

Research Paper

Unicellular cyanobacteria *Synechocystis* accommodate heterotrophic bacteria with varied enzymatic and metal resistance properties

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The interactions between heterotrophic bacteria and primary producers have a profound impact on the functioning of marine ecosystem. We characterized the enzymatic and metal resistance properties of fourteen heterotrophic bacteria isolated from a unicellular cyanobacterium *Synechocystis* sp. that came from a heavy metal contaminated region of Cochin estuary, southwest coast of India. Based on 16S rRNA gene sequence similarities, the heterotrophic bacteria were grouped into three phyla: namely Actinobacteria, Firmicute, and Proteobacteria. Overall Proteobacteria showed a higher level of enzyme expression while Actinobacteria and Firmicutes showed higher tolerance to heavy metals. Among Proteobacteria, an isolate of *Marinobacter hydrocarbonoclasticus* (MMRF-584) showed highest activities of β -glucosidase ($1.58 \pm 0.2 \mu\text{Mml}^{-1} \text{min}^{-1}$) and laminarinase ($1170.17 \pm 95.4 \mu\text{gml}^{-1} \text{min}^{-1}$), while other two isolates of *M. hydrocarbonoclasticus*, MMRF-578 and 581, showed highest phosphatase ($44.71 \pm 0.2 \mu\text{Mml}^{-1} \text{min}^{-1}$) and aminopeptidase ($33.22 \pm 0 \mu\text{Mml}^{-1} \text{min}^{-1}$) activities respectively. Among Firmicutes, the *Virgibacillus* sp. MMRF-571 showed exceptional resistance against the toxic heavy metals Cd (180 mM), Pb (150 mM), and Hg (0.5 mM). *Bacillus cereus*, MMRF-575, showed resistance to the highest concentrations of Co (250 mM), Cd (150 mM), Pb (180 mM), Hg (0.5 mM), Ni (280 mM), and Zn (250 mM) tested. Our results show that heterotrophic bacteria with varied enzymatic and metal resistance properties are associated with *Synechocystis* sp. Further studies to delineate the role of these heterotrophic bacteria in protecting primary producers from toxic effects of heavy metals and their potential application in bioremediation will be appreciated.

Keywords: Cyanobacteria / Bacteria / Enzyme / Heavy metal

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Introduction

Bacteria exist as free swimming or may be associated with other organisms in the estuarine and marine environment. The diversity of bacteria associated with many species of benthic and pelagic organisms of the estuarine and marine environment and their functional interaction with hosts have been well documented [1–4]. The association between heterotrophic bacteria and cyanobacteria are rarely reported, though their interaction can influence the physiology of both the partners

ecosystem diversity [5]. Recent studies also propose the importance of investigating the diversity and interactions of heterotrophic bacteria with cyanobacteria to engineer microbial consortia with desired properties [6]. The supportive role of heterotrophic bacteria in maintaining the low oxygen partial pressure inside the heterocyst of nitrogen-fixing filamentous cyanobacteria forms one of the initial studies to understand the primary producer bacteria interaction [7]. Later studies also investigated the structural shift of bacteria associated with different growth stages of phytoplankton bloom and positive chemotaxis of heterotrophic bacteria towards exudates of phytoplankton [8–12].

Unicellular cyanobacteria *Prochlorococcus* and *Synechococcus*, alone handle an estimated 20–40% of global

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carbon fixation, although they account for only one percent of photosynthetic biomass [13]. Also, cyanobacteria represent an important sink for metals in aquatic environments and have the capability of controlling the speciation of many biologically active metals [14, 15]. Several strains of cyanobacteria have shown the capability of accumulating, detoxifying or metabolizing heavy metals up to certain concentration range [14]. The potential applications of these cyanobacteria in bioremediation of heavy metal polluted environments have been well documented [16–18]. However, the association of cyanobacteria with other heterotrophic bacteria and their functional interactions are rarely reported [19]. In the present study, we report the identity, enzymatic and metal resistance properties of heterotrophic bacteria associated with a unicellular cyanobacterium, *Synechocystis* sp., isolated from heavy metal contaminated area of Cochin estuary, south west coast of India.

Materials and methods

Cyanobacteria and heterotrophic bacteria

The unicellular cyanobacteria used in the study were isolated from the water column of the heavy metal contaminated region of Cochin Estuary, southwest coast of India [20]. The water sample (2 L) was filtered sequentially through 200 μ nylon mesh, 70 and 30 μ cell strainers (Genetix Biotech Asia, India) and 0.2 μ nitrocellulose filter. Subsequently, the particles collected on 0.2 μ nitrocellulose filter was inoculated in to ASN III medium [21] and incubated at 28 ± 2 °C and with a light:dark cycle of 14:10 h. The growth of cyanobacteria was regularly monitored under the 40 \times objective of an inverted microscope and purified by serial dilution. Since the aim of the study was to isolate the associated microorganisms, antibiotics were not used in the medium. The pH of the medium was monitored once in a week and maintained at 7.8 ± 2 using 1N HCl or NaOH. Cyanobacteria was maintained by subculturing once in every 2 weeks.

Heterotrophic bacteria associated with cyanobacteria were isolated using serial dilution technique. Briefly, cyanobacteria from 10 ml of ASNIII medium were separated by centrifugation at 5000 rpm for 5 min and washed copiously with sterile saline with intermittent sonication in a water bath sonicator (50 Hz power for 30 s) to remove loosely attached heterotrophic bacteria. Further, the cells were macerated in saline, serially diluted up to six times. One hundred microliter of inoculum from each dilution was spread over the surface of ZoBell's 2216e media. The plates were incubated at 28 ± 2 °C for 72 h to form distinct colonies.

Heterotrophic bacteria were purified, maintained in ZoBell's marine agar slants and subcultured every 2–3 weeks. The purity of the isolates was confirmed by Gram staining and fatty acid profile analysis.

