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Full Length Research Paper

A gene expression study on strains of *Nostoc* (Cyanobacteria) revealing antimicrobial activity under mixotrophic conditions

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Cyanobacteria are well known for their production of a multitude of highly allelopathic compounds. These products have features such as incorporation of non-proteinogenic amino acids which are characteristics of peptides biosynthesized by non-ribosomal peptide synthetases (NRPSs). Some of these peptides have acetate-derived moieties, suggesting that their biosynthesis also involves polyketide synthases (PKSs). Among the photosynthetic microorganisms, cyanobacteria belonging to the genus *Nostoc* are regarded as good candidates for producing biologically active secondary metabolites. Aiming at the maximization in the production of natural product, we compared autotrophic, and mixotrophic growth at high light intensity of two *Nostoc* species in relation to the production of bioactive compounds with the antimicrobial activity at different source of sugar. Glucose was shown to be the best substrate for the production of high natural product when compared with sucrose. Also, the rate of biomass production and antimicrobial activity was reaching ~2.0 to 2.5 and ~1.5 times greater than that of the autotrophic and sucrose grown cultures, respectively. Also, we conduct a combined NRPSs and PKSs polymerase chain reaction (PCR). The sequences presented in this study was deposited in GenBank and had accession numbers JF795278 and JF795279 (NRPS A domains) and JF795280 and JF795281 (PKS KS domains). Computer modeling and phylogenetic analysis was conducted to predict the putative amino acid recognized by the unknown adenylation domain in the NRPS sequences. This study highlights the importance of environmental and nutrimental factors in maximization of antibiotic production of two *Nostoc* species.

Key words: Peptide synthetase gene, polyketide synthase gene, *Nostoc,* secondary metabolites, mixotrophic conditions.

INTRODUCTION

The genus *Nostoc* is an ecologically, morphologically and physiologically diverse group of microorganisms harboring in soil with the largest reservoir of potentially valuable natural compounds (Dembitsky and Rezanka, 2005). The ecological significance of the *Nostoc* species extends beyond their productivity, though, as many of these organisms are capable of modifying their habitats through the synthesis of biologically active natural products (Ehrenreich et al., 2005). These compounds demonstrate a diverse range of biological activities and chemical structures, including novel cyclic and linear

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lipopeptides, fatty acids, alkaloids and other organic chemicals (Dittmann et al., 2001). A large number of novel antimicrobial agents have been identified with cytotoxic (Bui et al., 2007), antifungal (Kajiyama et al., 1998), antibacterial (Jaki et al., 2000), immunosuppressive, enzyme inhibiting and antiviral (Kanekiyo et al., 2005; Zainuddin et al., 2002) activities.

A number of abiotic and biotic factors such as temperature of the incubation period, pH of the culture medium, incubation period, medium constitute and light intensity, are known to influence the production of bioactive compounds (Abedin and Taha, 2008). This prompted us to do an endeavor towards investigation of cultured *Nostoc* adaptive to their habitats, which might be correlated with maximum synthesis of natural products.

Polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) are large (200- to 2,000-kDa) and exclusively involved in the biosynthesis of these natural products. NRPSs possess a modular structure containing highly conserved regions, specifically involved in the activation and condensation of amino acids, whereas PKSs use acyl coenzyme A monomers. Moreover, additional modifications in the structure of non-ribosomal peptides and polyketides can be introduced by genetic manipulation in order to re-design substrate specificity, allowing the production of novel natural products. Therefore, the recognition of the detection of PKS and NRPS in the environment is important for future drug discovery and combinatorial biosynthesis efforts (Schwarzer and Marahiel, 2001).

The aim of the present work was to examine the combined effect of light intensity, temperature and different source of sugars in the solid culture and also, sequencing and analyzing the genes responsible for the production of secondary metabolites from two cyanobacterial strains. Result showed that high light intensity (100 to 150 µmol photon m^{-2} s^{-1}) + 30±2°C and glucose enhanced the biological activity when compared to autotrophic condition. Different methods reported so far for the screening of microbial cell extracts, mostly rely on autotrophic condition. This work is the first on the subject of culturing in solid media on mixotrophic condition at high light intensity and has more advantages in maximization of antibiotic production of two *Nostoc* species.

MATERIALS AND METHODS

Culture conditions, morphological characterisation and preparation of extracts for bioactivity analyses

Two *Nostoc* strains from the Tarbiat Moallem University, Tehran, Iran were selected on the basis of initial morphological characteristics and released polysaccharide (RPS) production under N_2-fixing conditions. In this connection, it is worth mentioning that *Nostoc* species ASN_M (JF272482) is a capsulated strain and *Nostoc* species FSN_E (JF795278) is a non-capsulated strain that produces a high amount of exopolysaccharides that are completely released into the culture media. In fact, they distribute on arid and semi-arid paddy fields of Iran with great economic value as biologic fertilizers. The strains were acclimated to autotrophic (liquid BG-11_0 medium, Rippka et al., 1979) and mixotrophic conditions in the presence of glucose and sucrose in aerated cultures maintained in logarithmic phase for several months before the beginning of the experiments. Cultures were carried out axenically in Petri dishes containing 20 to 25 ml of the appropriate solid medium (BG11_0) with glucose and sucrose separately at an initial concentration 4.5 g l^{-1}. After autoclaving and cooling, the pH of medium was about 7.4.

In this study, the solid medium was used for obtaining the exact amount of extraction and total biomass can be collected from the surface of the Petri dishes. Petri dishes were inoculated axenically with 1 ml of 10 to 25 day-old cultures and incubated under continuous illumination provided by an array of cool white fluorescent tubes giving a mean photon flux density of 150 µmol photon m^{-2} s^{-1} at the flask surface at 30°C (Liu and Chen, 2003).

Morphological observations were made on solid media. Thallus growth, filament shapes were evaluated by bright-field microscopy and by phase-contrast illumination of 10-day-old cultures using a Leica DM750 microscope.

