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LACTOBACILLUS SPECIES MTCC 10093 AS A MODEL BACTERIUM FOR THE BIOREMEDIATION OF ARSENIC IN AGRICULTURAL FIELD.

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ABSTRACT
The Soil inhabiting *Lactobacillus* readily isolated and characterized on the basis of biochemical and molecular study. It has found that this strain is also exhibits tolerance against Arsenic. It can grow at high concentrations of arsenite (AsIII) ranging from 2 ppm to 20 ppm (part per million). The positive silver nitrate ($AgNo_3$) test has revealed oxidizing capacity of strain. No extra band of protein in either stressed or non-stressed condition noticed in protein profile. We noticed maximum tolerance concentration (MTC)s of this bacterium is 20 ppm. This strain is also resistance against antibiotic Streptomycin and Nalidixic acid. The strain has been identified as *Lactobacillus rhamnosus* MTCC 10093 (NCBI Gene Bank Accession No: KT982211). Its arsenic oxidizing capacity could be exploited for bioremediation of arsenic in agricultural field.

INTRODUCTION

Arsenic is extensively distributed in the earth's crust along with other metals. The crust roughly contains about 3.4 ppm arsenic (Wedepohl., 1991). Arsenic in soil, exists in various oxidation states and in combination with other chemical species, depending upon soil pH and their oxidation-reduction potential. The most common species of inorganic arsenic are trivalent arsenite (AsIII) and pentavalent arsenate (AsV). Although arsenic is generally toxic to life, earlier workers have reported that microorganisms can use arsenic compounds as electron donors, electron acceptors, or possess arsenic detoxification mechanisms (Ahmann *et al*., 1994). Although the most communal oxidation states of arsenic in the environment are the less toxic pentavalent AsV and more toxic trivalent AsIII forms (Cullen and Reimer., 1989). Despite its noxiousness, a number of microorganisms are proficient of using either the oxidized form of inorganic arsenic AsV or the reduced form AsIII in their metabolism (Silver and Phung., 2005). The *Lactobacillus* strain MTCC 10093 was isolated from soil and shows adverse adaptability. It can grow at variable temperature and pH ranging from 12ºC to 42ºC and 5.2 to 10.5 respectively. It also utilizes a vast array of carbon sources (*viz* starch, arabinos, glucose etc). Further it can tolerate NaCl concentration up to 10% w/v. Antibiotic sensitivity study revealed that this is resistant to Streptomycin and Nalidixic acid while sensitive to ciprofloxacin and Azithromycin (Kumar and Ghosh, 2016). Based upon previous studies, the *Lactobacillus* spp. MTCC 10093 may have application in bioremediation of arsenic polluted soil and water. The bacterium may also help to overcome to counter act the toxic effect of heavy metals.

On this back drop, the present study has been carried out to test the sensitivity of this *Lactobacillus* strain MTCC 10093 against different concentrations of arsenic as well as transforming ability from arsenite to arsenate and its bioremediation role if any.

MATERIALS AND METHODS

Soil sample collection and bacterial isolation.

Soil sample was collected from wheat field (Lat:25.787941, long:84.777036) of the district of Saran, Bihar, India, during a survey carried out to study microbial flora of the region. Following the standard soil collection procedure and were taken from upper most layers approximately 4-6 cm depth and kept in 4ºC, methodology was followed as described by (Janssen *et al*., 2002 and Dutta *et al*., 2010). One gram of soil was mixed with 150 ml normal saline to make a suspension. The soil suspension was then serially diluted and transferred for plate count experiments to petri dishes containing medium MRS (de Man Rogsa and sharpe). This was followed by incubation at 37ºC for 48 to 72 hours in the dark. Sub culturing was done to isolate pure cultures (Kumar and Ghosh., 2016). Chemicals used in this study were purchased from (Sigma-Aldrich, USA). As (III) stock solutions were prepared freshly before use from Sodium arsenite ($NaAsO_2$) and were stored at 40 C in the dark. All were of analytical grade.

