* Effect of different concentrations of nitrate andphosphateon Geosmin and 2-Methylisoborneol production by some species of cyanobacteria

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Abstract

This study is considered the first of its kind in Iraq, It deals with compounds that never had been studied in Iraq called taste and odour compounds, mostfrequentweregeosmin and 2-MIB. Two species were identified as producers for taste and odour compounds, the first was *Phormidiumretzii* produced geosmin and *Microcoleusvaginatus* produced 2-MIB which were recorded for the first time in the world as a producer. The headspace solid phase microextraction method was used to extract these two compounds from aqueous solution, which were used for the first time in Iraq.

Four concentrations of nitrate and phosphate were used (97, 350, 861 and 1500 µgNO_3^--N/L) and(12, 40, 400 and 870 µgPO_4^--P/L), for observing its effect upon the production of geosmin and 2-MIB by two cyanobacterialspecies.Themaximalproductionofgeosmin was 281.38 ng/l occurred at 861µg NO3-N/L, whereas the higher production of 2-MIB was 296 ng/l occurred at the same concentration in the late of logarithmic phase. The high concentration of nitrate (1500 µgNO_3^--N/L) leaded to suppressed geosmin and 2-MIB productions.Themaximalproductionofgeosmin was 132.2 ng/l occurred at 870µg PO4-P/L, whereas the higher production of 2-MIB was 167.2 ng/l occurred at the same concentration in the late logarithmic phase.

Keywords :Cyanobacteria , Geosmin , Nitrate, Phosphate, Geosimin2-MIB

Botony Classification Qk 900 - 989

*The Research is apart on Ph.D. thesis in the case of the Third researcher

Introduction

Drinking water is an essential daily requirement for humans, for which there is no substitute[1]. The importance of the aesthetic qualities of drinking water can not understate the production of water of a high aesthetic quality which is a major goal of water authorities as consumer judge the quality of drinking water by its taste, odour and appearance. The presence of adverse taste and odour can give consumers the impression that water is not safe for drinking leading to increased consumer complaints [2].

Unpalatable taste and odour compounds in water may not be directly linked to health risks; nevertheless, it has major negative impacts on drinking water, recreational waters, and aquaculture.[3].A significant issue affecting the aquaculture and water industries is thepresence of off-flavour compounds in water, which cause problems by imparting an undesirable earthy/musty flavour and smell to water and fish. Two predominant off-flavour compounds are geosmin (GSM) and 2-methylisoborneol (MIB). These compounds are produced by several varieties of cyanobacteria and actinomycetes as metabolic products(exactly in secondary metabolites) and can be detected by humans at concentrations as low as $0.015 \mu g\ L^{-1}$[4].

Environmental factors such as light intensity, temperature, nutrients like nitrogen and phosphorus, etc. have been shown to modulate the production rate of odour compounds for both cyanobacteria and actinomycetes, but these alone cannot explain the substantial differences in concentrations often observed in surface waters under natural conditions[5]. Zhang *et. al*[6]mentioned that various endogenous and/or external factors are able to affect the synthesisof secondary metabolites. Furthermore, the relative amounts of extra and intracellular portions of odourants may also vary considerably with environment conditions [7, 8].

Materials and Methods:

Isolation , purification and culture of cyanobacteria

Samples for isolation Cyanbacteria and microscopic examination were picked from different sites included drinking water plant, paddy fields, rivers, ponds and soils in Al-Diwaniya city, with a sterile forceps and disposable syringe from the sampling sites and transferred to sterile 10 ml capacity screw cap test tubes. For isolation of predominant filamentous strains and get unialgal culture a Single-Cell Isolation by Micropipette method was used as decribed by [11].

For obtained axenic culture of cyanobacterial species two methods were used the first was a Unidirectional light (Phototactic movement), The surface of usually 3 day old agar plates was scored with parallel lines with a flamed rough glass triangle. Small pieces of young cyanobacterial vegetation were transferred to the center of scored agar plates. The plates were incubated at 25C° under unidirectional light, the scores parallel with incident light (10 $\mu E\ m^{-2}\ s^{-1}$). After overnight incubation, the plates were examined microscopically. The cyanobacterial filaments glided much further from the inoculum's site on scored agar because they glided directly along the scores towards the incident light, The direct gliding led to a rapid separation of cyanobacterial filaments from their adhering contaminants. The axenic filaments were picked on an agar block with a sterile injection needle and subcultured on fresh agar plates [12].

The second was a Density gradient centrifugation,An inoculum of cells (8-9 ml liquid) from the culture to be processed was placed in a thick-walled, 15 ml centrifuge tube and treated with alternate centrifugations at 3000 rpm for 5 minutes and washings with sterile liquid (either culture medium or distilled water, depending upon whether the organism can tolerate a change in molarity) this process repeated at least 12 time[13]. For ensuring the purity of the strains from contamination transfer to the Petri dish contain bacteriological nutrients agar medium and incubated for 24 hours at 37 C^o[9].

Obtained cyanbacterial species were identified according to classical algal classificationreferences[14, 15].

