

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.706.117>

Characterization of Cyanobacterial Isolates from Rann of Kutch for Salinity Tolerance

Yattapu Prasad Reddy*, Ravindra Kumar Yadav and G. Abraham

Centre for Conservation and Utilization of BGA, ICAR-Indian Agricultural Research Institute,
New Delhi-110012, India

*Corresponding author

ABSTRACT

Keywords

Cyanobacteria, Extreme environment, 16S rDNA

Article Info

Accepted:

06 May 2018

Available Online:

10 June 2018

Isolation and characterization of cyanobacteria from extreme environment is important in identifying novel molecules and understanding the mechanisms underlying the tolerance. Therefore in the present study an attempt was made to isolate, identify and characterize cyanobacteria from Rann of Kutch experiencing high levels of salinity. On the basis of microscopic, morphological and cultural characteristics the identified cyanobacterial strains included *Anabaena*, *Nostoc*, *Westiellipsoidis*, *Phormidium* and *Synechocystis*. Further analysis on the basis of 16S rDNA sequence and BLAST analysis confirmed their identity. Growth, cellular constituents and parameters of nitrogen assimilation in these strains were studied and showed significant differences. The present investigation helped in the identification of cyanobacterial strains tolerant to high salt concentration will help in selecting appropriate strains as bio-fertilizer.

Introduction

Cyanobacteria occupy one of the largest and diverse groups of photosynthetic organisms having a unique distribution pattern. They have been found to colonize successfully in several diverse types of ecosystems. The origin of cyanobacteria dates back to the Pre-Cambrian times and this long evolutionary history holds the key to their competitive success to thrive in a wide range of environment. They have the ability to tolerate high temperature, UV desiccation, water and saline stress (Gröniger and Hader, 2000; Whitton and Potts, 2000; Herrero and Flores, 2008). These organisms are important because they made the atmosphere oxygenic and

allowed other organisms to develop. The cyanobacteria are the simplest photosynthetic organisms and are the only prokaryotic organisms to carry out a higher plant type oxygen evolving photosynthesis. Their chloroplasts are remarkably similar to higher plant chloroplasts in structure and function. They are important as primary producers in soil, fresh water and marine environments.

Since, time immemorial the cyanobacteria played key role in maintaining the sustainability of ecosystems and improved soil fertility and crop production. Further advancement in the field of microbiology and biotechnology helped in the exploration of cyanobacteria for industrially important and

valuable products from them. The cyanobacteria are also rich sources of industrially important compounds. Burja *et al.*, (2001) reported a variety of cyanobacterial products from diverse cyanobacteria. In this context exploration of newer habitats, especially the extreme ones is important from a biotechnological prospective. Hence, search for these organisms from extreme environments would be an important strategy to isolate and identify potential organisms for exploitation. The cyanobacterial isolates from extreme habitats may constitute an important component of the vast an unexplored biological potential. The cyanobacteria are able to survive not very high salt concentrations prevailing in diverse hyper saline environments. Oren, (2000) reported that cyanobacteria from extreme environment can contribute significantly to the productivity of such environments. However, there have been very few reports on the cyanobacteria from extreme environment such as Runn of Kutch till date.

Materials and Methods

Soil samples were collected randomly from five different sites from the Runn of Kutch region of Western Gujarat during June, 2011. These soil samples were used to isolate cyanobacterial strains following standard enrichment culture techniques (Stanier *et al.*, 1971). BG-11 medium (+N for non heterocystous and -N for heterocystous strains) was used for the isolation procedure. The medium used for the isolation was made saline by the addition of sodium chloride at a concentration of 1-5% and the pH was adjusted to 8.0. The isolated and identified cultures were maintained in the culture room having light intensity of 52-55 μ mol photon/m²/S, 16/8 light and dark period and 28 \pm 2 °C temperature. Strains were examined microscopically and the morphological characters were compared according to

Desikachary (Desikachary, 1959). For molecular identification, the technique 16S r DNA gene sequencing was used for the identification of cyanobacterial strains. DNA extraction was carried out using DNeasy Tissue Kit and by following the Manufacturer's Protocol (Genetics, USA). Amplification of 16S rDNA gene fragment was done according to Weisburg *et al.*, (1991) and Lyra *et al.*, (1997). The amplified PCR products were electrophoresed, stained and visualized. The amplified product was sequenced partially. The 16S partial gene sequence was subjected to search for highly similar sequence using mega blast and sequences with the highest similarity indicated by BLAST were identified.