Arsenic tolerance capacity of the isolates

The MTCs of strain was determined on MRS medium. 5 ml of

culture medium was taken and added different concentrations, Control (0 ppm), 2ppm, 4ppm, 8ppm, 12ppm 16ppm and 20ppm of arsenite As (III) (Sigma-Aldrich, USA) was supplemented on each culture tubes. The culture medium with different concentration of arsenite (III) was incubated at 37ºC for 72 h in shaking incubator (Scigenics, India). The MTCs of the strain was noted when the isolate showed prominent growth on the tubes after incubation of 72 hrs (Shakoori *et al*.,2014). Optical densities of bacterial isolates were estimated by Spectrophotometer. It was recorded by reading the absorbance (OD) at 570 nm at every 2 hours of time interval (Hitachi double beam spectrophotometer U-2900). All experimental set-ups were prepared in duplicates.

Screening of arsenite Oxidizing bacteria

The isolates, that exhibited growth in media containing AsIII, was screened for arsenite oxidising ability. Pure culture of *Lactobacillus* strain inoculated MRS broth. Strain was grown up to 0.5–0.6 O.D of cell suspension at 570nm. Then culture was centrifuged at 10,000 rpm for 5 min and supernatant was taken for further analysis. Bacterial isolate was streaked on MRS agar plate containing 10 mg/L of arsenite. A plate was incubated at 37ºC for 48 h and was flooded with 0.1 M $AgNO_3$ as shown in Figure 2. Appearance of yellow precipitation in MRS agar plates was carefully observed (Majumdar.A.,2012)

Protein profiling

In conical flasks, 20 mL Luria Bertani broth were taken in triplicates and steam sterilized. Bacterial isolates were stressed with 20 ppm of arsenite and non-stressed for control incubated for 24 h at 37 ºC in shaking incubator and harvest the cells by centrifugation. Pellet was dissolved in 100μL of 1X loading dye then heat shock was given for 5 min, eppendorf was shifted on ice for 2 min, and then was centrifuged at 12000 rpm for 10 min. Supernatant was transferred to a new eppendorf, then final centrifugation was done at 12000 rpm for 10 min and supernatant was shifted to a new eppendorf. Initially gel was run at 40 mV after stake formation the voltage was increased to 80 mV. (Shakoori *et al*.,2014).

RESULTS AND DISCUSSION

The maximum tolerated concentration of arsenite As (III) was up to 20 ppm. In growth curve as shown in Figure 1, the lag, log, and exponential phases are less dissimilar in both with and without arsenic content. However, the differences noticed in the stationary phases. Slow growth was observed in lag phase. This could be due to toxic effect on the cell wall of bacterium. Earlier results in case of *Pseudomonas* sp. against heavy metal have been obtained by (Gikas *et al*., 2009).

Verification of transforming ability of the strains

AgNO3 method was performed as per the methods given by Valenzuela *et al*. (2009) to verify the transforming ability of bacterial isolates

The appearance of bright yellow precipitates indicated the presence of arsenite which is due to arsenate reducing bacteria. The agar plate was again flooded with 0.1M $AgNO_3$; a brownish precipitate observed it indicated the presence of arsenate in the medium proving this isolate to be arsenite oxidizing bacteria. This provides evidence that the isolated

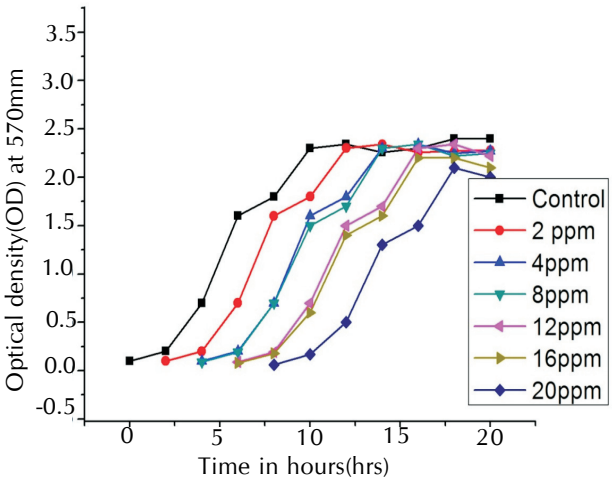

Figure 1: Arsenic resistance Lactobacillus spp. MTCC10093.

