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CHARACTERIZATION OF FIVE MARINE CYANOBACTERIAL SPECIES WITH RESPECT TO THEIR pH AND SALINITY REQUIREMENTS

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Abstract

Cyanobacteria a rich source for many useful natural products are used as feed and fertilizer. Optimum condition for their mass culture was evaluated. Here we characterize five marine cyanobacterial species isolated from different niches at Buleji, a rocky shore near Karachi and describe their pH and salinity requirements. Growth rates were determined at three pH values (6.5, 7.4 & 8.0) and four salinity values (5, 10, 20 & 40‰). According to both chlorophyll and protein estimations all cyanobacterial species appear to have preferred near neutral to alkaline pH. Although all cyanobactrial species were able to grow at all salinities, the best growth was observed at a particular salinity. Four out of five isolates of cyanobacteria were able to grow under nitrogen-limited conditions and therefore, may be considered as putative nitrogen fixing species.

Introduction

Cyanobacteria are prokaryotic organisms capable of oxygenic photosynthesis (Moore, 1981). They appeared to be a rich source for many useful products and are known to produce a number of bioactive compounds (Carmichael, 2001; Codd, 1997). During the last few decades, cyanobacteria have been described as potentially important source for vitamins, fuels, fine chemicals and many other pharmaceutical products (Chacon-de-Popioici, 1994; DeVries *et al*., 1993; Miura *et al.,* 1993; Pesando & Bouicha, 1991). Some of the species can fix dinitrogen and hence play a significant role in agriculture (Vaishampayan *et al*., 2001) and in global nitrogen budget (Carpenter & Romans, 1991). Cyanobacteria also hold great promise for bioremediation (Patterson, 1996).

S*pirulina* sp. is known species cultivated on commercial basis for use as food and feed. The growth of cyanobacteria is influenced by a number of factors, therefore it is necessary to evaluate the optimum condition for their mass culture. Pure culture of cyanobacteria is also essential for the study of various aspects of their biology and physiology. Due to their complicated requirements for salts, pH, light, temperature, vitamins, organic carbon, nitrogen and trace elements, marine cyanobacteria are difficult to isolate and culture. These requirements may differ from species to species (Van Baalen, 1967). The successful cultivation and growth of cyanobacteria require modification in the media composition as well as other physico-chemicals parameters (Rippka *et al.,* 1979; Allen & Stainer, 1968; Allen & Arnon, 1955). Marine cyanobacteria have additional requirement for Na^+, Cl^-, Mg^+ and Ca^+ ions for their optimal growth (Rippka *et al.,* 1979) therefore, several liquid media have been modified for the culture of marine and freshwater species eg., ASN-III, MN, and BG11 (Rippka *et al.,* 1979), ASP-2 and SAG1 (Provasoli *et al.,* 1957; Van Baalen, 1962; Rippka, 1988).

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A number of cyanobacterial species have been reported from coastal waters of Pakistan (Bano & Siddiqui, 2003; Siddiqui & Bano, 2001; Mansoor *et al.,* 2000; Shameel, 2000; Siddiqui *et al.,* 2000; Zaib-un-Nisa *et al.,* 2000; Bano, 1998; Shameel *et al*., 1996; Saifullah & Taj, 1995; Shameel & Tanaka, 1992). Some of these species have been tested for their biological activity (Siddiqui & Qasim, 2001). These organisms have been characterized on the basis of cell morphology, usually employed in botanical taxonomic assessment. Here we characterize five marine cyanobacterial species isolated from the rocky shore, Buleji near Karachi and describe their pH and salinity requirements.

Materials and Methods

Isolation and characterization: Samples of cyanobacteria were collected from various niches of a rocky shore, Buleji. Isolation was done using serial dilution and streaking plate method (Throndsen, 1969b; Rippka, 1988). Samples were diluted with sterilized ASN-III medium upto 10^{-25} dilution. Dilution tubes were incubated under constant light at room temperature 28 ± 2°C. The uni-algal cultures of cyanobacteria obtained in dilution tube were further purified on pre-washed ASN-III agar slants (Kantz & Bold, 1969; Rippka, 1988). Stock cultures were maintained at room temperature under diffused light. The species were identified according to previously described botanical mode of classification (Rippka *et al.,* 1979; Desikachary, 1959; Anagnostidis & Komarek, 1985, 1988, Komarek & Anagnostidis, 1986, 1989).

