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ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF SOIL INHABITING *LACTOBACILLUS SPECIES* MTCC 10093

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ABSTRACT
Lactobacillus species was readily isolated from wheat field of Chapra (Saran), Bihar, India, during a survey carried out to study microbial flora of the region. This strain is Gram's positive, rod shaped with size of 3-6μm and capable of growing optimally in the media MRS (de Man Rogsa and sharpe). It utilizes a vast array of carbon sources (viz starch, arabinose, glucose, etc). It grows at variable temperature and pH ranging from 12ºC to 42ºC and 5.2 to 10.5 respectively. 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. It has been found that the isolated strain MTCC10093 is similar to *Lactobacillus rhamnosus*. This strain is hitherto unknown and unreported from the area. This paper deals with the identification and characterization of soil inhabiting *Lactobacillus species*. We report here, immense capability and adaptability of this bacterium growing in diverse habitant and large spectrum of environment which makes it a successful member among the probiotics bacteria.

INTRODUCTION

Soils are among the most immense and the most interesting assortment of microbial habitats on earth (Teitelbaum *et al*., 2002). The structure, properties and population of bacteria present in a particular soil is always influenced by geographical location such as soil temperature, soil type, soil pH, organic matters, contents, cultivation, aeration and moisture content (kok *et al*., 2012). Bacteria belonging to the genus *Lactobacillus* are members of the lactic acid bacteria (LAB), a broadly defined group characterized by the formation of lactic acid as the sole or main end product of carbohydrate metabolism. Various species of LABs, *L. salivarius, L. rhamnosus, and L. paracasei* are also present in infants. Earlier workers have reported this bacterium from various sources such as plant origin, silage, fermented food (yogurt, cheese, olives, pickles, salami, etc.) (Blaiotta *et al*., 2001, Randazzo *et al*., 2004 and Omogbai *et al*., 2005). A large collection from diverse environments including acid mine drainage (Kishimoto *et al*., 1991), soils and sediments (Barns *et al*., 1999 and Dunbar *et al*., 1999), wastewater (Crocetti *et al*., 2002), soil crusts of sand dunes (Smith *et al*., 2004), water distribution systems (Martiny *et al*., 2005), hot springs (Hobel *et al*., 2005), peat bogs (Dedysh *et al*., 2006) have also confirmed the presence of large spectrum of these bacterium. It would be evident from the foregoing account that there are many unidentified useful bacteria which have not been investigated for their activity. The present paper deals with isolation of bacterium strain from soil and characterized it as *Lactobacillus rhamnosus* on the basis of its physiological morphological and biochemical features.

MATERIALS AND METHODS

LAB isolation and culture

Soil samples were collected from 5 different locations in the Chapra district and were taken from upper most layers approximately 4-6 cm depth. 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 NA and MRS separately. This was followed by incubation at 37ºC for 48 to 72 hours in the dark. Sub culturing was done to isolate pure cultures.

Characterization of the Isolates

Preliminary characterization was performed using morphological and cultural characteristics as described by Holt *et al*. (1994). Briefly, identification of the isolate was done under the compound microscope (Olympus CX21) and recorded the cell size, shape arrangement. Classical methodology for staining of bacteria followed as described by Antonio *et al*. (1999). All media components and other chemicals used were from HI- Media and Merck (India). All were of analytical grade.

Biochemical characterization

Bacteria strain was cultivated at 37ºC for 48 to 72 hours in the dark conditions on MRS agar (Difco), containing 5.0g calcium carbonate and 15 g agar l^{-1}.

After isolation, the strains were maintained in MRS broth (de Man Rogsa and Sharpe). Morphological, physiological and

biochemical characteristics were determined according to the methods of Holdeman *et al*. (1977), Gerhardt *et al*. (1981) and Okada *et al*. (1992). It was recorded by reading the absorbance (OD) at 570 nm at every 2 h (Hitachi double beam spectrophotometer U-2900). Carbohydrate fermentation tests were conducted in modified MRS broth containing 0.5% (w/v) of various carbohydrates. The gas producing capacity (H_2S) was assessed by culturing the microorganisms on a medium containing peptone and casein at 37ºC for two weeks.

For pH assay, the cultures were inoculated on a MRS medium at various pH ranges: 5.2, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, and 10.5. The ability of the isolates to excrete extracellular enzymes was tested through hydrolysis of starch and gelatine excreted intracellular enzymes ware determined through different biochemical tests viz Voges-Proskauer, hydrogen sulphide production, methyl red, citrate utilization, and catalase and oxidase test for utilization of carbon sources.

