| <i>AgRIB1</i>                         | Down regulation reduced riboflavin production but did not increase guanosine excretion  | Ledesma-Amaro et al. (2015)                |

Ag, *A. gossypii*; An, *A. niger*; Sc, *S. cerevisiae*; Sh, *S. halstedii*; Tr, *T. reesei*; Yl, *Y. lipolytica*.

several genes involved in the purine biosynthetic pathway such as *AgADE4* (Jiménez et al., 2005), *AgPRS2,4* and *AgPRS3* (Jiménez et al., 2008); (6) deregulating the transcription factor *AgBas1p* that constitutively activates the purine and glycine biosynthetic pathways (Mateos et al., 2006); and (7) blocking the *A. gossypii*'s *de novo* pyrimidine biosynthetic pathway by deletion of the *AgURA3* gene (Aguiar et al., 2014c) to increase the availability/accumulation of PRPP for the formation and/or accumulation of riboflavin precursors (GTP and ribulose-5P; Silva et al., 2015). Other strategies focused on improving riboflavin production from plant oils by overexpressing genes involved in the glyoxylate cycle, such as the *AgICL1* (Käsler et al., 1997) and the *AgMLS1* (encoding the malate synthase; Sugimoto et al., 2009), and on increasing the excretion of riboflavin into the medium by disrupting the gene encoding the vacuolar ATPase subunit A, *AgVMA1* (Förster et al., 1999).

An increasing number of evidences have shown that the triggering of riboflavin overproduction in *A. gossypii* is associated with the activation of intracellular stress signalling cascades in response to environmental changes (Kavitha and Chandra, 2009, 2014; Walther and Wendland, 2012; Nieland and Stahmann, 2013). Therefore, future attempts to

further improve riboflavin production will rely on the better understanding of the overall stress responses of *A. gossypii*. Meanwhile, the knowledge already gained with *A. gossypii* will serve as a basis to guide the improvement of riboflavin production by other cell factories.

#### 4.2. Recombinant protein production

Beyond riboflavin production, the exploration of *A. gossypii* as a host for the production of heterologous proteins has received much attention in the last years. An endo- $\beta$ -1,4-glucanase (*celA1*) from *Streptomyces halstedii* (Althöfer et al., 2001), two cellulases from *Trichoderma reesei*, endoglucanase I (EGI) and cellobiohydrolase I (CBHI) (Ribeiro et al., 2010), a  $\beta$ -galactosidase from *Aspergillus niger* (Magalhães et al., 2014) and a *S. cerevisiae*'s invertase (Aguiar et al., 2014b) are among the repertoire of heterologous enzymes already produced in this host system (Table 2).

*A. gossypii* possesses the ability to secrete native and heterologous enzymes to the extracellular medium and to recognize signal peptides of other organisms as secretion signals (Aguiar et al., 2014b; Althöfer et al., 2001; Magalhães et al., 2014; Ribeiro et al., 2010; Stahmann



et al., 1997). The amount and variety of native proteins secreted by this fungus are low, being its secretory potential more similar to that of yeast than to that of other filamentous fungi (Aguiar et al., 2014a). In line with this observation, the protease activity in *A. gossypii* culture supernatants is negligible (Ribeiro et al., 2010). Together, these features represent important advantages for heterologous protein production in this host, as secreted products are less likely to be contaminated or degraded by native proteins, thus allowing cost-efficient downstream processing of low- and medium-value enzymes.

*A. gossypii* is also able to perform protein post-translation modifications, such as glycosylation and other modifications required for the biological activity and stability of proteins (Aguiar et al., 2013; Magalhães et al., 2014; Ribeiro et al., 2010). Compared to the closely related yeast *S. cerevisiae*, one of the fungal hosts most commonly used for the production of heterologous proteins (Demain and Vaishnav, 2009), *A. gossypii* has the tendency to hyperglycosylate secreted glycoproteins less extensively, as disclosed by the glycosylation pattern of the heterologous EGI, CBHI (Ribeiro et al., 2010) and  $\beta$ -galactosidase (Magalhães et al., 2014). This is advantageous for the production of proteins whose properties may be adversely affected by extensive glycosylation. A recent characterization of its secreted *N*-glycome corroborates these data, as *A. gossypii* revealed a processing system substantially distinct from that of *S. cerevisiae*, which allows the production of much shorter *N*-glycans (Aguiar et al., 2013). In fact, this study showed that *A. gossypii* predominantly produces *N*-glycans that are more similar in extent to those produced by nonconventional yeast hosts such as *Pichia pastoris*, *Hansenula polymorpha* and *Yarrowia lipolytica* (Aguiar et al., 2013). Nevertheless, the extent of *N*-glycosylation and *N*-glycan phosphorylation in *A. gossypii* is highly influenced by the culture medium used (Aguiar et al., 2013).