Geosmin and 2-Methylisoborneol measurement

Spmemanual holder (57330-U, Supelco) with SPME fibre coated 50/30µm assemblyDivinylbenzene/Carboxen/Polydi methylsiloxane (DVB/CAR/PDMS)with length 2cm (57299-U, Supelco) was purchased from Sigma Aldrich company/ USA.

Head space solid phase microextraction (HS-SPME) procedure

The method described by[16] with following modifications was use to conduct analysis. Three grams of NaCl were placed in a 20 mL vial (medicinal vial contain tienam antibiotic with volume 20 ml washed by cleaning solution (H2SO4+sodium dichromate for remove any chemical substances then washed by distilled water to be ready to use), 12 mL of an aqueous sample (incubated cyanobacterial strains) was added to the vial and a magnetic bar (20x5mm) put in the vial. Then the vial was sealed with a rubber cap, and placed on a hotplate-magnetic stirrer. A 2cm Stable Flex coated with 50/30µm DVB/Carboxen/PDMS SPME fibre (Supelco) injected through the rubber septum and placed in the headspace of the vial and the sample was extracted for 30 minutes with rapid stirring 400 rpm and heated to 65 C^o then the fiber retracted in the Spme holder and removed from the vial and inserted into injection port of GC device.

Gas Chromatography (GC/MS and GC) analysis

GC/MS analysiswas carried out according to the [17]a GC/MS QP2010 Ultra Shimadzu/Japan coupled with Mass detector and fused silica capillary column InertCap 1MS (0.25 mm I.D. × 30 m × df = 0.25 µm, 100%

Methylpolysiloxane).Whereas GC Analysis was carried out according to the [18] a Gc-2014 Shimadzu/Japan coupled with flame ionization detector (GC-FID), fused silica capillary column InertCap 5MS/NP 0.25 mm I.D. × 30 m × df = 0.25 µm (5 % Phenyl - 95 % Methylpolysiloxane).

Growth Measurements:

Two methods were used to estimate the growth of cyanobacterial species :

Optical DensityAnalysis: As the photosynthetic pigments absorb the light energy from 400 to 700 nm, a wavelength of 750 nm, which was outside the pigment absorbance range, was used for the optical density measurements. Cell density, monitored as the optical density at 750 nm (OD750) of cyanobacterial suspensions, was measured with a spectrophotometer (UV-Visible; Apple, Japan)[19].

ChlorophyllaMeasurement:30ml of each Samplesweretakenfromeachincubationflaskt henfilteredbyGF/Cwhatman0.45µ milliporefilterpapersafter added drops of magnesium carbonate,the paper dried fine and then placed in the glass tube and added 4-5 ml of 90% acetone and Grind the sample filter vigorously for approximately 30 seconds then complete the volume to the 10 ml 90%acetone,and placed overnightat4°Cin darksoastoensurecomplete extraction.The optical density of the extract was measured (aftercentrifugationat3000rpmfor15min.) withaspectrophotometerat665and750nm after Add twodrops of 2N HCl. Theamount of chlorophyll a extracted was calculated according to the equation of Lorenzen[20].Chlorophyll A µg/ml = $11.9 \times 2.43 D_b - D_a \times V/L$

Effect of Environmental Factors on Geosmin and 2-MIB Production

The effect of nutrients at different concentrations on Cyanobacteria growth and odorous compounds production were evaluated according to[21]. The ranges of the environmental parameters selected were to cover the typical conditions in the Diwaniya river (mimic environment factors) because it considered the only source to supply the city with drinking water.The effect of two nutrient limiting factors, nitrogen and phosphorus, on the purified cyanobacterial species growth , geosmin and 2-MIB production in the experiments, phosphate and nitrate concentrations were tested 97, 350, 861 and 1500 (BG-11 medium)µgNO_3^--N/L for nitrogen and 12, 40(BG-11 medium), 400 and 870 µgPO_4^--P/L for phosphorus.

Statistical analysis

The results were statistically analyzed according to the statistical program (SPSS). All data were treated with the analysis of a Factorial Experiment to detect the variations of different variables between total, intra-extracellular concentrations of geosmin and 2-MIB and growth phases for all environmental factors were used. The significance at the probability (0.05) of each data was calculated.

Results and Discussion:

Isolation and identification of cyanobacterial species:

Two species isolated that produced taste and odour compounds were *Microcoleusvaginatus* produced 2-MIB and *Phormidiumretzii* produced GSM when analyzed by gas chromatography/mass spectrometry (Plate 1), these two species were identified as producer for the first time not in Iraq only but in the world.AWWA[22] indicated that not all cyanobacteria produce these compounds or other strong odours; in fact, fewer than 50 of the > 2000 species classified to date have been directly confirmed as geosmin and/or 2-MIB producers.