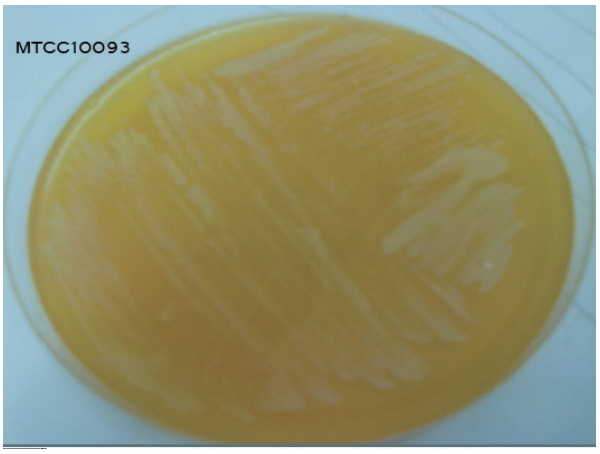

Figure 2: the presence of arsenate showing on MRS agar plates

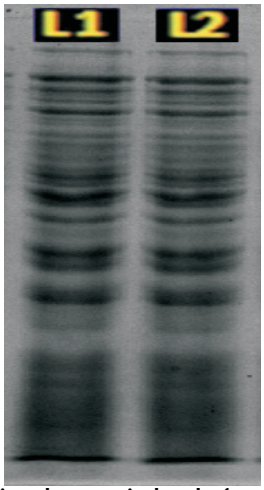

Figure 3: L1 showing the protein band of non-stressed bacterial culture after 6 h, While L2 indicating the protein band of arsenic stressed bacterial culture after same hours at 20 ppm of concentration.

Lactobacillus strain MTCC 10093 is arsenite oxidizing bacterium which transferred toxic trivalent arsenite (AsIII) to less toxic form pentavalent arsenate (AsV).

Protein profiling

A large number of microorganisms are involved in the biogeochemical cycle of arsenic. Arsenite metal stress to bacteria was given after their optical density reached to 0.3 indicating that bacteria have entered into log phase. In SDS-PAGE of stressed organisms did not indicate any new protein band (Chovanová *et al*., 2004 and Lacerda *et al*., 2007). This indicated that the arsenite resistance proteins in bacteria were constitutive proteins and they expressed in the non-stressed conditions as shown in Figure 3.

This bacterium is capable to transform toxic trivalent arsenite (AsIII) to less toxic form pentavalent arsenate (AsV). Arsenite oxidizing ability of this bacterium comes from positive test result by AgNo3 (Figure 2). The arsenite oxidase play important role in converting As III to As V as reported earlier in other bacteria which includes, *Herminiimonas arsenicoxydans* (Weeger *et al*., 1999), *Klebsiella pneumoniae* (Butt *et al*., 2011). We speculate the presence of this enzyme in this bacterium too. Earlier a number of bacterial species like Bacillus, *Pseudomonas*, *Acidthiobacillus* and *Disulfito* bacteria have been recorded as resistant against arsenic (Suresh *et al*., 2004). Lactobacillus strain could have evolved variety of mechanisms to develop resistance against arsenic in nature like *Aeromonas*, *Bacillus*, *pseudomonas*, *Ecchirichia etc* as described by Anderson and cook (2004). This strain of *Lactobacillus* also exhibit resistance to Nalidixic acid, Streptomycin (Kumar & Ghosh 2016) along with arsenic. Very similar dual resistances of bacterium against antibiotics and arsenic have been recorded in *Pseudomonas sp, Artrobacter sp* and *Corynebacter* sp by Owolabi and his co-workers (2014). It shows analogy to the present result. Moreover, the bacterium is capable to grow at high temperature 12ºC to 42ºC, variable pH 5.2 - 10.5 and high salt concentration 10% of NaCl (w/v), which make it more successful group amongst probiotics (Kumar and Ghosh 2016). This strain is biochemically, genetically characterized using ribotyping (16SrRNA) as *Lactobacillus rhamnosus*. (NCBI Gene Bank Accession No: KT982211). Its probiotics role, dual resistance against some antibiotics like Nalidixic acid, Streptomycin as well as arsenic have made this form more viable and economically important. Apart from this it can also play positive role in bioremediation to clear toxic effect of arsenic in the environment.

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