International Journal of Multidisciplinary Research and Development

International Journal of Multidisciplinary Research and Development

Volume: 2, Issue: 4, 215-220
April 2015
www.allsubjectjournal.com
e-ISSN: 2349-4182
p-ISSN: 2349-5979
Impact Factor: 3.762

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Growth pattern and Heterocyst frequency assessment of dizotrophic Cyanobacterium – *Anabaena doliolum* treated with Paper mill effluent.

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Abstract
Paper mill effluents are of large scale environmental concern because they diminish the quality of water bodies into which they are released. Their disposal into the environment creates adverse effects by altering the normal physiochemical properties of soil and water. Though many conventional physicochemical methods are currently being practiced, biotechnological methods are becoming attractive alternatives, as they are economical and eco-friendly. During present investigation the physic-chemical properties of paper mill effluent sample was analyzed and dizotrophic cyanobacterial strains were isolated from such effluent flooded fields, simultaneously the growth behavior and heterocyst frequency of *Anabaena doliolum* treated with graded concentrations of paper mill effluent was assessed and observed result of growth experiments was found very significant. Findings has been revealed that graded effluents at lower concentration promotes the Cyanobacterial population especially nitrogen fixers and finally enhance the agriculture production by increasing the soil fertility. The present investigation has also provided a cue of further investigation, which would confer that such dizotrophic Cyanobacteria can serve as the potential bio-remedial organism for industrial pollution.

Keywords: Paper mill effluent, dizotrophic micro-organism, Cyanobacteria, industrial pollution, *Anabaena doliolum*

1. Introduction
Due to increasing industrialization on one hand and exploding population on the other, the demands of water supply have been increasing tremendously. The quality of land water is being deteriorated by mixing up of industrial wastes and domestic sewage in our rivers [1]. Especially in urban areas, the careless disposal of industrial effluents and other wastes contributes greatly to the contamination of the water [2]. Increased pollution load in fresh water bodies increases the nutrient level of water [3] and causes a violent alteration in pH, reduction in oxygen content and high osmotic pressure.

As autotrophic and/or dizotrophic prokaryotes, Cyanobacteria are common inhabitants of water logged area throughout the world and very significant due to nitrogen fixing ability of its heterocystous forms in nature. However certain non-heterocystous forms of Cyanobacteria can dominate phytoplankton with in water reservoirs and also produce potent toxins. Cyanobacteria play spectrum of remarkable roles in the field of energy production, bio-fertilizer, human food, animal feed, polysaccharides, biochemical and pharmaceuticals and in cleaning up of the environment, etc. Cyanobacteria have both beneficial and detrimental properties when judged from a human prospective. They are also the source for substances of pharmaceutical interest such as antibiotics [4], [5], [6]. The properties that make the Cyanobacteria generally undesirable are also the qualifications for possible positive economic use. Blue-greens are the source of many valuable products [4] and carry promising physiological processes, including light-induced hydrogen evolution by bio-photolysis [5].

Cyanobacteria are capable of abating various kinds of pollutants and have advantages as potential biodegradation organism [7]. These organisms degrade various aromatic hydrocarbons and are useful for metal removal from polluted water. As these organisms have simple growth requirements, they could be attractive host for production of valuable organic products and are also known to produce a diverse array of toxic or other bioactive metabolites [8], [9], [10], [11]. Looking to the fossil record of nearly 3.5 billion years ago, these Cyanobacteria are known to produce a large number of biologically active compounds specially those of nitrogen-rich alkaloids and peptides [12].

2. Materials and Methods

Effluent sample was collected from Madhya Bharat Paper Industries Limited situated at Village Birgahani of Janjgir (Champa) district, Chhattisgarh in plastic jar, prior to the collection the samples jar were rinsed thoroughly with the sample. Temperature and pH of the samples were recorded immediately and then carried to the laboratory carefully. For analyzing the chemical parameter like Turbidity, Conductivity, Total alkalinity, Total acidity, DO, BOD,COD, chloride, Total Hardness, Calcium, Magnesium, Nitrate Nitrogen, Nitrite nitrogen, Total Phosphate, Total dissolved solids and Total suspended solids, Standard methods [13] were carried out.

