Int.J.Curr.Microbiol.App.Sci (2021) *10*(11): 170-187

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 10 Number 11 (2021)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2021.1011.020

Biocontrol of Toxin Producing Cyanobacterium *Microcystis aeruginosa* by Algicidal Bacterium *Exiguobacterium acetylicum* Strain TM2 Isolated from Mid-Altitudinal Himalayan Lake of Northern India

Surabhi Rawat[1], Neetu Shahi[1*], Sumanta Kumar Mallik[1], Richa Pathak[1], Bhupendra Singh[1] and Veena Pande[2]

[1]ICAR-Directorate of Coldwater Fisheries Research, Anusandhan Bhavan, Industrial Area, Bhimtal 263136, Nainital, Uttarakhand, India
[2]Department of Biotechnology, Bhimtal, Kumaun University, 263136, Nainital, Uttarakhand, India

**Corresponding author*

A B S T R A C T

Microcystis aeruginosa is a hepatotoxin producing cyanobacteria, found globally in freshwaters. In the present study, an algicidal bacterium against *M. aeruginosa* was isolated from Bhimtal Lake (29°20'39"N; 79°33'32"E) of Himalayan region of Uttarakhand, India. The isolated bacterium *Exiguobacterium acetylicum* strainTM2was identified by morphological characteristics, biochemical characteristics and partial 16S ribosomal DNA (rDNA) gene amplification (GenBank accession number: KX155561). Efficacy of *E. acetylicum* TM2, and its mode of algicidal activity was evaluated against *M. aeruginosa*. *E. acetylicum* TM2 showed intense anti-cyanobacterial effect against *M. aeruginosa*, and approximately 90.0 % death of *M. aeruginosa* cells were observed after 10 days of incubation. The bacterium attacked the *M. aeruginosa* cells directly by physically coming in contact and caused damaged to its membrane and internal organelles. Cell free filtrate of *E. acetylicum* TM2 did not exhibited algicidal activity, which indicates that mode of algicidal mechanism, is cell to cell contact, and not chemically mediated damage by algicidal compounds released from *E. acetylicum* TM2. Furthermore, *in vitro* and *in vivo* pathogenicity test confirm the non-virulence of *E. acetylicum* TM2 and so it could be potentially useful in mitigation of *M. aeruginosa* blooms in water.

Keywords

Microcystis, Algicidal bacteria, Algal bloom, Biological control, Himalaya

Article Info

Received:
05 October 2021
Accepted:
30 October 2021
Available Online:
10 November 2021

Introduction

Increase in cyanobacterial blooms and cyanotoxins in eutrophic freshwater have become a major crisis worldwide, mainly due to rise in global temperature, pollution and release of excess nutrients in water bodies by anthropogenic activities (Gobler 2021). Toxin producing cyanobacterial blooms harms the ecosystem, human health, animals, tourism

and local fisheries (Jeong *et al.,* 2000; Nagayama *et al.,* 2003). In lentic freshwater system, *Microcystis aeruginosa* has been associated with cyanobacterial blooms of economic and ecological impact (Sigee *et al.,* 1999; Carmichael 2001), worldwide. *Microcystis* blooms deteriorate quality of drinking water and cause human and animal health hazards by producing a massive range of toxins particularly microcystins which is a hepatotoxin (Ishii *et al.,* 2004; Zurawell *et al.,* 2005; Shahi *et al.,* 2012). Several methods have previously been developed and proposed to control cyanobacterial blooms, including chemical treatment (Haughey *et al.,* 2000; Nowack *et al.,* 2011; Paerl *et al.,* 2011), physical removal and biological control (Chio *et al.,* 2005; Ho *et al.,* 2007; Cai *et al.,* 2011; Li *et al.,* 2014).

In natural environment, cyanobacteria and bacterial flora co-exist (Su *et al.,* 2007).The physiological state of cyanobacteria can be influenced by bacteria. Some bacteria initiate bloom formation, whereas other can have antagonistic effects (Mayali and Azam 2004). And so, in recent year's biological control of cyanobacteria by bacteria isolated from natural environment have received immense consideration due to their potential efficacy, species specificity, and environmental friendliness (Chio *et al.,* 2005; Ho *et al.,* 2007; Manage *et al.,* 2009; Zheng *et al.,* 2012; Liu *et al.,* 2019; Jing *et al.,* 2020).

In the current study, an algicidal bacterium against *M. aeruginosa* was isolated and characterised from the fresh water lake of western Himalayan region of northern India.

This bacterium was identified as *Exiguobacterium acetylicum,* which exhibited cell-to-cell contact algicidal activity against *M. aeruginosa*. Upon *in vitro* and *in vivo* virulence assay, the bacterium was found to be non-virulent, and therefore in coming days could be used to mitigate *M. aeruginosa* bloom in freshwater.

Materials and Methods

Sampling and culture of *Microcystis aeruginosa*

Cyanobacterium *M. aeruginosa* used in our study was collected during May-October 2016 and May-October 2017 from surface water of Bhimtal Lake (29°20'39"N; 79°33'32"E; altitude 1370 meters above sea level), Uttarakhand, India(Fig 1A-D).Altogether, 15 water samples (200 ml each) of *M. aeruginosa* bloom were collected in sterile LDP bottles of 500 mL capacity, and within half an hour of its collection, the water samples were transported to Directorate of Coldwater Fisheries Research (DCFR), Bhimtal, India. One portion of the water sample (50 ml) was fixed in Lugol's iodine (HiMedia, India), whereas another portion (150 ml) was used for culturing *M. aeruginosa* and also for the isolation of algicidal bacteria. *M. aeruginosa* was identified by cell morphology and by PCR amplification of cyanobacterial 16S rDNA gene (Hotto *et al.,* 2007). The *M.aeruginosa* cells were picked, cultured and maintained in sterilized cyanophycean agar (HiMedia, India) and Blue-Green broth (BG-11broth) (HiMedia, India). A 12h light: 12h dark photo-cycle with a light intensity of 30 µmol photons m^{-2} $s^{-1}$was provided, which was maintained throughout the study period. Every day cells were monitored for growth, and cell count of *M. aeruginosa* was carried out under an inverted light microscope (Olympus IX53, Japan), every alternate days using a Sedgewick rafter cell.

Isolation and screening of algicidal bacteria

Unfixed water sample aliquots were serially diluted with 9 ml sterile phosphate-buffered saline (PBS) (HiMedia, India) pH 7.2, and

then diluted aliquots (100µl) of each dilution were spread onto trypticase soy agar (TSA) (HiMedia, India). Plates were incubated for 24-48h at 27ºC.Individual bacterial colonies were selected and picked according to their distinct colony morphology. Bacteria were purified for pure culture by streaking and restreaking. The bacterial strains were preserved for further use in freezing medial, bacterial (VWR Life Science AMRESCO's, USA), and stored at -80°C in deep freezer.

To examine the algicidal activity of bacterial isolates, freshly revived bacterial culture was grown overnight in tryptic soy broth (TSB) and then centrifuged at 6,000 rpm for 10 min at 4 °C. Bacterial pellet was washed two times with sterile PBS, and then re-suspended in fresh PBS. One ml of re-suspended bacterial cells was then added to 50 ml of axenic *M. aeruginosa* (10^8cells/ml) culture at the final concentration of ($1.25x10^8$ cfu/ml). All the tests were conducted in triplicate, unless otherwise mentioned.

Control contained *M. aeruginosa* cells without addition of bacteria. The experiment period was 15 days, and *M. aeruginosa* cell density and morphological changes were regularly monitored under an inverted light microscope (Olympus IX53, Japan) using a Sedgewick rafter cell. Algicidal activity was calculated using the equation mentioned in section 2.5. The bacterial strains with potent algicidal rate of > 80% (t=10 days) were further analysed.

