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Identification of microcystin producing Cyanobacteria by PCR based method.

KEYWORDS: Cyanobacteria, water toxicity, *mcy* gene, Cyanobacteria primers, cyanotoxins.

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ABSTRACT *Cyanobacterial blooms are one of the major environmental concerns that have worried people of a significant number of places worldwide. The blooms can prove to be hazardous to both the aquatic as well as nearby non aquatic lives, including humans. Microcystins are the most widespread cyanobacterial toxins and can bioaccumulate in common aquatic vertebrates and invertebrates such as fish, mussels, and zooplankton. Microcystin primarily affect the liver (hepatotoxin), but can also affect the kidney, and reproductive system. Several places in Bharat (India) have reported deaths of aquatic lives due to cyanobacterial blooms. The microcystin (mcy) gene cluster includes various mcy genes that are mcyA, mcyB, mcyC, mcyD, mcyE and mcyG. The current study intends to identify microcystin producing cyanobacteria in various water bodies of Ranchi, Jharkhand, by PCR based detection of mcyE gene sequence.*

1. Introduction

Cyanobacteria or the blue-green algae are single cellular, phototrophic organisms that are generally found in water bodies like lakes, ponds, dams, rivers and oceans. A significant number of species of this wonder organism like Nostoc, Anabaena sp., Oscillatoria etc. are advantageous, as they fix the atmospheric Nitrogen and contribute widely to the nitrogen cycle. But some species of cyanobacteria like *Microcystis aeruginosa*, *Microcystis flos-aquae*, *Cylindrospernum sp.* etc. are toxin producers in nature. Some of the toxins producing cyanobacteria also include the nitrogen fixing species like *Anabaena* and *Oscillatoria*. *Microcystis sp.* can produce neurotoxins and hepatoxins such as microcystin and cyanopeptolin.

Microcystin is one of the most common cyanotoxins produced by *Microcystis aeruginosa*. When ingested, microcystin is actively absorbed by fishes, birds and mammals. It primarily affects the liver, causing minor to widespread damage, depending upon the amount of toxin absorbed (Anonymous). Storm Lake in Iowa, United States experienced dramatic bloom events in 1952: associated with *Anabaena flos–aquae* blooms where estimated deaths of 5–7,000 gulls, 560 ducks, 400 coots, 200 pheasants, 50 squirrels, 18 muskrats, 15 dogs, 4 cats, 2 hogs, 2 hawks, 1 skunk, 1 mink, plus "numerous" songbirds were recorded (Stewart et al., 2008). Recently, cyanobacterial blooms caused Chile $800 million. The algal blooms caused death of over 23 million Salmon fishes. The loss is equivalent to 15-20 percent of the total annual production (Anonymous). Many places in Bharat (India), Muttukkadu Backwaters Southeast Coast (Balaji et al., 2012), Varanasi (Anil et al., 2011) and Nagpur (Lalita et al., 2008) have also been reported to have witnessed cyanobacteria bloom.

Cyanobacteria blooms have worried the scientific community of the world since a long time. It has affected the human society in numerous ways. An in-land fresh water body is a source of life support for all forms of life in the area including cattle, stray animals and humans. The toxic cyanobacterial blooms have caused deaths of many aquatic animals that are of great economic importance, thereby affecting the lives of fishermen who are totally depended on fishing.

The major reasons of the blooms are the increased level of dissolved nitrogen and other nutrients in the water systems. The water systems that have both the Nitrogen fixing species and the toxin producing species are the most potent sites for cyanobacterial blooms to occur. Health hazards for mammals include liver damage (Elise et al., 1998), sever genetic mutations (Bojana et al., 2002), liver cancer (Rie Matsushima-Nishiwak et al., 1992), allergic reactions (Geoffrey et al., 2010) and many other such reports have been witnessed.

There are many varieties of microcystin bio-molecule, about 80 analogs. These are cyclic proteins containing 7 amino acids with variable positions denoted as X, Z, R^1 and R^2. Different amino acids can be attached at the variable positions. This difference in the amino acid composition at the variable positions marks the basis of categorising the different varieties of Microcystin.

