

Evaluation of Guanidium Thiocyanate as a Chemical Mutagen on the Expression of Nitrate Reductase Gene (NR) in *Chlorella* Sp., NTMP04.

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Abstract: Nitrate is the predominant form of inorganic nitrogen utilized by higher plants, fungi and algae and its assimilation forms the major pathway for production of biologically useful nitrogen. *Chlorella* is a unicellular eukaryotic green alga, which offers a potentially useful system for the expression of heterologous proteins. In the present study the microalgal samples were isolated from stagnant fresh water habitat (Tiruchirappalli, Tamil Nadu) and identified as *Chlorella* sp. NTMP04, *Chlorella* sp. NTMP05, *Chlorella* sp. NTMP06. Chemical mutation was adopted using guanidine thiocyanate as mutagen and the mutants were assessed in the specific media such as SGII-NO₃ and SGII-NH₄ for nitrate reductase (NR) gene regulation. A strain *Chlorella* sp, NTMP04 was chosen for the biochemical and molecular analysis to confirm NR gene mutation. The genomic DNA has extracted from the wild and mutant strains and PCR amplification of NR gene was done using degenerative primers (NR-F and NR-R). The wild type of *Chlorella* sp NTMP04 showed the presence of NR gene, whereas mutant did not amplified the specific gene. The comparative analysis showed the evidence of chemical based mutation in the *Chlorella* sp., NTMP04.

Key words: *Chlorella* sp. % Nitrate reductase (NR) % Guanidine thiocyanate (GTC) Mutation % Sager-Granick Medium

INTRODUCTION

Nitrate reductase (NR) is a complex enzyme which containing most of the prosthetic groups. The enzyme able to converts nitrate to nitrite and reduce the level of enzyme activity, synthesis and degradation in plant [1], fungi [2] and green algae [3, 4]. The assimilation of nitrogen from the environment is the major source for inorganic nitrogen and then it was later converted into organic compounds. NR gene is a model tool for transformation marker and it has been successfully expressed in number of organisms including *Fusarium moniliforme* [5] and *Chlamydomonas reinhardtii* [6]. Nitrate reductase has been characterized in many of microalgae. *Chlorella* is a efficient model system to study the genetics and molecular biology of photosynthesis and chloroplast development. In the view of biotechnological approach, the alga has stability to play a major role in bioengineering for the production of pharmaceutical and industrial products [7, 8]. Nitrate and Ammonia are not only substrates which cause "induction or repression"

[9]. In *Chlorella* NR is encoded by a single gene which was located in a cluster of genes and they are involved in nitrate assimilation [10] and these nitrate have the ability to induce the individual strong promoter. This paper describes the mutational analysis in different isolates of *Chlorella vulgaris* using a guanidine thiocyanate as a chemical mutagen to detect the presence of NR gene. This led to the isolation of mutant strain using Sager- Granick medium. Mutants deficient in nitrate assimilation have been generated by using conventional techniques of mutagenesis and enrichment.

MATERIALS AND METHODS

Sample Collection: The microalgal samples were collected from the fresh water habitat in the outskirts (Mathur) of Tiruchirappalli, Tamilnadu.

Isolation and Identification: Microalgae were isolated using Bold's Basal medium by the means of streak plate method [11]. The inoculated plates were incubated for a

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period of 15–20 days; the individual colonies were isolated and inoculated into Bold's Basal medium (BBM) and incubated at $25 \pm 1^\circ\text{C}$ under 1,500–2,000 lux light intensity with 16:8 h light and dark photoperiod. The purity of the culture was ensured by regular observation under microscope. The isolated microalgae were identified based on the microscopic observation using standard monograph [12].

Mutational Analysis: The guanidine thiocyanate is used to mutate the *Chlorella vulgaris* NTMP04, NTMP05 and NTMP06. The cells were treated with guanidine thiocyanate of a concentration of about $125 \mu\text{g}/2 \text{ ml}$ for about 45 min. The treated cells (1 ml) were separately inoculated in the Sager–Granick medium containing ammonium (SGII– NH_4) medium was made by adding the following to 1 L of distilled water: 3.67 g of NaH_2PO_4 , 1.15 g of K_2HPO_4 and 2 g of sodium acetate (anhydrous); in addition 10 ml of each of the following stock solutions was added: 0.1% FeCl_3 , 0.4% $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 1.5% (wt/vol) MgSO_4 , 3% (wt/vol) NH_4NO_3 and trace elements (trace element stock solution contained 100 mg of H_3BO_3 , 100 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 40 mg of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 20 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 4 mg of CuSO_4 in 1 L). In Sager-Granick medium lacking ammonium (SGII– NO_3 medium), KNO_3 replaced NH_4NO_3 and incubated for 12–15 days at $25 \pm 1^\circ\text{C}$ under 1,500–2,000 lux light intensity with 16:8 h light photoperiod [13].