Biochemical and molecular identification of bacteria

Biochemical and molecular identification of isolated bacterial strain TM2 with potential algicidal activity was performed, following the procedure described earlier (Shahi *et al.*, 2018). Bacterial colony characteristic was observed after 24-48 h growth on TSA at 27°C. Biochemical characterization was performed with readymade commercial media (HiMedia, India).

For molecular identification, after extracting the genomic DNA by wizard genomic DNA purification kit (Promega, USA), PCR was carried out using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-GGTTACCTTGTTACGACTT-3'). PCR amplified 16S rDNA nucleotide sequence of 1400 bp was gel purified and Sanger sequenced at SciGenom Labs Pvt Ltd, Kochi, India. After sequencing, nucleotide sequence was Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.gov /BLAST) searched. The 16S rDNA sequence was aligned using clustal omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Aneighbor-joining phylogenetic tree was constructed by Mega Xtool (Kumar *et al.*, 2018). Nucleotide sequence of bacteria was deposited in GenBank of National Centre for Biotechnology Information (NCBI), and the accession number was obtained.

Transmission electron microscopy

For transmission electron microscopy (TEM), overnight grown TM2 was processed by following the standard procedure mentioned previously (Bozzola and Russell 1998). The processed sample was examined using Transmission Electron Microscope Tecnai, G 20 (FEI) at All India Institutes of Medical Sciences (AIIMS), New Delhi, India. Images were taken at 2550X magnification.

Algicidal effect and bacterial cell density

Algicidal effect of bacteriumTM2 was evaluated, as previously described (Jeong-Deong *et al.*, 2009). Two experimental groups, 1 treatment and 1 control were set up in duplicatein a rectangular glass tank of 35 l capacity. *M. aeruginosa* suspension at the density of 3.6×10^8 cells/ml was added to

treatment and control tanks, both. A cell suspension of TM2 in the logarithmic phase was washed and diluted in TSA medium, and 100 ml of bacterial culture at a concentration of 4.06×10^8cfu/ml was prepared and added to 16 l of *M. aeruginosa* suspension. *M. aeruginosa* suspension without bacterium served as control. Culture condition for *M. aeruginosa* was similar to as described in the section 2.1. The cell count of *M. aeruginosa* and TM2 was determined by Sedgewick rafter cell and total plate count method, respectively. Experiment was monitored visually and microscopically everyday for 30 days. After every 24 h, samples were analyzed to elucidate the algicidal activity of TM2. Algicidal effect on *M. aeruginosa* was photographed using inverted light microscope (Olympus IX53, Japan).

To evaluate the effect of bacterial cell density on algicidal activity of TM2, log phase bacterial culture at the concentration of 4.06×10^8 cfu/ml was added at the volume of 1%, 5%, and 10% concentration to *M. aeruginosa* cells (3.6×10^8 cells/ml). This experiment was conducted in similar manner as mentioned above. Algicidal activity percentage was calculated by using the formula:

Algal lytic activities (%) = N_C-N_T/N_Cx100

Where N_C represents the count of *M. aeruginosa* in control tank and N_T is count of *M. aeruginosa* in treatment tank.

Preparation of bacterial extracellular proteases of TM2

At lag phase, bacterial cells grown in TSB were harvested by centrifugation at 6,000 rpm for 10 min. The supernatant was collected and immediately filtered through 0.2 µm syringe filter to remove any bacterial cells. To detect the inhibiting activities of extracellular compound, 1 ml of filtered supernatant was inoculated into 50 ml of *M. aeruginosa* (10^8 cells/ml). To assess the algicidal activity of released extracellular compound, growth of *M. aeruginosa* was daily monitored until 15 days, under an inverted light microscope (Olympus IX53, Japan) and cell density were determined as described in the section 2.5 of materials and methods.

In vitro virulence assay of TM2

Sheep blood hemolysis test

To test the hemolytic activity of TM2, defibrinated sheep blood was washed three times with sterile PBS and pellet was re-suspended in 10% (v/v) PBS containing 10 mM Dithiothreitol (DTT). Approximately, 100µl of TM2 (1.5×10^8 cfu/ml) at log phase was serially diluted in a 96 well sterile plate. To each dilution, 100µl of sheep erythrocytes were added into wells.100µl of sterile PBS was used as negative control, and 100 µl of 0.2% Triton X100 was used as positive control. The plate was incubated at 37º C for 60 min. After incubation, plate was centrifuged in a mini plate spinner, supernatant was collected and transferred to a flat bottom 96- well plate. Absorbance of the supernatant was taken at OD 540 nm using a spectrophotometer (Multiskan Go, ThermoScientific, USA). Hemolysin percentage was calculated as:

Hemolysis %= $100\times (A_{sample} - A_{PBS}) / (A_{TritonX100} - A_{PBS})$

Where A represents (absorbance)

Quantification of biofilm formation

Biofilm formation of *E. acetylicum* TM2 was evaluated using the method described earlier by Salma *et al.,* (2018). In brief, bacterium was grown overnight in TSB to the cell

concentration of 8 $\log_{10}$ cfu/ml, then diluted 100 fold with sterile TSB and added to 96-well flat-bottom polystyrene plates at the volume of 200 µl/each well. After incubation, the bacterial suspension was removed and plate was washed thrice with 270 µl sterile PBS. Bacterial cells attached to plate were stained with 0.1% crystal violet (CV) and washed thrice with sterile PBS. After adding 100 µl of 95% ethanol to each well, absorbance was taken at 590 nm by using a microplate reader (Multiskan Go, ThermoScientific, USA). Sterile TSB was used as a negative control, and all the assay was performed in three technical replicates.

Detection of siderophore production

An overnight grown *E. acetylicum*TM2 was spot inoculated on Chrome Azurol-S (CAS) plates (HiMedia, India), and incubated for 48-72hat 27 °C (Schwyn and Neilands 1987). The result was observed visually, and a dark yellow to orange halo zone around the inoculums represents the siderophore production.

In vivo virulence assay of TM2

To evaluate the virulence of bacterium *in vivo*, 20 healthy common carp (average body weight 20.45 ± 6.76 g) was i.p injected (100 µl) with the bacterium TM2 at the concentration of 2.0 $\times 10^5$cfu/ml. Fish of control group, 20 common carp were injected with 100 µl of sterile PBS. Any abnormality, morbidity and mortality were recorded immediately. Throughout the experimental period of 30 days, feeding behavior, survival percentage, animal behavior, internal and external gross lesions was recorded for both the groups. Feeding behavior was recorded daily and scoring was on scale of 0-4. No feed consumed is 0; 25% feed consumed is 1; 50% feed consumed is 2; 75% feed consumed is 3 and 100% feed consumed is 4. Histopathology scoring was recorded on the scale of 0 to 5. As no changes is 0;<5% tissue affected is 1 (normal), 5-15% tissue affected is 2 (mild), 15-25% tissue affected is 3 (moderate), 25-50% tissue affected is 4 (marked) and >50% tissues affected is 5 (severe). Survival was monitored daily.

Statistical analysis of data

Data were statistically analyzed using SPSS version 20.0, and shown as mean ± standard deviation of two independent assays. Two-way ANOVA with Bonferroni post-test was used to compare the differences between the control and different parameters to be observed. Using GraphPad Prism 5.01 software (GraphPad Software Inc, San Diego, CA) a '*P*' value of <0.001 was considered as statistically highly significant whereas, <0.005 was considered statistically significant.

Results and Discussion

Isolation and screening of algicidal bacteria

From water samples of *M. aeruginosa* bloom, thirty eight bacterial strains were isolated from Bhimtal Lake in between 2016 to 2017. Among thirty eight strains, only one strain TM2 had efficacious algicidal activity against *M. aeruginosa*, and so used for subsequent characterization, evaluation of algicidal activity and pathogenicity test.

