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Biochemical And Molecular Characterization Of Probiotic Bacteria Isolated From Krishna Riverbanks

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Abstract

The current study aimed to isolate, screen, and characterize potential probiotic bacteria from the riverbanks of the Krishna River in Andhra Pradesh, focusing on both biochemical and molecular identification. The isolated bacterial strains were assessed for their suitability as probiotics in aquaculture. Biochemical tests, such as the Catalase and Voges-Proskauer (VP) tests, were conducted to evaluate the properties of the bacteria. Molecular identification was performed through sequencing the 16S rRNA gene. A critical characteristic for probiotics is their ability to survive in the gastrointestinal tract and colonize under acidic conditions. To assess this, the bacteria were incubated at various pH levels for different durations. Following the biochemical and molecular analyses, the bacterium was identified as Bacillus subtilis, which demonstrated strong resistance to low pH, indicating its potential as a reliable probiotic for aquaculture applications.

Keywords: Probiotics, aquaculture, 16S rRNA, *Bacillus subtilis*, Biochemical tests

Introduction

India, with its extensive 5,700 km coastline, holds significant potential for aquaculture. Due to increasing global demand, aquaculture has become a vital economic activity and a growing sector in the country. In India, aquaculture is practiced in two main types: freshwater and brackish water. Among the states, Andhra Pradesh leads in both coastal and freshwater aquaculture, contributing approximately 40% of the nation's total marine exports. On a global scale, aquaculture serves numerous purposes, including the production of high-nutrient food for human consumption, providing rural income and employment, enhancing capture and sport fisheries, cultivating ornamental species for decorative purposes, managing aquatic pests and weeds, and addressing soil salinization and other environmental challenges.

Despite its rapid expansion, the aquaculture industry faces significant challenges, particularly from a wide variety of diseases. Some of the bacterial diseases impacting aquaculture include bacterial necrosis larval mid-cycle disease (MCD) and fungal infections. Over the past decade, these diseases have had a devastating effect on global aquaculture, with losses that are difficult to quantify. The prevalence of diseases increases risks, discouraging investment and hindering economic growth. The overuse of antibiotics has also led to the emergence of multi-drug resistant strains of pathogens, further complicating disease management. In addition to reduced drug efficacy, there is a growing concern that the use of antibiotics may lead to the rejection of aquaculture products in international markets. The lack of effective therapeutic or preventive measures has worsened these issues. While fish farming has advanced rapidly in many Southeast Asian countries, challenges such as environmental pollution, poor management practices, and diseases continue to hinder successful production

Selecting an appropriate bacterial strain for use as a probiotic in aquaculture requires careful consideration to ensure its safety. It is essential to assess the strain's potential for transmitting antibiotic resistance or virulent plasmids. Additionally, the ability of the bacteria to survive and proliferate under culture conditions, as well as to effectively colonize the gut of aquatic species, is crucial. Managing the gut microbiota is key to preventing infections caused by enteric pathogens and ensuring efficient digestion, which in turn supports optimal growth performance. Probiotic bacteria can promote a favorable gut microbial composition by modulating the existing flora. Therefore, when selecting probiotics for aquaculture, it is important to consider their ability to antagonize pathogens through mechanisms such as competitive exclusion, their capacity for growth and attachment to intestinal mucus, and their production of beneficial metabolites. Given the varied environmental conditions in which aquatic organisms are cultured, factors like salinity and temperature range should also be considered when choosing a suitable probiotic strain. Isolating bacteria from their natural environment is an effective approach, as these strains are more likely to perform well when incorporated into aquaculture diets.

The objective of the present study was to isolate microorganisms from natural environments with potential probiotic properties that could enhance the health and productivity of aquaculture systems. The bacteria were isolated from the riverbanks of the Krishna River in Andhra Pradesh, India. The isolated strains were evaluated for their suitability as probiotics by testing their tolerance to low pH. Among the various isolates, the strain with the best potential for aquaculture was selected for further investigation. This included determining its biochemical characteristics and identifying the bacterium at the genus and species level using 16S rRNA sequencing. The 16S rRNA gene, which is approximately 1540 base pairs long, contains both conserved regions and variable regions that make it highly useful for microbial identification. The broad specificity of 16S rRNA gene probes, ranging from universal to species-specific, allows for precise phylogenetic analysis and accurate identification of microorganisms.

