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Microcystis flos-aquae as major contributor of Microcystin-RR content in fresh water reservoirs of Varanasi, North India

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ABSTRACT

The toxic *Microcystis flos-aquae* strain was isolated from the local freshwater blooms and batch culture characteristics were observed in the modified J-medium. The isolation of toxic species was carried out by serial dilution and solid-agar plate adopting plate transfer method. Among the four culture media evaluated, modified Parker's J-media was found most suitable for batch culturing of *Microcystis flos-aquae* strains. The use of Na_2S and Na_2SO_3 in solid and liquid medium was found effective in reducing the contamination in culture and for providing better growth conditions as required for *Microcystis flos-aquae* specifically. The culture of isolated *Microcystis flos-aquae* showed the typical batch culture characteristics and the specific growth rate was in range of 0.02-0.40 d^{-1}. The 14 days old batch culture of isolate was used to estimate the toxic components, and besides several other variants, the microcystin RR (MC-RR) was dominantly present (0.16 % of its dry weight biomass) as confirmed by LC-PDA/MS. Thus it was observed in this study that the strain *M. flos-aquae* was responsible for the MC-RR contents in the local ponds of northern India.

Introduction

The toxic cyanobacterial blooms are well recognized environmental problem in all over the world. These blooms are major cause of deterioration of fresh water bodies in terms of turbidity, reduction or complete loss of transparency, oxygen depletion and by producing cyanotoxins responsible for diverse range of negative effects as cytotoxic, hepatotoxic, neurotoxic, carcinogenic, enzyme inhibition, oxidative stress etc. in living beings in direct contact (Fu et al. 2009; Puerto et al. 2009, Sieber and Marahiel, 2005; Burja et al. 2001; Carmichael, 2001). Recently, significant cytotoxic effects of microcystin was observed on the subcellular organization of kidney cell lines and the results revealed that cell viability decayed markedly after 24h of exposure to toxin (Alverca et al. 2009). In a recent study, Nasri et al. (2008) reported mortality case of turtles during *Microcystis* spp. bloom, which revealed the hazards of toxic blooms over living beings. There are so many pathway by which human being may be exposed to these toxins but the direct accumulation of microcystins in edible plant product such as water chestnuts as noticed in a recent study is a potential hazards for human health (Xiao et al. 2009). Besides *Anabaena*, *Aphanizomenon*, *Nodularia and Planktothrix* among the most common potentially toxic bloom forming genera, the *Microcystis* blooms are most severe in terms of toxicity and degrading water quality (Maske et al. 2009; Skulberg 1996; Atkins et al. 2001; Fastner et al. 2001; Jayatissa et al. 2006). The local ponds of the Varanasi city, India frequently support the massive *Microcystis* spp. bloom formation (Thakur 1996; Parker et al. 1996; Singh et al. 2001, Tyagi et al. 2006). In our pervious study conducted on five local ponds for their microcystins contents, it was reaffirmed that local blooms have rich amount of microcystins and the toxin profile for studied ponds has persistent domination of toxic species (Prakash et al. 2009).

To understand the basic biology, molecular systematics and the mechanism for cyanotoxin production, isolation and axenic culturing of cyanobacteria is crucial and mandatory. It was established that a single reservoir may contain several strains responsible for its toxin profile, but it is necessary to identify the most toxic strains for better characterization of toxins and their consequences, since in our earlier study it was found that the local water bodies in Varanasi were heavily contaminated with *Microcystis* spp. specially *Microcystis aeruginosa*, we have made attempts for other toxin producing strains. However, there are several evidences for the presence of more than one morphospecies (toxic and non toxic) of *Microcystis* in water reservoirs (Komárek 1991). Therefore, the study regarding isolation, identification and culturing of local morphospecies of *Microcystis* and determination of their microcystin content may provide better insight to ascertain the particular toxin and their consequences. In the present study, isolation and culturing of toxic *Microcystis flos-aquae* from local ponds of Varanasi, was carried out and an estimation of the microcystin content was determined by LC-PDA/MS.

