RESEARCH ARTICLE

VARIABLE SALINITY TOLERANCE IN *ANABAENA* SP. BHUAR002 THROUGH REGULATION OF ION UPTAKE AND PRODUCTION OF OSMOPROTECTANT

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Abstract: Filamentous, heterocyst-forming, diazotrophiccyanobacterium*Anabaena* sp. BHUAR002 was isolated from usar (saline) land near Banaras Hindu Universitycampus, and grown routinely on Allen Arnonmedium. The growth of cyanobacteriumwas measured at various concentrations (upto 1000 mM) of different salt combinations, NaCl, NaCl+Na_2CO_3 (1:1) and NaCl+Na_2SO_4 (1:1) and found thatthe cyanobacterium tolerated the salinity of 500 mMNaCl, 700 mMNaCl+Na_2CO_3 and 1000 mMNaCl+ Na_2SO_4, indicating that elevated carbonate and sulphate support the growth of cyanobacterium under salinity and increase the tolerance range. Natural abundance ^{13}C-NMR spectra chemical shifts showed sucrose as the osmoticum synthesized in NaCl and NaCl+Na_2CO_3 (1:1). However, synthesis of sucrose was not found in case of NaCl+Na_2SO_4 (1:1). Intracellular Na^+concentration increases under different salt concentrations as compared to control. K^+concentration also increases with increase of different salt concentration as compared to control is also an indication of acclimatization against salt stress; this type of ionic ratio was found in all three salt stress conditions. Intracellular Cl^- concentration was found minimum in case of NaCl+Na_2SO_4 as compared to NaCl and NaCl+Na_2CO_3 incubated cells.

Keywords: Intracellular ion concentration, Osmotic, Salinity, Tolerance range

INTRODUCTION

As organisms originated millions of years ago (Brock 1973) have passed successfully through several conditions generated by theenvironment on the earth. Cyanobacteria shows diverse stress response and offer an excellent prospect for conducting studies particularly the ability of heterocystous cyanobacteria to tolerate stresses as deficiency of nutrient, salinity and temperature (Apte et al. 1987; Aparna Rai 2015). Salt tolerance have been shown by many cyanobacteria(Thomas and Apte 1984), salt tolerance enhanced by presence of certain nitrogenous compounds in the growth medium(Reddy et al. 1989) and all treatments which inhibit Na^+ influx (such as alkaline pH, K^+ above 25 mM, NO_3^-, NH_4^+) have shown an enhancement in the salt tolerance for brackish-water and also for the freshwater cyanobacterium(Apte et al. 1987).It has also found that cyanobacteriagetacclimatizes to salt stressby ion regulation and osmoprotectants formation(Pade and Hagemann 2014). Similar studies were conducted on plants where it shows that plant adopts SOS pathway for regulation of ion uptake(Gupta and Huang 2014) during salinity stress. Salt adaptation by freshwater cyanobacteria is composed of several mechanisms. Studies on the N_2 fixing cyanobacteriumNostocmuscorum grown at high NaCl concentrations revealed stimulation of photosynthetic activity and sucrose accumulation(E Blumwald and Telor 1982), photoautotrophic nitrogen-fixing cyanobacteria in general exhibits considerable tolerance to salt or osmotic stress(Thomas and Apte 1984). One of the cyanobacterial approaches for the problem of saline soil was proposed in the 1950's, wherein cyanobacteria fixing nitrogen from the atmosphere naturally were employed for the reclamation of saline/alkaline 'usar' soil typical of certain North Indian States (Singh 1950, 1961).

MATERIAL AND METHOD

Isolation and purification of cyanobacteria- Surface soils (3-4 inch of the upper layer) from the usar fields of thenearby locality were collected and brought to the laboratory. Soil samples were ground to the powder and mixed. A known amount of soil samples (5 g) was taken in an autoclaved tube and added sterile double distilled water (5 mL). The samples were mixed thoroughly by shaking and allowed to stand for half an hour. After settling the soil particles, thesupernatant was used to inoculate in combined nitrogen-free agar plates of Allen Arnon (AA) medium(Allen and Arnon 1955) containing different concentrations of NaCl (100mM, 300mM, 500mM, 700mM and 1000 mM) to isolate nitrogen-fixing cyanobacterial forms. Plates were placed in a culture room set at 28 ± 1˚C, illuminated with daylight fluorescent lamps at photon fluence rate of 95 µmol m^{-2} $s^{-1,}$ under 16: 8 h light-dark period to allow the growth of cyanobacteria. After a week cyanobacterial colonies appeared on the plates of 100 – 500 mMNaCl. However, no colonies appeared at 700 and 1000 mMNaCl containing plates.

