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PRODUCTION OF BIOTIN BY USING OVERPRODUCER BIOTINGENIC STRAINS OF *SPOROBOLOMYCES ROSEUS, SPOROBOLOMYCES LINDERAE* AND *CANDIDA PARARUGSOA*

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ABSTRACT

The objectives of this study are isolate of biotingenic yeast from different sources, showing the ability of isolated yeast in biotin production and carry out molecular characterization and differentiation between biotingenic and non-biotingenic strains. Infested specimens of samples of *Malus domestica*, *Vitis vinifera*, *Protulaca oleracea*, *Mangifer indica*, and *Eruca sativa*, were collected from local market in sterile plastic bags and then used to isolate the test microorganisms. Yeast extract agar plates were inoculated by moistened swab then incubated at 30°C for 48 hours. Three types of microorganisms were isolated and identified as *Sporobolomyces roseus*, *Sporobolomyces linderae* and *Candida pararugsoa*. The isolated microorganisms were subjected to produce biotin using Feed-Batch technique by flasks containing 50ml yeast extract peptone dextrose broth medium (YEPD). All flasks were incubated in shaker incubator at 30°C for one week. Biotin determined and measured by flourometrics techniques using 400-500 nm wavelengths. The concentrations were ranged from (148.03 mg/l and 0.00095 mg/l) which obtained by *S.roseus* and *S.linderae* respectively. *Candida pararugosa* showed as non-biotinogenic microorganism. This study was concluded that the *S.roseus*has ability to produce biotin in significant amount (1.48g/l) with cheep substrate and low cost, in one week production, and at pH 6.0.

KEYWORDS

Biotin, *Candida pararugosa*, *Sporobolomyces linderae*, *Sporobolomyces roseus*, Vitamins and Yeast Extract Peptone Dextrose Broth.

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INTRODUCTION

Biotin (2′-keto-3, 4-imidazolido-2-tetrahydrothiophene-n-valeric acid) is one of the essential vitamins for nutrition of animals, plants, and microorganisms, and very important as medicine or as a food additive[1].

Biotin is cofactor, responsible for carbon dioxide transfer in several carboxylase enzymes such as Acetyl-CoA carboxylase alpha, Acetyl-CoA

carboxylase beta, Methylcrotonyl-CoA carboxylase, Propionyl-CoA carboxylase, and Pyruvate carboxylase[2].

Individuals with hereditary disorders of biotin deficiency have evidence of impaired immune system function, including increased susceptibility to bacterial and fungal infections[3].

The quantitative screening procedure for biotin production was conducted on 129 yeast strains able to grow in a biotin-free medium. Production of biotin varied considerably from strain to strain even within a species. The best producers of biotin were strains of *Sporobolomyces roseus*[4].

Sporobolomyces roseus is a unicellular basidiomycete "red" yeast species, a member of the class Urediniomycetes, which occurs in many different habitats but is frequently associated with plant; it has the smallest known genome size among basidiomycetes, the *S.roseus* genome is only 70% of that of the ascomycete *Saccharomyces cerevisiae*[5].

Sporobolomyces linderae orange *Pucciniomycotina* (Basidiomycota) yeast species that was isolated from a dead leaf of *Linderaobtusiloba* in Japan. CBS 7893 is, to date, the only known strain of this species in existence. Little is therefore known about its ecology although it is likely a plant associate. *S.linderae* belongs to *Agaricostilbomycetes* (incertaesedis), although the current name does not reflect its phylogenetic position as true *Sporobolomyces* species are members of Sporidiobolales[5].

Sporobolomyces roseus was described morphologically as follows: the yeast appears asovalor cylindrical, with septate hyphae, pseudohyphae and/or no hyphae. It was reproducing asexually by budding, bipolar, sympodial, and annellidic (percurrent) budding[6].

Sporobolmyces linderae was described morphologically to appear as circular, budded cell, without hyphae. The same author described that *Sporobolomyces* exhibit variable colony colours such as red, pink, and orange. The colonies appeared as: smooth, venose, warty, and furrowed[7].

Other author described that in culture, *Sporobolomyces linderae* is non-fermenting budding yeast that can form pseudohyphae and ballistospores (i.e. forcibly discharged spores). The characteristics of this species that separate it from other closely related yeasts are the high maximum growth temperature (28-29°C) and inability to use raffinose as a carbon source[5].

