# Cylindrospermopsin toxin production by cyanobacterial isolates from Kor River and evaluation of their effect using bioassay technique

**M. Zare[1], N. Bahador[2]***

[1]Department of Microbiology, Science and Research Branch, Islamic Azad University, Fars, Iran
[2]Department of Microbiology, Faculty of Science, Shiraz Branch, Islamic Azad University, Shiraz, Iran

*Corresponding author, E-mail: bahador@iaushiraz.ac.ir

**Abstract**

ABSTRACT: Cyanobacteria are a group of photosynthetic prokaryotes distributed worldwide. They are able to produce a wide range of secondary metabolites with diverse biological effects. he aim of this study was isolation and identification of cyanobacteria from Kor River waters and estimation of cylindrospermopsin toxin production. In total, 30 water samples were collected from six stations from Kor River. The samples were serially diluted ($10^{-6}$) and then cultivated on BG 11 medium and incubated under illumination of 1500 to 2000 lux at 28 ± 2 °C with 12/12 h light/dark regime. Developed colonies were isolated on new media. For molecular identification, genome of each purified isolate was extracted by a modified DNG kit. A set of primers was used for PCR analysis, universal primer (CYA78IR/CYA359F) for Cyanobacter detection, and a set for detection of cylindrospermopsin synthesis (cynsulfF, cylnamR). In addition, the toxin was extracted and its effect was evaluated using bioassay technique. Cylindrospermopsin toxin was detected in one of three isolates. Toxicity testing on Wistar rats showed flaccid paralysis and dyspnea. Overall, as cyanobacteria toxin threatens human and animal life, it is necessary to be aware and evaluate waters of this geographical area.

**Key words:** cyanobacteria, cylindrospermopsin, molecular identification, bioassy, Kor River.

## Introduction

Cyanobacteria or blue-green algae are well known organisms and represent one of the first oxygen-producing organisms on earth. Cyanobacteria belong to a group of oxygenic photoautotrophic gram negative bacteria (Boopathi, Ki 2014) with diverse morphology, including solitary, free-living cells or colony-forming, filamentous forms (Catherin et al. 2013). They are an ecologically flexible group, and can grow in all conditions. They are common in almost all environments including soil, rivers and other freshwater basins, and springs (Tiwari et al. 2005).

Occurrence of cyanobacterial blooms has harmful effects on aquatic life because of toxin production by cells (Hakansonet al. 2007). Toxic cyanobacteria are mostly classified into three groups based on their mode of action: hepatotoxic, neurotoxic and dermatotoxic (Boopathi, Ki 2014). Depending on genera, the cyanotoxins include cyclic peptide hepatotoxins, such as nodularin and microcystins, and alkaloid neurotoxins like anatoxin-a, cylindrospermopsin and saxitoxin (Wiegand, Pflugmacher 2005). Microcystins cause hepatosis, skin irritations, diarrhea and several other injuries (Chernoff et al. 2002; Martins et al. 2005). These toxins inhibit protein phosphatase activity, especially types 1 and 2A, in a similar way to the action of okadaic acid (Dawson 1998). More than 70 different analogues of microcystins have been described from natural blooms or laboratory cultures of cyanobacteria (McElhuney, Lawton 2005).

On the other hand, cylindrospermopsin is a polyketide-derived alkaloid with a central guanidine moiety and a hydroxymethyl-uracil attached to the tricyclic carbon skeleton (Muenchhoff et al. 2010). Its toxicity is due to inhibiton of glutathion and protein synthesis as well as inhibition of cytochorome P450-related activity (Mihali 2007). After the tragedy involving cyanobacteria bloom toxicity in 1996 in Caruaru city, Brazil, the Brazilian Ministry of Health announced a guideline for cyanotoxin monitoring in water suppliers (Kujbida et al. 2006). As the presence of cyanotoxins in water, even at low concentration, can threat human life, the present study was initiated to isolate and identify cyanboacteria with potential capability to produce toxins from Kor River, Fars Province, Iran.

## Materials and methods

### *Sample collection and isolation of cyanobacteria*

In this study, 30 river water samples were collected from six stations in the Kor River located in Marvdasht, Fars province, Iran. The samples were collected in sterile glass tubes from a depth of 30 to 50 cm at each station during September to October, 2014. At collection times, temperature and pH of

water were measured by mercurial thermometer and pH meter, respectively. The samples were serially diluted ($10^{-6}$) and cultivated on Petri dishes on BG11 medium containing $MgSO_4$, $CaCl_2$, $NaNO_3$, $K_2HPO_4$, $Na_2CO_3$, $Na_2EDTA$ and composition of trace elements in a form of $H_3BO_3$, $MnCl_2$, $ZnSO_4$, $NaMoO_4$, $CuSO_4$ (Graham et al. 2008; Newcombe et al. 2009; Ebli et al. 2013). The dishes were incubated under illumination of 1500 to 2000 lux with 12/12 h light/dark regime at 28 ± 2 °C.

