MICROBIOLOGY ARCHIVES
Original Research Article | ISSN: 3041-590X
Journal homepage: https://microjournal.researchfloor.org/

Screening of a Cyanobacterium *Lyngbya bipunctata* Lemm. for Antibacterial Potential

Janardhan Namdeo Nehul

Department of Botany, Dada Patil Rajale Arts and Science College, Adinathnagar, Tal-Pathardi, Dist.-Ahilyanagar-414505 (MS) India

ARTICLE INFO

Citation: Janardhan Namdeo Nehul (2026). Screening of a Cyanobacterium *Lyngbya bipunctata* Lemm. for Antibacterial Potential.
Microbiology Archives, an International Journal.
DOI: https://doi.org/10.51470/MA.2026.8.1.183

Received 02 March 2026
Revised 05 April 2026
Accepted 08 May 2026
Available Online June 03, 2026

Corresponding Author: **Janardhan Namdeo Nehul**
E-Mail: **jnnehul@gmail.com**

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ABSTRACT

Cyanobacterium, Lyngbya bipunctata, was isolated from the soil samples collected from the Maharashtra state from the locations Ahilyanagar, India, in August 2024. For the identification, morphological variation, and taxonomical methods consistent. The axenic culture of Lyngbya bipunctata was obtained by way of the use of the techniques. The isolated Lyngbya bipunctata was autotropically cultured in BG-11 medium and incubated at 30±2°c. After 25 days, biomass was harvested by means of filtration through double-layered muslin cloth and dried with the help of air blower. The biomass of this Lyngbya bipunctata species was used for the evaluation of antibacterial potential against Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus flavus, and Proteus mirabilis. The antibacterial property was evaluated by the disc diffusion method. Methanol extract of Lyngbya bipunctata confirmed the antibacterial property against all the tested bacterial strains. Maximum zone of inhibition (22±1.4 mm) was observed in the methanol extract of Lyngbya bipunctata against Bacillus subtilis. The MIC for all the tested strains of bacteria was found in between 32 and 256 µg/ml.

Keywords: *Cyanobacteria, Lyngbya bipunctata, Antibacterial property, BG-11, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa*

Introduction

Infectious diseases are among the leading causes of death in both developing countries and the rest of the world, ranking second in terms of mortality—surpassed only by heart disease. The search for antibiotics began in the late 19th century, when scientists started looking for drugs capable of eliminating pathogenic bacteria. The primary objective was to discover a "magic bullet"—a drug that could destroy harmful microorganisms without harming the patient. Although most current diseases are caused by pathogens, many can be treated with available antibiotics. However, there is a constant need to discover and develop new, effective antibiotics, as some microorganisms have developed resistance mechanisms against existing drugs. Consequently, the search for new chemical substances that can serve as the basis for medicines has generated significant global interest. In recent years, interest in cyanobacteria has grown, as these organisms produce numerous compounds that are valuable and important for both medicine and industry [1]. This interest has spurred efforts to produce such compounds on a large scale under controlled conditions [2].

The first known antimicrobial substance discovered in algae came from a species of green algae called *Chlorella*. It has been found that this substance, known as "chlorelin," inhibits the growth of both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* [3]. A significant reduction in Gram-positive bacteria in water has also been reported during cyanobacterial blooms. This may be due to cyanobacteria releasing antibacterial substances into the water. Cyanobacteria (also known as blue-green algae) are prokaryotic, Gram-positive, photosynthetic bacteria [4]. They have a long history—spanning approximately 3.5 billion years—and play a role in oxygen production [5]. They inhabit diverse environments, such as soil, water, and rock surfaces, and are highly tolerant of fluctuations in pH, temperature, and salinity [6]. Their appearance varies by species; they can exist as unicellular organisms or form filamentous structures [7].

