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Enhanced FAD Production in *Eremothecium ashbyi* with Statistically Optimized Oxidative Stress Modulators

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Abstract: Riboflavin (vitamin B2) and flavin adenine dinucleotide (FAD) are valuable biological flavins mainly used as animal feed supplement and in pharmaceutical formulation. *Eremothecium ashbyi* is a natural overproducer of FAD along with riboflavin. Flavins overproduction was found to be strongly correlated with stress induction but stress response in *E.ashbyi* is completely unexplored. Hence, in the present study, flavins production in presence of four oxidative stress modulators (menadione, vitamin E, cAMP, H_2O_2) was investigated. Their levels were statistically optimized to maximize the flavins production. The regression model obtained from Central Composite Design indicated that maximum flavins production occurred when 1.09 μM menadione and 1.12 μM vitamin E were supplemented to 24-hour grown fungus. Upon supplementation, total flavins production was significantly increased by 1.34-fold and FAD production was increased by 2.18-fold over untreated control. Intracellular – reactive oxygen species (ROS) level was increased and reduced glutathione (GSH) and oxidized glutathione (GSSG) ratio was decreased indicating oxidative burst experienced by the cell. Furthermore, a concomitant increase in specific activity of glutathione reductase and FAD synthetase was observed. For the first time this study showed that among flavins, FAD was majorly increased along with FAD synthetase activity as a response to oxidative stress. These results indicated that the oxidative stress and flavins overproduction was centered on FAD regulation.

Keywords: *Eremothecium ashbyi*, Riboflavin, FAD, Central Composite Design (CCD), Glutathione, Oxidative Stress

1. Introduction

Flavins are important biological compounds with common isoalloxazine moiety of tri-heterocyclic structure called flavin ring. Riboflavin, FMN and FAD are major flavins involved in several metabolic reactions vital for life. Riboflavin, also known as vitamin B_2, is used as food and feed additive with nearly 10000 tons of production per year. FMN and FAD are co-enzymes for important oxidation/reduction metabolic reactions [1]. FAD has a pharmaceuticals application and 1000 times more expensive than riboflavin. It is used as an ophthalmic agent and multi-vitamin supplements. [2]. FAD is used in the treatment of genetically inherited diseases like Friedreich ataxia which is caused by the lack of the mitochondrial protein frataxin [3] and chronic granulomatosis which is caused by mutations in leukocyte NADPH oxidase genes [4].

Eremothecium ashbyi is a filamentous fungus belonging to *Saccharomycetaceae* family [5]. It is a natural overproducer

of riboflavin and was used for industrial riboflavin production [6]. A closely related fungus, *Ashbya gossypii* was preferred to *E.ashbyi* for industrial production of riboflavin as *E.ashbyi* was genetically unstable. Moreover, annotated genome sequence database as well as genetic engineering tools were developed for *A.gossypii* [7]. However, *E.ashbyi* in contrast to *A.gossypii* is reported to overproduce FAD [8] and this property has not been exploited so far.

The basis for over production of flavins in stationary to lysis stage by these filamentous fungi is still intriguing. Riboflavin production as response to stress has been well explored in *A.gossypii* and *Pichia guilliermondii* [9]. Amongst the different types of stress tested [10], oxidative stress strongly correlated with riboflavin production in *A.gossypii* [11]. cAMP supplementation which generates stress relieving signals, decreased the riboflavin production [12] while oxidative stress inducers like hydrogen peroxide and menadione increased it in *A.gossypii* [13]. Stress

response transcription factor Yap-1 mediated increase in riboflavin production was observed upon supplementation of hydrogen peroxide in *A.gossypii*. This provided a gene-level association between riboflavin production and oxidative stress [14]. Thus, flavins emerged as a group of potential anti-oxidants whose effects are synergistically attributed through mainly glutathione redox cycle [15]. The potential of *E.ashbyi* for production of pharmaceutically important metabolite FAD upon oxidative stress through a combination of stress modulators has not been explored.

In this study, the effect of oxidative stress modulators on the production of the flavins was studied for the first time in *E.ashbyi*. The effect of combination of five different factors on flavins production in *E.ashbyi* was statistically analyzed. The significant factors' levels were optimized through Central Composite Design and regression analysis. Since glutathione is a key regulator of oxidative stress, ratio of reduced to oxidized glutathione (GSH/GSSG) and activity of stress response enzyme glutathione reductase were determined. Glutathione reductase requires FAD to convert oxidized glutathione to its active reduced form. Thus, glutathione metabolism could be correlated to increased FAD levels upon oxidative stress.