Cyanobacterial growth experiment: Five isolates with fast growth under laboratory conditions were selected to study the effect of variable pH and salinity on their growth. The pH of ASN-III medium (Rippka *et al*, 1979) was adjusted to 6.5, 7.4 and 8.0. Salinity was adjusted at 5, 10, 20 and 40‰ by varying the amount of NaCl in the medium. Known volume (5ml) of well-homogenised culture of each isolate was inoculated in 25 ml of medium adjusted to respective pH and salinity levels. Samples were incubated under light/ dark cycle (12h/12h) at 30°C ± 2. Cultures were manually stirred twice for a few minutes every day. Triplicate culture tubes at each pH and salinity value were retrieved at the beginning of the experiment (zero-hour sample) and on 3, 7, 10 and 18 days of incubation. Cells were harvested by centrifugation for the estimation of growth as increment in chlorophyll and total protein contents.

The isolates were tested for nitrogen fixing ability by first growing in ASN-III medium with combined nitrogen. After a suitable growth has been obtained, cells were harvested and washed three times in phosphate buffered saline (PBS) and re-suspended in the ASN-III$^-$ media where nitrate was omitted and ferric ammonium citrate was replaced by ferric citrate. Cultures were incubated under light/dark (12h: 12h) cycle at 30°C ± 2 as above. Healthy growth of cyanobacteria maintained for at least one month under nitrogen limitation indicated their potential to fix dinitrogen and these species were taken as putative nitrogen fixing strains.

Chlorophyll a estimation: Chlorophyll was estimated as measure of growth using previously described method (Becker, 1994). Cells were resuspended in 90% methanol and allowed to stand in dark for 24 hours. The suspension was cleared by centrifugation and chlorophyll was estimated spectrophotometrically using equation: Chlorophyll *a* (µg L^{-1}) = $(16.5\times A_{665}) - (8.3\times A_{650})$; where A650 and A665 were absorbance of the sample recorded at 650 and 665 nm, respectively.

Total protein estimation: The pelleted sample remained after chlorophyll extraction was homogenized in 50 ml distilled water and used for the estimation of protein employing Lowry's methods (Lowry *et al.,* 1951). Bovine serum albumin was used as standard.

Results

Isolation and characterization: The cynobacterial species were isolated from different niches at rocky shore, Buleji. The selected isolates include one unicellular and four filamentous non-heterocystous species. Their characteristic features are given below.

Description of isolates

***Katagnymene accurata* Geitler**
(after Geitler1982) Anagnostidis & Komarek 1988; pp. 427

Cells wide; not constricted at the cross walls; apical cell non-capitate, rounded; sheath present, colourless; cell width 9.1 µm, cell length 3.9 µm; gliding movement.
Habitat: isolated from open seawater.

***Lyngbya contorta* Lemm.**

Phytoplanct. Sachs Teiche in Ploner Forscber., 6: 202, pl. figs. 10-13. 1898; Forti in De Toni, Sylloge Algarum, 5: 288, 1907: Fremy, Myxo. d' Afr. equat. franc., 202. Fig. 172, 1929; Geitler Kryptogamenflora, 1043, fig. 660a, b, 1932. Fremy Cyano. Cotes. d' Eur., 109, pl. 29 fig. 2, 1933. Desikachary 1959; pp. 290.

Filament straight or regularly coiled, slightly constricted at the cross walls. Apical cell non-capitate, rounded; cell width 2.6 µm, cell length 3.9 µm; bright green in colour; sheath absent; gliding and oscillating movement.
Habitat: isolated from rock pool waters.

***Pseudoanabaena lonchoides* Aanagnostidis**
(after Anagnostidis 1961) Anagnostidis & Komarek 1988; pp. 384, fig. 22 (10).

Cells dark green in colour; sheath not present; distinctly constricted at the cross walls; apical cells non-capitate, rounded; cell width 2.6 µm, cell length 3.9 µm; non-motile.
Habitat: epilithic at high tide.

***Spirulina major* Kutz. ex Gomont**

Kutzing, Phyc. gene., 183, 1843; Gomont, Monogr. Oscillariees, 251 pl. 7, fig. 29, 1892; Forti in De Toni, Sylloge Algarum, 5; 210, 1907; Fremy, Myxo. d' equat. Franc., 234, fig. 208, 1929; Geitler Kryptgamenflora, 930, fig. 595, 1932. Desikachary 1959 pp 196.