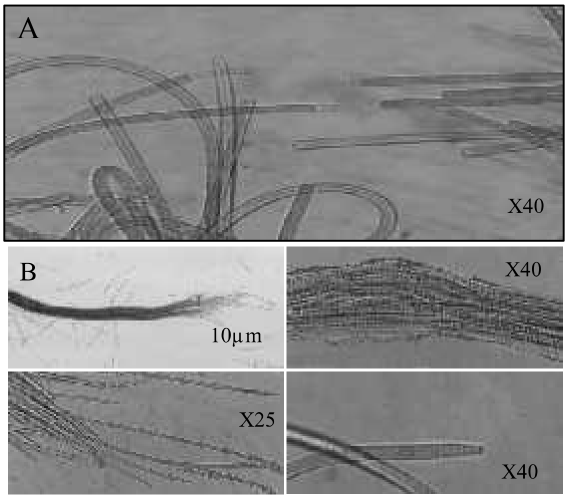


Plate (1) cyanobacterial species producing geosmin and 2-MIBA-*Phormidiumretzii* B-*Microcoleusvaginatus*

Geosmin and 2-Methylisoborneol Measurement

Head Space Solid Phase Microextraction (HS-SPME) Procedure

HS-SPME coupled with GC device was used in this study for the first time in Iraq which was gave good consequences for measured geosmin, 2-MIB standards and releasing compounds from two cyanobacterium*Phormidiumretzii* and *Microcoleusvaginatus* too. This method showed a good linearity from 1 to 1000 ng/L for geosmin and 2-MIB standards and detection limits within 1 ng/l (Figure 1).

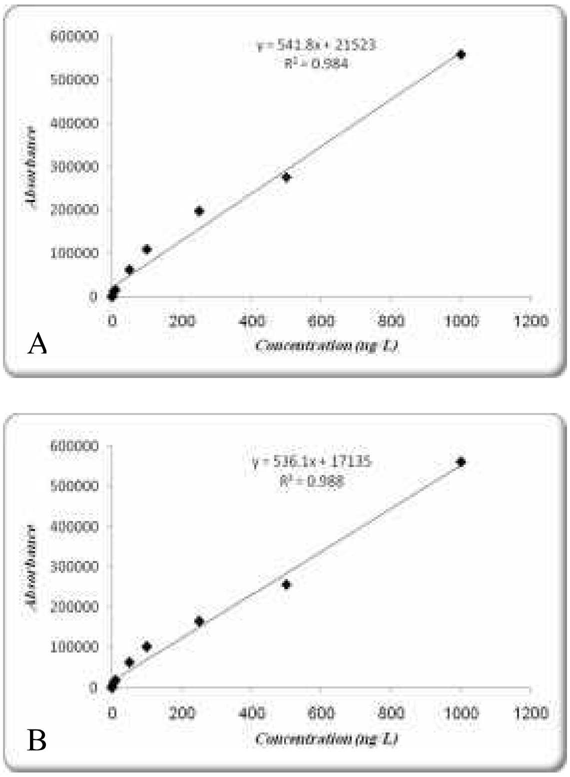


Figure (1) Calibration curve for A. Geosmin and B. 2-MIB standards

GC/MS Analysis :

GC/MS analysis was conducted in order to prove the production of geosmin and 2-MIB compounds by two cyanobacterial species *Phormidiumretzii*and *Microcoleusvaginatus* respectively. Under optimized conditions, these compounds were successfully separated within 20 min without any interfering peaks on the chromatogram. A GC/MS analysis showed a major peak with a retention time of approximately 13.6min for geosmin and 11.2min for 2-MIB (Figure 2). The of geosmin, with base peak at m/z 112 and 2-MIB m/z 95 these mass spectrum gave a similarity to mass spectrum found in the library of geosmin and 2-MIB compounds in the GC/MS device figure (3) and (4) respectively.

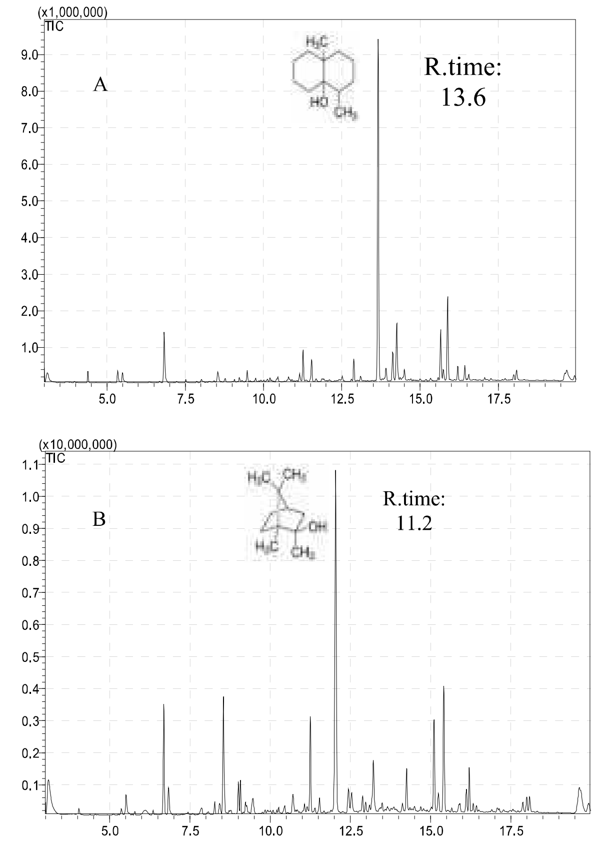


Figure (2) Gas chromatogram of the:A. Geosmin concentrated from a culture of *Phormidiumretzii* B. 2-MIB concentrated from a culture of *Microcoleusvaginatus*

