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RESEARCH ARTICLE

Effect of Stress Inducer on the Morphology of the Riboflavin producer *Eremothecium ashbyii*

Sampath Kumar Jadhav[2], S. Vijayalakshmi[1,2]

[1]Department of Biotechnology, School of Life Sceinces, VELS University, Pallavaram, Chennai-600117.
[2]Department of Biotechnology, School of BioEngineering, Faculty of BioEngineering, SRM University, Kattankulathur, Chennai-603103.
*Corresponding Author E-mail: **vijayaiitm@gmail.com**

ABSTRACT:

Eremothecium ashbyii is a filamentous hemiascomycete fungus known for the overproduction of riboflavin and is therefore an industrially important micro organism. Riboflavin is required in trace amounts, but at high concentration it is toxic to the cell, causing photo-induced damage of the macromolecules. Although riboflavin acts as a protective agent for spores against UV radiation, at high concentrations it causes the same damage to the cell as UV radiation. Production of riboflavin is initiated by stress such as UV rays or limiting substrate. Thus stress induced riboflavin production is seen in this organism. Preliminary studies have shown that as a first line of defence against toxicity due to production of excess riboflavin, morphological changes are produced. Sporulation in this fungus has also been correlated with riboflavin overproduction. Non sporulating *E.ashbyii* produces less riboflavin. The morphology and physiology of different *E.ahbyii* UV mutants varied and showed distinct changes during their growth in the riboflavin production medium. The physiological characteristics of UV mutants and that of wild type *E.ashbyii* differed in terms of increased glucose utilization and higher biomass production. The mutants also showed higher riboflavin production. Highly flavinogenic mutants show good mycelial integrity and sporulation. In this study for the first time the morphological changes in *E.ashbyii* under conditions of stress are reported. The organism was grown in the presence used two stressors – riboflavin itself and the chemical mutagen ethidium bromide. It was found that when riboflavin itself was used as a stress compound, production was initiated at an early stage, increased production was obtained and the organism was able to tolerate upto 0.8mM riboflavin, but when ethidium bromide was used,a toxic effect was observed in the organism leading to a decreased riboflavin production. Thus a feed forward effect on riboflavin production was observed when riboflavin itself was used as a stress inducer.

KEYWORDS: *Eremothecium ashbyii*, riboflavin, hemiascomycete fungus, stress, morphological changes.

INTRODUCTION:

Eremothecium ashbyii is a filamentous hemiascomycete fungus known for the overproduction of riboflavin also known as vitamin B_2[1] and is an industrially important micro organism for the biotechnical production of riboflavin[2]. Thus fermentation based production of riboflavin has overtaken the traditional chemical based method[3].

Riboflavin forms the main component of FAD and FMN and is required in many important processes in the cell. Until recently, the vitamins were produced predominantly by chemical synthesis. Currently fermentation processes using *B.subtilis*, *E.gossypii*, or *Candida* have progressively replaced the synthetic preparation method[1,3,4]. Being non toxic, riboflavin is used in many commercially available food products too as a food colourant on account of its yellow colour.

Riboflavin production by *E.ashbyii* begins early during exponential phase; however the excess amount is produced only during stationary phase just before sporulation. The morphology and physiological characters of the various *E.ahbyii* UV mutants shows

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varying and distinct changes during their growth in the riboflavin production medium. The physiological characteristics of UV mutants differed from that of wild type *E.ashbyii* in terms of increased glucose utilization and higher biomass production. The mutants also showed higher riboflavin production. Morphological changes also take place and variations are seen. Highly flavinogenic mutants show good mycelial integrity and sporulation. The initial growth of the organism in riboflavin production medium begins as a filamentous form, the organism produces long filaments during the initial stages of growth. During growth the filaments start swelling and give rise to bulbous forms with hyaline granules totally visible under the bright field microscope. The initial riboflavin synthesis begins intracellularly which can be easily seen as yellow riboflavin crystals inside the cells. When the maximum riboflavin is reached, it becomes toxic and cell lysis starts, with complete stoppage of riboflavin production[5].Since the production of riboflavin is triggered not because of the increased growth but because of the decreased growth, stress induced riboflavin production is possible[6]. Thus this organism when supplied with limited substrate in the production medium produces riboflavin at an early stage. *E.ashbyii* is sensitive to radiation, and riboflavin may protect the organism from radiation induced damage[7]. Hence UV induced riboflavin production also gives good amount of riboflavin[5]. In this study we undertook a preliminary investigation of the riboflavin production and morphological changes that the organism exhibits in the presence of stress inducing agents. We used two stress inducers – ethidium bromide and riboflavin itself to study their influence on the production of riboflavin and also analyse whether this is an indication of the stress response of the organism.

