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COMMENTARY

New biotechnological applications for *Ashbya gossypii*: Challenges and perspectives

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ABSTRACT

The filamentous fungus *Ashbya gossypii* has long been considered a paradigm of the White Biotechnology in what concerns riboflavin production. Its industrial relevance led to the development of a significant molecular and *in silico* modeling toolbox for its manipulation. This, together with the increasing knowledge of its genome and metabolism has helped designing effective metabolic engineering strategies for optimizing riboflavin production, but also for developing new *A. gossypii* strains for novel biotechnological applications, such as production of recombinant proteins, single cell oils (SCOs), and flavour compounds. With the recent availability of its genome-scale metabolic model, the exploration of the full biotechnological potential of *A. gossypii* is now in the spotlight. Here, we will discuss some of the challenges that these emerging *A. gossypii* applications still need to overcome to become economically attractive and will present future perspectives for these and other possible biotechnological applications for *A. gossypii*.

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Introduction

Ashbya gossypii (syn. *Eremothecium gossypii*) is a highly flavinogenic mold of the Saccharomycetaceae family that has been extensively exploited for the production of riboflavin (vitamin B2). Genetically improved *A. gossypii* strains have been used since 1990 for the industrial production of this vitamin by the chemical company BASF.[1] Given the biotechnological relevance of this filamentous fungus, significant efforts have been made in the last decades to develop molecular and systems biology tools for its engineering, which altogether allowed to greatly improve the productivity of the initial industrial strains developed by classical mutagenesis techniques.[2,3] The success of this strain improvement program ultimately led to the replacement at the industrial level of the chemical riboflavin production process by the biotechnological one, as the later became economically and ecologically more feasible than the first.[1-3] Thus, this biotechnological process with *A. gossypii* became a paradigm of the sustainable White Biotechnology business model.

Although the industrial production of riboflavin has come a long way, the recent advances in the field of systems biology for *A. gossypii*[2,4-7] have shown that the carbon flux of this fungus toward riboflavin production can be further improved.[8,9] Moreover, the *in silico* modeling tools that recently became available for *A. gossypii* are also boosting the emergence of other commercially valuable biotechnological applications for this fungus, such as SCO production,[10] flavour compounds production[11-13] and also recombinant protein production[14,15] (Fig. 1).

While important advances have been accomplished in what concerns these new *A. gossypii* applications (Table 1), several challenges still need to be overcome until these emerging applications can follow the successful commercial track of riboflavin production. Here, we will pin point some of these challenges and will indicate future perspectives for these new *A. gossypii* biotechnological applications.

Recombinant protein production

Among the new biotechnological applications for which *A. gossypii* has been explored, recombinant protein production was the first to emerge, motivated by

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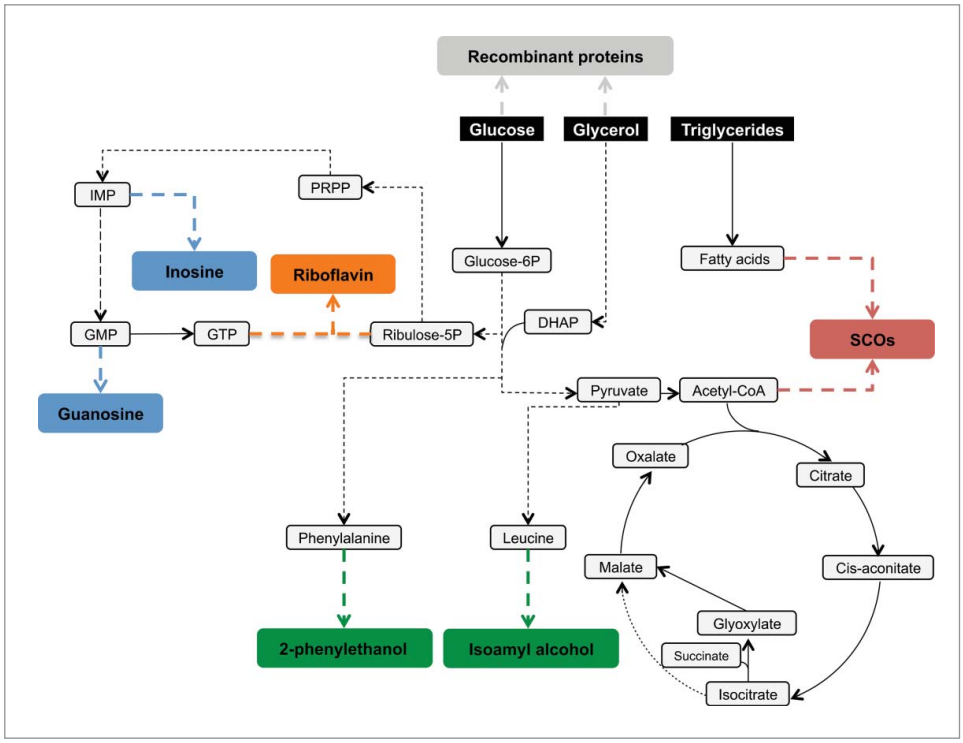

