THE EVALUATION OF NITROGEN FIXATION ACTIVITY OF SOIL CYANOBACTERIA VIA REDUCTION ASSAY AND REAL TIME- PCR

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Abstract

Cyanobacteria, especially the nitrogen fixation, are considered as one of the most important bacteria in all aquatic and terrestrial environments and have vital roles in the arid and semi-arid ecosystems. Therefore, in the present study, cyanobacteria obtained from the harsh terrestrial ecosystem were selected and the nitrogen fixation activities were assayed by acetylene reduction and Real-Time Polymerase Chain Reaction (RT-PCR). First, the soil cyanobacteria were isolated by using the BG11 medium. Then, all the isolates were transferred into the nitrogen-free medium of BG11 (BG0). In addition, the rate of nitrogen fixation was assayed by gas chromatography mass based on acetylene reduction assay. Next, the *nifH* gene was expressed in the nitrogen fixation isolates utilizing the RT-PCR. Based on the results, the highest level of ethylene production was observed in *Nodularia* sp. with 86.3% yielding. Further, the RT-PCR data indicated that all the selected isolates could express the *nifH* gene. Furthermore, the *nifH* gene expression of *Nodularia* sp. and *Trichocleus* sp. was considerable compared to the other genera. Additionally, the activity of nitrogenase and its increase in the gene expression of nitrogen fixation were extremely higher than those of the filamentous and heterocystous cyanobacteria compared to the other genera. Therefore, cyanobacteria play a crucial role in the nitrogen fixation of the soil.

Key words: Cyanobacteria, *NifH* Gene, Nitrogen Fixation, Real-time Polymerase Chain Reaction

Introduction. Nitrogen-fixing microorganisms are globally significant since they are the only natural biological source of nitrogen fixing in the biosphere [^{1, 2}]. In addition, cyanobacteria, as one of the most significant nitrogen fixing bacteria in all aqueous and terrestrial environments, have an essential role in arid and semi-arid ecosystems [³]. According to several studies, three main processes exist for nitrogen fixation including Haber-Bosch process (i.e., anthropogenic), lightning strikes (i.e., abiotic), and the biological response to N₂ and H₂ for producing the ammonia [^{4, 5, 6}]. In the biological process, the complex of nitrogenase enzyme, which is very sensitive to free oxygen, catalyzes the nitrogen reduction to ammonium. This enzyme contains the dinitrogenase and dinitrogenase reductase proteins, which are of MoFe and Fe proteins,

respectively [7]. Further, dinitrogenase reductase is considered a homodimer of about 60-70 kDa protein, which is encoded by the *nifH* gene [8].

Therefore, different PCR primers were developed for the *nifH* gene in order to understand the procedure of nitrogen fixation using the environmental samples. Using the *nifH* as a genetic marker, the researchers could recognize and describe the diversity and ecological aspects of the nitrogen-fixation bacteria and archaea [9]. Furthermore, the nitrogen fixation rate was measured by the acetylene reduction assay (ARA) and Real-time quantitative PCR (RT-qPCR) methods which are commonly used in both axenic and non-axenic cyanobacteria [10, 11, 12]. The first assay demonstrated the reduction of acetylene gas to ethylene gas [13]. As described by Bergersefn, the nitrogenase enzyme converts acetylene to ethylene using the same amount of energy, which is required to fix the N₂ [14]. Based on the above-mentioned discussions, the present study sought to assay the rates of biological nitrogen fixation and *nifH* expression from cyanobacteria, which were isolated from the terrestrial ecosystem. Although many studies have focused on the nitrogen-fixing cyanobacteria isolated from the aquatic environment, reports on arid and semi-arid terrestrial are rare. Accordingly, the current study aimed to determine the nitrogen fixation potential of cyanobacteria from the harsh environment.

Materials and methods. Site Sampling. In this study, Cyanobacteria of arid and semi-arid Khabar International Park soil samples were isolated and identified through the microbiological and molecular method. Based on the level of *nifH* gene expression, the highest nitrogen fixation isolates were selected and compared with each other. Thereafter, the isolates were grown in continuously supplied BG₀ medium and aerated by an aquarium pump [14, 15, 16].