Identification of cyanobacteria and heterotrophic bacteria

Morphological and molecular (16S rRNA gene sequencing) tools were used for the identification of unicellular cyanobacteria, while heterotrophic bacteria were identified using molecular tool only. The morphology of cyanobacteria was studied using light and scanning electron microscopy. For light microscopy, a 25 ml capacity culture flask containing cyanobacteria was placed on the stage of an inverted microscope and imaged using 40 \times objective. The images were collected using a CCD camera and processed using ImagePro software (Olympus, USA). The images of cyanobacteria were also taken using a scanning electron microscope. Cyanobacterial cells were separated from the culture medium by centrifugation at 5000 rpm for 10 min, followed by copious washing with sterile water to remove the salts. The cells were dehydrated and placed on a microscope sample holder and gold sputtered in an argon atmosphere. Adequate care was taken to obtain a homogenous cell gold coating. The cyanobacteria cells were imaged on a Neoscope JCM 5000 scanning electron microscope (JEOL, Japan).

Genomic DNA was extracted from overnight grown cultures following standard phenol-chloroform method and the quality of DNA was checked on 0.8% agarose gel [22]. The 16S rRNA gene of cyanobacteria (~1200 bp) were amplified using universal primers 378 F(GGGGAATYTTCCGCAATGGG) and 23S30R (CTTCGCCTCTGTGTGCCTAGGT) [23]. The 16S rRNA gene of the bacteria (~1465 bp) was amplified using universal primers [27F: AGAGTTTGATC(AC)TGGCTCAG and 1492R: GGTTACCTTGTTACGACTT] [24]. The reactions were carried out in a 25 μ l reaction volume containing 1 μ l DNA (50–100 ng), 1 μ l each of primers (10 pmol), 2.5 μ l 10 \times Taq polymerase buffer (NEB, Canada), 0.5U Taq DNA polymerase (NEB, Canada), and 200 μ M each dNTPs (NEB, Canada). PCR cycling conditions maintained were as follows; initial denaturation at 95 °C for 2 min, followed by cycle denaturation at 95 °C for 40 s, annealing at 54 °C for 40 s for cyanobacteria/55 °C for 40 s for heterotrophic bacteria, extension at 72 °C for 1.5 min for a total of 30 cycles and a final extension for 10 min at 72 °C. PCR products were purified using Nucleo-pore Genetix brand Sure Extract PCR clean up/Gel extraction kit (Genetix Biotech, India) and cloned into TOPO- PCR cloning vector (Life

technologies, USA), following the instruction manual. The recombinant plasmids were extracted using Nucleo-pore Genetix brand SureSpin plasmid mini prep kit (Genetix Biotech, India) and were used as a template for sequencing PCR. Cyanobacteria clones were sequenced using gene specific internal primer 1090R [GCTCGTTGCGGGACTTAACC] [25]. Heterotrophic bacteria clones were sequenced using three sets of primers 518R (ATTACCGCGGCTGCTGG), 1090R and 1492R. Sequencing PCR was done with ABI PRISM BigDye terminator ready reaction mix (Life Technologies, USA). The cycle extension products were purified following ethanol/EDTA/sodium acetate precipitation. The products were analyzed on an Applied Biosystems ABI 3730×1 DNA analyzer. Sequence data obtained were analyzed and edited using Sequencher V4.10.1 (GeneCodes, USA). Bacteria were identified based on sequence similarity (>99%) with their cultured representatives in Eztaxon (<http://eztaxon-e.ezbiocloud.net>) [26]. The sequences were submitted to NCBI Genbank (accession numbers: Cyanobacteria KU598870 and heterotrophic bacteria KT123191 to KT123204).

Enzyme expression profile

Aminopeptidase, phosphatase, laminarinase, and β -glucosidase activities of the isolates were studied using standard spectrophotometric assays. One hundred microliters of overnight grown isolates was inoculated into a 50 ml capacity conical flask containing 25 ml ZoBell's marine broth. Separate flasks were maintained for each substrate and were supplemented with 1% substrates such as casein, KH_2PO_4 , laminarin and starch. The flasks were incubated at $28 \pm 2^\circ\text{C}$ at 120 rpm for 48 h. The supernatants separated by centrifugation at 5000 rpm for 10 min at 4°C were stored at 4°C for further use as the crude enzyme. Enzyme assays were done in triplicate in a 96 well microplate. Aminopeptidase, phosphatase, and β -glucosidase activities were measured using fluorescent-conjugated substrates [27]. Here, each well containing 50 μl substrates was supplemented with 50 μl crude enzymes and incubated at $28 \pm 2^\circ\text{C}$ for 2 h and fluorescent signals were measured at every 10 min. Aminopeptidase assay was done with the fluorescent substrate L-leucine – AMC ($200 \mu\text{M ml}^{-1}$). The activity was measured as a function of release of fluorescent 7-amido-4-methyl coumarin hydrochloride (AMC) from the substrate by the action of crude enzyme. The fluorescence intensity was measured in a multimode microplate reader at an excitation/emission of $\lambda 380/\lambda 450 \text{ nm}$. The fluorescent substrate MUF-phosphate ($200 \mu\text{M ml}^{-1}$) was used as the substrate for phosphatase and the enzyme activity was measured as release of

fluorescent 4-methyl umbelliferyl (MUF) from the substrate. The fluorescence signals were measured at an excitation/emission of 380/450 nm. β -glucosidase activity was measured in wells supplemented with MUF-glucopyranoside ($200 \mu\text{M ml}^{-1}$), and the fluorescent measurement was made at an excitation/emission of 385/450 nm. Laminarinase was assayed following DNS assay [28]. Briefly, 50 μl of laminarin (0.25% w/v) was mixed with 50 μl crude enzymes in a microwell and incubated at $28 \pm 2^\circ\text{C}$ for 2 h. Subsequently 100 μl of 3,5-dinitrosalicylic acid (DNS) reagent (1% DNS prepared in a solution containing 0.5 N NaOH and 30% sodium potassium tartrate) was added and incubated at 95°C for 5 min, followed by cooling for 2 min in ice. DNS reacts with reduced sugar to give a yellow colored complex, which was measured at 540 nm. Aminopeptidase, phosphatase and β -glucosidase activities were expressed as $\mu\text{M ml}^{-1} \text{ min}^{-1}$ of dye released while laminarinase activity was expressed as $\mu\text{g ml}^{-1} \text{ min}^{-1}$ glucose released. Standard graph was prepared from fluorescence of serially diluted MUF (5, 10, 15, 20, 25, 30, and 35 $\mu\text{M ml}^{-1}$), AMC (2, 4, 6, 8, 10, 12, 14, and 16 $\mu\text{M ml}^{-1}$) and absorbance of different concentration of glucose (50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 $100 \mu\text{g ml}^{-1}$) reacted with 100 μl DNS reagent.

