



EVALUATION OF MEDIA COMPONENTS AND REPLACE THEM WITH SEA WATER FOR SCYTONEMIN PRODUCTION BY USING NOSTOCALES

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ABSTRACT

Scytonemin is a naturally occurring UV screening pigment, found in extracellular sheaths of most of the Nostocales. Accumulation of scytonemin in extracellular sheath is regulated by many of the nutrients and physiochemical parameters. The purpose of this study was to evaluate BG 11 media component by using Plackett-Burman statistical design and study their effective concentrations on the production of scytonemin, by using four isolated Nostocales *Scytonema sp.*, *Plectonema sp.*, *Spirulina sp.*, and *Lyngbya sp.* All four fresh water cyanobacteria were adopted for 3% salinity and BG 11 media is then replaced with sea water, by adjusting evaluated components and providing optimum physiochemical conditions for scytonemin production. As per our results, NaNO₃ (nitrate), K₂HPO₄ (phosphate + potassium), CaCl₂ (calcium) and MgSO₄ (Magnesium) has a significant effect on scytonemin production. NaNO₃ concentration varies from 0% to 0.015%. MgSO₄ at 10 times more concentration increases scytonemin yield by 2 folds. CaCl₂ presence decreases scytonemin yield, optimized concentration was 10 time less as that of original concentration. Optimized concentrations for pH, salinity and temperature were 7- 8, 2% and 28 – 30 °C, respectively. Scytonemin produced by using sea water culture was about 2.5% of dry weight of all 4 cyanobacteria.

KEY WORDS: Scytonemin, UV screening pigment, Nostocales, Plackett-Burman design.



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INTRODUCTION

In the development of algal product, one of the major target is to select suitable nutrient medium.^{1,2} The selection of medium mainly depends on several factors that include chemical composition of the medium.³ Considering these facts, Blue Green media (BG 11) were selected for fresh water cyanobacteria isolation and its components were evaluated for the scytonemin production. The main objective of the present investigation is to find out optimum values of physiochemical parameters for large scale production of scytonemin. Scytonemin is a lipid soluble alkaloid that is synthesized in response to ultra violet (UVA) radiation and accumulates within the extracellular sheaths of cyanobacteria. Due to which the organisms are protected from cell damage by absorbing the harmful solar radiation.^{4, 5, 6} The yellow-green pigment scytonemin was first reported as early as 1849⁷. However, the structure was unknown for over 100 years until 1993 when a complete elucidation of the chemical structure was provided.⁴ Scytonemin absorbs mostly in the UVA (325-425 nm, λ_{\max} = 370 nm) and UVC region (λ_{\max} = 250 nm), but it also absorbs substantially in the UVB region (280-320 nm).⁴ The maximum absorption wavelength of scytonemin is 370 nm in vivo. However, it shifts towards a longer wavelength of 384 nm in a solvent after isolation. It is reported that the molar extinction coefficient of scytonemin is large (250 L/g/cm) at wavelength 384 nm.⁸ Based on a molecular weight of 544 Da, it is calculated to be 136,000 L/mol/cm. Due to its large extinction coefficient, scytonemin acts as an efficient photo-protective compound.⁹ In addition to scytonemin's important function as a sunscreen it also possesses anti-proliferative¹⁰, anti-inflammatory¹¹, radical scavenging¹² and antioxidant properties¹³. It also alters tyrosinase activity and melanogenesis¹⁴. In 2002, the United States has patented methods of using scytonemin in treating disorders associated with cell proliferation, cell cycle progression, kinase activity, tissue hyperplasia or angiogenesis, as in cancer or inflammatory diseases¹⁵. Due to this different important commercial uses of Scytonemin one need to study its large scale production. Different cyanobacteria were identified for the presence of scytonemin. Four Nostocales (cyanobacteria) were isolated from the selected location having intense light falling on algae, namely *Scytonema sp.*, *Plectonema sp.*, *Spirulina sp.*, and *Lyngbya sp.* All of them produce scytonemin, which can be detected by

