International Journal of Technology and Applied Science (IJTAS)

E-ISSN: 2230-9004 ● Website: www.ijtas.com ● Email: editor@ijtas.com

Application of Plackett Burman Design to Evaluate Media Components Affecting Antibacterial Activity of Cyanobacteria Isolated from Salim Ali lake

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Abstract:
This study investigates the influence of medium components on the antibacterial activity of cyanobacteria isolated from Salim Ali Lake, Aurangabad, using the Plackett–Burman design. Three isolates, identified as Spirulina maxima, Microcystis aeruginosa, and Oscillatoria amphibia, were tested for antibacterial properties. Among these, methanol extracts of Microcystis aeruginosa showed the highest inhibitory effects against multiple bacterial strains. Statistical optimization revealed that magnesium sulfate and citric acid significantly influenced antibacterial compound production. The results highlight the potential of cyanobacteria as a natural source of bioactive compounds with pharmaceutical importance.

Keywords: Cyanobacteria, Antibacterial activity, Microcystis aeruginosa, Plackett–Burman design, Bioactive compounds.

1. INTRODUCTION

The cyanophyceae are an ancient and diverse group of organisms. They are a large and morphologically diverse group of phototrophic prokaryotes, which occur in almost every habitat of earth. This versatility may explain the remarkable lack of morphological change seen in 3.5 –billion-year-old fossilized cyanobacteria and their modern day counterparts. Through their long existence, cyanobacteria have radiated to an extremely broad range of habitats including hot springs, frigid Antarctic lakes and soils, and extreme euryhaline and eurythermal environments (Raven *et al.*, 1992). Cyanobacteria are widely distributed; some are extremely tolerant and survive in air-drying condition.

The cosmopolitan distribution of cyanobacteria indicates that they can cope with a wide spectrum of global environmental stresses such as heat, cold, desiccation, salinity, nitrogen starvation, photo-oxidation, anaerobiosis and osmotic stress, etc. They have developed a number of mechanisms to defend themselves against environmental stressors. Important among them are the production of photoprotective compounds such as mycosporine-like amino acids (MAAs) and scytonemin (Sinha *et al.*, 2001), enzymes such as superoxide dismutase, catalases and peroxidases, repair of DNA damage and synthesis of shock proteins (Bhagwat and Apte, 1989; Sinha and Häder, 1996).

The term 'bioactivemolecule' is a slang expression in common use and includes substances which may at low concentrations affect life processes – beneficial or harmful. Generally it refers to secondary metabolites that attract the attention of both scientists and industrialists.

The following groups of organisms are the main producers of the substances concerned: dinophytes, raphidophytes, haptophytes and cyanophytes. Once the structure and physiological effects of the phycotoxins are understood, some may serve as source material for useful drugs . The neurotoxic compounds among the cyanotoxins can be used as an example (Skulberg, 2000). They exert their effects on specific ion channels in nerve and muscle membranes. Their use in investigating the exitable properties of nerve cells determines the current interest attached to some selected substances (Carmichael, 1974).

The Salim Ali lake named after the famous Indian ornithologist Salim ali, earlier known as Delhi gate talab is situated near Himayat bagh at Longitude 75° 30' and Latitude - 19° 55' in the historical city Aurangabad on Aurangabad –Jalgaon – Agra - highway in Maharashtra State. It is an important wetland habitat in Aurangabad city, its shape is roughly rectangular and having an area about 54 acres.(Patil *et al*, 2008) . The lake is green throughout the year and always has a bloom of cyanobacteria. Cyanobacteria are the dominant phytoplankton group in eutrophic freshwater bodies (Davidson, F.F 1959.; Negri *et al*, 1995). Microcystis aeruginosa is the most commonly reported species causing hepatotoxicity and odor problems in lakes and water supplies (Sivonen et al 1990.,; (Azevedo et al 1994)

2. MATERIALS AND METHODS

2.1. Isolation, enrichment and Identification

2.1.1 Sample collection

Water samples were collected in presterilized plastic bottles from various sites from Salim Ali Lake Aurangabad. Approximately 200 ml of sample was collected from each site.

