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Analysis of Cyanide Bioremediation Using Cyanobacterium; *Chroococcus* Isolated from Steel Manufacturing Industrial Wastewater

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ABSTRACT

Cyanide and its derivates contamination of waste waters is one of the problems in steel manufacturing industries. Increasing usage of this toxic element is leading to its accumulation and contamination of the environment. In the present study, cyanide resistant cyanobacterium has been isolated from the waste water of steel manufacturing industrial area in Iran and identified based on the morphological characteristics. For this purpose, contaminated samples achieved from different parts of waste water containing lagoons and inoculated to BG11 culture medium. The antibiotics cycloheximide and imipenem used for removing the fungi and other bacteria. The isolated cyanobacterium has been identified based on the microscopic morphological characteristics and amplification of 16S rDNA sequence. For in vitro analysis of cyanide resistance, different concentration of potassium cyanide (5-15 mg L^{-1}) prepared in BG11 media and the microorganism inoculated to the media. The bacterial growth assessment was done based on the cell counting, culture turbidity, dry weight and the amount of chlorophyll a. The cyanide removal was estimated after 16 h incubation in 25°C and shaking at 140 rpm and the presence of light (16 h lightening in 3 Klux and 8 h darkness continuously), using cyanide ion characterization electrode. Also the purified cyanobacterium inoculated to the samples obtained from the lagoons and the cyanide removal has been analyzed in waste water samples. The isolated cyanobacterium identified as a species of *Chroococcus* in the family Chroococcaceae. The bacterium was able to grow in the presence of 5-30 mg L^{-1} KCN. The results of direct studies showed that the isolated *Chroococcus* sp. removed a mean of 14.06% of free cyanide from the steel manufacturing waste water samples.

Key words: Cyanide resistance, cyanide bioremediation, *Chroococcus* sp., molecular identification, steel manufacturing waste water, chlorophyll a content

INTRODUCTION

Cyanide and its derivations are among the toxic and dangerous substances that are used in industries such as insecticide production, electro plating, jewelry cleaners, organic chemicals production and photography. The increasing use of these toxic substances resulted in high concentration of them in the environment and increased levels of pollution (Fokunang *et al.*, 2001; Hamel, 2011). This compound has been found in some foods and seed in the amounts above the equivalent recommended by WHO and FAO (Gernah *et al.*, 2011; Okafor and Omodamiro, 2006;

Oyetayo and Omenwa, 2006). Cyanide is largely toxic for aerobic cell metabolism. Cyanide binds to the mitochondrial cytochrome oxidase a_3 enzyme. This enzyme which is located in the fourth complex of oxidative phosphorylation system, catalysis the reduction of oxygen to water. Binding of cyanide to the ferric ion in the cytochrome oxidase a_3 inhibits the terminal enzyme in the respiratory chain and halts the electron transport and oxidative phosphorylation which leads to intracellular hypoxia (Hall *et al.*, 2007).

Cyanobacteria have been found to remove and decrease harmful industrial materials from environment (El-Bestawy, 2008). Cyanobacteria are able to remove heavy metals such as Pb, Cd, Cu, Mn and Zn (Taghiganji *et al.*, 2005; Nagase *et al.*, 2005). Shing *et al.* (2008) showed that the concentrations of Cd^{2+} below approximately 8 mg L^{-1} have not any inhibition but stimulate the oxygen production by cyanobacterium *Anabaena torulosa*. Chay *et al.* (2009) designed a toxicity biosensor for Pb, using immobilized cells of *Anabaena torulosa* for the assessment of Pb toxicity in river water samples.

The photosynthetic cyanobacteria can destroy hydrogen cyanide by the nitrogenase enzyme, because they can fix nitrogen. Nitrogenase can also use hydrogen cyanide instead of its normal substrate, dinitrogen and convert it to methane and ammonia (Gantzer and Maier, 1999).

The aim of the present study was the isolation and characterization of cyanide resistant cyanobacteria from waste water storage lagoons in the steel plant and the study of cyanide bioremediation in these wastes using isolated microorganisms.

MATERIALS AND METHODS

Samples and isolation of bacteria: Cyanide contaminated waste water samples obtained from the waste water of steel manufacturing industry in the October and November 2011. The samples inoculated in the BG11 specific broth medium for cyanobacterial growth. The medium was enriched by the antibiotics cycloheximide (1.2%) and imipenem (0.5%) for the inhibition of fungal and bacterial growth. The solid medium prepared with 0.7% agar added to the broth medium. The growth of bacteria was estimated after 15 days on the presence of light (16 h lightening in 3 Klux and 8 h darkness continuously) in the 140 rpm shaking and 25°C (Ferris and Hirsch, 1991; Prescott, 1961). The isolated cyanobacterium was initially identified according to the standard keys (Rippka *et al.*, 1979).

