Journal of Research in Biology

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Microbial diversity and bioremediation of distilleries effluent

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ABSTRACT:

The role of cyanobacteria in distilleries effluent was studied in Kanchipuram Tamilnadu, India. Totally 12 species of cyanobacteria belonging to 6 genera falling under 4 families were identified. Six different genera of bacteria and 3 different genera of fungi were isolated and identified. Among the cyanobacteria isolated, *Nostoc muscorum* was selected to treat the effluent. Distilleries effluent was the potential source of cyanobacteria. *Nostoc muscorum* was found to be the most dominated genus in this effluent (heterocyst organism). The inoculation of *Nostoc muscorum* resulted in removal of various chemicals as well as nutrients such as nitrogen, ammonia and phosphorus from the effluent. It is concluded that *Nostoc muscorum,* a cyanobacteria found in the distilleries effluent, could be potentially employed for the treatment of distilleries effluent.

Keywords:
Distillery effluent, Microbial diversity, Bioremediation, Physico-Chemical analysis.

Article Citation:
Ganapathy Selvam G, Baskaran R and Mohan PM.
Microbial diversity and bioremediation of distilleries effluent.
Journal of research in Biology (2011) 3: 153-162

Dates:
Received: 22 Jun 2011 /**Accepted:** 02 Jul 2011 /**Published:** 07 Jul 2011

Journal of Research in Biology

INTRODUCTION

Bioremediation is a pollution control technology that uses biological systems to catalyse the degradation or transformation of various toxic chemicals to less harmful forms. Pollution of water and land due to hazardous toxic wastes has become a major global concern. The industrial, municipal and agricultural wastes which are legally or illegally discharged into the environment are responsible for the environmental pollution (Lang *et al.*, 1999 and Bakare *et al.*, 2003). Among the major industries in India, distillery is one of those that contribute to water pollution. There are nearly 290 distillery unit present in India, of which, 17 units are located in Tamilnadu. All these distillery industries use molasses obtained from sugar industries as raw material and generate large amount effluents. Alcohol is separated by distillation and the residual liquor is discharged as effluent. This effluent called as spent wash, is hot, pH is highly acidic, dark coloured nature. Estimated quantity of distillery waste generated in India is 8057 kilo litres per day. The waste is highly hazardous in nature because of very high COD and BOD levels. Satyawali and Balakrishnan (2008) studied the molasses-based distilleries are one of the most polluting industries generating large volumes of high strength waste water. Different processes covering anaerobic, aerobic as well as physico-chemical methods have been employed to treat this effluent. Anaerobic treatment is the most attractive primary treatment due to over 80% BOD removal combined with energy recovery in the form of biogas. Further treatment to reduce residual organic load and colour includes various: (i) biological methods employing different fungi, bacteria and algae, and (ii) Physico-chemical methods such as adsorption, coagulation / precipitation, oxidation and membrane filtration.

In recent years, urban people are facing many problems and water pollution is one among them. Environmentalist and government are looking for efficient, cheap and lasting solutions to waste treatment and recycling. Physico-chemical methods of waste water treatment are inevitably cost intensive and cannot be employed in all industries especially in developing countries like India. Hence, in recent years, the importance of biological treatment systems has attracted the attention of workers all over the world and has helped in developing relatively efficient, low cost waste treatment systems. Algal systems, more particularly the cyanobacteria, are not only useful in treating the waste but also producing a variety of useful byproducts from the biomass (Subramanian and Shanmugasundaram, 1986).

MATERIALS AND METHODS

Isolation and identification of Cyanobacteria

Effluent was collected from the Distilleries, Padappai, Kanchipuram (TN), India where over diluted effluent has been released from the factory. Standard microbiological methods were followed for isolation of Cyanobacteria from these samples. Algal samples were microscopically examined and plated on solid agar medium - BG11 (Rippka *et al.*, 1979). The inoculated plates were incubated in culture room (temperature maintained at 25 ± 2^{o}C fitted with cool white fluorescent tube emitting 2500 lux for 18 hrs a day) and were regularly examined for the growth of cyanobacteria. Colonies appearing on solid medium were picked up and transferred to liquid medium. By repeated streaking, cultures were made unialgal and maintained in BG11 liquid medium. Identification of algal forms was made with the help of keys given by Desikachary (1959) and Geitler (1932).

