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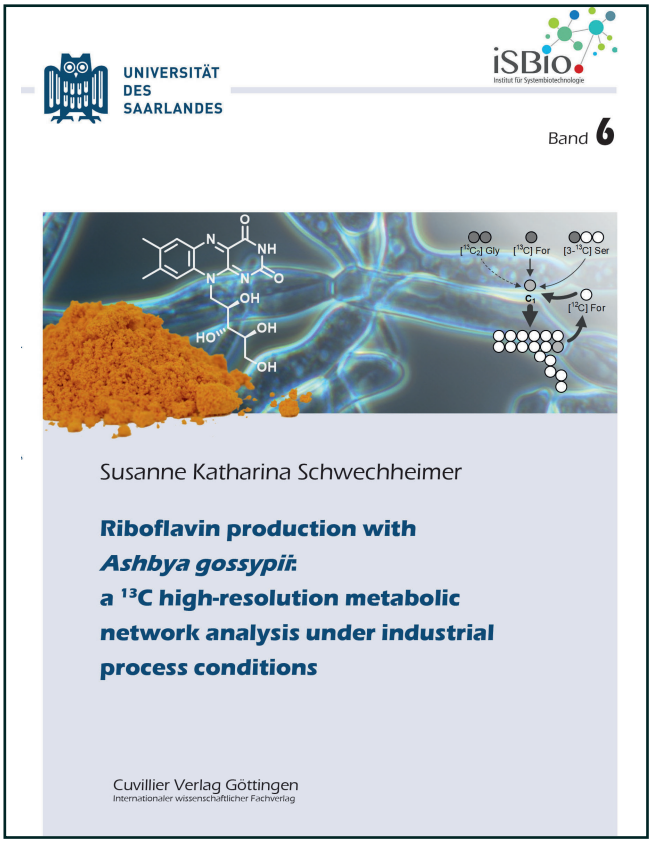
Riboflavin production with Ashbya gossypii

A 13C high-resolution metabolic network analysis under industrial process conditions



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1 INTRODUCTION

1.1 General Introduction

Riboflavin, also known as vitamin B_2, is a water-soluble compound, which can be synthesized by plants and microorganisms, but is essential for animals as they lack an endogenous biosynthetic pathway. It plays an important role in multiple cellular functions. Pioneering discovery and research dates back almost 150 years, and throughout the past decade, increasing interest has turned riboflavin meanwhile into one of the most important products in biotechnology. The two active forms of riboflavin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), act as cofactors for oxidoreductases as well as prosthetic groups for enzymes in the β-oxidation pathway (Massey, 2000). Moreover, riboflavin is part of a flavoprotein called cryptochrome, a photoreceptor in charge of the upkeep of the circadian clock (Banerjee and Batschauer, 2005; Miyamoto and Sancar, 1998). Natural sources of riboflavin are for example milk, eggs, and leafy vegetables (O'Neil, 2006). According to the Food and Nutrition Board (1998), the recommended daily allowance of riboflavin lies between 1.1 and 1.3 mg for women and men assuming a healthy person. In humans, riboflavin deficiency amongst other symptoms is associated with skin lesions and corneal vascularization. Riboflavin, which is exclusively synthesized biotechnologically using microorganisms, is mainly used as feed additive (about 70 % of today's market), whereas about 30 % are used as food additive and for pharmaceutical applications (Revuelta et al., 2016). The world market for riboflavin has more than doubled in a little over a decade from 4000 t a^{-1} to 9000 t a^{-1} in 2002 and 2015, respectively (Schwechheimer et al., 2016). Hereby, the production has come a long way from chemical synthesis on fossil fuels to the exclusive biotechnological production today. One of the industrial microbes for riboflavin is *Ashbya gossypii*, a natural overproducer. It accumulates up to 20 g L^{-1} riboflavin, explaining the high interest in this filamentous fungus (Abbas and Sibirny, 2011; Kato and Park, 2012; Lim et al., 2001; Revuelta et al., 2016; Schwechheimer et al., 2016; Stahmann et al., 2000). The chemical company BASF has installed a plant in South Korea, which is specialized in riboflavin production on industrial scale using *A. gossypii* (Schwechheimer et al., 2016). The biochemistry of riboflavin biosynthesis as well as the empirically derived fermentation set-up for *A. gossypii* are extremely complex. Several subcellular compartments contribute to the production of the vitamin, including the peroxisome, the mitochondrion, and the cytosol (Kato and Park, 2012). Furthermore, industrial production is based on a complex mixture of various raw materials: corn steep liquor, peptone, yeast extract in addition to the main carbon source vegetable oil (Epstein et al., 1979; Malzahn et al., 1959; O'Neil, 2006; Tanner et al., 1948). At

