



Effects of Differential Time Applications on Some Cyanobacterial Norharman Production Rates

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Abstract. Cyanobacteria are an important class of bacteria by their metabolic activities for biology, ecology and economy. They contain series of secondary metabolites produced under negative stress conditions and providing specialized functions. One of the metabolites which is biologically active and can be used as drug for antibacterial and antitumor properties is norharman which has (9H-pyrido 3, 4-b) indole structure. In this study, water samples were collected from Yeşilirmak river of Tokat province of Turkey and cyanobacteria were isolated under inverted microscope by micropipette and microinjection and were cultured for a month. Selection from cultures was done during predetermined time courses and produced norharman levels were determined by HPLC. At 16th day maximum norharman production was determined as 8.8167 and 0.712 µg/g from *Chroococcus minutus* and *Anabaena oryzae* respectively. Highest norharman production from *Nostoc linckia* determined as 1.191 µg/g at 20th day. Since *Geitlerinema carotinosum* began exponential growth phase faster than other strains the highest amount of norharman production was determined as 0.825 µg/g at 12th day.

Keywords: Cyanobacteria; Cultivation; Norharman; HPLC; Secunder Metabolite

Farklı Gün Uygulamalarının Bazı Siyanobakterilerde Norharman Üretimi Üzerine Etkisi

Özet. Siyanobakteriler, metabolizmalarından ötürü biyolojik, ekolojik ve ekonomik bakımdan önemli bakterilerdir. Özellikle çeşitli olumsuz şartlarda üretilmiş ve her biri özelleşmiş fonksiyonlara sahip bir dizi sekonder metabolit içerirler. Antibakteriyel, antikanser gibi biyolojik etkili ve ilaç olarak kullanılabilen metabolitlerden biri; 9H-Pyrido [3,4-b] indole yapısında Norharman'dır. Bu çalışmada Yeşilirmak Nehri (Tokat)'nden su örnekleri alınarak inverted mikroskop altında mikropipet ve mikro enjektör yardımıyla izole edilen siyanobakteriler ortalama bir aylık kültüre alınmıştır. Belirli zaman aralığında kültürlerden alınarak ürettikleri norharman miktarı HPLC ile tespit edilmiştir. *Chroococcus minutus* ve *Anabaena oryzae*' de sırasıyla 8.8167, 0.712 µg/g norharman 16. günde maksimum olarak üretilmiştir. *Nostoc linckia*'da en fazla norharman üretimi 1.191 µg/g olup 20. gündedir. *Geitlerinema carotinosum*'da ise diğer türlere göre daha erken logaritmik faza girdiği 12. günde maksimum norharman metaboliti 0.825 µg/g olarak üretilmiştir.

Anahtar kelimeler: Siyanobakteri; Kültivasyon; HPLC; Norharman; Sekonder Metabolit

INTRODUCTION

Cyanobacteria are a class of prokaryotic organisms lacking nuclei and plastids and are alternatively named as *Schizophyta* or *Myxophyta* [1]. Cyanobacteria differentiate from algae by being prokaryotic organisms. However, their shared features with algae are having chlorophyll-a also ability to perform photosynthesis [2]. These unique class of microorganisms has different names such as cyanoprokaryotes, cyanophytes and blue-green algae [3].

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Cyanobacteria contains many important and biochemically active compounds composed of peptides and depsipeptides. Cyanobacteria produce large quantities of secondary metabolites, especially genii of *Spirulina*, *Anabaena*, *Nostoc* and *Oscillatoria spp.* Cyanobacterial toxins contain different compounds with antitumor, antifungal, anti-inflammatory, siderophore, phytohormone and photoprotectant properties and have secondary metabolites with series of specialized functions and some of them having protease inhibitory effects [4, 5]. Some of these compounds are obtained by extraction from dissolved cyanobacteria biomass (intracellular). Also they can excrete various organic exometabolites into the environment (extracellular) [6].

Cyanobacteria can produce different allelochemicals such as peptides, lipids and alkaloids. Among cyanobacterial allelochemicals, norharman generally produced by strains of *Nodularia harveyana* and *Nostoc insulare* [7]. Norharman is an alkaloid exometabolite in the class of (9H-pyrido 3, 4-b) indole β -carboline.

One of the important factors affecting the metabolite production of cyanobacteria is the culture age. Cyanobacteria and bacteria in general have four different phases of growth (lag, log, stationary and death). Organisms can be directly measured by either cell counting or total biomass (wet or dry weights) changes [8]. Metabolite production rates were observed as the highest in different growth phases like exponential (logarithmic) and stationary phases. For some cyanobacteria, metabolite production rates increase accordingly with culture age [9]. In this study, cyanobacteria species previously proved as producing norharman were cultured for a month and their norharman productions in different times were determined.

