Screening of Thermophilic Cyanobacteria from some hot Springs of Iran

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Abstract

Hot springs have been a subject of intense discussion for biologists in the last decades. The polyphasic approach is the most progressive system that has been suggested for distinguishing and phylogenetically classifying cyanobacteria. In a revision of the cyanobacteria in hot springs of Iran, four hot springs of Iran were investigated. We developed a combined molecular and morphological approach to identification of cyanobacteria. In this study seventeen populations and 13 morphological characters were analyzed. Molecular study based on 16S rRNA gene sequence does not disrupt morphological information and it confirms the separation of studied taxa according to morphological characters. Based on their growth characteristics; seven species were selected for the production of antimicrobial agents. The results of this study showed that the methanol extracts exhibited high antimicrobial activity against some gram positive bacteria, moderate antibacterial activity against some gram negative organisms and moderate antifungal activity against some fungi. Four species were selected for salinity tolerance. The results showed high tolerance of these taxa to salinity.

Key words: Cyanobacteria, Hot Spring, Morphological diversity, 16S rRNA, Polyphasic study.

Introduction

The high temperatures associated with geothermal activity frequently result in surface and subsurface geothermal springs, commonly known as "hot springs" (Ghozzi et al., 2013). In general, these ecosystems inhabit with population of microorganisms with great commercial importance and interest to the researchers and industry working on enzymes, sugars, compatible solutes and antibiotics (Zakaria and Abdulrahman, 2007). Several investigations of microalgae and cyanobacteria flora for many sources of springs have reported a dominance of prokaryotic organisms (Brock, 1978; Castenholz, 2001; Ward et al., 2000; Sompong et al., 2005), and small eukaryotes (e.g., diatoms, unicellular algae) (Jonathan and John, 2009). Despite the possibility of the presence of novel taxa with high economic and industrial use very few reports are available on the cyanobacterial diversity of hot springs from the Iran.

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Material and Methods

Sampling and Morphological study

Water samples were collected from four hot springs in Geno, Khamir, Chahahmad and Ramsar (Table 1). Samples were streaking on agar nitrate BG-11 medium (Stanier et al., 1971), and incubated in a culture chamber at 25°C and a 12/12 h light-dark cycle at artificial illumination (37- 46 µmol $m^{-2}s^{-1}$) for two weeks. Isolation involved from removing colonies that developed in medium and observed under light microscope. Taxonomic determination was carried out by light microscopy (Olympus, Model BM-2) and based on Komarek and Anagnostidis (1989, 1998, 2005). Identification was carried out by morphometric method. Seventeen morphological characters and numerical taxonomic studies were used for classifying the various species of cyanobacteria. Taxonomic analysis was performed with cluster analysis and principal component analysis. A data matrix based on the coded multiple states of characters were used in this study (Table 3). Cluster analysis using the UPGMA method (unweighted pair-group method with arithmetical averages) was carried out. Pheneticrelationships among the species were constructed. All analyses were carried out using SPSS (Ver.16).

DNA extraction, PCR amplification and sequencing

The bulk of cyanobacterial culture isolation was extracted by genomic DNA extraction kit AccuPrep (Bioneer). 25mg of each sample in a 1.5ml tube with 200µl lysis bufferand 20µl of ProteinaseK were homogenized. The tubes were incubated for 1 h at 60°C. 200µl Binding Buffer was added. After mixing gently for 10 minutes, the samples were incubated at 60°C. 100µl Isopropanol was added. The supernatant was then transferred to a 2ml tube. After a final gentle mixing centrifugation for 5 min at 8,000 rpm, 150µl elution buffer was added. Extracted DNA was harvested at -20°C temperature.

