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Production and Molecular Characterization of riboflavingenic Strain of *Aashbya gossypii*

[1]Shami E. A. Bakhiet and [1] Engaz Mustafa Ibrahim Mohamed
[1]*Department of Microbiology & Molecular Biology – Faculty of Science and Technology - Al-Neelain University – Khartoum, SUDAN.*

Abstract: *Riboflavin, a yellow, water soluble solid, is wide distributed in plants and animals, and plays an important role in live organisms because it is the precursor of flavin mononucleotide (riboflavin 5'-monophosphate, FMN) and flavin adenine di-nucleotide (FAD), which are functioning as coenzymes for wide variety of enzymes in intermediate metabolism. The main objective of the current study is to produce riboflavin by Ashbya gossypii, estimate the concentration of riboflavin and molecular characterization and differentiation between riboflavinogenic and non-riboflavinogenic strains. This work was conducted by isolation of Ashbya gossypii using Potato dextrose agar medium. The production of riboflavin was achieved by inoculation of isolates in different flasks contain different types of media. The concentration of riboflavin was estimated by flourmeteric technique with wave length of 400-500nm. DNA of A.gossypii was extract. The DNA purity was measured using NanoDrop ND-1000 spectrophotometer system. After incubation period of 18 days, the concentration of riboflavin varied due to types of media and source of isolation. It was recorded as high in medium3 containing A.gossypii isolated from sickelpod 73 mg/l and low in medium1containing A.gossypii isolated from tomato 29 mg/l and intermediate concentration in medium2 A.gossypii isolated from cotton 47 mg/l. Ashbya gossypii has an ability to overproduce riboflavin and it can be used in large scale production with expectation of significant amounts. The best incubation period was 18 days and suitable pH was 6.5 which enhance production of riboflavin.*

Key words: *Ashbya gossypii, Flavin adenine di-nucleotide, Riboflavin, riboflavinogenic, Spectrophotometer.*

I. Introduction

Riboflavin, a yellow, water soluble solid, is wide distributed in plants and animals, and plays an important role in live organisms because it is the precursor of flavin mononucleotide (riboflavin 5'-monophosphate, FMN) and flavin adenine di-nucleotide (FAD), which are functioning as coenzymes for wide variety of enzymes in intermediate metabolism [1].

A daily dose of 0.3–1.8 mg of riboflavin is essential for humans in order to avoid deficiency symptoms like dermatitis [1]. Furthermore, the vitamin is used as animal feed supplement in the less pure form. The amounts used in animal feed additives make more than 80 % of industrial products of riboflavin [2].

Riboflavin is produced commercially by chemical synthesis or by biological synthesis, the latter being the preferred route because it is cheaper than the former [3].

Riboflavin is a unique vitamin that can be synthesized in large enough amounts by some fungi and bacteria to be exploited successfully on an industrial scale. Many microorganisms have been screened and studied for industrial production.

Ashbya gossypii is yeast like fungus plant pathogen on cotton and a citrus fruit belongs to the family Saccharomycetacea and about 95% of its genes have a homologue in *Saccharomyces cerevisiae* [4], and [5]. *Ashbya gossypii* otherwise known as *Nematospora gossypii*, has characteristics which accentuate its importance to man and its capacity to synthesize large amount of riboflavin, or vitamin B_2.

In the early 1980s, the world consumption of riboflavin was reported to be 1250t/year for human and animal uses [6]. The same author reported that the amounts of riboflavin production are speculated to be more than 3000 t/year. Riboflavin production by fermentation using microorganisms is estimated to be about 500t/year. This process has several merits *e.g.* economically less expensive, reducing waste and energy requirements, and using renewable resources like sugar or plant oil [2].

Perkin *et al*., [7] reported to obtain riboflavin production using *B. subtitles* requires at least the deregulation of purine synthesis and a mutation in flavokinase/FAD-syntheses. recombinant *B. subtitles* with these features is in use in large-scale fermentations and producing concentrations exceeding 15 g/L. *Ashbya gossypii* as a strong overproducer and has been yields in the range of 15–20 g/L.

