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Studies on Acid Stress Tolerant Proteins of Cyanobacterium

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Abstract: Cyanobacterial cultures isolated from diverse environment of acidic condition were studied for their tolerance mechanism. The identified predominant genera of *Anabaena*, *Westiellopsis* and *Nostoc* were taken for study. Among the acid tolerant cyanobacterial cultures, a protein of 15.7 kDa was identified in the cyanobacterium *Nostoc* sp. governing for acid tolerance mechanism. The N terminal sequencing of the desired protein were done to construct a suitable primer for the identification of desired gene. This may serve as a tool in engineering them onto suitable saline tolerant effective nitrogen fixers making them a good candidature for all type of soils thus directly influencing the productivity of rice.

Key words: Cyanobacteria, pH, protein profiling, kDa, N-terminal sequencing, degenerate primer

INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes that utilize oxygen evolving photosynthesis similar to higher plants (Bryant, 1987). Many cyanobacteria fix nitrogen under aerobic conditions in specialized cells called heterocysts which comprise 5-10% of cells in a filament (Fleming and Haselkorn, 1973). The unique feature of cyanobacteria is their versatility of occurrence (Brock, 1973). Cyanobacteria have been isolated from diverse habitats including saline soils (Amsaveni, 1995), hot springs (Ward *et al*., 1997) and coastal swamps (Komarek, 1998). The practice of utilizing cyanobacteria as an efficient source of biofertilizer for rice has been advocated and adopted in India (Venkataraman, 1981). Among the various soil factors affecting the distribution of cyanobacteria, pH is particularly important directly influencing cyanobacterial distribution and abundance in soil (Sardeshpande and Goyal, 1981). Cyanobacteria generally prefer neutral to slightly alkaline conditions (Singh, 1978).

Do acid-tolerant cyanobacteria exist? In a phytoplankton survey of 10 lakes in the Bavarian Forest as well as the lignite mining districts of Bavaria (Upper Palatine) and Lusatia, covering a pH gradient from 8.0 to 2.8, Steinberg *et al*. (1998) demonstrated that acid-tolerant cyanobacteria do exist. Later, Madhosoodhan and Dominic (1999) found that a large diversity of cyanobacteria are distributed profusely over a wide range of acidic pH regions of Kerala soils. Recently several promising cyanobacterial cultures were isolated from problem-specific soils in Tamil, Nadu India, which could definitely increase rice production in those soils. In India acid soils constitute a major component deficient in available nutrients. Only iron and aluminum are available in excess amounts but this causes toxicity to many microorganisms (Das, 1996). Previously Moore (1963) reported that many cyanophyceae were strongly related to soil acidity although acidity was a limiting factor. He also reported many nitrogen-fixing cyanobacteria in fairly acid soils. Aiyer (1965) reported that in acid soils many cyanobacteria flourish well even at an acidity of pH 3.8. However the predominant genera were recorded only in neutral or slightly alkaline soils (Fogg *et al*., 1973; Brock, 1973; Singh, 1978; Raghava Reddy *et al*., 1980). Jurgensen and Davey (1968) reported that cyanobacteria also survive under acidic conditions in field soils suggesting that they tolerate low pH levels. Gopalaswamy *et al*. (2002a) studied the biomass and biochemical constituents of acid tolerant cyanobacterium and found maximum acid tolerance at pH 5.

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Stress alleviation in cyanobacteria has been known to be achieved through the production of stress proteins (Webb and Sherman, 1994). Cyanobacteria are known to synthesize a variety of proteins in response to various stresses. The mechanism of tolerance to various stresses is governed by the genes and the proteins assisting them. The exact mechanism of acid tolerance in cyanobacteria is yet to be elucidated. In the present investigation attempts were made to elucidate these specific proteins governing for acid tolerance mechanism from isolated acid tolerant strains of cyanobacterium. The proteins thus conferring the acid stress tolerant property were identified, purified and characterized. The acid tolerant protein so identified will be used to identify the specific gene that will pave the way for future transformation using other promising and predominant neutrophilic and alkalophilic cyanobacteria thereby making them a good candidate biofertilizer for all type of rice soils.

