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Molecular Diagnosis of Microcystin-Producing Cyanobacteria of Anzali Lagoon

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Cyanobacteria bloom in territorial waters leads to the production of dangerous cyanotoxins such as Microcystin, which causes world-wide problems. We exerted an optimized PCR for detection of Microcystin-producing gene of Cyanobacteria from óAnzali Lagoon. The shallow coastal Anzali lagoon is located in the province of Gilan (a temperate region) in Northern Iran. It covers an area of about 200 km2 situated between 37°28 ì N and 49°25 ì E. It is26 km long and 2.0 - 3.5 km wide. For sampling, 20 stations were selected within western, central and eastern parts of the Lagoon. DNA extraction was performed by using DNG-Plus Kits. For two universal (23S30R, CYA106F) and two specific primers (mcyA-Cd1F, mcyA-Cd1R), PCR test was optimized, and its sensitivity and specificity were evaluated. Then the test was applied for samples from Anzali lagoon. Cloning of the two fragments was also performed. Application of the optimized PCR method to the DNA samples from Anzali Lagoon revealed that all of the stations contained Cyanobacteria, among which only ten indicated Microcystin-producing Cyanobacteria. In addition, a successful process of cloning and extraction of recombinant plasmids was achieved that its products were stored for future investigations. Anzali lagoon might be in danger of predomination of Cyanobacteria. Therefore, our optimized PCR method can be implemented as helpful tool for monitoring and controlling of Cyanobacteria, especially Microcystin-producing species in the lagoon.

Key words: Anzali lagoon, Cyanobacteria, Iran, Microcystin, PCR.

Cyanobacteria are blue-green prokaryotic and photosynthetic micro-organisms that grow in warm, superficial and eutrophic waters. A Cyanobacteria bloom in territorial waters leads to the production of dangerous toxins (cyanotoxins) which cause world-wide problems (de Figueiredo *et al.*, 2004). In addition, many of these species of Cyanobacteria cause the same issues in inlets, rivers, freshwater lakes, oceans, and storage and drinking waters (Cheung *et al.*, 2013). Therefore, to guarantee safety, drinking waters should be quantitatively and qualitatively monitored for possible removal of these dangerous toxins.

Microcystin is one of the most common and prevalent cyanotoxins mostly produced by species of Microcystis, Planktothrix and Anabaena genera of Cyanobacteria. This toxin is a monocyclic, nonribosomal hepapeptide that consists of several uncommon non-proteinogenic amino acids (such as dehydroalanine derivatives), and is dissolved and persistent in water (Botes *et al.*, 1982; Oliveira *et al.*, 2005). Microcystin is coded by mcy gene and produced by Microcystin synthetase (Dittmann *et al.*, 1997; Tillett *et al.*, 2000).

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For determining Microcystin-producing Cyanobacteria and detections of different kinds of cyanotoxins, several methods have been implemented, which include enzyme-linked immunosorbent assay (ELISA), protein phosphatase inhibition assay (PPIA), solid phase adsorption toxin tracking (SPATT), high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC/MS), and matrix-assisted laser desorption/ionization time-off-flight (MALDI-TOF) assays. However, these approaches harbor some disadvantages (Sangolkar *et al.*, 2006; Singh *et al.*, 2012; Zhao *et al.*, 2013). Fore instance, ELISA often shows false positive results, and or HPLC demands highly expensive tools. In addition, cell-culture-dependant methods are complicated and time-consuming (Singh *et al.*, 2012). Moreover, due to close genetic relationship between Microcystin-producing and non-producing strains, identification of Microcystin-producers by morphological criteria is impossible (Hisbergues *et al.*, 2003).

Recently, it is suggested that molecular methods, including polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), can be more effective tools for detection of Cyanobacteria and their toxin-coding genes (Tillett *et al.*, 2001; Hisbergues *et al.*, 2003; Rantala *et al.*, 2006). Hisbergues et al. (2003) suggested PCR as a powerful means to identify Microcystin-producing strains of the genera Anabaena, Microcystis, and Planktothrix. Then, Rantala et al. (2006) investigated the frequency and composition of potential Microcystin producers in 70 Finnish lakes with general and genus-specific Microcystin synthetase gene E (mcyE) PCR. Vaitomaa et al. (2003) determined the Microcystin concentrations and cyanobacterial cell densities of lakes of Tuusulanjärvi and Hiidenvesi, using quantitative real-time PCR. Furthermore, genetic analysis of Microcystis strains from environmental samples has revealed that only few number of these microorganisms possessed Microcystin synthetase and coding genes; however, most of them were not able to produce the toxin which can be caused by genetic mutations and variations (Dittmann *et al.*, 2001; Kaebernick *et al.*, 2001; Mikalsen *et al.*, 2003).

