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Assessment of Factors Influencing Growth and C-Phycocyanin Production of *Arthrospira platensis* from Meteoritic Crater Lake

Mohite Y.S. and Wakte P.S.

Department of Microbiology,
Dnyanopasak Shikshan Mandal's College of Arts, Commerce and Science,
Parbhani (M.S) India.
E mail: prashantwakte@rediffmail.com; yuvrajmohite@gmail.com

Abstract

The *Arthrospira platensis* was isolated from the unique habitat of Lonar Crater Lake, world's only crater in the basalt rocks. The isolate was primarily enriched in CFTRI and Zarrouk's medium and then monoalgal culture was established by Single cell isolation technique. The important physicochemical parameters influencing the growth of the isolate were analyzed. The optimum temperature for the growth was recorded 28° C with production of 27.22 mg $l^{-1}day^{-1}$ of the cell mass showing 2.88 mg/ml of chlorophyll- a on twelfth day. The maximum growth was found at pH 9±0.1 with 37.23 mg $l^{-1}day^{-1}$ of cell mass indicating 2.35 mg/ml of chlorophyll- a with light intensity of 2.5 Klux. The study revealed considerable influence of magnesium salts on C-Phycocyanin concentration but negligible effect on growth. The cell mass grown with $MgCl_2$ showed maximum C-PC concentration up to 0.413 mg/ml and minimum with $MgCO_3$ 0.356 mg/ml and $MgNO_3$ 0.404 mg/ml respectively. The isolate exhibited the siderophore production by hydroxyl group chelation up to 14.6 % without any pretreatment ensuring, its possible use in agricultural industry. The optimized media for the isolate was formulated for the maximum growth as well as C-PC production.

Key Words: *Arthrospira platensis*, Lonar Crater Lake, Growth profile, C-phycocyanin, Siderophore.

Introduction

The Lonar Crater Lake is among the worlds few basaltic meteoritic craters. The Lonar Crater is emplaced in the Deccan traps centered at 19°59′ N and 76°31′ E in Buldhana district of Maharashtra, India. The average diameter of the crater is about 1830 m and depth is about 150m with a hypersaline lake occupying the crater (Fedrikson et al., 1973) (Osae et al., 2005). The lake water acts as extreme hypersaline environment as the pH of the water ranges from 9-14, so the organisms are alkalophilic and salt tolerant. The microbial biota of the lake water comprises variety of microorganisms but mainly microalgae and cyanobacteria such as *Arthrospira, Euglena, Oscillatoria, Chlorella, Eudorina,* etc (Chakaravarthy, 2009). One of the major reasons for such a variety of Microalgal flora is the lake water chemical composition. The chemical analysis of the lake water revealed the presence of carbonates, bi-carbonates, nitrates, magnesium sulphate, and various macro and micro elements which serves as growth promoters for the microalgae (Babar & Wakte, 2007;Reddy & Wakte, 2007). The *Arthrospira platensis* is the most investigated cyanobacteria in recent years by the researchers worldwide. It is one of the few cyanobacteria that have the longest history of being utilized as a part of human nutrition. *Arthrospira* platensis was the first cyanobacterium to be mass cultivated using modern biotechnology. The use of *Arthrospira* has expanded from its original application as human food and animal feed to the production of fine chemicals for clinical diagnosis, biological research and in cosmetic industry. Recent studies of the therapeutic and health effects of *Arthrospira* and its secondary metabolites are expected to promote the application of this organism in pharmaceutical and nutraceutical industries (Belay, 2002). It's

industrial and variety of biotechnological applications makes it, the organism of immense important. The valuable secondary metabolites such as C-phycocyanin, allophycocyanin, poly β hydroxybutarate are also synthesized by *Arthrospira platensis*. *Arthrospira platensis* is endowed with oxygenic photosynthesis. The major influencing factors on growth include light intensity, temperature, salinity and alkalinity (Richmond & Grobbelaar, 1986). Our earlier investigation reveals the potential effect of alterations in concentration of various nutritional elements such as $NaHCO_3$, K_2SO_4 etc. on growth and morphological variations of the *Arthrospira platensis*, morphological variants showed different growth rate under different nutritional conditions (Mohite & Wakte, 2008). Further optimization of influencing growth factor would be the key element in successful large scale monoalgal cultivation of *A. platensis* for C-PC production.

