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Original Research Paper

Identification and Characterization of Zinc Solubilizing Bacteria Isolated from Mixed Sewage of East Kolkata Wetlands

Amrita Mukherjee, Rubina Islam, Aditya Bhattacharya, Sayantan Bose, Sandeep Kaur Matharu, Sitabja Mukherjee, and Arup Kumar Mitra

Department of Microbiology, St. Xavier's College, 30 Mother Teresa Sarani, Kolkata-700 016, West Bengal, India

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ABSTRACT

The eastern wetlands of Kolkata have been reservoirs of some of the most unique metal tolerant bacteria that have inexplicable bioremediation potentialities in immobilizing radionuclides and toxic metals, in the degradation of oil, as a bioleaching agent, and in chelating zinc in living systems. Four bacterial strains were isolated from mixed sewage and designated as L4(1), L4(2), L4(3) and L4(4). Gram staining was performed to determine the Gram nature and morphology of the strains. All were found to be Gram negative coccobacilli in character. The IMViC biochemical tests were performed for the characterization of the strains. Further, the L4(4) strain was found to grow on Eosin Methylene Blue agar medium, thereby indicating it to be of coliform origin. Different concentrations of zinc solutions i.e., 100ppm, 500ppm and 1000 ppm were prepared in which each of the four strains were inoculated, and incubated at 37°C for 4 days. The bacterial growth was measured spectrophotometrically at 610nm. Atomic absorption spectroscopy analysis was performed to determine the uptake of zinc from the medium. Maximum uptake at 1000 ppm concentration of zinc was observed by strain L4(4) [954.7 mg/L] and least by strain L4(1) [896mg/L]. At 500ppm concentration of zinc, maximum uptake was observed by the strain L4(3) [464.7mg/L], and minimum by the strain L4(4) [442.7mg/L]. L4(4) was identified to be *Escherichia coli*. L4(3) having maximum uptake at 500ppm concentration was identified with the help of 16S rDNA analysis and was found to be *Pseudomonas pseudoalcaligenes*. This organism on one hand acts as a potential source of zinc to fishes thereby acting as a probiotic. We project this organism for future treatment of marine oil spills, and it can be considered as one stop remedy for different kinds of marine pollution.

INTRODUCTION

Zinc is a micronutrient required for both human and plant growth, apart from its utility in the manufacture of galvanized steel. The total zinc content in our body fluids and tissues has been estimated to be ~30mmol (2g). Zinc influences our system since it participates in constituting a large number of enzymes required in the synthesis and degradation of carbohydrates, lipids, proteins, nucleic acids and in the metabolism of other micronutrients. It has shown stabilizing effect on the molecular structure of cellular components and membranes leading to the integrity of organs. It also plays an essential role in polynucleotide transcription and in regulating genetic expression. Zinc plays a pivotal role in providing cellular and humoral immunity (King & Turnlund 1989). Inadequate zinc uptake prohibits normal homeostatic regulation. This knowledge has led to the understanding of the role of zinc deficiency in the etiology of stunting and impaired immunocompetence. Zinc has also shown its effect on increasing white blood cells, that fight against cancer and help release antibodies by leucocytes (Shankar & Prasad 1998). The inevitable requirement of zinc in our system has led to the inception of an inquisitive thought as to whether the organism designated as L4(3), identified as *Pseudomonas pseudoalcaligenes*, can be used as a probiotic supplement in humans. It was also seen that this organism, enhances the growth rate of bony fish, *Labeo rohita*, when used in fermented feed as a probiotic. The chelated zinc in fish when consumed by humans also indirectly enters the body as a probiotic source. However it's efficiency as a natural supplementary source in the form of lyophilized lysates is yet to be revealed (Venkatakrishnan et al. 2004) .

Pseudomonas pseudoalcaligenes is known to have endowed with the inherent ability to oxidize petroleum hydrocarbons, mainly constituting of nitrobenzene and toluene, thus eradicating oil contamination. This organism is further known to show transformations by immobilization of radionuclides and removal of toxic metal wastes through chelation. Uranium and other toxic metals are removed from contaminated wastes by extracting with the chelating agent citric acid, after which the metal is recovered from citric acid extract following biodegradation and photodegradation.