Materials and Methods

Culture Media

Four culture media BG-11 (Allen, 1968), Chu-10 (Chu, 1942), MA (Ichimura, 1979) and modified Parker's J-medium (Parker, 1982) were evaluated for culturing efficacy of local *Microcystis* isolates. For selective screening of *Microcystis* spp. clones, the J-agar medium as suggested by Parker (1982) was used with slight modification. The pH of the medium was initially adjusted to 7.8. The five freshly prepared constituents; Na_2SO_3 (0.25, 0.50, 0.75 and 1.00 mM), $CuSO_4.5H_2O$

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(0.05 mg l^{-1}), Co $(NO_3)_2.6H_2O$ (0.15 mg l^{-1}), $Al_2K_2(SO_4)_4.24H_2O$ (0.45 mg l^{-1}) and Na_2S (1.2 mM solution) were autoclaved in separate and added to the medium aseptically after cooling to the room temperature. Four different concentrations of Na_2SO_3 were used to optimize its supportive concentration for *Microcystis* spp. growth. For the selective inhibition of contaminating bacteria, sodium sulphide (Na_2S) was added to the J-agar medium by applying it to the surface in petriplate, immediately after inoculation. For removal of fungal contaminants, cyclohexamide 25µg ml^{-1} was used in liquid and solid form of medium.

Isolation and purification of *Microcystis* species

Microcystis spp. blooms were collected from different freshwater ponds of Varanasi city (25.5º N 82.9º E, elevation 279 ft / 85 m) and were identified as per systematic keys given by Desikachary (1959). Adhering mud and other planktons were removed carefully, and the specimen was washed with sterile distilled water (Milli Q). In order to reduce contaminants, two-step centrifugation of inoculums (150×g for 30 min, at room temperature, followed by higher-speed centrifugation; 1,000 to 4,000×g for 5 min, at room temperature) and repetitive sub-culturing of single colony in solid and liquid culture media alternately, were made as suggested by Shirai et al. (1991). *Microcystis* colonies were disaggregated by vortex mixing in deionized water, as suggested by Parker (1982). After the two-step centrifugation of inoculums, the surface layer was withdrawn with micropipette and inoculated on modified J-agar medium. After incubation of 10 to 12 days in light (22 µE m^{-2} S^{-1}), a contaminant free individual colony was selected with the help of fine bore glass capillary, under binocular microscope and transferred to culture tubes containing 10 ml modified J-medium.

Incubation and maintenance of cultures

The culture was maintained by regular sub-culturing in Parker's J-medium. Cultures were incubated in culture rooms (25±1ºC) illuminated with full spectrum fluorescent lights. Fluorescent lamps (Sylvania 40-W cool white) were used in culture rack to provide the light energy necessary for photosynthesis. The average light intensity at the surface of the flasks was measured using a lux-meter (TES1332 digital lux-meter) and kept at 22.5 µE $m^{-2}s^{-1}$. In addition, for the microcystin determination the batch cultures of *Microcystis* sp. were maintained separately in 500 ml flask with 200 ml J-medium. The cultures were shaken manually 3 to 4 times each day.

Growth measurement

A batch culture experiment with isolated *Microcystis* spp. was conducted at room temperature in 100ml Erlenmeyer flasks (14×3 flasks), with working volume of 35 ml. Each flask was inoculated at 5×10^4 cells ml^{-1} of the prepared inoculums. Typical growth experiments were carried out in triplicates with the flasks being agitated manually 3 or 4 times in a day. To assure the same light conditions throughout the experiment, the flasks were placed at exactly the same distance from the light source and at the same height. Growth was monitored after 1 day interval by both cell counting in hemocytometer and by estimating Chl-*a*. One set of culture (three flasks) were used for estimating cell number and Chl-*a*, while other set was used to estimate the microcystin content. For Chl-*a* estimation, the cultured biomass were extracted in 5.0 ml of 80 % acetone and kept overnight at 4 ºC in refrigerator. Thereafter, it was homozenized and centrifuged at 4000 rpm for 10 min. The final volume of the supernatant was made to 25 ml with 80 % acetone and the absorbance of extract was read at 663 nm using Spectronic-20 colorimeter (Bausch and Lomb). Chlorophyll *a*, content was calculated according to the formula proposed by Talling and Driver (1963) with the modification in extinction coefficient (12.7) of Chl-*a* at 663 nm as given by Mackinney (1941).

$$\mu g\ Chl\text{-}a\ per\ sample = 12.7(D_{663})\ V/L,$$

Where, D_{663} = absorbance (OD) reading at 663 nm, **V** = volume of acetone extract in ml, and **L** = length of spectrophotometer cell *i.e.* 1 cm.