Materials and Methods

Isolation: For isolation of the *Arthrospira (Spirulina) species* the water samples were collected from the five different locations of the Lonar Crater Lake. The sample collected by using sterilized plastic containers were inoculated in the CFTRI (Venkantraman & Becker, 1985) and Zarrouk's (Zarrouk, 1966) medium for the enrichment purpose and kept under continuous illumination of 2500 flux at 27 ^{0} C with manual agitation twice a day for 5 minutes. The pH of the medium was kept 9±0.1; which preferentially inhibit the growth of the other microorganisms. The enriched culture was then utilized for the single cell isolation of the microorganism. The isolated culture was microscopically examined for morphological characters. The isolated culture was then cultivated and maintained in Zarrouk's medium and CFTRI medium for further study with 2500 flux and 12-12 hours cycle of light and dark phases. All the

experiments were carried in 500 ml Erlenmeyer Flasks with 200 ml volume of CFTRI medium.

Identification: Identification of the species of the *Arthrospira* isolate was done by 16S r-RNA sequencing carried out at NCCS laboratory, Pune and comparative analysis of the obtained sequence was done with the database of NCBI by performing the blast.

Experimental design: The culture of *A. platensis* in exponential growth phase was used for inoculation with 20% (v/v) inoculum size in 200 ml of the CFTRI medium in Erlenmeyer flasks (500 ml). The inoculated flasks were kept under growth conditions for 12 days. The mat formation was restricted by manual agitation thrice a day. The 20 ml media was used for each analytical test i.e. determination of dry weight, chlorophyll content and for C-PC concentration. The analysis was performed after 72 hrs of time interval.

Analytical Methods

Growth Measurements: Algal growth was spectrophotometrically measured as described by Payer (1971). The calculated mean of biomass (experiments in Triplicate) was used to obtain maximum specific growth rates (μmax) from the log phase of the growth curves by exponential regression. Productivities was calculated from the equation P= (Xi -X0) / ti, where P = productivity (mg L-1day-1), X0 = initial biomass density (mg L-1), Xi = biomass density at time i (mg L-1) and ti =time interval (h) between X0 and Xi (Colla et al., 2007).

Dry weight determination: Twenty ml from the different cultures were filtered using Whatman GF/C filter of 47 mm diameter in vacuum. The filtered cell mass was dried at 70° C for 30 mins (Rafiqul et al., 2005). The dried filter paper then kept in desiccators for 20 min for cooling and weighed.

Determination of Chlorophyll: One gram of *S. platensis* was homogenized in acetone (20 ml, 80%) and allowed to stand overnight in dark at 4°C for complete extraction followed by centrifugation at 10,000 xg for 5 min (El-Baky et. al, 2008). The contents of total chlorophyll (TChl), chlorophyll a (Chl-a) and chlorophyll b (Chl-b) in the supernatant were spectrophotometrically determined according to Vonshak and Richmond (1988) method.

Determination of C-PC: The content of C-Phycocyanin in the isolated *Arthrospira platensis* was determined by the method described by the *Boussiba* and *Richmond* (1979).

The analytical techniques were carried out up to 288 h (12^{th} day) at time interval of 72 h (3 days).The influence of factors such as pH, light intensity, inoculum size on growth of *A. platensis* isolate were studied. Analysis of C-PC production was performed on twelfth day for each influencing factor.

Siderophore Detection & Determination: The supernatant of cultures grown under iron limiting conditions was tested by the CAS assay as described by Schwyn & Neilands (1987). The assays for catechol or hydroxamate groups were performed as described by Arnow (1937), Csaky (1948) and Rioux et *al.* (1983).