Same author stated that riboflavin about 30% of the world's industrial riboflavin output is produced by direct fermentation with *A. gossypii*, Krishnan 1997 substituted for *E. Ashby*, although its use in industry has been limited to date.

Marvin *et al*., [8] reported that Gram-positive bacteria *Bacillus subtitles*, the yeast *Candida famate*, and the filamentous fungus *Ashbya gossypii* are mainly used for the biosynthesis of riboflavin via large-scale production.

Lim *et al*., [9] reported that riboflavin production using soybean oil as a sole carbon source. Riboflavin reached its highest value of 680 mg/L at culture time of 120 h, when soybean oil was consumed almost completely.

Sugimoto *et a*l., [10] reported that the optimal combination of media for riboflavin production is corn steep liquor 20g/l, osseocolla 25g/l and soybean oil 50g/l, with the other parts comprising the media being NaCl 2 g/l and KH_2PO_4 1g/l (pH6.8). This indicated that a higher riboflavin production could be expected when the optimized medium is used.

II. Methodology

2.1. Collection of sample

Tomato, cotton, and sickelpod samples were collected from local markets in clean plastic bags.

2.2. Preparation of culture media

All culture media were prepared under aseptic conditions.

2.2.1. Potato dextrose Agar (PDA)

An amount of 3.9 grams (PDA) were added to 100ml Distilled water in a conical flask, heated to boiling in water bath to obtain a homogeneous mixture then sterilized by autoclave at 121°C for 15 minutes dispensed into Petri dishes [11].

2.3. Preparation of production media

Production media include several and different substrates and they were prepared under aseptic conditions.

2.3.1. Preparation of Corn Steep Liquor

Corn steep liquor was prepared using a method modified from [12]. By soaking 250 g of well-washed healthy maize grains in 500Lof water for 48h, grinding in a well-washed blender and allowing this to stand further at room temperature. After another 48h, the suspension was mixed thoroughly and strained in a domestic sieve. The liquor obtained was made up to500L with more water and allowed to sediment for 2h.The resultant supernatant was decanted and used in degradation studies [12].

2.3.2. Preparation of Media 1:

An amount of 3 grams glucose, 1.5 g peptone, and 3g corn steep liquor were added to 300 ml of distilled water in a conical flask.

2.3.3. Preparation of Media 2:

An amount of 6 grams peptone, 1.5g yeast extract, 0. 6 g KH_2PO_4, , 0.45g. g $MgSO_4.7H_2O$, and 0.15g and 3g corn steep liquor added to 300 ml of distilled water in a conical flask.

2.3.4. Preparation of Media 3:

It has the same composition of medium 2, but supplemented with 0.0015 grams $FeCl_3$, oleic acid 0.45g and 0.06g glycine. The mixtures were added to 300 ml of distilled water in a conical flask. All the mixtures were heated to boiling in a water bath until a homogeneous mixture was obtained. The medium was sterilized in autoclave at 121 °C for 15 minutes.

2.4. Isolation of *Ashbya gossypii*

Serial dilutions were obtained by taking one ml of microbial suspension prepared previously into sterile test tube and diluted by adding 9 ml of previously sterilized distilled water, this step was repeated 10 times to obtain one 10^{th} of the previous dilution every times. From these serial dilution the 4^{th} dilution containing 1/1000 parts of microbial suspension was used for culturing yeast on Potato dextrose agar and incubated at 30ºC for 3 days. All microorganisms were identified microscopically by Gram stain and Lactophenol cotton blue stains [13].

2.5. Production of riboflavin

An isolated *Ashbya gossypii* was subjected to produce riboflavin and inoculated into flask contain media 1, 2 and 3 separately. The flasks were incubated at 30°C and examined daily until production of riboflavin observed as change of the medium color to yellow.

2.6. Detection of riboflavin

2.6.1. Preparation of sample solution

An amount of 10 ml of the sample solution were taken in flask (50 ml) an equal amount of standard hydrochloric acid were added. The solution was agitated vigorously. The mixture was heated in an autoclave at 121°C for 30 minutes and cool to room temperature. pH was adjusted to 6.5 with the sodium hydroxide solution [14].