2.1.2. Enrichment of alkaliphilic cyanobacteria

Samples collected from the Salim Ali Lake and were enriched in presence of light and BG.11 medium (Kaushik, 1987) containing (g/L) $NaNO_3$, 1.5; K_2HPO_4, 0.04; $MgSO_4.7H_2O$, 0.075; $CaCl_2.2H_2O$, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; Na_2CO_3, 0.02. Medium was amended with 1 ml trace solution of composition (g/L) H_3BO_3, 2.86; $MnCl_2$, 1.81; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.39; $CuSO_4.5H_2O$, 0.079; and $Co(NO_3)_2.6H_2O$, 0.0494. The medium was sterilized at 121°C for 15 min. In flat glass bottles, sterile enrichment medium (50 ml) was inoculated with 5 ml of sample collected from Salim Ali lake. The bottles were incubated under continuous light illumination (fluorescent tubes) at 24±2°C. The growth of cyanobacteria was judged on the basis of visual observations (7 day incubation). The enriched samples were transferred to same medium.

2.1.3. Isolation of cyanobacteria

Cyanobacterial colonies were isolated by streaking the enriched sample on respective growth medium-agar plates. Visibly distinct cyanobacterial colonies were reinoculated for further purification. In case of filamentous cultures, selective inoculation of single filament under aseptic conditions was the method of isolation.

2.1.4. Purification of cultures

Cultures were purified as per the methods described by Kaushik (1987). Isolates were purified by either number of transfers in broth media or solid agar plate method or using antibiotics in the media (chloramphenicol, 25 mg/l combined with penicillin 50 mg/l and antifungal antibiotics, fluconazole, 100 mg/l separately). The cultures were further maintained in the broth medium in which it had exhibited maximum growth during enrichment experiments.

2.1.5. Identification of cultures

On the basis of morphological characteristics cyanobacterial cultures (Fig. 3.2) were identified to genus and species level as described by Rippka *et al.* (1979) and Desikachary (1959).

2.2 Antibacterial Activity

2.2.1. Preparation of cell extract

Cyanobacterial biomass was harvested after 15 days of incubation by centrifugation at 5000 *g* for 15 min (Sorvall, USA, Model RC2). Water extracts were made by resuspending cyanobacterial biomass in distilled water (10 mg/L) and ultrasonicating at 20 KHz frequency (Sonics and Materials Inc., USA) for 2 min. The biomass was also extracted simultaneously with organic solvents viz. methanol, isopropanol and acetone. The cell mass was separated by centrifugation at 5000 *g* for 20 min and the extraction procedure was repeated twice. The supernatant was kept at 4°C until further use in antibacterial assays.

2.2.2. Test organisms

10 bacterial cultures from the laboratory repository viz. *S. aureus* NCIM 2127, *E.coli* NCIM 2545, *S. typhi* NCIM 2501, *P. aeroginosa* NCIM 2053, *P. vulgaris* NCIM 2857, *B. thrungensis* NCIM 2159, *B. megaterium* NCIM 2087, *B. subtilis* NCIM 2117, *B. licheniformis* NCIM 2051, *E.coli* wild type were used in study. 12 hours old cultures in nutrient broth were used as the inoculums in antibacterial assay.

2.2.3 Disc diffusion assay

Antibacterial activity of cell extracts against the test organisms was done by disc diffusion assay. Petri plate containing 15 ml of solidified nutrient agar was spread inoculated with 100 µl of 12 h old test bacterial cultures. Presterilized Whatman No.1 paper discs (6 mm) were saturated with 50 ml of cyanobacterial extracts and dried to be used in assays. The plates were kept at 4 oC for 2 h before they were incubated at 37 oC for 24 h. Antibacterial activity was assessed by measuring the diameter of growth inhibition zone around the discs.

2.2.4. Statistical design

The Plackett-Burman design based on the first order model (Plackett and Burman. 1946)) was used to screen and evaluate the important media components that influence the production of antibacterial compounds. All the experiments were carried in triplicate according to designed matrix (Table 1) using the equation 1:

$$Y = \beta 0 + \Sigma \beta i X i \ (i = 1, \ldots. k) \qquad \text{(Equation 1)}$$

Where, *Y* is the estimated target function Where, *Y* is the estimated target function, $\beta 0$ is a constant, $\beta 1$ *is* the regression coefficient, *X* is independent variable and *k* is number of variables. Total eight macronutrient from BG-11 were screened include $NaNO_3$, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, citric acid, ferric ammonium citrate, EDTA and Na_2CO_3. Each independent variable was investigated at a high (+1) and a low (-1) level which represents two different nutrient concentrations as shown in Table 1. The level of micronutrients in all experiments was kept constant as described earlier. The level of micronutrients in all experiments was kept constant as described earlier. From the experimental trials (T1 - T12) cell mass was extracted with methanol and used in antibacterial disc diffusion assay (Table 1.)The diameter of zone of inhibition was recorded as response value. The student's *t*-test was performed to determine the significance of each variable employed. The regression coefficients were determined by least square method. All the statistical analysis was done using MINITAB 3.13.