Results and Discussion

Influence of pH: Cyanobacteria generally found in water bodies, so it's obvious that pH plays the important role in growth and pigment synthesis. Table No 1 illustrates the impact of pH on the growth of *A. platensis*. Current investigation reveals that the *A.platensis* isolated from extreme haloalkalophilic habitat of Lonar Crater Lake showed optimum growth at pH 9±0.1 with specific growth rate (μ max) of 0.093 & cell mass productivity reached up to 37.23

mg L^{-1} day^{-1} on 288 h (12^{th} day). It registered 2.58, 2.80 & 3.78 mg/ ml of Chl a, Chl b and total chlorophyll respectively confirming the alkalophilic nature of the isolate. The findings revealed that the most incremental period of the growth was between 216 h to 288 h. Among the various pH taken for study, the cyanobacterium registered least growth in terms cell mass productivity & chlorophyll content at pH 7. The productivity of cell mass was 34 mg L^{-1} day^{-1} with µ max 0.093, Chl a 2.27 mg/ml, Chl b 3.8 mg/ml & total Chl 2.1 mg/ml. The isolate exhibited specific growth rate of 0.086 with 36.23 mg L^{-1} day^{-1} of cell mass, the total chlorophyll concentration was found to be 2.45 mg/ml on twelfth day. The isolate was able to survive up to pH 12, but the growth was registered up to pH 11.5.

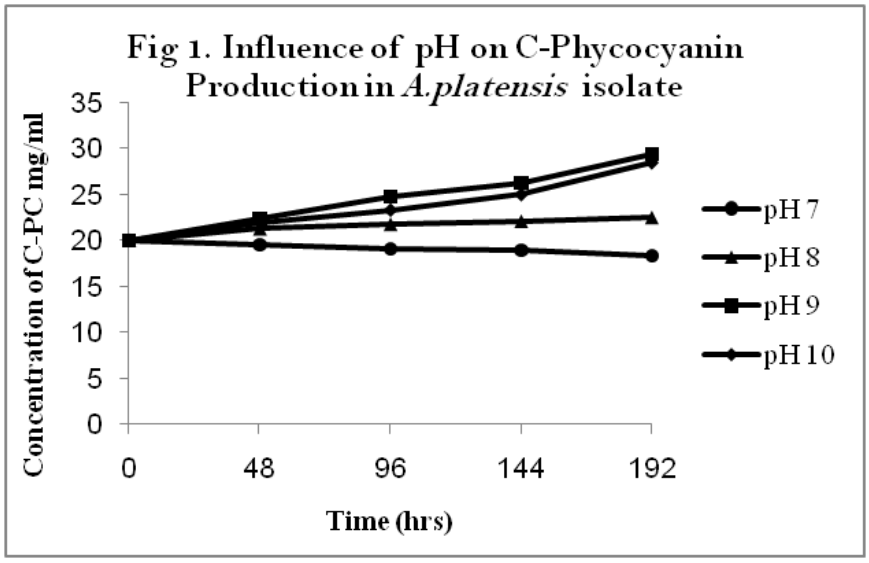
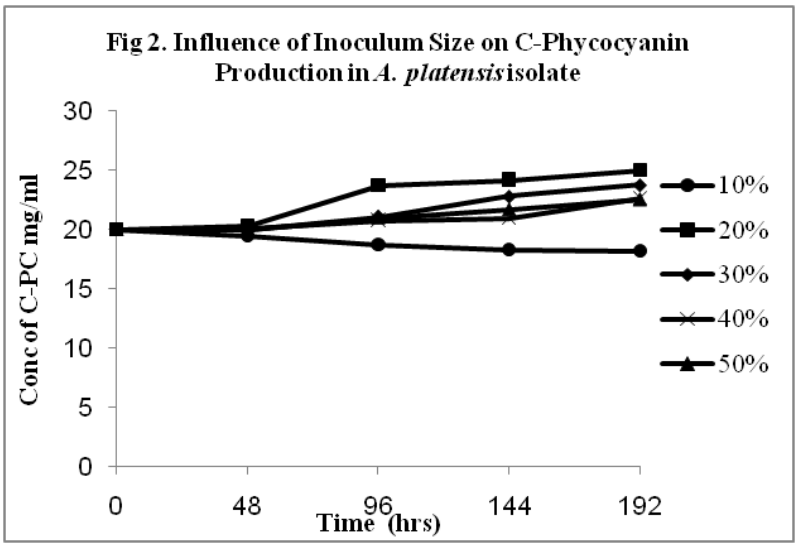


Figure 1 illustrates the influence of pH on C-PC production in *A. platensis*; pH of the medium has definite effect on the C-PC synthesis in the cyanobacterium. At pH 7 the C-PC concentration was found to be lowest 18.33 mg ml where as the optimum C-PC concentration 29.43 mg ml at pH 9±0.1.

