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Identification and structure elucidation of antimicrobial compounds from *Lyngbya aestuarii* and *Aphanothece bullosa*

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Abstract

Cyanobacteria are known to produce array of compounds. In an earlier report, we reported antibacterial and antifungal activities in methanolic crude extracts of laboratory grown *Lyngbya aestuarii* and *Aphanothece bullosa* isolated from Chilka Lake and local paddy field respectively. In this report the same methanolic crude extracts were subjected to TLC purification twice by altering the solvents and UV-illuminated bands bioassayed. Such UV illuminated potent bands obtained after 2nd TLC were subjected to spectroscopic analysis (UV, IR, ^{1}H NMR and LCMS/MS). We have screened malyngolide and dragonamide C from *L. aestuarii* and a diterpenoid and majusculoic acid from *A. bullosa*. Dragonamide C and malyngolide were found to be antifungal while majusculoic acid and a diterpenoid as antibacterial. As far as our knowledge goes, this is the first ever report where fresh water *A. bullosa* was found to be a source of diterpenoid and majusculoic acid. Likewise, *L. aestuarii* was also established as a source of malyngolide and dragonamide C. This again indicated that cyanobacteria are inherently endowed with the capacity to produce metabolites according to niche and species specific manner emphasising fresh water cyanobacterial strain are as important as marine one.

Key words: *Lyngbya aestuarii*, *Aphanothece bullosa*, TLC purification, spectroscopic analysis, antibacterial, antifungal.

Introduction

Increased drug resistance in bacterial pathogens such as *Staphylococcus aureus* (1), *Streptococcus pneumoniae* (2) and *Pseudomonas aeruginosa* (3) indicated loss of efficacy of conventional antibiotics and has necessitated their replacement. Therefore, search of new drug/ biomolecule/ lead molecule, is a major thrust area at the moment. Modern approaches to drug discovery could not accelerate the pace of newer drug development because of little exploration of natural resources especially microbes. Metabolites produced by actinomycetes, fungi and unicellular bacteria along with cyanobacteria contributed 45%, 38% and 17% respectively (4). Cyanobacteria are photosynthetic O_2-evolving proarch-conservates. These colonize on soil, fresh, marine and hot water habitats including tree-bark, antarctic regions as well as various other extreme conditions. These are potential source for array of secondary metabolites (5,6,7,8). Presence of NRPS and PKS genes made cyanobacteria a treasure of secondary metabolites (9). However, secondary metabolites produced from the cyanobacteria are found to be antibacterial, antifungal, antialgal, anticancerous, antimalarial with many more activities (10,11,12,13).

Among the cyanobacterial genera screened, *Lyngbya* is distributed throughout tropical and subtropical regions and proved as prodigious producer of secondary metabolites (14,15). *Aphanothece* sp. is an unicellular exopolysaccharide producing fresh water cyanobacteria. Exopolysaccharide of *Aphanothece* was reported to be sulphated (16) and have antitumor and antiproliferative properties (17). *Aphanothece* sp. is also little explored with regard to screening of bioactive molecules. Therefore, we have chosen *L. aestuarii* (brackish water) and *Aphanothece bullosa* (fresh water) isolates as a target strains to screen antibacterial and antifungal biomolecules after bioassay of crude extracts as well as TLC purified ones and subjected to spectroscopic analysis for identification of biomolecules.

Materials and methods

Cyanobacterial strains and growth conditions

Lyngbya aestuarii (a brackish water strain from Chilka lake, Orissa, India) and *Aphanothece bullosa* (a fresh water strain from paddy field, around Banaras Hindu University, Varanasi, India), were grown in ASN-III and BG-11 medium, respectively (18) for screening of biological activity. Cultures were maintained at 28±2°C, with a light intensity of 14.40 $Wm^{-2,}$ provided by cool white fluorescent tubes with a light/dark cycle of 18/6 hrs.

Preparation of methanolic extracts

Biomass of *L. aestuarii* and *A. bullosa* were harvested after 40 and 60 days respectively. The harvested biomass was centrifuged at 10,000 rpm for 15 min (Remi, India) and lyophilized (Christ-Alpha 1-2, Germany). Lyophilized cyanobacterial biomass (5 g) was extracted twice in 300 mL methanol (100%) by keeping it on shaker (150 rpm for 48 hrs) and centrifuged at 15,000 rpm (15 min). Supernatants were dried in a rotary evaporator (Perfit, India) at 40 °C redissolved in 3 mL 100% methanol (19) to be used for purification.

TLC purification of crude extracts

Dried methanolic extracts (100 mg) of *L. aestua-*

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rii and *A. bullosa* dissolved in methanol (1mL) for purification using TLC silica plates (TLC 60 M erck, Germany). Separations of crude extracts were done using carbon tetrachloride and methanol (9:1) as mobile phase. Spots developed on such TLC plates were observed under UV illumination. The illuminated orange spots were eluted separately and dissolved in methanol (1 mL). Each eluates again subjected to TLC purification using hexane: ethyl acetate (1:1). Now all spots obtained at second stage were bioassayed for antibacterial and antifungal activities.

Bioassay

The designate spots in 2nd TLC were dried in rotary evaporator. This dried spots were redissolved in 25%, 50%, 70% and 100% of methanol separately. Antibacterial and antifungal bioassays of such samples were performed. The methanol alone (25%) was not able to kill target bacterium and fungi therefore, rest work was done in 25% methanol.

Antibacterial

Antibacterial bioassay was performed using non-pathogenic *Enterobacter aerogenes* MTCC 2822 (IMTECH, India) grown over night in Luria Broth (LB) medium (25 mL). This was carried out on 3.8% Mueller Hinton (MH) agar (Sigma-Aldrich) plates. The target bacterium was suspended in 0.91% NaCl and turbidity adjusted to 10^7-10^8 CFU mL^{-1}, corresponding to 0.5 McFarland standards according to NCCLS 1997 guidelines/ CLSI guidelines (Clinical and Laboratory Standards Institutes) (20). The wells of 4 mm diameter were made using a steel borer and bottom was sealed off soft agar (0.8%). Each MH plates were swabbed with target bacterium aseptically. The spots eluates obtained after second TLC were dissolved in 25% methanol and filled in well along with respective solvents (25% methanol) and Rifampicin (50 µg mL^{-1}) as controls, and incubated at 37°C for 24 hrs thereafter. The diameters of the zones of inhibition were measured in mm.