2. Materials and Methods

2.1. Strain and Growth Conditions

Eremothecium ashbyi strain-NRRL Y-1363 was maintained on yeast extract-malt extract-broth (YM broth- yeast extract- 3 gm, malt extract- 3 gm, peptone- 5 gm, glucose-10 gm per liter). A 48-hour pre-inoculum of 0.5%, grown on YM-broth at 180 rpm at 30°C, was used to inoculate 50 ml YM broth prepared in 250 ml brown colored Erlenmeyer conical flask for all experiments.

2.2. Oxidative Stress Modulators Addition to YM Broth

Stock solutions of the following were prepared as follows.
a) Vitamin E (Sigma) (MW: 472.8 gm): A stock solution of 10 mM was made in absolute ethanol. 1mM working stock was made from 10mM stock in absolute ethanol.
b) Dibutryl cAMP (SRL) (MW: 491.37 gm): A stock solution of 50 mM was prepared in sterile water.
c) Menadione (Sigma) (MW: 376.23 gm): A stock solution of 10 mM stock was prepared in sterile water.
d) Hydrogen peroxide (Merck) (H_2O_2) – 30% w/v stock was diluted to desired concentration just before use.

All stock solutions were filter sterilized with 0.2 μm nylon syringe filters.

2.3. Estimation of Biomass and Residual Glucose

Fungal mycelium was harvested by filtering through Whatman[®] grade No. 1 filter paper followed by multiple washing and drying at 90ºC until constant weight was obtained. Residual glucose was estimated using 3, 5-Dinitrosalicylic acid (DNS) method.

Estimation of Flavins and FAD.

Total flavins and extracellular flavins were measured fluorometrically using the ISI-standard procedure (IS: 5399 1969) as described by Kavitha and Chandra, 2009 [11]. Briefly, extracellular flavins were measured by filtering the broth through Whatman[®] grade No. 1 filter paper. To estimate total flavins, broth was treated with four volumes of 0.02 N HCl, autoclaved at 121°C to break vacuoles and release intracellular flavins in supernatant and filtered to remove cell debris. Individual flavins - riboflavin, FMN and FAD were estimated as per Patel and Chandra, 2019 [16], through reverse phase HPLC gradient elution with column: HiQ Sil C18HS (4.6 mm × 250 mm, 5 μm). For total FAD estimation, the samples were autoclaved as mentioned earlier and treated with 10% Trichloroacetic acid (TCA) to remove proteins before injecting in HPLC.

2.4. Preparation of Cell-Free Extracts (CFE)

The cell-free extract was made by the method of Ayar-Kayali *et al.*, 2002 [17]. A constant amount of washed *E.ashbyi* mycelium was frozen at -20°C. The cells were suspended in 20 mM sodium phosphate buffer, pH 7.0 and grinded with sterile acid-washed glass beads in a mortar for 15 min over ice bath. The total volume was made up to 5 ml with sodium phosphate buffer and centrifuged at 10,000 rpm for 10 min to remove cell debris. The protein content in the extract was determined by Bradford method.

2.5. Reduced and Total Glutathione Estimation in CFE

The reduced glutathione was estimated as per Tietze, 1969 [18]. 5, 5'-dithiobisnitrobenzoic acid (DTNB) reacts with reduced glutathione to form the colored product 5-thio-2-nitrobenzoic acid (TNB) which is measured at 412 nm. CFE (containing 0.5–1.0 mg of protein/ml) was treated with 10% TCA to remove the proteins. 1ml assay reaction mixture contained 200 mM sodium phosphate buffer, pH-8, 6 mM DTNB in phosphate buffer and treated CFE. The reaction mixture was incubated for 10 minutes and colour developed was determined at 412 nm. The reduced glutathione was expressed as nmoles/mg of protein.

For measuring total glutathione, the oxidized glutathione (GSSG) present in the sample was converted to GSH by the highly specific glutathione reductase (Sigma) and NADPH (Sigma). The formation of the yellow colored compound TNB upon reaction of GSH with DTNB was measured at 412 nm as stated earlier. Reaction mixture contained 200 mM sodium phosphate buffer pH-8 (with 0.248 mg/ml of NADPH), 6 mM DTNB in phosphate buffer, 5 μl of 266 U/ml of glutathione reductase and treated CFE.

2.6. Glutathione Reductase (GR) Assay

The assay was performed as per Mavis and Stellwagen, 1968 [19]. Glutathione reductase catalyzes the NADPH dependent reduction of glutathione disulfide (GSSG) to glutathione (GSH). The oxidation of NADPH to NADP is accompanied by a decrease in absorbance at 340 nm. One Unit (U) of the enzyme was defined as 1.0 μmole of NADPH

oxidized per minute at 25 °C. at pH 7.0. The total reaction contained –100 mM potassium phosphate buffer pH-7, 0.2 mM GSSG, 0.02 mM of NADPH and CFE (0.1-0.2 mg of protein/ml).