the starting point of biosynthesis, the major carbon precursors of the vitamin are ribulose 5-phosphate and guanosine triphosphate (GTP). These intermediates are converted into riboflavin in a total number of seven steps. Glucose has been described as alternative carbon source (Demain, 1972; Tanner et al., 1949) and the sugar is the dominant substrate used for research of *A. gossypii* (Bacher et al., 1985; Ledesma-Amaro et al., 2015c; Schlüpen et al., 2003; Silva et al., 2015). So far, classical strain improvement by random mutagenesis and selection (Schmidt et al., 1996a; Schmidt et al., 1996b; Sugimoto et al., 2010) and empirical optimization of the fermentation process (Sahm et al., 2013; Storhas and Metz, 2006) have displayed the dominant strategies towards better production (Abbas and Sibirny, 2011; Kato and Park, 2012; Lim et al., 2001; Revuelta et al., 2016; Schwechheimer et al., 2016; Stahmann et al., 2000). Only a few success cases have managed to improve riboflavin production using rational approaches, such as metabolic engineering (Buey et al., 2015; Jiménez et al., 2008; Monschau et al., 1998; Sugimoto et al., 2009). This might result from the fact that the molecular processes involved in production of the vitamin are only partly understood. Admittedly, a huge complexity is faced inside and outside the cells: a multi-compartment biosynthesis meets a multi-substrate process environment. Without doubt, tailored strain and process engineering, strongly desired to keep up with market demands, will benefit from a better understanding of riboflavin production in *A. gossypii*.

1.2 Objectives

The aim of this work was the resolution of carbon fluxes of growth and riboflavin production by *A. gossypii* under industrial process conditions. Such flux studies typically require well-defined conditions, such as minimal media and only one carbon source. In this regard, the industrial process, i.e. medium with complex compounds and several carbon sources, posed an additional level of complexity. In order to tackle this challenge on a systems biology level, reproducible data are of utmost importance. To this end, a reliable cultivation and sampling scheme should be developed prior to the flux studies. A number of technical developments provided an experimental approach for tailored ^{13}C isotope studies on a complex medium with a deep assessment of ^{13}C labeling in various metabolites by complementary gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), and nuclear magnetic resonance (NMR) approaches. The integration of the data gained from the various analytical methods should provide deeper insight into the riboflavin metabolism in *A. gossypii* and optimally offer new starting points and targets for process optimization.

