Morphological and Genetical Characterization of Selected Cyanobacterial species from Lesser Cholistan and their Potentials

By

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(Ph.D.)

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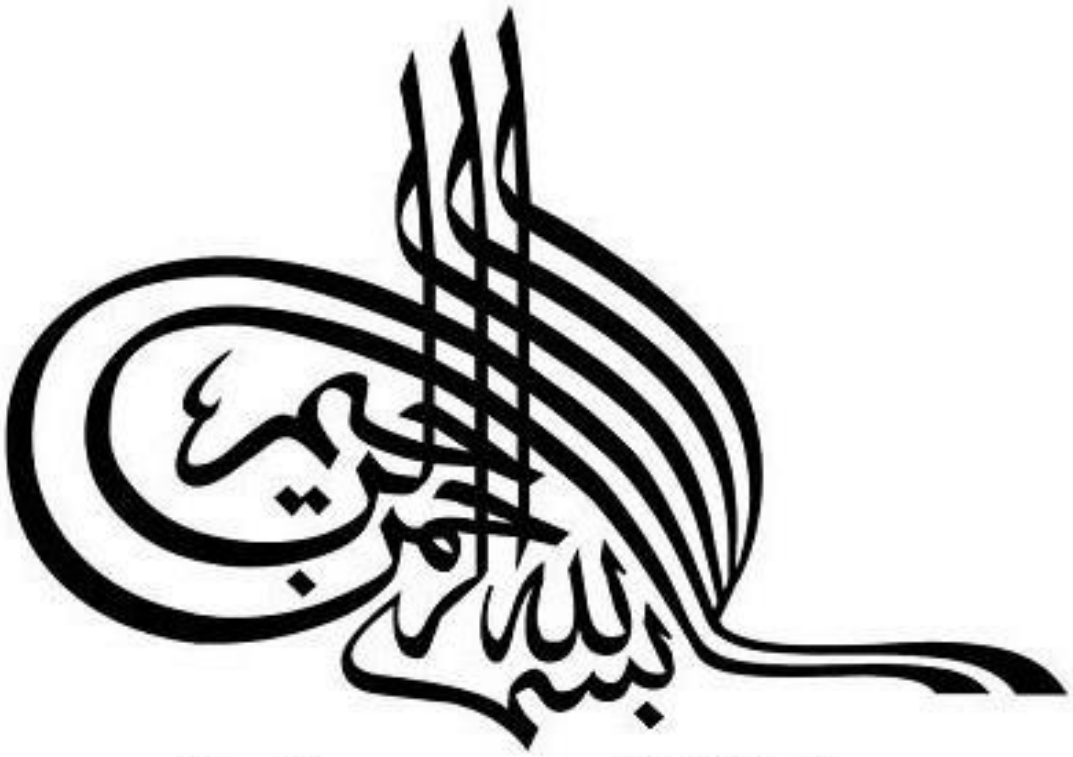

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the Most Beneficent,
the Most Merciful

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Preamble

This thesis is submitted for the degree of Doctor of Philosophy in the Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. No content or segment of this thesis has been submitted for any degree at any other institution or university. This thesis work is original according to the best of the author's knowledge unless reference is taken from the published work. This thesis has been published or submitted in international journal articles written originally by the author. A list of related publications is given below:

1. Inam, F., Mumtaz, A. S., Kaleem, M., & Sajid, I. (2022). Morphogenetic variation and assorted biological activities in true branching Nostocales strains of Cholistan oasis, Pakistan. *Journal of Basic Microbiology*, *62*(5), 634-643.

Thesis Dedication

Dedicated to my wonderful wife and dear father-in-law, my kids, parents, and respected teachers who all enable me to achieve this goal.

Acknowledgments

All praises belong to **Almighty ALLAH**, the most merciful, beneficent, creator, and source ofall knowledge and wisdom, who enabled me to complete this grand task successfully. I also pay my respect to **Hazrat Muhammad (Peace and Blessing of Allah Be Upon Him**), His faithful companions, who are forever a genuine torch of guidance for humanity.

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Farooq Inam

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List of Abbreviations

1	PARC	Pakistan Agriculture Research Council
2	PCR	Polymerase Chain Reaction
3	ATCC	American Type Culture Collection
4	AZRI	Arid Zone Research Institute
5	PCSIR	Pakistan Council of Scientific and Industrial Research
6	NCBI	National Center for Biotechnology Information
7	DNB	Dera Nawab Barrage
8	USA	United States of America
9	ATCC	American Type Culture Collection
10	Ec	Electrical conductivity
11	pH	Power of hydrogen ions concentrations
12	Rpm	Revolution per minute
13	°C	Degree centigrade
14	mm	Mili-meter
15	OD	Optical density
16	NJ.	Neighbour joining
17	L.W	Length,Width
18	DNA	Deoxyribonucleic Acid
19	$NaNO_3$	Sodium nitrate
20	$KN0_3$	Potassium Nitrate
21	$NaCO_3$	Sodium Carbonate
22	$MgCl_2$	Magnesium dichloride
23	dNTPs	Deoxynucleotide triphosphates
24	HCL	Hydrochloric acid
25	BG^0	Blue-Green media (Nitrogen free)
26	BG_{11}	Blue Green Media
27	BBM	Bold and Basal Media
28	Sp. media	Spirulina media
29	NGR	No Growth Respose

Abstract

The objectives of this study were to explore and collect cyanobacterial samples from different substrates and localities in the lesser Cholistan desert; characterize the isolates morphologically and genetically using the polyphasic approach; and assess the antibacterial, cytotoxic and anticancer potential. For this purpose expeditions were commissioned to Cholistan desert. In the field ecological data was recorded and collated with lab-based morphological and molecular analyses to infer species identification and diversity. This involved culturing of several species too. Axenic cultures obtained in BBM, BG_{11}, BG_0 and SpM media, corresponding to seven major taxa of Cyanobacteria, were recorded. Among field samples, the green types were observed frequently contributing a share of 48.5% while 37% blue green types were observed in the collections. Among green algal types, members of the genus *Scenedesmus* were found most frequently. Other green taxa were represented by either one or two species only. Among blue green types, *Phormidium* was observed most frequent. Among unique types, *Neowestiellopsis* was found at three sites. *Anabaena* was observed on sites found in close vicinity of agricultural land. In conclusion, Cholistan desert has huge cyanobacterial diversity including the unicellular forms; the polyphasic approach has clarified the identification/taxonomic position of morphologically closely related species. This included *Nodularia, Desertifilum, Neowestilliopsis* and *Nostoc* sp. with subtle morphological differences. For the first time, four genera *Neowestilliopsis, Desertifilum, Klisinema,* and *Aliinostoc* were reported from the Cholistan Desert, Pakistan. The biological activity analyses revealed strong antimicrobial, cytotoxic and anticancer potential in *Neowestiellopsis*, *Tolypothrix* and *Desertifilum*, and all these warrant further analyses.

INTRODUCTION

Cyanobacteria have long been considered ‘blue-green algae’, but being prokaryotic and autotrophs, these bacteria-like organisms are known as Cyanobacteria (Stanier, 1977; Demoulin et al., 2019). Members of this group are present in aquatic, terrestrial and atmospheric environments. These fascinating organisms evolved during the great oxygenation event of the early Precambrian era (Hamilton et al., 2016; Garcia-Pichel et al., 2019). Cyanobacteria are considered a diverse group of autotrophs with the closest resemblance to purple bacteria (Woese et al., 1990; Cavalier-Smith, 2002). These organisms also resemble land plants due to the presence of chlorophyll (Moore et al., 2019), although they are contained in simple thylakoids. Cyanobacteria lack nuclei and organelles found otherwise in eukaryotes, allowing their microscopic distinction from most other microalgae. Cyanobacteria have a variety of shapes and sizes, including spherical, ellipsoid, barrel, cylindrical, conical and disc shaped. Flagella are found in many phytoplanktonic taxa, but not in cyanobacteria. However, many cyanobacteria, particularly filamentous species, exhibit gliding motility (Hoiczyk, 2000; Read et al., 2007).

A traditional taxonomic treatment accepted:1300 species, 145 genera, 20 families, and three orders within Cyanophyta (Geitler, 1932). Since the classification system considered multiple morphological traits of field specimens and it had a pivotal taxonomical position in that era (Bourelly, 1970; Desikachary, 1959; Elenkin, 1938-1949; Fritsch, 1959; Golubic, 1976; Kondrateva, 1968; Starmach, 1966) An alternative classification system of the same era advocated that the diversity of morphology in natural samples of cyanophytes was, in fact, different varieties of the same taxa (Drouet, 1981).Therefore, this system catered a smaller number of genera and species. As this system underestimated the prevailing genetic diversity, it was not fully accepted (Palinska and Surosz, 2014).

A school of thought supports the use of bacteriological system of classification for these prokaryotes. An axenic cultured strain is the basic unit in the bacteriological system, and the comparison of similar strains formed a species. This system utilized Geitlerian designations and several cultural properties to define a genus (Castenholz and Waterbury, 1989). Recently, as mentioned in Burgey's manual, information is now collected through both physiological and bacteriological approaches. The physical characteristics of field-collected specimens assessed by light microscopy have traditionally been used to classify cyanobacteria (Geitler, 1932; Desikachary 1959). The species is named for specimens with comparable phenotypic traits (Waterbury, 2006). Taking this argument further, the proposed combination of morphology, genetics, ultrastructural and environmental information, is appropriate enough for characterizing and confidingly identify different taxa. However, at times this may result in discrepancies between traditional and bacteriological approaches (O'Neil et al., 2012). The polyphasic approach, a term introduced by Colwell (1970), provides a consensus of all available methods. Hence a morphospecies was defined by the "polyphasic approach" as a group of individuals that are genetically similar and can be recognized as a phenotype (Komarek, 2011). This technique has been frequently employed in recent cyanobacterial investigations such as those by Butte et al. (2005); Ballot et al. (2010); Berrendero et al. (2008); Dadheech et al. (2012); Heath et al. (2010) and Zapomelova et al. (2009).

Komárek et al. (2014) proposed a classification of taxa based on complete genome sequences, in addition to the ultrastructural characteristics, such as the distribution of thylakoids. These characteristics cannot be observed through light microscopy. Hence less useful for routine testing of the field samples. Classical and morphological features, such as filaments, the presence of sheath etc. and similar characteristics readily observed through microscopy, are less important in this system. Based on the multidisciplinary recommendations/polyphasic approach, the latest proposed systematic classification allocates

cyanobacteria into eight orders, some of which are still provisional and polyphyletic (Komárek et al., 2014; Mareš 2018).

1.1 The Polyphasic Approach and Definition of New Taxa

Since long, the taxonomic classification of cyanobacteria was based on morphology with very rare ecological considerations. However, the morphology has proved an insufficient tool in the modern taxonomy (Castenholz, 1992; Komárek, 2005). The combined approach describes organismal diversity, catering unicellular forms which are simpler to the multicellular types, based on cyto-morphological, molecular and ecological methods (Komárek, 2016). The molecular sequencing data is used as the basic approach along with other criteria (morphological and ecological) which highlight distinction for cyanobacterial populations. Employing such methods different strains of *Cylindrospermum* collected from different regions of the world were assessed. Five *Cylindrospermum*. strains satisfied the criteria for species foundation. Three strains showed positions distant from the main clade, which are deemed new to science. These species are *C. badium*, *C. moravicum*, and *C. pellucidum*. A *Cylindrospermum* strain, described earlier as *Cronbergia* was shown to be synonymous with *Cylindrospermum* in this study (Johansen et al., 2014). Strain CCM-UFV059 was investigated using a polyphasic approach as well as physiological and ecological traits. Taxonomic characters were similar to the main *Nostoc* clade but genetically it was showing relationship with main clade of *Desmonostoc*, but not showing any relation with the reported species of *Desmonostoc* taking in consideration the new ecological features, new species of *Desmonostoc* was described as *D. salinum* from alkaline saline environment (De Alvarenga et al., 2018). Another strain isolated from the Banxia mountain of southern China morphologically resembled the genus *Desmonostoc* but genetically showed no relationships with the previously described species. Therefore, a new species, *D. damxiaense* has been described (Cai et al., 2018). Another example of a new genus *Roholtiella*, from

morphogenetic study of 16 strains showed morphological features of both *Tolypothrichaceae* and *Rivulariaceae* with false branching and tapering trichomes respectively, along with typical feature of Arthrospores (Bohunicka et al., 2015).

Scytonematopsis is a new genus in the Nostocales, with a tropical distribution of *S. kiseleva.* A new species of *Scytonematopsis*, *S. contorta*, from the Hawaiian Islands of Oahu, was described on a morphological and molecular basis. Morphologically, it was different, with a spirally contorted trichome within a single filament; phylogenetically, it was placed with *Rivularia* and *Calothrix* strains rather than within the *Scytonema* clade (Vaccarino & Johansen, 2011). A new family, Getlereacea and new genus *Geitleaus*, was established to accommodate a species with a true branching pattern lacking heterocysts. This species was isolated from limestone caves of Israel and was also reported from France, Cook Islands, Costa Rica, Florida, Spain, and Romania (Kilagore et al. 2018).

Desmonostac is a new genus that has been morphologically and genetically described. This genus was distant from the main Nostoc clade, and its morphological features also deviated from those of the genus *Nostoc*. The diversity of the natural population of the genus *Anabaenopsis* was taxonomically reviewed, with the development of heterocysts as the main generic character (Komarek, 2005). Eight new Nostocalean species have been morphologically reported in rice fields of Iran. *Tolypothrix conglutuonata, Aulosira, Cylindrospermum,* and *Stagnale* are dominant in all seasons, whereas *Gloeotrichia longiarbiculata* and *Cylindrospermosis* are common only in spring and summer (Siahalaei et al., 2011). Four strains of *Anabaena* and *Aphanozomenone* were analyzed using a polyphasic approach to assign these strains to *Anabaena* and *Aphanozomenone* species (Stuken et al., 2009). The genus *Anagnostidinema* was described in *Geitlernema pseudacutissimum* based on its molecular and morphological characteristics (Johansen et al., 2017).

1.1.1 16SrRNA and its diagnostic value in polyphasic studies

With the advent of genetic analysis, the classification of cyanobacteria has undergone major revisions and is expected to be restructured even more in the upcoming years owing to further phylogenetic analysis. The 16S rRNA locus has huge diagnostic value. In the following text several examples prove this proposition. For instance: a study deployed the polyphasic approach on the heterocystous cyanobacteria belonging to the *Calothrix* and *Tolypothrix* genus. The morphology of the obtained environmental samples was compared with the phylogeny prepared using the 16S rRNA gene sequences. Experimentation showed strains from several rivers and streams with higher levels of tapering in nutrient rich medium while lower level of tapering in nutrient poor environment. Strains resembling both genera were observed, with characteristics like lower tapering and false branching that resemble *Tolypothrix* while other characteristics which resemble *Calothrix*. The phylogenetic analysis corresponded with the phenotypic characterization in which the strains and environmental samples of both genera could be easily differentiated based on their morphology. The members of the *Tolypthrix* owing to their similar morphology, development and 16S rRNA DNA sequences were placed into the same cluster. However, although morphologically similar at times, members of the *Calothrix* genus were placed into separate clades due to their clear genetic divergence (Berrendero et al., 2011).

Initially based on morphology, it was placed in the genus *Calothrix*. However, based on the subsequent phylogenetic analysis the strains of the clade of *Calothrix* that lacked terminal hairs were re-described as *Dulcicalothrix* gen. nov., and *Dulcicalothrix necridiiformans* sp. nov. It was further supported by their ability to form necridia. The strain differed from other members of the clade based on secondary structure analysis which suggests that the groups marine/freshwater *Calothrix*, freshwater/ terrestrial *Calothrix Dulcicalothrix* and *Macrochaete* need to be placed into separate generas.

Evidence based on phylogenesis suggests that Calotrichaceae family needs to be separate from the existing family Rivulariaceae (Saraf et al., 2019).

Using the polyphasic approach (Kabirnataj et al., 2020) characterized five cyanobacterial strains from Iran which had *Nostoc*-like morphology. Three strains clustered with the *Aliinostoc* clade and the remaining OTU clustered within the genera *Desmonostoc* and *Desikacharya*. The 16S rRNA gene pointed in the direction of representing newly described species of the genera *Desmonostoc*, *Desikacharya* and *Aliinostoc* and further analysis showed that all strains represented novel species previously unknown. As per the International Code of Nomenclature for algae, fungi and plants, genus *Aliinostoc* was said to have three novel species and genus *Desmonostoc* and *Desikacharya* had one specie each.

A novel cyanobacterial strain (CHAB7200) was isolated from the alkaline pool in Zheiang, China which has an identical 16S rRNA gene sequence with *Desertifilum tharense* belonging to *Desertfilum* genus that contains filamentous cyanobacteria. Despite the identical 16S rRNA gene sequences, the cellular morphology, ultrastructure, and ecology were distinct, and their growth ability differed in different alkaline conditions showing optimum growth at 1% salinity portraying stronger resistance to alkaline and saline conditions as compared to *D. tharense*. The name of the novel species was proposed to be *Desertifilum salkalinema*. sp. nov. (Cai et al., 2018).

1.2 Cyanobacteria in the Desert Environment

Microbial life in desert ecosystems is abundant and diverse. In such environments, microorganisms endure several physicochemical stresses. These may include low water potential, nitrogen and carbon starvation and severe temperatures (Leung et al., 2020). Sparse vegetation is usually found in arid and semiarid lands. Nevertheless, a community of highly

specific organisms, such as mosses, lichens, liverworts, algae, fungi, cyanobacteria, and bacteria, occupy the ground surface in open spaces between vascular plants. These communities are usually called biological soil crusts (BSCs) or microphytic crusts of soils (Belnap 2006; West, 1990). BSCs have been found in desert areas worldwide because of their extraordinary capacity to avoid drying and survive in extreme temperatures (up to 70 °C), high pH, and saline environments.

BSCs play a vital role in ensuring that desert ecosystems are meticulously organized and functional. These organisms are involved in vital functions such as: soil formation, stability, and fertility; wind or water-borne prevention of soil erosion, plant root colonization and sand dune stabilization (Eldridge and Greene, 1994; Belnap, 2003; Zhang et al., 2006; Li et al., 2009; Lange, 2001). Recently, several desert ecologies have been investigated for their resilience in crust recovery affected by factors such as global warming and increased anthropogenic activities (Belnap et al., 2001; Belnap et al., 2008). As a result, Belnap (2003) recognized the preservation of BSCs as the top priority for desert area management. It is intriguing to note that such desert environments are rich in cyanobacterial species. For instance, one study reported that the Rajasthan Desert of India has 79 morphotypes belonging to 21 genera of cyanobacteria. Among these, Oscillatoriales, such as *Phormidium* species, were dominant, followed by *Oscillatoria* species, whereas *Lyngbya* species were less frequent. *Anabaena* species were the most abundant in the order Nostocales, followed by *Nostoc*, *Scytonema* and *Calothrix* species (Bhatangar et al., 2008).

The application of the polyphasic approach revealed new species in such unique ecologies. For instance, *Desertifilum tharense* gen. et sp. nov. was reported based on physiological ultrastructural morphology and phylogenetic analyses (Dadheech et al., 2012). The diversity of cyanobacteria in Rajasthan was physiologically characterized from soil samples. Members of the genus *Nostoc* were the most diverse, along with *Anabaena*, *Westiellopsis* and

Phormidium species (Tiwari et al., 2005). The genus *Allinostoc* and the species *A. morphoplasticum* were morphologically described and isolated from Jabalpur, India. Careful observation revealed that the characteristics of motile hormogonia with gas vesicles were different from those reported in *Nostoc* sp. Phylogenetic studies including *rbc*L, *psb*A. *rpo*CI and *tuf*A loci studied and compared with *Mojavia*, *Rphidiopsis*, *Desmonostoc, Nostoc*, *Anabaena*, *Allosira*, *Cylindrosphaero spermopsis*; with compelling evidence to describe these taxa as a new species (Bagchi et al., 2017).

Soil biocrusts were also analyzed for cyanobacterial growth in Utah's Calorado Plateau, USA (reference). Cyanobacterial filaments from natural populations and cultures were morphologically and genetically assessed. *Microcoleus vaginatus* was found to be common in most samples, as confirmed by morphogenetic analysis. Similarly, a new taxon, Xeronema, was found to be phylogenetically distant and morphologically similar to *Phormidium* and *Schizothrix*. Geologically, gypsum crust populations deviate from soil crust populations residing in sand, silt, and shale (Pichel et al., 2001). Another example of a change in genus is *Schizothrix adunca* from Atacama Desert, Chile, which considered the morphological, molecular and ecological data. The polyphyletic position of *Phormidium* was confirmed from molecular and morphological data and species isolated from different regions of the Arctic and Antarctica. Different strains showed large distances between the clades (Comte et al., 2007).

Based on morphological and ecological data, twenty species belonging to eleven genera have been reported from the desert are in Himachal Pradesh, India (Singh et al., 2014). These groups are established to accommodate the diversity. 1st group was characterized by high nutrient and high temperature with *N. punctiforma*, *Nodularia sphaerocarpa*, *Nostoc linckia*, *Geitlerinema acutissimnium*, *Limnothrix redekii*, *Planktothrix caloathrata* and *P. agardhii*. A second group showed high pH and low temperature with oligo-isotrophic water. *Gloeocapsa,*

pleurocapsoides, Leptolyngbya anthractiea, L. frigida, Psedoanabeana frigida, and Nostoc spongiaeforme. The last group was not associated with any environmental conditions and was present in all the groups. After a detailed study, the species isolated from the Antarctic region suggested a shift from the genus *Phormidium* to *Phormidesmis*. Tropical species showed special morphological features such as single or rarely two to many trichomes in a single sheath, moniliform trichome, undifferentiated terminal cells without calyptra genetically it is more closely related to Pseudoanabanaceae family rather than *Phormidium* main cluster. The parietal arrangement of thylakoids was also different from the main pattern of *Phormidium*. The same was true for the Antarctic strain, which showed deviations from the genus *Phormidium* (Komárek et al., 2009).

1.3 The True-branching Cyanobacteria

Taxonomical variety in true-branched cyanobacteria remains underestimated. It has been less explored and warrants attention; very few accounts recognized these as distinct groups within the order Nostocales. Phylogenetically, the branched heterocystous cyanobacteria were described as a separate order called Stigonematales (Anagnostidis and Komarek, 1990). The representatives of the true branching cyanobacteria were also distinct morphologically (Springstein et al., 2020), however, their taxonomic disposition remains unclear, making their identification more challenging. Recently, several proposals have been put forth for a comprehensive revision of this group (Gugger and Hoffmann, 2004; Kaštovský and Johansen, 2008: Komárek and Mareš, 2012; Dagan et al., 2013). However, the bases for such revisions are unclear with no consensus, whatsoever, to clearly categorize true branching cyanobacteria. This has led to confusion for a group which has huge taxonomic diversity. An attempt, employing polyphasic approach (employing molecular sequence data, colony & cell phenotypes and the ecology) by (Komárek et al., 2014) to revise the taxonomic position of true branching heterocystous cyanobacteria, revealing their monophyletic nature. This

comprised of following families: Stigonemataceae (true branching, multiseriate), Hapalosiphonaceae (true branching 'T') and Symphyonemataceae (true branching 'Y') (Golubic et al., 1996). On the contrary, Gugger and Hoffmann (2004) concluded that the true branching cyanobacteria are polyphyletic. Among the true branching forms, members of the family Hapalosiphonaceae were assigned to six genera including: *Fischerella*, *Hapalosiphon* and *Westiellopsis* (Komárek et al., 2014). However, *Neowestiellopsis* was recognized as a distinct taxon from *Westiellopsis* based on phylogeny (Kabirnataj et al., 2020). The later study emphasized the need for widespread exploration of the branched heterocystous types and their diversity.

1.4 Methods for Cyanobacterial Culturing

The isolation of morphologically different cyanobacteria and generating biomass in cultures is tremendously important. Monocultures enable researchers to precisely investigate the genetics, biochemistry, and eco-physiology of strains (Komarek & Johansen 2015). Thus, it is imperative to have basic knowledge of cyanobacterial taxonomy, physiology and ecology (Pulz and Gross, 2004), as the initial culturing conditions may influence success of their final application. Though, there are no clear rules for the purification and culturing of cyanobacteria, but an intuition is required by researchers (Castenholz, 1988). The primary aim of cultivation is to optimize the growth for biomass and product yielding through the culture conditions. The principles of microbial cultivation are also applicable to the cyanobacteria; however, microalgae hold its own capability to harvest light energy for growth yields (Lee and Shen, 2004).

The choice of cultivation method is determined by the type of species and its culturing requirements (MacIntyre and Cullen, 2005). At constant conditions cells eventually acclimatize to an environment and physiological responses attain an equilibrium. Similarly,

for cultures responding to subtle changes in media, the equilibrium may be dynamic, and growth remains balanced. Cultures subjected to systematic changes in conditions rarely attain equilibrium, and growth becomes unbalanced (MacIntyre and Cullen, 2005). Gerloff et al. (1950) was a pioneer attempt to record the nutritional and physiological needs for *in vitro* culturing of cyanobacteria. The study recognized paucity of knowledge for *in vitro* culturing of a group of organisms which otherwise has widespread prevalence. Microalgae are recognized as sensitive indicators of ecological changes in their vacinity (Kelly et al., 1998). In natural conditions, algae exist as mixed assembly of organisms that include other non-algal taxa too. The maintenance of *in vitro* cultures is thus important for gaining an understanding of microbial ecology (Kumar et al., 2019). (Hay & Fenical, 1988) urged compilation of comprehensive ecological information while establishing new cultures for cyanobacteria. A previous study by Duy et al., (2000) suggested insignificant differences between natural and laboratory cultures. However, research undertaken initially by Neilan et al., (1995) and later by Reynolds et al., (2002) and Sekar et al (2004) refuted these claims, recognizing that there are considerable difficulties in differentiating field samples from those in culturing.

1.5 Significance of Cyanobacteria

Cyanobacteria are among the most ancient lineages of extant organisms, appearing approximately 3.5 billion years ago (Planavsky et al., 2014; Nutman et al., 2016). These are the only prokaryotes that can perform oxygenic photosynthesis owing to their complex pigment composition (Schopf, 2002). Cyanobacteria of the early Earth were responsible for the creation of an oxygen-rich atmosphere suitable as aerobic organisms, including plants and animals (Schirrmeister et al., 2015). Cyanobacteria thrive all around the globe due to their

ability to colonize all types of habitats and survive extreme conditions (Whitton and Potts, 2000 and 2012).

The occurrences of cyanobacteria in a broad range of ecological niches rely on the maintenance of cell function and structure, with little energy required for their growth (Maavara et al., 2017). Cyanobacteria are major components of phytoplankton and periphyton communities. They are either free-floating organisms in open water or attached to rocks and other substrates. The ecological significance of cyanobacteria lies in their involvement in global carbon, oxygen, and nitrogen cycles through production of organic compounds utilized by other organisms, and stabilization of sediment and soil (Whitton and Potts, 2000; Pichel et al., 2003). Many cyanobacteria from freshwater, marine and terrestrial habitats are diazotrophs capable of converting dinitrogen gas to two ammonium ions because of nitrogenase activity (Letelier et al., 2004). The converted nitrogen is later available for utilization in amino acids (proteins and peptides), two nucleic acids, and other cellular parts that are rich in nitrogen (Zehr et al., 2001; Bothe et al., 2010).

Nitrogen fixation by cyanobacteria provides a beneficial resource for symbiotic relationships with non-diazotrophic organisms; thus, cyanobacteria are often involved in symbiosis with diverse organisms in which they act as nitrogen suppliers. Nitrogen-fixing cyanobacteria (e.g., members of the genus Nostoc) are frequently involved in consortia with liverworts, hornworts, moss, ferns, cycads, and angiosperms (Rai et al., 2000). Nitrogen fixation in the often nitrogen-limited marine environment is utilized by both free-living (Bergman et al., 2013) and symbiotic (Zeev et al., 2008) diazotrophic cyanobacteria of diverse phylogenetic lineages. Marine cyanobacterial endosymbionts are associated with diatoms and other microalgae species. (Momper et al., 2015, Stenegren et al., 2018). The ability to fix atmospheric nitrogen gives nitrogen-fixers a competitive advantage over other microorganisms in environments with nitrogen source deficiencies (Huisman et al., 2018).

1.6 Cyanobacteria: a source of Bioactive compounds

Cyanobacteria accumulate high value metabolites; for instance, the cyclic depsi-peptides (Matern et al., 2003); alkaloids, polyketides and non-ribosomal peptides (Zainuddin et al., 2002). Only a very few of such compounds have been characterized. The allelopathic interactions between cyanobacterial species are well recognized, however truly little is known on how these compounds are produced and released (Le Flaive and Ten-Hage, 2007). Several authors have adopted the view that allelopathy has evolved as a secondary course or as a by-product of other ecological processes. There is no easy way to detect such allelopathic interactions under laboratory conditions, as organisms have greater adaptability to co-exist; however, physicochemical stress is preliminary evidence for such interactions (Le Flaive and Ten-Hage, 2007). Naturally, these substances regulate the microalgal populations and the formation of metabolites with biological activity (Mundt et al., 2001). A clear view of the impact of stress factors on such interactions within communities remains unclear. Smith and Doan (1999) proposed that allelopathy should explain, to an extent the process of succession.

The growth phase significantly influences the synthesis of secondary metabolites which eventually are involved in stimulating interactions among organisms (Le Flaive and Ten-Hage, 2007). It is the stationary phase during which the Microbe produce secondary metabolites (Namikoshi and Rinehart, 1996); however, others disagree to this proposition (Repka et al. 2004). Chetsumon et al. (1995) observed copious amounts of antibiotic peptides accumulation in cells of *Scytonema* sp. during its growth phase. However, Cannel et al (1988) disputed this proposition and supported the findings of Namikoshi and Rinehart's (1996). In a similar study, Borowitzka (1995) observed abundant production of metabolites in the stationary phase. Hence there is an influence of growth phase and culture conditions on secondary metabolite production in microorganism including cyanobacteria (; Morton and Bomber, 1994) Van Dar and Elo (1985).

Reliable extraction techniques are required to explore biosynthesis and functioning of cyanobacterial metabolites (Morrison et al., 2006). In this context, gauging efficiency of extraction solvents is quite complex, and might consider factors such as water content in cells, cell contents and differences between field samples and laboratory cultures (this may be called matrix effect) (Fastner et al., 1998). Consequently, the solvents used in extraction process, are important to ascertain isolation of bioactive metabolites. For instance, Fastner et al. (1998) noted that even pure methanol used as solvent did not extract microcystin from freeze-dried samples. However, sequential extraction with methanol and water proved efficient. Similarly, a series of dilutions of methanol were initially employed to extract secondary metabolites (Monaghan and Tkacz 1990; Smith and Doan 1999). Thus, it is important to understand the rationality of production of biologically active metabolites (Monaghan and Tkacz, 1990). Similar preselection rationality is also required for cyanobacteria because strains of the same species may be highly variable in exhibiting biological activities (Ördög et al., 2004). Bioassay screening through application of stresses that might impact growth parameters is normally taken as a primary mechanism at any stage of organism's life cycle to find compounds with the desirable characteristics (Monaghan and Tkacz, 1990). Such techniques are employed most often since naturally produced compounds are considered environment friendly (Ozdemir & Florosa, 2004). Luescher-Mattli (2003) proposed that microalgal metabolites form the basis of novel therapy system for treatment against viruses. Others believe that bioactive metabolites may serve as important compounds in several industrial applications (Burjà et al., 2001; Bokesch et al., 2003). The synthetic means to produce metabolites are economically not viable (Degachi and Ben, 2021) because of significant energy inputs required in the process. Local cyanobacterial blooms may prove invaluable source of bioactive compounds against bacteria, fungi, and viruses.

Over the last few years, there has been a lot of research into the toxicity impact of cyanobacterial metabolites and their implications ecologically (Ferrão-Filho et al., 2000: Bloom et al., 2006: Lürling and Beekmann, 2006; Wilson et al., 2006; Sarnelle and Wilson, 2005). Because several cyanobacterial metabolites (for example microcystins and anatoxin-a) have been linked to serious health concerns in humans and animals, their presence in drinking water has been closely monitored globally (Chorus and Bartram, 1999). However, because cyanobacterial metabolites come in a large variety of structural variations (Welker et al., 2006; Van Wagoner and Drummond, 2007), it is likely that certain harmful structures remain unnoticed. Furthermore, only approximately 30% of cyanobacterial peptides are thought to have a recognized chemical structure (Welker and Dohren, 2006). Biological effects are known in only a small percentage of cases. As a result, it's critical to use a sophisticated testing procedure to assess potential human health concerns (Solis et al., 1993). Model organisms, such as *Artemia salina* or *Daphnia* spp., are commonly employed to test cyanobacterial toxicity. A viable approach for evaluating the cytotoxic activity of plant extracts has also been proposed using the *A. salina* assay as well as a quick preliminary screening tool for toxic cyanobacteria (Lahti et al., 1995). According to the published data, there is a strong link between toxic activity in the brine shrimp assay and cytotoxicity against some tumor cells (Anderson et al., 1991) and hepatotoxic activity (Kiviranta et al., 1991). As a result, the brine shrimp assay is frequently employed to replace cell line assays as a low-cost and simple cytotoxicity test (Piccardi et al., 2000).

Numerous studies have revealed that cyanobacterial metabolites have inhibitory or even fatal effects on invertebrates. Microviridin J and microcystin, two cyanobacterial peptides, have been found to be harmful to eukaryotic species such as *Daphnia pulicaria* and *Daphnia galeata*. (Rohrlack et al., 1999, Rohrlack et al., 2004). Non-peptide substances such as cryptophycin, tolytoxin, calothrixins, and pahayakolide have also been discovered to have

substantial fatal effects on invertebrates (Berry et al., 2004; Biondi et al., 2004; Rohrlack et al., 2005).

1.7 Research Hypotheses

Hypothesis 1: Cyanobacterial species from Cholistan Desert are expected to exhibit morphologically diverse species, adapted to harsh desert conditions.

Hypothesis 2: Genetic characterization of cyanobacterial species from Cholistan Desert will reveal genetic diversity and unique genetic signatures, suggesting genetic adaptations for survival in the extreme desert environment.

Hypothesis 3: Cyanobacterial species from Cholistan Desert with unique morphological and genetic makeup may exhibit potential bioactivity.

1.8 Aims and Objectives

The objectives of this study are:

- To explore and collect cyanobacterial samples from different substrates and localities in the lesser Cholistan desert.
- To isolate, purify and culture cyanobacteria and characterize the isolates morphologically and genetically using the polyphasic approach.
- To produce the biomass of selected (culture responding) isolates for antibacterial, cytotoxic and anticancer potential.

MATERIALS AND METHODS

The algal samples were collected from the Cholistan Desert situated in the Punjab province, Pakistan (Fig. 2.1). Further experiments were conducted at the Genetics Laboratory, Department of Plant Sciences, Quaid-e-Azam University Islamabad. The study area, methodology and plan of work adopted for the study are presented here:

2.1. Description of Study Area

The Cholistan desert is locally known as „Rohi". It occupies an area of 26000 km^2 in the southern part of Punjab, Pakistan. It is situated along latitudes 27.42 and 29.45 North and longitudes 69.52 and 75.24 East. According to the topography, Cholistan desert is classified into two geomorphic regions: the lesser Cholistan which covers 7770 km^2 in the North and the greater Cholistan which occupies 18,130 km^2 in the south. Greater Cholistan is a hot, sandy, and wind-resorted desert with large dunes. Due to unstable sand dunes, the vegetation cover is poor. In the Cholistan desert soil is divided into saline and saline-sodic due to pH which ranges from 8.2 to 8.4 and from 8.8 to 9.6 respectively. The average rainfall ranges from less than 100 mm in the west to 200 mm in the East part mostly falling during monsoon (July and August every year). The average summer temperature is 34 to 38^oC with the maximum reaching over 51 to 60^oC. There was no regular source of surface water in this area. Natural accumulation of water is least due to meagre rainfall, maximum rate of infiltration, and a fast rate of evaporation. The scarce vegetation of this desert is influenced by bush-forming persistent shrubs with dispersed little trees. After rain substantial number of ephemeral and annual species emerge which complete their life cycle in a limited time and dry up after the dispersal of seeds

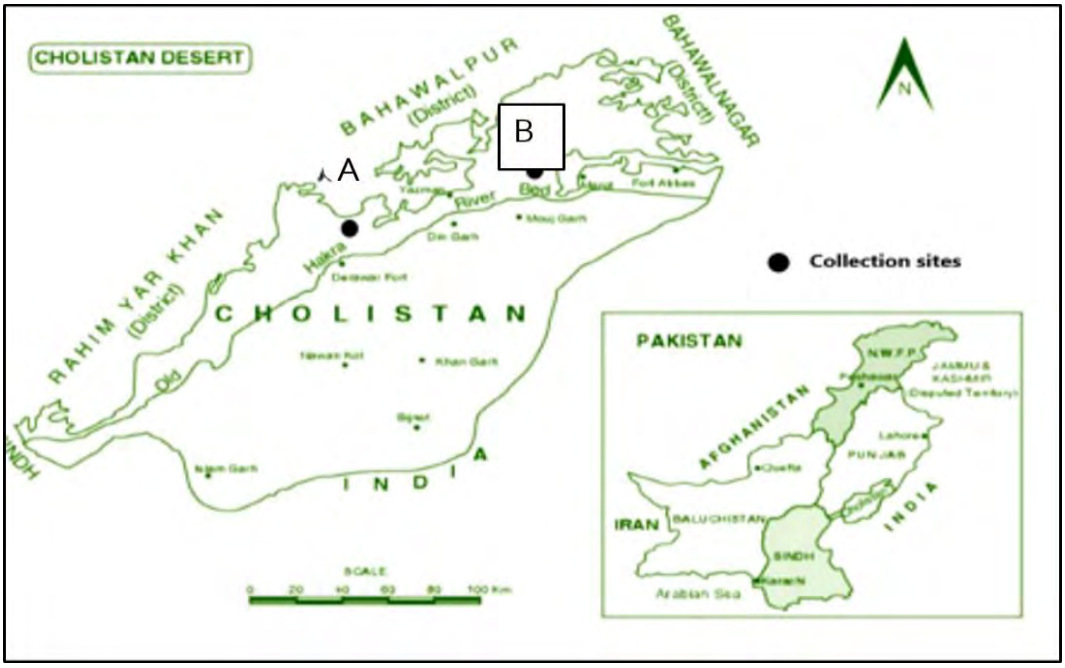


Fig. 2.1. The location map showing two routes (A and B) in the Cholistan Desert.