Despite these advantages, the production levels reported for the first heterologous proteins secreted by *A. gossypii* (EGI and CBHI) were low, although comparable to those obtained with *S. cerevisiae* (Ribeiro et al., 2010). In fact, the obtainment of reasonable amounts of these cellulases by recombinant microbial hosts in a biologically active form has been a challenging task (Ribeiro et al., 2010). Therefore, further investigation of *A. gossypii* as a recombinant protein production host went through the implementation of random engineering techniques to improve its general secretory capacity. In this context, the recombinant *A. gossypii* strain expressing EGI was exposed to the chemical mutagen EMS (Ribeiro et al., 2013). The following screening strategy was initially based on the diameter of the haloes formed around the mutant colonies on screening media for different enzymatic activities (Table 1). Among the activities tested, only cellulolytic activity gave a good correlation and allowed the selection of the most promising mutants (Ribeiro et al., 2013). Subsequently, these mutants were grown in submerged cultures and extracellular activities were determined for heterologous cellulase (*T. reesei* EGI), native  $\alpha$ -amylase and native  $\beta$ -glucosidase. One mutant exhibiting a superior performance (of 1.4- to 2-fold) in all extracellular enzymatic activities was identified, suggesting a global improvement in its secretory capacity (Ribeiro et al., 2013). Other mutants also presented partial improvements (of 2- to 3-fold): two in EGI or  $\beta$ -glucosidase activity, and one in EGI and  $\alpha$ -amylase activities (Ribeiro et al., 2013). Another attempted to increase the general secretory potential of this fungus involved the deletion of *AgGAS1* homologues (encoding a  $\beta$ -1,3-glucanosyltransglycosylase involved in cell wall assembly) to enhance the permeability of the cell wall, but the resulting mutants presented serious growth defects (Ribeiro et al., 2013).

Transcriptomic analysis performed in *A. gossypii* cells under recombinant protein secretion conditions and dithiothreitol-induced secretion stress subsequently provided useful information about possible strategies that could be used to further improve its productivity (Aguiar et al., 2014a). Among these, the use of stronger promoters was found to be of utmost importance, as the heterologous *S. cerevisiae* *PGK1* constitutive promoter was proved to be inefficient in driving the overexpression of the EGI-encoding gene (Aguiar et al.,

2014a). This study also revealed the existence in *A. gossypii* of alternative regulatory mechanisms to cope with protein secretion stress, other than the conventional unfolded protein response (UPR), which may offer novel ways of improving protein secretion in this fungus (Aguiar et al., 2014a).

The strength of several native (*A. gossypii* *TEF* and *GPD*) and heterologous (*S. cerevisiae* *PGK1* and *ADH1*) constitutive promoters was compared when producing a heterologous protein of other origin, the  $\beta$ -galactosidase from *A. niger* (Magalhães et al., 2014). Given the ease of detection of this secreted enzyme, the screening of transformants was facilitated. Moreover, as this protein had already been efficiently secreted by recombinant *S. cerevisiae* strains (Domingues et al., 2002, 2005; Oliveira et al., 2007; Ramakrishnan and Hartley, 1993), it provided a good model protein to further explore the potential of *A. gossypii* as a heterologous protein producer. The strong *A. gossypii*'s *TEF* promoter drove the highest production levels of this enzyme in *A. gossypii* and *S. cerevisiae* laboratory strains, followed by the other native promoter tested, *GPD* (Magalhães et al., 2014). Using the *TEF* promoter, *A. gossypii* secreted up to 37-fold more active  $\beta$ -galactosidase than two different *S. cerevisiae* strains transformed with the same plasmids (Magalhães et al., 2014). In similar culture conditions, *A. gossypii* also produced 2.5-fold more extracellular  $\beta$ -galactosidase than that previously reported for a  $\beta$ -galactosidase high-producing *S. cerevisiae* strain transformed with a different plasmid under the control of the *S. cerevisiae*'s *ADH1* promoter (Domingues et al., 2002; Magalhães et al., 2014). However, much can still be improved just by manipulating the production conditions, as demonstrated by the 1.5-fold increase in  $\beta$ -galactosidase secretion when glucose was replaced by glycerol in the culture medium, which allowed reaching extracellular activity levels (1127 U/mL) within the range of those reported for nonmodified *A. niger* strains (152 to 3000 U/mL) (Magalhães et al., 2014).