Screening of Mutants: The NR mutants were confirmed by their ability to grow in the presence of ammonia in the culture medium and they were fails to grow when nitrate was present in the culture medium [13].

DNA Extraction: The genomic DNA was extracted from the wild and mutant strains, according to the method of [14]. Briefly, the cells were harvested by centrifugation at 14,000 rpm for 5 min, then the medium was decanted and the resulting pellet was suspended in 500 μl of extraction buffer (200 mM Tris Cl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and vortexed. After that resulting suspension was extracted with 350 μl of ice cold phenol: chloroform: isoamyl alcohol (25:24:1) by vortexing for 1 min followed by centrifugation at 15,000g for 45min in a microcentrifuge. The upper phase was transferred to a fresh 1.5 ml tube containing 1 mg/ml final concentration of RNase and incubated at 37°C for 10 min. 350 μl of phenol:chloroform:isoamylalcohol (25:24:1) was added to this solution, mixed thoroughly and spin at 1,5000 rpm for 10 min. Genomic DNA was precipitated from the

supernatant by adding 250 μl of cold isopropanol. Finally, the DNA pellet was washed using 70% ethanol, air dried and resuspended in 50 μl of TE buffer (10 mM Tris Cl, pH 8.0 & 1 mM EDTA) and stored at -20°C . The extracted DNA was separated by 0.8% agarose gel electrophoresis.

Primer Designing for NR Gene: We have designed the degenerative primers for nitrate reductase gene using the online Primer 3 Software and the reference sequence was retrieved from NCBI.

NR—F 5' CTTTCAGCTGAGCTTGAGACACT—3'
NR—R 5' ACACTTGCAGGCTAGGTAGACAC—3'

PCR Amplification of NR Gene: The NR gene amplification was done using the designed primers NR–F & NR–R, in a Thermal cycler (Applied Biosystems, USA) following the instruction of the manufacturer of Prime Taq Premix (GeNet Bio). The reaction was carried out in 40 μl volumes [20 μl of premix, 1 μl (50 pmol) of each primer, 2 μl (50 ng) of template DNA and 16 μl of Milli Q water]. The PCR condition was optimized for the amplification of NR gene. The reaction was began with 1st cycle of 5 min of initial denaturation at 94°C , followed by the amplification with 40 cycles of denaturation at 94°C for 1 min, Primer annealing at 50°C for 1 min and extension at 72°C for 1 min, followed by 10 min of final extension at 72°C and hold at 4°C . The amplified product was separated on 1.2% low melting Agarose (Sigma, USA), stained with Ethidium bromide and recorded using a CCD camera in UVP gel documentation system (UVP, England). A ready to use standard molecular marker of 1 kb ladder was used as marker DNA.

RESULTS

The microalgal samples were collected from fresh water habitat (Mathur) and isolated using Bolds Basal medium (Fig. 1). The isolated strains showed the cell size of (NTMP04 width- 3.7 μm , length-3.7 μm), (NTMP05 width- 2.96 μm , length- 3.7 μm) (NTMP06 width- 3.7 μm , length-3.7 μm). The strains were identified as *Chlorella vulgaris* NTMP04, *Chlorella vulgaris* NTMP05 and *Chlorella vulgaris* NTMP06 (Fig. 2).

The mutation was carried out in all the strains using guanidine thiocyanate. Among the three strains of *Chlorella vulgaris* only one strain NTMP04 showed the successful mutation. The treated cells were inoculated in SG II– NO_3 and SGII– NH_4 and incubated for 12–15 days at $25 \pm 1^\circ\text{C}$ under 1,500–2,000 lux light intensity with 16:18 h

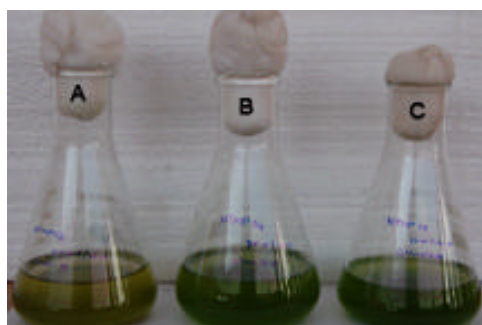


Fig. 1: Growth of microalgae in Bold's basal medium. (A) *Chlorella vulgaris* NTMP04 (B) *Chlorella vulgaris* NTMP05 (C) *Chlorella vulgaris* NTMP06.

