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Study on destruction of extracted toxins from isolated Cyanobacteria using titanium dioxide

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ABSTRACT

Cyanobacteria are major group of microorganism with capability to produce different types of toxin. Indeed, anthropogenic eutrophication of surface water is the main reason for acceleration of their growth and ecological problems. Therefore, the present study tried to isolate toxic cyanobacteria from Kor River, Fars province. For this purpose, 18 water samples were collected from three sampling stations of Kor River, namely: Rah-Ahan Bridge, Esfandaran and Khan Bridge stations. The samples were serially diluted and cultivated on BG11 media under illumination of 1500 to 2000 lux with 12/12 h light/dark regime at $28 \pm 2^\circ\text{C}$. Then the colonies were purified and molecularly identified using three sets of primers. The toxins were extracted according to Anjos protocols and confirmed by HPLC. Finally, effect of titanium dioxide under ultraviolet lights with a wavelength of 450-330 nm for half an hour evaluated. The results obtained from this study indicated that 4 different isolates were detected from sampling sites. The isolates had capability to produce toxins (Microcystin, Cylindrospermopsin) and their presence were confirmed by HPLC. In addition, the toxins were affected by titanium dioxide. Although the titanium dioxide has detoxifying ability, evaluation of different concentration of titanium dioxide on different types of toxins is suggesting.

1. Introduction

Cyanobacteria (blue-green algae) belong to the group of organisms which share the same characteristics with bacteria and algae. These photosynthetic microorganisms are found in different environments including oceans, freshwater, rocks and soil, hot springs, deserts and polar territories (Whitton et al., 2012). The organisms are photosynthetic, with simple, non-nucleated cells and contain bacterial characteristics. These old organisms cooperating in atmospheric oxygen and like algae contain chlorophyll and other pigments, which may

range from blue to green (Rasmussen et al., 2008). The colors might include light cyan, grass green, dark brown pigments and when expose to bright sunlight it shows gray and white pigments.

Microscopically, cyanobacteria can be seen as mono cellular or chain organisms, some single-cell cyanobacteria, such as microcystis, can grow and appear in the form of colony. Colonies may be irregular and uncovered, which are continual and several millimeters thick, in a row. Some cyanobacteria can fix nitrogen by

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specific cells. In addition, some species can control their specific flotation by using intracellular gas vacuoles (Porat et al., 2000). The organisms are commonly seen in aquatic systems and under favorable environmental conditions, these cells can be replicated rapidly in the form of planktonic or benthic sprouts. These cells can be survived in environmental stress or during starvation and when the conditions of growth are appropriate, they can sprout again (Jüttner et al., 2007). During last decades, Microcystins considered as promoter of cancerous tumors in the liver (Tillett et al., 2001). Hence, their presence in water bodies is potential hazard and serious threat to the aquatic biota, human and animal health (do Carmo Bittencourt-Oliveira et al., 2011). While in 1996, in Brazil, 76 people were died due to direct exposure of high concentration of microcystins (Vecchione et al., 2002), presence of toxic cyanobacteria in aquatic environment were an interest subject of researchers. On the other hand, in Australia scientists worked on other cyanobacterial toxin; Cylindrospermopsin which cause human hepatotoxicity (Falconer et al., 2006; Griffiths et al., 2003), and they found that CYN is genotoxic in *in vitro* systems and carcinogenic in mice (Falconer et al., 2006). Therefore, the present study tried to isolate toxic cyanobacteria from Kor River, Fars province, Iran using three set of primers. Subsequently effect of titanium dioxide were tested on extracted toxins.

2. Materials and Methods

2.1. Sample Collection

In the present study, 18 water samples were collected from three stations of Kor River, namely: Rah-Ahan Bridge, Esfandaran and Khan Bridge stations during June-July, 2017. The samples were collected in sterile glass tubes from a depth of 30 to 50 cm at each, and then they were transferred to the laboratory via cold chain in a cold box. Simultaneously, the temperature and the pH of each site were specified.

2.2. Isolation and Purification

The samples were serially diluted (10^{-6}) and cultivated on BG11 medium containing: MgSO₄, CaCl₂, NaNO₃, K₂HPO₄, Na₂CO₃,

Na₂EDTA and composition of trace elements in a form of H₃BO₃, MnCl₂, ZnSO₄, NaMoO₄, CuSO₄ (Zare et al., 2015). The plates 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 and the culture purity was investigated under a light microscope (Karimi et al., 2013; Zare et al., 2015). After 2 to 3 weeks, the colonies were identified based on colony shape, cell diameter and mucilage presence of the isolates (Zarrini et al., 2011).