In summary, the potential of *A. gossypii* as a host for heterologous protein production is still underexplored and much could be learned about possible strategies to improve its productivity, among which are as follows: (1) culture medium optimization; (2) screening for better promoters and secretion signal sequences; and (3) development of better expression strategies, preferably through the integration of stable expression cassettes.

#### 4.3. Single cell oil

The adaptation of *A. gossypii* for SCO production is a perfect example of how the molecular (see Section 3) and *in silico* toolbox made available in recent years for this fungus allowed to rationally capitalize its natural abilities (see Section 2.4). SCO is an alternative source to the oil industry, which has seen its main sources (crude oil, animal fat and plant oil) linked to negative environmental and economical effects (Beopoulos et al., 2011). In spite of being considered a non-oleaginous microorganism (Vorapreeda et al., 2012), *A. gossypii* was metabolically engineered to fit the requisites of a candidate for SCO production (minimum of 20% lipid content in relation to its cell dry weight) (Ledesma-Amaro et al., 2014a). Two different strategies were used (Ledesma-Amaro et al., 2014a): first, the genes *AgPOX1* and *AgFOX2*, which are involved in the  $\beta$ -oxidation pathway, were disrupted to avoid FA degradation; second, a heteromeric ATP-citrate lyase (*AcIp*) from *Y. lipolytica* was overexpressed in *A. gossypii* to enable the conversion of citrate into acetyl-CoA and thus increase the pool of this crucial FAs building block.

Single mutants (*pox1* $\Delta$  or *fox2* $\Delta$ ) with the  $\beta$ -oxidation pathway blocked were able to improve the accumulation of FAs in media containing only glucose or glucose and oils (Ledesma-Amaro et al., 2014a). In turn, the double mutant strain (*pox1* $\Delta$ *fox2* $\Delta$ ) did not present any advantage in relation to the single mutants. When oils were supplied as the sole carbon source, all of these strains presented growth limitation (Ledesma-Amaro et al., 2014a). The *pox1* $\Delta$  mutant revealed to be the most efficient strain, reaching a lipid content of approximately 40% (after 3 days of growth) and 70% (after 7 days of growth) of its dry

weight (Ledesma-Amaro et al., 2014a). The heterologous overexpression of *YIACL1/YIACL2* (encoding both subunits of AcIp from *Y. lipolytica*) also fulfilled the goal for which it was originally proposed, i.e., increase the cellular pool of acetyl-CoA (Ledesma-Amaro et al., 2014a). Thus, the *A. gossypii* *YIACL1/YIACL2* strain presented superior lipid accumulation, fulfilling the requirements of oleaginous microorganisms in terms of lipid accumulation. However, the *A. gossypii* *pox1Δ,YIACL1/YIACL2* strain did not translate the higher levels of acetyl-CoA available into more lipid accumulation in comparison with the *A. gossypii* *pox1Δ* (Ledesma-Amaro et al., 2014a). The *pox1Δ* strain revealed a metabolic imbalance that increased the acetyl-CoA pool independently of the AcIp presence and some other cellular mechanisms benefited from these higher levels of acetyl-CoA (Ledesma-Amaro et al., 2014a). Hence, as suggested by the authors, this increase in acetyl-CoA pools can be valuable in the future to explore the production of other relevant products beyond SCO, such as biobutanol.