Trial	Level and concentration of variable (g/l)							
	X_1 $NaNO_3$	X_2 K_2HPO_4	X_3 $MgSO_4.7H_2O$	X_4 $CaCl_2.2H_2O$	X_5 Citric acid	X_6 Ferric ammonium citrate	X_7 EDTA	X_8 Na_2CO_3
1	+1 (2.25)	-1 (0.02)	+1 (0.1125)	-1 (0.018)	-1 (0.003)	-1 (0.003)	+1 (0.0015)	+1 (0.03)
2	+1 (2.25)	+1 (0.06)	-1 (0.0375)	+1 (0.054)	-1 (0.003)	-1 (0.003)	-1 (0.0005)	+1 (0.03)
3	-1 (0.75)	+1 (0.06)	+1 (0.1125)	-1 (0.018)	+1 (0.009)	-1 (0.003)	-1 (0.0005)	-1 (0.01)
4	+1 (2.25)	-1 (0.02)	+1 (0.1125)	+1 (0.054)	-1 (0.003)	+1 (0.009)	-1 (0.0005)	-1 (0.01)
5	+1 (2.25)	+1 (0.06)	-1 (0.0375)	+1 (0.054)	+1 (0.009)	-1 (0.003)	+1 (0.0015)	-1 (0.01)
6	+1 (2.25)	+1 (0.06)	+1 (0.1125)	-1 (0.018)	+1 (0.009)	+1 (0.009)	-1 (0.0005)	+1 (0.03)
7	-1 (0.75)	+1 (0.06)	+1 (0.1125)	+1 (0.054)	-1 (0.003)	+1 (0.009)	+1 (0.0015)	-1 (0.01)
8	-1 (0.75)	-1 (0.02)	+1 (0.1125)	+1 (0.054)	+1 (0.009)	-1 (0.003)	+1 (0.0015)	+1 (0.03)
9	-1 (0.75)	-1 (0.02)	-1 (0.0375)	+1 (0.054)	+1 (0.009)	+1 (0.009)	-1 (0.0005)	+1 (0.03)
10	+1 (2.25)	-1 (0.02)	-1 (0.0375)	-1 (0.018)	+1 (0.009)	+1 (0.009)	+1 (0.0015)	-1 (0.01)
11	-1 (0.75)	+1 (0.06)	-1 (0.0375)	-1 (0.018)	-1 (0.003)	+1 (0.009)	+1 (0.0015)	+1 (0.03)
12	-1 (0.75)	-1 (0.02)	-1 (0.0375)	-1 (0.018)	-1 (0.003)	-1 (0.003)	-1 (0.0005)	-1 (0.01)

Table1 Plackett-Burman experimental design matrix for screening composition of growth medium.

3. RESULTS

3. 1. Culture isolation from Salim ali Lake

Enrichment of salim Ali Lake samples yielded 3 cyanobacterial isolates. All the three cultures were enriched using BG-11 medium. Only one of the three Isolate was unicellular (*Microcystis aeruginosa)* and all other isolates were filamentous. All the isolated cultures from Salim ali lake were of non-heterocystous nature. (Table 2.)

Sr. No.	Isolate No.	Morphology	Enrichment medium	Identification
1	SA-1	Fil, NH	BG-11	*Spirulina maxima*
2	SA-2	Uni	BG-11	*Microcystis aeruginosa*
3	SA-3	Fil, NH	BG-11	*Oscillatoria amphibia*

Fil, Filamentous, NH, Non-heterocystous, Uni, Unicellular

Table 2. Morphological characterisation of cyanobactrerial isolates for Salim ali lake