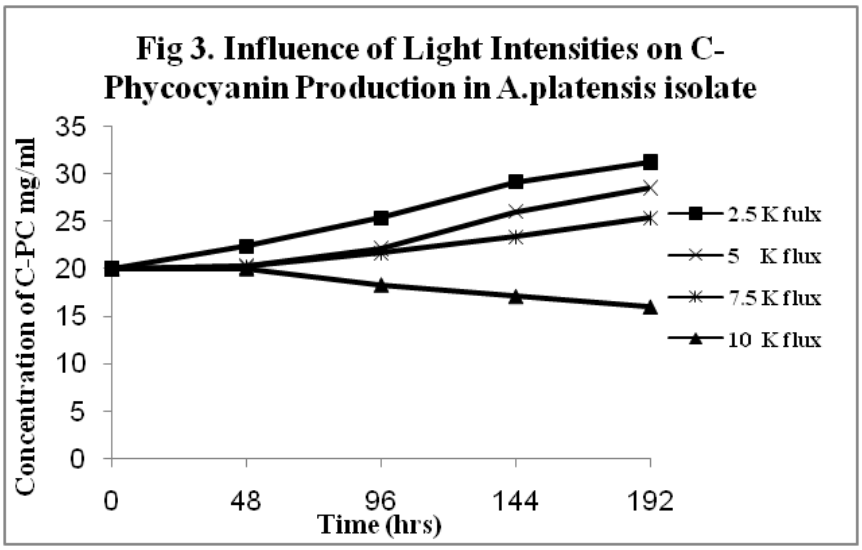
Influence of Inoculum Size: Inoculum size plays a key role in growth of the microalgae. Surely it depends on the size of the

bioreactor or pond in case of open harvesting of the *A.platensis***.** In the present investigation five different inoculum sizes for 200 ml of the growth medium were studied as depicted in Table No 2. The inoculum 20 mg (10% *v/v*) exhibited µ max 0.095 with 23.46 mg L^{-1} day^{-1} cell mass productivity showing concentration of Chl a 2.45 mg/ml, Chl b 2.51mg/ml and total chl. 2.63 mg/ml. In 40 mg (20% *v/v*) inoculum size the isolate recorded maximum growth & cell mass productivity, the µ max and cell mass production were 0.097 day^{-1} & 45.67 mg L^{-1} day^{-1} showing concentrations of Chl a 2.87 mg/ml, Chl b 2.76 mg/ml & 3.11 mg/ml of total Chl. respectively. In case of 100 mg (50% *v/v*) of the inoculum size increase in minimal growth was observed up to 216h (9 day) but at 288 h the decrease was registered. The specific growth rate (µ max) 0.068 with 101.83 mg L^{-1} day^{-1} cell mass production were recorded. The concentrations of Chl a Chl b and total Chl were found to be 1.62 mg/ml, 1.50 mg/ml & 1.70 mg/ml respectively. Figure 2 illustrates the impact of inoculum size on C-PC concentration of the isolate. The optimum C-PC concentration 25.00 mg ml^{-1} was recorded at 20% (*v/v*) inoculum size, the least concentration was registered by 10% inoculum 18.19 mg ml^{-1} and slight declination were recorded at 30%, 40% and 50% sizes of inoculum. We can interpret from the findings that 20% (*v/v*) inoculum size was found to be optimum for increased synthesis of C-PC in the isolate.



