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Alkaliphilism and Carbonate Transport in Alkaliphilic Cyanobacteria (*Phormidium* sp.) Isolated from Alkaline Lake Lonar, India

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ABSTRACT

The alkaline lake Lonar is situated in buldhana District of Maharashtra, India. The lake is around 50-60 thousand years old and water is alkaline (pH 10.36). It is the third largest meteorite crater in basaltic rock in the world, with a diameter of 1,800 m. While Lake Bosmatvi in Ghana, has a diameter of 10,000 m and lake New Quebec in Canada with a diameter of 3,500 m. The alkaliphilic cyanobacteria in this lake have developed mechanisms for their survival in alkaline environments. The alkaliphilic cyanobacteria *Phormidium* sp. isolated from Lonar lake was able to tolerate a concentration of 1M Na₂CO₃ this revealed its existence in the lake throughout the year. In order to reveal its carbonate transport system a variation of NaCl along with Na₂CO₃ was done which resulted that a small amount of NaCl is need for the transport of carbonates. The cyanobacterial sheath under carbonate stress was isolated and FTIR of the sheath material was done. The FTIR spectra gave a characteristic features of the sheath isolated from *Phormidium* sp at 1M Na₂CO₃ concentration between the range of 1000-3000 cm⁻¹. It was established that the investigated cyanobacterium possessed two carbonate transport systems operational at pH 9 and 10. This reflects the adaptation of the cyanobacteria to seasonal variation in the alkaline- saline lake Lonar. Thus the alkaliphilism or adaptation of cyanobacteria in the alkaline lake lonar was understood which depends on the seasonal salinization and desalinization cycles.

Keywords

Cyanobacteria,
Lonar Lake,
Alkaliphiles,
Sodium carbonate,
FTIR, Phormidium.

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Introduction

A characteristic feature of the majority of soda lakes is the presence of permanent or seasonal colored blooms of phototropic microorganisms. Extreme alkaliphiles live in soils laden with soda or in soda lakes where the pH can rise to 12. The soda lakes are the most stable and productive naturally occurring alkaline environments in the world, with pH values greater than 10 (Jhingran and Rao, 1954). One of the striking facts about

soda lakes is that, despite apparently inhospitable caustic conditions, these environments are extremely productive because of high ambient temperatures, high light intensities, and effectively unlimited supplies of CO₂ via the HCO₃⁻/CO₃⁻/CO₂ equilibrium (Grant and Tindall, 1980).

The alkaline lake Lonar is situated in buldhana District of Maharashtra, India. The

lake is around 50-60 thousand years old and water is alkaline (pH 10.36). It is the third largest meteorite crater in basaltic rock in the world, with a diameter of 1,800 m. While Lake Bosmatvi in Ghana, has a diameter of 10,000 m and lake New Quebec in Canada with a diameter of 3,500 m (Castanier *et al.*, 1999). Soda lakes are characterized by the presence of large amount of sodium carbonate (or complexes of these salts) formed by evaporative concentration under particular condition of geology, geography, and climate (Grant, 2004). The soda lake habitats often have a combination of Ca^{2+} and carbonate levels. The higher the levels of Ca^{2+} than carbonates lowers alkaline conditions and lower the level of Ca^{2+} than carbonates higher alkalinity develops (Giordane *et al.*, 2005).

The saline alkaline Lake Lonar experiences different periods of annual cycles which results in change in carbonate concentration and pH as well. The main aim of the study was to elucidate the effect of these factors on the *Phormidium* sp. isolated from Lonar Lake.

Materials and Methods

Organism and culture conditions

The cyanobacterium *Phormidium* sp. used in this study was isolated from the water sample of alkaline Lake Lonar. The identification of the blue-green algae was done as per the monograph, 'Cyanophyta' (Desikachary, 1959). The cyanobacterium was maintained on BG-11 media (Kaushik, 1987). Each liter of BG-11 medium contains (g L^{-1}) of NaNO_3 , 1.5; K_2HPO_4 , 0.04; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.075; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; Na_2CO_3 , 0.02 and 1 mL of trace solution A₅ containing (g L^{-1}) of H_3BO_3 , 2.86; MnCl_2 , 1.81; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.222; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.39; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.079 and $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.0494. The pH of the media

was adjusted at 10.36 (lake water pH). The flask was exposed to a light intensity of 1200-1500 lux with a light/dark cycle of 16/8 h at $24 \pm 2^\circ\text{C}$.