MATERIALS AND METHODS

Bacterial Strains

The present investigation was conducted in Algal Biotechnology Lab, Department of Plant Molecular Biology and Biotechnology, Tamil Nadu, Agricultural University (TNAU), Coimbatore, India during the period of June 2003 to October 2005. The cultures were isolated earlier, characterized and maintained in the laboratory. Six cyanobacterial cultures viz., *Westiellopsis*-ARM 48 (non acid tolerant culture), *Westiellopsis* AT-TGK-4A2 (acid tolerant), *Westiellopsis*-AT-TGK-4A7 (acid tolerant), *Westiellopsis*-AT-TGK-5B2 (acid tolerant), *Nostoc*-GG-SK-A7 (acid tolerant) and *Anabaena*-GG-SK-A7 (reference) were taken for the study.

Culture Conditions

The pure cyanobacterial cultures were inoculated at the rate of 0.00125-0.00150 g mL^{-1} sterile N-free BG-11_0 broth kept in 250 mL conical flasks. Seven days old cyanobacterial cultures were inoculated in all the flasks. The flasks were incubated under 3000 lux light intensity at 28±1°C, for 16/8 h day/night cycle for a period of 21 days. The cultures were grown on different pH of 4.0, 5.0, 6.0 and neutral condition.

Stress Treatments

The cultures in the flask were maintained to be in acidic medium using citrate buffer. The addition of the buffer were done regularly at an interval of 16 h, since the secretion of ammonia in the medium by the culture turns over the condition from acidic towards the normal/saline condition. The amount thus added for different pH was also standardized during the period of experimentation.

Sample Preparation and Protein Analysis

Total proteins were extracted from the acid tolerant and reference cultures. The total protein content of the cyanobacterial cultures was estimated by following the method of Lowry *et al*. (1951). The separation of protein was done by SDS-PAGE following the method described by Maniatis *et al*. (1987). Fifteen microgram of total protein were loaded onto each well to study the protein profile of the cultures.

Elution and Concentration

The size of molecular weight of the unknown band was determined using graphical method employing calibration curve (Weber and Osborn, 1969). To excise the identified protein of 15.7 kDa size and elute the protein from gel the electro elution method was adopted as described by Maniatis *et al*. (1987). The eluted samples in the vials were lyophilized based on Halliday and Baker (1985).

Blotting and Sequencing

The concentrate protein sample was ran in SDS-PAGE and were kept without staining and destaining for transferring to the PVDF membrane. The protein was blotted onto PVDF membrane (Pal corporation) using the Semi-dry blotting apparatus (Bio-Rad, USA). The N-terminal amino acid sequence was determined using the facility available at the Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala following the Edman degradation method (Niall, 1973).

The analysis was carried out on a Shimadzu model PPSQ-21 protein sequencer using a Wako pack -PTH amino acid column (C18) maintained at 40°C. The coupling and conversion reactions were carried out at 45 and 65°C, respectively.

RESULTS

Cyanobacterial Protein Estimation

The total protein content of the standard reference cultures (adapted to neutral pH) and the acid tolerant cyanobacterial cultures was estimated 21 days after inoculation by Lowry *et al*. (1951) (Table 1).

The protein content of both acid and non acid tolerant cyanobacterial cultures varied significantly based on their tolerance to their pH. A higher protein content of 1280 μg mL^{-1} was observed in non acid tolerant *Westiellopsis* ARM 48 which drastically reduced to pH with 1090 μg mL^{-1}. This shows that the growth and tolerance of *Westiellopsis* ARM 48 is affected by shift in pH towards acidity. A reverse trend was observed in acid tolerant cyanobacterial cultures which showed increased total content of protein towards the shift of acidity except *Anabaena*-GG-SK-A7. Since this culture can grow well at pH of 6.0 and gets reduced towards acidity. The protein content was 1720 μg mL^{-1} at pH 6.0 and reduced to 920 μg mL^{-1} at pH 4.0. At pH of 4.0 the highest total protein content was observed in acid tolerant *Westiellopsis*-AT-TGK-4A2 followed by *Westiellopsis*-AT-TGK-4A7, *Nostoc*-GG-SK-4B3 and *Westiellopsis*-AT-TGK-5B2, respectively. At pH of 7.0, the highest protein content was observed in *Anabaena*-GG-SK-A7 (Reference) and non acid tolerant *Westiellopsis* ARM 48 cultures.

From the above results it's clear that acid tolerant cultures showed a higher total protein content at acidic pH and reduced towards salinity whereas non acid tolerant cultures showed increased protein content in neutral while comparatively reduced towards the shift of acidity.