MATERIALS AND METHODS:

Organism and culture conditions: Maintenance of culture:

Eremothecium ashbyii (NRRL 1363) was cultured onto Potato Dextrose Agar slants and incubated at 25°C.

Media composition:Potato Dextrose Agar Medium:

Potato infusion	–	200g
Dextrose	–	20g
Agar	–	20g
Distilled water	–	1L
pH	–	5.6 ± 0.2

Revival of the cultures was done every ten days by subculturing onto new slants.

Stress induced riboflavin production:

Two different stress compounds, the chemical mutagen (ethidium bromide) and riboflavin itself were used in this study. Concentration of ethidium bromide used was 1µl, 0.5µl and 0.25µl. The above concentration was made in duplicates with one flask for the control. 5ml of above mentioned concentration was used in the production medium for the production of riboflavin. Concentration of riboflavin used was 0.1mM, 0.2mM, 0.4mM, 0.6mM, 0.8mM, and 1.2mM in each flask. The mentioned concentration was used in duplicates along with one flask for the control.

Preparation of pre- inoculum – Day1:

Pre-inoculum was prepared before the initiation of production of riboflavin, so as to obtain the good amount of culture.

Basic composition for the pre-inoculum medium used was

Glucose	=	30g/l
Yeast Extract	=	2g/l
Peptone	=	8g/l

100 ml of sterile GYP broth of the above composition was inoculated with growth from one PDA slant and the flask was incubated at 120 rpm and 30°C in an incubator shaker overnight.

Preparation of production medium – Day2:

Production medium was used with composition as mentioned by[3] with some modification

Glucose	=	20g/L
Yeast extract	=	2g/L
Peptone	=	2g/L
Sodium Molybdate	=	0.01g/L
Magnesium Sulphate	=	0.1g/L
Copper sulphate	=	0.1g/L
Zinc sulphate	=	0.1g/L
Pottasium dihydrogen phosphate	=	2g/L
Sodium Chloride	=	1g/L
pH	=	6

For experiments using ethidium bromide as the stressor, 150ml of the above mentioned production medium was prepared in 6 different flasks and sterilized. 1% of a 24-36 hr old preinoculum treated under sterile conditions with different concentrations of ethidium bromide mentioned above was inoculated into each flask in duplicates. For experiments using riboflavin as the stressor, 150ml of the production medium was prepared in 13 different flasks and sterilized. 1% of a 24- 36 hr old preinoculum treated under sterile conditions with different concentrations of riboflavin mentioned above was inoculated into each flask in duplicates. One flask containing150ml of the production medium was inoculated with 1% of a 24- 36 hr old untreated preinoculum served as the control.

Time course analysis:

A time course analysis of the biomass production, extracellular riboflavin, intracellular riboflavin, and pH was observed every 24 hours for 9 days. Alsohysiological and morphological characteristics were observed under the bright field microscope.

Biomass determination:
2 ml of samples were collected from the flask in a preweighed 2ml eppendorf tubes and centrifuged at 10,000rpm for 20 min. The supernatant was discarded and the above step was repeated twice. The pellet was air dried in a hot air oven for 1hr and the tube was weighed again.

Cell disruption for Intracellular Riboflavin:
100 ml of medium was passed through a high pressure homogenizer at 1000bar pressure thrice. The disrupted cells were collected in a beaker and subjected to centrifiguation at 10,000 rpm for 20 min. The supernatant was collected in a different beaker and used for estimation of intracellular riboflavin.

Riboflavin Estimation:
Riboflavin was estimated fluorimetrically using the ISI standard procedure (IS 1374, 1979). From the supernatant collected above, 10 ml of a suitable dilution was taken in two tubes marked A and B, 1 ml of the riboflavin standard (1 µg/ml) was added to tube A and 1 ml of distilled water was added to tube B. The solutions were then acidified using 1 ml of glacial acetic acid followed by the addition of 0.5 ml of 4 % $KMnO_4$ to each tube in order to oxidize the impurities. After 2 minutes 0.5 ml of 3 % H_2O_2 was added to both the tubes in order to oxidize the residual $KMnO_4$. The fluorescence of the solutions was measured using an ELICO Fluorometer Model CL-53 to give readings A (Standard + Sample) and B (Sample alone). Into tube B, 20 mg of Sodium dithionite was added and the fluorescence measured within 10 seconds (reading C). The riboflavin concentration in the original sample was calculated using the formula :

$$\frac{(B-C)}{(A-B)} \times \frac{1}{10} \times \text{Dilution factor} = \text{Riboflavin conc (µg/ml)}.$$

RESULTS:

Stress induced riboflavin production:
The current study focused on the chemical mutagen Ethidium bromide and riboflavin itself as stress inducing compounds.