2.7. FAD Synthetase Assay

The assay was performed as per Yatsyshyn *et. al.*, 2014 [2]. The assay mixture contained 75mM potassium phosphate buffer (pH -7.5), 1.5 mM $MgCl_2$, 0.065 mM FMN and 2mM ATP. The reaction mixture was incubated at 30°C for 30 minutes. The reaction was stopped by incubating the assay mix at 65°C for 10 min and immediately transferring to the ice. The FAD produced is quantified with reverse phase HPLC as stated earlier. One Unit (U) of enzyme activity was defined as 1.0 µmole of FAD produced per minute at 30°C at pH 7.5.

2.8. Reactive Oxygen Species (ROS) Assay

Intracellular ROS was assayed by slightly modifying the method given by Rastogi *et. al*. 2010 [20]. 2',7 – dichloro fluoresce in diacetate (DCFDA) is a fluorogenic dye which can diffuse through cell membrane and gets deacetylated by cellular esterase which is then oxidized to fluorescent compound 2', 7' –dichloro fluorescein (DCF) by ROS. The DCF can be quantified by spectro-fluorimeter with excitation wavelength of 495 nm and emission wavelength of 529 nm after quenching with 10mg/ml of sodium dithionite. The mycelium was harvested after exposing to oxidative stress modulators - menadione and vitamin E for 6 hours. The assay mixture for ROS determination contained 100 mg of washed mycelium in phosphate buffer saline (PBS) with 5.0 µM of DCFDA. ROS levels are reported indirectly as amount of DCF in nmoles/mg of protein.

2.9. Factorial Design of Experiment and Response Surface Methodology

Factorial design of experiments was carried out to identify important oxidative stress modulating factors and interaction among them. Five factors were considered: A.) Menadione–superoxide radical generator. B.) H_2O_2–peroxide radical generator C.) Vitamin E an antioxidant and a pro-oxidant in rare cases D.) Dibutryl-cAMP – negative stress regulator E.) Incubation time ie. time of supplementation of stress modulators post-inoculation. Extracellular flavins, total flavins and percentage excretion were recorded as responses in 120-hour grown culture. Half factorial design with 5 factors and level 5 resolution was used to screen significant factor for above mentioned responses. The limits of each variable were chosen from literature data and preliminary

investigations carried out with individual factor. As the design level 5 resolution, effect of individual factor was confounded with four factor interactions and two factor interaction effect was confounded with three factor interactions. The data was analyzed with MINITAB 14 software. The factors significant at 95% confidence interval ($p < 0.05$) were considered significant for total flavins production.

Experiments for optimization by Central Composite Design (CCD) were performed with the two most significant factors. Central composite design which is widely used response surface optimization strategy was employed to optimize supplement concentration of menadione and vitamin E. Both the factors' levels were decided from the above two runs of factorial design. Both factors were studied at five different levels (coded units: -1, +1 (corner point), -1.414, +1.414 (star points) and 0 (center point). Contour plots and surface response plots were drawn using MINITAB 14. Multiple regression analysis was done on the experimental data to obtain a model with high R^2 (multiple coefficient of regression) and low p-value. The model was then used to predict maximum flavins production. The model's prediction was checked and the model was validated.

3. Results and Discussion

3.1. Effect of Individual Stress Modulators on Flavins Production

To analyse the preliminary response of *E.ashbyi* against oxidative stress, the growth media was individually supplemented with three different concentration of H_2O_2 (1.0, 2.5 and 5.0 mM), menadione (0.5, 1.0 and 2.5 µM) and vitamin E (0.5, 1.0 and 2.5 µM). The total flavins were estimated at 72 and 120-hour of growth. Maximum total flavins and FAD production was obtained with 1.0 µM menadione at 120-hour compared to untreated control (Table 1). FAD was also increased by 1.21-fold with 1.0 µM vitamin E and 1.35-fold with 1.0 µM menadione at 120-hour (Table 1). Vitamin E and H_2O_2 supplement had no significant effect on total flavins production and 5mM H_2O_2 level was toxic. *A.gossypii* was reported to significantly increase flavins production with H_2O_2 [13]. This indicated that, although *A.gossypii* and *E.ashbyi* are closely related species, they responded quite differently to oxidative stress. It was of interest to study combined effect of oxidative stress supplements as they may act synergistically to enhance flavins rather than individual supplement by plausible activation of multiple pathways leading to enhanced expression of flavin genes.