Color bright green; spiral very close to each other; spirals width 1.3 µm to 2 µm; Oscillating or gliding movement.
Habitat: edaphic.

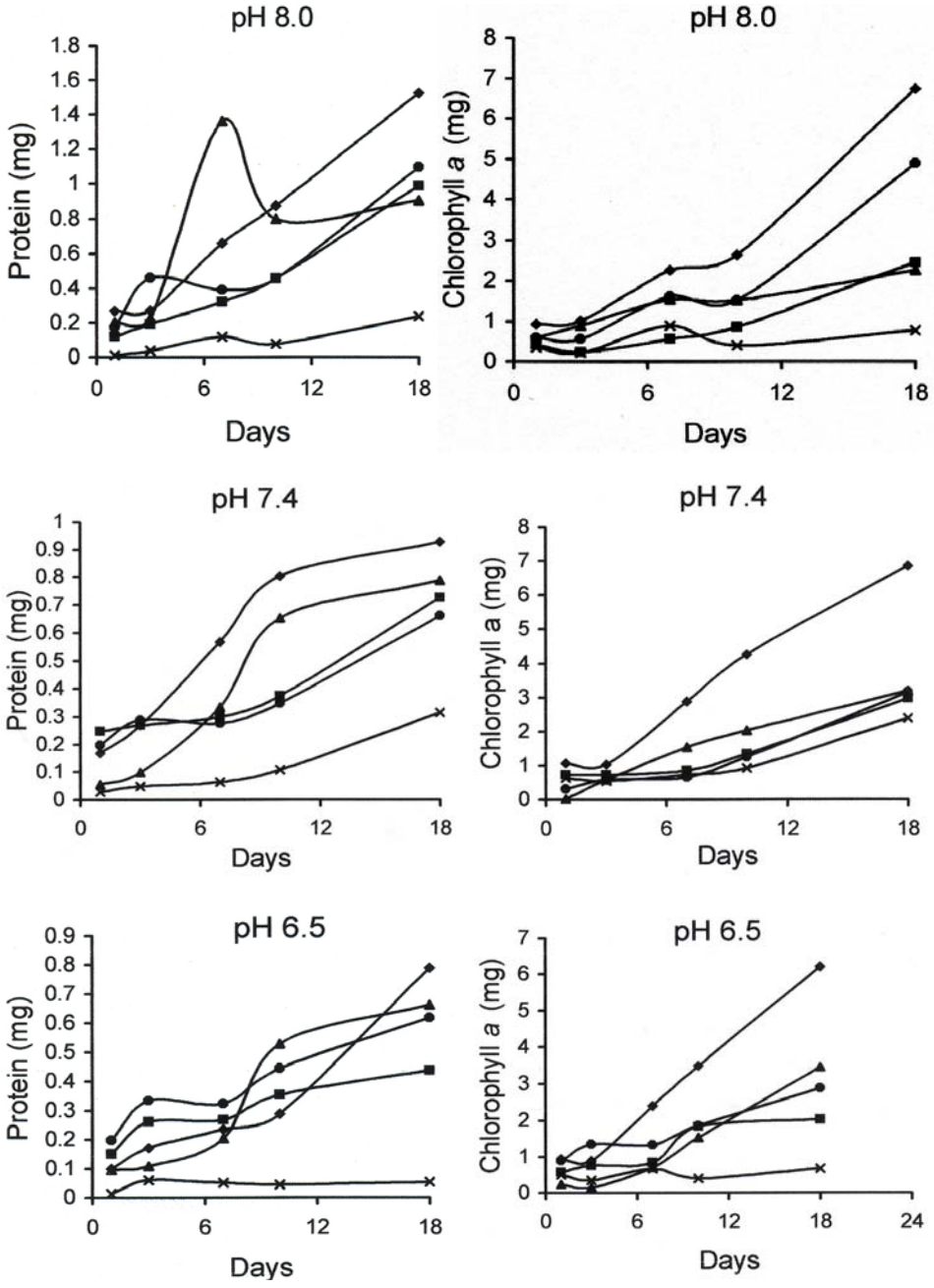

Fig. 1. Effect of pH variation on the growth of five marine cyanobacterial species. Growth was measured as increments in protein and cholorophyll *a* contents. ◆ *Pseudoanabaena lonchoides,* ■ *Lyngbya contorta*, ▲ *Spirulina major*, × *Synechocystis aquatilis*, ● *Katagnymene accurata.*

Table 1. Growth as increment in chlorophyll and protein contents (µg/d^{-1}) of marine cyanobacteria under different pH conditions. Values are growth rates obtained using linear regression. Correlation coefficients are given in parenthesis.

