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## SCREENING OF CYANOBACTERIA STRAINS FROM DELHI AND NCR FOR ANTI-BACTERIAL POTENTIAL

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**ABSTRACT:** Cyanobacteria or blue green algae have an extraordinary potential of producing secondary metabolites which can be an ultimate source of newer drugs for future. The purified strain *Anabaena* showed best photoautotrophic growth at 28°C with specific growth and generation time were 0.098 and 10.0 hr respectively. The better biomass production and antibacterial activity on 30<sup>th</sup> days of culture. 16 cyanobacterial strains collected from different sites of Delhi and NCR (National Capital Region, INDIA) identified as *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc*, *Phormidium* spp were examined for antibacterial properties. The crude methanolic intracellular extracts of 8 strains showed activity against non-pathogenic *Enterobacter aerogens* and all 16 given potential against pathogenic *Escherichia coli*. In which purified *Anabaena* spp given maximum antibacterial activity against *E. coli* while *Anabaena* (Johad) and *Microcystis* (water tank) from Manesar (Gurgaon, India) against *E. aerogens*. The same strain from different origin has different antibacterial activity. This interpretation indicates the biosynthesis of bioactive molecules is niche and species specific.

**INTRODUCTION:** Cyanobacteria or the blue green algae exhibiting the general characteristics of Gram negative bacteria, inhabiting earth since proteozoic era.

These are responsible for the transition of primitive reductive atmosphere of the earth to the present oxidative atmosphere<sup>1</sup>.

The photosynthetic oxygen-evolving proarch-conservate are found in almost all the ecological habitats which may be psychrophilic to thermophilic, acidophilic to alkalophilic, planktonic to barophilic, freshwater to marine and hypersaline to tree barks<sup>2, 3</sup> with morphological diversity (unicellular to multicellular, coccoid to branched filaments).

Because of diversity, cyanobacteria produce an unparallel array of bioactive secondary metabolites including alkaloids, isoprenoids, polyketides and non-ribosomal peptides; show a broad spectrum biological activity including toxins, antibiotics, fungicides and algacides as reviewed exhaustively<sup>4</sup>, some of which are potent toxins<sup>5</sup>.

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Increasing multi-drug resistance in pathogenic bacteria and limitation of workers to go for newer bioactive molecule/ drugs without indication of the concerned lead molecules is of paramount concern. The importance of bacterial natural products as a replacement for new antibiotics is emphasized<sup>6</sup> because of rich structural diversity and complexity of natural products of microbes according to habitats.

Therefore, the domain of natural product research deserves to be expanded significantly. It seems that, isolation, characterization and synthesis of natural product would open the door to a new era in academia and industry. Rich opportunity exists for drug discovery from cyanobacteria as the expected rate is far lower than for other-studied better group of organisms<sup>7</sup>.

Although cyanobacteria with the logic that unique biochemical pathways might be uncovered and may lead to the emergence of niche-specific bioactive molecules that could have favored the evolution of indigenous species through novel biochemical adaptations<sup>8</sup>, and recent report of presence of secondary metabolite genes in cyanobacteria represented three family (Nostocales, Chroococcales and Oscillatriales)<sup>9</sup>.

The present study has been focused to screen cyanobacterial strains from various sites of Delhi and NCR regions for antibacterial potential.

**MATERIAL AND METHODS:** The Purified strains of *Anabaena* and *Microcystis* were grown in Chu-10 medium<sup>10</sup> lacking combined nitrogen with periodic shaking and continuous illumination (tungsten + fluorescent,  $5.1 \text{ Wm}^{-2}$ ) at  $28 \pm 1^\circ\text{C}$ , and the specific growth rate was calculated according to Kartz and Myers<sup>11</sup>.

The 1 L capacity culture vessels were shaken periodically. Purity of the cultures was routinely checked by transferring aliquots to Luria Broth medium and incubating in the dark at  $37^\circ\text{C}$  for 24 h. As the cyanobacterium was slow growing, 35-40 d old batch cultures ( $10 \text{ g fresh wt L}^{-1}$ ) were used for processing.