spectrophotometric techniques. *Scytonema sp.* was selected for evaluation of media components by using Plackett – Burman design. This design allows reliable short listing of medium components in fermentation for further optimization and allows one to obtain unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations. By using the results of evaluated media components for *Scytonema sp.*, effects of each component were determined by varying its concentrations for all isolated Nostocales. As evaluated media components such as CaCl₂, MgSO₄, salinity etc. for scytonemin production shows similarities that of sea water; cyanobacteria possibly can grow in sea water¹⁶. Cyanobacteria can be adapted to the salinity (hyper-saline environments), such as the Great Salt Lake in Utah¹⁶. Halotolerant and halophilic cyanobacteria can adapt to the high salinity in four ways

- I. Active export of inorganic ions in the protoplasm leading to relatively unchanged internal salt concentrations¹⁶.
- II. Organic osmoprotective compounds accumulation, such as glycine, glucosylglycerol²⁹, and betaine (trimethylglycine) to maintain the osmotic equilibrium¹⁷.
- III. Expression of a set of salt-stress proteins such as the protein flavodoxin¹⁶.
- IV. Synthesis of trehalose to stabilize the phospholipid membrane bilayers of the cell¹⁶.

Due to ability of Nostocales for adaptation to salinity, one can replace media by sea water. Using this phenomenon of adaptation, sea water was tested as a replacement for BG 11 media by determining its components.

MATERIALS AND METHODS

(i) Isolation and culturing of Nostocales^{3, 19, 20}

Four Nostocales namely *Scytonema sp.*, *Plectonema sp.*, *Spirulina sp.*, and *Lyngbya sp.* were isolated³ and identified¹⁹ from soil nearby Old Swimming pool in Savitribai Phule Pune University, Pune, India as per standard protocols²⁰. All Nostocales were maintained in BG 11 medium by providing 24 h light condition at 28°C.

(ii) Extraction and measurement of Scytonemin²¹

For extraction of scytonemin, samples were dried at 50 °C. Cells were suspended in 100% acetone

and kept overnight at 4°C. After centrifugation sample was filtered through Whatman filter paper number 1 and absorbance were measured on UV-visible spectrophotometer. Absorbance of filtrate was measured at 384 nm (scytonemin maximum), 490 nm (pooled carotenoid), 663 nm (chl. a). The value of absorbance at 750 nm was subtracted from all measured absorbance. The cellular scytonemin content was calculated using the trichromatic²¹. (Extinction coefficient in 100% acetone is 112.6 L g⁻¹ cm⁻¹)

$$A_{384}^* = 1.04A_{384} - 0.79A_{663} - 0.27A_{490}$$

A_{λ} is the measured absorbance at λ .

In the absence of absorption coefficient for scytonemin, the unit represents the absorbance of 1 mg dry weight of material extracted in 1 mL

acetone in a cuvette with 1 cm path length. Specific content was expressed as ($A_{\lambda} \cdot \text{mg}^{-1}$).

(iii) Evaluation of media components by using Plackett-Burman design^{22, 23}

For the large scale production of *Scytonema sp.*, media was optimized based on first order model²² and 8 trails for 7 variables were conducted as per standard protocol²³ by using following equation,

$$Y = \beta_0 + \sum \beta_i X_i (i = 1, \dots, k)$$

Where, Y is the estimated target function, β_0 is a constant, β_1 is the regression coefficient, X is independent variable and k is number of variables. Experiment conducted is described in table-1.

Table 1
Plackett - Burman statistical design

Variables	Nutrients	High values (+)	Low values (-)
		In g/100 mL	
A	NaNO ₃	1.5	0.015
B	K ₂ HPO ₄	0.04	0.0004
C	MgSO ₄	0.075	0.00075
D	CaCl ₂	0.0036	0.000036
E	Na ₂ CO ₃	0.002	0.0002
F	Citric acid	0.006	0.0006
G	EDTA	0.001	0.0001

Trial	Variations						
	A	B	C	D	E	F	G
1	+	+	+	-	+	-	-
2	-	+	+	+	-	+	-
3	-	-	+	+	+	-	+
4	+	-	-	+	+	+	-
5	-	+	-	-	+	+	+
6	+	-	+	-	-	+	+
7	+	+	-	+	-	-	+
8	-	-	-	-	-	-	-

1 denotes a high level values; -1 denotes a low level values,
F- Citric acid and G- EDTA are dummy variables.