Comparison of Non Acid Tolerant and Acid Tolerant Proteins of Cyanobacteria

About 15 μg of protein extracts from the acid tolerant and non acid tolerant cyanobacterial cultures derived from different pH were loaded in each well and the resulted profiles are presented in Fig. 1-6.

The comparison studies revealed no particular difference among the profile except that for non stress proteins, wherein unique band of 23 kDa was present which was absent in acid tolerant *Westiellopsis*- AT-TGK-4A2. These results revealed that these proteins govern specially for non acid tolerant condition. The cyanobacterial culture *Anabaena*-GG-SK-A7 was used as a reference culture for the study. In this present study, similar protein of 52±2 kDa was found to occur in *Anabaena*-GG-SK-A7 under acidic condition. Under similar conditions, *Nostoc*-GG-SK-A7 expressed a protein of

Table 1: Total protein content of the non-acid stress and acid stress cyanobacteria (μg mL^{-1})

Cultures	pH 4.0	pH 5.0	pH 6.0	pH 7.0
Westiellopsis ARM 48	1090	1200	1280	1280
Westiellopsis-AT-TGK-4A2	2100	1460	1030	840
Anabaena-GG-SK-A7	920	1150	1720	2180
Westiellopsis-AT-TGK-4A7	1960	1680	1410	500
Nostoc-GG-SK-4B3	1290	1190	1040	740
Westiellopsis-AT-TGK-5B2	1130	1350	1080	570

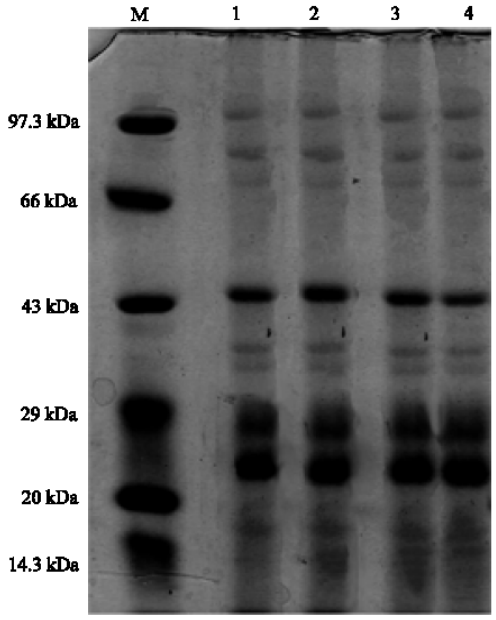

Fig. 1: Protein profile of *Westiellopsis*-ARM-48 at different pH, M: Medium range marker, Lane 1: *Westiellopsis*-ARM 48 at pH 4.0, Lane 2: *Westiellopsis*-ARM 48 at pH 5.0, Lane 3: *Westiellopsis*-ARM 48 at pH 6.0, Lane 4: *Westiellopsis*-ARM 48 at pH 7.0

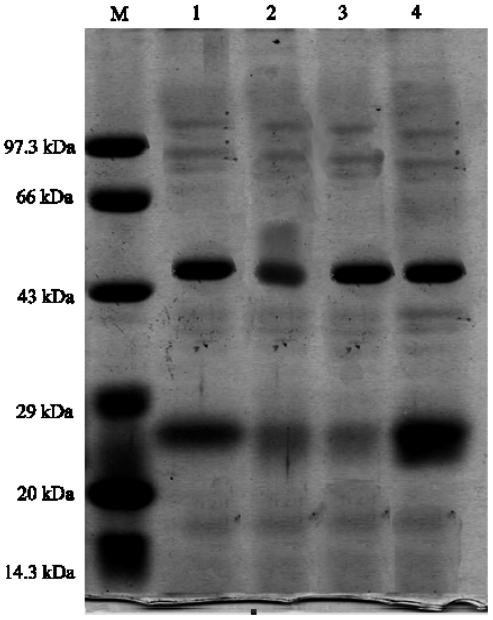

Fig. 2: Protein profile of *Westiellopsis* AT-TGK-4A2 at different pH, M: Medium range marker, Lane 1: *Westiellopsis*-AT-TGK-4A2 at pH 4.0, Lane 2: *Westiellopsis*-AT-TGK4A2 at pH 5.0, Lane 3: *Westiellopsis*-AT-TGK-4A2 at pH 6.0, lane 4-*Westiellopsis*-AT-TGK-4A2 at pH 7.0