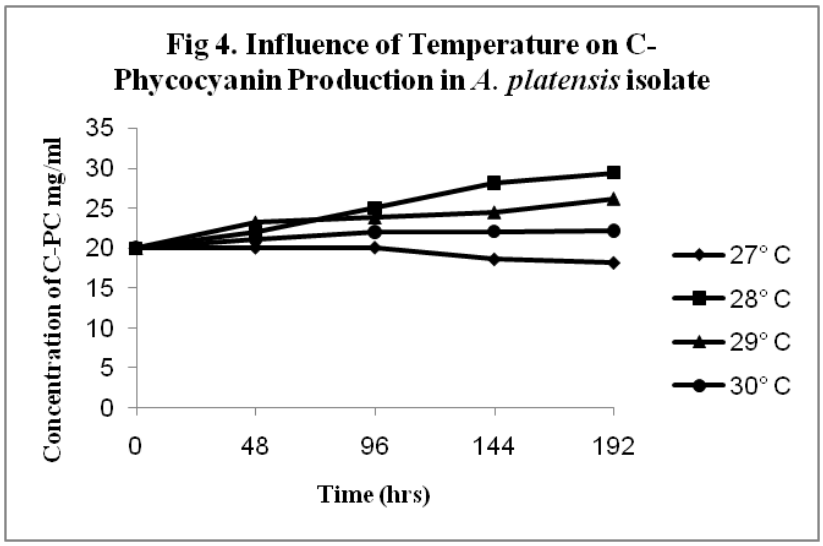
Influence of Light intensity: Table No. 3 depicts the influence of light intensities on growth of *A. platensis* isolate. The isolate showed the optimal growth at light intensity of 2500 flux with µ max 0.095, 30. 87 mg L^{-1} day^{-1} cell mass productivity, concentrations of Chl. a, Chl. b and total Chl were 3.24 mg/ml, 3.15 mg/ml & 3.86 mg /ml respectively. Light intensity of 5000 flux and 7500 flux showed growth rate 0.080 & 0.059 & 23.17, 24.87 mg L^{-1} day^{-1} cell mass productivity respectively indicating decline in growth. The maximum decrease in growth and total Chl. concentration was measured at 10000 flux light intensity. The growth rate 0.061 and cell mass productivity 20.40 mg L^{-1} day^{-1} with 1.16 mg/ml of total Chl. were recorded. Figure 3 reveals the influential behavior of light intensities on C-PC production; C-PC synthesis was found optimum at 2500 flux registering 31.22 mg ml^{-1}.

Declination was recorded as the light intensity was increased. The minimum C-PC concentration 16.00 mg ml^{-1} was recorded at 10000 flux.



Influence of Temperature: Temperature is vital factor for optimum growth of any microalgae. The isolate illustrated luxuriant growth at 28° C with growth rate 0.095 and 27.22 mg L^{-1} day^{-1} cell mass productivity. The concentrations of Chl. a, Chl. b & total Chl. were 2.88, 2.91 & 3.49 mg/ml were registered respectively. The isolate showed great sensitivity towards the temperature, at 29° C & 30° C the growth rates 0.085 & 0.084 were recorded with minimum increase in cell mass productivity 22.11 & 21.12 mg L^{-1} day^{-1} respectively. The Chlorophyll content on twelfth day at 29° C were 1.64 mg/ml Chl a, 1.70 mg/ml Chl b and total Chl 1.25 mg/ml were recorded. The concentration of chlorophylls, Chl a 2.79 mg/ml, Chl b 2.91 mg/ml & total Chl 1.56 mg/ml were recorded at 30° C respectively.

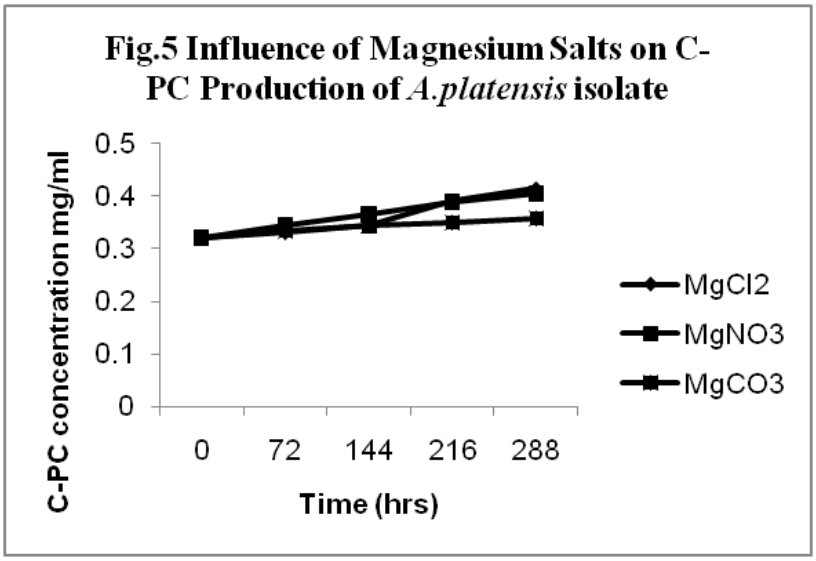
Figure 4 reveals the influential capacity of the temperature on C-PC production by *A. platensis* isolate. The maximum production was recorded at 28° C with 29.38 mg ml^{-1} of C-PC. Steep declination was recorded as the temperature increases.