Table 1. Effect of individual oxidative stress modulators on flavins production.

Sample	Total Flavins 72-hour (mg/L)	Total Flavins 120-hour (mg/L)	Total FAD 120-hour (mg/L)
Control	49.75±2.15	118.01±3.00	13.63±0.79
H_2O_2			
1.0 mM	52.6±2.30	128.38±2.87	-
2.5 mM	55.25±2.35	130.26±5.00	16.13±0.32*
5.0 mM	40.1±1.30	106.51±2.50	-

Sample	Total Flavins 72-hour (mg/L)	Total Flavins 120-hour (mg/L)	Total FAD 120-hour (mg/L)
Menadione			
0.5 µM	56.25±1.24	129.13±3.38	-
1.0 µM	56.85±1.15	137.88±4.13*	18.40±0.81*
2.5 µM	51.9±1.10	127.76±3.75	-
Vitamin E			
0.5 µM	53.05±1.82	127.12±1.50	-
1.0 µM	64.04±1.19*	129.18±0.92	16.49±0.53*
2.5 µM	60.91±1.45	123.59±2.01	-

Control = Untreated culture, * = p-value <0.05.

3.2. Screening of Combination of Stress Modulators Affecting Flavins Production

Factorial experimental design was made with MINITAB 14 with five different factors and extracellular as well as total flavins and percentage excretion were recorded as responses (Table 2). Pareto chart obtained from the experiment showed menadione, vitamin E and time of supplementation to be most significant to increase the total flavins production (Figure 1). cAMP, a well-known negative stress regulator significantly decreased the total flavins production suggesting oxidative stress indeed played an important role in modifying regulation on flavins synthesis in *E.ashbyi*. cAMP was also reported previously to suppress riboflavin production in *A.gossypii* [12].

Menadione is most significantly affecting the total flavins production followed by vitamin E (Figure 1A). Vitamin E had profound effect on flavins excretion which could be observed when percentage excretion was recorded as response (Figure 1B). Vitamin E was also reported to increase the excretion in closely related fungus *Ashbya gossypii* [11]. Increased extracellular flavins is a desirable property due to ease and decreased loss during product recovery. Similar to *A.gossypii, E.ashbyi* also seems to respond with very low concentration of vitamin E compared to *Ustilago maydis* which was supplemented with 232 µM vitamin E to be protected from fungicide toxicity [21]. Adjusted R^2 – value for obtained factorial DOE model was 0.637.
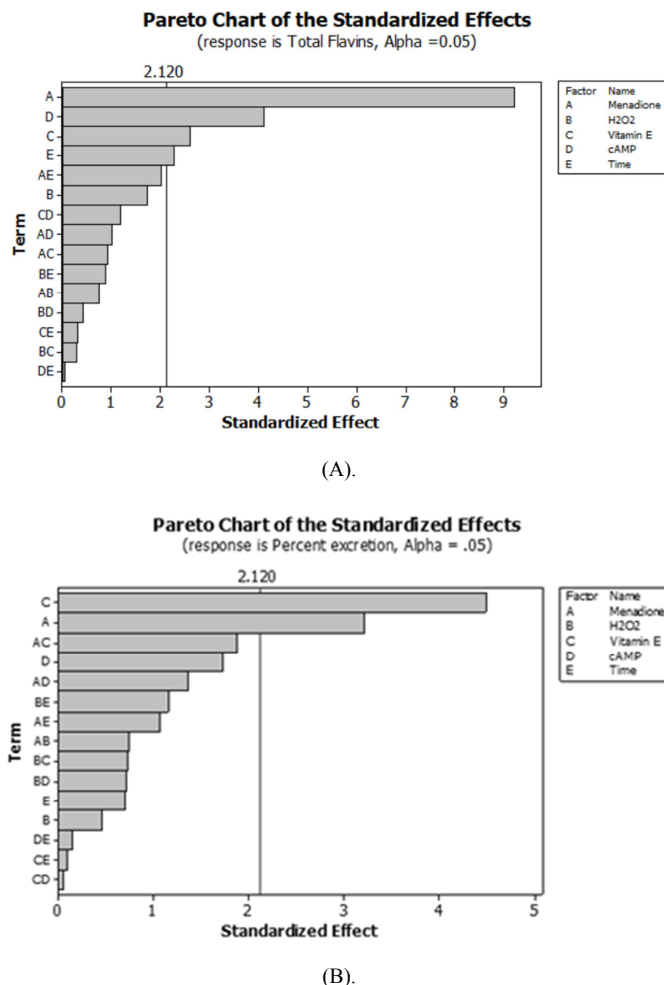

(A).