2 THEORETICAL BACKGROUND

2.1 Vitamin B_2: discovery and pioneering chemical synthesis

Riboflavin was first mentioned by Blyth in 1879, who isolated a yellow-fluorescing substance from milk whey, which he called lactochrome (Northrop-Clewes and Thurnham, 2012). However, it took almost half a century until the vitamin was isolated, its structure described, and its nutritional function revealed. Early micronutrition studies with rats showed growth impairment upon ariboflavinosis. These findings led to more intensive research in the field of vitamins in general and of riboflavin in particular. In the early 1930s riboflavin was successfully isolated from egg white (Kuhn et al., 1933b), milk whey, and vegetables (Eggersdorfer et al., 2012; Kuhn et al., 1933a), which was followed by the discovery of its structure (then called lactoflavin): a methylated isoalloxazine ring with a ribityl-sidechain (Figure 1) (Karrer et al., 1935; Kuhn, 1936). Soon afterwards, vitamin B_2 officially became "riboflavin" (from Latin *flavus* for yellow and "ribo" for the ribityl-sidechain) by the Council of Pharmacy and Chemistry of the American Medical Association (Northrop-Clewes and Thurnham, 2012). The high interest in the molecule was a major driver to derive it by chemical synthesis (Karrer et al., 1935; Kuhn, 1936). The initially developed and still major multi-step large-scale chemical route starts either from D-glucose or D-ribose (Figure 1). Glucose is first oxidized to arabonate, which is subsequently epimerized to ribonate and transformed into ribonolactone. This intermediate is reduced to D-ribose using amalgam. After addition of xylidine, the product ribitylxylidine and an aniline derivative together form phenylazo-ribitylxylidine. The final reaction step of the chemical synthesis is a cyclocondensation of phenylazo-ribitylxylidine with barbituric acid, which yields riboflavin as a product (Wolf et al., 1983). For many years, with a few alterations, this was the only way to synthesize riboflavin. Nowadays, the production of riboflavin is exclusively done using fermentation as it is economically and ecologically more feasible.

While the production of riboflavin via fermentation was only 5 % of the annual production in 1990, the percentage of biotechnological production has increased to 75 % of the market volume in 2002 within just twelve years due to metabolic engineering of the production strains (Schwechheimer et al., 2016). The two dominating processes employ the Gram positive bacterium *Bacillus subtilis* and the hemiascomycete *Ashbya gossypii*. Today, due to further optimization of the bio-based production in the past years, chemical synthesis has been replaced completely. Most of today's riboflavin market is used as feed additive (Sahm et al., 2013), but also as food fortification, dietary supplement, pharmaceutical applications as well as food colorant (E-101) in yoghurt and drinks.