Isolates	pH					
	6.5		7.4		8.0	
	Chlorophyll	Protein	Chlorophyll	Protein	Chlorophyll	Protein
Katagnymene accurata	112 (0.96)	15.7 (0.9)	165 (0.96)	26 (0.92)	253 (0.95)	47 (0.92)
Lyngbya contorta	92 (0.92)	15.4 (0.95)	135 (0.95)	28 (0.95)	126 (0.94)	51 (0.98)
Pseudoanabaena lonchoides	327 (0.99)	39 (0.96)	355 (0.99)	47 (0.95)	345 (0.972)	77 (0.99)
Spirulina major	199 (0.98)	37 (0.95)	184 (0.99)	45 (0.96)	181 (0.99)	42 (0.93)
Synechocystis aquatilis	9.7 (0.49)	1.2 (0.44)	106 (0.92)	16 (0.95)	27 (0.603)	12 (0.94)

Table 3. The growth of marine cyanobacterial species in media devoid of combined nitrogen source. The species which maintained healthy growth in N$^-$media were considered as putative nitrogen fixers.

Isolates	Growth in N$^-$media
Synechocystis aquatilis	+
Pseudoanabaena lonchoides	-
Lyngbya contorta	+
Katagnymene accurata	+
Spirulina major	+

***Synechocystis aquatilis* Sauv**

Bull. Soc. Bot. France, 39: 121, pl. 6. fig. 2 1892; Forti in De Toni, Sylloge, Algarum, 5: 26, 1907; Geitler, Kryptogamenflora, 270, 1932; Desikachary 1959; pp.144.

Cell single, spherical, yellowish green in colour, cell content is homogenous; sheath not present; cell size 2.6 µm, rotatory movement.

Habitat: Isolated from rock pool waters.

Cyanobacterial growth: Growth of cyanobacteria at different pH conditions are shown in Fig. 1 and their growth rates in Table 1. According to both chlorophyll and protein estimations all cyanobacterial species appear to have preferred near-neutral to alkaline pH. The growth was comparatively lower at pH 6.5; *Spirulina major* appeared to have higher growth rate at acidic pH on the basis of chlorophyll estimation. Similarly, all cyanobactrial species were able to grow at all salinities but have shown highest growth at a particular salinity level (Fig. 2 and Table 2). On the basis of increment in both chlorophyll and protein contents, the highest growth rates were obtained for *S. aquatilis* at 5-10‰, *K. accurata* at 10‰, *P. lonchoides* and *S. major* at 20‰ and *L. contorta* at 40‰. *Synechocystis aquatilis* appeared to be slow growing species where as *Pseudoanabaena lonchoides* was the fastest growing species.

Four isolates, out of five cyanobacteria studied, were able to grow under nitrogen-limited conditions (Table 3) and therefore may be considered as putative nitrogen fixing species. *Psedonabaena lonchoides* failed to grow in ASN-III$^-$ medium and died within a few days of transfer. Healthy growth of nitrogen fixing species was maintained under nitrogen limiting condition for at least four weeks.