2.1 Isolation of Cyanobacteria –

A number of N_2-fixing and non N_2-fixing Cyanobacterial forms were collected from the effluent flooded area that identified and isolated in the form of pure culture. Morphological observations of isolated Cyanobacterial forms were made through compound light microscope and Micro Imaging Photography system (MIPS), Model No. ML-TR (Olympus). Based on characterization, systematic identification of the Cyanobacterial species were performed using morphological variation studies and taxonomical approaches according to [14], [15]. Isolation of axenic culture of marked strains was raised by alternatively inoculating between solid and liquid media (BG-110 and Chu-10) employing standard procedure adopted after [16], [17].

2.2 Selection of test organisms-

Selection of experimental material was done from amongst the N_2-fixing forms. For this all the N_2-fixing Cyanobacterial species were critically examined for their relative efficiency to from - (i) discrete and conspicuous colonies on agar medium for facilitating to produce clonally population (ii) Homogeneous culture suspension in liquid medium for facilitation the assessment of their growth kinetics, heterocyst frequency.

The trichomous, heterocystous and N_2-fixing Cyanobactrial strain - *Anabaena doliolum* collected from paddy fields during the present course of investigation was found to perform best according to the criteria, as mentioned earlier for the selection of test material and hence the same was used during the entire course of the present investigation for assessing the effects of the effluents.

2.3 Effluent treatment –

Thirty minutes treatments period was given on the test organism and 25%, 50%, 75% and 100% gradation effluent were taken, using sterilized liquid nutrient medium. Each bioassay was consisted of treatment four effluent levels and one control culture.

The experimental stock culture of *Anabaena doliolum* was maintained on the 5mM NO_3- growth non-heterocystous culture, so that the same may be harvested and subjected to a 30 min effluent treatment for evaluating whether effluents interfere with the normal heterocyst differentiating mechanism of the organism on transfer to a fresh N_2 (n-free, i.e., at the cost of molecular nitrogen) medium.

The Cyanobacterial sample treated with the different graded concentrations were transferred to fresh liquid N_2, 5mM KNO_3, 5mM KNO_2 or 1mM NH_4CL containing medium with or without a carbon source i.e., 3mM glucose. The growth and heterocyst forming activity of the control (untreated) and the variously treated samples of the organism were accessed every alternate day till the completion of 10 days of inoculation.

2.4 Growth Measurement –

In view of the homogeneous culture suspension formed by the test organism in liquid medium the direct optical density readings for acetone soluble chlorophyll pigments. A systronic colorimeter with red filter at 663 nm was employed for the measurement of optical density (O.D.).

2.5 Heterocyst frequency –

Heterocyst frequency was determined in terms of the average number of heterocyst per hundred vegetative cells 12 randomly assorted Cyanobacterial trichomes, through microscopic examination. In case of massive fragmentation, heterocyst frequency was determined as a number of heterocyst per hundred vegetative cells under a microscopic field.

3. Results and Discussion:

The quality and the concentrations of potentially toxic elements in effluent are summarized in Table -1. The influence of paper mill effluent which was found to be slightly alkaline in nature has been evaluated on the growth pattern, as relation to heterocyst frequency.

Observation against the effect of effluent on the growth and heterocyst frequency of isolated organism (WT) – *Anabaena doliolum* has been mentioned in tables (2 & 3) and graphically present in figures (1 to 5).

Data on growth of heterocystous& filamentous isolate – *Anabaena doliolum* growing in N_2, NO_3^-, NO_2^-& NH_4^+ supplemented media at different concentration (25%, 50%, 75% & 100%) of the paper mill effluent (N) un-supplemented and un-supplemented with 5mM glucose are incorporated in TABLE – 2 and 3 respectively and growth pattern is graphically presented in figures – 1 to 4. Apparently, the 25% and 50% effluent treated samples exhibited a lag – phase of two days after and samples have shown an usual better growth pattern in N_2, NO_3^- and NH_4^+ growth media. The growth yield of these treated samples in all the four nitrogen media was significantly higher when compared to their untreated counts parts within the period of ten days. It is import to point out here that the 75% & 100% effluents treated samples have shown a sharply lower growth yield than that of other three samples and after 4 days went down ultimately leading to the death of the culture.