Influence of Magnesium Salts: $MgSO_4$ is the important constituent of the nutrient media routinely utilized for the cultivation of *A. platensis*. In the present investigation the effect of $MgCl_2$, $MgNO_3$ and $MgCO_3$ on growth & C-PC production of the isolate was analyzed, the effect on growth of the isolate was negligible under the studied concentrations of magnesium salts exhibiting cell mass productivity up to 37.22 mg $l^{-1}day^{-1}$. The maximum C-PC concentration up to 0.413 mg/ml of buffer extract in presence of $MgCl_2$ (1 g l^{-1}) was recorded where as $MgNO_3$ registered 0.404 mg/ml and the least concentration was observed for $MgCO_3$ with 0.356 mg/ml on twelfth day (288 h) as depicted in figure 5.

Siderophore Production: The isolate exhibited the siderophore production potential revealing its possible use in agricultural industry. The isolate showed 14.6 % of hydroxy group chelation in iron limiting conditions. Response of cyanobacterium *Oscillatoria tenius* to low iron environments producing both hydroxymate type and catechol type siderophore have been earlier reported (Brown and Trick, 1992). Further investigation is expected to reveal more

detailed and interesting findings regarding the siderophore production by *A. platensis* isolate.



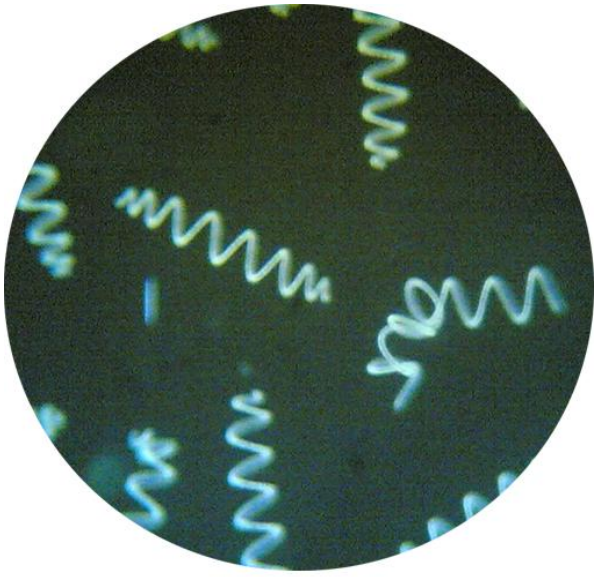


Image 1-Microscopic image of ***A. platensis*** isolate.

Table No. 1: Chlorophyll profile of *Arthrospira platensis* MWL- 01 at various pH with light intensity of 2500 flux.

pH	Time (Hours)	G. R μmax day^{-1}	CMP mg L^{-1} day^{-1}	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	TC (mg/ml)
	00	0.065	20.00	0.54	0.59	0.60
	72	0.077	23.89	0.58	0.59	0.72
7	144	0.084	28.23	0.90	0.11	1.0
	216	0.090	30.22	1.00	1.77	1.2
	288	0.093	34.00	2.27	3.8	2.1
	00	0.065	20.00	0.54	0.59	0.60
8	72	0.072	25.12	0.91	1.11	0.78
	144	0.077	31.29	1.15	2.04	1.2
	216	0.084	35.72	2.07	1.94	2.00
	288	0.086	36.23	2.65	2.35	2.45
	00	0.065	20.00	0.54	0.59	0.60
9	72	0.075	28.43	0.89	0.68	0.93
	144	0.078	35.35	1.78	1.74	1.55
	216	0.085	36.09	1.86	1.78	2.68
	288	0.093	37.23	2.35	2.80	3.78
	00	0.065	20.00	0.54	0.59	0.60
	72	0.073	24.43	0.69	0.65	0.87
10	144	0.078	34.55	1.13	1.44	1.75
	216	0.082	35.09	1.26	1.57	2.18
	288	0.083	36.21	1.28	1.84	2.38

Table No. 2: Effect of Inoculum size on Chlorophyll profile of *Arthrospira platensis*.