DNA extraction and 16S rDNA amplification: DNA content of media was extracted using Roche Applied Science DNA extraction kit. Forward and reverse primers (MAZ.B1, 5-GCGGTGAAA TGCGTAGAG-3 and MAZ.B2, 5-CAGGCGGGAAACTTAACG-3) were designed according to 16S rDNA sequence of the cyanobacterium *Chroococcus* using Oligo program. The primers amplified a 200 bp fragment in the cyanobacterium 16S rDNA sequence (Table 1).

Growth parameters analysis: Cyanobacterial growth analysis achieved by assessment of the number of cyanobacteria by means of direct microscopic count, the Optical Density (OD) of cultured cells in 650 nm, the dry weight (after centrifugation and drying in 80°C) and the content of chlorophyll a (MacKinney, 1941; Chinnasamy *et al.*, 2009). For estimation of chlorophyll a concentration, 1 mL of culture media has been centrifuged at 13000 rpm for 5 min. After washing the pellet with distilled water, 1 mL of methanol 90% was added to the pellet and incubated in 70°C for 20 min. The optical density of the pellet in 650 and 665 nm was

Table 1: The sequence data of 16S ribosomal RNA gene achieved from NCBI

Sequence characteristics	*Chroococcus* sp. 16S ribosomal RNA gene. 843 bp linear DNA
Identification code	Accession: JQ003585.1
Sequence	GAGGAAGGCTCTTGGGTTGTAAACCTCTTTTGCTTGGGAAGAAGATCTGACGGTACCAAGCGAATCAGC ATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATTATTGGGC GTAAAGCGTCCGCAGGTGGTTATTCAAGTCTGCTGTTAAAGACTGAAGCTTAACTTCGGAAAGGCAGTG GAAACTGAAAGACTAGAGGTCAGTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTGAAATGCGTAGAGA TTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTGAATCTGACACTGAGGGACGAAAGCTAGG GGAGCAAAAGGGATTAGATACCCCTGTAGTCACTAGCCGTAAACGATGGAAACTAGGCGTGGCTTGTA TCGACCCGAGCCGTGCCGAAGCTAACGCGTTAAGTTTCCCGCCTGGGGAGTACGCACGCAAGTGTGAA AGCTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAA GAACCTTACCAGGGCTTGACATGTCGCGAATCCCCTTGAAAGGGGGAGTGCCTTCGGGAGCGCGAAC ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA CCCTCGTCCTTAGTTGCCAGCAGGTAAAGCTGGGCACTCTGAGGAGACTGCCGGTGACAAACCGGAGG AAGGTGGGGATGACGTCAAGTCAGCATGCCCCTTACGCCCCTGGGCTACACACGTACTACAATGGTTGA GACAATGGGCAGCTGAGCTTGCGA

determined in the supernatant after centrifugation at 13000 rpm for 5 min. The chlorophyll a concentration calculated by the following equation (MacKinney, 1941):

$$\text{Chlorophyll a (mg L}^{-1}\text{)} = 16.5(A_{665})\text{-}8.3(A_{650})$$

The effect of pH on the isolation and growth: A range of different pH values between 7 and 10 were prepared for the growth of bacteria in BG11 medium to determine the best pH value in which the cyanobacteria have the best growth and the growth of fungi and other bacteria had been minimized.

Growth of cyanobacteria in cyanide: The concentrations of 5-150 mg L^{-1} of KCN in 3 repeats were added to the BG11 medium. The media equally inoculated with precultured cyanobacteria in the BG11 medium without cyanide component. The amount of 24×10^6 cell inoculated to every 100 mL of culture media. The growth of bacteria in control medium (without cyanide) and in the presence of cyanide was estimated every 24 h in the presence of light (16 h lightening in 3 Klux and 8 h darkness continuously) in the 140 rpm shaking. The parameters including direct microscopic count, OD in 650 nm, dry weight measurement and the chlorophyll a concentration were analyzed for the growth estimation. Also the control bacterium, *Pseudomonas aeruginosa* ATCC 1074 was cultivated in the presence of 0.1 to 1.5 mg L^{-1} of KCN in 3 repeats, in Mueller Hinton broth media and incubated in darkness in 37°C. The growth of control bacterium determined based on direct microscopic count and optical density in 650 nm.