Characterization of bacteriumTM2

Colony of TM2 was yellow, round, convex, shiny, opaque and 3-4 mm in diameter after 48 h of incubation in TSA plates (Fig 2A) at 27 - 28 °C. Upon Gram-staining, strain TM2 was found to be Gram-positive, rod-shaped bacteria (Fig 2B). Biochemical, growth and other characteristic of this strain is mentioned in table 1. TM2 could survive and grow in a wide range of temperature and salinity and did

not produce protease, lipase, DNase and enzyme for hydrolysis of carbohydrate (table 1).

PCR amplification (Fig 3A) and nucleotide sequence similarity search of 16S rDNA region of this bacterium (GenBank accession number: KX155561) reveals that, this strain shares 99.92% nucleotide sequence similarity with *E. acetylicum* strain V12 (GenBank accession number: MW041273) and *E. acetylicum* strain KNUC604 (GenBank accession number: HM047519).

In phylogenetic tree strain TM2 clustered with strain V12 and KNUC604 (Fig 3B). Therefore, based on the morphological, biochemical and molecular characteristic, strain TM2 was designated as *E. acetylicum* strain TM2.

Transmission electron microscopy

Negatively stained electron micrograph of *E. acetylicum*TM2 cells confirm the elliptical rod shaped bacterium with peritrichous flagella, and also hair-like structure called pili (Fig 4).

The length of the flagella was in between 4-6µm. Further, two bacterial cells were directly connected with a long thread-like structure, which is similar in appearance to sex pili.
Algicidal activity of *E. acetylicum*TM2

Bacterium *E. acetylicum* TM2 had efficacious anti-cyanobacterial activity against *M. aeruginosa* (initial cell density was 3.6×10^8 cells/ml), in comparison to control. Rapid decolourization and disintegration of *Microcystis* cells after addition of TM2 confirms the attack of cyanobacteria by TM2 bacterium (Fig 5A). Approximately 90.0 % reduction in *M. aeruginosa* cell count was observed after 10 days of incubation (Fig 5B) with bacterium TM2.

The algicidal activity percentage of TM2 (10^8 cfu/ml) for 1% bacterial culture within 24, 48 and 72 h were 11.5%, 19.5% and 29%, for 5% concentration these values were 23%, 25%, 54% whereas, for 10% algicidal percentage reached at 36%, 55%, 94.5% respectively (Fig 5C). At different bacterial concentrations the algicidal activity percentage varied significantly. At high bacterial concentrations (10%) the algicidal activity is significantly high.

In comparison to *M. aeruginosa* without TM2 (Fig 6A), a very distinct morphological changes were observed in *M. aeruginosa* exposed to TM2 (Fig 6B).

The bacterium harm the cells of *M. aeruginosa* by direct physical contact, and thereafter these cells were killed by damaged to its internal organs (Fig 6C, D).In control the cell number of *M. aeruginosa* was gradually increasing.

Algicidal mode of *E. acetylicum* TM2

The cell free supernatant of TM2 did not exhibit algicidal activity against *M. aeruginosa* till 15 days of incubation (data not shown). This suggests that the TM2 attack and kill the cell directly by cell to cell contact, and not by secretion of extracellular protease or other algicidal compound.

In vitro pathogenicity assay of *E. acetylicum* TM2

Detection of hemolytic activity

In the present study, hemolytic activity was quantified by liquid hemolytic assay test and percentage haemolysis was calculated. Hemolytic activity was not shown by TM2, and the amount of hemolysin produced was 0%, indicating that algicidal strain TM2 could be considered to have non-hemolytic (Fig 7A).

Assessment of biofilm formation

BacteriumTM2 did not produced the biofilm (values < 0.1) after 24 h and 48 h of incubation (Fig. 7B, C), which further confirms the non-pathogenicity of our algicidal bacterium.

Siderophore production

Siderophore production by TM2 was not detected till 72 h of incubation.

In vivo pathogenicity assay of TM2

Throughout the experimental period of 30 days, feeding behaviour, survival rate, animal behaviour was normal and similar in between treatment and control group of common carp. Feeding behaviour was normal and regular and score was 4. No gross external or internal lesions such as skin discoloration, severity of dermal lesion and gill pallor were observed. Gasping of air, flashing, hyperactivity, lethargy, loss of equilibrium and abnormal pigmentation was not observed in TM2 injected common carp. Histopathology of liver, kidney, spleen and gall bladder was normal (data not shown). Histopathology score of all the organs were 0-1, which is considered not biologically important. Survival percentage of common carp in treatment and control group was > 90% the end of experiment.

Due to rise in global temperature and excessive eutrophication of water bodies by anthropogenic activities, cyanobacterial blooms of freshwater system have become a serious concern over the last few decades. Cyanobacterial blooms can cause damage to aquatic fauna, makes the water unfit for human and animal use. It also decreases the aesthetic value of the system, which hampers the tourism and other regular activities. In warm and eutrophic freshwater, *Microcystis* is the major bloom producing cyanobacteria (Zurawell *et al.,* 2005). *Microcystis* produce microcystins, a potent hepatotoxin which cause severe damages to liver (Ishii *et al.,* 2004). Since microcystins, cannot be degraded by proteases such as pepsin and trypsin due to its cyclic chemical nature, a safe and effective biological control method of limiting the growth of *Microcystis* is very much required.

In this study, altogether thirty eight bacterial strains were isolated from Bhimtal Lake during *M. aeruginosa* bloom, and their algicidal activity were evaluated under experimental conditions. Out of thirty eight bacterial strains, only one bacterium, TM2 had efficacious (>90% algicidal activity; t=10 days) algicidal activity in preliminary screening, and so was selected for further characterization and evaluation of mode of algicidal activity.

Generally majority of the algicidal bacteria are classified under the cytophaga-Flavobacterium-Bacteroidetes group or the γ-Proteobacteria group (Mayali and Azam, 2004). However in our study by biochemical tests and molecular characterization the bacterium was identified as *Exiguobacterium acetylicum*, and designated as *E. acetylicum* strain TM2. The genus *Exiguobacterium* comes under the coryneform bacteria, and are Gram-positive, oxidase and catalase positive motile rods. *Exiguobacterium* spp. has been previously reported from a wide range of habitat including soil, glacier water and deep sea sediments (Chaturvedi and Shivaji, 2006; Govindan *et al.,* 2009; Singh *et al.,* 2013; White *et al.,* 2019). In India this bacterium has been previously reported from soil of North West Himalaya (Chaturvedi and Shivaji, 2006; Govindan *et al.,* 2009; Singh *et al.,* 2013).

Table.1 Colony and biochemical characteristic of the bacterium *Exiguobacterium acetylicum* strain TM2

Characteristic	*E. acetylicum* strain TM2
Colony characteristics	
Colony colour	Yellow
Elevation	Raised
Surface	Smooth & glistering
Transparency	Opaque
Texture	Moist
Margin	Entire
Biochemical characteristics	
Gram Staining	Gram-positive
3% KOH	-
Bacterial morphology	Long rod
Spore shape	ND
Spore swollen	ND
Motility	+
Oxidase	+
Catalase	+
H_2S	-
Indole	-
Methyl red	+
Vogus-Proskeur	-
Citrate	+
***In vitro* virulence test**	
Casein	+
Gelatin	+
Nitrate	-
Urease	+
Esculin	-
DNAse	-
Lipase	-
Lecithinase	-
Starch	+
Haemolysin	-
Growth condition	
Temperature range (°C)	4-37
Optimal temperature (°C)	19-32
NaCl concentration (% w/v)	1-7
Optimal NaCl concentration (% w/v)	1
pH range	5-8.5
Optimal pH	7.2

+ positive; - negative; ND no data

Fig.1 (A & B) Sampling site for *Microcystis aeruginosa* bloom and algicidal bacterium. (C) Bhimtal lake. (D) *M. aeruginosa* bloom in Bhimtal Lake during warmer period