2.2. Sampling strategy and collection

In desert, small ponds and biocrusts form regularly following the rainy season which provides a unique opportunity for the collection of algae. Water samples from ponds as well as the soil in the vicinity of natural ponds were collected to observe cyanobacteria. Samples with spores were subjected to *in vitro* culturing under laboratory conditions to assess the diversity. Several samples were collected from water reservoirs (locally called **Toba**) in falcon tubes and dried samples were collected in plastic zipper bags for further analysis. The falcon tubes were filled with ¼ of the total volume to provide space for oxygen, thus enhancing the life of the samples (Mutanda et al., 2011, Minhas et al 2023). Field based initial data were summarized in Table 2.1.

A sampling at each site was conducted during the spring season (2016-2017). In the case of soil substrate, the top 1mm soil layer was collected. Coarse material, including plant debris, pebbles, and sand was removed on site using filtration and the algal samples were stored in falcon tubes for transportation to the laboratory. The algal samples were collected from different sites along two routes in the Cholistan desert (Pakistan), (Fig. 2.2). With the help of a digital camera, images of collection sites and samples were also captured, and coordinates were recorded by using Global Positioning System, Garmin, 12^{TM} (USA).

Route A (A-series of sites): Shahiwala to Fort Derawar

Route B (B-series of sites): Kudwala Bangla to Fort Morot.

These were traditional routes followed by the locals to access the area. The area has partial access through the metaled road followed by walkways leading deep into the desert. Both routes and description of sites along these routes are given below (Fig. 2.2).

Fig. 2.2. A Map illustrating the sampling sites along route A) Shahiwala to Fort Derawar; and route B) Kudwala Bangala to Fort Morot.

2.3.1. Sites along Route A

The route A (Shahiwala to Fort Derawar) spans a total stretch of 41 Kms. It included seven sites, selected based on availability of water and substrate types encountered (Fig. 2.3), description of these sites is given in Table 2.1.

Table 2.1: Sites description along Route A

Sample code	Description of site and substrates
A1	Dry (top 1mm) soil sample from a dry water channel along roadside.
A2	An irrigated area with a water channel, along the roadside. The channel was mostly dry with some leftover water, appearing green with thick algal growth
A3	A small ditch developed from seeping water from a water pipe, named RD-65000, a project developed by the Pakistan Agriculture Research Council (PARC) in collaboration with Arid Zone Research Institute (AZRI) for the distribution of drinking water for animals in Desert areas. These pipes pump ground water from Shahiwala to Fort Derawar.
A4	A temporary pond developed due to the seepage of water from water pipe that carries water from Shahiwala to Fort Derawar
A5	Samples were taken from the wall of a water tank. Another sample was taken from the crust formed along the soil surface
A6	There was a small ditch with prominent yellowish water. The source of water was nearby newly developed fishpond.
A7	A pond formed naturally on a large low-lying area in front of Fort Derawar. The main source of water was rain. This pond was filled with water coming from adjoining areas during the rainy season and it dries up in winter.
A8	The size of the pond was small and was filled with rainwater.

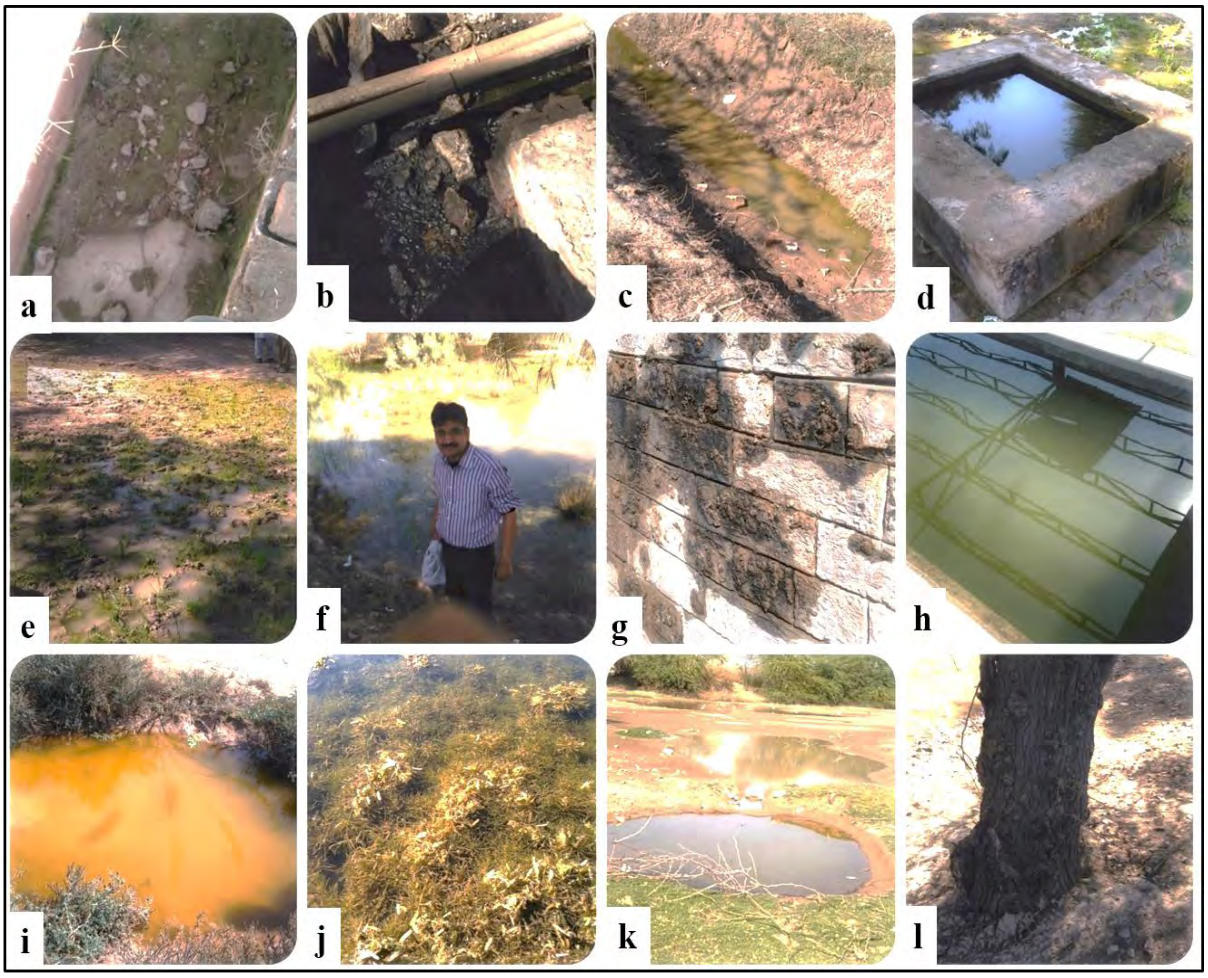


Fig. 2.3. (a) Roadside dry water channel (b) small ditch on roadside, Sahiwala (c) Drying canal water with dense Algal growth (d) Water tank created on the roadside, water pipe RD 65000 (e) and (f) A temporary pond formed as a result of water seepage from a water pipe (g) Crust formed on the pumping station's wall (h) An open water tank designed for animal consumption (i) Rainfed ditch along the roadside, near Fort Derawar (j) Drying Pond on the north side of Fort Derawar (k) A rain-fed pond south of Fort Derawar (l) Dry soil under the tree.

2.3.2. Sites along route B

This route comprised of nineteen sampling sites, stretching an area of 90 Km. The details on sites visited and samples are given in Table 2.2 and Figure 2.4.

Table 2.2: Site description along Route B

Sample code	Substrate
B1	This site was a small ditch on the roadside outside the village in 42 BC. Such types of ditches were developed to store water for animal drinking in these areas. These water bodies are filled with canal water and time to time. Soil sample as well as the liquid was sample collected from the site.
B2	This is the second site, a small village Chak41 DB, 3.4 km away from the B-1 site towards Kudwala. A small ditch was present for animal drinking. No prominent algal growth was observed. Liquid samples as well as soil samples were taken.
B3	This site was on the main road near Chak 46 DB, some 9.3 km from Chak 41 DB. A small size pond was present with crystal clear water. Some Cyanobacterial growth was prominent in shallow water. Soil sample as well as the liquid sample was collected from the site
B4	This site was 6.2 km ahead of the previous site (B-3). At Kudwala Bangala there was a barrage on a canal (DNB) Deranawab Barrage. As the doors of the barrage were closed, some water was present at the time of collection with very thick algal growth. Thick algal growth was observed in front of the Barrage doors. A liquid sample was collected.
B5	This site was located outside Kudwala Bangla, on Kudwala Fort Abbas Road, at a distance of 2.8 km from the previous site. A liquid sample was collected from small water course outside the agricultural field.
B6	This site was typically a desert habitat with large sand dunes and was very dry. It was 18.4 km away from Chak 68/DB which was the previous site
B7, B8, B9, B10	This series of B sites were 2.4 km away from B6 site and known as Kalay Pahar. These sites different ponds were developed by PCSIR and were used for animal drinking as well as human drinking. Normally people come from different areas of the Cholistan for water but also for different economic activities.
B11	This site was a roadside field on road leading to Fort Mauj Garh from Kalay Pahar previous sites (B7, B8, B9, B10). As the soil was dry and

	there were patches of dry crust on the surface. These crusts were collected.
B12	Small Pond on the main Kudwala Fort Abbas Road, area called Thandi Khui. The water was turbid. This pond was rain fed and was developed for animal drinking. Water samples were collected from this site.
B13	From the B-12 site, this site was present on the south side of Thandi Khui deep within the desert some 4 kilometer away. A rain fed pond was present with highly turbid water. Liquid as well as soil samples were collected from this site.
B14	This pond was in close vicinity of site B-13 with the same physical and environmental parameters. Liquid and soil samples were collected.
B15	This site was a temporary pond developed underside of canal in an irrigational area of Fort Abbass on road leading to Fort Marrot. The water in the pond was turbid with some algal growth. Liquid and soil samples were collected from the area.
B16	This site contains a large pond developed outside village on road which leads to Fort Marrot. The pond was the sewerage pond and was black in colour. The water sample was collected.
B17	B17 was the wall of the mosque on the front side of the Fort. On the wall dark black colour crust was present which was collected in falcon tube
B18	On the other side of the Fort Marrot, near the pond, a bio-crust was collected from soil under *Capparis decidua*.
B19	This was the only large pond present outside the Fort Marrot. Water was highly turbid. The soil sample was taken from the side of the pond.

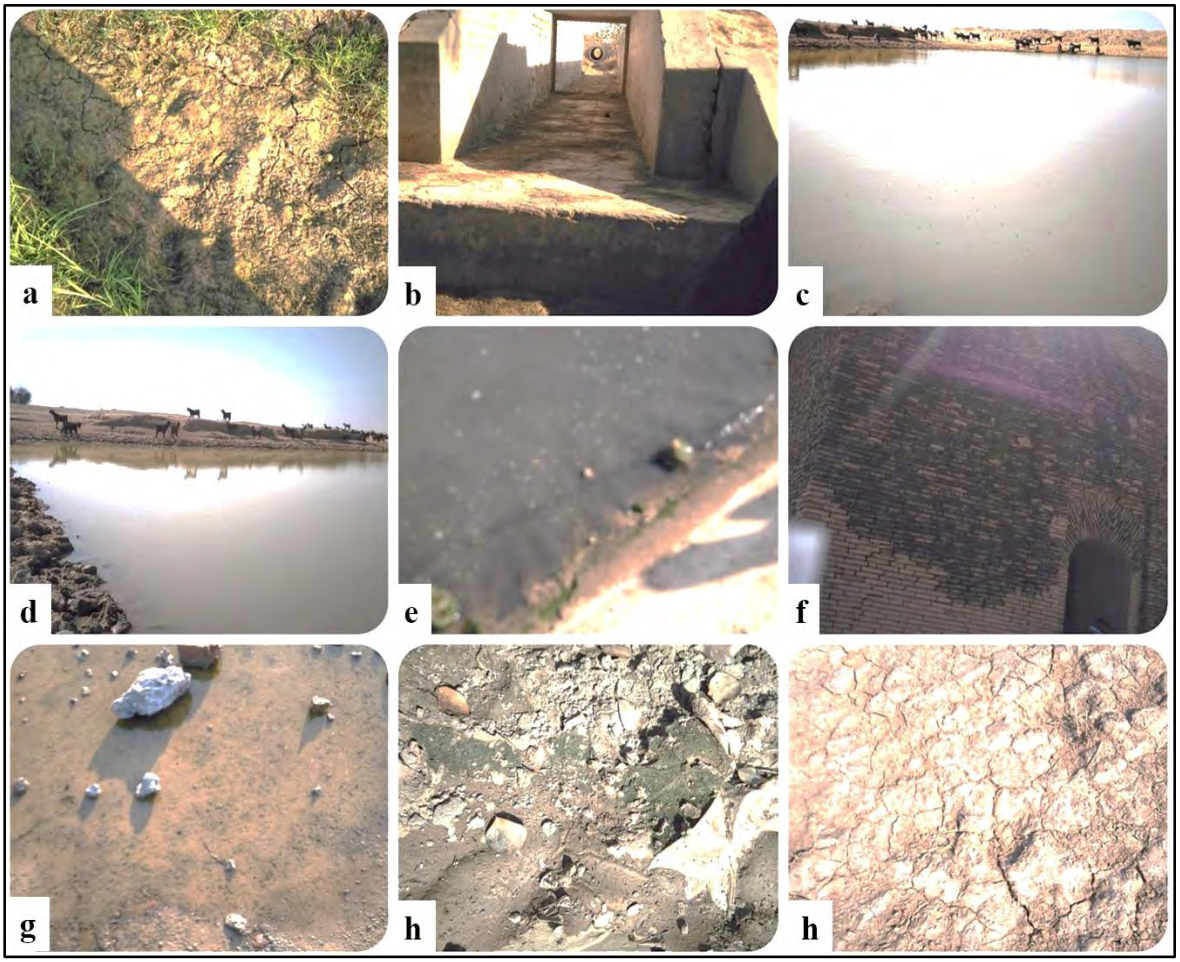


Fig. 2.4. (a)Village 46 BC, roadside pond (b) Opening of rain-fed pond along the road on Kalay Phar (c) A roadside pond one kilometer from Kalay Phar (d) Ponds on the east side of site J, 3 km away from Kalay Phar (e) Roadside Pond developed by villagers for animal Drinking (f) Dry crust on wall of Local Mosque. (g), (h)and (i) dry crust on desert soil.

2.4. Material description

The passport information including notes on habitat, substrate type, temperature, humidity, vegetation, etc. were recorded in the field. After collection, samples were brought to the laboratory and their pH and electrical conductivity (EC) were measured. Algal samples were kept in a growth room at 25±2˚C under continuous light for further analysis.

2.5. Microscopic observations

The samples were examined under a light microscope. A binocular light microscope (Leitz Wetzler, made in Germany) was used to observe morphological characters. The observations were made with a 10x eyepiece and 4x, 10x, 40x, and 100x (with emersion oil) objectives. An ocular micrometer was used to measure the size of individual cells, colonies, and filaments. The specimens were identified using authentic literature and algae base data bank.

2.5.1. Slide preparation

Slides were prepared by mounting a small portion of specimen in water and observed under a microscope. For the preparation of permanent slides, a drop of fixing jelly was added to the specimen and a cover slip was placed on it. The mixture was gently heated on a hot plate and mixed thoroughly. The solution was allowed to cool down and incubated at 4°C overnight for future use (Jaffer et al., 2019).

2.6. Isolation and purification

The standard microbial technique, solid agar plate method, was used for isolation and purification.

2.6.1. The solid agar plate method

To isolate various species of cyanobacteria from soil samples solid plating technique was used. For this purpose, solid BG_0, BG_{11}, BBM and SP media were prepared by adding 2% agar and on cooling poured in autoclaved Petri plates. The soil sample was sprinkled on the plates. To maintain an aseptic environment, the procedure was performed in a laminar flow hood. Plates were incubated in a growth chamber at 25^0 C and 25 µmol photon m2s1 light intensity. After five days of incubation, plates were observed under a light microscope (Fig. 2.5). The pure colonies were picked with the help of a sterilized loop and re-inoculated in broth media. Nitrogen source $NaNO_3$ or

KNO_3 should be omitted for the isolation and growth of N_2 fixing species. Isolation efforts with BG_{11} medium have shown that omission of nitrogen source sometimes gives better results (Markou et al., 2011)

2.7. Media used for *in vitro* culturing

Four types of culture media were used to get maximum growth of isolated species of microalgae and cyanobacteria.

- **BG_{11} Media**

Three stocks of the culturing medium BG_{11} were prepared according to the recipe. About 10 ml of these 3 stocks were taken into an Erlenmeyer flask and 1ml of the 4^{th} stock which was the stock of trace metals was added. The total volume of the medium was made up to 1000 ml by adding distilled water. The carbohydrates and nitrates were provided by the addition of 0.02g of $NaCO_3$ and 1.5g of $NaNO_3$. The pH of the medium was adjusted to 7.3 and after it was autoclaved at 121˚C and 15 psi for 45 min.

- **BG_0 media**

All the three stock solutions of BG_{11} (10ml of each) media and nutrient (1ml/L) media are used for the preparation of BG_0 media. 0.02g of $NaCO_3$ was also added. The only difference was that $NaNO_3$ will not be added.

- **BBM Media**

For BBM medium preparation, five stock solutions were prepared. About 10 ml of stock 1 and 1ml of the remaining 4 stocks were taken in an Erlenmeyer flask. The medium was made up to 1000ml by adding distilled water. The pH was adjusted to 6.8 and the medium was autoclaved at 121˚C and 15 psi for 45 min. BBM medium support maximum strains of green microalgae.

- **SP media**

SP stands for *Spirulina*. SP medium was used for the growth of *Spirulina* and other blue-green algae. Two stock solutions with different pH were prepared. Stock 1 was prepared in 500ml of distilled water with a pH of 9.0 ± 0.2 and stock 2 was prepared in 500ml distilled water with a pH of 3.8 ± 0.2. Both stocks were autoclaved separately and mixed after cooling. 1ml of the trace metal solution was added and the final pH was adjusted to 9.2 and again autoclaved.

2.8. Bulk culturing of species

Once strains of cyanobacteria were isolated and purified, these were used as inoculum for bulk biomass production. Initially, algal strains were cultured in a sterilized glass test tube to assess their acclimatization in liquid media. After 2 to 4 days, algal species were observed under a microscope to rule out the contamination. At that stage, the growth response of the algal species was observed by measuring absorbance at 630nm through spectrophotometer.

2.9. Biomass production of selected species of Cyanobacteria

After confirmation of contamination free growth, the samples were shifted from test tubes to 500ml and 1 L conical flasks for batch culturing. The inoculated flasks were kept in a growth room at constant temperature, maintained at 25˚ ± 2˚C under continuous white light conditions.

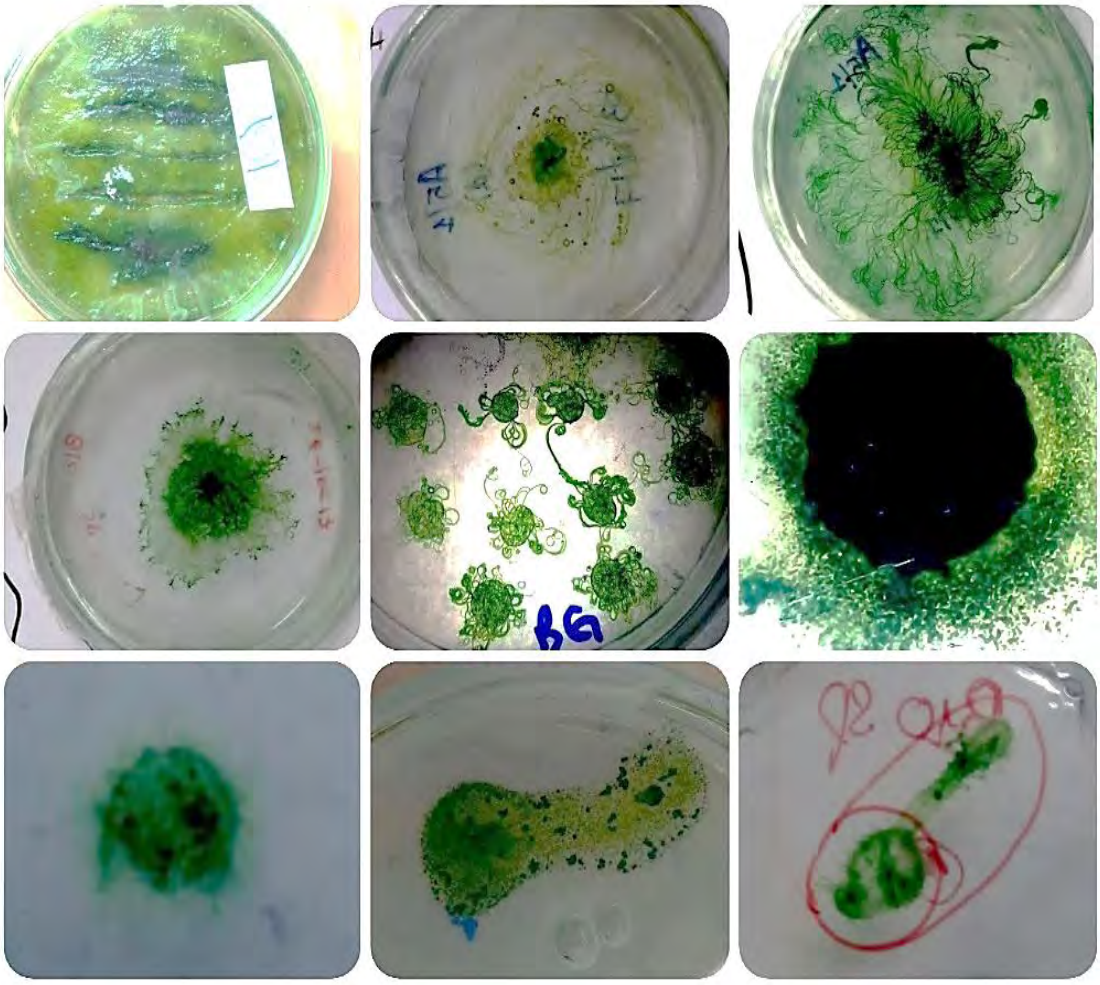


Fig. 2.5. The *in vitro* growth of cyanobacteria in petri plates with agar-based media

2.9.1. Harvesting of concentrated algal biomass

After incubation of 20-25 days of batch culturing, biomass was harvested through centrifugation. The concentrated biomass of algal strains was centrifuged at 12000rpm at 18˚C for 6 min. After centrifugation, the thick slurry of algae was washed with distilled water and again centrifuged. The algal paste achieved at the end of centrifugation was concentrated further by removing the supernatant. Some of the algae paste was air dried for phyco-chemical assessment.

2.10. Morphological characterization

The algal specimens in culturing (Andersen, 2005) were observed under microscope on regular basis. The algal species were observed based on their morphological characteristics. For this purpose, the key characters and character states observed in the samples are given in Table 2.3.

Table 2.3: Morphological characters assessed in Cyanobacteria of Cholistan Desert

Sr. no.	Character	Character states	Classification
1	Colony Shape	Uniformly Circular; Irregular	Binary
2	Colony Size	Length (mm); Width (mm)	Quantitative
3	Colony Sheath	Absent (0); Present (1)	Binary
4	Colony	Solitary & Loose; Multiple & Compact	Qualitative
5	Colony texture	Hard; Soft; Gelatinous	Qualitative
6	Filament	Straight, Bent; Curved; Coiled	Qualitative
7	Filament Polarity	Homopolar or Heteropolar	Qualitative
8	Uniformity of cells in a filament	Uniform across length; slightly tapering; Clearly tapering	Qualitative
9	Filament Size	Mm	Quantitative
10	Terminal cell	Not Attenuated; partially attenuated; Attenuated; Attenuated and elongated	
11	Branching	True or False or Absent	Qualitative
12	Secondary trichomes	Remain or not remain in mother sheath	Binary
13	Cell shape	Cylindrical; Cylindrical and constricted. Barrel shaped and constricted. Elliptical and constricted; spherical	Qualitative
14	Cells in filament	All one type; differentiated types	Qualitative
15	Vegetative Cell Shape	L=W; L>W; L<W	Qualitative
16	Vegetative cell Size	Mm	Quantitative
17	Akinetes	Present or Absent	Binary
18	Akinete Shape	L=W; L>W; L<W	Qualitative
19	Akinete Size	L; W	Quantitative
20	Akinete formation	All at once; in a series; one by one from terminals, from the center	Qualitative
21	Heterocyst	Present; Absent	Binary
22	Heterocyst Shape	L=W; L>W; L<W	Quantitative
23	Heterocyst size	Mm	Quantitative
24	Heterocyst position	Intercalary; Terminal; Terminal on both sides	Binary
25	Heterocyst frequency	One or Few; Frequently present	Qualitative
26	Heterocyst Sheath	Present; Absent	Binary

2.11. Molecular Identification

2.11.1. DNA extraction

Total genomic DNA was extracted from each sample using a previously described method (Elshire et al., 2011), with slight modification done for optimization purpose and described in Inam et al (2022).

2.11.2. Amplification and sequencing of 16S locus

PCR amplification of 16S rRNA locus was performed using previously designed primers CYA106F and CYA781R (Nubel et al.,1997). In each 25µl PCR, reaction mixture was prepared by adding: 14.5µl PCR water, 2.5µl 10X buffer, 2µl $MgCl_2$ (25mM), 2µl dNTPs (25mM), 1µl (10pmol each) of forward and reverse primers, 0.2µl (0.2U/reaction) of Taq polymerase, and 2µl (50ng/µl) of template DNA (Lee et al., 2014). Preheating at 99°C for 1min, DNA was denatured at 94°C for 2min, followed by 30 cycles of 94°C for 1min; 55°C for 1min and 72°C for 3min, and a final extension at 72°C for 10min, with optional holding at 4°C. The amplified products were resolved on 1.5% agarose and visualized under UV using a gel documentation apparatus (Bio- Rad). The PCR samples were purified using Thermo-Scientific GeneJET PCR Purification Kit, following the manufacturer's instructions. Purified PCR samples were sequenced commercially from Eurofins.

2.11.3. Phylogenetic analysis

The sequences obtained were inspected in BioEdit software website (Hall, 1999) for any errors and were compared with the pre- existing sequences in the GenBank database using the option of NCBI BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast). Contigs of sequences were assembled using CLC Main Workbench version 8.1 (QIAGEN Aarhus A/S), followed by phylogenetic analysis as described in Inam et al. (2022).

2.12. Biological Applications

2.12.1. Extraction and antibacterial activity

The antibacterial activity was carried out with agar well diffusion method (Murray et al., 2022). The dried algal samples were ground to powder. Methanol was used to macerate dried material for 72 h. The concentrated extract was then treated with 5% HCl and the aqueous solution of ammonia. The extracts were screened for their efficacy against local strains of *Pseudomonas*, *Klebsiella, Bacillus* and *Streptococcus.* All experiments were carried out with three biological replications.

2.12.2. Brine **shrimp microwell cytotoxicity assay**

Dehydrated eggs of *Artemia salina* (0.5g) were revived in 400 ml of artificial seawater with bubbling air for 24 h at room temperature. After aeration, the suspended particles were allowed to settle down. The active larvae were exposed to illumination in a separating funnel. The phototrophic larvae were gathered near an illumination source, which were collected and transferred carefully to a microtiter plate, each well with 0.2 ml of saline. Since larvae are sensitive to culturing conditions, some may die during incubation. Therefore, at the initial experimental stage, the dead larvae (N) were counted. To the active larvae, a solution of 20 µg each of the crude extract and 10 µl of dimethyl sulfoxide (DMSO) were added. These were then incubated in the dark at room temperature. After 24 hrs of incubation, the number of dead individuals (A) in each well was counted again. Conseqeuntly, the surviving larvae were also killed by the adding 0.5 ml methanol to obtain “G” (i.e. total number of larvae) in the sample. A blind sample containing pure DMSO was also included in each row. As positive control actinomycin in a concentration 10 µg/ml was used. The mortality rate “M” (percent of the dead larvae after 24 h) was then calculated by tabulating the data as follows:

$$M= (A-B-N) / G- N \times 100$$

Here, A implies = the number of dead larvae after 24 h of incubation.

B = average number of dead larvae in the blind samples after 24 h of incubation; and

N = the number of dead larvae at the beginning of each test.

2.12.3. The ***in vitro*** **MTT antitumor assay**

For this purpose, the HCT- 116 (ATCC CCL- 247) human colorectal cancer cell lines were obtained from American Type Culture Collection (ATCC), which is a global bioresource center. The cell lines were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics (penicillin and streptomycin), as monolayers followed by sub culturing two times a week while incubating at 37°C with 5% CO_2 and 100% relative humidity to ensure viability. The procedure followed here was originally described previously (Vichai and Kirtikara, 2006) with some modifications as described in Jubeen et al., (2019). Modifications were made to optimize growth conditions in the laboratory. After 24 h of incubation, absorbance was recorded at 570 nm using a spectrophotometer. The growth inhibition was calculated using a formula as follows:

$$\text{Cell viability} = \text{OD (control well)} - \text{OD (treated well)} \times 1$$

RESULTS

3.1 The sites and substrate description

In Cholistan the major sources of samples along sites were: (a) the agricultural fields with irrigation canals/ponds; (b) rainwater harvesting ponds in the deep desert; and (c) soil crusts, stone and wall crevices in the ruins of historical remains. The rainwater harvesting ponds locally called as 'Toba' were an active source of indigenous cyanobacterial species in the desert. Water condition in most cases was turbid while few were found clear for instance at sites A2 and A3 (Table 3.1). In majority of cases, the sample electrical conductivity (EC) was found in the range: 110-500 ds/m, some sites had 500-700ds/m while 6 sites showed an EC range from 700-1880 ds/m. Low EC values were observed at sites deep in the desert (A8, B7 and B10, 11, 12, 13 & 14) whereas higher EC values were encountered at sites along agricultural land. Similarly, pH of samples ranged from (5.82) - 6.2 to 7.3 - (8.6) while the temperature ranged from 15-34°C at different sites with humidity levels 10 - 71% (Table 3.1).

While collecting along the sites, mostly standing water in small ditches (brief and natural collection of water) and ponds was encountered. In some cases, especially in summer, the water drains or evaporates quickly leaving hard soil crusts. Furthermore, it was observed that water in most cases was turbid. Deep in the desert, rain is the only source of water, which was harvested in local man-made ponds, known as 'Toba'. The rainwater harvesting meets the requirements of indigenous population, especially the household utilities, and for drinking purpose by human and livestock alike.

Table 3.1: The passport information of samples including sites, nearest settlement, description of habitat and ecological data

Site code	Nearest Settlement	Habitat/Soil Type	Sample type	Turbidity	EC	Temp (°C)	Humidity (%)	pH	GPS Data	
									N	E
A2	Zakhira Mor (1 km from Shahiwala	Sandy/ Irrigated land	Standing water	Turbid	350	25	27	6.2	29.00306N	71.30083E
A3	Roadside waterpipe (RD65000) road leading to QD (Qila Dilawar)	Sandy/ Irrigated land	Standing water	Slightly turbid	540	27	49	6.2	28.92936N	71.33981E
A4	Roadside temporary pond, road leading to QD	Sandy/ Irrigated land	Standing water	Clear	550	34	27	6.40	28.91414N	71.34003E
A5	Water pumping Station	Sandy/ Irrigated land	Standing water	Turbid	770	29	38	6.55	28.86972N	71.33447E
A6	Pond on roadside, near QD	Sandy/ Rainwater	stagnant water	Turbid	1880	28	10	7.18	28.82441N	
A7	Northern side of mosque near QD	Sandy/ Rainwater	a small ditch	Turbid	900	32	21	6.5	28.7675N	71.33765E
A8	Pond on southern side of QD	Sandy/ Rainwater	Stagnant water in a pond	Clear	270	30	23	**8.6**	28.76908N	71.33752E
B1	Ada 41	Sandy loam	a small pond	Slightly turbid	640	18	60	7.2	29.23871N	71.75524E
B2	1 km from Ada 41	Roadside pond	Pond	Turbid	470	18	55	**5.95**	29.23405N	71.76E
B3	Chak 46	Roadside pond	Pond	Slightly turbid	490	16	71	6.34	29.20936N	71.85871E
B4	Kudwala Bangla	Closed Canal	-	Highly turbid	540	18	65	6.29	29.18059N	71.8680E
B5	Sanghar	Roadside stagnant water	-	Slightly turbid	400	15.8	68	6.52	29.19104N	71.92411E
B6	1.5 km from Sanghar	Sand dunes/dry soil sample	-	-	-	15.8	68	–	29.17185N	71.98679E
B7	Kalay Pahar	Rainwater Pond	Stagnant water	Turbid	300	21	45	6.49	29.17406N	72.06455E
B8	Kalay Pahar	Rainwater Pond	Stagnant water	Turbid	210	19	45	6.79	29.17525N	

									72.06757E
B9	Kalay Pahar	Pond	-	Turbid	410	19	45	6.7	
B10	Kalay Pahar	Pond	-	Slightly turbid	230	28	31	6.7	29.17605N 72.06102E
B11	Thandi Kohi	Dry soil from roadside	-	-	170	28	31	–	29.17958N 72.14920E
B12	Thandi Kohi	Toba	-	Turbid	170	28	31	7.31	29.18596N 72.14697E
B13	Jalwala	Toba	-	Turbid	110	30	45		
B14	Jalwala	Toba	-	Turbid	150	30	45		
B15	318 Pul	Roadside pond	-	Turbid	970	29	25	8.01	29.22076N 72.40318E
B16	2 km from 318 Pul	Roadside pond	-	Highly turbid	600	29	128	6.55	29.21204N 72.40294E
B17	Fort Marot ruins	A dry mosque wall	-	-	-	32	–	–	29.17781N 72.43531E
B18	Fort Marot ruins	Dry soil sample other side of road by fort Marot under *Capparis decidua*	-	-	-	34	128	–	29.18016N 72.43340E
B19	Fort Marot ruins	Pond	-	Highly turbid	340	34	128		29.18016N 72.43240E

3.2 Composition, Morphology and Identification of Algae and Cyanobacteria in the Field Samples

3.2.1 Chlorophyte and chlorophyte-like taxa

Based on the morphological observations, following algal species were identified mostly to the genus level; however, some of the obvious types were also being identified to the species level. The morphological notes taken during these observations are given as follows:

3.2.1.1 ***Scenedesmus quadricauda*:** Colonial form with four cells. Cells are oblong cylindrical in shape with round ends, arranged in a linear order. Each pole of the terminal cell has one long, slightly curved spine. Ends of the spine or terminal cell were pointed. The upper two cells have pyrenoids on the right side while the lower two cells have pyrenoid on their left.

3.2.1.2 ***Scenedesmus obliquus*:** Cells arranged in fours, fusiform in shape with straight sides. The outer side of the terminal cell is slightly convex. Cell wall is smooth in appearance with tiny spines.

3.2.1.3 ***Scenedesmus tropicus***: Colony four-celled and subquadrate. Cells biconvex in the middle, attenuated towards the ends and with inflated poles. Adjacent cells connected to each other by two narrow processes leaving a linear intercellular perforation. Poles of terminal cells provided with a long-recurved spine. Chloroplast is parietal and with a single pyrenoid.

3.2.1.4 ***Scenedesmus denticulatus*:** Colonies usually with 4 cells; Cells ovoid to oblong and arranged in sub-alternating form. The end cells have small growth on the blunt side called as teeth.

3.2.1.5 ***Scenedesmus armatus*:** Colony of four cells. Cells are oblong and ellipsoid in shape with acute apices. Cells arranged in a linear order.