Anzali Lagoon is a coastal liman in the Caspian Sea with an area of 15,000 hectares located near the northern port city of Bandar Anzali in Gilan, a province of in the northern part of Iran. The lagoon is habitat of both the Selke Wildlife Refuge and the Siahkesheem Marsh, and is ecologically affected by many microorganisms, especially toxin-producing Cyanobacteria (Khatib-Haghighi and Khodaparast, 2011). However, the genes responsible for these toxins in óAnzali Lagoon are not studied.

As mentioned above, PCR is a helpful technique to determine poisonous strains of Cyanobacteria and their toxins such as Microcystin; therefore, this tool was implemented to determine the Microcystin-producing gene of Cyanobacteria from óAnzali Lagoon.

MATERIALS AND METHODS

Sample preparation

For sampling, 20 stations were selected within western, central and eastern parts of the Lagoon (Fig 1). Geographic Latitude and Longitude of these stations are given in Table 1. Surface water from each station was taken using distinct two-liter sterile plastic bottles, which then, were immediately put into ice-gel containing box, and were transferred to the laboratory in a light-free condition. Afterwards, each sample was centrifuged (8000 rpm for 12 minutes), and after removal of supernatant, remaining pellet was mix with 500 ¼l of deionized water.

DNA extraction

DNA extraction was performed by using DNG-Plus Kits (Sinagene Co., Iran), according to the manufacturer2 s instructions. In details, for each sample, 100 ¼l of sample-containing deionized water was mixed with 400 ¼l of DNG solution in 1.5 ml micro-tubes, heated on heater block for 4 hours, agitating every 30 minutes. Then, the mixture was centrifuged (1200 rpm for 5 minutes) and its supernatant was transferred into a new 1.5-ml micro-tube and was mixed with 500 ¼l Chloroform solution, agitated and centrifuged (1200 rpm for 10 minutes). The resultant supernatant was mixed with 300 ¼l cold ethanol in a new 1.5-ml micro-tube and mixture was lightly inverted ten times, and then, was centrifuged (1200 rpm for 15 minutes), decanted, mixed with 500 ¼l of 70% ethanol, agitated, and centrifuged (1200 rpm for 5 minutes) and decanted for second time. Finally, each micro-

tube was dried on heater block for removal of alcohol, and was mixed with 100 ¼l of deionized water and incubated for on heater block (65 °C degree for 5 minutes).

PCR and Electrophoresis

Using standard DNA of *Microcystis aeroginosa* strain PCC7806, and universal primers for Cyanobacteria detection (23S30R, CYA106F) (Sueoka *et al.*, 1997; Namikoshi *et al.*, 2003) and also the primers specific for Microcystin-coding gene (mcyA-Cd1F, mcyA-Cd1R) (Hisbergues *et al.*, 2003) (Table 2), optimization of PCR test for each pair primer was achieved and, the sensitivity and specificity of the test was evaluated. After making sure of optimization, the optimized PCR was exerted for detection of the Microcystin-producing gene of Cyanobacteria from óAnzali Lagoon.

Then, 10 ¼l of each PCR product together with 2 ¼l of 6X loading buffer was added onto 1.5% agarose gel that was previously mixed with CYBR Green dye, and electrophoresis was performed for 45 minutes at 70 V. Finally, electrophoresis gel was visualized under Ultraviolet transilluminator.

For cloning, PCR product was purified, transfected into pTZ57R plasmid vector (Fig 2), and then transformed into E.coli JM107, using T/ Acloning kit (Fermentas Co.USA).

Purification of PCR products

For purification, 50 ¼l of PCR product was mixed with 5 ¼l of 3Molar Sodium acetate and 125 ¼l of cold absolute alcohol within an Eppendorf tube, and incubated in a freezer (-20 °C degree for one hour). Then, it was centrifuged (1200 rpm for 15 minutes), decanted, mixed with 200 ¼l of cold 70% alcohol, inverted ten times, and centrifuged (1200 rpm for 10 minutes) for second time. After removal of supernatant, pellet was desiccated within 30-40 minutes at 37 °C degree, and then, was dissolved in 20 ¼l distilled water.