Antifungal

Antifungal assay was performed by using *Candida albicans*. The target strain was grown on Sabouraud Dextrose Agar (4%, SDA) at 22°C (48 hrs). For antifungal bioassay sterile petri plates, having 4% SDA were prepared. The wells were prepared as described for antibacterial assay. *C. albicans* was suspended in 0.91% NaCl and turbidity was adjusted to 10^7-10^8 CFU mL^{-1}, corresponding to 0.5 McFarland standards according to NCCLS 1997/ CLSI guidelines (20). SDA plate was now swabbed with testing fungal strain aseptically. The TLC spots dissolved in 25% methanol (20µl) were poured in each well along with fluconazole (50 µg mL^{-1}) as controls. The petri plates were kept aseptically in laminar hood for 20 min and incubated at 22°C (24 hrs).

Screening and identification of prospective compounds

UV spectra of antifungal (G1 and L5) and antibacterial bands (F1 and L3) after TLC (2nd TLC) purification from target cyanobacterial extract were recorded in methanol over scanning range of 195-500 nm using double beam spectrophotometer (U-2910, Hitachi, Japan). IR (infra red) spectra of the same spots were obtained using Perkin Elmer spectrum version 10.03.05 as film in KBr disc. ^{1}HNMR (proton nuclear magnetic resonance) spectra of the same spots were recorded on JEOL AL300 FTNMR spectrophotometer (JEOL Ltd., Tokyo,Japan) in downfield from TMS (tetra methyl silane) in DMSO. D6 (dimethyl sulfoxide-d_6) at 300 MHz. LC-MS/MS (liquid chromatography mass spectrometry/ mass spectrometry).

Preparation of unknown samples for LC-MS/MS experiments

1 mg mL^{-1} stock solution of each samples (G1, F1, L5 and L3) were prepared in 50% MeOH containing 0.1% formic acid and was diluted in 50% MeOH containing 0.1% formic acid to reach the final strength of 100 ng mL^{-1}, for LC-MS/MS. For blank run, 50% MeOH containing 0.1% formic acid, was used. Mass grade formic acid, acetonitrile and methanol were purchased from Merck, Germany. Water was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvents were one of the highest analytical grades available.

Information-dependent acquisition (IDA) mode by LC-MS/MS

Chromatography separation was achieved using Thermo Accela UHPLC system (Thermo Electron Corp, Waltham, MA, USA) with a quaternary pump connected to an online degasser, autosampler and photodiode array detector (PDA). Chromquest software version 4.1 was used to control all parameters of UHPLC system. Analytical separation of the unknown compound was achieved on a Purospher STAR RP-18 endcapped (3 µm particles, 55 x 4mm size) column from Merck (Darmstadt, Germany) while column chamber was kept at ambient temperature. The mobile phase consisted of acetonitrile containing 0.1% formic acid (A) and water containing 0.1% formic acid (B) with the following linear gradient program: 0 min 100% B, 30 min 0% B. Mobile phase was pumped at the rate of 0.5 mL min^{-1}. The autosampler tray was kept at ambient temperature. Twenty micro liter of the target sample was injected into the UHPLC with a run time of total 30 min. To identify compound in unknown samples by LC-MS/MS, an IDA method consisting of enhanced mass scan (EMS), enhanced resolution (ER), and enhanced product ion (EPI) scan mode were developed. LC-MS/MS IDA experiment for unknown compounds was carried out on Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer 4000Q TRAP AB Sciex instrument (ABS, Foster City CA, USA) equipped with a Turbo Ion Spray (TIS) source operated in the positive ion mode. Following IDA mass parameters were set for all experiments: Curtain gas= 20; Collisional activated dissociation (CAD) = High; Ion Spary voltage = 5.5 kV; Ion source Temperature= 450°C; Ion source gas-1= 30; and Ion source gas-2= 60. The differential potential (DP) was set at 50. The Collision Energy (CE) was set at 10. Internal heat was kept on. The EMS and EPI was performed with a scan rate of 4000 Da/s. For ER scan, a scan rate of 250 Da/s was performed. All IDA experiments were performed in the range of *m*/*z* 100 to *m*/*z* 1400 with a Q3 entry barrier of 8 V and disabled Q_0-trapping. Linear Ion Trap (LIT) fill time was set at 20 msec. Dynamic

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Table 1. Bioassay of 2nd TLC spots (25µg mL^{-1}) from *L. aestuarii* and *A. bullosa* against bacterial and fungal strain.

Designated		Inhibition zone (mm)	
Spots in 2nd TLC	R*f* value	*E. aerogenes*	*C. albicans*
F1	0.841	3.36±0.185	0.0
G1	0.841	0.0	5.56±0.348
I1	0.0	1.26±0.145	0.0
L1	0.428	0.90±0.208	0.0
L2	0.560	1.16±0.166	2.0
L3	0.645	2.5±0.288	0.0
L4	0.784	0.0	0.0
L5	0.831	0.0	5.13±0.592
25% Methanol	-	0.0	0.0
Rifampicin		11.0±0.564	-
Fluconazole	-	-	9.0±0.577

fill time was kept on. Data acquisition and integration was performed by Analyst 1.4.2. software (ABS, Foster City CA, USA).

Results

Antibacterial and antifungal activity

1st TLC of methanolic crude extracts from both cyanobacteria is represented in Figure 1. The UV illuminated designate bands A, B, C, D, E, F, G from *L. aestuarii* and H, I, J, K, L from *A. bullosa* were prospective potent bands. These potent bands were subjected to 2nd TLC and UV illuminated designate bands (Figure 2) were bioassayed against bacterial and fungal strains. The bioassay revealed that band F1 from *L. aestuarii* and L3 form *A.*

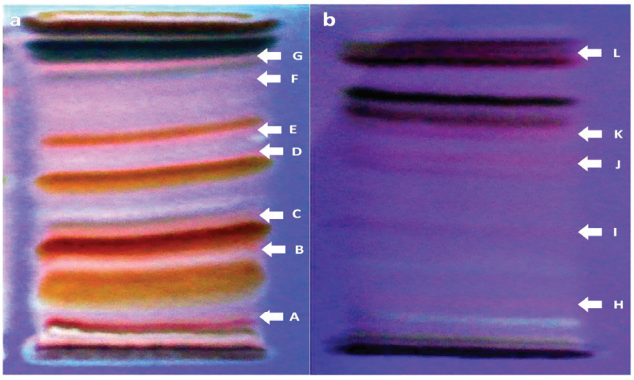

Figure 1. TLC pattern of methanolic crude extracts from *L. aestuarii* (a) and *A. bullosa* (b) using carbon tetrachloride and methanol (9:1) as mobile solvent.

