Cytogenetic Study on Mitotic Cell Division in Allium cepa by Lead (Pb) and Chromium (Cr) Containing Bacterial Strain Isolated from Tannery Effluents of Bangladesh

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ABSTRACT

In the present study, a bacterial strain capable of Pb and Cr detoxification was isolated from tannery effluents which was identified as Myroides sp. through 16S rRNA gene sequencing. In the cytogenetic experiment, 100 and 600 μg/ml of lead and chromium were used as treatment for the root tips of Allium cepa and caused many chromosomal abnormalities such as abnormal chromosome position, damaged nucleus, breaks of chromosome bridges and fragments also occurred. Notably, sticky metaphase was found where sticky chromosomes indicated highly toxic, usually irreversible consequences leading to cell death. However, Myroides sp. treated supernatant, collected after day 7, used to treat Allium cepa tips showed less mitotic aberrations, nuclear degeneration and observed normal anaphase and telophase stage indicating possible metal detoxifying ability of the isolated strain. Furthermore, LC50 value was 64.63 μl/ml for Myroides sp.

INTRODUCTION

Heavy metals are the most recurrent inorganic pollutants in water [1] and are highly distributed over the earth’s crust [2]. Epidemiological evidence suggested that a long-term exposure to heavy metals was highly associated with increased risk of development of several diseases including cancers [3]. In vivo and in vitro assays have shown that heavy metals induce chromosomal aberrations and micronucleus in plants [4]. Lead and chromium are considered to be of highest concern because of their genotoxicity in microorganisms, animals and humans. Acute lead poisoning results in a well characterized syndrome manifested in adults by colic, anemia, headache, fatigue, gum line and peripheral neuropathy [5] and has also adverse effects on oxidative stress [6]. Moreover, toxicity of chromium has been associated with cancer and disruption to cellular functions [7]. It was reported that chromium concentrations should not be exceeded 80 μg/ml in sea water and 520 μg/ml in rivers and lakes [8]. Thus, our present investigation was planned to determine bacterial detoxifying ability at 100 μg/ml and 600 μg/ml concentrations of Pb and Cr salt, separately.

Genotoxicity identifies compounds present in the environment having the potential to cause mutation by damaging the DNA which can result into point
incubated at 37°C for 5 days and bacterial colonies were isolated by plating from the old bacterial suspension of enrichment and selection. Pb and Cr degrading bacterium was isolated from the waste water sample collected from the discharge effluents of a leather processing industry in Dhaka, Bangladesh, during January 2016.

**Nitrate [Cr(NO₃)₃] at different concentrations (100 μg/ml, 200 μg/ml, 300 μg/ml, 400 μg/ml, 500 μg/ml, 600 μg/ml) for enrichment and selection. Pb and Cr degrading bacterium was isolated by plating from the old bacterial suspension of the liquid medium into an agar solidified MS medium by the standard pour plate method supplemented with different concentrations of lead and chromium. The plates were then incubated at 37°C for 5 days and bacterial colonies were found to grow on the medium. Isolated bacterial colony was purified by re-streaking further on nutrient agar medium and incubated for 18–24 hour at 37°C. Then the isolate was preserved in 30% glycerol at -20°C.

**Molecular identification of isolated bacterium

16S rRNA identification is considered the most reliable tools for identification of microorganism species [11]. The chromosomal DNA was extracted from the bacterial culture and the extracted DNA was used as template for PCR to amplify 16S rRNA gene. 8F(5’AGA GTT TGA TCM TGG CTC AG3’) and 1492R(5’GTT TAC CTT GTT AGC ACT T3’) were used as forward and reverse primer, respectively. After PCR amplification the gel was run and desired band was found. Next the PCR product was purified and the concentration of purified product was measured using the Nano Drop ND-2000 Spectrophotometer and diluted with sterile water to 50 ng/ml before samples were sequenced. Sequencing was done from both forward and reverse ends using either 8F or 1492R primers and carried out in ABI Prism 310 machine. 16S rRNA gene sequence of selected bacterial isolate was compared with other reference sequences as available in the NCBI database using the Basic Alignment Search Tool (BLAST) algorithm.