Microcystin variety.	X position amino acid.	Z position amino acid.	Molecular weight (KDa).
Microcystin LA	Leucine (L)	Alanine (A)	910.06
Microcystin YR	Tyrosine (Y)	Arginine (R)	1045.19
Microcystin RR	Arginine (R)	Arginine (R)	1038.2
Microcystin LR	Leucine (L)	Arginine (R)	995.17

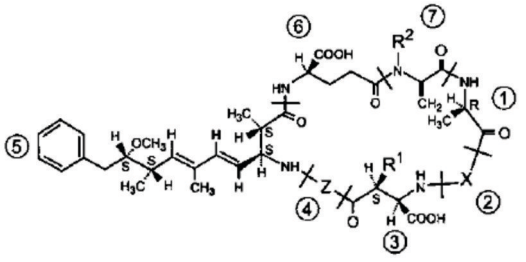

Figure: General structure of Microcystin.

The study was conducted to identify the potential toxin producing species of Cyanobacteria present in the water bodies of Ranchi (Jharkhand, Bharat), with major focus on the microcystin producing species. The genomic DNA of each purified cyanobacterial strain was subjected to PCR amplifica-

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tion and thus, detection of the gene sequence of *mcyE* gene. Morphological identification has become an old technique and can only be used to isolate the different species of the cyanobacteria for pure culture. For identification on genotypic level molecular-biology techniques are best to use. Detection based on PCR amplification is the most reliable method as it confirms the presence or absence of the specific protein or molecule under investigation on genetic level.

1. Materials and Methods

1.1. Sample collection

Water samples containing cyanobacteria were collected from different water reservoirs of Ranchi city, Jharkhand, in the months of November and December 2015 (Table 1). The samples were inoculated in BG12 media and allowed to grow in liquid as well as on solid media. The strains were isolated by the serial dilution method. The culture was incubated at 23ºC. Regular microscopic observations were made to ensure pure culture.

Table-1: Sites of sample collection.

Sl No.	Site of collection.	Cyanobacterial Species Identified and Isolated.	Geographical Location of source.
1	Dhurwa Dam, Ranchi	*Oscillator sp., Pseudoanabaena, Trebouxia, Microcystis floss-aquae, Microcystis aeruginosa, Anabaena sp.*	23.2937-85.259
2	Lalpur, Ranchi	*Tolypothrix, Pseudoanabaena, Oscillatoria sp., Nostoc sp., Chroococcus turgidus*	85.33774-23.37569
3	Ranchi Lake(Bada Talab)	*Anabaena sp.*	23.3668-85.318

2.2 DNA Isolation

DNA was isolated from purified cultures of cyanobacteria, using CTAB method (Deepak *et al*, 2014).

2.3. PCR

Primer Synthesis: Primers were synthesized using the NCBI Primer Designing Tool.

Amplification was performed in a 20 µL reaction volume, consisting of 9µL nuclease free water, 1µL Taq polymerase, 10 pM concentration of forward and reverse primers and 30ng template, in a PCR machine (BioEra). The thermal cycle was programmed for 2 minutes at 94 °C for initial denaturation, followed by 35 cycles of 30 seconds at 94 °C for denaturation, 30 seconds at 54°C for annealing, 40 seconds at 72°C for extension, and 5 min at 72°C for the final extension. PCR products were examined by Agarose Gel Electrophoresis at 100 volts for 30 min in a 1% (w/v) agarose gel in 1 x TAE buffer. PCR amplification of G3PDH gene in plant DNA that gives an amplification of 200bp (standardised at Aakriti Biotechnology laboratory) was used as a marker as the expected amplification is approximate 190bp for the test samples.