2.6.2. Estimation of riboflavin

An amount of 10 ml of the sample solution were taken and added to two tubes. To one of these tubes, one milliliter of the standard riboflavin solution was added and mixed. To another, one milliliter water was added and mixed. One milliliter of acetic acid was added to both the tube and mixed. An amount 0.5 ml of potassium permanganate solution was added with mixing. The mixture was allowed to stand for two minutes. To each of the tubes, 0.5ml of the hydrogen peroxide solution was added with mixing. Whereupon the permanganate colour gets destroyed within ten seconds. The tubes were shaked vigorously until excess of oxygen is expelled.

2.6.3.1. In the fluorometer

The fluorescence of tube one was measured and called this reading as A. Tube two was measured and called reading as B. Tube two with addition of 20 mg of powdered sodium hydrosulphite was measured with five seconds, and called C. All test tubes were measured using 400-500 nm wavelength.

2.6.3.2. Calculation

According to the Indian Standards Institute [14], the riboflavin content of the samples was calculated on the basis of aliquots taken as follows

Mg of riboflavin /ml of the final sample solution $= \frac{B-C}{A-B} x \frac{1}{10} x \frac{1}{1000}$

(Value of $= \frac{B-C}{A-B}$ shall not be less than 0.66 and not more than 1.5)

2.7. DNA Extraction from *Ashbya*

An amount of 0.1g fresh frond material was grinded with 500 µL extraction buffer and incubated for 1 hour in room temperature. An amount of 500 µL of 24:1 chloroform:isoamyl alcohol was added and vortexed on a low setting for 1 minute. Centrifuged for 10 minutes 15, 000 x g then an aqueous layer was transferred to a fresh tube. The volume of an aqueous phase was estimated. Sequential addition was performed as 0.08 volumes of cold ammonium acetate and 0.54 volumes of cold isopropanol were added to precipitate the DNA. Mixed well. Let sit in freezer for overnight. Centrifuged 15, 000 x g for 6 min to recover DNA. The pellet was saved and an aqueous layer was discarded. The pellet was washed with 700 µL 70% cold ethanol mixed well and centrifuged at maximum speed for 3 minutes. The liquid was pipetted off. The excess ethanol was drought off in the vacuum dryer/speed for 20 minutes. Then re-suspended in ddH20 (50 µL). The product was allowed to re-suspend for 1 hour at 55ºC or overnight in the freeze [15].

2.8. Nanodrop to measure purity of DNA

The purity and concentration of DNA was measured by using NanoDrop ND-1000 spectrophotometer as follows:-

The upper and lower optical surfaces of the microspectrophotometer sample retention system was cleaned by pipetting 2 µL of clean deionized water onto the lower optical surface. The lever arm was closed and taped a few times to bathe the upper optical surface. The both optical surfaces were wiped with paper tissue. The NanoDrop software was opened and nucleic acid module was selected. The Nanodrop was initialized by placing 1µl clean water onto the lower optic surface. Then the lever arm was lowered and 'intialize' option in Nanodrop was selected. After completeness of initialization (10 seconds), the both optical surfaces were cleaned. After that the device was set as blank by adding 1µl deionized water and the both optical surfaces were cleaned. The nucleic acid sample was measured by loading 1µl of sample by selecting 'measure' option. Once the measurement was completed, the both optical surfaces were cleaned using paper tissue [16].

III. Result

3.1. Isolation of samples

As can be seen from table 1, three *Ashbya* species were isolated from tomato, cotton, and sicklepod samples using potato dextrose agar medium. The isolated species were identified using lactophenol technique to see their morphology. *Ashbya* exhibited circular, flat to slightly raised, membranous, myceloid with advancing edge filamentous, moist, appraised hyphal growth with short, pointed, matted hair –like outgrowths over the surface. Growth appears which changes to a moist, translucent, dirty-white folded and wrinkled plechtenchyma. Swollen hyphal element, termed, Bulb-forms are typical of the flavinogenic strains of *Ashbya gossypii*.

Ashbya under microscope Hyphae hyaline, often vacuolated or containg a granular material and numerous hyaline droplets, at first non-septate.in flavinogenic strains,riboflavin observed within certain cells a yellowish oily fluid , or needle –like orange crystals.