For DNA amplification, the 16S rRNA gene regions, approximately 600 bp in length, were amplified by PCR using the A2 (5- AGAGTTTGATCCTGGCTCAG-3) and S8 (5-TCTACGCATTTCACCGCTAC-3) primers (Giovannoni et al., 1988). Each reaction contained 2.8µl $MgCl_2$, 150 mMd NTPs, 0.5µM of each primer, approximately 50 ng template DNA, 3µl Taq polymerase in a total volume of 100µl. For PCR amplification cycle using the cyanobacterial primers was 4 min at 95°C, then 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 59°C, 2 min extension at 72°C, and a final extension of 8 min at 72°C.

Phylogenetic analysis

Multiple alignments were created with ref-

erence to the selected GenBank sequences using BioEdit which implements the Clustal W multiple alignment algorithm. Sequences were aligned with the software MEGA (version 5). The Neighbor-joining method was used to compute evolutionary distances in present study. In this program, bootstrap analysis was used to evaluate the tree topologies by performing

Table 1. Some physio-chemical parameters of four hot springs of Iran

Parameters	Geno	Chahahmad	Khamir	Ramsar
Location	Hormozgan province, Bandar Abbas city	Hormozgan province, Khamir city	Hormozgan province, Khamir city	Mazandaran province, Ramsar city
Latiude/Longitude	26º 56′ 55″ N 55º 32′ 27″ E	26º 56′ 31″ N 55º 26′ 17″ E	26º 56′ 46″ N 55º 33′ 23″ E	36º 55′ 20″ N 50º 39′ 30″ E
Temp (°C)	40	39	37	50
Ph	7/2	6/9	7	6/8
EC (ms cm^{-1})	14/02	20	20	13.3
Na^{+}(mg L^{-1})	1600	2400	2200	410
K^{+}(mg L^{-1})	580	400	200	240
Ca^{++}(mg L^{-1})	800	3000	1440	1100
Mg^{-}(mg L^{-1})	475	950	710	1100
Cl^{-} (mg L^{-1})	4560	18520	9210	3504
PO_4^{-3} (mg L^{-1})	14/02	300	400	400
SO_4^{-2} (mg L^{-1})	1600	152	1374	1800
NO_3^{-} (mg L^{-1})	0.7-1.0	1.1- 1.3	1.3-1.4	0.8-13.6
Total alkalinity (mg L^{-1})	135	150	160	244

Table 2. Total variance of factors according to principal component analysis on morphological characters.

Total Variance Explained

	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
Component	Total	%of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	5.144	39.566	39.566	5.144	39.566	39.566	3.256	25.045	25.045
2	2.131	16.392	55.958	2.131	16.392	55.958	3.172	24.401	49.446
3	1.696	13.046	69.004	1.696	13.046	69.004	1.730	13.305	62.751
4	1.372	10.557	79.561	1.372	10.557	79.561	1.690	12.997	75.748
5	1.135	8.734	88.295	1.135	8.734	88.295	1.631	12.547	88.295
6	.875	6.730	95.025						
7	.282	2.167	97.192						
8	.230	1.770	98.962						
9	.086	.659	99.622						
10	.041	.316	99.938						
11	.007	.055	99.993						
12	.001	.007	100.000						
13	-7.371E-16	-5.670E-15	100.000						

Extraction Method: Principal Component Analysis.

1000 resamplings. The tree was rooted using the *Bacillus subtilis* 16S rRNA sequence as an out-group.

Experiments for physiological characteristics

Six different salinities (0%, 3%, 6%, 9%, 12% and 15%,) were obtained by preparing BG-11 medium in various ratios of NaCl in distilled water. Four cyanobacterial isolation *Oscillatoria subbrevies, Chroococcus minimus, Synechocystis aquatilis, Synechococcus* elongates were selected and were cultured in different salinities media. All cultures for salinity tolerance were incubated in a culture chamber. The growth rate was measured over a period of five days.