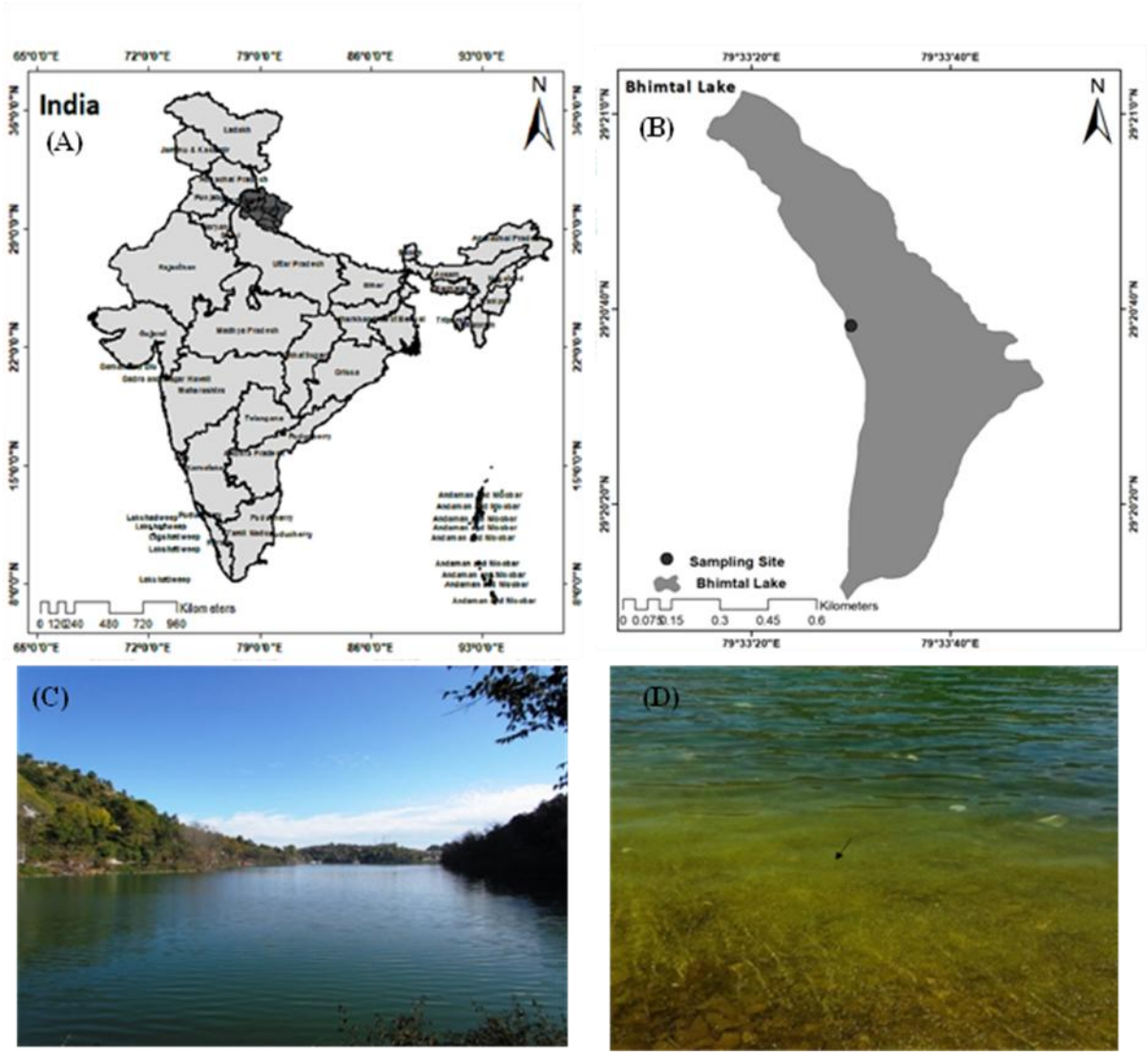


Fig.2 (A) Yellow pigmented colony of the *Exiguobacteriumacetylicum*TM2 on TSA plate, incubated at 27 ℃ (B) Gram-stain of *E. acetylicum*TM2 shows the Gram-positive, long rod bacterium (at 1000X oil immersion). At the bottom right side of the image, scale bar is given

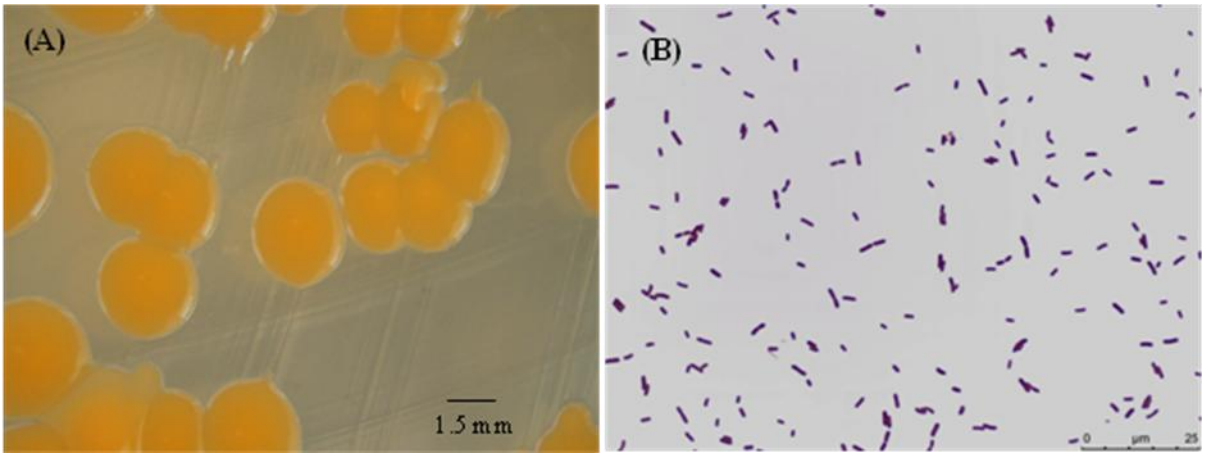


Fig.3 (A) PCR amplification of 16S rDNA sequence of *Exiguobacterium acetylicum* TM2. (B) Phylogenetic tree constructed from partial 16S rDNA nucleotide sequences, showing clustering of *E. acetylicum* TM2 (GenBank accession no: KX155561) to other 15 *Exiguobacterium* taxa. Bootstrap values is shown above the branches of the phylogenetic tree. Scale bar below the tree represents 0.01 substitutions per nucleotide position

