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# Impact of pH on growth and survival of wild type , MHR (multiple herbicide resistant) and various class of salt/osmotolerant strains of diazotrophic cyanobacterium *Anabaena variabilis*

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**Abstract**

Cyanobacteria,an ubiquitous group of soil micro-organism, specially in flooded rice fields. The rice field ecosystem provides a favourable environment for the growth of cyanobacteria. In rice paddy fields, they contribute to soil fertilization by supplying nitrogen derived from atmospheric nitrogen fixation. Soil pH also has a selective effect on the distribution and predominance of algae. Although pH specific cyanobacteria growing at pH as low as 3.8 and high as 9.5 are not rare. Most of them have a wide pH range showing good growth at 6.5 – 8.5. The pH of natural habitats is known to vary from 1 to 11 and the organisms growing there are expected to have evolved mechanisms of pH homeostasis to overcome the adversity of external pH on their growth and survival. Our results indicate that pH of 6.0 and 12.0 proved lethal for wild type, MHR(multiple herbicide resistant) and salt/osmotolerant strains, whereas at pH 7.0 gradual increase in growth was observed. On the basis of all parameters it can be concluded that the sensitivity of wild type and MHR (multiple herbicide resistant) strains of *Anabaena variabilis* towards acidic and alkaline pH is higher as compared to other salt/osmotolerant strains, which promotes their application as biofertilizer where the soil is acidic and alkaline.

Cyanobacteria are known to have survived a wide spectrum of environmental stresses such as heat and cold shock, anaerobiosis and oxygeny, photo-oxidation, nitrogen starvation, salinity and osmotic stress[2]. Most major groups of microorganisms have at extremely low pH values[16].

Conspicuously absent from lists of such organisms are cyanobacteria (Blue green algae) and other phototrophic prokaryotes. Cyanobacterial growth appears to be inhibited completely in habitats with pH values below 4-5[3], as does the growth of anoxygenic phototrophic bacteria[21,22]. Most cyanobacteria have growth optima between pH 7.5 and about 10 and accrodingly alkalophiles.

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Cyanobacteria, an ubiquitous group of soil micro-organism, specially in flooded rice fields. The rice field ecosystem provides a favourable environment for the growth of cyanobacteria. In rice paddy fields, they contribute to soil fertilization by supplying nitrogen derived from atmospheric nitrogen fixation[27,34]. Soil pH also has a selective effect on the distribution and predominance of algae. Blue green algae or cyanobacteria grow extensively on alkali or "usar" soils in India[29] and on the salted "takyr" soils of the USSR[5,29,30] claimed, and it was subsequently reaffirmed that blue green algae can be used to reclaim alkali soils[10,36].

Virgin alkali (sodic) soils have a high pH and high exchangeable Na and are often barren. Blue green algae, however, tolerate excess Na and grow extensively on the soil surface in wet seasons. Soil salinity and alkalinity are major problems associated with soil management in arid and semi-arid regions worldwide[37]. Alkali (sodic) soils have a high pH, high exchangeable Na, and measurable amounts of carbohydrates, and undergo extensive clay dispersion, leading to poor hydraulic conductivity and reduced soil aeration[6]. As a consequence, crop production in these soils is poor. The reclamation of sodic soils involves chemical amendment (*e.g.*, with gypsum or Fe pyrites) and leaching to remove excess salts. Reclamation by biological methods is much slower and depends on the incorporation of green manures.

The primary function of the pH homeostasis mechanisms is to maintain the cytoplasmic pH close to neutrality by regulating the production and consumption of $H^+$ within the cell or the exchange of $H^+$ across the cellular membranes[23]. The electrogenic system of $K^+$ uptake associated with activation of $H^+$ extrusion by the $H^+$ pumping respiratory chain in the pH range below 8 and the activation of $Na^+$ exporting/$H^+$ importing in the pH range at or above 9 are two known basic mechanisms of pH homeostasis in bacterial systems[9,11,14] The absence of cyanobacteria from low pH environments might reflect and inherent sensitivity of photosynthesis (oxygenic and bacterial) in prokaryotic cells. Low external pH values might limit growth by lowering the intracellular pH, by increasing the maintenance energy requirement, by affecting solute transport, or cell wall biosynthesis[8].