**Cytogenetic study

The test material used was onion bulbs from a population of commercial varieties of *Allium cepa*. At first, commercially available onions were soaked into distilled water for 2 or 3 hours, were put on the test tube, filled with distilled water and kept in a dark place at room temperature for growing roots and after 48 hours of exposure, *Allium cepa* roots were observed. The most important macroscopic parameter was the root growth. When *Allium cepa* roots were observed after 48 hours, the length of the root bundle was measured using a calibrated ruler in mm. Three longest roots in each bulb were selected and used for the experiment out of ten onions. Here, the isolated strain was used as test sample to determine the detoxifying capability of the bacterium towards lead and chromium. Before each test, bacterial cultures were centrifuged and 10 ml supernatant was then diluted with 10 ml water which was used as the treatment solution supplemented with different concentrations (100 μg/ml and 600 μg/ml) of Pb and Cr up to 7 days. After 48 hours, the onions were set up for 24 hours in each test solution. At the same time, some of the onions were grown in control medium (Distilled water) for 24 hours.

From both of control and treatment medium roots were collected by a pair of fine forceps and were fixed in aceto-alcohol (1:3) solution. After 24 hours of fixation, root tips were transferred from fixative to 70% ethanol and stored in the refrigerator until they were used in the laboratory. After removing from 70% ethanol the root tips were washed with distilled water for 10 minutes. Washed root tips were then transferred to 1N HCL for 7 minutes and the root tips were washed again with distilled water for 5 minutes. The mordanting was done with 2% aqueous solution of iron alum (Ferric ammonium sulfate) for 7 minutes. The root tips were fixed in 3% glutaraldehyde solution for 2 hours and infiltrated with 1% osmium tetroxide (in 0.1 M phosphate buffer pH 7.2) for 2 hours. After washing in distilled water for 5 minutes, the root tips were dehydrated in graded series of ethanol and embedded in Epon resin. 40–50 μm thick sections were cut and mounted on glass slides. The sections were stained with 2% aqueous solution of uranyl acetate for 1 hour and dehydrated in graded series of ethanol. The slides were air dried and coated with carbon and examined under JEM 1200EX transmission electron microscope.
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washed again with frequent changes of distilled water for 7 minutes in order to complete removal of the mordanting fluid from the tissues. Then root tips were stained with 0.5% hematoxylin for 10 minutes. Finally, root tips were washed with distilled water for 5 minutes. The stained root tip was then taken on a clean slide and meristematic zone (1-1.5 mm portion) of it was cut with a razor blade and a drop of 0.5% acetocarmine was added. The cells were then covered with a cover glass and slight pressure was applied by a pencil eraser over the cover glass. The slide was warmed over an alcohol flame and again a slight pressure was applied by finger tips over the cover glass keeping the slide duly wrapped in blotting paper. All slides were marked and examined under a microscope (LABOMED CXL, USA) with 40X magnification. Then, mitotic abnormalities in treatment medium were recorded and compared to control.

**Cytotoxicity test of isolated bacterium culture**

In our present study, toxicity test of bacterial supernatant was performed through brine shrimp lethality bioassay to make confirmation about bacterial toxic protein secretion and its impacts on disrupting cellular functions. Brine shrimp lethality bioassay is considered a useful tool for preliminary assessment of toxicity [12]. Brine shrimp eggs were hatched in simulated seawater to get nauplii. For test sample, 10 ml of bacterial culture were taken in 10 eppendorf tubes and were centrifuged at 7000 rpm for 10 min. Supernatants were collected which was further used as test sample and specific volumes of test sample were transferred to the respective vials to get, final concentrations of 25 μl/ml, 50 μl/ml, 75 μl/ml, 100 μl/ml, 125 μl/ml, 150 μl/ml, 175 μl/ml and 200 μl/ml, respectively. Survivors were counted after 24 hours and these data were processed in a simple program for probit analysis to estimate LC₅₀ value with 95% confidence intervals.

**RESULT**

**Molecular identification of isolated bacterium**

After gene sequencing, the sequence was checked against the 16S rRNA gene sequences of other organisms that had already been submitted to NCBI Gene bank database. Isolated bacterium showed 98% identity with *Myroides* sp. (Accession no. MG733977). The band of PCR product of isolated bacterium was shown in figure 1.

