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Estimation of biological nitrogen fixation by genetically characterized local strains of cyanobacteria

Zainulabdeen H. A. Al-Khafaji[1*], Nathir G. M. AlMaathidy[1], Yousef J. I. Al-Shahery[1], Rasha Ayad Nadhim[1]

[1] Department of Biology, College of Education for Pure Science, University of Mosul, Mosul, Iraq
* Corresponding author's e-mail: zainulabdeen.hamzah@uomosul.edu.iq

ABSTRACT

The cyanobacteria are an extremely varied group of gram-negative bacteria with different chemical and physical features. Phycocyanin is a pigment found in every member of this group. It helps with the process of converting atmospheric nitrogen into organic nitrogen, specifically ammonia (NH_3). Two different cyanobacteria species were studied for their ability to fix organic nitrogen in order to achieve the goals of this research. The strain of *Crinalium magnium* (Oscillatonales Oscillatonales) that does not cause heterocytosis and the strain of *Fischerella muscicola* SAG 1427-1 that does produce heterocytosis are the two strains that in question. In the course of the investigation, it was discovered that both categories of organisms have the ability to fix nitrogen from organic matter. In terms of the fixation of organic nitrogen, the findings revealed that the strain *F. muscicola* had a considerable advantage (563 mg/l) when compared to the strain *C. magnium*, which had a value of 395 mg/l when cultivated in the medium Chu 10, which is devoid of nitrogen. The findings also demonstrated that the *F. muscicola* strain significantly outperformed the *C. magnium* strain. When compared to the carbohydrates content (480 mg/L) and the protein content (465 mg/l) of the strain *C. magnium*, it was observed that the value of the carbohydrates content (540 mg/l) and the protein content (505 mg/l) for the strain *F. muscicola* increased after 15 days in the medium Chu 10, which is nitrogen-free. This was the case when comparing the two strains. In contrast to the strain of *C. magnium*, this was demonstrated to be the case.

Keywords: fixing, atmospheric nitrogen, ammonia, blue green algea, *Crinalium magnium.*

INTRODUCTION

Cyanobacteria is a group that is variant chemically and morphologically of the gram negative bacteria, all cyanobacteria contain phycocyanin pigment (AL-Khafaji and Dwaish, 2020). Which has the ability to fix the atmospheric nitrogen and changes it into an organic nitrogen in the form of ammonia (NH_3). This process takes place inside cells that are known as the heterocystes, such as the species *Nostoc* sp. and *Anabaena* sp. The cyanobacteria have certain physiological strategies that allow it to fix the nitrogen in conditions with a good oxygen status even without heterocystes and that is done through the compatibility between the activity of the netrogenase and the photosynthese through the spatial or the temporal separation between the two incompatible processes to protect the netrogenase from the oxygen (O_2) such as *Oscillatoria* sp. and *Lyngbya* sp. (Inomura *et al.,* 2017). The cyanobacteria fixes the nitrogen without getting into a coexistence relationships as the case of legume plants (Heddam *et al.*, 2019). The high protein content of some types of algae and cyanobacteria like *Nostoc* sp., *Anabaena* sp. and *Spirulina* sp., drew the attention of the world to use it as a food directly or as an alternative food source and in addition to that, it is rich with vitamins, amino acids, glycosides, resins and fatty acids (Mohammed *et al.,* 2024; Al-Asady, 2023). Moreover, the algae and cyanobacteria helps the production of growth stimulating materials such as the auxin and the gibberllin hormones. Additionally, its cells possesses bioaccumulation and biosorption mechanisms like the use of plant residuals including walnut peels and husk as an environmentally sound materials used for bio-remediation (Khalaf *et al.,*2025), which

in general means the employing of low cost materials often with high affinity and capacity for binding metal ions; some of these biosorbents are algae, fungi, bacteria, waste biomass of corps, which are naturally abundant, especially cyanobacteria as a new biosorbents (Taha *et al.,*2023). On the other hand, the benefit of cyanobacteria and methanogens to produce bioenergy (Al-Asad *et al.,* 2023) Thus, it is of great benefit to the environment in terms of removing the heavy metals pollutants (Dubey *et al.,* 2019; Al-Khafaji, 2022; Al-Khafaji and Saeed, 2024). Because all what has been mentioned above, the cyanobacteria gained a vast scientific attention in all the fields and the objective of the current study was the isolation and purification of cyanobacteria, which is morphologically variant and then identifying it genetically and identifying the best strains of the bacteria that fixes the atmospheric nitrogen by means of estimating the amount of the nitrogen fixed by each strain.