Materials and Methods

Isolation of Bacteria:

Soil samples were collected from the riverbanks of the Krishna River in Andhra Pradesh, India. The samples were taken from the upper soil layers, where bacterial populations are most concentrated. Approximately 3 grams of soil was collected using a sterilized spatula and placed in a clean, dry, and sterile polythene bag. A 1-gram portion of the soil sample was then subjected to serial dilution, with dilution factors ranging from 10^{-4} to 10^{-8}. The diluted samples were streaked onto nutrient agar plates. After incubation, single colonies were selected and subcultured to obtain pure bacterial cultures.

Biochemical Characterization of isolated bacteria:

Various biochemical and molecular techniques were employed to characterize and identify the bacteria at both the genus and species levels.

Biochemical Tests:

Gram staining:

After 24 hours of bacterial growth, a sample of the culture was taken on a slide and heat-fixed. The smear was then flooded with crystal violet and allowed to incubate for 1 minute. The slide was rinsed gently under a stream of tap water for 2 seconds. Next, iodine mordant was applied to the slide, which was incubated for 1 minute. The slide was washed again with a gentle stream of tap water for 2 seconds. Following this, the slide was counterstained with safranin and then washed with 95% ethanol. Finally, the slide was examined under a microscope.

Catalase test:

Catalase production was tested by adding hydrogen peroxide to a nutrient agar slant that had been incubated with the bacterial culture. The presence of catalase was indicated by the formation of oxygen bubbles. Catalase is an enzyme found in aerobic and facultative anaerobic bacteria that contain cytochrome. Bacteria

lacking cytochrome also lack catalase and are unable to break down hydrogen peroxide. To test for catalase activity, a nutrient broth slant inoculated with the isolated bacterial culture was incubated at 37°C for 24 hours. After incubation, 1 ml of hydrogen peroxide was added to the slant, and the presence of bubbles was observed, indicating catalase production.

Indole production test:

Indole is a nitrogen-containing compound produced by some bacteria through the degradation of the amino acid tryptophan. The enzyme tryptophanase catalyzes the breakdown of tryptophan, converting it into indole, skatol, and indole acetic acid. To test for indole production, tryptone broth was prepared and its pH adjusted to 7.2. A 5 ml aliquot of the broth was dispensed into tubes, sterilized at 121°C for 15 minutes at 15 lbs pressure, and allowed to cool. The tubes were then inoculated with the bacterial isolate, while a control tube was maintained without the bacterial addition. Both tubes were incubated at 37°C for 48 hours. After the bacteria had grown, 1 ml of Kovac's reagent was added to each tube, and the contents were mixed thoroughly. The results were observed after a 5-minute incubation period..

Voges Proskauer (vp) test:

The Voges-Proskauer (VP) test is used to assess an organism's ability to ferment glucose and produce neutral or non-acidic end products, such as acetyl methyl carbinol. MR-VP broth was prepared and sterilized in tubes, which were then inoculated with the bacterial culture and incubated at 37°C for 24 hours. After incubation, two drops of Barritt's reagent were added to each tube. The tubes were then allowed to incubate for an additional 10 minutes, and the results were recorded.

Molecular level Characterization: 16s rRNA Studies:

DNA was extracted from the culture incubated overnight. The culture was centrifuged, and the pellet was collected, then suspended in extraction buffer and incubated in a water bath at 60°C for 20 minutes. Afterward, the mixture was centrifuged again, and the supernatant was collected. An equal volume of isopropanol was added to the supernatant, followed by another round of centrifugation to collect the DNA pellet. The pellet was then dissolved in TE buffer for further analysis. The quality of the extracted DNA was assessed spectrophotometrically and by running the sample on an agarose gel. The DNA was stored in TE buffer for further use.

PCR amplification of 16s rRNA gene:

The extracted DNA was used for amplification of the 16S rRNA gene. Universal primers specific to the 16S rRNA gene were employed in the PCR reaction. The primers used for amplification were 16S F (5´-AGA GTT TGA TCC TGG CTC AG-3´) and 16S R (5´-GGT TAC CTT GTT ACG ACT T-3´).