3.2.1.6 ***Scenedesmus bijugatus*:** Colony four celled. Cells oblong, fusiform with acute ends and arranged in a sub alternating series, usually in two planes, two cells above and two cells below. Terminal cells have four spines each.

3.2.1.7 ***Scenedesmus bijugatus* var *graevenitzii*:** Cell body spindle-shaped four to eight cells attached side by side (in 1 or 2 rows); without spiny projections; outer cells slightly curved inward and the inner side straight or slightly concave, ends of cells stumpy and with apical nodules.

3.2.1.8 ***Scenedesmus dimorphous*:** Colony of two cells. Cells are ovoid to cylindrical, cells narrow and the spines longer than cells.

3.2.1.9 ***Scenedesmus abundance*:** Colonies usually two to four celled. Cell is twice as long as broad. Poles of terminal cell with single spine. Colony 16.5µ Wide, 33µlong: Cylindrical Cell- 6.6µ - 8.9µ wide, 16.5 long; spine 8.25- 9.9µ long.

3.2.1.10 ***Scenedesmus* sp.:** Colony with four cells arranged in a sub-alternate manner. Cells are ovoid to oblong to ellipsoidal with 1-2 teeth along each pole.

3.2.1.11 ***Chlorella vulgaris*:** Cells spherical in outline arranged in groups to form stratum. Pyrenoid was present.

3.2.1.12 ***Chlorella* sp.:** Cells single, without mucilage and free floating. Single cup shaped chloroplast with pyrenoid.

3.2.1.13 ***Schroederia* sp.:** Cell solitary, free floating, curved. Chloroplast single, parietal and have a single pyrenoid.

3.2.1.14 ***Korshikoviella*** **sp.:** Cells long, spindle-shaped, straight, pointed. Two to three pyrenoids present.

3.2.1.15 ***Sphearoplea*** **sp.:** Characteristic long cylindrical cells with red color oospores.

3.2.1.16 ***Genicularia*****:** Solitary cylindrical cell with band shaped chloroplast and spiral chromatophores. Ends of the cylinder are slightly narrow.

3.2.1.17 ***Chlamydomonas*** **sp.:** Both cells are unicellular. Cell on left has smooth, transparent, and thin cell wall with chloroplast covering most of the cell. Cell on the right has thick and rough cell wall with cup shaped chloroplast.

3.2.1.18 ***Chlamydomonas*****:** Reproductive stage of *Chlamydomonas*.

3.2.1.19 ***Spirogyra*** **sp.:** Simple filaments with cylindrical cells slightly replicate and ribbon like single chromatophore embedded in the cytoplasm. One to two pyrenoids present. Cells are longer than broad.

3.2.1.20 ***Zygnema*** **sp.:** Only spore form was observed. Zygote globose, spore wall blue black and marked with pits.

3.2.1.21 ***Cosmarium*** **sp.:** Cell divided into two semi cells produced by constriction in the middle. Semi cells semicircular with truncated apex.

3.2.1.22 ***Microspora*** **sp.:** Filamentous algae without branching; cell body mostly cylindrical. Cell wall shows H-shape in transverse section. Chloroplasts reticulated without pyrenoids.

3.2.1.23 ***Ulothrix*** **sp.:** Filaments unbranched, vegetative cells cylindrical or barrel shaped, chromatophore band shaped and occupy whole of cell circumference with more than one pyrenoids with spore.

3.2.1.24 ***Stigeocolonium*** **sp.:** Filaments branched; vegetative cells slightly swollen, chromatophore transversely zonate with several pyrenoids. Side branches are tapering towards the end. Cells enclosed in mucilage.

3.2.1.25 ***Cylindrospermosis*** **sp.:** Trichomes single straight or mildly curved. Heterocyst is present on both sides and elongated-conical. The cells are almost square.

3.2.2 Cyanobacteria

3.2.2.1 ***Haplosiphone*** **sp.:** Thallus caspitose, thin aquatic filaments free and continuously branched. Branches irregularly lateral, arising from one side of the filament.

3.2.2.2 ***Leptolyngbya*** **sp.:** Filaments straight and long with loose arrangement. Ends neither attenuated nor capitate with thin, hyaline sheath. Cells are isodiametric with homogenous contents.

3.2.2.3 ***Chroccoccus*** **sp.:** Cell subspherical, hemispherical after division in group of two. Blue green in colour. Sheath of the individual cells firm and homogenous.

3.2.2.4 ***Oscillatoria limosa*****:** Trichome straight, brown in colour and slightly constricted at cross walls. Cross walls are not prominent with granules spreading in the cytoplasm. End cell flatly rounded with slightly thickened membrane.

3.2.2.5 ***Oscillatoria*** **sp.:** Filaments are straight, elongate and not constricted at the joints Apices of trichome straight, briefly tapering with a sharp pointed hook like structure.

3.2.2.6 ***Lyngbya*** **sp.:** Trichomes brownish green, mostly straight, end cell flatly rounded. Trichrome not constricted at the cross walls.

3.2.2.7 ***Anabaenopsis*** **sp. 1:** Free floating trichomes, tightly coiled and spirally arranged. Cells are barrel shaped and highly granulated. Mucilaginous envelop absent with clear constrictions at the cross walls. Heterocyst spherical in shape.

3.2.2.8 ***Anabaenopsis*** **sp. 2:** Free floating trichomes, spirally coiled, without mucilaginous envelops and constricted at cross-walls. Cells cylindrical and slightly constricted at the ends. Heterocyst spherical.

3.2.2.9 ***Nodularia*****:** Filaments free with a sheath. Vegetative cells uniform in breadth. Cells short, depressed, and discoid.

3.2.2.10 ***Phormidium autumnale*****:** Filaments mostly straight, sheath firm and mucilaginous. Cells not constricted at cross wall. End cells briefly attenuated and prominently capitated.

3.2.2.11 The undetermined species: Cells aggregated in a mucilaginous colony.

3.2.3. The composition of species in field (fresh) samples

Initial observations revealed a total of 34 species, besides few undetermined types. Among these, the green types contributed a share of 70% while 30% were blue green types. Among green algal types, members of the genus *Scenedesmus* were found most abundant. Other taxa were represented by either one or two species only. Among blue green algal types, the genus *Anabaenopsis* was represented by two species while the rest of genera were represented by one species each.

3.3 The *in vitro* culturing and isolation of species

3.3.1 Culturing of dry soil samples

Various cyanobacteria readily responded to the Blue Green Medium with full nutrients (BG_{11}) as well as to Blue Green Medium with no additional nutrients (BG_0). Samples of all sites except those obtained from site B-7 showed growth response. B-7 did not show any growth either in BG_{11} or in BG_0 media. B-19 showed response only to BG_{11} medium, while there was no growth in BG_0 medium. The colonies of different sites were observed under Stereo microscope (Fig. 3.1) and shifted to respective broth media. In the BG_{11} broth medium mixed growth of cyanobacteria and green algae was observed. For comparison purpose, the no. of species isolated from BG_{11}, BG_0 media are shown in Table 3.2.

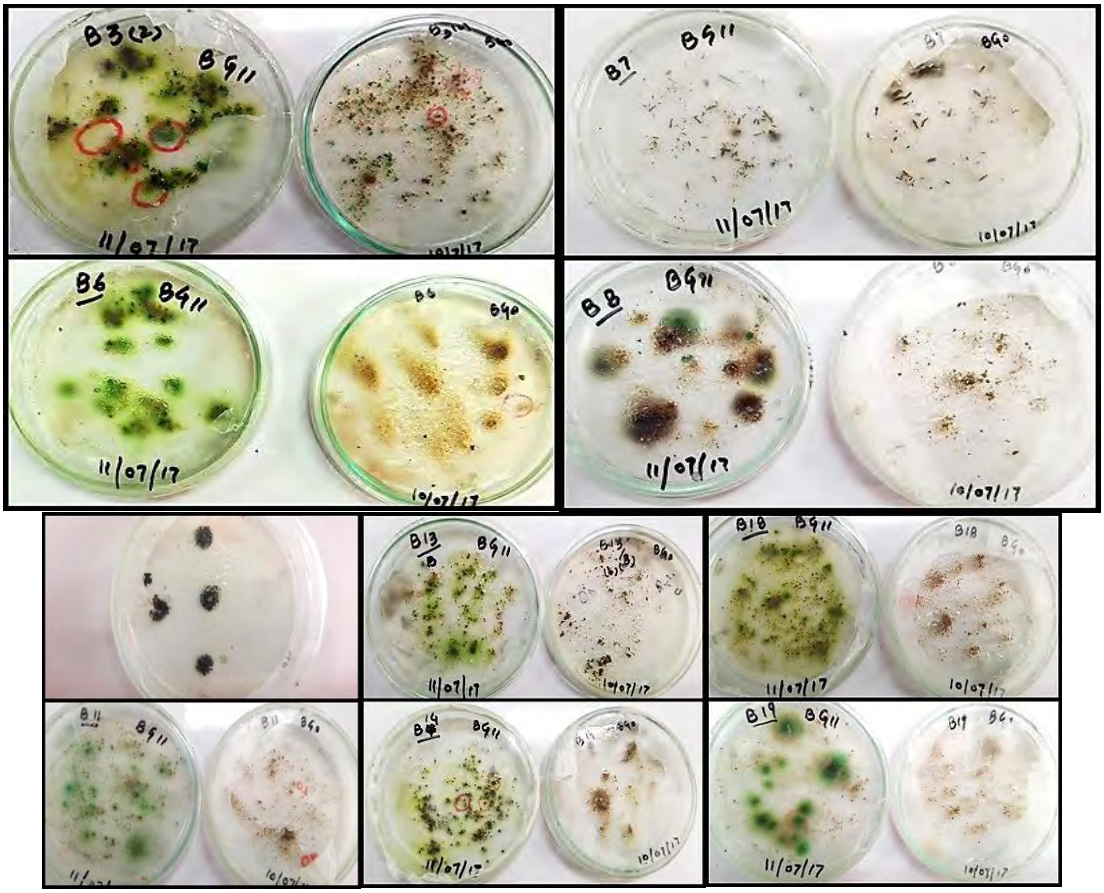


Fig. 3.1: BG_{11} and BG_0 Agar-base plate culturing of soil samples of sites A-1, A-8, B-3, B-6, B-7, B-8, B-9, B-11, B-13 B-14, B-17, B-18 and B-19.

Table 3.2: The soil samples collected from dry substrates such as wall crevices and stone surfaces encountered in the Cholistan. With no initial growth, samples were subjected to *in vitro* culturing using BG_{11} and BG_0 media.

S. No.	Sites	BG_{11} medium	BG_0 medium	Total
1	A1	**NGR**	**NGR**	**0**
2	A8	*Leptolyngbya* sp.	*Tolypothrix*	2
3	B3	*Arthrospira*; *Oscillatoria*; *Lyngbya*	*Anabaena*	4
4	B6	*Oscillatoria*	*Aliinostoc*	2
5	B7	**NGR**	**NGR**	0
6	B8	*Cylindrospermum*	*Goleocapsa* sp.	2
7	B11	*Lyngbya*	NGR	1
8	B13	*Desertifilum*	*Nostoc*	2
9	B14	*Phormidium*	*Calothrix; Nodularia*	3
10	B17	*Kalisinema*	*Cylindrospermum*	2
11	B18	*Oscillatoria; Phormidium*; *Leptolyngbya*	*Nostoc*; *Scytonema*	5
12	B19	*Leptolyngbya*	**NGR**	1
	species	**8**	**8**	**17**

Note back:

- **Abbreviations: NGR**: no growth response; **A**: sites along route A; **B**: sites along route B; sp.: species.
- Counting all common and unique types once, the total no. of species recorded in all media were 17.

3.3.2 The *in vitro* culturing and species observations in liquid samples

3.3.2.1 Observations of species at site A2

Field samples: *Euglena,* the green algae *Scenedesmus* and *Nostoc* like cyanobacteria.

***In vitro* culturing:** The BG_{11} and BBM showed mixed growth of green and blue green initially. Among green types only *Scenedesmus* sp. was observed in both cultures, however, *Euglena* sp. did not show any response. In BG_0 medium two heterocystous cyanobacteria i.e. *Calothrix* and *Nodularia* species appeared. In the SP (modified) medium, a strain of *Leptolyngbya,* which is a non heterocystous filamentous cyanobacteria showed growth (Fig. 3.2 and Fig. 3.2.1).

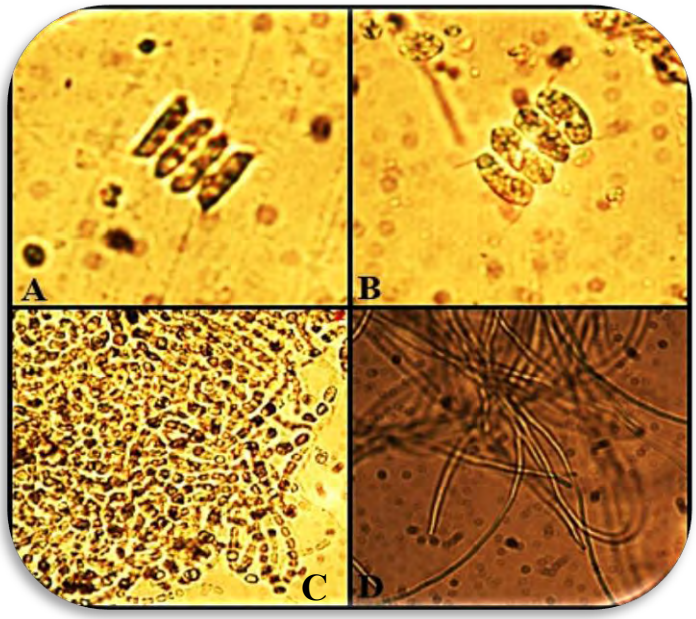
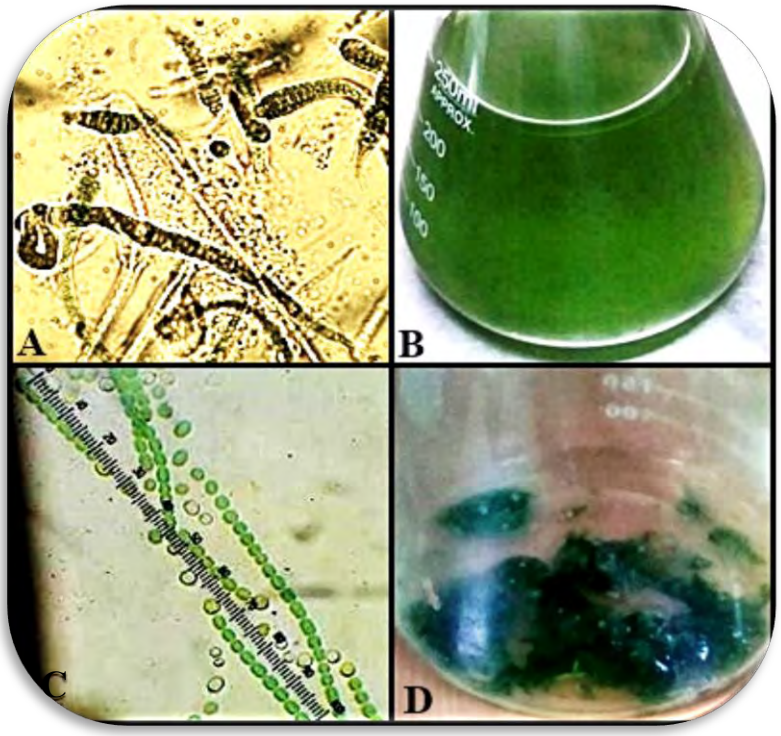


Fig 3.2 A: Mix growth in BBM media of *Scenedesmus* and filamentous cyanobacteria. **B:** Mixed growth of cyanobacteria and *Scenedesmus* sp. in BG_{11} media. **C:** Culture of *Nodularia* sp. in BG_0. **D:** Culture of *Leptolyngbya* sp. in SP Medium.(50X)



Fi 3.2.1 A & **B)** culturing condition of *Calothrix* species; **C** & **D)** appearance of *Nodularia* in culturing.. **A** and **C** (50X and 100X)

3.3.2.2 Observations of species at site A-3

Field samples: *Spirogyra* sp. was observed along with the cyanobacteria.

***In vitro* culturing**: BG_{11} and BBM showed mixed response of greens and blue greens. *Spirogyra* did not respond to any culturing media. In BG_{11} *Phormidium* was observed; in BG_0 *Nostoc commune* was purified and in SpM *Chroococcus* species was purified (Fig. 3.3).

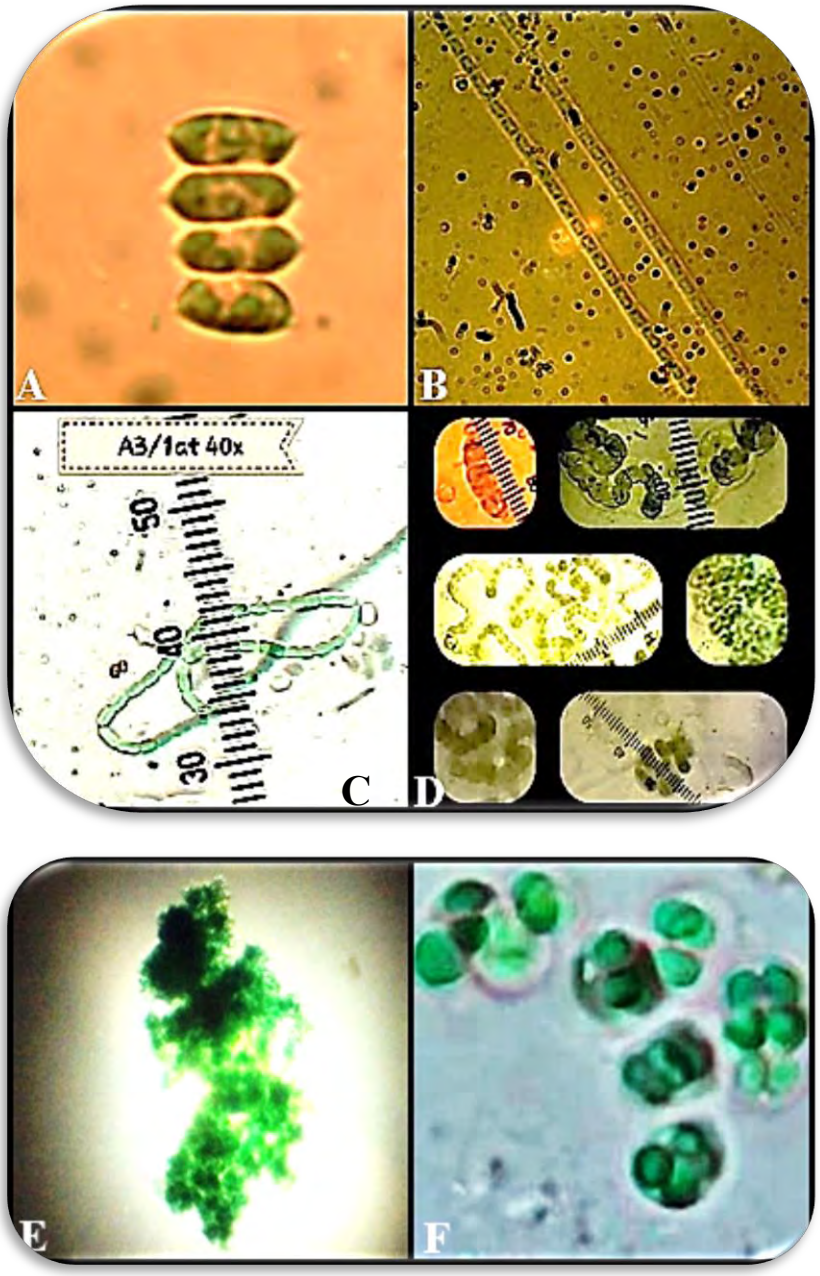


Fig. 3.3: A) *Scenedesmus* (50X) was equally produced by BG_{11} and BBM; **Fig B)** *Phormidium* sp.(1000X) in BG_{11}; **C & D)** *Nostoc* species(400X) in BG_0. **E & F)** Mass and individual colonies of C*hroococcus* sp. in SP Media.(100X and 1000X).

3.3.2.3 Observations of species at Site A-4

Field samples: At this site *Chara* was dominant in growth in initial observation along with some cyanobacteria. *Zygnema* sexual spore were also observed.
in vitro* culturing: Neowestiellopsis* and *Chlamydomonas* were observed in BG_{11} and *Chlamydomonas* and a *Phormidium* like species was observed in BBM. *Neowestiellopsis Nostoc* sp. and *Chroccodiopsis* were purified in BG_0 medium whereas, SpM (the modified medium) showed mixed growth of green and blue greens (Fig. 3.4).

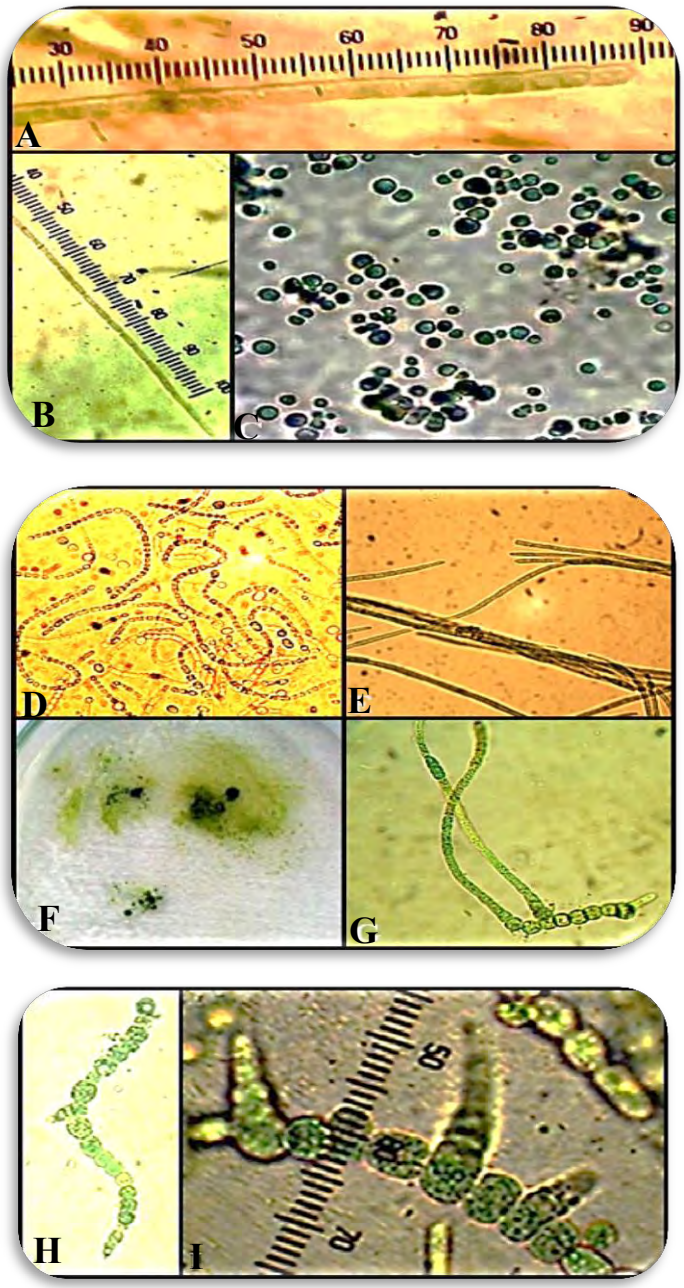


Fig 3.4 A & B) *Phormidium* sp.(100X and 1000X) cultured in both BG_{11} and BBM media; **C)** *Chroccodiopsis* sp.(100X) produced in BG_0; **D)** *Nostoc* sp.(50X) cultured in BG_0; **E)** Sp. of *Phormidium* (50X)in BG_{11}; **F, G, H & I)** *Neowestiellopsis* in BG_0 media.(100X and 400X)

3.3.2.4 Observations of species at site A-5

Field samples: Initial observation showed the presence of *Spirogyra* sp. Filamentous and unicellular colonial cyanobacteria i.e. *Gloeocapsa* and *Merismopedia*.
***in vitro* culturing**: BG_{11} and BBM showed mixed growth of greens and blue greens i.e. *Scenedesmus, Chlamydomonas* and *Oscillatoria* species. *Nodularia, Cylindrospermosis* and *Pleurocapsa* were purified in BG_0 culture while *Microcoleus* and *Desetifulium sp.* were purified in Sp medium (Fig. 3.5).

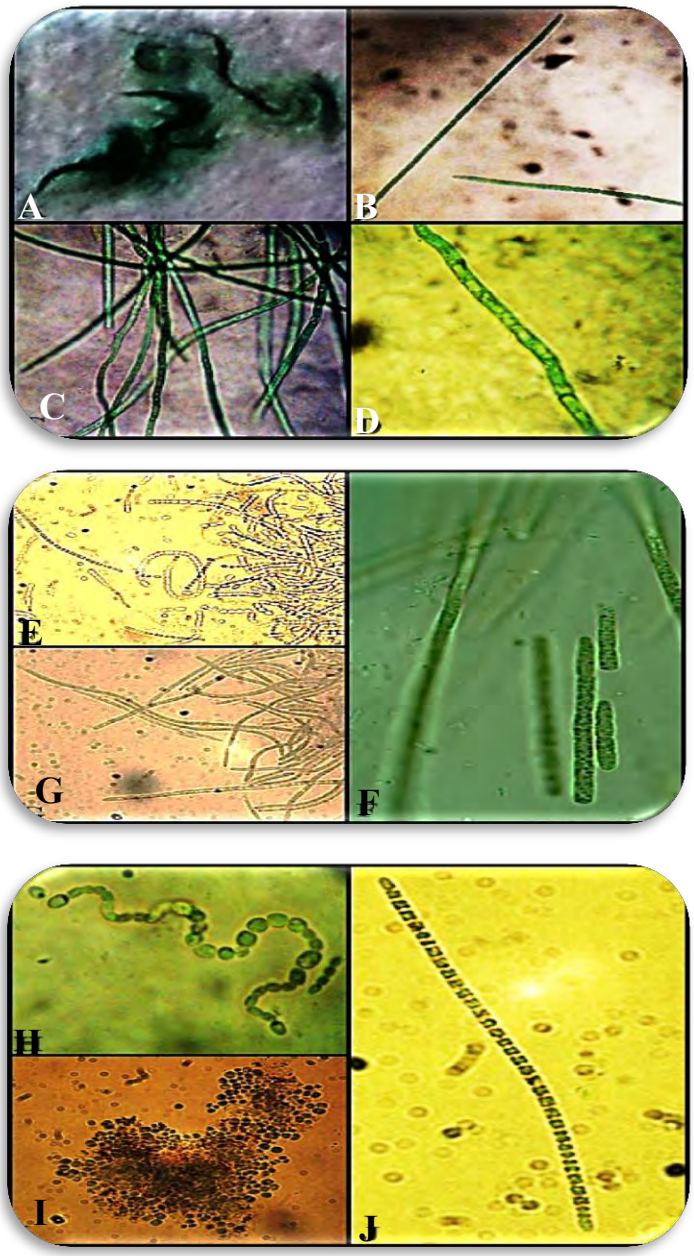


Fig. 3.5 A & B) *Desertifilum* sp(on petri plate and (400X) in BG_{11} media. and purified in SP media**; C & D)** *Oscillatoria* sp.(100X and1000X) in BG_{11}; **E)** *Anabaenopsis* sp.(50X) in BG_0 media. **F)** *Desertifilum* sp.(50X) in SP media. **G:** *Microcoleus* species in SP media. **H)** *Nodularia* sp.(400X)in BG_0 media. **I)** *Pleurocapsa* sp.(50X) in BG_0 media; **J)** *Cylindrospermosis* sp(1000X) in BG_0 media.

3.3.2.5 Observation of species at Site A-6

Field samples: *Scenedesmus* was common along with some blue greens like *Spirulina*, *Merismopedia* and two species of *Anabaenopsis*.
***in vitro* culturing**: the BG_{11} and BBM showed mixed growth of species, while none of the species responded to the BG_0 medium. In SpM medium a filamentous cyanobacteria identified as *Leptolyngbya* sp. showed growth (Fig. 3.6).

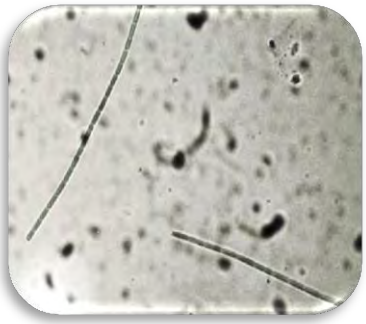


Fig. 3.6: *Leptolyngbya* sp. purified in SP Media and shown here at (400X) magnifications.

3.3.2.6 Observation of species at Site A-7

Field samples: *Euglena* and *Sheoride* species were observed along with some filamentous cyanobacteria.
***in vitro* culturing:** *in vitro* culturing revealed mixed growth of species in BG_{11}, BBM and SPM media. In 2nd attempt *Synechocystis* sp appeared in BG_{11} and BBM media while *Synechococcus* sp. in SP media. An unknown species was cultured in BG_{11} (Fig. 3.7).

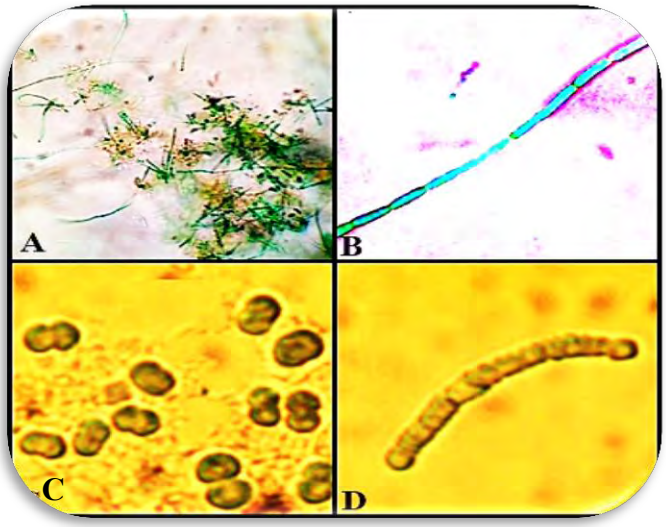


Fig 3.7 A & B) *Synechococcus* sp.(100X and1000X) in SP media at 5.74 pH **C)** *Synechocystis* sp (50X)produced in BG_{11}, BBM and SP Media**. D)** Unknown sp cultured in BG_{11}.(50X)

3.3.2.7 Observation of species at Site A-8

Field samples: *Chlorella, Cosmarium,* and *Oedogonium* sp were observed with some members of blue greens *Nodularia* and *Merismopedia.*
***in vitro* culturing:** BG_{11} and BBM produced mixed growth of greens and blue greens in 1st culturing. A *Nostoc* like species was produced and purified in BG_0 and Sp Media produced *Synechocystis* sp. (Fig. 3.8).

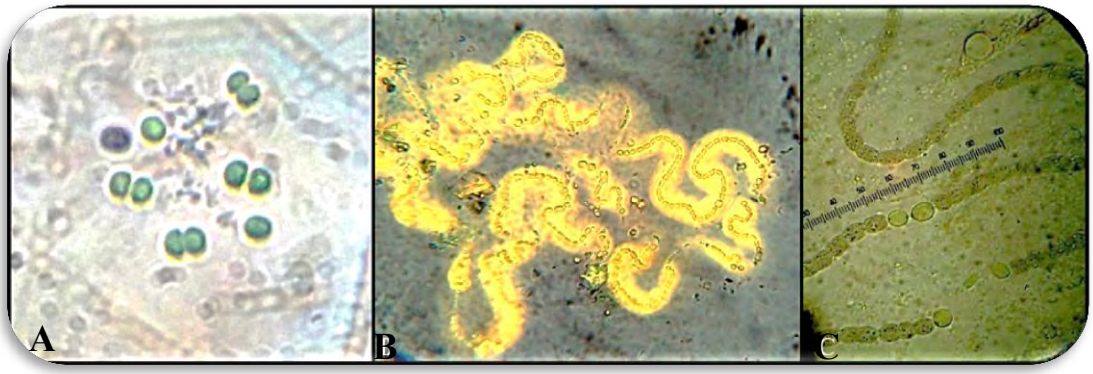


Fig 3.8 A) Sp medium produced *Synechocystis* sp(1000X).; **B & C)** colony(100X) and individual filaments of *Nostoc* sp.(400X)

3.3.3 Site B

3.3.3.1 Observation of species at Site B-1

Field samples: *Scenedesmus* sp. were common along with some *Phormidium* like sp.
***in vitro* culturing:** BG_{11} showed the growth of *Scenedesmus* sp, *Leptolyngbya* sp and a *Chlorella* sp. whereas BBM showed the growth of *Scenedesmus* sp. along with *Ankistrodesmus* sp and some *Nostoc* sp. *Nostoc* like sp. developed in BG_0 and SP M developed *Leptolyngbya* sp. (Fig. 3.9).

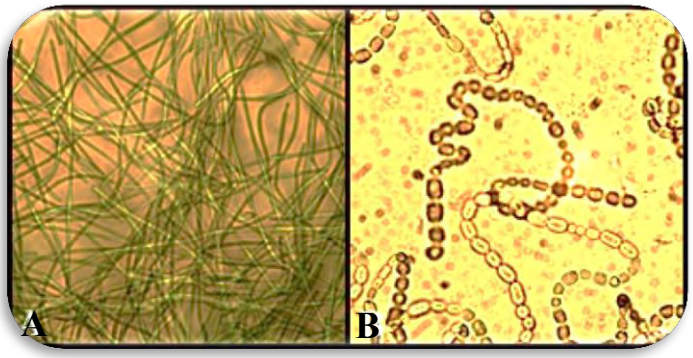


Fig 3.9 A) *Leptolyngbya* sp.(200X) cultured in SP Media**. B)** *Nostoc* like species (200X) in BG_0 media.

3.3.3.2 Observation of species at Site B-2

Field samples: *Scenedesmus* sp and *Euglena* were observed.
***in vitro* culturing:** BG_{11} showed the growth of *Gloeocapsa, Lyngbya* and *Leptolyngbya* sp. BBM showed *Scenedesmus* sp along with *Euglena* sp. and *Nostoc* sp. BG_0 showed the growth response of *Nostoc* sp. *Chlorella* species was developed by SpM and purified (Fig. 3.10).

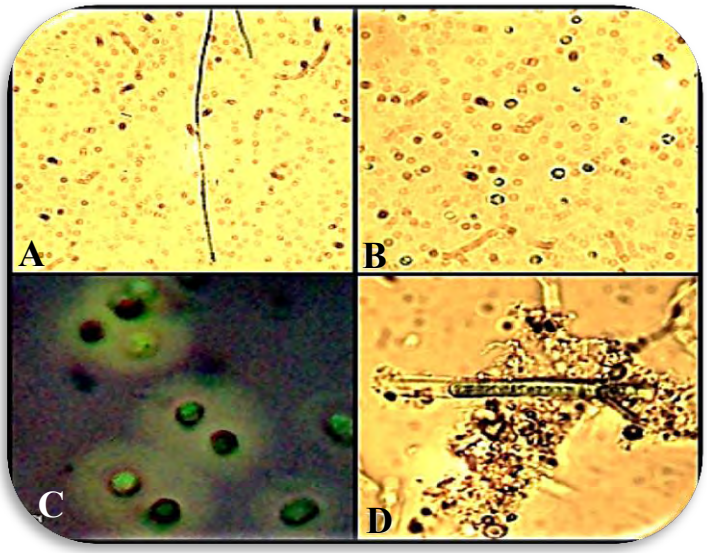


Fig 3.10. A) *Leptolyngbya* sp (50X)cultured in SP Media; **B)** *Chlorella* sp.(50X) in SP Media; **C)** *Gloeocapsa* sp.(100X) in BG_{11} media; **D**) *Lyngbya* sp.(50X) in BG_{11} media.

3.3.3.3 Observations of species at Site B-3

Field samples: A mix presence of *Scenedesmus* sp. and *Phacus* sp. and *Arthrospira* sp.
***in vitro* culturing:** BG_{11} media developed a mixture of *Arthrospira* sp. *Microchaete* sp. *Oscillatoria* sp., *Leptolyngbya* sp., *Anabaena* and *Chlorella* sp. Whereas, BBM supported the growth of *Scenedesmus* sp. *Oscillatoria* and *Arthrospira* sp. were commonly supported by BG_{11} and BBM. *Anabaena* sp. and *Nostoc* sp. were purified by BG_0 and *Arthrospira* sp. was purified by Sp M (Fig. 3.11).

