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Research Article

Growth and norharmane production of *Chroococcus minutus* under various stress conditions

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ABSTRACT

Cyanobacterium samples were collected from fresh water of Tokat city in Turkey, and then isolation and cultivation of *Chroococcus minutus* were achieved successfully. TLC (Thin layer chromatography) and HPLC (High Performance Liquid Chromatography) analyses revealed that the *C. minutus* consisted of norharmane as a major product. So amount of norharmane was determined during the growth process. Growth and norharmane production of *C. minutus* were executed under salt stress and pH stress conditions. The most growth and the highest production of norharmane were detected at 16th day. Therefore inoculation process was performed at 16th day. Salt stress was evaluated at 0.5, 1.0, 3.0 and 5.0 M concentrations. The most norharmane was synthesized by *C. minutus* at 5 M concentration. The norharmane production and the growth were higher at pH 9 than that of the pH 5. Most norharmane was produced at pH 7.

Keywords: *Chroococcus minutus*, cyanobacteria, HPLC, norharmane.

Chroococcus minutus'un değişik stres koşullarında büyüme ve norharmane üretimi

ÖZ

Siyanobakteri örnekleri Türkiyede Tokat şehrinin tatlı sulardan toplandı ve *Chroococcus minutus*'un isolasyonu ve kültürü başarılı bir şekilde gerçekleştirildi. İTK (İnce tabaka kromatografisi) ve HPLC (Yüksek basınçlı sıvı kromatografisi) analizleri *Chroococcus minutus*'un ana ürün olarak norharman içerdiğini gösterdi. Böylece *C. minutus'un* gelişim döneminde norharman miktarı belirlendi. Tuz stresi ve pH stresi şartlarında *Chroococcus minutus*'un gelişimi ve norharman üretimi belirlendi. En fazla büyüme ve en çok norharman üretimi 16. günde gözlendi. Bu bakımdan inokulasyon işlemi 16. günde gerçekleştirildi. Tuz stresi 0.5, 1.0, 3.0 ve 5.0 M konsantrasyonlarında gerçekleştirildi. En fazla norharman 5 M konsantrasyonunda *C. minutus* tarafından sentezlendi. Norharman üretimi ve büyüme pH 5' teki şartlara göre pH 9' da daha yüksekti. En fazla norharman üretimi pH 7' de gerçekleşti.

Anahtar Kelimeler: *Chroococcus minutus*, syanobakteri, HPLC, norharman.

1. INTRODUCTION

Cyanobacteria are able to adapt to wide environmental conditions. The abiotic effects including pH and salinity are helpful to create the suitable conditions for optimizing cyanobacteria growth. Cyanobacteria have to deal with altering environmental conditions by adapting to stress situations via syntheses of secondary metabolites. The optimum pH growth of cyanobacteria species are almost between 7.4-8.0[1] but some species prefer growing at alkaline and acidic medium.[2] Salt stress has a significant effect on cyanobacteria growth and development process. Salt stress is resulted in an increase in the intracellular lipid content of green alga, *Dunaliella tertiolecta*.[3] Sodium chloride stress also causes an increase of antioxidative enzymes in the fresh water alga, *Chlamydomonas reinhardtii*.[4] Cyanobacterial natural products reveal a wide range of biological and pharmaceutical properties that play a significant role in the drug discovery process.[5]

Cyanobacterial compounds mostly have fascinating chemical structures and strong biological effect due to the particularity of freshwater environment.[6] Algae are simple organisms with chlorophyll including one cell or

living together in colonies, or as organisms with many cells and collaborating together as simple tissues. Algae are found in the sea, rivers, and lakes, on soil and walls in animal and plants as symbiotic; in fact almost everywhere in which there is a light to execute photosynthesis.[7] Algae are classified as macroalgae and microalgae. The former inhabits in littoral area, contained green algae, brown algae and red algae. The later is found in both benthic and shore zone as well as in ocean as phytoplankton.[8] Phytochemical investigations on algae have been resulted in the isolation of pharmaceutically and medicinally valuable secondary metabolites revealing a broad spectrum of biological activities such as antiinflammatory[9], immunosuppressive[10], antifungal[11], antineoplastic[12], antiviral[13], hepatoprotective[14], antioxidant[15], antidiabetic[16], anticancer[17], anti-HIV[18] activities. Norharmane, 9H-pyrido (3, 4-b)indole is a derivative of *β*-carboline alkaloids which have several pharmacological effects including the inhibition of various enzymes such as monoamine oxidase[19], indoleamine 2,3-dihydroxygenase, and nitric oxide synthesis.[20]