Some authors found that, to overcome the many disadvantages faced by the biotin industry, efficient fermentation processes to produce biotin are in great dem and, the consensus is that any fermentation process has to be able to produce more than 1 g biotin per liter and with a cheap substrate in order to be cost-effective[8,9].

One author reported that fungi and *Streptomyces* tested, generally accumulated larger amounts of biotin while, in contrast, most of the bacteria and yeast tested accumulated relatively small quantities of biotin, An exception among then bacteria was the best producer of biotin, the Gram-positive bacterium, *Bacillus sphaericus.* Addition of pimelic acid to cultures of this micro-organism could enhance biotin vitamins accumulation 0·07 to ~200 mg/l. In contrast, the biotin production of *Escherichia coli* was improved from 'trace' levels to 0·07 mg/l, an amount significantly less than that observed for *B. sphaericus*[10].

One hundred twenty nine (129) different yeasts have screened for their biotin production. The best producers of biotin were species of *Rhodotorula* and *Sporobolomyces,* while the best producers of total vitamers were *Rhodotorula* and *Yarrowia*[11].

Production of biotin using a microorganism, there have heretofore been known various processes using a microorganism such as those of the genus *Streptomyces* or *Micromonospore*, *Sporobolomyces*, *Bacillus*, *Escherichia*, *Serratia*, and *Brevibacterium*[11].

An author reported that it has been possible, by cloning the biotin operon on multicopy plasmids, to increase biotin synthesis to 1 μg/ml in microorganisms transformed with these genes[12].

The same authors were cloned, sequenced, and characterized the biotin synthase (BIO2) gene from the food yeast *C. utilis*. By integrating the biotin synthase gene into the chromosome of *C. utilis*, the biotin level reached above 1.8 mg/l of the growth medium this level (1.8 mg/l) was obtained by using the integrating plasmid.

On comparison of rate of biotin production to the cost of the substrate, one of the authors reported that any fermentation process has to be able to produce more than 1 g biotin per liter and with a cheap substrate in order to be cost effective through over-expression of extra copies of biotin-biosynthesis genes and selection for mutants resistant to analogues or other chemicals, various high biotin-producing strains had been generated during the past decades[9].

The mass spectrometry (MS) has been shown to be an accurate method for biotin determination on proteins by comparing the protein's mass before and after biotinylation. Unfortunately, mass determination of proteins larger than about 60 kD is problematic and assay of a large number of samples may be cost prohibitive[13].

The objectives of this study are isolate of biotingenic yeast from different sources, showing the ability of isolated yeast in biotin production and carry out molecular characterization and differentiation between biotingenic and non-biotingenic strains.

METHODOLOGY

Area of study

This work was conducted at the department of Microbiology and Molecular biology, Faculty of Science and Technology, Al-Neelain University, Khartoum - Sudan. All experiments were performed under aseptic conditions and vigilance supervision.

Collection of sample

Samples of *Malus domestica, Vitis vinifera, Protulaca oleracea, Mangiferindica L, Eruca sativa,* were collected from local market in sterile plastic bags[14].

Preparations of culture media

An amount 3.5gs of yeast extract agar (YEA) medium were taken in conical flask, dissolved in 100ml distilled water. The mixture was placed in a water bath for 15 minute to be homogenized. The flask containing medium was autoclaved at 121°C for 15minutes. The sterilized medium was poured in clean sterile plates aseptically. All plates were let to solidify. The culture media were supplemented with antibiotic to inhibit the growth of commensal and saprophytic microorganisms[15].

Fed-Batch Biotin Production

Yeast extract peptone dextrose broth medium (YEPD) was prepared using the following formula: Yeast extract 1g, bacteriological peptone 2g, glucose 2g, and 100 ml distilled water.

An amount of 150 ml YEPD broth media was prepared in three 100 ml-conical flask. Each flask contained 50 ml YEPD. A loop-full of each test microorganism was inoculated into the flasks containing production media. All flasks were incubated in shaker incubator at 30°C for one week. 10ML of fresh sterile YEPD medium were added to each flask; to feed fermentation process and dilute accumulated waste. This step was repeated daily and under aseptic conditions[14].

