In vitro evaluation of bioremediation capacity of a commercial probiotic, Bacillus coagulans, for chromium (VI) and lead (II) toxicity

Pranoti Belapurkar, Pragya Goyal, Anand Kar

ABSTRACT

Introduction: The bioaccumulation of heavy metals including chromium (VI) (Cr (VI)) and lead (II) (Pb (II)) causes fatal toxicity in humans. Some naturally occurring bacterial genera such as Bacillus and Pseudomonas help in bioremediation of these heavy metals and some of the species of Bacillus are proven probiotics. However, no study has been conducted on Bacillus coagulans, which is a proven probiotic species of genus Bacillus.

Objectives: The primary objective of the present study was to assess the potential of a proven probiotic, B. coagulans, marketed as “Sporlac-DS,” to survive in the presence of Cr (VI) and Pb (II) and its ability to reduce its concentration in vitro. Materials and Methods: The Minimum inhibitory concentration (MIC) of the organism for Cr (VI) and Pb (II) was determined followed by its biochemical and morphological characterization. Its antibiotic sensitivity and probiotic efficacy were assessed. Further, its bioremediation capacity was observed in vitro by determining the residual Cr (VI) and Pb (II) concentration after 72 h. Results: B. coagulans could tolerate up to 512 ppm concentration of Cr (VI) and had an MIC of 128 ppm for Pb (II). After 72 h, the organism reduced 32 ppm Cr (VI) and 64 ppm Pb (II) by 93% and 89%, respectively. When B. coagulans was studied before and after growing on Cr (VI) and Pb (II) for 24 h, an increase was seen in sensitivity toward the tested antibiotics whereas no change was observed in morphological and biochemical characters. It also showed no change in their bile and acid tolerance, indicating that it retains its probiotic efficacy. Conclusion: The tested probiotic B. coagulans may have a potential role in bioremediation of Cr (VI) and Pb (II), in vivo.

KEY WORDS: Bacillus coagulans, bioremediation, chromium, lead, probiotics

Various human activities such as mining, agriculture, transportation, and industrial production lead to the release of large amounts of heavy metals including chromium (VI) (Cr (VI)) and lead (II) (Pb (II)) into the biosphere. In cities especially of developing countries, heavy metal contamination is a major cause of environmental pollution. Some other factors which aid heavy metal contamination are: use of contaminated water for field irrigation, use of fertilizers and metal based pesticides, harvesting process, and industrial emissions. Through these processes, heavy metals enter the food chain most commonly through leaves where the heavy metal accumulation is very high and thereby threatening human life. These heavy metals also enter the human body through inhalation and skin absorption. If the heavy metals accumulate in the body tissues faster than their detoxification, it leads to their gradual buildup causing toxicity leading to diseases, especially cardiovascular, renal, bone, and nervous diseases. They are further responsible for causing impairment of immune system, intrauterine growth retardation, and upper gastrointestinal tract carcinomas.

Many microorganisms including lactic acid bacteria (LAB) are naturally present in environment that tolerate these heavy metals and even utilize them for their growth. The common strategies used by microorganisms for survival under heavy metal stress are (i) biosorption (ii) bioaccumulation, and (iii) bioremediation. This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

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(iii) transformation to less toxic form. Some species of LABs are also associated with human gut flora and are known probiotics. According to WHO, probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit to the host.” They are used either as live microbial dietary supplements or food ingredients that impart a beneficial effect on the host. They influence the composition as well as metabolic activity of the flora of gastrointestinal tract. There are some genera of microorganisms in nature including Bacillus whose few species have the property of bioremediation of heavy metals while their other species have also shown probiotic efficacy.

The present study is an attempt to evaluate the potential role of some commercial strains of genus Bacillus that are established Pb, in bioremediation of Cr (VI) and Pb (II).