Ligation

For ligation, 4 µl of purified PCR fragment, 3 µl of plasmid vector pTZ57R, 6 µl of 5X ligation buffer, 3 µl of PEG 4000 solution, 1 µl of T4DNA Ligase (5u), and 13 µl of deionized water were added into a tube. Afterwards, the tube was incubated at 22 °C degree for 4 hours to let the ligation process successfully occur. In order to optimize ligation, 7 ¼l of insert was taken from each purified DNA product onto electrophoresis gel, and only those that revealed only specific bands on the gel, were used for the process. In addition, at the time of PCR performance, we had let elongation phase continue for 25 minutes more at 72, to increase the number of target DNAs that have extra Adenine nucleotide at their tail, which previously was reported that had augmented the number of recombinant clones three or four times.

Transformation

Competent host, E.coli JM107, was prepared for transformation. Briefly, 2 ml of TransformAid C-Medium was poured into a 15-ml tube, several clones of E.coli JM107 were added, and then the tube was shaken at 37 °C degree for 2 hours. Afterwards, 1.3 ml of this suspension was transferred into a 1.5-tube and centrifuged (12000 rpm for one minute). After removal of the supernatant, 300 ¼l of cold T solution (solution A 500 + solution B 500, equally) was added to the pellet, and the resultant suspension was incubated on ice for 5 minutes, and then was centrifuged (12000 Rpm for one minute). Again, 120 ¼l of the T solution was added to the pellet, and suspended. This competent bacteria suspension was incubated on ice for 5 minutes.

Ligation mixture-containing tube was put on ice for 2 minutes, 10 ¼l of which was then added to 50 ¼l of competent bacteria suspension and incubated on ice for 5 minutes. Finally, 40 ¼l of it was cultured on the plate containing LB Agar + Amp medium with 0.1M IPTG and 20mg/ml X Gal, and incubated at 37 °C for 24 hours.

Clone Selection

Several white clones were selected and re-cultured on other LB+Amp-containing plates, from which appropriate clones were selected and plasmid DNAs were extracted using boiling method. In detail, a single clone was adopted and dissolved in 50 ¼l of distilled water, mixed with 50 ¼l mineral oil in a tube and its cap was fastened and covered by Parafilm. The tube was heated in boiled water for 15 minutes, and then was centrifuged (1200 rpm for 10 minutes). Afterwards, 45 ¼l of the liquid underlying the oil that contained plasmid was taken to another tube, which further was used as a template for monitoring the presence of insert when applying optimized PCR test. After presence of desired fragment was proven, the

related clone was amplified. Finally, extracted plasmids from resultant clones were stored for further investigations.

RESULTS

PCR optimization

Specific bands of amplicon resulted from Cyanobacteria-specific primers and Microcystin-gene-specific primers were 487 bp and 297 bp in length, respectively (Fig. 3). Thermal cycling profile was optimized using Gradient PCR, which gave out 56 as an optimum annealing temperature.

Table 1. Geographic Latitudes and Longitudes of sampling station of Anzali Lagoon

Location	Longitude	Latitude	Station
East	49º,32," 28.29	37º,49, "27.68	S1
East	49º,23, " 28.21	37º,50, "27.45	S2
East	49º,51, "32.88	37º,11, "20.11	S3
East	49º,28, " 26.78	37º,12, "21.97	S4
East	49º,22, "27.10	37º,46, "26.27	S5
East	49º,42, "26.73	37º,7, "24.24	S6
Centre	49º,32, "26.22	37º,8, "26.17	S7
Centre	49º,43, "23.13	37º,19, "27.28	S8
centre	49º,11, "23.57	37º,29, "26.96	S9
centre	49º,48, "23.06	37º,27, "27.34	S10
centre	49º,24, "21.00	37º,59, "27.53	S11
centre	49º,47, "22.01	37º,18, "27.16	S12
West	49º,9, "17.49	37º,58, "30.90	S13
West	49º,40, "16.54	37º,57, "29.25	S14
West	49º,56, "17.49	37º,17, "30.27	S15
West	49º,4, "19.35	37º,24, "30.86	S16
West	49º,22, "17.52	37º,15, "29.84	S17
West	49º,58, "16.25	37º,2, "31.47	S18
West	49º,12, "18.85	37º,45, "30.21	S19
West	49º,52, "17.69	37º,59, "30.30	S20

Sensitivity and specificity of PCR assay

Sensitivity of PCR for detection of Microcystin, the minimum number of bacteria used to detect the gene, was calculated as 10 cyanobacteria or copies of the genome (Fig 4a). Moreover, the primers were able to accurately detect the Cyanobacteria and Microcystin-coding gene, while they did not showed any specificity for other control microorganisms such as, staphylococcus and streptococcus species, *E.coli*, *Legionella pneumophila*, *Pseudomonas aeroginosa*, and also for human and mice genomes (Fig 4b and 4c).