Isolates		*Spirulina maxima*				*Microcystis aeruginosa*				*Oscillatoria amphibia*			
		Mean Diameter of zone of inhibition (cm)											
Bacteria cultures	Extracts	Ac	Mt	Ip	Aq	Ac	Mt	Ip	Aq	Ac	Mt	Ip	Aq
S. aureus 2127		0.5	0.7	0.8	R	0.7	1.2	0.9	0.5	0.6	0.8	0.5	0.6
E.coli 2545		0.9	0.8	0.5	0.8	0.8	1.1	1.3	0.8	0.7	R	0.7	1.0
S. typhi 2501		0.5	0.6	0.5	0.8	0.6	1.2	0.6	0.6	0.6	0.8	R	R
P. aeroginosa 2053		R	0.9	0.5	0.6	0.8	0.8	0.9	0.5	0.8	1.0	0.5	R
P. vulgaris 2857		1.0	R	1.1	R	0.5	1.1	0.7	0.8	0.6	R	0.5	R
B. thrungensis 2159		R	0.8	R	R	0.7	0.9	1.0	1.0	R	0.5	0.7	0.5
B. megaterium 2087		R	0.5	R	0.7	0.5	1.0	0.5	0.6	1.2	0.6	0.8	0.5
B. subtilis 2117		0.5	0.1	R	0.6	0.6	0.1	0.7	0.9	R	R	1.0	R
B. licheniformis 2051		0.5	0.5	R	0.9	0.5	0.7	0.6	0.8	0.8	0.5	0.6	1.0
E.coli wild		R	0.6	R	0.9	0.5	0.7	0.5	0.8	0.7	0.5	0.5	0.5
Cumulative Inhibition		3.9	5.5	3.4	5.3	6.2	8.8	7.7	7.3	6	4.7	5.8	4.1

3.2 Antibacterial activity

Antibacterial activities of all the three cyanobacterial isolates extracted with acetone, isopropanol and methanol along with aqueous extracts were tested using disc diffusion method (Table 1).

Based on cumulative antibacterial activity *Microcystis aeruginosa* proved to be the most promising among the cyanobacterial isolates, with the highest cumulative inhibition of 8.8, 7.7 and 7.3. Among the four solvents, aqueous extraction did not show highest inhibition in any of the cultures.

3.3 Statistical optimization

Based on the antibacterial activity methanol extract of *Microcystis aeruginosa* was chosen for further experiments. A Plackett-Burman design matrix (Table 1) with eight variables of BG-11 medium was applied to find out variables influencing antibacterial activity of *Microcystis aeruginosa*. Since the data observed was inhibitory against all the test organisms a mean of cumulative inhibition was considered (Table 3). Highest mean of cumulative antibacterial activity was obtained for trial T_4 followed by T_2. The mean cumulative antibacterial effect was further processed by using the statistical software MINITAB 13.13. Significance of each variable was determined using student t-test. The components were screened at the confidence level of 95% on the basis of their effects.

Bacteria cultures	Trials	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
S. aureus 2127	Mean diameter of zone of inhibition (cm)	0.6	1.2	1.6	0.7	0.5	1.7	0.8	1.2	1.2	0.5	0.9	1
E.coli 2545		0.4	0.6	1.1	1.5	0.8	0.7	1.2	1.8	0.5	0.7	0.9	1.1
S. typhi 2501		0.5	0.6	0.6	0.7	0.7	0.5	1.3	1	0.6	1.6	0.8	0.5
P. aeroginosa 2053		0.9	1.3	1.1	1.9	1	0.5	0.3	0.6	0.7	0.8	1.5	0.5
P. vulgaris 2857		0.8	1	0.5	0.7	0.6	0.5	0.8	0.8	1.2	1.2	0.9	0.7
B. thrungensis 2159		0.6	1.1	0.7	1.2	0.5	1.5	0.6	0.5	0.7	0.8	1.2	0.8
B. megaterium 2087		1.4	1.6	0.8	1.9	0.5	0.6	0.4	0.6	0.8	0.5	0.5	0.5
B. subtilis 2117		0.5	0.7	0.4	1.6	0.6	1.8	0.9	1.2	1	1.6	0.5	0.8
B. licheniformis 2051		0.6	1.5	1	0.9	1	0.5	0.8	0.8	1.8	1.4	1.2	0.9
E.coli wild		1.2	1.6	1.8	0.6	0.9	1.1	0.7	0.8	1.6	1.2	0.5	0.5
Mean Cumulative Inhibition		7.5	11.2	9.6	11.7	7.1	9.4	7.8	9.3	10.1	10.3	8.9	7.3

Table3: Antibacterial activity of the 3 cyanobacterial extracts using different solvents against selected bacteria cultures

Table 3 Effect of media components on antibacterial activity of *Microcystis aeruginosa* tested as per the Plackett-Burman design

