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In Vitro Antioxidant and Antibiofilm Activities of *Microcystis* Sp. Against Multidrug-Resistant Human Pathogens

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Abstract

The biofilm of drug-resistant pathogenic bacteria is a serious issue worldwide. The natural growth of blue-green algae *Microcystis* sp. has the potential to antagonize the bacterial pathogens in a novel approach. In the present study,*Microcystis* sp. was isolated and its mass production was optimized by Response Surface Methodology (RSM) and also, the *invitro* antioxidant and antibiofilm potentials against the drug-resistant pathogens were investigated. The experimental design was carried out on mass production of *Microcystis* sp. with pH, and mineral salts as independent variables.The maximum biomass yield (1.5 g/L) was achieved with $NaNO_3$ (20mM) andK_2HPO_4 (10mM) at pH 7.5. The *Microcystis* sp. exhibited remarkable antioxidant (65%) and antibiofilm activities against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The Gas chromatography-mass spectrometry (GC-MS)of *Microcystis* sp. extract analysis revealed the presence ofsixteen bioactive potential compounds which proved the synergistic activity against the bacterial pathogens. Therefore, the potential bioactive compoundsin*Microcystis* sp. could be an effective biocontrol agentagainst the challenging drug-resistant bacterial pathogens.

Keywords: Antioxidant activity; Biofilm; Cyanobacteria; *Microcystis* sp., Response surface methodology

Introduction

The microalgae, including cyanobacteria (blue-green algae),is a photosyntheticeukaryote which grows in a wide range of aquatic environment including marine, rivers, polluted water, ponds and even lakes (1, 2).Microalgae and cyanobacteria are an eco-friendly, cost-effective, and sustainable natural resource that is used in various fields as bio-potential compounds, food industries and biofuels production (3).Cyanobacteria are an eco-friendly, cost-effective and sustainable natural resource that is used in food and biofuel production industries as biopotential compounds. Recently, it has been considered as an attractivenatural source of potentcompositein cosmetics, pharmaceuticals, agriculture, aquaculture, and bioremediation and also as food supplements for animal and human beings(4). Several microalgae possess bio-potent composites such as lipids, proteins, fatty acids, lutein, astaxanthin, β-carotene, vitamins, and pigments which exhibit anti-inflammatory, antioxidant, antimicrobial, antidiabetic and anticancer activities (5-

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9).Cyanobacterial metabolic compounds may be used as a potential source for the development of novel bactericidal and antibiofilm drugs, because of their ability to combat pathogenic organisms found in lakes(3).

Biofilms are complicated rigid structures produced by bacteria to resist against different antibiotics, ecological stress, and enhance the pathogenicity (10). The formation of biofilm is one of the potential characteristics of pathogenic bacteria extending into multidrug-resistant (MDR)and caused over 75% of human biofilm-related infection (11, 12). At this regard, the U.S National Institutes of Health (NIH) have bewilderedwith the emergence of MDR pathogenic bacteria as a worldwide concern (NIH, 2014). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are well known bacterial pathogens alive inside biofilms and cause major threat such as soft tissue and nosocomial infections to human beings (11, 13). Scientists have turned towards investigating the antibiofilm composites from a variety of sources like medicinal plants, edible mushrooms, metal nanoparticles, marine algae and edible vegetables (14-18).Moreover, green microalgae and cyanobacteria like *Spirulina platensis*, and *Oscillatoria subuliformis* were also reported against bacteria. Therefore considering these facts, the present study is to explore the isolation of blue-green algae from freshwater and to investigate it is*in vitro* antioxidant and antibiofilm activities against both Gram-positive and Gram-negative human pathogenic bacteria (19).