Isolation and identification of fungi

Standard serial dilution method was used for the isolation of fungus, 1ml of diluted sample was plated in petridishes containing Potato Dextrose Agar medium accumulated with Streptomycin sulfate 100µg/ml (PDA) to inhibit the bacterial growth. The fungus were purified and mounted over a clean slide, stained with lactophenol and cotton blue and observed under the microscope. The fungus were identified by using standard manuals, such as Manual of soil fungi (Gillman, 1957), Dematiaceous Hyphomycetes (Ellis, 1971), More Dematiaceous Hyphomycetes (Ellis, 1976), Hyphomycetes (Subramanian, 1971).

Isolation and identification of Bacteria

Bacteria were isolated by standard method using nutrient agar. (Difco Manual, 1953). The purified bacterial cultures were used for identification. The bacteria were identified based on colony characteristics, gram staining methods and by various biochemical studies as given by Bergey and Buchanan (1974).

Treatment of distilleries effluent by *Nostoc muscorum*

Sterilized Erlenmayer flasks (250 ml) were used for the experiments. The initial physico-chemical analysis of effluent was made following the Standard Methods (APHA, 1975). The following treatments were employed in order to

study the interaction of Cyanobacterium with the distilleries effluent. Effluent uninoculated was taken as, control for physicochemical analysis (C). BG_{11} medium inoculated with *Nostoc muscorum* for the estimation of growth (CO). Inoculation was made by adding 3.0 ml uniform suspension of *Nostoc muscorum* separately (0.03 mg ml^{-1}). The experiment was conducted under controlled conditions (Temperature 25 ± 2°C with light intensities of 2500 lax provided form overhead cool with fluorescent tubes) for one month. Since the growth of *Nostoc muscorum* was very slow in the effluent, a long duration was provided to get a culture of exponential growth. The cultures were harvested on every 5th day by filtration through filter paper and washed repeatedly with distilled water. The filtered effluents (inoculated and control) were used for physicochemical analysis. *Nostoc muscorum* obtained both from the medium and effluents were used for the estimation of growth (estimation of chlorophyll a).

Estimation of Chlorophyll 'a'

The cultures were centrifuged at 5,000 x g for 10 minutes. The pellets were washed with distilled water; suspended in 80 per cent methanol and vortexed thoroughly. Then the tubes were covered with aluminium foil to prevent solvent evaporation and incubated in a water bath set at 60°C for 1 hr, in dark with occasional shaking. After 1 hr the contents were cooled and centrifuged at 5,000 x g for 5 minutes. The supernatant was saved and the above procedure was repeated twice to ensure complete extraction of the pigment. The pooled supernatant was made upto a known volume with 80 per cent methanol (to compensate the solvent loss during heating). The absorbency was measured at 663 nm in Spectronic 20 against methanol blank.

Physico-chemical Analysis of distilleries effluent

The physico-chemical properties of the distillery effluent like pH, Carbondioxide, alkalinity (Carbonate and Bicarbonate), Dissolved oxygen, Biological Oxygen Demand, Chemical Oxygen Demand (COD), Nitrate, Nitrite, Ammonia, total phosphorus, inorganic phosphate, Organic phosphate, Calcium, Magnesium and Chloride were analyzed following the procedure of Apha (1975).

Biochemical analysis of *Nostoc muscorum*

Biochemical analysis of *Nostoc muscorum* such as Carbohydrate (Dubois *et al.*, 1956), Protein (Lowry *et al.*, 1951), aminoacid. (Jayaraman, 1981) and Lipids (Sato and Murata, 1988), were analyzed for the estimation of biochemical compound variation in effluent treated and control culture.