**Cytogenetic study on Allium cepa**

Control samples indicated normal mitotic cell division. The figure 2(A) clearly showed the prophase stage in which the chromatin shortens and thickens into double stranded chromosomes and the nuclear membrane disintegrates. Normal metaphase is shown in figure 2(B) where the spindle fibers align as double stranded chromosomes in single file along the equator of the cell, metaphase plate. In anaphase (Figure 2C), the spindle fibers pull on the centromeres of the chromosomes separating the sister chromatids of each double stranded chromosome. Each single strand is pulled to an opposite pole of the cell. Consequently in telophase (Figure 2D) the chromatids reach the opposite poles of the cell and the spindle detaches and new nuclear membranes form around the chromatids where cytokinesis begins even before telophase completes with the formation of a cleavage furrow (indentation of the cell membrane). In control experiment, 30% dividing cells (Prophase-17.5%, Metaphase-4.5%, Anaphase-3%, Telophase-5.5%) were found among 2000 cells.

The cyto morphological study indicated adverse effects of chemical on the root tip cells compared to the control untreated group (Figure 3). Low dose of lead (100 μg/ml)
on day 1 caused the appearance of many chromosomal abnormalities (Figure 3A) as reported earlier [13]. Lead also caused development of extensive vacuoles in the root tip cells growing in 100 μg/ml (Figure 3C). The vacuoles of root tip cells are the major sites of metal sequestration under chronic exposure scenario and it is thought to be a detoxification pathway for preventing cell damage by retaining metals in specific vacuoles [14]. *Allium cepa* root tip cells in the highest dose (600 μg/ml) exposed group, after 24 hours exhibited nuclear disruption and condensation of the chromatin material, indicating apoptotic death of root tips cells (Figure 3B). A similar finding of apoptotic death of root tips cells after aluminium exposure was reported in barley [15]. Root tip of *Allium cepa* treated with supernatant, containing low dose of lead (100 μg/ml) that was treated with bacterium for 7 days, showed less mitotic aberration and absence of vacuoles (Figure 3D). High dose (600 μg/ml) of the lead sample also gave the similar result, decreasing the presence of nuclear condensation and abnormal positioning of the chromosome (Figures 3E, 3F).

In case of treatment with bacterial supernatant containing chromium, 20% dividing cells (Nuclear degeneration–9%, Abnormal shape of nucleus–8%, Sticky metaphase–3%) were found among 2000 cells at day 1 of exposure whereas 27.5 % dividing cells (Normal metaphase–9%, Normal telophase–7.5%, Less nuclear degeneration–11%) were observed among 2000 cells at day 7 of exposure.

**Figure 3** The figure shows - A) Sticky telophase and abnormal position and shape of cells B) Improper metaphase and C) Vacuolation at day 1 of exposure (24 hr after treatment). D) Normal structure of nucleus with less abnormality E) Less nuclear condensation and abnormal positioning of chromosomes and F) Normal structure of the cells at day 7 of exposure (168 hr after treatment).

**Figure 4** The figure shows - A) Nuclear degeneration B) Abnormal shape of nucleus and C) Sticky metaphase at day 1 of exposure (24 hr after treatment). D) Similar distributions of the cell with less abnormalities and nuclear degeneration E) Normal metaphase and F) Normal telophase at day 7 of exposure (168 hr after treatment).

Cytotoxicity test of isolated bacterium culture

The cytotoxic effect of isolated bacterial supernatant was studied at concentrations of 25 μl/ml, 50 μl/ml, 75 μl/ml, 100 μl/ml, 125 μl/ml, 150 μl/ml, 175 μl/ml and 200 μl/ml. From the result, it was found that LC50 was 64.63 ± 0.13 μl/ml and the regression equation was, \( Y = 1.963x + 1.444 \), while the 95% confidence limits were 39.34 to 106.19 μl/ml for the 48 hours of exposure (Table 1, figures 5, 6).
DISCUSSION

Environmental pollution by heavy metals is always considered as a serious threat to living organisms in an ecosystem [16,17]. Industrial tannery wastewater is a major source of heavy metal contamination in our environment as heavy metal is of economic significance in industrial use [18]. Several approaches were undertaken to isolate and characterize heavy metal tolerant and degrading bacteria from industrial effluents [19]. Microorganisms with catabolic potential and their products such as enzymes and bio surfactant are directly used to enhance and boost their remediation efficacy [20]. The *Myroides* sp. are prevalent as environmental bacterial organisms [21]. Like other bacteria, *Myroides* sp. can adsorb metal ions by ionizable groups of the cell wall or capsule (Carboxyl, Amino, Phosphate and Hydroxyl groups) [22]. In the present investigation, lead and chromium detoxifying bacterial strain (Yellow colored colony) was isolated from tannery effluents and cultured on MS agar solidified medium supplemented with metals (Lead and chromium) by using streak plate and pour plate methods.