Materials and Methods

Material

Commercially available strain of probiotic “Sporlac-DS” was procured from local chemist shop at Indore, India. Each 200 mg tablet contained 120 million spores of Lactobacillus sporogenes. This species was originally isolated and described by Horowitz-Wlassowa and Nowotelnov, which was later reclassified as Bacillus sporogenes. This species is evidenced to share some characteristics of Bacillus coagulans and therefore, the species has been moved into B. coagulans group. According to 8th edition of Bergey’s manual of Determinative Bacteriology, spore-bearing rods, producing lactic acid, facultative or aerobic, and catalase-positive are to be classified within the genus Bacillus.

Determination of minimum inhibitory concentration (MIC)

The spores were inoculated by spread plate technique on de Man, Rogosa and Sharpe (MRS) medium and incubated at 37°C for 24 h. MIC of B. coagulans for Cr (VI) and Pb (II) was determined by methodology of Mistry et al.[22] For this, MRS plates were prepared having two-fold concentration range of 1–512 ppm for potassium dichromate and lead nitrate as source of Cr (VI) and Pb (II) respectively and inoculated with 24 h old culture of the organism and incubated at 37°C for 24 h.

Morphological and biochemical characterization of resistant bacteria

To check whether the heavy metals have caused any changes to the culture under study, the bacteria were morphologically and biochemically characterized before and after growth on Cr (VI) and Pb (II) for 24 h.

Similarly, both were tested for antibiotic sensitivity by standard disc diffusion method[31] against Kanamycin (30 µg), Norfloxacin (10 µg), gentamycin (10 µg), chloramphenicol (30 µg), amoxycillin (10 µg), ciprofloxacin (5 µg), and teicoplanin (30 µg). The antibiotic discs were procured from HiMedia.

Further, B. coagulans was characterized for its probiotic efficacy after growth on Cr (VI) and Pb (II) for 24 h. It was determined by bile and acid tolerance assays; both the tests were performed in triplicates.

Bile tolerance was determined by broth assay using methodology of Jacobsen et al.[14] with some modifications. MRS broth with 0.2, 0.3, and 0.4% (w/v) bile salts was inoculated with overnight cultures and tubes were incubated for 6 h at 37°C under aerobic conditions. The control and test cultures were adjusted to pH 6 using either 1N HCl or NaOH. Turbidity was monitored hourly at 650 nm using UV-visible spectrophotometer (Systronics-118). MRS broth without bile salt was used as control.

The tolerance to acidic pH was evaluated according to the methodology of Ehrmann et al. with some modifications.[35] The test cultures were incubated at three different pH, i.e., 2, 3, and 4 for 30 min to 4 h. They were first grown in MRS broth at 37°C for 24 h and then refreshed in 10 ml MRS broth for another 24 h. The culture was then centrifuged at 8000 rpm for 15 min at 4°C followed by washing of bacterial pellet by phosphate-buffered saline (PBS). The pellet was then diluted twenty times with PBS and its pH was adjusted to 2, 3, and 4 and incubated for 0.5, 1, 2, 3, and 4 h at 37°C. The cells were enumerated by plating on MRS agar plates and incubating them for 24 h at 37°C.

Determination of residual chromium (VI)

The Cr (VI) reduction by Cr-resistant B. coagulans was done at 32 ppm. For this, the culture was grown overnight at 37°C in MRS broth and harvested at 8000 rpm for 15 min in cold conditions. The supernatant obtained was used for Cr (VI) estimation by diphenylcarbazide (DPC) method as given in the standard protocol by the American Public Health Association.[36] To 1.5 ml of supernatant, two drops of H2PO4 were mixed and pH was adjusted to 1.0 ± 0.3 with 0.2 N H2SO4 and the volume was made up to 100 ml and 2 ml of DPC was added to it. The mixture was allowed to stand for 5–10 min for color development and optical density (OD) was measured at 540 nm.