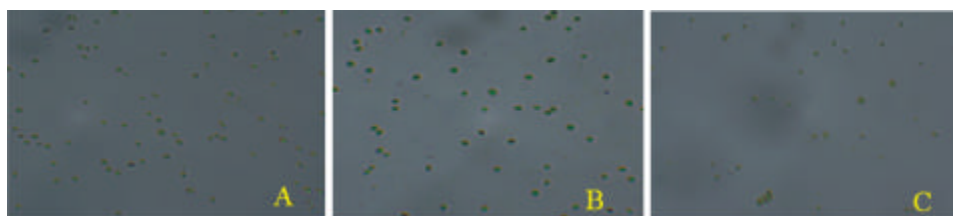


Fig. 2: Microphotograph of (A) *Chlorella vulgaris* NTMP04. (B) *Chlorella vulgaris* NTMP05 and (C) *Chlorella vulgaris* NTMP06.

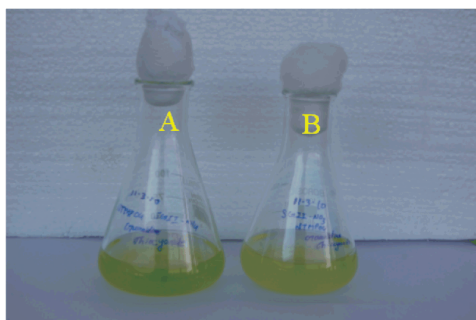


Fig. 3: Growth of mutant strains of *Chlorella vulgaris* NTMP04 in (A) SGII-NH₄ and (B) SGII-NO₃



Fig. 4: Genomic DNA from (W) wild and (M) mutant strains of *Chlorella vulgaris* NTMP04.

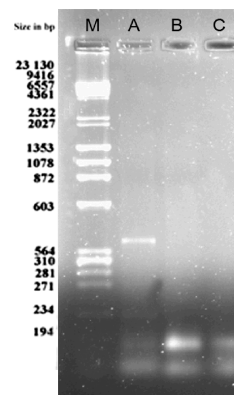


Fig. 5: NR gene amplification in wild and mutant strains of *Chlorella vulgaris* NTMP04. M Marker (1 kb), A wild strain, B mutant strain

light photoperiod. After 15 days, OD value 0.223 was observed in *Chlorella vulgaris* NTMP04 in SG II-NO₃. Mutant strain was confirmed by their ability to grow in the SG II-NH₄ medium in the increased value of 0.257 and it was subjected to further evaluation. The result was shown in (Fig. 3).

The molecular weight of mutant and wild strains DNA was similar in agarose electrophoresis (Fig. 4). The extracted DNA from the mutant and wild strains were subjected to PCR amplification using the specific primers (NR-F and NR-R), the amplicon of wild strain alone showed the banding pattern in 570 bp (Fig. 5) NR gene

was mutated in the mutant strain and hence the amplification was failed in the above strain. It reveals the occurrence of chemical mutation in the *Chlorella vulgaris* NTMP04.

DISCUSSION

In the present study, guanidine thiocyanate was used as mutagen, the mutation was confirmed by their ability to grow in the SGII-NH₄ medium and comparatively low growth in SGII-NO₃ medium (Fig. 3). Similar mutational study was previously reported [13] in NR gene of *Chlorella sorokiniana* using chemical mutagen, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. Nitrate reductase gene was present in all types of nitrogen fixers and also in green plant, but the expression of the particular gene has varied. No more studies found on guanidium thiocyanate based mutation of NR gene. So, these may be the new attempt on GTC based chemical mutation of NR gene. According to Wang *et al.*, [15], amplified cDNA of NR gene from *Chlorella ellipsoidea* using the degenerative primer has length of below 1 kb. The amplified product showed the band in 2,600 bp. Among the strains, the significant band was observed only from *Chlorella vulgaris* NTMP04. The size of the band was observed at ~570 bp (Fig. 5). Dawson *et al.* 1996 stated that the NR gene of *Chlorella* sp was encoded by a single gene located in a cluster of genes involved in nitrate assimilation and has also reported about the presence of 18 intron regions in the NR genes of *Chlorella vulgaris*. Studies of Gruber *et al.*, [16] and Merchant *et al.*, [17] showed the presence of ten intron regions in *Volvox carteri* and 15 introns in *Chlamydomonas reinhardtii*. Cramer and Myers *et al.*, [8] Stated the importance of light in promoting the synthesis of NR is probably indirect and linked to carbon metabolism. It has also been known that *Chlorella* will assimilate nitrate in darkness, if provided with a source of carbon. The mutant strains of *Chlorella vulgaris* was failed to yield the amplification in the 1.2% agarose gel electrophoresis (Fig. 5). Based on the above study realized the confirmation of mutation in *Chlorella vulgaris* NTMP04.

CONCLUSION

In conclusion, we are proposing the guanidium thiocyanate as a chemical mutagen to mutate the NR gene in *Chlorella vulgaris* NTMP04. The effect of mutation was successfully evidenced by the PCR technique. Further study will be based on the co- transformation of

NR gene in mutant strain to check the nitrate assimilation and NR enzyme activity because the enzyme for nitrate assimilation is the major factor for their survival in the environment.

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