Naturally occurring cyanobacteria contribute to the fertility of flooded rice fields by reducing $N_2$ to $NH_3$. Increases in rice yield have been attributed to cyanobacteria in several studies under controlled conditions[24,38,42]. However, the contribution of natural populations of cyanobacteria to rice field is limited because fixation of $N_2$ is coupled to growth of the cyanobacteria and N is not immediately available for plant growth. Fixed N is released gradually by mineralization of organic N in the cyanobacterial biomass[42]. Nitrogen fixing cyanobacteria are being used as nitrogen biofertilizers in rice fields in countries where rice is the major staple diet[39,40].

Thus the application of cyanobacteria in agriculture is well documented, where they are used in field condition exposed to natural environment. In this multitude of environmental, agricultural application the cyanobacterial inocula encounters diverse macro and micro environmental stress.

In order to maintain the cyanobacterial biofertilizer programme successful, it is essential to use species which have the greater capabilities to survive these stresses.

Diazotrophic cyanobacteria are capable of profuse growth in saline/alkaline soil and in their use has been advocated for reclamation of such soil. The use of cyanabacterial strain in such soil has been found to improve the physico-chemical properties of the soil.

*Organisms and growth conditions :*
*Source of strains :*

The axenic clonal culture of $N_2$-fixing cyanobacterium *Anabaena variabilis*, a rice field isolate[32], its multiple herbicide resistant mutant *A. variabilis* (MHR)$^{Ar, Al, B, 2, 4D}$ exhibiting resistance to herbicides- Arozin, Alachlor, Butachlor, and 2,4-D [4,33], salt and osmotolerant variants of *A. variabilis* i.e.*A.v.*.(PLiCl-R) *A. v.* (PNaCl-R), *A. v.* (PSucrose-R) resistant mutants of LiCl,NaCl and sucrose respectively, salt and osmotolerant variants of *A. variabilis*(MHR) i.e.*A.v.* (MLiCl-R) *A. v.* (MNaCl-R), *A. v.*(MSucrose-R) resistant mutants of LiCl, NaCl and sucrose respectively and *A.v.* M(LiCl+NaCl+sucrose) R, salt and osmotolerant variants of *A. variabilis* (MHR)which shows resistance for LiCl, NaCl and sucrose[28] were routinely grown in $BG^{-}_{11}$ medium devoid of any combined nitrogen source (called as $N_2$-medium).

*Growth medium :*

$BG_{11}$ medium[25,26] without combined nitrogen source was used as a basal medium (hereafter designated as $N_2$ or CNF medium) for routine cultivation of cyanobacterial strains.

The composition of the growth medium ($BG^{-}_{11}$) is given below:-

| Macronutrients | gL-1 | Micronutrients | gL-1 |
|---|---|---|---|
| $FeSO_4$ $7H_2O$ | 0.006 | $CO(NO_3)_2$. $6H_2O$ | 0.0049 |
| $K_2HPO_4$ | 0.04 | $CuSO_4$. $5H_2O$ | 0.0079 |
| $MgSO_4$. $7H_2O$ | 0.075 | $H_3BO_4$ | 0.268 |
| $CaCl_2$ $3H_2O$ | 0.036 | $MnCl_2$ $6H_2O$ | 0.181 |
| $Na_2CO_3$ | 0.02 | $Na_2MoO_4.2H_2O$ | 0.039 |
| Citric Acid | 0.006 | $ZnSO_4$ $7H_2O$ | 0.022 |
| EDTA. $Na_2$ | 0.001 | | |

pH after autoclaving and cooling- 7.6
Note: All the micronutrients were dissolved together in a separate container.

The culture medium, glass wares and chemicals were steam sterilized by autoclaving at pressure of 15 Ibs inch$^2$ at 121°C for 15 minutes. For the preparation of solid medium 2-3 % agar-agar was employed. All the chemicals used in the present investigation were of analytical grade and were product of either British Drug House (India), Qualigens

(Glaxo), India, Sigma chemical company (U.S.A.) or E. Merck (Germany).