At the stationary phase of growth (15 days), the collected biomass was freeze-dried and re-suspend in methanol (1 ml) and collected in 2 ml plastic tubes containing approximately 200 ml of 500 mm glass beads (Scientific Industries, New York) using Fast Prep homogenizer (FP120, Bio 101, Savant) at speed value of 6.5 mm/s. After centrifugation at 20000 rpm for 5 min, supernatant at concentration of 500 mg ml^{-1} was collected, stored refrigerated at 4°C until bioactivity analyses were performed.

Evaluation of antimicrobial activity

The microorganisms used in antibacterial assay were collected from the Iranian Research Organization for Science and Technology (IROST). Extracted compounds of the two strains were examined for bioactivity against the yeast *Candida albicans* (ATCC 10231), the fungus *Aspergillus flavus* (ATCC 16404), three Gram-positive bacteria *Staphylococcus aureus* (PTCC 1112), *Bacillus subtilis* (PTCC 1023), *Bacillus cereus* (PTCC 1015) and three Gram-negative bacteria *Escherichia coli* (PTCC 1047), *Pseudomonas aeroginosa* (PTCC 1310), *Salmonella typhimurium* (PTCC 1609) by disk diffusion assays.

Müller-Hinton agar plates were inoculated with a standardized quantity of suspension containing 1.5×10^8 cfu ml^{-1} bacteria corresponding to 0.5 MacFarland standards according to NCCLS (now CLSI) (1997). Final inoculums density of 1.0×10^7 cfu ml^{-1} yeasts and 1.0×10^5 cfu ml^{-1} filamentous fungi was calibrated by using a hemacytometer cell counting chamber. 100 µl of the methanolic cyanobacterial extracts and 100 µl MeOH as negative controls were pipetted into the 6 mm diameter filter paper discs in assay plates. Plates were incubated overnight at 37°C for a period of 18 to 24 h for bacteria and at 27°C, for 24 to 48 h for fungi. The diameter of the zones with complete inhibition of growth were measured to the nearest millimeters using a ruler, and expressed in mm.

All the tests were performed under sterile conditions and repeated three times. The antibacterial agent tetracycline (30 µg) and gentamicin (10 µg) and antifungal agent nystatin (10 µg) were included in the assays as positive standard antibiotic control (Espinel-Ingroff, 2007). The data of all the parameters were statistically analyzed using the one-way analysis of variance (ANOVA) with 95% confidence limits ($P<0.05$) and expresses as mean ± SE. The following formula was used for comparison of the antimicrobial activity of the sample with that of the standard (antimicrobial index):

$$\text{Antimicrobial index} = \frac{\text{Inhibition zone of sample}}{\text{Inhibition zone of the standard}} \times 100$$

Determination of stability of antimicrobial activity

Filter discs with methanol extracts were stored in Petri dishes at room temperature for 10 days and the inhibition zones were measured at weekly intervals (Qstensvik et al., 1998). For determination of stability of biomass in the next generation, we collect the biomass for the second time and after 2 weeks, the antibiogram bioassays were applied again.

Quantifying antimicrobial effects, estimating minimum inhibitory concentration (MIC)

Glucose grown cultures extracts, recording maximum inhibitions in disc diffusion assay were used in further bioassays for more evaluation of results about the quantity of the compound with antimicrobial activity by MIC of µg ml^{-1}, according to the standard reference method (NCCLS, 2008).

MIC of methanolic extracts was determined by broth dilution method. The MIC for fungi was carried out in 10 tubes (16×160 mm) containing 1 ml Saubouraud's dextrose broth with 1 ml of various dilutions of cyanobacterial extracts and 100 µl of suspension containing 10^4 spore ml^{-1} of fungi cultures. The required concentration of the extract was dissolved in 1 ml of Saubouraud's dextrose broth and dilutes to give serial two-fold dilution ranging from 500 to 0.97. The tube of 11 and 12 were considered as control containing 1 ml Saubouraud's dextrose broth with 1 ml of cyanobacterial extracts (500 mg ml^{-1}) and 1 ml Saubouraud's dextrose broth with 100 µl of fungi cultures, respectively. Tubes were incubated at 27°C for 2 days. Fungi growth in tubes was assessed visually. One microliter (1 µl) of supposed tested broth was placed on the sterile Saubouraud's dextrose agar as the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate. If there is no visible growth, MIC is considered as MBC.

For bacteria, the MIC was determined according to the standard of CLSI guidelines. Mueller-Hinton agar plates with active principle (500 to 0.97 µg ml^{-1}) were spotted with 2 µl bacterial inoculum (10^7 cfu ml^{-1}) and incubated (37°C, 20 h). The MIC was determined as the lowest concentration of cyanobacterial extract resulting in complete inhibition of fungi and bacteria growth after the incubation time. There were three replicates per assay and each test was repeated at least twice.

Genomic DNA extraction

Genomic DNA of tow *Nostoc* strain was extracted utilising the E.Z.N.A® SP Plant DNA kit (Omega Bio-tek). The microtube containing 100 mg wet cells was supplemented with 300 mg two differently sized glass beads (acid-washed, 180 µm and 425 to 600 µm, Sigma-Aldrich) as well as lysis buffer and RNase solution, both as provided by the kit. In order to ensure proper disruption of cells, tubes were homogenised three times for 20 s at a speed of 6.5 ms^{-1} with a FastPrep instrument (Savant Instruments). The extraction procedure was continued according to the kit's protocol supplied by the manufacturer. DNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc).

Polymerase chain reaction (PCR) amplifications

PCRs were performed by Primus advanced Thermal Cycler (MWG, Germany) with 50 µl reaction mixtures each containing 1.5 mM $MgCl_2$, 0.2 mM of dNTP, 1× PCR buffer, 20 pmol of each primer, 1.25 U of *Taq* DNA polymerase and 1 µg template DNA. The reactions were run with the following holds and cycles: 94°C for 5 min; 35 cycles of 94°C for 1 min, 51°C for 1 min, 72°C for 1.5 min and 72°C for 7 min. PCR products were checked by electrophoresis on 0.7% agarose gel (Promega, Mannheim, Germany) followed by 1 µl ethidium bromide staining and visualization on an ultraviolet (UV) transilluminator. Gels were documented with a Kodak DC290 camera and the Kodak 1D imaging program, version 3.5.0. The sizes of fragments were estimated by comparison to fragments of the size marker. Purification of amplicons was performed with the Geneclean Turbo kit (Q-Biogene) before sequencing. PCR product concentration was determined by a Nondrop (ng/µl) and sequencing PCR was performed with 10 µl reaction mixtures each containing 1× PCR buffer (1 µl), 2 µl of sequencing buffer, 1 µl of big dye and 3.3 µl PCR product. The PCR programme followed by 25 cycles consisting of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. Sequence data were obtained for both strands using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed on the ABI 310 Genetic Analyzer.