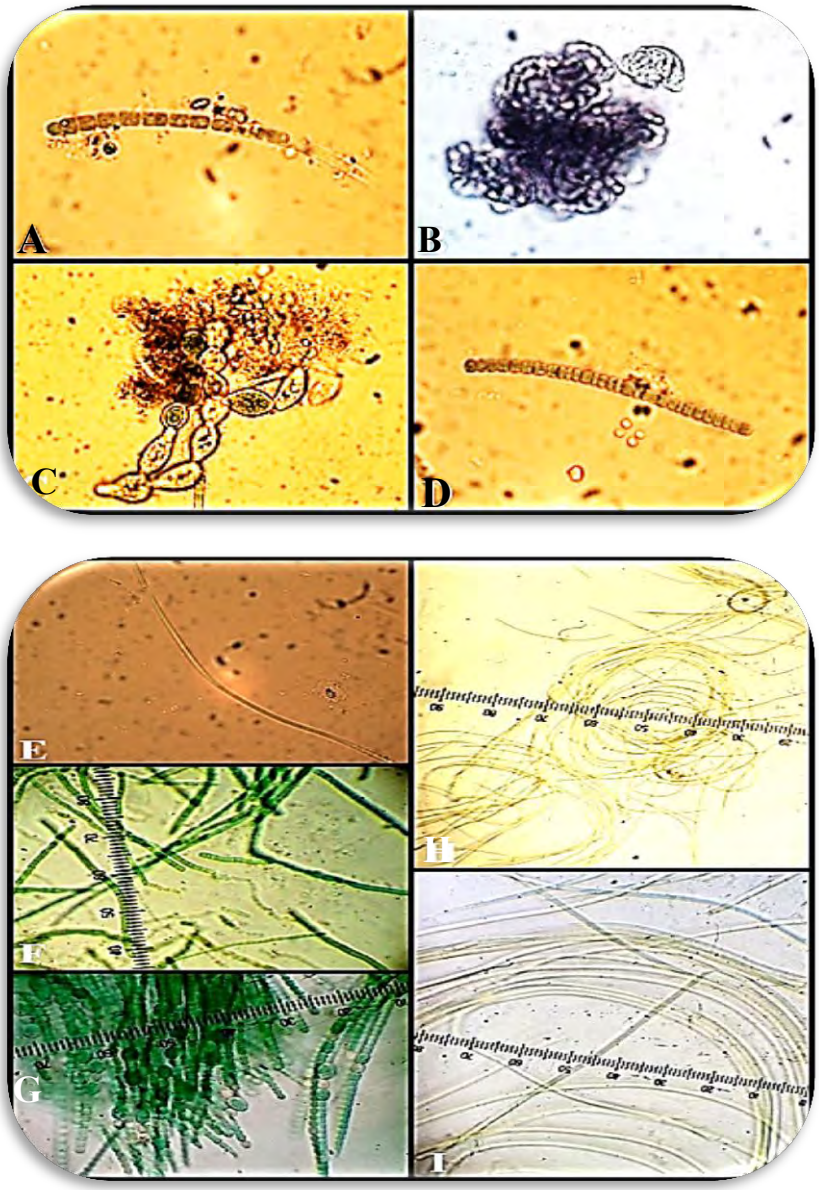


Fig 3.11: A) *Microcheate* sp in both BG_{11} and BBM media; **B)** *Nostoc* sp developed in BG_0 media**; C)** Unknown Uni-green with *Arthrospira* sp in BG_{11}; **D)** *Anabaena* sp. in BG_{11} media (50X); **E)** *Leptolyngbya* sp (50X) in BG_{11} media; **F)** *Arthrospira* sp purified in SP media; **G)** *Anabaena* sp cultured in BG_0. **H & I)** *Oscillatoria* sp. in solid and liquid BG_{11} culture media.(100X)

3.3.3.4 Observations of species at Site B-4

Field samples: Initial observations revealed *Scenedesmus, Euglena, Actinastrum, Golenkinis* and *Phormidium* besides the blue greens.
***in vitro* culturing:** BG_{11} and BBM showed mixed growth whereas there was no response shown by BG_0 as there was non-heterocystus in sample. *Leptolyngbya* was purified in Sp M (Fig. 3.12).

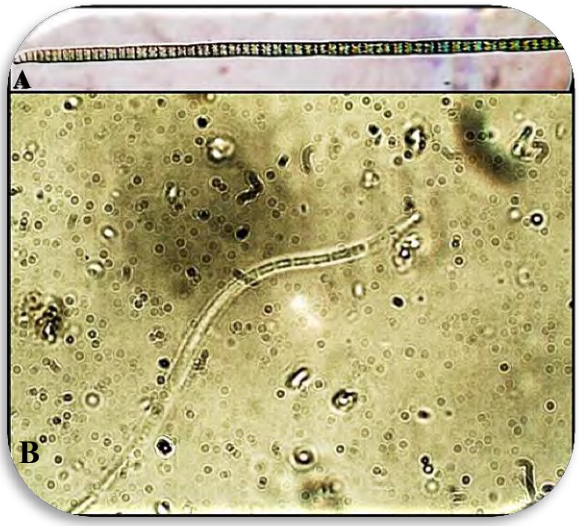


Fig 3.12: A) *Phormidium* sp. (1000X) in BG_{11} media; **B)** *Leptolyngbya* sp. (200X) purified in SP Media.

3.3.3.5 Observations of species at Site B-5

Field samples: *Closterium* and *Chlorella* species.
***in vitro* culturing:** BG_{11} cultured *Phormidium* sp. and *Synechococcus* sp while BBM produced *Nodularia* and *Phormidium* species. BG_0 produced axenic cultures of *Anabaenopsis* species. Sp media produced *Chlorella* species (Fig. 3.13).

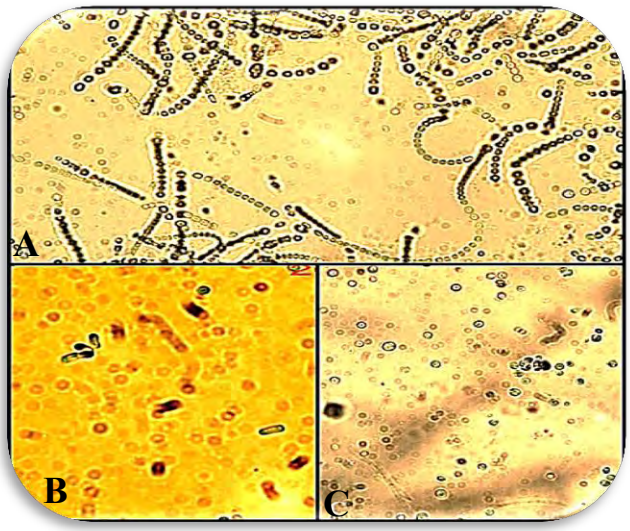


Fig. 3.13: A) *Nodularia* sp (50X) in BG_0 media; **B)** *Synechococcus* sp.(50X) in BG_{11}; **C)** *Chlorella* sp. (50X)) in SP media.

3.3.3.6 Observations of species at Site B-7

Field observations: *Euglena* and diatoms were observed.
***in vitro* culturing:** in BG_{11} produced *Chlorella* and *Haematochoccus* species while BBM produced *Scenedesmus* species. There was no response of BG_0 suggesting absence of heterocystous sp. SpM produced an axenic culture of *Oscillatoria* species (Fig. 3.14).

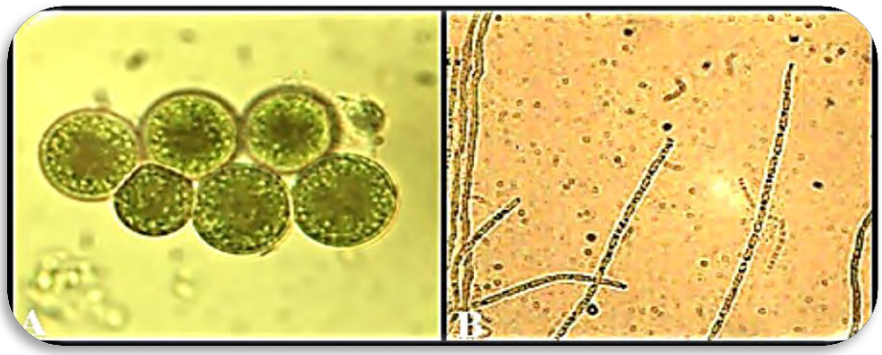


Fig 3.14: A) *Haematochoccus* sp. (50X) in BG_{11} medium**; B)** *Oscillatoria* sp. (50X) in SP medium.

3.3.3.7 Observations of species at Site B-8

Field sample: A dry soil sample with no initial observations.
***in vitro* culturing:** in BG_{11} solid media, there were three species i.e. *Phormidium* sp, *Gloeocapsa* sp. and *Cylindrospermum* species. *Gloeocapsa* axenic culture was obtained in BG_0 (Fig. 3.15).

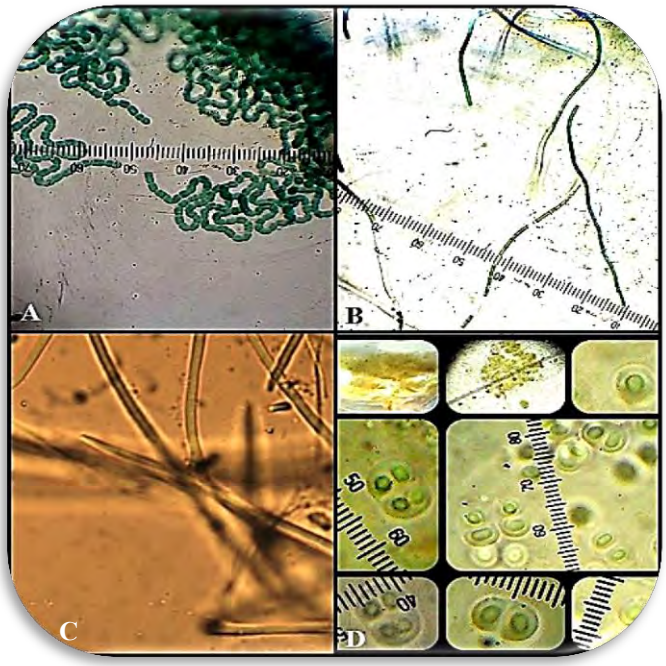


Fig 3.15: A) *Cylindrospermum* sp. (100X) in BG_{11}; **B & C**) *Phormidium* sp.(100X)and(50X) in BG_{11}; **D)** *Gloeocapsa* sp. purified in BG_0. (1. petri plate. 2. 100X. 3-7 1000X)

3.3.3.8 Observations of species at Site B-9

Field sample: *Cylindrospermosis* and diatoms species were observed. ***In vitro* culturing:** in BG_{11} *Cylindrospermosis* was grown without heterocyst. BG_0 media produced two types of species of *Nostoc*. SpM produced *Phormidium* like species (Fig. 3.16).

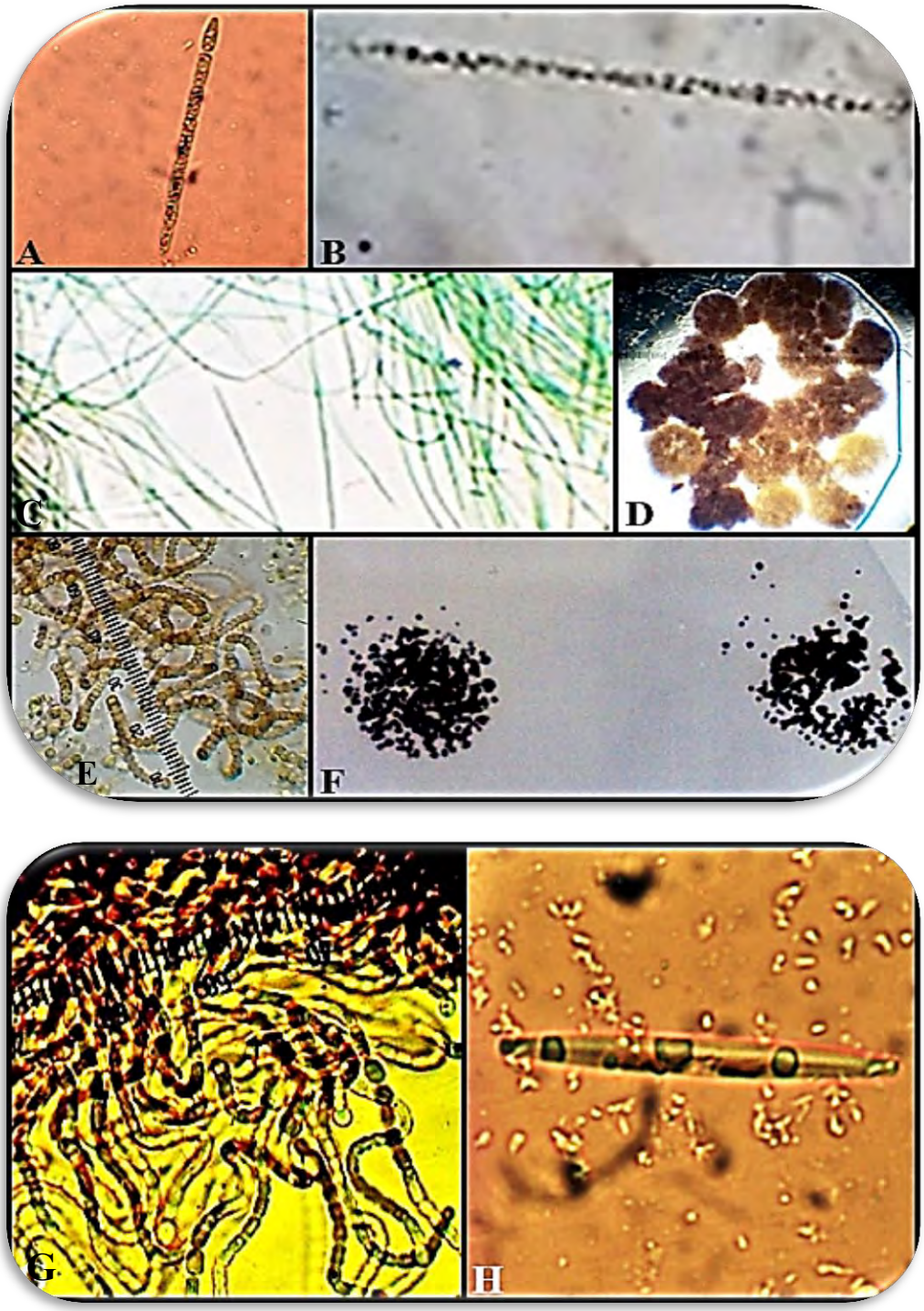


Fig 3.16: A & B) *Cylindrospermosis* sp. (50X) and (100x). in BG_{11}; **C)** *Phormidium* sp. (100X) in SP Media; **D** & **E)** Colonial(100X) and filamentous (400X) form of *Nostoc* sp. in BG_0; **F** & **G)** Colonial (petri plate) and filamentous(50X) form of *Nostoc* sp. in BG_0; **H)** Diatoms (50X) in BG_{11} media.

3.3.3.9 Observations of species at Site B-10

Field Sample: *Chara* was observed in shallow water of pond bank.
***in vitro* culturing:** in BG_{11}, two types of *Phormidium* sp were observed. BG_0 produced two axenic cultures of *Neowestiellopsis,* and *Nostoc*. SpM produced another axenic culture of *Pseudoanabaena* and *Chlorella* sp. (Fig. 3.17).

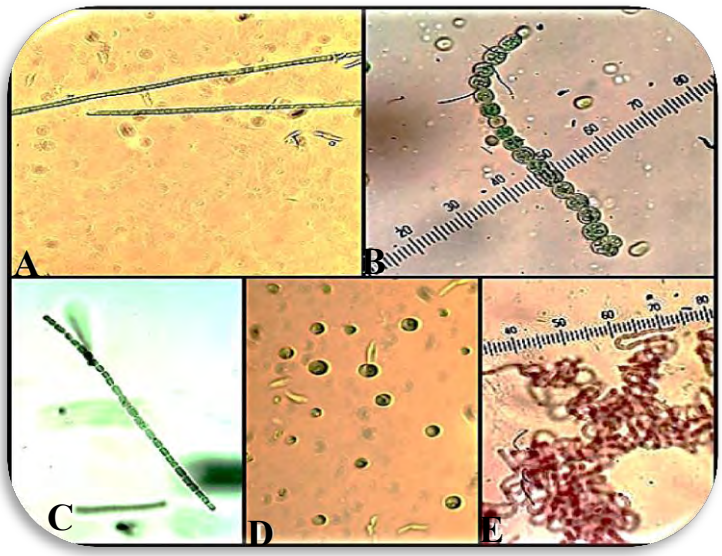


Fig 3.17: A) *Phormidium* sp. (50X) in BG_{11}; **B)** *Neowestiellopsis* (100X)in BG_0; **C**) *Pseudoanabaena* sp. (100X) in SP Media; **D)** *Chlorella* sp. (50X). in SP media; **E)** *Nostoc* sp. (100X) purified in BG_0 media.

3.3.3.10 Observations of species at Site B-11

This sample was biocrust, when grown on solid liquid BG_{11} media produced number of species i.e. *Oscillatoria* and *Phormidium* sp., BG_0 produced *Dulcicalothrix* sp. while BBM and SpM did not show any response (Fig. 3.18).

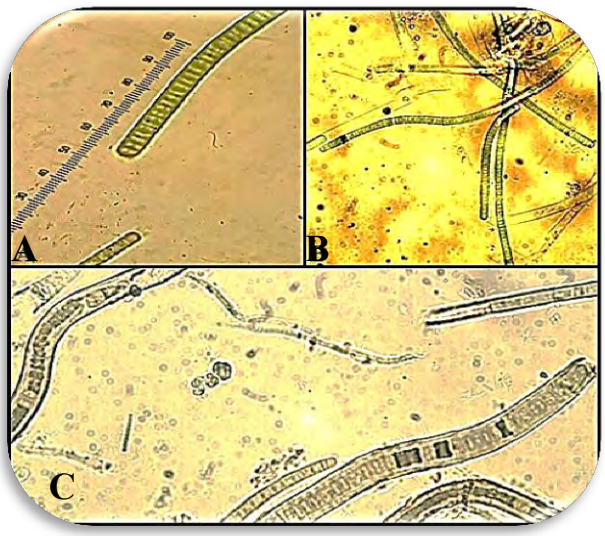


Fig 3.18: A) *Oscillatoria* and *Phormidium* sp. (400X) in BG_{11}; **B)** Species of *Phormidium* (50X) in BG_{11}; **C)** *Dulcicalothrix* sp. (50X) in BG_0 media.

3.3.3.11 Observations of species at Site B-12

Field samples: In initial observation the water sample was semi turbid. When observed under microscope some spore like structures were observed.
***in vitro* culturing:** BG_0 media produced *Neowestiellopsis* sp. and Sp Media produced *Chlorella* sp. (Fig. 3.19).

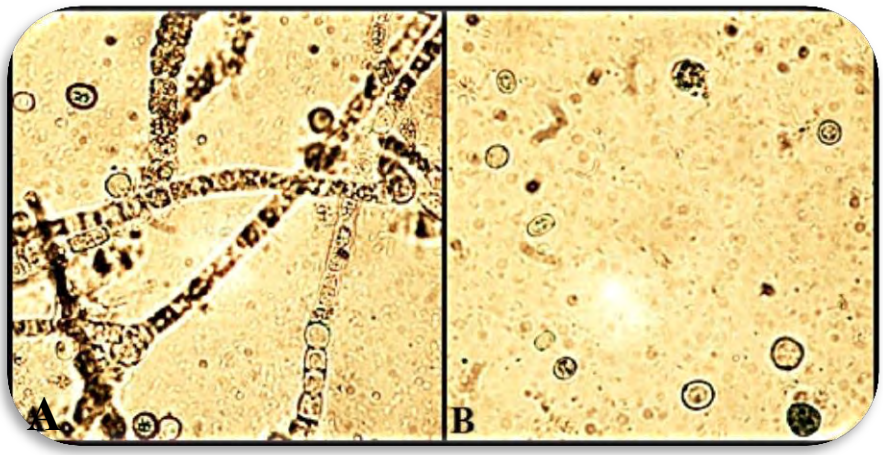


Fig 3.19: A) *Neowestiellopsis* sp. (50X) in BG_0 media; **B)** Spores of *Neowestiellopsis* sp. (50X) in BG_{11}.

3.3.3.12 Observations of species at Site B-13

Field sample: The sample was dry soil crust.
***in vitro* culturing:** On BG_{11} solid media, produced a few species i.e., *Leptolyngbya*, *Calothrix* and unicellular colonial cyanobacteria. BG_0 produced two species like *Microcheate* and *Nostoc* species (Fig. 3.20).

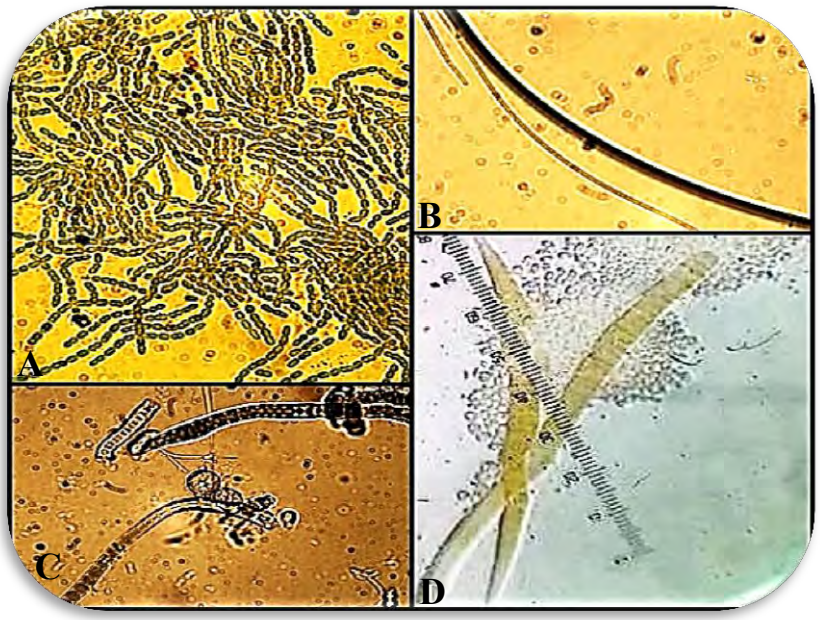


Fig 3.20: A) *Nostoc* sp.(50X) in BG_0 media; **B)** *Leptolyngbya* sp. (50X) in BG_{11} media; **C)** *Microcheate* sp. (50X) in BG_0 media; **D)** *Calothrix* sp. (100X) in BG_{11} media.

3.3.3.13 Observations of species at Site B-14

Field Sample: This sample was also dry soil crust.
***in vitro* culturing:** On BG_{11} *Phormidium* sp, *Anabaena* sp, *Calothrix* sp. and some filamentous cyanobacteria species were observed. Unknown colonial green algae were observed on solid media. SpM produced axenic culture of *Leptolyngbya* species (Fig. 3.21).

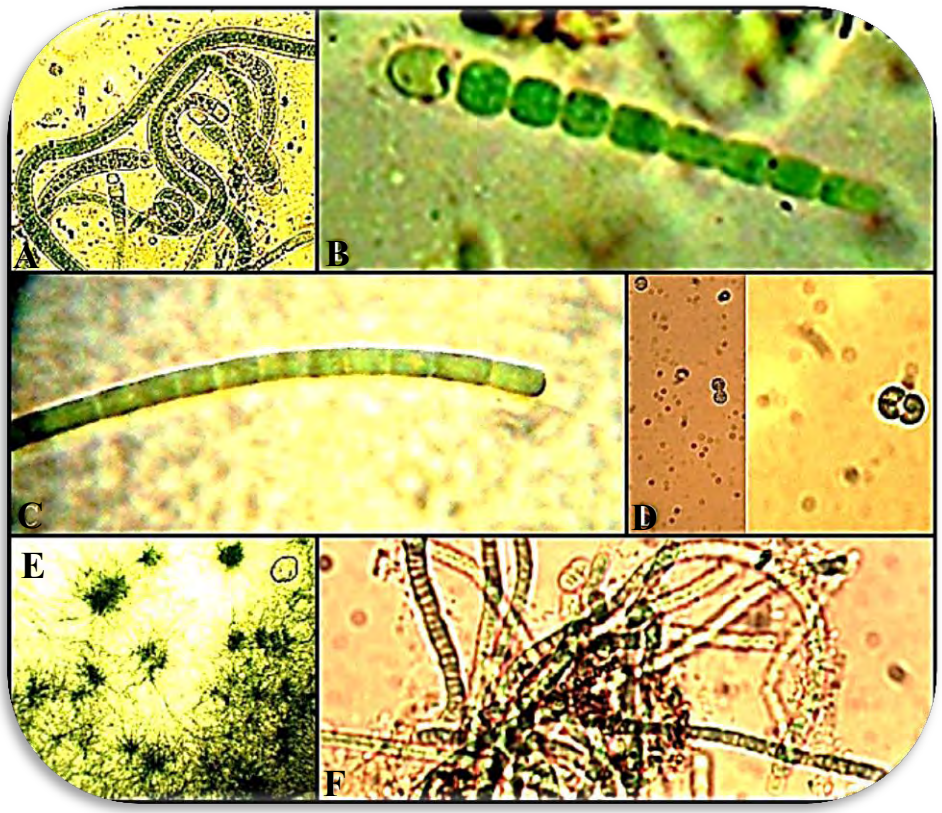


Fig 3.21: A) *Calothrix* sp. produced in BG_{11} (50X) and purified in BG_0 media; **B)** Young trichome of *Calothrix* sp(50X).; **C**) *Phormidium* sp in BG_{11} media (200X); **D)** Spores of cyanobacterium (50X); **E & F)** Colony (50X) and filamentous growth of *Leptolyngbya* sp. (200X).

3.3.3.14 Observations of species at Site B-15

Field samples: Initial observation in this site showed rich algal growth including *Spirogyra*, *Phormidium* sp, *Anabaena* sp, *Nodularia* sp, and *Pinocchia* sp were observed. ***in vitro* culturing:** BG_{11} produced *Nodularia* sp, *Anabaenopsis* and *Phormidium* species. BBM showed mixed growth of Unigreen and *Stigeoclonium* along with *Leptolyngbya* sp. BG_0 purified *Nodularia* sp. SpM produced axenic culture of *Leptolyngbya* (Fig. 3.22).

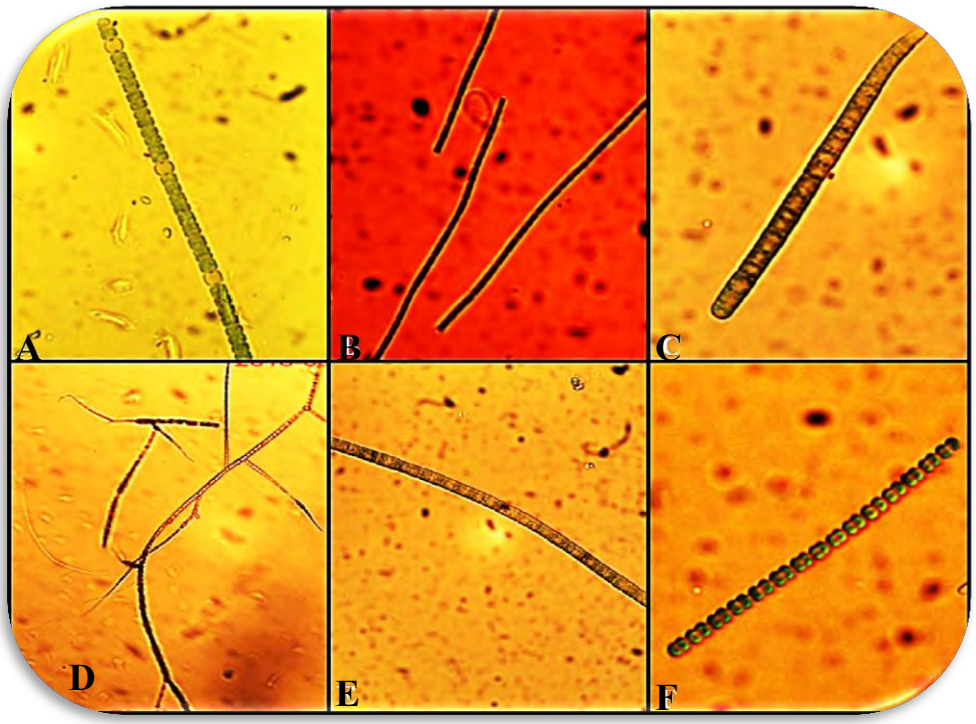


Fig 3.22: A) *Nodularia* sp. cultured in BG_{11} and purified in BG_0 media; B) *Leptolyngbya* sp. was produced in BG_{11} and purified in SP media. **C & E)** Species of *Phormidium* cultured in BG_{11} media; **D)** *Stigeoclonium* sp. and *Leptolyngbya* produced in BBM media; **F)** *Anabaenopsis* sp. in BG_{11} media. A-F (50X).

3.3.3.15 Observations of species at Site B-16

Field samples: filamentous greens: *Oscillatoria*, *Phormidium* sp. and some diatoms.
***in vitro* culturing:** BG_{11} produced *Phormidium* species whereas BBM showed some mixed growth of filamentous cyanobacteria and unicellular green types. BG_0 and SP media did not show any response (Fig. 3.23).

Fig 3.23: A) Unigreen in BG_{11} media (50X)**. B)** *Phormidium* sp in BG_{11} media (200X); **C)** Germinating spore of *Spirogyra* in BG_{11} media (50X).

3.3.3.16 Observations of species at Site B-17

Field samples: This sample was dry black crust on the wall of the Mosque.
***in vitro* culturing:** When this crust was grown on solid BG_{11} medium Cyanobacteria *Chroococcus* sp., and *Anabaena* sp., *Klisinema persicum*, *Cylindrospermum* sp. was purified when grown on BG_0 medium while *Klisinema* was purified when grown on BG_{11} (Fig. 3.24).

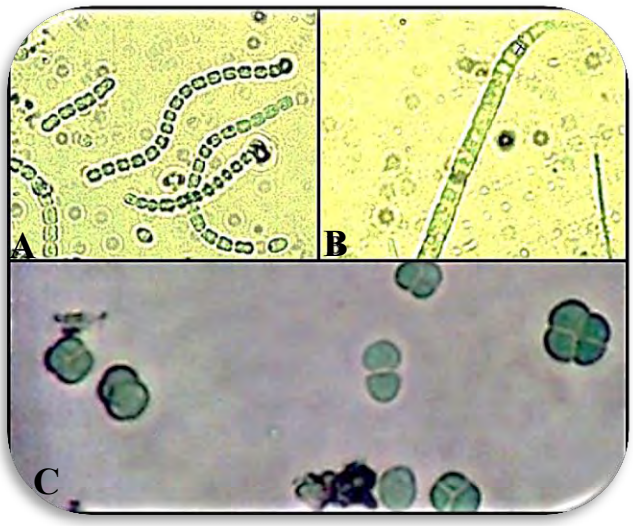


Fig 3.24: A) *Cylindrospermum* sp in BG_0 media (200X); **B)** *Klisinema* sp. purified in BG_{11} media (200X); **C)** Colonial *Chroococcus* sp. in BG_{11} media (400X).

3.3.3.17 Observations of species at Site B-18

Field samples: Biological crust sample with no initial growth observation.
***in vitro* culturing:** on BG_{11} solid media produced *Oscillatoria* sp, *Phormidium* and *Leptolyngbya* sp. BG_0 produced *Nostoc* sp and *Scytonema* species (Fig. 3.25).

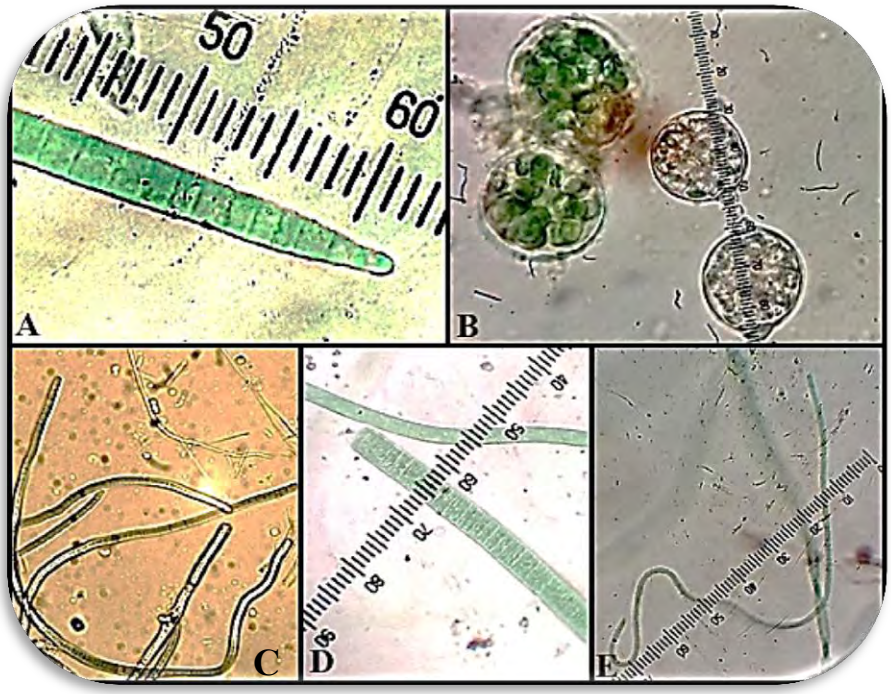


Fig 3.25: A) *Phormidium* sp in BG_{11} media (400X). **B)** *Nostoc* sp in BG_0 media (400X). **C)** *Scytonema* sp. in BG_0 media (200X). **D)** *Oscillatoria* species culture in BG_{11} media (400X). **E**) *Leptolyngbya* sp in BG_{11} (400X).

3.3.3.18 Observations of species at Site B-19

Field samples: Dry soil crust
***in vitro* culturing:** *Leptolyngbya* sp. showed growth response on solid BG_{11} media only (Fig. 3.26).

Fig 3.26: An axenic culture of *Leptolyngbya* sp. in BG_{11} medium (400X)

3.4 The composition of species examined during *in vitro* culturing

Among samples, green types were observed most frequently contributing a share of 48.5% while 37% blue green types were observed in the collections. Among green algal types, members of the genus *Scenedesmus* were found most frequently with nine species. Other green taxa were represented by either one or two species only. Among blue green types, the genus *Anabaenopsis* was represented by two species while the rest of genera were represented by one species only. Among blue green types, *Phormidium* was observed most frequent. Among unique types, *Neowestiellopsis* was found at three sites. *Anabaena* was observed on sites found in close vicinity of agricultural land. The site wise summary of species is given in Tables 3.3 and 3.4.

Table 3.3: Species observed in samples collected along route A and through *in vitro* culturing.

Sites	Field (fresh) samples	BG_{11}	BBM	BG_0	Sp	F+C
A2	*Euglena* *Scenedesmus* *Nostoc* *Diatoms*	*Scenedesmus* Uni green *Nostoc* sp.	*Scenedesmus* Uni green *Nostoc* sp.	*Calothrix* sp. *Halotia*	*Leptolyngbya.*	*4+3 = 7*
A3	*Spirogyra* Cyanophytes Diatoms	*Scenedesmus* sp. *Nostoc commune*	*Scenedesmus* *Chrococcus* sp.	*Nostoc commune* *Nostoc careum*	*Leptolyngbya* sp.	*3+3 = 6*
A4	*Chara* *Zygnema*	*Neowestiellopsis*	*Phormidium*	*Neowestiellopsis* *Nostoc punctiformae*	Mixed growth of *Phormidium* and *Nostoc*	2+3 = 5
A5	*Spirogyra* *Gloeocapsa* *Merismopedia*	*Desertifilum* sp *Oscillatoria* sp	*Oscillatoria* sp	*Nodularia* sp *Cylindrospermosis* sp *Pleurocapsa* sp	*Desertifilum* sp c *Desertifilum* sp b *Desertifilum* sp d	*3+5 = 8*
A6	*Scenedesmus* *Spirulina* *Merismopedia* *Anabaenopsis*	**X**	**X**	**X**	*Leptolyngbya*	*4+1 = 5*
A7	***Euglena*** ***Shroderia***	**Filamentous cyanobacteria** **Unicellular green types**	**Mixed growth of cyanobacteria and green types**	**X**	**Mixed growth**	**2+? = ?**
A8	***Chara*** ***Chlorella*** ***Cosmarium*** ***Oedogonium***	**No growth**	**No growth**	***Synecocystis* sp.**	***Chlorella* sp.**	***4+1 = 5***

Notes: *Scenedesmus* sp. were observed at most sites and cultured frequently in BG_{11} and BBM; followed by *Spirogyra* and *Cosmarium* types

Euglena in BBM is rare to culture. *Arthrospira* strain of site 3 has the potential to be cultured more lucidly. *Chlorella* responded well in the SP medium and rarely also responded in BG_{11}. In BBM medium mostly mixed growth was observed. BG_{11} being a preferred medium has supported growth of 2-5 species.

Table 3.4: The composition of species observed in field fresh samples collected along route B and those observed in *in vitro* culturing using different media.