Pseudomonas pseudoalcaligenes also acts as a cyanotrophic microorganism and can effectively utilize several nitrogen sources including cyanide, cyanate, cyanoalanine, cyanoacetamide and nitroferricyanide under alkaline conditions, thereby preventing the formation of

volatile HCN (pKa = 9.2). This bacterium has a unique ability to grow in heavy metal cyanide containing waste effluent, generated by jewellery industries. The organism has developed a cyanide resistant mechanism by inducing an alternative oxidase and a siderophore based mechanism for iron acquisition in the presence of cyanide. These findings indicate the marvelous ability of this organism as a potent bio-remediator.

MATERIALS AND METHODS

Isolation of bacteria: Mixed sewage was obtained from the eastern wetlands of Kolkata. The sample was serially diluted with distilled water. For this purpose 6 test tubes were filled with 9mL of sterile distilled water and autoclaved at 121°C for 15 minutes. After this 1mL of the sample was aseptically transferred to one of the test tubes. This gave the dilution 10^{-1}. From this test tube 1mL of solution was added to the next test tube to get the 10^{-2} dilution and the process was repeated to get dilutions 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Aliquots of 1mL were plated in Petri plates containing sterilized nutrient agar medium by the pour plate method. The media composition was according to Sherman and Cappuccino (?). All plates were incubated at 37°C for 24 hours. Four colonies were isolated from the plates of dilution 10^{-4}, 10^{-5} and 10^{-6}. The colonies were aseptically transferred to nutrient agar slants by streaking. Thus, pure cultures of these organisms were obtained and were designated as L4(1), L4(2), L4(3) and L4(4).

Growth on EMB agar: The composition of Eosin Methylene Blue agar was as per Sherman and Cappuccino (?).

The four organisms were streaked onto 4 Petri plates containing sterile EMB agar medium and incubated for 48 hours.

Microscopic observation: Gram staining was performed to study morphology of the organisms.

Biochemical tests: Standard biochemical tests were performed for characterization of the bacteria. The tests performed included indole production test, methyl red test, Voges-Proskauer test and citrate utilization test.

Zinc tolerance by the bacterial isolates: The ability of the bacterial isolates to tolerate high concentrations of zinc was determined under *in vitro* condition by inoculating them in nutrient broth containing different concentrations of zinc chloride ($ZnCl_2$). Standard nutrient broth was prepared and zinc chloride was added in such a way so that the effective zinc concentrations become 100ppm, 500ppm and 1000ppm. The medium was autoclaved at 121°C and distributed into sterilized test tubes. Eight test tubes were maintained for each concentration of zinc. The four isolates were inoculated in each of them and replicates were made. An uninoculated control was also maintained. The cultures were incubated for four days and then growth was estimated by measuring the optical density at 610nm. The growth of the bacteria in zinc containing medium indicated their tolerance to high concentration of zinc.

Measurement of zinc uptake: Cultures showing appreciable growth at 500ppm and 1000ppm were centrifuged at 3000 rpm for 5 minutes and the supernatant was collected. The amount of zinc uptake by the bacterial cells was measured by atomic absorption spectroscopy after digesting the samples with nitric acid (APHA 1998).

Identification of bacterial isolates by 16S rDNA analysis: Bacterial isolates showing maximum uptake of zinc were identified using 16S rDNA analysis.

a. DNA was isolated from the slant culture provided with. Its quality was evaluated on 1.2% agarose gel, a single band of high molecular weight DNA was observed.
b. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500bp was observed when resolved on agarose gel (Fig. 1).
c. The PCR amplicon was purified to remove contaminants.
d. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492 R primers using BDT v3.1 cycle sequencing hit on ABF 3730 × 1 genetic analyser.
e. Consensus sequence of 1419 bp 16S r DNA gene was generated from forward and reverse sequence data using aligner software.
f. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genebank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programme Clustal W. Distance Matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4 (Fig. 2).
g. The evolutionary relationship of 11 taxa was established using the neighbour joining method (Fig. 3).

RESULTS

Gram characteristics of the bacterial strains: All the four bacterial strains isolated, L4(1), L4(2), L4(3) and L4(4) were observed to be Gram negative cocco- bacilli in nature.

Biochemical tests: The biochemical tests were performed as per Sherman & Kappuchino (2007), and the results of the IMViC biochemical tests are given in Table 1.