Since our interest was to isolate salt tolerant cyanobacterial forms, therefore, individual colonies appearing on plates of 500 mMNaCl were picked up

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and subcultured in liquid mediumusing standard microbiological techniques.A single strain of cyanobacterium was obtained in unialgal culture condition. Morphological characters (filament sheath, length, width, shape and size of vegetative cells and heterocysts, etc.) and keys of Desikachary(Desikachary 1959)were used to identify the cyanobacterial strain as *Anabaena* sp. Further 16s ribosomal RNA partial sequencing of isolated DNA and the sequences were submitted in NCBI where it is identified as new species named *Anabaena* sp. BHUAR002 (Accession no. bankit1353506 HM235817)(A Rai and Rai 2011). Axenic nature of isolated cyanobacteria was ensured periodically by plating the diluted cultures on caseinate-glucose agar AA medium (free of combined nitrogen) supplemented with (w/v) casamino acids (0.05%), glucose (0.5%) and agar (1%)], and incubated under standard growth conditions. To eliminate bacterial contamination, if any, clean microcolonies were transferred to AA liquid medium and allowed to grow under standard growth conditions.

Design of simulated saline condition- Salt of 100mM,300mM, 500mM, 700mM and 1000mM concentrations of NaCl and salt mixture of these concentrations using combinations of either two salts NaCl+Na_2CO_3andNaCl+Na_2SO_4, mixed at a 1:1 molar ratio(Yang et al. 2007), for the salt treatment concentrations referred to the total salt concentrations of NaCl +Na_2CO_3 or NaCl+Na_2SO_4. Therefore, in 100mM solution, a mixture of 50mMNaCl and 50mM Na_2CO_3 would result in total ion concentrations of 150mM Na+50mMCl+50mM CO_3. For 100mMNaCl+Na_2SO_4 solution, a mixture of 50mMNaCl and 50mM Na_2SO_4 result in total ion concentrations of 150mM Na+50mMCl +50mM SO_4like this the five concentration treatmentsfor each pair were applied .

Stress treatment- Exponentially growing cyanobacterial cells were subjected to stress treatment by inoculating it at 20:1 (20 is the medium and 1 is inoculum) ratio on different concentrations of salts, different combinations of salts and also in control.

Growth estimation -Salt tolerance in cyanobacteria has been studied mainly for their ability to tolerate different salt levels. Very few studies describe their ability to tolerate or grow in different salts and /or their combinations. We measured the growth of cyanobacteria at different salt combinations and concentrations. Exponentially growing acyanobacterial strain of *Anabaena sp*. BHUAR002 were inoculated in combined N-free sterile AA medium supplemented with NaCl, NaCl + Na2CO3 (1:1) and NaCl + Na2SO4 (1:1) (Yang et al. 2007) to get final salt concentrations of 100 mM, 300 mM, 500 mM, 700 mM and 1000 mM. Growth was measured by recording the absorbance of the cultures daily at 650 nm in a spectrophotometer (Milton Roy, Spectronic 20).

Measurement of osmoticum – In an attempt to find out the osmoticum synthesized by cyanobacteria to counteract the effect of salts, cells of *Anabaena* sp.BHUAR002wereincubated in Allen Arnon medium with different salt concentrationsof NaCl, NaCl + Na2CO3 (1:1) and NaCl + Na2SO4 (1:1) under standard growth conditions. Sampling was done at regular intervals for 96 h and estimated the osmoprotectants such as sucrose, trehalose and glycine betainespectrophotometrically. Sucrose was measured as described by(Handel 1968), trehalose as (Lillie and Pringle 1980) and glycine betaine as(Wall et al. 1960) at different salt combinations and concentrations.

Ion estimation: Exponentially growing cells of *Anabaena* sp.BHUAR002 was incubated at different concentrations of salts of different combinations under standard growth conditions. After 24 h, the cells were collected by centrifugation and analyzed the intracellular ion content employing atomic absorption spectrometer(Association of Official Analytical Chemists 1984; Rodkey and JR 1963).

OBSERVATION AND RESULT

Growth estimation- *Anabaena*sp. BHUAR002, tested grew well in AA medium. However, the growth was maximal when the medium contained 100 mMNaCl; growth (yield) increased by13% over the control. 300 mMNaClconc, also stimulated the growth as well as yield, it is 8% over the control. Cyanobacterial strain tolerated the salinity of 500 mMNaCl, but with reduced yield. Yield reduction at 500 mMNaClwas observed 15% to that of control,however,cyanobacterium could not tolerate NaCl concentrations of 700 mM and beyond. This indicates that *Anabaena* sp.BHUAR002 is salt tolerant upto 500mM in case of NaCl as a salt.