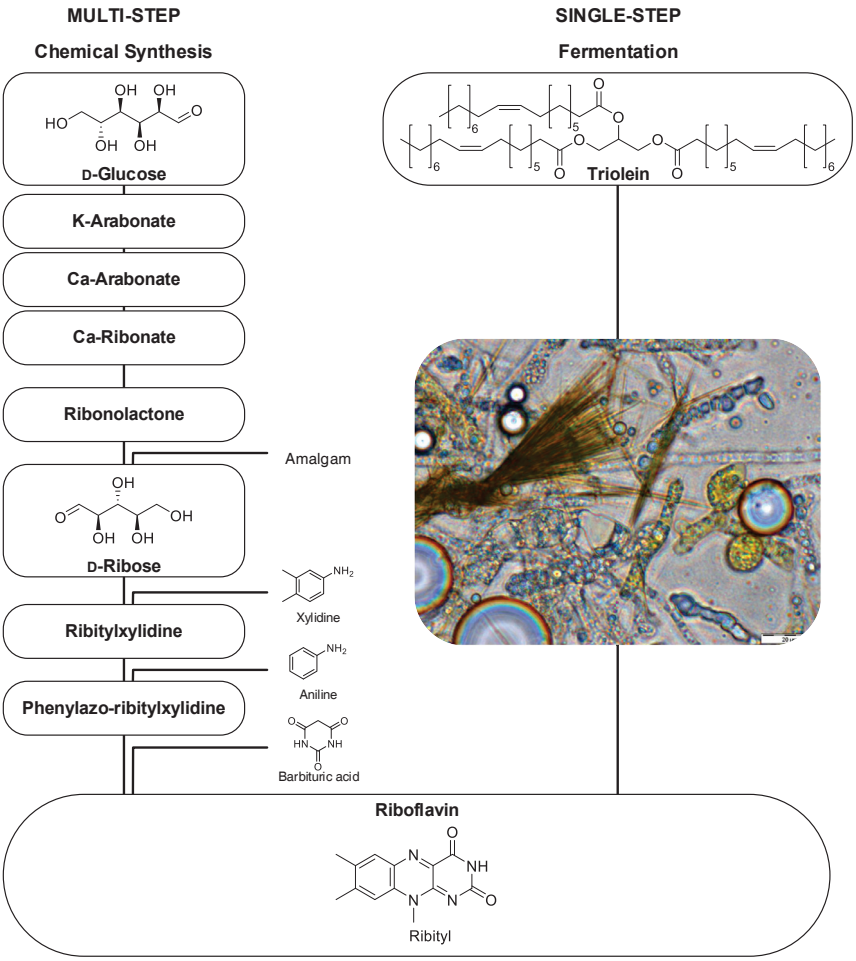

Figure 1: Chemical versus biotechnological riboflavin synthesis by fermentation. The microscopic image depicts a wild type *A. gossypii* in a vegetable oil medium. Mycelia, oil droplets, and riboflavin crystals are shown in a 600-fold magnification (Schwechheimer et al., 2016).

2.2 *Ashbya gossypii* – a fungal riboflavin overproducer

The filamentous hemiascomycete *A. gossypii* was first isolated from cotton as phytopathogen causing stigmatomycosis in 1926 in the British West Indies (Ashby and Nowell, 1926). Later, it was also discovered on other crops in tropic and sub-tropic regions (Pridham and Raper,

1950). The use of insecticides efficiently reduced the role of *A. gossypii* as phytopathogen, because the fungus relies on insect vectors for transmission. It is incapable of penetrating intact plant cell walls, which can be explained by the low amount of extracellular enzymes secreted by *A. gossypii* (Aguiar et al., 2014b). Its ability to produce large amounts of volatile aroma compounds might be advantageous when attracting insects for transmission (Ravasio et al., 2014; Wendland et al., 2011).

The life cycle of *A. gossypii* starts from a needle-shaped spore. After germ bubble and germ tube formation, a mycelium is generated by lateral branching of the initial hyphae (Figure 2A), which also leads to dichotomous tip branching. Each hyphal cell, which is separated by septa from neighboring cells, contains several haploid nuclei. In the late growth phase, sporangia usually containing eight spores each are formed by fragmentation of old hyphae. These spores, which are linked by actin filaments, are then released (Figure 2C) (Wendland and Walther, 2005). The transition from vegetative growth to sporulation is linked to nutrient depletion, however, the exact underlying mechanisms are still poorly understood. Recent studies on the genome suggest that *A. gossypii* displays two different mating types and thus, may also exhibit sexual reproduction (Dietrich et al., 2013).

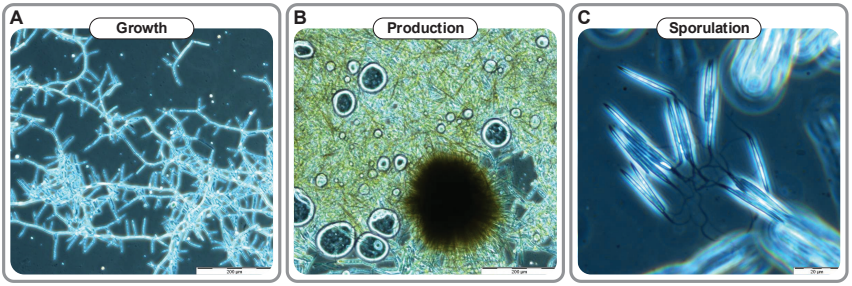

Figure 2: Microscopic images of *A. gossypii* B2 grown on complex medium and vegetable oil (A, B) or glucose (C). 6 h after inoculation, lateral branching can be observed in the growing culture (A). The low solubility of riboflavin leads to formation of vitamin crystals after 72 h (B). At the end of cultivation on glucose, the needle-shaped spores, which are connected by actin, are released from sporangia (C). Images A and B are shown in a 100-fold magnification. The spores in image C are 600-fold magnified.

A. gossypii belongs to the family of Saccharomycetaceae and shares more than 90 % synteny of protein coding genes with the budding yeast *Saccharomyces cerevisiae*. Due to its very small genome size (9 Mb), close relationship with *S. cerevisiae*, and readily available molecular tools, it has become a model organism for filamentous growth (Wendland and Walther, 2005).