Sequence analyses

Nucleotide sequences were translated into peptide sequences by using BioEdit version 7.0.9.0 (Hall, 2004). Peptide sequences were first aligned with Clustal W (Thompson et al., 1994) with the default settings and then manually edited in BioEdit. A BLASTX search was used to detect similar sequences deposited in the GenBank™ database of NCBI (http://www.ncbi.nlm.nih.gov/). Identifications of the predicted amino acid activated by a specific unknown NRPS A module was performed using software located at http://www.tigr.org/ Jravel/nrps (Challis et al., 2000). The activation of amino acids by the identification A-domain motif will indicate their presence, unmodified or modified in the final natural product structure. Unrooted phylogenetic trees using the neighbor-Joining method (Poisson correction model) and maximum Likelihood (Jones–Taylor–Thornton model) analyses were constructed using MEGA5 software package (version 5.0); complete deletion handling of gaps and confidence levels were calculated via bootstrapping using a resampling number of 1,000. Reference sequences were obtained from GenBank (NCBI).

Nucleotide sequence accession numbers

The sequences presented in this study was deposited in GenBank and had accession numbers JF795278 and JF795279 (NRPS A domains) and JF795280 and JF795281 (PKS KS domains).

RESULTS

Morphological characteristics and assaying *Nostoc* glucose (G) grown cultures and sucrose (S) grown cultures extracts for antimicrobial activity

In sucrose grown cultures, *Nostoc* species filaments tended to be spiral form, but in the glucose grown cultures, filaments appeared to be directed (Figure 1).

Nostoc spesies can grow mixotrophically at high light intensity using glucose, sucrose as substrates and a greater production of antibacterial and antifungal can be reached compared with the autotrophic growth using only mineral medium (Figures 2, 3 and 4).

Preliminary results of bioactive components' stability indicated that MeOH extracts in both species lost about 85% of the inhibitory effect against of *Aspergillus niger*

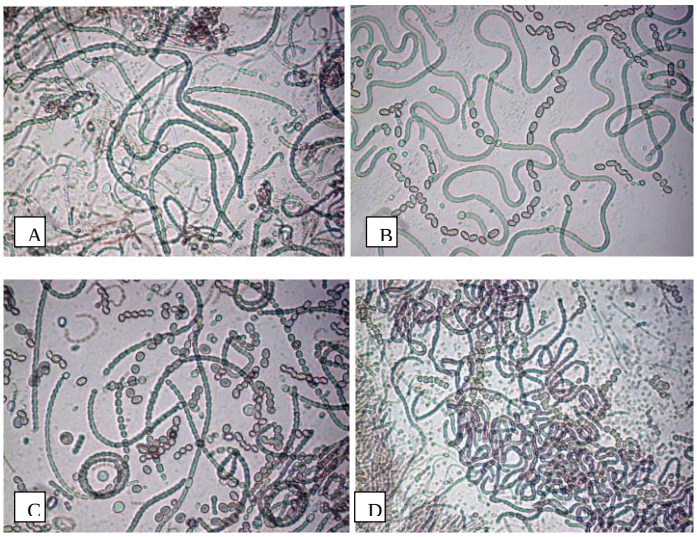

Figure 1. Microphotographs of *Nostoc* species FSN_E (A and B) and *Nostoc* species ASN_M (C and D), grown in solid BG-11_0 medium in the presence of glucose (left) and sucrose (right) (x 400).

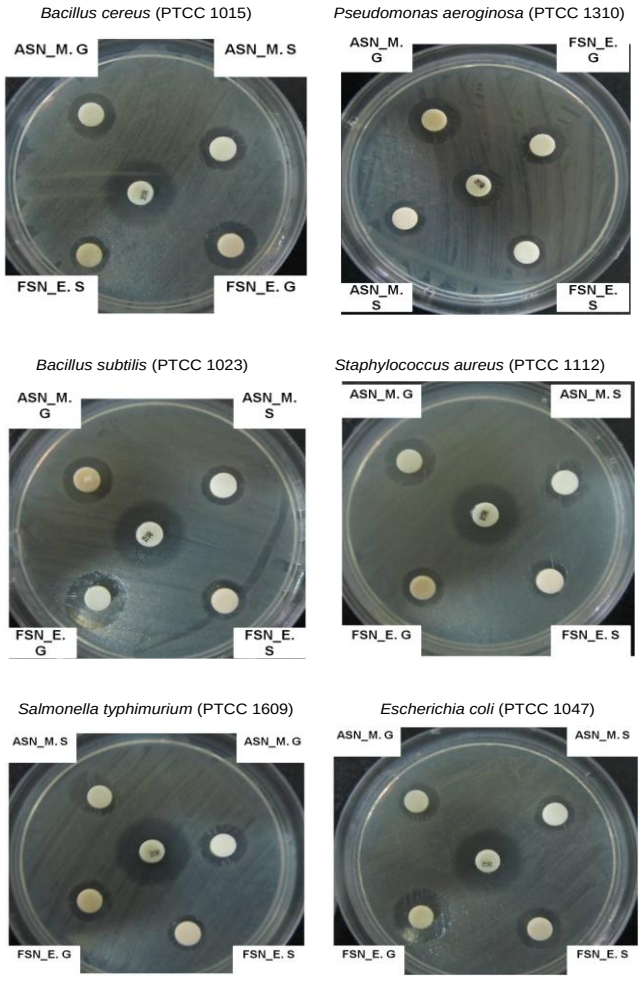

Figure 2. Zone of inhibition exhibited by extracts grown in glucose (G) and sucrose (S) medium against different bacteria.