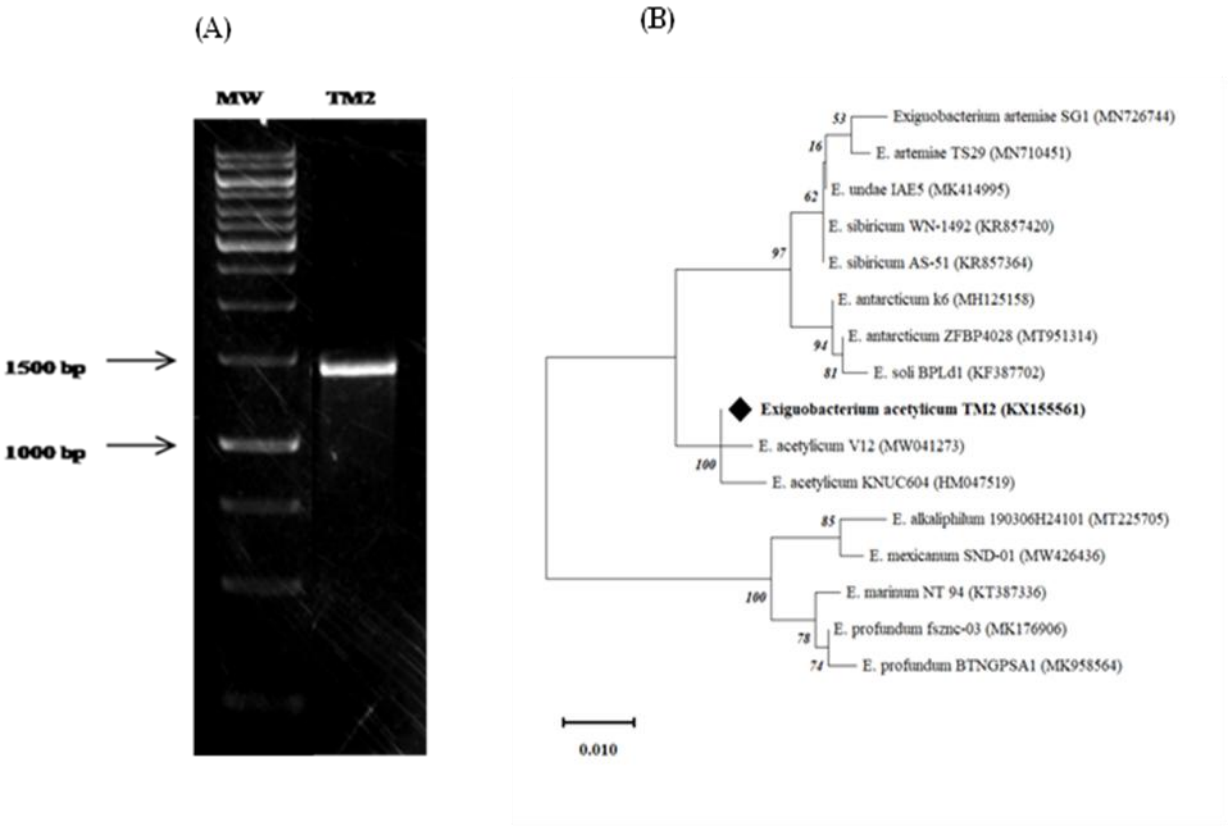


Fig.4 Transmission electron microscopy (TEM) image of algicidal bacterium *Exiguobacterium acetylicum* TM2 isolated from Bhimtal Lake, Uttarakhand, India. The thick outer capsule and finger like projection, which is lined to virulence and host attachment, is not detected. Scale bar is shown at bottom left side of the image and magnification is X2550

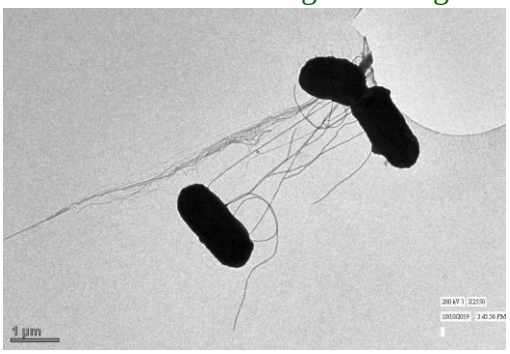


Fig.5 (A) Visual detection of change in color of *M. aeruginosa* in presence of *Exiguobacterium acetylicum* TM2 (treatment) in comparison to control group (Control). (B) Changes in cell number of *M. aeruginosa* till 10 days of experiment. (C) Algicidal activity of *Exiguobacterium acetylicum* TM2 against *M. aeruginosa* at different volume fraction of 1%, 5% and 10%. Results are expressed as the mean ± SE from triplicate assays

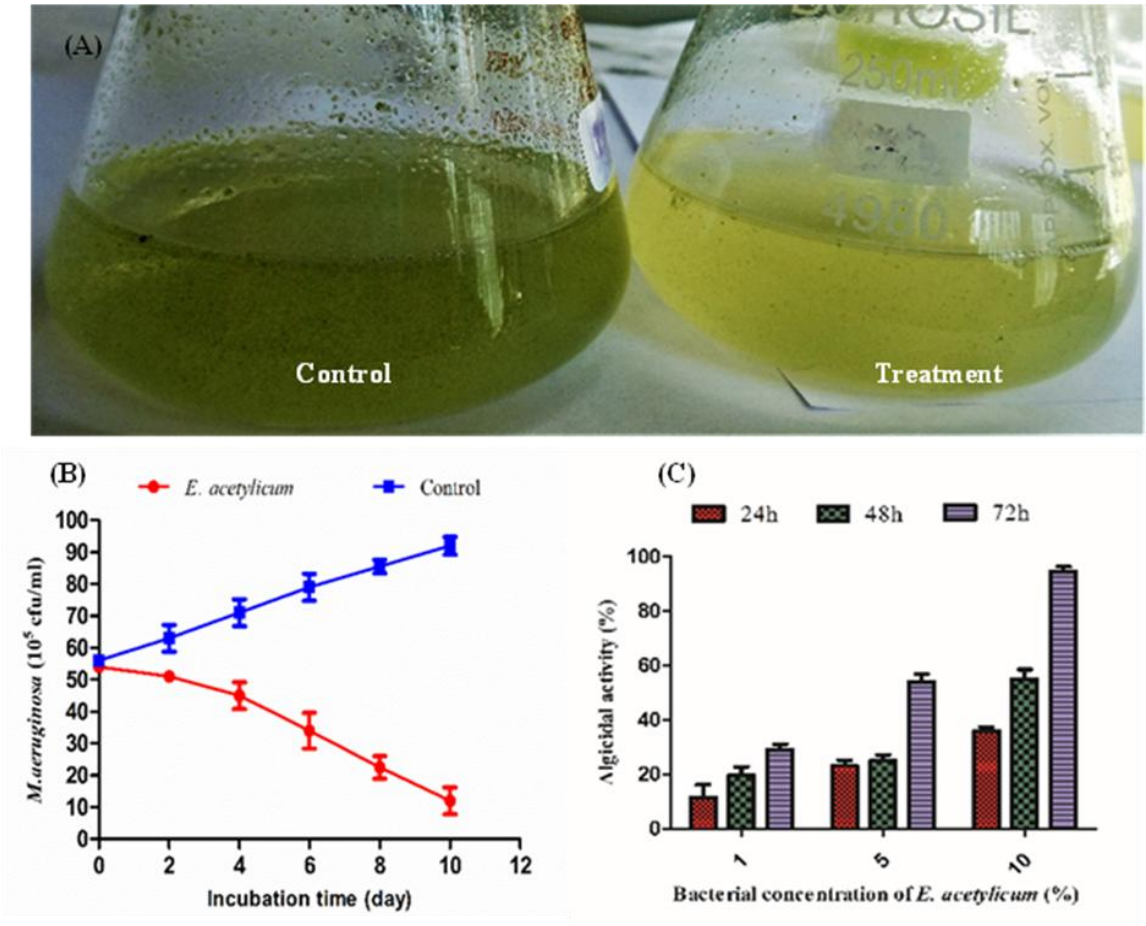


Fig.6 Morphological changes in *Microcystis aeruginosa* exposed to *Exiguobacterium acetylicum* TM2 for 7 days. (A) Control *M. aeruginosa* with normal appearance. (B) *Microcystis aeruginosa* exposed to *Exiguobacterium acetylicum* TM2 at 24 h. (C) *Microcystis aeruginosa* exposed to *Exiguobacterium acetylicum* TM2 at 72 h. (D) *Microcystis aeruginosa* exposed to *Exiguobacterium acetylicum* TM2 at 10 days. Images were taken by inverted microscope at X400, and scale bar is shown at bottom right side of the image