Determination of residual lead (II)

For determination of residual Pb (II) concentration, sample was prepared by the method of Fifield and Haines and was determined by colormetric method.[38] For this, Pb-resistant B. coagulans was inoculated at 64 ppm Pb (II) MRS broth and incubated overnight. The cells were harvested in cold conditions at 8000 rpm for 15 min. For estimation of Pb (II), the supernatant was used. A small amount of supernatant was added to a flask containing 5% KMnO4 and 2–3 drops of concentrated H2SO4 and refluxed for 4 h. After this, hydroxylamine hydrochloride acid was added to reduce KMnO4 followed by addition of ammonial citrate cyanide solution and dithizone. The whole system was shaken vigorously for 50 s and OD was taken at 750 nm using MRS broth without Pb (II) as blank.
Results

The culture of *B. coagulans*, when tested for MIC against Cr (VI), showed good growth up to 512 ppm, which is way beyond the permissible limit; hence, no MIC was obtained. For Pb (II), the growth was observed up to 64 ppm and the MIC was obtained at 128 ppm. These concentrations, i.e., 512 ppm for Cr (VI) and 64 ppm for Pb (II), are referred to as high heavy metal concentration in this study.

On comparison, the morphological and biochemical characteristics of the culture under study before and after growing at high concentration of Cr (VI) and Pb (II) showed no appreciable change suggesting that even such high concentration of heavy metals did not alter its basic characteristics [Table 1]. The culture showed increased sensitivity against most of the antibiotics tested for, even after growing on heavy metals [Table 2 and Figures 1, 2], which is in corroboration with earlier reports of Mishra *et al.* and Khusro *et al.*[33,39]

As the organism used in the study is a probiotic, therefore, to observe for any change in its probiotic efficacy, the bacteria grown on high heavy metal concentration for 24 h was tested for its acid and bile salt tolerance. For any bacteria to have a potential to work as probiotic, it must tolerate very low pH of the stomach and should also be able to tolerate and survive in the presence of bile salts. The cultures were found to be most resistant at 0.3% bile salt concentration and also showed a good survival rate at pH as low as 2.

The percentage reduction of 32 μg/ml of Cr (VI) in MRS broth after inoculation with log phase *B. coagulans* after 24, 48, and 72 h was found to be 58%, 76%, and 93%, respectively. The Pb (II) resistant *B. coagulans* reduced 64 μg/ml of Pb (II) in broth by 52%, 71%, and 86% after 24, 48, and 72 h, respectively [Figure 3].

Discussion

Our findings clearly indicate that the probiotic *B. coagulans* has a potential role in *in vitro* bioremediation of Cr (VI) and Pb (II). The good growth of *B. coagulans* up to 512 ppm of Cr (VI) is much beyond the permissible limit of 0.1 mg/L limit of total Cr for drinking water.[40] According to USEPA, the maximum permissible limit for Pb (II) is zero in drinking water. The culture tested in the study showed great resistance toward both heavy metals beyond these limits, thus confirming...

![Figure 1: Comparative antibiotic sensitivity of *Bacillus coagulans* before and after its growth on Pb (II)](image1)

![Figure 2: Antibiotic sensitivity of *Bacillus coagulans* after its growth on Cr (VI)](image2)

![Figure 3: Percentage reduction of 32 ppm Cr (VI) and 64 ppm Pb (II) in MRS broth by Cr (VI) and Pb (II) resistant *Bacillus coagulans*](image3)

| Table 1: Morphological and biochemical characterization of *Bacillus coagulans* before and after its growth on chromium (VI) and lead (II) |
|-----------------------------------------------|
| Morphological and biochemical properties | Characteristics of *B. coagulans* and its Cr (VI)- and Pb (II)-resistant strains |
| Colony characteristics | Creamish, opaque, elevated, small-medium sized |
| Cell shape and arrangement | Squarish end bacillus, single or in chain |
| Gram’s staining | Gram-positive |
| Endospore staining | Positive |
| Catalase test | Positive |
| Mannitol fermentation | Acid and gas production |
| Sucrose fermentation | Acid and gas production |
| Sorbitol fermentation | Acid and gas production |
| MR test | Positive |
| VP test | Negative |