RESULTS AND DISCUSSION

Isolation of cyanobacteria:

In the present investigations totally 12 species (**Fig-1 and Table -1**) of cyanobacteria distributed in five genera falling under four different families were identified from the distilleries effluent polluted habitat. Among the genera, *Oscillatoria* with six species followed by *Phormidium* two species, *Lyngbya, Microcystis, Nostoc* and *Plectonema* with single species each. This is attributed to favorable conditions of oxidizable organic matter, less DO and high calcium content; (Table 4) an observation which supports Venkateswaralu (1969) who observed that high orthophosphate levels favored the development of cyanobacterial bloom. Similar observations were made in the present study with reference to various nutrients (Table-4). Heterocystous cyanobacteria have not been recorded in polluted waters rich in nitrogen (Boominathan *et al.,* 2007). In contrary to that present study heterocystous cyanobacteria have already been recorded and is because of the presence of lower concentration of various forms of nitrogen effluent as suggested by Vijayakumar *et al.*, (2007).

Isolation of fungi and Bacteria:

In the present investigation, a total of three different genera of fungus were isolated and identified. Among the genera *Aspergillus* with four

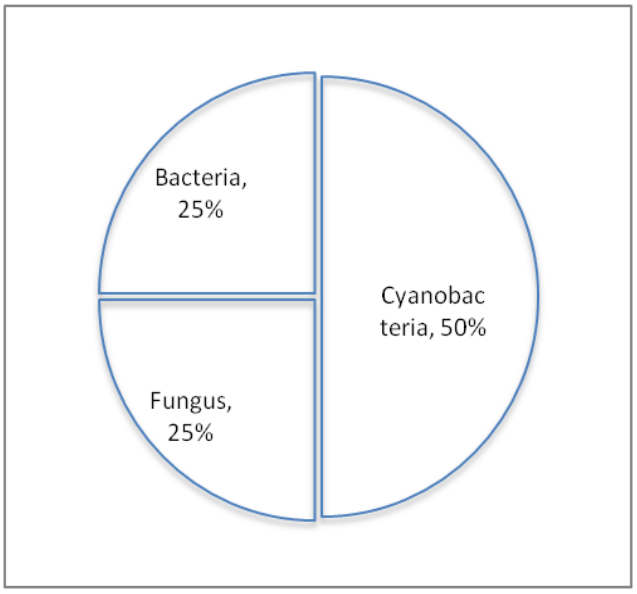

Fig-1 Microbial diversity of distillery effluent

Table 1 Cyanobacteria from distilleries effluent

Sl. No.	Name of cyanobacteria species
	CHROOCOCCACEAE
1.	*Microcystis aeruginosa* Ktz
	OSCILLATORIACEAE
2.	*Oscillatoria acuta* Bruhl et Biswas, Orth. Mut. Geitler
3.	*Oscillatoria earlei* Gardner
4.	*Oscillatoria late-virens* (Crovan) Gomont
5.	*Oscillatoria princeps* Vaucher ex Gomont
6.	*Oscillatoria terebriformis* Ag ex Gomont
7.	*Oscillatoria willei* Gardner em.Drouet
8.	*Phormidium fragile* (Meneghini) Gomont
9.	*Phormidium tenue* (Menegh) Gomont
10.	*Lyngbya majuscula* Harvey ex Gomont
	NOSTOCACEAE
11.	*Nostoc muscorum* Ag ex Born. et Flah.
	SCYTONEMATACEAE
12.	*Plectonema notatum* Schmidle

species dominant given in (Fig-1 and **Table-2**). Jaganathan (2006) isolated 21 species from oil refinery effluent polluted habitat with *Aspergillus* as the dominant genus. From the distilleries effluent, six different species of bacteria were isolated (Fig-1 and **Table-3**). Pursuing the literature, there has not been much work regarding the isolation and identification of bacteria from dye and other related effluent samples. Contrary to that, Boominathan *et al.* (2007) isolated nine different species of bacteria were reported. Jain *et al.* (2001) isolated three different bacterial strains from the distillery sludge to treat the predigested distillery waste water.