Exponentially growing (15th day of incubation) cultures were used for the determination of growth and other physiological variables. The dry weight determination was done according to (Sorokin, 1973). Chlorophyll was determined by the protocol given by (McKinney, 1941) and the carotenoids were estimated according to (Jensen, 1978). Freezing thawing protocol was used for the study of phycobiliproteins (Bennett and Bogorad, 1973). Total protein was estimated by the method of (Lowry *et al.*, 1951). Total carbohydrates were estimated by the method of (Spiro, 1966). The activity of nitrate reductase enzyme was estimated according to (Herrero *et al.*, 1981) and the enzyme activity is expressed as μ mol NO₂ formed per mg protein by using standard curve of nitrate. Activity of glutamine synthetase was estimated according to (Shapiro and Stadtman, 1970). Determination of nitrogenase activity was performed by acetylene reduction assay of (Stewart *et al.*, 1968). The results were analyzed by using the statistical package SPSS 10.0. Duncan's multiple range test (DMRT) was employed to compare the mean performances of different treatments for the parameters used in the

study. The rankings have been denoted by superscripts in appropriate tables.

Results and Discussion

The morphological attributes of the cyanobacterial isolates from Rann of Kutch identified on the basis of cultural parameters and microscopic observations is shown (Table 1 and 2). Cyanobacterial forms identified on the basis of morphology belong to *Anabaena*, *Nostoc*, *Phormidium* and *Westiellopsis*. The cyanobacterial strains of *Anabaena* sp., *Nostoc* sp., *Phormidium* and *Westiellopsis* sp. (YPR-4, YPR-6, YPR-8 and YPR-10) grew well at 1% NaCl. However, at 2% NaCl, strains of *Westiellopsis* (YPR-5) were recorded. The strains found to grow at 3% salinity consisted of *Phormidium* sp., *Anabaena* sp. and *Nostoc* sp (YPR-1, YPR-2, YPR-3, YPR-7 and YPR-9). 16S rDNA gene sequencing was used for the identification of cyanobacterial isolates. The extracted and quantified DNA from cyanobacterial isolates was subjected to 16S rDNA gene amplification with the primers FD1 and RP2 (Plate 1). Single amplified product of 1500 bp for 16S rDNA was observed in the all the strains examined (Plate 2). The amplified product was used for sequence analysis and the BLAST analysis established the homology in terms of % similarity (Table 3). The cyanobacterial strains of *Synechocystis*, *Phormidium*, *Anabaena*, *Nostoc* and *Westiellopsis* showed close similarity with *Synechocystis* sp (98%), *Phormidium inundatum* (98%), *Phormidium priestleyi* ANT.LACV5.1 (99%), *Anabaena bergii* 09-02 (99%), *Anabaena oryzae* (97%), *Nostoc elgonense* TH3S05 (97%), *Nostoc commune* VB516200 (99%), *Nostoc punctiforme* (99%), *Westiellopsis prolifica* (99%), *Westiellopsis* sp. 1590-2 (97%) respectively.

These strains have been subsequently analyzed for growth and physiological

parameters. The growth (dry weight) of these cyanobacterial isolates showed significant variation amongst them (Table 4). Chlorophyll ranged between 0.010 to 2.22 $\mu\text{g/ml}$. YPR-2 showed maximum chlorophyll content whereas YPR-10 recorded the minimum chlorophyll content.

Table 5 shows the phycocyanin, phycoerythrin, allophycocyanin and total phycobiliprotein content of cyanobacterial isolates. Highest phycobiliprotein content was observed in YPR 9 and lowest was observed in YPR-4. Allophycocyanin, phycocyanin and phycoerythrin were highest in YPR-9.

Lowest phycocyanin and phycoerythrin contents were recorded in recorded in YPR-4 whereas lowest allophycocyanin content was recorded in YPR- 6. The range recorded for phycocyanin was to be in the range of 8.05 to 1.003 $\mu\text{g/ml}$ and allophycocyanin content varied in the range of 0.0237 to 0.284 $\mu\text{g/ml}$. The phycoerythrin content showed variation in the range of 0.24 to 1.93 $\mu\text{g/ml}$. However, highest and lowest carotenoid content was observed in YPR-2 and YPR-6, respectively. Carotenoids ranged between 0.22 and 5.82 $\mu\text{g/ml}$.

