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Biochemical evaluation of some cyanobacterial strains isolated from the lime sludge wastes of a Paper Mill in Southern Assam (India)

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Abstract

Disposal of wide range of industrial wastes in the environment has been a major cause of concern. Cyanobacteria are conjectured to be important primary colonizer on the deposited spoil materials. The present work was undertaken with an aim to analyze some isolates from the unexplored area of lime sludge waste dumps generated by a paper mill at Panchgram, situated in the Hailakandi district of Southern Assam. Five cyanobacterial species of the genus *Phormidium*, *Lyngbya*, *Anabaena*, *Calothrix* and *Fischerella* were isolated from the lime sludge waste. Growth kinetics and different biochemical constituents were evaluated in terms of total carbohydrate, soluble protein, chlorophyll *a*, carotenoid and phycobiliprotein contents. The isolates *Lyngbya holdenii* and *Anabaena doliolum* showed higher accumulation of chlorophyll *a*, phycocyanin, carbohydrates and protein than the rest.

Key Words: Biochemical, Cyanobacteria, Lime sludge wastes, Paper Mill, Southern Assam

Introduction

A significant component of many different ecosystems, cyanobacteria occupy almost every niche of the earth, including fresh and salt waters, rice fields, hot springs, arid deserts, and polar regions. Cyanobacteria are considered as most diverse and ubiquitous organisms occupying almost all ecological niches (Wilkie *et al.*, 2011). Owing to high genome plasticity they can withstand extreme environmental stressed conditions fairly well (Tiwari *et al.*, 2005). Disposal of wide range of industrial wastes in the environment is a major concern now-a-days (Suriyanarayanan *et al.*, 2010). Pertinent here is to mention that the pulp and paper industry is branded as one of the largest and major polluter in the world (Kumar *et al.,* 2004). This industry is categorized as one of the twelve most polluting industries in India (Singh and Thakur, 2004). Lime sludge waste is one of the largest solid by-products generated by the kraft paper mill recovery unit (Gaskin, 2004) and constitutes a major source of soil pollution (Battaglia *et al.*, 2003; Geng *et al.*, 2006). This is generated as a byproduct during the causticizing process where calcined lime is used for regeneration of caustic soda by conversion of soda ash (Backwell, 1987; Dorris, 1985). Analysis and alternative utilization of lime sludge wastes from a paper mill at Nagaon (Assam) has been documented (Deka and Yasmin, 2006). Cyanobacteria play a crucial role as modifiers of the degraded ecosystems including various industrial solid waste dumps (Schwabe *et al.*, 1971) through reclamanation of the waste substratum by binding large amount of metals (Metting *et al.*, 1981) and thus helps in vegetational succession. The bioactive compounds produced by algae enhance the physical and chemical properties of soil and thus affect the other components of soil biota (Schwabe *et al.*, 1971). This apart, cyanobacteria are recognized to be prolific producers of bioactive compounds drawing interests as a source of various nutraceuticals, biomass and pigments (Cifferi *et al.*, 1985; Pulz *et al.*, 2004). Protein rich cyanobacteria like *Anabaena, Nostoc* and *Spirulina* are widely used as health food for human beings and also as animal nutritional supplements (Spolaore *et al.*, 2006). The cyanobacterium *Phormidium valderianum,* for instance, has been recently shown to be rich in phycocyanin content (Singh *et al.*, 2012). Annually more than 3000 tons of dry weight of *Spirulina platensis* is produced worldwide for extracting phycobiliproteins (Spolaore *et al.*, 2006). Cyanobacteria also play a potential role in metal removal from the disturbed soil by acting as chelating agent (Misra and Kaushik,1989). Worthwhile to note in this regard that studies on biochemical evaluation of soil cyanobacteria growing on paper mill lime sludge wastes with remarkably high soil pH are scanty. Accordingly it was deemed fit to undertake the present study to assess the occurrence and biochemical contents of some cyanobacteria isolated from such highly alkaline waste.

Materials and methods

The samples were collected from the crusts of lime sludge dump spread over about 1 sq. Km from near the Paper Mill, located at Panchgram in Hailakandi district of Southern Assam. Geographic details of the study sites were recorded using GPS (Germin eterex). The study area (Fig.1) is located at the longitude of 92° 36´ E and the latitude 24° 52/ N at an elevation of 68 feet. A temperature in the range of 41.6˚C to 27˚C, relative humidity of 93% (max) and 64% (min) and a very strong alkaline soil (pH ~ 12) were noted during the study period.