Cyanide bioremediation analysis: After the growth of cyanobacteria in BG11 media containing KCN, the media was centrifuged at 3000 rpm for 10 min. The supernatant was acidified and distilled for releasing of HCN. The free cyanide concentration analyzed using ASM method, number 98-2036D by using selective cyanide ion characterization electrode (Mensi *et al*., 2008). Also in direct examination, 24×10^6 cyanobacterial cell added to each 100 mL of waste water from steel manufacturing industry. The amount of cyanide removal assayed according to control media after 16 days growing in the presence of light (16 h lightening in 3 Klux and 8 h darkness continuously) in the 140 rpm shaking by the method of Mensi *et al*. (2008).

RESULTS

The isolated cyanobacterium: Based on the characteristics in BG11 medium including green cells with 3-4 mm in diameter, location of bacterial cells in the sheets as individual cell or aggregation of 2 or 4 cells, unicellular or colonial, the isolated cyanobacterium identified as a species of *Chroococcus* in the family of Chroococcaceae (Fig. 1).

Molecular identification of the isolated cyanobacterium: For molecular identification of the isolated cyanobacterium, a 200 bp fragment in 16S rDNA of the *Chroococcus* sp. was amplified using specific primers MAZ.B1 and MAZ.B2 (Fig. 2).

The growth curve of purified cyanobacterium: The growth curve used for determination of the exponential growth time. As shown in Fig. 3, the number of cyanobacterial cells

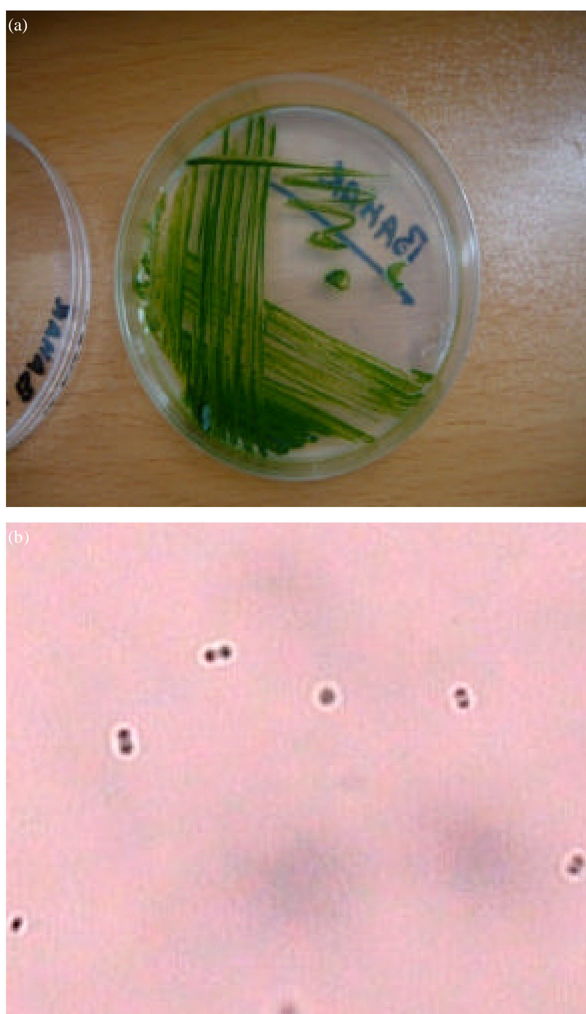

Fig. 1(a-b): (a) The growth of cyanobacterium *Chroococcus* in BG11 solid medium and (b) *Chroococcus* cells in microscopic view with 40X magnification