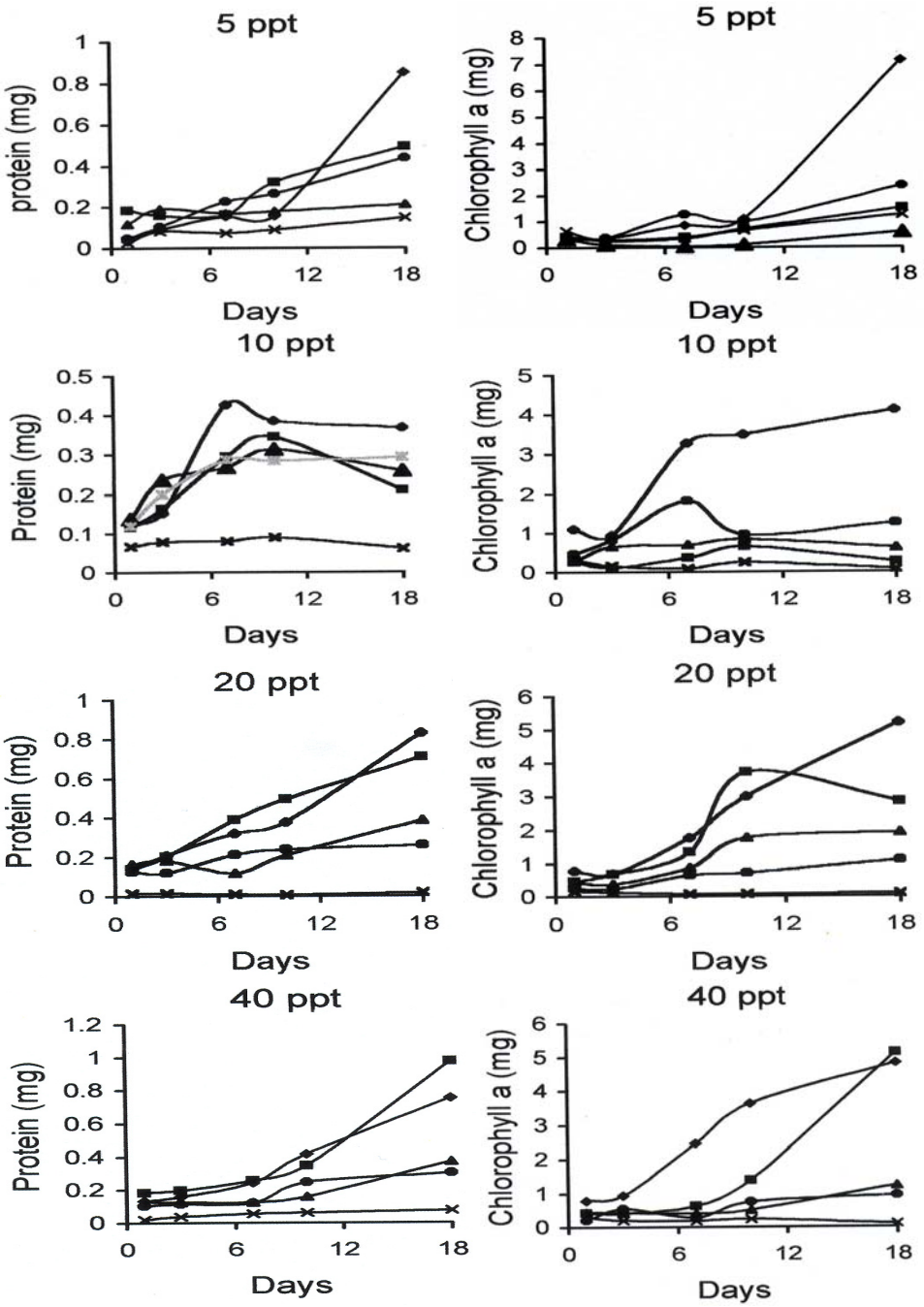

Fig. 2. Effect of salinity variation on the growth of five marine cyanobacterial species. Growth was measured as increments in protein and cholorophyll *a* contents. ◆ *Pseudoanabaena lonchoides,* ■ *Lyngbya contorta*, ▲ *Spirulina major,* × *Synechocystis aquatilis*, ● *Katagnymene accurata.*

Table 2. Growth of marine cyanobacteria under different salinity conditions measured as increment in chlorophyll and protein contents (µg/d^{-1}). Values are growth rates obtained using linear regression. Correlation coefficients are given in parenthesis.

Isolates	Salinity							
	5‰		10‰		20‰		40‰	
	Chlorophyll	Protein	Chlorophyll	Protein	Chlorophyll	Protein	Chlorophyll	Protein
Katagnymene accurata	46.1 (0.452)	12.8 (0.952)	299 (0.99)	21.7 (0.987)	53 (0.98)	16.1 (0.93)	38.4 (0.85)	12.6 (0.939)
Lyngbya contorta	71 (0.929)	20.1 (0.0.965)	48.2 (0.86)	26.6 (0.99)	151 (0.99)	33.9 (0.991)	254 (0.98)	46.4 (0.935)
Pseudoanabaena lonchoides	90.7 (0.98)	13.8 (0.0.93)	196 (0.89)	15.1 (0.71)	277 (0.98)	397.8 (0.98)	254 (0.98)	37.6 (0.987)
Spirulina major	20.4 (0.5985)	3.9 (0.764)	15. (0.48)	6.1 (0.6299)	100.5 (0.92)	12 (0.835)	46.2 (0.89)	3.2 (0.899)
Synechocystis aquatilis	46.9 (0.985)	5.9 (0.94)	52.1 (1)	2.2 (0.944)	1.9 (0.993)	000.2 (0.976)	5.8 (0.821)	3.16 (0.95)