Table 1:Riboflavin production, pH and biomass when *E.ashbyii* was subjected to various concentrations of Ethidium bromide

Ethidium bromide conc (µl/ml)	Parameter measured	Time of growth					
		24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
0.25	**Riboflavin (µg/ml)**	6	16	79	82	89	91
	pH	5.5	5.8	6.1	6.0	6.2	6.2
	Biomass (g/l)	0.78	0.82	1.06	1.14	1.18	1.16
0.5	**Riboflavin (µg/ml)**	6	16	44	52	51	51
	pH	5.9	6.2	6.4	6.4	6.4	6.4
	Biomass (g/l)	0.8	0.84	0.99	1.02	1.02	1.02
1.0	**Riboflavin(µg/ml)**	6	9	16	16	16	16
	pH	6.1	6.1	6.1	6.1	6.1	6.1
	Biomass (g/l)	0.82	0.84	0.88	0.88	0.88	0.88
Control	**Riboflavin (µg/ml)**	16	26	111	196	290	310
	pH	5.0	5.3	5.8	5.8	6.0	6.0
	Biomass (g/l)	4.8	7.9	10.5	8.3	7.8	7.5

Table 2: Time course analysis of the morphological changes in the organism subjected to various concentration of ethidium bromide

Ethidium bromide conc (µl/ml)	Time of growth (hrs)					
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
Control	Filamentous growth.	Maximum biomass and riboflavin production began.	Hyaline granules and bulbous forms observed, pH increased from 5.0.to 5.5.	Maximum riboflavin production was observed.	pH increased to 6.0. Growth stagnant.	Constant pHat 6.0 and cessation of growth.
0.25	Negligible filamentous growth.	Decrease in viable cells. A few hyaline granules and beginning of bulbous forms.	Riboflavin production and beginning of cell lysis.	Increased pH upto 6.0. Stagnant growth.	Extensive cell lysis.	Constant pH at 6.2 and cessation of growth alongwith cell lysis.
0.5	Very few long filamentous growth.	Hyaline granules and few bulbous forms. Low conc of riboflavin.	An increase in pH upto 6.4. Decrease in growth	Stagnant pH at 6.4. Cessation of growth.	Dead cells.	Dead cells.
1.0	Long filamentous growth, thin filaments compared to others.	Beginning of cell lysis was observed. pH constant at 6.1. No riboflavin production.	Extensive cell lysis and cell death.	-	-	-

Time course analysis of the riboflavin production and morphological changes in the organism subjected to various concentration of ethidium bromide:

When *E.ashbyii* was treated with ethidium bromide, a toxic effect alongwith reduced riboflavin production was observed. No filamentous growth was observed and no hyaline granules were visible. The number of viable organismsdecreasedwith an increased concentration of ethidium bromide. Biomass in the production medium also decreasedwith time. Thus ethidium bromide had an inhibitory effect on the production of riboflavin (Tables 1, 2, Fig 1).

Time course analysis of the riboflavin production and morphological changes in the organism subjected to various concentrations of riboflavin:

Riboflavin production by *E.ashbyii* was affected by the addition of external riboflavin in the production medium. At 0.1 mM concentration the organism showed similar characteristics to that in control. Long filamentous growth was observed at 24 hrs of growth followed by bulbous growth with visible hyaline granules and mass riboflavin production at 96 hrs. At concentration above 0.4 mM some changes in the physiological and morphological characteristic was observed, bulbous growth was reduced and overall less riboflavin was produced. At 0.2 mM concentration, early riboflavin production was initiated with hyaline granules clearly visible at 48 hrs. Intracellular riboflavin was visible at 72 hrs. itself. Thus the total amount of riboflavin produced was maximum when riboflavin at 0.2 mM concentration was added to the production medium. At higher concentrations very less riboflavin was produced and at 1.2mM concentration it became toxic to the cell (Tables 3, 4; Figs 2,3a, 3b).