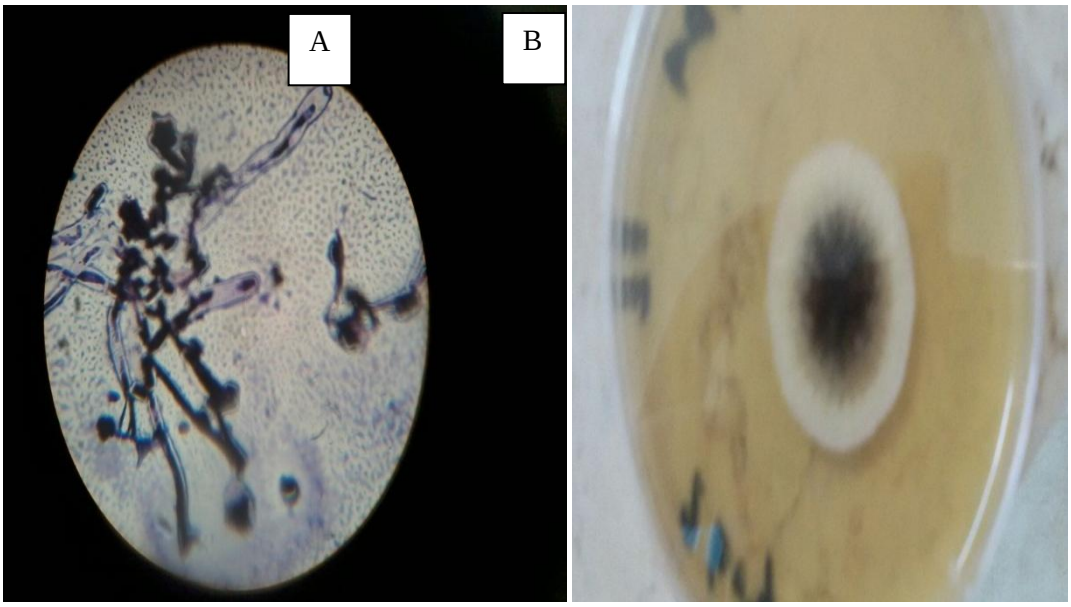
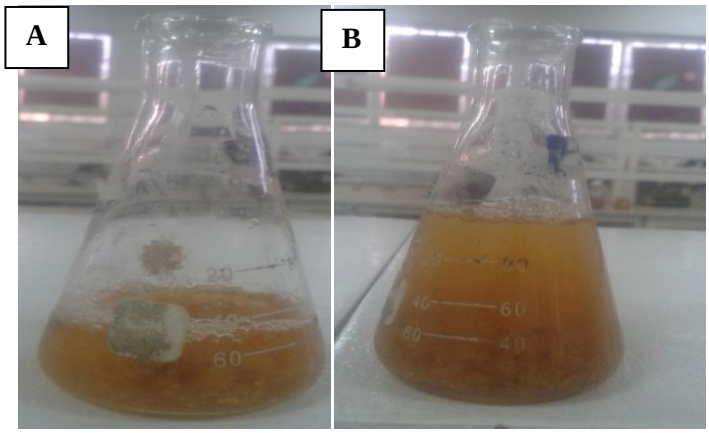


Fig. 1: (A) the morphological appearance of *Ashbya gossypii* under microscope using x40 objective lens; **(B)** the culture characteristics of *Ashbya gossypii* inoculated onto potato dextrose agar medium.