Cyanobacteria and Microcystin detection in Anzali Lagoon

When optimized PCR for detection of Cyanobacteria was applied to the DNA samples form Anzali Lagoon, along with positive and negative controls, it was observed that Cyanobacteria existed in all twenty studied stations (Fig 5a). In addition, Application of optimized PCR for detection of Microcystin-coding gene to these samples revealed that only ten of the stations contained Microcystin-producing Cyanobacteria (Fig 5b). These stations included; six stations in eastern, three in central, and one station in western parts of the lagoon.

Cloning

PCR assay on DNAs that were extracted from randomly selected white clones revealed that all of the clones contained desired-recombinant plasmids; heavy recombinant plasmids that harbored Cyanobacteria and or Microcystin gene segments moved slower than the non-recombinant plasmid pTZR57 on electrophoresis gel. These findings implied a successful process of cloning and extraction of recombinant plasmids. Th cloning products were stored for future investigations.

Table 2. Universal primers for Cyanobacteria detection and the primers specific for Microcystin-coding gene

PCR product length	Primer sequence	Gene
487	5'-GGGGAATYTTCCGCAATGGG-3'5 '-GACTACWGGGGTATCTAATCCCWTT-3'	CYA359FCYA781R
297	5'-AAAAGTGTTTTATTAGCGGCTCAT-' 3'5'AAAATTAAAAGCCGTATCAAA-3	mcyA-Cd1RmcyA-Cd1F

DISCUSSION

Determining the presence of Cyanobacteria and their poisonous-toxins in water is becoming the future world-wide issue. Cyanobacteria bloom in eutrophic waters brings about to many issues. For instance, it is calculated that in Alberta Lake or in other eutrophic lakes in Canada, 59% of the bloom which happened during the summer, were poisonous (Rantala *et al.*, 2006). In addition, it is reported that the bloom of a cyanophyte, Nodularia spumigena, in Caspian Sea leads to accumulation of forty-six species of Phytoplankton, the event that has detrimental effects on the sea and habitats of other entities (Nasrollahzade *et al.*, 2011). Microcystin, with sixty-five isoforms, is the most studied cyanotoxin (Bagu *et al.*, 1995); consumption of waters contaminated with this toxin can be dangerous for human health (Nishiwaki-Matsushima *et al.*, 1992; Carmichael *et al.*, 1997; Sturgeon and Towner, 1999). For example, Teixeira Mda et al. (1993) reported that Anabaena and Microcystis species cause 88 child-deaths in Bahia city of Brazil.

Several methods have been introduced for detection of Microcystin; however, they are some complication with these methods (Zeck *et al.*, 2001; Rapala *et al.*, 2002; Hilborn *et al.*, 2005; Sangolkar *et al.*, 2006; Singh *et al.*, 2012; Zhao *et al.*, 2013). Thus, molecular methods such as, PCR has been of great importance to detect