8 trails for 100 mL culture were conducted. Micronutrient concentration was same in all trials, pH was 7.5, salinity was 1%, and kept at 25 °C for 10 days providing 24 h light conditions. Each trial was conducted in triplicate and mean was considered. At the last day chlorophyll contain, scytonemin contain and fresh weight was recorded.

(iv) Effect of media nutrients and physiochemical parameters on biomass and scytonemin production^{24,28}

Media nutrients have great impact on transcription and translation of a cell. To study

these effects on isolated 4 cyanobacteria, BG 11 media were used. Effect of nitrate, phosphate + potassium, calcium and Magnesium were determined by varying concentrations of nutrients.²⁴ Different concentrations of nutrients were added by preparing stock solutions. Final volume of each tube was 10 mL with same other nutrients and physiological conditions. 1 mL of culture was inoculated in each experimental tube and incubated for 15 days at 28°C providing 24 h light conditions. Each experiment was conducted in triplicate; concentration is listed in table 2.

Table 2
Concentrations of nutrients varied

Sr. No.	Nutrients	Concentrations					
1.	NaNO ₃	0.0%	0.015%	0.15%	1.5%		
2.	K ₂ HPO ₄	0.0%	0.0004%	0.004%	0.04%		
3.	MgSO ₄	0.0%	0.00075%	0.0075%	0.075%		
4.	CaCl ₂	0.0%	0.000036%	0.00036%	0.0036%		
Sr. No	Parameters	Variations					
1.	pH	5	6	7	8	9	10
2.	Salinity	0%	2%	4%	6%	8%	10%
3.	Temperature	4 °C	10 °C	28 °C	RT	37 °C	50 °C
4.	Light	white	Blue	Green	Red	Yellow	Black

Each experiment was replicated in duplicate. All other physiochemical parameters (e.g. pH, salinity etc.) were measured daily and kept constant. Also added micronutrient concentrations were same in all experiments. At the last day sample were centrifuged at 8000 rpm for 10 min at 4 °C and fresh weight was recorded and scytonemin contain was estimated by tricometric equation¹⁹. (Extinction coefficient in 100% acetone is 112.6 L g⁻¹ cm⁻¹)

(v) *Sea water culturing of cyanobacteria for scytonemin isolation*

For large scale production of isolated cyanobacteria, sea water collected from Dadar, Mumbai, Maharashtra, India was used. Salinity of water was 2.5% and pH was adjusted to 7.5. Calcium and Magnesium ion concentrations were determined and adjusted according to optimum culture. 10 L of sea water having optimum salinity, pH, Calcium and Magnesium ion concentrations was autoclaved and pour into UV sterilized plastic trays. 100 mL of cyanobacterial inoculum was added and kept at 25 °C under 24 h light conditions for 15-20 days. Bubbler at 60-100 bubbles/min was adjusted in tray to provide air. Control was kept as respective optimized media for all 4 Nostocales. At the last day, grown cultures were filtered with Whatman paper and fresh weight was recorded.

(vi) *Calcium and Magnesium ion estimation*²⁵

Calcium ion concentration of sea water was estimated by EDTA²⁵. To the 50 mL of sample 3-4 drops of polyvinyl alcohol and 10-20 drops of 40% NaOH was added. Sample was boiled for a few minutes. 1-2 drops of Hydroxy naphthol indicator was added and titrated with 0.01 M EDTA. End point was clear blue color. Magnesium ion concentration of sea water was estimated by EDTA²⁵. To 50

mL of sample 2-3 drops of concentrated HCl was added and boiled for few minutes. 2 drops of methyl red and 0.3 M NaOH drop wise added till color changes. Add 2-3 drops of Eriochrome black T indicator and titrate with 0.01 M EDTA. Color changes from red to pure blue. It gives total hardness of water. Magnesium ion concentration was calculated by subtracting Calcium ion concentration from hardness.