S. no.	sites	Field (fresh) samples	*in vitro* culturing (media)				Species recorded at each site
			BG_{11}	BBM	BG_0	Sap	
1	B1	***Scenedesmus*** *Phormidium*	*Scenedesmus Leptolyngbya Chlorella*	*Scenedesmus Nostoc Ankistrodesmus*	*Nostoc*	*Leptolyngbya*	2+4 = 6
2	B2	***Scenedesmus*** *Euglena*	*Gloeocapsa Anabaena Leptolyngbya*	*Scenedesmus Nostoc Lyngbya Euglena*	*Nostoc*	*Chlorella*	2+6 = 8
3	B3	***Scenedesmus Arthrospira*** *Phacus*	*Gleocapsa Arthrospira Oscillatoria Leptolyngbya Anabaena Microchateae Chlorella*	*Scenedesmus*	*Anabaena Nostoc*	*Arthrospira*	3+7=10
4	B4	*Scenedesmus Euglena Actinastrum*	Mixed growth	X	X	*Leptolyngbya*	3+1 = 4
5	B5	***Chlorella*** *Closterium*	*Phormidium Synecochoccus*	*Phormidium Anabaenopsis*	*Anabaenopsis*	*Chlorella*	2+3 = 5
6	B6	Bio-crust/Dry soil	X	X	X	X	0
7	B7	*Euglena Diatoms*	*Chlorella Haematochoccus*	*Scenedesmus*	X	*Oscillatoria*	4+1+1 = 6
8	B8	Bio-crust/Dry soil	*Phormidium Gloeocapsa Cylindrospermosis*	X	*Gloeocapsa*	X	0+3 = 3
9	B9	***Cylindrospermosis*** *Diatoms*	*Cylindrospermosis*	X	*Nostoc*	*Phormidium*	3+1 = 4
10	B10	*Chara*	*Phormidium*	X	*Neowestiellopsis Anabaena* species	*Pseudoanabaena Chlorella*	2+4 = 6
11	B11	Bio-crust/Dry soil	*Microcheate*	X	X	X	0+1 = 1
12	B12	No fresh growth visible	X	X	*Fischrella*	*Chlorella*	1+1 = 2
13	B13	Bio-crust/Dry soil	*Leptolyngbya* Unigreen	*Microcheate Anabaenopsis*	X	X	1+3 = 4
14	B14	Bio-crust/Dry soil	*Phormidium Anabaena Calothrix* Unigreen	X	X	*Leptolyngbya*	1+4 = 5

15	B15	*Spirogyra* *Anabaena* ***Phormidium*** ***Nodularia*** ***Pinnochia***	*Nodularia* *Phormidium*	Unigreen *Stigeocoloniu m* *Pinnochia*	*Nodularia* sp.	*Pinnochia* sp.	4+3 = 7
16	B16	*Oscillatoria* ***Phormidium*** *Diatoms*	*Phormidium* sp	Mixed green & cyanobacteria	X	X	1+1+1 = 3
17	B17	Bio-crust/Dry soil	*Chroococcus* *Anabaena* *Klisinema*	X	*Anabaena*	X	0+3 = 3
18	B18	Bio-crust/Dry soil	*Leptolyngbya* *Oscillatoria* *Phormidium*	X	*Nostoc* *Scytonema* sp	X	0+5 = 5
19	B19	Bio-crust/Dry soil	*Leptolyngbya*	X	X	X	0+1 = 1
		Total no of Genera	21+11= 32			Total no of species	84

Note back:

- no observations at sites B6; while others were soil samples with no initial observations as shown in case of sites B8, B9.
- After successful culturing of isolated species, biomass was produced by shifting species of cyanobacteria to respective broth cultures.

3.5: Polyphasic analysis of species

3.5.1 The Nostocales

3.5.1.1- *Nostoc punctiformae* B10: (Family Nostocaceae)

Morphological description: Small size colonies, mostly irregular in shape, present in mucilage. Thallus dark brown in colour; trichomes compactly arranged, appearing dark green or brown in colour; terminal cells large in size, 7µm long and 4µm wide; vegetative cells 5µm in length and 3µm in width; heterocysts sub-spherical in shape and intercalary, 6 µm long and 5µm in width (Fig. 3.5.1).

Diagnostic characters: Punctate colonies; densely entangled filaments; cells cylindrical (i.e. longer than broad); Akinetes not observed normally.

Site details and habitat: At site B10, a cyanobacterium strain was isolated. Soil samples were taken from a rainwater harvesting pond (Kaly pahar) with turbid water. Other taxa found in the sample were: *Phormidium*, *Nostoc*, *Anabaena*, *Pseudoanabaena* and *Chlorella*.

Culturing conditions: *N. punctiformae* responded in BG_0 medium.

Genus originally described by: Hariot 1891

Fig. 3.5.1: A) The microscopic colonies of *N. punctiformae* (7mm) on BG_0 agar medium. B) Highly entangled and compact filaments (50X). C) Filaments with vegetative cells (5µm x 3µm) at 1000X and heterocysts.

Phylogenetic Analysis: Phylogenetic analysis of strain B10 revealed a close resemblance with: *Nostoc punctiformae* and *N. microscopicum*. This clustering pattern suggests a clear position of this strain with in *Nostoc* clade. The topology finds a robust support with a bootstrap value of 67 (Fig. 3.5.2). A similar phylogenetic position was attained through the maximum likelihood and Maximum Parsimony analyses.

Sequence similarity and query cover: Sequence length achieved was 634 bp (61~694). The NCBI blast analysis showed similarity of generated sequences with *N. punctiformae* KT818630; *N. microscopicum*, MH144147).

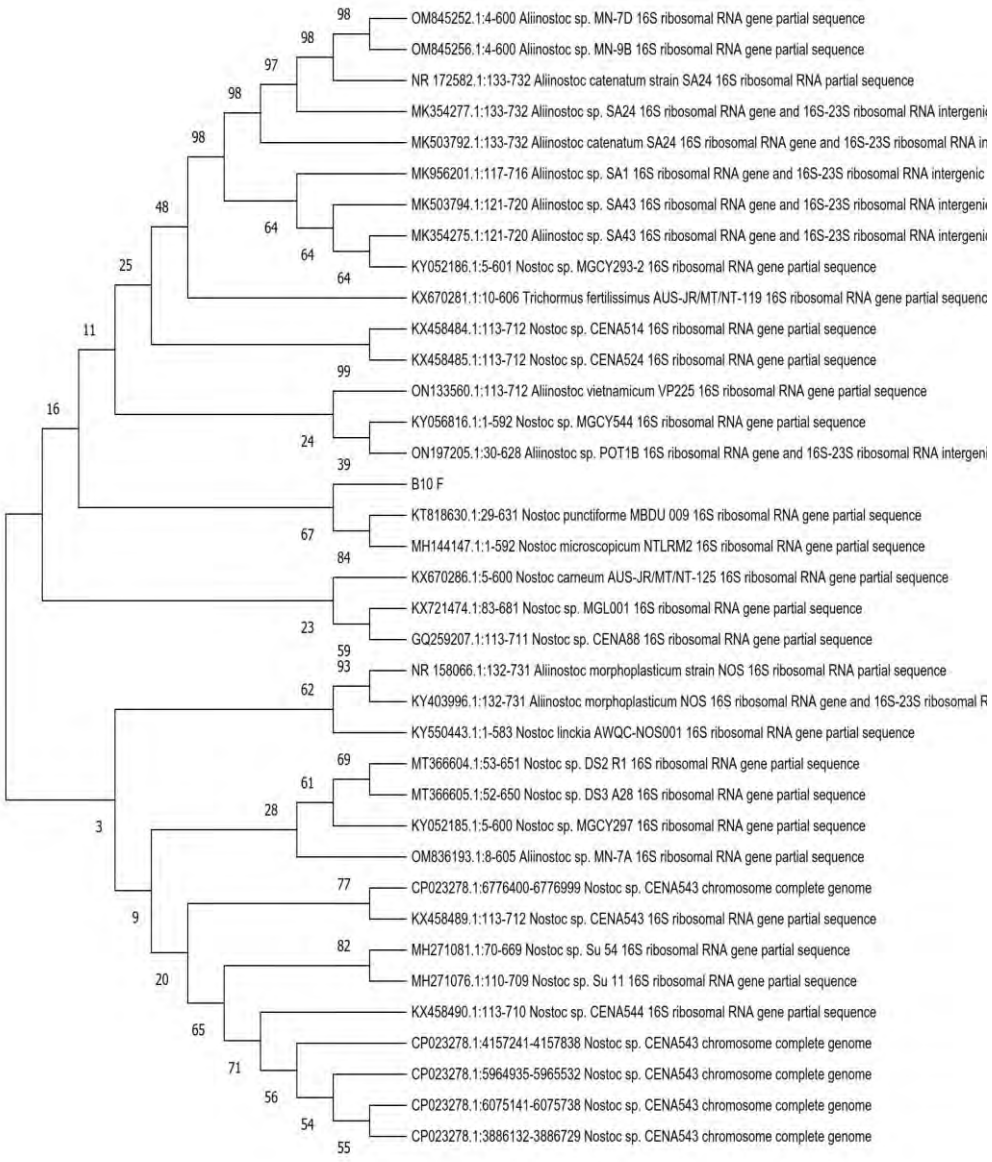


Fig. 3.5.2: Phylogenetic analysis strain B10 based on neighbor joining (NJ) method.

3.5.1.2 ***Aliinostoc morphoplasticum*** F6C.

Description: Colonies absent. Filaments brownish green and **loosely arranged** with diffluent **mucilage** layer. Cells **barrel-shaped** to sub quadrate with **prominent constrictions at the cross walls**; terminal cells slightly longer and wider than the intercalary cells. Intercalary cells 5×4µm in length and width. Heterocytes circular to slightly cylindrical with one end **tapering**; present mostly at the **terminal position**; 3.7-6 µm in length to 3.2-6µm in width.

Reproduction: Akinetes oblong, 5.69–6.11 µm in length to 4.48–4.63 µm in width, in series, brown to green outer cell wall (Fig. 3.5.3).

Diagnostic characters: The sample differs from *Nostoc* due to the absent of colonies; vegetative cells and heterocysts of variable shapes. Filaments are straight and show low degree of coiling.

Site and habitat: From site B6, soil Bio-crust sample, with no prominent growth in the natural habitat. Other species found in the sample were *Oscillatoria, Leptolyngbya* and a unicellular green alga.

Culturing conditions: Growth response in BG_{11} and SP media were recorded.

Genus originally described by: Bagchi, Dubey & P. Singh

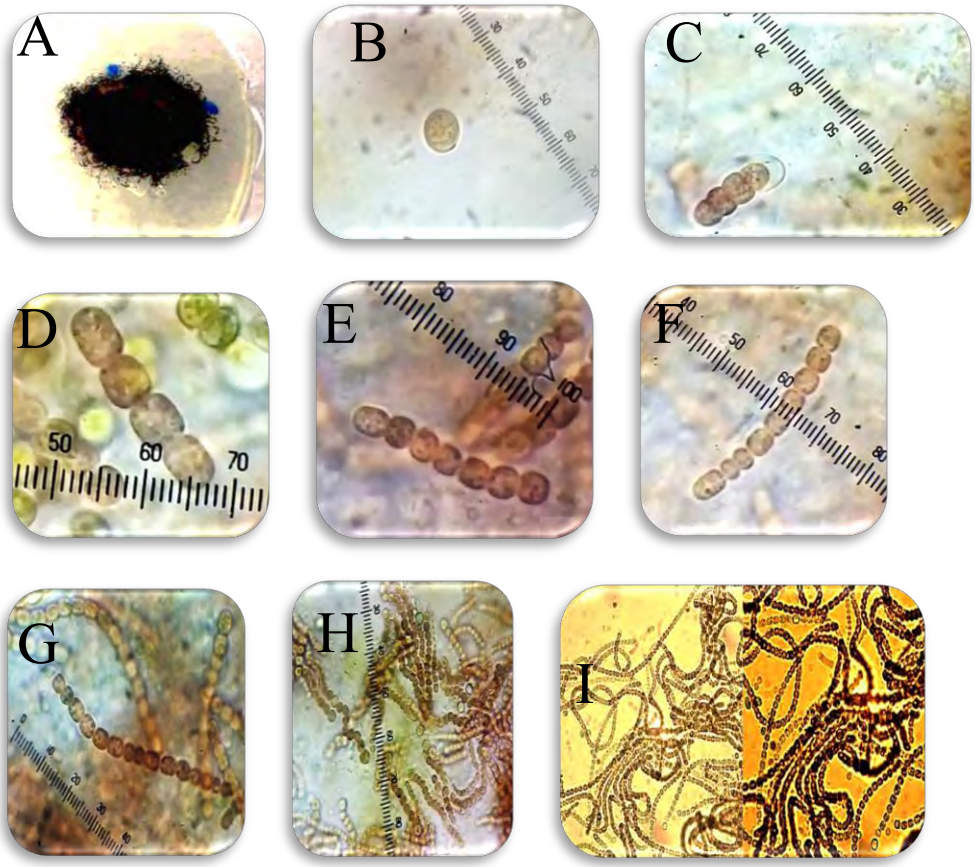


Fig. 3.5.3: A) Colony with partially irregular shape (20mm), B) Akinete (6 µm x 4.6 µm LW) emerging form main colony; C) Development of akinete to form a three cell (5µmx4µm LW) trichome emerging out form parent wall; D) Developing trichome with dividing cells centrifugally; E)

Development of heterocyst on terminal position and subsequent development of akinete; H and I) Partially interwoven filaments.

The phylogenetic analysis revealed proximity of strain F6C with *Allinostoc morphoplasticum* with bootstrap value of 56. In the clustering, other *Allinostoc* species were also found. The Maximum likelihood and Maximum parsimony analyses also revealed same clustering patterns with bootstrap value of >50 (Fig. 3.5.4).

Sequence, similarity and query cover: A 620 bp sequence was generated amplifying 16S ribosomal RNA gene. *A. morphoplasticum, A. catenatum, A. magnakinelifex* and APCC8976 were the top four matches revealed in NCBI blast analysis. The pairwise similarity of these accessions was 98.54%, 96.92%, 96.27% and 95.63% respectively.

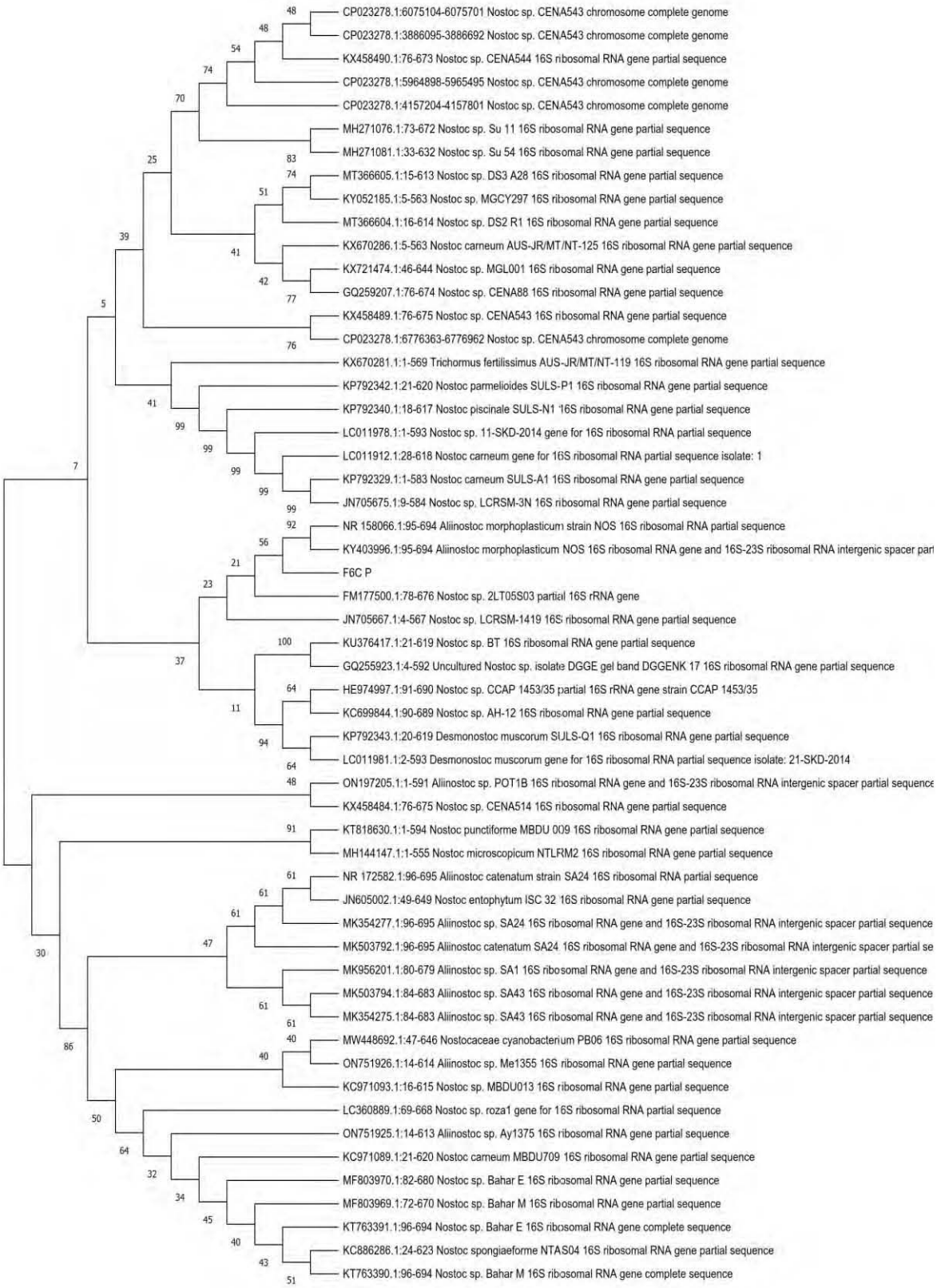


Fig. 3.5.4: Phylogenetic analysis strain F6C based on neighbor joining (NJ) method.

3.5.1.3 *Nodularia harveyana* B15P

Morphological Description: Filaments are mostly straight; cells are discoid 2×5µm in length and width respectively. Heterocysts are intercalary and terminal, sometimes two heterocysts are present on both sides of a single cell, 6×7µm in length and width. Akinetes are produced in series, as many as up to 11 recorded, 9µm in width (Fig. 3.5.5).

Diagnostic characters: Straight filaments; Cells shorter than long; Mucilage absent; Presence of intercalary and terminal heterocysts; Akinetes develop amphoterically. The reported species was differentiated from *N. spumigena* by having a diffluent mucilage and heterocysts frequently present on terminal positions. *N. spumigena* has a clear sheath and the absence of terminal heterocyst.

The reported species resemble morphologically with *N. harveyana* more than *N. sphaerocarpa* in traits such as: the width of vegetative cells. In case of *N. spherocarpa* cells are broader than the *N. harveyana*. Similarly, the shape of terminal cell was different in both species. *N. sphaerocarpa* have blunt ends while these are conical in *N. harveyana. N. harveyana* strains commonly have terminal heterocytes and the intercalary heterocytes are often in pairs.

Site and habitat: Reported as a benthic species; in the present study it is isolated from stagnant water on the soil at site B15, alongside other species: *Pinnochia polymorpha, Phormidium*, *Pseudoanabaena* sp. and *Stigeocolonium*.

Culturing conditions: this strain showed growth response in BG_0 medium.

Genus originally described by: Thuret ex Bornet & Flahault

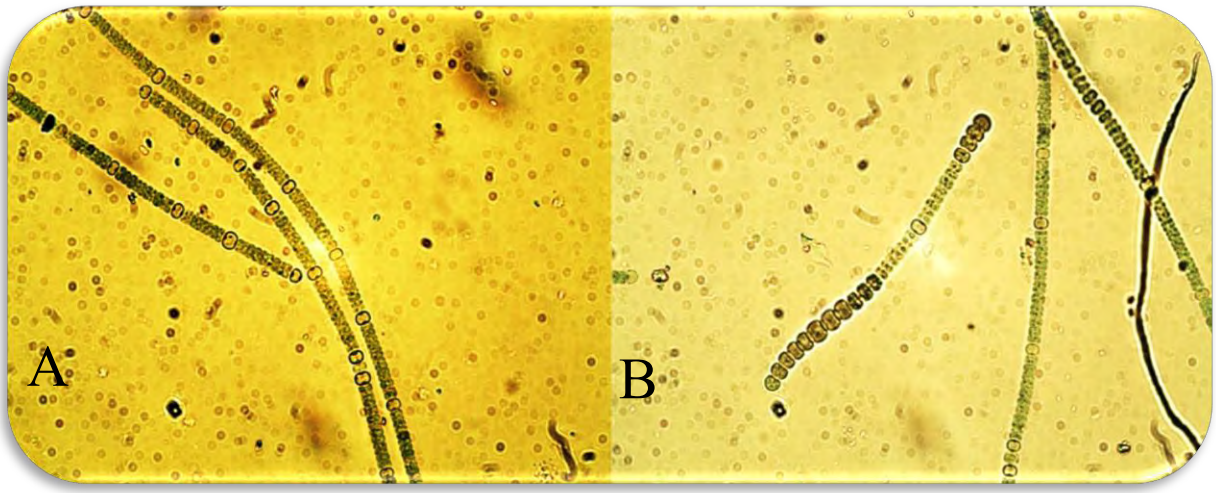


Fig. 3.5.5: a) Parallel arrangement of filaments with frequent heterocysts (6µmx7µm LW) on terminal and intercalary positions, cell broader than long (2µm x 5µm LW) having discoid shapes; b) development of akinetes (9µmW) amphoterically.

Phylogenetic analysis: Phylogenetic analysis of (B15 P) based on NJ clustering revealed proximity with *Nodularia harveyana* acc. no. AJ781145 with a bootstrap support of 53. The Maximum likelihood and Maximum Parsimony analyses showed similar clustering with stronger bootstrap support of 84 and 89 (Fig. 3.5.6).

Sequence, similarity and query cover: The 16S ribosomal RNA gene amplified a nucleotide length of 620 bp. The blast analysis revealed sequence similarity of the strain B15 P with *Nodularia harveyana* and with *Anabaenopsis* sp. with pairwise similarity of 98.22% and 96.05% respectively.

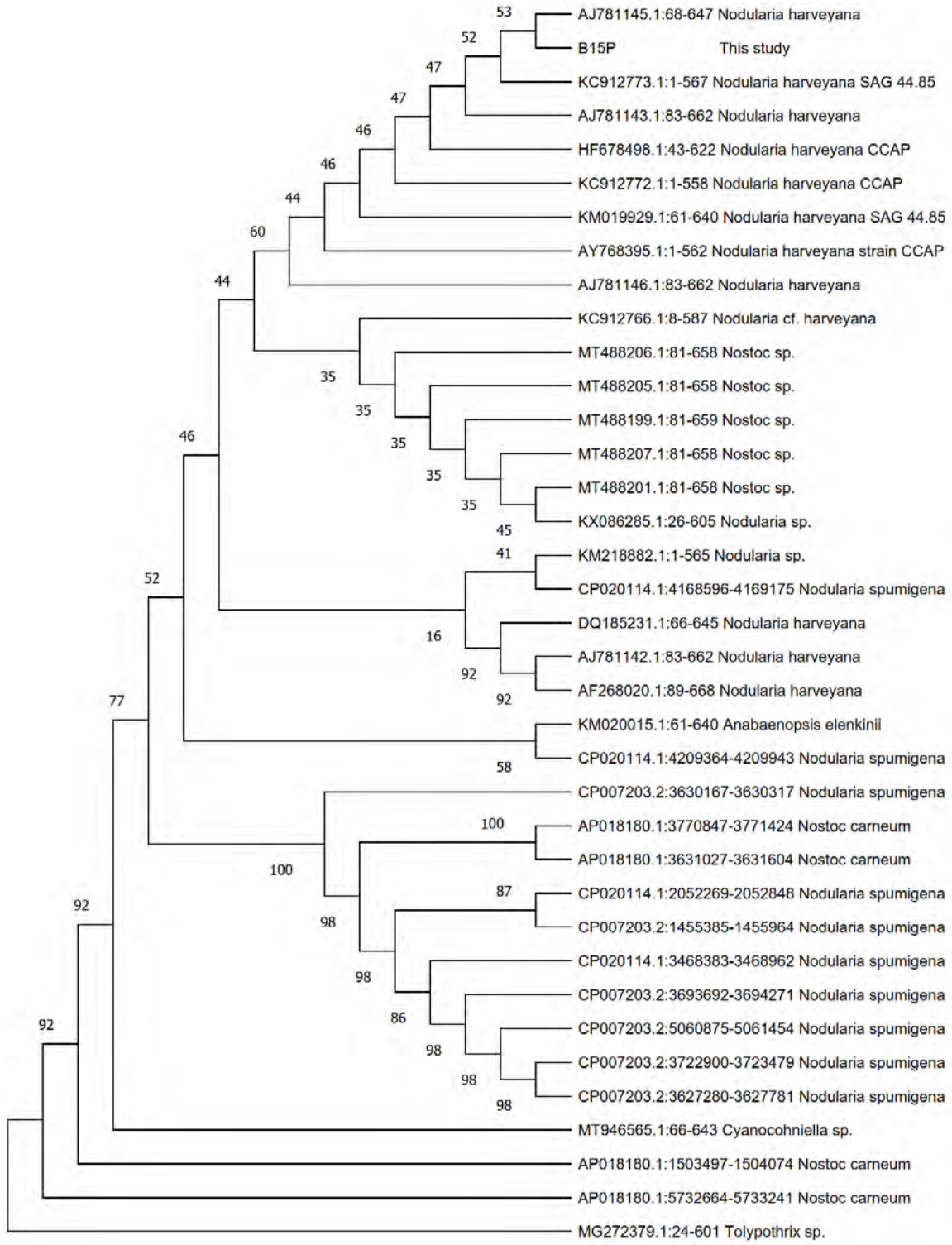


Fig. 3.5.6: Phylogenetic analysis strain B15P based on neighbor joining (NJ) method.

3.5.1.4 *Nodularia* sp. A2aCom

Morphological Description: Filaments: blue green in colour, straight or slightly bent in the early stage of growth, unbranched with diffluent mucilage. Cells: barrel shaped, isodiametric, 5µm in length and 4µm in width. Heterocysts: mostly terminal on both sides in young culture and very rarely intercalary in older cultures, 4-5µm in length and 3-5 µm in width. Akinetes grayish in early life stage while this turn yellow in older stage, 5-7 µm in diameter (Fig. 3.5.7 & 3.5.8).

Diagnostic Characters: Vegetatively this strain is similar to *N. harveyana*. However, during akinete formation cells size begin to increase and eventually all cells transform into akinetes; Frequency of intercalary heterocysts was low. At times pair of heterocysts may be seen on terminal position.

Site and Habitat: A sample isolated from site (A2a4), in a water channel with thick algal growth. Other species isolated from the same site were: *Leptolyngbya* and *Scenedesmus*.

Culturing conditions: BG_{11} and BBM supported the growth of this species along with other species. Re-plating and re-culturing in solid and liquid BG_0 media with continuous light under anaerobic conditions helped purify and isolate this species.

Genus originally described by: Thuret ex Bornet & Flahault

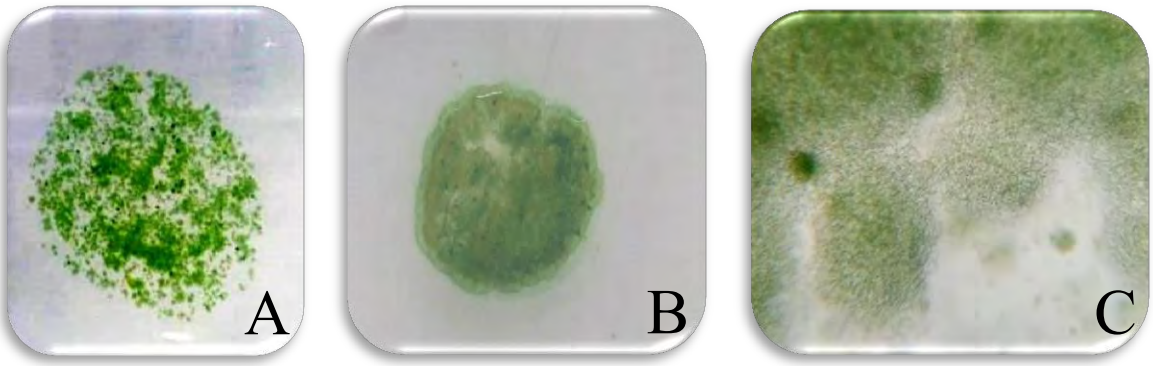


Fig. 3.5.7: A) Initial stage of colony growth after 7 days. B: Full growth of colony after 15 days. C: Growth of filaments on solid media with absence of individual mucilage (100X).

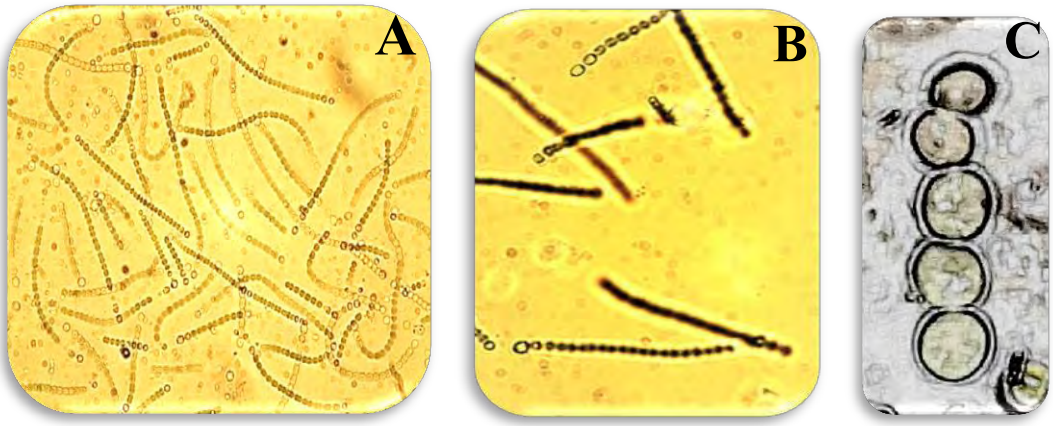


Fig. 3.5.8: A) Straight and curved filaments of *Nodularia* without mucilaginous sheath. B) Individual Trichomes showing vegetative cells (5µmx4µmLW) and heterocysts (4-5µmx3-5µmLW) present on both terminals. C) formation of akinetes (5-7µm) in a series.

The phylogenetic analysis of strain A2a Com using neighbor joining method revealed its close clustering with *Nostoc* sp. IMU16, *Nodularia* sp. IMU17 and *Nostoc* sp. HCC1088 with bootstrap values: 91, 93 and 66 respectively. The sister cluster contained species of *Nostoc* (IMU9, IMU21, IMU27); *Nodularia* (AL6) and *Anabenopsis*. The maximum likelihood analyses also showed similar clustering with a bootstrap support of up to 70 and the Maximum Parsimony analysis showed close clustering with *Nostoc* species IMU16 with bootstrap value of 81 and with *Nodularia* species IMU17 with bootstrap value of 58 (Fig. 3.5.9)

Sequence, similarity and query cover: The 16S ribosomal RNA gene sequencing revealed a nucleotide length of 628bp. The NCBI blast analysis revealed top matches with *Nostoc* sp. HCC10, *Anabaenopsis* sp. *Nostoc* sp BEA, *Nodularia* sp. IMU17, *Nodularia* sp. AL6 with percentage identities: 99.03%, 98.87%, 98.87%, 98.55% and 98.39% respectively.

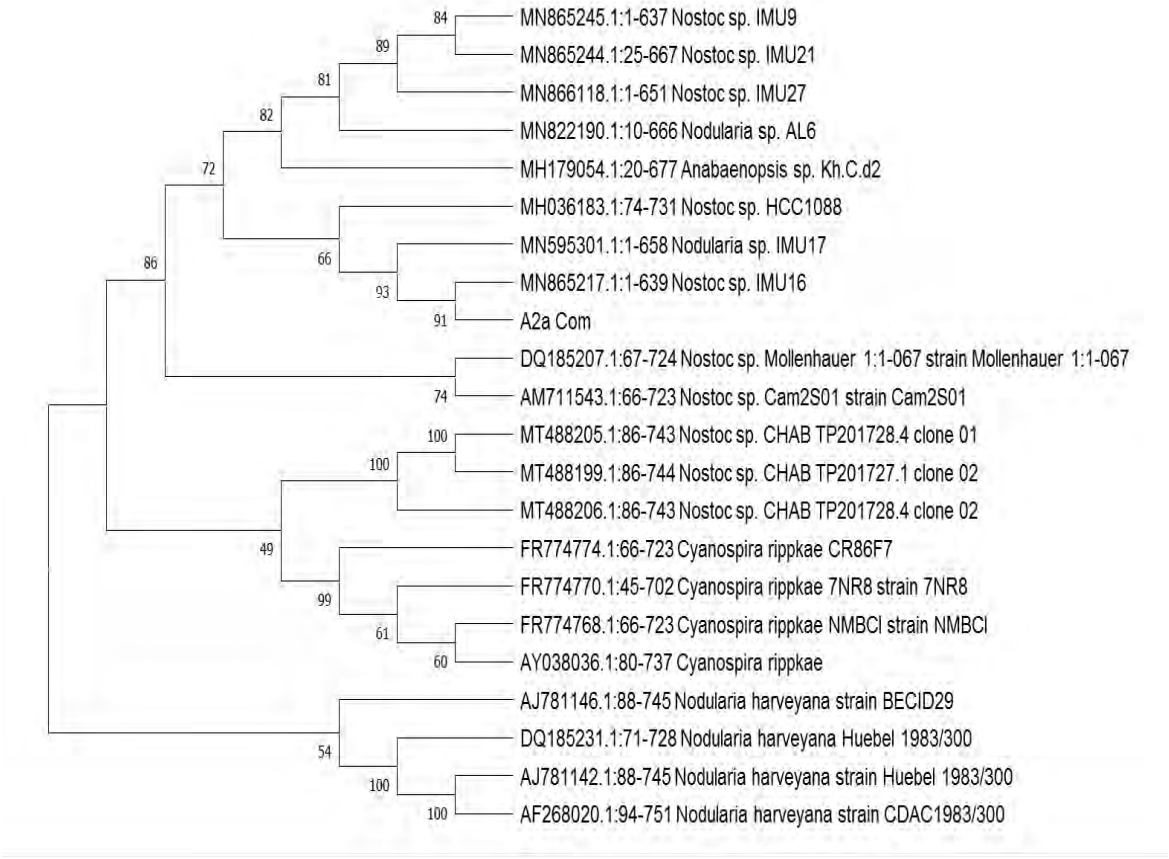


Fig. 3.5.9: Phylogenetic analysis of strain A2a Com based on neighbor joining (NJ) method.

3.5.1.5 *Nodularia* sp. A51T

Morphological Description: Trichomes are uniseriate, isopolar, short and unbranched. Vegetative cells are constricted, quadratic or longer than wide (5.8µm in width and 7.2µm in length) and contain aerotops. Heterocytes both intercalary (rare) and terminal (very frequent), usually spherical or sub-spherical to oblong (10.4-18.2µm in length and 10.4-15.6 in width) (Fig. 3.5.10).

Diagnostic Characters: Heterocysts at intercalary position not found; cell length only slightly greater than width; Akinetes form through merging of two adjacent vegetative cells.

Habitat and Site: Obtained from a drinking vat at site A5.

Culturing Conditions: Species responded both to BG_{11} and BG_0.

Genus originally described by: Thuret ex Bornet & Flahault

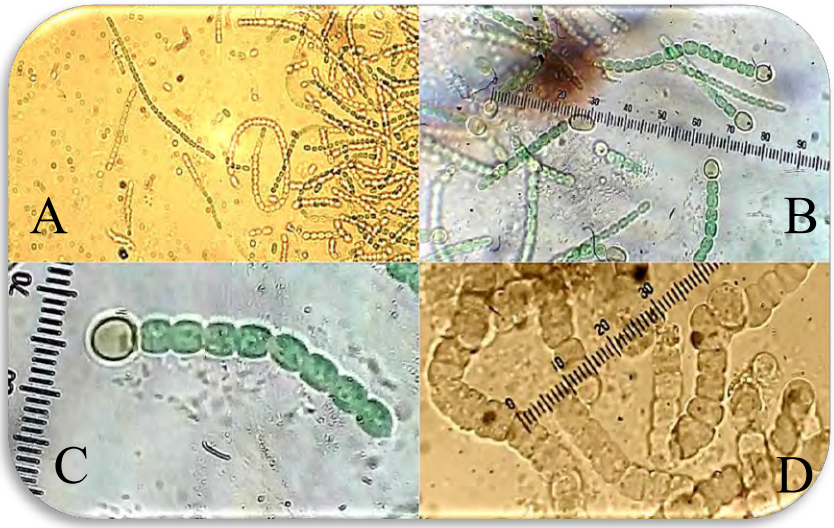


Fig. 3.5.10: A) Growth of filaments in liquid media B) Filaments showing terminal heterocysts; C) Eight cell(7.2µmx5.8µmLW) filaments with terminal heterocysts (10.4-18.2µmx10.4-15.6µm LW) only D) Reproductive stage with the development of Akinete.

Phylogenetical analysis

Phylogenetic analysis of strain using the NJ method revealed close clustering with *Nodularia* sp. IMU17 and *Nostoc* sp IMU16 with bootstrap values of 50 and 94 respectively. The Maximum Parsimony revealed similar topology by clustering with *Nodularia* sp. IMU17 with

a bootstrap value of 96; *Calothrix* and *Nostoc* sp. with bootstrap value of 85 and 86 respectively (Fig. 3.5.11).