MATERIALS AND METHODS

In the present study local isolates of the cyanobacteria were isolated, which were: *Fischerella muscicola* and *Crinalium magnium*. The samples were cultured in Chu10 ($MgSO_4 7H_2O$ 0.025g؛ Ca $(NO_3)_2$ 0.4g؛ K_2HPO_4 0.1g؛$FeCl_2$ 0.02g؛ Na_2SiO_3 0.25g؛ Na_2CO_3 0.2g) ،The samples were obtained from the environment of Mosul city and cultured in the medium for the purpose of purification and preparing the vaccination for the algae (Al-Khafaji, 2023).

Characterization of nitrogen fixing cyanobacteria

Two methods of cyanobacteria species were employed, which are: the Morphological identification and the molecular identification in order to get an accurate description of the genres. The morphological diagnosis was conducted using the light microscope.

Detection by PCR

The genomic DNA of cyanobacteria was subjected to a targeted polymerase chain reaction after separation and purification. The primers used were specific to the *nifH* gene, which is involved in nitrogen fixing. Table 1 contains the primer sequences for *F. muscicola* while Table 2 has the sequences for *C. magnium*. The reaction protocol was as followed: initial denaturation at 95 °C for 10 min followed by 30 cycles of 95 °C 30 sec, (60 °C 1 min for *F. muscicola* and 58 °C for *C. magnium*), and 72 °C 30 sec; following that 5 min at 72 °C for final extension;

After isolating the genomic DNA of the local isolates of the cyanobacteria and after performing the electrical relay of the selected DNA using the agarose with a concentration of 1.5%, the agarose was subjected to the ultraviolet ray at a waveband of (270 nm) in the UV transilhuminator to detect the genomic DNA bands in the samples.

Analysis methods

Measuring the growth average of the strains by using the optical density method. Estimating the biomass of the strains in question by measuring the dry weight as described by (Yousef *et al.,* 2021a). Then, the final pH measurement by means of using pH meter and the determination of carbohydrate con using the method described by (Yousef *et al.,* 2021b).

Determination of protein content

The determination of the protein content was performed using the method described by (Abdulrazzaq *et al.,* 2020).

Table 1. Primers used for *F. muscicola*

Gene	Primer	Sequences	Temperature	CG%	Size
nifH	F	5'- CGTAGGTTGCGACCCTAAGGCTGA-3'	60	52.6	370 bp
	R	5'- GCATACATCGCCATCATTTCACC-3'	60	47.3	

Table 2. Primers used for *C. magnium*

Gene	Primer	Sequences	Temperature °C	CG%
nifH	F	5'-AGCGCGGGGTTATGTTGAT-3'	58	52.6
	R	5'-ATGGCAAAACCACCGCAAA-3'	58	47.3

Determination of nitrogen content

The determination of the nitrogen content was conducted by using the method described by (Abdulrazzaq *et al.,* 2020), which involves three basic stages, which are: the digestion, distillation and calibration and by relying on the following equation:

$$N\ \% = \frac{volume\ of\ H_2So_4 \times AC\ (N) \times EW}{1000} \times \frac{TWE}{VEU} \quad (1)$$

where: *AC* – acid calibration, *EW* – equivalent weight, *TWE* – total volume of the extract, *VEU* – volume of the extract used

RESULTS AND DISCUSSION

Isolation and characterization of microscope cyanobacteria

The colonies isolated are characterized with the blue green color and they were examined using the compound microscope and morphologically diagnosed (Prescott, 1973). So, the strains *C. magnium* (doesn't contain heterocyst) and *F. muscicola* (contains heterocyst) were obtained (Figure 1).