Isolation of yeasts

Infested specimens of *Malus domestica, Vitis vinifera, Protulaca oleracea, Mangiferindica L, Eruca sativa* were swabbed using sterile cotton swab. Yeast extract agar plates were inoculated by moistened swab then incubated at 30°C for 48 hours. After incubation period, observed pure colony which expected to be biotin producer was picked and inoculated on new plates containing YEA+ antibiotic. All plates were incubated at 30°C for 48hrs[16].

Biotin detection and measurement

Biotin was detected and measured in accordance of the following.

Sample preparation

An amount of 10 ml of the sample solution were taken in flask (50 ml) an equal amount of standard hydrochloric acid was added. The solution was agitated vigorously. The mixture was sterilized in an autoclave at 121°C for 30 minutes and cooled to room temperature. The pH was adjusted to 6.5 with the sodium hydroxide solution[16].

Estimation of biotin

An amount of 10 ml from each samples solution were taken in two tubes. To the first tube of each sample 1ml of the standard biotin solution was added, then mixed; to second tube of each sample 1ml water was added, then mixed. To all tubes 1ml of acetic acid was added, then were mixed. An amount 0.5 ml of potassium permanganate solution was added with mixing. The mixtures were 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 get destroyed within ten seconds. The tubes were shaken vigorously until excess of oxygen is expelled[17].

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 within five seconds, and called C. All test tubes were measured using 400-500 nm wavelength.

According to[17], the biotin content of the samples was calculated on the basis of aliquots taken as follows

mg of biotin/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)

DNA extraction from biotingenic and non-biotingenic yeast strain

An amount 1.5ml of liquid culture of yeast growing for 20-24 h in YEPD was taken in microcenterfuge tube. The cells were pelleted by centrifugation at 20,000g for 5 minutes. An amount of 200µl of Harju-buffer was added to each tube. Tubes were immersed in dry ice-ethanol bath for 2 minutes. Tubes were transferred to 95°C water bath for 1 minute. This step was repeated 3 times. The tubes were vortexed for 30 seconds and 200µl chloroform were added. The test tubes were vortexed for 2 minutes. Then were centrifuged for 3 minutes at 15000 rpm. The upper aqueous phases were transferred to new micro centrifuge containing 400µl ice cold absolute ethanol. The tubes were mixed by inversion and incubated at room temperature for 5 minutes. DNA was pelleted by centrifugation for 5 minutes at15000rpm. Supernatant was removed. The pellets were washed with 0.5ml 70% ethanol. The tubes were centrifuged for 5 minutes at 15000rpm Supernatant was removed. The rest of the pellets were air-dried at room temperature. Resuspended in 25 µl TE buffer (pH 8.0)[18].

Nano Drop for measurement of DNA purity

The purity and concentration of DNA was measured by using Nano Drop ND-1000 spectrophotometer[19].

RESULTS AND DISCUSSION

Isolation of biotinogenic and non-biotinogenic yeasts

After 2 days incubation period at 30°C on YEA medium, a yeast colony inoculated from *Malus domestica* exhibited red, smooth, large, veinous and warty colonies. Dark in center, slightly light in edges which were determined as *S.roseus*, Figure No.1 (a). Its morphology was showed using Gram's stain technique and appeared as oval cells without hyphae, Figure No.1 (b).

The second yeast colonies were isolated from *Vitis vinifera* and they exhibited large orange waxy colour colonies, which were determined as *S.linderae*, Figure No.2 (a). Its morphology was showed using Gram's stain technique and appeared as single bugging large cell, Figure No.2 (b).

The third yeast colonies were isolated from *Portulaca oleracea* and they were showed small, smooth, white colony, which were determined as *C.pararugsoa*, Figure No.3 (a). Its morphology was showed using Gram's stain technique and appeared as single cell with pseudohyphae, Figure No.3 (b).

The second and third yeast isolates were incubated for 3 days to obtained their significant growth and typical colonial appearance.

Production of biotin

Biotin determined by change of medium color from clear bright brown to heavy trapped brown this indicate the concentration of biotin Figure No.4.