2.3. Molecular identification of the strains

For molecular identification, DNA of the isolates was extracted by a DNG Kit according to manufacturer instructions (Yekta Tajhiz, Iran). Then, spectrophotometry was used to determine purity of the extracted DNA. Primers (Table 1) of target genes (Rantala et al., 2004; Vaitomaa et al., 2003; Zare et al., 2015) and the thermal cycling conditions were 95°C for 3 min followed by 94°C for 10 s, 54°C for 30 s and 72°C for 1 min, repeated for 30 cycles with a final extension of 72°C for 2 min and the same program has been done for the *Anabaena mcyE* gene. In this procedure the *Anabaena* (IBRC-M5002) was used in as standard isolate with capability to produce microcystin. In addition PCR program of Sulfotransferase of cylindrospermopsin synthesis shown in Table 2. Finally, amplified PCR products were separated by electrophoresis on an agarose gel 2% in TAE buffer. The electrophoresis was performed for approximately 45 min at 80 mA and visualized by staining with ethidium bromide solution (0.5 µg/mL⁻¹) and the PCR product was investigated by transilluminator device.

2.4. Extraction of bacterial toxins

50 mg lyophilized samples were extracted by adding 1mL acetic acid (0.03 N), sonicated at 35 kHz for 60 s in an ice bath, centrifuged at 11000 rpm for 5 min, and the supernatant filtered with a single-use syringe-filter (0.2 µm). Finally, 10 mL of the extract was injected in the HPLC system (Dos Anjos et al., 2006).

2.5. Effect of titanium dioxide on extracted toxins

To study the effect of titanium dioxide on extracted toxins, 0.02 g of titanium dioxide powder were mixed with 1 ml of deionized water and they were added to 2 ml extracted toxins. For the effectiveness of the titanium on the extracted toxins, samples were placed on a shaker under ultraviolet lights with a wavelength of 450-330 nm for half an hour. Then, they were

analyzed by HPLC with an optical diode detector (Beck man 166) and isolation was performed in ODS-Spherisorb (25 cm in 4.6 ml and 5 μ m particle size). The mobile phase contained 50% CH3OH and 50%, 20 mM Ammonium acetate buffer. Chromatograms at 238 nm were capable of being analyzed (Huang et al., 2007).

Table1. Lists of primers used for PCR

| Genes | Primers | Sequence (5' to 3') | PCR product length |
|------------------------------|---------------------|------------------------|--------------------|
| <i>mcyE</i> Gene | <i>mcyE</i> -F2 | GAAATTTGTGTAGAAGGTGC | 820bp |
| | <i>mcyE</i> - Ra4 | AATTCTAAAGCCCAAAGACG | |
| <i>mcyE</i> Gene Anabaena | <i>mcyE</i> -F2 | GAAATTTGTGTAGAAGGTGC | 247bp |
| | Mic <i>mcyE</i> -R8 | CAATGGGAGCATAACGAG | |
| Sulfotransferase Gene | Cynsulf F | ACTTCTCTCCTTTCCCTATC | 579 bp |
| | Cylnam R | GAGTGAAAATGCGTAGAACTTG | |

Table 2. PCR program of sulfotransferase of cylindrospermopsin synthesis

| Step | Temperature © | Time (minutes) | Number of cycles |
|----------------------|---------------|----------------|------------------|
| Primary denaturation | 95 | 3 minutes | 1 |
| Denaturation | 94 | 10 seconds | 35 |
| Annealing | 57 | 25 seconds | |
| Extension | 72 | 50 seconds | |
| Final extension | 72 | 2 minutes | 1 |

3. Results

3.1. Detection of isolates

Totally, 4 isolates of cyanobacteria were collected from the sampling sites. All of the isolates were belonged to the Khan bridge at

temperature of 24 and pH 7.5. All isolates were morphologically spherical and had no trichome. Fig 1 shows the colonies obtained from the sampling areas and Figs 2 illustrates the microscopic profile of the isolates.