Overproduction of riboflavin by *A. gossypii* was first reported in 1946. The production starts once vegetative growth has ceased and cells start sporulating (Figure 2B). Originally, it was hypothesized that overproduction of riboflavin protected the hyaline spores against ultraviolet (UV) light (Stahmann et al., 2001). However, since also non-sporulating cells accumulate riboflavin, these two events are not exclusively connected (Nieland and Stahmann, 2013; Walther and Wendland, 2012). Over the past years, it was shown that nutritional and oxidative stress induce riboflavin overproduction in the fungus (Kavitha and Chandra, 2014; Schlösser et al., 2007). Walther and Wendland (2012) even suggested that riboflavin plays a role in stress defence against oxidative burst of plants upon infection with *A. gossypii* cells.

In recent years, the potential of *A. gossypii* as host for other high-value products has been investigated and its spectrum was tremendously broadened (Aguiar et al., 2017). This entails flavor compounds derived from the Ehrlich pathway (2-phenylethanol and isoamyl alcohol) (Ravasio et al., 2014) and purine biosynthesis (inosine and guanosine contribute to the *umami* flavor) (Ledesma-Amaro et al., 2015a; Ledesma-Amaro et al., 2016), but also single cell oil (SCO) production (Díaz-Fernández et al., 2017; Ledesma-Amaro et al., 2015b; Ledesma-Amaro et al., 2014b; Lozano-Martínez et al., 2016). The expression performance of recombinant cell wall degrading enzymes was inspected (Aguiar et al., 2014a; Ribeiro et al., 2010). In contrast to other filamentous fungi, *A. gossypii* secretes only a small amount of extracellular enzymes, which makes it a promising host for recombinant protein production. However, current titers still leave room for improvement. Most recently, the overproduction of folic acid (vitamin B_9) was reported (Serrano-Amatriain et al., 2016). Genetic engineering towards vitamin B_9 production seems obvious, considering the high potential of the purine biosynthetic pathway in this organism and the shared precursor, GTP, with riboflavin.

2.3 Riboflavin biosynthesis – pathways and regulations

Many microorganisms are capable to synthesize riboflavin. *Candida famata*, *Clostridium acetobutylicum*, and *Lactobacillus fermentum* are able to overproduce the vitamin (Demain, 1972). It is, however, easy to understand that most of the knowledge on riboflavin biosynthesis has been collected for the two major industrial producers: *A. gossypii* (Figure 5) and *B. subtilis* share two important precursors for riboflavin biosynthesis: ribulose 5-phosphate (Ru5P) is derived from the pentose phosphate (PP) pathway and GTP originates in the purine biosynthesis (Bacher et al., 2000).

2.3.1 Terminal biosynthesis

The terminal riboflavin biosynthetic chain comprises a total of seven enzymatic steps starting from two different branches: the purine biosynthesis and the PP pathway (Figure 3). The GTP cyclohydrolase catalyzes the cleavage of GTP with release of formate. This step is encoded by *RIB1* in *A. gossypii* and by *ribA* in *B. subtilis*. The first reaction is followed by a reduction reaction in the fungus, carried out by the gene product of *RIB7* (DARPP reductase), and a subsequent deamination (*RIB2*, DarPP deaminase). In *B. subtilis*, these latter two steps are in reverse order and are catalyzed by a bifunctional enzyme encoded by *ribG*. The phosphatase that cleaves ArPP (2,5-diamino-6-ribityl-amino-2,4(1H,3H)pyrimidinedione 5'-phosphate) into ArP (5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione) is the only, still unknown, enzyme in the riboflavin biosynthetic pathway. Ribulose 5-phosphate is converted to DHBP (3,4-dihydroxy-2-butanone 4-phosphate) by the DHBP synthase (*RIB3* or *ribA* for *A. gossypii* and *B. subtilis*, respectively). At this point the two different branches of the riboflavin pathway merge into one. The condensation of DHBP and ArP yields one molecule of DRL (6,7-dimethyl-8-ribityllumazine) and is catalyzed by the lumazine synthase (*RIB4* for *A. gossypii*, *ribH* for *B. subtilis*). In the final step of the riboflavin biosynthetic pathway the enzyme riboflavin synthase converts two mole DRL into one mole riboflavin and one mole ArP, which is recycled in the previous step (Fischer and Bacher, 2005).