They are found in a variety of animals and microbes such as bacteria, viruses, and fungi. Their ability to adapt to different environments and respond to different stress and nutrient levels is due to their flexible metabolism. This flexibility may help explain the diversity of chemical compounds found in cyanobacteria [8,9]. Many useful bioactive secondary metabolites have been isolated from cyanobacteria, which are very promising in research and drug discovery [10,8,11,12,13,14,15,16]. Cyanobacteria can produce many secondary metabolites with pharmaceutical potential. These include compounds such as alkaloids, aromatic compounds,

peptides and terpenes, many of which have biological activity [17]. They can also produce a variety of toxins, about 40% of which are lipopeptides. According to Burja [18] and Singh [19], cyanobacterial lipopeptides include compounds with different activities, such as being toxic to cells (41%), anti-cancer (13%), anti-viral (4%), and antibiotic (12%). Other activities include fighting malaria, fungus, reversing drug resistance, serving anti-feedants, herbicides, and suppressing immune responses (18%). Recently, it has been reported that several cyanobacterial metabolites exhibit anti-inflammatory, antiprotozoal, anticancer, antioxidant, antifungal, antibacterial, antiviral and antialgal activities [13].

The process of isolating bioactive compounds from cyanobacteria has two main goals. One is to discover new substances for use in medicine, agriculture, or to control harmful organisms. The other is to better understand how these organisms interact with their environment and other living things. For both of these goals, it is important to screen new cultures to find out how often and where these useful strains occur. There are many reviews about how marine, freshwater, and land-based cyanobacteria from different families can be a source of antibacterial substances. This study describes the results of testing *Lyngbya bipunctata* against harmful bacteria.

Materials and Methods

Collection, isolation, and identification of cyanobacteria

Lyngbya bipunctata was taken from soil samples collected from various places in Ahilyanagar district of Maharashtra state, India. The isolated *Lyngbya bipunctata* was cultured in BG-11 medium according to the methods of Rippka [20] and kept at a temperature of 30±2°C. The identification was done by looking at the shape and structure of the organism and using taxonomical methods according to Desikachary [21] and Prescott [22]. The axenic culture was obtained by way of the use of the technique recommended by Bolch and Blackburn [23]. *Lyngbya bipunctata* was cultured in BG-11 culture medium to make a large amount of biomass. After 25 days, the biomass was collected by passing it through two layers of muslin cloth and then dried with an air blower. The biomass of *Lyngbya bipunctata* was used to test its antibacterial property.

Extraction procedure

Five grams of finely ground biomass of *Lyngbya bipunctata* was extracted using a Soxhlet apparatus with 50 ml of hexane, chloroform, methanol, and water at 40°C for 24 hours. The filtered extract was then concentrated under reduced pressure at 40°C. The final volume of the extract was adjusted to 1ml using the respective solvents.

Standard antibiotic

The standard antibiotic disc, which contained 10 µg/ml of streptomycin, used in this study was bought from HiMedia (India). These discs were placed on the nutrient agar medium that had a known amount of bacteria.

Test organisms

Pure cultures of *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus Aureus*, *Pseudomonas Aeruginosa*, *Micrococcus Flavus*, and *Proteus Mirabilis* were obtained from the National Collection of Industrial Microorganisms (NC). The cultures were maintained according to NCIM specifications (NCL, Pune).

Preparation of culture medium

The chemicals needed to make the nutrient agar media were bought from Hi-media Laboratories in Pune, India. The composition of the culture medium is as follows.

Ingredient g L^{-1}
Peptone 10.00
Beef 10.00
NaCl 5.00
Agar 20.00

The pH was adjusted to 7.5 using 0.1 N HCL or 0.1 N NaOH, as measured by a standard pH meter. The culture medium was sterilized in an autoclave at 1.06 kg cm^{-2} pressure for 20 minutes. All the required equipment, like Petri dishes, conical flasks, forceps, Pipettes, etc., were also sterilized in an autoclave at 1.06 kg cm^{-2} pressure for half an hour.

Preparation of Inoculum

Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus,* and *Micrococcus) and Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa,* and *Proteus mirabilis*) were first cultured overnight in nutrient broth using a shaker at 37°C. Subsequently, they were centrifuged for five minutes at 10,000 rpm. The resulting pellet was suspended in water, and the cell density was adjusted using a spectrophotometer at 610 nm.

Antibacterial Test

The antibacterial test was done using agar diffusion assay. Paper discs (Whatman No. 41) with a diameter of 6.4 were made and then sterilized in an autoclave. A total of 10 ml of liquid nutrient agar was cooled to 45°C. To this, 20 microliters of bacterial culture with a concentration of about 1.5 X 10^8 colony forming unit (CFU) was added and poured into a sterile Petri dish. The mixture was left to solidify, and then each plate was labeled with the type of bacteria used. Four discs were grouped and soaked in an extract solution at a concentration of 400µg/ml. The solvent was left to evaporate. Once the agar solidified, the discs were placed in the Petri plates with equal spacing. For each type of bacteria, two duplicate plates, one standard plate, and one control plate with only solvent were prepared. The standard plate used the antibiotic streptomycin at a concentration of 10µg/ml. The plates were first kept at 4°C for 8 hours to let the samples spread and then incubated at 37° C for 24 hours. After 24 hours, the size of the clear area around each disc, called the inhibition zone, was measured in millimeters. The activity of the test extract was then compared with that of the standard. All the tests were done under sterile conditions and repeated three times.

