

# The characterization of lactic acid bacteria from intestine of rohu (*labeo rohita*) for their potential use as probiotics from chandrapur district.

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## ABSTRACT

The Lactic acid bacteria isolated from intestine of Rohu (*Labeo rohita*) has the probiotic potential, which enhance and improve the immune system by restoration of normal intestinal microflora balance in the digestive tract of fishes. Fish samples were collected from centrally situated Ramala lake of Chandrapur city. The bacterial culture where isolated from fish intestine ,among them *Bacillus coagulans*, which is non pathogenic Gram positive bacteria where isolated and studied as probiotic and considered depending on the biochemical and molecular characterization techniques. The probiotic characterization tests such as acid tolerance, bile salt tolerance, and antimicrobial activity profiles also identified. The probiotic bacteria where normal inhabitant in gut of freshwater fish *Labeo rohita*, were isolated and molecular characterization by using the 16s rDNA sequencing method, Polymerase Chain Reaction (PCR) etc. The overall complete study revealed, the isolated *Bacillus coagulans* species fulfills the required criteria for probiotic such as tolerance to perishable, harsh conditions in the intestine of fish like low PH and high bile salt concentration and it also produce inhibitory substance extra cellularly which inhibit pathogenic microorganisms. These isolated microbes were used for potential probiotics.

**Keywords:** Lactic acid bacteria, Probiotics, *Labeo rohita*, Acid tolerance, Bile salt tolerance, *Bacillus coagulans*, Ramala lake, 16s rRNA sequences.

## INTRODUCTION

The centrally placed "Ramala lake" of Chandrapur city is the main source of fresh water fishes for whole Chandrapur district of Maharashtra state. The fish market occupies most of Rohu (*Labeo rohita*) fishes are brought from the Ramala lake. The term Probiotics ("for life") are environmental friendly microorganisms generally Described as health promoting bacteria. According to WHO probiotics are define as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". Probiotic concept introduced in the early 20 th century by Elie Metchnikoff, gained much popularity recently with considerable and a significant advances in functional and health food market across the world. India is also fast emerging as a potential market for probiotic foods. Lactic acid bacteria are well known microorganisms that have probiotic properties. Lactic acid bacteria are normal microflora of gastrointestinal tract of healthy animals like mammals and aquatic animals with no harmful effects [7,10].

The colonization of the intestine by probiotic bacteria which prevents harmful bacteria from growth by competition exclusion and by the production of organic acid and antimicrobial compounds [8]. The acid tolerance and bile salt tolerance are fundamental probiotic properties that indicate the ability of probiotic microbes to survive the intestinal tract, specially, acidic conditions of stomach and presence of bile salt in the intestine [4,6]. Lactic acid bacteria are normal native microbiota of aquatic animals from temperate regions. [9] The present study is based on the study of probiotic properties of Lactic acid bacteria isolated from the intestine of Rohu (*Labeo rohita*) which is normal edible fish in the local market of Chandrapur district, Maharashtra state, of India.

## METHODOLOGY

### 1) Collection of the fresh water fish and Isolation of Lactobacillus Species:

The Rohu fish (*Labeo rohita*) was collected from centrally located freshwater reservoir namely **Ramala lake** of Chandrapur city from where edible fresh water fishes are supplied to common fish market of Chandrapur district, Maharashtra state. The collected

fish was brought to the laboratory in transparent plastic jar containing lake water. The body surface of fish was cleaned thoroughly with distilled water, then disinfected with alcohol (70%); dissected under aseptic condition. Intestine taken out and washed three times with normal saline (NaCl 0.85%) and homogenized with a mechanical homogenizer. The pieces of gastrointestinal tract were homogenized with sterile distilled water and centrifuged for 10 minutes at 13,000 rpm. One ml of supernatant was serially diluted with sterile distilled water in 6 test tubes. Among serially diluted samples  $10^{-5}$  and  $10^{-6}$  sample selected and 0.1 ml of diluted sample was inoculated on lactic acid bacteria selective agar (Himedia, M1072). Then incubated at 37°C for 48 hours. The isolated colonies developed on lactic acid bacteria selective agar were picked up and cultivated on slants of selective agar to obtain pure culture [1].