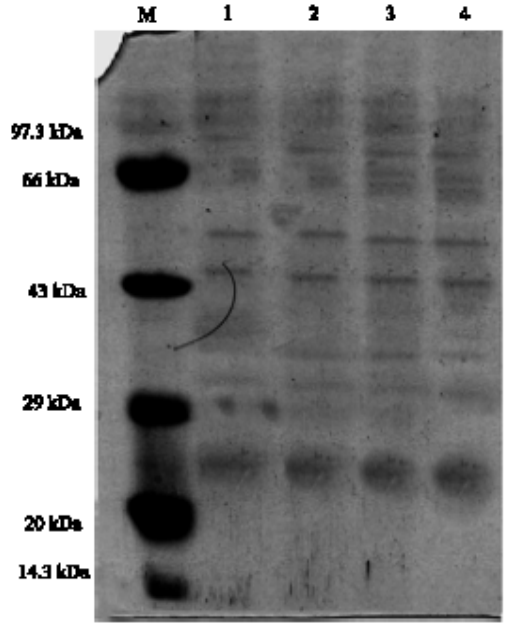

Fig. 3: Protein profile of *Anabaena*-GG-SK-A7 at different pH, M: Medium range marker, Lane 1: *Anabaena* GG-SK-A7 at pH 4.0, Lane 2: *Anabaena* GG-SK-A7 at pH 5.0, Lane 3: *Anabaena* GG-SK-A7 at pH 6.0, Lane 4: *Anabaena* GG-SK-A7 at pH 7.0

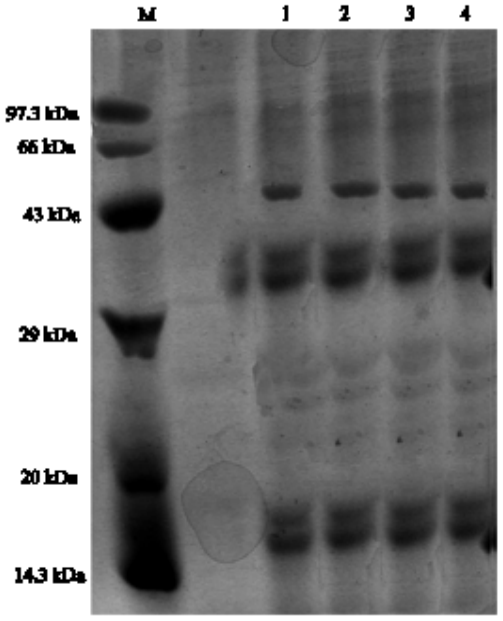

Fig. 4: Protein profile of *Westiellopsis* At-TGK 4A7 at different pH, M-Medium range marker, Lane 1: *Westiellopsis* At-TGK-4A7 at pH 4.0, Lane 2: *Westiellopsis* At-TGK-4A7 at pH 5.0, Lane 3: *Westiellopsis* At-TGK-4A7 at pH 6.0, Lane 4: *Westiellopsis* At-TGK-4A7 at pH 7.0

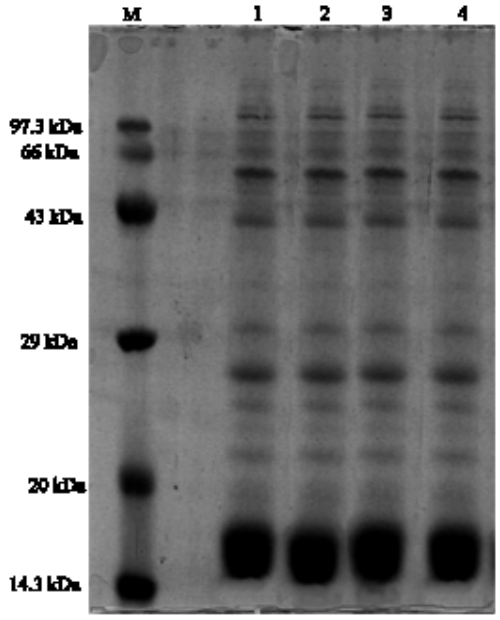

Fig. 5: Protein profile of *Nostoc*-GG-SK-4B3 at different pH, M: Medium range marker, Lane 1: *Nostoc*-GG-SK-4B3 at pH 4.0, Lane 2: *Nostoc*-GG-SK-4B3 at pH 5.0, Lane 3: *Nostoc* GG-SK-4B3 at pH 6.0, Lane 4: *Nostoc*-GG-SK-4B3 at pH 7.0