2. Materials and Methods

2.1. Sample collection and isolation of cyanobacteria

The fresh blue-green algae containing water sample was collected in sterile borosilicate glass bottles from different sites of the Nangavallilake (11.75°N 77.88°E), Salem District, Tamil Nadu, India. The water sample was brought to the laboratory, inoculated into sterile BG 11 broth medium (Hi-Media, Mumbai, India)and incubated in a growth chamber at 28 ± 2ºC with the illumination of 54-67µmol photons/m^2/s by cool white 40 W fluorescent tubes (Philips, Bangalore, India) for 14 days. After incubation, the growth was microscopically observed and loopful of culture was streaked on BG 11 agar plate medium (20). The isolated blue-green algae were identified based on the standard protocol and inoculated in fresh BG 11 broth medium and incubated for 14 days at the growth chamber. After blue-green algae cultivation, the culture broth was centrifuged at 10,000 rpm (REMI, Mumbai, India) and the algal biomass was washed thrice with sterile distilled water, freeze-dried (Lyophilizer-Lark, Chennai, India) at -80°C and stored at 4°C until further use.

2.2. Optimization cyanobacteriacultivation

The cyanobacteria were cultivated using BG 11 broth medium to optimize to the biomass production. The major impacts of response surface methodology (RSM) were used to optimize interactionsof three different variables at different levels. The maximum yield of blue-green algae biomass has been achieved by using the Box-Behnken design (BBD) tool. The experimental design was formulated with 19 experiments with three variables at three various levels. (Table 1) describes the experimental parameters of $NaNO_3$(10-30 mM), K_2HPO_4(5-15mM), and pH (7.0-8.0) as variables. The correlation coefficient (R^2) and analysis of variables (ANOVA) of the blue-green algae biomass production in percentage were studied. The universal form of the experimental equation is

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$$Y = \beta 0\, \Sigma\, \beta iXi + \Sigma\, \beta iXi\beta ij + \Sigma\, XiXj \quad (1)$$

Where, Y is the predicted reaction, β0, βi, and βij are continuous regression coefficients of the BBD model, and Xi and Xj stands for independent variables (21, 22).

Table 1: Response surface methodology of the *Microcystis* sp. for growth optimization for biomass production

Run	***Carbon source (mM)***	***Nitrogen source (mM)***	***pH***	***Biomass yield (g/L)***
1	30	10	7	1.2
2	30	15	7.5	1.25
3	20	15	8	1
4	20	10	7.5	1.5
5	10	10	7	0.8
6	10	10	8	0.79
7	20	5	8	0.95
8	20	10	7.5	1.5
9	20	10	7.5	1.51
10	30	5	7.5	1.35
11	20	5	7	1.22
12	20	10	7.5	1.51
13	20	10	7.5	1.5
14	30	10	8	1.15
15	20	15	7	1.2
16	10	15	7.5	0.9
17	10	5	7.5	0.85

2.3. Preparation of blue-green algae extract

After the mass cultivation, the biomass was collected and dried at 40°C for five days and sequentially extracted with 100% methanol. The suspension was sonicated using an ultra sonicator (UP400ST, Bangalore) with the number of cycles ensuring complete breakdown of cells. The extracted sample wascentrifuged at 7000 rpm for 10 min and the dried the pellets and usedfor*in vitro* antioxidant and antibiofilm activity (18).

2.4. *In vitro* antioxidant activity of Cyanobacteria

The antioxidant activity of cyanobacteria biomass extract was determined by radical-scavenging activity (RCA) according to 2,2-Dipyenyl-1-picrylhydrazyl (DPPH) method (23) with slight modifications. DPPH solution (3ml) was mixed with varyingconcentrations (20-100 µg/mL) of algal extract and incubated at room temperature for 30 min with ascorbic acid as the standard. The optical density was measured byUV-vis spectrophotometrically (Shimadzu 1800) at 517 nm. The analysis was repeated thrice and the EC_{50} (half-maximal effective concentration) was calculated by the DPPH method. The percentage of RCA was calculated using the following formula:

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Scavenging activity (%) = $[(A_0 - A_1)/A_0] \times 100$ (2)

Where A_0denoted as the absorbance of the control and A_1denoted the absorbance of the samples.