The enzymes of nitrogen assimilation were studied in the isolates (Table 6). The nitrate reductase enzyme activity was observed among the strains and the activity ranged between 5.21 to 94.91 $\mu\text{ moles NO}_2$ formed per mg protein. Maximum level of nitrate reductase activity was shown by YPR-4 and lowest activity was observed in YPR-8. Activity of glutamine synthetase varied in the range of 107.372 to 232.41 n mol γ -glutamyl hydroxamate formed min^{-1} mg protein $^{-1}$. Similarly the strain YPR-10 showed higher levels of GS activity as compared to YPR-2 which showed a low level of activity. Acetylene reduction activity was performed among the heterocystous isolates.

Table.1 List of cyanobacterial isolates and growth from Rann of Kutch

Sl. No.	Salt concentration (%)	Strain	Taxonomic group
1	3	YPR-1	<i>Anabaena</i> sp.
2	3	YPR-2	<i>Anabaena</i> sp.
3	3	YPR-3	<i>Phormidium</i> sp.
4	1	YPR-4	<i>Westiellopsis</i> sp.
5	2	YPR-5	<i>Westiellopsis</i> sp.
6	1	YPR-6	<i>Synechocystis</i> sp.
7	3	YPR-7	<i>Anabaena</i> sp.
8	1	YPR-8	<i>Phormidium</i> sp.
9	3	YPR-9	<i>Nostoc</i> sp.
10	1	YPR-10	<i>Nostoc</i> sp.

Table.2 Cultural characteristics of the cyanobacterial isolates from Rann of Kutch

Sl. No.	Taxonomic	Designation	Cultural characteristics			
			Life form	Colour	Pattern of growth	
					Liquid	Solid medium
1	<i>Anabaena</i> sp.	YPR-1	Benthic	Light Green	Uniform	Mucilaginous,
2	<i>Anabaena</i> sp.	YPR-2	Benthic	Dark Green	Spreading	Spreading
3	<i>Phormidium</i> sp.	YPR-3	Benthic	Blue Green	Spreading	Spreading
4	<i>Westiellopsis</i> sp.	YPR-4	Benthic	Dark Green	Uniform	Bead like
5	<i>Westiellopsis</i> sp.	YPR-5	Benthic	Dark Green	Spreading	Spreading
6	<i>Synechocystis</i> sp.	YPR-6	Benthic	Dark Green	Uniform	Bead like
7	<i>Anabaena</i> sp.	YPR-7	Benthic	Light green	Floccose	Mucilaginous,
8	<i>Phormidium</i> sp.	YPR-8	Benthic	Blue Green	Spreading	Spreading
9	<i>Nostoc</i> sp.	YPR-9	Planktonic	Blue Green	Spreading	Spreading
10	<i>Nostoc</i> sp.	YPR-10	Benthic	Light green	Spreading	Spreading

Table.3 Morphologically identified cyanobacterial strains and their close match as revealed by BLAST analysis

Salt (%)	Strain	Morphologically identified genera	Close match based on 16SrDNA sequence	Score (%)
3	YPR-1	<i>Anabaena</i> sp.	<i>Anabaena bergii</i>	98%
3	YPR-2	<i>Anabaena</i> sp.	<i>Anabaena oryzae</i>	97%
3	YPR-3	<i>Phormidium</i> sp.	<i>Phormidium inundatum</i>	98%
1	YPR-4	<i>Westiellopsissp.</i>	<i>Westiellopsis prolifica</i>	99%
2	YPR-5	<i>Westiellopsissp.</i>	<i>Westiellopsis</i> sp.	97%
1	YPR-6	<i>Synechocystissp.</i>	<i>Synechocystis</i> sp	98%
3	YPR-7	<i>Anabaena</i> sp.	<i>Nostoc elgonense TH3S05</i>	99%
1	YPR-8	<i>Phormidium</i> sp.	<i>Phormidium priestleyi</i> ANT.LACV5	99%
3	YPR-9	<i>Nostoc</i> sp.	<i>Nostoc punctiforme</i> VB62229	95%
1	YPR-10	<i>Nostoc</i> sp.	<i>Nostoc commune</i> VB516200	99%

Table.4 Selected physiological attributes of the cyanobacterial isolates from Rann of Kutch showing mycosporine like amino acids