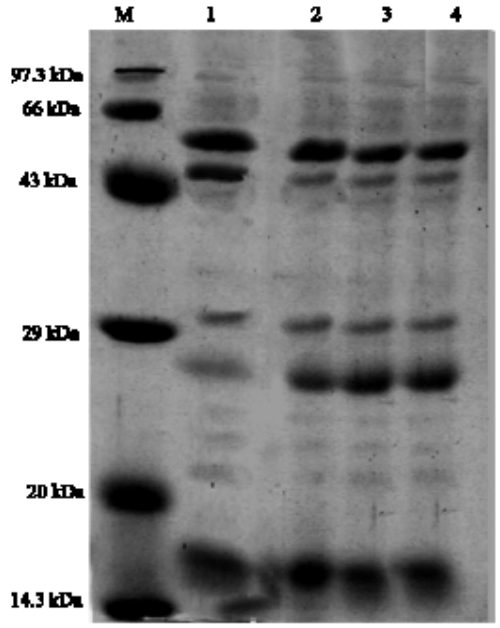

Fig. 6: Protein profile of *Westiellopsis* At-TGK 5B2 at different pH, M: Medium range marker, Lane 1: *Westiellopsis*-At-TGK-5B2 at pH 4.0, Lane 2: *Westiellopsis*-At-TGK5B3 at pH 5.0, Lane 3: *Westiellopsis*-At-TGK-5B3 at pH 6.0, Lane 4: *Westiellopsis*-AT-TGK-5B3 at pH 7.0

Table 2: N-terminal amino acid sequence of the acid tolerant protein

Single letter	Three letter code	Amino acid
T	Thr	Threonine
F	Phe	Phenylalanine
S	Ser	Serine
P	Pro	Proline
Q	Gln	Glutamine

Single letter notation T F T S P P P P P Q. Three letter notation, Thr, Phe, Thr, Ser, Pro, Pro, Pro, Pro, Pro, Gln

Table 3: Degenerate nucleotide sequence for acid tolerant protein

THR	PHE	THR	SER	PRO	PRO	PRO	PRO	PRO	GLN
ACU	UUU	ACU	AGU	CCU	CCU	CCU	CCU	CCU	CAA
ACC	UUC	ACC	AGC	CCC	CCC	CCC	CCC	CCC	CAG
ACA		ACA		CCA	CCA	CCA	CCA	CCA	
ACG		ACG		CCG	CCG	CCG	CCG	CCG	
AC	TT	AC	AG	CC	CC	CC	CC	CC	
(A/C/G/T)	(C/T)	(T/C/A/G)	(T/C)	(T/C/A/G)	(T/C/A/G)	(T/C/A/G)	(T/C/A/G)	(T/C/A/G)	CA(A/G)
ACN	TTY	ACN	AGY	CCN	CCN	CCN	CCN	CCN	CAR

same size. This protein was not expressed in other cultures viz., *Westiellopsis*-ARM 48, *Westiellopsis*-AT-TGK-4A2, *Westiellopsis*-AT-TGK-4A7 and *Westiellopsis*-AT-TGK-5B2. The comparison studies revealed no particular difference among the profile except that for non stress proteins, wherein one unique band of 23 kDa was present, which was absent in *Anabaena*-GG-SK-A7. These results were found to be similar for these proteins as in *Westiellopsis*-AT-TGK-4A2. These results confirm that the proteins govern specially for non acid tolerant condition. On subjecting *Westiellopsis*- AT-TGK-4A7 to acidic stress condition, induction of 15.7 kDa acid tolerant protein was found to be expressed. The same size proteins were also found to occur in *Nostoc*-GG-SK-A7 and *Westiellopsis*-AT-TGK-5B2. This protein was absent in other cultures. The result revealed the presence of a unique 15.7 kDa acid tolerant protein, which was absent in non-acid *Westiellopsis*-ARM 48. The result also revealed the presence of two more unique bands of size ranging 22-23 kDa that was absent in non-acid *Westiellopsis*-ARM 48. The results revealed the presence of a unique acid tolerant protein of size ranging 52±2 kDa in size also found in *Anabaena*-GG-SK-A7, which were absent in the non-acid *Westiellopsis* - ARM 48. The profile also revealed the presence of unique acid tolerant proteins of size ranging from 102-103 kDa. The presence of all this unique proteins confers that these proteins may govern for acid tolerance in acid tolerant cyanobacteria. On subjecting *Westiellopsis*-AT-TGK-5B2 to acidic stress condition, induction of 15.7 kDa acid tolerant protein was found to be expressed. The result also revealed the presence of unique band of size ranging 29±2 kDa. These proteins were found to be absent in non acid tolerant *Westiellopsis*-ARM 48.