2.5. *In vitro* antibiofilm activity of cyanobacteria against human pathogens

The biofilm inhibitory activity of methanolic extract (20-250 µg/ well) was determined according to Frassinettiet al., (2020) (24) with slight modification. Briefly, the human pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* were grown in 200 µL of trypticase soy broth (TSB) using 96 well plates and incubated at 37°C for 24h. After incubation, the plate was washed with phosphate buffer saline (Hi-Media, Mumbai, India) to remove non-adherent cells and exposed to different concentration of biomass extract (20-250 µg/ well) and incubated at 37°C for 24 h. After 24 h, the 96 well plates were washed thrice with PBS, dried and stained with 0.1% crystal violet for 20 min. Themicrotitre plate waswashed in sterile PBS and allowed to dry at 60°C for 1 h.The biofilm production in the microtitre plate was estimated by adding 30% acetic acid for 15 min and analyzed the absorbance at 595nm using a microtiter plate reader (Thermo Scientific-Multiskan Ex,). The percentage inhibition of biofilm (25) was calculated by the following formula,

Biofilm inhibition(%)= [Control OD-Test OD/Control OD] ×100 (3)

2.6. GC-MS analysis of the cyanobacterial extract

The extractedblue-green algal biomass was analyzed by using Clarus 680 Gas Chromatography instrument equipped with a fused silica column, packed with Elite-5MS and the component was separated using a carrier gas helium with a constant flow of 1 mL/min. The bioactive composite obtained in the spectrum was compared with the GC-MS National Institute of Standards and Technology (NIST - 2011) library.

Results and Discussion

Optimization of biomass production

Based on the morphological features and habitat the cyanobacteria species isolated from the Nangavallilakewas identified as *Microcystis* sp. based on morphology and other identification test.

The optimum biomass for the cultivation of *Microcystis* sp.was identified by the RSM approach and the analysis of variance (ANOVA) was calculated to identify the significance of variables, and quadratic conditions to the responses. ANOVA was used to make sure a high-quality model as explained in (Table 2). The quadratic model is significant and the reports recommended that the F-value (36.73) was high with incredibly low p-value (< 0.0001). Also, the correlation coefficient (R^2) value of 0.9793 was in balanced agreement with the adjusted R^2 value (0.9526). The predicted and adjusted values were closer to 1.0 and are in a balanced agreement representing the enhanced fitness of the model to gain experimental information. The quadratic model in the experiment of implied variables is expressed as an equation.

Biomass yield (g/L) = + 0.4081 + 0.1965A + 0.0019B-0.0610C - 0.0335AB - 0.0075AC + 0.0170BC - $0.2305A^2$ - $0.1138B^2$ - $0.2118C^2$ (4)

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Where, Y (not there in the equation)represented the *Microcystis* sp. yield (%) and A, B, and C indicate the encoded values of carbon source, nitrogen source, and pH respectively. A flat surface of three-dimensional (3D) plots indicated optimum conditions for maximum biomass yield as shown in (Fig. 1).Each 3D graphs represented a mixture of two different parameters with others kept at zero levels.The maximum biomass yield of 1.5 g/L was achieved for the $NaNO_3$(20 mM), K_2HPO_4 (10 mM) and pH 7.5.The interactions between $NaNO_3$ and K_2HPO_4revealthe enhanced biomass yield after 10 days of cultivation.