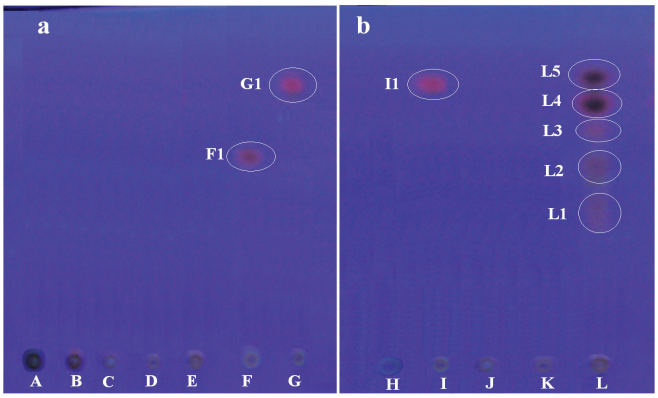

Figure 2. TLC pattern of 2nd TLC spots of *L. aestuarii* (a) and *A. bullosa* (b) using ethyl acetate and hexane (1:1) as mobile solvent.

bullosa were antibacterial while G1 of *L. aestuarii* and L5 of *A. bullosa*, antifungal at 25 µg mL^{-1}. The spots F1 and L3 produced 3.36±0.185 and 2.5±0.288 mm inhibition zones against *E. aerogenes* (Table 1). The purified spots G1 and L5 have 5.56±0.348 and 5.13±0.592 mm inhibition zones. Rifampicin (50 µg mL^{-1}) and fluconazole (50 µg mL^{-1}) were used as positive controls with inhibition zone sizes of 11.0±0.564 and 9.0±0.577mm respectively (Table). The bigger inhibition zones obtained with controls over TLC purified cyanobacterial extracts may be attributed to their purity in nature. Therefore, we have spectroscopically analysed the potent bands obtained after 2nd TLC.

Spectroscopic analysis of potent spots obtained after 2nd TLC

UV absorption of F1 spot showed λ_{max} at 203.5 nm indicated the presence of carboxylic acid/ acetic acid (Figure 3a), IR absorption spectrum of target spots having 3419.06 cm^{-1} implied presence of hydroxyl moiety, however, 1634 (carbonyl, C=O), 2922.20 (CH_3), 2851.83 (C-H or methoxy), 1414.90 (C=C) and 1106.58 (C-O-C) for other groups (Figure 3b). The signal for proton in ^{1}H NMR spectrum revealed shift as: δ 3.393 (2H, d, J= 12 Hz) for isolated oxygen bearing methylene (CH_3-O), δ 2.490 (1H, s, J=30Hz) for carbonyl (C=O), δ 5.306 (1H, s) and δ 1.971 (1H, s) for OH moiety. The other signals as: δ 1.218 (2H, d), δ 0.838 (2H, d), δ 0.817 (3H, t) led towards methyl moiety of the aliphatic chain. The chemical shift δ 8.467 revealed the benzene ring (Figure 3c.). The m/z values of such target spots were as: 958.9, 537.7, 433.5, 415.4, 313.4 and 149.0. The m/z value, 541.44 coincided with described compound malyngolide from cyanobacteria as screened from the data bank available on web (Figure 3d). UV absorption of G1 spot dissolved in methanol showed λ_{max}= 204.5 nm indicating carbonyl or acetic acid (Figure 4a). The IR absorption spectrum of target spot showed 1633.93 and 1384.76 cm^{-1} for amide group (N-H stretching) however, 1102.49 (C=O), 3441. 73 (O-H), 2925.18 (C-H) and 1417.32 cm^{-1} (C=C) for other stretching (Figure 4b). The signal for proton in ^{1}H NMR spectrum at the chemical shifts, δ 2.278 (3H, t, J=7.367) δ 2.993 (1H, s,) of same spot revealed the methane moiety. δ 3.354 (m, H, J=6.840) and δ 3.332 (s, 1H, J=6.840) indicated the amide moiety and other signals δ 0.853 (d, 2H, J=6.833), δ 1.234 (s, 1H), δ 1.604 (s, 1H, J=7.367) and δ 2.278 (t, 3H, J=6.833) for methyl moiety as well as aliphatic nature of compound (Figure 4c). LCMS/MS

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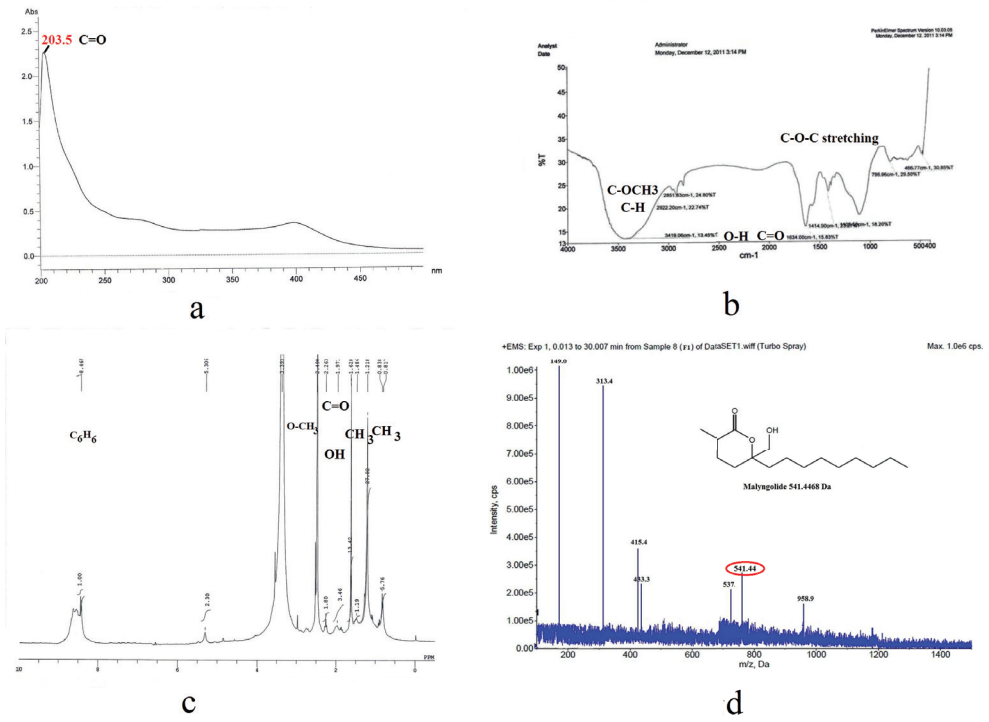

Figure 3. The spot F1 eluate derived from *L. aestuarii* was subjected to spectroscopic analysis. UV Absorption spectrum (a); IR spectrum (b); ^{1}H NMR spectrum (c); and LC MS/MS (d).