Specific growth rate (µ) of the batch culture was determined using following equation, according to its definition (APHA, 1998)

$$\mu.d^{-1} = \ln(X_2\text{-}X_1)/\ t_2\text{-}t_1$$

Where X_2 = biomass concentration *i.e.* (cells no. ml^{-1}) or Chl-*a* (µg ml^{-1}) at the end of selected time interval; X_1 = biomass concentration at the beginning of selected time interval; $t_2 - t_1$ = elapsed time between selected interval, d.

Characterization and quantification of microcystin

The batch culture of the *Microcystis* sp. in exponential phase was used for microcystin extraction. After centrifugation (5000 rpm; 10 min) of the culture the pellet was extracted with 80% methanol (5 ml). For proper extraction three times extractions were performed and supernatant were pooled before vacuum evaporation. The samples with methanol were sonicated for 5 min and subsequently shaken for 45 min during each extraction step. After centrifugation (at 5000 rpm, 15 min.) the supernatants were pooled and concentrated at 42 ºC by vacuum centrifugation. The extracts were re-dissolved in 80 % HPLC grade methanol and before HPLC analysis the samples were filtered through 0.22 µm cellulose filter.

Identification and quantification of microcystins by LC-PDA/MS

The extracted samples were analyzed by HPLC with photodiode array (PDA) and mass (MS) detection. A Waters Alliance 2695 solvent delivery system equipped with a 2996 PDA and ZQ 2000 MS detector (in series) was used for all analysis (Waters, Elstree, UK). Separation was carried out on a Sunfire C_{18} column (2.1 mm id × 150 mm long; 5 µm particle size) which was maintained at 40 ºC. Mobile phase was Milli-Q water (A) and acetonitrile (B) both containing 0.05 % trifluoroacetic acid (TFA). Test samples (20 µl) were separated using a gradient increasing from 15 % to 60 % B over 25 min at a flow rate of 0.3 ml min^{-1} followed by ramp up to 100 % B and re-equilibration over the next 10 min. Eluent was monitored from 200-300 nm with a resolution 1.2 nm and peptides were quantified by external standard at 238 nm. MS analyses were all performed in positive ion electrospray mode, scanning from *m/z* 100 to 1600 with a scan time of 2s and inter-scan delay of 0.1s. Ion source parameters, sprayer voltage, 3.07 kV; cone voltage, 70 eV; desolvation temperature, 300 ºC; and source temperature, 100 ºC. Instrument control, data acquisition and processing were achieved using Masslynx v4.0. Cone-induced dissociation (CID) mass data were obtained using a cone voltage of 100 eV to obtain sufficient fragmentation for identification.

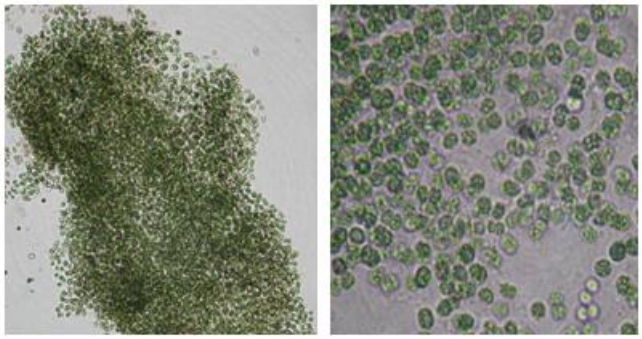
Results

Isolation of *Microcystis* sp. from local pond

Unialgal culture of isolates was obtained by single colony isolation procedure for *Microcystis flos-aquae via* repeated isolation and subsequent sub-culturing in solid and/or liquid medium. A series of steps has been taken to ensure pure culture of *Microcystis flos-aquae* species. This includes a two-

step centrifugation as mentioned in material methods, Disaggregation of *Microcystis* colonies by vortex mixing in deionized water, and addition of Na_2S solution drops over the surface of J-agar medium to eliminate mucilage bound bacteria and other contaminations. In addition Cyclohexamide, was also selectively inhibited the fungal contaminations specifically. As compared to 0.25 and 0.50 mM concentration, 0.75 mM solution of Na_2SO_3 was found effective for promoting the growth of *Microcystis flos-aquae*. Identification of cultured organisms was done by culturing in N_2 deficient medium as well as by microscopic observation. The microscopic view of the isolated organisms was similar to that of *Microcystis flos-aquae* as per Desikachary (1959). Besides *Microcystis flos-aquae*, few strains were also isolated which were identified as *Aphanocapsa* sp., *Aphanothece* sp. and two other unidentified unicellular cyanobacterium. The photographs of cultured organisms in solid and liquid medium are given in Fig.1. The microscopic views of isolated *Microcystis flos-aquae* are depicted in Fig.2.