Microbial Study

The antimicrobial activity of cyanobacteria extracts isolated from hot spring were individually tested against a panel of microorganisms, including; *Bacillus subtilis* (ATCC 465), *Enterococcus fecalis* (ATCC 29737), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 85327), *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763). Bacterial strains were cultured activity. Overnight at 37°C in Mueller Hinton agar (MHA). Yeast Data on antimicrobial activity in terms of inhibition was cultured overnight at 30°C in Sabouraud's dextrose zones exhibited by the cyanobacteria methanol extract agar (SDA). Antimicrobial screening by the Disk Diffusion Method was applied for determination of antimicrobial activity of the prepared methanol extracts.

Extracts were dissolved in dimethyl sulfoxide (DMSO). A suspension of tested microorganism (0.1 ml of 108 cells/ml) was spread over surface of agar plates (MHA and SDA). Filter papers having a diameter of 6 mm, soaked with 25µl of each extracts were placed on the inoculated agar plates. then all Petri dishes were incubated at 37°C for 24 h for bacteria and at 30°C for 48 h for yeast. The diameters of the inhibition zones were measured in millimeters. Minimum Inhibitory Concentrations (MIC) were determined by broth micro dilution assay recommended by the [NCCLS].

Statistical Analysis

The statistical analysis was performed with stat graphics (Centurion XV) and Excel software. The multi-factorial ANOVA analysis was followed by the Tukey multiple comparison tests for statistical comparisons. P-value of less than 0.05 was assumed for significant differences.

Results

In morphological study, 43 distinct morphospecies of cyanobacteria were characterized using light microscopy. The lowest species diversity was observed in Nostocales and the highest species diversity belonged to Oscillatoriales. Among the genera were identified, *Oscillatoria*, *Phormidium*, *Chroococcus* and *Spirulina* showed the highest number of species. *Oscillatoria subbrevis*, *Jaaginema metaphyticum* (*Syn.: Oscillatoria angusta*) and *Spirulina subsalsa* was observed in 3 stations of each hot spring. In total 17 morphospecies of cyanobacteria from 4 hot springs by using 13 most stable quantitative and qualitative characters used for clustering analysis (Table 3). Results of this study showed that the selective morphologi-

Table 3. Rotated component matrix according to PCA analysis of morphological characters.

	Component				
	1	2	3	4	5
VAR00001	.267	.309	.844	-.027	.045
VAR00002	.872	.367	.110	-.009	.210
VAR00003	-.072	.030	-.110	.938	.004
VAR00004	.943	.203	.178	.035	.148
VAR00005	.092	.973	.051	-.030	.076
VAR00006	.685	.599	.181	.097	.345
VAR00007	-.041	-.345	.660	.335	.370
VAR00008	.097	.898	.105	.275	.156
VAR00009	-.045	.075	.169	-.179	.694
VAR00010	.836	-.463	.064	-.061	-.175
VAR00011	.459	.625	.598	.001	-.081
VAR00012	.101	.181	.291	.755	-.174
VAR00013	.347	.157	-.064	.061	.854

Extraction Method: Principal Component Analysis.

Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 9 iterations.

Table 4. GenBank code of samples.

	Sample	**GenBank code**
1	*Synechocystis sp.*	HQ900669.1
2	*Synechocystis sp.*	AB039001.1
3	*Synechocystis sp.*	HQ900668.1
4	*Synechococcuselongatus str. Ramsar*	JQ771323.1
5	*Synechococcus sp.*	AF448077.1
6	*Oscillatoria sp.*	EF150796.1
7	*Phormidiumirriguum f. minor*	FN813342.1
8	*Lyngbya sp.*	AY049752.1
9	*Lyngbya sp.*	AY049751.1
10	*Spirulina sp.*	DQ058861.1
11	*Phormidium sp.*	HM217057.1

cal characters separate genera but they did not separate them in species rank. Although, single cell strains were separated completely from filamentous forms. Some of filamentous samples due to their morphological similarities were not completely separated from each other. In numerical taxonomic study according to morophological char acters, it was considered that the species *Jaaginema metaphyticum* (Syn: *Oscillatoria angusta*) and *Geitlerinema amphibium* (Syn: *Oscillatoria amphibian*) are grouped to their pervious genus *Oscillatoria* (Fig. 1).