MATERIALS & METHOD

Sampling area and Isolation

Water samples were collected from 4 different pelagic areas determined by geographical positioning system (GPS) in Yesilirmak river of Tokat province of Turkey (Table 1). Samples were filtered (GF/C filter papers, Whatman) and transferred into petri dishes by trompe. Then, isolated under inverted microscope by using micropipette and microinjection [10].

Table 1. GPS Coordinates of water sampling areas.

| Sampling Areas | GPS Coordinates |
|----------------|--------------------------------------|
| Station 1 | 49° 19' 49.12" N - 36° 34' 2.06" E |
| Station 2 | 40° 19' 45.655" N - 36° 33' 45.06" E |
| Station 3 | 40° 17' 40.19" N - 36° 19' 28.81" E |
| Station 4 | 40° 19' 43.77" N - 36° 28' 22.26" E |

Morphological Identification of Cyanobacteria and Cultivation

Following the mechanical isolation, strains were purified on 1.5% agar by line inoculation method. Samples were examined under light microscope for recognition and purification checks. Steps were repeated until only one strain purified. Morphological identifications of cyanobacteria were done according to previous studies [1, 11, 12].

Cultivations were done for each strain by 10% respective strain inoculation into 250 mL conical flasks containing 235 mL appropriate growth medium (Bristol, BG11). Each cultivation was performed thrice. Cultures were ventilated once in daily by gently mixing using sterile Pasteur pipettes. Species were illuminated by fluorescent light for 12 hours at 26 °C [13].

HPLC Analyses

Samples were taken from cultures in 4 day intervals and organisms were separated from media by centrifugation. After centrifugation process concluded, 0.05 g was weighted and put into 1 mL glass tube and was dissolved in 50% (v/v) methanol and chloroform. Dissolved material was vortexed for a minute than laid in supersonic bath for 10 minutes. Following bathing, materials were vortexed for a minute then filtrated by using polytetrafluoroethylene (PTFE) syringe (ChromTech, 0.45 μm \times 13 mm). 20 μL extract obtained by filtration was injected into colon [14]. HPLC analyses were performed by using HPLC – DAD (High Pressure Liquid Chromatography – Diode Array Detection, Shimadzo Co., Japan) equipment. For separation purposes C18-120A inverted phase colon (Thermo Scientific, 4.6 \times 150 mm, 3 μm particulate) was used. Ultrapure water (A), 0.1% (v/v) formic acid (FA) and acetonitrile (B) were used for mobile phase. Metabolites were fragmented in C18 inverted phase colon at a rate of 1mL per minute at 40 °C.

Statistical Analyses

All data were analyzed by using SPSS 22.0 package software (IBM Corporation, Armonk, New York, USA). For comparisons of more than two groups one-way ANOVA (Robust Test: Brown – Forsythe) was employed together with bootstrapping results. For post hoc analyses, tests of LSD, Dunnett and Games Howell were employed. According to ANOVA results, interactions of produced norharman by cyanobacteria in different times were objected into variance analyses and statistical comparisons were made between mean values. Quantitative values were represented as mean \pm standard derivations in tables while categorical values were represented as number (n) and percentages (%). All data were analyzed in 95% confidence interval and $p < 0.05$ was accepted as statistically significant.

RESULTS

Purified Strains

During the morphological examinations, cyanobacteria were identified under light microscope in accordance with diagnosis books and their morphological traits of whether they were filamentous or single celled and if they were filamentous whether they have heterocyst or have not were taken into preliminary consideration. Morphological sizes were also measure by micrometer apparatus incorporated into ocular. General features were listed below:

***Chroococcus minutus* (Kütz.) Nägeli:** Morphological features were ovoid or spherical. They were observe as singular or as clusters of 2 to 4 cells. Diameters of large enveloped cells were measured as 6 – 15 μ , and of non-enveloped cells were measured as 4 – 10 μ and they were colorless (Figure 1).

***Geitlerinema carotinosum* (Geitler) Anagnostidis:** Trichomes were observed as orderly and slightly coiled and both ends were observed as thinner. Cells were ordered as fascicules. Sizes for widths and for lengths were observed as 1.5 – 3 μm and 3 – 9 μm respectively (Figure 1).