**Dry weight determination:** Cells were concentrated by centrifugation, washed and dried ( $40^\circ\text{C}$ ) to constant weight (expressed as  $\text{g l}^{-1}$ ).

**Protein estimation:** Protein content of the cyanobacterial culture was estimated using the method adopted by Asthana et al<sup>10</sup>. The reagents employed were reagent 'A' (1.0 N NaOH), reagent 'B' including (i) 5.0%  $\text{Na}_2\text{CO}_3$  and (ii) 0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 1% sodium potassium tartarate and solution (i) and (ii) were mixed in the ratio 25:1 (v/v). Reagent 'C' (1.0 N Folin-Phenol reagent (Sisco, India).

A 0.5 mL of 1.0 N NaOH was added to 0.5 mL culture, incubated for 5 min in a boiling water bath. After cooling, 2.5 mL of reagent 'B' was added and the reaction mixture incubated for 10 min at  $37^\circ\text{C}$ . This was followed by the addition of 0.5 mL Folin-Phenol reagent and incubation for another 15 min. The intensity of the resulting blue color was read at 650 nm in Spectrophotometer (Systronic, India) and the protein amount quantified as  $\mu\text{g mL}^{-1}$  culture with reference to a standard calibration curve obtained with Bovine Albumin Serum (BSA) (Sigma- Aldrich).

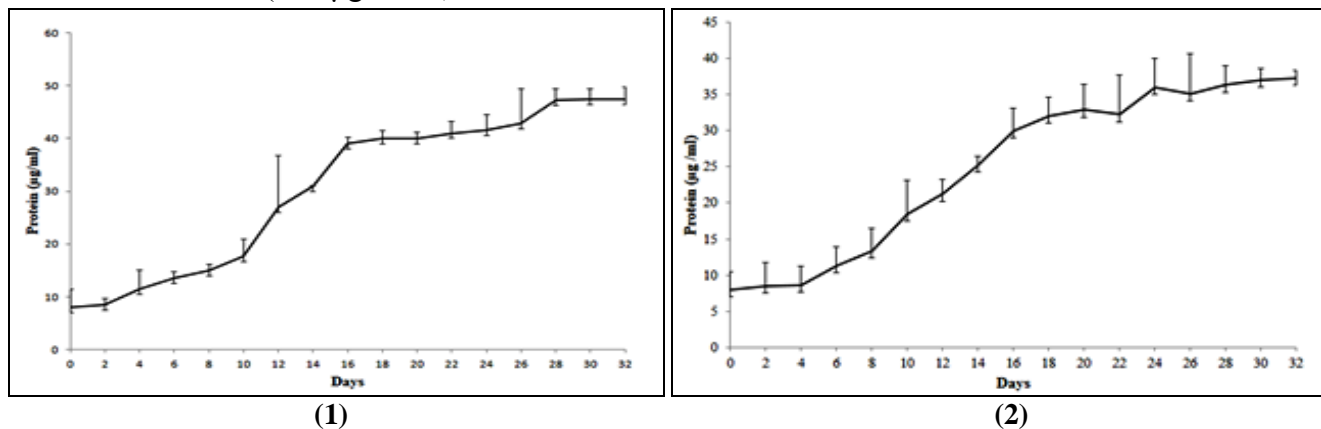
**Extraction and isolation of bioactive molecule:** Extraction was done according to Doan et al<sup>12</sup>. Cyanobacterial biomass (10g, fresh wt) was pelleted, washed with deionised water and oven dried at  $40^\circ\text{C}$ . The biomass (1g dry wt) was extracted twice with 100 mL methanol, centrifuged ( $20,000 \times \text{g}$ , 30 min), the supernatant evaporated to dryness and residue redissolved in methanol (2.5 mL).