RESULTS AND DISCUSSION

1. *Media components evaluation by Plackett-Burman design for scytonemin production*

From the Main effect plot (Graph 1) and Pareto chart (Graph 2) of standardized effects, it can be seen that MgSO₄, CaCl₂, NaNO₃ and K₂HPO₄ have a significant effect on Scytonemin production when compared to NO₂CO₃, EDTA and citric acid. K₂HPO₄ and MgSO₄ gave higher yield at its higher concentration, yield obtained using NaNO₃ and CaCl₂ was found to be greater at its lower concentrations. Hence it can be inferred that NaNO₃, K₂HPO₄, MgSO₄ and CaCl₂ has significant effect for scytonemin production by *Scytonema sp.* The analysis of yield using Plackett - Burman design is shown in Table 3. In this investigation the maximum yield of scytonemin was 42.19 mg/g of biomass for a medium composition in which the concentrations of NaNO₃ and MgSO₄ were high and minimum yield of scytonemin was 7.28 mg/g of biomass for a medium with low concentration of NaNO₃, MgSO₄ and CaCl₂. Hence to optimize scytonemin production it is necessary to optimize the concentrations of NaNO₃, K₂HPO₄, MgSO₄ and CaCl₂ using different concentrations whereas other components of the medium can be kept constant.

Table 3
Yield of scytonemin by trials 1 to 8 performing Plackett - Burman design

Trial	Scytonemin in mg/g of biomass	Trial	Scytonemin in mg/g of biomass
1	29.48	5	07.28
2	35.44	6	42.19
3	38.55	7	27.80
4	30.81	8	17.37

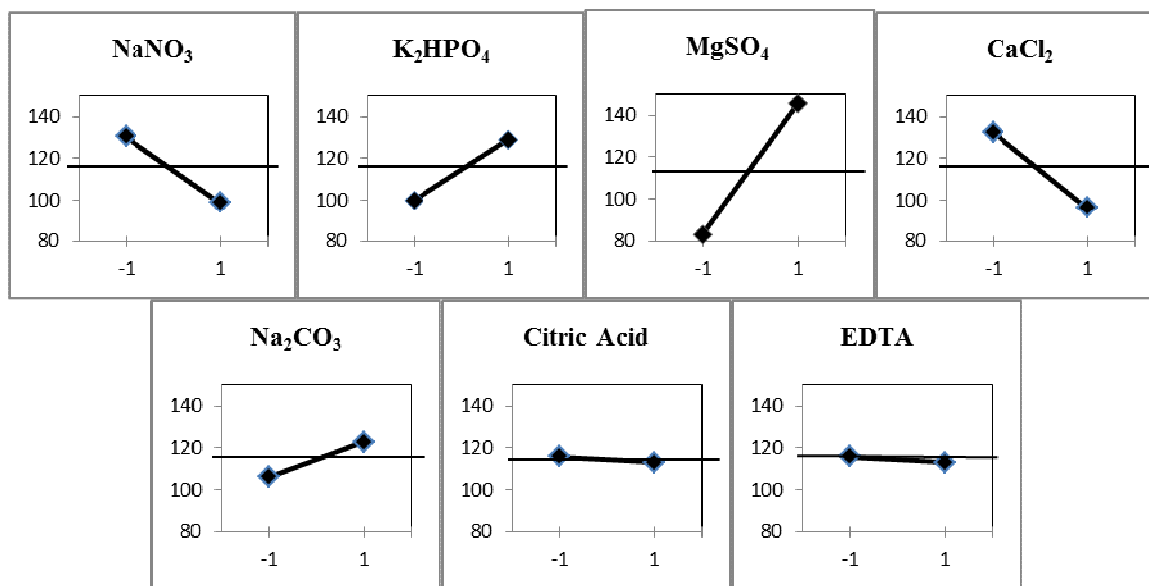
Table 4
Analysis of yield for Plackett - Burman media optimization design