Tolerance to carbonates

The cyanobacterium *Phormidium* sp. was tested for its tolerance to carbonates in BG-11. A series of flasks containing sterile growth medium amended with increasing carbonate concentrations (0.2 – 1 M) were inoculated with equal inoculum. The bottles were incubated at $24 \pm 2^\circ\text{C}$ for 15 days. The carbonate tolerance was judged on the basis of visible growth.

Effect of NaCl with carbonates

In order to elucidate the effect of carbonate concentration on the physiology of the *Phormidium* sp., it was varied with the NaCl concentration. A combination of sodium carbonate along with sodium chloride was prepared in such a way that the total molarity of the medium remains at the level of 1 M. The flasks were incubated for 15 days at $24 \pm 2^\circ\text{C}$. After incubation the flasks were harvested, chlorophyll a and phycocyanin content was measured (Kaushik, 1987).

Isolation of sheath

The sheath was isolated as according to procedure described by Stuart and Ando (1997). The filamentous cyanobacterium *Phormidium* sp was harvested and washed twice in 20 mM ammonium acetate buffer (pH 7.0). The filaments were resuspended in the buffer and broken by ultrasonication (Sonics and Materials Inc., USA). The crude sheath was isolated by centrifugation (15 min at 750 X g) (Sorvall, USA, Model RC2). Further purification of the sheath was done with lysozyme. The enzyme was added to a protein/sheath ratio of 1: 5 (dry weight/wet

weight) and the digestion was performed at 37°C for 24 h. After incubation the sheath was washed and extracted with sodium dodecyl sulphate (SDS; 4% [wt/vol] in acetate buffer at 60°C for 15 min). Finally the sheathed material was repeatedly washed with distilled water.

FTIR spectroscopy

In order to study the functional group of the isolated sheath under carbonate stress the spectroscopic analysis of the purified sheath was done by means of Fourier transform infrared (FTIR) spectroscopy. About 50 mg of sheath material was used for the analysis. The spectra were recorded using Nicolet 380 FTIR spectrometer in the wavelength of 500-4000 nm.

Effect of pH

In order to reveal the transport systems in the lonar lake isolate the culture was exposed to a pH range of 8 to 12 keeping the concentration of carbonate to 1M in each flask. The flasks were incubated for 15 days at 24±2°C. After incubation the flask were harvested and the remaining concentration of carbonates in each flask was measured (APHA, 1995).

Results and Discussion

The cyanobacteria are globally important contributors to the biogeochemical cycling of carbon and to primary productivity. The ability of this diverse group of photoautotrophs to efficiently assimilate CO₂ from the environment relies upon the concurrent activity of a biophysical CO₂-concentrating mechanism (CCM) (Badger *et al.*, 2006, Garcia-Pichel, 1998, Joshi *et al.*, 2008). Calcium carbonate (CaCO₃) precipitation is a common phenomenon found in environments such as marine water, freshwater, and soils (Douglas and Beveridge, 1998). It is a rather straightforward chemical