Supplementation of growth medium with an exogenous carbon i.e., glucose could not protect the organism against the effluents induced growth inhibition a lethality. Growth with the exogenous source was not doubt marginally higher in all inorganic nitrogen media, but the pattern of effluents induced growth inhibition could not at all be reversed with this agent.

Data on heterocyst frequency of *Anabaena doliolum* has been computed in TABLE – 3 and its respective histogram presented in figure – 5.

Table 1: Mean values of Different parameters of effluent of Orion Paper mill.

Parameters	Mean values
Temperature (°C)	33.75 ± 5.40
pH	8.0 ± 0.27
Turbidity (NTU)	28.39 ± 4.49
Electric Conductivity (dSm^{-1})	1.12 ± 0.13
Total Alkalinity (mg L^{-1})	257.8 ± 98.32
Total Acidity (mg L^{-1})	24.6 ± 9.47
DO (mg L^{-1})	1.00 ± 0.58
BOD (mg L^{-1})	310.28 ± 46.75
COD (mg L^{-1})	572.6 ± 99.32
Chloride (mg L^{-1})	461.6 ± 32.96
Total Hardness (mg L^{-1})	280.8 ± 42.39
Calcium (mg L^{-1})	102.8 ± 24.75
Magnesium (mg L^{-1})	43.1 ± 5.91
Nitrate Nitrogen (mg L^{-1})	67.8 ± 8.27
Nitrite Nitrogen (mg L^{-1})	58.0 ± 8.45
Phosphate (mg L^{-1})	35.7 ± 3.54
TDS (mg L^{-1})	2525.8 ±422.38
TSS (mg L^{-1})	784.2 ± 140.12

The effluents of Paper mill industry apparently interface with the timely appearance of heterocyst along the Cyanobacterial filament in N_2 medium although the growth of the samples was promoted at lower concentration and inhibited in proportion to the high concentration or absolute used during the treatment phase as noted above of course, in culture treated with lower concentration shown the heterocyst suppression shown the heterocyst suppression. TABLE – 3 present the data on the heterocyst frequency of test (wild type) organism (*Anabaena doliolum*) untreated or treated (for 30 min.) with graded concentration (25%, 50%, 75% & 100%) of paper mill effluent.

4. Conclusion:

Findings of the present study reveals that the paper mill effluent causes water pollution especially in adjoining fields that affect to agricultural practices. By inhibiting the growth of natural bio-fertilizers like Cyanobacterial flora, warmly affect to the soil economy and ultimately agriculture production, however graded effluents at lower concentration promotes the Cyanobacterial population especially nitrogen fixers and finally enhance the agriculture production by increasing the soil fertility. In the present study all the effluents showed considerable amounts of nitrates and phosphates, with increased level of BOD and COD along with very low DO level. This could be reason for the flourishing growth of Cyanobacteria in the effluent investigated.

5. Acknowledge:

Author is grateful to the Principal, Govt. E. Raghavendra Rao Postgraduate Science College, Bilaspur (C.G.) for providing laboratory facilities and encouragement.

Table 2: Growth of WT organism (*Anabaena doliolum*) untreated and treated * with graded concentrations (25%, 50%, 75% and 100%) of the Paper mill effluent in N_2, NO_3^-, NO_2^- and NH_4^+ media, unsupplemented or supplemented with 5mM glucose.