	A	B	C	D	E	F	G
$\sum H$	130.28	100.00	83.26	132.60	106.12	115.72	115.82
$\sum L$	98.64	128.92	145.66	96.32	122.80	113.20	113.10
$\sum H - \sum L$	31.64	-28.92	62.40	36.28	-16.68	2.52	2.72
Effect	7.91	-7.23	-15.60	9.07	-4.17	0.63	0.68
Mean Square	125.14	104.55	-486.72	164.53	34.78	0.79	0.92
Mean Square for error = 0.8593							
F test	145.63	121.66	566.41	191.47	40.47	0.79	0.92
p value	0.0245	0.0275	0.0101	0.0205	0.0563	0.6243	0.5839

Significant at 95% level ($p < 0.05$)

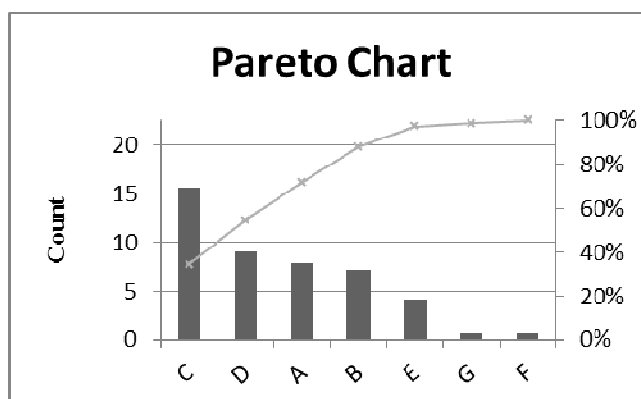
A: NaNO_3 , B: K_2HPO_4 , C: MgSO_4 , D: CaCl_2 , E: Na_2CO_3 , F: Citric Acid, G: EDTA.

Graph 1
Main effect plot for A – G⁺ variable responses



*A: NaNO_3 , B: K_2HPO_4 , C: MgSO_4 , D: CaCl_2 , E: Na_2CO_3 , F: Citric Acid, G: EDTA.