*Growth conditions:*

The axenic clonal culture of cyanobacterial strains were maintained in a bacteria free state by routinely transferring (at intervals of 7 days) the exponential phase cultures to 100ml fresh sterile $N_2$ medium in 250ml Erlenmeyer flask under a laminar flow hood (Klenzaids, Bombay, India).

The cultures were grown photoautotrophically in a air conditioned culture room maintained at 25±1°C and illuminated with cool day florescent lights (photon flux density 45µE $m^{-2}$ $s^{-1}$) for 18 hours a day.

Culture flasks were hand shaken thrice a day to ensure proper distribution of nutrients, air and light to the cells for better growth.

*Measurement of growth :*

Growth was measured at regular interval of one day.Following method was employed for growth estimation:

*Chlorophyll a estimation following extraction in methanol* [18]:

A known aliquot (5ml) of algal sample was centrifuged (3000x g,5 minutes) and supernatant solution was discarded. The pellet was resuspended in the same amount of methanol and shaken thoroughly. The tubes were kept for 15 minutes in a hot water bath maintained at 60°C and was centrifuged to discard pellet. The optical density of chlorophyll *a* and carotenoids solution was read at 665 nm and 460 nm respectively against methanol blank in systronics UV-VIS Spectrophotometer (model 118 Systronics, India).

*Extinction coefficients :*

$A_{665}$ X 13.42= µg$ml^{-1}$ Chlorophyll *a*

*Heterocyst frequency :*

Heterocyst frequency was determined microscopically and in expressed in percentage as total number of heterocyst occurring per 100 vegetative cells of each cyanobacterial culture.

$$\text{Heterocyst Frequency} \frac{\text{No. of heterocyst}}{\text{No. of vegetative cells}} = \text{X } 100$$

*Specific growth rate constant ($K_t$) and Generation time (G) :*

Method of [13] was followed for the calculation of Specific growth rate constant and generation time. Growth rate constant ($K_t$) values was calculated from the equation:

$K_t = \log_{10} (N_t/N_o)$.

Where, $K_t$ = Growth rate constant

t = Growth period

$N_t$ = Absorbance at time t

$N_o$ = Absorbance at time o

$$\text{Generation time} = \frac{24}{\text{Specific growth rate constant}}$$

*pH stress on growth and survival of Cyanobacterial strains :*

Diazotrophic cyanobacterium *Anabeana variabilis* and its various class of salt/osmo

tolerant strains were analysed under different pH up to 12 days of growth. Nine sets of eight 250ml conical flasks containing 100ml of $BG^{-11}$ medium were used 1st set for wild type strain of *Anabaena variabilis*, 2nd set for *A.v* MHR, 3rd set for *A.v* P LiCl-R, 4th set for *Av*. MLiCl-R, 5th set for Av. PNaCl-R 6th set for Av. MNaCl-R 7th set for Av. P Sucrose-R, 8th set for Av. M sucrose-R and 9th set for Av. M (NaCl + LiCl + sucrose) R. The pH of media was adjusted to 6.0, 7.0,7.5,8.0,9.0,10.0, 11.0, 12.0 using 1.0 N NaOH or HCl.

Impact of varying pH (6.0-12.0) on growth of *Av* (wild type), MHR, *Av* PLiCl-R, *Av* MLiCl-R, *Av* PNACl-R *Av* MNaCl-R, *Av* PSucrose-R, *Av* M sucrose-R and Av M(NaCl + LiCl + sucrose)R were determined in terms of chlorophyll *a* content. The growth kinetics of all above mentioned strains were shown in figs. 1-9

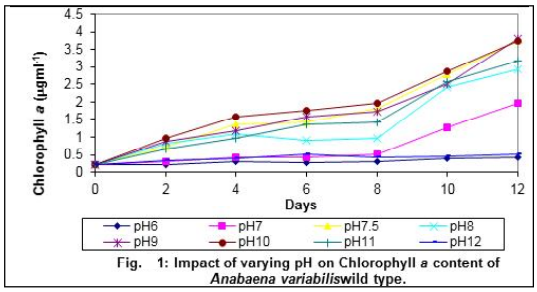

Fig. 1: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis*wild type.