To find out the growth of cyanobacterium at elevated CO_2concentration,NaCl+Na_2CO_3 increased the growth of cyanobacterial strain at low salt concentrations of 100 and 300 mM NaCl+Na_2CO_3. Salt concentration of 100 mM increased the yield of *Anabaena* sp.BHUAR002 by 93%, over the control. Growth stimulation of cyanobacteriumwas also evident at 500 mM NaCl+Na_2CO_3, and the yield was found to be increased by 19%, over the control. cyanobacterium was able to sustain even at salt concentration of 700 mM NaCl+Na_2CO_3 with reduced growth rate and yield. Growthat 700 mM salt concentration was identical to that of control. The data thus indicated that elevated carbonate supported the growth of *Anabaena sp*. BHUAR002strain under salinity and increased the tolerance range. However, 1000 mM NaCl+Na_2CO_3 inhibited the cyanobacterial growth completely.

Further it was found that with the salt combination of NaCl+Na_2SO_4 the yield was increased at lower salt concentration of 100 and 300 mM. Cyanobacterium was found to grow even at 1000 mM salt

concentration, although the yield was reduced. This indicated that presence of SO_4 in the saline environment not only protected the cyanobacterial cells from salt toxicity, albeit increased the range of salt tolerance to 1000 mM(Figure-1, Table-1).

Osmoticum - Sucrose and trehalose were synthesized maximum at salt concentration of 200 mM of NaCl and NaCl+Na_2CO_3at 24 h of incubation (Figure-2).

Since methods used for estimation of sucrose and trehalose was based on anthrone test, we confirmed the compounds by ^{13}C-NMR using standard sucrose, trehalose and glycine betaine. Natural abundance ^{13}C-NMR spectra chemical shifts coincided with that of sucrose standard and not with trehalose and glycine betaine proving sucrose as the osmoticum synthesized by *Anabaena* sp.BHUAR002 (Figure-3, Figure-4and Table -4).

Presence of NaCl in the nutrient solution induces the synthesis of sucroseand trehalose, both were found 165.18% and 101.68% respectively when compared to control at 200 mM NaCl. Further, when*Anabaena* sp.BHUAR002 exposed to the different concentrations of NaCl+Na_2CO_3, sucrose and trehalosesynthesized maximum at 200 mM salt concentration and both were found to be 130.63% and 24.35% respectively to that of control. Presence of 200 mM of NaCl+ Na_2SO_4 does not induce the synthesis of sucrose (Table-2). It was further confirmed by ^{13}C-NMR spectra which do not show any chemical shifts towards the standard of sucrose in case of NaCl+Na_2SO_4 (Table-4).

Intracellular ion concentrations- Increasing salt (NaCl, NaCl+Na_2CO_3, NaCl+Na_2SO_4) concentration in the medium, increases the intracellular Na^+ content accordingly. Intracellular K^+ ion at various selected concentrations of NaCl have lesser values in comparison to control but the K^+ content have shown an increasing trend from 100 to 1000 mM concentration of NaCl. It was observed that upto 700 mM NaCl + Na_2CO_3intracellular level of potassium ion gradually increases but at 1000 mM sudden drop was observed. Organism growth was also arrested at 1000 mM NaCl + Na_2CO_3concentration. Intracellular K^+ content under NaCl + Na_2SO_4increases with the increase in concentration of salt from 100 mM to 1000 mM. Increasing level of intracellular potassium ion content upto 1000 mM indicated and supported the growth of organism upto 1000 Mm.

Salinity increases the calcium content in *Anabaena* sp. BHUAR002 upto 1000 mM NaCl concentrations. On observing the pattern of intracellular Ca^{2+}in the case of NaCl + Na_2CO_3, it was found that Ca^{2+} concentration gradually increases from 100 to 700 and decreases at 1000 mM. This indicated that increase of intracellular calcium content from 100 to 700 mM NaCl + Na_2CO_3 help organism to survive and tolerate such a high degree of salt stress and 1000 mM was found unfavorable for organism growth. As similar to intracellular potassium ion content, intracellular calcium ion content was also found increasing upto 1000 mM NaCl + Na_2SO_4 salt stress. So it is concluded that intracellular calcium ion content also helps the cyanobacterial cells to tolerate NaCl + Na_2SO_4 upto 1000 mM. (Table-3)

