



POTENTIAL ASSESSMENT OF *BACILLUS COAGULANS* FOR BIOREMEDIATION OF ZN (II) AND NI (II): AN *IN VITRO* STUDY

Pranoti Belapurkar^{1*}, Pragya Goyal² and Anand Kar³

¹Associate Professor, Dept. of Biotechnology, IPS Academy, Rajendra Nagar, Indore.

²Assistant Professor, Dept. of Biotechnology, IPS Academy, Rajendra Nagar, Indore.

³Professor, School of Life Sciences, DAVV, Indore.

***Corresponding Author: Pranoti Belapurkar**

Associate Professor, Dept. of Biotechnology, IPS Academy, Rajendra Nagar, Indore.

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ABSTRACT

To understand the microbe-metal relationship in terms of the bioremediation strategies in nature, a study was undertaken to assess the potential of *Bacillus coagulans*, a probiotic species of genus *Bacillus*, for bioremediation of Zn (II) and Ni (II) *in vitro*. The minimum inhibitory concentration of *B. coagulans* against Zn (II) was found to be 16 ppm while it was 256 ppm for Ni (II), indicating that the test organism can tolerate Ni (II) better than Zn (II). When Zn (II) and Ni (II) tolerant culture was grown on nutrient agar medium, no variation was observed in its colony morphology, staining and biochemical properties as compared to the unexposed culture. Both exposed and unexposed cultures of *B. coagulans* were tested for their antibiotic sensitivity against seven antibiotics. The Zn (II) tolerant culture showed increased sensitivity against most of the antibiotics especially against ciprofloxacin, teicoplanin and norfloxacin. However, no change in sensitivity was obtained against kanamycin. Ni (II) tolerant culture showed significant increase in sensitivity against almost all antibiotics tested against it. When Zn (II) tolerant culture was tested for any change in its probiotic efficacy, it showed highest bile tolerance at 0.3% bile (w/v) after 3 hrs and showed acid tolerance at pH 3. Similar results were obtained for Ni (II) tolerant culture. These results indicate that even under heavy metal stress *B. coagulans* did not undergo any change and maintained its probiotic nature, thus suggesting its great potential to bioremediate the two test heavy metals, possibly in *in vivo* condition as well.

KEYWORDS: Bioremediation, Probiotic, Heavy Metals, MIC, Antibiotic Sensitivity.

INTRODUCTION

Humans across the globe are often exposed to heavy metals such as cadmium, nickel, zinc, arsenic and mercury. These heavy metals enter the food chain due to the anthropogenic activities. This problem is thought to be more in developing countries where screening and treatment of soil and water sources are not readily available.^[1-3] Due to high toxicity, carcinogenicity and mutagenicity of these heavy metals,^[4] they cause many disorders in humans like disruption of hormone and lipid metabolism, infertility, memory loss, cancers and affect the functioning of multiple organs of the human body.^[5-6] Microbes interact with heavy metals present in contaminated soil and water. This interaction has led to development of coping mechanisms in them which allows them to survive at toxic levels of these heavy metals.^[7-9] The mechanisms include biosorption, bioaccumulation, biotransformation and biomineralization for bioremediation of heavy metals.^[10-13] Various genera including *Bacillus* have shown the capacity of bioremediation of heavy metals present in the environment. Similarly, intestinal

microbiota of humans have also been known to sequester heavy metals. This is supported by studies where it was observed that clinical samples had lower concentration of heavy metals as compared to concentration of heavy metals ingested/consumed.^[14,15] The present study focuses on determination of tolerance of a species of the *Bacillus* genus i.e. *B. coagulans*, an established probiotic, for Zn (II) and Ni (II) *in vitro*, so that it can be used for potential bioremediation, *in vivo*.

MATERIAL AND METHODS

Material

The organism for the study, *Bacillus coagulans*, was procured in the form of commercially available tablet, Sporlac-DS from the local chemist. It is a Gram positive motile rod, which is facultatively anaerobic. This test organism was studied for its tolerance against two common heavy metal contaminants: Zn (II) and Ni (II). The metals were obtained as zinc sulphate and nickel chloride salts from Merck, Germany. Seven different antibiotics [Kanamycin (30 µg), Norfloxacin (10 µg), gentamycin (10 µg), chloramphenicol (30 µg),

amoxycillin (10 µg), ciprofloxacin (5 µg), and teicoplanin (30 µg)] were obtained from HiMedia, India.

Determination of minimum inhibitory concentration (MIC) of Zn (II) and Ni (II) tolerant bacteria

The MIC of the test culture was determined against Zn (II) and Ni (II) by method of Mistry *et al* (2010).^[16] Two-fold ppm dilutions of respective heavy metals were prepared ranging from 1-512 ppm in nutrient agar (NA) medium. All the plates were inoculated with 24 hr old test culture and incubated at 37°C for 24 hrs.