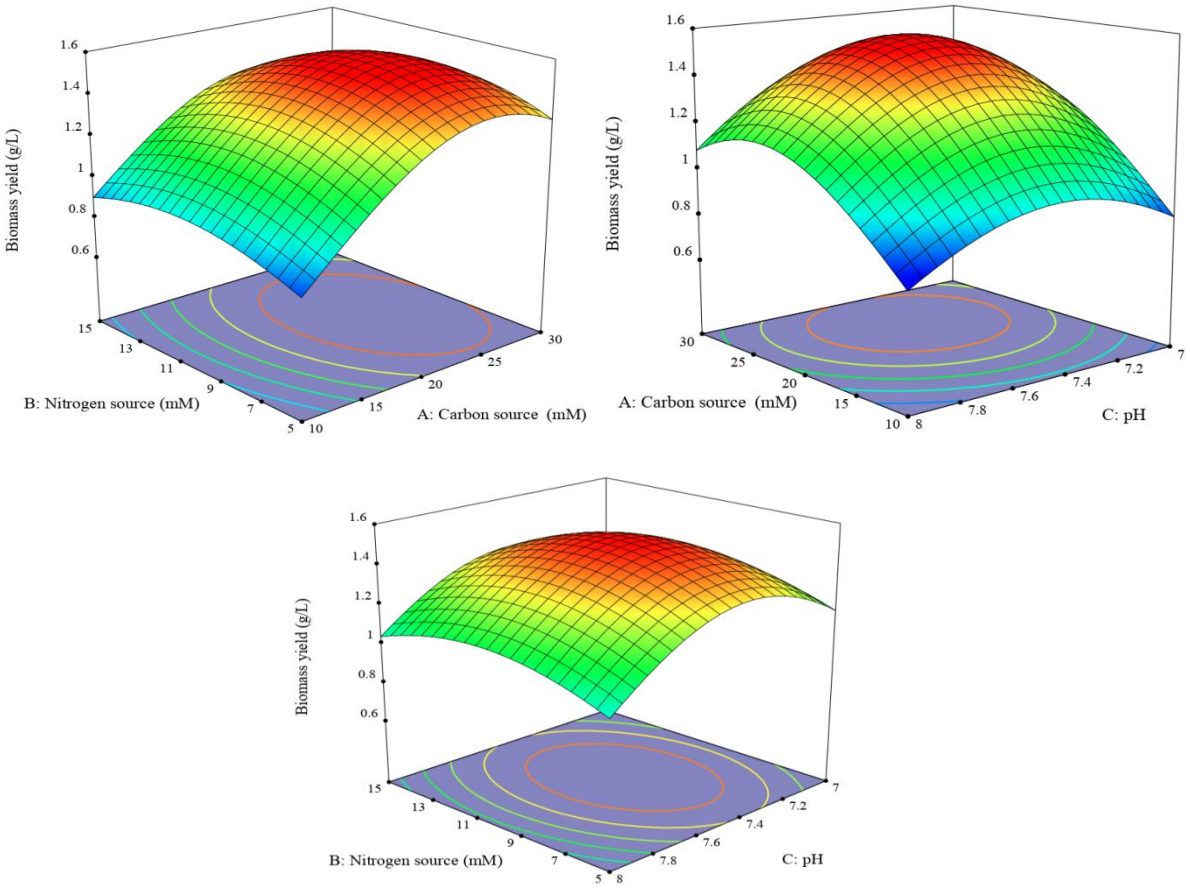

Figure 1: 3D contur plots of variables influence on biomass production

Table 2:ANOVA for response surface quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.8611	9	0.0957	36.73	<0.0001	significant
A-Carbon source	0.3089	1	0.3089	118.59	< 0.0001	
B-Nitrogen source	0.0000	1	0.0000	0.0107	0.9204	
C-pH	0.0297	1	0.0297	11.41	0.0118	
AB	0.0045	1	0.0045	1.73	0.2303	
AC	0.0002	1	0.0002	0.0863	0.7775	
BC	0.0011	1	0.0011	0.4414	0.5277	

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A^2	0.2237	1	0.2237	85.86	< 0.0001	
B^2	0.0545	1	0.0545	20.93	0.0026	
C^2	0.1890	1	0.1890	72.54	< 0.0001	
Residual	0.0182	7	0.0026			
Lack of Fit	0.0182	3	0.0061	457.59	< 0.0001	significant
Pure Error	0.0001	4	0.0000			
Cor Total	0.8793	16				

In-vitro antioxidant activity

The *Microcystis* sp.extract was assessed for their radical scavenging activity at different concentrations (20-100 µg/mL). The *Microcystis* sp.extract showed dose-dependent DPPH radical scavenging activity as compared to a positive control (ascorbic acid) (Fig. 2). The *Microcystis* sp.extract showed the highest DPPH radical scavenging potency, at 100 µg/mL concentration and scavenged 65% of DPPHradicals. Renugadeviet al., (2018) reported that DPPH scavenging activities of *Geitlerinema*sp TRV57 were in the range of 68–78% (200 µg/mL) which coincides with the results of the present study (26).