Indole Production Test: Test tubes inoculated with the bacterial cultures were separately treated with the Kovac's reagent. A red coloured ring formation was observed in case of L4(2) and L4(4) strains. L4(1) and L4(3) were found to be Indole negative, and L4(2) and L4(4) to be Indole positive.

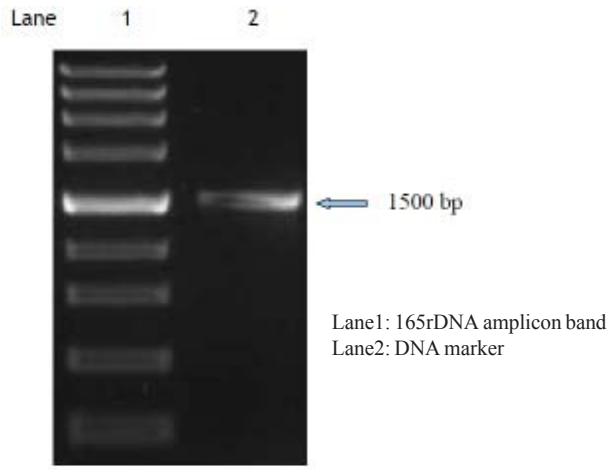

Fig. 1: Gel documentation image of 16S rDNA amplicon for sample L4-3.

Methyl Red test: Test tubes inoculated with the bacterial cultures were separately treated with methyl red indicator. Following results were obtained.

L4(1) was found to be Methyl red negative.
L4(2) was found to be Methyl red positive.
L4(3) was found to be Methyl red negative.
L4(4) was found to be Methyl red positive.

Voges-Proskauer test: Test tubes inoculated with the bacterial cultures were separately treated with Barritt's reagent A and Barritt's reagent B. Following results were obtained.

L4(1) was found to be Voges-Proskauer positive.
L4(2) was found to be Voges-Proskauer negative.
L4(3) was found to be Voges-Proskauer positive.
L4(4) was found to be Voges-Proskauer negative.

Citrate Utilization Test: 48hrs after inoculation of the bacterial cultures on the Simmon's Citrate Agar medium and incubation at 37degree celcius, the green colour of the medium changed to prussian blue for the L4(2) and L4(4) strains.

L4(1) was found to be Citrate utilization test positive.
L4(2) was found to be Citrate utilization test negative.
L4(3) was found to be Citrate utilization test positive.
L4(4) was found to be Citrate utilization test negative.

Eosin Methylene Blue Agar Medium: The bacterial strain L4(4) was found to grow on Eosin Methylene Blue Agar medium. The colonies were small, pinpoint, shiny with a characteristic metallic sheen, typical of *Escherichia coli.*

Atomic Absorption Spectroscopy: The atomic absorption spectroscopy experiments conducted revealed that the bacterial strain L4(4) showed the maximum uptake of zinc at a concentration of 1000ppm, whereas the strain L4(3) showed the maximum uptake at 500ppm concentration of zinc. Moreover, L4(3) showed the second highest uptake at

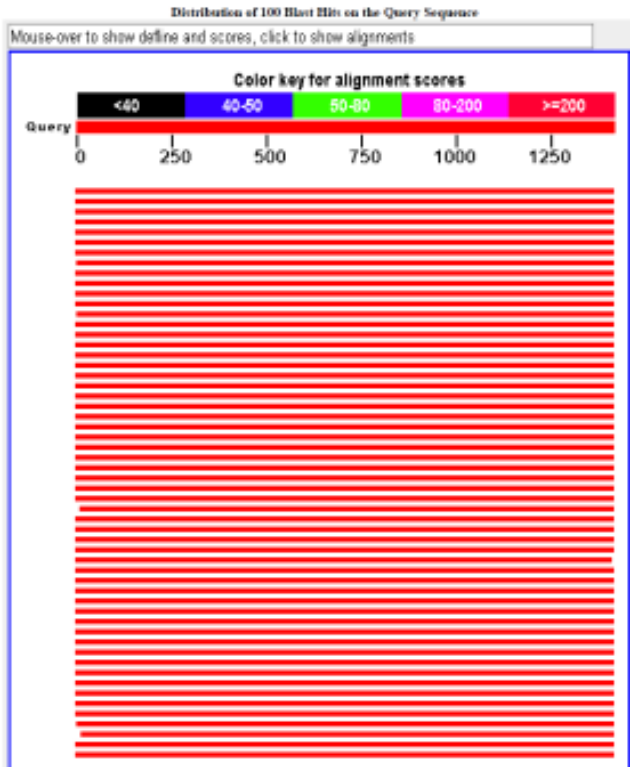

Fig. 2: Blast data analysis showing alignment view using combination of NCBI GenBank.