The lowest concentration of the metal that inhibited the visible growth on the NA plate was considered as MIC for the respective metal.

Characterization of Zn (II) and Ni (II) tolerant bacteria

To check whether the heavy metals have caused any changes to the culture under study, the Zn (II) and Ni (II) tolerant bacteria were morphologically and biochemically characterized.

Morphological characterization

The Zn (II) and Ni (II) tolerant *B. coagulans* was characterized according to cell shape, size and arrangement and staining properties like Gram staining reaction and endospore staining by Barthelomew-Mittwear protocol.

Biochemical characterization

Catalase production

Slants of NA were inoculated with the heavy metal tolerant *B. coagulans* and incubated at 37°C for 24 hrs. Two to three drops of H₂O₂ were added on the visible growth and observed for vigorous bubbling within 10s.

Fermentation of carbohydrates

Fermentation broth was prepared by adding carbohydrate to be tested (glucose, sucrose, lactose) to nutrient broth (NB) along with phenol red as pH indicator. Inverted Durham's tube was placed inside each tube. Each heavy metal tolerant culture was added to the individual nutrient broth tubes and incubated at 37°C for 24-48 hrs and observed for colour change and gas production. An uninoculated NB tube was labelled as control.

Methyl Red-Voges Proskauer (MRVP) test

To determine glucose fermentation products, MRVP test was performed in glucose phosphate (GP) broth tubes. Two tubes of GP broth were inoculated with Zn (II) and Ni (II) tolerant *B. coagulans* while one uninoculated tube was kept as control. All the tubes were incubated for 72-96 hrs at 37°C. For MR test 5 drops of methyl red indicator was added and observed for development of red colour immediately. For VP test 12 drops of VP reagent I and 2-3 drops of VP reagent II were added to and the broth was exposed to air by removing the caps and

mixed intermittently and observed for half an hour for colour change from pink to crimson.

Antibiotic sensitivity

This test was performed using unexposed *B. coagulans* as well as heavy metal tolerant *B. coagulans* to observe whether the heavy metal exposure has caused any changes in its sensitivity towards antibiotics. For this both unexposed and tolerant cultures were tested for seven different antibiotics mentioned above by Kirby-Bauer disc diffusion method.^[17] The inoculated plates were incubated at 37°C for 24hrs and results were compared on the basis of their zone of inhibition.

Characterization for probiotic efficacy

Bile tolerance

The bile tolerance of the exposed cultures was estimated by the methodology of Hassanzadazar *et al.*, 2012^[18] with some modifications. The tolerant culture was grown overnight at 37°C in NB. A saturated bile solution was prepared which was used as stock. It was filter sterilized and added to the tolerant cultures to make three working concentrations (0.2, 0.3 and 0.4%). 0% served as control sample. The cultures were incubated for 3 hrs at 37°C and every hourly they were monitored for growth by measuring the absorbance at 600 nm. The experiments were done in triplicates.

Acid tolerance

The tolerant culture was grown overnight at 37°C in NB. To test tolerance to acidic pH, the methodology of Hassanzadazar *et al.* (2012) was followed with some modifications.^[18] 0.1ml of each tolerant culture was adjusted to pH 2, 3, and 4 with 5N HCl. The cultures were incubated for 3 hrs at 37°C and every hourly the viable number of bacteria were enumerated by pour plate technique. The experiments were done in triplicates.

RESULTS AND DISCUSSION

Minimum inhibitory concentration (MIC)

Many studies performed on environmental strains of *Bacillus* genus have shown tolerance to various heavy metals.^[19,13,20] In present study, the MIC of *B. coagulans* for Zn (II) was found to be 16 ppm while that for Ni (II) was 256 ppm indicating that the culture tested showed greater resistance towards Ni (II) as compared to Zn (II). Similar studies conducted earlier have shown that *Bacillus* sp. has lower tolerance for zinc^[20] as compared to Ni (II)^[21] which indicates that this probiotic species might have some potential in bioremediation of both the heavy metals especially nickel.

Zn (II) tolerant *B. coagulans*

After growth in the presence of Zn (II), the Zn (II) tolerant *B. coagulans* were characterized morphologically and biochemically, the results of which are shown in Table 1.

Table. 1: Comparison of morphological and biochemical characteristics of *B. coagulans* before and after exposure to Zn (II) and Ni (II).