process governed by four key factors: (1) the calcium (Ca²⁺) concentration, (2) the concentration dissolved inorganic carbon (DIC), (3) the pH (pK₂ (CO) = 10.3 at 25°C) and (4) the availability of nucleation sites (Castanier *et al.*, 1999; Kile *et al.*, 2000). Numerous different bacterial species have previously been detected and assumed to be associated with natural carbonate precipitates from diverse environments. The primary role of bacteria in the precipitation process has subsequently been ascribed to their ability to create an alkaline environment (high pH and [DIC] increase) through various physiological activities (Douglas and Beveridge, 1998; Castanier *et al.*, 1999). Although microbiological carbonate precipitation (MCP) has been investigated extensively both in natural environments and under defined laboratory conditions, the exact mechanisms of precipitation and the function of this process within the microbial ecology of the precipitating organism remains unresolved (Ferrer *et al.*, 1988; Rivadeneyra *et al.*, 1994; Yates and Robbins, 1999). Microbial associations with natural carbonate deposits have been described for seawater (Morita, 1980), saline lakes (Douglas and Beveridge, 1998), soils (Boquet *et al.*, 1973) and geological formations (Peckman, 1999). The formation of alkalinity in the soda lake environment requires a combination of geographical, topographical and climatic conditions: firstly, the presence of geological conditions which favour the formation of alkaline drainage waters; secondly, suitable topography which restricts surface outflow from the drainage basin; and thirdly, climatic conditions conducive to evaporative concentration. Such conditions are found in arid and semi-arid zones of tropical or subtropical areas (Ulukanli and Digrak, 2002).

The Buldhana district of Maharashtra receives rains through the south west monsoon (from June to September). The average rainfall is

about 500 to 900 mm, the climate is dry and hot. The lake water is alkaline, having an average pH of 9.0–10.0. Alkalinity of the lake is attributed to the high content of sodium carbonate (Jhingran and Rao, 1954). Previous reports of chemical analysis of Lonar lake suggest that lake water is highly alkaline and rich in various salts, carbonate and bicarbonate (Thakker and Ranade, 2002; Joshi *et al.*, 2008). The alkaline lake lonar experiences a significant variability of pH (Fig. 1) and specifically during the rainy season as the pH starts to fall due to shift in the carbonate/ bicarbonate equilibrium. Generally during the rainy season in natural environments there is a decrease in pH by approximately 2.5 units (Garcia-Pichel *et al.*, 1998).

The *Phormidium* sp. isolated from the alkaline lake Lonar was able to tolerate a concentration of 1M Na₂CO₃. At this concentration of 1M the *Phormidium* sp. was well adapted to the media as compared to lower concentration of Na₂CO₃.

Na⁺ ions play an important role in transportation of constituents across the cell membrane.

Raven (1970) suggested that if the rate of photosynthesis by an alga is markedly higher at an alkaline pH, for a given CO₂ concentration, than at a more acid pH it can be concluded that the bicarbonate ion crosses the cell membrane and contributes C for photosynthesis. Miller *et al.*, (1990) found that Na⁺ is required for growth, photosynthesis and pH regulation in alkali tolerant cyanobacterium *Synechococcus leopoliensis*. In the present study the effect of NaCl on the carbonate transport was elucidated keeping the total concentration in the medium to 1M. The Chl.a and phycocyanin content decreased as the concentration of NaCl increased than the

concentration of Na₂CO₃ and vice-versa (Fig. 2 and 3). These result suggested that for the transportation of carbonates a very less amount of NaCl is needed, similar reports were obtained by Mikhodyuk *et al.*, (2008) using the cyanobacterium *Euhalothece* sp isolated from Lake Magadi.

The FTIR analysis showed different bands in the range of 500-4000 nm (Fig. 4). The most characteristic features of the sheath isolated from *Phormidium* sp at 1M Na₂CO₃ concentration was observed between the range of 1000-3000 cm⁻¹.

The spectrum of the purified sheath was dominated by the amide I feature at ~ 1658.97 and 1641.08 cm⁻¹ (band 5 and 6, Table 1). This band is sensitive to the protein's secondary structure, to ligand interactions and to its folding characteristics (Zeroual *et al.*, 1995); Brandenburg and Seydel, (1996); Panick and Winter, (2000). Some lipid vibrations can be found between 2800- 3000 cm⁻¹ (bands 3 and 4 Table 1). These distinctive bands at 2851.21 and 2920.23cm⁻¹ correspond to the symmetric and asymmetric vibrations of CH₂ methyl and CH₃ methylene groups.