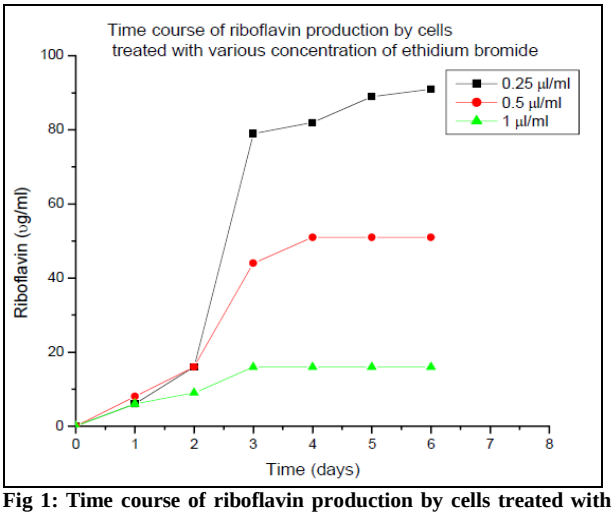


Fig 1: Time course of riboflavin production by cells treated with various concentration of ethidium bromide.

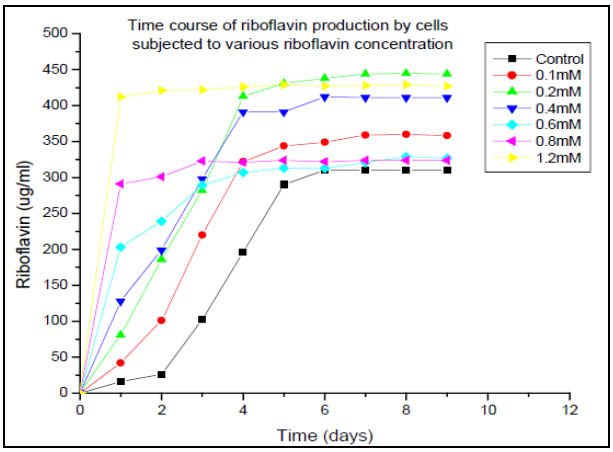


Fig 2: Time course of riboflavin production by cells treated with various concentration of riboflavin.

Table 3:Riboflavin production, pH and biomass when *E.ashbyii* was subjected to various concentrations of Riboflavin

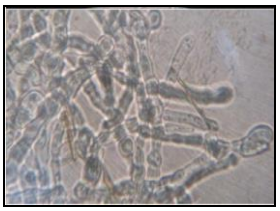
Riboflavin conc (mM)	Parameter measured	Time of growth					
		24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
0.1	Riboflavin (µg/ml)	42	101	220	322	344	349
	pH	5.0	5.2	5.6	5.7	5.9	5.9
	Biomass (g/l)	0.68	1.19	1.4	1.5	1.5	1.5
0.2	Riboflavin (µg/ml)	81	186	282	413	431	438
	pH	4.7	5.2	5.9	6.1	6.1	6.1
	Biomass (g/l)	0.66	1.0	1.46	1.5	1.5	1.5
0.4	Riboflavin (µg/ml)	128	199	298	391	391	411
	pH	5.8	5.9	6.1	6.1	6.1	6.1
	Biomass (g/l)	0.64	1.12	1.59	1.59	1.59	1.59
0.6	Riboflavin (µg/ml)	203	239	289	307	313	313
	pH	5.8	5.9	6.1	6.1	6.1	6.1
	Biomass (g/l)	0.62	1.07	1.42	1.42	1.4	1.4
0.8	Riboflavin (µg/ml)	291	301	323	321	321	323
	pH	5.9	5.9	6.1	6.1	6.1	6.1
	Biomass (g/l)	0.64	1.06	1.4	1.42	1.42	1.42
1.0	Riboflavin (µg/ml)	370	386	394	392	392	392
	pH	5.9	5.9	6.0	6.1	6.1	6.1
	Biomass (g/l)	0.64	1.06	1.4	1.4	1.42	1.42
1.2	Riboflavin(µg/ml)	421	426	429	429	429	429
	pH	5.8	5.9	6.2	6.1	6.1	6.1

	Biomass (g/l)	0.73	1.03	1.36	1.42	1.43	1.42
Control	**Riboflavin (µg/ml)**	16	26	111	196	290	310
	pH	5.0	5.3	5.8	5.8	6.0	6.0
	Biomass (g/l)	4.8	7.9	10.5	8.3	7.8	7.5

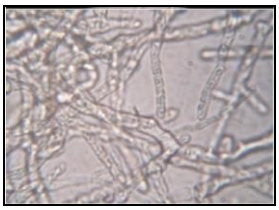
Table 4: Time course analysis of the morphological changes in the organism subjected to various concentration of riboflavin