(B).

Figure 1. *Factorial design of experiments for screening significant factors affecting flavins production. Pareto charts for standardized effects on A.) Total flavins production. B.) Percentage excretion.*

***Table 2.** Factorial design of experiment.*

Run Order	Menadione (µM) (A)	H$_2$O$_2$ (mM) (B)	Vitamin E (µM) (C)	cAMP (µM) (D)	Time (hour) (E)	Extracellular flavins (mg/L) 120-hour	Total flavins (mg/L) 120-hour	Percent excretion
1	0	0	0	0	24	57.92±0.34	118.35±5.85	49.07±2.13
2	1	0	0	0	0	66.49±1.22	128.95±1.10	51.58±1.37
3	0	2.5	0	0	0	56.4±1.170	121.27±3.41	46.52±0.32
4	1	2.5	0	0	24	71.12±1.95	137.60±0.24	51.71±1.33
5	0	0	2.5	0	0	64.30±0.97	121.64±2.8	52.87±0.40
6	1	0	2.5	0	24	75.41±0.88	145.16±3.66	51.96±0.69
7	0	2.5	2.5	0	24	67.66±1.51	124.32±5.24	54.50±1.09
8	1	2.5	2.5	0	0	74.59±0.73	139.31±0.49	53.55±0.32
9	0	0	0	25	0	52.16±1.02	112.25±5.36	46.61±3.16
10	1	0	0	25	24	66.30±0.93	130.78±0.00	50.70±0.69
11	0	2.5	0	25	24	54.21±1.02	118.35±2.92	45.85±1.98
12	1	2.5	0	25	0	63.08±1.02	126.02±2.56	50.11±1.81
13	0	0	2.5	25	24	59.23±0.97	115.42±4.39	51.44±2.78
14	1	0	2.5	25	0	67.81±0.58	126.63±0.97	53.54±0.06
15	0	2.5	2.5	25	0	58.99±0.34	119.57±0.24	49.30±0.39
16	1	2.5	2.5	25	24	71.81±1.56	134.80±2.07	53.30±2.01

3.3. Optimizing Flavins Production with CCD

Central composite design is shown in the Table 3 with Total flavins recorded as response. Multiple regression analysis with the experimental data gave a second-order polynomial equation as follows in terms of uncoded units:

$$Y = 68.52 + 131.93M + 29.7V - 77.72M^2 - 29.73V^2 + 26.36MV$$

Where, Y = predicated response, M = menadione and V = vitamin E coded values.

***Table 3.** Central composite design matrix with two factors - menadione and vitamin E.*

Run	Menadione (µM)	Vitamin E (µM)	Experimented Total flavins (mg/L) 120-hour	Predicted value
1	0.50	0.50	134.80	129.80
2	1.20	0.50	143.09	138.89
3	0.50	1.20	128.71	128.00
4	1.20	1.20	151.13	150.00
5	0.36	0.85	120.42	119.44
6	1.34	0.85	136.75	141.42
7	0.85	0.36	134.80	139.63
8	0.85	1.34	146.99	146.21
9	0.85	0.85	151.13	149.47
10	0.85	0.85	153.08	149.47
11	0.85	0.85	146.25	149.47
12	0.85	0.85	148.20	149.47
13	0.85	0.85	148.69	149.47
14	0.50	0.50	131.63	129.80
15	1.20	0.50	145.52	138.89
16	0.50	1.20	123.10	128.00
17	1.20	1.20	148.69	150.00
18	0.36	0.85	117.98	119.44
19	1.34	0.85	139.92	141.42
20	0.85	0.36	133.34	139.63
21	0.85	1.34	149.91	146.21