Figure1. Pure batch cultures of isolated *Microcystis flos-aquae* in solid and liquid J-medium.



(A) (B)

Figure2. Microscopic view and colony characteristics of Microcystis flos-aquae (Magnification 10× and 40×).

Growth of *Microcystis* sp. in culture

In batch culture experiment, only J-media found to be supportive for the growth of *Microcystis flos-aquae*. The growth curve plotted for biomass estimation (Chl-*a* estimation and cell count per ml) showed typical growth curve pattern of batch culture (Fig. 3). The first three days after inoculation represent no marked increase in biomass which represented the lag phase of the characteristic batch culture. Thereafter, a steady growth in biomass was observed indicating that the culture enters into an exponential growth phase. During 8 days of exponential phase, biomass increased in steady state as a function of time (days). Though, steep increase in the biomass recorded during late exponential phase (10 to 12 days). Specific growth rate for the batch culture was calculated by the APHA (1998) method, and was found minimum (0.02 d^{-1}) for stationary phase and maximum (0.40 d^{-1}) for exponential phase (Table 1).

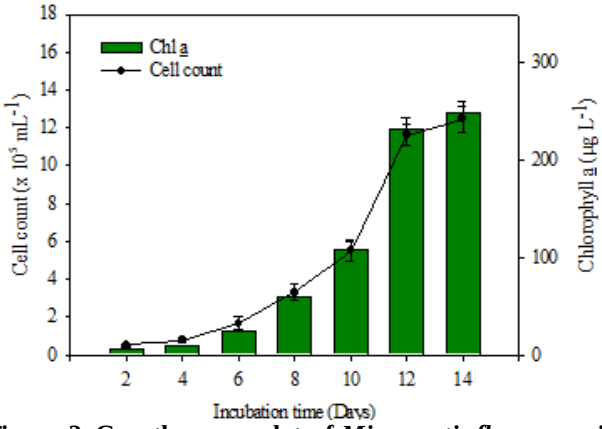


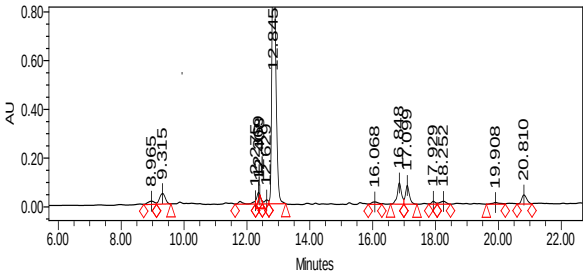
Figure 3. Growth curve plot of *Microcystis flos-aquae* in batch culture.

Table 1. Specific growth rate of *Microcystis flos-aquae* in batch culture.

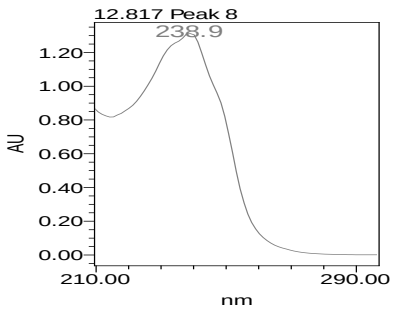
Incubation time (days)	2nd	4th	6th	8th	10th	12th	14th
Specific Growth rate (µ)	0.21	0.18	0.40	0.32	0.30	0.36	0.02

Detection of microcystins by LC-PDA/MS

LC-MS analysis of the batch culture samples confirmed the presence of MC-RR as the foremost toxin in isolated *Microcystis flos-aquae* strain. The LC-PDA chromatograms for standard mixture and culture samples were given in Fig. 4. The presence of prominent peak at retention time 12.8 min and characteristic PDA spectrum of that peak led to the identification of microcystin as MC-RR. In the ESI-MS (Fig. 5) of the sample, the characteristic molecular ions having 1038 and 520 *m/z* confirmed the identity of MC-RR. The amount of MC-RR was determined by peak area calculation against standard MC-LR, and found 1618 μg^{-1} dry weight of the culture sample.