Characterization of cyanobacteria isolates depending on the nitrogen-fixing genes "nif H gene"

The results of the chain reaction of the DNA selected from the cyanobacteria isolates were appeared positive band with 370 bp for *F. muscicola* and C. magnium as shown in Figure 2. In contrast to the nitrogen base sequences shown in Figure 3, which pertain to DNA samples from *C. magnum*, Figure 4 displays the sequence of DNA nitrogen bases from *F. muscicola*. The sequences of the PCR products were then obtained using

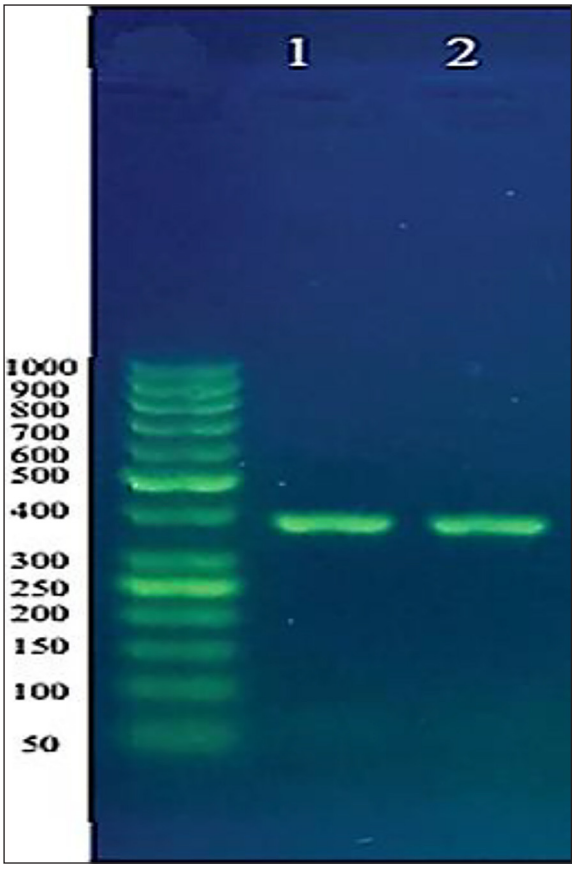

Figure 2. Visualization of *nifH* gene by 1.5% agarose gel analysis. The shown bands are representative of PCR products (370 bp) amplified from the cyanobacteria isolates (lane 1 for *F. muscicola* and lane 2 for *C. magnium*), and lane M represents the 50 bp DNA ladder

Sanger method, the obtained sequences were then aligned with gene sequences from NCBI using BLAST analysis. Due to the accuracy of the results of molecular methods, it is recommended to apply in diagnosis of many organisms in various biological fields. In addition to the environmental