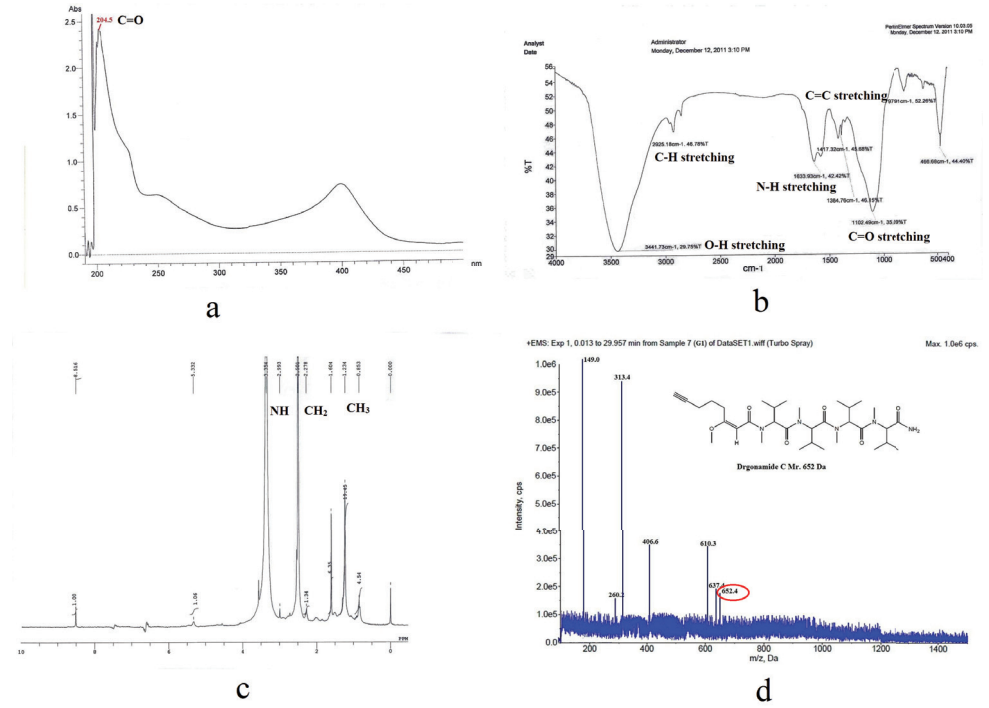

Figure 4. The spot G1 eluate derived from *L. aestuarii* was subjected to spectroscopic analysis. UV Absorption spectrum (a); IR spectrum (b); ^{1}H NMR spectrum (c); and LC MS/MS (d).

data revealed m/z values as: 652.4, 637.4, 610.3, 406.6, 313.4, 260.2 and 149.0. The m/z value indicating 652.4 was fully consistent with the literature values for the known metabolite 'dragonamide C' (Figure 4d).

UV absorption spectrum of L3 spot had λ_{max} at 219.5, 222.5 and 231.5 nm predicting the carboxylic acid and benzene moiety in the compound (Figure 5a). The IR absorption spectrum revealed moiety as: 3436.88 (O-H, hydroxyl), 1638.18 (C=O, carbonyl) and 1605.19 cm^{-1} (aromatic ring) stretching. The other signals as 2925.93 (C-H stretching), 2852.41 (C=C stretching) and 1384.65 showed (C-O-C) stretching (Figure 5b). The ^{1}H NMR spectrum suggested the proton environment as chemical shifts, δ 0.736, 0.835, 0.853 and 1.233 indicated methyl (tertiary), 1.653 and 1.673 for methyl (secondary) group, δ 6.375 (1H, s, J=8.4), δ 6.485 (1H, s, J=0.715), δ 7.385 (1H, s, J=1.9), δ 7.445 (1H, s, J=2.4) and δ 7.586 (1H, s, J=6.6) for aromatic ring moiety. Other chemical shifts δ 3.369 (1H, m, J=6.2) and δ 3.571 (1H, s, J=1.3) pointed towards one proton attached to oxygenated carbon (Figure 5c). The m/z values were found as 758.4, 689.8, 610.3, 443.5, 437.5, 433.3, 415.4, 371.3, 355.5, 288.3, 149.2 and 149.0. The peaks 415.4, 443.5, 437.5 and 433.3 led the possibility of a diterpenoid in the sample. Thus data from spectroscopic analysis confirmed presence of a diterpenoid type of compound in *A. bullosa*

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(Figure 5d).

The UV absorption spectrum of spot L5 had λ_{max} at 220, pointing towards cyclopropane moiety in the compound (Figure 6a). The IR absorption spectrum of same spot showed as: 1634.10 (C-H, cyclopropane), 3438.62 (O-H, hydroxyl), 2105.62 (X-C, halogen-carbon stretching), 1384.50 (C=O, carbonyl) and 1105.49 cm^{-1}(R-O-R, ether bond) for various functional groups (Figure 6b). The proton signal in ^1H NMR spectrum suggested the environment as: δ 0.858 (2H, d) for cyclopropane, δ 6.637 (1H, m, J=7.597) for 1-ethylene, δ 3.347 (3H, t, J=7.012) for methyl, δ 2.502 (1H, s) for methylene, δ 1.589 (3H, t) and 1.234 (3H, t) for methyl (Figure 6c). The m/z values were recorded as 758.3, 637.3, 610.3, 437.4, 415.4, 371.3, 359.4, 350.5, 355.2, 313.4, 298.6 and 149.0. The 313.4m/z value provided clue regarding the majusculoic acid in the sample (Figure 6d).

Discussion

Identification of newer natural resources of known and unknown lead molecules is as important as discovery of new lead molecules. Low yield of biomolecules from cyanobacteria (11) compelled to screen every species of potent cyanobacteria for such biomolecules. Easy accessibility and generation time also adds importance to such efforts. From the present investigation we, here in report first time *A. bullosa* (fresh water) as a source of diterpenoid and majusculoic acid and *L. aestuarii* as malyngolide and dragonamide C. We have already re-