Graph 2
Pareto chart of standardized effects



2. Effect of nutrients and physiochemical parameters on scytonemin production

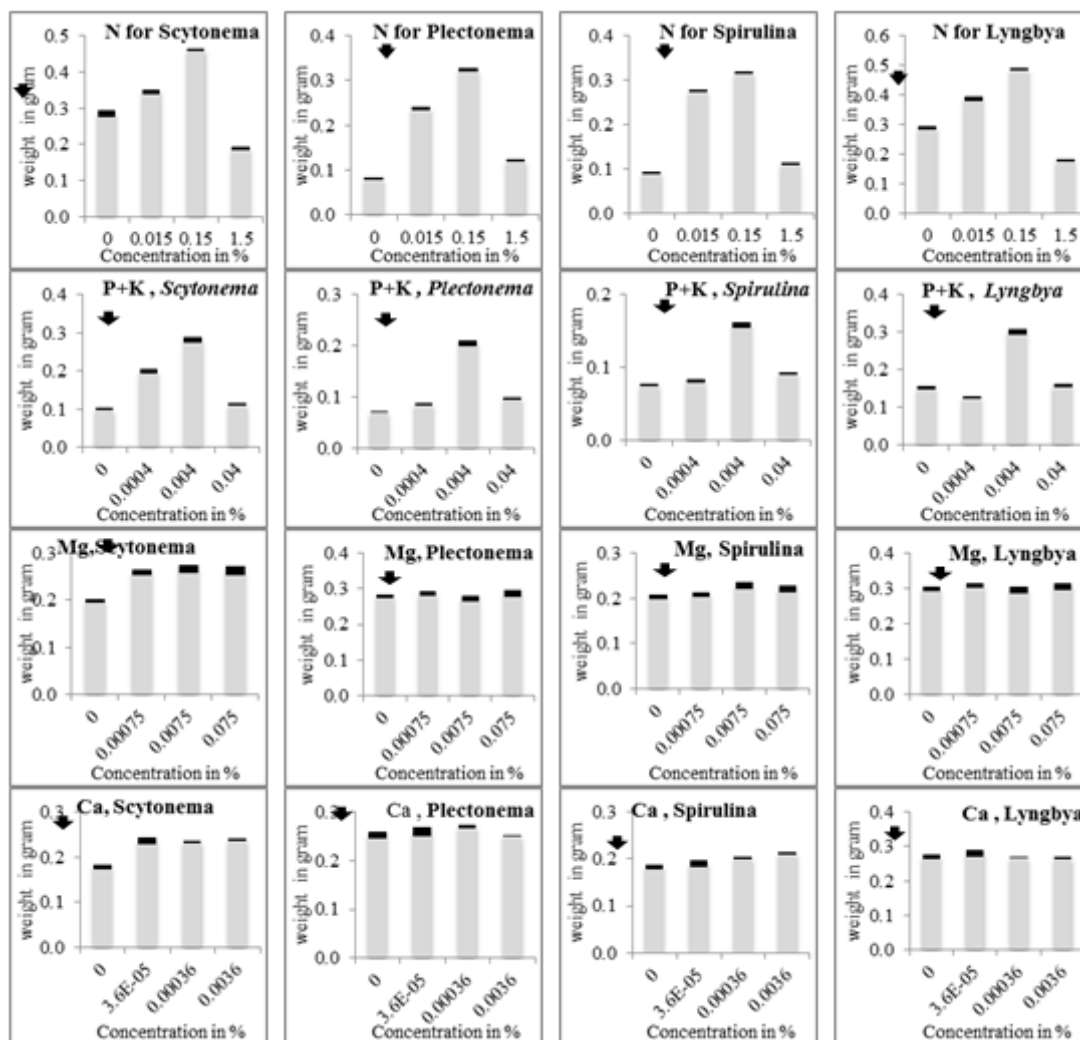
2.1 Study the effects of nutrients on scytonemin production

NaNO₃ concentration required for scytonemin yield varied among Nostocales i.e. 0% - 0.15%. Optimized concentrations of NaNO₃ required for scytonemin yield with more biomass is 0% for *Scytonema sp.*, 0.15% for *Plectonema sp.* and *Spirulina sp.* and 0.015% for *Lyngbya sp.* Although there is maximum growth of organisms at 0.15% of NaNO₃ concentration we select 0% for *Scytonema sp.* and 0.015% for *Lyngbya sp.*, as there is more production of

scytonemin at those respective concentrations. There is an optimum yield of scytonemin at 0.004% concentration of K₂HPO₄. MgSO₄ promote scytonemin production, optimized concentration is 0.075% for all 4 Nostocales, while CaCl₂ inhibit scytonemin production, and estimated concentration is 0.000036% for all 4 Nostocales. MgSO₄ at 10 times more concentration increases scytonemin yield by 2 folds. CaCl₂ presence decreases scytonemin yield, optimized concentration is 10 times less as that of original concentration of BG 11 medium (Graph 3).

Graph 3

Effect of NaNO₃, K₂HPO₄, MgSO₄ and CaCl₂ on scytonemin and biomass production



Effect of NaNO₃, K₂HPO₄, MgSO₄ and CaCl₂ on scytonemin and biomass production for all 4 isolated cyanobacteria. (Biomass production is indicated by grey bars while Scytonemin production is indicated by black bars above respective grey bars.)