The purified sheath of *Phormidium* sp showed a prominent features corresponding to complex carbohydrates. For *Phormidium* sp, the carbohydrate bands are the main distinguishing features in the "fingerprint" region (1500 - 600 cm⁻¹) (Diem, M, 1993; Stuart and Ando, 1997). These features exhibited characteristic C-O derived carbohydrate absorption bands in the wavenumbers between 1300 and 1000 cm⁻¹ (bands 8 and 9, Table 1). At the wavenumbers between 500 and 1000 cm⁻¹, some additional weak bands were observed.

Several transport systems with different pH optima mediates the process of carbonate transport in cyanobacteria (Badger *et al.*,

2006).

Table.1 Frequency and band assignment for FTIR spectra derived from *Phormidium* sp. purified sheath at 1 M Na₂CO₃

Band	Wavenumber (cm ⁻¹)	Assignments	Main group with source	Comment
1	3532.98	(Str)O-H and (str) N-H	Water ^(4,11)	Water/Amide
2.	3350.69	(Str)O-H and (str) N-H	Water ^(4,11)	Water/Amide
3.	2920.23	(str ^{as}) CH ₂	Lipids ⁽⁵⁾	Methylene group
4.	2851.21	(str) CH ₂ /-CH ₃	Lipids ^(4,5)	Methyl and Methylene groups in fatty acids.
5.	1658.97	(str) C=O	Amide I ⁽¹⁻⁸⁾	Protein (mostly β-helical structure; the secondary structure of the protein can affect the position – 1695 to 1637 cm ⁻¹⁽⁴⁾)
6.	1641.08	(str) C=O	Amide I ⁽¹⁻⁸⁾	Protein (mostly β-helical structure; the secondary structure of the protein can affect the position – 1695 to 1637 cm ⁻¹⁽⁴⁾)
7.	1629.64	(str) O-H	Water ⁽¹⁰⁾	Water
8.	1214.76	(str) C-O	Polysaccharides ^(2,4,9)	Dominated by ring vibrations in polysaccharides
9.	1068.13	(str) C-O	Polysaccharides ^(2,4,9)	Main region for carbohydrate peaks, dominated by ring vibrations in polysaccharides

Str= stretching, as= asymmetric; 1: Hendrick *et al.*, (1991); 2: Zeroual *et al.*, (1995); 3: Williams and Fleming (1996); 4: Nauman *et al.*, (1996); 5: Sturat and Ando (1997); 6: Lewis and Mcelhaney (1996); 7: Giordano *et al.*, (2001); 8: Nelson (1991); 9: Wong *et al.*, (1991); 10: Sokrates (2001); 11: Keller (1986).