The samples were collected in triplicate from five different site (S1-S5) of the waste dump area during the month of April to August in 2012, when algal patches were found to form on the crust. The pH and conductivity were measured by electrometric method. Bulk density was estimated by soil core method (Brady and Weil, 2004) while the moisture content was determined by oven drying method (Gupta, 1999). Organic carbon was determined by Walkey and Black's rapid titration method (Jackson, 1958). The texture of the solid waste was analyzed by Bouyoucos soil hydrometer method (Allen, 1989). The cyanobacterial axenic cultures were developed by repeated sub culturing method using BG11 (Rippka *et al.*, 1979) media with and without combined nitrogen. A total of five species were isolated and identified. They were maintained under fluorescent white light 3000 Lux at 24±1° C in 12:12 (light: dark) photoperiod regime until their harvest during exponential period for conducting biochemical analysis. Identification was made using morphological keys given by Desikachary (1959) and Prescott (1951). The algal growth was estimated in terms of chlorophyll *a*, carotenoid and phycobiliprotein contents. Chlorophyll *a* and carotenoid was estimated by taking absorbance of the homogenized culture suspension spectrophotometrically in a UV-1800 model following the method of Strickland and parson's *et al.*, (1984). The concentration of phycobiliproteins was determined spectrophotometrically using the equation given by Bennett and Bogorad (1973). The total soluble protein content was measured following the method of Lowry *et al.*, (1951) and Anthrone method (Spiro, 1966) was used for determination of total carbohydrates.

Statistical analysis

All the experiments were replicated three times. The data obtained were subjected to one way ANOVA followed by post hoc comparison conducted using SPSS V-19. Significant differences were indicated at $p < 0.05$.

Results

A total of five filamentous cyanobacterial species under 5 genera, including both heterocystous and non-heterocystous forms, belonging to 5 families and 2 orders, were isolated from the lime mud deposited area located near the paper mill. The nature of soil where the algae are thriving is found to be alkaline in nature with low organic carbon content and low bulk density (Table 1). Five algal species isolated are *Phormidium angustissimum*, *Lyngbya holdenii, Anabaena doliolum, Calothrix marchica* and *Fischerella muscicola* (Table 2). The results showed that the selected strains growing under similar cultural condition expressed significant differences in their growth pattern and metabolic activities. The growth kinetics of the isolates expressed in terms of specific growth rate (K) and generation time (G) showed wide differences in their values (Table 3). Result showed maximum specific growth rate for *Lyngbya holdenii* Forti (0.409 day^{-1}) followed by the species *Phormidium angustissimum* W.*et al.* G.S. West (0.346 day^{-1}). The growth rate of the remaining three species was similar, with that of *Calothrix marchica* v. *crassa* Var. Intermedia Rao, C.B. being the lowest with 0.104 day^{-1}. However, this species exhibited highest value of generation time as 8.57 days. In case of both the filamentous isolates, exponential growth (Fig. 2) commenced very rapidly, with almost no log phase apparently evident. A one-way ANOVA was conducted to compare the biochemical content of the selected isolates. There was a significant difference of the means of Chl a [$F (4, 10) = 194.482$, $p = 0.000$], Carotenoid [$F (4, 10) = 543.111$, $p = 0.000$], Protein [$F (4, 10) = 2162.000$, $p = 0.000$], Carbohydrate [$F (4, 10) = 5720.250$, $p = 0.000$] PC [$F (4, 10) = 3.799$, $p = 0.040$], APC [$F (4, 10) = 5.279$, $p = 0.015$] and PE [$F (4, 10) = 18.041$, $p = 0.000$] at the $p<0.05$ level for the three isolates. Post hoc comparisons using the Tukey HSD test indicated that the maximum mean values were significantly different. Table 4 presents data on carbohydrate, protein, carotenoid and phycobilliproteins content. The total carbohydrate content analysis showed that the highest contents in *Anabaena doliolum* (296.14 μg ml^{-1}) followed by the non-heterocystous form *Phormidium angustissimum* (240.10 μg ml^{-1}) while the species *Calothrix marchica* and *Fischerella muscicola* (Borzi) Gomont 1985 species exhibited significantly low (P<0.05) carbohydrate content (103.22 μg ml^{-1} and 112.57 μg ml^{-1}, respectively) in comparison to the other strains examined. It was *Lyngbya holdenii* followed by *Phormidium angustissimum* which exhibited significantly (P<0.05) higher amount of soluble protein (183.72 μg ml^{-1} and 149.02 μg ml^{-1}, respectively), followed by *Anabaena doliolum* (120.05 μg ml^{-1}), *Fischerella muscicola* (98.55 μg ml^{-1}) and *Calothrix marchica* (73.74 μg ml^{-1}). Of the strains examined, *Calothrix marchica* (5.26 μg ml^{-1}) was significantly (P<0.05) efficient in terms of carotenoid production and in the remaining four species carotenoid content ranged from 2.31 μg ml^{-1} to 4.82 μg ml^{-1}. The results of phycobilin analysis of investigated cyanobacteria showed phycocyanin (PC) to be the most dominant-phycobiliproteins with maximum value recorded in *Anabaena sp.* and lowest in the *Calothrix sp.* On the contrary, *Calothrix marchica* recorded highest phycoerythrin (35 mg/g of PE).