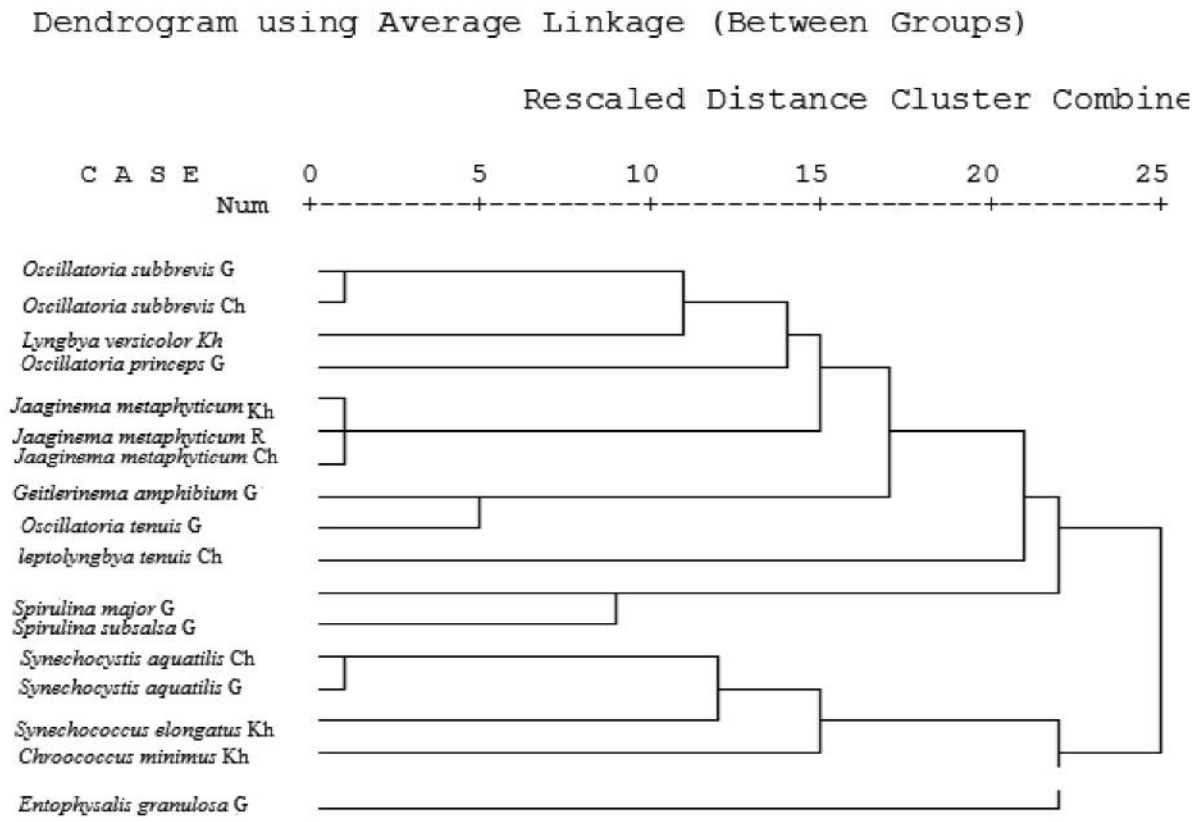

Fig. 1. Hierarchical cluster analysis dendrogram of cyanobacteria taxa based on morphological characters by UPGMA method.