Variable	Effect	Coefficient	SE Coefficient	*t*-value	*p*-value
Constant		9.1833	0.1848	49.68	0.000
X_1 ($NaNO_3$)	0.7000	0.3500	0.1848	1.89	0.155
X_2 (K_2HPO_4)	1.1667	0.5833	0.1848	3.16	0.051
X_3 ($MgSO_4.7H_2O$)	-1.4667	-0.7333	0.1848	3.97	0.029*
X_4($CaCl_2.2\ H_2O$)	0.7000	0.3500	0.1848	1.89	0.155
X_5(Citric acid)	1.7667	0.8833	0.1848	4.78	0.017*
X_6 (Ferric ammonium citrate)	-0.5000	-0.2500	0.1848	-1.35	0.269
X_7 (EDTA)	0.1333	0.0667	0.1848	0.36	0.742
X_8 (Na_2CO_3)	0.4333	0.2167	0.1848	1.17	0.326

SE, standard error; *Significant at 95% level (p<0.05)

Table 4. The effects and coefficients of variables estimated using Plackett-Burman design

Table 4. represents the result of Plackett-Burman trials with respect to *t*-value, *p*-value and confidence level of each component. The significant values indicate that the component was effective in antibacterial activities. Out of eight media components tested, only magnesium
sulphate and citric acid had a significant effect on antibacterial activity of the cyanobacterial cultures, when cumulative effect against all test organisms was considered.

4. DISCUSSION

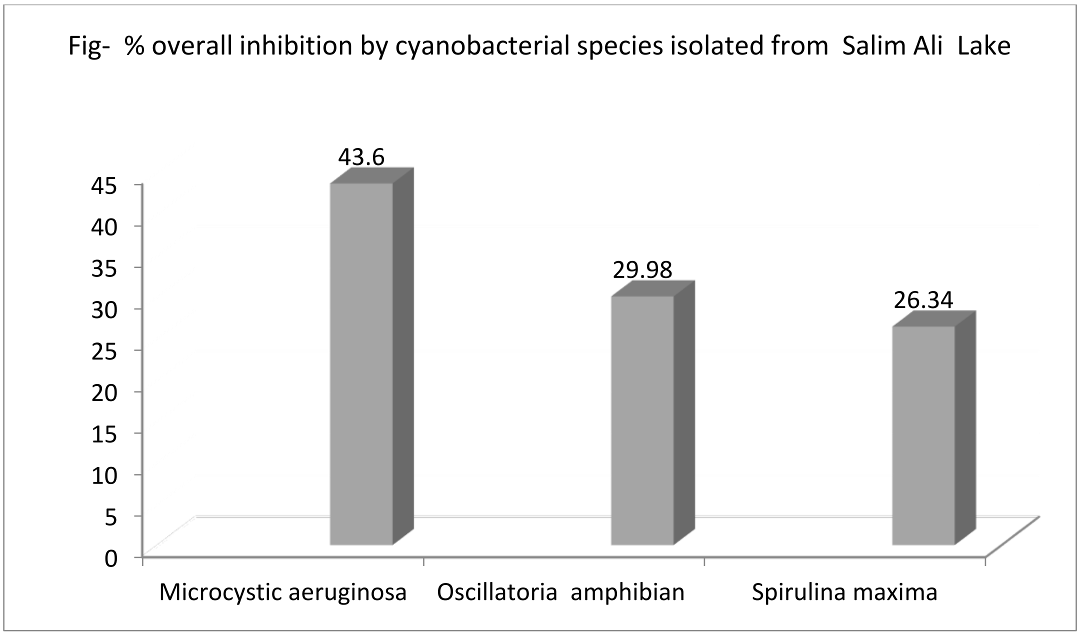
The primary objective of any isolation program is the establishment of a clonal culture from a single cell so that one has a genetically uniform strain. The preferred method is to isolate a single cell immediately from its habitat before any enrichment (Brand, 1990). A method often found useful is plating and streaking of the sample onto agar plates, allowing colonies to develop from individual cells assuming they can survive and grow on agar plates.

Three cyanobacterial cultures were isolated from Salim Ali Lkae, Aurnagabad The observed diversity of cyanobacteria in these samples and culturability did not match. The reduction in observed diversity and cultural diversity could be due to many reasons. i) Many planktonic species of microalgae cannot survive solid agar conditions. ii) Agar, which is routinely used as a solidifying agent in bacteriological media, is known to contain impurities (Allen and Gorham, 1981), and some of these are suspected to be responsible for the inhibition of cyanobacterial growth. iii) Out numbering of slow growers during enrichment, iv) Non-optimal (artificial) growth conditions never substitute natural habitats.