(A)



(B)

Figure 4. Analysis of aqueous methanolic extract by HPLC-UV (A) and PDA-spectra (B) for the *Microcystis flos-aquae* culture. PDA spectra represents typical spectrum for MC-RR

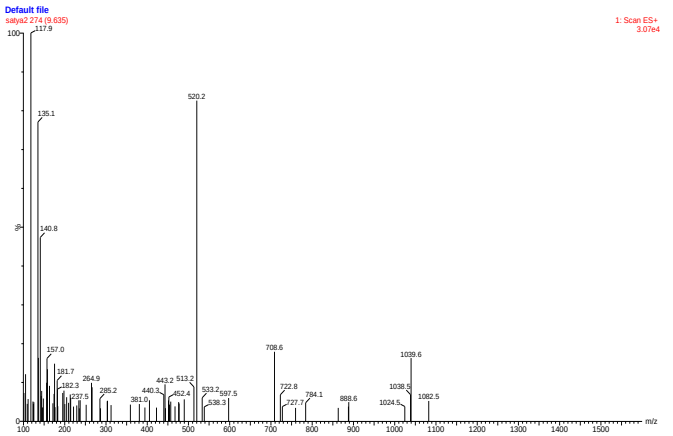


Figure 5. CID mass spectra of *Microcystis flos-aquae* culture with characteristic fragment ions at *m/z* 520 [M+2H] $^{2+}$ and 1038 [M+H] $^{+}$, corresponding to standard MC-RR.

Discussion

Water bodies throughout the world suffer with cynobacterial toxin and associated consequences to the living beings. In India, water reservoirs are often open for all community utilizations, in addition to irrigation, thus contamination of water with cyanotoxins and related toxicosis are more frequent in this part of the world. A survey in this regard has been done in the present study to find out the toxigenic cyanobacterial strains, in the local water reservoirs of Varanasi district, in northern India. These local water reservoirs was found heavily contaminated by *Microcystis* spp. which was successfully isolated and cultured in laboratory from a sample collected from the Luxmikund pond. The isolate was identified as *Microcystis flos-aquae* on the basis of the liquid culture behavior and microscopic view as per standard taxonomic keys such as the dispersed cells, cell size and non clatharate colonies which are identical to the *Microcystis flos-aquae* as per Desikachary (1959). The pure culture of *Microcystis* sp. is always problematic because the copious and tenacious capsule surrounding *Microcystis* colonies traps contaminants this make pure isolation of *Microcystis* strains very tedious (Shirai et al. 1991) and implicated. Thus the disaggregation of the capsule is a crucial and determining step for isolation and cloning procedure. In the present study repeated washings with sterile medium and repeated passages yielded a unialgal strain grown in the form of single cells. The disaggregations of the mucilaginous capsule of *Microcystis* colonies by double distilled water as suggested by Parker (1982) enabled the elimination of trapped contamination and facilitate the isolation. Two step centrifugation as suggested by Shirai et al. (1991) also effective in the removal of contamination. The first slower centrifugation step allows the cells containing gas vesicles to float on the surface of the medium and thus facilitate reduction in contamination. The contaminations were further reduced after the second step (higher speed) of centrifugation. But the higher speed centrifugation without prior low speed centrifugation was ineffective as it decreases the number of *Microcystis* cells in the surface layer. Due to positive buoyancy of *Microcystis* spp. (Castenholz, 1989) the two steps of centrifugation left sufficient number of *Microcystis* cells at surface and spun down the contaminations (Shirai et al. 1991). The preferential survival of re-contaminated culture over the isolated culture also make difficult to the cloning of cyanobacteria (Lange, 1970). The addition of sodium sulfite (Na_2SO_3) into J-agar media facilitates the *Microcystis* growth. According to Parker (1982) addition of Na_2SO_3 in the J-media stimulates the *Microcystis* sp. growth and overcome the need of heterotrophic bacteria (*E. coli*) in medium. The additional stimulation of growth by sodium sulfite might be due to protection of cyanobacteria against photo-oxidation (Kruger et al. 1981) and lowering of reduction potential of the medium which also has been observed in enhancement of the growth of certain cyanobacteria (Weller et al. 1975). Paerl and Kellar (1978) also advocated the need of reducing environment around the cyanobacteria for their proper growth which was maintained by heterotrophic bacteria associated with them. The selective inhibition of contamination through the use of sodium sulfide (Na_2S) solution and cyclohexamide in solid medium confirmed isolation of pure *Microcystis flos-aquae* in the present study. The rich Na_2S region of the media associated with poor growth of contaminations were preferential site for the growth of *Microcystis* clones, since Na_2S formed a concentration gradient as it diffused into the agar, this made easier selection of contamination. Cyclohexamide being a potent antibiotic of protein synthesis in eukaryotes inhibited the growth of fungal contaminations specifically. The use of several other approaches were also used to produce pure axenic cultures Such as the mechanical separations of the cyanobacteria from bacterial contaminants; by micromanipulation (Bowyer and Skerman, 1968), differential filtration (Heaney and Joworski, 1977; Meffert and Chang, 1978) and repeated transfer of cells (Vaara et al. 1979). In the present study repeated transfer of cells was tried and it was found significant in removal of contaminations. The second approaches involved the use of an agent which is relatively harmless to the cyanobacteria but is toxic to the bacterial contaminations. The previous studies also suggested to use agents like phenol (Carmichael 1974; McDaniel et al. 1962), sodium hypochlorite (Fogg, 1942), detergents (McDaniel et al. 1962), elevated temperature (Allen and Stanier, 1968; Wieringa, 1968), UV radiations (Allison and Morris, 1930; Kraus, 1966) and antibiotics (Pinter and Provasoli, 1958; Rippka, 1988; Vaara et al. 1979) for the effective removal of contaminations. In the present study we used sodium sulphide and it was found useful to select contamination free isolate. In the present study, batch culture was chosen to study the culture conditions of *Microcystis flos-aquae*. Several literatures evidenced the use and advantages of batch cultures. In batch culture cells can be exposed to the same potential growth stress with multiple limitations as in nature and furthermore, a large number of parallels can be run in a short time (Lyck, 2004). Long et al. (2001) pointed out that batch culture available obvious conditions during exponential phase at m^{max} and during stationary phase at m^0. In nature, cyanobacterial populations often maintain high growth rates for longer period, because continuous cell division would be limited by exhaustion of available nutrients, CO_2 or unavailability of photo- synthetically active radiation (PAR) (Lyck, 2004). In the present investigation *Microcystis flos-aquae* batch culture provid a typical growth curve having three to four days of preparatory phase (lag phase) followed by eight days exponential growth (log phase). The least generation time (nearly 1.5 days) during the exponential phase represented the characteristic of cyanobacterial growth (Vander Westhuizen and Eloff, 1985). LC-MS analysis of the samples confirmed the presence of MC-RR as the foremost toxin variant in the culture. The LC-PDA spectrum of the prominent peak identified microcystin variant as MC-RR. The ESI-MS of the sample on behalf of characteristic molecular