***Nostoc linckia* PACC 5085:** Cell widths were observed as 3 – 4 μm and cells were observed as barrel shaped with blue – green colored and having brown envelopes. Heterocyst sizes were observed as 5 – 6 μm . Akinete shape were observed as spherical and have sizes of 6 – 7 μm for width and 7 – 10 μm for length (Figure 1).

***Anabaena oryzae* F.E. Fritsch:** Trichomes were observed as linear and non-enveloped. Cells were observe as barrel shaped and have sizes of 2.5 – 4 μm for width and 4 – 8 μm for length. Heterocyst shapes were observed as either spherical or ellipsoidal and have sizes of 3.5 – 6 μm for width and 6 – 8 μm for length and were positioned near the ends (Figure 1).

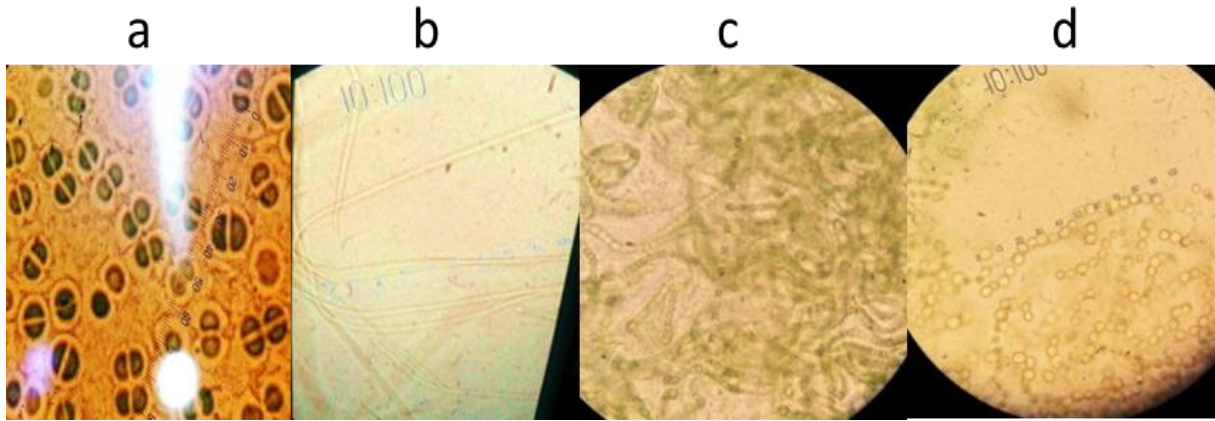


Figure 1: Light microscopy images of purified strains in following order: a) *Chroococcus minutus* b) *Geitlerinema carotinosum* c) *Anabaena oryzae* d) *Nostoc linckia*

Norharman Production of Cyanobacteria

Cyanobacteria biomasses (g/L) were recorded in 4 day intervals (Figure 2). Norharman metabolite quantities ($\mu\text{g/g}$) were measured by using Gauss method on calibration curve lined on absorbance of standard at 247 nm wavelength. Metabolite quantities produced by each strain were associated into time intervals according to HPLC results (Figure 3). As seen in table 2, letters located in right indices of values indicate average metabolite quantity differences for a given particular time and letters located in left indices of values indicate average metabolite quantity differences produced through different time intervals from top to bottom.