Strains	Dry weight (mg/ml)	Protein (µg/ml)	Sugar (µg/ml)	Chlorophyll (µg/ml)
YPR-1	255.62 ^{fg}	773.84 ^b	605.9 ^b	0.658 ^e
YPR-2	332.02 ^{ab}	949.61 ^a	563.79 ^b	2.219 ^a
YPR-3	316.93 ^{bc}	716.64 ^b	1467.9 ^a	0.372 ^f
YPR- 4	349.68 ^a	106.55 ^d	1377.8 ^a	1.499 ^b
YPR-5	259.28 ^{fg}	327.54 ^c	118.89 ^b	0.276 ^f
YPR- 6	276.94 ^{ef}	144.93 ^d	1363.8 ^a	0.118 ^g
YPR-7	307.51 ^{cd}	716.64 ^b	371.90 ^b	0.778 ^d
YPR-8	248.48 ^g	86.33 ^d	1413.4 ^a	0.359 ^f
YPR-9	291.52 ^{de}	724.95 ^b	563.79 ^b	1.020 ^c
YPR10	266.97 ^{fg}	79.665 ^d	497.11 ^b	0.010 ^g

Table.5 Selected physiological attributes of the cyanobacterial isolates from Rann of Kutch showing mycosporine like amino acids

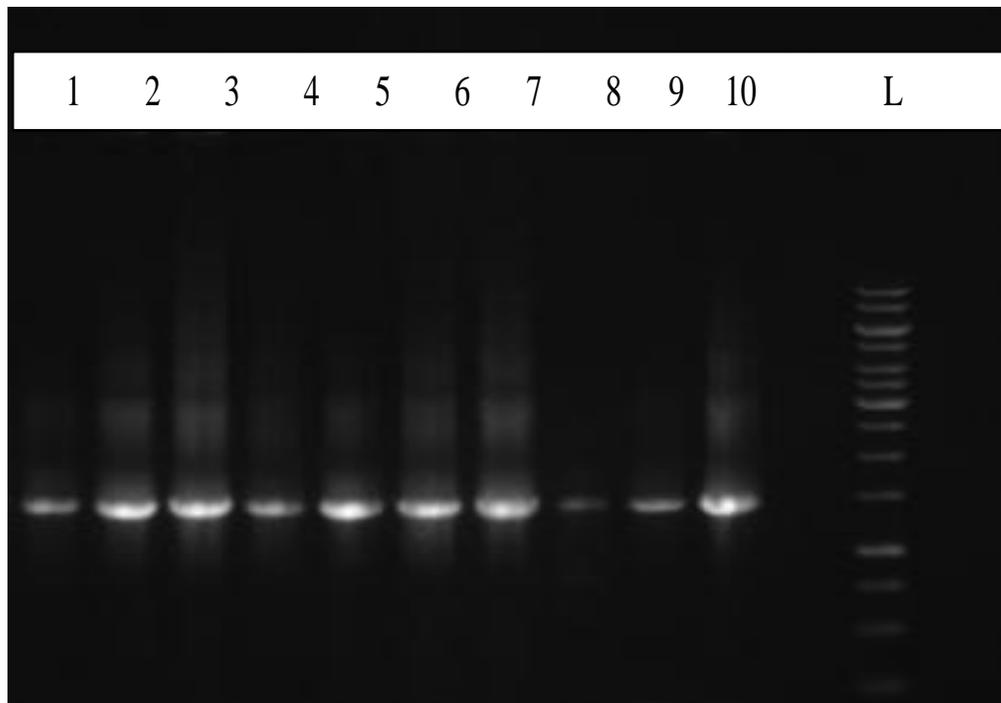
Strains	Carotenoids (µg/ml)	Phycocyanin (µg/ml)	Allophycocynin (µg/ml)	Phycocerythrin (µg/ml)
YPR-1	2.421 ^{bcd}	6.430 ^b	0.133 ^{cd}	1.592 ^{ab}
YPR-2	5.828 ^a	5.640 ^c	0.097 ^d	1.400 ^{abc}
YPR-3	1.577 ^{bcd}	4.270 ^d	0.086 ^d	1.059 ^{bcd}
YPR- 4	3.28 ^{abcd}	1.003 ^f	0.159 ^c	0.239 ^f
YPR-5	3.986 ^{ab}	3.630 ^d	0.156 ^c	0.894 ^{cde}
YPR- 6	0.774 ^{cd}	7.746 ^a	0.027 ^e	1.930 ^a
YPR-7	2.484 ^{bcd}	1.31 ^{ef}	0.226 ^b	0.311 ^{ef}
YPR-8	1.444 ^{bcd}	2.016 ^e	0.120 ^{cd}	0.494 ^{def}
YPR-9	3.600 ^{abc}	8.05 ^a	0.282 ^a	1.418 ^{abc}
YPR10	0.244 ^d	7.483 ^a	0.081 ^d	1.861 ^a