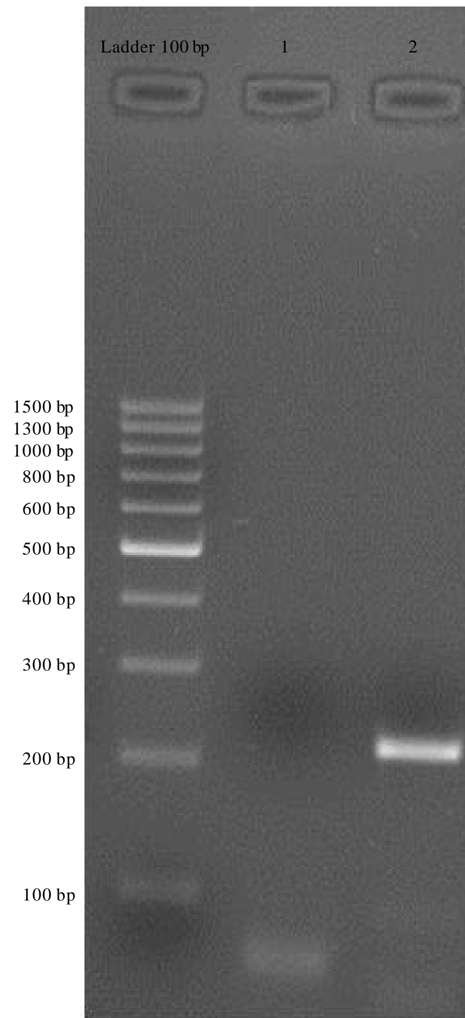

Fig. 2: Amplification of the 200 bp fragment by PCR, using the specific primers designed for detection of cyanobacterium *Chroococcus* sp. (lane 2), Lane 1: Negative control

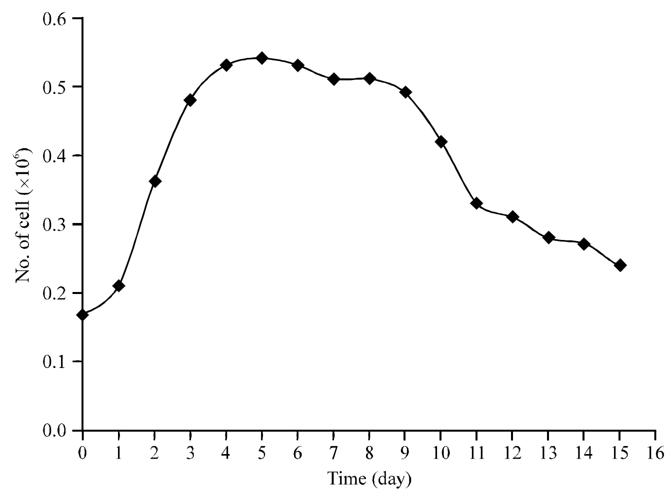

Fig. 3: The growth curve of cyanobacteria in BG11 medium based on the number of cells counted

Table 2: The growth of the *Chroococcus* sp. in different pH values in BG11 medium according to direct cell counting

pH	No. of the bacteria ($\times 10^6$)
7.0	1.02
7.5	3.15
8.0	6.78
8.5	9.13
9.0	9.06
9.5	8.92
10	7.03

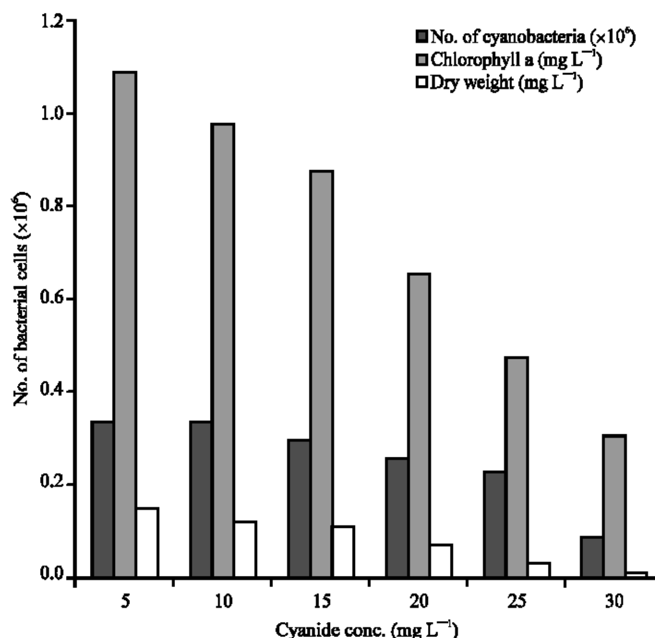

Fig. 4: The growth of isolated *Chroococcus* sp. in the 5-30 mg L^{-1} cyanide in the BG11 medium based on cell count, chlorophyll a content and the dry weight of the cells

increased in the first 5 days (exponential phase), remained almost constant in the second five days (stationary phase) and decreased in the third 5 days (death phase).

The effect of pH on the cyanobacterial growth: The best pH for the growth estimated as 8.5, at which highest bacterial counts were observed (Table 2).