MR: Methyl Red, VP: Voges-Proskauer. *B. coagulans*: *Bacillus coagulans*, Cr (VI): Chromium (VI), Pb (II): Lead (II)
Table 2: Antibiotic sensitivity of original *Bacillus coagulans* before and after its growth on chromium (VI) and lead (II)

| Antibiotics | Zone of inhibition of original *B. coagulans* (mm) | Zone of inhibition of *B. coagulans* resistant to 64 ppm Pb (II) (mm) | Zone of inhibition of *B. coagulans* resistant to 512 ppm Cr (VI) (mm) |
|-------------|--------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| Chloramphenicol (30 µg) | 23 | 24 | 28 |
| Kanamycin (30 µg) | 12 | 10 | 25 |
| Amoxicillin (10 µg) | 14 | 12 | 12 |
| Teicoplanin (50 µg) | 12 | 12 | 19 |
| Ciprofloxacin (5 µg) | 07 | 07 | 23 |
| Norfloxacin (10 µg) | 07 | 06 | 26 |
| Gentamycin (10 µg) | 10 | 14 | 22 |

*B. coagulans*: *Bacillus coagulans*, Cr (VI): Chromium (VI), Pb (II): Lead (II)

that it is naturally resistant to Cr (VI) and Pb (II). Many studies performed on environmental strains of *Bacillus* genus have shown resistance as well as tolerance to heavy metals like Cd, Zn, Cu, and Pb.[41] Similar results were reported earlier,[42] indicating tolerance of *Bacillus cereus* toward Cd and Cr. The species of *Bacillus* under study, though probiotic, showed good ability to grow at high concentration of Cr (VI) and Pb (II), which corroborates with the earlier reports of environmental species of *Bacillus*.[41,42]

There was no morphological and biochemical change in the organism under study even after growing at such high concentration of heavy metals. These observations are in corroboration with Mishra et al.[33] wherein it is reported that the Cr-resistant probiotic LAB strains showed similar biochemical parameters as their respective normal strain.

It has been reported earlier that heavy metal resistance in bacteria exists together with antibiotic resistance.[43] Certain environmental conditions such as metal stress make the bacterial strain to acquire the antibiotic resistance plasmid either by mutation or natural selection.[44,45] However, *B. coagulans*, after growth on Pb (II), showed negligible change in sensitivity to the antibiotics tested against while the organism showed increased sensitivity to almost all antibiotics after growing at high Cr (VI) concentration of 512 ppm [Table 2]. Khusro et al. have reported similar finding for *Bacillus subtilis* strain KPA where high metal resistance has not lead to increase in antibiotic resistance.[39] Similar increase in antibiotic sensitivity by Cr-resistant LAB strains has been reported earlier.[33] These results are against reports of Basu et al. and Kamala-Kannan and Lee.[45,46] In fact, the bacteria, after growing on high concentration of heavy metals showed bile and acid tolerance, indicating that it has not lost its probiotic efficacy and can still tolerate and survive at very low pH of stomach in presence of bile salts.

Heavy metals such as Cr and Pb are common environmental pollutants. They are released by different industries. Due to demerits of existing conventional methods, newer eco-friendly biological methods are being widely used. In nature, *Bacillus* species is reported to have high biosorptive power attributing to its high teichoic acid and peptidoglycan content in cell wall.[16,46] Since the strain under study, i.e., *B. coagulans* is a proven probiotic strain belonging to *Bacillus* genus, it might have a potential to biosorb heavy metals such as Cr and Pb entering the gut as reported now. Since probiotic remain for short duration of 2–3 days in human gut, this strain can be used as a probiotic bioremediator as it reduced Cr (VI) and Pb (II) to a great extent (93% and 86% respectively in 72 h).

**Conclusion**

This study has exhibited the potential of heavy metal bioremediation by probiotic species of genus *Bacillus*, *B. coagulans*. Although similar finding has been reported for LABs, the potential of probiotic species of genus *Bacillus* for bioremediation is yet unexplored. *B. coagulans* along with various other probiotic species of this genus can also be studied for bioremediation of different heavy metals along with their mechanisms. Such probiotic organisms can be genetically engineered to increase their efficacy of heavy metal bioremediation, in *vitro* as well as in *vivo*.

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**Conflicts of interest**

There are no conflicts of interest.

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