Metal resistance profile

Heavy metal resistance profile of cyanobacteria associated heterotrophic bacteria were determined by the standard plate diffusion method [29]. Briefly, 1 cm diameter wells with 4 mm depth were prepared at the center of Nutrient agar plates and were individually filled with 500 μl of different concentrations of metal salt solution. The metal salt solution was allowed to diffuse into surrounding media for 24 h at room temperature. The heavy metal salt solutions used in the study were $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (25, 50, 100, 150, 200, and 250 mM), $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ (5, 10, 20, 30, 60, 90, 120, 150, and 180 mM), $\text{Pb}(\text{CH}_3 \text{COO})_2$ (30, 60, 90, 120, 150, and 180 mM), HgCl_2 (0.005, 0.025, 0.05, 0.1, 0.2, and 0.5 mM), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (5, 10, 35, 70, 140, and 280 mM) and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (25, 50, 100, 150, 200, and 250 mM). The stock solutions were prepared and maintained at 4°C for not more than 1 month. The glassware used for metal stock preparation were leached in 2 N HNO_3 and rinsed several times with distilled water before use to avoid metal contamination. On each plate, two isolates were inoculated in radical streaks and triplicate. Plates were then incubated at $28 \pm 2^\circ\text{C}$ for 48 h. After incubation, the area of growth inhibition (in mm) was measured as that from the edge of the central well to the leading edge of the growing streak. The percentage of bacterial

tolerance was calculated in terms of the ratio of the length of the grown in mm Vs length of total inoculated streak.

Fatty acid profile of heterotrophic bacteria

The fatty acid profile of heterotrophic bacteria grown in the presence and absence of heavy metals were tested following gas chromatography technique. Here we prepared two sets of media for each metal, one without any heavy metals and the other supplemented with heavy metal. The concentration of metal supplemented to the media was 1 mM of Ni, Co, Pb and Zn, 0.05 mM Cd and 0.005 mM Hg. Isolates were inoculated into control and heavy metal supplemented plates and incubated at $28 \pm 2^\circ\text{C}$ for 48 h. Subsequently 40 mg of cells were harvested using a sterile nichrome wire loop, transferred into a clean glass tube and treated with 1 ml methanolic base reagent (15% w/v Sodium hydroxide in 50% methanol solution). The tubes were repeatedly exposed to heating (5 min in boiling water) and cooling cycles for 25 min to separate the fatty acid from cell lipids and convert to their sodium salts. The fatty acids were methylated by treating with methylation reagent (6 N HCl in 50% methanol) at $80 \pm 2^\circ\text{C}$ for 10 min. Subsequently, the fatty acid methyl esters were dissolved in 1.25 ml extraction solvent (hexane: Methyl tert-butyl ether, 1:1 (v/v) with gentle tumbling on a clinical rotator for 10 min. The organic layer containing fatty acid methyl esters was transferred to a fresh tube and washed copiously with 3 ml base wash solution (10% w/v sodium hydroxide) with 5 min tumbling. Approximately, 2/3 of the organic phase was pipetted into a GC vial and analyzed in a gas chromatography system (Agilent GC 6950).

Results

The unicellular cyanobacterium used in the study was identified as *Synechocystis* sp. based on morphological characteristics and 16S rRNA gene sequence similarities (NCBI accession No KU598870). The microscopic images confirm the unicellular morphology of the cyanobacterium used in the study (Fig. 1a). The cells were approximately $2\ \mu\text{m}$ in size, spherical with grain like appearance on the surface (Fig. 1b). The SEM image also showed the attachment of rod-shaped heterotrophic bacteria on the surface of cyanobacteria. Fourteen heterotrophic bacteria were isolated from cyanobacteria, and were deposited to the Marine Microbial Reference Facility (MMRF) maintained at CSIR-National Institute of Oceanography Regional Centre Cochin, India, and were assigned with culture numbers MMR-

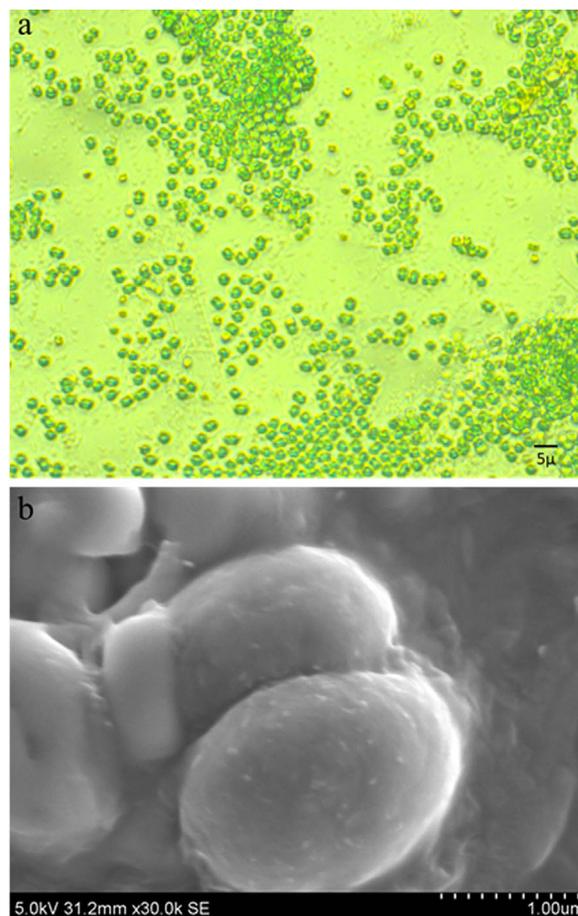


Figure 1. The light (a) and scanning electron micrograph (b) of unicellular cyanobacteria. Scale bars for light and SEM micrograph are 5 and $1\ \mu\text{m}$, respectively.