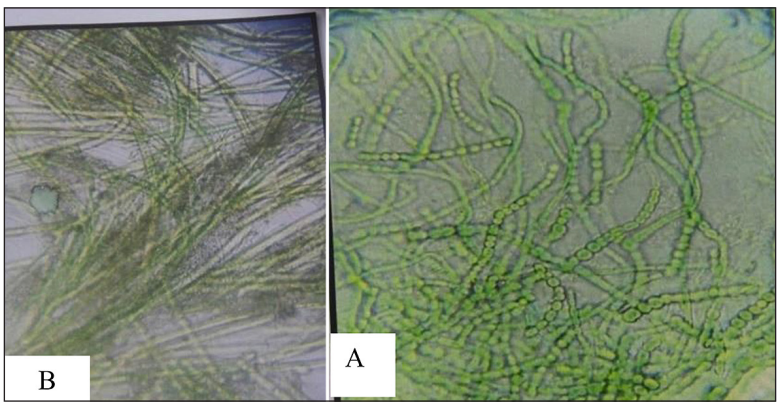

Figure 1. The photo (A) is *F. muscicola* and photo (B) is *C. magnium*

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   1 ggatgaacgc tggcggtctg cttaacacat gcaagtcgaa cggaactaga aatagtttag
  61 tggcggacgg gtgagtaacg cgtgagaatc trgcttgagg ttcgggacaa ccactggaaa
 121 cggtggctaa taccggatgt gccgaaaggt aaaaggttta ccgcctgaag atgagctcgc
 181 gtctgattag ctagttggtg tggtaagagc gcaccaaggc gacgatcagt agctggtctg
 241 agaggatgat cagccacact gggactgaga cacggcccag actcctacgg gaggcagcag
 301 tggggaattt tccgcaatgg gcgaaagcct gacggagcaa gaccgcgtga gggaggaagg
 361 ctcttgggtc gtaaacctct tttctctggg aataagaaag tgaaggtacc agaggaatca
 421 gcatcggcta actccgtgcc agcagccgcg gtaatacgga ggatgcaagc gttatccgga
 481 atgattgggc gtaaagagtc cgtaggtggt gattcaagtc gattgttaaa gagcggggct
 541 taaccccgta gcagcagtgg aaactgaatc actagagaga ggtaggggta gagggaattc
 601 ccggtgtagc ggtgaaatgc gtagagatcg ggaagaacac cagtggcgaa ggcgctctac
 661 tggacctcat ctgacactga gggacgaaag ctaggggagc gaaagggatt agataccccc
 721 gtagtcctag ccgtaaacga tggatactag gtgttgtctg tatcgacccg gacagtgccg
 781 tagctaacgc gttaagtatc ccgcctgggg agtacgcacg caagtgtgaa actcaaagga
 841 attgacgggg gcccgcacaa gcggtggagt atgtggttta attcgatgca acgcgaacaa
 901 ccttaccagg gcttgacatg tctggaatct tggggaaacc taggagtgcc ttcgg
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Figure 3. Comparison of the nitrogen bases sequences of isolate DNA pieces *Crinalium magnum* MSYYJA 16S

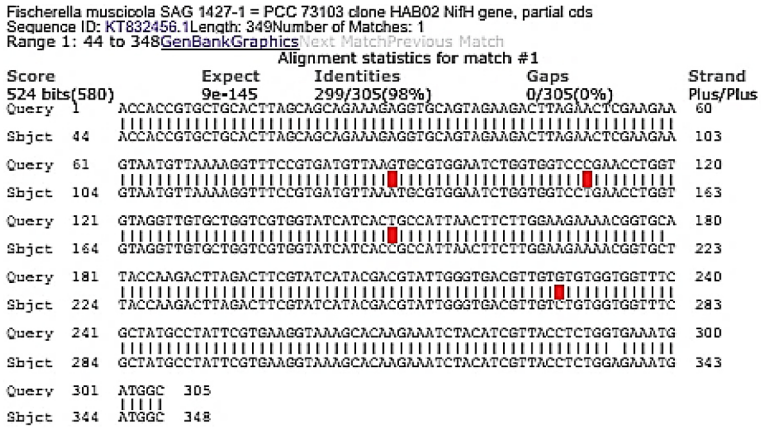

Figure 4. Comparison of the nitrogen bases sequences DNA pieces of the isolate *Fischerella muscicola* SAG 1427.1 C1: *Crinalium magnum*; C2: *Fischerella muscicola*

aspect, it has been used in the agricultural and medical fields, there are many authors recommend to used PCR in their reports (Saleh *et al.,* 2020; Hassoon, 2022; Al-Humairi *et al.,* 2022 Bassi and Al-Rubaii, 2024) and other.

Crinalium magnum MSYYJA 16S ribosomal RNA gene, partial sequence GenBank: OQ955250.1 FASTA Graphics, ORIGIN

In order to make a comparison for the strains to identify the optimum period of the incubation for the growth, biomass, carbohydrate content, protein content and the nitrogen content of the cyanobacteria, the various studied strains were subjected to various incubation periods (3, 5, 7, 9, 12, 15, and 17 days). Results in the Tables 3 and 4 showed that there is a clear increase in the growth of cyanobacteria isolates with the increase of the period of incubation in the medium Chu10, which is provided with the nitrogen in the fifteenth day of incubation with values of (0.830, 0.898 nm) for the isolates *Fischerella muscicola* and *Crinalium magnium* respectively. While the growth values were (0.639, 0.663) nm in the Chu10 medium which is free from the nitrogen source. Results of the biomass (Tables 3 and 4) also showed that the biomass increase with the longer incubation period as the highest value of the biomass was (1332, 1800 mg/L) in the fifteenth day of incubation for the strains *Fischerella muscicola* and *Crinalium magnium* respectively and the biomass value decreased after that. When using the medium free of the nitrogen, a decrease was observed (Tables 3 and 4) in the values of the biomass compared to the medium provided with nitrogen and the highest value was for the fifteenth day of incubation (1200, 1406 mg/l). These values reflect the ability of isolates to grow in a nitrogen-free medium and the presence of the organic nitrogen through their capability of fixing the atmospheric nitrogen (Loaiza *et al.,* 2016). The values of the biomass were consistent with the values of the growth of the strains in question as the relationship was direct between the value of light density for growth and the biomass. As for the final pH value (Tables 3 and 4) of the medium that was provided with the nitrogen source, it increased with the increase of the period of incubation for the