Norharmane has significant properties in pharmacology as well as cyanobacterial life condition. Therefore, we determined the norharmane production under temperature and light conditions in previous work.[21] Herein, we aimed to determine the norharmane production of *C. minutus* under salt and pH conditions. There is only a report revealing the existence of norharmane in *C. minutus.*[22]

2. MATERIALS AND METHODS

2.1. General experimental procedure

Cyanobacteria genomic DNA isolation was executed with ZRFungal/Bacerial DNA kit according to the manufacturer's instructions. PCR amplification of specific primer 16S rRNA (27F-1492R) was carried out by Roche FastStart Taq DNA polymerase kit. All chemicals and solvents were supplied from Sigma-Aldrich (Darmstadt, Germany).

2.2. Collecting, isolation and cultivation of *Chroococcus minutus*

C. minutus was collected from Yesilirmak river, Tokat, Turkey (49° 19' 49.12" N, 36° 34' 2.06" E). It was filtered by filter paper (Whatmann, Germany) then was collected in petri dish. *C. minutus* was isolated by micropipette and micro injector under the inverted microscope and sample was streaked onto agarised Bristol medium (1.5% agar) (Table 1). During the cultivation process, incubation was kept for 2 weeks at 26 °C ± 2 for 12/12 h (light/dark). The light intensity was 155 µmol s^{-1} m^{-2}.

Table 1. Composition of culture media

ml	Stock Solution	g/400 ml H_2O
10	$NaNO_3$	10.0
10	$CaCl_2 \times 2H_2O$	1.0
10	$MgSO_4 \times 7H_2O$	3.0
10	K_2HPO_4	3.0
10	KH_2PO_4	7.0
10	NaCl	1.0

2.3. Morphological identification

C. minutus was identified under the light microscope by taking the photograph of sample as a micrometer. The cells existing as single or groups are in ovoid or spherical shape. The cells with big cover are 6–15 µm diameter and 4-10 µm diameter without cover.[23]

2.4. Molecular identification

Thermal cyclic conditions were 4 min at 95°C for initial denaturation, 95°C for 1 min, 60°C for 45 s, 72°C for 1 min for 30 cycle and 72°C for 7 min for final extension step. PCR product was imagined by 1.5% agars jell electrophoresis and UV transilluminator (Figure 1). Sequence analysis of PCR product was carried out at REFGEN (METU Techno Center), Gene Research and Biotechnology Ltd. Co.

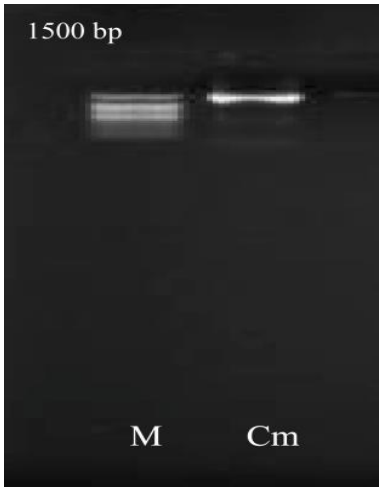

Figure 1. Digital photograph of an agarose gel with 27F-1492R primer PCR product from *C. minutus* (Cm), Marker (M).

2.5. Stress experiments

For salt stress experiments, nutrient media concentrations were 0.5, 1.0, 3.0 and 5.0 M. The control cultures were kept in the Bristol's Medium without sodium chloride (pH 7). For pH stress, pH values were adjusted to 5 and 9 in the culture medium.[24] To the nutrient media (235 ml) in each Erlenmeyer flask (250 ml), a stock culture (10%) was inoculated at 12-16th days of growth which had the most norharmane production. During the growth process, the cultures were shaken three times a day to homogenize the medium and accelerate the growth process. All stress trials were executed under the same temperature and light.

2.6. Cell number and biomass

Cell number was counted with a hemacytometer. Cell size was measured under an Olympus CX4 Boeco (Germany) light microscope. Culture samples (15 ml) were centrifuged at 5000 × g for 10 min. After washing the pellets with distilled water (pH 4), they were dried at 50°C for 6-8 h, and then weighed.[25]