1000ppm concentration. At 1000ppm concentration of zinc L4(1) showed the least uptake, whereas L4(4) showed the least uptake at 500ppm concentration of zinc.

The results showing bacterial growth at 1000 ppm, 500 ppm and 100 ppm concentration of zinc, at 610 nm are given in Table 2. The results of the atomic absorption spectroscopy are given in Table 3.

16S rDNA molecular study: Bacterial strain L4(3) was identified to be *Pseudomonas pseudoalcaligenes* strain JM4 (Genbank Accession Number FJ472856.1), based on nucleotide homology and phylogenetic analysis.

DISCUSSION

The aim of this study was to determine whether the isolated bacterial species could be used as probiotic to supplement zinc or could be used in the treatment of marine oil spill. The bacterial strain L4(3) showed higher growth at a concentration of 1000ppm as compared to 500ppm of zinc (Table 2). This may be attributed to the fact that the strain solubilises zinc to a greater extent and thus utilizes it for its growth. It was seen that the identified bacterial species, *Pseudomonas pseudoalcaligenes*, was used in fermented feed as a probiotic for the bony fish, *Labeo rohita*. The chelated zinc in fish when consumed by humans also indirectly enters the

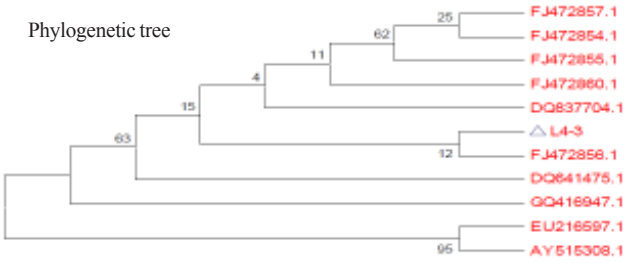

Fig. 3: Phylogenetic tree showing molecular evolution of L4-3.

Table 1: Results of the IMViC biochemical tests for the bacterial samples.

Bacterial Sample	Indole	Methyl Red	Voges-Proskauer	Citrate Utilization
L4(1)	Negative	Negative	Positive	Positive
L4(2)	Positive	Positive	Negative	Negative
L4(3)	Negative	Negative	Positive	Positive
L4(4)	Positive	Positive	Negative	Negative

Table 2: Bacterial samples showing absorbance for 500 ppm and 1000 ppm concentration of zinc at 610 nm.

Bacterial Sample	L4(1)	L4(2)	L4(3)	L4(4)
Absorbance for 500ppm concentration of zinc at 610nm	0.04	0.12	0.09	0.06
Absorbance for 1000ppm concentration of zinc at 610nm	0.07	0.14	0.14	0.12

Table 3. Bacterial samples showing zinc uptake at 500ppm and 1000 ppm concentration in (mg/mL).

Bacterial Sample	L4(1)	L4(2)	L4(3)	L4(4)
Zinc uptake at 500ppm concentration in (mg/mL)	453.2	464.5	464.7	442.7
Zinc uptake at 1000ppm concentration in (mg/mL)	896	933.5	937.5	954.7

body as a probiotic source. Moreover, studies pointed to the ability of this bacterium to degrade toluene and nitrobenzene. *Pseudomonas pseudoalcaligenes* also acts as a cyanotrophic microorganism and has developed a cyanide resistant mechanism by inducing an alternative oxidase and a siderophore based mechanism for iron acquisition in the presence of cyanide (Luque-Almagro et al. 2005). It is used in immobilizing radionuclides and toxic metals, as a bioleaching agent and in chelating zinc in the human system (Francis 2006). This bacterium has a remarkable ability to take up zinc from a zinc containing medium. Since it was isolated from mixed sewage, it can be concluded that the bacterium takes part in the treatment of marine oil spill and in eradication of different types of marine pollution.

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