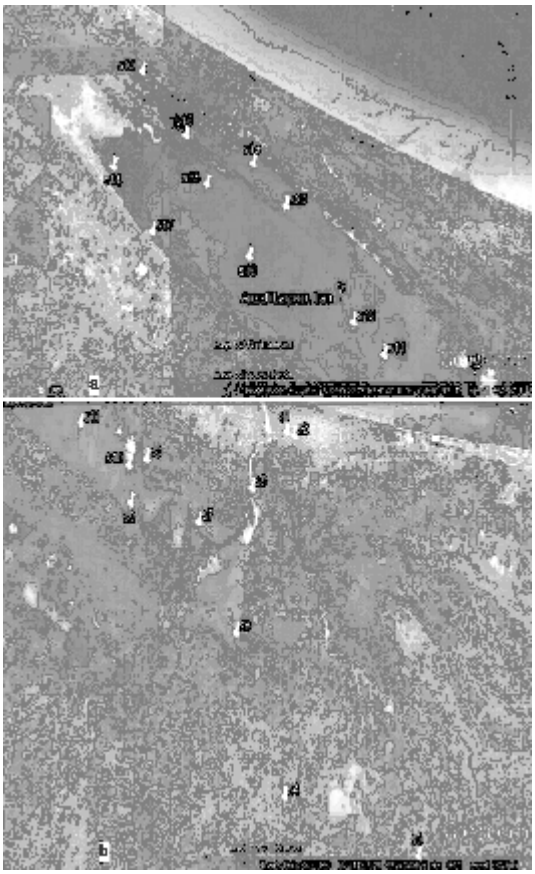
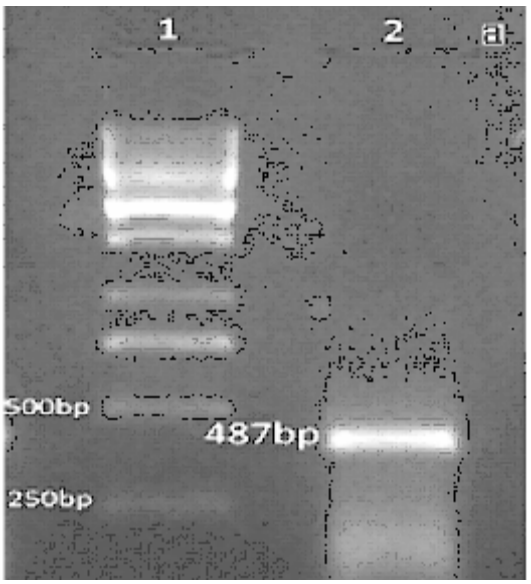


Fig 1. Satellite imagery of western (a), and central and eastern (b) stations of Anzali Lagoon that were selected for sampling



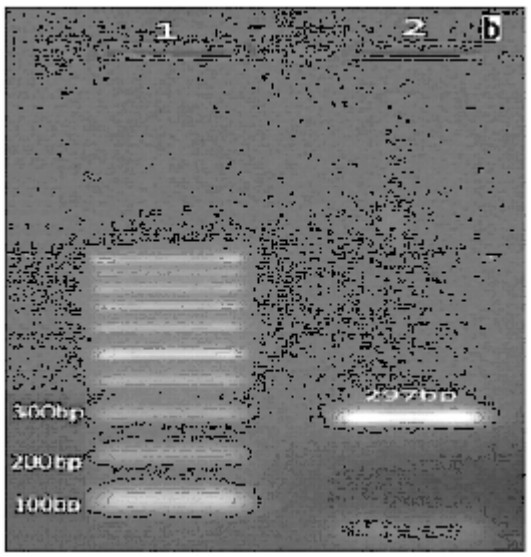


Fig 2. Optimized PCR assay for detection of Cyanobacteria (a) and Microcystin-coding gene (b)

Cyanobacteria and their toxin-coding genes (Tillett *et al.*, 2001; Hisbergues *et al.*, 2003; Rantala *et al.*, 2006). For instance, in order to redress the number of false positive results of ELISA, in 2010, Valerio et al. (2010) devised a new PCR technique for coinciding detection of three gene areas of Microcystin-producing strains, and they declared that this method can be served as a reliable approach for monitoring these kinds of environmental samples.

In the present study, we designed and performed an optimized PCR assay for detection of Microcystin-producing Cyanobacteria in twenty stations of Anzali lagoon for the first time. Our results implied that the optimized PCR method is able to detect Microcystin in very low