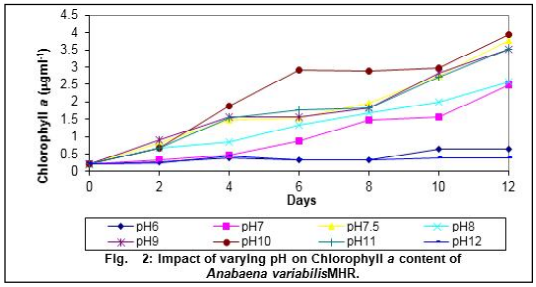

Fig. 2: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis*MHR.

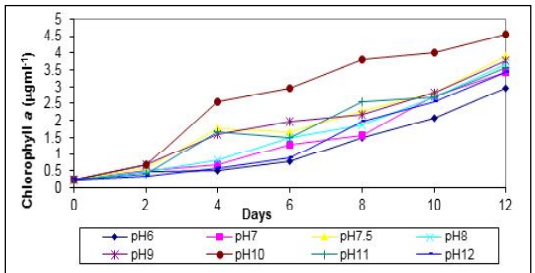

Fig. 3: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis* PLiCIR.

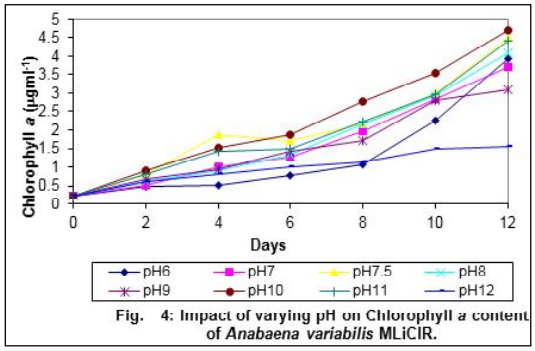

Fig. 4: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis* MLiCIR.

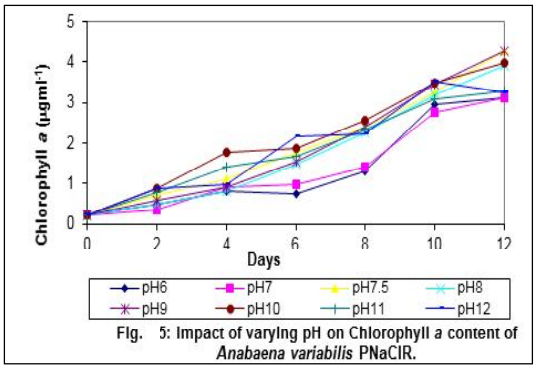

Fig. 5: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis* PNaCIR.

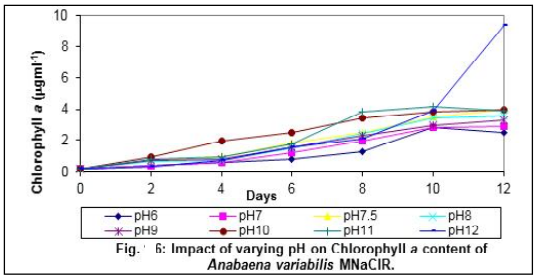

Fig. 6: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis* MNaCIR.

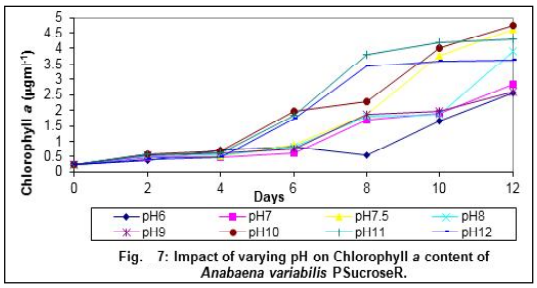


Fig. 7: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis* PSucroseR.

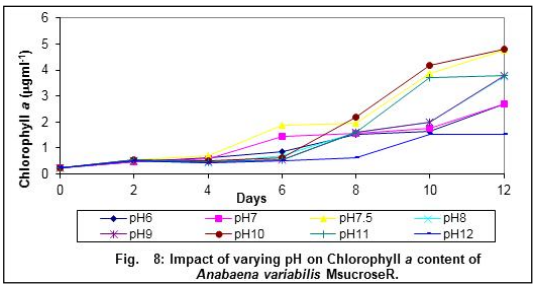


Fig. 8: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis* MsucroseR.