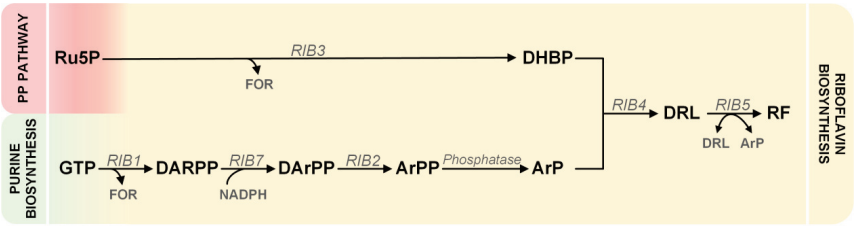

Figure 3: Terminal biosynthesis of riboflavin in *A. gossypii*. Ru5P is converted into DHBP and condensed with ArP to yield DRL. In the final step, riboflavin synthase catalyzes the conversion of DRL to riboflavin. Note that the pentose phosphate pathway branch is undergone twice, since two molecules DHBP are needed for one molecule of riboflavin. ArP, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione; ArPP, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5-phosphate; DARPP, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5-phosphate; DarPP, 2,5-diamino-6-ribitylamino-pyrimidinone 5-phosphate; DHBP, 3,4-dihydroxybutanone 4-phosphate; DRL, 6,7-dimethyl-8-ribityllumazine; FOR, formate; GTP, guanosine triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; PP pathway, pentose phosphate pathway; Ru5P, ribulose 5-phosphate; RF, riboflavin; *RIB1*, GTP cyclohydrolase II; *RIB2*, DarPP deaminase; *RIB3*, DHBP synthase; *RIB4*, lumazine synthase; *RIB5*, riboflavin synthase; *RIB7*, DARPP reductase.