Bioremediation of distilleries effluent

Now-a-days the use of algae, particularly cyanobacteria for biomonitoring of pollution is being stressed. The isolated dominant taxa, encounted in the present study, *Nostoc muscorum* was selected for the treatment of distilleries effluent (Fig-2). Many of the studies conducted in various laboratories have shown that under defined

Table 2 Cyanobacteria from distilleries effluent

Sl. No.	Name of fungal organisms
1.	*Aspergillus flavus* Link
2.	*Aspergillus luchuensis* Inui
3.	*Aspergillus niger* Vantieghem
4.	*Aspergillus terreus* Thom
5.	*Fusarium oxysporum* Schlectenndhal
6.	*Penicillium janthinellum* Biourge

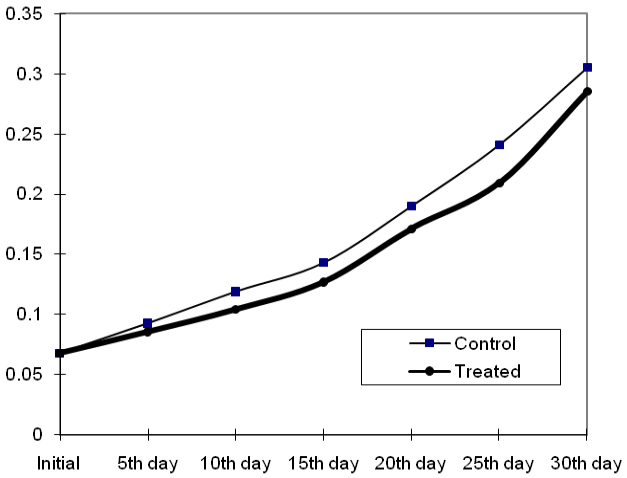

Fig-2. Growth of *Nostoc muscorum* treated and control

conditions indicator species activity take part in the degradation of organic matter (Gupta and Rao, 1980; and Vijayakumar *et al.*, 2005).

Manoharan and Subramanian (1992 and 1993) found a rise in pH value upto 10th day in various effluents inoculated with free BGA and after that it was declined. Similarly, Tang *et al.* (1997) reported a rise in pH while tertiary waste water was treated with cyanobacteria. In the present study, the initial pH of the effluent was 3.7 and it rose till 6.7 after the effluent inoculated with *Nostoc muscorum* was observed for a period of 30 days (Table-4).

There was no carbonate in the effluent, but, fairly high level of free Co_2 and bicarbonate was recorded. Gradual removal of both CO_2 and bicarbonate was observed from 5th day onwards. On 30th day CO_2 and bicarbonate was respectively 80 and 74 percent removal of CO_2 and bicarbonate was noticed. The relative proportion of CO_2 and HCO_3 depends on the pH of the medium. It was found that HCO_3 with 6 to 7 pH is predominant. Thus cyanobacteria may have competitive advantage over chlorophytes since the former are capable of assimilating HCO_3 as source of inorganic carbon for photosynthesis (Colman, 1989). In general, the removal of these carbon sources effectively by cyanobacteria, as expected, was observed in the present investigation.

Chemical oxygen demand is generally considered as a major indicator of organic pollution in water (Dash and Mishra, 1999). The strength of the waste water is determined by the level of BOD and COD. Inoculations of cyanobacteria reduced the BOD and COD levels considerably when compared to control (Table-4). More than 53 per

Table 3.Biochemical characteristics of isolated bacteria from distilleries effluent