Figure 1. Main products and metabolic reactions involved in the new biotechnological applications for which *A. gossypii* has been explored. Dashed arrows indicate several reactions. DHAP, dihydroxy-acetone-phosphate; PRPP, phosphoribosyl pyrophosphate; IMP, inosine-5′-phosphate; GMP, guanosine-5′-phosphate; GTP, guanosine-5′-triphosphate.

several interesting features presented by this fungus, such as: (a) ability to secrete native and heterologous enzymes to the extracellular medium and to recognize signal peptides of other organisms as secretion signals;[15-17,23] (b) ability to perform protein post-translation modifications, such as glycosylation (producing *N*-glycans similar in extent to those produced by nonconventional yeast, such as *Pichia pastoris*) and other modifications required for the bioactivity and stability of recombinant proteins;[16,17,24] (c) secretion of low amount and variety of native proteins and negligible extracellular protease activity, which facilitates downstream processing and recovery of low- and medium-value secreted products, as these are less

Table 1. Production levels of some biotechnological relevant compounds produced by *A. gossypii*. The production levels of these bioproducts by other microorganisms are also presented for comparison.

Species	Product	Genetic Background	Culture Conditions Optimization	Production levels	Reference(s)
Recombinant proteins					
A. gossypii	Endoglucanase I	Engineered	No	Up to 400 μmol/min.L	14,16
Saccharomyces cerevisiae		Engineered	No	Up to 1000 μmol/min.L	
A. gossypii	β-galactosidase	Engineered	No	Up to 1127 U/mL	17
Aspergillus niger		Wild Type	No	Up to 3000 U/mL	
SCOs					
A. gossypii	Single cell oil	Engineered	No	Up to 70% of CDW	10
Yarrowia lipolytica		Engineered	Yes	Up to 88% of CDW	
A. gossypii	Linoleic acid	Engineered	No	16% of total fatty acids	18
Y. lipolytica		Wild type	Yes	31% of total fatty acids	19
Flavour compounds					
A. gossypii	2-phenylethanol	Wild type	No	Up to 0.2 g/L	11
S. cerevisiae and *Kluyveromyces marxianus*		Wild type	Yes	0.25 – 20 g/L	20
A. gossypii	Isoamyl alcohol	Engineered	No	0.5 – 0.6 g/L	11
S. cerevisiae		Mutants	Yes	0.4 – 0.5 g/L	21
A. gossypii	Inosine	Engineered	No	Up to 2.2 g/L	12,13
Bacillus subtilis		Engineered	No	6 – 7 g/L	
A. gossypii	Guanosine	Engineered	No	Up to 0.14 g/L	12
Bacillus amyloliquefaciens		Engineered mutant	No	Up to 13.5 g/L	22

likely to be contaminated or degraded by native proteins;[15,16] (d) high genetic tractability, with a rich molecular toolbox available for its manipulation;[2,25] (e) remarkable genomic similarities with the budding yeast *S. cerevisiae*, which facilitates the transfer of accumulated know-how on this model yeast genetics and cell biology to *A. gossypii*;[2] (f) ability to grow in cheap waste-derived substrates to high cell densities;[26-27] (g) and demonstrated suitability for use in large-scale industrial fermentation processes.[1,3]