### 2) Identification of Lactobacillus Species:

Selective colonies were characterized and identified following Bergey's Manual of systematic Bacteriology [11] for their morphology, gram staining, motility test, cultural and Biochemical tests [5]. Two types of lactobacillus species were isolated from the gut of *Labeo rohita* fish collected from Ramala lake.

### 3) Study of probiotic characters of lactic acid bacteria isolated from intestine of *Labeo rohita* :

In vitro tests was carried out to screen out potential probiotic characters of isolated two lactobacillus species. These tests were based on the gut environment of fish which they mimic under in vitro conditions for screening potential probiotic strains.

1. Acid tolerance (Resistance to gastric acidity)
2. Bile salt resistance.
3. 16S rDNA Sequencing ,PCR Method

The acid tolerance test was carried out as per the method of Dhanasekaran et al., [2-3] whereas the bile salt resistance was determined by a method given by Salminen et al., [10]

## RESULTS AND DISCUSSION

The isolated and homogenized gut sample from *Labeo rohita* fish was serially diluted with sterile distilled water in 6 test tubes up to  $10^{-6}$  dilution and the

diluted sample from  $10^{-6}$  dilution was inoculated on the Lactic acid selective agar medium (Himedia-M1072). After incubation the colony characteristics developed were noted and prepared the slant culture. The morphological identification of isolated Lactobacillus species was done by studying Gram staining, motility test, and the Biochemical identification by IMViC test and sugar fermentation. (Table-1). Two lactobacillus species were identified based on cultural, morphological and biochemical characterization. Dhanasekaran et al., [2-3] reported similar findings and noted, maximum population of lactobacillus species in the gut of fresh water fishes.

In the present study the identified, Lactobacillus species 1 was found to be Gram positive, having long rods, colony appearance creamy white on selective media, ferment glucose, lactose with acid and gas production, show Indole test negative, MR test negative, VP test positive and CU test positive. Whereas Lactobacillus species 2 was found to be Gram positive having long rods, colony appears to be creamy white on selective media, ferment glucose lactose with acid and gas production, show Indole negative MR negative, VP positive and CU test positive. Further probiotic characteristic of isolated lactobacillus species was done by acid tolerance test and bile salt tolerance test.

**TABLE 1**

CHARACTERISTICS		SPECIES 1	SPECIES 2
<b>Cultural Characteristics</b>	Colour Margin Shape Opacity Pigmentation	Creamy white Undulate Irregular large Opaque No	Creamy white Undulate Irregular large Opaque No
<b>Morphological characteristics</b>	Gram Stain Reaction Cell Shape	+Ve Rods	+Ve Rods
<b>Biochemical Characteristics</b>	Catalase test MR VP Indole test Citrate Utilisation test	+Ve -Ve +Ve -Ve +Ve	+Ve -Ve +Ve -Ve +Ve
<b>Carbon Source</b>	Glucose Lactose Mannitol	+Ve +Ve -Ve	+Ve +Ve -Ve

**1. Acid tolerance test:**

The isolated lactobacilli species gave promising results to in vitro selection probiotic criteria such as pH and bile salt tolerance tests. The much more growth was observed at pH 3, pH 4 and highest at pH 5 and growth decreases after pH 6. (Fig.- 1). One of the most important criteria for the probiotics organism is the potential viability at low pH. In this study, survival and growth at low pH confirm that these lactobacilli species survive under harsh condition of stomach, hence serve to possess probiotics properties.

**2. Bile salt tolerance:**

Bile salt tolerance to various detrimental concentrations ranging from 0.0%, 0.15% and 0.30% at 2 hrs incubation period recorded for lactobacillus species. Result shows activity as well as growth in all three concentrations for 2 hrs incubation period (Fig.- 2). Bile salt tolerance is required for probiotics bacteria to grow and survive in fish intestine (Salminen et al., 2004)[10]. The probiotics that have capacity to tolerate low pH and bile salt indicate that they are capable of inhabit and survive under stress conditions.

In the present study, given strain of Lactobacilli isolated show acid and bile salt tolerance capacity and appear to have high potential of probiotics properties.