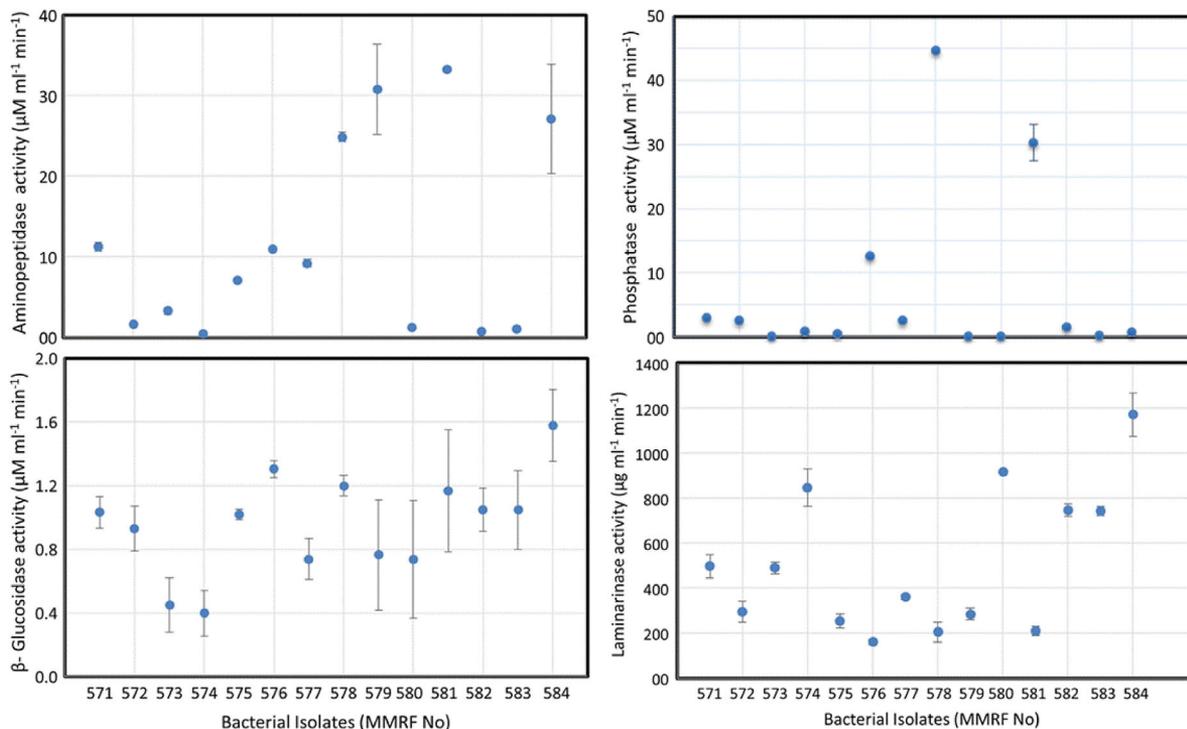
F571-MMRF-584. The heterotrophic bacteria were grouped into three phyla based on the similarities of their 16S rRNA gene sequence with that of cultured representatives available in Eztaxon (<http://eztaxon-e.ezbiocloud.net>) [26] (Table 1). Two isolates (MMRF-573 and 574) were grouped as phylum Actinobacteria and were identified as *Arthrobacter creatinolyticus*. The members of Firmicutes were identified as *Virgibacillus* sp. (MMRF-571), *Bacillus cereus* (MMRF-575, 576 and 577), *Bacillus* sp. (MMRF-580) and *Bacillus aquimaris* (MMRF-583). *Nitratireductor* sp. (MMRF-572) and *Marinobacter hydrocarbonoclasticus* (MMRF-579, 581, 582, and 584) were the Proteobacteria isolated from *Synechocystis* sp. Figure 2 shows the aminopeptidase, phosphatase, β -glucosidase, and laminarinase activities of the isolates. The isolates of phylum Proteobacteria exhibited high enzymatic properties compared to Firmicutes and Actinobacteria. High levels of aminopeptidase and phosphatase activities were exhibited by the isolates of *M. hydrocarbonoclasticus*

Table 1. 16S rRNA based phylogenetic identity of heterotrophic bacteria isolated from unicellular cyanobacteria. The bacteria were identified by 16S rRNA gene sequence similarity (>99%) with cultured representatives in Eztaxon (<http://eztaxon-e.ezbiocloud.net>).

Group	MMRF No	NCBI accession No	Sequence length (bp)	Identity	% similarity (EZtaxon)
Actinobacteria	573	KT123193	1287	<i>Arthrobacter creatinolyticus</i>	99.77
	574	KT123194	1419	<i>A. creatinolyticus</i>	99.71
Firmicutes	571	KT123191	799	<i>Virgibacillus</i> sp.	99.87
	575	KT12195	1426	<i>Bacillus cereus</i>	100
	576	KT123196	1443	<i>B. cereus</i>	100
	577	KT123197	1444	<i>B. cereus</i>	100
	580	KT123200	791	<i>Bacillus</i> sp.	99.49
	583	KT123203	1247	<i>B. aquimaris</i>	99.68
	572	KT123192	770	<i>Nitratireductor</i> sp.	100
Proteobacteria	578	KT123198	1430	<i>Marinobacter hydrocarbonoclasticus</i>	99.72
	579	KT123199	1456	<i>M. hydrocarbonoclasticus</i>	99.66
	581	KT123201	1445	<i>M. hydrocarbonoclasticus</i>	99.65
	582	KT123202	1447	<i>M. hydrocarbonoclasticus</i>	99.72
	584	KT123204	1454	<i>M. hydrocarbonoclasticus</i>	99.72