ion fragments having *m/z* 1038 and 520, confirmed the identity of MC-RR. In previous studies conducted on five local ponds including the present one, it was observed that the MC-RR was the predominant toxin (Prakash et al. 2009) and the amount of MC-RR observed in batch culture was found comparable to that of field study, so it can be assumed that the isolated strain *i.e. Microcystis flos-aquae* might be the major responsible cause of MC-RR production in ponds. Further, it was also observed that the local fresh water blooms are producing other microcystin variants such as MC-LR, MC-AR, MC-WR, MC-AHar and [DHa^7] MC-RR in addition to MC-RR; further studies will be needed to explore the presence of other species/strains of *Microcystis* in local ponds. As in field bloom samples, *Microcystis aeruginosa* is also the dominant species, the isolation of *Microcystis aeruginosa* in culture must be undertaken to observe its microcystin variants. Further, ahead to single toxic variant, the diverse microcystin variants profile, which has been observed as a typical trend of local blooms, could grant additional vulnerability in comparison to recognized most toxic variant MC-LR as noticed in some recent studies (Ghazali et al. 2009). A series of reports narrating apoptosis and necrotic potential of MC-RR (Huang et al. 2009) and its bioaccumulation in aquatic food wave (Zhang et al. 2009) as well as in body tissues such as liver, brain, kidney, ovary, muscles etc. (Papadimitriou et al. 2009); alarming WHO to reconsider the tolerable intake limit for these toxins.

The successful isolation and culturing of the local toxic isolate revealed that *Microcystis flos-aquae* is causal victim to produce high amount of microcystin particularly MC-RR. Our results especially the isolation of toxic *Microcystis flos-aquae* is additional as previous studies have accounted only *M. aeruginosa* for the appearance for local blooms. The study confirms that *M. flos-aquae* are the responsible strain for the higher content values of MC-RR in local ponds, besides *M. aeruginosa*. The level of toxin observed in batch culture was in higher range and provided an alarm for potential hazards after long term chronic exposure of toxic blooms to local population. The study also opens the probability for further studies regarding the development of molecular markers for local isolate of *Microcystis flos-aquae* and also for identification of environmental factors responsible for higher production of microcystins.

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