As can be seen in Table No.1, biotin was produced in various concentrations in various genera and even within the species in the same genus. The concentration ranged fromm148.03 mg/l and 0.00095 mg/l which obtained by *S.roseus* and *S.linderae* respectively. *C.pararugosa* showed as non-biotinogenic microorganism. The present study is in disagreement with an author[4] who used *S.roseus* for production of biotin**,** medium used of 25 g/l sucrose, KH_2PO_4, $MgSO_4.7H_2O$, $ZnSO_4$ (0.2% solution) and 10% sodium phosphate buffer 0.1M, pH 7.0. But was adjusted pH7.0 and assayed biotin production in the culture broth after 48h and

production range 0.54 g/l. The concentration of biotin in the present study was 1.5g/l which is in agreement with author[8] who reported that any fermentation process has to be able to produce more than 1 g biotin per liter and with a cheap substrate in order to be cost effective, Also the present study is in agreement with author[20] who reported that the best results (more than 1 g biotin per liter of culture medium) were achieved in *B.subtilis* with strains resistant to 5-(2-thienyl) pentanoic acid and over expression of various biogenes.

The present study is in agreement also with author[12] who cloned, sequenced, and characterized the biotin synthase (BIO2) gene from the food yeast *C. utilis*. By integrating the biotin synthase gene into the chromosome of *C. utilis*, the biotin level reached above 1.8 mg/l of the growth medium, this level (1.8 mg/l) was obtained by using the integrating plasmid.

Also the present study is in disagreement with author[21] who reported that in *E.coli*, biotin synthesis has reached 10 mg/l by transforming a multicopy plasmid harboring biotin operon and deregulating at least one enzyme of the fatty acid biosynthetic pathway in the host.

The present study was revealed less concentration of biotin than that obtained by author[22] who reported that a strain of *E.coli* resistant to threonine analogue can produce biotin even up to 970 mg/l in the fermentation broth.

The findings in this study were in disagreement with author[10] who reported that the best producer of biotin is the Gram-positive bacterium, *Bacillus sphaericus.* Addition of pimelic acid to cultures of this micro-organism could enhance biotin vitamins accumulation 0·07 to ~200 mg/l. In contrast, the biotin production of *Escherichia coli* was improved from 'trace' levels to 0·07 mg/l.

Purity of DNA by using Nano Drop system

The purity of DNA was measured using Nano Drop ND-1000 with wave length of 260/280. To all isolated yeast samples, the results showed that all samples were in pure form as 0.997~1 and 1 which indicates the purity of DNA with references. The concentration of DNA varied from 91-107 ng/l which obtained from Table No.2. Accordingly, the yeast genome was amplified with the 28s rRNA primer and distinguish between the three isolated yeasts to determine the biotingenic and non-biotingenic microorganisms and to confirm the conventional methods to identify the isolates.

Table No.1: Concentration of biotin by different isolated yeasts

S.No	Character	Concentration of biotin mg/l		
		S.roseus	*S. linderae*	*C.pararugosa*
1	YEPD	148.03	0.00095	0.00

Table No.2: Purity and concentration of DNA

S.No	Types of sample	Purity of DNA 260/280	Concentration of DNA 260/280
1	*S.roseus*	1.00	107 ng/l
2	*S.linderae*	0.998	97 ng/l
3	*C.pararugsoa*	0.997	91 ng/l