Table 3. The effect of the daily growth on the bio-nitrogen fixation and some cell components of the species *Grinalium magnum* MSYYJA

Growth period (day)	Final pH		Absorption (nm)		Biomass (mm/l)		Carbohydrates content		Protein content		Nitrogen content	
	Medium with N	Medium without N	Medium N+	Free of N	Medium N+	without N	N+	N-	N+	N-	N+	N-
3	7.88 (0.01)	7.80 (0.40(	0.371 (1.00(	0.300 (0.22(	290 (0.31(	110 (0.00(	95 (0.00(	53 (0.90(	45 (0.01(	31 (0.00(	30 (0.95(	25 (1.00(
6	7.98 (0.40(	7.85 (1.11(	0.560 (1.00(	0.395 (0.05(	567 (0.12(	330 (0.33(	394 (0.42(	101 (0.01(	130 (0.04(	79 (0.22(	90 (0.21(	60 (0.11(
9	8.21 (0.30(	7.99 (0.33(	0.691 (0.24(	0.590 (0.31(	905 (0.00(	590 (0.11(	666 (1.00(	290 (0.91(	490 (0.00(	200 (0.11(	350 (0.19(	139 (0.11(
12	8.53 (0.03(	8.00 (0.51(	0.751 (0.11(	0.601 (0.92(	1220 (0.11(	905 (0.55(	690 (0.11(	401 (0.91(	550 (1.00(	90 (0.22(	370 (0.01(	290 (0.33(
15	8.98 (0.01(	8.01 (0.11(	0.830 (0.91(	0.839 (0.91(	1332 (0.91(	1200 (0.00(	720 (0.15(	480 (1.00(	660 (0.90(	465 (0.81(	395 (0.22(	300 (0.55(
18	8.71 (0.91(	8.05 (0.50(	0.801 (0.55(	0.569 (0.91(	1110 (0.22(	790 (0.42(	705 (0.11(	390 (0.90(	590 (0.11(	430 (0.00(	300 (0.21(	290 (0.33(
21	8.22 (1.00(	7.90 (0.30(	0.701 (0.33(	0.511 (0.90(	905 (0.22(	707 (0.55(	550 (0.31(	300 (0.22(	580 (0.10(	290 (0.10(	210 (0.11(	195 (0.00(

Table 4. The effect of the daily growth on the bio-nitrogen fixation and some cell components of the species *Fischerella muscicola SAG 1427-1*

Growth period (day)	Final pH		Absorption (nm)		Biomass (l/mm(		Carbohydrate content (mm/l)		Protein content (mm/l(		Nitrogen content (mm/l)	
	N+	N-	N+	N-	N+	N-	N+	N-	N+	N-	N+	N-
3	7.91 (0.01)	7.90 (1.00)	0.290 (0.18)	0.222 (0.00)	293 (1.00)	190 (0.22)	120 (0.22)	100 (0.11)	80 (0.52)	43 (0.22)	63 (0.11)	25 (0.22)
6	7.99 (0.22)	7.91 (0.21)	0.330 (1.11)	0.282 (0.00)	405 (1.80)	222 (0.23)	290 (0.42)	130 (1.12)	200 (0.51)	79 (0.41)	170 (0.42)	70 (0.21)
9	8.80 (0.11)	8.11 (0.44)	0.509 (0.11)	0.400 (0.39)	890 (0.31)	580 (0.91)	430 (0.91)	220 (1.00)	390 (0.12)	170 (0.12)	301 (0.33)	111 (0.11)
12	9.11 (1.11)	8.90 (0.11)	0.799 (0.90)	0.600 (0.22)	1490 (1.00)	1111 (0.99)	770 (0.91)	460 (0.55)	590 (0.00)	360 (0.11)	488 (0.22)	310 (0.00)
15	9.55 (1.90)	9.22 (0.42)	0.898 (0.11)	0.663 (0.12)	1800 (0.22)	1406 (0.59)	840 (0.92)	540 (0.42)	800 (0.00)	505 (0.09)	563 (0.11)	395 (0.11)
18	9.31 (0.11)	9.10 (0.33)	0.808 (0.09)	0.509 (0.01)	1700 (0.00)	1305 (1.00)	700 (0.55)	670 (0.23)	661 (0.11)	500 (0.22)	501 (0.19)	422 (0.22)
21	8.90 (0.12)	8.72 (0.12)	0.700 (0.11)	0.490 (0.91)	1330 (0.81)	999 (0.01)	600 (0.09)	505 (0.11)	520 (0.11)	490 (0.91)	401 (0.12)	388 (1.00)