**Antibacterial assay:** The crude extracts were bioassays for antibacterial potential against *Enterobacter aerogenes* MTCC 2822 (IMTECH, India) and *Escherichia coli* ATCC 25992 using slightly modified Kirby Bauer disc diffusion method<sup>13</sup> on 3.8% Mueller-Hinton (MH) agar (Sigma-Aldrich). The inoculum from LB grown cells ( $37^\circ\text{C}$ , 18 h) was suspended in 0.85% NaCl and turbidity adjusted to  $10^8 \text{ CFU mL}^{-1}$  that corresponds to 0.5 MacFarland standard according to NCCLS (now CLSI)<sup>14</sup>. The cell suspension in each case was inoculated on MH agar with the help of sterile, non-toxic cotton swab and incubated ( $37^\circ\text{C}$ , 20 min). Thereafter, 15  $\mu\text{l}$  each of methanolic extract (crude) was spotted on plates keeping methanol as control and incubated ( $37^\circ\text{C}$ , 18 h). Antibacterial potential was based on the inhibition zone size (mm).

**RESULTS:**

**Photoautotrophic growth:** It was necessary to determine the optimum growth temperature of the cyanobacteria for desired biomass. Photoautotrophic growth was compared at two different temperatures (28 and 35°C) (Fig. 1 and 2). The initial concentration (8.0  $\mu\text{g mL}^{-1}$ ) of the starter

culture was common to all the three sets. The test organism was grown at showed slow initial trend of growth at least for the first 72 h, followed by the subsequent rise in biomass up to 6 day i.e., ~1.4-fold (11.33  $\mu\text{g mL}^{-1}$ ) over the initial inoculum. This followed a slow pace of the growth to attain 32.06  $\mu\text{g mL}^{-1}$  on 18<sup>th</sup> day.



**FIG. 1: PHOTOAUTOTROPHIC GROWTH OF PURIFIED ANABAENA STAIN AT 28°C [1] & 35°C [2] FOR 32 DAYS AND ERROR BAR INDICATES STANDARD DEVIATION**

The calculated specific growth and generation time for 35°C were 0.08 and 12.5 hr respectively when taken the values between 10 to 16<sup>th</sup> day of growth period. The organism showed the best growth at 28°C with for the first 6 day as evident from a 2.16 fold increment in the biomass (17.7  $\mu\text{g mL}^{-1}$ ). Interestingly, the biomass reflected a rising trend from 6 day onwards ending up to 16<sup>th</sup> day (39  $\mu\text{g mL}^{-1}$ ) and after that slow pace of growth, this may be referred as decreasing log growth phase.

The calculated specific growth and generation time for 28°C were 0.098 and 10.0 hr respectively when taken the values between 10 to 16<sup>th</sup> day of growth period.

**Solvent vs. bioactivity:** Antibacterial property of crude extract of selected cyanobacterium *Microcystis* were also examined using different

solvents such as water, and DMSO in addition to methanol against non-pathogenic bacterium *E. aerogenes* using slightly modified Kirby Bauer Disc Diffusion Susceptibility Method. The diameter range of inhibition zone as achieved by various extracts was in the sequence: 9 mm (methanol) >5 mm (DMSO) while no inhibition zone in case of water. Therefore, only methanolic extract of these cyanobacteria were adopted for further bioassay (Table 1).

**Biomass age vs. antibacterial activity:** Subsequent to identification of antibacterial potential, it was imperative to determine the exact cell age as secondary metabolite (intracellular) production is a function of age. Antibacterial bioassay of lipophilic (methanolic) extract of cyanobacterial biomass for intracellular metabolites is represented in (Table 1).

**TABLE 1: RELATIVE AFFECTIVITY OF THE 'ACTIVE MOLECULES' IN DIFFERENT SOLVENTS AND ON DIFFERENT AGES AGAINST E. AEROGENES**

Strains	Inhibition zone (mm)				
	Different Solvent				
	Water	DMSO	Methanol		
<i>Anabaena</i>	n.d.	5.0 Opaque zone	9.0		
	Biomass Age				
<i>Anabaena</i>	10 day n.d.	20 day 3.0	30 day 13.0	60 day 5.0	90 day 3.0

\*n.d.- not detected

The bacterial sensitivity the intracellular metabolites were measured in terms of inhibition zones formed taking *E. aerogenes* as the target. Methanolic extracts from 20 and 30 day old biomass produced 3 mm and 13 mm inhibition zone on the bacterial lawn respectively. For 60<sup>th</sup> day sets, the extract had showed impact (5 mm zone) compared to the cells grown until 90<sup>th</sup> day (3 mm). While for 10 days biomass no inhibition zone was formed. Therefore, 30 day culture age was selected for optimal intracellular antimicrobial activity.