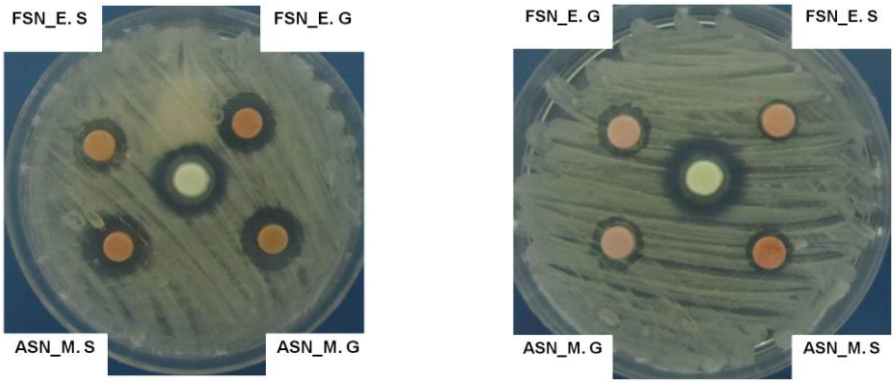

Figure 3. Zone of inhibition exhibited by extracts grown in glucose (G) and sucrose (S) medium against *C. albicans* ATCC 10231. The zone of inhibition extracts in the first collection is shown in the left figure and in the second collection is shown in the right figure.

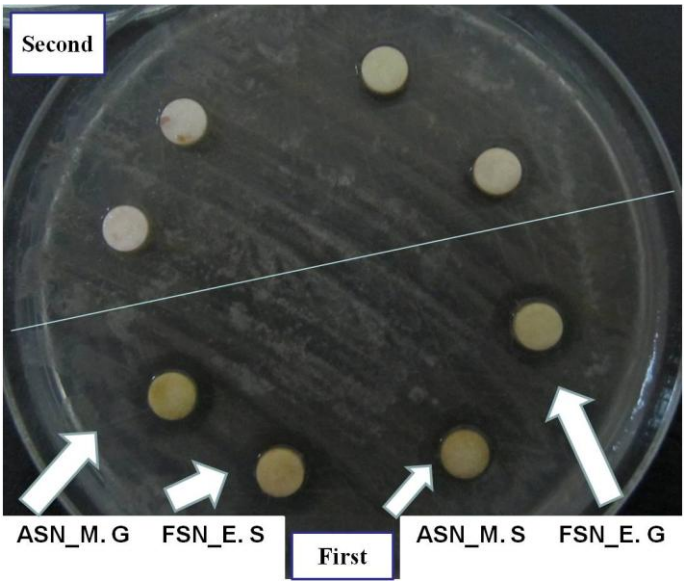

Figure 4. Zone of inhibition exhibited by extracts grown in glucose (G) and sucrose (S) medium against *A. niger* ATCC 16404. The zone of inhibition extracts in the first collection is shown down and in the second collection it is shown in the up of the figure.

and *C. albicans* after 5 days and ~50% against of bacteria after 10 days (expressed as reduction of zone size) storage at room temperature (data not shown).

The response of inhibitory zone of extracts grown in glucose and sucrose medium against bacterial and fungal was different in both strains (Tables 1 to 6). Zone of inhibition in glucose grown cultures was reaching ~2.0 to 2.5 and ~1.5 times greater than that of the autotrophic and sucrose grown cultures, respectively (Tables 1 to 6). This case proves that glucose is the best used carbohydrate in *Nostoc* species. The statistical analysis revealed a significant decrease in the inhibitory effect of the biomass from second collection (Figures 3 and 4).

As mentioned, the quantity of the compound produced antimicrobial activity measured by MIC of active crude extracts and results are shown in Tables 7 and 8. The results indicated that the MICs of methanol extract in glucose grown cultures of *Nostoc* species FSN_E were 3.9 μgml^{-1} against *B. subtilis* and *P. aeroginosa* whereas it was more than 7.81 against *B. cereus*, *E. coli*, *S. aureus* and *S. typhimurium.* The MICs of *Nostoc* species ASN_M were 3.9 μgml^{-1} against *B. cereus*, *S. aureus* and *S. typhimurium,* whereas it was more than 7.81 against *B. subtilis, E. coli* and *P. aeroginosa*. Also MIC of methanol extract of *Nostoc* species against *A. niger* and *C. albicans* is shown in Tables 9 and 10.

Table 1. Antibacterial activity of *Nostoc* species FSN_E and *Nostoc* species ASN_M extracts (glucose and sucrose grown cultures) in first collection as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses).

Microorganism	FSN_E. S	FSN_E. G	ASN_M. S	ASN_M. G	Standard (mm)
B. subtilis (PTCC 1023)	12.3± 0.6 (72.54)	14.3± 1.3 (84.29)	10.9±0.16 (64.11)	12.16±0.16 (71.56)	17
B. cereus (PTCC 1015)	10.83±0.16 (61.90)	12.5±0.76 (71.42)	12.16±0.44 (69.52)	12.93±0.34 (73.90)	17.5
E. coli (PTCC 1047)	11.66±0.33 (70.70)	13±2.51 (78.78)	10.3±0.88 (62.62)	10.83±0.44 (65.65)	16.5
S. aureus (PTCC 1112)	9.3± 0.33 (58.33)	12.5±1.040 (78.12)	12.33±0.33 (77.08)	13.5±0.28 (90.62)	16
P. aeroginosa (PTCC 1310)	12± 0.57 (70.58)	13.8±0.44 (81.37)	9.83±0.44 (57.84)	12.5±0.28 (73.52)	17
S. typhimurium (PTCC 1609)	11.83±0.83 (67.61)	13.5±0.76 (77.14)	12.5±0.76 (71.42)	13.9±0.28 (88.57)	17.5

Results are means ± SE.

Table 2. Antibacterial activity of *Nostoc* species FSN_E and *Nostoc* species ASN_M extracts (glucose and sucrose grown cultures) in second collection as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses).