Determining the antibiotic Susceptibility assay.

Antibiotics resistance test was determined by the method described by Curragh and his co workers (1992). Impregnated discs containing Nalidixic acid (10 mcg), Streptomycin (10 mcg), Azithromycin (10 mcg), and Ciprofloxacin (10 mcg) were used. The diameter of the clear zone was measured in centimetres from the centre of the well after 18 hrs of incubation.

DNA isolation

Total genomic DNA from isolate was extracted and purified as described by Gevers *et al*. (2001). Isolation of plasmid DNA was based on the alkaline lysis method of Anderson and McKay (1983).

RESULTS AND DISCUSSION

The isolated strain was purified and kept on MRS medium for morphological, physiological and biochemical studies. Most colonies were able to grow within 24 to 48 hrs of incubation at 37ºC. The bacterium was flat, smooth or rough and off white in colour. The gram staining results indicates that the isolated bacteria was gram's positive, micro aerophilic or facultative anaerobic rod-shaped of 3-6μm as shown in Fig. 1 and can grow up to 10% NaCl concentrations (w/v). Growth curve was sigmoid and were recorded at every 2 hrs of intervals as shown in Fig. 4. It can grow at different pH ranging from 5.2 to 10.5 as well as varying temperatures from 12ºC to 42ºC.A wide range of habitat and probiotics role of *Lactobacillus spp*. may be due to its adaptability in growing at different pH and temperatures result shown in Table 1. As it is evident from the findings of earlier workers its probiotics role in human gastrointestinal. (Delgado *et al*., 2005). The isolated bacterium is catalase negative and could not mediate the decomposition of H_2O_2 to produce O_2. The present study shows analogy to the earlier findings by Robinson 1990. Furthermore this strain exhibits positive report in some biochemical test like methyl red test, casein and starch hydrolysis etc., Table 2. It also utilize vast array of carbon sources which include sucrose, arabinose, glucose, mannose, raffinose, melibiose, salicine, and fructose as shown in Table 3, which show that the isolated bacteria could ferment maltose, lactose, sucrose and glucose,

Table 1: Charecteristics of bacterial strain

Taxonomic classification:	
Family	Lactobacilaceae
Genus	Lactobacillus
Morphological and culture characteris:	
Colour	Off white
Shape	Long rods
Gram coloration	Yes
Physiological characteristics:	
pH	5.2 -10.5
Temperature (°C)	12 º- 42º
Growth on NACl (%)	2- 10

Table 2: Biochemical characterization

Methyl red test	Yes
Voges Proskauer test	No
H_2Sproduction	No
Casein hydrolysis	Yes
Citrate	No
Gelatin hydrolysis	No
Starch hydrolysis	Yes
Nitrate reduction	No
Catalase test	No
Oxidase test	No

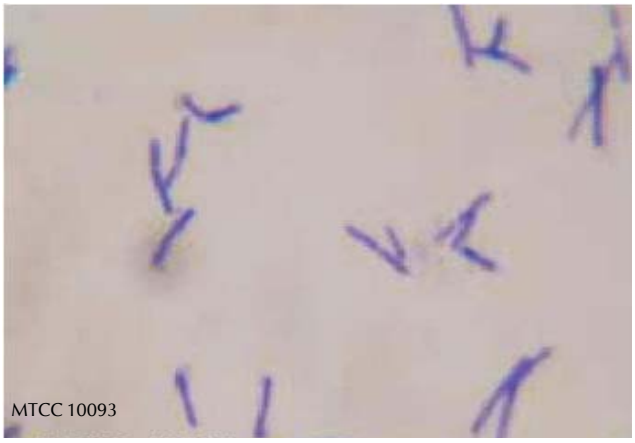

Figure 1: Gram's positive *Lactobacillus*

Figure 2: Antibiotic resistance plate assay showing sensitivity with Azithromycin (AZ) and Ciprofloxacin (CL) at 2.8 cm and 3.0 cm respectively

Table 3. Carbohydrates and Antibiotics susceptibility tests

Arabinose	Yes	Xylose	No
Galactose	No	Sucrose	Yes
Glucose	Yes	Rhamnose	No
Mannose	Yes	Melibiose	Yes
Salicin	Yes	Raffinose	Yes
Fructose	Yes		
Resistance to antibiotics	Nalidixic acid (NA) and slightly to Streptomycin (S)	Sensitive to antibiotic Azithromycin (AZ)	Highly to Ciprofloxacin (CL) and