Table.6 Activity of enzymes involved in nitrogen metabolism in the cyanobacterial isolates from Rann of Kutch

Strains	ARA (μ mol/mg chl/h)	NR (μ mol/mg protein/h)	GS (μ mol/mg protein)
YPR-1	17.949 ^b	16.559 ^{cd}	181.248 ^{bc}
YPR-2	4.277 ^e	18.421 ^{bc}	107.372 ^e
YPR-3	-----	28.914 ^b	135.865 ^{cd}
YPR-4	6.29 ^e	94.914 ^a	186.069 ^{bc}
YPR-5	35.28 ^a	12.913 ^{cd}	204.143 ^{abc}
YPR-6	-----	14.801 ^{cd}	130.496 ^{de}
YPR-7	14.42 ^c	15.379 ^{cd}	166.12 ^{cd}
YPR-8	-----	5.206 ^d	211.625 ^{ab}
YPR-9	8.97 ^d	22.82 ^{bc}	182.9389 ^{bc}
YPR-10	8.556 ^d	91.303 ^a	232.401 ^a

ARA= Acetylene reduction activity, NR= Nitrate reductase activity and GS= Glutamine synthetase activity

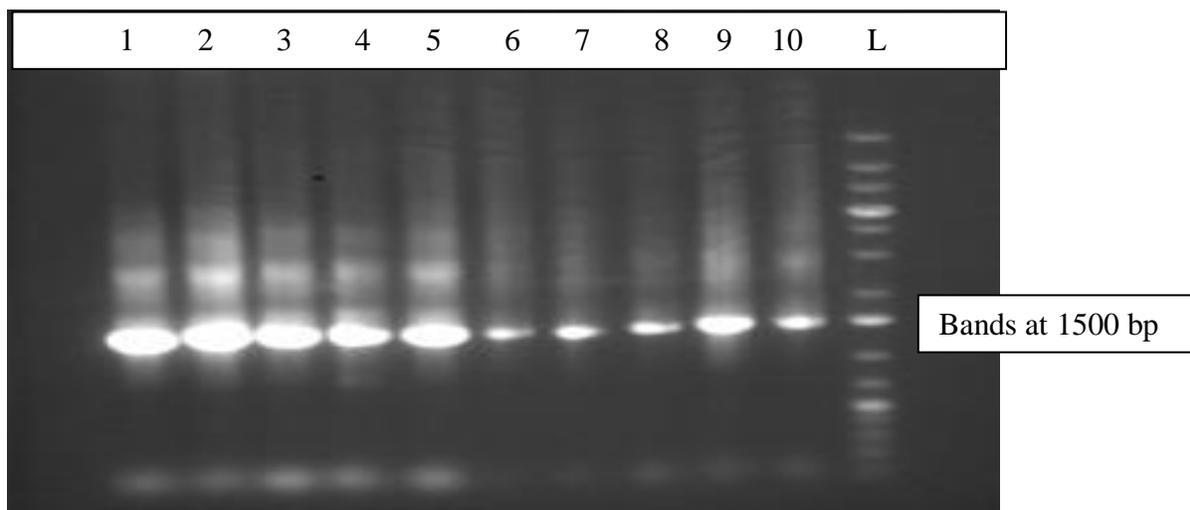
Plate.1 Isolated genomic DNA by Dneasy tissue kit manufacturer's protocol with certain modifications



L= 1 Kb ladder

1.YPR-1, 2.YPR-2, 3.YPR-3, 4.YPR-4, 5.YPR-5, 6.YPR-6, 7.YPR-7, 8.YPR-8, 9.YPR-9, 10.YPR-10

Plate.2 16S rDNA amplified product in cyanobacterial isolates from Rann of Kutch



1.YPR-1, 2.YPR-2, 3.YPR-3, 4.YPR-4, 5.YPR-5, 6.YPR-6, 7.YPR-7, 8.YPR-8, 9.YPR-9, 10.YPR-10, L= ladder

The nitrogenase enzyme activity was found to be in the range of 4.277 to 35.28 μ moles/mg chl/h. The strain YPR-5 showed highest ARA activity as compared to YPR-2 which showed low levels of activity.