ANOVA test (analysis of variance) was carried out for determining the optimal value. Goodness of fit results obtained from ANOVA test indicated that 89.8% could be explained by response model. Thus, the obtained model showed fair correlation between the experimental and predicted values with further improvement in R^2 – value of 0.90. The results from ANOVA test is shown in Table 4 indicating effect of individual factor as well as interaction are significant for flavins production. Contour plots and response-surface plots derived from CCD revealed optimal concentration of supplements for flavins production centered near 1.09 µM menadione and 1.12 µM vitamin E (Figure 2).
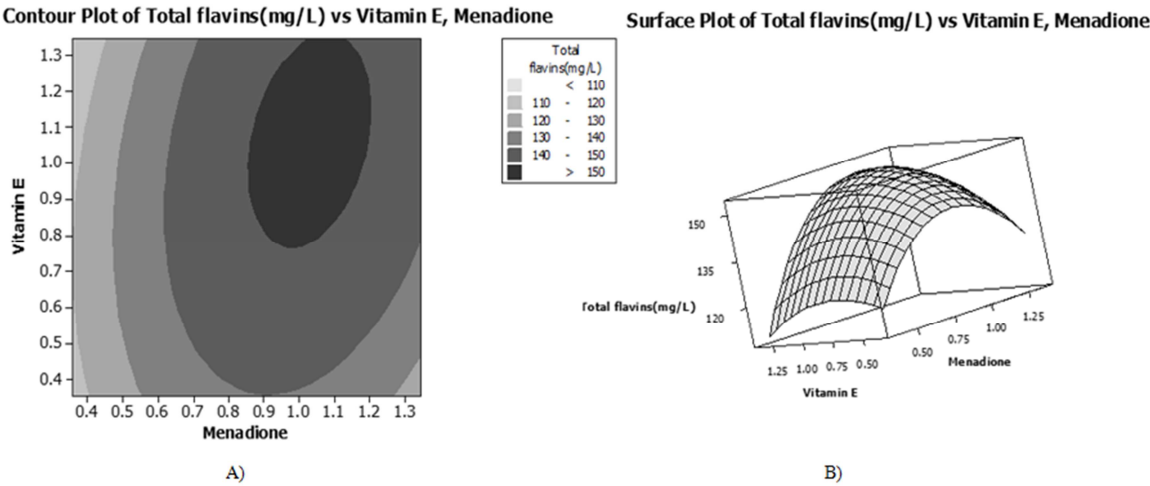
Figure 2. *Central composite design for quantification of optimum levels of supplements. A.) Contour plot of total flavins production as a function of menadione and vitamin E. B.) Response surface plot of total flavins production as a function of menadione and vitamin E.*

Table 4. *Regression analysis and Analysis of variance (ANOVA) test for the model obtained with CCD design for total flavins production.*

Source	Degree of freedom	Sum of squares	Mean square	F-value	P-value	Significance
Regression	5	2455.8	491.16	35.28	<0.001	Significant
Linear	2	1052.62	526.31	38.8	<0.001	Significant
Square	2	1319.78	659.89	47.4	<0.001	Significant
Interaction	1	83.4	83.4	5.99	0.024	Significant

3.4. Time Course Analysis of Flavins Production with Optimized Oxidative Stress Modulators

A time course analysis with optimized concentration of menadione plus vitamin E was followed against control (Figure 3). Menadione and vitamin E were supplemented after 24-hour of culture inoculation during mid-log phase. Significantly higher residual glucose and decrease in biomass production was observed at 48-hour of total incubation. Higher residual glucose (1.81-fold) was observed in treated (1.47±0.18 g/L) Vs control (0.86±0.10 g/L), Pattern of biomass production and auto-lysis were different in the treated cells (Figure 3A). Mycelia in untreated flasks showed rapid lysis from 72-hour onward. But treated mycelia were stable even up to 120-hour without undergoing autolysis unlike the control.
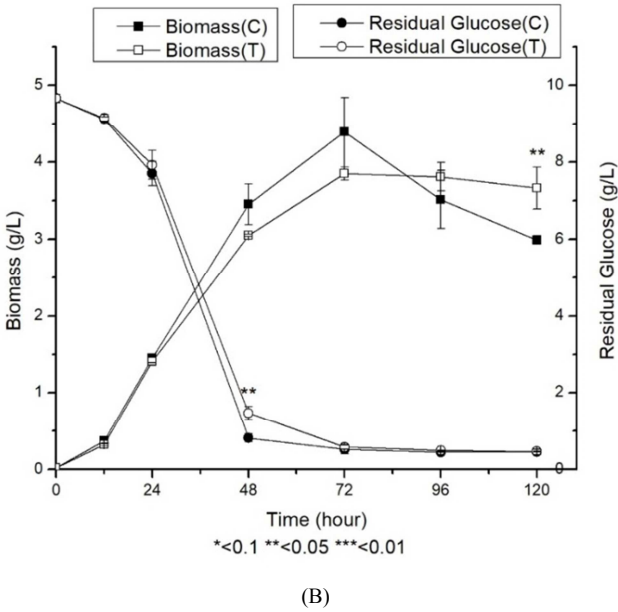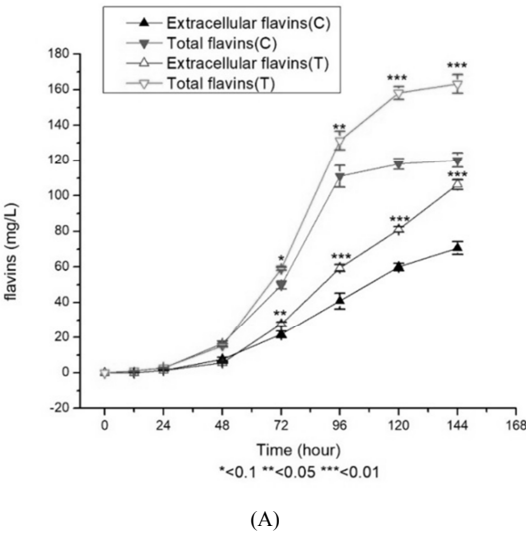