Microorganism	FSN_E. S	FSN_E. G	ASN_M. S	ASN_M. G
B. subtilis (PTCC 1023)	11.9±0.1 (70)	13.5±±.31 (79.41)	9.9±0.46 (58.23)	11.1±0.28 (65.29)
B. cereus (PTCC 1015)	10.1±0.02 (57.71)	11.7±0.187 (66.85)	11.5±0.18 (65.71)	12±0.134 (68.57)
E. coli (PTCC 1047)	11.04±0.1 (66.90)	12±0.1 (72.72)	9.56±0.37 (57.93)	10.1±0.61 (61.21)
S. aureus (PTCC 1112)	8.9±0.04 (55.62)	12±0.12 (75)	12±0.15 (75)	13.9±0.61 (86.87)
P. aeroginosa (PTCC 1310)	11.5±0.12 (67.64)	12.9±0.18 (75.88)	9±0.2 (52.94)	11.3±0.5 (66.47)
S. typhimurium (PTCC 1609)	10.9±0.45 (62.28)	12.1±0.2 (69.14)	12±0.49 (68.57)	13±0.006 (74.28)

Results are means ± SE.

Table 3. Antibacterial activity of *Nostoc* species FSN_E and *Nostoc* species ASN_M extracts (Autotrophic condition) as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses).

Microorganism	FSN_E	ASN_M
B. subtilis (PTCC 1023)	10.16± 0.72 (59.76)	8.5± 0.29 (50.29)
B. cereus (PTCC 1015)	8.14± 0.09 (46.51)	8.4± 0.25 (48)
E. coli (PTCC 1047)	10.5± 0.28 (63.63)	9.2± 0.14 (56.18)
S. aureus (PTCC 1112)	9.16± 0.16 (57.25)	11.9± 0.3 (74.43)
P. aeroginosa (PTCC 1310)	11.06± 0.520 (65.09)	8.6± 0.2 (50.58)
S. typhimurium (PTCC 1609)	10.5± 0.28 (60)	11.6± 0.2 (66.62)

Results are means ± SE.

Table 4. Antifungal activity of *Nostoc* species FSN_E and *Nostoc* species ASN_M extracts (glucose and sucrose grown cultures) in first collection as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses).

Microorganism	FSN_E. S	FSN_E. G	ASN_M. S	ASN_M. G	Standard (mm)
A. niger ATCC 16404	11.3±0.12 (68.48)	12.4±0.42 (75.39)	11.14±0.61 (67.51)	13.55±0.18 (82.12)	16.5
C. albicans ATCC 10231	11.3±0.45 (66.64)	13.3±0.49 (78.70)	10.2± 0.5 (60.41)	12.33±0.37 (72.52)	17

Results are means ± SE.

Identification of KS and NRPS regions and phylogenetic analysis of gene homologues

BLAST analysis in GenBank (tBlastn) confirmed that the sequenced clones (Figure 5) were cyanobacterial NRPS A or PKS KS domains, as all sequences were most similar to cyanobacterial A or KS domains already present in GenBank. BLASTx sequence analysis of these clones showed varying similarities to other known cyanobacterial NRPS and PKS sequences.

Table 5. Antifungal activity of *Nostoc* species FSN_E and *Nostoc* species ASN_M extracts (glucose and sucrose grown cultures) in second collection as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses).

Microorganism	FSN_E. S	FSN_E. G	ASN_M. S	ASN_M. G
A. niger ATCC 16404	9.7±0.1 (58.78)	9.98±0.21 (60.48)	10.73±0.31 (65.03)	10.9±0.1 (66.06)
C. albicans ATCC 10231	11±0.13 (64.70)	11.5±0.46 (67.64)	10.2±0.15 (60)	10.5±0.18 (61.76)

Results are means ± SE.

Table 6. Antifungal activity of *Nostoc* species FSN_E and *Nostoc* species ASN_M extracts (Autotrophic condition) as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses).

Microorganism	FSN_E	ASN_M
A. niger ATCC 16404	9.5±0.28 (57.57)	10.4±0.2(63.13)
C. albicans ATCC 10231	10.98±0.006 (64.58)	10.2±0.36(60.19)

Results are means ± SE.

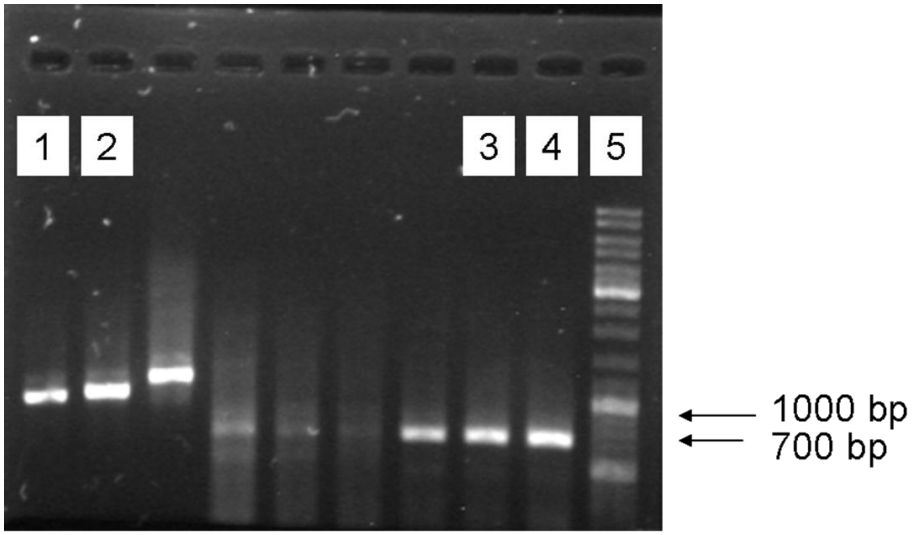

Figure 5. Agarose gel electrophoresis of degenerate PCR products from DNA isolated from representative *Nostoc* strains. Lanes 1 and 2, Selective amplification of ~1 kb fragments using primers specific for NRPS adenylation sequences of *Nostoc* species FSN_E and *Nostoc* species ASN_M, respectively; lanes 3 and 4, selective amplification of ~700 bp fragments using specific primers for PKS KS domains of *Nostoc* species FSN_E and *Nostoc* species ASN_M, respectively. Lane 5, 1 kb DNA ladder.