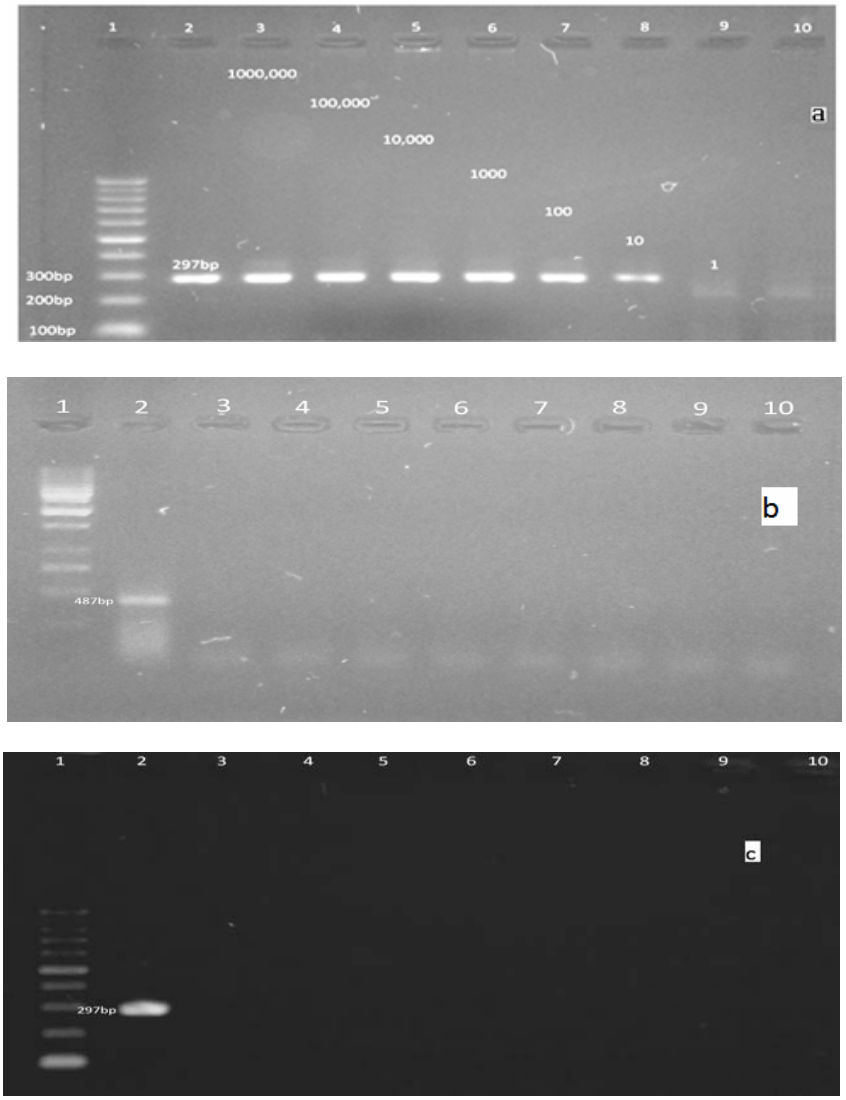


Fig .3. Sensitivity of PCR for detection of Microcystin (a), and also specificity of PCR for detection of Cyanobacteria (b) and Microcystin (c). (a): 1, Vivantis 100bp DNA Ladder 2,Positive control 3,1000000 Genume 4, 100000 Genume 5, 10000 Genume 6, 1000 Genume 7, 100 Genume 8, 10 Genume 9, 1 Genume 10, Negative control b : 1, Fermentas 1Kb DNA Ladder; 2, positive control; 3, staphylococcus sp.; 4, streptococcus sp.; 5, *Legionella pneumophila*; 6, *E.coli*; 7, *Pseudomonas aeroginosa*; 8, human genome; 9, Mice genome; 10, negative control,c: 1, Vivantis 100bp DNA Ladder 2, Positive control 3, *Fischerella spp* 4, *Synechococcus spp.* 5, *Pleurocapsa spp.* 6, *Cylindriospermopsis spp.* 7, *Aphanothece spp.* 8, *Schizothrix spp.* 9, *Microcystis wesenbergii* 10, Negative Control

concentration of the Cyanobacteria, which represents higher sensitivity for this method in comparison with other known tools. Moreover, our optimized PCR showed remarkable specificity for Cyanobacteria genes as it did not detect any other microorganism genes and also human and Mice genes. Furthermore, we could successfully clone and amplify two of important cyanobacteria gene fragments, which can be used in future investigations.

We applied PCR method to samples from different stations of the lagoon and found that Cyanobacteria were present in all of the stations, which can be an alarming sign and should be taken under consideration by governmental experts and scientists. In addition, we observed that Microcystin was produced in ten of the studied stations.

According to Kurmayer et al. (2002) the ability to produce Microcystin depends on the presence of mcy genes within a Cyanobacterium, that is, cells unable to produce Microcystin lack the mcy genes. Therefore, based on our results, it is estimated that almost 50% of water samples from Anzali lagoon contained the Cyanobacteria that might dispossess the Microcystin-coding genes.