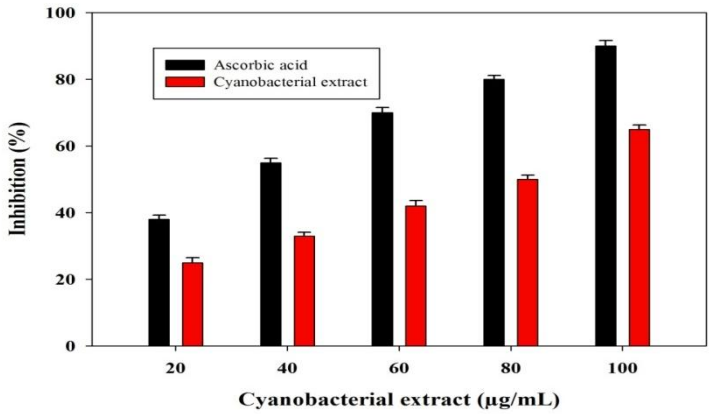

Figure 2: Radical scavenging activity of *Microcystis* sp.

Antibiofilm activity

The antibiofilm activity of *Microcystis* sp.extract was screened against *S. aureus* and *P. aeruginosa* (Fig. 3). Both strains, biofilm formation was significantly inhibited between 75 and 80% at a concentration of 250 µg/mL, while 20 and 22 %(20-22) inhibition was observed at the initial concentration of 25 µg/mL, indicating the biofilm inhibition in a dose-dependent manner.The inhibition of biofilm formation by *Microcystis* sp.extracts with high antibiofilm activity against both *S. aureus* and *P. aeruginosa*, was confirmed by microscopic visualization.The microscopic image showsthe changes in the surface morphology and the architecture of the *Microcystis* sp. treated biofilm. The biofilm formation remarkably reduced on the surface of the algal treated cells. According to Brown et al., (1990), biofilm-forming bacteria are more resistant to the antibiotic, hence the changes of culture morphology architecture proved the *Microcystis* sp. inhibits the bacterial pathogens. The changes of morphology and distraction of cellular architecture will prove the presence of antagonistic agents on the *Microcystis* sp. extract (27).

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Figure 3: Antibiofilm activity by CV assay

Bioactive compound analysis using GC-MS

The GC-MS results showed that the main components of the blue-green algalextract. Sixteen bio-potent compounds namely 11-Tricosene, Cis-9,10-Epoxyoctadecan-1-Ol, Lauroyl Peroxide, 17-Pentatriacontene, 11-Tricosene, 4-Tetradecanol, Cyclotrisiloxane, Hexamethyl-Trimethyl[4-(2-Methyl-4-Oxo-2-Pentyl)Phenoxy]Silane, 5-Methyl-2-Trimethylsilyloxy-Acetophenone,, Cyclotrisiloxane, Hexamethyl-, 1,1,1,3,5,5,5-Heptamethyltrisiloxane, 1,2-Bis(Trimethylsilyl)Benzene, Hexestrol DI-TMS, 2,4,6-Cycloheptatrien-1-One, 3,5-BIS-Trimethylsilylsi, 1,2-Benzenediol,3,5-BIS(1,1-Dimethylethyl)-, Tetrasiloxane, Decamethylsi were identified through GC-MS spectrum (Figure 4 and Table 3).

The bioactive compounds of *Microcystis*sp influenced the antagonistic activity of microbial pathogens. Overhageet al., (2006) stated that the bioactive compounds target on the inhibition of motility of *P.aeruginosa* and other multidrug-resistant organisms (28-30).