2.3.2 Precursor supply

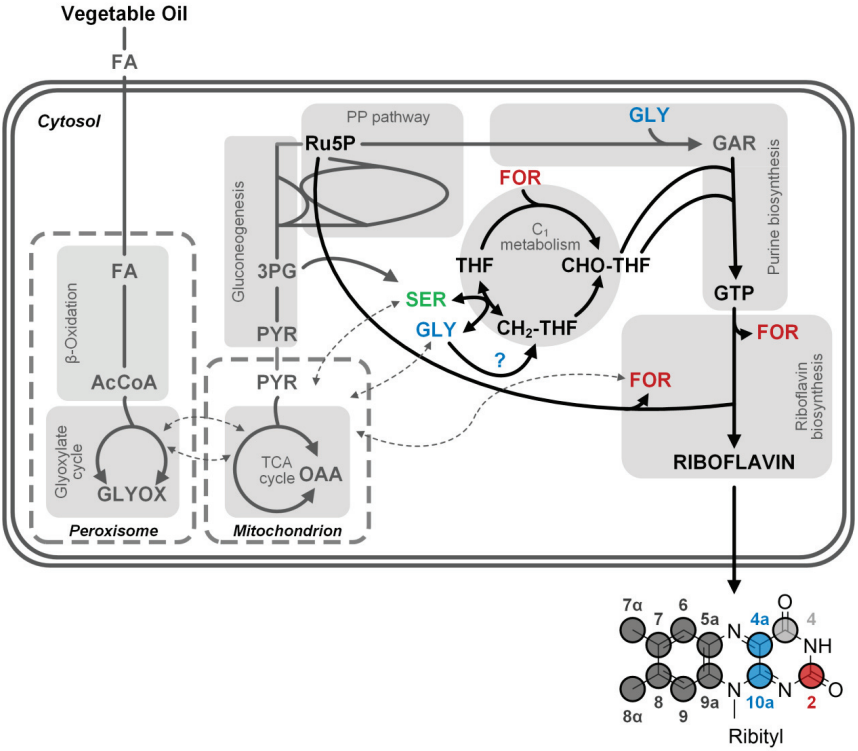

Figure 4: Schematic riboflavin biosynthesis from vegetable oil in *A. gossypii*. The multi-compartment process ends with the terminal riboflavin biosynthesis starting from ribulose 5-phosphate and GTP. Dashed lines indicate exchange of metabolites between different compartments. Note that the one-carbon metabolism is only drawn in the cytosol. It can be assumed, however, that there is also a one-carbon metabolism in the mitochondrion. The circles in the riboflavin molecule designate the carbon atoms and their respective molecular origin: ribulose 5-phosphate (dark grey), carbon dioxide (light grey), glycine metabolism (blue), one-carbon metabolism (red). The ribityl side chain originates from ribose 5-phosphate. 3PG, 3-phosphoplycerate; CH_2-THF, 5,10-methylenetetrahydrofolate; AcCoA, acetyl-CoA; CHO-THF, 10-formyltetrahydrofolate; FA, fatty acid; FOR, formate; GAR, glycineamide ribonucleotide; GLY, glycine; GTP, guanosine-5'-triphosphate; OAA, oxaloacetate; PYR, pyruvate; Ru5P, ribulose 5-phosphate; SER, serine; THF, tetrahydrofolate.

For the overproduction of riboflavin, *A. gossypii* prefers oil as substrate, which is cleaved into fatty acids and glycerol by an extracellular lipase (Stahmann et al., 1997). The fatty acids are transported into the cell and oxidized into acetyl-CoA via the β-oxidation pathway, located in the peroxisome. Acetyl-CoA is further metabolized via the glyoxylate shunt, gluconeogenesis, and the PP pathway (Figure 4, Figure 5). In addition, supplementation with glycine is frequently

used for riboflavin production (Demain, 1972; Malzahn et al., 1959). The amino acid is supposed to have two functions. One molecule glycine is incorporated into the pyrimidine ring of riboflavin (Plaut, 1954a). Furthermore, glycine is linked to the folate-dependent carbon-one (C_1) metabolism, which supplies 10-formyltetrahydrofolate (CHO-THF) as essential C_1 donor for the biosynthesis (Pasternack et al., 1996; Schlüpen et al., 2003). In more detail, the enzyme serine hydroxymethyltransferase (SHMT) forms serine from glycine. The terminal carbon atom of serine then contributes to the C_1 pool via 5,10-methylenetetrahydrofolate (CH_2-THF), which in turn is converted into CHO-THF, the immediate C_1 donor for riboflavin (Figure 4). Moreover, a potential glycine cleavage system (GCS) has been suggested as alternative route in the fungus (Schlüpen et al., 2003), but to date has neither been proven nor disproven experimentally.