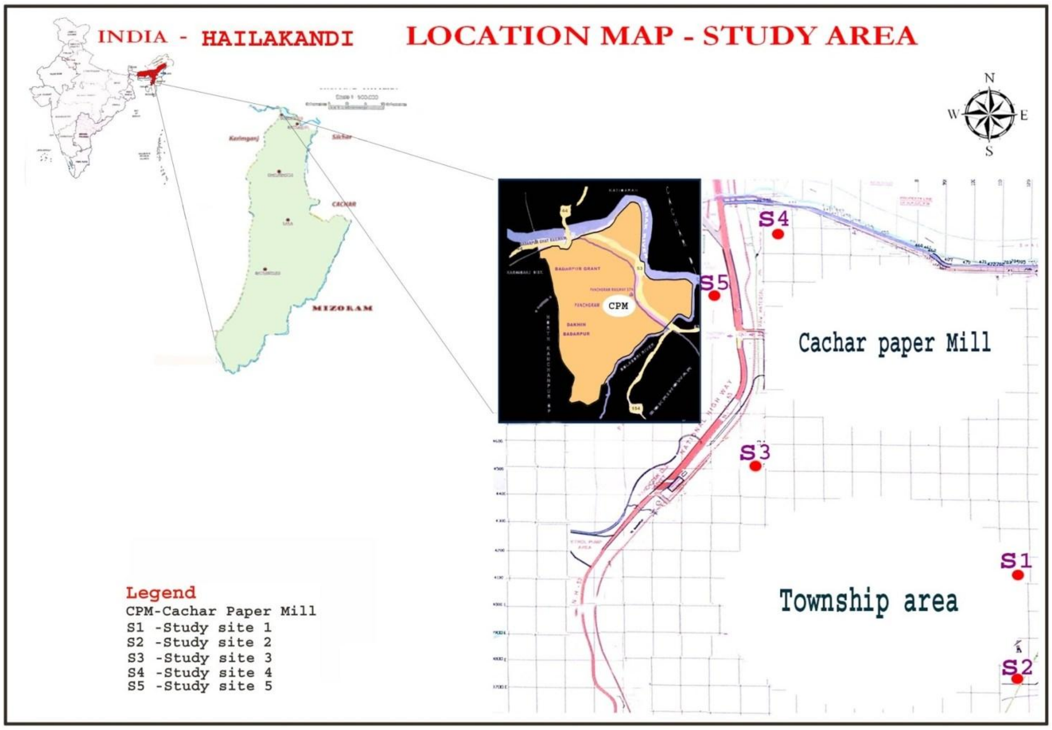

Fig. 1 Map of the study area showing different study site

Table 1. Physico-chemical parameters of lime sludge waste of the study area

Parameters	Average ± Standard Deviation
pH	12.03±0.4
Conductivity (ms)	17.99±2.98
Moisture content (%)	18.22±1.92
Bulk Density (gm cm^{-3})	0.90±0.52
Organic Carbon (%)	0.35±0.06
Textural class	Sandy