(MMRF-578, 579, 581, 582, and 584). MMRF-581 showed higher aminopeptidase activity ($33.22 \pm 0 \mu\text{M ml}^{-1} \text{ min}^{-1}$), followed by MMRF-579 ($30.7 \pm 5.6 \mu\text{M ml}^{-1} \text{ min}^{-1}$), MMRF-584 ($27.12 \pm 6.8 \mu\text{M ml}^{-1} \text{ min}^{-1}$), and MMRF-578 ($24.88 \pm 0.6 \mu\text{M ml}^{-1} \text{ min}^{-1}$). Phosphatase activity was relatively low among the isolates, *M. hydrocarbonoclasticus* MMRF-578 and 581 showed highest activities of 44.71 ± 0.2 and $30.29 \pm 0.3 \mu\text{M ml}^{-1} \text{ min}^{-1}$,

respectively. Eight out of fourteen isolates showed β -glucosidase activity of more than $1.0 \mu\text{M ml}^{-1} \text{ min}^{-1}$. High levels of β -glucosidase activity was exhibited by *M. hydrocarbonoclasticus* MMRF-584 ($1.58 \pm 0.2 \mu\text{M ml}^{-1} \text{ min}^{-1}$), *B. cereus* MMRF-576 ($1.3 \pm 0.1 \mu\text{M ml}^{-1} \text{ min}^{-1}$), *M. hydrocarbonoclasticus* MMRF-578 ($1.2 \pm 0.1 \mu\text{M ml}^{-1} \text{ min}^{-1}$), and *M. hydrocarbonoclasticus* MMRF-581 ($1.17 \pm 0.4 \mu\text{M ml}^{-1} \text{ min}^{-1}$). All the isolates showed laminarinase

**Figure 2.** Enzyme expression profile of heterotrophic bacteria isolated from unicellular cyanobacteria. The activity is expressed as average \pm SD ($n = 3$).

activity with highest expression in *M. hydrocarbonoclasticus* MMRF-584 ($1170.17 \pm 95.4 \mu\text{g ml}^{-1} \text{ min}^{-1}$) followed by *Bacillus* sp. MMRF-580 ($917.1 \pm 8.8 \mu\text{g ml}^{-1} \text{ min}^{-1}$), *A. creatinolyticus* MMRF-574 ($845.5 \pm 82.6 \mu\text{g ml}^{-1} \text{ min}^{-1}$), *M. hydrocarbonoclasticus* MMRF-582 ($745.4 \pm 27.5 \mu\text{g ml}^{-1} \text{ min}^{-1}$) and *B. aquimaris* MMRF-583 ($744.0 \pm 19.7 \mu\text{g ml}^{-1} \text{ min}^{-1}$). The *M. hydrocarbonoclasticus*. (MMRF-584) expressed high levels of aminopeptidase, β -glucosidase and laminarinase activities. Two other isolates of *M. hydrocarbonoclasticus* (MMRF-578 and 581) expressed a higher level of aminopeptidase, phosphatase, and β -glucosidase.

Many heterotrophic bacteria showed interestingly higher tolerance to heavy metals tested (Fig. 3). All heterotrophic bacteria except some isolates of *A. creatinolyticus* (MMRF-574) and *M. hydrocarbonoclasticus* (MMRF-578 and 582) showed tolerance to 250 mM Co (Fig. 3), with maximum resistance index exhibited

by *B. cereus* MMRF-575 (0.38 ± 0.06) followed by *Nitratireductor* sp. MMRF-572 (35 ± 0.07), and *B. cereus* MMRF-577 (35 ± 0.09). Maximum metal resistance index towards 250 mM Zn was expressed by the isolates of *B. cereus* (MMRF-575 (0.5 ± 0.09) and MMRF-577 (0.34 ± 0.12), *Virgibacillus* sp. (MMRF-571 (0.32 ± 0.05) and *M. hydrocarbonoclasticus* (MMRF-581 (0.34 ± 0.08) and MMRF-584 (0.32 ± 0.10)) (Fig. 4). The isolates of *M. hydrocarbonoclasticus* MMRF-578, 579, and 582 could tolerate only up to 100 mM Zn. The *Virgibacillus* sp MMRF-571 showed interestingly higher tolerance up to 180 mM Cd (metal resistance index: 0.31 ± 0.05 at 180 mM) (Fig. 3). *A. creatinolyticus* MMRF-574 and *B. cereus* MMRF-575 showed a metal resistance index of 0.21 ± 0.07 and 0.25 ± 0.09 , respectively at 150 mM Cd. The isolates of *A. creatinolyticus* (MMRF-573 and 574), *B. cereus* (MMRF-577 and 580) and *M. hydrocarbonoclasticus* (MMRF-584) showed metal resistance index of more than 0.20 at 90 mM Cd while all