Sequence, similarity, and query cover: Amplification of 16S ribosomal RNA gene revealed a sequence length of 581 bp. The NCBI blast analysis showed sequence similarity with *Nostoc* (99.03%), with *Anabenopsis* (98.87%) and with *Nodularia* species IMU17 (98.78%).

Fig. 3.5.11: Phylogenetic analysis strain A5/1T based on neighbor joining (NJ) method.

3.5.1.6 *Nodularia* sp. B14.

Morphological Description: Colonies not visible; Cells were compact or loosely compact, longer than broad, more prominent so at later stage of life cycle. Heterocysts were absent altogether. Akinete visible, develop through fusion of two adjacent cells, a trait also noted in case of A51T. A uniform pattern of Akinete development was observed at regular interval, through fusion of adjacent cells (Fig. 3.5.12).

Diagnostic characters: Sub-spherical cells, akinete developed by fusion of two adjacent cells.

Site and Habitat: pond site soil in pure desert environment.

Culturing conditions: Responded in BG_0 medium.

Genus originally described by: Thuret ex Bornet & Flahault

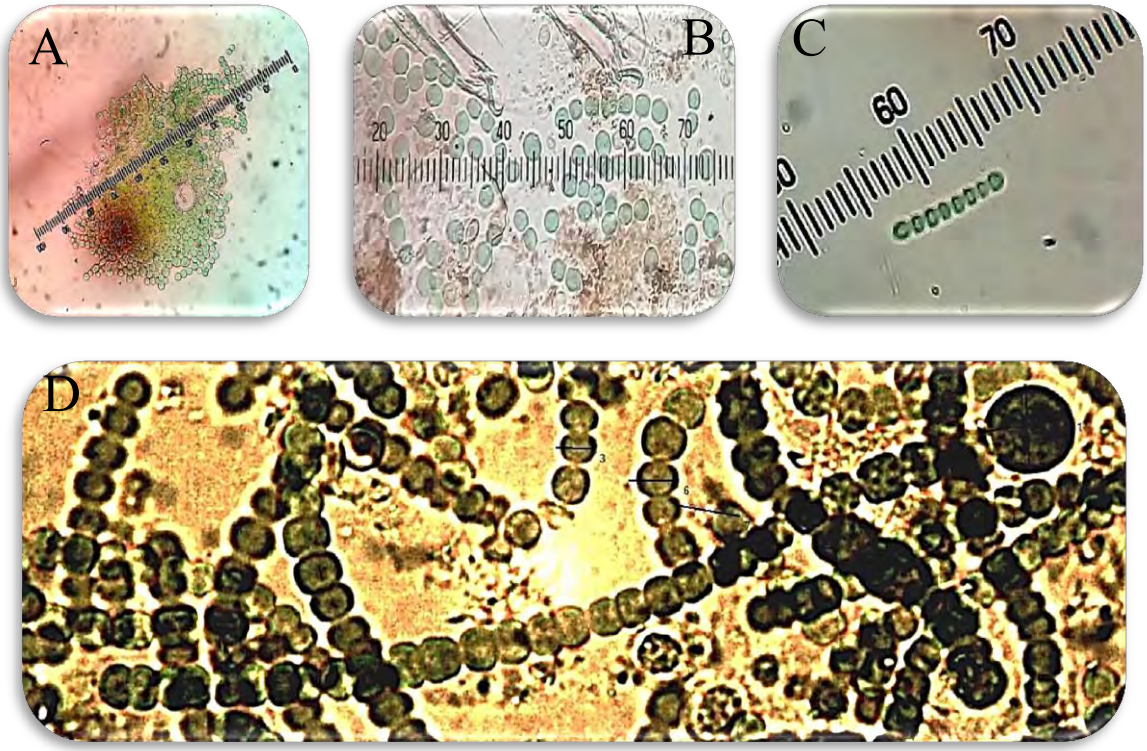


Fig. 3.5.12: A) Colonies of *Nodularia* with akinetes isolated from soil; B) developing akinete into short trichomes C & D) vegetative cells fused to form akinete

Phylogenetic Analysis: Phylogenetic analysis of strain B14 revealed clustering with *Nodularia* sp. IMU17 with a high bootstrap support of 98 which then clustered with *Nostoc-Anabaenopsis* (Fig. 3.5.13).

Sequence, similarity, and query cover: Sequence of 16S ribosomal RNA gene achieved a sequence length of 625 bp. The NCBI Blast analysis revealed top similarity matches with *Nodularia* sp IMU17 with pairwise similarity of 99.32%; *Anabaenopsis* with pairwise similarity of 96.32% and *Nostoc* sp. strains NIES-2098 with pairwise similarity of 95.69%.

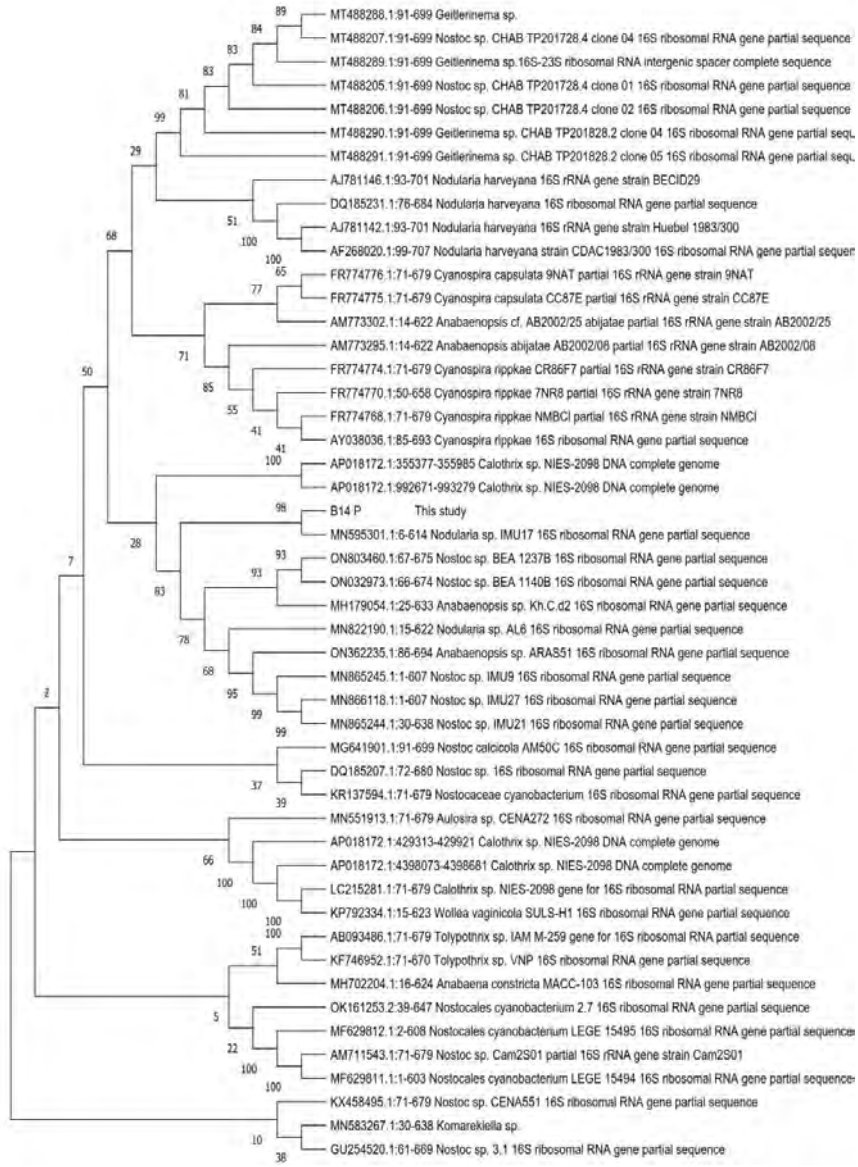


Fig. 3.5.13: Phylogenetic analysis strain B14 based on neighbor joining (NJ) method.

3.5.1.7 *Calothrix* sp. A2aR. (Family: Rivulariaceae)

Morphological Description: Colony in the form of tuft of filaments forming a solid mass, olive green in color, small hormogones i.e. cells with basal heterocyst radiating out from the main thallus. Heterocysts basal, 5µm in length and width. Cell adjacent to the heterocysts are discoid, 3µm in length and 7 µm in width. The cells gradually become tapered in the apical region but not hair like endings. End cell conical in shape, 3µm in length and 4 µm in width. Intercalary heterocyst present. It is the point where short filament grows and form pseudo-branching pattern. These short branches form short hormogones, which are liberated apically. These hormogones aggregate at their base to form a new colony. *Calothrix* also reproduce by spore formation. Spore is produced endogenously. When the filament becomes mature the protoplast of the filament condenses and transform into a spore. The spores are liberated from the basal portion of the filament by the disintegration of the heterocyst. The spore is spherical in shape, 8-12µm in length and width (Fig. 3.5.14).

Diagnostic Characters: Presence of heteropolar filaments with a wider basal part; Compared with the genus *Rivularia* which have the heteropolar filaments with a narrower base; Development of akinete just above the basal heterocysts; *Calothrix* and *Tolypothrix* both have firm sheath around, but *Calothrix* has colourless sheath while *Tolypothrix* has yellow to brown sheath; *Calothrix* has high degree of tapering cells towards the apex as compared to a less degree of tapering in *Tolypothrix*; Cell length of the reported species is 3µm as compared to 4.23µm.

Habitat and Site: This species (A2aR) was present in canal water along with *Scenedesmus*, species of *Nostoc* and *Leptolyngbya.*

Culturing Conditions: Responded in BG_0, under continuous illumination and anaerobic conditions.

Genus originally described by: G. H. Schwabe

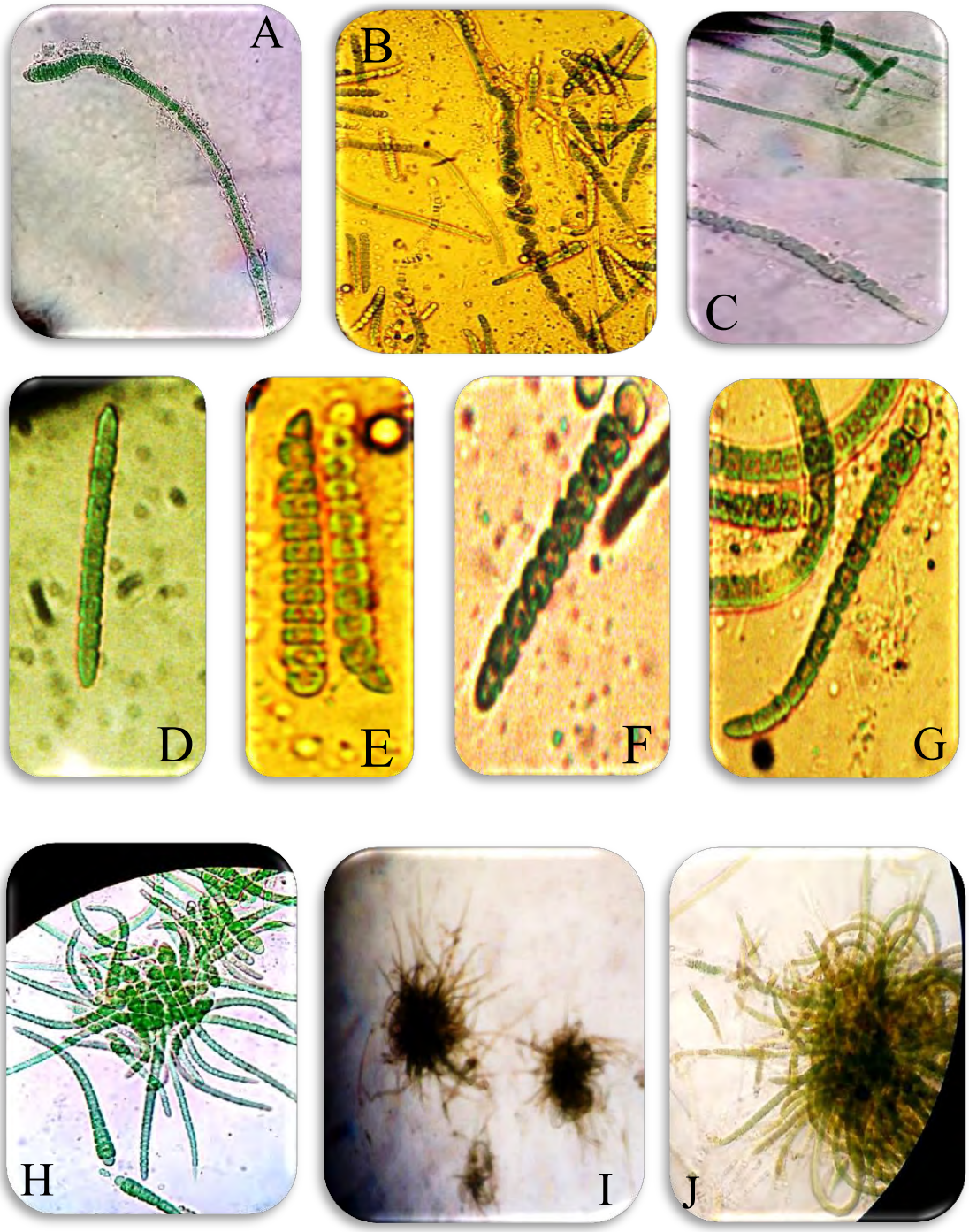


Fig. 3.5.14: (A) Filament grown full length with basal heterocyst (b) Short trichome separating from the main filament (c) small trichome with undifferentiated cells; d) differentiation of basal heterocyst and apical cell of conical shape; E) fully developed heterocyst(5µmLW) and conical cell at the apex; F) germinating trichome; G & H) Fully developed trichome aggregating to start forming colony (vegetative cells 3µmx7µmLW, 3µmx4µmLW; I & J) Developed colonies with emerging juvenile trichomes.

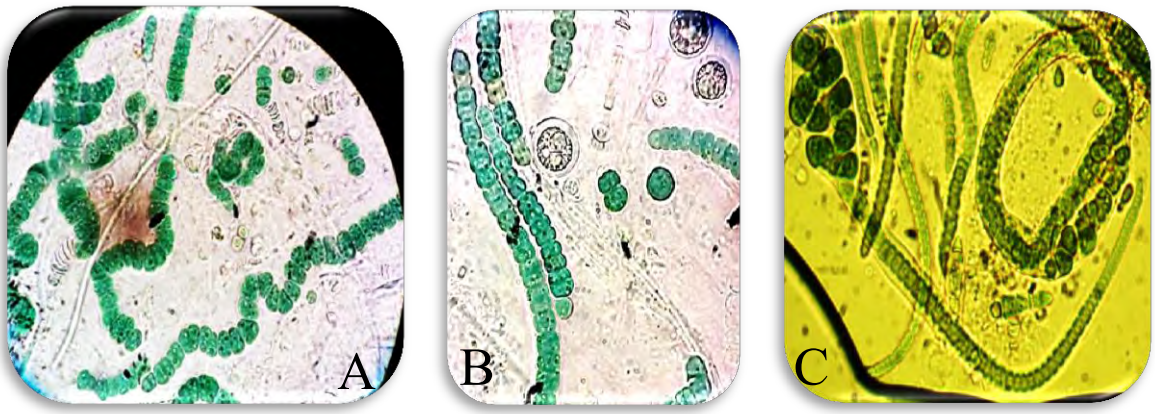


Fig. 3.5.15: (A) potentially all cells develop into akinetes (8µmx12µmLW). (B) All cell drift towards base and (C) Formation of Akinete and germination of Akinete to form Trichomes

Phylogenetic Analysis (A2aR): The Phylogenetic analysis of strain A2aR using Neighbor Joining method showed close clustering with *Calothrix desertica* clade including strains KSU-AQ1Q13, PCC7716, and HK06 and *Rivularia* IAM-M261. The M.L and M.P methods also revealed close clustering with two strains of PCC7716 with bootstrap value of 100 (Fig. 3.5.16).

Sequence, similarity, and query cover: Amplification of 16S ribosomal RNA gene revealed a sequence length of 419 bp. Top match in the NCBI blast analysis were: Calothrix p*arietina* sp and *Desertica* sp with 98.61% and 85% Query cover. *Desertica* sp showed 98.33% % identity with 99% query cover.

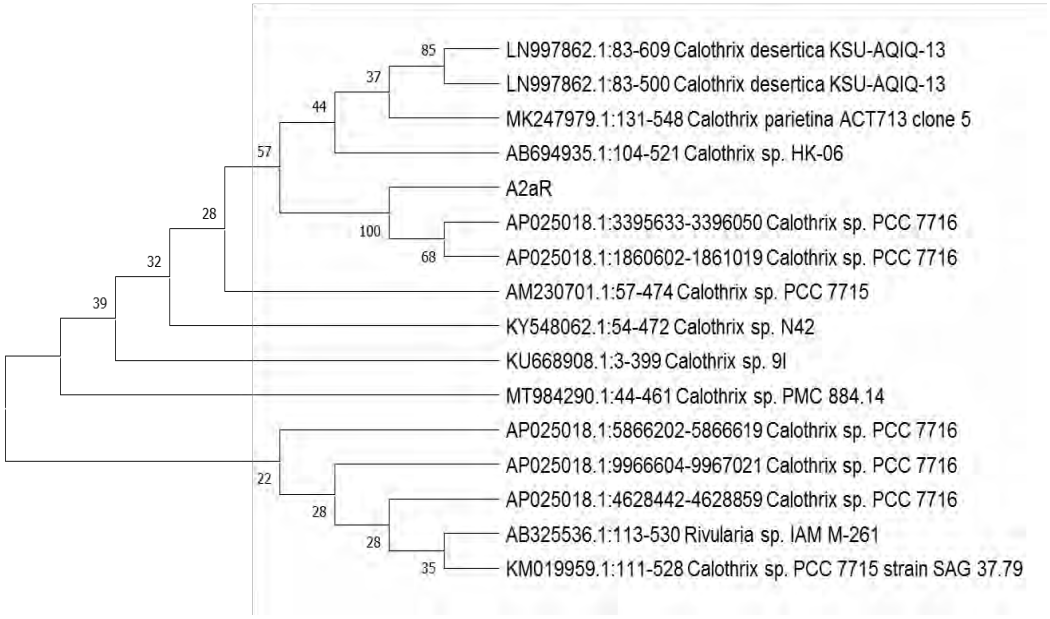


Fig. 3.5.16: Phylogenetic analysis strain A2aR based on neighbor joining (NJ) method.

3.5.1.8 *Tolypothrix* sp. A8 (Family: Tolypothrichaceae)

Morphological description: Colony appeared as tiny brownish to black spots on the BG_0 solid agar media, as the colony grew in size, the air bubbles began to develop within the colony. In the BG_0 liquid medium, the colonies grew in size with large air bubbles inside (Fig. 3.5.17). Filaments embedded in thick mucilage, visible only when ink was applied. Filaments are olive green to dirty green in colour. Cells oval to barrel shaped and highly granulated, 6.5µm wide. Heterocyst oval to sub-spherical in shape, olive green in colour when young and became transparent at a later stage, 5.1µm wide. Akinete is barrel to oval in shape, 10µm in length and 8µm in width (Fig. 3.5.17 & 3.5.18).

Diagnostic characters: Presence of cells with uniform width in filament with conical cells at the apex; presence of thick mucilage around trichomes; potential development of all vegetative cells into akinetes even before emerging from the parent filament.

Habitat and Site: Dry bio-crust along a pond site A8.

Culturing conditions: Species responded in BG_0 medium with continuous illumination.

Genus originally described by: Kützing ex Bornet & Flahault

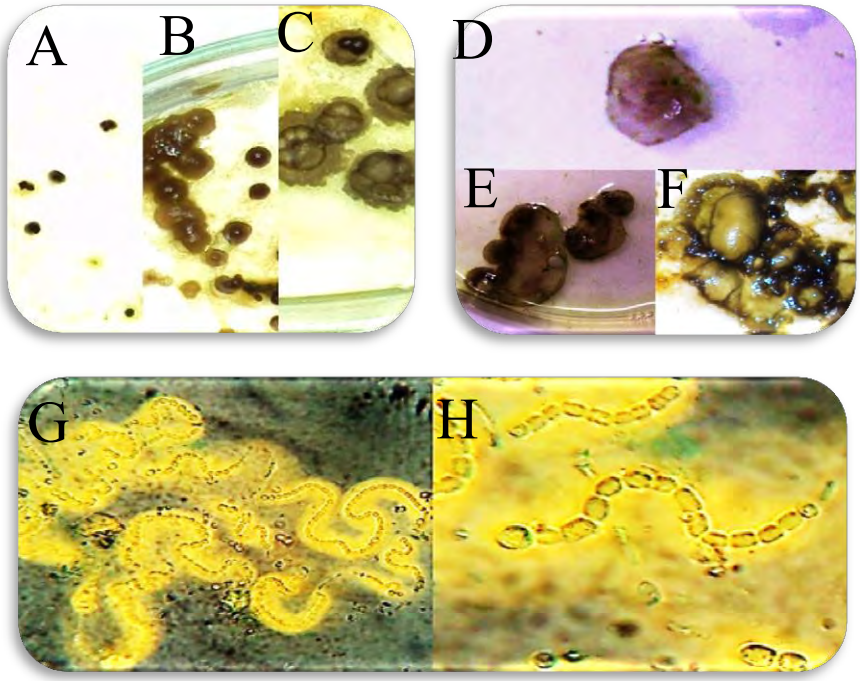


Fig. 3.5.17: (A), (B)and (C) Development and enlargement of colonies in gradual manner (D), (E) and (F) growth of colony (G) filaments of *Tolypothrix* sp with thick mucilage (enhanced through treatment with indian ink) (H) individual filament showing heteropolarity.(5.1µmW vegetative cells While 6.5µm wide heterocyst).

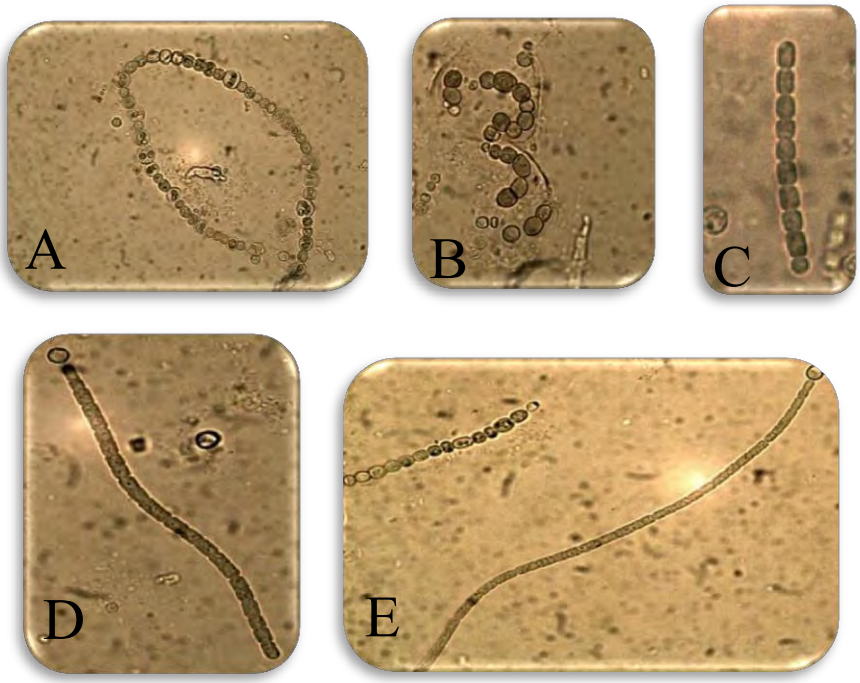


Fig. 3.5.18: Reproductive cycle with different Stages (A) Chain of Akinetes with the development of short trichomes with in the akinetes.(10µmx8µmLW) (B) trichomes soon after liberation from akinete develop again into akinetes (C) and (D) Akinetes develop into short trichomes. (E) trichome change into filament with the development of basal heteocysts.

Phylogenetical Analysis (*Tolypothrix* sp A8): Phylogenetic analysis of strain A8 using NJ method revealed clustering with ***Tolypothrix*** sp OF-23 with a bootstrap value of 59. This small cluster forms a sister clade with *Calothrix* sp NIES-2100 etc. with bootstrap value of 35. The Maximum likelihood method also revealed close clustering with *Tolypothrix* sp. OF-23 with a bootstrap value of 53 and with *Tolypothrix* sp 0F23 with bootstrap value of 44 (Fig. 3.5.19).

Sequence, similarity and query cover: Amplification of 16S ribosomal RNA gene generated a sequence of 581 bp. The NCBI blast analysis showed 97.08%, 95.73%, percentage similarity with *Tolypothrix* and *Calothrix* respectively.

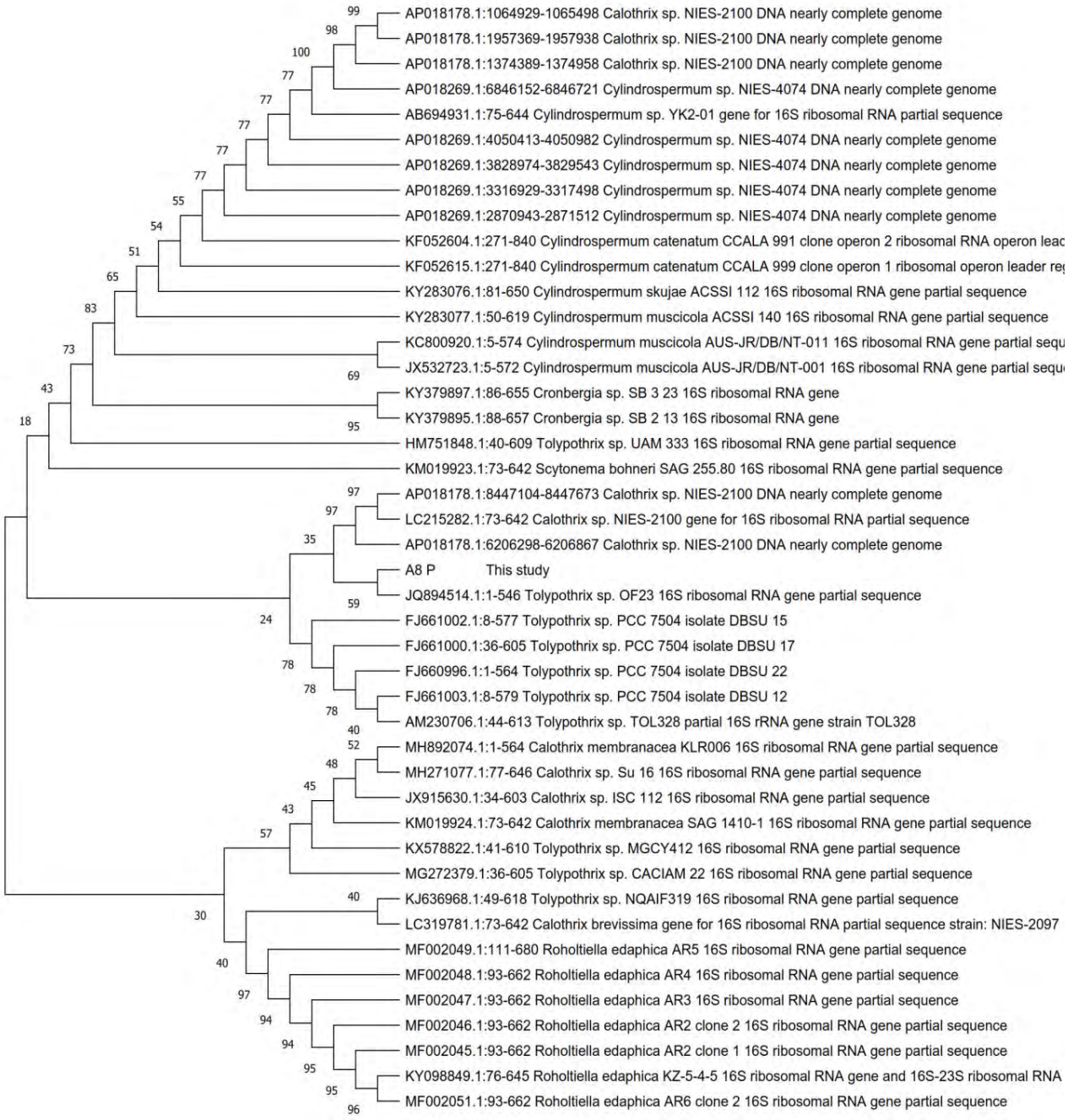


Fig. 3.5.19: Phylogenetic analysis strain A8 based on neighbor joining (NJ) method.

3.5.1.9 *Neowestiellopsis* sp. (Family: Haplosiphonaceae)

Morphological Description: Colony morphology: The strain B-10 showed smaller size colonies when cultured on $BG11_0$ agar medium, however, prolific growth was seen in broth (Fig. 3.5.20). Growth of main filaments was visible in the central part depicted by darker regions while the lateral branches radiate out from the center. On the contrary, the *in vitro* culturing of strain A-44 was sporadic in $BG11_0$ agar medium with smaller dark blue green colonies, and a loose growth was noticed in the broth (Fig. 3.5.20).

The filaments: Imaging though microscope revealed unilateral arrangement of branches in strains isolated from site B-10. This character was unique for this strain, thus profuse T-type branching pattern was evident with lateral branches often originating from consecutively adjacent cells (Figs. 3.5.21 and 3.5.22). The primary filaments comprised of single layer of cells in the filament (described as uniseriate); the cells were usually broad compared to the secondary branches with much narrower cells. The secondary branches were tapering and erect. On the contrary, the secondary branches in the strain A-44 were clearly bilateral originating from the primary filaments (Fig. 3.5.22). The heterocysts: In the strain of B-10 the heterocysts were irregularly shaped and loosely square to spherical. Hormocytes were also observed. On the contrary, the strain A-44 only grew vegetatively, producing spores in cells within the filament, despite maintaining optimum culturing conditions even for much longer time, it did not produce hormocytes.

Diagnostic characters: The bi-seriate arrangement of cells in the main filament and the branching pattern were indeed important diagnostic characters that distinguished both strains (Figs. 3.5.21 and 3.5.22).

Genus originally described by: Kabirnataj, 2018

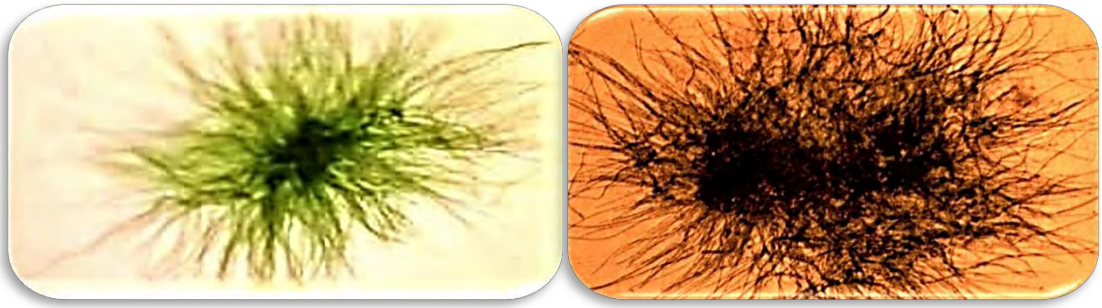


Fig. 3.5.20: Comparison of colony morphology obtain on Agar-based BG_0 medium; the strain of site B-10 (on left side); strain derived from the site A-44 (on right side).

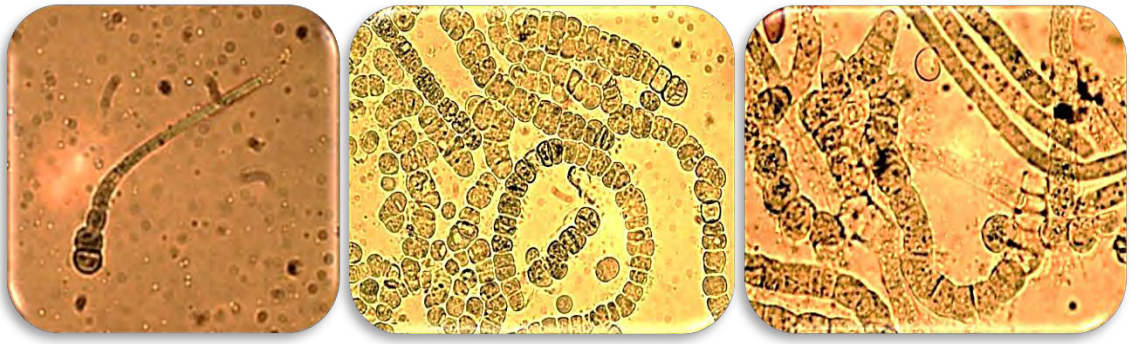


Fig 3.5.21: Life stages for strain derived from B-10 site. a) germination of tapering filament; b) the uniseriate and biseriate primary filaments with intercalary heterocyst; c) mature filaments with sparse growth of lateral branches.

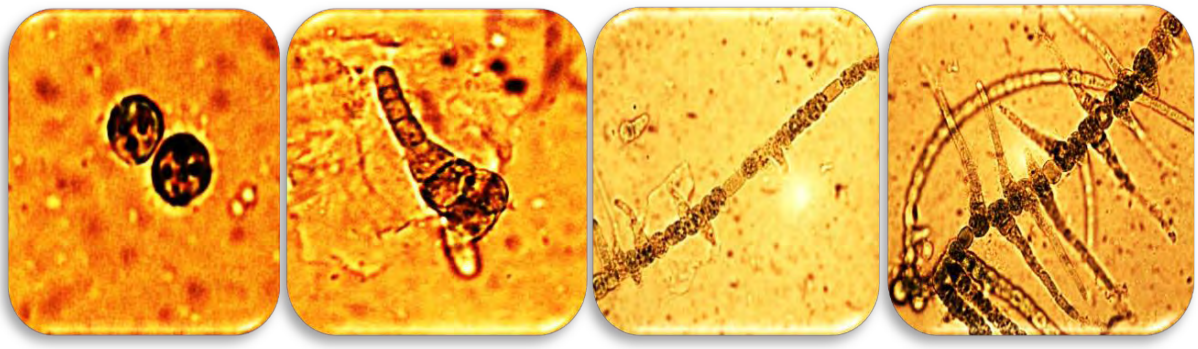


Fig. 3.5.22: Life stages for *Neowestiellopsis* strain derived from site A-44. a) Initial spore stage; b) germination of spore into a filament; c) Uniseriate primary filament with clearly visible heterocysts; d) Profuse growth of secondary branches on both sides of primary filaments.

Phylogenetic analysis: The neighbor joining (Nj) analysis of B-10 (OM831953) showed its close clustering within the *Neowestiellopsis* clade especially with the *Neowestiellopsis persica* SA33, with a bootstrap support of 93. The phylogeny based on neighbor joining also revealed clustering of A-44 (OM831955) with the *Neowestiellopsis* clade; including *N. persica* and *N. bilateralis,* with high bootstrap support (Fig. 3.5.23).

Sequence, similarity, and query cover: The similarity search for both strains at NCBI revealed 99.84% similarity with *Neowestiellopsis bilateralis* SA16 (MF066911), *Hapalosiphone* sp. SAG2376 (MK963008.1) and *Fischerella* sp. F29 (MG385060.1).

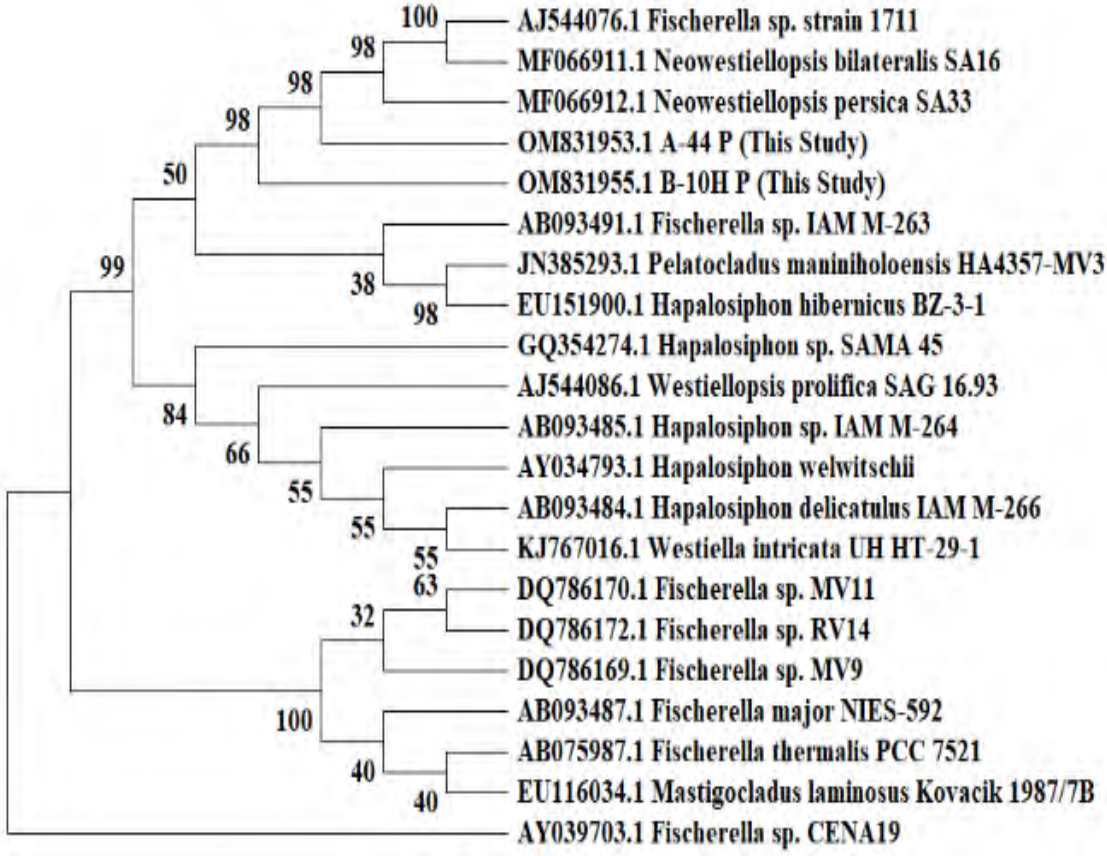


Fig. 3.5.23: Phylogenetic inference based on 16S rRNA gene data The information on branches showed bootstrap support.