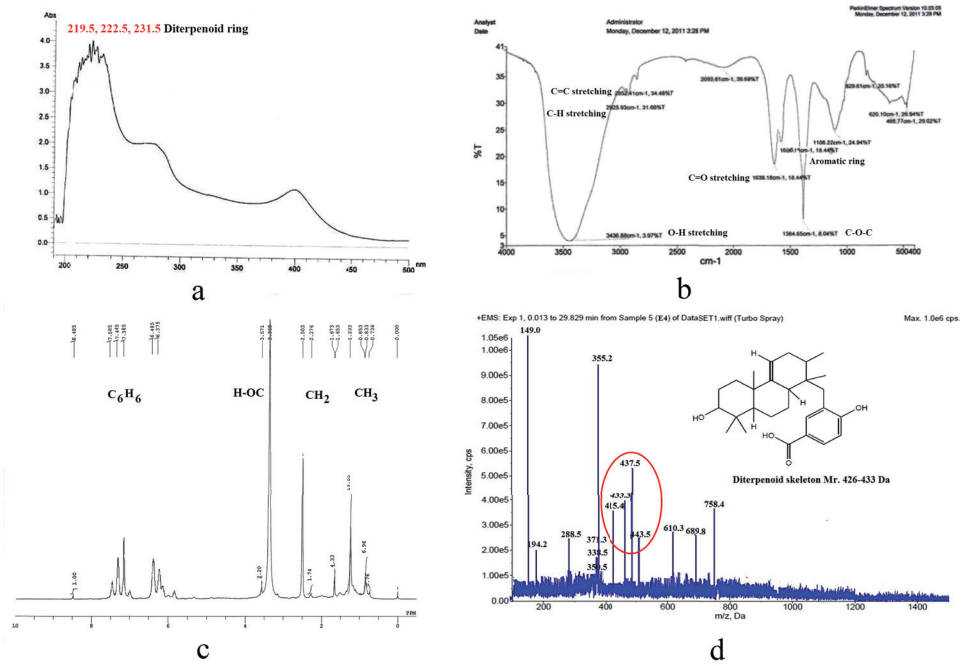

Figure 5. The spot L3 eluate derived from *A. bullosa* was subjected to spectroscopic analysis. UV Absorption spectrum (a); IR spectrum (b); ^1H NMR spectrum (c); and LC MS/MS (d).

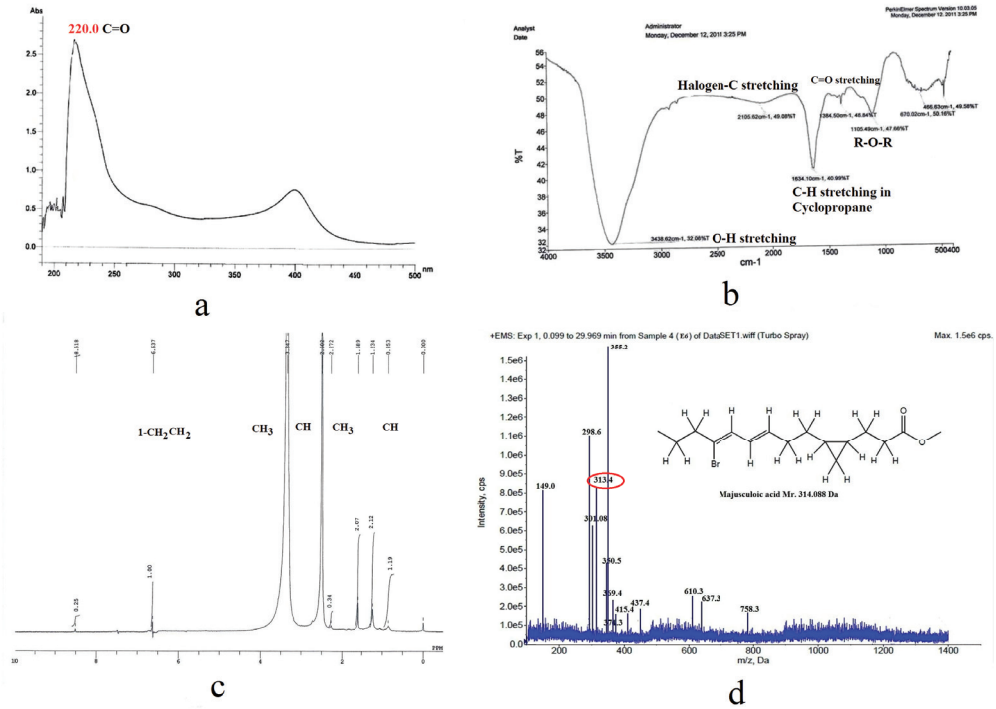

Figure 6. The spot L5 eluate derived from *A. bullosa* was subjected to spectroscopic analysis. UV Absorption spectrum (a); IR spectrum (b); ^1H NMR spectrum (c); and LC MS/MS (d).

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ported that methanolic crude extracts (100 µg mL^{-1}) of test cyanobacteria against 20 bacterial strains (17 Gram -ve and 3 Gram +ve) possessed broad spectrum antibiotic activity, keeping rifampicin (100 µg mL-1) as control (21). Presence of bioactive molecule against fungi and protozoa (*Leishmania donovani*) were also documented by the same extracts (22).

Therefore, we took non pathogenic strain *E. aerogenes* one of the test strains taken earlier alongwith *C. albicans* for identification of potent antibacterial, antifungal bands in TLC purified extracts of both test cyanobacteria, respectively. Thus, bands having potent antibacterial and antifungal biomolecules were identified (Table) and processed for spectroscopic analysis. Spectroscopic analysis of spot F1 showed the structure close to malyngolide compound. However, presence of UV λ_{max}, 203.5 nm indicated the carboxylic/acetic acid moiety. The same compound malyngolide showed UV absorption at 208 nm (23). IR absorption at 3419.06 cm^{-1} was in tune with report of Cardllina and Moore (24) where 3410 cm^{-1} as absorption maxima was for hydroxyl group in malyngolide. Similarly, IR spectra at 1643 cm^{-1} showed C=O stretching confirming the lactone carbonyl group (25). Likewise, ^{1}H NMR showed presence of carbonyl and hydroxyl moiety of malyngolide. The characteristic oxygen bearing methylene group was found at δ 1.218 and carbonyl group at δ 2.490 as reported previously by Cardllina and Moore (24). The other methyl signal revealed at δ 0.832 and δ 0.817 corresponding to the aliphatic chain in tune with report of Gutierrez *et al.* (26). Malyngolide is an antibiotics naturally occurring from marine strain *L. majuscula*. Its molecular weight was 563.4288. The LCMS/MS value indicating Mr = 541.44 was close with the known metabolite malyngolide. Malyngolide was reported as antibacterial against *Bacillus subtilis*, *S. aureus*, *Escherichia coli* and *P. aeruginosa* (27) as our previous report coincided with the same property of malyngolide.