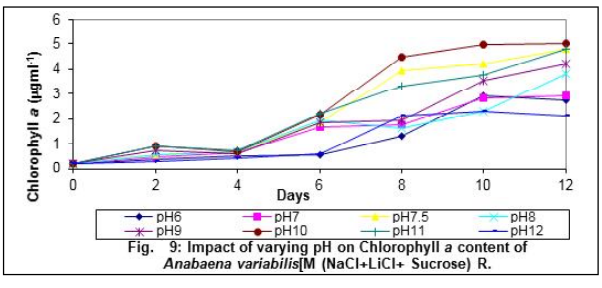


Fig. 9: Impact of varying pH on Chlorophyll *a* content of *Anabaena variabilis*M (NaCl+LiCl+ Sucrose) R.

Highest growth rate was shown by the salt osmotolerant mutant *Av* M(NaCl + LiCl + Sucrose)R (1.34) followed by the *Av* M sucrose R. (1.26), *Av* PLiClR (1.25) *Av* Psucrose R (1.24), *Av* MNaClR (1.23) *Av* MLiClR (1.19), *Av* P NaClR (1.11), *Av* MHR (1.11), *Av* wild type (1.03) at pH 10. (Table 1).

Table-1. Specific Growth rate of Different Cyanobacterial Strains at varying pH

| pH | *Av* Wild type | *Av* MHR | *Av* PLiCl -R | *Av* MLiCl -R | *Av* PNaCl -R | *Av* MNaCl -R | *Av* P Sucrose -R | *Av* M Sucrose -R | *Av* M (NaCl+LiCl Sucrose)R |
|---|---|---|---|---|---|---|---|---|---|
| 6 | 0.264 | 0.456 | 0.963 | 1 | 1.11 | 1.1 | 0.860 | 0.857 | 1.11 |
| 7 | 0.745 | 0.841 | 1.07 | 1.1 | 1.0 | 1.1 | 0.922 | 0.889 | 1.09 |
| 7.5 | 1.08 | 1.08 | 1.09 | 1.12 | 1.16 | 1.19 | 1.22 | 1.23 | 1.33 |
| 8 | 1.02 | 0.942 | 1.07 | 1.11 | 1.14 | 1.17 | 0.915 | 0.942 | 1.22 |
| 9 | 0.972 | 1.09 | 1.09 | 1.09 | 1.18 | 1.11 | 0.938 | 0.948 | 1.26 |
| 10 | 1.03 | 1.11 | 1.25 | 1.19 | 1.11 | 1.23 | 1.24 | 1.26 | 1.34 |
| 11 | 0.983 | 1.08 | 1.07 | 1.11 | 1.13 | 1.26 | 1.26 | 1.21 | 1.32 |
| 12 | 0.252 | 0.238 | 1.05 | 1.09 | 1.12 | 1.23 | 1.20 | 0.824 | 0.96 |

The specific growth rate of wild type, MHR and salt osmotolerant mutant strains was observed highest at pH 10. Whereas these strains showed slower growth rate at pH 6 and pH 12 as compared to other. pH range. The generation time of *Av* wild type, MHR, and salt osmotolerant strains at different pH. is shown in (Table-2).

Table-2. Generation time of Different Cyanobacterial Strains at varying pH.