(B)

Figure 3. *Time course analysis of flavins production by E.ashbyi (A) biomass production and residual glucose (B) extracellular flavins and total flavins production. Treated (T) - 1.09 µM menadione and 1.12 µM vitamin E supplemented at 24-hour, Control (C) - Untreated culture. p-value *<0.1, **<0.05, ***<0.01.*

Extracellular flavins production was significantly increase by 1.35-fold and total flavins by 1.34-fold on 120-hour of incubation (Figure 3B). Thus, with menadione and vitamin E combination, higher flavins production was achieved compared to menadione treatment alone (1.15-fold). Vitamin E could act as pro-oxidant at low levels and induce

(A)

membrane peroxidation which resulted in increased extracellular flavins. Membrane peroxidation has been reported to affect membrane fluidty and transport earlier [22]. Similar pro-oxidant activity of vitamin E at lower levels of 1-5 μM was reported to increase extracellular riboflavin by 1.70-fold (56.2±3.6 mg/L in control Vs. 95.8±13.8mg/L in 2.5 μM vitamin E treatment) in *A.gossypii* [11].

HPLC analysis of samples indicated significant increase in FAD, the end product of riboflavin biosynthetic pathway. Increase of 2.18-fold in total FAD was observed at 120-hour of incubation (Table 5). Major increase in FAD production suggested that correlation between stress and flavins metabolism is linked through FAD level regulation. Interestingly, flavins production was induced most significantly by superoxide radical generator menadione. Superoxide radical induced glutathione metabolism could have direct corelation with flavins metabolism which was studied further.

3.5. Effect on Glutathione Metabolism

As glutathione is a key regulator of oxidative stress [23] levels of reduced (GSH) and oxidized (GSSG) glutathione were measured in the cell free extract. At 72-hour incubation GSH was significantly decreased by 1.42-fold and GSSG was increased by 1.35-fold causing significant decrease in GSH/GSSG ratio (10.27±0.74 Vs. 5.41±0.85 for control Vs. treated) (Table 5). Activity of glutathione reductase was also significantly increased by 2.34-fold at 72-hour and 1.64-fold at 120-hour incubation suggesting cells have sensed oxidative stress and increased the production of counter response enzyme (Table 5). Though activity of glutathione reductase was increased, GSH/GSSG ratio was lower than control indicating high production of ROS in chain reactions.

Table 5. Effect of menadione and vitamin E supplementation on glutathione and related enzymes.

Parameters	Control		Treated cells	
	72-hour	120-hour	72-hour	120-hour
Total FAD (mg/L)	0.94±0.01	13.63±0.79	2.36±0.65*	29.73±2.22*
GSH (mmoles/mg)	242.99±7.88	258.88±36.43	171.36±15.28*	322.69±28.87
GSSG (mmoles/mg)	23.79±1.91	30.63±5.25	32.34±5.17	51.26±8.77
GSH/GSSG	10.27±0.74	8.56±1.05	5.41±0.85*	6.39±0.58*
GSH reductase (μU/mg)	45.53±2.62	71.79±7.19	106.44±12.47*	117.8±2.82*
FAD synthetase (μU/mg)	25.33±2.24	76.85±10.44	54.96±6.15*	235.29±11.19*

Control = Untreated culture, * = p-value <0.05.

FAD a coenzyme for glutathione reductase which plays a key role in conversion of oxidized glutathione to its reduced form, which is the active form reacting with ROS [19]. Concomitant increase in glutathione reductase enzyme activity and FAD levels suggest a dynamic link between oxidative stress and flavins production in *E.ashbyi*. Direct response of oxidative stress was measured through estimation of ROS by DCFDA conversion which was increased by 1.91-fold in mycelium treated for 6-hour (3.51±0.73 nmol/mg of protein) vs. control (1.83±0.12 nmol/mg of protein).