While the first heterologous proteins secreted by *A. gossypii* (endoglucanase I (EGI) and cellobiohydrolase I (CBHI) from *Trichoderma reesei*) were produced at very low yields,[16] latter on it was found that the expression vector and promoter (from *ScPGK1*) initially used were inefficient in promoting gene overexpression in *A. gossypii*.[14,15,17] After removal of the *ScADH1* terminator sequence from the initial vector (because it had been reported to display autonomous replicating sequence activity in *A. gossypii*), a 2-fold improvement in EGI production was achieved.[14] Similar enhancements were also obtained by random mutagenesis of the recombinant *A. gossypii* EGI-producing strain.[14] However, when the *ScPGK1* promoter was substituted by the native *A. gossypii* promoters from *AgTEF* and *AgGPD*, the recombinant secretion of a β-galactosidase from *A. niger* was improved by up to 8-fold, thus leading to production levels (248 to 1127 U/mL) within the range of those reported for unmodified *A. niger* strains (152 to 3000 U/mL).[17] Interestingly, when glycerol was used instead of glucose as carbon source, the recombinant production of β-galactosidase by *A. gossypii* was 1.5-fold higher,[17] which indicates that further optimization of culture media and operation conditions will most likely enhance even more recombinant protein production by *A. gossypii*. Therefore, *A. gossypii* shows up as promising recombinant protein production host. Nevertheless, the potential of this fungus as a host for heterologous protein production is still underexplored. The screening for better promoters and secretion signal sequences, together with the development of better expression strategies, preferably through the integration of stable expression cassettes, hold the potential to maximize heterologous protein secretion by *A. gossypii*, ultimately clarifying whether *A. gossypii* will ever be able to meet the demands of the biotechnology industry in what concerns recombinant protein production.

Optimization of the *A. gossypii* natural metabolism

The natural ability of *A. gossypii* to overproduce riboflavin is well known, but this fungus also has a naturally attractive metabolism in what concerns biolipid accumulation,[10] nucleosides excretion[12] and aroma alcohols formation.[11] With the increasing number of genomic, transcriptomic, proteomic, fluxomic and bioinformatic data that became available for *A. gossypii* in the last years,[2,4-7,15] several systems metabolic engineering strategies have been designed and implemented to improve its metabolic flux toward the production of these commercially valuable bioproducts.[10-13]

SCO production

Microbial lipids (often referred to as SCOs) constitute a promising renewable source for the sustainable production of biodiesel and other value-added oleochemicals, as they do not affect food costs and security, and can be obtained in large amounts by fermentation of waste-derived substrates under controlled conditions that are not dependent on climate or soil conditions. SCOs can also be interesting as food supplements, as some microorganisms are able to produce high-value polyunsaturated fatty acids (PUFAs), such as those belonging to the omega-3 and omega-6 families.

Although not considered an oleaginous microorganism, *A. gossypii* was metabolically engineered to fit the requisites of a candidate for SCO production, being one of the engineered strains (*pox1*Δ) able to accumulate up to 70% of its dry cell weight (CDW) as lipids.[10] *A. gossypii* naturally produces mostly unsaturated fatty acids: 55% oleic acid, 20% palmitoleic acid, 14% palmitic acid, 3% linoleic acid, 1.1% stearic, and around 4% of very long-chain fatty acids.[18] Since the potential applications of SCOs are strictly connected with the fatty acid profile of the strain, metabolic engineering was also applied to the *A. gossypii* elongase and desaturase systems and some of the generated strains were able to accumulate more linoleic acid (which is a precursor of both omega-3 and omega-6 PUFAs; strains *GPD-DES589* and A586), cerotic acid (which is the main component of high-melting point waxe; strain *GPD-ELO586*), or nervonic acid (which is a major component of high-temperature lubricants and engineered nylons, and also important for pharmaceuticals and nutraceuticals; strain *GPD-ELO624*),

or presented optimized features for the production of biodiesel (strains *elo586*Δ and *elo624*Δ).[18,28]

Despite the substantial improvements achieved in biolipid production with *A. gossypii*, both in terms of yield as in enrichment in desired fatty acids, given the importance of the cost/benefit ratios in microbial biotechnology processes, production costs must be minimized as much as possible. Therefore, is important to obtain good product yields and productivities from low cost substrates, such as raw carbon sources derived from industrial wastes. Given that in sugar-based media *A. gossypii* strains were only able to produce up to 9% of their CDW as lipids,[10] biolipid production from waste/low-cost sugars must be greatly improved both by medium optimization and strain engineering (this may also include expanding the range of substrates that *A. gossypii* can degrade and increasing its resistance to toxic compounds present in cheap raw materials). On the other hand, given the increased rate of lipid accumulation by *A. gossypii* in oil-containing media, the conversion of cheap oils into high-value oils should be further explored. Additionally, future studies should also consider engineering strains with improved fatty acid secretion to facilitate their recovery and reduce extraction costs.