PCR reaction mixtures:

The PCR conditions used were as follows: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 10 minutes, with a final hold at 10°C. The PCR reaction mixture consisted of 1 µl of forward primer, 1 µl of reverse primer, 30 µl of PCR master mix, and 2 µl of the isolated DNA. A total of 30 PCR cycles were performed using parameters that were specifically developed and optimized for the isolated DNA sample.

Sequencing the 16s rRNA gene:

The amplified product was gel-purified and subsequently sequenced using the ABI 3730xl Sequencer, employing Sanger's method with internal primers specific to the entire 16S region. The sequencing was carried out through the automated DNA sequencing service provided by Bioserve Laboratory (Pvt.) Ltd., located in Hyderabad, India.

Test for resistance to low pH:

The four isolated bacterial cultures were evaluated for their potential use as probiotics in aquaculture. A key factor for probiotics is their ability to withstand low pH, as they must be capable of colonizing the gut of aquatic animals, where the environment is acidic. To assess this, an acid tolerance test was conducted to determine the bacteria's resistance to low pH. The bacterial cultures were incubated at pH levels of 2, 3, and 6 for durations of one hour, two hours, and six hours to evaluate their ability to tolerate acidic conditions.

Results and Discussion

Isolation of Bacteria: The bacterial colonies were observed on nutrient agar plates, where they grew well. The morphology of the colonies was noted, and a single colony was selected and subcultured. The resulting cultures were labeled as ProBt Is-1, ProBt Is-2, ProBt Is-3, and ProBt Is-4. Among these four isolates, ProBt Is-1 was chosen for further biochemical and molecular characterization, as well as for evaluating its potential as a probiotic in aquaculture, specifically for its resistance to low pH. The pure culture of ProBt Is-1 is shown in Image 1. The colonies of this bacterial isolate were round, opaque, and light cream-colored, while microscopic examination revealed that the bacteria were Bacillus-shaped.

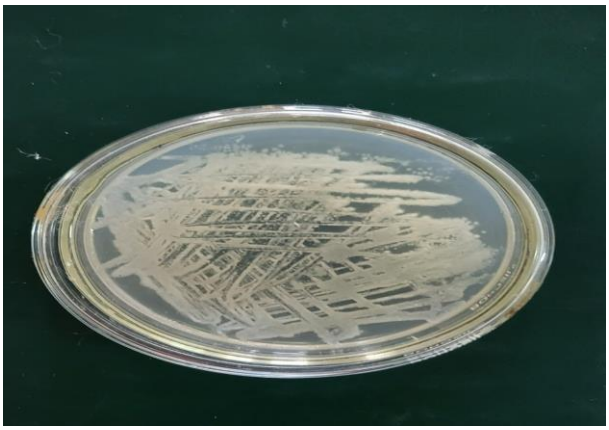

Image: 1. Bacterial isolate ProBt Is- 1.

Acid tolerance test:

Survival under low pH is a critical characteristic for any probiotic bacterium, particularly those intended for gut health. All the isolates were subjected to an acid tolerance test under various incubation times. The results indicated that all isolates were able to withstand low pH at levels of 2 and 3 for both 1 hour and 6 hours. The survival rates of the isolates at pH 2 and pH 3 after 1 hour and 6 hours of exposure are presented in Table 1.

Table 1: Percentage of Survival Rates of Isolated Bacterial Strains Under Acidic Conditions

Strain	Survival rate at pH 2 (1hr)	Survival rate at pH 3 (1hr)	Survival rate at pH 3 (6hr)
Pro Bt IS-1	92 ± 1.5	90 ± 2.0	87 ± 1.8
Pro Bt IS-2	78 ± 2.5	75±2.3	72 ± 2.2
Pro Bt IS-3	85 ± 3.0	83 ± 2.5	65 ± 3.5
Pro Bt IS-4	66 ± 4.0	57 ± 4.0	42 ± 7.0

Isolate ProBt Is -1 exhibited highest survival rate at both pH 2 and 3 after 1hr of exposure with values of 92 ± 1.5 and 90 ± 2.0 and 87 ± 1.8, respectively. In contrast, ProBt IS -4 demonstrated the lowest survival rate 42 ± 7.0, 66 ± 4.0 and 57 ± 4.0 (Table 1). Among the four strains ProBt IS -1 showed highest survival rate at pH 2 for 1 hour incubation period (92 ± 1.5) while ProBt Is – 4 had the lowest survival rate at pH 3, after 6 hours of incubation period which is (42 ± 7.0).