2.2 Study the effects of physiochemical parameters on scytonemin production

For *Scytonema sp.* and *Spirulina sp.* optimum pH is 8 while for *Plectonema sp.* and *Lyngbya sp.* optimum pH is 7. The pH required for

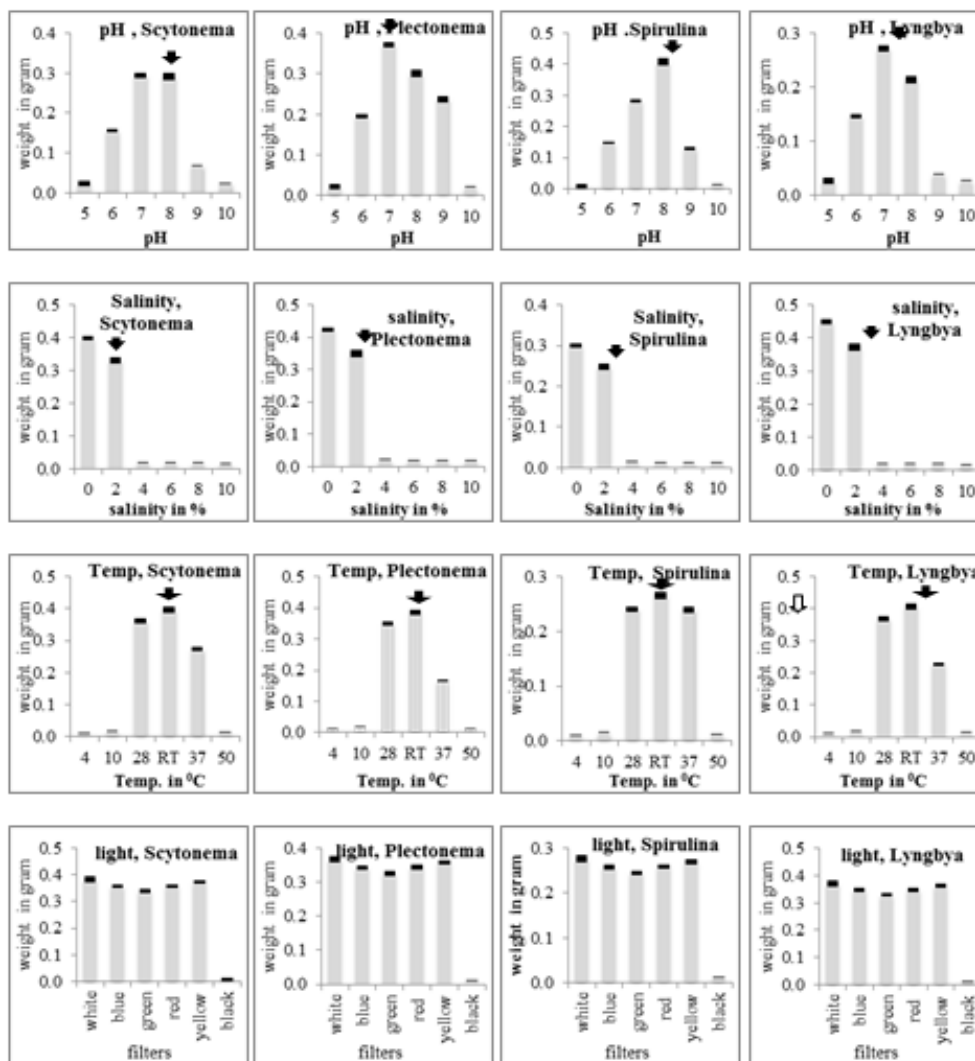
scytonemin production varies from 7 to 8. Although at pH 5, all Nostocales shows higher scytonemin production but they were not selected because of less biomass production. Optimum salinity is 2% for all 4 Nostocales.

Although biomass reduces at 2% salinity, scytonemin yield is higher as compared to 0% and other salinity. At room temperature (28 °C-30 °C) good yield of scytonemin is observed with higher biomass for all 4 Nostocales. There is no any significant effect of light filters on

biomass production but White light shows better scytonemin production as compare to others. Growth of Nostocales is inhibited in black filter and scytonemin production is very less for all 4 Nostocales (Graph 4).

Graph 4

Effect of pH, salinity, temperature and light filters on scytonemin and biomass production



Effect of pH, salinity, temperature and light filters on scytonemin and biomass production for all 4 isolated cyanobacteria. (Biomass production is indicated by grey bars while Scytonemin production is indicated by black bars above respective grey bars).