Fig1. Cyanobacterial colonies

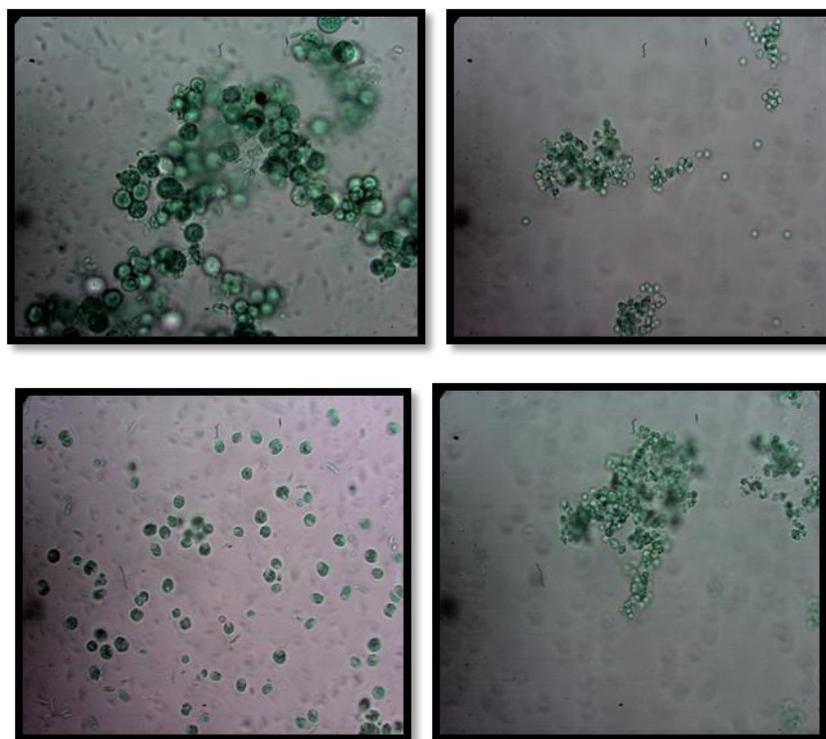


Fig 2. Microscopic identification (100X) of the isolates

3.2. confirmation of toxic strains

According to Fig. 3, the results indicated that among the isolates, strain no. 1 includes all the evaluated three toxin genes. However, the isolate no. 2 have both sulfotransferase as well

as *Myc E* gene and compared to the standard strain, isolate no. 3 contains two sets of *myc E* genes, while it lacks Sulfotransferase gene and the isolate no. 4 does not show any toxin.