The trees obtained from the neighbor-Joining method were congruent to that using the maximum likelihood method. Phylogenetic analysis was performed on the putative peptide synthase protein sequences from the two *Nostoc* species screened from the present study, and additional sequences of enzymes from species that showed high similarity to the sequences identified (Figure 6). This analysis indicated two major clusters that grouped separate and relatively further from known peptide sequences (the clade comprising sequences *Nostoc* species ASN_M and the clade *Nostoc* species FSN_E) (Figure 6).

The phylogenetic analysis clustered the NRPS sequence of *Nostoc* species FSN_E in a strongly supported monophyletic group (bootstrap value of 70/84% (NJ/ML)), while *Nostoc* species ASN_M sequence falls into a subclade also highly supported (bootstrap value of 96/96% (NJ/ML)).

Identifications of the predicted amino acid activated by NRPS A module and the probably name of the compound was performed using software located at http://www.tigr.org/ jravel/nrps. Analysis showed that two

Table 6. Antifungal activity of *Nostoc* species FSN_E and *Nostoc* species ASN_M extracts (Autotrophic condition) as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses).

Microorganism	FSN_E	ASN_M
A. niger ATCC 16404	9.5±0.28 (57.57)	10.4±0.2 (63.13)
C. albicans ATCC 10231	10.98±0.006 (64.58)	10.2±0.36 (60.19)

Results are means ± SE.

Table 7. MIC of *Nostoc* species FSN_E (glucose grown cultures) extracts.

Microorganism	250	125	62.5	31.25	15.62	7.81	3.9	1.95	0.97	MIC
B. subtilis (PTCC 1023)	-	-	-	-	-	-	-	+	+	3.9
B. cereus (PTCC 1015)	-	-	-	-	-	-	+	+	+	7.81
E. coli (PTCC 1047)	-	-	-	-	-	-	+	+	+	7.81
S. aureus (PTCC 1112)	-	-	-	-	-	-	+	+	+	7.81
P. aeroginosa (PTCC 1310)	-	-	-	-	-	-	-	+	+	3.9
S. typhimurium (PTCC 1609)	-	-	-	-	-	-	+	+	+	7.81

(-), No growth observed; (+), growth observed. Concentration of extracts, in µg ml^{-1}.

Table 8. MIC of *Nostoc* species ASN_M (glucose grown cultures) extracts.

Microorganism	250	125	62.5	31.25	15.62	7.81	3.9	1.95	0.97	MIC
*B. subtilis (*PTCC 1023)	-	-	-	-	-	-	+	+	+	7.81
B. cereus (PTCC 1015)	-	-	-	-	-	-	-	+	+	3.9
E. coli (PTCC 1047)	-	-	-	-	-	-	+	+	+	7.81
S. aureus (PTCC 1112)	-	-	-	-	-	-	-	+	+	3.9
P. aeroginosa (PTCC 1310)	-	-	-	-	-	-	+	+	+	7.81
S. typhimurium (PTCC 1609)	-	-	-	-	-	-	-	+	+	3.9

(-), No growth observed; (+), growth observed. Concentration of extracts in µg ml^{-1}.

Table 9. MIC of *Nostoc* species FSN_E (glucose grown cultures) extracts.

Microorganism	250	125	62.5	31.25	15.62	7.81	3.9	1.95	0.97	MIC
A. niger ATCC 16404	-	-	-	-	-	-	-	+	+	3.9
C. albicans ATCC 10231	-	-	-	-	-	-	-	-	+	1.95

(-), No growth observed; (+), growth observed. Concentration of extracts in µg ml^{-1}.

Table 10. MIC of *Nostoc* species ASN_M (glucose grown cultures) extracts.

Microorganism	250	125	62.5	31.25	15.62	7.81	3.9	1.95	0.97	MIC
A. niger ATCC 16404	-	-	-	-	-	-	+	+	+	7.81
C. albicans ATCC 10231	-	-	-	-	-	-	-	+	+	3.9

(-), No growth observed; (+), growth observed. Concentration of extracts in µg ml^{-1}.

strains screened possessed only 1 A-domain, while the remaining possessed 2 or more. These A-domains can result in the production of one compound, or multiple independent compounds. The signature sequence, the name of the compound and the predicted amino acid of two species were the same (Table 11). Clustering of