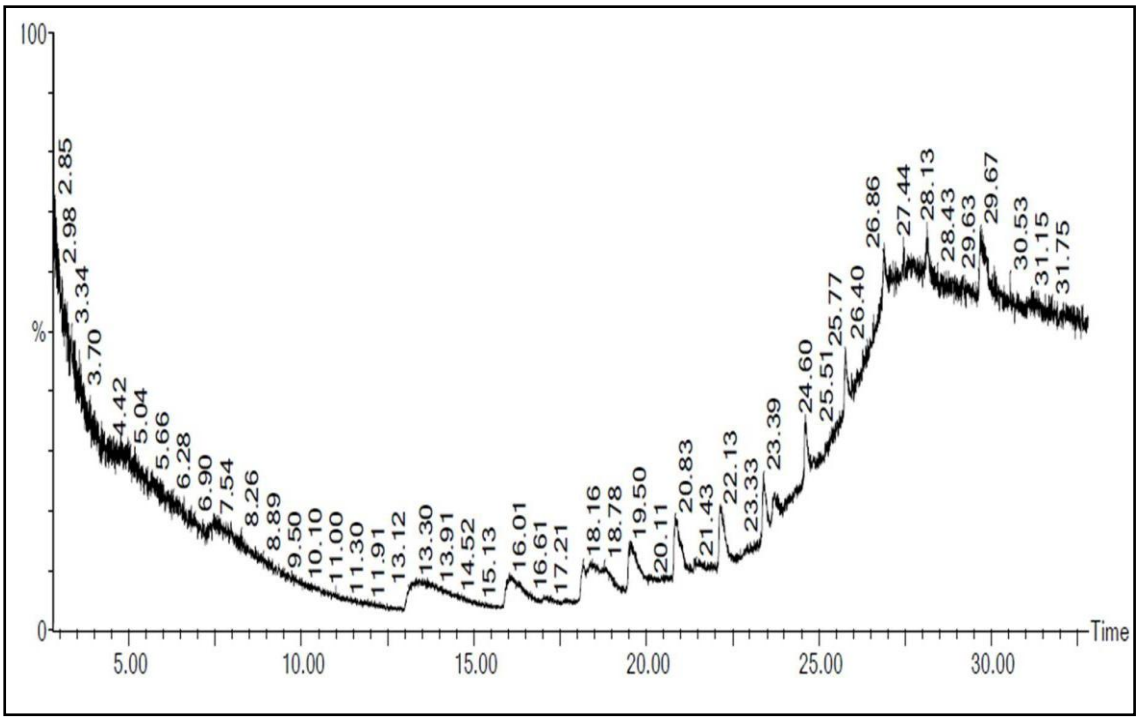

Figure 4: GC-MS spectrum of the methanolic extracted metabolites from *Microcystis* sp.

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Table 3: GC-MS analysis of intracellular metabolites of *Microcystis* sp.

S.No	RT	Name of the compound	Area %	Molecular weight	Structure
1	18.060	11-Tricosene	4.967	322	
2	18.380	Cis-9,10-Epoxyoctadecan-1-Ol	2.226	284	
3	20.82	Lauroyl Peroxide	18.225	398	
4	22.157	17-Pentatriacontene	15.444	490	
5	23.387	11-Tricosene	8.254	322	
6	24.603	4-Tetradecanol	7.834	214	
7	25.768	Cyclotrisiloxane, Hexamethyl-	6.199	222	
8	26.888	Trimethyl[4-(2-Methyl-4-Oxo-2-Pentyl)Phenoxy]Silane	9.650	264	
9	27.444	5-Methyl-2-Trimethylsilyloxy-Acetophenone	5.214	278	
10	27.564	Cyclotrisiloxane, Hexamethyl-	2.848	222	
11	27.669	1,1,1,3,5,5,5-Heptamethyltrisiloxane	3.120	222	
12	28.129	1,2-Bis(Trimethylsilyl)Benzene	4.371	222	

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13	28.429	Hexestrol DI-TMS	3.649	414	
14	29.266	2,4,6-Cycloheptatrien-1-One, 3,5-BIS-Trimethylsilylsi	2.299	250	
15	29.674	1,2-Benzenediol,3,5-BIS(1,1-Dimethylethyl)-	3.107	222	
16	29.715	Tetrasiloxane, Decamethylsi	2.594	310	

Conclusion

The present study concluded that cyanobacteria *Microcystis* sp. isolated from NangavalliLake was optimized for biomass production with different Physico-chemical characters using the RSM approach. The biomass production of 1.5 g/L can be achieved at amaximum with $NaNO_3$ (20 mM), K_2HPO_4 (10 mM) and pH 7.5. The *Microcystis* sp.extract showed the highest antioxidant potential and antibiofilm activity against both Gram-positive and Gram-negative pathogenic bacteria, due to the presence of bioactive compounds, as determined using GC-MS.These findings may direct future studies on the development of cyanobacterial based pharmaceutical products.

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