Growth of the isolated *Chroococcus* sp. in culture media: The isolated cyanobacterium was able to grow in the concentration up to 30 mg L^{-1} of potassium cyanide according to different growth parameters as shown in Fig. 4. Also, the control bacterium, *Pseudomonas aeruginosa* ATCC 1074 was able to tolerate up to 1 mg L^{-1} potassium cyanide. There was no growth in the presence of 1 mg L^{-1} potassium cyanide and upper concentrations.

Bioremediation of cyanide by the isolated *Chroococcus* sp.: The cyanobacterium was removed 14.53% to 29.8 of cyanide from culture media (Table 3) and in the steel manufacturing waste water was removed 14.2% to 17.1% (mean of 14.06%) of free cyanide (Table 4).

Table 3: Cyanide removal in culture media after 15 days growth of cyanobacterium

Initial cyanide conc. (mg L^{-1})	Cyanide conc. (mg L^{-1}) after cyanobacterial growth	Cyanide removal (%)
10	7.02	29.80
15	11.86	20.93
20	16.32	18.40
25	20.06	19.76
30	25.64	14.53

Table 4: Cyanide removal in steel manufacturing waste water after 15 days growth of cyanobacterium

Waste water samples No.	Initial cyanide conc. (mg L^{-1})	Cyanide conc. (mg L^{-1}) after cyanobacterial growth	Cyanide removal (%)
1	2.18	1.87	14.2
2	4.59	4.09	10.9
3	6.32	5.24	17.1

DISCUSSION

Cyanide poisoning is a serious environmental hazard which is a problem resulted from different industrial and environmental activities. As cyanide is an inhibitor for the last protein complex of electron transport chain, it is an inhibitor for enzymatic reduction by the enzymes such as nitric oxide reductase (Abdel-Banat *et al*., 2008, 2009). Cyanide can be removed using chemical, physical and biological methods. The biological treatment methods are more cost-effective. The degradation of cyanide into less toxic products can be done by several microbial species. These microorganisms use cyanide as nitrogen and carbon sources and convert it to ammonia and carbonate in appropriate aerobic or anaerobic conditions (Dash *et al*., 2009).

Cyanobacteria have remarkable adaptability to wide ranges of environmental factors. For example, Nagasathya and Thajuddin (2008) isolated 61 species of cyanobacteria in different saltpans of southeastern coast of India. Also cyanobacteria have important role in compost production (Baftehchi *et al*., 2007). In the present study we isolated the cyanobacterium *Chroococcus* sp., which was resistant to potassium cyanide, based on cell count, dry weight and chlorophyll a concentration. The highest growth was obtained in alkaline pH 8.5 in which the growth of other bacteria have been lowered. Luque-Almagro *et al*. (2005) has been shown that the biological treatment of industrial cyanide contaminated effluents requires an alkaline pH for prevention of the formation of volatile HCN (pKa = 9.2). So cyanide bioremediation using alkaliphilic microorganisms such as cyanobacteria is preferred. Cyanobacteria have the ability to survive in different concentrations of cyanide, because of the presence of cyanide-resistant respiration and oxygen uptake pathways (Shing *et al*., 2008).

The steel manufacturing waste water samples contained an average of 4.36 mg L^{-1} cyanide. This concentration is much higher than world standards for these wastes for entering to the environment which determined up to 0.2 mg L^{-1} (Dash *et al*., 2006). The isolated cyanobacterium *Chroococcus* sp. was able to grow in 30 mg L^{-1} potassium cyanide and removed an average of 14.06% of free cyanide in steel manufacturing waste water which was lower than the removal in culture medium. For elevated removal we propose the usage of microbial consortia with other microorganisms and the addition of nutrients to waste waters for better bioremediation.

CONCLUSION

The results of the present study showed that cyanobacteria such as *Chroococcus* sp. which isolated and identified in our study, as well as other cyanobacteria are able to grow in high

concentrations of cyanide. Also, the *Chroococcus* sp. removed a range of 14.53-29.8% of cyanide from culture media and 14.2-17.1% of free cyanide in the samples obtained from steel manufacturing waste water. The difference in the results of culture media and waste water is probably because of poor nutritional conditions in the waste water.

As the cyanobacteria can grow in minimal nutritional conditions and in the presence of light as sole energy source, the usage of these phototrophic bacteria in biological reactors for bioremediation of hazardous materials such as cyanide is proposed as a cost effective approach. Also the usage of phototrophic microbial consortia will probably elevate the bioremediation quality in waste waters.

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