Intracellular chloride ion content increases with increase in NaCl concentration from control to 1000 mM NaCl. Accumulation of Intracellular chloride ion content under NaCl + Na_2CO_3 stress and NaCl + Na_2SO_4stress was similar to NaCl stress i.e., level of Intracellular chloride ion content increases with increasing concentration of NaCl + Na_2SO_4 but its value was low as compared to NaCl and NaCl + Na_2CO_3. (Table-3)

DISCUSSION

The data obtained from this investigation strongly suggest that the very rapid NaCl entry into the cell triggers the adaptive response of the cyanobacteria to salt and that both organic and osmoregulatory mechanism are involved in this process. Osmoregulation can be accounted by the intracellular concentrations of sucrose, K^+, and residual Na^+ ions. For 200mM NaClgrown cells, there is arelatively rapid accumulation of sucrose followed by a more gradual accumulation of K^+ suggesting that the initial osmoregulatory response of the cells is a ‘compatible ‘ solute, i.e. a solute which is not disruptive of macromolecular interaction(Eduardo Blumwald et al. 1983). However, when salt is NaCl+Na_2SO_4 there is avery small accumulation of sucrose; it means some other regulatory mechanisms also operate regarding the salt tolerance under sulphate supplement combined salt stress of NaCl+Na_2SO_4.

The growth of cyanobacteria in NaCl salt stress found up to 500mM, but when carbonate was given as a supplement with NaCl, the tolerance level was found to be increased upto 700mM NaCl+Na_2CO_3 concentrations.However, when salt was NaCl+Na_2SO_4,thegrowth of cyanobacteria became luxurianteven at 1000mMconcentration. These results show that carbonate and sulphate both are growth inducer in*Anabaena* sp.BHUAR002. Primarily carbonate gives some sort of tolerance (up to 700mM), one reason behind this is CO_2 concentration, which increases the rate of photosynthesis and organism grow properly.However, when we think about the factors responsible for increased salt tolerance in cyanobacteria,twoaspectscan be considered; one is that the intracellular ions play amajor role for the survival of organism athigher salt concentration and the second is that the formation of anosmoprotectant.

In case of NaCl+Na_2CO_3, osmoprotectantplaymajor role in cyanobacterial growth under high salt concentrationof 700m Min *Anabaena* sp. BHUAR002 in comparison to intracellular ion concentrations. However, incase of NaCl+Na_2SO_4, intracellular ion concentration and osmoprotectant

both play certain role for the survival of organism at ahigh salt concentration(1000mM) but some other mechanism is also involved that helps organism to survive so luxuriantly under high salt concentration which is not known.As mentioned elsewhere in result section thatosmoprotectant formed in NaCl and $NaCl+Na_2CO_3$ is sucrose but when salt is $NaCl+Na_2SO_4$ sucrose is not formed. Same way K^+ accumulation is also found less in case of $NaCl+Na_2SO_4$ as compared to NaCl and $NaCl+Na_2SO_4$.

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Table 1. Showing percentage increase/decrease yield of *Anabaena* sp. BHUAR002 under different concentrations and combinations of salts. (AA represent Allen Arnon medium as control).

Conc (mM)	**Yield (%)**		
	NaCl (mM)	**$NaCl+Na_2CO_3$ (1:1)**	**$NaCl+Na_2SO_4$ (1:1)**
Control(AA)	100	100	100
100	112.5	193.1	108.386
300	108.3	128.7	119.7
500	85.4	118.81	101.8
700	14.6	98.02	76.64
1000	2.1	8.912	76.64

Table 2. Percentage increase/decrease in the content of sucrose and trehalose in *Anabaena* sp. BHUAR001 and *Anabaena* sp. BHUAR002 with varying concentrations of salts.

% increase/decrease of osmoprotectant				
Salt concentration	*Anabaena* sp. BHUAR002			
	Sucrose	**Trehalose**	**Sucrose**	**Trehalose**
NaCl			**($NaCl+Na_2CO_3$)**	
Control	100	100	100	100
100 mM	212.74	160.22	209.5	86.6
200 mM	265.18	201.68	230.63	124.35
300 mM	246.69	167.05	221.97	103.35
400 mM	147.08	142.97	186.29	98.52
500 mM	98.54	134.95	132.21	98.32

Table 3. Showing different intracellular cations and anion in *Anabaena* sp. BHUAR002 under different concentrations and combinations of salts.