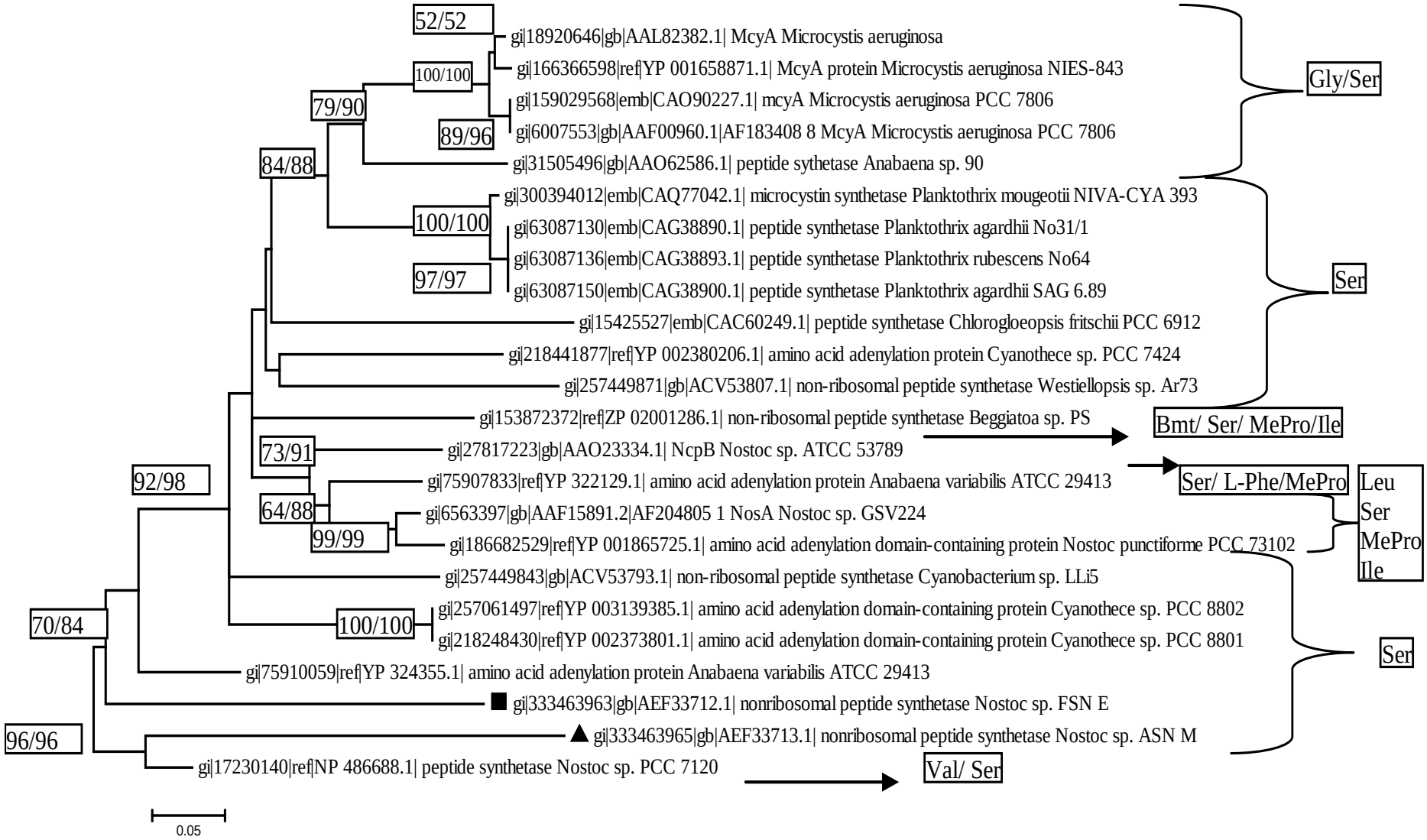

Figure 6. Phylogenetic analysis of two translated A domains sequenced in this study or taken from the GenBank. The PCR-fragment is ~1 kb long. The significant bootstrap percentages obtained from 1,000 replicates using neighbour-joining (NJ) as well as from 100 replicates using maximum likelihood (ML), greater than 50%, indicated in the near of nodes, respectively. The studied *Nostoc* species ASN_M and *Nostoc* species FSN_E are shown in full triangle and full square, respectively. The scale indicates 0.05 mutations per amino acid position.

Table 11. Cyanobacterial NRPS sequences analyzed using the BLASTp tool for natural product biosynthesis.

Strain	Accession numbera [a]	Compound [e]	Signature sequencec [c]	Predicted amino acid [d]
***Nostoc* sp. FSN_E**	**AEF33712**	**Nostopeptolide synthetase**	**D V W H I S L I**	**NosA-M2-Ser**
***Nostoc* sp. ASN_M**	**AEF33713.1**	**Nostopeptolide synthetase**	**D V W F I S L I**	**NosA-M2-Ser**
Nostoc sp. PCC 7120	NP_486688	Tyrocidine synthetase 3	D A F W I G G T	TycC-M4-Val
		Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Anabaena variabilis ATCC 29413	YP_322129.1	Nostopeptolide synthetase	D V Q F I A Q L	NosA-M3-mePro
		Tyrocidine synthetase 1	D A W T I A A V	TycA-M1-D-/L-Phe
		Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Planktothrix rubescens NIVA-CYA 98	CAQ48259.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Beggiatoa sp. PS	ZP_02001286.1	Nostopeptolide synthetase	D V W H I S L V	NosA-M2-Ser
Nostoc sp. GSV224	AAF15891.2	Microcistin synthetase B	D A W F L G N V	McyB-M1-Leu
		Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
		Nostopeptolide synthetase	D V Q F I A H L	NosA-M3-mePro
		Anabaenopeptilide synthetase D	D A F F L G X X	AdpD-M1-Ile
Cyanothece sp. PCC 8802	YP_003139385.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Cyanothece sp. PCC 8801	YP_002373801.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Nostoc sp. ATCC 53789	AAO23334.1	Cyclosporine synthetase	D A W T I G G V	CssA-M5-Bmt
		Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
		Nostopeptolide synthetase	D V Q F I A H -	NosA-M3-mePro
		Anabaenopeptilide synthetase D	D A F F L G V T	AdpD-M1-Ile
Anabaena sp. 90	AAO62586.1	Microcistin synthetase A	D L F N N A L T	McyA-M2-Gly
		Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Chlorogloeopsis fritschii PCC 6912	CAC60249.1	Nostopeptolide synthetase	D V W L I S L I	NosA-M2-Ser
Nostoc punctiforme PCC 73102	YP_001865725.1	Microcistin synthetase B	D A W F L G N V	McyB-M1-Leu
		Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
		Nostopeptolide synthetase	D V Q F I A H L	NosA-M3-mePro
		Anabaenopeptilide synthetase D	D A F F L G V T	AdpD-M1-Ile

Table 11. Contd.