Period (Days)	Gradation of Effluent (Paper mill)	Growth (Optical Density at 663 nm)							
		N_2		NO_3		NO_2		NH_4^+	
		NIL	(+) Glucose	NIL	(+) Glucose	NIL	(+) Glucose	NIL	(+) Glucose
0	Initial OD	0.14 ± 0.022	0.14 ± 0.020	0.14 ± 0.015	0.14 ± 0.016	0.14 ± 0.016	0.14 ± 0.022	0.14 ± 0.022	0.14 ± 0.020
2	0	0.181 ± 0.012	0.211 ± 0.015	0.195 ± 0.020	0.214 ± 0.014	0.211 ± 0.014	0.217 ± 0.015	0.212 ± 0.021	0.218 ± 0.012
	25	0.192 ± 0.014	0.218 ± 0.022	0.198 ± 0.015	0.221 ± 0.016	0.226 ± 0.020	0.232 ± 0.016	0.225 ± 0.014	0.234 ± 0.015
	50	0.196 ± 0.015	0.215 ± 0.016	0.201 ± 0.021	0.224 ± 0.021	0.228 ± 0.015	0.235 ± 0.012	0.227 ± 0.014	0.236 ± 0.018
	75	0.156 ± 0.022	0.162 ± 0.014	0.161 ± 0.016	0.172 ± 0.014	0.164 ± 0.022	0.175 ± 0.014	0.176 ± 0.017	0.184 ± 0.012
	100	0.117 ± 0.014	0.212 ± 0.016	0.119 ± 0.017	0.127 ± 0.016	0.121 ± 0.018	0.132 ± 0.017	0.121 ± 0.016	0.13 ± 0.014
4	0	0.298 ± 0.021	0.336 ± 0.015	0.316 ± 0.022	0.372 ± 0.014	0.332 ± 0.012	0.386 ± 0.020	0.318 ± 0.012	0.364 ± 0.018
	25	0.316 ± 0.016	0.358 ± 0.014	0.328 ± 0.016	0.398 ± 0.015	0.352 ± 0.014	0.408 ± 0.022	0.334 ± 0.017	0.381 ± 0.012
	50	0.324 ± 0.014	0.384 ± 0.020	0.351 ± 0.017	0.452 ± 0.014	0.368 ± 0.016	0.436 ± 0.017	0.342 ± 0.015	0.405 ± 0.015
	75	0.103 ± 0.016	0.142 ± 0.020	0.118 ± 0.012	0.156 ± 0.020	0.123 ± 0.022	0.165 ± 0.012	0.131 ± 0.017	0.177 ± 0.018
	100	0.074 ± 0.020	0.097 ± 0.014	0.079 ± 0.017	0.108 ± 0.012	0.083 ± 0.017	0.114 ± 0.017	0.081 ± 0.019	0.112 ± 0.014
6	0	0.428 ± 0.014	0.475 ± 0.012	0.457 ± 0.019	0.488 ± 0.014	0.483 ± 0.012	0.522 ± 0.016	0.488 ± 0.022	0.531 ± 0.016
	25	0.431 ± 0.012	0.497 ± 0.022	0.468 ± 0.012	0.511 ± 0.019	0.495 ± 0.022	0.538 ± 0.012	0.592 ± 0.012	0.551 ± 0.022
	50	0.452 ± 0.012	0.516 ± 0.019	0.412 ± 0.012	0.515 ± 0.019	0.475 ± 0.019	0.516 ± 0.022	0.498 ± 0.019	0.547 ± 0.019
	75	0.071 ± 0.017	0.095 ± 0.017	0.068 ± 0.019	0.082 ± 0.022	0.076 ± 0.017	0.089 ± 0.012	0.074 ± 0.012	0.083 ± 0.019
	100	0.026 ± 0.019	0.038 ± 0.012	0.028 ± 0.012	0.033 ± 0.019	0.031 ± 0.019	0.036 ± 0.020	0.029 ± 0.019	0.033 ± 0.012
8	0	0.516 ± 0.020	0.578 ± 0.022	0.531 ± 0.017	0.591 ± 0.020	0.544 ± 0.019	0.617 ± 0.019	0.548 ± 0.022	0.624 ± 0.019
	25	0.539 ± 0.012	0.596 ± 0.019	0.557 ± 0.018	0.614 ± 0.022	0.576 ± 0.017	0.627 ± 0.019	0.579 ± 0.015	0.633 ± 0.012
	50	0.561 ± 0.017	0.598 ± 0.012	0.582 ± 0.022	0.632 ± 0.019	0.591 ± 0.015	0.658 ± 0.022	0.596 ± 0.019	0.667 ± 0.014