| pH | *Av* Wild type | *Av* MHR | *Av* PLiCl -R | *Av* MLiCl -R | *Av* PNaCl -R | *Av* MNaCl -R | *Av* P Sucrose -R | *Av* M Sucrose -R | *Av* M (NaCl+LiCl Sucrose)R |
|---|---|---|---|---|---|---|---|---|---|
| 6 | 90.9 | 52.6 | 24.9 | 24 | 21.6 | 21.8 | 27.9 | 28.0 | 21.6 |
| 7 | 32.2 | 28.5 | 22.4 | 21.8 | 24 | 21.8 | 26.0 | 26.9 | 22.01 |
| 7.5 | 22.2 | 22.2 | 22 | 21.8 | 20.6 | 20.16 | 19.6 | 19.5 | 18.04 |
| 8 | 23.5 | 25.47 | 22.4 | 21.6 | 21 | 20.5 | 26.2 | 25.47 | 19.67 |
| 9 | 24.6 | 22.0 | 22.0 | 22 | 20.3 | 21.6 | 25.58 | 25.3 | 19.04 |
| 10 | 23.3 | 21.6 | 19.2 | 20.16 | 21.8 | 19.5 | 19.35 | 19.0 | 17.9 |
| 11 | 24.4 | 22.2 | 22.4 | 21.6 | 21.2 | 19.0 | 19.0 | 19.8 | 18.18 |
| 12 | 95.2 | 100.8 | 22.8 | 22.06 | 21.4 | 19.5 | 20 | 29.1 | 24.76 |

Heterocyst frequency of different cyanobacterial strains (wild type, MHR, and other salt osmotolerant strains of *Anabaena variabilis*) are shown in (Tabl-3).

Table-3. Impact of varying pH on Heterocyst frequency (%) of cyanobacterial strains

| pH | *Av* MHR | | *Av* Wild type | | *Av* P LiCl-R | | *Av* M LiCl-R | | *Av* P NaCl-R | | *Av* M N aCl-R | | *Av* P Sucrose-R | | *Av* M Sucrose-R | | *Av*M (NaCl+LiCl + Sucrose)R | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0day | 8th day | 0 day | 8th day | 0 day | 8th day | 0 day | 8th day | 0 day | 8th day | 0 day | 8th day | 0 day | 8th day | 0 day | 8th day | 0 day | 8th day |
| 6 | 5.4 | 5.8 | 5.1 | 5.9 | 5.2 | 5.6 | 5.3 | 5.6 | 5.0 | 5.1 | 5.1 | 5.9 | 5.6 | 6.2 | 5.6 | 5.2 | 6.0 | 6.2 |
| 7 | 5.4 | 6.2 | 5.2 | 7.2 | 5.2 | 5.8 | 5.3 | 7.0 | 5.0 | 5.6 | 5.2 | 7.2 | 5.6 | 7.1 | 5.6 | 7.6 | 6.0 | 7.2 |
| 7.5 | 5.4 | 6.8 | 5.2 | 5.6 | 5.2 | 7.6 | 5.3 | 7.6 | 5.0 | 6.8 | 5.2 | 7.8 | 5.6 | 7.8 | 5.6 | 8.2 | 6.0 | 8.8 |
| 8 | 5.4 | 5.6 | 5.2 | 6.6 | 5.2 | 7.6 | 5.3 | 7.4 | 5.0 | 6.2 | 5.2 | 7.6 | 5.6 | 7.6 | 5.6 | 7.8 | 6.0 | 8.6 |
| 9 | 5.4 | 6.7 | 5.2 | 6.6 | 5.2 | 7.9 | 5.3 | 7.4 | 5.0 | 6.3 | 5.2 | 7.6 | 5.6 | 7.6 | 5.6 | 7.8 | 6.0 | 8.6 |
| 10 | 5.4 | 6.7 | 5.2 | 6.2 | 5.2 | 7.6 | 5.3 | 7.8 | 5.0 | 6.8 | 5.2 | 8.2 | 5.6 | 7.8 | 5.6 | 7.6 | 6.0 | 8.5 |
| 11 | 5.4 | 6.8 | 5.2 | 6.4 | 5.2 | 7.8 | 5.3 | 7.1 | 5.0 | 7.5 | 5.2 | 7.4 | 5.6 | 7.8 | 5.6 | 7.8 | 6.0 | 7.8 |
| 12 | 5.4 | 5.2 | 5.2 | 6.1 | 5.2 | 5.9 | 5.3 | 5.2 | 5.0 | 5.2 | 5.2 | 5.1 | 5.6 | 7.1 | 5.6 | 5.6 | 6.0 | 5.2 |

Maximum heterocyst frequency was shown by the strain *Av* M (NaCl + LiCl + Sucrose) (8.8%) at pH 7.5 on 8th day of diazotrophic growth followed by the *Av* M Sucrose R (8.2%) *Av* MNaCl-R (7.8%), *Av* PSucrose-R (7.8%), *Av* MLiCl-R (7.6%), *Av* PLiCl-R (7.6%), *Av* PNaCl-R (6.8%), *Av* (MHR) (6.8%) and *Av* wild type (5.6%). As evident from the result that heterocyst frequency of all strains are favoured most by pH 7.5 where as lowest heterocyst frequency was observed when cultures were grown in pH 6 and pH 12.