Nucleoside production

As a riboflavin overproducer, *A. gossypii* displays a naturally enhanced metabolic flux through the purine biosynthetic pathway, the central pathway for purine nucleotide and nucleoside biosynthesis (Fig. 1). Recently, several *A. gossypii* wild strains were screened for their natural ability to produce purine nucleosides and all were found to excrete high levels of inosine to the culture medium, and also guanosine.[12] These nucleosides are very appreciated by the food industry, as they are the direct precursors of the *umami* flavour enhancers IMP and GMP, and have various beneficial effects associated to them, such as antioxidant and immunomodulatory properties.[12,13]

Through rational metabolic engineering of key genes in the *A. gossypii* purine biosynthetic pathway, inosine excretion could be increased by up to 150-fold.[13] This particular improvement was achieved by disrupting only 2 genes: *AgADE12*, which encodes the enzyme that catalyzes the conversion of IMP into adenylosuccinate, and *AgPNP1*, which encodes the enzyme that catalyzes the conversion of the nucleosides inosine and guanosine into the nucleobases hypoxanthine and guanine, respectively. Of all the metabolic engineering strategies tested, this was the most efficient in redirecting the metabolic flux of *A. gossypii* toward the production of inosine, allowing the obtainment of up to 2.2 g/L inosine in rich medium containing 40 g/L glucose.[9,12,13] Guanosine excretion was also increased in this strain (Δ*ade12*/Δ*pnp1*), but only by up to 2-fold.[13]

In view of these results, *A. gossypii* became the first eukaryotic microorganism presenting good potential for the industrial production of inosine, which traditionally has been accomplished with *B. subtilis* and *Escherichia coli* modified strains. However, in order to reach the inosine excretion levels reported for the best engineered bacterial strains (6–7 g/L), additional manipulations of the central metabolism and of the purine salvage pathway, of global metabolic regulators, and of transporters may be necessary.[9,12.13] On the other hand, given that the deletion (or underexpression) of the *AgADE12* gene efficiently redirected the metabolic flux toward the GMP purine branch, future studies should explore the production of other GMP-derived metabolites, such as folates (vitamin B9), by these strains.[8,9,12,13]

Aroma alcohols production

Many microorganisms naturally synthesize flavour compounds as secondary metabolites during fermentation on nutrients that include precursors or intermediates for their biosynthesis/bioconversion, as is the case of *A. gossypii*.[11] Traditionally, complex mixtures of aromatic alcohols (known as fusel alcohols) and esters have been obtained as part of food or beverage fermentation processes (e.g. cheese, beer, wine) at low concentrations, where they contribute to the overall flavour of the fermented products.[29] The strong interest of the fermented food and beverages sector in new products with interesting flavour/aroma perceptions has supported the continuous search for novel producing organisms.[2,11] Moreover, the increasing demand for natural flavour compounds such as isoamyl alcohol (banana/fruity aroma) and 2-phenylethanol (rose/flower aroma), which can be produced from amino acids via the Ehrlich pathway in microbial-based bioconversion processes (Fig. 1), has been boosting the design of new strains and fermentation processes capable of generating high amounts of specific flavour compounds for subsequent purification and use as additives.[29]

Recently, the potential of *A. gossypii* for flavour compounds production was explored and the analysis of its aroma alcohol profile revealed that *A. gossypii* produces large amounts of isoamyl alcohol and 2-phenylethanol.[11] Through overexpression of the *AgARO80* gene, which encodes a transcriptional regulator for some of the Ehrlich pathway *ARO*-genes, isoamyl alcohol and isobutanol production could be increased by 2.5-fold, whereas the levels of 2-phenylethanol remained unchanged, possibly due to phenylalanine limitation in the culture medium.[11] This modification also resulted in a 50% increase in overall volatile compound production.[11]

Envisioning the development of commercial production of 2-phenylethanol by yeast, some of the organisms identified as possibly good sources of natural 2-phenylethanol have been the target of optimization strategies that have included the overexpression/deletion of other *ARO*-genes.[20] Therefore, future attempts to improve 2-phenylethanol production by *A. gossypii* may follow similar strategies. Nevertheless, the use of metabolic engineering approaches to improve yields of this compound is very sensitive due to the complexity of the hosts' metabolic regulation. On the other hand, one of the major bottlenecks associated with the production of high amounts of these aromatic alcohols is their toxicity to the producer strains,[20,29] and so it may be necessary to consider engineering strains with improved resistance to these compounds.