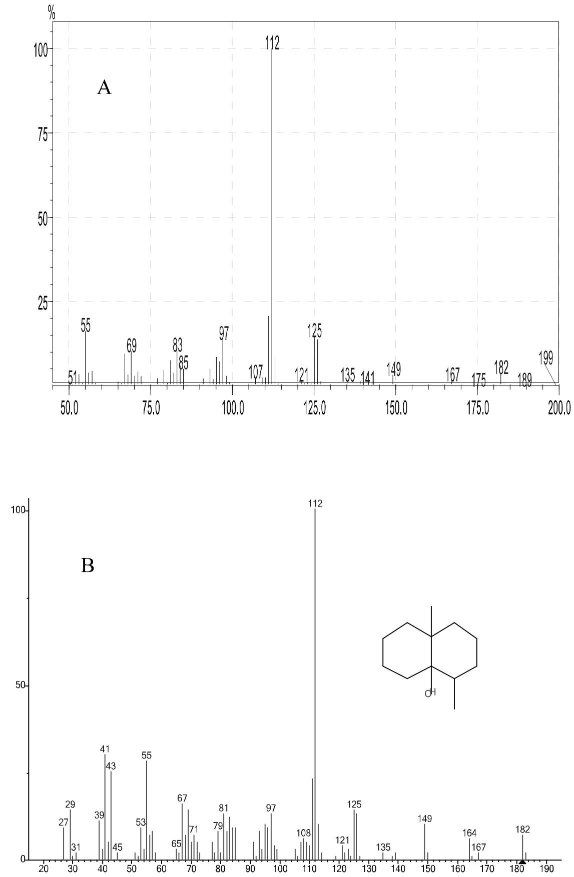


Figure (3) Mass spectrum of the: A. Geosmin concentrated from a culture of *Phormidiumretzii* B. Geosmin from library GC/MS device

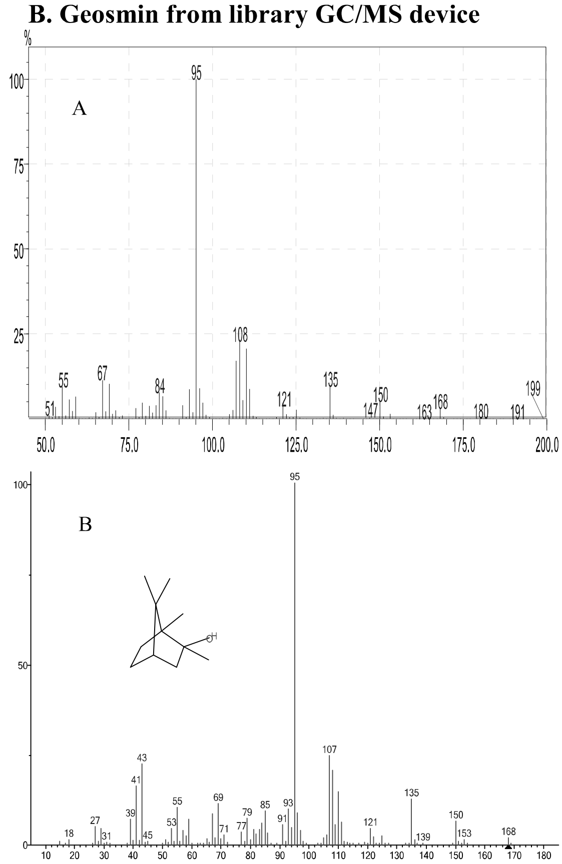


Figure (4) Mass spectrum of the: A. 2-MIB concentrated from a culture of *Microcoleusvaginatus*
B. 2-MIB from library GC/MS device

GC Analysis:

Under optimized conditions, the compounds were successfully separated with retention time 9.4min for geosmin production by *phormidiumretzii* and 4.9min for 2-MIB production by *Microcoleusvaginatus*, the gas chromatographic retention data were consistent with those of the standard geosmin and 2-MIB analysis by the same device (Figure 5 and 6).