3. Result and Discussion

3.1. Sample isolation: Cyanobacteria samples were grown under provided condition as mentioned above. Single isolates were grown in liquid as well as solid BG 12 media. Regular microscopic observation confirmed the single type of cyanobacteria isolated (Figure: 1 and 2). Genomic DNA was isolated from these isolated cyanobacteria. An estimated 30ng of genomic DNA from each of the pure culture of cyanobacteria was used for PCR amplification of *mcy*E gene sequence. A G3PDH gene amplification was used as a gel marker for 200 bp amplification (primer are designed to amplify 200bp DNA fragment). 190bp amplification of *mcy*E gene from isolated cyanobacteria DNA was obtained which indicate the presence of *mcy* genes in the obtained isolates (Figure 3 and 4). Active expression of the *mcy* genes for microcystin production is possible from these strains, which need to be confirmed by expression profile studies.

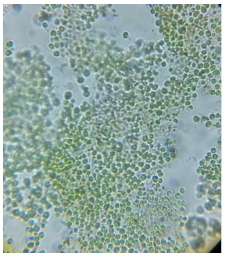

Fig-1: Pure isolate of *Microcystis aeruginosa.*

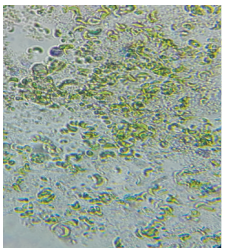

Fig-2: Pure isolate of *Microcystis floss-aquae.*

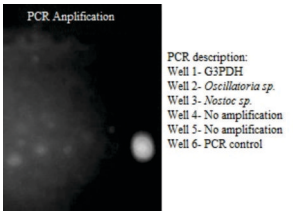

Fig-3

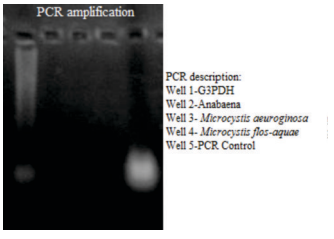

Fig-4

Cyanobacterial blooms can prove to be extremely hazardous to the local environment. However, fortunately the water reservoirs of Ranchi have not faced such situations till date. Since, our previous study reported that the reservoir houses Nitrogen-fixing species of Cyanobacteria, and in current study we identified toxin producing Cyanobacteria species also present in the reservoir, it can be a potential algal bloom site (Rahul et al., 2016). Further investigation about the nutrient content and other parameters such as pH and temperature variations need to be done in order to accurately predict the occurrence of algal bloom. Cyanobacteria presence in other water reservoirs needs to be investigated. . Although a report by Karl E H, 2008 indicates that cyanobacteria bloom elevates pH which can give a sub-lethal/lethal impact on fish population (Karl et al., 2008). The transparency of bloomed lake or reservoir reduces, which adversely affects the aquatic plants', phytoplankton's light requirement. Toxin producer leave lethal toxin effect on fish, zooplanktons, macro-invertebrate and aquatic vertebrates. The bloom and the toxin producing cyanobacteria give ultimate impact on the aquatic life of lakes and rivers. These toxins are lethal to probiotic bacteria which lead to the digestion related problems in the case of Humans (Ganesh et al, 2016).

A large percentage of population of Ranchi gets its household water supply from Hatia Reservoir in Ranchi, Jharkhand. If the level of microcystin in the water rises to an alarming level and not taken into consideration during purification process, it can prove to be an epidemic. Supply and thereby use of contaminated water can cause allergic reactions, tumor initiation and other severe implications depending upon response in different individuals, and health of a major part of population will be compromised.

There is a severe health risk, as pisciculture is carried in a

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floating fishing-facility in the waters of Hatia Reservoir. If total microcystin concentration ingested by a person reaches to 1.5µg/L (estimated on average water intake by an average person) by regularly having microcystin-infected fish, it can cause severe health issues (Anonymous). Moreover, high microcystin concentration can cause death of the fishes leading to economical loss to State (Francesca et al., 1994).

Proper measures need to be employed to keep an eye on the Cyanobacterial concentration per unit volume of water to avoid any unwanted incidents.

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We are thankful to Dr. A.S. Khalkho, Head of the Department, Centre for Biotechnology, Marwari College, Ranchi for inspiring us to work on toxicology of cyanobacteria. We also express our sincere gratitude towards her for allowing us to work under the esteemed and ever-inspiring guidance of Dr. Deepak Kumar, Director, Aakriti Biotechnology.

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