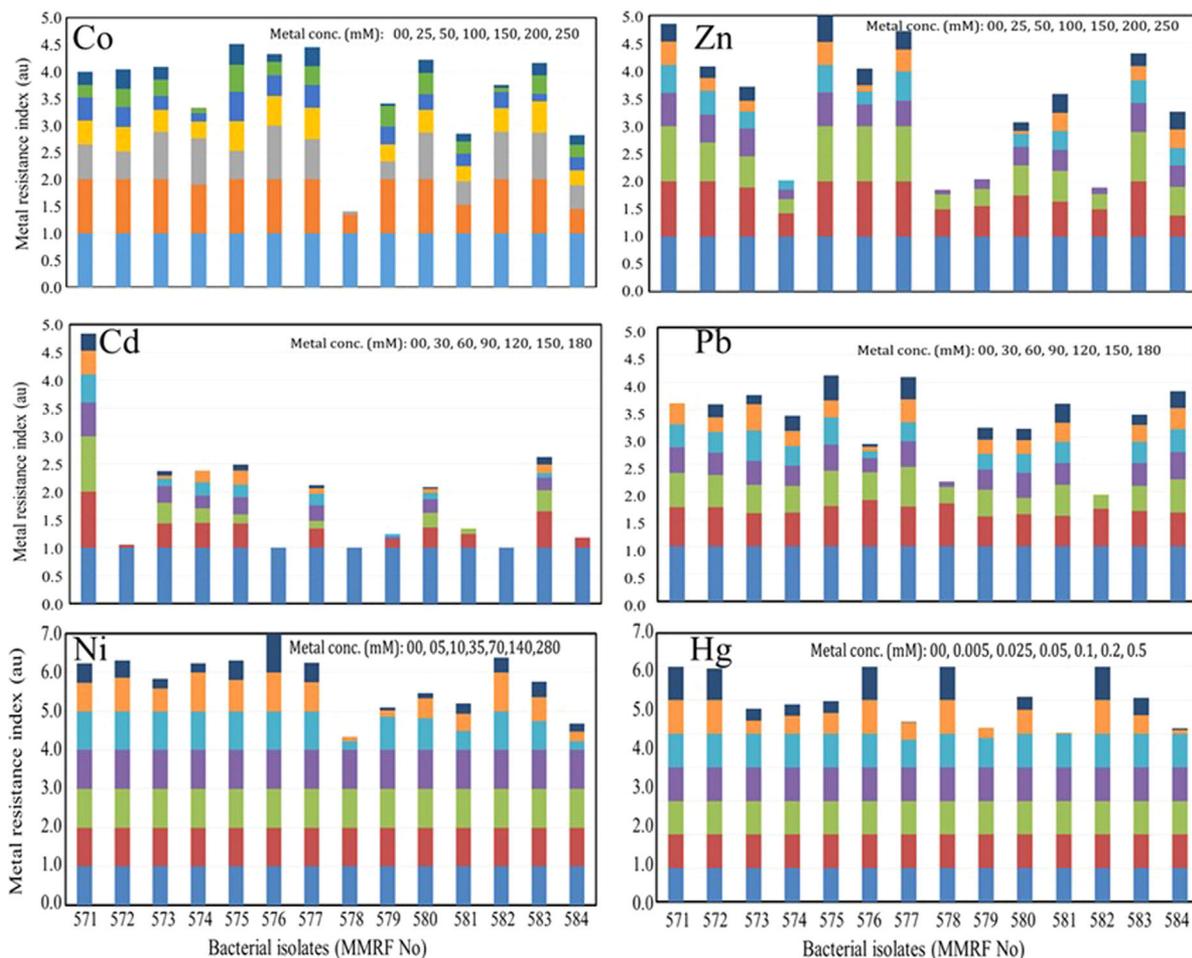


Figure 3. Two dimensional column graph showing the resistance index of heterotrophic bacteria against increasing concentration of heavy metals. Different sections of the column indicated in different color from bottom to top indicates increasing concentrations.

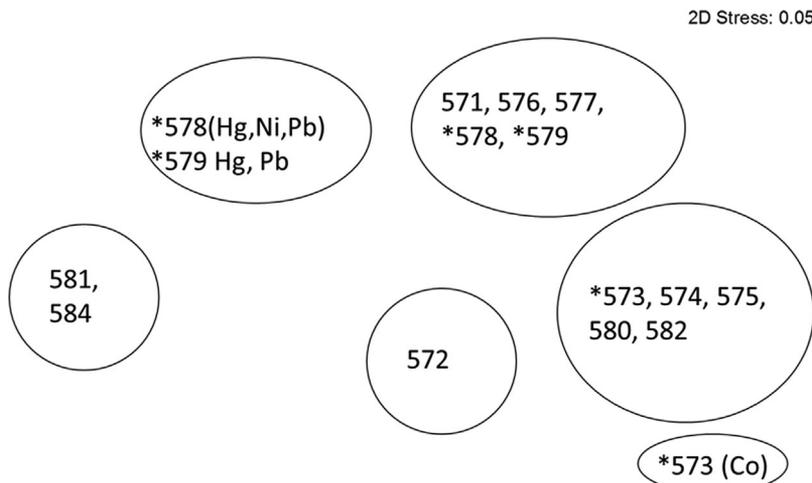


Figure 4. Non-metric multidimensional scaling (MDS) ordination plot showing the clustering of isolates based on Bray Curtis similarities of fatty acid profile on exposure to different heavy metals. Fatty acid profile of those isolates exposed to heavy metals forming different clusters are highlighted with dots.

other isolates could tolerate below 30 mM Cd. High tolerance up to 180 mM Pb was exhibited by isolates of *B. cereus* (MMRF-575 (0.45 ± 0.1) and *M. hydrocarbonoclasticus* (MMRF-581 (0.35 ± 0.06) and MMRF-584 (0.31 ± 0.09). All the isolates exhibited tolerance up to 140 mM Ni, and maximum tolerance up to 280 mM was exhibited by *B. cereus* MMRF-575 (0.51 ± 0.01), MMRF-577 (0.5 ± 0.03) and *Virgibacillus* sp. MMRF-571 (0.5 ± 0.02). All isolates of Firmicutes except *B. cereus* MMRF-577 exhibited tolerance up to 0.5 mM Hg. The isolates of *Virgibacillus* sp. MMRF-571, *B. cereus* 576 and *M. hydrocarbonoclasticus* (MMRF-578 and 582) exhibited complete tolerance (i.e., metal resistance index of 1) at all the concentrations of Hg tested. Table 2 shows the EC_{50} values prepared based on metal resistance index of each isolate against individual heavy metals. The *B. cereus* MMRF-575 showed high EC_{50} value against heavy metals Co, Zn, Pb, and Ni, while *Virgibacillus* sp. MMRF-571 showed high EC_{50} against Zn, Pb, Ni, and Hg.

Heterotrophic bacteria were grouped into six major clusters based on the similarity of their fatty acid profile (Fig. 4). The fatty acid profile of control and heavy metal exposed ones were grouped into the same cluster in a majority of heterotrophic bacteria, except some isolates of *A. creatinolyticus* (MMRF-573) and *M. hydrocarbonoclasticus* (MMRF-578 and 579). The fatty acid profile of MMRF-578 exposed to Hg, Ni and Pb and MMRF-579 exposed to Hg and Pb formed a different cluster compared to their control group (Table 3). In both the cases, the fatty acids 13:0 iso, 15:0 iso and 17:0 iso were reduced while the expression of 16:0 was increased (Table 3). MMRF-573 exposed to Co formed different clusters, in which over-expression of 15:0 iso and anteiso fatty acids were observed.