Table 2. Taxonomic identification of the cyanobacteria from the lime sludge waste

Classical designation	Order	Place of collection
Phormidium angustissimum	Oscillatoriales	Lime sludge dump of paper mill at Panchgram, Assam
Lyngbya holdenii Forti	Oscillatoriales	-Do-
Anabaena doliolum	Nostocales	-Do-
Calothrix marchica	Nostocales	-Do-
Fischerella muscicola	Nostocales	-Do-

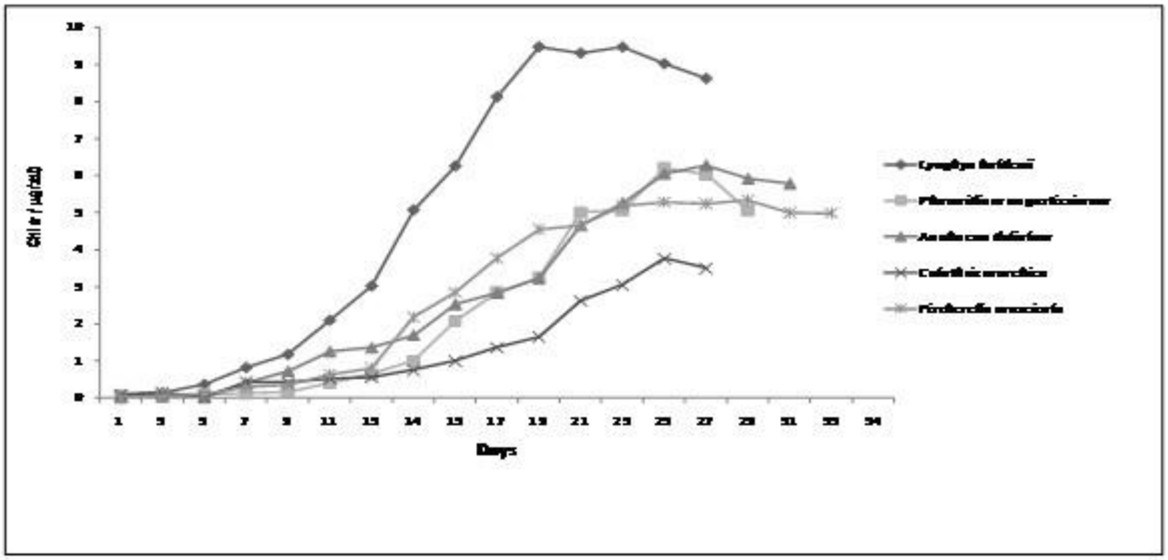

Fig. 2: Growth curves of the isolates

Table 3: Specific growth rate (K) and generation time (G) of the isolates

Species isolated	K (µ day^{-1})	G (day)
Lyngbya holdenii	0.409	3.782
Phormidium angustissimum	0.346	3.840
Anabaena doliolum	0.310	4.343
Calothrix marchica	0.104	8.577
Fischerella muscicola	0.130	6.681

Table 4: Pigments and nutritive value of five cyanobacterial isolates from the lime sludge deposits

Species	Chlorophyll *a* (μg ml^{-1})	Carotenoid (μg ml^{-1})	Protein (μg ml^{-1})	Carbohydrate (μg ml^{-1})	Phycocyanin (μg ml^{-1})	Allophycocyanin (μg ml^{-1})	Phycoerythrin (μg ml^{-1})
Lyngbya holdenii	9.48±0.03	1.77 ±0.02	183 ±0.72	207.40 ±1.30	43±9.5	28±2.0	10±6.6
Phormidium angustissimum	6.06 ±0.50	1.00 ±0.01	149.02 ±1.13	240.10 ±1.38	36±7.1	33±1.2	12±7.1
Anabaena doliolum	6.20 ±0.11	0.82 ±0.05	120.05 ±1.36	296.14 ±1.01	48±4.7	36±0.73	14±0.64
Calothrix marchica	3.77 ±0.24	2.26 ±0.02	73.74 ±2.52	103.22 ±3.65	32±3.5	40±7.6	35±1.5
Fischerella muscicola	5.32 ±0.11	1.31 ±0.07	98.55 ±1.62	112.57 ±0.20	34±1.3	30±0.8	9±0.36