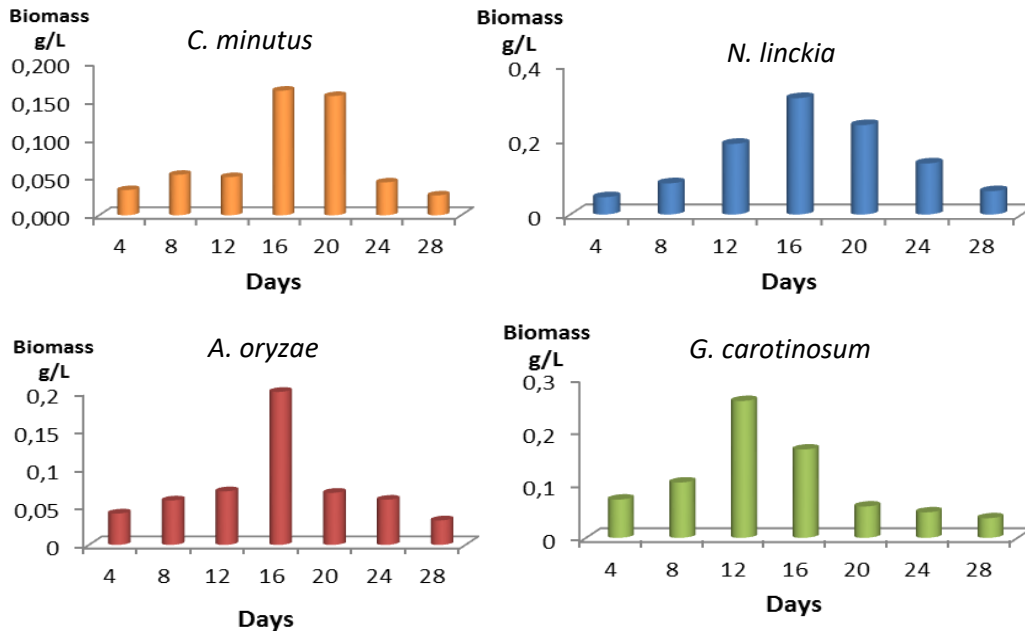


Figure 2: Biomasses of cyanobacteria recorded in different time intervals.

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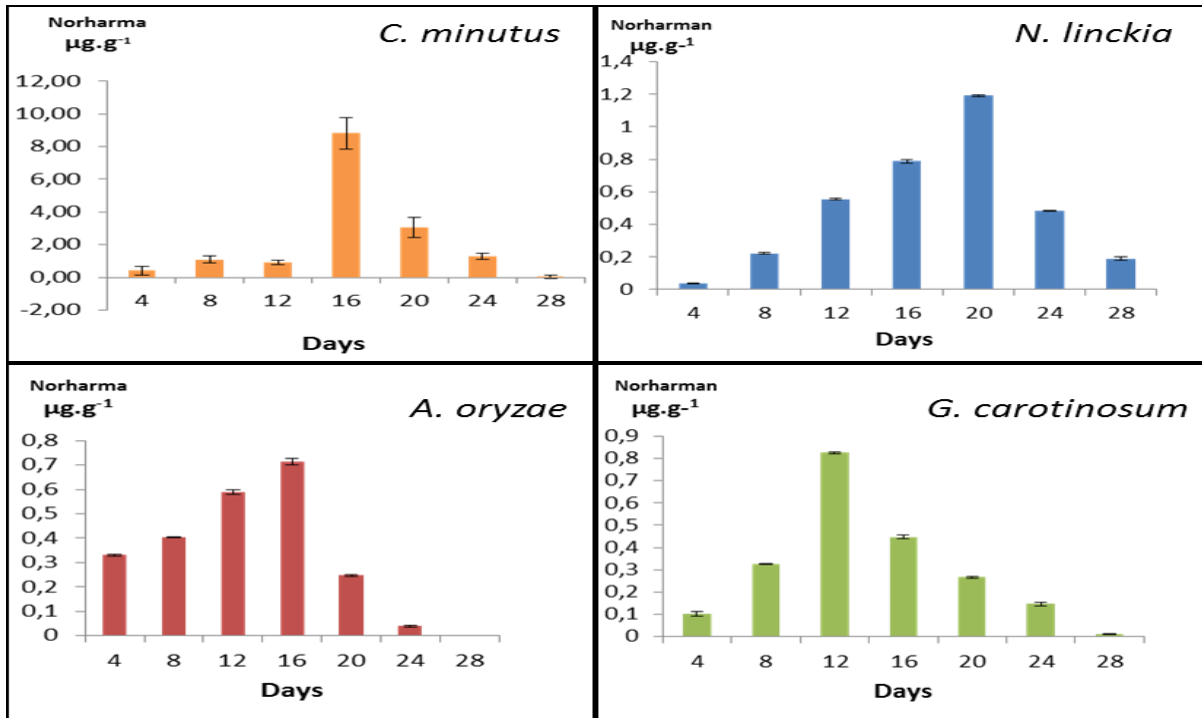


Figure 3: Norharman levels produced by cyanobacteria in different time intervals.