Continuous sub-culturing was carried out on the same medium to obtain pure cultures. The culture purity was investigated under a light microscope (Mehrjardi et al. 2011; Rajeshwari et al. 2011; Karimi et al. 2013). After 2 to 3 weeks, colonies of cyanobacteria appeared on the medium and colonies differing in morphology were selected and purified through by cultivation on the new media.

*Identification of isolated cyanobacteria*

For phenotypic identification of the isolates, colony shape, cell diameter and mucilage presence of the isolates were evaluated. In addition, the identity of isolates was confirmed using gram-specific staining (Zarrini et al. 2012).

For molecular identification, DNA of the isolates was extracted by a DNG Kit according to manufacturer instructions (Sinagen Company, Iran). Then, spectrophotometry was used to determine purity of the extracted DNA. 16S rRNA gene analysis were performed for molecular identification (Namikoshi et al. 2003; Zarrini et al. 2012). For this purpose two types of primers (CYA359F, CYA781R) with 487 bp length were used for general identification of cyanobacteria (Maniglia et al. 2010). Primers, target genes and the required thermal profiles of PCR procedure are shown in Table 1. Synthesis of cylindrospermopsin by the isolates was determined with cynsulfF and cylnamR primers (578 bp) in the PCR process (Kokocinski et al. 2013; Table 1). Finally, amplified PCR products were separated by electrophoresis on an agarose gel 1.5% in TAE buffer. The electrophoresis was performed for approximately 45 min at 70 mA and visualized by staining with ethidium bromide solution (0.5 μg $mL^{-1}$). The PCR product was investigated by a transilluminator device.

*Toxin extraction and bioassay technique*

Extraction of the toxin was performed using pure cultures. For this purpose, 500 mg of cells was collected from BG11 agar plates, grown at 28 ± 2 °C for 7 days, and then the cells were lysed by freeze-thawing in an extraction solvent (0.05 M acetic acid) and subjected to sonication (Rositano et al. 1998).

Toxicity was tested by intra peritoneal injection (0.5 mL) of cell lysate of cyanobacteria to Wistar rats. The animals were observed for 24 h and the response to injected toxin was evaluated (Agarwal et al. 2012). The prior approval of the Ethics committee of the Islamic Azad University was obtained to perform experiments with laboratory animals.

## Results

*Morphological characterization of the isolated cyanobacteria*

In this study, various phenotypes were identified based on their morphological characteristics such as colony shape, cell size, presence or absence of heterocysts and akinetes. In total, three morphotypes were isolated from Kor River and they were coded as C1, C5 and C8 (Fig. 1 A). After transferring and purification of the colonies on fresh BG11 medium (Fig. 1 B and C), the colonies were observed under light microscope. C1 colonies had blue-green, convex, circular, shiny, smooth cells with spherical shape. C5 had elliptical cells with a mucilaginous sheath that showed a symmetrical division by binary fission. In contrast, the colonies of C8 were pale blue-green, string-like on medium with filamentous form (Fig. 1).

*Molecular analysis*

The results obtained from PCR using the primers CYA359F, CYA781R with 487 bp length confirmed the presence of cyanobacteria (C1, C5 and C8) in Kor River (Fig. 2). In addition, cylindrospermopsin synthesis gene PCR results are shown in Fig. 3. Among the isolates, the presence of cylindrospermopsin toxin synthesis was confirmed only in isolate C8.

**Table 1.** Primers for cyanobacteria and toxin synthesis detection and respective PCR conditions

| Target gene | Primers & amplicons | Function | Temperature profile |
|---|---|---|---|
| 16S rRNA | CYA359F 5'-GGGGAATYTTCCGCAATGGG-3' | Cyanobacterial | 94 °C 3 min |
| | CYA781R 5'-GACTACWGGGGTATCTAATCCCWTT-3' | detection | 94 °C 20 s |
| | 487 bp | | 50 °C 20 s 40 cycles |
| | | | 72 °C 40 s |
| | | | 72 °C 5 min |
| Gene for | cynsulfF 5'-ACTTCTCTCCTTTCCCTATC-3' | Cylindrospermopsin | 94 °C 3 min |
| sulfotransferase | cylnamR 5'-GAGTGAAAATGCGTAGAACTTG-3' | synthesis | 94 °C 10 s |
| | 578 bp | | 57 °C 20 s 35 cycles |
| | | | 72 °C 60 s |
| | | | 72 °C 7 min |