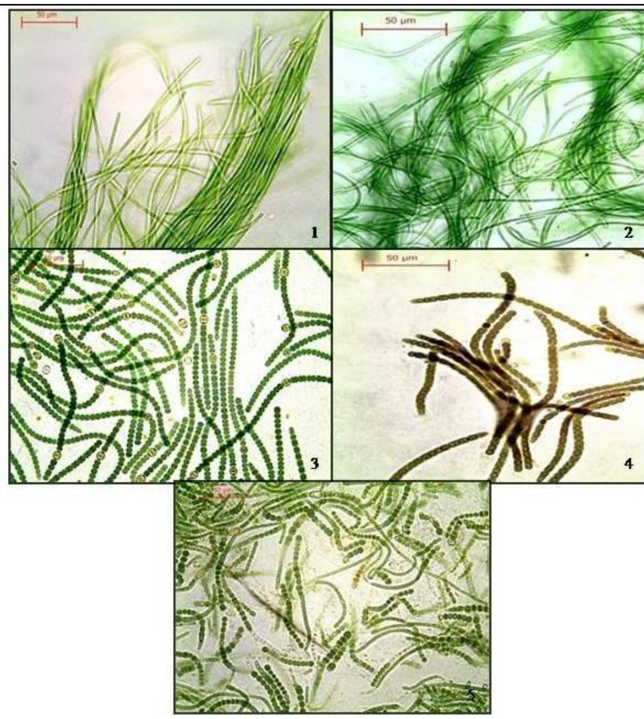

Plate.1. Microphotographs of the cyanobacterial isolates. 1: *Lyngbya holdenii*; 2: Phormidium *angustissimum*; 3: *Anabaena doliolum*; 4: *Calothrix marchica*; 5: *Fischerella muscicola*

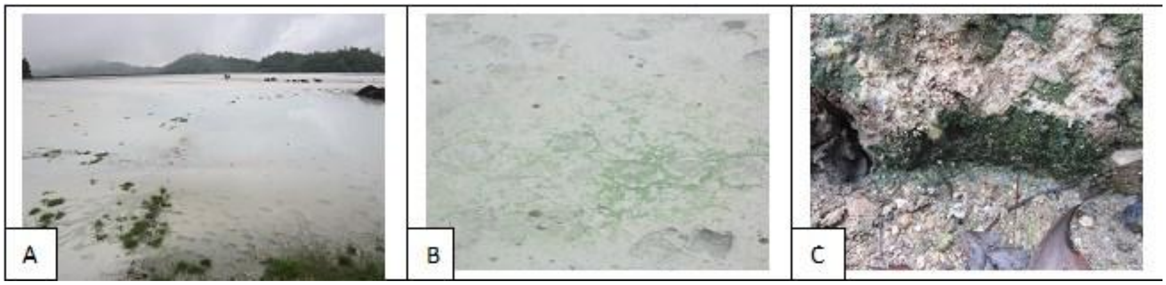

Plate 2.

Discussion

Cyanobacterial strains isolated from the study area were examined for growth kinetics and biochemical contents. A remarkable variation was observed with respect to these attributes amongst the isolates studied. Study of biochemical parameters showed higher chlorophyll in *Phormidium* than *Calothryx* sp. (Tiwari *et al.*, 2005). *Calothrix* exhibited highest carotenoid content which is in agreement with the result obtained by Narayanan *et al.* (2006). Phycocyanin was found to be the major phycobiliproteins in the investigated cyanobacterial species. Similar variations in the concentrations of phycobilin contents in different species as found in this study, has been documented by others (Gantt, 1980; Moreno *et al*., 1995).

On an average, lime sludge waste generated per day is approximately 60 MT from the paper mill. As observed in the present study, a very vast area is under continuous landfill due to the regular deposition of this lime sludge**.** The lime sludge waste dump is characterized by a unique substrate quality, like very high alkaline pH (~12). The nature of algal flora in different habitats is the result of a complex influence of soil properties and climatic conditions (Metting, 1981; Lukesˇova´, 1993; Starks, 1981). Although the spectrum of algal flora narrows down sharply in the dry and stressed region, yet the present site with high alkaline pH (~12) condition showed several algal species growing in the crusts**.** Differential mechanisms of tolerance towards stressed environment have been reported in literature, which might be responsible for the variability observed in the parameters examined amongst the isolates (Potts & Bowman, 1985; Potts, 1994**).** Reports on the occurrence of cyanobacteria in highly alkaline pH have appeared sporadically (Kroll, 1990; Singh *et al.*, 1995; Gimmler and Degenhardt 2001). The nature of the effluents can be assayed by algae since the response can be measured in terms of biomass production or through metabolic response generated (APHA, 2005). All the cyanobacteria in the present site appear to show a rather high tolerance as indicated by their natural occurrence at such high pH. Amongst the strains analyzed, *Lyngbya holdenii* and *Anabaena doliolum* showed higher production of the pigments, proteins and carbohydrates.