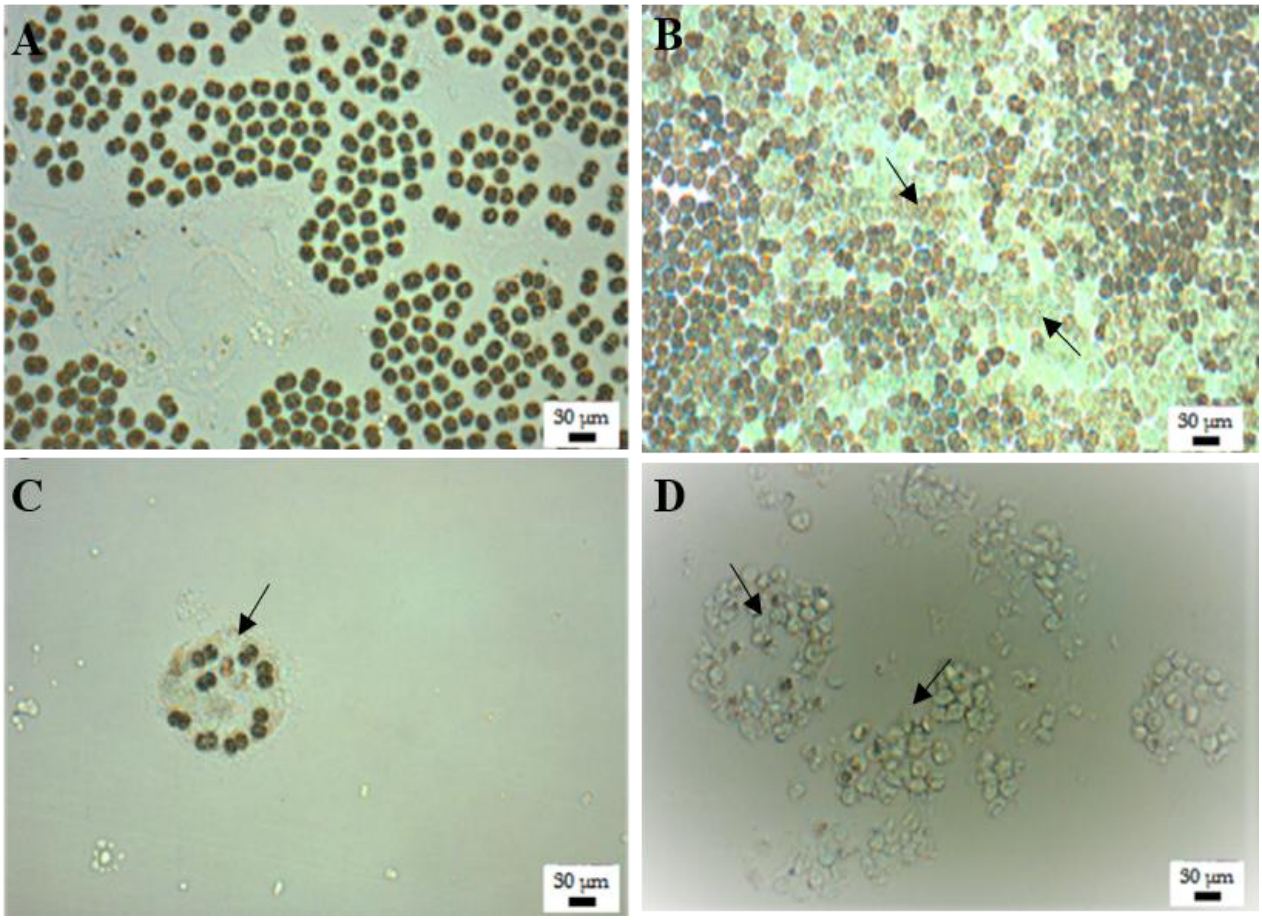
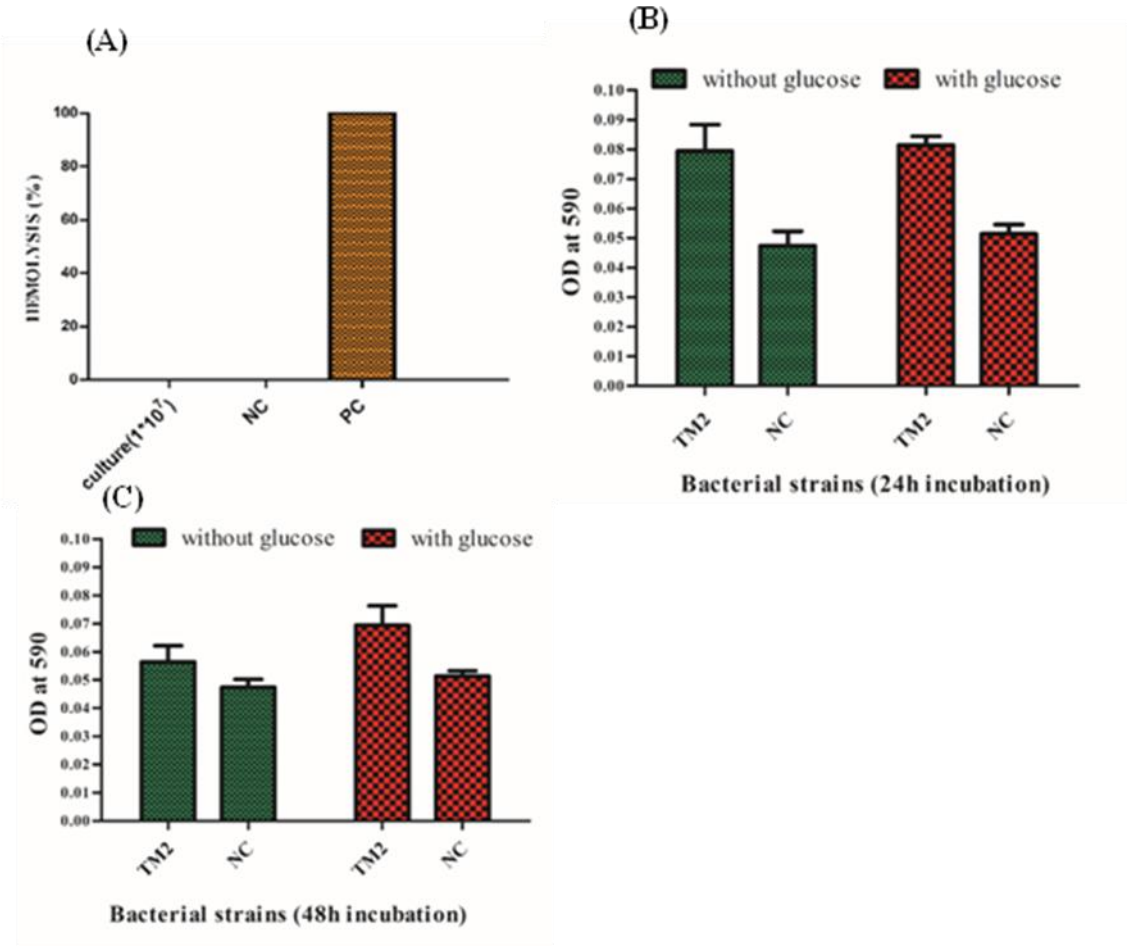


Fig.7 (A) *In vitro* hemolytic assay to assess the cytotoxicity of bacteria. Bar represents the non-hemolytic activity of *E. acetylicum* (TM2) in sheep RBCs. Data shown are averages of two replicates ± standard deviation. NC and PC are negative and positive controls respectively. (B). Bar graph showing negative biofilm formation (values < 0.1) by *E. acetylicum* TM2 after 24 h of incubation in media supplemented with or without glucose. NC (negative control) contains broth only. (C) Bar graph showing negative biofilm formation (values < 0.1) by *E. acetylicum* TM2 after 48 h of incubation in media supplemented with or without glucose. NC (negative control) contains broth only



In our study the bacterium *E. acetylicum* TM2 was isolated from water samples during declining phase of *M. aeruginosa* bloom, which was never reported previously. However, another closely related species of this genus *Exiguobacterium chiriqhucha* strain RW2 was isolated from cyanobacterial bloom from the Pavilion Lake in south-eastern BC (White III *et al.*, 2019). The *Exiguobacterium chiriqhucha* strain RW2 showed potential for detoxification of heavy metal by secretion of several enzymes (White III *et al.*, 2019). To the best of our information, this is the first report of algicidal bacteria against *M. aeruginosa* belonging to the genus *Exiguobacterium*.