Fig.1 pH recorded from the water samples of the saline alkaline Lake Lonar

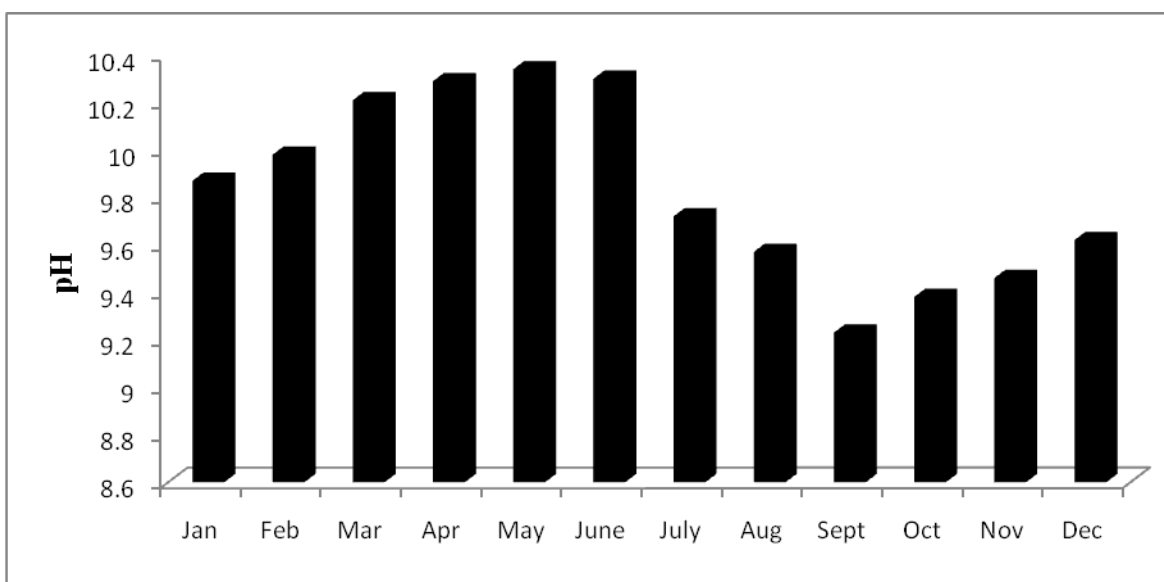


Fig.2 Combined effect of NaCl and carbonates on Chl.a, the total molarity of the medium was kept at 1M. 1- 0.2M Na₂CO₃ + 0.8 M NaCl, 2- 0.4M Na₂CO₃ + 0.6 M NaCl, 3- 0.6M Na₂CO₃ + 0.4 M NaCl, 4-0.8M Na₂CO₃ + 0.2 M NaCl

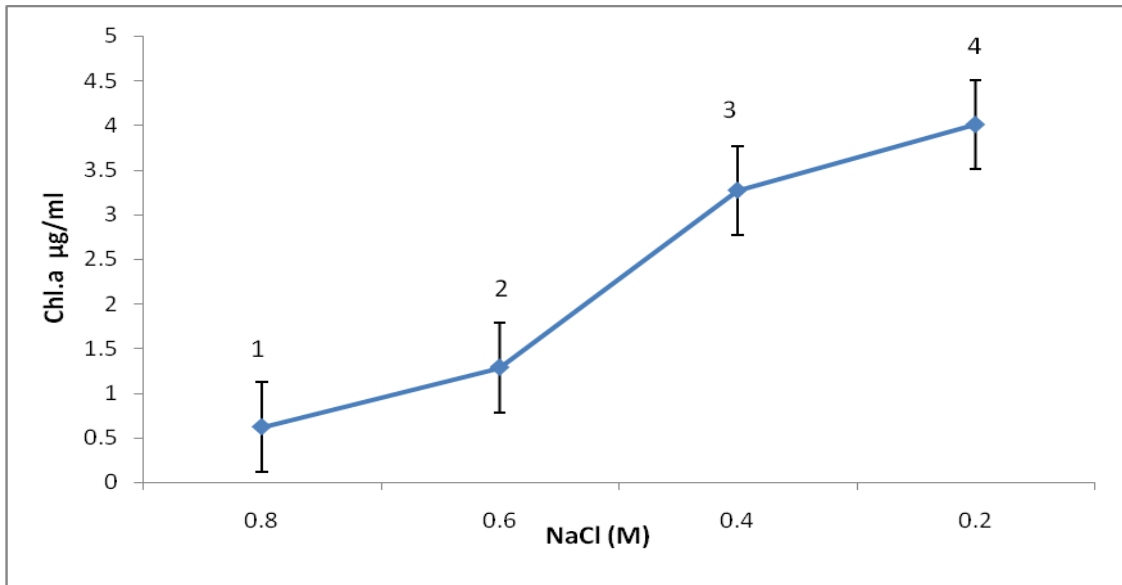


Fig.3 Combined effect of NaCl and carbonates on PC, the total molarity of the medium was kept at 1M. 1- 0.2M Na₂CO₃ + 0.8 M NaCl, 2- 0.4M Na₂CO₃ + 0.6 M NaCl, 3- 0.6M Na₂CO₃ + 0.4 M NaCl, 4-0.8M Na₂CO₃ + 0.2 M NaCl