Measurement of minimum inhibitory concentration (MIC)

Crude extracts from the biomass of *Lyngbya bipunctata* were tested for antibacterial effects against both Gram-positive and Gram-negative bacteria. The micro broth dilution method used was, as described by Sahm and Washington [24]. The crude extracts were diluted in nutrient broths to concentrations ranging from 1 to 400 micrograms per milliliter using dimethylsulphoxide (DMSO) as the solvent. These diluted extracts were then placed into 96-well plates. Bacterial cultures were prepared at a concentration of about 1.5 × 10^8 colony-forming units (CFUs) per milliliter and added to each well. The plates were incubated at 37°C for 24 hours. The MIC was determined as the lowest concentration where no bacterial growth was visible, measured by the level of turbidity using absorbance at 600nm.

Streptomycin was used as the positive control, and DMSO was used as the negative control.

Results and Discussion

The antibacterial activity of *Lyngbya bipunctata* was assessed using the disc diffusion method. Table 1 shows the antibacterial activity of various extracts from *Lyngbya bipunctata* (at a concentration of 400 µg/ml) against Gram-positive and Gram-negative bacteria, determined by measuring the zone of inhibition. Streptomycin was used as a positive control. The methanolic extract of *Lyngbya bipunctata* exhibited varying levels of growth inhibition, depending on the susceptibility of the tested bacteria. This extract proved effective against all tested bacterial strains except *Escherichia coli*. The largest zone of inhibition (18 ± 1.6 mm) was recorded for *Bacillus subtilis* when the methanolic extract was used. The chloroform extract of *Lyngbya bipunctata* showed activity against *Bacillus subtilis* and *Staphylococcus aureus*, although the effect against *Staphylococcus aureus* was minimal. The hexane extract was effective only against *Micrococcus flavus* at a concentration of 400 µg/ml. The aqueous extract showed no antibacterial activity.

Antibiotics are vital tools for combating bacterial infections and have contributed significantly to the treatment of numerous health issues. However, their efficacy has declined in recent decades, as many commonly used antibiotics are no longer effective against certain diseases. This phenomenon is attributed to both toxic reactions and the emergence of drug-resistant bacteria. Consequently, the search for new drugs that trigger lower levels of resistance has become crucial. Research into various pharmacological compounds indicates that a single drug can perform multiple functions and prove useful across a wide range of medical fields.

The antibacterial activity of *Lyngbya bipunctata* extract was tested against six types of pathogenic bacteria. Extraction was performed using hexane, chloroform, methanol, and water. Of the six bacterial strains tested, five showed inhibition when treated with the methanolic extract. The highest level of inhibition—measured by the size of the inhibition zone (18 ± 1.6 mm)—was observed against *Bacillus subtilis* using the *Lyngbya bipunctata* methanolic extract. Analysis of the methanolic extract revealed significant inhibition of *Bacillus subtilis*, whereas the hexane extract showed lower activity. It is logical that the methanolic extract demonstrated greater potential, as it exhibited a higher level of antimicrobial activity compared to the other extracts. Similar results were reported by Rosell [25] and Moreau [26], who noted that methanol extraction yields better antimicrobial activity than extraction with hexane or other solvents. However, some studies have reported that chloroform is more effective than methanol or benzene [27]. Organic solvents are more effective at extracting compounds with antimicrobial activity than water-based methods [28, 29].