Salt (mM)	**Intracellular ion content (mmol.µg chl-1) of *Anabaena* sp. BHUAR002**			
	Na^+	K^+	Ca^{2+}	Cl^-
NaCl				
Control	0.95	2.77	0.48	1.53
100	6.53	1.64	0.71	7.20
300	25.81	1.91	1.68	25.67
500	27.73	1.17	1.72	27.53
700	43.30	0.70	1.37	41.67
1000	55.14	0.09	0.09	54.00

$NaCl+Na_2CO_3$				
100	6.39	1.75	0.84	7.20
300	25.55	2.19	1.39	26.33
500	26.90	1.95	1.47	27.33
700	40.80	2.10	1.50	40.83
1000	51.00	1.27	1.20	53.33
$NaCl+Na_2SO_4$				
100	5.06	0.56	0.55	1.68
300	13.30	0.90	1.37	4.42
500	37.09	1.21	2.04	9.82
700	52.97	1.92	2.44	11.01
1000	94.37	1.96	5.14	23.37

Table 4. ^{13}C-NMR spectrum of sucrose, trehalose and glycine betaine (standards) and its chemical shifts (δppm) in *Anabaena*sp. BHUAR002 at different salt combinations.

Std Sucrose	**StdTrehalose**	**Std Glycine betaine**	**Control**	**NaCl**	**NaCl +Na_2CO_3 (1:1)**	**NaCl +Na_2SO_4 (1:1)**
			*Anabaena*spBHUAR002			
49.9	49.9	49.9	49.9	49.9	49.9	49.9
		54.853				
61.084						
	61.521					
		64.521				
70.166						
	70.669					
	73.084					
73.323						
	73.504					
73.529				73.529		
74.946					74.946	
77. 361						
82.298				82.298		
93.086						
	94.149					
104.6				104.6		
		167.83				