Riboflavin conc (mM)	**Time of growth (hrs)**					
	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**	**144 hrs**
Control	Filamentous growth.	Maximum biomass and riboflavin production began.	Hyaline granules and bulbous forms observed, pH increased from 5.0.to 5.5.	Maximum riboflavin production was observed.	pH increased to 6.0. Growth stagnant.	Constant pHat 6.0 and cessation of growth.
0.1 mM	Filamentous growth.	Hyaline granules and beginning of bulbous forms.	Extensive production of bulbous forms.	Maximum riboflavin production was observed.	Increased pH upto 5.8. Stagnant growth.	Constant pH at 5.8 and cessation of growth.
0.2 mM	Long filamentous growth.	Hyaline granules and many bulbous forms. High conc of riboflavin.	Maximum biomass was produced.	An increase in pH upto 6.2.	Stagnant pH at 6.2. Cessation of growth.	Dead cells.
0.4 mM	Long filamentous growth, bulbous forms,faint hyaline granules visible.	Low riboflavin production with good amount of biomass.pH increased from 5.8 to 6.1.	Riboflavin production was observed but biomass was limited.	Growth stopped and riboflavin production was also not observed.	-	-
0.6 mM	Long filamentous growth.	Bulbous growth, hyaline granules not clearly visible.	Maximum biomass was produced but no riboflavin was produced.	Onset of cell lysis.	-	-
0.8 mM	Long filamentous growth, thin filaments compared to others.	No bulbous growth was observed, hyaline granules not visible, pH increased from 5.9 to 6.1, very less riboflavin production.	Cell lysis was observed.	-	-	-
1.2 mM	Long filamentous growth, thin filaments compared to others.	Cell lysis was observed.	-	-	-	-

Day 1
Long filaments



Day 2
Bulbous forms

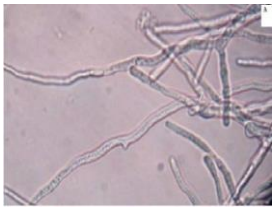


Day 1
Long filaments

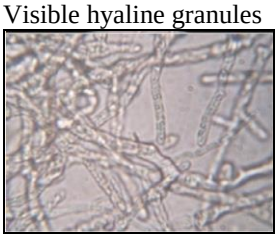
Day 3
Bulbous forms

Day 3
Visible hyaline granules

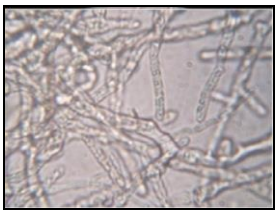
Day 4
Intracellular riboflavin



Day 4
Visible hyaline granules



Day 5
Intracellular riboflavin



DISCUSSION:

The effect of two stress inducers, the chemical mutagen ethidium bromide and riboflavin itself; on the riboflavin overproduction and morphological changes in the hemiascomycete fungus *Eremothecium ashbyii*, was studied as a time course. It was found that the chemical mutagen was toxic to the cells resulting in a rapid degradation of the fungal hyphae (Tables 1, 2, Fig 1). The absence of visible hyaline granules in the hyphae correlated with the inhibitory effect on riboflavin production. Ethidium bromide is a chemical mutagen known to damage DNA by intercalation leading to deletions or insertions. Such damage is usually repaired by the Base Excision Repair (BER) mechanism[8]. The susceptibility of *E.ashbyii* to ethidium bromide induced damage indicates that this DNA repair mechanism is probably not active in *E.ashbyii.* However in the hyphal cells exposed to riboflavin no such damage was observed. Instead it was observed that Riboflavin production does work on a feedforward and feedback mechanism, and it acts as a stress signal at a concentration of 0.2 mM. At higher concentrations of added riboflavin very less riboflavin is produced and at 1.2mM concentration it becomes toxic to the cell (Tables 3, 4; Figs 2, 3a, 3b). This indicates that there is probably a repair mechanism which operates at low concentrations of riboflavin. Riboflavin induced damage to the DNA is thought to be repaired by the Nucleotide Excision Repair (NER) mechanism and the absence of toxicity at low riboflavin concentrations points to a possible role for the NER pathway in repairing DNA damage in *E.ashbyii* at the concentrations indicated in this study.

The observation of morphological changes during riboflavin production may also be developmentally regulated both in the control as well as the treated cells. A developmental regulation of diploid cells of the budding yeast *Saccharomyces cerevisiae* into a filamentous growth form has been reported during nitrogen starvation, during growth on poor carbon sources such as starch, various alcohols, by products of amino acid metabolism[9]. Strains of *Aspergillus* and *Trichoderma* exposed to high doses of PAHs significantly vary in their growth rates and sporulation characteristics[10].However no such reports exist for *E.ashbyii* and this is the first such report. Further studies aimed at understanding the repair mechanisms in repairing riboflavin induced DNA damage and correlating it to the observed morphological changes could be the focus of future studies on *E.ashbyii.*

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