The presence of this compound was supported with the data obtained from other spectroscopic analysis as UV, IR and ^{1}H NMR. The spectroscopic data of G1 spot showed similarity with dragonamide, however, presence of carbonyl group, carboxyl group and nitromethane can be ascribed because of UV absorption λ_{max} at 204.5 nm (28). IR absorption at 1633.93 cm^{-1} was in tune with respect to results reported by Gunasekera *et al.* (29). These workers have reported 1636 cm^{-1} as absorption maximum indicating amide in dragonamide. Out of many IR ν_{max}, 1632 cm^{-1} also indicated closeness to dragonamide compound (28). The linearity of the compound dragonamide was evident with lack of chemical shifts between 4-6 ppm. The characteristic triplet for a triplet acetylenic proton (δ 2.278 3H, t, J= 6.8) was comparable with that of report (δ 2.20, J=2.7Hz) of Gunasekera *et al*. (29). Presence of chemical shifts as δ 3.332 (1H, s) and δ 3.354 (1H, m) confirmed the amide group in the compound. Dragonamide has different types varying in molecular weight as dragonamide C (642) (29), dragonamide D (628) (30) and dragonamide E (652) (28). LCMS/MS peak with 652 indicated the presence of dragonamide as one of the compound within mixture. Mr is the average of isotopic means of the compounds therefore, this mass may fluctuate with presence of varying neutrons. This led us to report here the presence of one of the compounds as dragonamide species. Dragnomide is strong antileishmanial in character and also active against *Plasmodium falciparum*, *L. donovani* and *Trypanosoma cruzi* (28). We have reported antileishmanial activity of crude extract from *L. aestuarii* (22)

UV λ_{max} at 219.5, 225.5, 231.5 and 400 nm suggested the conjugated and isolated carbonyl, aromatic and hydroxyl group which create the base of diterpenoid (31). The IR absorption at 3436.88 cm^{-1}, 1638.18 cm^{-1} and 1605.19 cm^{-1} confirm the hydroxyl, carbonyl and aromatic moiety in the sample L3, similar to diterpenoid (32). IR of diterpenoid also revealed the similar results as hydroxyl at 3300cm^{-1}, carboxyl at 1710 cm^{-1} and aromatic at 1604 and 1520 cm^{-1} (33). Similarly, Norabictane a diterpenoid showed the IR absorption for aromatic rings at 1605, 1580 and 1520 cm^{-1}, the carbonyl moiety at 1723 and hydroxyl group at 3388 cm^{-1} (23). The ^{1}H NMR of spot L3 support the methyl moiety at chemical shifts δ 0.736, 0.835, 0.853, 1.233, 1.653, 1.673 and aromatic moiety at δ 6.375, 6.485, 7.385, 7.445 and at 7.586. Four tertiary groups of methyl were also observed at δ 0.67, 0.88, 0.89, 1.11 and secondary at δ 6.74, 7.70 and at 7.80 by Jaki *et al*. (32). We could identify a proton attached with oxygenated carbon as observed at δ 3.369 and at δ 3.571 of spot L3. Such oxygenated carbon was observed in noscomin at δ 3.19 (32). Tertiary and secondary methyl groups were also observed in commonstin A at similar chemical shifts as 0.86, 0.94, 0.99, 1.00 and 0.98 while aromatic ring was also observed at similar shift at δ 6.93, 7.75 and 7.89 (30). ^{1}H NMR of norbietane, a diterpenoid from *Microcoleus lacustris*, revealed methyl groups at chemical shifts at δ 1.14, 0.93 and at 0.98 (23). Diterpenoid of similar molecular weight were also reported as, tolypodiol [$M+H^+$ 456.56] a diterpenoid from *Tolypothrix nodosa* (34), Norabietane [$M+H^+$ 326.4091] (23) and Noscomin [$M-H^-$ 425.2] (32). Comnostin from *Nostoc commune*, showed various $[M-H]^-$ values as 427 (comnostin A), 425 (comnostin B), 441 (comnostin C), 471 (comnostin D) and 425 (comnostin E) (33) Similar 415.4, 433.3, 437.5 and 443.5 m/z values showed the molecular weight of diterpenoid compound in spot L3. Diterpenoids from cyanobacteria were found active against *S.aureus*, *Staphylococcus epidermis, Salmonella typhi, Vibrio cholera, B.subtilis, B.cereus, E.coli* and *Klebsiella pneumoniae* (32,23)

All the spectroscopic data of spot L5 lead to the confirmation of majusculoic acid as reported previously by MacMillan and Molinski (28) from cyanobacterial mat community dominated by *Lyngbya*, *Anabaena*, *Phormidium* sp. and others. UV λ_{max} at 206 of spot L5 confirm the cyclopropane moiety in compound which is base confirmation of majusculoic acid (35). IR absorption at 1634.10 cm^{-1} confirmed the cyclopropane (C-H stretching) and 1105.49 cm^{-1} for carbonyl (C=O stretching) in the compound as similar results were obtained by MacMillan and Molinski (36). The other IR absorption peaks at 3438.62 (O-H stretching), 1384.50 cm^{-1}(R-O-R) and 2105.62 cm^{-1} (halogen-carbon stretching) supported the basic structure of majusculoic acid. ^{1}H NMR of spot supports the cyclopropane moiety at δ 0.854 and other aliphatic chain at δ 6.637, 3.347, 2.502, 1.589 and 1.234. LCMS/MS value at 313.4 showed the presence

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of one Br atom (equal intensity $^{79}Br/^{81}Br$ isotope peak). The LCMS/MS peak at 313 suggested that compound was similar to majusculoic acid (314.0881) active against *C. albicans* (36). Unknown peaks by LCMS/MS obtained by us using TLC purified compounds in addition to the compounds screened, suggested that our compounds do have mixture of the unknown compounds. Although, these compounds were known from different cyanobacterial species. Here we reported such compounds from the either source studied. *A. bullosa* is having half the generation time (48 hrs) of *L. aestuarii* therefore, it can act as a better source of the biomolecules as far as over production is concerned. Thus cyanobacteria from different origins can be screened for various metabolites which can act as a lead molecule for synthetic biochemist and fresh water cyanobacteria do add the biomolecules of specific interests.

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Other articles in this theme issue include references (37-52).

References

1. Reynolds, R., Potz, N., Colman, M., Williams, A., Livermore, D. and MacGowan, A., Antimicrobial susceptibility of the pathogens of bacteraemia in the UK and Ireland 2001-2002: the BSAC bacteraemia resistance surveillance programme. *J. Antimicrob. Chemother*. 2004, **53**:1018-1032.

2. Karchmer, A.W., Increased antibiotic resistance in respiratory tract pathogens: PROTEKT US- an update. *Clin. Infect. Dis.* 2004, **39**:S142-S150.

3. Paterson, D.L., The epidemiological profile of infections with multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species. *Clin. Infect. Dis*. 2006, **43**:S43-S48

4. Berdy, J., Bioactive Microbial Metabolites. In: *The Journal Antibiotics*. 2005, **58** (1):1–26.

5. Singh, S., Kate, B.N. and Banerjee, U.C., Bioactive compounds from cyanobacteria and microalgae: an overview. In: *Critical Reviews in Biotechnology*. 2005, **25**:73-95.