Microcystis aeruginosa	AAL82382.1	Microcistin synthetase A Phosphitricin synthetase	D L F N N A L T D V W H F S L I	McyA-M2-Gly PhsB-M1-Ser
Cyanobacterium sp. LLi5	ACV53793.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Cyanothece sp. PCC 7424	YP_002380206.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
M. aeruginosa NIES-843	YP_001658871.1	Microcistin synthetase A Phosphitricin synthetase	D L F N N A L T D V W H F S L I	McyA-M2-Gly PhsB-M1-Ser
M. aeruginosa PCC 7806	CAO90227.1	Microcistin synthetase A Phosphitricin synthetase	D L F N N A L T D V W H F S L I	McyA-M2-Gly PhsB-M1-Ser
A. variabilis ATCC 29413	YP_324355.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Westiellopsis sp. Ar73	ACV53807.1	Nostopeptolide synthetase	D V W H I S L V	NosA-M2-Ser
Planktothrix mougeotii NIVA-CYA 393	CAQ77042.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
P.x rubescens No64	CAG38893.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
Planktothrix agardhii SAG 6.89	CAG38900.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
P. agardhii No31/1	CAG38890.1	Nostopeptolide synthetase	D V W H I S L I	NosA-M2-Ser
M. aeruginosa PCC 7806	AAF00960.1	Microcistin synthetase A Phosphitricin synthetase	D L F N N A L T D V W H F S L I	McyA-M2-Gly PhsB-M1-Ser

[a], Accession numbers corresponding to the NCBI website for amino acid sequences; [c], eight variable amino acids of the signature sequences; [d], Nomenclature of the reference compounds; [e], the compound here is for illustrative purposes, in that the domain is similar. This does not imply the strain makes this exact compound. Sequences identified in this study are marked in bold and the other sequences were retrieved from the GenBank.

these NRPS sequences, illustrates a lack of taxonomic affiliations between cyanobacteria and A-domains (Table 11). For example, few instances of close relationship between the taxonomic status and the predicted compound were evident (Figure 6).

Clustering was also performed according to substrate-binding pocket specificity (Figure 6). These include valine (Val), glycine (Gly), serine (Ser), leucine (Leu), isoleucine (Ile), methylproline (MePro), L - phenylalanine (L- Phe) and non-protein amino acids such as 2(S)-amino-3(R)-hydroxy-4(R)-methyl-6(E)-octenoic acid (Bmt). As shown in Figure 6, the activation of serine is predicted for all of the modules from the different cyanobacterial isolates (Table 11).

DISCUSSION

In two decades, cyanobacteria of the genus *Nostoc* are known to produce a wide range of new bioactive compounds having pharmacological significance. These active compounds exhibit a wide range of bioactivities that may be relevant to the natural environment such as antibacterial, antifungal, antiviral and cytotoxic activities, and sometimes immunomodualtion and protease inhibition activities which are not related to ecological significance (Biondi et al., 2004; Juttner et al., 2001; Kaushik et al., 2009; Portmann et al., 2009; Dahms et al., 2006).

Many different active substances, mainly

belonging to the chemical classes of cyclic peptides, alkaloids and macrocycles, have been already identified in *Nostoc* species (Rezanka and Dembitsky, 2006; Dembitsky et al., 2005).

The production of bioactive compounds and expression of antimicrobial activity depends on physiological factors such as the presence of organic compounds, stage ofgrowth and culture conditions. Two-fold increase in antibiotic production under optimum light conditions has been reported by Chetsumon and et al. (1994). These results go in harmony with those obtained by Radhakrishnan et al. (2009) and Borsari et al. (2007).

There is a growing interest in isolating antimicrobial substances from cyanobacteria. In different studies, the antimicrobial effects of *Nostoc* (Asthana et al., 2009), *Phormidium* (Fish and Codd, 1994), *Anabaena*, *Oscillatoria*, *Pseudoanabaena*, *Synechocystis* (Kreitlow et al., 1999), *Oscillatoria anguistisima* and *Calothrix parietina* (Issa, 1999) extracts on some pathogenic organisms have been reported. They have also reported that the extracts in different solvents were effective against both Gram-positive and Gram-negative organisms. This is in agreement with our findings in two methods' agar- well diffusion and MIC that *Nostoc* species ASN_M had the highest antimicrobial activity range against *B. cereus*, *S. aureus, S. typhimurium and A. niger* while *B. subtilis, E. coli, P. aeroginosa and C. albicans* have proven the most sensitive target for antimicrobial activity in the *Nostoc* species FSN_E (Figures 2, 3 and 4) (Tables 1 to 10).

The variation in antifungal and antibacterial activities could be due to different permeabilities of bioactive substances into the test organisms. However, when compared with the standard, tetracyclines, gentamicin and nystatin, all methanol extracts exhibited lower antimicrobial activity (Figures 2, 3 and 4).

It is worth mentioning that although studies have been carried out in the past on antimicrobial compounds with inhibitory effects on bacteria and fungi, no data on the effect of extreme mixotrophic condition on maximum bioactive compound production in solid media were available in the literature and this screening program is among the first studies done for assessment of antimicrobial activity of Iranian paddy field cyanobacteria.

The success of the method lies in the fact that preliminary screening results give more zone inhibitory diameters. In addition, Skulberg (2000) has emphasized on exercising imagination and creativity in investigating organisms from untried biotopes and develop new methodologies to exploit them.

It has been emphasized that cyanobacterial secondary metabolites are produced through large multienzyme complexes, constituted by NRPS and PKS modules and responsible for the addition of an amino acid or chain-elongation step, respectively. Several authors have performed similar screening of these two genes in cyanobacteria as relates to a specific compound (Lerena et al., 2007; Moffitt and Neilan., 2001; Zhao et al., 2008; Burns et al., 2005; Genuario et al., 2010; Christiansen et al., 2001).

The phylogenetic analysis using neighbour-joining method revealed the same topology and branch lengths as the maximum likelihood tree. Clustering revealed that *Nstoc* species FSN_E falls into a nearly supported major clade (bootstrap value of 70/84% (NJ/ML)). However, *Nostoc* species FSN_E positioned in a separate internal branch within this clade; the *Nostoc* species ASN_M formed a distinct cluster well supported by high bootstrap value (bootstrap value of 96/96% (NJ/ML)) and distantly related to *Nostoc* species FSN_E (Figure 6).

The results of this study showed that two *Nostoc* species assayed for natural products had both NRPS and/or PKS genes with biologically detectable activities. However, until now, there is not any evidence to determine if NRPS and/or PKS genes underlie the detected activities. Ehrenreich and et al. (2005) found that both *Lyngbya* species strain PCC7419 and *Cyanothece* species strain WH8904 were negative for NRPS and PKS genes but both had detectable activities. Actually, further studies have to be made on connecting the biochemical activities and the existence of the NRPS and PKS genes.

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