3.5.2 The Oscilatoriales

3.5.2.1 *Desertifilum tharenseA5/1b* (Family: Desertifilaceae)

Thallus thin, bright blue green forming lumps in broth, filaments mostly densely entangled, varying in length, motility present, sheath diffluent, colorless, attached to trichome, slightly constricted at the cross wall, 2µm wide and 4µm in length. Cells cylindrical, slightly elongated and attenuated end cell. Cell content amphorus, gas vesicles absent (Fig. 3.5.24).

Diagnostic characters: Thin filaments with less than 1um width

Site details and Habitat: Soil sample obtained from site A5/1b, with filamentous growth

Culturing conditions: Cultured in SP medium.

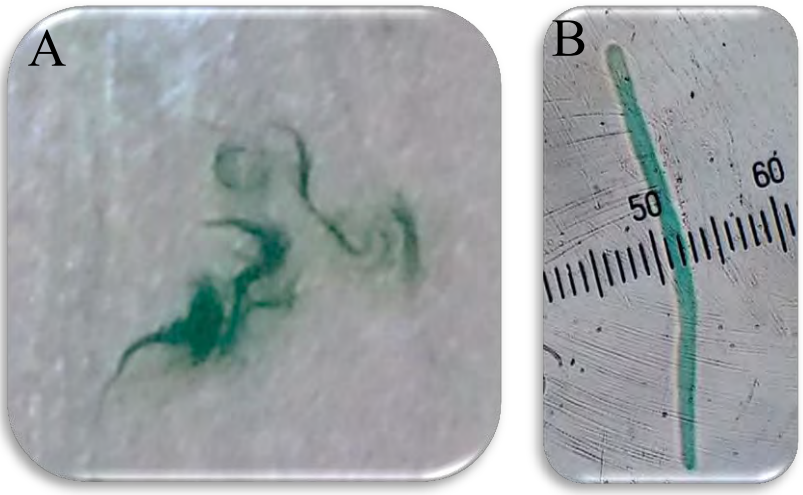


Fig. 3.5.24: A) Aggregating filaments; **B**) individual filament showing partial constrictions. Vegetative cells (4µmx2µm LW)

Phylogenetic Analysis: Phylogenetic analysis of strain under study A51b using NJ method showed close clustering with two strains of *Desertifilum* sp. (*D. tharense* MAR314) with bootstrap value of 92. The sister clades also have *D. tharense* species IPPAS-B- 220. The Maximum likelihood method also revealed similar clustering pattern (Fig. 3.5.25).

Sequence, similarity and query cover: Amplification of 16S ribosomal RNA gene revealed a sequence length of 610 bp. The NCBI blast analysis of *A51b* showed top match with the

Desertifilum sp. NapGtcm17 *Desertifilum* sp. IPPAS-B-1220(KU556389.1) and D. sp IPPAS-B-1220. The percentage identity was 99.35% and query cover was 99% in each case.

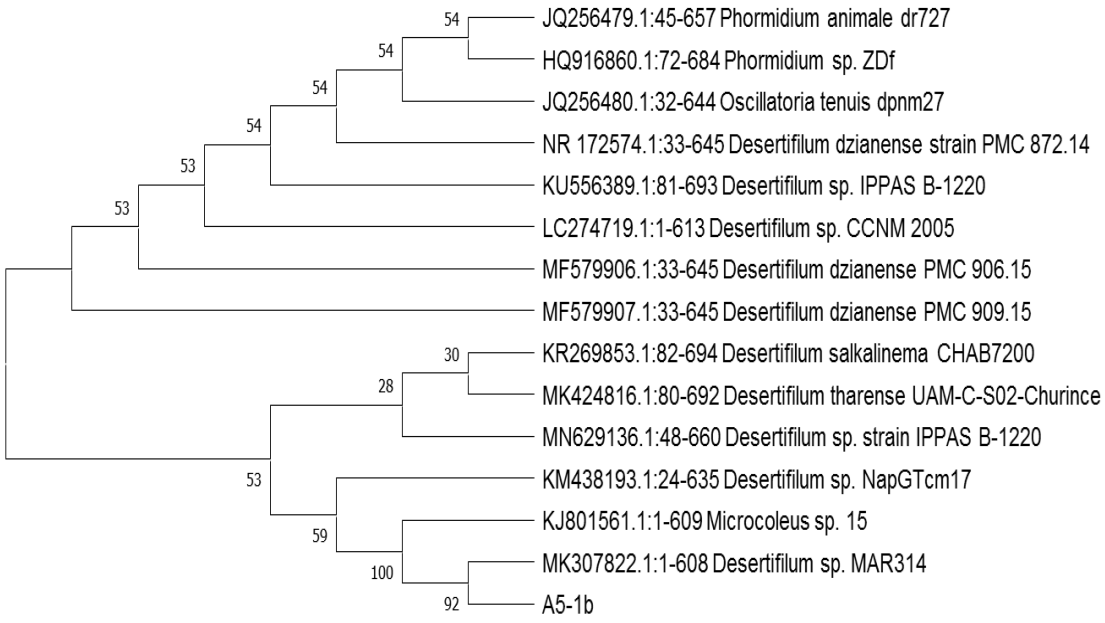


Fig. 3.5.25: Phylogenetic analysis strain A5/1b based on neighbor joining (NJ) method.

3.5.2.2 *Desertifilum tharense*: A5/1 C

Filaments are motile, unbranched, long and embedded in common mucilage. Hormogones with heavily granulated contents. Sheath is colorless. Ends are straight and attenuated. End cells are conical and slightly bended in some filaments. Contents of cell is granulated. Cell width is 4µm. Filament color is light green. Thallus color is blue green (Fig. 3.5.26).

Diagnostic characters: Hormogones with aerotops

Site details and Habitat: Obtained from site A5

Culturing conditions: Purified and isolated in SP medium

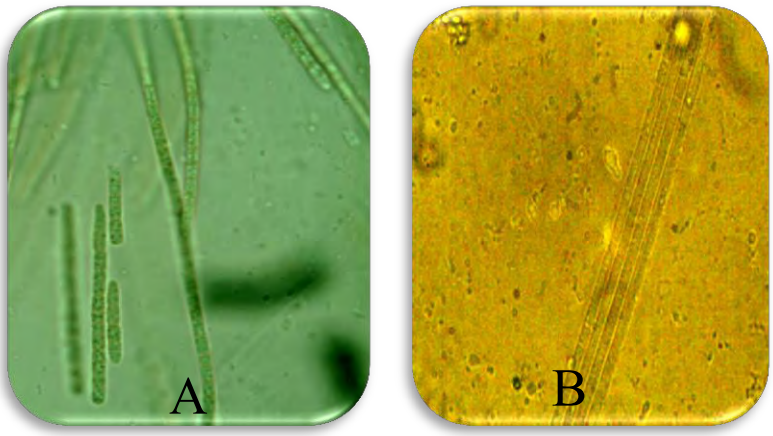


Fig. 3.5.26: A5/1C) hormogones with aerotops. These hormogones are different from A51b (absence of aerotops in A51b) and presence of achlorphyllous cells B. Cells (4µm W) are parallel arranged and not interwoven, however showing difference in cell constriction (fully constricted)

Phylogenetic analysis of strain under study showed close clustering with *Oscillatoria* sp., *O. earlei* strain NRMC-F0140, *Kamptonema cortianum* NRMCF-0138, *Getilerinema splendidum* NRMCF-0141, *Phormidum acula* NRMCF48, *Oscillatoria lunis* NTAPD02 etc. while the sister clade included strains of *Oscillatoria*, *Geitlorinema* and *Desertifilum* sp with bootstrap value of 40 as compared to *D. tharence* UAM-C with a bootstrap value of 41. The maximum likelihood analysis of strain under study A51C showed clustering with the clade including *Oscillatoria amoena* NRMCF-0135, O. sp50A, *O.tanuis* M4, D.spUPMC-A0092, *Phornidium* animale PMC239-04, *O.tenuis* NTApD02 *D.clzianense* PMC, D.tharense UAM-C showed clustering with strain under study A51C with Maximum bootstrap value of 57.

Sequence, similarity, and query cover: The NCBI blast data showed percentage identity of strain under study with two species of *Kamptonema animale* NTLRM10 and *Cortianum* NRMC-F83); species of *Oscillatoria* (O.amoena NRMC-F-0135, *O. tenuis* NATPD02 and O. tenuis M4), three species of *Phormidium* (sp CBC, *P. animale* M8 and *P. animale* PMC239.04). One species each one of *Desertifilum* spUPMC- A0092 and Geitlermema

calcuttense NRMC-F83. The percentage identity were 100% and last three strains were 99%, 99% and 97% respectively.

3.5.2.3 *Desertifilum tharense* A51/D

Morphological description: Filaments are unbranched and motile. Hormogones present in mucilage. First cell of the hormogone is large in length, 10µm in length as compared to 3µm of normal cell. Sheath is diffluent. End cells are rounded. Content of cell is homogenus. Cell width is 4µm. Cell length is variable. Filament color is pale olive green. Thallus color is dark green (Fig. 3.5.27)

Diagnostic characters: Slightly less constricted at cross walls as compared to strains A5/b and A5/C strains.

Site details and Habitat: Same as for A5/b and A5/C

Culturing conditions: Purified in SP medium

Genus originally described by: P. K. Dadheech & L. Krienitz, 2012

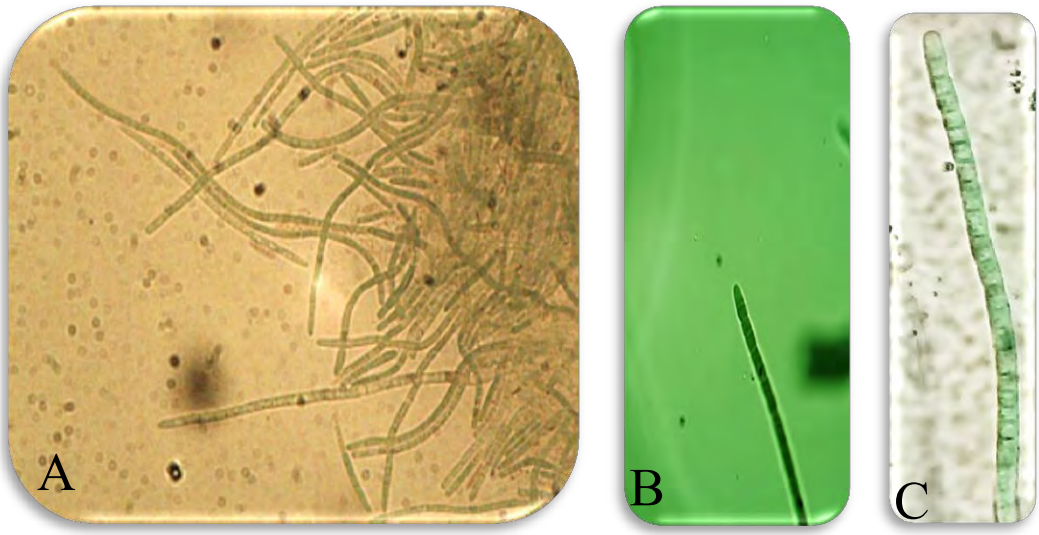


Fig. 3.5.27: A) Mature filaments of *Desertifilum* strain B) hormogone; C) Individual filament with slight constrictions. Vegetative cells 3µmx4µmLW.

Phylogenetic analysis of strain under study A51D showed close clustering with the *Desertifilum* clade, including *Desertifilum tharense* UAMC-S02, *D. tharense* NTLRM5,

D.tharense PD2001/TDC4, *D. tharense* Kh eb. *D. tharense* EA01, EA04, *Desertifilum* sp. The likewise Maximum analysis showed same results. Maximum Parsimony method analysis showed close clustering of strain under study with *D. tharense* (EA04 and NTLRM5) and D.sp NAGTcmm17 with bootstrap value of 97 in each case (Fig. 3.5.28 & Fig. 3.5.29).

Sequence, similarity, and query cover: On NCBI data, percentage identity of strain under study showed similarity with *Desertifilum tharense* (EA04, EA01, Kheb, PD2001/TDCP4, UTMC-502). The percentage identity was 99.81% in first four strains while the last strain showed 99.62% with 100% query cover in all. The *Desertifilum* strains (EAZ03, IPPAS-B-1220 and MAR314CO22801, L. sp Doroninskoye, L. valderiana, BEA1439B, L. sp CY-021 and L. sp CY-073 with 99.05% identity and 100% query cover.

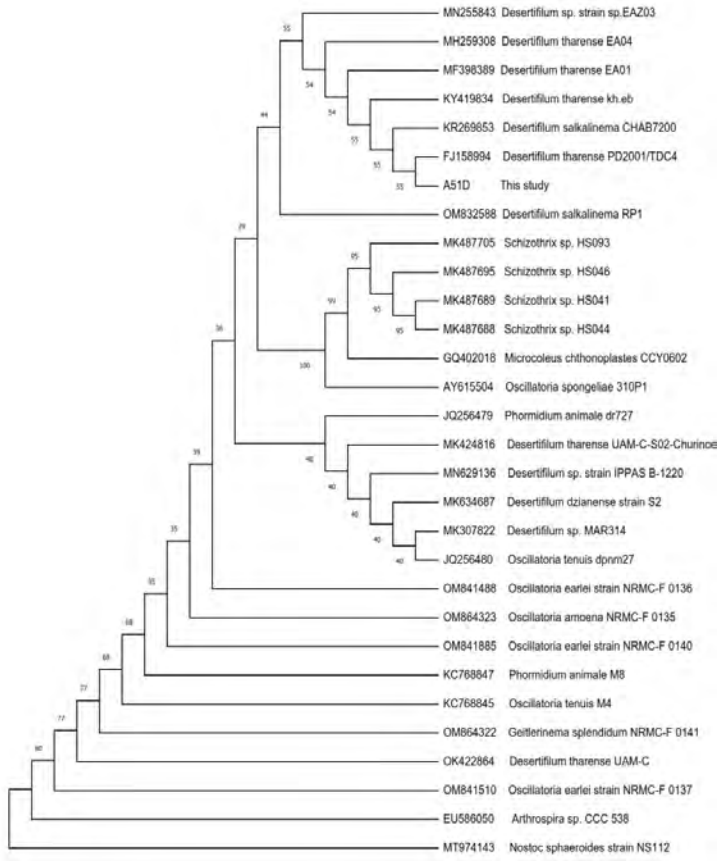


Fig. 3.5.28: Phylogenetic tree based on 16S rRNA data of D strain isolated from site A5/1. The bootstrap values less than 50 are ignorable and show unreliable clustering.

Combined phylogenetic Analysis for A5/1b; /1C; /1D

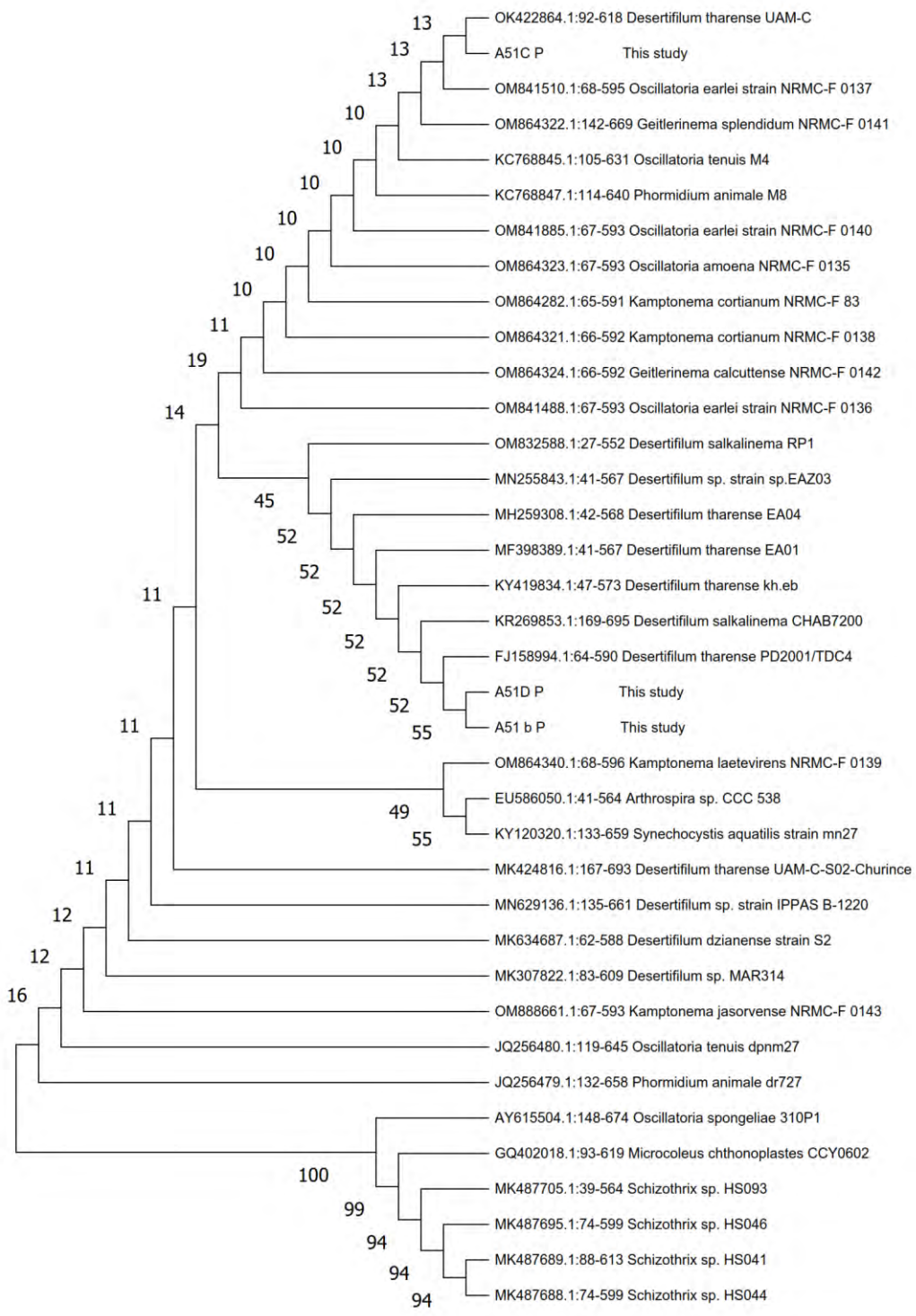


Fig. 3.5.29: Phylogenetic analysis strains A5/1b; A5/1C; A5/1D based on neighbor joining (NJ) method.

3.5.2.4 *Klisinema persicum* B7/O(Family: Phormidiaceae)

Morphological Description: Trichomes are always solitary, 3 - 5 µm in width, blue green in colour; mostly straight, long and without sheaths, intensely motile; Constrictions absent or only slightly present. Cells isodiametric or more long than wide; 3 - 5 µm in length. Terminal cell conical and quite elongated (Fig. 3.5.30).

Diagnostic characters: Pointed hook like end

Site details and Habitat: This strain was recovered from (B17) an aerial sample obtained from a wall in ruins.

Culturing conditions: Purified in BG_{11} medium.

Genus originally described by: Heidari & Hauer 2018

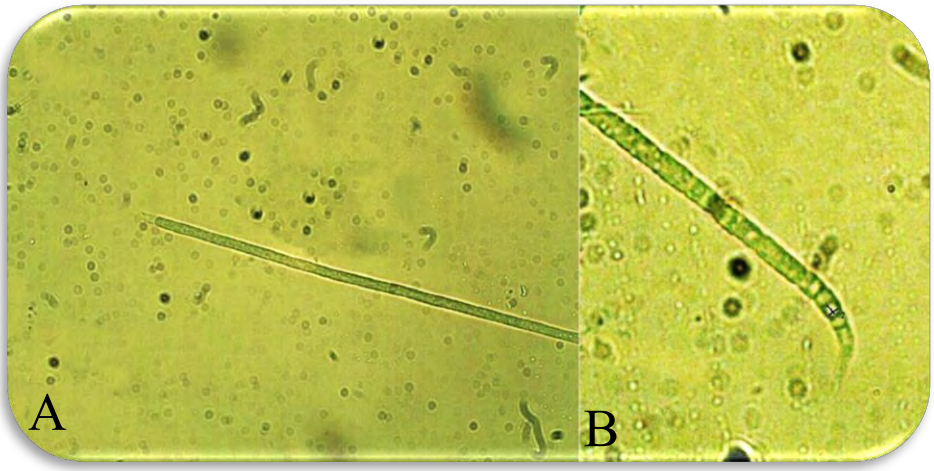


Fig. 3.5.30 A) Small size filaments having sheath (similar to Oscillatoria) on one end; B) Filament showing Oscillatoria like single filaments, hooked with elongated tip.
The vegetative cells are 3µmx5µm in LW.

Phylogenetic analysis of strain B7 using NJ method showed close clustering with the *Klisinema persicum* including strain HA7 with bootstrap value of 92. Other strains included in this clade were *K. persicum* SHAFAS10; *Geitlerinema* sp ZD; *K. persicum* cl2; *Geitlerinema* sp PUPCC 110.6 (Fig. 3.5.31).

Sequence, similarity, and query cover: The NCBI blast data revealed similarities between the strain and *Geitlerinema* sp PUPCCC1106, *Klisinema persicum* SHAFAS10 clone cl2, *K.persicum* SHAFAS10 clone cl1, *Geitlerinema* sp ZD persieum, *Klisinema persicum* SHAFAs10 and clone cl3, K. persie 99.32 percent, 99.15 percent, and 99.99 percent, with high (99) query cover.

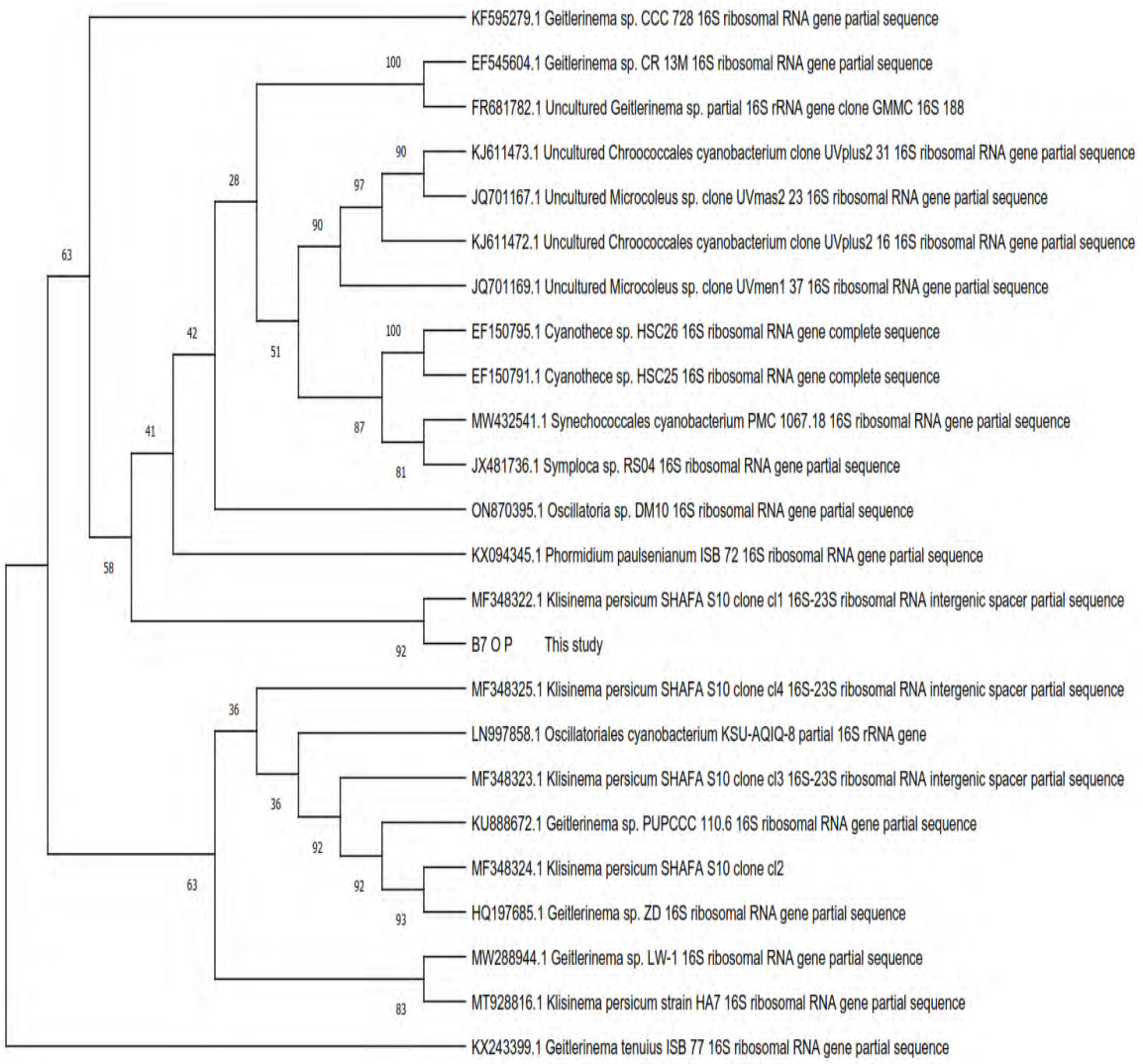


Fig. 3.5.31: Phylogenetic analysis strain B7/O based on neighbor joining (NJ) method.

3.5.3 The Synechocales

3.5.3.1 *Leptolyngbya valderiana* B1Sp (Family: Leptolyngbyaceae)

Morphological description: Thallus dull green to yellow green, rarely blue-green, lubricous, expanded, firm, leathery, slippery, up to 3 cm thick. Filaments variously curved, flexuous, densely packed and entangled. Sheath thin, firm, distinct or diffluent, mucilaginous. Trichomes pale to bright blue green, not or somehow constricted at the cross-walls, not attenuated at the ends, straight. Cells isodiametric, or thrice as long as wide, 1.6-2.5 µm wide, 2.5-8 µm long; cell content homogeneous, with one or two granules on either side of cross walls, rarely without granules. Apical cells rounded, sometimes hemispherical, not capitate, without calyptra or thickened outer cell wall (Fig. 3.5.32).

Diagnostic characters: Prominent sheath; in *Lygnbya* group their size is smallest; morphologically these are not diverse in nature

Site details and Habitat: **(B1/Sp)** Agricultural site next to human settlement.

Culturing conditions: Purified in SP medium.

Genus originally described by: (Gomont) Anagnostidis & Komárek 1988

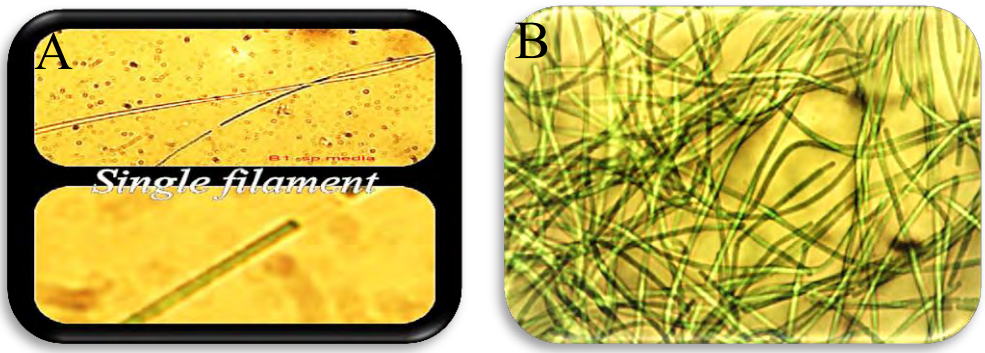


Figure 3.5.32: A) The small size **(**>1 µm) filaments with sheath; **B)** Densely entangled filaments.

Phylogenetic analysis of the strain B1sp using NJ method revealed close clustering with *Sodaleptolyngbya stromatolitin* strain PMC 867 with a bootstrap value of 81, while the sister clade revealed two strains of *Kammptonema okenu* (SABC011902 and WUC1509) with a bootstrap value of 80 and *Leptolyngbya* sp (CY013, CY011) *Lyngbya* sp. (CY-021) *Leptolyngbya valderiana* (BEA 1439B and SABC 022801) *Anagonistidnema* amphibian ISB 67 and *Toxifilum* sp. The Max likelihood and Maximum Parsimony analyses revealed the similar topology with a strong bootstrap support of 86 and 90 (Fig. 3.5.33).

Sequence, similarity and query cover: The NCBI blast analysis revealed a 99.29% similarity with *Sodaleptolyngbya stromatolitii* PMC867.14, with a query cover of 100%. The two other close matches were: *Keptonema okenii* strains, WUC1509 and SABCO11092, with 99.29 percent percentage identity and 100 percent query cover.

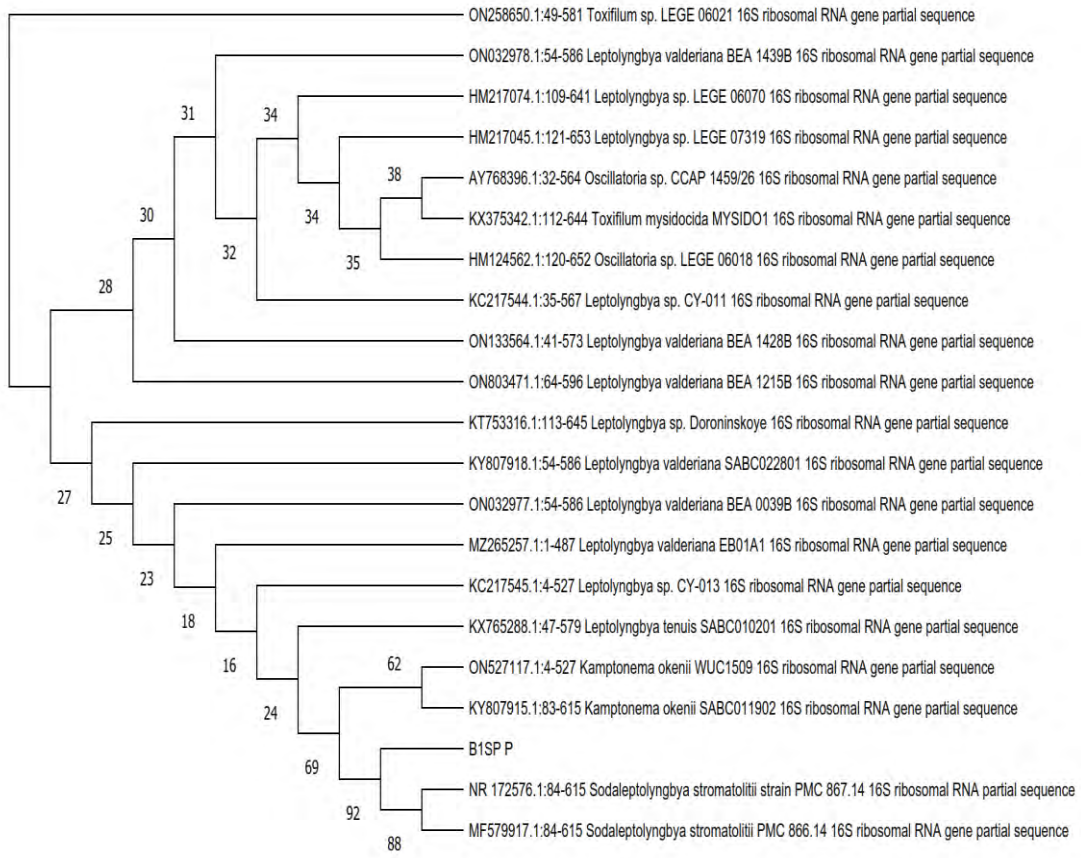


Fig. 3.5.33: Phylogenetic analysis strain B1sp based on neighbor joining (NJ) method.

3.5.3.2 *Leptolyngbya* sp. B15/N(Family: Leptolyngbyaceae)

Morphological Description: Trichomes solitary or arranged in colony (mats), a thin sheath present, usually colourless. Individual trichomes straight or slightly bent, normally constricted at crosswalls; 75 µm in length with 2 to 34 cells long. Cells blue green, with distinctive chromatoplasma, polar aerotopes usually present, cells connected with hyaline bridges, cells with small granules, 1 - 3 µm wide and 1 - 9 (to 12) µm long, cell length significantly varies within filament. Terminal cells often more long than broad; up to 12 µm in length, pointed, conical, or rounded. Reproduction occurs through hormogones (Fig. 3.5.34).

Diagnostic characters: Cell constrictions are clear, uniform appearance of filaments due to the presence of sheath. Presence of air bubbles (aerotops) at both ends of filaments. These differ from *Pseudaleptolyngbya* by having less wider cells and prominent sheath. This species is derived from genus *Pseudoanabaena*, a genus identified by the presence of aerotopes present on both terminal positions of the filament. These differ from *Pseudaleptolyngbya* by having less wider cells and prominent sheath.

Site details and Habitat: This species was isolated from (B15) site; from fresh water source.

At the site it was present with species of *Anabaenopsis,* and *Nodularia harveyana.*

Culturing conditions: Purified in SP medium.

Genus originally described by: (Gomont) Anagnostidis & Komárek 1988

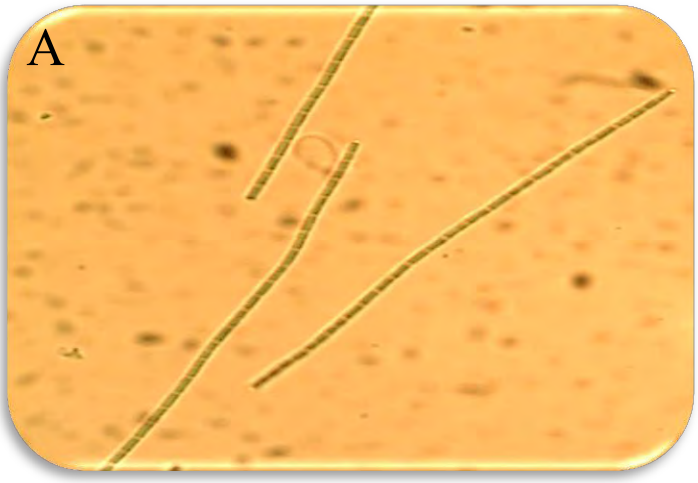


Figure 3.5.34: A) Morphology of filament. Cell constrictions are clear, uniformity due to the presence of sheath. Presence of air bubbles (aerotops) at both ends of a filament. Vegetative cells are 1-9µmx1-3µm in LW.

Phylogenetic analysis of the strain B15P using the NJ, Maximum Likelihood and Maximum Parsimony analyses revealed its closely proximity with *Leptolyngbya* sp. OF29, ISC-83 and NK-1 18, but with a low bootstrap support (value of 32) (Fig. 3.5.35).

Sequence, similarity, and query cover: The strain under study showed 99.80 percent pairwise similarity with *Leptolyngbya* sp. UIC 10065 with 94.29 percent and 93.15 percent similarity.