S. No.	Characteristics	Unexposed <i>B. coagulans</i>	Ni (II) tolerant and Zn (II) tolerant <i>B. coagulans</i>
1.	Colony characteristics	Creamish, opaque, small	Creamish, opaque, small
2.	Gram's staining	Gram positive	Gram positive
3.	Endospore staining	Endospore present	Endospore present
4.	Catalase test	Positive	Positive
5.	Glucose	Acid formation and gas production	Acid formation and gas production
6.	Sucrose	Acid formation and gas production	Acid formation and gas production
7.	Lactose	Acid formation and no gas production	Acid formation and no gas production
8.	MR test	Positive	Positive
9.	VP test	Negative	Negative

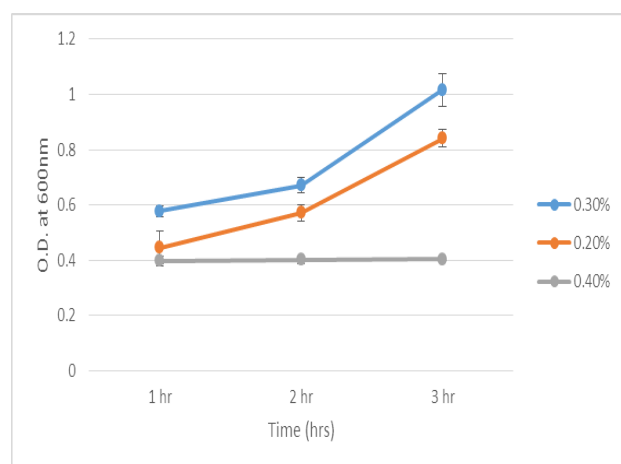
To investigate if Zn (II) tolerant *B. coagulans* developed resistance against antibiotics, the antibiotic sensitivity test of exposed and unexposed bacteria was carried out using antibiotics such as kanamycin (30 µg), norfloxacin (10 µg), gentamycin (10 µg), chloramphenicol (30 µg), amoxycillin (10 µg), ciprofloxacin (5 µg), and teicoplanin (30 µg). Interestingly the culture showed an

increase in antibiotic sensitivity for almost all the tested antibiotics especially for ciprofloxacin, teicoplanin and norfloxacin where the size of zone of inhibition increased from 10 mm to 13 mm, 7 mm to 12 mm and 7 mm to 11 mm respectively (Table 2). However, no change in sensitivity was obtained against kanamycin (Table 2).

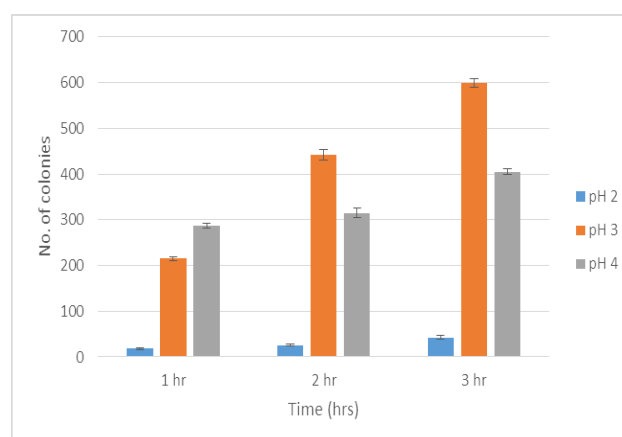
Table. 2: Diameter of zone of inhibition (mm) of Zn (II) and Ni (II) tolerant *B. coagulans* for different antibiotics.

S. No.	Antibiotic	Zone of inhibition of unexposed <i>B. coagulans</i> (mm)	Zone of inhibition of Zn (II) <i>B. coagulans</i> (mm)	Zone of inhibition of Ni (II) <i>B. coagulans</i> (mm)
1.	Ampicillin	6	12	30
2.	Chloramphenicol	6	10	24
3.	Ciprofloxacin	20	26	30
4.	Amoxycillin	12	22	30
5.	Norfloxacin	14	26	20
6.	Kanamycin	22	22	24
7.	Teicoplanin	14	24	8

As the culture under study is probiotic in nature, the Zn (II) tolerant *B. coagulans* was evaluated for its probiotic efficacy by checking for its bile tolerance and acid tolerance. This culture showed maximum optical density and hence highest bile tolerance at 0.3% bile (w/v) after 3 hrs (Fig 1).

**Fig. 1: Bile tolerance of Zn (II) tolerant *B. coagulans*.**

For acid tolerance, the maximum number of colonies was observed at pH 3 after 3 hrs (Fig 2).