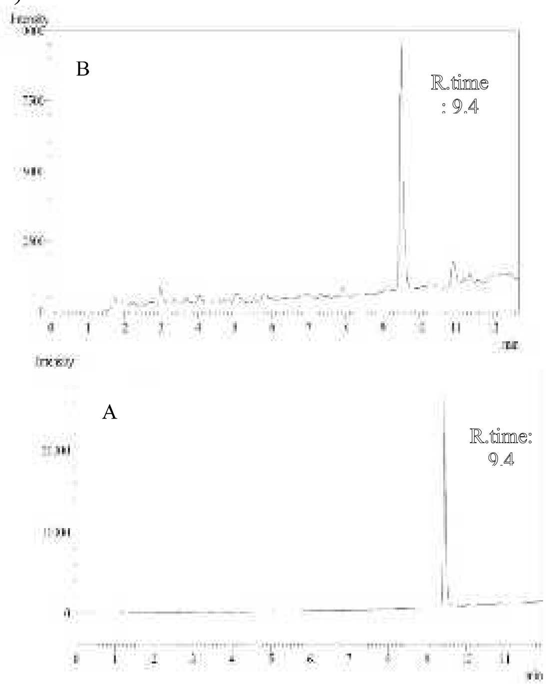
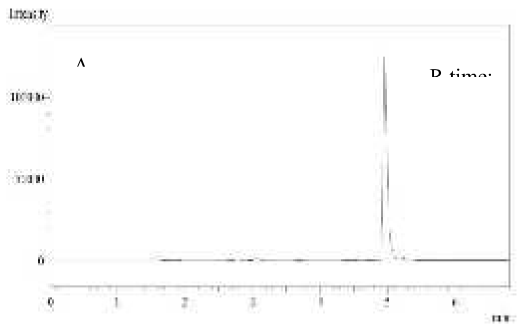


Figure (5) Gas chromatogram of the:A. Geosmin standard B. Geosmin concentrated from a culture of *Phormidiumretzii*



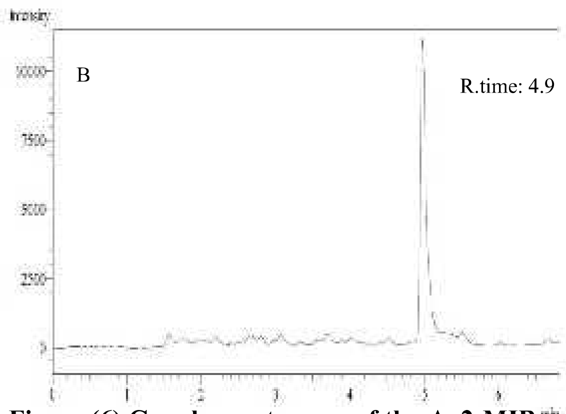
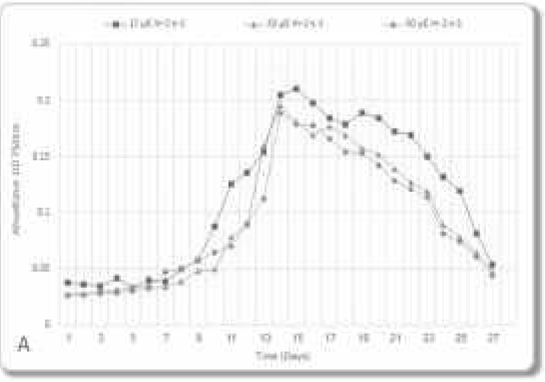


Figure (6) Gas chromatogram of the:A. 2-MIB standard B. 2-MIB concentrated from a culture of *Microcoleusvaginatus*

Growth Measurements:

The optical density and chlorophyll a were used to estimate the biomass of Cyanobacteria under three light intensities incubation (17, 33, 50 µE m^{-2} s^{-1}), 25 C^{o} and BG-11 medium were chosen for each one of these three light intensities for giving optimum growth for cyanobacterial species *Phormidiumretzii* was produced geosmin and *Microcoleusvaginatus* was produced 2-MIB. Because of the light intensity found in nature reached about 1000 µE m^{-2} s^{-1} which was measured by lux meter in Al-Diwaniya river area, a far outweigh the light intensity for getting the optimal growth of cyanobacteria as well as no one of the published researches used a similar light intensities for getting optimal growth. Therefore, in this study was adopted from [23] in order to study the effect of light intensity on growth of cyanobacterial species. The cell harvested for 27 days from culture of*Phormidiumretzii* and 29 days from culture of*Microcoleusvaginatus* until the growth began decrease and cell die. The results of these two methods came identical giving a same growth phases with differences in the obtained data. The lag phase represented by 7 days, logarithmic phase 7 and 9 days, the stationary phase 7 days and the rest of days (6) represented by death phase for *PhormidiumretziiandMicrocoleusvaginatus* respectively (Figure 7 and 8).



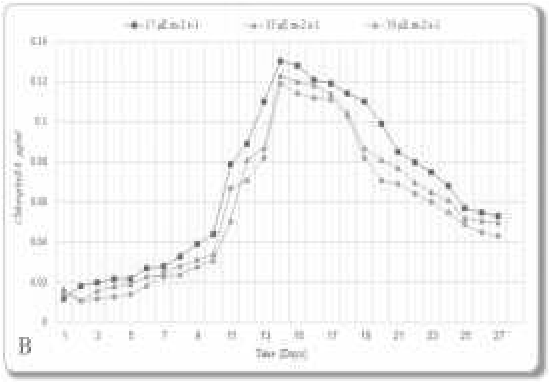


Figure (7) Growth curve for cyanobacterium*Phormidiumretzii*under three light intensities measured by: A. Optical density B. Chlorophyll a