3.6. Effect on FAD Synthetase Enzyme Activity

FAD is a coenzyme for many of the stress response and metabolic enzymes. Promoter study of *FAD1* gene (encoding for FAD synthetase enzyme) of closely related yeast *Saccharomyces cerevisiae* with YEASTRACT toolbox showed presence of binding sites for stress response transcription factors - three *YAP1* binding sites at -418, -552 and -695 positions and *MSN2* binding site at -8. Further, FAD synthetase activity was studied in 72-hour and 120-hour grown culture of *E.ashbyi* to analyze its role as stress response enzyme. Interestingly, FAD synthetase activity was increased by 2.16-fold in 72-hour grown culture and 3.06-fold in 120-hour grown culture (Table 5).

De novo synthesis of FAD through fermentative route provides a great advantage over enzymatic or microbial biotransformation which uses expensive intermediates such as FMN and ATP. *De novo* synthesis of FAD was reported in yeast and fungi so far while biotransformation was reported mainly in bacteria. Metabolic engineering of *Candida famata* with overexpression of *FMN1* and *FAD1* genes, resulted in 70 mg/L FAD titer on YPD broth compared to 4 mg/L in WT and media optimization through Central Composite Design increased it 451 mg/L FAD [2]. Overexpression of *FMN1* gene in *A.gossypii* increased FAD production by 14.02-fold with maximum titer of 86.5 mg/L [16]. Nearly 194 mg/L FAD was obtained in earlier literature with *E.ashbyi* with culture and complex media components optimization [24]. In the present study, maximum FAD titer of 29.73±2.22 mg/L was obtained with YM broth with wild type strain. Flavins production was nearly 16.5-fold higher with complex media components like molasses and peanut seed cake compared to simple sugars like glucose [25]. Further studies can be conducted to improve FAD titer through media and culture conditions optimization using complex medium components along with oxidative stress supplements.

YAP1 transcription factor was reported to regulate glutathione reductase (*GSR*) gene in *Saccharomyces cerevisiae* [26] and it is also reported to regulate riboflavin metabolism (*RIB*) genes in *Ashbya gossypii* [14]. Putative role of Yap-1 in FAD1 gene regulation can be studied further. Moreover, presence of binding sites for stress response transcription factors like *YAP1* and *MSN2* in *FAD1* gene promoter indicated FAD synthetase itself as one of the major putative stress response enzymes and warrants further studies on *FAD1* gene expression under oxidative stress. Present experimental findings suggest oxidative stress increase flavins in particular FAD synthesis by upregulating FAD

synthetase and increased FAD helps in glutathione mediated oxidative stress response. Accordingly, putative mechanism of FAD synthetase as oxidative response enzyme is shown in Figure 4.
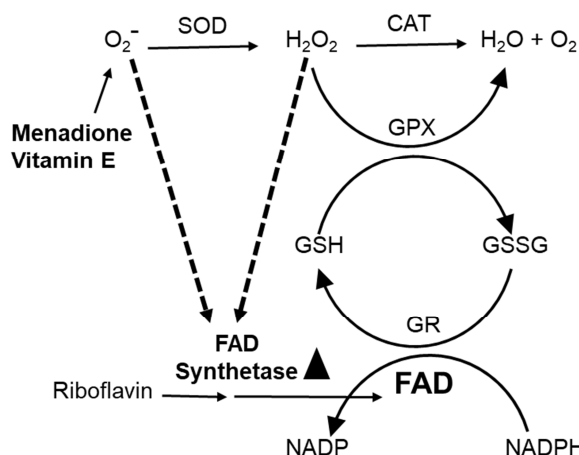

Figure 4. *Putative mechanism for enhanced FAD production upon oxidative stress. Compiled from Kavitha and Chandra, 2014 [13].*

Flavins production in response to oxidative stress can be of evolutionary as well as ecological importance. Being a plant parasite, *E.ashbyi* may encounter an oxidative burst as a defensive action mechanism developed by plants in response to microbial invasion [27]. Upregulation of riboflavin and FAD production can aid fungal own defense system in quickly scavenging the free radicals. Increased flux towards FAD synthesis can be due to its direct role as electron mediator in stress response enzymatic reactions.

4. Conclusion

This is a first report on statistical optimization of oxidative stress modulators for increasing flavins production in *E.ashbyi*. Menadione and vitamin E combination at low levels enhanced flavins production by 1.34-fold against untreated control and 1.15-fold against menadione treatment alone. FAD production was significantly increased with these supplements' combination by 2.18-fold over untreated control. For the first time present study proved that among the flavins, FAD levels increased majorly upon oxidative stress. FAD synthetase specific enzyme activity was also concurrently increased with stress response enzyme glutathione reductase. This finding suggested that the FAD synthetase can also be a stress response enzyme. As FAD is a coenzyme for several redox reactions, increased flux towards FAD can help in oxidative stress response including glutathione mediated response as observed here.

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