2.3.3 Regulation

Regulation of the riboflavin biosynthetic pathway is not completely solved for the hemiascomycete *A. gossypii*. However, a few studies have dealt with unraveling the regulatory mechanisms behind riboflavin overproduction, which has been linked to nutritional (Schlösser et al., 2007) as well as oxidative stress (Kavitha and Chandra, 2009; Kavitha and Chandra, 2014). It was reported that the beginning of riboflavin oversynthesis and sporulation were linked, which was proven by addition of the second messenger cyclic adenosine monophosphate (cAMP) that is known to inhibit sporulation in fungi: riboflavin biosynthesis as well as sporulation were negatively affected (Stahmann et al., 2001). Supplementation of riboflavin to spore suspensions had a positive effect on spore viability upon UV light exposure (Stahmann et al., 2001). The regulation of pathway-specific enzymes was also investigated. DHBP synthase (encoded by the gene *RIB3*) catalyzes the first step in riboflavin biosynthesis starting from the ribulose 5-phosphate derived branch and carries twice the metabolic burden compared to the GTP branch of this pathway. Therefore, its regulation was of special interest. *RIB3* exhibited increased mRNA levels during the riboflavin production phase, caused by induction of the promoter (Schlösser et al., 2001). In a different study, upregulation of the three *RIB* genes involved in the PP pathway branch (*RIB3*, *RIB4*, *RIB5*) were upregulated upon cessation of growth due to nutrient depletion and entry into the riboflavin production phase (Schlösser et al., 2007). A more recent study, however, reported that there is no significant increase at the transcriptional level for all *RIB* genes except *RIB4* during the riboflavin biosynthetic phase (Ledesma-Amaro et al., 2015c). Two transcription factors have been described in the literature as having direct or indirect regulatory functions in the production of riboflavin: *BAS1* and *YAP1*. The Myb-related factor *BAS1* is involved in the adenine-dependent transcriptional control of the genes *SHM2* and *ADE4*, which play important roles in the glycine and purine metabolism, respectively, both of which contribute to the riboflavin precursor GTP.

A C-terminal deletion of the *BAS1* gene resulted in increased riboflavin titers due to constitutive activation of *SHM2* and *ADE4* (Mateos et al., 2006). The second transcription factor, *YAP1*, is known to be involved in oxidative stress response and targeted exposure of *A. gossypii* to oxidative stress led to increased riboflavin titers in a Yap1-dependent manner. The Yap-regulon comprises more than 100 genes, amongst others *RIB4*. This gene of the riboflavin pathway contains three Yap-binding domains and is transcriptionally controlled by *YAP1* (Walther and Wendland, 2012).

2.3.4 Biosynthesis in *B. subtilis*

Since *B. subtilis* is the other major industrial riboflavin producer next to *A. gossypii*, its riboflavin biosynthesis should be discussed briefly. Starting from its preferred carbon source glucose *B. subtilis* forms glucose 6-phosphate, which then enters into the PP pathway toward riboflavin biosynthesis. The bacterial riboflavin biosynthesis is organized in the so-called *rib* operon, which entails five genes (*ribGBAHT*) (Abbas and Sibirny, 2011; Perkins et al., 1999; Yakimov et al., 2014). The riboflavin biosynthesis in *B. subtilis* includes two bifunctional enzymes: the gene product of *ribA* shows GTP cyclohydrolase II activity as well as DHBP synthase activity (Hümbelin et al., 1999). The second bifunctional enzyme is the above mentioned gene product of *ribG*: the combined reductase and deaminase (Richter et al., 1997). As for *A. gossypii*, the regulation of riboflavin biosynthesis is not fully understood in *B. subtilis*, either. The *rib* operon of *B. subtilis* itself at least seems to be regulated by a “riboswitch” (Mironov et al., 2002). A conserved sequence within the 5‘-untranslated region of the *rib* operon is likely to fold into a secondary structure. FMN, which is the product of a kinase reaction of the gene product of *ribC*, is able to directly bind to this secondary structure, thus repressing transcription of the *rib* operon (Mack et al., 1998; Mironov et al., 2002). RibR, a protein that is not part of the *rib* operon, is believed to act as a regulatory protein since it seems to be able to bind to this riboswitch (Higashitsuji et al., 2007). The gene *ribR* is part of a transcription unit that entails gene products involved in sulphur uptake and degradation. Recently it was shown that when sulphur is present, *ribR* expression increases, the FMN demand of the cell rises, and the *rib* operon is expressed even with high FMN levels (Pedrolli et al., 2015).

2.4 Biotechnology and industrial production of riboflavin

2.4.1 Metabolic engineering of *A. gossypii*

The availability and advance in the development of molecular tools have made it very easy to modify and engineer prokaryotes and eukaryotes like *B. subtilis* and the model organism for filamentous fungi *A. gossypii* (Figure 5, Table 1). In *A. gossypii*, riboflavin production was increased in the wild type by medium supplementation with riboflavin precursors such as