As reported earlier for genus *Exiguobacterium*, adaptation to wide range of temperature and salinity (White *et al.*, 2013;

White *et al.*, 2019) was observed for strain TM2 also. Cosmopolitan distribution of *Exiguobacterium* may be due to their highly adaptable physiology and tolerance to wide range of abiotic factors.

Nucleotide sequence of 16S rDNA gene confirms the bacterium as *E. acetylicum* with 99.92 % sequence identity with *E. acetylicum* strain V12 isolated from cut vegetables in China (GenBank accession number: MW041273), and *E. acetylicum* strain KNUC604 isolated from water in South Korea (GenBank accession number: HM047519). Presence of peritrichous flagella was observed in TEM, which confirm the mechanism of motility of this bacterium. In contrast to our study, in case of *E. chiriqhucha* strain RW2, flagella were not observed in the scanning electron microscopy (SEM) (White *et al.*, 2019).

The algicidal activity of *E. acetylicum* TM2 was evaluated against *M. aeruginosa* isolated from the same lake. Rapid decolourization and disintegration of *Microcystis* cells after addition of TM2 confirms the attack of cyanobacteria by TM2 bacterium. It appears that this bacterium inhibit the metabolic activity of the algae. The algal lytic ability of the bacterium was concentration and time dependent. At the end of 10 days of inoculation, the *Microcystis* cell density was reduced to 90%. The algicidal effect of TM2 on *Microcystis* was not dependent on physiological status of the algae, as reported in case of other *Microcystis* killing bacterial strains (Manage *et al.*, 2009).

In general, algicidal bacteria act directly or indirectly against cyanobacteria (Lin *et al.*, 2014). In direct attack, algicidal bacteria comes in contact with cyanobacteria, and then kill them by various mechanisms, while in indirect attack algicidal chemicals are secreted by bacteria which kills the cyanobacteria (Mayali and Azam, 2004). The algicidal chemicals secreted by algicidal bacteria can be peptides or enzymes, biosurfactants, pigments and antibiotic-like substances (Lin *et al.*, 2014; Li *et al.*, 2014; Jing *et al.*, 2020). In our study, significant decrease in *M. aeruginosa* cell density was observed when *E. acetylicum*TM2 was added directly to the *Microcystis* cells. However when cell free bacterial filtrate was added to *Microcystis* cell, algicidal effect was not observed visually and microscopically, both. This suggests that the algicidal mode of action of *E. acetylicum* TM2 was by direct contact, and not by secretion of algicidal extracellular protease. In contrast to our study, there are several reports where algicidal bacteria are known to inhibit the growth of *Microcystis* spp. by release of algicidal substance (Sigee *et al.*, 1999; Chio *et al.*, 2005; Li *et al.*, 2014; Liu *et al.*, 2019; Jing *et al.*, 2020). According to the results obtained in this study, it is confirmed that bacterium *E. acetylicum* induced a high level of algicidal activity against *M. aeruginosa* (within 10 days) *via* direct attack.

Additionally, *in vitro* pathogenicity test by evaluating the haemolytic activity and by biofilm formation, it is confirm that *E. acetylicum* is non-pathogenic, and so there are scopes of using this bacterium in natural water system for mitigating the *M. aeruginosa* bloom. *In vivo* pathogenicity assay, by injecting the bacterium to common carp and observing it for 30 days also establishes that this bacterium is non-virulent. No internal or external gross lesions, histopathological changes or other behavioural abnormality was observed in common carp during the experimental period.

Collectively, our study supports that *E. Acetylicum* TM2 holds a great promise for the prevention of *M. aeruginosa* blooms inconfined water bodies as well as in natural conditions. To our knowledge, this is the first

report of algicidal property of *E. Acetylicum* against *M. aeruginosa.*

The discovery of algicidal bacterium *E. acetylicum* TM2 against *M. aeruginosa*is reported in our study. The algicidal mode was through direct cell to cell contact. Additionally, in terms of biological safety *E. acetylicum* TM2 was found to be non-pathogenic. Therefore, present study provides the evidence that isolated algicidal strain possesses efficacious algicidal activity, and may be used as an attractive bioagent for controlling *Microcystis* blooms.

Acknowledgements

We gratefully acknowledge Department of Biotechnology (DBT), Ministry of Science and Technology, India for their financial support (Grant no.102/IFD/SAN/3632/2014-15) to Neetu Shahi. We are thankful to the Director, ICAR-Directorate of Coldwater Fisheries Research (ICAR-DCFR), Bhimtal, India, for providing necessary facilities to carry out the present work. We also thank Krishna Kala and Shivam Singh of ICAR-DCFR for their support throughout the study period.

Funding

This work was funded by Department of Biotechnology (DBT), Ministry of Science and Technology, India (Grant no.102/IFD/SAN/3632/2014-15).

Availability of data and material

The data that support the findings of this study may be obtained from corresponding author upon request.

Conflict of interest

The authors declare that there is no conflict of interest.

References

Bozzola J. J, Russell L D (1998) Electron microscopy, Principals and Techniques for Biologists. 2nd eds. Jones and Bartlett Publishers, Sudbury, Massachusetts, p. 641.

Cai W, Wang H, Tian Y, Chen F, Zheng T (2011) Influence of a bacteriophage on the population dynamics of toxic dinoflagellates by lysis of algicidal bacteria. Appl. Environ. Microbiol. 77,7837– 7840. https://doi.org/10.1128/AEM.05783-11.

Carmichael W W (2001) Health effects of toxin-producing cyanobacteria: "The CyanoHABs". Hum. Ecol. Risk Assess. 7, 1393–1407.https://doi.org/10.1080/20018091095087.

Chio H J, Kim B H, Kim J D, Han M S (2005) *Streptomyces neyagawaensis* as a control for the hazardous biomass of *Microcystis aeruginosa* (Cyanobacteria) in eutrophic freshwaters. Biol. Control. 33, 335–43.https://doi.org/10.1016/j.biocontrol.2005.03.007.

Chaturvedi P, Shivaji S (2006) *Exiguobacterium indicum* sp. nov., a psychrophilic bacterium from the Hamta glacier of the Himalayan mountain ranges of India. Int. J. Syst.Evol.Microbiol. 56, 2765-2770. https://doi 10.1099/ijs.0.64508-0.

Gobler C J (2020) Climate change and harmful algal bloom; Insights and perspective. Harmful Algae 91. https://doi.org/10.1016/j.hal.2019. 101731.

Govindan S, Piyush J, Sehar N, Pankaj K M, Samaresh K, Gupta H S (2009) E*xiguobacterium acetylicum* strain 1P (MTCC 8707) a novel bacterial antagonist from the North Western Indian Himalayas. World J.Microbiol. Biotechnol. 25,131–

137.https://doi.org/10.1007/s11274-008-9874-4.

Haughey M A, Anderson M A, Whitney R D, Taylor W D, Losee R F (2000) Forms and fate of Cu in a source drinking water reservoir following CuSO4 treatment. Water Res. 34, 3440–3452. https://doi.org/10.1016/S0043-1354(00)00054-3.

Hotto A M, Satchwell M F, Boyer GL(2007) Molecular characterization of potential microcystin-producing cyanobacteria in Lake Ontario embayments and nearshore waters. Appl. Environ. Microbiol. 73 (14): 4570-4578. https://doi: 10.1128/AEM.00318-07.

HoL, Hoefel D, Saint C P, Newcombe G (2007) Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter. Water Res. 41:4685–4695. 10.1016/j.watres.2007.06.057.