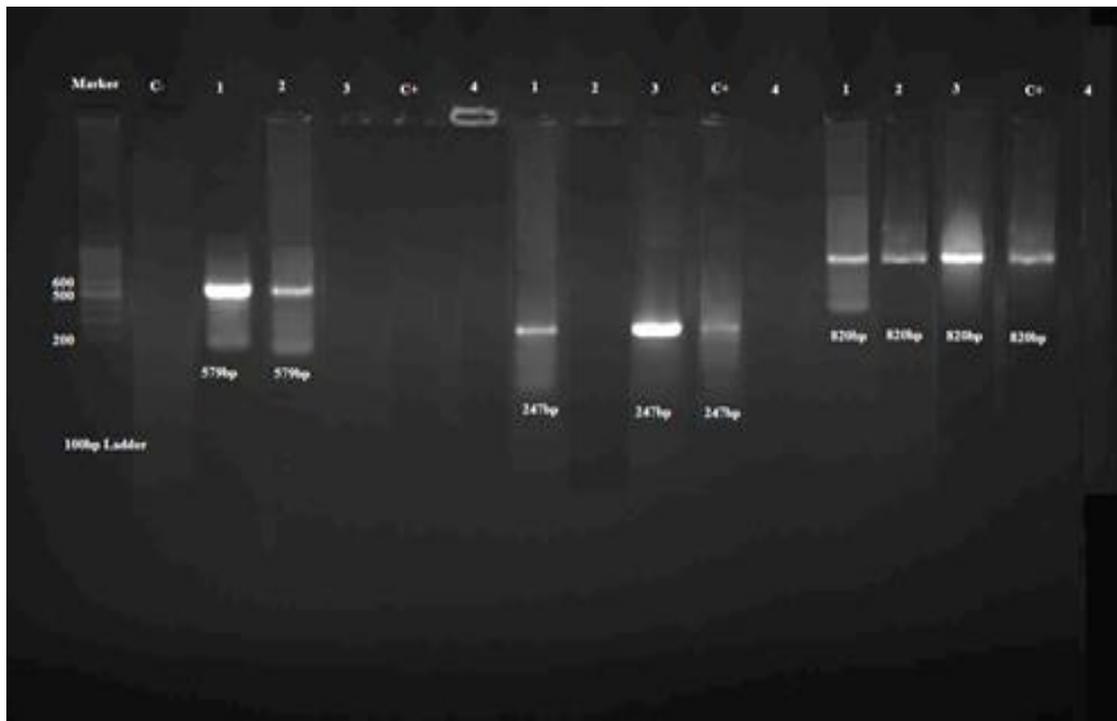


Fig3. The results of PCR product, Line M: Marker: the ladder with 100bp, lines 5, 10, 15: positive control (Anabaena (IBRC-M5002), other lines the isolated cyanobacteria from Kor River

3.3. Effect of titanium dioxide on extracted toxins

As shown in Fig. 4 presence of the extracted toxin by the standard sample (Anabaena: IBRC-M5002) confirmed by the HPLC. In addition,

Fig 5. Illustrating that the toxins were affected by titanium dioxide. The results indicate that titanium dioxide was able to destroy the toxin within half an hour.

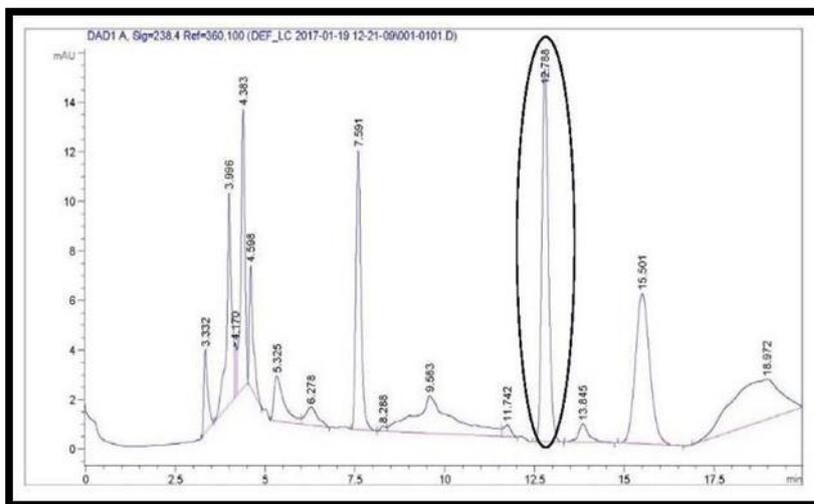


Fig 4. Standard chromatogram of extracted toxin from Anabaena (IBRC-M5002)