Table 5. Antimicrobial activity of cyanobacteria methanol extract isolated from hot springs of Iran

	OS.SUB		OS.TEN		OS.LIM		OS.ANG		OS.ARTI		SYN.AQU		SYN.CER	
	IZ[a]	MIC[b]	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
B.SUB	20	3.75	18	3.75	19	3.75	16	7.5	19	3.75	22	3.75	20	3.75
B.PUM	22	3.75	20	3.75	18	3.75	17	7.5	20	3.75	20	3.75	19	3.75
E.FAE	14	15	10	15	11	15	0	-	15	7.5	16	7.5	14	7.5
S.AU	16	7.5	12	7.5	14	7.5	12	15	15	7.5	17	3.75	15	7.5
S.EP	24	3.75	20	3.75	17	3.75	14	7.5	19	3.75	23	3.75	22	3.75
E.coli	15	7.5	11	15	14	15	10	>15	14	15	17	7.5	15	3.75
P.AER	0	-	0	-	0	-	0	-	0	-	0	-	0	-
K.PNE	0	-	0	-	0	-	0	-	0	-	0	-	0	-
CAN	0	-	0	-	0	-	0	-	0	-	12	15	0	-
SAC	12	15	0	-	10	>15	0		12	15	16	7.5	14	7.5

[a] Inhibition Zone includes diameter of disc (6 mm). [b]Minimum inhibitory concentration values as mg/ml. abbreviations: OS. SUB (*Oscillatoria subbrevis*), OS. TEN (*O. tenuis*),OS.LIM (*O. limentica*), OS. ANG (*O. angusta*), OS. ARTI (*O. articulate*), SYN. AQU (*Synechocystis aquatilis*), SYN. CER (*Synechoccous cerdorum*). B. SUB(*Bacillus subtilis*), B. PUM (*B. pumulis*), E.FAE (*Enterococcus faecalis*), S.AU (*Staphylococcus aureus*), S.EP (*S. epidermidis*), E. COLI (*Escherichia coli*), P. AER (*Pseudomonas aeruginosa*), K. PNE (*Klebsiella pneumonia*), CAN (*Candida albicans*) and SAC (*Saccharomyces cerevisiae*).Inactive (-); moderately active (7 - 14); highly active (> 14); nt, not tested. Values are given as mean ± standard deviation.

To discover the most variable characters among the several morphological features, PCA carried out. The analysis revealed that the first five factors comprise about 88.2% of total variance. In the first factor with about 25% of total variance, characters of trichome, vegetative cell, form of spiral and kind of proliferation possessed the highest positive correlation. In the second factor with about 24% of total variance, characters of apical cell shape and filament width possessed the highest positive correlation (Table 5). In phylogenetically analysis, for drawing cladogram by using the analysis of data of sequence of genome in 16S rRNA region, the genome sequences of 22 samples were used. Among the studied samples, 11 samples belonged to cyanobacteria of hot springs from four regions of Geno, Chahahmad, Khamir and Ramsar. In the phylogenetic tree by algorithm neighbor-joining based on gene sequence 16S rRNA the distinctive primary clustering of the sample of out-group from taxa of photosynthetic prokaryotes from cyanobacteria were analyzed. In clusters, the cluster containing all of the analyzed cyanobacteria, two minor groups are recognizable. In one of minor clusters, all the unicellular samples belonged to *Synechococcus, Synechocystis, Chroococcus* with the bootstrap value of 99% placed in a unique group, and in other cluster *Spirulina* and *Oscillatoria* were presented (Fig. 2).

Based on cyanobacteria growth characteristics; seven species namely *Oscillatoria subbrevis*, *O. tenuis*, *O. limentica O. angusta*, *O. articulate*, *Synechocystis aquatilis* and *Synechoccous cerdorum* were selected for antimicrobial activity. Data on antimicrobial activity in terms of inhibition zones exhibited by the cyanobacteria methanol extract were shown in Table 5. Result showed that all cyanobacteria extracts were found to have high activity against *B. subtilis*, *S. epidermidis* and *B. pumullis P. aeruginosa* and *K. pneumoniae* were resistant to all tested algal extracts. All extracts inhibited slightly the growth of *E. coli*, *S. aureus* and *E. fecalis* (Table 5).