2.7. Quantitative analysis of norharmane in *C. minutus* methanol extract

TLC (Thin layer chromatography) and HPLC analyses revealed the existence of norharmane in methanol extract by comparing the standard (norharmane). Then, quantitative analysis was executed on methanol extract to determine the amount of the norharmane variation under various stress conditions including pH and salt stresses. HPLC analyses were carried out by Shimadzu UV-260 spectrometer with diode array detector (Thermo Finnigan, Cambridge, England). Norharmane was purchased from Sigma-Aldrich (Steinheim, Germany, Product number: N6252). The samples with various concentrations were vortexed for 1 min then kept into the ultrasonic bath. After filtration by PTFE syringe (Chrom Tech, 0.45 0.45 µm 13 mm), 20 µl aliquot was injected to the HPLC column at 40 °C. C18 120A reverse phase column (4.6 × 150 mm, 3 µm particle size) was used. The flow rate was adjusted to 1 ml/min using a gradient system of A, water with 0.1% formic acid and B, acetonitrile (Figure 2). The gradient program was fixed as follows: 0-14 min, 100% A; 15-29 min, 80% A, 30-32 min, 60% A, 33-34 min, 0% A. UV spectra were measured at 247 nm.[26] The amount of norharmane was calculated by the calibration curve using the Gauss method.

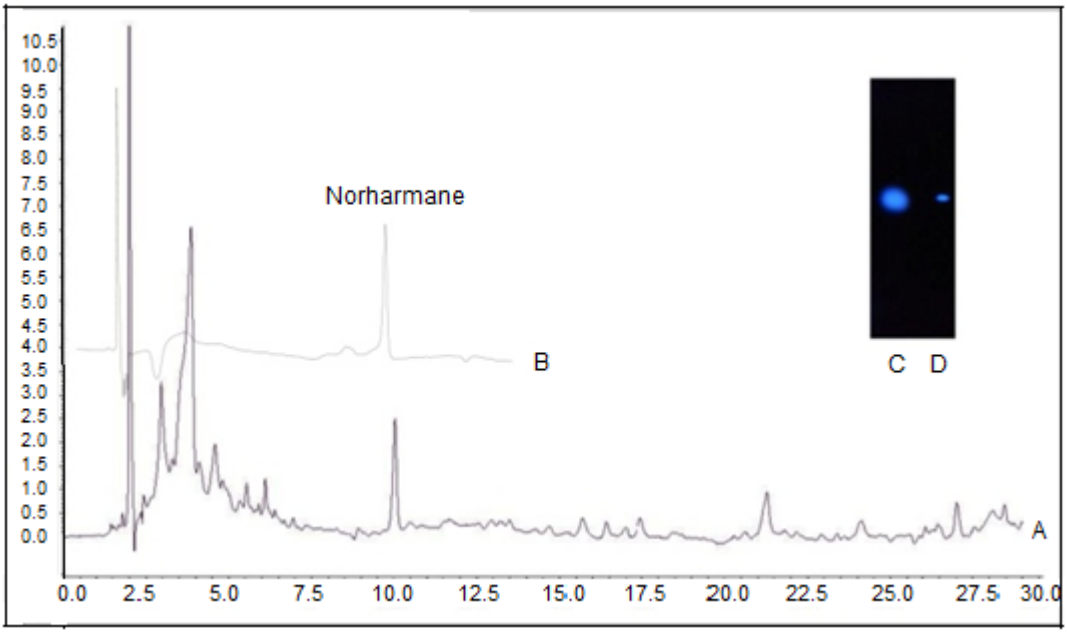

Figure 2. HPLC chromatograms of extract (A), standard, norharmane (B), TLC (UV 254 nm) of *C. minutus* extract (C), and norharmane (D), EtOAc as a mobile phase.

2.8. Statistical Analyses

All experiments were executed with three times. The statistical analysis were carried out by ANOVA and using the SPSS software (SPSS Inc., version 20).

3. RESULTS AND DISCUSSION

The identification of *C. minutus* was carried out by NCBI-BLAST analysis. According to 16 S rRNA analysis, a partial sequence of 1437/1437 base pairs (bp) similarities were 100%. Nucleotide sequence accession number was GQ375047.1. The samples taken for every 4 days were calculated by thoma lame. The growth curve of *C.minutus* was presented (Table 2). To determine time and amount of norharmane production, HPLC analyses were executed. The most growth rate was observed at 12-16 days and the maximum production of norharmane was also detected at same time interval. Algae can live within a certain range of enhanced salt concentrations. The salt stress was evaluated at 0.50, 1, 3, and 5 M concentrations of nutrient media (Table 3).