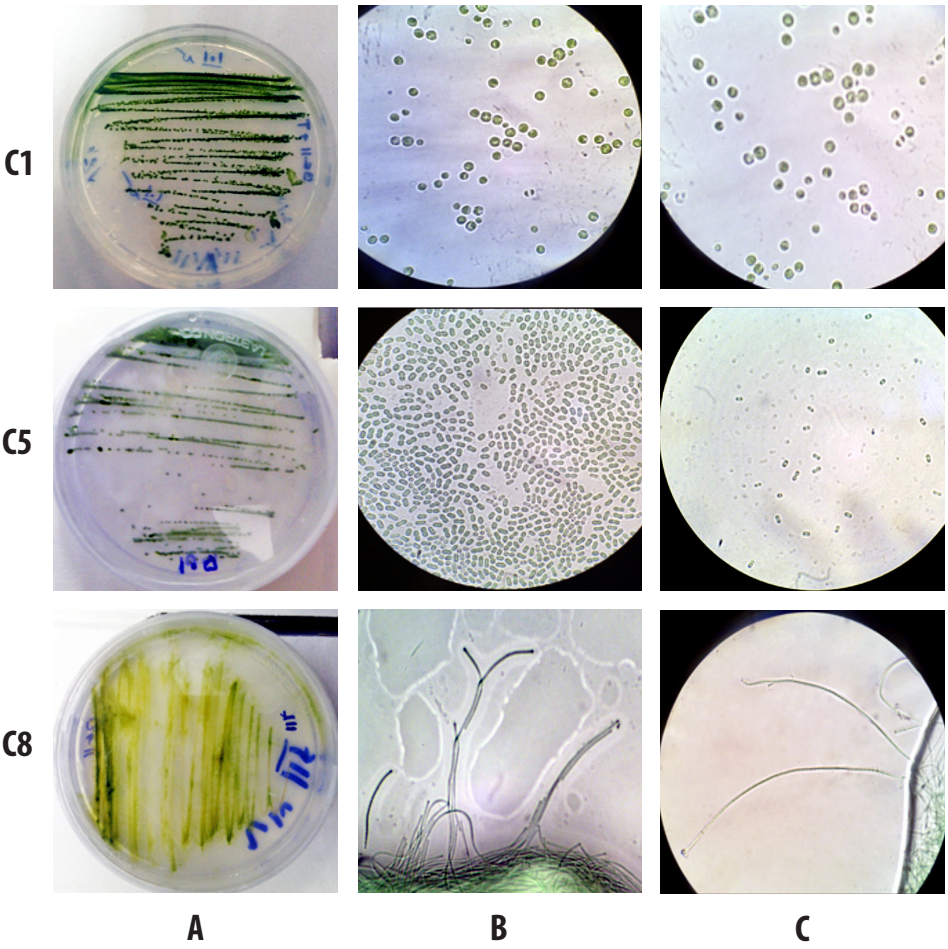


**Fig. 1.** Colonies (A) and microscopic morphology of pure cultures (B, C) of cyanobacteria isolated from Kor River. C1, C5, C8, different isolates.

*Bioassay*

In the present study, rat bioassay was used for testing toxicity of cyanotoxins by intra-peritoneal injection of the cell lysate of cyanobacteria. The results obtained indicated that after first minutes of injection the test group showed paraplegic paralysis, severe giddiness, imbalance and also breathing disorder. After 15 min, the rat fainted in a corner of the cage, and closed his eyes. Finally, the stage of fainting gradually passed, and during the next 24 h no death was observed in the experiment group. No changes were observed in the control group of rats.

## Discussion

Cyanobacteria are an interesting group of ancient phototrophic prokaryotic organisms. The organisms are