Determination of N₂ fixation by the acetylene reduction assay. The isolated cyanobacteria were inoculated into 100 ml tubes containing 50 ml of BG₀ broth medium and incubated in 34-38°C. After 48 hours, 20% of the headspace was replaced with acetylene gas by using a 2.5 ml syringe and incubated for 24 hours with the same temperature. Then, 2 µL of air was injected into the each tube and GC/MS data were acquired by gas chromatograph (Agilent 7890B).

RNA extraction. RNA was extracted from 0.15 grams of cyanobacteria isolates. For this reason, Cyanobacterial cells were harvested *via* centrifugation at 500×g for 10 min (JA 20 rotor, Beckman) in three replicates. Pellets were immediately vortexed to resuspend the cells in 1mL ice cold RNXTM –PLUS solution and then dipped in liquid nitrogen. To break down the cyanobacterial

cell wall, 5 cycles of snap freezing in liquid nitrogen, thawing in 70°C was carried out. Total RNA was extracted using phenol/chloroform extraction method [17].

Quantity and quality of extracted RNA. Quantity and quality of extracted RNA were assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific) and agarose gel electrophoresis, respectively. The RNA was quantified using the spectrophotometer at 260 and 280 nm wavelength. The quality of RNA was checked in a 2% w/v agarose gel. The electrophoresis was performed at 85 V in 0.5x Tris-borate EDTA (TBE) buffer for 45 min. The RNA migration was visualized after GelRed™.

DNase treatment and cDNA synthesis. All RNA samples for cDNA synthesis were adjusted to the same concentration. In order to remove potential contamination of genomic DNA and achieve pure cDNA, all samples were treated with DNaseI enzyme (Fermentase). According to the manufacturer protocol, cDNA was synthesized using the Prime Script™ RT Reagent (Takara kit Perfect Real Time). The total volume of the reaction was 10 µL, containing 500 ng of RNA, 2 µL of 5X buffer, 0.5 µL RT enzyme, 0.5 µL oligo dT primer and 0.5 µL random hexamer and reached to the final volume with DEPC water.

Real Time PCR. Real time PCR was performed using Sybr® Premix Ex Taq™II Takara kit. The Applied Biosystems™ StepOne™ Real time- PCR system was used to record changes in the fluorescence dye. Reactions were performed in a total volume of 12.5 µl, containing 4 µl cDNA template (diluted 1:20), 6.25 µl 2X SYBR green master mix and 0.1 µM of each forward and reverse primers of *nifH* or *16S rRNA* according to the table. 1 [18, 19]. The relative expression of *nifH* was assessed in comparison to *16S rRNA*, as an internal control reference. The experiments were carried out twice and each time in triplicate. All controls were excluded of primers. The real time- PCR was conducted according to this program: initial activation phase at 95°C in 1 minute, denaturation phase at 95°C in 5 seconds, annealing phase at 60 °C for 34 seconds, and was repeated for 40 times. The gene expression of *nifH* were determined in different groups using $2^{-\Delta\text{ct}}$ method.

Table 1. The primers of Cyanobacteria used in RT-qPCR

Name	Primer	Primer Sequence (5' to 3')	PCR Product Length
<i>nifH</i>	Forward	CGTAATGCTCGAAGGGTTTGAA	79 bp
<i>nifH</i>	Reverse	CACGACCAGCACAACTCACT	
<i>16S rRNA</i>	Forward	CAGTAGCTGGTCTGAGAGGATG	150 bp
<i>16S rRNA</i>	Reverse	GTTTACAACCCAAGAGCCTTCC	

Statistical and analysis. Statistical analysis was performed using SPSS software (version 22).

The data were presented as mean $\pm$ standard deviation (SD) and the ANOVA test was employed to determine the significance of the observed differences between groups. P value less than 0.05 was considered statistically significant.

Result. A total of nine cyanobacterial isolates including *Chroococcidiopsis* sp. (n=5), as well as *Leptolyngbya* sp., *Synechococcus* sp., *Nodularia* sp., and *Trichocleus* sp. (one out of each genus) was tested. Additionally, all the isolates were identified by both morphological and molecular methods.