Discussion

The present study reports the enzymatic and metal resistance properties of heterotrophic bacteria isolated from unicellular cyanobacteria in culture. The cyanobacteria were initially isolated from heavy metal polluted regions of Cochin Estuary, southwest coast of India. Light microscopy image confirms the purity and unicellular morphology of the cyanobacteria while the attachment of heterotrophic bacteria on the surface of unicellular cyanobacteria was observed in SEM image. While the structural relationship between filamentous cyanobacteria and heterotrophic bacteria were demonstrated earlier using combined use of light and electron microscopy [30], the interaction with unicellular cyanobacteria is rarely reported [31]. Fourteen heterotrophic bacteria isolated from cyanobacteria were distributed into three phyla namely Proteobacteria, Firmicutes and Actinobacteria. The samples were washed copiously with saline aided with short cycles of sonication in water bath sonicator, and the heterotrophic bacteria attached to unicellular bacteria are expected to be retained after this procedure [32]. The colonization of heterotrophic bacteria inside the heterocyst of filamentous cyanobacteria, and their role in maintaining low oxygen partial pressure required for nitrogenase enzyme activity are reported earlier [7]. However, such internalization is not reported from unicellular cyanobacteria. The possibilities of bacterial internalization are limited due to the relatively small size of unicellular cyanobacteria (i.e., $\sim 2 \mu$). Heterotrophic bacteria are reported to attach to the surface mucilage of unicellular cyanobacteria and play a functional role in benthic and pelagic production [33]. The

Table 2. EC₅₀ of each metal against the heterotrophic bacteria associated with unicellular cyanobacteria. The value is calculated from metal resistance index.

Isolate	EC ₅₀ value for each metal					
	Co	Zn	Cd	Pb	Ni	Hg
MMRF-571	75	150	120	90	280	>0.5
MMRF-572	50	100	15	75	280	>0.5
MMRF-573	88	100	25	60	140	0.18
MMRF-574	83	21	26	60	225	0.2
MMRF-575	200	150	25	90	280	0.3
MMRF-576	100	88	7	60	>280	>0.5
MMRF-577	100	150	25	90	280	0.2
MMRF-578	19	25	20	48	55	>0.5
MMRF-579	43	25	17	60	105	0.16
MMRF-580	88	50	20	30	140	0.4
MMRF-581	25	50	18	60	70	0.15
MMRF-582	90	25	6.5	42	245	>0.5
MMRF-583	100	100	47	60	210	0.2
MMRF-584	25	50	18	90	55	0.16

heterotrophic bacteria form mutualistic interaction with young cells of unicellular cyanobacteria while the interaction becomes commensalism or parasitism with moribund colonies [33]. It is estimated that heterotrophic bacteria associated with the unicellular cyanobacteria, *Microcystis* sp., constituted 19–40% of total bacterial abundance, and they contribute 7–30% of the total bacterial production in the water column of Lake Vallentunasjön in Sweden [33]. A recent study reported

the gene sharing between *Bacillus subtilis* and *Synechococcus leopoliensis* for supporting the growth of the later in solid medium [31].

We separated five isolates of *Marinobacter* sp. (MMRF-578, 579, 581, 582, and 584), one *Nitratireductor* sp. (MMRF-572), five *Bacillus* sp. (MMRF-575, 576, 577, 580, and 583), one *Virgibacillus* sp. (MMRF-571) and two *Arthrobacter* sp. (MMRF-573 and 574) from the unicellular cyanobacteria. The *Marinobacter* sp. and *Nitratireductor* sp. belong to phylum Proteobacteria, *Bacillus* sp. and *Virgibacillus* sp. belong to Firmicutes and *Arthrobacter* sp. is an Actinobacteria. The association of these groups of bacteria with phytoplankton blooms have been reported earlier [5, 34–36]. Proteobacteria is a dominant group of bacteria associated with cyanobacteria, as estimated by Berg *et al.* [37] using 212 cyanobacterial bloom samples collected from different environments ranging from drinking water treatment systems to the Baltic Sea. Proteobacteria are also isolated from cyanobacteria grown in culture conditions [38]. The coexistence of *Marinobacter* sp with phytoplankton [39] and their role in the release of dissolved iron from organic matter and degradation of crude oil are reported [40, 41]. Although the coexistence of Firmicute and Actinobacteria with cyanobacteria are found in literature, their functional interaction needs further investigation.

Heterotrophic bacteria are responsible for processing approximately one-half of the organic carbon fixed by

Table 3. Fatty acid profile of MMRF-573, 578, and 579 exposed to different heavy metals. Results are expressed as percentage of total fatty acids. The fatty acid profile of these isolates exposed to heavy metals formed different clusters compared to their unexposed controls.

Bacteria	Treatment	Proportion of fatty acid (%)						
		13:0 iso	15:0 iso	15:0 anteiso	16:0 iso	16:0	17:0 iso	17:0 anteiso
MMRF-573	Control		12.65	45.83	12.53	8.24	Nil	12.51
	Cd	Nil	13.01	40.06	10.82	8.58	Nil	11.3
	Co	Nil	41.87	58.13	Nil	Nil	Nil	Nil
	Hg	Nil	21.39	42.8	11.03	4.46	6.0	11.65
	Ni	Nil	16.81	40.5	9.63	4.77	5.85	13.26
	Pb	Nil	18.85	25.27	5.04	13.17	5.18	7.12
	Zn	Nil	25.65	36.56	10.27	2.48	9.09	13.77
MMRF-578	Control	10.02	28.5	Nil	7.8	6.54	11.24	Nil
	Cd	6.97	19.12	Nil	6.18	14.38	8.83	Nil
	Hg	Nil	0.64	Nil	Nil	29.39	Nil	Nil
	Ni	0.2	0.49	Nil	0.15	25.23	0.41	Nil
	Pb	0.19	0.36	Nil	Nil	25.52	0.39	Nil
	Zn	6.02	21.15	Nil	6.01	13.53	9.51	Nil
MMRF-579	Control	10.83	28.68	Nil	6.58	5.12	8.07	Nil
	Cd	3.45	11.54	Nil	4.01	19	5.04	Nil
	Co	2.98	8.65	Nil	3.3	19.53	4.69	Nil
	Hg	0.19	1.01	Nil	0.22	24.95	0.85	Nil
	Ni	7.67	22.83	Nil	6.86	11.16	8.73	Nil
	Pb	Nil	0.54	Nil	Nil	31.43	Nil	Nil
	Zn	6.04	21.99	Nil	4.46	11.83	9.59	Nil