Microscopic observations of the samples collected from Rann of Kutch revealed the presence of several cyanobacterial isolates and the identified forms belonged to *Anabaena*, *Nostoc*, *Westiellopsis* and *Phormidium*. Although, there are several reports on the occurrence of cyanobacteria in saline soils the type of cyanobacteria found in such saline soils are different (Ali and Sandhu, 1972; Kaushik, 1989; Shah *et al.*, 2000). In less saline conditions, the heterocystous cyanobacteria have advantage over non heterocystous cyanobacteria due to the presence of glycolipid envelope of the heterocyst (Stal, 1991). According to (Srivastav *et al.*, 2009) low salinity favours heterocystous cyanobacteria where as high salinity supports growth of non-heterocystous genera. It is difficult to characterize the cyanobacteria on the basis of observing structures such as hormogonia, heterocysts and akinetes (Nubel *et al.*, 2000) and hence the molecular identification is important. In

the present study to support the morphological identification of the cyanobacteria, 16SrRNA gene amplification was carried out. BLAST analysis was employed to establish homology in terms of percentage similarity and revealed that the identified cyanobacteria had close similarity with cultured cyanobacteria. (Rudi *et al.*, 1997) used 16S rRNA for individual strain characterization and identification of cyanobacteria. Similar observations were also made by (Srivastav *et al.*, 2009).

The variation in the growth rate of several species of *Anabaena* collected from a geographical habitat was observed by (Meeks *et al.*, 1983). Ambient physiological conditions also play a role in the growth and cellular constituents. Biochemical constituents of cyanobacteria depend upon the nature of strains, physiological conditions and the environment (Maslova *et al.*, 2004; Rosales *et al.*, 2005). (Mekonnen *et al.*, 2002) reported variation in chlorophyll content within the genus as well as species of cyanobacteria characterized. Significant variation with respect to the carotenoids and phycobiliproteins was also observed in the study. *Anabaena* strains isolated from

different geographical locations showed significant differences in the phycobiliprotein content as shown by (Prasanna *et al.*, 2006). The chlorophyll and carotenoids in the organisms is related to the physiological competence of the cells in case of cyanobacteria isolated from hyper-saline environments. Significant differences in the sugar content was observed and those isolates growing at high concentrations of salinity showed very high sugar content as compared to their counterparts growing at low levels of salinity. Cyanobacteria exposed to stress conditions respond by the synthesis of osmotically active compounds such as sugars to counter the salinity stress (Ponti *et al.*, 2007). Increase in the sugar content observed could therefore, be related to the survival of the organisms at higher concentrations of salt. (Asthana *et al.*, 2008) observed enhanced trehalose content in the cyanobacterium *Anabaena* 7120 under salinity stress.

Significant variation with respect to the enzymes involved in nitrogen metabolism such as nitrate reductase, glutamine synthetase and nitrogenase was observed. Increase in the nitrate reductase activity under salinity is due to increased nitrogen demand of the cyanobacteria (Rai and Abraham, 1993). Difference in the nitrate reductase and glutamine synthetase activity has been reported in cyanobacterial strains isolated from extreme Antarctic environment by (Pandey *et al.*, 2004). The sensitivity of the nitrogen fixing makes it difficult for cyanobacteria to survive in extreme habitats (Reed and Stewart, 1985). (Moisander *et al.*, 2002) observed decreased nitrogenase activity in cyanobacterial strains isolated from an estuarine water. The study of these enzymes is important keeping in view of the potential of cyanobacteria as bioinoculants. (Prasanna *et al.*, 2006) also studied these parameters from *Anabaena* isolates collected from different biogeographical habitats.

The studies therefore showed that several cyanobacterial strains isolated from Runn of Kutch exhibited distinct patterns of growth, cellular constituents and nitrogen fixation. The responses are adaptive in nature and help the organism to grow and survive under inhospitable habitats. Further detailed analysis needs to be conducted to decipher the exact nature of tolerance mechanisms studies using molecular tools.

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How to cite this article:

Yattapu Prasad Reddy, Ravindra Kumar Yadav and Abraham G. 2018. Characterization of Cyanobacterial Isolates from Rann of Kutch for Salinity Tolerance. *Int.J.Curr.Microbiol.App.Sci.* 7(06): 994-1003. doi: <https://doi.org/10.20546/ijcmas.2018.706.117>