Table 2. Cell number, biomass and norharmane production of *C. minutus* under stress conditions. Values are means ± standard deviation (n= 3)

Stress conditions	Cell number ($\times 10^4$ ml^{-1})	Biomass (g l^{-1})	Total norharmane (µg g^{-1})
5 (pH)	63.333 ± 4.163^a	0.028 ± 0.005^a	0.146 ± 0.002^a
9 (pH)	133.667 ± 4.041^c	0.194 ± 0.003^c	3.192 ± 0.008^c
7 (pH) Control	124.000 ± 3.000^b	0.160 ± 0.011^b	8.816 ± 0.323^b
0.5 M	124.667 ± 4.163^c	0.178 ± 0.004^d	9.823 ± 0.421^b
1.0 M	133.667 ± 3.512^d	0.210 ± 0.004^e	10.803 ± 0.055^c
3.0 M	88.667 ± 8.327^b	0.085 ± 0.004^b	12.720 ± 0.087^d
5.0 M	67.667 ± 3.215^a	0.074 ± 0.004^a	16.066 ± 0.208^e
Control	124.000 ± 3.000^c	0.160 ± 0.011^c	8.816 ± 0.323^a

*Means followed by different letters (a, b, c, d, e) are significantly different at $p < 0.001$. M indicates the salt concentration.

At 0.5 M the growth was almost same as the control (133.6×10^4 cell ml^{-1}). The growth was better than control at 1.0 M (133.6×10^4 cell ml^{-1}). The growth decreased at 3 and 5 M concentrations since the *C. minutus* did not tolerate the salt at these concentrations. There was a direct proportion with the production of norharmane and salt concentrations. All norharmane production values were higher than that of the control. The production of norharmane was 9.823 µg g^{-1} at 0.5 M concentration. At the 1.0 and 3.0 M the norharmane productions were 10.80 µg g^{-1} and 12.72 µg g^{-1} respectively. The maximum norharmane production was observed as 16.07 µg g^{-1} at the 5.0 M.

Table 3. Cell number, biomass and total norharmane of *C. minutus* for days. Values are means ± standard deviation (n= 3)

Days	Cell number ($\times 10^4$ ml^{-1})	Biomass (g l^{-1})	Total norharmane (µg g^{-1})
0-4	48.00 ± 0.00^a	0.033 ± 0.012	0.410 ± 0.265
4-8	56.67 ± 1.16^b	0.053 ± 0.025	1.086 ± 0.212
8-12	82.00 ± 2.00^d	0.050 ± 0.020	0.910 ± 0.132
12-16	126.00 ± 2.00^f	0.163 ± 0.042	8.816 ± 2.665
16-20	105.33 ± 1.16^e	0.157 ± 0.047	3.063 ± 0.625
20-24	84.67 ± 3.06^d	0.043 ± 0.006	1.273 ± 0.186
24-28	64.00 ± 2.00^c	0.026 ± 0.006	0.035 ± 0.007

*Means followed by different letters (a, b, c, d, e) are significantly different at $p < 0.001$.

The norharmane production and growth were investigated at pH 5 and 9. The growth was 133.66 × 10^4 cells ml^{-1} and 63.33 × 10^4 cells ml^{-1} at pH 9 and 5, respectively. *C. minutus* preferred a basic medium for growth. The best norharmane production was observed as 8.82 µg g^{-1} at pH 7. The norharmane production was 0.146 µg g^{-1} at pH 5 whereas; it was 3.19 µg g^{-1} at pH 9 (Figure 2).

4. CONCLUSION

Cyanobacteria have been considered to be a promising source of highly valuable compounds for the pharmaceutical industry. The optimum norharmane synthesis requirements by *C. minutus* were depicted under the stress conditions. Recently, the identification of cyanobacteria has been executed by combination of morphologic and molecular aspect.[27] Therefore, the morphological characterization of cyanobacteria was supported by molecularly, 16S rRNA gene sequence in our research. The sample matched with 1437/1437 bp region of *C. minutus* at 100%. Cyanobacteria need the water, light, carbon dioxide, and inorganic compounds for survival.[28] Cyanobacteria can flourish under particular environmental conditions. The specific nutrient media are available for cultivation of cyanobacteria such as Bristol and BG-11.[29] Bristol medium has been preferred for cultivation of *C. minutus* for a month period. Maximum growth of *C. minutus* was observed at 12^{th} and 16^{th} days interval. In addition, *C. minutus* excreted the most norharmane at the same time interval. It was reported that the amount of metabolites has been increased proportionally with the culture age.[30] The exposure of salt stress of *C. minutus* resulted in the high norharmane production. The most norharmene production was observed at 5.0 M caused by the synthesis of secondary metabolites of *C. minutus* for adaptation to the environment. Life condition of *C. minutus* was presented. The conditions of most norharmane production were depicted. The cultivation conditions of *C. minutus* were revealed to isolate the most norharmane. Due to the containing of pharmaceutically significant compounds of cyanobacteria, isolation of corresponding compounds should be carried out in the further work.

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Conflict of interest

Authors declare that there is no a conflict of interest with any person, institute, company, etc.

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