6. Sivonen, K. and Börner, T., In: *The Cyanobacteria*; *Molecular Biology, Genomics and Evolution,* Herrero, A. and Flores, E. (eds.), Caster Academic Press, Norflok, United Kingdom, 2008, pp. 159-197.

7. Prasanna, R., Sood A., Jaiswal, P., Nayak, S., Gupta, V., Chaudhary, V., Joshi, M. and Natarajan, C., Rediscovering cyanobacteria as valuable sources of bioactive compounds. In: *Applied Biochemistry and Microbiology.* 2010, **46** (2):119-134.

8. Uzair, B., Tabassum, S., Rasheed, M. and Rehman, S.F., Exploring marine cyanobacteria for lead compounds of pharmaceutical importance. In: *The Science World Journal* 2012, 1-10

9. Kehr, J.C., Picchi, D.G. and Dittmann, E., Natural product biosyntheses in cyanobacteria: A treasure trove of unique enzymes Beilstein. *J. Org. Chem*. 2011, **7**:1622–1635.

10. Ezanka, T. and Dembitsky, V.M., Metabolites produced by cyanobacteria belonging to several species of the family *Nostocaceae. Folia Microbiol* 2006, **51** (3):159–182.

11. Asthana, R.K., Deepali, Tripathi, M.K., Srivastava, A., Singh, A.P., Singh, S.P., Nath, G., Srivastava, R. and Srivastava, B.S., Isolation and identification of a new antibacterial entity from the Antarctic cyanobacterium *Nostoc* CCC 537. *J. Appl. Phycol.* 2009, **21**:81–88

12. Tan, L.T., Bioactive natural products from marine cyanobacteria for drug discovery. In: *Phytochemistry* 2007, **68**:954–979.

13. Asthana, R.K., Srivastava, A., Singh, A.P., Deepali, Singh, S.P., Nath, G., Srivastava, R. and Srivastava, B.S., Identification of an antimicrobial entity from the cyanobacterium *Fischerella* sp. isolated from bark of *Azadirachta indica* (Neem) tree. *J. Appl. Phycol.* 2006, **18**:33–39.

14. Gerwick, W.H., Tan, L.T. and Siachitta, N., Nitrogen containing metabolites from marine cyanobacteria. In: *The Alkaloids*; academic Press: San Diego CA USA, 2001, **75**-184.

15. Liu, L. and Rein, K., New peptides isolated from *Lyngbya* species. In: *Marine Drugs* 2010, **8**:1817-1837.

16. Okajima-Kaneko, M., Ono, M., Kabata, K. and Kaneko, T., Extraction of novel sulfated polysaccharides from *Aphanothece sacrum* (Sur.) Okada, and its spectroscopic characterization. *Pure Appl. Chem*. 2007, **79**:2039–2046.

17. Riou, D., Colliec-Jouault, S., Pinczon du Sel, D., Bosch, S., Siavoshian, S., Le Bert, V., Tomasoni, C., Sinquin, C., Durand, P. and Roussakis, C., Antitumor and antiproliferative effects of a fucan extracted from *Ascophyllum nodosum* against a non-small-cell bronchopulmonary carcinoma line. *Anticancer. Res*.1996, **16**:1213-1218.

18. Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y., Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol*. 1979, **111**:1-6.

19. Doan, N.T., Rickards, R.W., Rothschild, J.M., and Smith, G.D., Allelopathic actions of the alkaloid 12-*epi*-Hapalindole E isonitrile and calothrixin A from cyanobacteria of the genera *Fischerella* and *Calothrix. J. Appl. Phycol*. 2000, **12**:409–416.

20. Rex, J.H., Alexander, B.D., Andes, D., Skaggs, B.A., Brown, S.D., Chaturvedi, V., Ghannoum, M.A., Espinel-Ingroff, A., Knapp, C.C., Zeichner, L.O., Pfaller, M.A., Sheehan, D.J. and Walsh, T.J., In: Reference method for Broth Dilution Antifungal Susceptibility testing of yeast (2008). Approved Standard- Third Edition; 28: M 27- A3, ISBN1-56238-662-2, ISSN 0273-3099.

21. Kumar, M., Tripathi, M.K., Srivastava, A., Gour, J.K., Singh, R.K., Tilak, R. and Asthana, R.K. Cyanobacteria, *Lyngbya aestuarii* and *Aphanothece bullosa* as antifungal and antileishmanial drug resources. *Asian .Pac. J. Trop. Biomed*. 2013, **3**(6):458-463.

22. Kumar, M., Tripathi, M.K., Srivastava, A., Nath, G. and Asthana, R. K., A Comparative Study of Antibacterial Activity of Brackish and Fresh Water Cyanobacterial Strains. Asian J. Exp. Biol. Sci. 2012, **3** (3):548-552.

23. Gutiérrez, M., Tidgewell, K., Capson, T.L., Engene, N., Almanza, A., Schemies, J., Jung, M. and Gerwick, W.H., Malyngolide dimer, a bioactive symmetric cyclodepside from the Panamanian marine cyanobacterium *Lyngbya majuscula*. *J. Nat. Prod*. 2010, **73**(4), 709-711.

24. Cardllina, J.H., and Moore, R.E., Structure and absolute configuration of Malyngolide, an antibiotic from the marine blue-green alga *Lyngbya majuscula* Gomont. *J. Org. Chem*. 1979, **44**:4039-4042.

Copyright © 2014. All rights reserved.