	75	0.063 ± 0.018	0.079 ± 0.019	0.061 ± 0.015	0.084 ± 0.015	0.063 ± 0.019	0.089 ± 0.014	0.067 ± 0.017	0.093 ± 0.022
	100	0.014 ± 0.014	0.028 ± 0.018	0.011 ± 0.022	0.022 ± 0.014	0.012 ± 0.020	0.023 ± 0.014	0.014 ± 0.019	0.026 ± 0.019
10	0	0.541 ± 0.014	0.613 ± 0.020	0.565 ± 0.014	0.651 ± 0.019	0.574 ± 0.022	0.666 ± 0.014	0.578 ± 0.017	0.671 ± 0.017
	25	0.569 ± 0.020	0.624 ± 0.014	0.587 ± 0.014	0.673 ± 0.015	0.592 ± 0.019	0.705 ± 0.014	0.597 ± 0.022	0.691 ± 0.014
	50	0.585 ± 0.015	0.597 ± 0.017	0.598 ± 0.015	0.676 ± 0.019	0.605 ± 0.015	0.694 ± 0.017	0.611 ± 0.017	0.698 ± 0.019
	75	0.016 ± 0.014	0.037 ± 0.015	0.018 ± 0.017	0.048 ± 0.017	0.019 ± 0.018	0.045 ± 0.014	0.021 ± 0.015	0.047 ± 0.022
	100	0.008 ± 0.017	0.011 ± 0.014	0.008 ± 0.017	0.012 ± 0.015	0.009 ± 0.017	0.014 ± 0.017	0.008 ± 0.017	0.014 ± 0.018