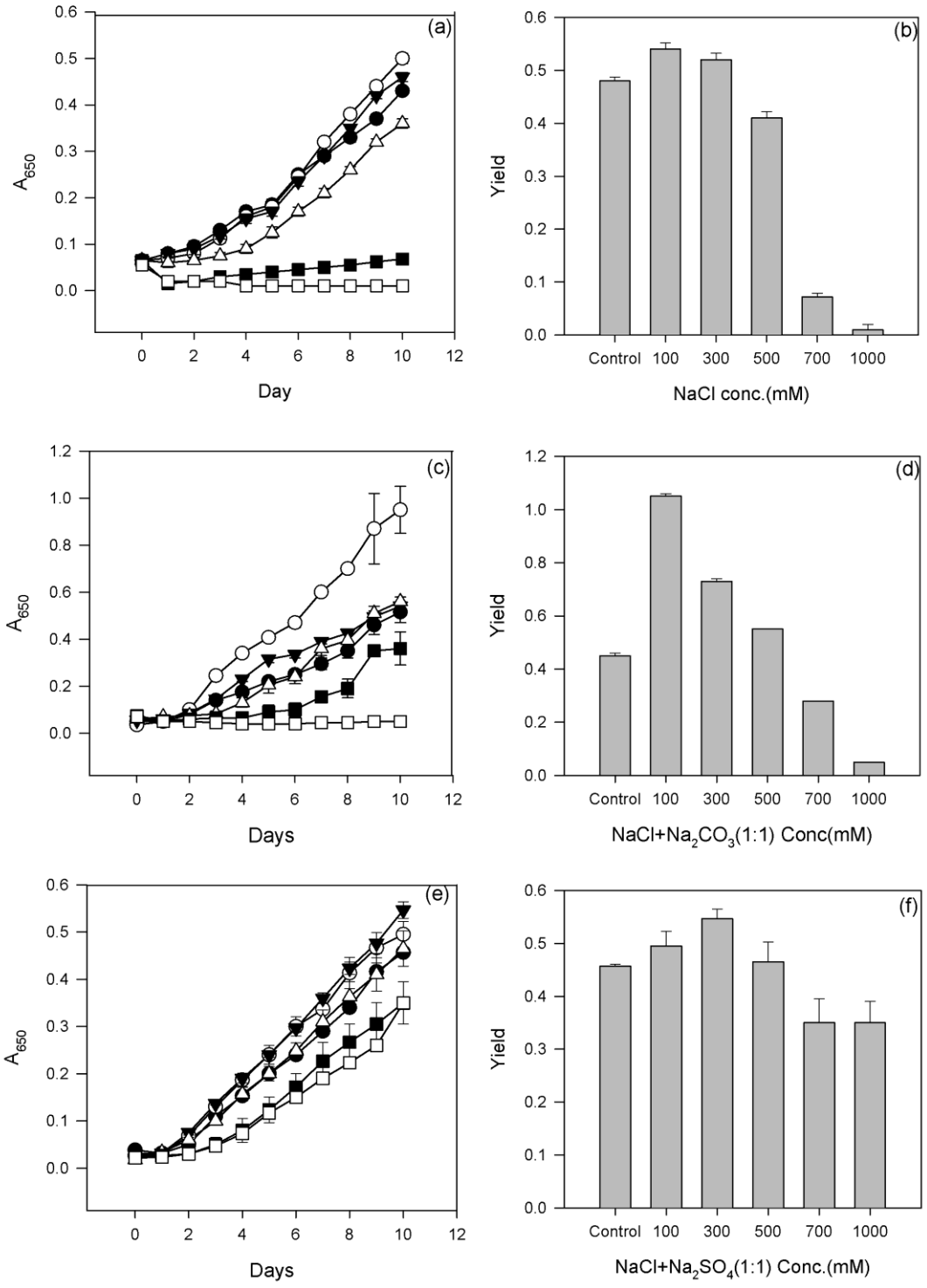

Figure 1.0. (a, b) Showing growth and yield of *Anabaena* sp. BHUAR002 at 10th day of growth under different NaCl concentrations respectively likewise (c, d) showing growth and yield (at 10th day) respectively when salt is NaCl+Na_2CO_3 (1:1), and (e, f) also showing growth and yield (at 10th day) respectively of *Anabaena* sp. BHUAR002whensalt is NaCl+Na_2SO_4 (1:1) : control (●), 100 mM (○), 300 mM (▼), 500 mM (△), 700 mM (■), 1000 mM (□)