**Antibacterial activity:** The crude methanolic extract of 16 identified cyanobacterial strains (*Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc*, *Phormidium* spp) from different part of Delhi and NCR showed different activity against both non-pathogenic and pathogenic target bacteria i.e., *E. aerogenes* and *E. coli* respectively. The strains from different part of Delhi *Microcystis* (Water logging, Najafgarh, Delhi) and *Microcystis* (Johad, Rani Khera, Delhi) showed activity against both targets and produced inhibition zone on agar plate of 10 and 27.5 or 45 and 22 mm respectively.

The *Oscillatoria* from open field (India gate, Delhi) given impact for 28 mm against *E. coli* only and same as for *Nostoc* from Yamuna River (Lok Nayak Setu, Delhi) 33 mm. The strains from different habitat of Manesar (Gurgaon, NCR, India) were active against both target bacteria. *Microcystis* from Johad (a pond for multipurpose human activity) had given impact against *E. aerogenes* (47 mm) and *E. coli* (32 mm) as inhibition zone.

Respectively, other strains from Manesar *Nostoc* (Water logging) 10 and 32 mm, *Anabaena* (Water tank) 47 and 30 mm, and only *Anabaena* (Water tap) were active against *E. coli* (25 mm). The Purified strain from different research institute *Anabaena* and *Nostoc spongiforme* (AMITY university, Noida), *Syneocystis* (Banaras Hindu university, Varanasi, India) showed activity against both target bacterial strain 5, 37 cm and 10, 25 cm and 10, 25 cm respectively. Among purified strains, only *Fischerella* spp (BHU, Varanasi) active against *E. coli* only (26 mm) (**Table 2**).

**TABLE 2: BIOASSAY OF DIFFERENT CYANOBACTERIAL METHANOLIC EXTRACT AGAINST NON-PATHOGENIC *E. AEROGENES* AND *E. COLI***

S. No.	Collection Sites	Habitat	Identified Strains	Inhibition zone (mm)	
				<i>E. aerogenes</i>	<i>E. coli</i>
1	India Gate (Delhi)	Open field	<i>Oscillatoria</i>	n.d.	28
2	Yamuna river (Delhi)	Loknaya setu	<i>Nostoc</i>	n.d.	33
3	Manesar (Gurgaon)	Pond (Johad)	<i>Microcystis</i>	47	32
4	Manesar (Gurgaon)	Water logging	<i>Nostoc</i>	10	32
5	Manesar (Gurgaon)	Water tank	<i>Anabaena</i>	47	30
6	Manesar (Gurgaon)	Water tap	<i>Anabaena</i>	n.d.	25
7	Nazafgardh (Delhi)	Water logging	<i>Microcystis</i>	10	27.5
8	Rani Khera (Delhi)	Pond (Johad)	<i>Microcystis</i>	45	22
9	Rani Khera (Delhi)	Pond (Devali)	<i>Microcystis</i>	n.d.	23
10	Satya nicketan (Delhi)	Pot	<i>Oscillatoria</i>	n.d.	20
11	Hauz Khas (Delhi)	Pond	<i>Microcystis</i>	n.d.	22
<b>Purified Culture:</b>					
12	AMITY University (Noida)		<i>Anabaena</i>	5	37
13	AMITY university (Noida)		<i>Nostoc spongiforme</i>	10	25
14	Banaras Hindu university		<i>Fischerella</i> spp	n.d.	26
15	Banaras Hindu university		<i>Syneocystis</i>	10	25

**DISCUSSION:** There are reports of cyanobacterial production of biomolecules intracellular by mesophilic by *N. commune*<sup>15</sup>, *N. linckia*<sup>16</sup>, *N. muscorum*<sup>17</sup> and *Nostoc* ATCC 53789<sup>8</sup>. In batch cultures, *N. insulare* had its metabolism dependent

on the cell age<sup>18</sup>. Cyanobacterial metabolites with antimicrobial activity may either be produced constitutively throughout the exponential and linear phase, or specific to pre-stationary or stationary phase<sup>19</sup>.