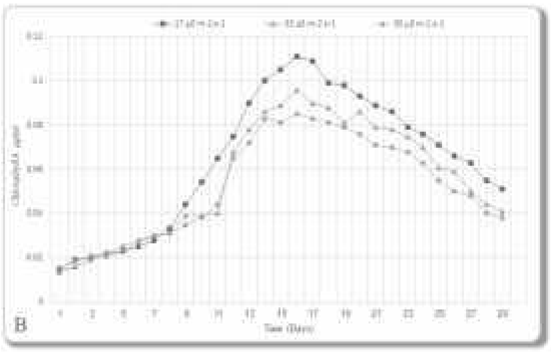
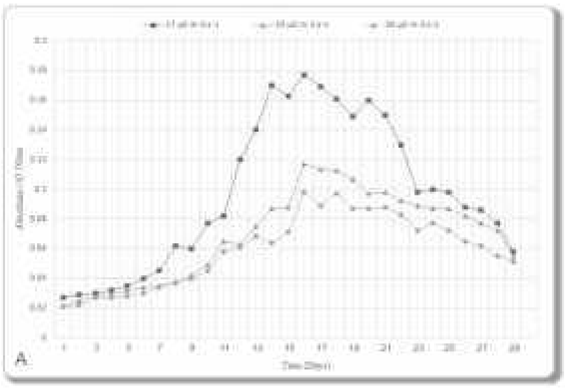


Figure (8) Growth curve for cyanobacterium*Microcoleusvaginatus*under three light intensities measured by: A. Optical density B. Chlorophyll a

Effect of Environmental Factors on Geosmin and 2-MIB Production

The production of these compounds by cyanobacteria may effect by many Environmental factors. Furthermore, these metabolites seem to be governed by environmental variable such as nutrient concentration, water temperature, light intensity and water quality [24].It is recognized that the physical factors influence on environmental conditions and vice versa, both influence the source and presence of taste and odour compounds which may lead to taste and odour events that will develop a greater understanding of these complex interactions will lead to a better understanding of the potential taste and odour events and also the development of more specific management options[25].

The effect of environmental factors on geosmin produced by *Phormidiumretzii* and 2-MIB produced by *Microcoleusvaginatus* were discussed as below:



Effect of Nutrients

Nutrients are present in several forms in aquatic systems, including dissolved inorganic, dissolved organic, particulate organic, and biotic forms. Only dissolved forms are directly available for algal growth: for nitrogen these include ammonia, nitrate, nitrite, and andfor phosphorus,orthophosphate as well as dissolved CO_2, and dissolved silica, etc. [24].In both natural and engineered systems, algae can be exposed to a variety of environmental conditions that affect growth rate and cellular composition. , the amount of carbon fixed in lipids and carbohydrates (e.g., starch) is highly influenced by environmental factors and nutrient availability [25]. Environmental factors such as nutrient enrichment and low nitrogen: phosphorus ratios favor cyanobacterial dominance thereby increasing the risk of taste-and-odour episodes caused by geosmin and 2-MIB.[26].

Theeffectoftwonutrientlimiting factors,nitrogenandphosphoruson thepurified*PhormidiumretziiandMicrocoleusvaginatus* were examined. Intheexperiments, nitrateandphosphatewereused,the temperaturetestedwas25C°andthelightintensitywas17 μE m^{-2} s^{-1}. The results of this study illustrated that the geosmin and 2-MIBconcentrationwereaffectedbytheconcentrationofnitrate. Themaximal productionofgeosmin was 281.38 ng/l occurred at 861μg $NO3^{-}$-N/L, whereas the higher production of 2-MIB was 296 ng/l occurred at the same concentration.The high concentration of nitrate (1500 μgNO_3^--N/L) leaded to suppressed geosmin and 2-MIB productions. (Figure 9).

This is in consistency with the observation by [23] who suggested that high nitrogen concentration was not necessary to promote 2-MIB production by *Oscillatoria* spp. in his experiments, nitrate concentration range tested was 0.8 - 81 mg $NO3^{-}$-N/L for nitrogen and maximal production of 2-MIB, as much as 31 μg/ml, occurred at 8 mg $NO3^{-}$-N/L. [29]alsofoundthatlowergeosminvaluesoccurredat higher $NH4^{+}$-Nand $NO3^{-}$-Nconcentrations for *Anabaena* sp. and more chl. a was synthesis, geosmin decreasing two fold when $NO3^{-}$-N was increased 10 fold from 24.7 to 247μg/l and from 2.8 to 1.47 μg/l. geosmin/biomass values increased to a maximum of 107.6 ng/mg at the low concentration of 123.53 μg/l$NO3^{-}$-N/l.

Nitrate concentration 861 μg $NO3^{-}$-N/L was differed significantly from another concentrations 97, 350, and 1500 μg $NO3^{-}$-N/L in its effect on geosmin and 2-MIB production at $P< 0.05$.

It was concluded that elevated concentrations of geosmin were complexly interrelated with nutrient dynamics (concentrations of inorganic nitrogen), type and density of cyanobacterial species, water temperature[30].Geosminconcentrationand algal biomass registered high levels of 3.8 x 10^4 cells/ mL and 1.1 x 10^4ng/L, respectively achieved at 0.5 mg/l in the study of [31] when they were test a several concentrations of nitrate as nitrogen source

0.05, 0.17, 0.5, 1 and 2 mg/l on the growth of *Anabaena spiroides* isolated from Yanghe reservoir/China.