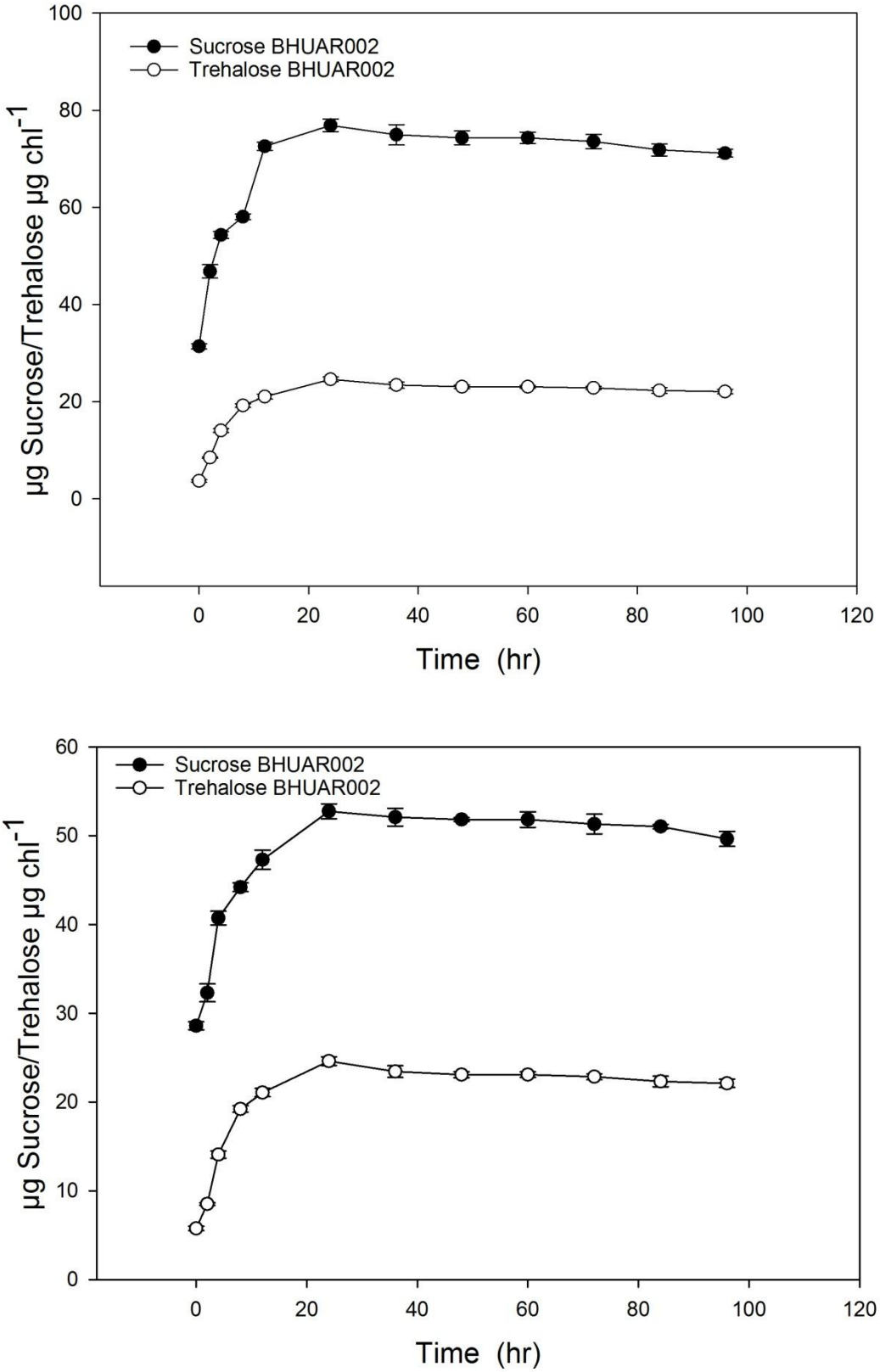

Figure 2. Level of osmoprotectants in*Anabaena*sp. BHUAR002at different time interval with 200 mMNaCl (a) and NaCl+Na_2CO_3 (1:1) (b).

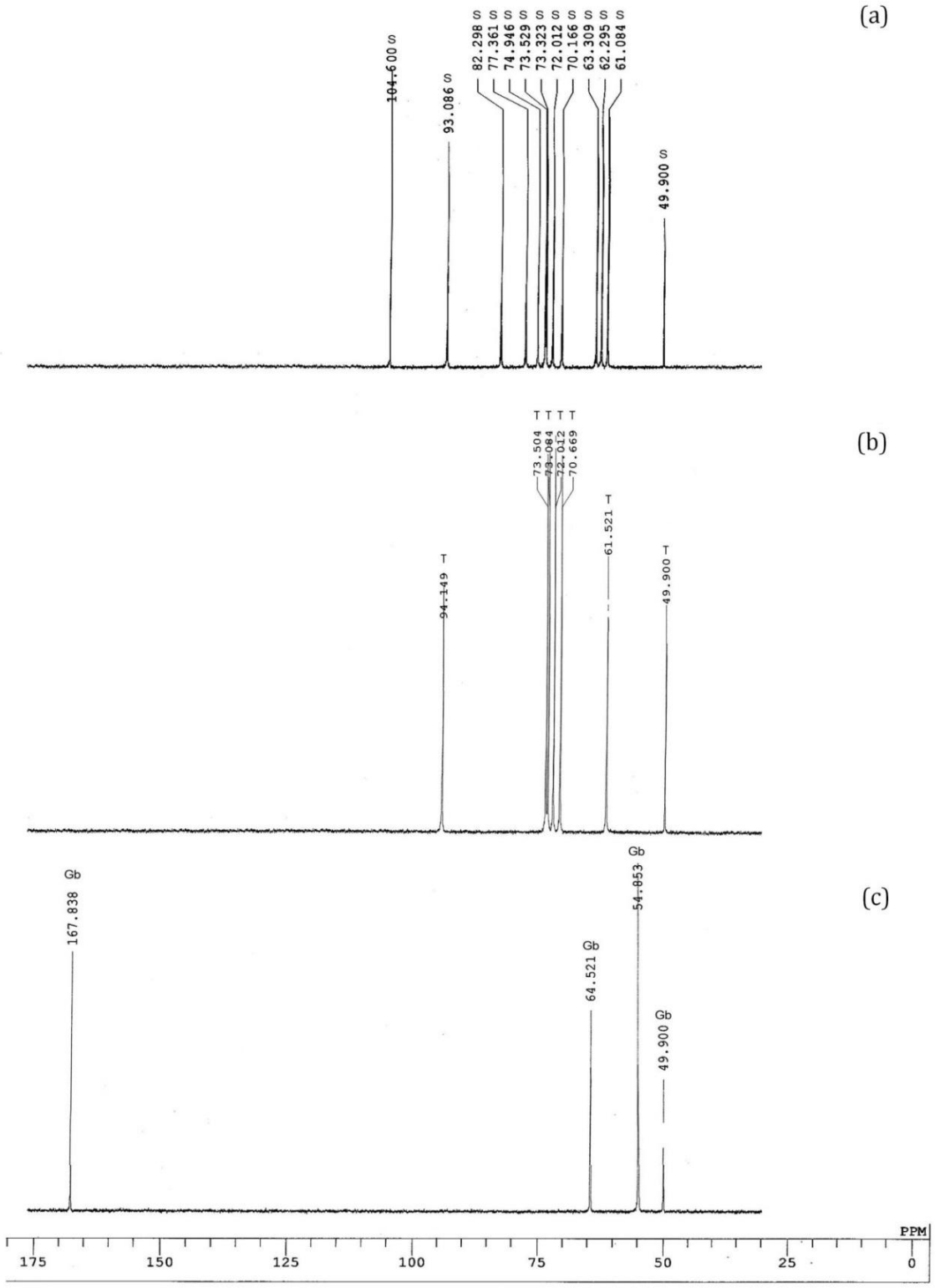

Figure 3.0. δppm shift (^{13}C-NMR Scale) of thestandard of Sucrose (a), Trehalose (b) and Glycine betaine (c).