Table 2: Effects of different time applications on secondary metabolite productions of cyanobacteria.

| Days | Cyanobacteria | | | | | | | | Mean | Standart Deviation | Sig. (p) |
|--------------------|-------------------------|-------|---------------------|------|--------------------|------|-----------------------|------|--------|--------------------|----------|
| | <i>C. minutus</i> | | <i>N. linckia</i> | | <i>A. oryzae</i> | | <i>G. carotinosum</i> | | | | |
| | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. | | | |
| 0-4 | a.410 ^{abc} | .265 | a.037 ^a | .003 | c.330 ^c | .005 | b.101 ^b | .011 | a.219 | .197 | 0.0001 |
| 4-8 | ab1.0867 ^{abc} | .212 | b.221 ^a | .005 | d.405 ^c | .002 | e.325 ^b | .003 | ab.509 | .366 | 0.0001 |
| 8-12 | a.910 ^{abc} | .132 | d.554 ^a | .005 | e.590 ^b | .009 | g.825 ^c | .006 | b.720 | .168 | 0.0001 |
| 12-16 | c8.8167 ^{abc} | 2.665 | f.787 ^c | .012 | f.712 ^b | .013 | f.448 ^a | .010 | d2.691 | 3.867 | 0.0001 |
| 16-20 | b3.063 ^{ab} | .625 | e1.191 ^b | .006 | b.247 ^a | .004 | d.266 ^a | .006 | c1.192 | 1.226 | 0.0001 |
| 20-24 | ab1.273 ^{bcd} | .189 | c.484 ^c | .002 | a0.04 ^a | .004 | c.146 ^b | .008 | ab.486 | .511 | 0.0001 |
| 24-28 | a0.035 ^{ab} | .007 | a.107 ^c | .010 | a.000 ^a | .000 | a0.011 ^b | .002 | a.039 | .046 | 0.0001 |
| Mean | 2.338 ^b | | .483 ^a | | .332 ^a | | .303 ^a | | .846 | 1.723 | 0.0001 |
| Standart Deviation | 2.338 | | .389 | | .251 | | .259 | | | | |
| Sig. (p) | 0.0002 | | 0.0001 | | 0.0001 | | 0.0001 | | 0.0001 | | |

DISCUSSION & CONCLUSION

Basic conditions for cyanobacteria survival are generally water, illumination, carbondioxide and simple organic compounds [15]. However, cyanobacteria can provide quick growth in determined conditions [16]. There are synthetic media for specialized culturing of cyanobacteria. For culturing from water samples two different media were prepared which were Bristol solution and BG11 medium. Strains were incubated at 25 °C under 2465 lux illumination in Bristol solution for *C. minutus* and *G. carotinosum* and in BG11 medium for nitrogen fixating *N. linckia* and *A. oryzae*. For some cyanobacteria strains, produced metabolite quantities are in proportion to culture age [9]. Therefore samples were taken from cultures in 4 day intervals and growth phase curves determined by counting cells. Each sample was centrifuged and weighted on sensitive scales.

C. minutus was in lag phase at 4th day and started to adapt into medium. Growth phase initiation for this specie is at 4th to 8th days. From 8th to 12th days, divisions proceed and biomass was observed as highest at 12th to 16th days. At 16th day stationary phase initiated and biomass was gradually decreased until 28th day. *N. linckia* PACC 5085 and *A. oryzae* Ind3 were presented similar growth pattern as *C. minutus* and highest biomasses were measured at 12th to 16th days. However, reproduction of these strains requires dissolution of fibers which decreases biomass compared to *C. minutus*. For *G. carotinosum* AICB 37 strain, reproduction was notably slower and growth concludes faster which resulted in highest biomass measures at 8th to 12th days.

Norharman production was found to be highest for *C. minutus* (8.8167 µg/g) when the biomass is at highest levels. Norharman production outside of exponential growth was notably lower and norharman levels started to decrease after exponential growth concluded. According to results, maximum norharman production interval for *C. minutus* is concluded as 12th to 16th days. While *N. linckia* strain exhibits similar growth pattern as *C. minutus*, maximum norharman production (1.191 µg/g) was observed during transition into stationary phase and then observed as decreasing gradually. Therefore maximum norharman production interval for *N. linckia* is at 16th to 20th days. *A. oryzae* strain also exhibits similar growth pattern as *C. minutus* and provide maximum norharman levels (0.712 µg/g) during highest biomass available which indicate 12th to 16th days. However, norharman levels even in maximum production is notably lower than *C. minutus*. *G. carotinosum* strain initiates exponential growth faster than other strains and therefore achieves highest biomass quicker at 8th to 12th days and this interval is determined as the maximum norharman production (0.825 µg/g) period of this strain. Statistics on norharman productions of cyanobacteria in different time intervals revealed that there are both significance ($p < 0.05$) and great significance ($p < 0.001$) between mean values. As a general conclusion on these strains, norharman production is evident and gradually increasing from the start of lag phase into the start of stationary phase. *C. minutus*, *A. oryzae* and *G. carotinosum* produces highest norharman metabolites during their highest biomasses while *N. linckia* produces highest norharman metabolites during their stationary phase.

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