Conclusion

Occurrence of cyanobacteria and their biochemical attributes in lime sludge wastes has been investigated. In this study the luxuriant growth of some cyanobacterial strains growing on the disturbed and stressed environment has been observed. These species can eventually be utilized for biological treatment of solid waste. Further, the two strains, *Lyngbya holdenii* and *Anabaena doliolum,* owing to higher biochemical contents, can be considered as superior strains and are of relevance for possible biotechnological applications.

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Allen, S. E. 1989. Chemical Analyses of Ecological Materials. Blackwell Scientific Publication, Oxford

APHA. 2005. Standard Methods for Examination of water and wastewater.21st ed.*pub*. APHA, AWAA, WPCF, Washington DC, USA.

Battaglia, A., N. Calace, E. Nardi, B. M. Petronio, and M. Pietroletti, 2003. Paper mill sludge-soil mixture: Kinetic and thermodynamic tests of cadmium and lead sorption capability. *Microchem. J.* **75**: 97-102

Bennet, A. and L. Bogorad, 1973. Complementary Chromatic Adaptation in a Filamentous Blue Green Alga. *J Cell Biol* **58**(2):419-435.

Back Well, B. 1987. *Pulp Paper Can.* **88**(6): T181.

Brady, N. C. and R. R. Weil, 2004. Elements of the nature and properties of soils. Pearson Education. 606pp.

Ciferri, O. and O. Tiboni, 1985. The biochemistry and industrial potential of *Spirulina*. *Annu. Rev. Microbiol.* **39**: 503-526

Deka, S. and S. Yasmin, 2006. Utilization of lime sludge waste from paper mills for fish culture. *Curr. Sci.* **90 (8)**: 1126-1130

Desikachary, T.V. 1959. Cyanophyta. Monograph. ICAR, New Delhi. India.

Dorris, G. M. and L. H. Allen, 1985. "The effect of Reburried Lime structure on the rates of slaking, causticizing and Lime mud setting". *J. Pulp Pap. Sci.* **II** *(4): J89-97*

Eroglu, H., H. H. Acer, O. Ucuncu, and S. Imamoglu, 2006. Soil Stabilization of Forest Roads Sub-base Using Lime Mud Waste from the Chemical Recovery Process in Alkaline Pulp Mill. *J App Sci* **6** (5): 1199-1203.

Gantt, E., 1980. Structure and function of phycobilisomes: light harvesting pigment complexes in red and blue-green alga. *Int. Rev. Cytol.* 66: 45-80.

Gaskin, J. 2004. Land application of pulp mill lime mud. Bulletin 1249, Georgia, USA

Geng, X., J. Deng, and S.Y. Zhang, 2006. Effects of hot-pressing parameters and wax content on the properties of fiberboard made from paper mill sludge. *Wood Fiber Sci*. **38**(4): 736-741.

Gimmler, H., B. Degenhardt, L. C. Rai and J.P. Gaur, 2001. Alkaliphilic and alkali-tolerant algae, algal adaptation to environmental stresses. Springer.

Gupta, P. K. 1999. Soil, Plant, Water & fertilizer analyses. Exobios (India).

Jackson, M. I. 1958. Soil chemical analysis. Prentice Hall, Englewood Cliffs New Jersey.

Roll, R.G. 1990. Alkalophiles. Microbiology of Extreme Environments. McGraw Hill Publishing Co, pp. 53-92.

Kumar, A., V. Singhal, B. D. Joshi, J.P.N. Rai, 2004. Lysimeteric approach for ground water pollution control from pulp and paper mill effluent using different soil textures. *Ind. J. Sci. Res.* ***63****:429-438*

Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the Folin – phenol reagent. *J. Biol. Chem*., **193**: 265 – 275.