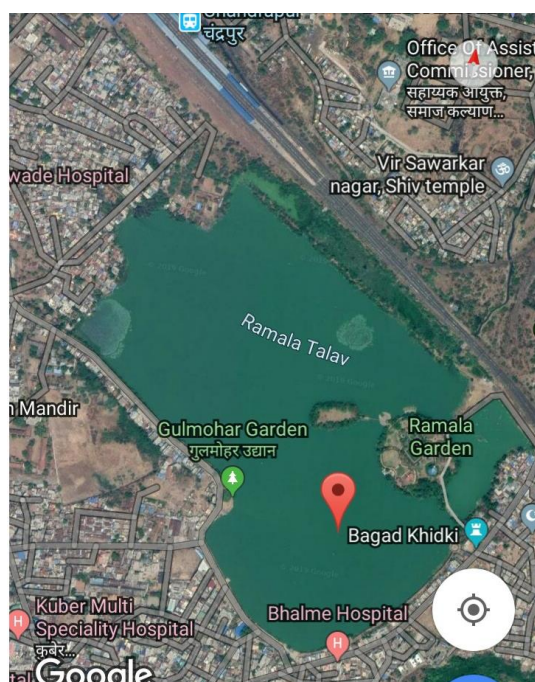


Figure 1: Location of Ramala lake chandrapur



Figure 2: Isolated species 1 and 2 colony and biochemical tests

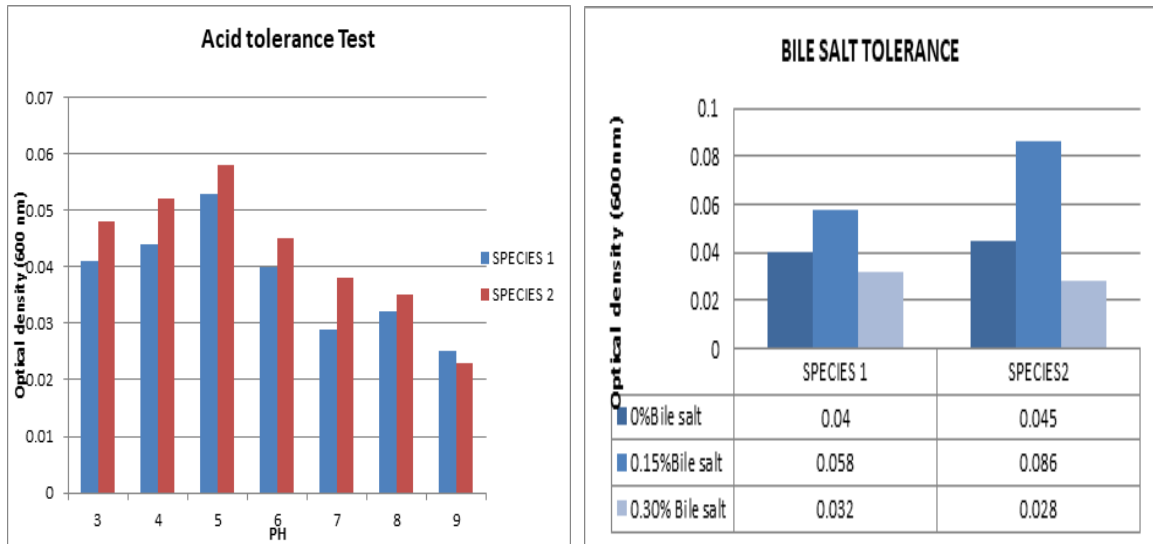
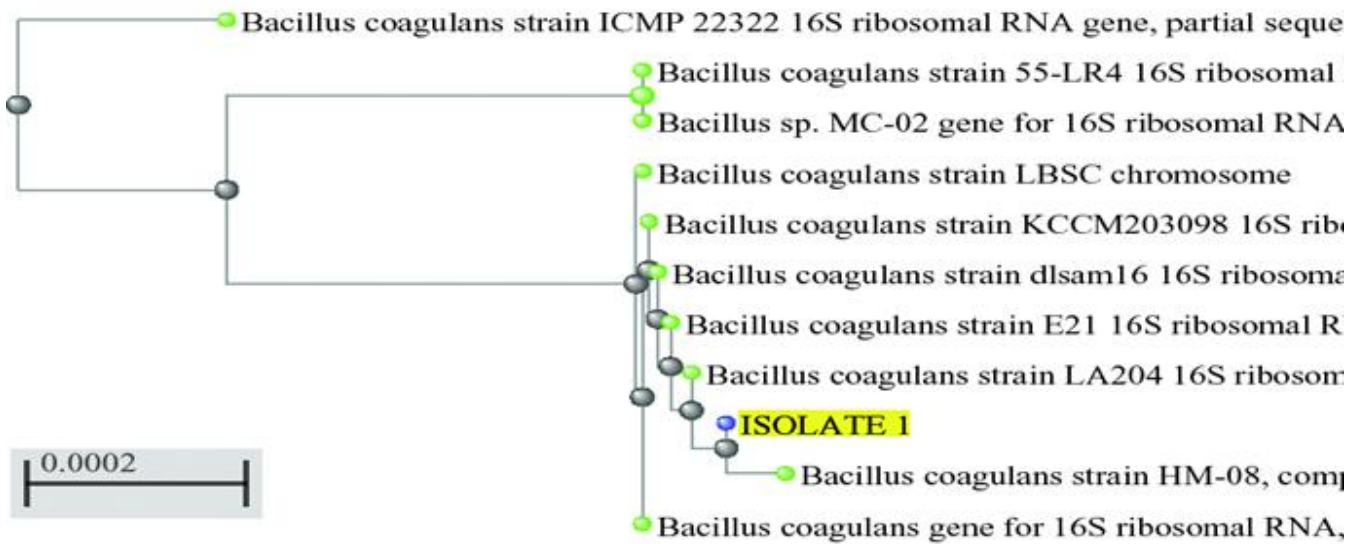
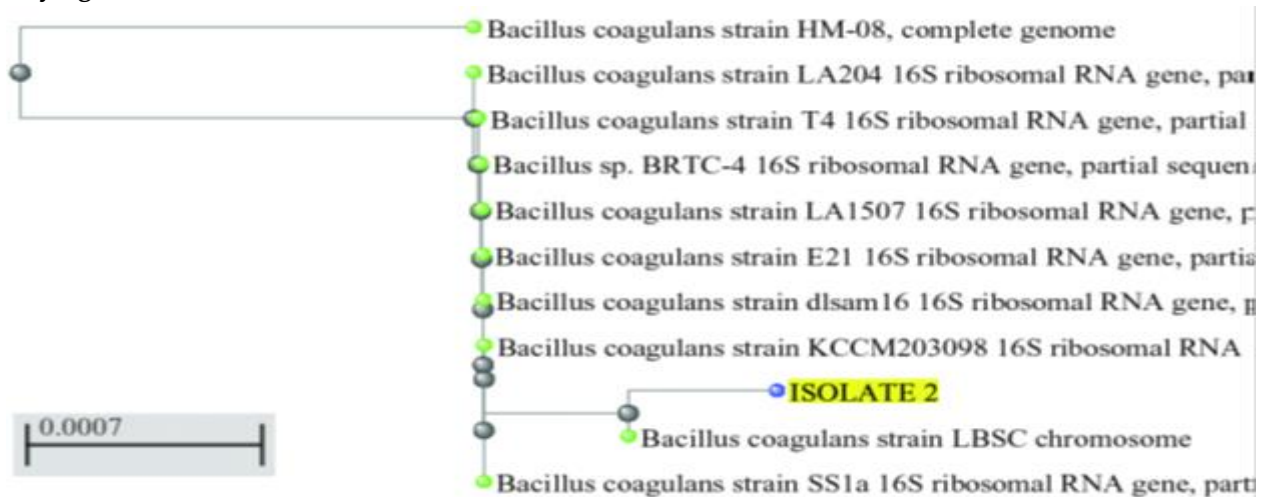


Fig.216S DNA SEQUENCING BY PCR METHOD

Phylogenetic tree



Phylogenetic Tree:



**16S DNA SEQUENCING BY PCR METHOD**

**Sample :** ISOLATE-1

The ~1.5kb, 16S-rDNA fragment was amplified using high-fidelity PCR polymerase. The Sample was found to Bacillus coagulans gene for 16S ribosomal RNA, partial sequence, strain: NTUIOB YUN2  
Sequence ID: [AB696800.1](#)

