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Short communication

Antagonistic effect of myo-inositol on riboflavin production in two riboflavinogenic fungi *Ashbya gossypii* and *Eremothecium ashbyi*

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ABSTRACT

Ashbya gossypii and *Eremothecium ashbyi* are riboflavin over producing fungi. Guanosine tri-phosphate (GTP) cyclohydrolase II, encoded by *RIB1* gene, catalyses the rate limiting step of the riboflavin biosynthetic pathway. We report here the antagonistic effect of myo-inositol on riboflavin production in these two fungi by adapting them in Yeast Malt Agar (YMA) media containing 0.1% inositol. The adapted *E. ashbyi* showed 92% reduction and the adapted *Ashbya* showed 39% increase in the total riboflavin production, when compared to that produced by *E. ashbyi* and *Ashbya* respectively. Reverse transcription-polymerase chain reaction (RT-PCR) study indicated that myo-inositol reduced *RIB1* gene expression in adapted *E. ashbyi* but enhanced the same in adapted *Ashbya*.

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Riboflavin or vitamin B_2 is the precursor molecule of the co-enzymes FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide). About 75% of the 4000 ton riboflavin production is via the cultivation of microorganisms as it is cost effective, eco-friendly and its production can be genetically altered (Karos et al. 2004). *Ashbya gossypii* and *Eremothecium ashbyi* are two natural riboflavin overproducing fungi which can produce riboflavin from cheap agro-industrial wastes such as molasses, whey etc (Abbas and Sibirny 2011). Unlike bacteria and yeasts, both can tolerate iron in the medium (Demain 1972). Riboflavin biosynthetic (*RIB*) pathway is catalyzed by 6 enzymes encoded by *RIB1* to *RIB5* and *RIB7* genes. GTP cyclohydrolase II (GCH II) which is encoded by *RIB1* gene, catalyzes the initial rate limiting step of riboflavin biosynthesis, hydrolyzing one molecule of Guanosine tri-phosphate (GTP) to formate 2, 5-diamino-6-hydroxy-4-5-phosphoribosyl amino pyrimidine and diphosphate (Sengupta and Chandra 2010).

Inositol receptors mediate several calcium-regulated signal transduction events, control gene expression, and regulate cell proliferation and cell death (Krizanova and Ondrias 2003). It also transcriptionally regulates the yeast phospholipid structural genes (Chen et al. 2007). However, the effect of inositol on *RIB* gene expression and riboflavin production, in any organism, has not been studied so far. Hence, in the present paper we analyzed the effect of myo-inositol (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol), the most prominent naturally-occurring form of inositol, on riboflavin production in *A. gossypii* and *E. ashbyi*.

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1. Strains, media and oligonucleotides

Ashbya gossypii (NCAUR-1056) and *E. ashbyi* (NCAUR-1363) were obtained from National Centre for Agricultural Utilization Research (NCAUR) Illinois, USA. The two cultures were grown on Yeast Malt Agar medium (YMA) containing (per liter) yeast extract 3 g, malt extract 3 g, peptone 5 g, glucose 10 g and agar 20 g. All the synthetic oligonucleotides were synthesized from MWG-Biotech (Ebersberg, Germany). All DNA manipulations and microbiological experiments were carried out by standard methods (Sambrook and Russell 2001).

2. Effect of inositol on riboflavin production by both fungi in plate cultures

Ashbya gossypii and *E. ashbyi* cultures were maintained and transferred 10 times in each medium of YMA and YMA containing 0.1% inositol (adapted culture). The study was to analyze whether inositol influenced riboflavin production in these two fungi. Subculturing was done every two weeks onto fresh medium.

Eremothecium ashbyi produced copious amounts of riboflavin in YMA media (Fig. 1a), as seen by the dark orange colonies indicative of high riboflavin production but produced much less riboflavin when 0.1% inositol was added to the media (Fig. 1b). *Ashbya gossypii* in YMA media produced white colonies (no riboflavin production) (Fig. 1c), but riboflavin production was recovered on addition of 0.1% inositol (Fig. 1d). The study indicated that inositol stimulated riboflavin production in *A. gossypii*, but inhibited that in *E. ashbyi* in YMA media.