In addition, the nitrogenase activity of the bacteria was detected in a BG₀ medium using a *nifH* marker gene. Then, the species were grown in nitrogen-free medium, followed by nitrogen fixation assay through measuring the ethylene production. Among the isolated bacteria, *Nodularia* sp. could convert a large percentage of acetylene to ethylene and was introduced as the best nitrogen-fixation bacterium. The production of ethylene was detected in peak at 0.968 min. Further, the highest level of ethylene production was observed in *Nodularia* sp., yielding 86.3%. Furthermore, *Trichocleus* sp. converted a lower percentage of the acetylene to ethylene. For this second strain, ethylene was detected in peak after 1.096 min. The yield of ethylene for *Trichocleus* sp. was 73.6%.

RNA extraction result

The quantity of RNA measured by the nanodrop2000 indicated a high yield of RNA. Additionally, the purity of RNA was estimated by calculating the ratio between the absorbance at 260 and 280 nm. The A260/A280 ratio was around 2 for the majority of RNA samples (three replicates), which represents a sufficient quality of RNA without any protein contamination.

RT-PCR result

The *nifH*, as a marker gene, was used to study the diversity and ecology of nitrogen-fixing bacteria. The results demonstrated that the *nifH* gene expression in the *Nodularia* sp. was more than that of the other strains. In addition, the highest level of *nifH* gene expression was observed in *Nodularia* sp., followed by *Trichocleus* sp., *Synechococcus* sp., and *Leptolyngbya* sp. while the lowest level of *nifH* gene expression belonged to *Chroococcidiopsis* sp. (Ch28A). As shown in Fig. 1, there is a significant difference between the samples in terms of the *nifH* gene expression ($P < 0.05$).

Further, the melting curve of the RT-PCR reactions has a sharp peak of T_m of the products, which represents that all the RT-PCR reactions amplify the DNA target.

Fig. 1. The comparison of *nifH* gene expression in cyanobacterial isolates. Note. The expression of the *nifH* gene is the highest in *Nodularia* sp. while it is the least in *Chroococcidiopsis* sp. (Ch28A). Furthermore, the results demonstrate $SD \pm P\text{-value} < 0.05$ of the three replicates.

Discussion. Cyanobacteria are regarded as one of the natural biological sources of nitrogen fixing in nearly all the habitats. Based on the quantitative results regarding *nifH* gene expression and N_2 fixation obtained from the RT-PCR analysis, significant differences were found between the samples, indicating that the expression of the *nifH* gene was higher in *Nodularia* sp. and *Trichocleus* sp. compared to the other species. Based on the reports, the rate of expression of *nif* operon in the filamentous and heterocyst cyanobacteria was higher than that of the other

microorganisms [19]. Additionally, in this study, the heterocystous cyanobacteria exhibited more nitrogenase activity compared to the other species isolated from the arid and semi-arid areas.

Bauer reported the dominance of non-heterocystous cyanobacteria in most of the studied sites and the maximum levels of nitrogenase activity during the dark period and found that the oxygen level was the minimum in the cells [20].

In addition, it was found that the expression of the *nifH* gene and nitrogen fixation in the filamentous and heterocyst cyanobacteria was greater in hot and intact areas compared to the other cyanobacteria areas of the desert. This can be attributed to the presence of various factors such as organic matter and carbon, nitrogen, the amount of light irradiation absorbed on the surface, and the temperature of the region [3].

The highest level of ethylene production was observed in *Nodularia* sp. Several studies reported that *nifH* is used as the most important functional gene for studying the structural and functional diversity in different environments [16, 19]. Further, the high levels of ammonium produced by the high activity of the nitrogenase increase further NH₄ + stabilization.

Conclusion

In general, the analysis of the Real-time Polymerase Chain Reaction revealed significant differences between *nifH* gene expression and nitrogen fixation among the strains, which was confirmed by measuring the total nitrogen fixation in the sample using the acetylene reduction assay method.

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