Ishii H, Nishijima M, Abe T(2004) Characterization of degradation process of cyanobacterial hepatotoxins by a gram-negative aerobic bacterium. Water Res. 38:2667–2676. 10.1016/j.watres.2004.03.014

Jeong-Dong K, Kim J Y, Park J K, Lee CG(2009) Selective control of the *Prorocentrum minimum* harmful algal blooms by a novel algal-lytic bacterium *Pseudoalteromonas haloplanktis* AFMB-008041. Mar.Biotechnol. 11, 463–472. https://doi.org/10.1007/s10126-008-9167-9.

Jeong J H, Jin H J, Sohn C H, Suh K H, Hong Y K (2000) Algicial activity of the seaweed *Corallinapilulifera* against red tide microalgae. J. Appl. Phycol. 121, 37–43.https://doi.org/10.1023/A:1008139129 05

Yang J, Qiao K, Lv J, Liu Q, Nan F, Xie S, Jia Feng J (2020) Isolation and identification of two algae-lysing bacteria against *Microcystis aeruginosa*. Water, 12, 9, (2485) https://doi.org/10.3390/w12092485.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35,1547-1549.doi: 10.1093/molbev/msy096.

LiZ, Lin S, Liu X, Tan J, Pan J, Yang H (2014) A freshwater bacterial strain, *Shewanella sp*. Lzh-2, isolated from Lake Taihu and its two algicidal active substances, hexahydropyrrolo[1,2-a] pyrazine-1,4- dione and 2, 3-indolinedione. Appl.Microbiol.Biotechnol. https://doi.org/10.1007/s00253-014-5602-1.

Lin S, Pan J, Li Z, Liu X, Tan J, Yang H (2014) Characterization of an algicidal bacterium *Brevundimonas* J4 and chemical defense of *Synechococcus* sp. BN60 against bacterium J4. Harmful algae. 37, 1-7. http://dx.doi.org/10.1016/j.hal.2014.05.002.

Liu J, Yang C, Chi Y, Wu D, Dai X, Zhang X, Igarashi Y, Luo F (2019) Algicidal characterization and mechanism of *Bacillus licheniformis* Sp34 against *Microcystis aeruginosa* in Dianchi Lake. J. Basic Microbiol. 59(11) 1112-1124. https://doi: 10.1002/jobm.201900112.

Mayali X, Azam F (2004) Algicidal bacteria in the sea and their impact on algal blooms. J.Eukaryot.Microbiol. 51:139–44.https://doi.org/10.1111/j.1550-7408.2004.tb00538.x.

Manage P M, Edwards C, Singh B K, Lawton L A (2009) Isolation and identification of novel microcystin-degrading bacteria. Appl. Environ. Microbiol. 75:6924–6928. https://doi.org/10.1128/AEM.01928-09.

Nagayama K, Shibata T, Fujimoto K, Honjo T, Nakamura T (2003) Algicidal effect of phlorotannins from the brown alga *Eckloniakurome* on red tide microalgae. Aquaculture 218, 601–611.https://doi.org/10.1016/S0044-8486(02)00255-7.

Nowack B, Krug H F, Height M (2011) 120 years of nanosilver history: implications for policy makers. Environ. Sci. Technol. 8, 48-67.https://doi.org/10.1021/es103316q.

Paerl H W, Xu H, McCarthy M J, Zhu G W, Qin B Q, Li Y P, Gardner W S (2011) Controlling harmful cyanobacterial blooms in a hypereutrophic lake (Lake Taihu, China): the need for a dual nutrient (N&P) management strategy. Water Res. 45, 1973–1983. https://doi.org/10.1016/j.watres.2010.09.018.

Salma A L, Brownlie J, Barlow R (2018) Characterization of biofilm-forming capacity and resistance to sanitizers of a range of *E. coli* O26 pathotypes from clinical cases and cattle in Australia. BMC Microbiol. 18,41.https://doi.org/10.1186/s12866-018-1182-z.

Schwyn B, Neilands J B (1987) Universal chemical assay for the detection and determination of siderophores. Anal.Biochem. 160, 40–47. https://doi.org/10.1016/0003-2697(87)90612-9.

Shahi N, Sahoo M, Mallik S K, Sarma D, Das P (2021) The microcystins-induced DNA damage in the liver and the heart of zebrafish, *Danio rerio*. 92(2). https://doi.org/10.1080/02772248.2011.646111

Shahi N, Mallik S K, Sahoo M, Chandra S, Singh A K (2018) First report on characterization and pathogenicity study of emerging *Lactococcus garvieae* infection in farmed rainbow trout, *Oncorhynchus mykiss*(Walbaum), from India. Transbound. Emerg. Dis.65, 1039-1048.https://doi/abs/10.1111/tbed.12843 23/02/2018.

Singh N K, Raichand R, Kaur I, Kaur C, Pareek S, Mayilraj S (2013) *Exiguobacterium himgiriensis* sp. nov. a novel member of the genus *Exiguobacterium*, isolated from the Indian Himalayas. Antonie van Leeuwenhoek 103, 789–796. doi: 10.1007/s10482-012-9861-5.

Shen Y, Liu Y, Wu G, Ao H, Qiu C. Mechanical removal of heavy cyanobacterial bloom in the hyper-eutrophic lake Dianchi. Acta Hydrobiol Sin. 2004;28:131-136

Sigee D C, Glenn R, Andrews M J, Bellinger E G, Butler R D, Epton H A S, Hendry R D (1999) Biological control of cyanobacteria: principles and possibilities. Hydrobiologia 395,161–172.DOI: 10.1007/978-94-017-3282-6_15.

Su J Q, Yang X R, Zheng T L, Hong H S (2007) An efficient method to obtain axenic culture of *Alexandrium tamarense*—a PSP-producing dinoflagellate. J.Microbiol.Methods. 69,425-430.https://doi.org/10.1016/j.mimet.2006.07.005**.**

White III R A, Grassa C J, Suttle C A (2013) Draft genome sequence of *Exiguobacterium pavilionensis* strain RW-2, with wide thermal, salinity, and pH tolerance, isolated from modern freshwater microbialites. Genome Announc.1:e00597-13. doi: 10.1128/genomeA.00597-13.

White III R A, Soles S A, Gavelis G, Gosselin E, Slater G F, Lim DSS, Leander B, Suttle, C A (2019) The complete genome and physiological analysis of the eurythermal firmicute *Exiguobacterium chiriqhucha* strain RW2 isolated from a

freshwater microbialite, widely adaptable to broad thermal, pH, and salinity ranges. Front. Microbiol. 9: 3189.doi: 10.3389/fmicb.2018.03189.

Zheng X, Zhang B, Zhang J, Huang L, LinJ, Li X, Zhou Y, Wang H, Yang X, Su J, Tian Y, Zheng T (2012)A marine algicidal actinomycete and its active substance against the harmful algal bloom species against *Phaeocystis globosa*. Appl. Microbiol. Biotechnol. 97 (20):9207-9215.https://doi.org/10.1007/s00253-012-4617-8.

Zurawell R W, Chen H, Burke J M, Prepas E E (2005) Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. J.Toxicol. Environ. Health B Crit. Rev. 8, 1–37.https://doi.org/10.1080/10937400590889412.

How to cite this article:

Surabhi Rawat, Neetu Shahi, Sumanta Kumar Mallik, Richa Pathak, Bhupendra Singh and Veena Pande. 2021. Biocontrol of Toxin Producing Cyanobacterium *Microcystis aeruginosa* by Algicidal Bacterium *Exiguobacterium acetylicum* Strain TM2 Isolated from Mid-Altitudinal Himalayan Lake of Northern India. *Int.J.Curr.Microbiol.App.Sci.* 10(11): 170-187.
doi: https://doi.org/10.20546/ijcmas.2021.1011.020