Discussion

Uni-algal culture of cyanobacteria is essential for studying many aspects of their biology. No earlier studies are available from Pakistan on the culture of cyanobacterial isolates and their cultural characteristics. Information on requirements for optimal growth of cyanobacteria is necessary for the propagation of these species in mass cultures. Although a large number of species were recorded from any given habitat on a rocky shore (Bano & Siddiqui, 2003), the number of isolates obtained so far is low (data not given) and we selected only five isolates for present study. The general inability of cyanobacterial species to grow as uni-algal culture has been noted in the literature and this has been attributed to the fact that laboratory environment is quite different and limited compared to natural marine habitat (Rippka, 1981). The variety of biotic interactions that can exist among species may be critically important for the survival of cyanbacterial species. This study indicates that isolation and culture of all forms of cyanobacteria is difficult.

The test isolates depict an optimum for pH between 7.4 and 8.0. The data is in good agreement with previous reports (Rippka *et al.,* 1979; Rippka, 1981). The fact that all cyanobacteria were able to grow in acidic (pH 6.5) medium, reflects that cyanobacteria can adapt to variable pH conditions as suggested earlier (Buck & Smith, 1995; Burja *et al.,* 2002). The cyanobacteria possess different mechanisms for maintenance of pH homeostasis depending their natural habitat (Buck & Smith, 1995).

Only *Spirulina major*, showed comparatively higher growth rates at all pH values. However on the basis of chlorophyll content, best growth was obtained at pH 6.5, whereas highest growth was at pH 8.0 when protein content was taken into account. Earlier results, however, suggested a complete absence of cyanobacteria in the environment (pH < 4-5) (Rippka *et al.,* 1979). This may suggest that slightly acidic environment is not deterrent to the growth of cyanobacteria, and in some cases it preferred pH for higher growth. This conclusion is also supported by the fact that beside *Spirulina major*, all other species were also able to grow in pH 6.5 but at lower rates.

The changes in salinity also affect the growth of cyanobacteria. All species showed growth at all salinities tested, but an optimum requirement may be clearly seen through the data. Many fresh water species are reported to withstand higher salinities (Carr & Wyman, 1986). Similarly, many marine forms can survive at lower salinity, but for their optimum growth they express specific requirements for additional salts (Rippka *et al.,* 1979). Therefore, cyanobacteria have been regarded as halotolerant and halophilic forms. For example, *P. lonchoides, L. contorta* and *S. major* appear to be halophilic. Two species *S. aquatilis* and *K. accurata* demonstrate their halotolerant behavior as they grew well at low salinities but could sustain higher salinity. It may be noted from earlier literature that although cyanobacteria can adapt to the variations in salinity and other trace metal concentrations but all cyanobacteria are not halotolerant (Yopp *et al.,* 1978; Blumewald & Tel-Or, 1982). However, there are strains that can grow well at salinities ranging from 0-99‰ (Thajuddin & Subramanian, 1992), and a *Nodularia* species has been shown to grow in salinity range between 0-20 gL^{-1} (Moisander *et al*., 2002). Therefore, all cyanobacteria may not be distinctly classified as marine or fresh water forms.

Information on salinity and pH requirement of cyanobacteria is very important for mass culture for industrial purposes. Cyanobacteria possess many secondary metabolites

that are of pharmaceutical importance and growth condition influence the secondary metabolites production (Burja *et al*., 2002).

Diazotrophic activity in the marine environment has a significant role in the production of new nitrogen and this activity has been attributed largely to nitrogen fixing bacteria and cyanobacteria (Herbert, 1999). In this study 4 out of 5 isolates of cyanobacteria have shown a capacity to fix dinitrogen. These isolates were obtained from a variety of habitat such as epiphytes, planktonic and edaphic. Diazotrophic cyanobacteria have been reported from similar habitats from various parts of the world including planktonic (Carpenter & Romans, 1991), epiphytic (Capone, 1982), and edaphic environments (Zuberer & Silver, 1978). Nitrogen fixing cyanobacteria has significant role in the production of new nitrogen as well as primary production in marine environment (Herbert, 1999).

This is the first preliminary report on the isolation and cultural characterisation of marine cyanobacteria from Pakistan. Availability on the characterisation of isolates will enhance the appreciation of the role of cyanobacteria in the coastal areas and also in its intensified culture for fine chemicals.

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