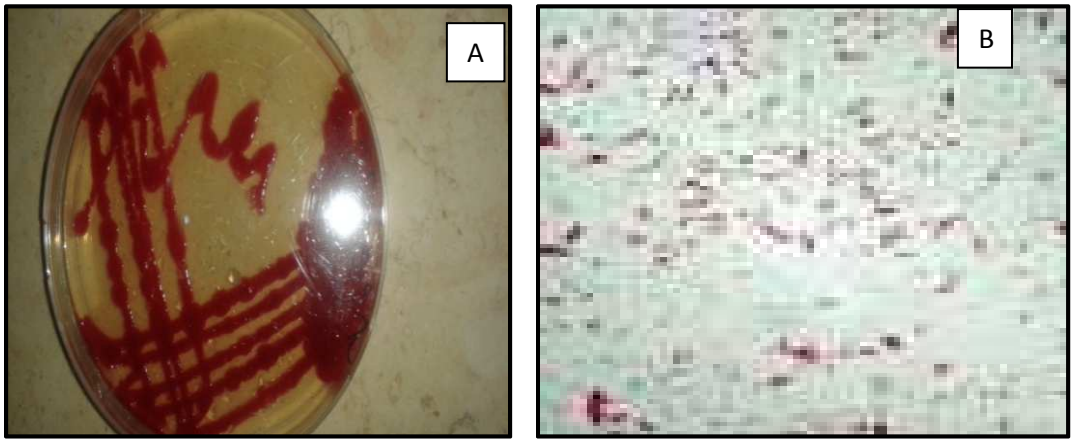

Figure No.1: (a) *S.roseus* in YEA, **(b)** *S.roseus* under microscope 100x

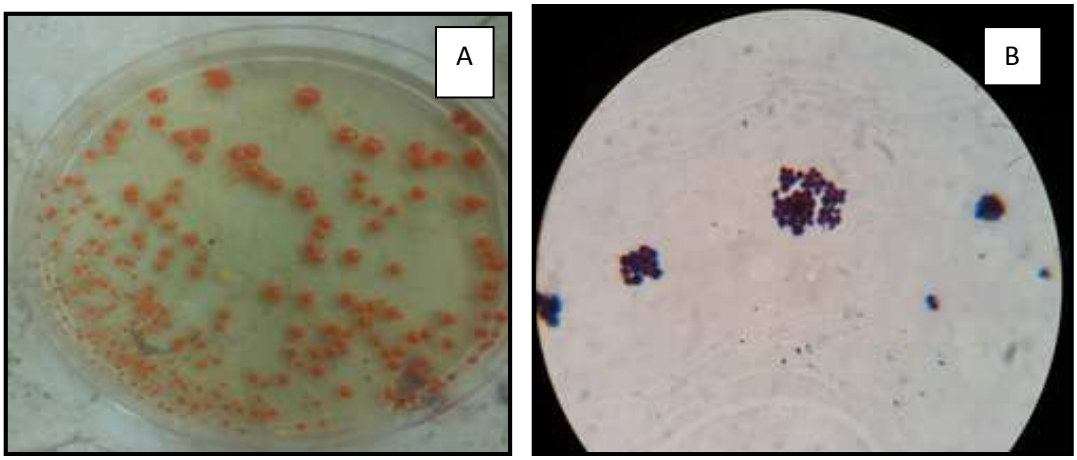

Figure No.2: (a) *S.linderae* in YEA, **(b)** *S.linderae* under microscope 100x

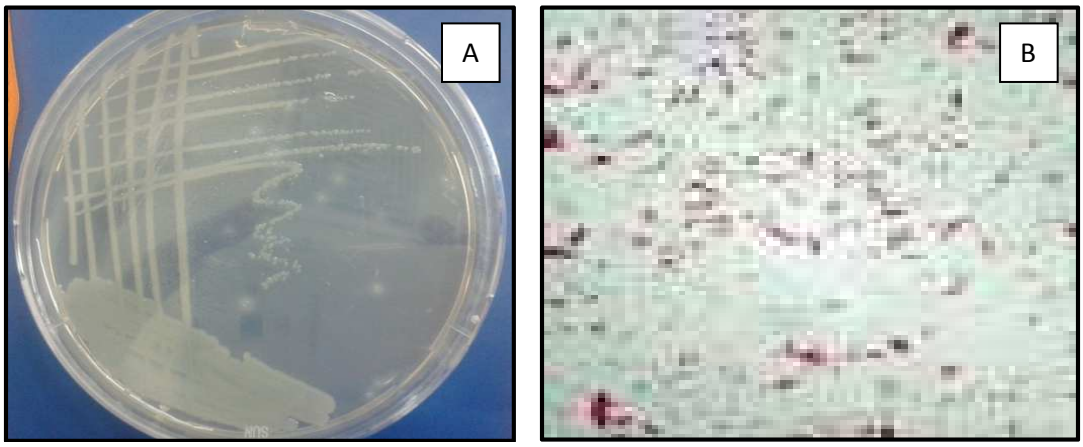

Figure No.3: (a) *C.pararugsoa* in YEA, **(b)** *C.pararugsoa* Under microscope 100 x

Figure No.4: Changing in colour of medium due to biotin production; (c) ≡ control media

CONCLUSION

Sporobolomyces roseus has ability to produce biotin in significant amount (1.48g/l) with cheep substrate and low cost, in one week production, and at pH 6.0. So it can be used for production in large-scale under controlled circumstances. The authors recommend further investigation under controlled circumstances will be needed to discover more biotin producing strains.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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