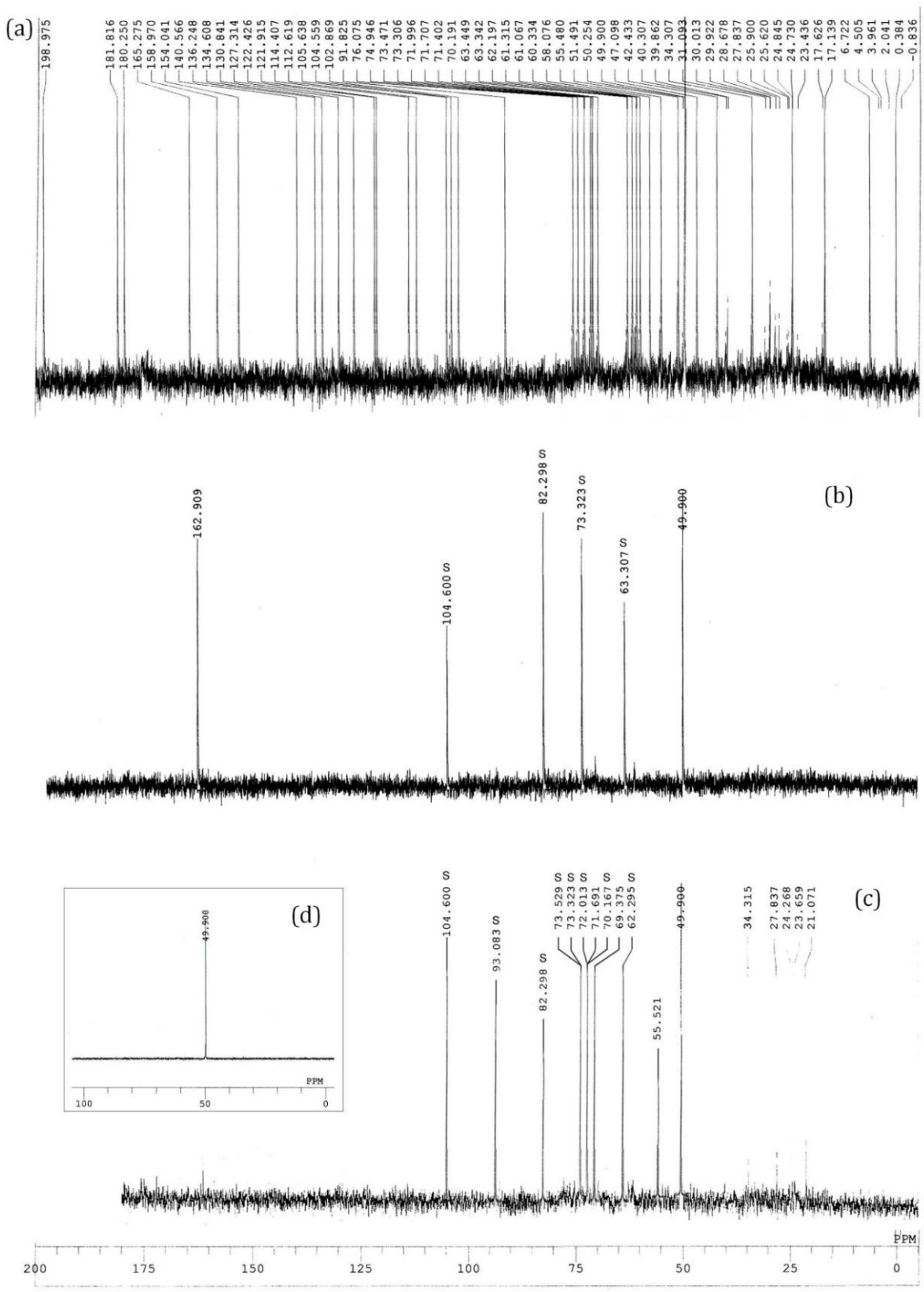

Figure 4.0. a, b, c, d are showing ^{13}C-NMR results (δppm shift) of NaCl+Na_2SO_4 (1:1) stressed, NaCl+Na_2CO_3 (1:1) stressed, NaCl stressed, and control samples respectively.

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