Optimization of production conditions

Although significant advances have been given in terms of *A. gossypii* strain engineering for new biotechnological applications, rational engineering and systems biology will necessarily have to be combined with process development and optimization in order to drive these new *A. gossypii* applications through the successful commercial track of riboflavin production.

While some improvements have been achieved in the production of recombinant proteins, SCOs and inosine by changing the origin/concentration of the carbon source used in the fermentation medium,[13,17,18] systematic optimization of the culture medium composition or of fermentation conditions has not yet been assessed. Therefore, it is foreseen that much can still be improved in terms of strain productivity and product yields by optimization of production conditions. But to make these biotechnological processes commercially attractive, when designing optimization strategies, efforts must be made to reduce production costs to a minimum.

A. gossypii already possesses interesting characteristics that confer it biotechnological advantages in terms of production costs, such as (a) ability to grow by pellet fermentation, which reduces culture viscosity and improves mixing and mass transfer, saving both costs and energy,[10] (b) at late growth phases or at low temperature its hyphae undergoes autolysis, thus facilitating the downstream recovery of intracellular metabolites,[2] and (c) its biomass can be easily separated from the culture broth by gravity sedimentation or simple filtration, which are very convenient separation process for use at industrial scale.[10]

On the other hand, one important way to lower production costs is by using inexpensive carbon sources and nutrients, preferably from widely available industrial residues. In this sense, the known ability of *A. gossypii* to grow in some industrial by-products and low-cost oils[2,26,27] may be easily explored. Additionally, the expansion of the carbon source range that *A. gossypii* can utilize by metabolic engineering (e.g., xylose utilization) may open new perspectives in terms of the raw substrates that may be used in these processes (such as lignocellulosic biomass).

Solid-state fermentation (SSF) has also been used as a mean to improve the economical feasibility of secondary metabolite production processes.[29] The main advantages of SSF over submerged fermentation are: (a) higher energy efficiency and reduced water consumption, (b) lower cost due to efficient utilization and value-addition of solid wastes, and (c) generally higher product yields and better product characteristics.[29] With this in mind, and given that filamentous fungi generally have a higher metabolic potential in SSF,[29] future studies should assess the production of secondary metabolites (e.g. 2-phenylethanol) by *A. gossypii* using this cultivation mode.

Concluding remarks

Over the past few years, with the expansion of the *A. gossypii* molecular and *in silico* modeling toolbox, the potential of this fungus as a cell factory has been thoroughly explored. Supported by the increasingly high number of genomic,[5,30] transcriptomic,[4,15] proteomic[15] and fluxomic[7] data that is becoming available for *A. gossypii*, in the forthcoming years systems biology is expected to continue driving the improvement of current and the emergence of novel

biotechnological applications for this fungus. The (re-) construction and investigation of progressively more accurate genome-scale metabolic models will certainly lead to the identification of novel targets for *A. gossypii* metabolic engineering; and to facilitate and speed up systems metabolic engineering approaches, the development of efficient synthetic biology tools for use in *A. gossypii* will be necessary.

While the existing molecular toolbox has been effective in generating stable *A. gossypii* strains with improved traits for pinpointed biotechnological applications, the development of new and more flexible tools will be crucial to boost the engineering capabilities of this fungus. In this regard, the establishment of flexible and highly efficient genome editing methods (e.g., CRISPR-Cas9 system) for *A. gossypii* engineering is of great interest. Also, as the number of characterized promoters currently available for *A. gossypii* is limited, the screening, identification and design of other promoters with desired characteristics will settle the basis for the development of new and more flexible gene expression tools.

On the other hand, the systematic exploration of this fungus natural biodiversity will help identify new strains with better biotechnological potential, and should therefore be considered as a standard procedure in the development of novel applications. However, the success of any emerging biotechnological application will strongly depend on the combination of strain and process optimization. In this context, the development of cost-effective high-throughput screening methodologies will be of upmost importance to guarantee the rapid screening and characterization of wild, mutant and engineered strains under different process conditions.

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