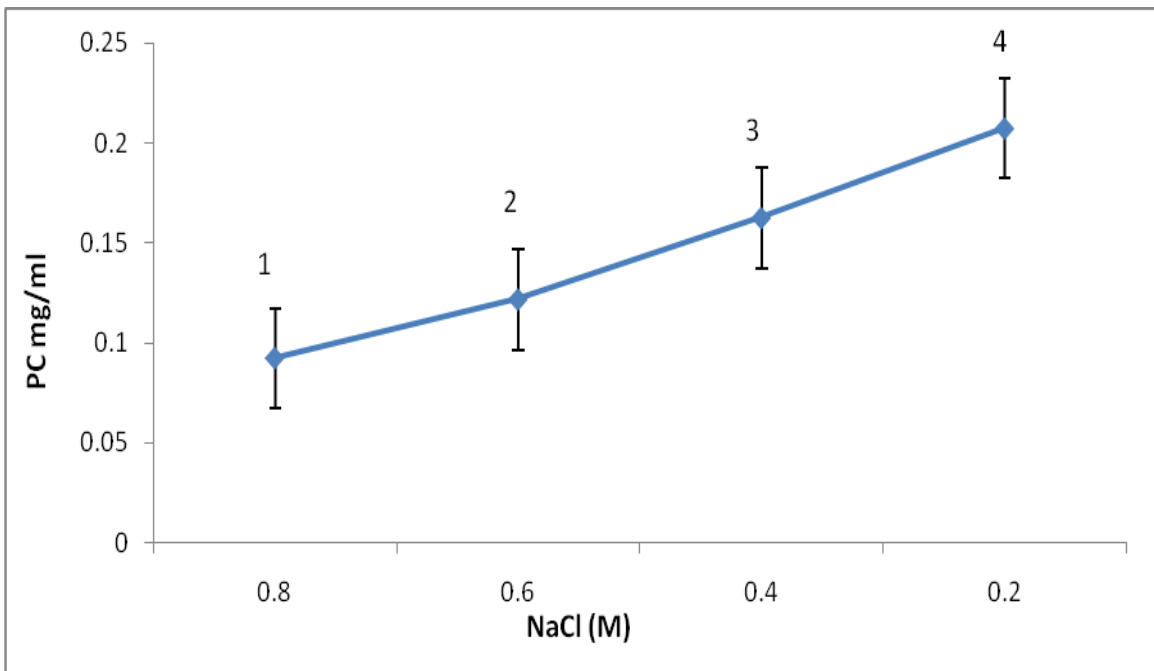


Fig.4 The FTIR- Spectra of the purified sheath of *Phormidium* sp. at 1M Na₂CO₃

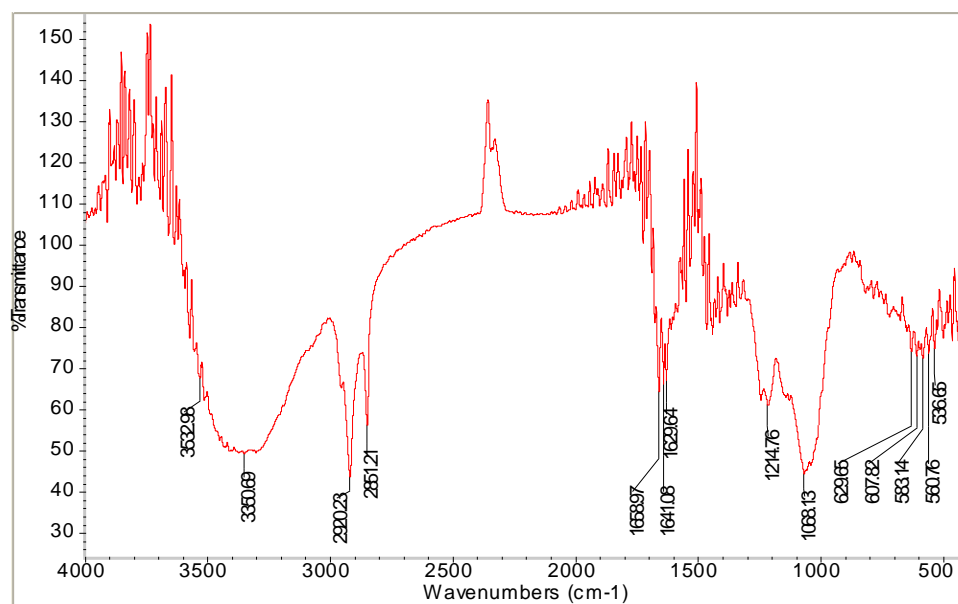
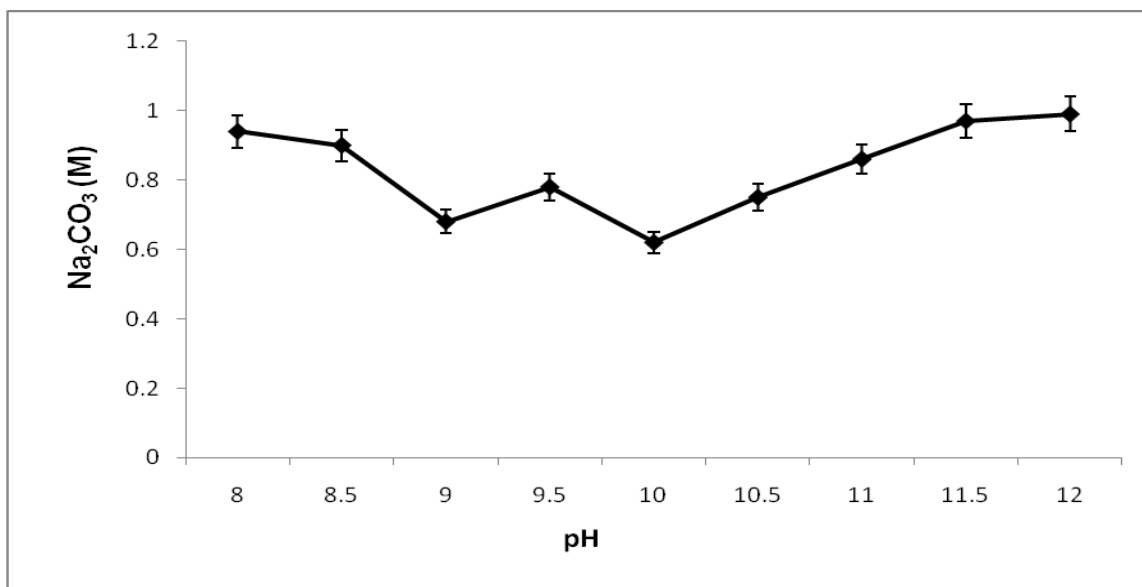


Fig.5 Amount of Na₂CO₃ left in the medium at varying pH. The total carbonate concentration of the medium in each flask is 1M



In alkaliphilic cyanobacteria, the intracellular pH remains stable upon change in the pH of the medium. Thus change in the pH level of the medium do not significantly affect the intracellular metabolic processes, but primarily affect processes occurring in the near cellular space and in cell membranes

(e.g. carbonate transport). Difference between the internal pH and environmental pH affect the transportation of ions and substances through the cell membrane. Thus the alkaliphily is associated with the cell surface (Kupriyanova *et al.*, 2003). In this study it was observed that at pH 9 and 10 there was a

significant decrease in the remaining carbonate concentration in the medium i.e. 0.68 and 0.62 M respectively (Fig. 5). This suggest that the *Phormidium* sp isolated from Lonar Lake contains two carbonate transport systems, one of it operates at pH 9 and the other at pH 10. Similar reports of two carbonate systems were observed by Mikhodyuk *et al.*, (2008) in *Euhalothece* sp. isolated from Lake Magadi.

Presence of more than one transport systems points to the fact that the cyanobacteria residing in the saline alkaline Lonar Lake experience a fluctuation in the carbonate concentration due to desalinization during rainy season. This is the reason that the two transport systems found in the *Phormidium* sp isolated from Lonar Lake differ in their pH optima. During the process of desalinization the pH may shift to lower values (Alcocer *et al.*, 1999). Thus during the rainy season as desalinization takes place the TS-I, whose pH optima is lower, becomes the main transport system and during the period of drought the carbonate content in the lake increases which results in the increase in the pH level. Under these conditions the TS-II becomes the dominant transport system. Thus the alkaliphilism or adaptation of cyanobacteria in the alkaline lake lonar depends on the seasonal salinization and desalinization cycles.

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