In the study conducted on salim ali lake, 3 different cyanobacteria were isolated. These isolates were classified into 3 species belonging to 3 genera, identified as per Rippka *et al.* (1979) and Desikachary (1959).. The present study is an attempt to screen cyanobacterial cultures isolated from Salim Ali Lake, Aurnagabad. The results indicated that *Microcystis aeruginosa* was the dominant among other cyanobacteria (Table1.).

Cyanobacteria of the genus *Microcystis* (Chroococcales) belong to the most common bloom-forming microorganisms in many freshwater bodies worldwide. They also occur in coastal areas of the brackish Baltic Sea. A number of cyanobacteria and eukaryote algae, particularly macroalgae, produce various biologically active compounds (Kulik, 1995). These include antibiotics which in laboratory tests inhibited bacteria and fungi that elicit diseases of humans. The production of bioactive compounds and expression of antimicrobial activity depends on physiological factors such as stage of growth and culture conditions (Schlegel *et al*., 1999). Several species of cyanobacteria shown to produce substances with antibiotic activity include *Tychonema bourrellyi, Aphanizomenon flos-aquae, Cylindrospermopsis* (Østensvik et al., 1998), *Anabaena, Calothrix, Leptolyngbya, Lyngbya, Microcystis, Nostoc, Phormidium, Pseudoanabaena, Synechococcus, Synechocystis, Tolypothrix* (Ördög et al., 2004). Cyanobacteria are still largely unexplored, thus representing a rich opportunity for discovery of new bioactive compounds. The expected rate of rediscovery is far lower than for other better-studied group of organisms (Olaizola, 2003). Cyanobacteria produce a wide variety of toxins and other bioactive compounds, which include 40% lipopeptides, 5.6% amino acids, 4.2% fatty acids, 4.2% macrolides and 9% amides (Singh et al., 2005).

The dominance of *Microcystis aeruginosa* in salim ali lake, is justified by the production of certain bioactive compounds or toxin which has a growth inhibitory effect on all the bacterial species. In the present study all the cyanobacteria associated with Salim ali lake, proved their bioactive potentials the pattern of antibacterial activity with an overall performance was *Microcystis aeruginosa*> *Oscillatoria amphibian* > *Spirulina maxima. (*Fig 1). Methanol was proved to the best solvent for the extraction of bioactive compounds.



The methodology of Plackett–Burman is a powerful and useful tool in searching for the key factors rapidly from a multivariable system. This method does not determine the exact quantity, but it can provide some important information about each factor by relatively few experiments (Plackett & Burman1946;Demeo *et al.* 1985; Roseiro et al. 1992; Yu et al. 1997; Kalil et al. 2000; Long-Shan *et al*. 2003).

From the Plackett Burman design carried out for the enhancement of the bioactive compounds from *Microcystis aeruginosa*. The result suggested the that the methanolic extracts of *Microcystis aeruginosa* gave the highest cumulative inhibition of 8.8 and when the similar method was run using the Plackett burman design of 12 trials it gave the highest cumulative inhibition of 11.8. Thus combination of media components had an influence on the production of bioactive compounds from *Microcystis aeruginosa* . A 3% increase in the production of bioactive compounds was reported when the media was optimized using the plackett Burman design.

The Plackett Burman design gave two media components viz. X_3 ($MgSO_4.7H_2O$) and citric acid which gave a 95% significance. Both these compounds have a potential of inhibitory effect. The role of magnesium and citric acid in increasing antibacterial activity could be correlated to change in regulation of branched metabolic pathway for tetrapyrole compounds in cyanobacteria. The three major tetrapyrrole end products chlorophyll (Chl), 3 heme, and phycobilins are synthesized in a branched metabolic pathway with protoporphyrin IX (PIX) as the last common precursor. The magnesium branch magnesium catalyzes the chelation of Mg^{2+} into PIX, there by directing tetrapyrroles into Chl synthesis. Cyanobacteria and plants accumulate various tetrapyrrole species in different quantities in the cell. The influence of magnesium and citric acid on antibacterial activity of *Microcystis aeruginosa* has indicated to the tetrapyrolic nature of antibacterial compound. However, these results emphasize further studies on purification and characterization of antibacterial principles in *Microcystis aeruginosa* .

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