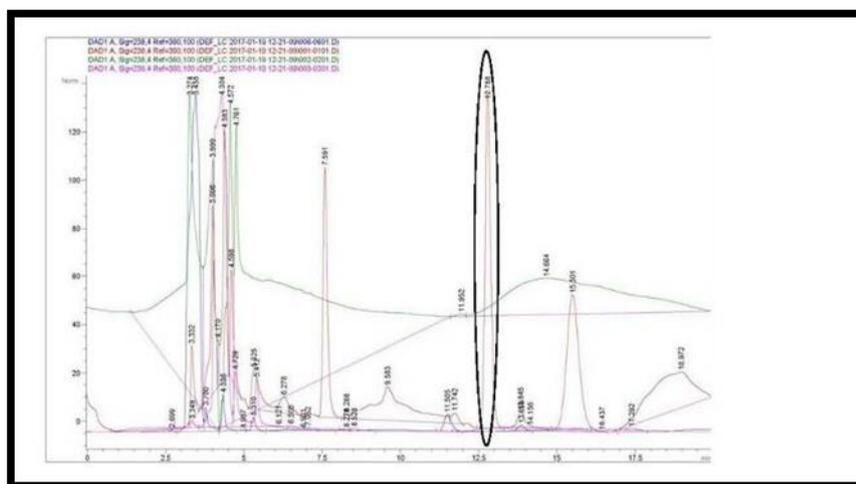


Fig5. Effect of titanium dioxide on the extracted toxins from the isolates

4. Discussion

Cyanobacteria are a large group of organisms that exist in different environments, including fresh and salty waters (Madhumathi et al., 2011). This group of organisms consists of a variety of bacteria with the ability to fix nitrogen and are highly adaptable to the various environmental conditions (Babichenko et al., 2000). Among the cyanobacteria, some of them have ability to produce toxins. The production of cyanotoxin threatens the health of humans and animals, which, depending on their concentration in aquatic environments, which can lead to severe poisoning, chronic illness and even death. Cyanotoxins are important chemical compounds in terms of ecotoxicology, toxicology, and environmental chemistry (Paerl et al., 2011). On the other hand, hepatotoxins are circular peptides composed of 5 amino acids, such as nodularin or, like, microcystin, including seven amino acids isolated from various cyanobacteria of freshwaters. This group of toxins with an effect on the liver cells leads to rupture of the liver structure by hypovolemic shock and excessive accumulation of blood in the liver. Furthermore, they have ability to inhibit protein phosphatase 1 or 2 and disrupt the structure and function of the liver cells and essential components, which ultimately stimulate tumors and cancers (Pearson et al., 2010). Hataitai and Wellington in 2011 demonstrated the production of microcystin toxin by *Anabaena* in New Zealand, which is

considered as a major hepatotoxic toxin (Weller, 2011).

In addition, cylindrospermopsin is regarded as a bicyclic alkaloid of cyanobacteria in freshwaters. In 2000, Saker and Griffiths isolated cylindrospermopsin toxin produced by *Cylindrospermopsis raciborskii* from Australia and observed mice liver damage. Similarly, Zare and Bahador in 2015, were able to extract cylindrospermopsin from the isolated cyanobacteria in Kor River, Fars province and *in vivo* testing of the toxin showed crippling and shortness of breath in the mice (Zare et al., 2015).

As the blooms of these cyanobacteria were in most of the drinking waters, researchers believed in using some methods to eliminate these toxins, although some studies reported that the methods were ineffective, they found that these toxins are severely degraded in the presence of high levels of chlorine and ozone. On the other hand, researchers have shown that blooms derived from the vast growth of cyanobacteria have the potential to make changes such as taste, smell and opacity that the produced toxins can have negative effects on aquatic organisms and accordingly on human health (Dehghaniet al., 2006; Jakubowska et al., 2013). Therefore, given that the removal of cyanobacteria from aquatic environments is difficult due to their small size and gravity, various technologies such as ultraviolet ray ozone, chlorine dioxide, chlorine, sodium permanganate, filtration, coagulation and other advanced technologies

were used, which are costly and lead to secondary contaminants (Chen et al., 2005; Plummer et al., 2002). Hence during the years, researchers conducted different approaches to remove cyanobacterial toxins from environments. For this purpose photocatalytic investigation of dichloromethane, chloroform, carbon tetrachloride, trichloroethylene, phenol, chlorine and benzene were studied, but the results showed that only 6.4% of these toxins were degraded (Hsiao et al., 2003). Then, Linda *et al* (Lawton et al., 1999), showed that photocatalyst titanium dioxide is a reliable method for detoxification of water contaminated with cyanotoxins. But the significance of toxins and their effects on the surrounding environment led to the use of photocatalytic oxidation of titanium dioxide in recent years due to its low risk and its appropriateness confirmed by the Food and Drug Administration. Accordingly, in the present study, detoxification of all three samples which produced the microcystin and cylindrospermopsin were evaluated by the effect of titanium dioxide in the presence of ultraviolet ray for half an hour. Although the effect of 500 mg/L was investigated by Wu and his colleagues (Wu et al., 2015), in the present study 20 g/L showed destructive effects.

Since cyanobacteria are a large group of organisms that can be observed in different environments, and they have ability to produce different metabolites, the present study alarmed with finding three important isolates from the Kor River, which have capability to produce toxic substances such as microcystin and cylindrospermopsin. In addition, because of the importance of these organisms and their destructive effects on humans and other creatures the study tried to examine effect of titanium dioxide on extracted toxins. Although the titanium oxide had ability to destroy the toxin, monitoring of such locations is necessary and the organizations which is working with ministry of health should design some annually project for evaluating the of quality of surface and fresh waters.

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