**Fig. 2: Acid tolerance of Zn (II) tolerant *B. coagulans*.**

Ni (II) tolerant *B. coagulans*

Upon evaluation of the morphological and biochemical characters of Ni (II) tolerant *B. coagulans*, it was observed that it did not show any change in its colony morphology, staining properties and biochemical

characters indicating that it did not undergo any change under heavy metal stress (Table 1). When the culture was tested for its antibiotic sensitivity for various antibiotics listed above, it showed an increase in zone of inhibition for kanamycin (22 mm to 24 mm), ampicillin (6 mm to 30 mm) and norfloxacin (14 mm to 20 mm), ciprofloxacin (20 mm to 30 mm) and chloramphenicol (6 mm to 24 mm) but a reduction in its sensitivity for teicoplanin (14 mm to 8 mm) (Table 2). When this Ni (II) tolerant strain was tested for its bile tolerance it was observed that it showed maximum absorbance at 0.3% bile after 3 hrs (Fig 3).

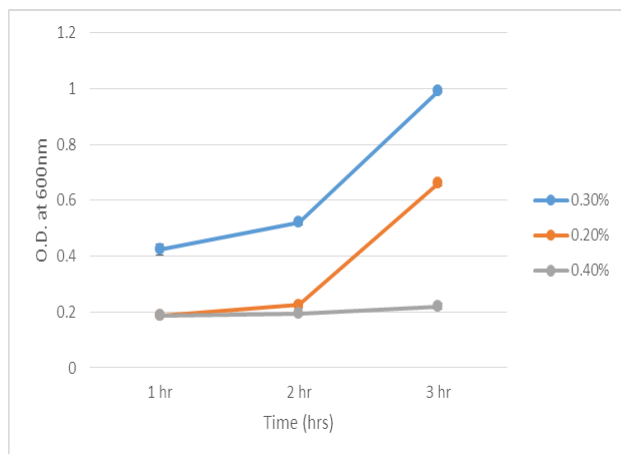


Fig. 3: Bile tolerance of Ni (II) tolerant *B. coagulans*.

Similarly, it showed no change in its acid tolerance i.e. maximum number of colonies was recorded at pH 3 after 3 hrs (Fig 4).

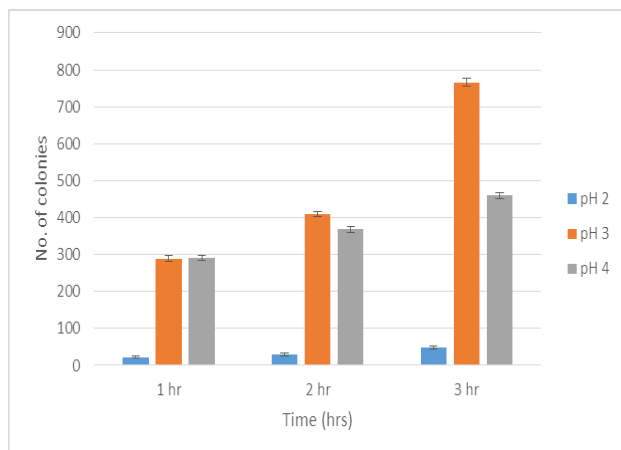


Fig. 4: Acid tolerance of Ni (II) tolerant *B. coagulans*.

Heavy metals are known to be mutagenic in nature^[22] and exposure to them can cause change in characteristics of the organism under heavy metal stress.^[23,24] However, the above results indicate that the organism under study did not undergo any change and retained its morphological and biochemical characteristics.

Both antibiotic resistance and ability to withstand heavy metal stress are plasmid encoded.^[6,25] Under environmental stress, it has been found that

microorganisms adapt faster by spread of R-factors than by mutation or natural condition.^[26,27] However, both Zn (II) and Ni (II) tolerant cultures showed an increase in their susceptibility towards the antibiotics tested against, which is in corroboration with results obtained in previously conducted studies.^[28,29]

An important characteristic of a probiotic bacteria is its ability to survive, grow and function in the gut,^[30] where it encounters varying conditions. This is critical to their function. The range of gastric pH varies from pH 2.5-3.5^[31] while the bile salt concentration in the intestine ranges from 0.2%- 2.0%.^[32] For an organism to be categorized as probiotic it must survive a critical bile concentration of 0.3%,^[33,30,34] while the pH for acid tolerance has been standardized as pH 3.^[35] The organism under study is an already established commercially available probiotic. Even under Zn (II) and Ni (II) stress it does not lose its probiotic efficacy, indicating that *B. coagulans* has the potential to be used in heavy metal bioremediation *in vivo*.

CONCLUSION

The study undertaken has proved that a commercially available probiotic *B. coagulans* can tolerate heavy metals such as Zn (II) and Ni (II). As indicated by its MIC, its potential for bioremediation of Ni (II) is more as compared to that of Zn (II). Despite exposure to high concentration of these heavy metals, it remained unchanged morphologically and biochemically. The heavy metal stress does not affect its probiotic efficacy as well. Hence, it appears that *B. coagulans* has the potential for bioremediation in the human gut. However, further studies are needed to prove its role *in vivo*.

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