Results obtained from disc diffusion method, followed by measurements of MIC, indicated that *S. epidermidis* was the most sensitive among tested organisms, since the methanol extract of cyanobacteria showed lowest MIC value (3.75 mg/ml) (Table 5).

Results of salinity tolerance showed despite *Oscillatoria subbrevies* peresence in all of the hot springs but have best growth rate in culture media without salinity. Three unicellular cyanobacterial samples in 3% and 6% salinity have better growth rate than without salinity culture media. Moreover, the results indicated that unicellular cyanobacterial isolated (*Synechococcus elongates, Synechocystis aquatilis*) with higher growth rates in NaCl treatments, have the maximum level of antimicrobial activity.

Discussion

Temperature is one of the most important parameter for cyanobacterial species diversity in microbial mat of hot springs. The studies revealed that cyanobacterial diversity and complexity decreased with increasing temperature (Ferris, 1996; Ferris and Ward, 1997; Hongmei et al., 2005). Skimisdottir (2000) showed that in thermal gradients from 50˚C to 75˚C, the layered mats are characterized by the presence of

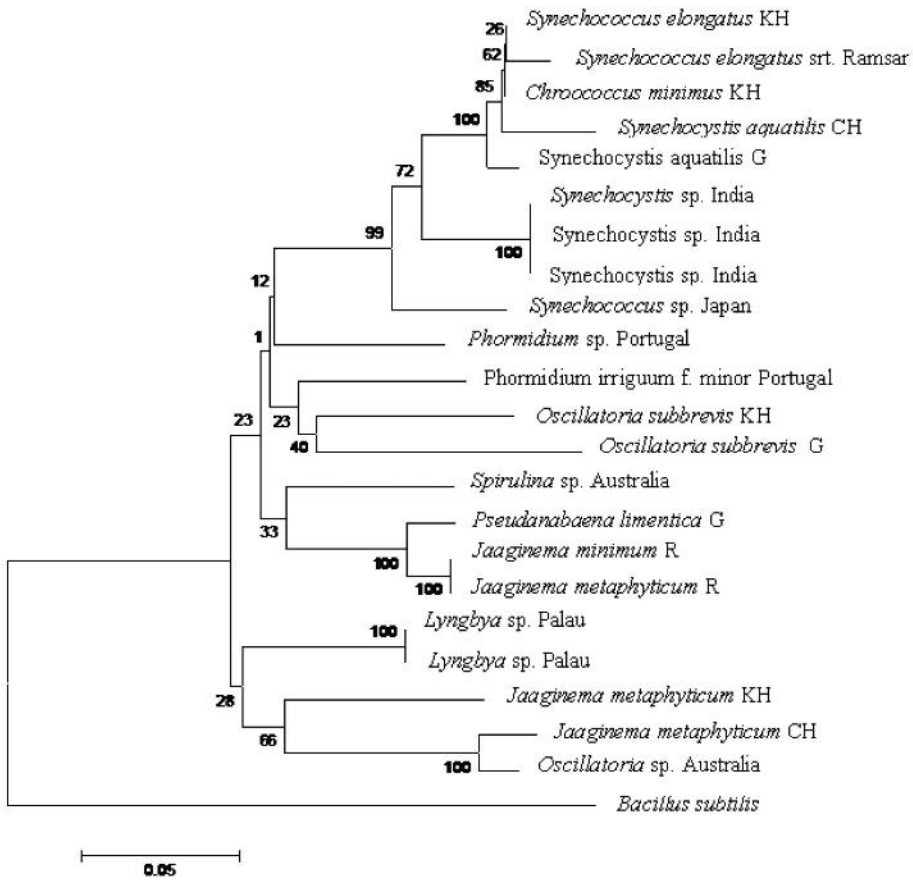

Fig. 2. Phylogenetic relationship for the cyanobacteria taxa was constructed using partial 16S rRNA gene sequences.