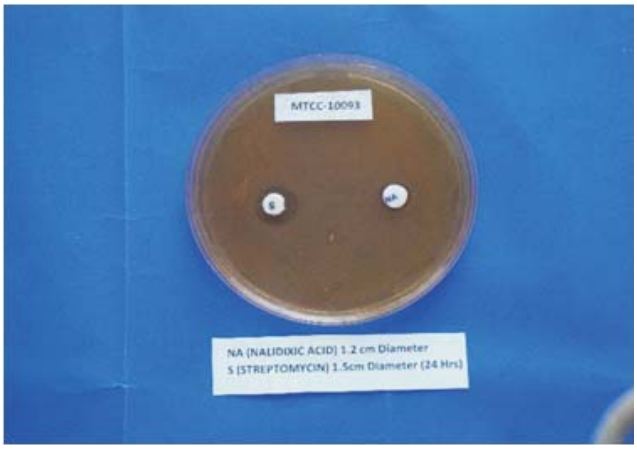

Figure 3: Showed mild resistance with Streptomycin (S) and completely resistant towards Nalidixic acid (NA) at 1.5cm and 1.2 cm.

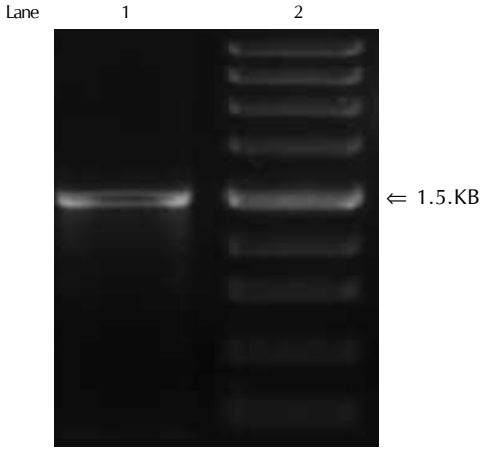

Figure 5: Lane 1: DNA bandLane; 2: DNA marker 1.5kb band

but not galactose, xylose and rhamnose. Antibiotics sensitivity tests shown that the isolate was resistance to streptomycin (S) with inhibition zone of 1.5 cm and nalidixic acid (NA) of 1.2 cm while sensitivity to azithromycin (AZ) and ciprofloxacin (CL) with inhibition zone of 2.8 cm and 3.0 cm respectively as shown in Fig. 2 and 3. Resistance of this strain against nalidixic acid and streptomycin may be due to production of some secondary metabolites as recorded in some species of the bacterium (Todorov 2008 and Sarika *et al.,* 2010) and soil inhabitating *Streptomyces spp*. (MTCC324) produces kanamycin as reported earlier by (Ghosh and Prasad, 2010).

The present results matched with the *Lactobacillus species* as

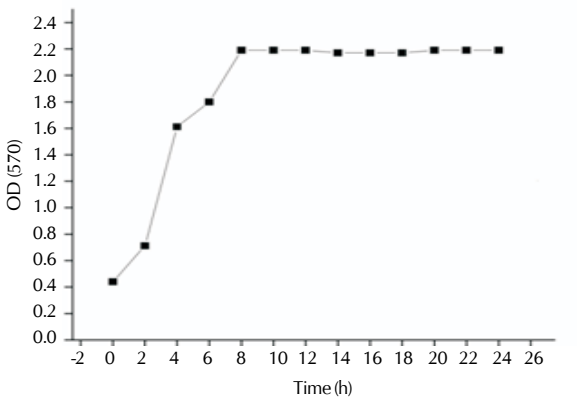

Figure 4: Growth curve taken at OD-570 nm, inoculums media-MRS, inoculums - 10^7 cells by using the counting chamber. Growth changes at every 2 hr intervals (0 hrs, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26hrs)

per the Bergey's manual of determinative bacteriology (Holt *et al.,* 1994 and Paul *et al.,* 2011). DNA homology studies also provide a key for identification of the *Lactobacillus strain* (Giorgi *et al.,* 1987). A compact single band of DNA with 1.5KB of this bacterium shown in Fig. 5. The isolated strain MTCC10093 shows proximity to *Lactobacillus rhamnosus*. However, molecular identification through 16 S rRNA study will further add authenticity and conformation of the identification. Further work on this direction is going on.

This strain was also identified as *Lactobacillus species* and accredited no 10093 by Institute of Microbial Technology (IMTECH) Chandigarh India.

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