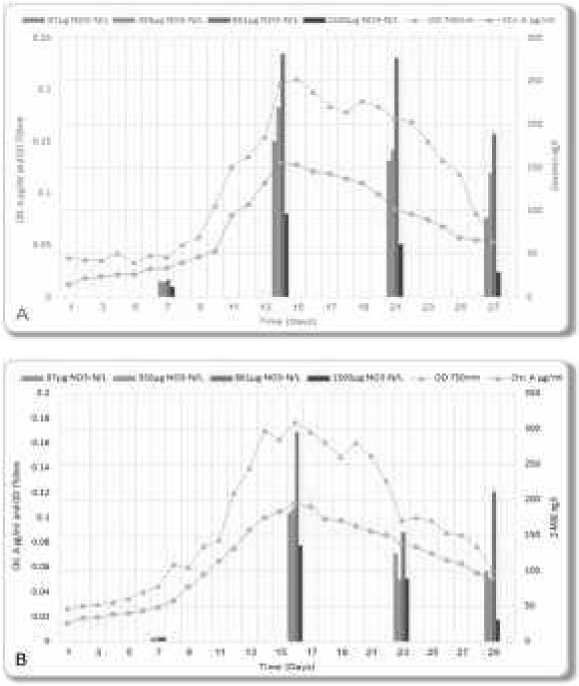
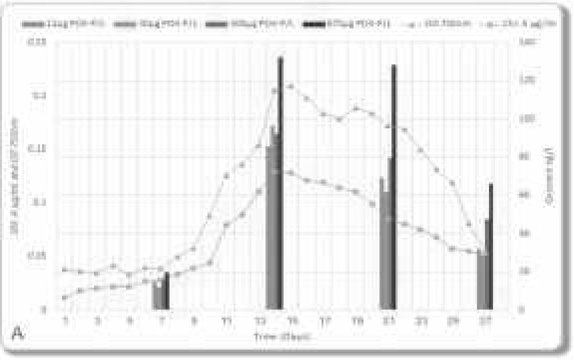


Figure (9) Odour compounds production under four nitrate concentrations (97, 350, 861and 1500 µgNO3$^-$-N/L) A. Total geosmin production by *Phormidiumretzii* B. Total 2-MIB production by *Microcoleusvaginatus*

Saadoun*et al.*[29]suggested that a decrease demand for phytol under low nitrate-N concentrations could shift isoprenoid precursors to geosmin synthesis and that at higher NO3-N levels, geosmin production is suppressed. Lower nitrogen concentration would induce the synthesis of geosmin using the isoprenoid precursors rather than the demand of chlorophyll accumulation[32].A decrease in geosmin and pigment content were observed during transition from light to nitrogen-limited growth. However, geosmin increased relative to phytol (chl. a) and β-carotene which may indicate that a lowered demand for phytol and β-carotene during N-limited growth allows isoprenoid precursors to be directed to geosmin rather than to pigment synthesis. Synthesis of chl. a and β-carotene at the expense of geosmin was suggested for the observed start of increase in geosmin production only at the time that chl. a and β-carotene had reached their light-limited steady state[33].

Phosphorus effect on geosmin and 2-MIB productionexperiments, The results showed that the geosmin and 2-MIBconcentrationwasaffectedbytheconcentrationofphosphate.Themaximal productionofgeosmin was 132.2 ng/l occurred at 870µg PO4$^-$-P/L, whereas the higher production of 2-MIB was 167.2 ng/l occurred at the same concentration (Figure 10).

In this study, the results were similar to those obtained by [29] who indicated that at low phosphate concentrations, no increase in biomass/geosmin was observed at 2 to 118 µg PO4$^-$-P/l, with possibly retained in cells below detection level at low phosphate concentrations. Increasing phosphorus from 235-491 µg PO4$^-$-P/l content enhanced growth and could also enhance geosmin production which was increasing significantly when tested on *Anabaena* spp.[31]Indicated the growth of *Anabaena* sp. could be promoted significantly until phosphorus level attained 0.12 mg/L continuous to 1.8 mg/l , when they were test a several concentrations of phosphate as phosphorus source 0.04, 0.12, 0.4, 1 and 1.8 mg/l on the growth of *Anabaena spiroides* isolated from Yanghe reservoir/China.