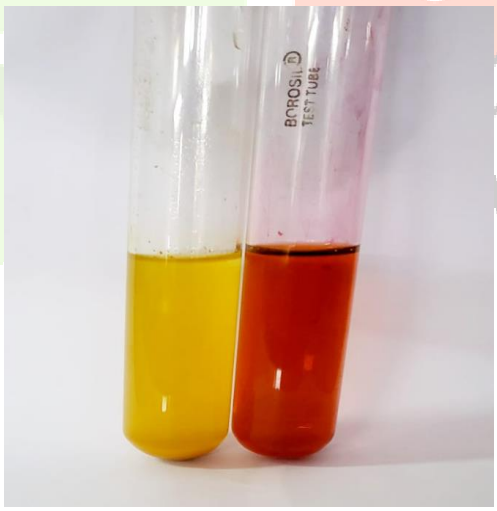

Image-2 Voges proskauer (vp) test

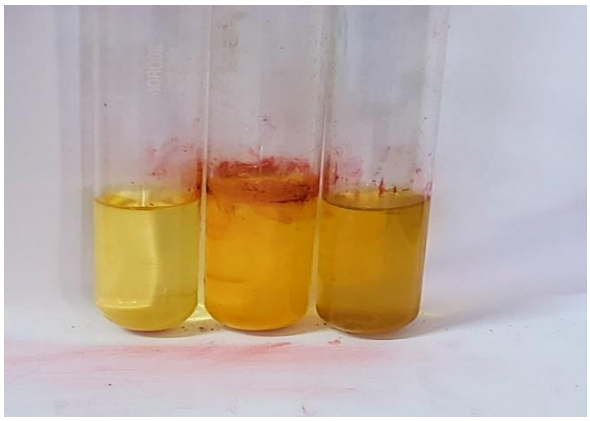

Image – 3: Indole production test: Test tube 1- Control,

Test tube – 2 indole positive,

Test tube-3 isolate

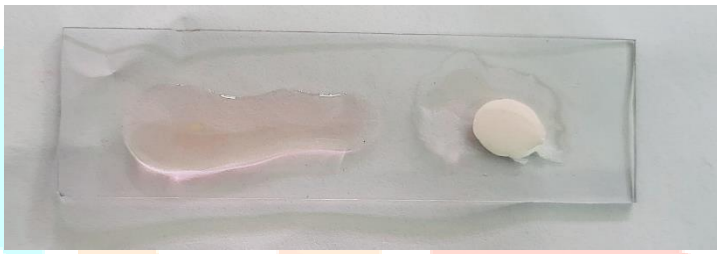

Image – 4 Catalase test

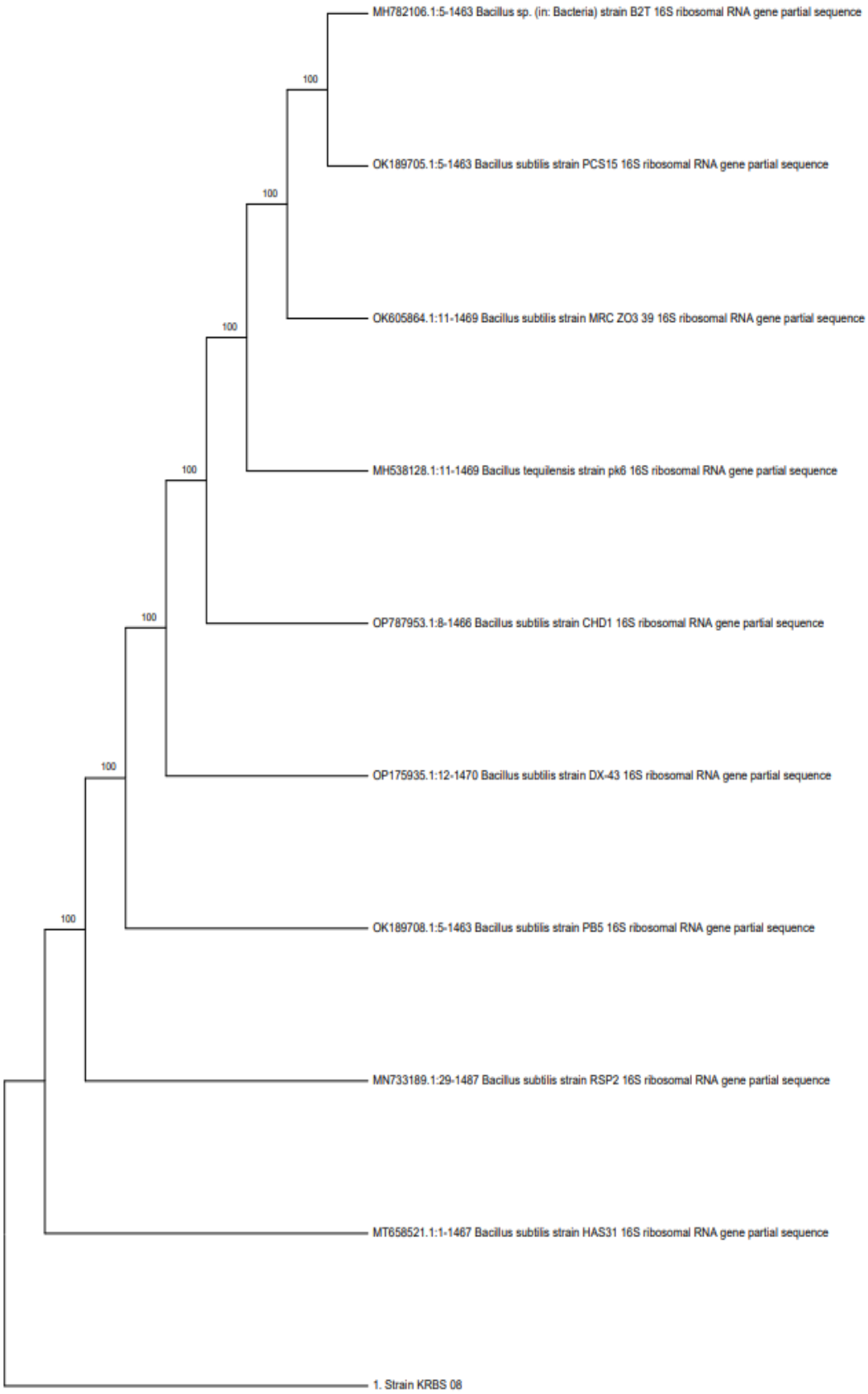

Image -5 Phylogenetic tree

Summary and conclusion: In this study, bacteria were isolated from their natural habitat and assessed for their potential use as probiotics in aquaculture. The isolated strain was identified at the genus and species levels, and various biochemical tests were conducted to evaluate the biochemical properties and efficacy of the bacterial isolate.

Based on the results obtained, the isolated microorganism is identified as *Bacillus subtilis*.

The classification of the organism is as follows:.

Domain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Bacillaceae

Genus: Bacillus

Species: subtilis

Bacillus subtilis is a Gram-positive, rod-shaped, aerobic bacterium that forms spores. It is commonly found in soil and can also inhabit the root zones of certain plants, where it exhibits both antibacterial and antifungal properties. Various biochemical tests, including those found in the Analytical Profile Index (API), are used for its identification and classification. B. subtilis is positive for amylase, lipase, and protease activities. It also demonstrates multiple mechanisms of nitrate reduction, gas production from glucose, and acid production from different carbon sources, such as arabinose, mannitol, xylose, glucose, and lactose. Additionally, B. subtilis can produce acetyl butanediol (ABD) from acetoin, which is confirmed by a positive Voges-Proskauer test.

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