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequencing of the 15.7 kDa protein was done for ten cycles and the resultant amino acid sequence is shown in Table 2. On back translation of the ten amino acids, a degenerate nucleotide sequence was obtained and is shown in Table 3.

DISCUSSION

Does an acid tolerant cyanobacterium exist? This remained a major contradiction and challenging perspective in phycology. As a result the occurrence of the phytoplankton mass was reported in acidic lake at pH of 8.0 to 2.8 (Steinberg *et al*., 1998). In a study conducted among the 240 composite samples 68, 89, 65 and 18 respectively, were reported from pH range 6-5, 5-4, 4-3 and 3-2.8 (Madhosoodhan and Dominic, 1999). Later few reports have been made on the isolation of cyanobacteria from acidic soils of India (Gopalaswamy *et al*., 2002a). Few off cyanobacterium cultures were studied for their growth and biomass production and were found to be best at pH 4.0 (Gopalaswamy *et al*., 2002b). This confirms that cyanobacteria do live on acidic condition but the exact

molecular mechanism remains yet to be revealed. Stress alleviation in cyanobacteria has been known to be achieved through the production of stress proteins (Webb and Sherman, 1994).

Huang *et al*. (2002) reported that Lethal acid stress at a pH below 4.4 results in the formation of aggregates of denatured proteins observed as granules near the cell periphery the disruption of the transmembrane pH gradient cell color change to blue and damage to photosystem II. In support to this in non acid tolerant *Westiellopsis*-ARM 48 only fewer protein were found to occur. There was much variation in other acid tolerant cyanobacterial cultures which produced other protein(s). But a unique band of 23 kDa was present which was absent in *Westiellopsis*-AT-TGK-4A2 that showed chlorosis at pH 7.0. This might infer that the specific protein may govern for saline tolerance mechanism. From the above result much of the present study can be correlated with various protein observed during stress conditions. On subjecting *Westiellopsis*-AT-TGK-4A7 to acidic stress condition induction of 15.7 kDa acid tolerant protein was found to be expressed. The same size proteins were also found to occur in *Nostoc*-GG-SK-A7 and *Westiellopsis*-AT-TGK-5B2. Roy *et al*. (1999) observed a similar trend in 16 kDa protein upon heat-shock treatment in the thermophilic cyanobacterium *Synechococcus vulcanus*.

Also a chloroplastic drought-induced stress protein 32 kDa was observed when induced by environmental and oxidative stress (Broin *et al*., 2002). The protein can be accorded to the protein of 29±2 kDa found in *Westiellopsis*-AT-TGK-5B2 specifically due to acid stress condition. The present study also revealed the presence of 52±2 kDa in *Anabaena*-GG-SK-A7 acidic condition under acidic condition. Under similar conditions *Nostoc*-GG-SK-A7 expressed a protein of same size. Similarly stress responsive protein of about 58 kDa was reported by Weissman *et al*. (1996) in *Synechococcus* sp. strain PCC 7942 in response to light temperature and metal stress. Eriksson *et al*. (2001) characterized *clpB* gene that encodes a 97 kDa protein with novel features in *Synechococcus* sp. strain PCC 7942 under heat shock. Recent study by Akira *et al*. (2001) revealed protein SynNhaP with a molecular weight of 129 kDa was in *Synechocystis* sp. PCC 6803 when subjected to an acidic pH of 5.0 in *Synechocystis* sp. PCC 6803. A similar trend was observed in the present study wherein a 103±2 kDa protein was found to occur at the same range in acid tolerant *Nostoc*-GG-SK-A7. Thus it is clear that this protein might be in response to the acidic stress condition. The above results confirm that the above revealed protein(s) are produced due to stress and govern for acid tolerance mechanism.

The mechanism of tolerance to various stresses is governed by the genes and the proteins assisting them. With the help of derived a degenerate nucleotide, primer sequence can be constructed to identify the desired fragment or gene governing for acid tolerance. This may be designed that could pave the way for the cloning and sequencing of the gene encoding the acid stress protein. The gene could be cloned and used for transformation to other promising and predominant neutrophilic and alkalophilic cyanobacteria thereby making them good candidate biofertilizers for all types of rice soils for wider adaptability.

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