Sl. No.	Name of the test	*E. coli*	*E.aerogenes*	*Lactobacillus* sp.	*P .aeruginosa*	*P .vulgaris*	*S.sonnei*
1.	Gram staining	Gram –ve Rods	Gram –ve Rods	Gram –ve Rods	Gram –ve Rods	Gram –ve Rords	Gram – ve Rods
2.	Motility	+	+	-	+	+	-
3.	Spore staining	-	-	-	-	-	-
4.	Indole	+	-	+	-	+	-
5.	Methyl red	+	-	+	-	+	+
6.	Voges Proskauer	-	+	-	-	-	-
7.	Citrate utilization	-	+	+	+	+	-
8.	Triple sugar iron	+	+	+	-	+	-
9.	Gas production	+	+	+	-	+	-
10.	H_2S production	-	-	-	-	+	-
11.	Catalase	+	+	-	+	+	+
12.	Oxidase	-	-	+	+	-	-
13.	Urease	-	-	-	-	+	-
14.	Lysine	+	+	-	-	-	-
15.	Phenyl alanine	-	-	+	-	+	-
16.	Nitrate reduction	+	+	-	+	+	-
17.	Carbohydrate utilization test						
18.	Glucose	+	+	+	+	+	+
19.	Maltose	+	+	+	-	+	+
20.	Lactose	+	+	+	-	-	-
21.	Mannose	+	+	-	-	-	+
22.	Sucrose	+	+	+	-	-	

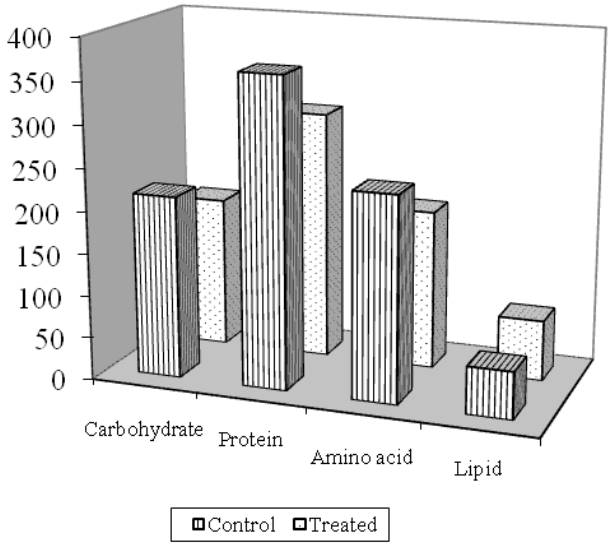

Fig- 3. Total carbohydrate, protein, amino acid and lipid content in *Nostoc muscorum*

cent reduction of BOD and 68 per cent reduction of COD were observed with both cyanobacteria. Use of acclimatized algal cultures in considerably reducing BOD and COD with different effluents has been reported (Sharma *et al.*, 2003 and Vijayakumar *et al.*, 2005).

Determination of the amounts of O_2 dissolved in water at study sites is undoubtedly of treatment importance since it is considered as one of the best parameters in evaluating pollution stress of aquatic habitats, unless it contains toxic substances (Lester, 1975). As revealed from the results obtained in the present investigation, the initial DO content of distilleries effluent was very low (1.2 $mg1^{-1}$). From 5^{th} – 10^{th} day onwards a gradual increase in DO was noticed and it was 1.2 and 2.1 effluent with *Nostoc muscorum* respectively (Table-4). Increase in DO level when treated with

Table 4. Characteristics of treated and non treated distilleries effluent

Sl.No.	Parameters	Non Treated effluent (mg/ml)	After 30 day Treated effluent (%)
1.	Colour	Dark Brown	-
2.	pH	6.7	6.7
3.	Temperature	29°C	29°C
4.	Free CO_2	100	80
5.	Carbonate	Nil	Nil
6.	Bicarbonate	158	74.68
7.	BOD	327	53.51
8.	COD	232	68.53
9.	Nitrate	583	52.83
10.	Nitrite	115	66.08
11.	Ammonia	423	58.86
12.	Total phosphorus	59	55.93
13.	Inorganic phosphorus	36	36.11
14.	Organic phosphorus	23	86.93
15.	Calcium	84.10	73.78
16.	Magnesium	62.42	69.91
17.	Chloride	179.9	48.36

Except pH and temperature all other parameters are in mg/l.

different cyanobacteria with different effluents has already been reported by Vijayakumar *et al.* 2005 and Boominathan *et al.*, 2007. This raise in DO level in effluents with cyanobacteria might be due to oxygenic photosynthetic nature of cyanobacteria. The correlation between increase in DO and removal of BOD and COD observed in this study agree with observations of Kankal *et al.*, (1987); and Vijayakumar *et al.*, (2005).