*** Treatment time was 30 minutes.**

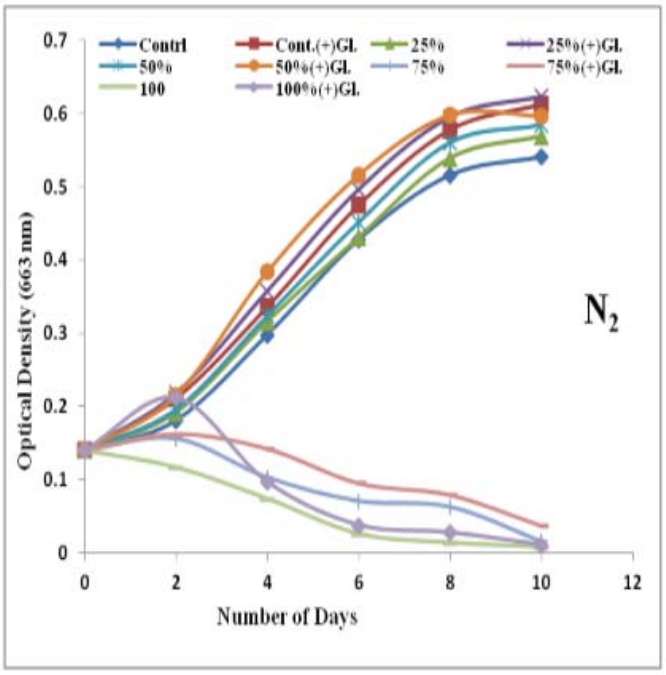

Fig.: 1

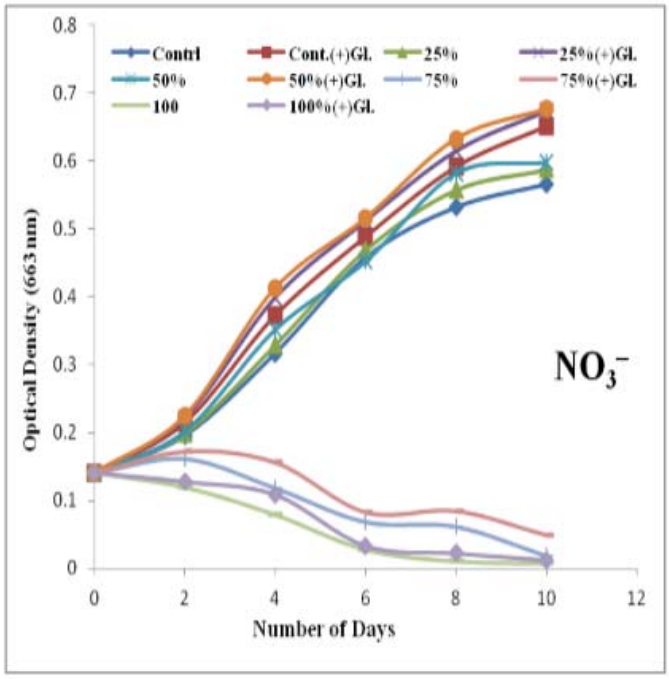

Fig.: 2

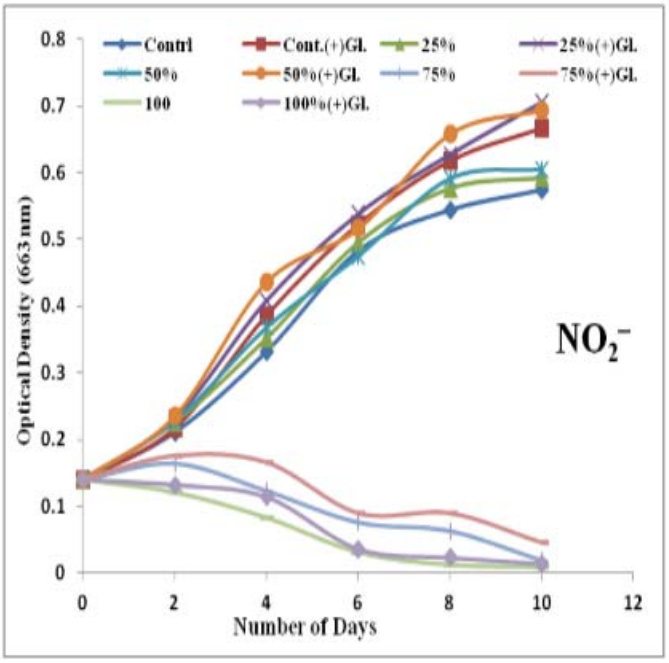

Fig.: 3

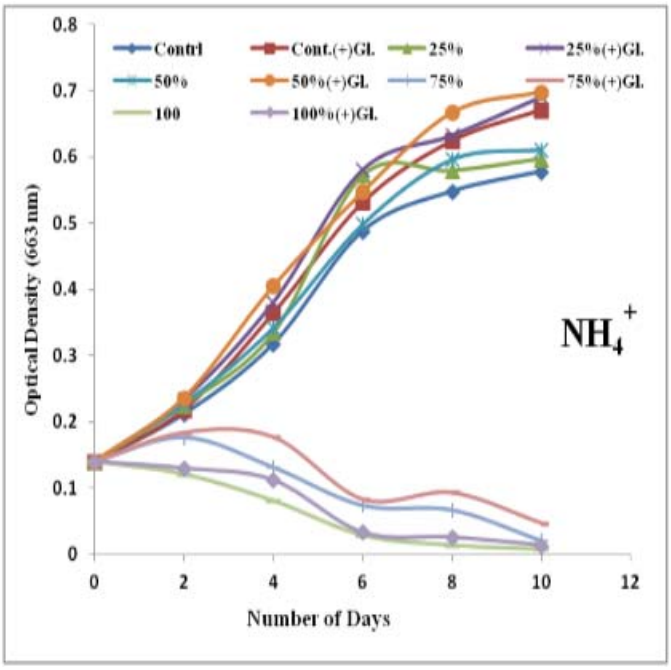

Fig.: 4

Fig : 1-4 Growth Pattern of *Anabaena doliolum* untreated and treated * with graded concentrations (25%, 50%, 75% and 100%) of the Paper mill effluent in N_2, NO_3^-, NO_2^- and NH_4^+ media, unsupplemented or supplemented with 5mM glucose.