Cannell [30] tested organic solvent extracts from various cyanobacteria to see if they had antibacterial effects against *Escherichia coli*, *Bacillus subtilis*, *and Staphylococcus aureus.* They found that five different cyanobacterial cultures showed some antibacterial activity. In the chloroform extract, the highest level of antibacterial effect was seen in *Lyngbya bipunctata* against *Staphylococcus aureus* with an inhibition zone of 10±1.3 mm. This extract also showed some activity against *Bacillus subtilis* and *Staphylococcus aureus,* but the effect was not as strong. This suggests that the amount of the active compound in the extract may be low. The chloroform extract was not as effective as the methanolic extract. The hexane extract did not show much antibacterial activity against the tested bacteria. It did not affect *Escherichia coli*, *Bacillus subtilis*, *and Staphylococcus aureus*, but it did show some activity against *Micrococcus flavus* at a concentration up to 400µg/ml.

Table 1: Antibacterial activity of different extracts of Lyngbya bipunctata at 400µg/ml concentration against Gram-positive and Gram-negative bacteria

Bacterium	Diameter of effective zone of inhibition (mm)				
	Methanol extract	Chloroform extract	Hexane extract	Aqueous(water) Extract	Streptomycin (10 µg/ml)
Escherichia coli	-	-	-	-	17±2.4
Bacillus subtilis	18±1.6	10±1.5	-	-	25±1.3
Staphylococcus aureus	18±1.7	10±1.3	-	-	23±1.7
Pseudomonas aeruginosa	13±1.4	-	-	-	22±1.6
Micrococcus flavus	12±1.7	-	8±1.2	-	20±1.2
Proteus mirabilis	13±1.4	-	-	-	19±1.2

Falch and their team [31] did multiple steps to extract compounds using solvents that become more polar, like petroleum ether, dichloromethane, ethyl acetate, methanol, and others. The different extracts had varying abilities to stop bacterial growth, as tested using a bioautographic method with bacteria such as *B. subtilis*, *E. coli,* and *Micrococcus luteus*. From our experiments, we saw that methanol extracts were more effective than chloroform and hexane in stopping both Gram-positive and Gram-negative bacteria.

Minimum inhibitory concentration (MIC)

The lowest amount of substance that stops visible bacterial growth is considered the MIC. For a specific type of bacteria, the MIC was either the same or slightly different across tests. In this study, the extracts from biomass were made using methanol, chloroform, hexane, and water, and tested against various Gram-positive and Gram-negative bacteria. The MIC values changed depending on the solvents used for Lyngbya *bipunctata*. The MIC for all the bacteria tested was between 128 and more than 400 micrograms per milliliter. The methanol extract of *Lyngbya bipunctata* had lower MIC values, specifically 128 micrograms per milliliter against *Bacillus subtilis* and 256 micrograms per milliliter against *Staphylococcus aureus*. The chloroform extract showed its best performance at an MIC of 325 micrograms per milliliter. Hexane had higher MIC values, while methanol had lower ones. The chloroform extract had MIC values between 325 and more than 400 micrograms per milliliter. The aqueous extract of *Lyngbya bipunctata* didn't stop any bacterial growth, even at the highest tested concentration of 400 micrograms per milliliter.

Table 2: Minimum inhibitory concentration (MIC) of different extracts of Lyngbya bipunctata against tested pathogenic bacteria. Concentration of extracts is expressed in terms of µg/ml

Bacterium	Concentration of extracts in µg/ml.			
	Methanol extract	Chloroform extract	Hexane extract	Aqueous(water) Extract
Escherichia coli	>400	>400	>400	>400
Bacillus subtilis	128	325	>400	>400
Staphylococcus aureus	256	325	>400	>400
Pseudomonas aeruginosa	256	>400	>400	>400
Micrococcus flavus	256	>400	325	>400
Proteus mirabilis	325	>400	>400	>400

Screening processes provided some clues about the type of compound responsible for the antibacterial activity of *Lyngbya bipunctata* which showed positive results.During this study the best strain of *Lyngbya bipunctata* that produced the most antibacterial metabolites showed a variety of activity against different bacteria, including *B. subtilis*, *S. aureus* and *Pseudomonas aeruginosa*. These results match findings from other studies that suggest cyanobacteria can be a rich source of biological compounds [32,33,34,35].

Conclusion

The cyanobacterium *Lyngbya bipunctata* is a good source of bioactive antibacterial compounds. The methanol extract was more effective than the chloroform, hexane, and aqueous extracts. The chloroform extract was less effective against all tested bacteria. The bioactive compounds are not soluble in water, so the aqueous extract did not show any activity against the tested bacteria even at a concentration of up to upto 400 µg/ml. The lowest minimum inhibitory concentration (MIC) was found in the methanol extract.

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