The potential applications of SCO are strictly connected with the FA profile of the strain, which should be manipulated according to the requisites of a particular industry. This FA profile is largely defined by the action of enzymes such as the elongases and desaturases, which are responsible for the length and unsaturations of FAs, respectively (Hashimoto et al., 2008). In this regard, Ledesma-Amaro et al. (2014c) performed a functional characterization of selected genes of the elongation (*AgELO586* and *AgELO624*) and desaturation (*AgDES589* and *AgDES079*) system of *A. gossypii*, which had already been identified by Hashimoto et al. (2008). After testing the functionality of the respective proteins in *S. cerevisiae*, only *AgDES079* did not match its predicted function, not showing any notable role in FA desaturation (Ledesma-Amaro et al., 2014c). The regulation of these genes by the carbon source used (glucose or soybean oil) was also checked by analysis of their transcription levels. Once again, *AgDES079* was the only gene that did not present any changes (Ledesma-Amaro et al., 2014c). Finally, all genes were independently overexpressed or deleted in *A. gossypii*. Excluding *AgDES079*, the manipulation of the other three genes generated a repertoire of strains that can be applied in different industries according to their FA profile (Table 2). For instance, the engineered strains *A. gossypii* *elo586Δ* and *A. gossypii* *elo624Δ* fulfilled the restrictive requirements established by the European biodiesel quality standard EN 14214, and are therefore very promising for application in this industry (Ledesma-Amaro et al., 2014c).

The adaptation of *A. gossypii* for SCO presented very consistent and promising results. According to Ledesma-Amaro et al. (2014a), the 70% of lipid accumulation achieved in the engineered *A. gossypii* strains are among the highest accumulation levels reported so far in both oleaginous and nonoleaginous microorganisms. Moreover, the strains created by metabolic engineering of the elongation and desaturation systems have a wide application spectrum in other industrial sectors, such as cosmetics, food, paper and pharmaceutical industry (Ledesma-Amaro et al., 2014c).

#### 4.4. Flavour compounds' production

The fermented beverages sector invests strongly in new products that can offer a new variety of flavour/aroma perceptions, which are determinant features for product quality. Therefore, there is a continuous effort for screening the biodiversity in order to find new natural producers. In this regard, there is a growing interest on non-*Saccharomyces* species as natural flavour producers, which many believe to remain largely underexplored (Domizio et al., 2010).

The Ehrlich pathway plays an important role in the metabolism of flavour/aromatic compounds and Wendland and Walther (2011) noticed significant genetic differences between *A. gossypii* and its closely related *E. cymbalariae* regarding this pathway. *E. cymbalariae* harbours only one gene related with this pathway (*ARO8a*), whereas *A. gossypii* encodes four genes (*AgARO8a*, *AgARO8b*, *AgARO10* and *AgARO80*). In

comparison with *S. cerevisiae*, *A. gossypii* lacks *ARO9* (Ravasio et al., 2014). Therefore, Ravasio et al. (2014) decided to characterize and explore the importance of this pathway in flavour production by *A. gossypii*. The profile of volatile compounds produced by *A. gossypii* is characterized by high levels of 2-phenylethanol (rose/flower aroma), which are residual in *E. cymbalariae*, and of isoamyl alcohol (banana/fruity aroma), also elevated in *E. cymbalariae*. Indeed, the production of 2-phenylethanol by *A. gossypii* was determined to be higher than that in *S. cerevisiae* strains, namely *Weihenstephan* lager yeast and laboratorial CEN.PK2 (Ravasio et al., 2014). *A. gossypii* single mutants for the *ARO* genes revealed a strong impairment in the production of 2-phenylethanol and also isoamyl alcohol (with exception for the *AgARO8a*) (Ravasio et al., 2014). As *AgARO80* encodes a transcriptional regulator of some of these genes, these authors overexpressed this gene. Although it was not possible to note significant differences in the production of 2-phenylethanol (possibly due to phenylalanine limitation in the culture medium), the engineered strain was able to produce about 2.5-fold more isoamyl alcohol and isobutanol than the parent strain (Ravasio et al., 2014). This work reinforced the idea that the exploration of non-*Saccharomyces* strains for flavour enhancement of fermented beverages may result in a new wide line of products.