strains *Fischerella muscicola* and *Crinalium magnium* respectively. The values were (8.98, 9.55) in the medium provided with the nitrogen, while the values were (8.01, 9.22) nm in the medium free of nitrogen. The value of pH is directly affected by the process of photosynthesis, which consumes CO_2 and the continuous consumption of CO_2, especially in the high growth of cyanobacteria leads to the formation and release of the basal causers (Saeed *et al.,* 2020). The carbohydrate content increased with the increase of the incubation period (Tables 3 and 4) and the highest value of the carbohydrate content was in the fifteenth day of incubation (840, 720 mg/l) for the strains in question respectively and then the value dropped. When culturing the cyanobacteria in a medium that is free of nitrogen, the highest value (Table 3 and 4) was obtained in the fifteenth day (540, 480 mg/l). Therefore, it is obvious that carbohydrate content is affected directly by the average daily growth and the biomass and the nutrient stored in the cyanobacteria cells is carbohydrates and several researches assert that this nutrient is connected to the protein (Du *et al.,* 2019). The protein content of cyanobacteria cells of the strains studies in the medium that is provided with the nitrogen was of the values (800, 660 mg/l) respectively, (Tables 3 and 4), in the fifteenth day of incubation and then these values decreased. From the other hand, the protein content (Tables 3 and 4) when the cyanobacteria was cultured in a medium without nitrogen (505, 465 mg/l) respectively for the strains *F. muscicola* and *C. magnium*. The results of the Tables 3 and 4 show that the nitrogen content of the cyanobacteria cultured in the medium provided with the nitrogen increases with the increase of the incubation period. The highest values of the nitrogen content were (563, 395 mg/l) respectively in the fifteenth day of incubation for

the strains studied. While, the nitrogen content value of the cyanobacteria cultured in the medium free of nitrogen was the maximum in the fifteenth day of incubation (602, 300 mg/l) for the strain *Fischerella muscicola* and this is because this species possesses variant heterocyst that is considered the agent that fixes the atmospheric nitrogen, while the strain *Crinalium magnium* is trichomas without heterocystis and this reduces it efficiency in fixing the atmospheric nitrogen (Riemann *et al.,* 2010). The process of nitrogen digestion in the cyanobacteria rests on a delicate organizing network that depends on the cellular 2-Oxoglutarate that reflects the balance of N/C to organize the gene expression of the protein activities and consequently the paths of the metabolism (Esteves-Ferreira *et al.,* 2017). The rise in the values of the protein and nitrogen contents of the cyanobacteria in the medium that is free of nitrogen source compared to the values in the medium provided with nitrogen for the fifteenth day of incubation explains the ability of cyanobacteria to substitute the lack of the organic nitrogen, which results from the lack of the nutrients necessary for growth and this is due to its ability to fix the organic nitrogen and them encoding the protein (Zheng and O'Shea, 2017).

CONCLUSIONS

Throughout the results of the research, it obvious that it is possible to gen pure strains of the morphologically-variant cyanobacteria that fixe the atmospheric nitrogen. Results showed that *Fischerella muscicola* is better than the strain *Crinalium magnium* in terms of fixing the nitrogen as it includes heterocystes.

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