**Aligned Sequence Data of Sample (1325bp) :**

```
CTATACATGCAGTCGTGCGGACCTTTTAAAAGCTTGCTTTTAAAAGGTTAGCGG
CGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGATCGGGATAACGCC
GGGAAACCGGGGCTAATACCGGATAGTTTTTTCCTCCGCATGGAGGAAAAAGG
AAAGACGGCTTTTGTGTCACTTACAGATGGGCCCCGGCGCATTAGCTAGTT
GGTGGGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACATTGGGACTGAGACACGGCCAAACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCCGGTGAGT
GAAGAAGGCCTTCGGGTCGTAAACTCTGTTGCCGGGGAAGAACAAGTGCCGT
TCGAACAGGGCGGCGCCTTGACGGTACCCGGCCAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTG
GGCGTAAAGCGCGCGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTTGCGGT
CAACCGCAAGCGGTCATTGGAAACTGGGAGGCTTGAGTGCAGAAGAGGAGAG
TGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGT
GGCGAAGGCGGCTCTCTGGTCTGTAAGTCTGACGCTGAGGCGGAAAGCGTGGG
GAGCAAACAGGATTAGATAACCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCG
CCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTTAATTCGAAGCAACGCGAAGAACCCTTACCA
GGTCTTGACATCCTCTGACCTCCCTGGAGACAGGGCCTTCCCCTTCGGGGGAC
AGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTGACCTTAGTTGCCAGCATTTCAGTTGGG
CACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTAC
AAAGGGCTGCGAGACCGCGAGGTTAAGCCAATCCAGAAAACCATTCAGTT
CGGATTGCAGGCTGCAACCCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCG
ATCAGCAT
```

**Sample:** ISOLATE 2

1. The Sample was found to Bacillus coagulans strain SS1a 16S ribosomal RNA gene, partial sequence  
Sequence ID: [MH169742.1](#)

**Aligned Sequence Data of Sample (~1321bp) :**

```
CGCGCGGGGCACCCCCCTGCGACATCCGCATCACGCCCTACTCCGGGGCTA
ATACCGGATAGTTTTTTCCTCCGCATGGAGGAAAAAGGAAAGACGGCTTTTGC
```

TGTCACCTTACAGATGGGCCCCGCGGCATTAGCTAGTTGGTGGGGTAACGGCT  
 CACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGG  
 ACTGAGACACGGCCCAAACCTCTACGGGAGGCAGCAGTAGGGAATCTTCCGC  
 AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGCCTTCGG  
 GTCGTAAACTCTGTTGCCGGGGAAGAACAAGTGCCGTTTGAACAGGGCGGC  
 GCCTTGACGGTACCCGGCCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC  
 GGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCG  
 CAGGCGGCTTCTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTC  
 ATTGGAAACTGGGAGGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGT  
 AGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCT  
 CTGGTCTGTAACCTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTA  
 GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTAGAGGGTTT  
 CCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG  
 GCCGCTAGGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG  
 AGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCCTTACCAGGTCTTGACATC  
 CTCTGACCTCCCTGGAGACAGGGCCTTCCCCTTCGGGGGACAGAGTGACAGGT  
 GGTGCATGGTTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAAAGTCCCGCAA  
 CGAGCGCAACCCTTGACCTTAGTTGCCAGCATTGAGTTGGGCACTCTAAGGTG  
 ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC  
 CCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCGA  
 GACCGCGAGGTTAAGCCAATCCCAGAAAACCATTCCCAGTTCGGATTGCAGGC  
 TGCA

## CONCLUSION

In the present study, the isolated Lactobacilli species 1 and species 2 isolated from the gut of *Labeo rohita* fish collected from Ramala lake of Chandrapur city show high acid and bile salt tolerance capacity and appears to have high potential of probiotics properties. The molecular biology study ,16s DNA Sequencing ,PCR study shows that given strains of lactobacilli are found to be potential probiotic species of *Bacillus Coagulans*.

**Conflicts of interest:** The authors stated that no conflicts of interest.

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