Although cyanobacteria are known since long to be inhabiting alkaline equatic habitats[30] but very little is known about the mechanism of pH homeostatic in any cyanobacterium. Studies show pH homeostasis mechanism maintain cytoplasmic pH close to

neutral by regulating the production and consumption of $H^+$ within cell or exchange of $H^+$ across cell membrane[23]. The electrogenic system of $K^+$ uptake associated with activation of $H^+$ extrusion by $H^+$ pumping respiratory chain in pH range below 8.0 and activation of $Na^+$ exporting/$H^+$ importing antiporter in pH range at or above 9.0 are the known basic mechanism of pH homeostasis in bacterial system[9,12,14].

The majority of freshwater cyanobacteria, including *C. raciborskii* T3, are alkaliphilic, growing naturally and preferentially at pH > 8. In alkaliphilic bacteria, the principle active process employed for the maintenance of cytoplasmic pH neutrality involves the cycling of ions (mainly $Na^+$ and $K^+$) across cell membranes[7,15]. In this study, the predicted imbalance of total cellular $Na^+$ and $K^+$ induced by applied pH and $Na^+$ stresses was verifed. In cyanobacteria, however, $K^+$ is thought to play a minor role and intracellular pH neutrality is achieved by net $H^+$ accumulation coupled to $Na^+$ efflux as mediated by the $Na^+$ /$H^+$ antiporter[17,19,41]. This process is energized by an imposed proton-motive force[1,35], with uptake of $Na^+$ required in alkaline conditions. $Na^+$ uptake can be achieved by general $Na^+$/ solute symporters, cation channels[15,20] or pH-gated $Na^+$ channels[17].

Supporting results were also found with *Synechococcus leopolien* and *Haplosiphon hybernicus west* concluding cyanobacterial pH homeostasis is regulated by $K^+/H^+$ antiporter system induced alkalization of its cytoplasm at external pH below 7.0 and by $Na^+/H^+$ antiporter system induced acidification of its cytoplasm at external pH of 10.0 and that regulatory mechanism of alkalization and acidification are operational under to the control of two separate genetic determinants[31].

Result shows that salt/osmotolerant mutants exhibited tolerance potential at pH 6.0 (acidic) as well as pH 12.0 (alkaline) may be due to regulatory mechanisms of alkalization and of acidification. pH homeostasis is regulated by $K^+$ induced alkalization of its cytoplasm at an external pH 6.0 (acidic) and by $Na^+$ induced acidification of its cytoplasm at an external pH of 12.0. The lack of alkaline pH – regulated $Na^+$-dependent extrusion of $Na^+$ appears to be the reason why *Av* wild type and *Av* MHR lacks a cytoplasmic pH homeostatic mechanisms in the alkaline environment of pH 12.0 where it fails to grow and survive. Heterocyst frequency was found in the following order at the 8$^{th}$ day of diazotrophic growth *Av* M (NaCl + LiCl + Sucrose) R > *Av* M Sucrose-R > *Av* MNaCl-R *Av* P Sucrose R > *Av* PLiCl-R, *Av* MLiCl-R> *Av* MHR, *Av* NaCl-R> *Av* wild type.

Our results indicate that pH of 6.0 and 12.0 proved lethal for both wild type and MHR strain, whereas at pH 7.0 gradual increase in growth was observed. On the basis of all parameters it can be concluded that the sensitivity of wild type and MHR strains of *Anabaena variabilis* towards acidic and alkaline pH is higher as compared to other salt/ osmotolerant strains, which promotes their application as biofertilizer where the soil is acidic and alkaline.

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