autotrophs in the ocean [34, 42], and is mediated through the secretion of extra cellular enzyme. Overall, the Proteobacterial isolates showed maximum enzyme activity in the present study. Genome analysis showed that many of the Proteobacteria associated with particulate organic matter are copiotrophic, which increases the carbon and nutrient availability to their oligotrophic counterparts through sustained enzyme activity [34, 43]. During the early stages of life, phytoplankton releases soluble low molecular weight (LMW) compounds that attract heterotrophic bacteria to them [12, 44]. On the contrary, phytoplankton secretes high molecular weight (HMW) compounds during the warning stages of their growth [34, 45, 46]. It was confirmed by bacterial growth model studies that the substrate for bacterial growth was provided by a combination of algal exudates and lysis products [47]. The high molecular weight biopolymers constitute an important source of carbon, nitrogen, and energy for biosynthesis and respiration of bacteria [47]. Reduction of HMW polymers into micromolecules of less than 600 Da and its further absorption by microorganisms is a vital process in sustaining primary productivity in marine environment [20, 48]. The high enzyme activity shown by Proteobacteria in the present study indicates their possible functional role in the processing of HMW exudates of unicellular cyanobacteria. Laminarin forms one of the major high molecular weight polymer of cyanobacterial exudates and hence the bacteria showing high activities of laminarinase and β -glucosidase could be participating in the processing of cyanobacterial exudate. Similarly, organisms with high aminopeptidase and lipase may also participate in the processing of protein and lipid portions of exudates. Largely, isolates of phylum Proteobacteria showed higher enzyme expression indicating their possible functional role in the processing of cyanobacterial exudates and making the nutrients available for the better growth of their oligotrophic counterparts.

One of the interesting observations recorded in the present study is the multiple metal resistance of heterotrophic bacteria associated with cyanobacteria. All the isolates used in the study showed tolerance to multiple metals, with a difference in the pattern of tolerance. The isolates showed tolerance to relatively higher concentrations of heavy metals like Co, Zn, Pb, and Ni while their tolerance to Hg and Cd was comparatively low. Firmicute and Actinobacteria isolates showed higher metal resistance profile compared to Proteobacteria. The *B. cereus* MMRF-575 showed high resistance against all the metals tested, while *Virgibacillus* sp. MMRF-571 showed exceptional resistance compared to other isolates against

the toxic metals Cd, Pb, and Hg. The high level of heavy metal tolerance in the bacteria in the present study may be attributed to the pre-exposure of unicellular cyanobacteria to heavy metal pollutants. We isolated the unicellular cyanobacteria from the heavy metal impacted regions of Cochin Estuary, southwest coast of India. In a previous study, we have reported the high metal tolerance of heterotrophic bacteria isolated from the same region of Cochin Estuary [20]. The bioavailability of heavy metals are reported to have a significant impact on benthic microbial loop functioning of the coastal and marine environment [49]. High level of Cd and Pb in Cochin estuary due to industrial effluent discharge is reported [20]. Hence the development of bacterial resistance to these pollutants is natural. Interestingly, many isolates showed resistance against Hg, which is not a major pollutant reported from Cochin estuary. Different bacteria, such as *Pseudomonas*, *Proteus*, *Xanthomonas*, *Alteromonas*, *Aeromonas*, and *Enterobacteriaceae* isolated from the coastal and marine environment are reported to be tolerant up to 50 ppm Hg [50]. Cyanobacteria isolated from heavy metal polluted environments have shown the ability to accumulate, detoxify or metabolize heavy metals up to certain concentration range [14]. Hence, cyanobacteria are used for bioremediation of industrial effluents containing heavy metals. However, the autotrophic and stringent growth conditions and requirement of large ponds are the limitations in their wide application. In the present study, we propose heterotrophic bacteria isolated from unicellular cyanobacteria as potential candidates for possible application in bioremediation of heavy metal pollution. The application of heterotrophic bacteria can reduce the cost of bioremediation compared to cyanobacteria because of their fast growth rate and minimal growth conditions required.

The fatty acid profile of isolates exposed to different metals were studied to understand the cellular adaptations of isolates. The MDS plots prepared based on Bray Curtis similarities grouped the organisms according to their similarities in the proportion of fatty acids. The *A. creatinolyticus* (MMRF-573) exposed to Co, isolates of *M. hydrocarbonoclasticus*, MMRF-578 exposed to Ni, Pb and Hg and MMRF-579 exposed to Pb and Hg formed different clusters due to the difference in the proportion of fatty acid compared to their unexposed controls. These isolates showed a major difference in the proportion of fatty acids 15:0 iso, 15:0 anteiso, 16:0 iso, 16:00, and 17:0 iso. Such difference in the proportion of fatty acid profile of different isolates of *Pseudomonas* sp. exposed to silver nanoparticle and naphthalene have been reported earlier [51, 52]. The changes in the proportion of membrane fatty acid composition in

Salmonella enterica was also reported as an adaptation to protect cells from toxic effects of plant derived terpenes [53]. The fatty acids, 15:0 and 17:0 are generally used as the biomarkers for bacteria, iso and anteiso isomers of 15:0 for Gram positive bacteria, β -hydroxy fatty acids (β 10:0, 12:0) for Gram negative bacteria which are present in lipid portion of the lipopolysaccharides in the cell wall [54]. Microorganisms adjust the cell membrane fluidity by altering the fatty acid proportion, which further adjust the homeostasis between the intra and extracellular levels of heavy metals and other toxicants [54].

In short, our results show that unicellular cyanobacteria also accommodate a consortium of microorganisms with varied functional roles. Proteobacteria associated with cyanobacteria showed higher expression of different enzymes, which may contribute to the processing of high molecular weight cyanobacteria exudates. Firmicute and Actinobacteria showed high tolerance to heavy metals including Pb, Cd and Hg and these bacteria may accumulate metals, saving their counterparts from toxic effect. The metal tolerance of heterotrophic bacteria may also find applications in bioremediation of heavy metal polluted environments, where they may replace already known properties of cyanobacteria. Further studies on the role of cyanobacteria in designing the diversity of associated heterotrophic bacteria and their mutual interaction may be appreciated.

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Conflict of interest

The authors have declare no conflict of interest.

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