In the present context, also cyanobacterial biomass at 30 d old was processed for antibacterial bioassay. Since secondary metabolites are produced in the pre-stationary phase/stationary phase, it was imperative to harvest the cyanobacterial biomass for intracellular biomolecule at selected time. The present organisms were routinely grown at 28°C (ref **Fig. 1**). Extracts from cyanobacterial biomass at 30 d, produced larger inhibition zones, reflecting that the duration was optimal for procuring the intracellular metabolites.

Preliminary screening revealed that in contrast to DMSO or aqueous extract of cyanobacterial extract, only methanolic one exhibited maximum antibacterial activity against *E. aerogenes*; an observation in agreement with others<sup>10, 20</sup>. Falch et al<sup>21</sup> tested a total of 80 lipophilic and hydrophilic extracts from 20 samples of freshwater and terrestrial cyanobacteria and out of 54 extract, 78% were antibacterial and 45% antifungal.

The antiviral activity of methanol: water (3:1) extract of *Spirulina maxima* is also on record<sup>22</sup>. The methanolic extraction of oven dried cyanobacterium was preferred as the minimum volume of methanol (5 µL) was non-toxic to *E. aerogenes* growth on test plate (ref **Table 1**). This perhaps justifies the adoption of methanol as the ideal solvent in the present case.

A fairly low level (15 µl) of methanolic crude extract of almost all collected cyanobacteria was effective against non-pathogenic *E. aerogenes* and pathogenic *E.coli* (ref. **Table 2**). Taton et al<sup>23</sup>, Biondi et al<sup>8</sup> prepared 126 extracts from 48 strains of cyanobacteria from the lakes there and found that extracts from 16 cyanobacteria were antimicrobial (the Gram (+) *S. aureus*), antifungal (*Aspergillus fumigatus*) and the yeast (*Cryptococcus neoformans*), and 25 were cytotoxic towards Hela cells. Jaki et al<sup>24</sup> screened 86 methanolic (lipophilic) and hydrophilic extracts from out of 43 samples of cultured and field collected freshwater and terrestrial cyanobacteria, only 18.3% were active against Gram-positive and 5.8% against Gram-negative bacteria.

Zornitza et al<sup>25</sup> reported a broad-spectrum antibiotics produced by *Nostoc* sp. that inhibited the growth of bacteria, notably the MDR strains of *S. aureus* and *P. aeruginosa*.

Goud et al<sup>26</sup> reported high antibacterial activity in extracts of *Nostoc*, *Lyngbya*, and even the green algae (*Mougeotia* and *Pithophora* sp.). Metabolites of fifty isolated strains were extracted (extracellular) in ethyl acetate and methanol, out of them 24 isolates showed antimicrobial activity against several pathogenic bacteria and one yeast.

The cyanobacterial strains *Cylindrospermopsis raciborskii* 339-T3, *Synechococcus elongatus* PCC7942, *Microcystis aeruginosa* NPCD-1, *M. panniformis* SCP702 and *Fischerella* sp. CENA19 provided the most active extracts<sup>27</sup>. Lagunamides A (1) and B (2) are new cyclic depsipeptides with antitumor as well as antibacterial activities isolated from the marine cyanobacterium *Lyngbya majuscula* obtained from Pulau Hantu Besar, Singapore<sup>28</sup>.

Despite their potent biological activities, very few cyanobacterial compounds have entered clinical trials and no cyanobacterially derived compound has been approved by the Food and Drug Administration.

In our opinion, the pharmaceutical potential of cyanobacteria deserves more scientific attention and interdisciplinary research. Further, to find novel compounds, cyanobacterial strains from still unexplored and extreme habitats should also be studied.

**CONCLUSION:** Antibacterial property of methanolic extract of *Microcystis* sps., from various niches shown different activity against non-pathogenic bacterium *E.aerogens*. This interpretation indicates the biosynthesis of bioactive molecules is niche and species specific.

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