Suspended cultivation of microalgae is one of the biological processes which have been employed to eliminate residual inorganic nutrients as a tertiary treatment step from secondary treated effluents (Prakasham and Ramakrishnan, 1998). Inorgornic compounds such as nitrite, nitrate, ammonia and phosphate are the essential requirements for the growth of cyanobacteria. They have high nutrient capabilities as they can accumulate inorganic phosphate and cynophycin respectively (Fay, 1983). In the present investigation, in distilleries effluent, all form of nitrogen were observed in appreciable quantities (Table-4) and cyanabacteria removed more than 55 per cent of inorganic nitrogen from the effluent. However, *Nostoc muscorum* was marginally better in removing all form of nitrogen. Suspended, cultivation of micro algae is one of the biological process has been employed to eliminate residual inorganic nutrients as a tertiary treatment step from secondary treated effluents (Oswald, 1978). Studies with *Spirulina* (Boominathan *et al.,* 2007), Anabaena (Mallick and Rai, 1994), *Oscillatoria* (Manoharan and Subramanian, 1992 and 1993) concluded that cyanobacteria can efficiently eliminate inorganic nitrogen compounds from waste waters. In general, the removal of inorganic nitrogen compounds by cyanbacteria is governed by growth conditions and physiological conditions of the state of the organism like pH, light, temperature, inoculum size and subtracted concentration.

The capacity of cyanobacteria to remove large amount of phosphorus from industrial waste water has been demonstrated by several workers (Monoharan and Subramanian (1992 and 1993), Boominathan *et al.* (2007) and Vijayakumar (2005) found a total or near total removal of all forms of phosphate by *Oscillatoria* and *Aphanocapsa* from different effluents. Tang *et al.*, (1997) also reported

a higher phosphate uptake, despite low biomass of cyanobacteria. Dash and Mishra (1999) observed 100 per cent removal of phosphate from paper mill effluent while testing with *Westiellopsis prolifica*. However in the present study more than 55 percent removal of total phosphorous and 36 per cent removal inorganic phosphate were observed in effluent with *Nostoc muscorum*. The major phosphate reserve of cyanobacteria is polyphosphate, which accumulates as discrete granules in the cytoplasm of the cell wall when phosphate is in excess (Fay, 1983). The intracellular accumulation of phosphate is energy dependent, being higher in the light than in the dark and depends strictly on the pH of the cytoplasm. All the above findings confirm that cyanobacteria can absorb phosphate in excess amount as it is required and this could be the reason for maximum removal of all forms of phosphate from the effluent.

The total hardness is constituted by calcium and magnesium. In the present study more than 68 per cent removal of calcium and magnesium were observed in effluent with *Nostoc***.** Uma and Subramaniam (1990) studied the effective use of cyanobacteria in ossein effluent which has high levels of calcium. They found more than 50 per cent reduction of calcium within 4 days when the effluent was treated separately with *Oscillatoria* and *Aphanocapsa*. Similarly, Manoharan and Subramanian (1992 and 1993) reported the reduction of calcium and magnesium in domestic sewage, ossein and paper mill effluents by *O. pseudogeminata* var. *unigranulata*. They observed more than 70 percent reduction of calcium and magnesium with retention time of 15 days. Dash and Mishra (1999) observed 50 per cent reduction of calcium in paper mill effluent by *Westiellopsis* (retention time of 15 days). On the other hand Vijayakumar *et al.* (2005) reported more than 90 percent removal of calcium and magnesium from dye effluent when treated with *Oscillatoria* sp. Although, calcium is undoubtedly required for cyanobacterial growth substantial reduction in calcium and magnesium are known to be essential for flocculation and would coflocculate (Richmond and Becker, 1986).) observed the excretion of organic acids by microbes and especially cyanobacteria and their capacity to solubilize magnesium in the waste water, which could explain observed reduction.