Lukešova, A. 1993. Soil algae in four secondary successional stages on abandoned fields**.** *Algol. Stud.* **71**: 81–102.

Metting, B. 1981. The systematics and ecology of soil algae. *Bot. Rev*. **47**: 195–312

Moreno, J., H. Rodriguez, M.A. Vargas, J. Rivas, and M.G. Guerrero, 1995. Nitrogen-fixing cyanobacteria as source of phycobiliprotein pigments. Composition and growth performance of ten filamentous heterocystous strains. *J. Appl. Phycol.* 7: 17-23.

Misra, S., and B. D. Kaushik, 1989. Growth promoting substances of cyanobacteria. II. Detection of amino acids, sugars and auxins. *Proc. Indian Natn. Sci. Acad.* 55: 499 – 504.

Narayan, K.P., Shalini, S. Tiwari, S. Pabbi and D. W. Dhar, 2006. Biodiversity analysis of selected cyanobacteria. *Curr. Sci*. 91(7)

Rippka, R.., J. Deruelles, J.B. Waterbury, M. Herdman, and R.Y. Stanier. 1979. Generic assignment, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol*.111: 1-61.

Saikia, M. K., S. Kalita, and G. C. Sarma, 2011. An experimental investigation on growth stimulation (+) and inhibition (-) of algae (*Oscillatoria chlorina* and *Scenedesmus quadricauda*) treated with pulp and paper mill. *Int. J. Appl. Biol. Pharm.* **2**(4): 87-94

Titovaa, N., P. Hrouzekc, and V. Drabkovad, 2008. Toxins and other bioactive compounds produced by cyanobacteria in Lake Ladoga; *Est. J. Ecol.* **57**(2): 100-110

Parson, T. R. and J. D. H.Strickland, 1965. Particulate organic matter III. I. pigment analysis III, I.I. Determination of Phytoplankton pigments. *J. Fish. Res. Bd. Canada* **18**: 117-127

Prescott, G.W. 1952. Algae of Western Great Lakes Area. Ottokoeltz. Sci Publisher West Germany, pp.977

Potts, M. and M. A. Bowman, 1985. Sensitivity of *Nostoc commune* UTEX–584 (cyanobacteria) to water stress. *Arch. Microbiol.* **141**: 51–56.

Pulz, O. and W. Gross, 2004. Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.* **65**: 635–648.

Spiro, R. G. 1966. Analysis of sugars found in glycoproteins. *Methods Enzymol.* **8**: 3-26

Singh, B. V., S. K. Goyal, & F. Tasneem, 1995. pH tolerance in cyanobacteria. *Phykos* **34**: 97-103.

Singh, O. K., G. Oinam, and O. N. Tiwari, 2012. New Record of Potential Cyanobacteria from Indian Region Falling Indo-Burma Biodiversity Hotspots (North-East Region of India) and Partial Characterization for Value Additions. *Philipp. J. Sci.* **141**(1): 57-66.

Singh, P. and I. S. Thakur, 2004. Removal of colour and detoxification of pulp and paper effluent by micro organisms in two step bioreactor, *J. Sci. Ind. Res.* **63**. 944-948

Spolaore, P., C. Joannis-Cassan, E. Duran, and A. Isambert, 2006. Commercial applications of microalgae. *J. Biosci. Bioeng.* **101**: 87–96

Starks, T. L. and L. E. Shubert, 1981. Colonization and succession of soil algae. *Phycol*ogia **18**: 99–107.

Suriyanarayanan, S., A. S. Mailappa, D. Jayakumar, K. Nanthakumar, K. Karthikeyan, and S. Balasubramanian, 2010. Studies on the Characterization and Possibilities of Reutilization of Solid Wastes from a Waste Paper Based Paper Industry, *Global J. Environ. Res.***4** (1): 18-22

Tiwari, O.N., B.V. Singh, U. Mishra, A. K.Singh, D. Wattal, and P. K. Singh, 2005. Distribution and physiological characterization of cyanobacteria isolated from arid zones of Rajasthan. *Trop Ecol* **46** (2): 165–171

Wilkie, A. C., S. J. Edmunson, and J. G. Duncan, 2011. Indigenous algae for local bioresource production: phycoprospecting. *Energy for sustainable*