Table 4

Optimized media for large biomass production and scytonemin isolation

Components	BG 11 media	Scytonema sp.	Plectonema sp.	Spirulina sp.	Lyngbya sp.
NaNO ₃	0.15%	0%	0.15%		0.015%
K ₂ HPO ₄	0.004%				
MgSO ₄	0.0075%	0.075%			
CaCl ₂	0.00036%	0.000036%			
pH	7.5	7		8	
Salinity	2%				
Temperature	28 °C – 30 °C				
Light	White				

3 Biomass production for scytonemin

The optimized media (Table 4) was used for large biomass production, which was further used for scytonemin isolation. Biomass (fresh weight) obtained by small scale sea water open pond culture varies from 24 g – 35 g. Scytonemin isolated varies from 22 – 33 mg/g of fresh weight of biomass, about 2% to 3% that of collected biomass. Biomass and

scytonemin obtained by freshwater culturing (control) and sea water culturing (test) are similar, not showing any variations (Table 5). Our results suggest that sea water acts as a good media for cultivation of cyanobacteria, and hence for production of scytonemin in small scale. One needs to study the similar effects on a large scale for commercialization.

Table 5
Amount of Scytonemin isolated

	Scytonema sp.		Plectonema sp.		Spirulina sp.		Lyngbya sp.	
	A	B	A	B	A	B	A	B
Fresh weight in g	24.453	26.898	24.006	27.953	35.748	39.026	34.455	34.407
Dry weight in g	2.54	2.67	2.49	2.81	3.49	4.02	3.45	3.48
Scytonemin in mg/g of dry weight	30.11	31.16	21.90	21.99	32.51	33.2	22.29	23.01

Amount of Scytonemin isolated from 4 cyanobacteria.

A is respective control values (freshwater) and B is Test values (sea water)

CONCLUSION

Scytonemin is a UV screening secondary metabolite accumulated in extracellular sheath²⁶ of *Scytonema sp.*, *Plectonema sp.*, *Spirulina sp.*, and *Lyngbya sp.* whose biosynthesis are highly regulated by media components. BG 11 media components when evaluated by using Plackett- Burman design, it reveals that nitrate (as NaNO₃), phosphate + potassium (as K₂HPO₄), calcium (as CaCl₂) and Magnesium (as MgSO₄) has significant effect on scytonemin production. All these ions may act as a cofactors or inhibitors during scytonemin biosynthesis, which need to be study. Among all these, Calcium and Magnesium ions concentration has great impact on biomass production and scytonemin accumulation which shows similar results in all four isolated Nostocales namely *Scytonema sp.*, *Plectonema sp.*, *Spirulina sp.*, and *Lyngbya sp.* *ScyA* and *ScyC* both ThDP (thiamine diphosphate) dependent enzymes are required in the biosynthesis of indole alkaloid i.e. Scytonemin. Mg⁺² act as a cofactor for these enzymes. Which may be the reason for ten times more concentration of MgSO₄ than normal BG 11 medium is required to synthesize scytonemin in higher amount. As per our results, isolated all four freshwater Nostocales i.e. *Scytonema sp.*, *Plectonema*

sp., *Spirulina sp.*, and *Lyngbya sp.* are adapted to sea water. Small scale sea water culturing for isolated four Nostocales is successfully done in laboratory, but need to optimize on large scale for commercialization. Salinity acts as a major factor in adaptation of freshwater cyanobacteria to conform in the sea water environment. Possible reasons for adaptation to salinity are mentioned in the introductory part. To overcome problems related to freshwater culturing one can go for sea water culturing; only problem is to adapt cells for salinity. Use of sea water for cyanobacterial culture is of great importance as it is easily available in large quantity, economically affordable and also already having half of the media components required for cyanobacterial growth.²⁷ Further studies required to find out effects of adaptation and other sea water components effects of cells and scytonemin productions.

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