Chlorides are generally considered as one of the major pollutants in the effluent which are difficult to be removed by conventional biological treatment methods. However, in the present investigation *Nostoc* was efficient by removing chloride from the sample (Table-4). Uma and Subramanian (1990) observed nearly 50 and 25 per cent removal when ossein effluent which has very high level of chloride was treated with *Oscillatoria* and *Aphanocapsa* respectively. Manoharan and Subramaniam (1992 and 1993) also observed more than 40 percent removal of chloride from various effluents by *Oscillatoria* sp. A similar observation attributing 50 percent chloride reduction under laboratory conditions by *O. brevis* was also reported in dye effluent (Vijayakumar *et al.*, 2005), oil refinery effluent (Boominathan *et al.*, 2007) and sugar mill effluent (Gopalakrishnan, 2007).

Biochemical studies on *Nostoc muscorum*

Growth was measured in terms of chlorophyll 'a' as a biomass component. This was estimated both in BG_{11} medium (control) and effluent. Maximum growth of *Nostoc* was recorded in the control than in effluent inoculated (**Fig.2**). In recent years, scientists have been increasingly concentrated more on the influence of these systems on the removal of nutrients from the effluent but only a few have investigated the effect of effluents on the biochemistry of the cyanobacterial systems. To develop suitable and efficient treatment system, it is obligatory to understand the mutual influence and interactions between the effluents and the organisms, so that manipulation to improve the treatment system becomes feasible and hence the present investigation on the biochemistry of effluent grown cyanobacteria was carried out.

Distilleries effluent has decreased the total carbohydrate content of *Nostoc muscorum* to a considerable level (**Fig.3**) when compared to BG_{11} medium (control). The reduction was around 16 percent. Contrary to the present observation, increase in the carbohydrate level of cyanobacteria with various effluents has been reported by many workers (Reddy *et al.*, 1983 and Vijayakumar, 2005, 2007). Nitrogen limitation caused photo assimilated carbon to be directed towards the synthesis of carbohydrate instead of proteins and chlorophyll. This response is widely observed in many algal species (Turpin, 1991). Protein and chlorophyll decreases and carbohydrate increases by CO_2 enrichment have been observed previously in a number of species (Loehle, 1995). But in the present investigation, considerable levels of all forms of inorganic nitrogen were observed; more over the carbohydrate content was also reduced by the effluent. There were no CO_2 recorded in the

effluent throughout the study period and hence this could not explain the observed variation in carbohydrate content.

Similarly carbohydrate, more than 19 percent reduction of protein and 21 percent reduction of amino acid were also recorded in cyanobacteria grown in effluent (Fig.3). Similar observations with decrease protein level in cyanobacteria with different effluents from paper mill have already been established. (Vijayakumar *et al*, 2005, 2007). Reddy *et al.* (1983) also observed that increasing concentration of oil refinery effluent significantly decreased the biochemical constituents including proteins and amino acid. Similarly, observed an increased concentration of tannery effluent (less diluted) decreased the biochemical contents such as proteins and carbohydrate in blue green algae as compared to control. In the present study, 100 per cent effluent (without dilution) was used to grow cyanobacterium. This could be the reason for the reduced biochemical components of test organism. The significant reduction of the above biochemical metabolites suggested that the effluent affects algal metabolism at multiple sites (Reddy *et al.*, 1983).

From the distilleries, the total lipids except other constituents in cyanobacteria showed an increase with the effluent. Increase was more than 20 per cent over control (Fig.3). Contrary to this, Manoharan and Subramanian (1992 and 1996), reported a decrease in the level of lipid content of cyanobacteria with different effluents. Alteration in the lipid content of an organism is more important in response to environmental stress (Vijayakumar *et al*, 2007). Variation in lipid contents and composition under different environmental conditions including light and dark has been observed in a number of cyanobacteria (Al-Hasan *et al.*, 1989). As already pointed out that the changes in biochemical contents of cyanobacteria might be due to the effluent which affects the algal metabolism at multiple sites (Reddy *et al.*, 1983).

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