25. Cardillo, G., Orena, M., Porzi, G. and Sandri, S., Synthesis of malyngolide, an antibiotics from the marine blue-green algae *Lyngbya majuscule* Gomont. *J. Org. Chem*.1980, **43**:2439-2442.
26. Gutiérrez, R.M.P., Flores, A.M., Solis, R.V. and Jimenez, J.C. Two new antibacterial norabietane diterpenoids from cyanobacteria, *Microcoleous lacustris*. *J. Nat. Med.* 2008, **62**:328-331.
27. Machiya, K., Ichimoto, I., Tonari, K., Kirihata, M. and Ueda, H., An efficient synthetic method for (±)-Malyngolide and its optical resolution. *Agric. Biol. Chem*. 1985, **49**(6):1767-1773.
28. Balunas, M.J., Linington, R.G., Tidgewell, K., Fenner, A.M., Uren˜a, L.D., Togna, G.D., Kyle, D.E., Gerwick, W.H. and Dragonamide E., A Modified Linear Lipopeptide from *Lyngbya majuscula* with Antileishmanial Activity. *J. Nat. Prod*. 2010, **73**:60–66.
29. Gunasekera, S.P., Ross, C., Paul, V.J., Matthew, S. and Luesch, H., Dragonamides C and D, Linear Lipopeptides from the Marine Cyanobacterium Brown *Lyngbya polychroa. J. Nat. Prod*. 2008, **71**:887–890
30. McPhail, K.L., Correa, J., Linington, R.G., Gonzalez, J., Ortega Barria, E., Capson, T.L. and Gerwick, W.H. Antimalarial linear lipopeptides from a Panamanian strain of the marine cyanobacteria *Lyngbya majuscule J. Nat. Prod*. 2007, **70**:984-988
31. Kuo, S.W. and Chang, F.C., Miscibility Behavior and Specific Interaction of Phenolic Resin with Poly (acetoxystyrene) Blends. *Macromol. Chem. Physiol*. 2002, **203**:868-878.
32. Jaki, B., Orjala, J. and Sticher, O., A novel extracellular diterpenoid with antibacterial activity from the cyanobacterium *Nostoc commune*. *J. Nat. Prod*. 1999, **70**:984-988.
33. Jaki, B., Orjala, J., Heilmann, J., Lindne, A., Vogler, B. and Sticher, O., Novel extracellular diterpenoids with biological activity from the cyanobacterium *Nostoc commune*. *J. Nat. Prod*. 2000, **63**:339-343.
34. Prinsep, M.R. and Thomson, R.A., Tolypodiol, an antiinflammmatory diterpenoid from cyanobacterium *Tolypothrix nodosa*. *J. Nat. Prod*. 1996, **59**:786-788.
35. Jorgenson, M.J. and Leung, T., Cyclopropyl conjugation in olefinic esters. Conformation effects on ultraviolet absorption. *J. Am. Chem. Soc.* 1968, **3**:3769-3774.
36. MacMillan, J.B. and Molinski, T.F., Majusculoic acid, a brominated cyclopropyl fatty acid from a marine cyanobacterial mat assemblage. *J. Nat. Prod*. 2005, **68:**604-606.
37. Singh, V. K., Singh, M. P. Bioremediation of vegetable and agrowastes by *Pleurotus ostreatus*: a novel strategy to produce edible mushroom with enhanced yield and nutrition. *Cell. Mol. Biol.* 2014, **60 (5)**: 2-6. doi: 10.14715/cmb/2014.60.5.2
38. Vishnoi, N., Singh, D. P., Biotransformation of arsenic by bacterial strains mediated by oxido-reductase enzyme system. *Cell. Mol. Biol.* 2014, **60 (5)**: 7-14. doi: 10.14715/cmb/2014.60.5.3
39. Srivastava, A. K., Vishwakarma, S. K., Pandey, V. K., Singh, M. P., Direct red decolorization and ligninolytic enzymes production by improved strains of *Pleurotus* using basidiospore derived monokaryons. *Cell. Mol. Biol.* 2014, **60 (5)**: 15-21. doi: 10.14715/cmb/2014.60.5.4
40. Kumari, B., Rajput, S., Gaur, P., Singh S. N., Singh D. P., Biodegradation of pyrene and phenanthrene by bacterial consortium and evaluation of role of surfactant. *Cell. Mol. Biol.* 2014, **60 (5)**: 22-28. doi: 10.14715/cmb/2014.60.5.5
41. Pandey, V. K., Singh, M. P., Biodegradation of wheat straw by *Pleurotus ostreatus. Cell. Mol. Biol.* 2014, **60 (5)**: 29-34. doi: 10.14715/cmb/2014.60.5.6
42. Pathak, V. V., Singh, D. P., Kothari, R., Chopra, A. K., Phycoremediation of textile wastewater by unicellular microalga *Chlorella pyrenoidosa*. *Cell. Mol. Biol.* 2014, **60 (5)**: 35-40. doi: 10.14715/cmb/2014.60.5.7
43. Pandey, A. K., Vishwakarma, S. K., Srivastava, A. K., Pandey, V. K., Agrawal, S., Singh, M. P., Production of ligninolytic enzymes by white rot fungi on lignocellulosic wastes using novel pretreatments. *Cell. Mol. Biol.* 2014, **60 (5)**: 41-45. doi: 10.14715/cmb/2014.60.5.8
44. Ayaz E., Gothalwal, R., Effect of Environmental Factors on Bacterial Quorum Sensing. *Cell. Mol. Biol.* 2014, **60 (5)**: 46-50. doi: 10.14715/cmb/2014.60.5.9
45. Singh, M. K., Rai, P. K., Rai, A., Singh, S., Alterations in lipid and fatty acid composition of the cyanobacterium *Scytonema geitleri* bharadwaja under water stress. *Cell. Mol. Biol.* 2014, **60 (5)**: 51-58. doi: 10.14715/cmb/2014.60.5.10
46. Singh, M. P., Pandey, A. K., Vishwakarma, S. K., Srivastava, A. K., Pandey, V. K., Singh, V. K., Production of cellulolytic enzymes by *Pleurotus* species on lignocellulosic wastes using novel pretreatments. *Cell. Mol. Biol.* 2014, **60 (5)**: 59-63. doi: 10.14715/cmb/2014.60.5.11
47. Chandra, P., Singh, D. P., Removal of Cr (VI) by a halotolerant bacterium *Halomonas* sp. CSB 5 isolated from sāmbhar salt lake Rajastha (India). *Cell. Mol. Biol.* 2014, **60 (5)**: 64-72. doi: 10.14715/cmb/2014.60.5.12
48. Tewari, S., Arora, N. K., Talc based exopolysaccharides formulation enhancing growth and production of *Hellianthus annuus* under saline conditions. *Cell. Mol. Biol.* 2014, **60 (5)**: 73-81. doi: 10.14715/cmb/2014.60.5.13
49. Arun, N., Vidyalaxmi, Singh, D. P., Chromium (VI) induced oxidative stress in halotolerant alga *Dunaliella salina* and *D. tertiolecta* isolated from sambhar salt lake of Rajasthan (India). *Cell. Mol. Biol.* 2014, **60 (5)**: 90-96. doi: 10.14715/cmb/2014.60.5.15
50. Prakash, S., Singh, R., Lodhi, N., Histone demethylases and control of gene expression in plants. *Cell. Mol. Biol.* 2014, **60 (5)**: 97-105. doi: 10.14715/cmb/2014.60.5.16
51. Singh, A. K., Singh, M. P., Importance of algae as a potential source of biofuel. *Cell. Mol. Biol.* 2014, **60 (5)**: 106-109. doi: 10.14715/cmb/2014.60.5.17
52. Dixit, S., Singh, D. P., Role of free living, immobilized and nonviable biomass of *Nostoc muscorum* in removal of heavy metals: An impact of physiological state of biosorbent. *Cell. Mol. Biol.* 2014, **60 (5)**: 110-118. doi: 10.14715/cmb/2014.60.5.18

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