Very recently, *A. gossypii* became the first eukaryotic microorganism presenting good potential for industrial nucleoside production, particularly inosine and guanosine (Ledesma-Amaro et al., 2015). These flavour active compounds (umami flavour) are used by the food industry with various beneficial effects associated to them. Some wild strains have the ability to naturally secrete high levels of inosine and guanosine (Ledesma-Amaro et al., 2015). Furthermore, as a natural riboflavin overproducer, *A. gossypii* has strong metabolic flow through the purine biosynthetic pathway, the central biosynthetic pathway of these two compounds. Based on this, a series of *in silico* simulations by the GSMM iRL766 (Ledesma-Amaro et al., 2014b) allowed defining a set of target genes (Table 2) for disruption or overexpression in order to redirect the flux through inosine and/or guanosine biosynthesis. An engineered strain (overexpressing a deregulated *AgADE4*; Jiménez et al., 2005) with increased flux through the purine biosynthetic pathway, which served before to enhance riboflavin production, was used as background strain to introduce more specific metabolic shifts (Ledesma-Amaro et al., 2015). From a total of eight new engineered strains, the strain deleted for *AgADE12*, encoding the enzyme responsible for the conversion of inosine-5'-phosphate (IMP) into adenylosuccinate (sAMP), and for *AgPNP1*, encoding the enzyme responsible for the conversion of the nucleosides (inosine and guanosine) into nucleobases (hypoxanthine and guanine), was able to produce and excrete 24-fold more inosine (0.27 g/L) and 2-fold more guanosine (0.14 g/L) than the wild type strain (Ledesma-Amaro et al., 2015).

The filamentous fungus *A. gossypii* demonstrated once again to possess a very interesting natural potential and the machinery needed to create improved engineered strains for different biotechnological applications, in this particular case as a natural flavour producer for the fermented beverages and food industries. The advent of systems biology has allowed leveraging this natural biotechnological potential of *A. gossypii* (Ledesma-Amaro et al., 2015) and will continue playing a major role in the development of this fungus as a cell factory.

With a dominant position in what concerns industrial riboflavin production, *A. gossypii* has benefited from constant and sustainable improvement of its natural ability, thus keeping the commercial competitiveness of the process. Production of riboflavin by *A. gossypii* will always be a landmark for the White Biotechnology and its improvement will continue to be pursued. However, there is no doubt that the trend in the last years show that *A. gossypii* holds the potential to expand its biotechnological significance way beyond riboflavin production. Following the promising results obtained with the production of recombinant proteins, SCOs and flavour compounds, future studies should assess if the optimization, scale-up and intensification of these bioprocesses can support the obtainment of economically

sustainable product yields. On the other hand, further strain engineering should also focus on expanding the range of substrate utilization by this fungus and address the utilization of low-cost substrates derived from renewable raw materials in the abovementioned bioprocesses.

## 5. Conclusions

The filamentous fungus *A. gossypii* has long been exploited for the industrial production of riboflavin. The fruitful synergy resulting from the strong investment in both applied and basic research puts *A. gossypii* in a leading position in the establishment of the knowledge-based biotechnology envisaged by the White Biotechnology. The wealth of molecular and *in silico* tools available for this microorganism has allowed the rational exploitation of its full biotechnological potential, which nowadays goes far beyond the production of riboflavin. It is now time to take advantage of all the knowledge acquired during these years and go further in the search for new commercially valuable applications for this emerging cell factory, such as the recently reported production of recombinant proteins, single cell oil and flavour compounds. To drive these new *A. gossypii* applications through the successful commercial track of riboflavin production, newer and more flexible tools for its rational engineering and synthetic biology will necessarily have to be combined with process development and optimization. Furthermore, given the physiological and genetic heterogeneity observed between *A. gossypii* strains, future exploration of this fungus natural biodiversity holds the potential to unravel new strains for the industrial applications described above or for yet unexplored novel applications.

## Acknowledgements

The authors thank the financial support from the Fundação para a Ciência e a Tecnologia (FCT), Portugal, through the Strategic Project of UID/BIO/04469/2013 unit (Post-Doc fellowship UMINHO/BPD/36/2015 to T. Q. Aguiar), the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462) and the project "BioInd - Biotechnology and Bioengineering for improved Industrial and Agro-Food processes", REF. NORTE-07-0124-FEDER-000028, co-funded by the Programa Operacional Regional do Norte (ON.2 - O Novo Norte), QREN, FEDER.

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