3.2. Production of riboflavin

After incubation period of 18 days, the concentration of riboflavin varied due to types of media and source of isolation. It recorded as high in medium3 containing *A.gossypii* isolated from sickelpod 73mg/l and low in medium1containing *A.gossypii* isolated from tomato 29 mg/l and intermediate concentration in medium2 *A.gossypii* isolated from cotton 47 mg/l. These findings were in agreement with Lim *et al*., [17] who reported that *A.gossypii* produce riboflavin in different concentration due to the type of media such as glucose (40 - 100 mg/l), corn oil and soybean oil (500mg/l), soybean oil and bone fat (500mg/l), finally soybean oil (55mg/l). Also the present study is in agreement with Wei *et al*., [18] who reported *A. gossypii* produced riboflavin on optimized medium as 63.8 mg/l and reaches 81.2 mg/l with pH adjusted to the range of 6.0-7.0. The concentration of riboflavin in this study which was recorded as 29 mg/l was in agreement with Lim, [9] who stated *A.gossypii* produced riboflavin with concentration of 29 mg/l when cultured in agitated fermentor. The present study in disagreement with Ertrk *et al*., [19] who stated the quantities of riboflavin produced by *A.gossypii* in whey after 8 days of Erlenmayer flask fermentation with bran, soybean flour, glycine + peptone and sucrose as supplements; were 389.5, 120.7, 101.7 and 87.5 mg/l, respectively. The same author reported the lowest yields were obtained using peptone and soybean oil (23.2 and 17.5 mg/l respectively) and supplements which is almost similar to the present study.



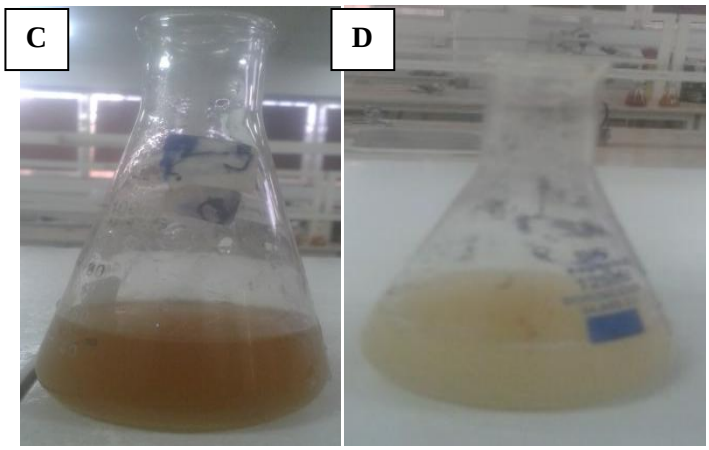


Fig. 2: Production of riboflavin by different isolates; **(A)** *A.gossypii* isolated from tomato, **(B)** *A.gossypii* isolated from cotton **(C)** *A.gossypii* isolated from Sickelpod , and **(D)** Control medium without inoculation.

Table 1: concentration of riboflavin in different media

Test microorganism / **Type of media**	**Concentration of riboflavin (PPB) /medium**		
	Media1	**Media2**	**Media3**
Ashbya from *Solanum lycopersicum* (tomato)	0.000029	0	0
Ashbya from *Gossypium hirsutum* (cotton)	0	0.00047	0
Ashbya from *Senna obtusifolia* (sickelpod)	0.000073	0	0

3.3. Detection and measurement of riboflavin

Riboflavin determine by change of medium color from beige to deep yellow in some medium and yellow in another medium with pale yellow in the last medium this indicate the concentration of riboflavin.

3.4. Purity of DNA by using NanoDrop system

The purity of DNA was measured using NanoDrop ND -1000 with wave length of 260/280 in different samples. The results showed that the all samples were in pure form as 0.997 □ 1 and 1 which indicate the purity of DNA with references. The concentration of DNA was varied from (142 ng/l – 149 ng/l) depending on the type of isolates and source which obtained from (Table 2).

Table 2: Purity and concentration of DNA

Types of samples	**Purity of DNA 260/280**	**Concentration of DNA 260/280**
Cotton isolate	0.997 □ 1	142ng/l
Sickelpod isolate	0.997 □ 1	142ng/l
Tomato isolate	1.00	149ng/l

IV. Conclusion

Ashbya gossypii has an ability to produce riboflavin by significant amount, the best incubation period was 18 days and suitable pH was 6.5 which enhance production of riboflavin. This amount of riboflavin will decrease the production cost and increase revenue. Also using of cheap and degradable materials will improve the environment clarification and decrease the wastes. Further investigation under controlled circumstances will be needed to discover more riboflavinogenic strain of *Ashbya gossypii*. Sudan has great useful undiscovered microbial populations which need governmental concern and support to supplement the scientific outcome. Resettlement of the microbiological production industries by introduction of the newest Products is of concern. Experimental research finding *in vivo* to study physiological changes during vitamin production will be conducted.

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