In the present study, sequencing was done by 16S rRNA gene for molecular identification of the isolated bacterial strain. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique [23]. Furthermore, 16S rRNA gene (1.5 kb in length) has emerged as a useful molecular tool since it is present in all bacteria, either as a single copy or in multiple copies as well as it is highly conserved over time within a species [24]. Sequencing result of 16S rRNA gene confirmed that the bacterial isolate showed 98% significant alignments with *Myroides* sp. Although Sanger sequencing of the 16S ribosomal RNA gene is used as a molecular method, identification of bacteria to the species level is not always possible due to high sequence similarities between some species [25]. Moreover, sequencing results are relative rather than absolute in which the actual quantity of a particular bacteria is uncertain. In addition, 16S rRNA gene sequencing could be a result of uncontrollable PCR biasness because of each 16S rRNA gene may not amplify with equal efficiency during PCR reactions due to differential primer affinity and GC content [26].

Generally, induction of structural and numerical changes in chromosomes is a common criterion for recognizing potential genetic hazards of a particular agent. Genotoxic and mutagenic action of heavy metals were evaluated using different cytogenetic techniques applied to the *Allium cepa* test organism [27]. According to a spectrum of morphological abnormalities such as bridge chromosome, improper metaphase, vacuolation and pycnotic nuclei were observed in root meristems after exposure to lead [28]. Several in vivo and in vitro studies have also shown that chromium compounds damage DNA in a variety of ways, including DNA Single And Double-Strand Breaks (SDSBs) generating chromosomal aberrations, micronucleus formation, sister chromatid exchanges, formation of DNA adducts, and alteration in DNA replication and transcription [29]. According to studies carried out by Seth, et al. [30] microbial sequestration of heavy metals show significant improvement in bioremediation of these heavy metals. Abbas, et al. [31] reported the heavy metals (Pb, Cr, Cu, Fe, Mn) detoxifying (50%) ability of *Pseudomonas* sp. within 7 days. In our study we also found that, by treating Pb and Cr with this bacterial isolate for about 7 days significant changes are found in *A. cepa* root cells, including less occurrence of chromosomal aberrations and nuclear condensation. Our findings showed similarity with the referenced data and
from the result we can consider that the high levels of heavy metals induce the genotoxic effects observed in root cells of *A. cepa*. At the same time, we tried to find out toxicity of *Myroides sp.* at cellular level on brine shrimp.

Cytoxic compounds generally show significant activity in the brine shrimp bioassay and this assay can be recommended as a guide for the detection of toxic compounds because of its simplicity and low cost [32]. In our present study, the test was done to confirm whether the bacterium had any effect on mitotic abnormalities because if the bacterium showed toxicity it might secrete extracellular toxic proteins in supernatant. Bacterial protein secretion systems have been an important spotlight in case of bacterial pathogenesis and our detoxifying bacterium was isolated bacterial supernatant enunciated that it can be used as a guide for the detection of toxic compounds because of its simplicity and low cost [32]. In our present study, the test was done to confirm whether the bacterium had any e

inhibit the genotoxic effect of cellular damage that is occurred by the metal poisoning. The results from the present study indicated Pb and Cr detoxifying capability of *Myroides sp.* by reducing the mitotic abnormalities in root cells of *Allium cepa* which may affect the normal growth and development at field level. Therefore, the cytoxic effect of isolated bacterial supernatant enunciated that it can be selected for further experimental assay as it had a little toxic effect at cellular level on *Artemia salina*.

CONCLUSION

The potential application of heavy metals detoxification in the forms of toxic solid and liquid wastes by microorganisms has several advantages. By isolating and using bacteria we can able to reduce the amount of metals from the contaminated water or soil and can reduce the effect of cellular damage that is occurred by the metal poisoning. The results from the present study indicated Pb and Cr detoxifying capability of *Myroides sp.* by reducing the mitotic abnormalities in root cells of *Allium cepa* which may affect the normal growth and development at field level. Therefore, the cytoxic effect of isolated bacterial supernatant enunciated that it can be selected for further experimental assay as it had a little toxic effect at cellular level on *Artemia salina*.

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References