Genes coding for Microcystin have been widely used for primer design and thereby for detection of Cyanobacteria (Schatz *et al.*, 2000; Baker *et al.*, 2002; Ouellette *et al.*, 2006). In 2010, Valerio and his colleagues exerted a multiplex PCR technique to detect mcyA, mcyA-B and mcyB gene areas at a time (Valerio *et al.*, 2010); the primers they used for mcyA area were the same as we implemented in our study. Moreover, recently, Pedro et al. (2011) applied PCR method, using both mcyA-Cd1F and mcyA-Cd1R primers for detection of significant number of Microcystin-producing Cyanobacteria in Mosambik Lake, and found that 33% of the thirty-three water sample contained these Cyanobacteria. However, by using the same primers, we could detect Microcystin-producing Cyanobacteria in 50% of water samples from twenty stations of Anzali lagoon.

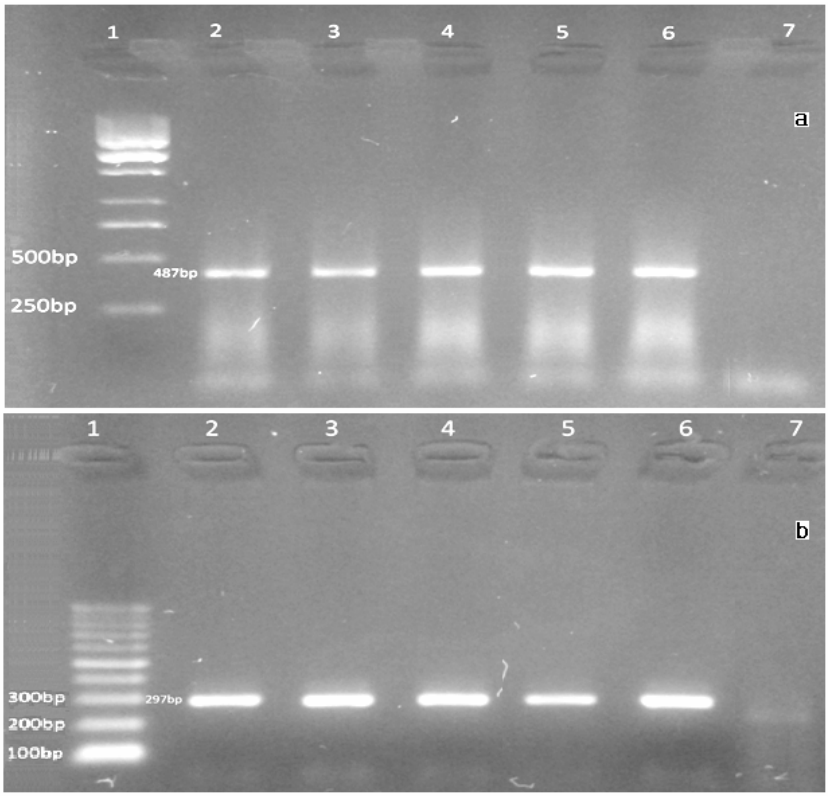


Fig. 4. Detection of Cyanobacteria (a) and Microcystin (b) in four stations of Anzali Lagoon, using optimized PCR assay: 1, DNA Ladder; 2, positive control, 3-6, (stations 1,2, 3, 4, respectively); 7, negative control

There are different methods for extraction of DNA from microorganisms by various DNA kits, or without kits, like salting out extraction method (Miller *et al.*, 1988). However, Kits are more efficient tools as they yield higher quantity of DNA, which is pivotal for achieving higher sensitivity. In the present study, our DNA yield was considerable. Another remarkable factor is the time that takes for DNA extraction. For example, Lin et al. (2011) investigated distribution and diversity of Microcystis species in Lake Ulungur of China, using molecular approaches. These scientists performed two types of DNA extractions that were so time-consuming, while the time we spent for extraction did not exceed four hours. In addition, Hotto et al. (2007), in their study of Microcystin-producing Cyanobacteria, used 47-mm glass fiber filters to entrap Cyanobacteria, and different solutions including Phenol–chloroform and Chloroform-Isoamyl, and also they spent higher amount of time for DNA extraction. But, we used lower amount of solutions and no filters for extraction process.

CONCLUSION

Results of the present study indicated that Anzali lagoon might be in predomination of toxic Cyanobacteria. In addition, our optimized PCR method can be implemented as helpful tool for monitoring and controlling of Cyanobacteria, especially Microcystin-producing species in the lagoon or other water sources.

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