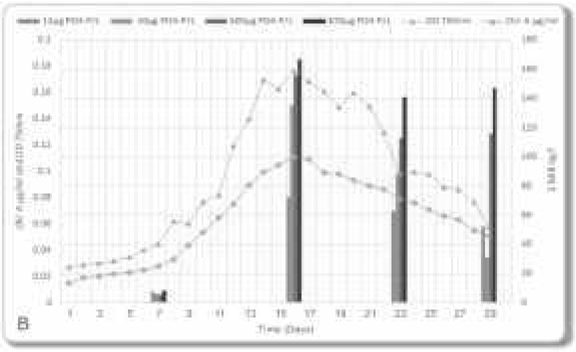


Figure (10) Odour compounds production under four phosphate concentrations (12, 40, 400 and 870 µgPO_4^--P/L) A. Total geosmin production by *Phormidiumretzii* B. Total 2-MIB production by *Microcoleusvaginatus*

Whereas the results of this study differed from[23] who suggested that in a wide range of phosphate concentrations, 0.07 - 7.3 mg $PO4^-$-P/L, no significant difference of 2-MIB production by *Oscillatoria* spp. was observed. These results may be implied that in the phosphate concentration range tested, phosphate is not a limiting factor for both 2-MIB production and cell growth. A similar result was also suggested by [34], who showed that for *Anabaena.* sp. both growth rate and odour production did not change at various phosphate concentrations.Thegeosmin and 2-MIB production showed significant relationship with phosphate concentration 870 µgPO_4^--P/L comparative with 12, 40, 400 and µgPO_4^--P/L

The metabolic pathway involved in geosmin and MIB production is intrinsically linked to phosphate. Both geosmin and MIB are secondary metabolites synthesised via the isoprenoid pathway [35]. The precursors of geosmin and 2-MIB are farnesyl pyrophosphate and geranyl pyrophosphate, respectively. The conversion of these terpenes to odour metabolites requires the phosphate group to be removed [35, 36]. The phosphate group may then be used in other metabolic processes within the cell. The possibility that geosmin and MIB may be an accidental by-product of an organisms' need to sequester phosphate for essential metabolic processes during times of phosphorus limitation needs to be considered and warrants further investigation [25].

This study noticed that the concentration of 2-MIB higher than of geosmin concentrated which was 296, 281.38 registered at the stationary phase in 861µg $NO3^-$-N/L (Figure 9).

Iwaseand Abe[37] indicated that the concentration of 2-MIB in *Phormidium* NIES-512 was 200 times higher than that of geosmin in *Phormidium* M-71 at late exponential phase of growth. [38] remember that 2-MIB concentrations were always higher than geosmin concentrations, but both followed similar seasonal trends.[39]remined that *Oscillatoriatenuis* produced geosmin about twice as much as that done by *Anabaena* *macrospora* and *A. viguieri* isolates. The ratio of geosmin to 2-methylisoborneol produced by *O. tenuis* was about 1.8.[40] showed in their study upon *phormidium* exist in Sagami and Tsukui reservoirs, Japan that the concentration of 2-MIB was more than 20 times higher than geosmin, except for 1999, in which geosmin concentration became as high as 300 ng/liter in the beginning of March, thereafter however, 2-MIB dominated.

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• تأثير تراكيز مختلفة من النترات والفوسفات على Geosmin و 2-Methylisoborneol المنتج من بعض أنواع الطحالب الخضر المزرقة

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الخلاصة

تعد هذه الدراسة الأولى من نوعها في العراق، والتي تناولت مركبات تدعى مركبات الطعم والرائحة إذ لم تدرس فيه سابقا. ويعد الـ geosmin و 2-MIB من أكثر مركبات الطعم والرائحة شيوعاً في مياه الشرب إذ يسببان طعما ترابياً و عفناً في تجهيزات مياه الشرب وخزاناتها وينتجان بصورة رئيسية بواسطة الطحالب الخضر المزرقة Cyanobacteria.

في هذه الدراسة تم عزل نوعين من هذه الطحالب منتج للمركبات المسببة للطعم والرائحة في المياه، النوع الأول هو *Phormidiumretzii* والذي ينتج مركب الـ geosmin والنوع الثاني هو *Microcoleusvaginatus* والذي ينتج مركب 2-MIB اللذين سجلا في هذه الدراسة لأول مرة في العالم كمنتجين لهاتين المادتين. تعد طريقة الحيز أو الفراغ الأمامي- الاستخلاص الدقيق بواسطة الطور الصلب HS-SPME من الطرق المستخدمة في استخلاص مادتي geosminو2-MIB من محاليلها المائيةوالتي استخدمت للمرة الأولى في العراق.

استعملت أربعة تراكيز للنترات والفوسفات (97 و 350 و 861 و 1500 مايكروغرام نترات/لتر)و (12و 40 و 400 و 870 مايكروغرام فوسفات/لتر). إذ سجل أعلى إنتاج للـ geosminوكان 281.38 نانوغرام/لتر عند 861 مايكروغرام نترات/لتر، بينما كان أعلى أنتاج لـ 2-MIB هو 296 نانوغرام/لتر عند نفس التركيز في نهاية الطور اللوغارتيمي. أن التركيز العالي للنترات (1500 مايكروغرام نترات/لتر) أدى إلى كبح أنتاج geosmin و 2-MIB.

أن أعلى انتاج للـ geosmin كان 132.2 نانوغرام/لتر عند 870 مايكروغرام فوسفات/لتر، بينما أعلى أنتاج للـ 2-MIB كان 167.2 مايكروغرام/لتر عند نفس التركيز في نهاية الطور اللوغارتيمي.

*البحث مستل من أطروحة دكتوراه للباحث الثالث