Inoculum size (v/v)	Time (Hours)	G R μmax day^{-1}	CMP mg L^{-1} day^{-1}	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	TC (mg/ml)
	00	0.058	20.00	0.43	0.49	0.51
	72	0.077	21.43	0.94	0.59	0.57
	144	0.086	22.12	2.24	1.2	1.8
10 %	216	0.095	22.87	1.01	1.80	1.9
	288	0.095	23.46	2.45	2.51	2.63
	00	0.068	40.00	0.43	0.49	0.51
	72	0.073	40.85	0.75	0.86	0.90
20 %	144	0.078	42.94	1.48	1.66	1.49
	216	0.085	43.88	2.05	1.92	2.15
	288	0.097	45.67	2.87	2.76	3.11
	00	0.060	60.00	0.43	0.49	0.51
	72	0.069	60.85	0.65	0.95	0.84
30 %	144	0.076	62.34	1.44	1.64	1.48
	216	0.088	63.88	2.35	2.22	2.15
	288	0.089	64.27	3.07	1.28	2.41
	00	0.060	80.00	0.43	0.49	0.51
	72	0.083	81.59	1.23	1.21	1.27
40 %	144	0.086	81.78	1.34	1.45	1.54
	216	0.087	82.11	1.95	1.87	1.98
	288	0.085	82.19	2.20	2.50	2.37
	00	0.067	100.00	0.43	0.49	0.51
50%	72	0.068	100.23	0.54	0.67	0.70
	144	0.073	101.48	1.10	1.06	1.22
	216	0.070	101.89	1.67	1.53	1.73
	288	0.068	101.83	1.62	1.50	1.70

Table No.3 Effect of Light intensities on Chlorophyll Profile of *Arthrospira platensis* isolate.

Light Intensity (Flux)	G R μ max day^{-1}	CMP mg L^{-1} day^{-1}	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	TC (mg/ml)
	0.068	20.00	0.45	0.43	0.51
	0.074	23.18	0.97	0.59	0.72
2500 flux	0.079	27.83	1.72	1.57	1.58
	0.082	29.77	2.60	2.41	2.65
	0.095	30.87	3.24	3.15	3.86
	0.065	20.00	0.45	0.43	0.51
	0.074	21.18	1.16	0.83	0.59
5000 flux	0.075	22.83	2.16	1.57	1.61
	0.079	22.87	2.88	3.11	3.21
	0.080	23.17	0.33	0.37	3.57
	0.067	20.00	0.45	0.43	0.51
	0.069	23.21	0.57	0.42	0.55
7500 flux	0.072	23.83	0.85	0.71	0.75
	0.067	24.77	1.22	1.25	1.18
	0.059	24.87	1.34	1.87	1.25
	0.065	20.00	0.45	0.43	0.51
	0.065	21.18	0.55	0.44	0.58
10000 flux	0.067	21.33	1.37	1.15	1.14
	0.065	21.37	1.69	1.60	1.24
	0.061	20.40	1.59	1.49	1.16

Table No.4 Effect of Temperature on Chlorophyll Profile of *Arthrospira platensis* MWL- 01

Temperature	G R μ max day^{-1}	CMP mg L^{-1} day^{-1}	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	TC (mg/ml)
	0.066	20.00	0.67	0.58	0.60
27° C	0.068	20.43	0.76	0.68	0.72
	0.073	21.32	1.42	1.07	1.08
	0.078	21.48	1.6	1.41	1.65
	0.085	23.22	1.24	1.15	1.77
	0.066	20.00	0.67	0.58	0.60
28°C	0.074	21.43	0.92	0.87	0.95
	0.079	23.32	2.16	2.43	2.59
	0.085	25.48	2.36	2.57	2.81
	0.095	27.22	2.88	2.91	3.49

	0.066	20.00	0.67	0.58	0.60
	0.072	20.73	0.85	0.71	0.85
29°C	0.077	21.37	0.96	0.85	0.64
	0.081	21.68	1.12	0.89	1.00
	0.085	22.11	1.64	1.70	1.25
	0.066	20.00	0.67	0.58	0.60
	0.079	20.13	2.67	1.45	1.14
30°C	0.078	20.42	2.69	1.60	1.28
	0.082	20.88	2.71	1.70	1.35
	0.084	21.12	2.79	2.91	1.56

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