Table 3: Heterocyst Frequency * of WT organisms (*Anabaena doliolum*) untreated or treated ** with graded concentration of the Paper mill effluents in niterogen (N_2) medium unsupplemented or supplemented with 3mM Glucose. (on Zero days frequency was zero, since the source of inoculate was 5mM NO_3 growth het-cultures).

Period (Days)	Supplements	Untreated	Paper Mill effluents			
			25%	50%	75%	100%
2	Nil	5.44 ±0.14	4.36 ±0.17	3.92 ±0.18	2.43 ±0.12	Fragmentation
	(+) Glucose	5.82 ±0.17	4.42 ±0.16	3.98 ±0.14	2.61 ±0.17	Do
4	Nil	5.67 ±0.21	4.63 ±0.15	4.05 ±0.14	2.61 ±0.17	Fragmentation & Lysis
	(+) Glucose	6.1 ±0.14	4.84 ±0.18	4.16 ±0.12	2.72 ±0.21	Do
6	Nil	5.88 ±0.12	4.83 ±0.14	4.32 ±0.17	2.68 ±0.12	Fragmentation & Lysis
	(+) Glucose	6.28 ±0.15	4.92 ±0.16	4.43 ±0.21	2.7 ±0.18	Do
8	Nil	5.82 ±0.18	4.71 ±0.12	4.58 ±0.15	2.14 ±0.17	Cellular Lysis
	(+) Glucose	6.12 ±0.14	4.88 ±0.12	4.66 ±0.14	2.32 ±0.12	Do
10	Nil	5.75 ±0.12	4.98 ±0.17	4.71 ±0.18	1.82 ±0.21	More Lysis
	(+) Glucose	6.08 ±0.21	5.08 ±0.16	4.84 ±0.12	1.96 ±0.14	Do

* Number of heterocyst per hundred vegetative cells; heterocyst frequency was not determined for the fragmented, lytic or chlorotic or dead cultures as it was not possible to have a correct assessment.

**** Treatment time- 30 minutes.**

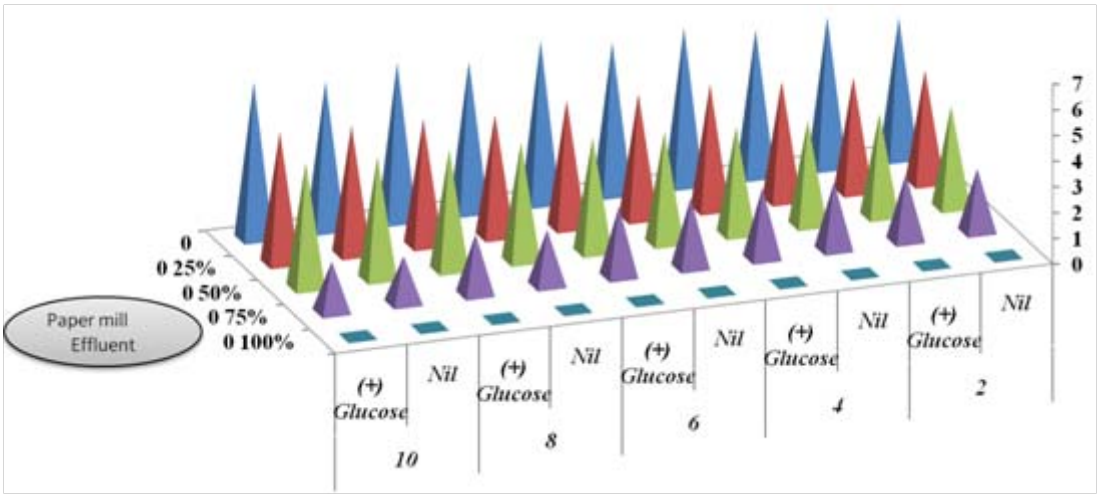

Fig.: 5 - Heterocyst Frequency of *Anabaena doliolum*

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