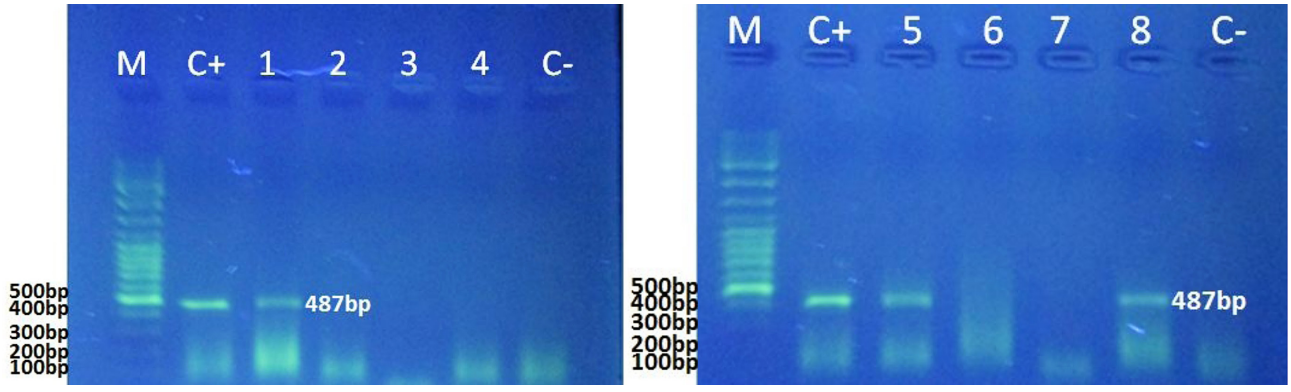


**Fig. 2.** Agarose gel electrophoresis of PCR products from fragment of 487 bp, using the CYA78IR/CYA359F primers. M, 100 bp ladder; C+, positive control *Microcystis aeruginosa*, C–, negative control. 1 to 8, separate cyanobacteria isolates.

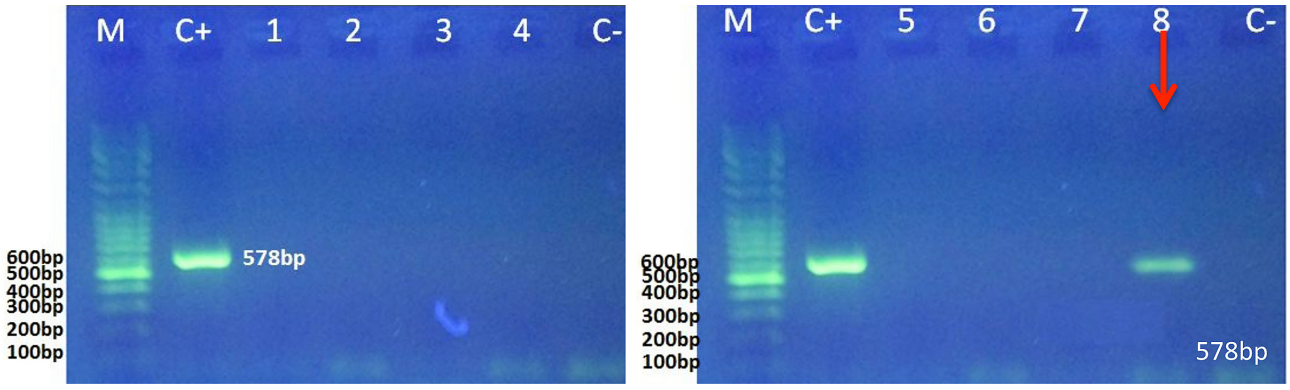


**Fig. 3.** Agarose gel electrophoresis of PCR products from fragment of 578 bp, using the cynsulfF/cylnamR primers. M, 100 bp ladder; C+, positive control *Cylindrospermopsis raciboroskii*, C–, negative control. 1 to 8, separate cyanobacteria isolates. An arrow indicates a toxin-synthesizing isolate.

found in several different habitats and are characterized by capability to produce toxic secondary metabolites. Among the cyanobacterial species, *Microcystis, Anabaena, Nodularia, Planktothrix, Aphanizomenon, Cylindrospermopsin* and *Lyngbya* are the most important toxin-producing genera (Agarwal et al. 2006). As the toxins can be potentially harmful for aquatic animals, fishs, cattle and even humans (Roegner et al. 2014), the present study was initiated to isolate cyanobacteria from Kor River and evaluate the presence of toxins. For this purpose, 30 water samples were collected from different locations of Kor River. One of three isolates had capability to synthesize cylindrospermopsin, which was confirmed by molecular techniques.

It has been reported that tricyclic alkaloid cylindrospermopsin is produced by Cylindrospermopsis raciborskii strains from Australia (Saker, Griffiths 2000) and Thailand (Li et al. 2001). This compound causes severe liver damage in mouse bioassay (Hawkins et al. 1997) with symptoms clearly distinguishable from those of some other cyanobacterial hepatotoxins, such as nodularin and microcystin. The toxin poses pathological symptoms in the liver, spleen, thymus, kidney and heart (Terao et al. 1994; Hawkins et al. 1997; Kokocinski et al. 2013). Furthermore, several studies have shown genotoxicity of cylindrospermopsin causing DNA strand breakage (Kokocinski et al. 2013) and DNA injury in non-liver cells (Zegura et al. 2011). Additionally, mutagenic and carcinogenic effects of cylindrospermopsin also have been demonstrated (Falconer, Hampage 2001; Bazin et al. 2010). Therefore, a water quality guideline value of 1 μg $L^{-1}$ has been proposed for the toxin (Shaw et al. 2000). Moreover, the bioassay test in this investigation showed different responses including paraplegic paralysis, severe giddiness, imbalance and also breathing disorder after first minutes of injection. Consequently, the presence of cyanobacteria with capability to produce toxin in this geographical area indicated the necessity to perform detailed studies including more sites during several seasons.

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