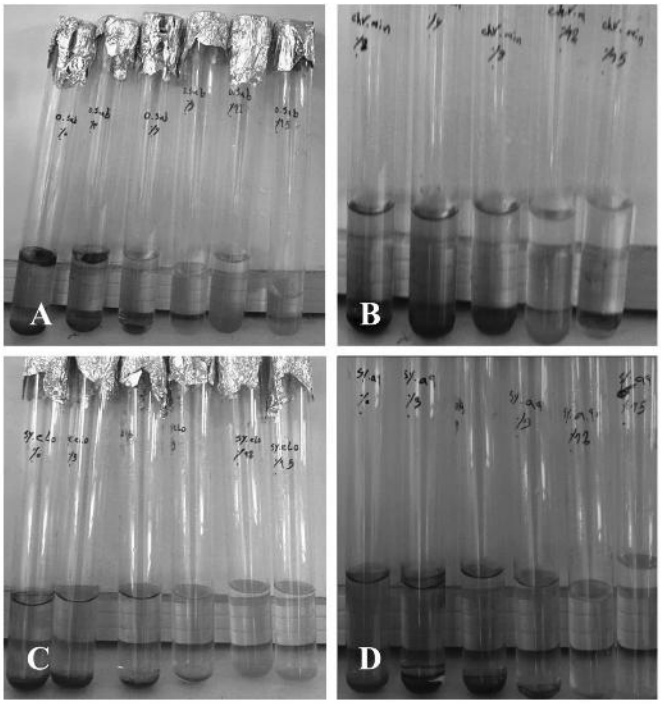

Fig. 3. A: *Oscillatoria subbrevies*, B: *Chroococcus minimum*, C: *Synechococcus elongates*, D: *Synechocystis aquatilis*.

unicellular forms like *Synechococcus*. The cyanobacterial mats occurring at the lower end of thermophily (40-50˚C) are often dominated by morphologically defined filamentous cyanobacteria like *Phormidium, Oscillatoria, Pseudanabaena, Calothrix* and *Fischerella* (Ward and *Castenholz,* 2000; Marteinsson et al., 2001; Sompong et al., 2008). However, Norris et al. (2002) reported that cyanobacteria such as *Synechococcous* also co-occurs with other unicellular and filamentous forms at lower temperature. In the present study, *Synechococcous* have been found in the mats that grow between 30-40˚C. Some workers also focus on the role of pH and combined nitrogen (especially ammonium), on the species distribution in cyanobacterial mat community below 60˚C (Ward and Castenholz, 2002; Sompong et al., 2005). Results of this study also support the previous studies. Diazo trophic cyanobacteria are able to colonize in the springs where nitrogen levels are lower than proper condition for the other taxa. Conversely, they may be out-competed by non diazotrophic cyanobacteria in spring with sufficient combined nitrogen (Ward and Castenholz, 2002).

According to this fact that the grouping of taxa according to morphological characters is not sufficient, especially in complex taxa such as *Jaaginema metaphyticum*, 16S rRNA gene sequencing was used for better sample recognition.

In conclusion, we report here that the molecular study does not disrupt results of morphological classification. Genomic sequences also don't provide the necessary information for separation of taxa from pervious groups (*Oscillatoria angusta, Oscillatoria amphibian, Oscillatoria limentica* and *Phormidium tenue*) and the separated taxa remain close to their pervious groups.

It can be concluded from this study that the extract obtained from some cyanobacteria strains isolated from the hot spring showed antimicrobial activity against the pathogens used in the present investigation. Regarding physiological responses of cyanobacteria isolated to NaCl as shown in Figure 3, the growth rate decreased with increase in salinity though it continued in NaCl 6%. These data were also seen in *Nostoc* sp. (Sekar and Subramanian, 1999). Further researches should be made to identify and purify natural products from these cyanobacteria with antimicrobial activity and high salinity tolerance.

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