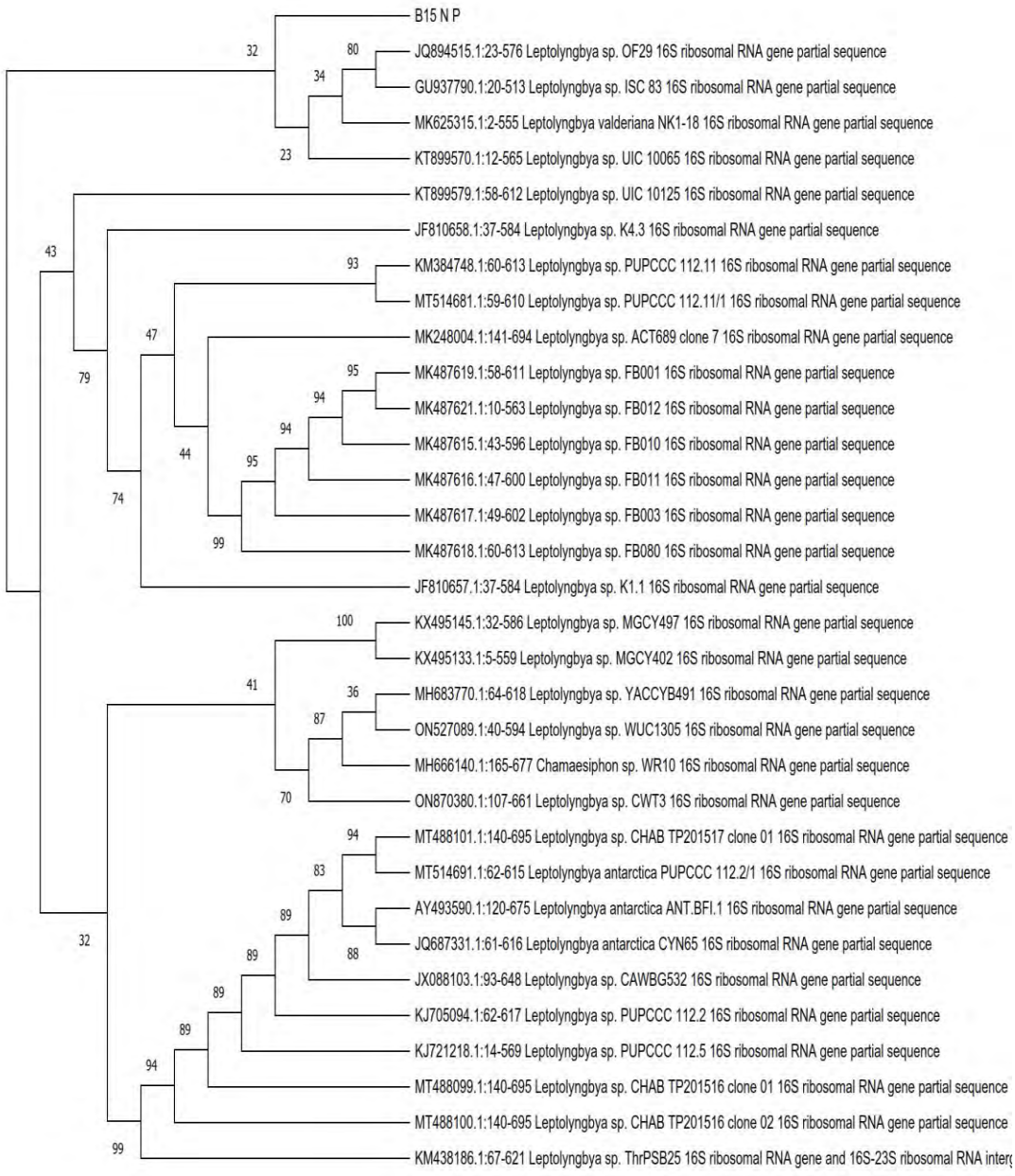


Fig. 3.5.35: Phylogenetic analysis strain B15N based on neighbor joining (NJ) method.

3.6: Biological screening and the *in vitro* bioactivity potential assessment of cyanobacteria

3.6.1 Antimicrobial activity

The antimicrobial efficacy of the extracts of eight cyanobacterial species/strains was assessed against three test bacteria including: *Pseudomonas*, *Klebsiella,* and *Streptococcus aureus* (Figure 3.6.1). It is important to mention that a fourth bacterial species: *Bacillus* was also used for this analysis. However, none of the extracts was found active against the *Bacillus* strain; hence those results were ignored in the present analysis. The extracts of seven species/strains showed inhibition against *Klebsiella* while six showed inhibition again the rest of two bacterial species.

The maximum activity was recorded as a holozone of 18mm for *Neowestiellopsis* sp. against *Pseudomonas*, while *Pseudoanabaena* showed least activity against *Klebsiella* sp. depicted as by a zone of 8mm in diameter. Among the strains assessed for antibacterial activity, *Neowestiellopsis* sp. *Neowestiellopsis persica* and *Pseudoanabaena* showed activities again all three bacterial test strains. Some, for instance *Klisinema*, *Aliinostoc* and *Nostoc* sp. showed activity against one of the three bacteria strains tested. Intriguingly, *Neowestiellopsis bilateralis* did not show activity against any of the tested bacterial strains.

The qualitative *in vitro* analysis has been shown in Figure 3.6.1 and a comparative data presentation is made in Table 3.6.1 and Fig. 3.6.2.

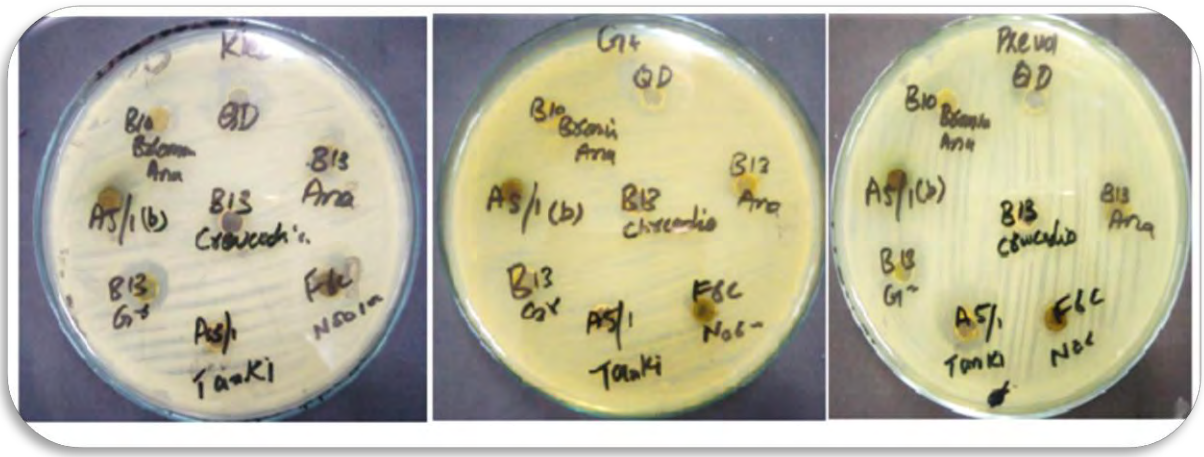


Figure 3.6.1: Qualitative analysis of antibacterial activity illustrated as holo-zones when subjected to the methanolic extracts of different strains.

Table 3.6.1. The zone of inhibition (ZOI) restricting bacterial growth due to the application of methanolic extracts of different cyanobacterial strains. Each value is an average of three observations ± standard deviation.

Species	*Pseudomonas* sp.		*Klebsiella* sp.		*Streptococcus aureus*		*Bacillus* sp
	ZOI	% Inhibition	ZOI	% Inhibition	ZOI	% Inhibition	ZOI
Neowestiellopsis persica	18 ± 0.02	70	15 ± 0.12	55	18 ± 0.15	70	0
***Neowestiellopsis* sp**	11 ± 0.01	35	11 ± 0.01	35	17 ± 0.05	65	0
Neowestiellopsis bilateralis	0	0	0	0	0	0	0
Nostoc punctiforme	0	0	11 ± 0.16	35	0	0	0
***Nostoc* sp**	0	0	10 ± 0.05	30	13 ± 0.07	45	0
***Nostoc* sp A51T**	0	0	0	0	12 ± 0.25	40	0
***Tolypothrix* sp**	9 ± 0.05	25	11± 0.05	35	0	0	0
Aliinostoc morphoplasticum	11 ± 0.15	35	0	0	0	0	0
***Pseudoanabaena* sp**	14 ± 0.05	50	8 ± 0.05	20	18 ± 0.05	70	0
Desertifilum tharense	0	0	17 ± 0.5	65	11 ± 0.5	35	0
Kelsinema persicum	13 ± 0.09	45	0	0	0	0	0
Control	20 ± 0.05	100	20 ± 0.05	100	20 ± 0.05	100	0

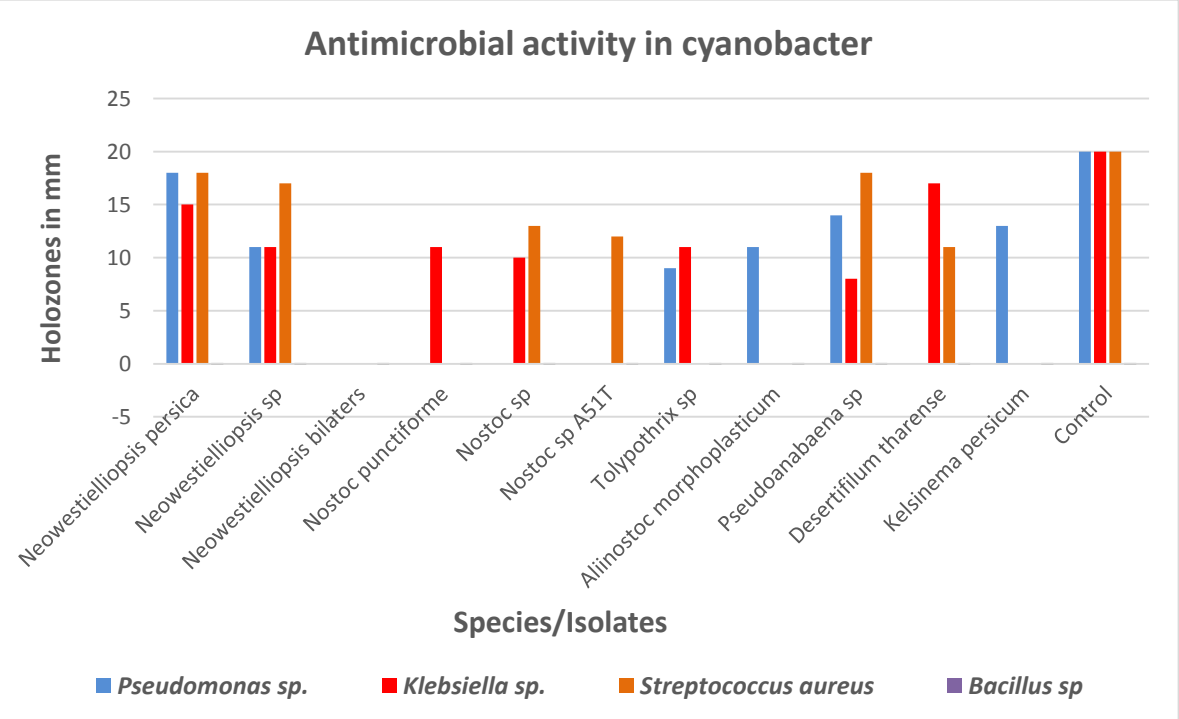


Figure 3.6.2: The antimicrobial efficacy of sample extracts expressed as mean (n=3) with standard error. (A) Zone of Inhibition (mm) of cyanobacterial extracts against *Pseudomonas*, *Kebsiella* and *Streptococcus aureus*, while no activity was observed in case of *Bacillus* sp.

3.6.2 Brine Shrimp (*Artimia salina*) Cytotoxicity Assay

The cytotoxic assay of the cyanobacterial strains revealed moderate cytotoxicity, revealed by less than 50% mortality of the tested brine shrimp larvae, exposed for a period of 24h. In some cases, the larval mortality was found more than 35%. For instance, in case of *D. tharense* extracts the mortality exceeded 44%, exhibiting maximum % mortality in brine shrimps. Similar mortality rate was observed in case of *Tolypothrix* sp. (i.e. 40%) and *Nodularia* sp. (36%). On the contrary, extracts of *Neowestiellopsis* caused minimum (0.7%) mortality to the brine shrimps. While few others acceded less than 10% mortality, as found in case of *Neowestiellopsis persica*, *Pseudoanabaena*, *Nostoc* and *Calothrix* (Table 3.6.2).

Table 3.6.2: Percentage mortality of brine shrimp larvae caused by the toxicity of extracts in lethality test

Methanolic Extract	Site	Extract conc. µg/ml	Dead Nauplii	No. of surviving Nauplii after 24 h			Avg. survival	% Mortality
				T1	T2	T3		
Neowestiellopsis persica	B10 H	10	3	47	46	45	92	8.0
Neowestiellopsis sp.	B12 H	10	4	46	45	44	91.3	8.7
Pseudoanabaena sp.	B10	10	1	49	48	48	97.3	2.7
Neowestiellopsis bilateralis	A-44	10	1	50	49	49	99.3	0.7
Nostoc sp.	B-17	10	8	42	41	39	83.3	16.7
Nostoc punctiforme	B-10	10	11	39	40	38	79.3	20.7
Tolypothrix sp.	A-8	10	19	31	29	30	60.0	40.0
Desertifilum tharense	A-5	10	13	37	35	36	72.2	27.8
Nodularia sp.	B-6	10	18	32	31	32	63.2	36.8
Desertifilum tharense	A-5	10	22	28	27	25	55.2	44.8
Nostoc edaphicum	B-18	10	2	48	46	45	94.3	5.7
Calothrix sp.	A-2	10	3	47	45	44	92.3	7.7
Control (distilled water)			1	49	50	50	99.3	0.7

Note back:

- Distilled water was used as blank control. The death counts were made after 24 h of exposure to the extracts.
- Extracts causing 50% mortality after 24h of exposure were considered lethal. Species showing causing up to 35% mortality are considering potential types with cytotoxic effects.

3.6.3 MTT Assay to assess cell viability

The percent inhibition of cancer cells assessed at different concentrations i.e. 25, 50, 100 and 200 µg/ml against HCT-116 (Figure 3.6.3) show a diverse pattern in the inhibition capacity of the extracts. Considering the minimum criterion of 50% inhibition, seven different species of cyanobacteria showed convincing evidence for an anticancer potential. For instance, *Tolypothrix* sp. showed more than 45% of inhibition at 25 µg/ml which increased to more than 73% at 200 µg/ml; followed by *Nostoc* sp. (68%)**,** *Pseudoanabaena* sp (65%), and Nodularia sp. (61%). It was further observed that the efficacy of extracts is dose- dependent activity. For instance, at a concentration of 200µg/ml, an inhibition of 73.6% of HCT- 116 cancerous cells were observed as the best rate. Whereas at 100, 50, and 25µg/ml concentrations a growth inhibition of 65%, 48% and 45%, respectively, was observed (Table 3.6.3). On the contrary, the *Calothrix* extract achieved lowest value of inhibition (19.54%) even at the top concentrations of extract; followed by the extract of *Desertifilum tharense* (24.48%).

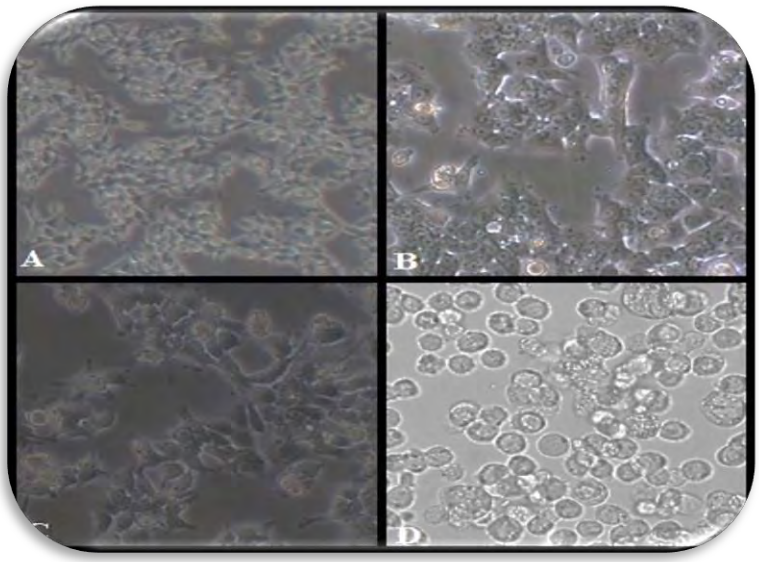


Figure 3.6.3: Microscopic images of the HCT 116 (Colorectal Carcinoma Cells) showing the growth inhibition at different concentrations of cyanobacteria extracts, A: 25µg/ml, B: 50µg/ml, C: 100µg/ml, D: 200µg/ml.

Table 3.6.3: The % growth inhibition of HCT 116 cancer cells determined by MTT assay using different concentration of cyanobacteria extract.

	Sample ID	**% Inhibition of cancer cells HCT-116** with **different concentrations**			
		25 µg/ml	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**
Neowestiellopsis persica	B10 H	10.21	33.72	40.23	50.15
Neowestiellopsis sp.	B12 H	15.73	20.52	33.67	37.075
Pseudoanabaena sp.	B10	40.99	53.24	57.75	65.15
Neowestiellopsis bilateralis	A4/4	15.05	21.43	21.05	30.06
Nostoc sp.	B17	37.36	45.49	61.56	68.68
Nostoc punctiforme	B10	27.14	33.27	37.59	51.65
Tolypothrix sp.	QD	45.86	51.31	65.93	73.68
Desertifilum tharense	A5/1(B)	5.32	14.96	21.60	24.48
Nodularia sp.	B6	31.88	48.01	55.15	61.53
Desertifilum tharense	A5/1 T	8.92	10.20	27.68	53.91
Nostoc edaphicum	B18	4.73	14.75	22.76	43.16
Calothrix sp.	A2a(4)	0	10.37	18.75	19.54

DISCUSSION

Cyanobacteria thrive naturally in deserts and contribute a major share in hypolithic consortia. These bio-crusts play a key role in stabilizing desert soils (Cameron, 1962). Among hot deserts, the Cholistan Desert of Pakistan holds a rich diversity of the cyanobacteria as revealed in the present study, aimed at exploring cyanobacterial diversity in world's 7th biggest desert. This is a pioneer effort to study various biological features associated with cyanobacterial diversity in Cholistan desert. The life-supporting features of the desert habitat, including rainfall, temperature, and edaphic characteristics, such as electrical conductivity and pH, support filamentous cyanobacteria and coccoid chlorophytes as abundant colonists. The Cholistan Desert is a dry and rain-scanty terrain. The obvious constraint to life in the Cholistan Desert is fresh water. Here rains are rare and far between, even in the current time of floods (which has affected 90% of land in the country), Cholistan has received poor rainfall and therefore freshwater resources remain critical even for survival.

During visits to various parts of Cholistan, water was found critically limited and the available drinking water in Toba's was turbid while the ground water in occasional wells is brackish. Alternatively, there are areas where agriculture is being introduced and fields are irrigated through canals linked to a nearby river. This created a unique opportunity to study and compare impacts of anthropogenic activity on to the diversity of algae and cyanobacteria endemic to the area. With agricultural practices, including the use of synthetic fertilizers and introduction of invasives through irrigation water, enormous impact was observed. For instance, in the pond water used for irrigation, the electrical conductivity (EC) was found in the range of 700-1880 ds/m which was 3 to 4-fold higher than that found in pure desert sites (where it was found in the range: 110-500 ds/m. Similarly, the pH ranged from 5.9-8.6 and the atmospheric temperature was 15-34°C at different sites with humidity

levels of 10–71% (Wariss et al., 2021). Hence the life detection methodology developed for Desert microflora program (Wynn-Williams, 2002) are still relevant.

4.1 The Diversity of Algae and Cyanobacteria in Cholistan

With the introduction of agriculture in the Cholistan Desert, several green algae with unique biodiversity have been introduced too. The present data suggest that *Chlamydomonas*, *Chlorella* and *Spirogyra* were among the introduced types in Cholistan. Since the last two decades, several such algal introductions have been noted at several sites near the settlements along both routes (Table 3.1). Most of the samples with introduced green microflora did not overlap with pure desert samples. Many of these strains potentially represent species that were also recorded in Lewis and Lewis (2005). The cyanobacteria notably recorded from such sites were: *Aliinostoc*, *Nodularia*, and *Nostoc*. Very few of such species (except *Nodularia spungina* (reference) have ever been reported from desert environment, hence these types were introduced to the Cholistan. *Neowestiellopsis* which is reported here for the first time at country level was previously reported from rice fields in Iran (Kabirnataj et al., 2018). In the present study this species has been found at two sites (B-10 and A-44), both of which pure desert sites and therefore, this type were indigenous to Cholistan (Inam, et al. 2022). Furthermore, other types such as *Klissinima*, *Nodularia* and *Aliinostoc* are among the types not previously reported from desert environments here in Pakistan.

In Cholistan, cyanobacteria showed a predominance of filamentous types such as: *Phormidium*, *Oscillatoria*, *Calothrix*, and *Desertifilum*, in addition to the branched *Neowestiellopsis* sp. The globular forms such as *Nostoc* were common too. Tiwari et al. (1999) reported a similar taxon distribution in the desert region in India. On the contrary, the desert soils from western Negev showed dominance of the branched cyanobacterium

Scytonema along with *Microcoleus*, *Schizothrix* and *Nostoc* (Danin, 1991). Several substrates were sampled to maximize the diversity records. This included the soil crust besides the stone crevices and drying ruin sites of old/historical places such as a fort ruins found in the Cholistan. In desert crusts from the Colorado Plateau, *Microcoleus vaginatus* and *Schizothrix*-like morphotypes have been reported. While *Scytonema* and *Nostoc* were frequent (Garcia-Pichel et al., 2001). In the present case *Leptolyngbya*, *Calothrix*, *Lyngbya* and *Nostoc* were found frequent at most sites.

Several species were found to co-exist here. For instance, *Leptolyngbya* was found to co-exist with *Calothrix*. Similarly, *Leptolyngbya* and *Phormidium* were also found together. A probable reason for this is the presence of exopolysaccharide sheath in case of *Leptolyngbya*. Another probable reason for holding several species (that co-exist) at these sites was the presence of spores in the sand dunes. These hides have the capacity to catch the air-borne spore that develop so due to extreme temperatures which cause such small structure to become air borne and, on the way, held by the vegetation that stand high on such dunes. Furthermore, the sand dunes and biological crust soils were found to hold several unique types, for instance, *Chroococcus* and *Gloeocapsa* were found in the crusts and soil samples (see initial observations given in sections 3.2.1 and 3.3).

4.2 The *in vitro* culturing and assessment of species diversity

Investigation of naturally occurring samples and the cultured samples is an important criterion in taxonomic studies. Unfortunately, cultivation of the natural samples has some drawbacks, and hence, attempts must be made to grow these samples into simplified and standardized culture conditions in short and more simplified cycles. It has been mentioned that wild strains, cultured under ‘natural’ conditions, are sometimes more important for this type of investigation, than the transfer into monospecific culture under standardized

conditions, where the morphology and life cycles are often modified, atypical or restricted (Koma´rek, 2013). The taxonomic consequences of these different stages during the life cycles and vegetation cycles are still to be elucidated properly. It is usually not very definite as to what extent do the wild morphological forms affect the taxonomic affiliation.

Several cyanobacteria and green algal types responded differentially to *in vitro* culture. This culturing tendency did not appear in the environmental samples offered an additional advantage of scoring species diversity in the true sense. To our estimates, culturing has added number of species to our records which might have been over looked if the *in vitro* methods were not adopted. For culturing of cyanobacteria different techniques and basic culture media were used. Axenic cultures for microalgae were reported for the first time by a Dutch microbiologist Beijernck (1890). Over time, advancements in various other fields of biology has helped refine the culturing practices (Rosenberg et al., 2008). The standard conditions for growth of samples, such as light intensity, temperature, and pH, were employed for purification purposes. The solid agar plating technique (Lee & Shen, 2004) and different media were employed for culturing algal and cyanobacterial species. It was observed that, the non-heterocystous cyanobacteria were more responsive to BG_{11} and BBM, whereas among these BG_{11} media elicited responses for the unicellular species such as *Chroococcus* and *Chroocoodiopsis*. Heterocystous cyanobacteria responded readily to the BG_{11}, whereas BG_0 was exclusively helpful in isolating heterocystous cyanobacteria from natural populations such as *Nostoc*, *Nodularia*, and *Cylindrospermosis*. Furthermore, the SpM was originally employed to culture *Spirulina* was also found suitable for *in vitro* culturing of non heterocystous cyanobacteria like *Desertifilum*, *Leptolyngbya, Pseudanabaena* and the green algae such as *Chlorella* and *Chlamydomonas* and therefore more indigenous species were recorded.

4.3 Species identification using polyphasic approach

4.3.1 The Nostocales

Due to the morphological plasticity, complex life cycle and huge diversity, the taxonomy of genus *Nostoc* (Family Nostocaceae) remains problematic (Mollenhauer et al. 1999; Komárek et al. 2014; Singh et al. 2016). Molecular data showed evidence of huge genetic diversity in the genus exceeding its morphological diversity, this depicts its polyphyletic nature (Rajaniemi et al. 2005a, 2005b; Řeháková et al. 2007; Hrouzek et al. 2013; Komárek and Genuário, 2015; Bagchi et al. 2017; (Maurer et al. 2017). Consequent to the recent efforts made to reevaluate the genus *Nostoc* sensu stricto was recognized off a clade with *Nostoc commune* as its type species and excludes species that fall outside this clade. This excluded certain taxa that are given the generic name *Nostoc* but still violates the monophyly of the genus when assessed phylogenetically (Hrouzek et al. 2013; Komárek and Genuário, 2015; Bagchi et al. 2017; Saraf et al. 2019; Cai et al. 2019). *Nostoc*-like taxa are difficult to differentiate based on morphology. Recently, many studies have emphasized the importance of ecological investigation in the cyanobacterial taxonomy (Komarek et al. 2014; Komarek 2016; Bagchi, Dubey and Singh 2017; Saber et al. 2017). In the present study, *Nostoc punctiforme* was confidently identified with the help of morphological and molecular methods. Based on the phylogenetic analysis, and collation of morphological and ecological data, it was found that both morphology and molecular data were congruent at genus and species level. Hence assignment of these samples to the *Nostoc punctiforme* is possible.

An allied genus *Aliinostoc* has also been reported previously from different habitats, namely eutrophic ponds, lakes and rice fields which usually have alkaline pH and higher concentration of dissolved ions etc. (Papaefthimiou et al. 2008; Bagchi, Dubey and Singh

2017; Genuario et al. 2017). In the present case, we encountered *Aliinostoc morphoplasticum*, a species previously known to occur in desert environments (Castenholz 2015). Due to its ubiquity and survival characteristics, *Aliinostoc* is found on crustal surfaces. The dew-absorbing quality of the crust allows the trichomes to remain dormant between the rainy seasons. The morphological analysis of strains B10 and B18 revealed that these belong to *Nostoc* genus, whereas strain B6 (also referred to as F6C) was identified as member of *Aliinostoc*.

Nodularia (family Aphanizomenonaceae) includes both planktic and benthic species (Hasler et al., 2011). *Anabaenopsis* and *Cyanospira* are the genera most closely related to *Nodularia.* According to botanical classification, members of the genus *Nodularia* have vegetative cells that are shorter than two-thirds of their width and may be cuboid but not oval (Komárek, 2013). In case of *Nodularia*, morphology has an important role. For instance, in the present case straight filaments; development of akinetes amphoterically; terminal cells conical in N. *harveyana* etc. differentiate these from other closely related species. Furthermore, the 16s rRNA further confirms these finding by being phylogenetically heuristic. Despite this, other morphotypes were also encountered for example: a strain morphologically similar to *N. harveyana* but differ in akinete formation, cells size begins to increase and eventually all cells transform into akinetes; frequency of intercalary heterocysts was low and optional pair of heterocysts may be seen on terminal position. Strains exhibiting variation were obtained from diverse substrates and ecologies etc.

The genus *Calothrix* (Family Rivulariaceae) was first described in C. Agardh (1824). Taxonomically, this genus is classified as a member of Nostocales Rivulariaceae (Anagnostidis and Komarek, 1990). Recent molecular studies have placed this genus in a clade with *Rivularia*, *Gloeotrichia*, *Tolypothrix*, and other closely related genera (Knoll,

2008). Molecular studies in the past (Berrendero et al., 2008) have suggested that the genus is polyphyletic, but more recent research indicated that it is monophyletic. In the present samples Calothrix was found in canal water along with *Scenedesmus*, species of *Nostoc* and *Leptolyngbya*. When compared with closely related taxa, several characters were found diagnostic, for instance: presence of heteropolar filaments with a wider basal part; compared with the genus *Rivularia* which have the heteropolar filaments with a narrower base; development of akinetes just above the basal heterocysts; *Calothrix* and *Tolypothrix* both have firm sheath around filaments, the earlier has a colourless sheath while *Tolypothrix* has yellow to brown colour sheath; *Calothrix* has high degree of tapering cells towards the apex, while less degree of tapering cells was found in *Tolypothrix*. Morphologically species of *Calothrix* have a basal heterocyst. Species of this genus occur in both salt and freshwater environments, as well as in sub-aerial and aerial environment (Uher, 2007). *Tolypothrix* thallus is composed of filaments with a firm, thick or thin sheath frequently with yellow to deep brown colour (Whitton, 2002). The strain under study showed characteristics similar to *Tolypothrix*, for instance on an agar plate, it showed filaments of uniform growth. The presence of a colorless, very thick sheath that transformed it into yellow-colored mucilage clearly visible under microscope. Characters which are different from *Tolypothrix* were: (a) side branches absent; the development of new filaments starts within the parent filament by the development of heterocyst. At this point, the new filament is separated by disintegration of the parent filament (c) The filaments in the liquid culture showed uniform growth but with large filaments. The 16srRNA data clearly clustered this isolate into the *Calothrix* clade, whic33h was in line with the morphological description of this strain.

4.3.2 The Oscillatoriales

Desertifilum (Family Desertifilaceae) is a newly described genus of Oscillatoriales, was recently established based on isolates from the desert in western India (Dadheech et al.,

2012). *D. tharense,* the type species, was found in black and dark-blue biological crusts on dry and moist sand dunes, with an annual precipitation of 25cm (Singh et al., 1999). The trichomes are isodiametric with a colorless sheath. Despite its benthic habitat, this species has gas vesicles. Another *Desertifilum* species, *Desertifilum fontinale* was later described from a warm spring in East Africa. The habitat, morphology, and molecular characteristics of the latter species differ from those of *D. tharense*. As a result, the *Desertifilum* genus now has more diversity of morphology, physiology, and ecological habitats (Dadheech at al., 2014). In the present study, three strains with subtle differences in morphological traits were encountered. Among these the morphological characteristics of strain A51b were consistent with *D. tharense*, which had a 2mm filament width, a slightly constricted cross wall, end cell alternated, and no gas vesicles. Phylogenetically, it showed similarity with the *D. tharense* clade. Another strain A51c displayed cells longer than broad and the absence of wall constrictions. These characters were consistent with *D. tharense* as described previously (González Resendiz et al., 2019). However, this strain differed in few characteristics for instance, the width of the filaments in strain A51c was 4µm and the presence of hormogones with areotopes. The filament color is blue green. Strain A51d olive green filament with a width of 4µm. The filament width matches that of *Microcoleus*, but it did not form aggregates because the *Microcoleus* cells are longer than broad. The combine phylogenetic analysis revealed clear and close similarity of All three strains with *D. tharense*. Hence, molecular data suggest that any morphological differences are subtle and in congruent with the genetic data.

4.3.3. The Synechocales

The genus *Leptolyngbya* (family Leptolyngbyaceae) is one of the most common and taxonomically most difficult cyanobacterial genera, containing numerous morpho- and eco-types (species), which are very common in the soil substrate, in periphyton and metaphyton

in a variety of freshwater and saline environments (Albertano and Kováik, 1994). Several species are known to occur in thermal and mineral waters and grow subaerially on wet rocks. Identification of species (more than 140 have been described) is difficult due to indistinct morphological differences. Whitford and Schumacher (1969) reported *L. lagerheimii, L. subtilis, L. angustissima* and *L. tenuis* under the generic name of *Phormidium. L. bijahensis, L. cartilaginea, L. geysericola, L. rubra, L. subterranea, L. vesiculosa* and *L. yellowstonensis*, from mineral and thermal waters in Yellowstone National Park. In Oscillatoriales, the highest strains belonged to the genus *Leptolyngbya*. These *Leptolyngbya* strains were ecologically diverse and were found in irrigated water, ponds, fresh tube well water, and in pure desert areas. Although this genus is widely distributed ecologically and is frequently found in different habitats throughout the earth's biosphere (Komerak, 2007), further research is needed to determine its ecological relevance in local communities. In the present study, the strains encountered were morphological diverse with following characteristics: the isopolar trichome, grouped in mats or clusters, and most crucially, thin, fine, and hard mucilaginous coatings. The morphological data of strain B1Sp. suggest its resemblance with species of the genus *Leptolyngbya*. While the 16 S rRNA analysis show sequence similarities with the *Leptolyngbya* clade contains B1Sp, which is morphologically similar to *D. valadriana*, but is phylogenetically more closely related to *Sodaleptolyngbya* stromatolite (Anagnostidis and Komarek, 1990).

4.4 Potential Assessment of Cyanobacteria

Microalgae are being thoroughly studied because of their fast growth and vast set of potential applications, especially in biogas production as promising feedstock’s for biofuel synthesis wastewater treatment and for human food, nutraceuticals products, and animal feed (Ahmad et al., 2022). Microalgae possess an outstanding significance for providing important neutral compounds like proteins, pigments, vitamins, minerals, medicines,

biofertilizers and biofuels. Hence, it is crucial to investigate and document accessible microalgae to exploit their significance to meet ever-rising human needs (Chandra et al., 2019). This multidirectional research on algae engages in a set of aspects to be studied. The most convenient feature is taxonomic study, which is an indispensable prerequisite for any applied and modern research. The first report on the antibiotic potential of algae was published Pratt et al., (1994). There is an extensive account of the algal applications for therapeutic practices in disease management and the withdrawal utilization of microalgal biologically active compounds, of which most antibiotics were confirmed during the early 1950 (Aketo et al., 2019).

Cyanobacteria synthesize metabolites that show varied biological activities, such as antibacterial (Safavi et al., 2019), antifungal (MacMillan et al., 2002), and cytotoxic activities (Litchman et al., 2003). In the present study, cyanobacterial extracts were tested against antibacterial, anticancer, and cytotoxicity. Among gram positive bacteria, *S. aureus* was the most sensitive to all the cyanobacterial extracts. The highest antibacterial activity (indicated as zone of inhibition) was recorded for *Newwestilliopsis persica* (70%), *Pseudoanabaena* sp (70%) and *Nostoc* sp (45%), respectively, while none of the cyanobacterial extracts showed inhibition activity against *Bacillus* sp. Similarly, in the cytotoxicity assay, 46% and 44% mortality rates were achieved with extracts of strains B13 and A5/1, respectively, while strains B10 and B10H showed the lowest mortality rates of 2% and 6%, respectively. This trend was analogous to that observed in *Mastigocladus laminosus*, where capsular polysaccharides were found to be cytotoxic (Walton and Berry, 2016). A similar study previously reported inhibition of cell migration and invasion in human epithelial and colon cancer cell lines (Zhu et al., 2018), the QD strain showed up to 73% inhibition of the HCT116 cancer cell line at 200µg/ml concentration, while strain A5/1 showed a 24% inhibition rate with similar concentration. Previous studies revealed presence

of westiellamide, a substance cytotoxic to human epithelial (KB) and colon (LoVo) cancer cell lines, isolated from *Westiellopsis prolifica* (Prinsep et al., 1992).

4.5 Conclusions

This study is concluded as follows:

- The Cholistan desert has huge cyanobacterial diversity including the unicellular forms like: *Chroococcidiopsis;* unicellular colonial forms like: *Gloeocapsa, Chrococcus Cyanosarcina* and *Aphanocapsa*; non heterocystous filaments forms like: *Desertifilum, Phormidium, Leptolyngbya, Lyngbya, Oscillatoria* and *Phormidium* sp.; heterocystous filamentous forms like *Nostoc, Calothrix, Tolypothrix, Neowestielliopsi*s and *Nodularia* sp.
- The culturing experiments suggest: BG_{11} and BG_0 were successful media for growth of cyanobacterial species especially BG_0 purified heterocystous cyanobacteria like *Nodularia* and *Nostoc* in first attempt. SpM showed a response to non-heterocystous filamentous forms like *Desertifilum* and unicellular green algae like *Chlorell*a and *Chlaymydomonas*. The filamentous green algae did not show any response in *in vitro* culturing except *Stigeoclonium*
- *Leptolyngbya, Neowestielliopsis, Nostoc* and *Nodularia* sp were present both in natural population and lab cultures.
- Employment of culturing techniques improved the assessment of species diversity as unique types appeared in *in vitro* culturing. The types scored/recorded in cultures only were *Cylindrospermosis, Cylindrospermum, Dulcicalothrix, Calothrix, Tolypothrix, Desertifilum* sp. etc.
- *Anabaenopsis* sp*, Merismopedia* sp*, Cyanosarcina sp* and *Nodularia spungena* did not appear in Culturing.

- The polyphasic approach helped clarify the identification/taxonomic position of morphologically closely related species. This included *Nodularia, Desertifilum, Neowestilliopsis* and *Nostoc* sp. with subtle morphological differences.
- The biological activity analyses suggest strong antimicrobial, cytotoxic and anticancer potential in *Neowestiellopsis*, *Tolypothrix* and *Desertifilum*, which warrant further analysis. Cyanobacterial research has the potential to lead to the development of new drugs, biofuels, and sustainable agriculture practices. However, more research is needed to fully understand the potential of these microorganisms and to optimize their production and applications.

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