3. Effect of inositol on riboflavin production in submerged cultures

All the wild type and adapted cultures of *A. gossypii* and *E. ashbyi* were next grown in submerged YM broth for 5 consecutive days for fermentation studies. A time course analysis of the biomass and total riboflavin production was carried out with the adapted cultures after 10 successive transfers in their respective agar media. The pre-inoculum for each of the wild type and adapted cultures of *A. gossypii* and *E. ashbyi* was prepared and grown under conditions as described earlier in *E. ashbyi* (Sengupta and Chandra 2010). One percent of 48 h old pre-inoculum was added to each of the 50 ml of broth. The experiment was done in triplicates. The analyses were done by sacrificing one set of inoculated broth, every 24 h after inoculation. The total riboflavin produced by each of the cultures was estimated fluorimetrically using the standard procedure (Sengupta et al. 2012).

In YM media, after 5 days of growth, the total riboflavin produced by *E. ashbyi* and the adapted *E. ashbyi* cultures were 413.3 and 33.5 mg/L respectively. Thus riboflavin production by *E. ashbyi* was significantly reduced by 92%, when 0.1% inositol was added to the media as seen in Table 1. Under similar conditions, the total riboflavin produced by *A. gossypii* and the adapted *A. gossypii* cultures were 98.2 and 136.7 mg/L respectively. Thus riboflavin production by *Ashbya* was increased by 39.2% as seen in Table 1. The biochemical study confirmed that inositol enhanced riboflavin production in *A. gossypii*, but significantly reduced that in *E. ashbyi* under same media conditions.

4. Transcriptional analysis to study the effect of inositol on RIB genes

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was next done to investigate whether inositol affected riboflavin production by influencing the *RIB1* gene expression in the adapted cultures. Total mRNA was extracted from 24 h to 48 h old wild type and adapted cultures of *E. ashbyi* and *A. gossypii*. Total mRNA from each culture was analyzed for the expression of both *TEF* and *RIB1* genes encoding translation elongation factor and GCH II respectively. *TEF* is a strongly expressed constitutive gene which was taken as the positive control. *RIB1*-specific PCR reaction was carried out using the forward primer (5′-TTCTCGCCTAGCCCG ATGCC-3′) and reverse primer (5′-GACAACAAGGAACA CCTAGC-3′). *TEF*-specific PCR reaction was carried out using the forward primer – (5′-CGAGCTCGCCATCTTGATCATTGC TGG-3′) and reverse primer – (5′-CGAGCTCTTGACTTCAGTG GTGACACC-3′). The primers were designed based on the sequences available at the *A. gossypii* genomic database (http://agd.vital-it.ch/index.html). The PCR mix composition and reaction conditions carried out were same as previously described in (Sengupta and Chandra 2010).

RT-PCR analysis of *TEF* and *RIB1* genes extracted from 24 h to 48 h old wild type and adapted cultures of *E. ashbyi* and *A. gossypii*, showed different *RIB1* gene expression patterns. *Eremothecium ashbyi*, after 24 h growth in YM, expressed the *RIB1* gene which was strongly expressed after 48 h (Fig. 1e). However in adapted *E. ashbyi*, there was expression of *TEF* gene but *RIB1* gene showed no expression after 24 h of growth. After 48 h, adapted *E. ashbyi* showed expression of both *TEF* and *RIB1* genes, but the *RIB1* gene showed a faint expression indicating lower rate of transcription (Fig. 1e). On the other hand, *A. gossypii* in YM media showed expression of both *TEF* and *RIB1* genes, but the *RIB1* gene showed a faint expression after 24 h indicating lower rate of transcription (Fig. 1f). In adapted *A. gossypii* cultures, there was strong expression of *RIB1* gene after 24 h of growth. Thus it can be inferred that in the presence of inositol the initiation of *RIB1* gene expression was significantly delayed in *E. ashbyi* which in turn resulted in reduced riboflavin production whereas *RIB1* gene expression was enhanced in *A. gossypii*.

Ashbya gossypii and *E. ashbyi* are the only two natural riboflavin overproducers used for the industrial production of riboflavin. The other microbes used in industrial production of riboflavin, i.e *Candida famata* and *Bacillus subtilis* are not natural overproducers but are genetically modified to do so (Stahmann et al. 2000). Hence, regulating the expression of specific genes encoding the rate limiting enzymes for enhancing riboflavin production in these two fungi will be highly desirable. The present study demonstrated the antagonistic effect of myo-inositol on riboflavin production by *A. gossypii* and *E. ashbyi*. Inositol stimulated *RIB1* gene expression in *A. gossypii*, leading to enhanced riboflavin production. It

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