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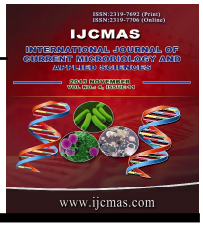


**Original Research Article**

# Isolation and Identification of Probiotic Bacteria from River Banks of Krishna by Biochemical and Molecular Level Characterization

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**A B S T R A C T**

The present study was focused on isolation, screening, biochemical and molecular level characterization of potential probiotic bacteria from the river banks of Krishna, Andhra Pradesh. The isolated bacteria potentiality was tested to be used in aquaculture as probiotic. The bacteria was tested for its biochemical ability and identified it at the molecular level by using 16s r RNA encoding gene sequence. The different biochemical tests conducted include catalase test, Vogesproskauer (VP) test. Survival in the gut and colonization at low pH is an essential requirement for any bacteria to be used in aquaculture as probiotic. To evaluate this potentiality the isolated bacteria was incubated at different pH and time period. The isolated bacteria after biochemical and molecular level characterization was identified as *Bacillus pumilus* and it showed good resistance to low pH, making it as the reliable source of probiotic in aquaculture.

## Introduction

India is the potential land for aquaculture with its 5700 km coastline. Due to its International demand aquaculture in India became a very important economic activity and a flourishing sector. Aquaculture is mainly practiced as two types in India as Fresh water aquaculture and brackish water aquaculture. Within India, Andhra Pradesh ranks first in coastal and fresh water aquaculture. It is estimated that Andhra Pradesh contributes nearly 40 per cent of the total marine exports of the country. Globally aquacultures have great importance which serves a variety of purposes majority of which include to produce high nutritional value food for human consumption, to contribute rural income and employment through farming and related activities, to enhance capture and sport fisheries, to cultivate ornamental species for aesthetic purposes, to control aquatic weeds or pests hazardous or crops and to salinization and other forms of soil etc.

Despite rapid growth in aquaculture sector there are problems associated with wide

range of diverse diseases. A few of the bacterial diseases in aquaculture include bacterial necrosis (Aquacop, 1977), larval mid- cycle disease (MCD) (Brock, 1983) and fungal diseases (Anderson, 1988) etc., from last decade, diseases in aquaculture have a devastating impact on world aquaculture farming. Losses due to diseases are enormous and difficult to estimate. Diseases increase risk, deterring investment and economic growth. Usage of antibiotics leads to development of multi drug resistance strains of disease causing pathogens. Apart from lose of sensitivity to drugs there is also a potential risk of Aqua products rejection in International market if antibiotics are used. Inadequate availability of effective therapeutic or prophylactic measures has aggravated the situation. Though shrimp culture has under gone rapid development in most Southeast Asian countries, successful production is increasingly hampered by environmental pollution, poor management, diseases, among others (Bachere, 2000).

Much care should be taken in selection of a bacterial strain as a probiotic such that the safety of strains must be carefully assessed, as well, and transmission of antibiotic resistance or virulent plasmids must not take place. Of further great importance are the survival and growth of beneficial bacteria in culture conditions and their ability to colonize the gut of the aquatic animals.

The management of gut flora is important for the ability to prevent infections with enteric pathogens and to guarantee a well-functioning and effective digestion of nutrients that result in good growth performance parameters. Probiotic bacteria modulate the gut microflora towards a favorable composition (Tuohy *et al.,* 2003; Ling *et al.,* 2012). Hence, selection criteria of probiotics for aquaculture should be based on their antagonism towards pathogens (through competitive exclusion), their growth, attachment to intestinal mucus and production of beneficial compounds. Since aquatic animals are cultured under different conditions salinity and optimum temperature range should also be considered for selection of the right probiotic strain.

All these criteria can be fulfilled by isolating the bacteria from their natural habitat, such that best results can be obtained when supplemented along with the diets in aquaculture.

The present study was aimed to isolate the potential microorganism from natural environments having probable properties of probiotics which improves the health and gives best benefits to aqua culture. The bacteria was isolated from natural habitat i.e., from the river banks of the Krishna, Andhra Pradesh, India.

The isolated bacterium was tested for its potentiality to be used as probiotic strain in aquaculture by testing the ability of isolated strain to tolerate low pH. Among isolated samples the strain best suited for aquaculture was selected for further studies of identifying the possible biochemical characters and also to identify the isolated strain at Genus and species level by 16s rRNA studies.

The 16S rRNA gene is nearly 1540 bases long and includes variable regions while the general structure is highly conserved. Because the probes have the broadest specificity ranging from universal to species specificity, it is possible to use 16S rRNA gene to study phylogenetic relationships between microorganisms and identify them more accurately (Cakir, 2003; Holzapfel *et al.,* 1998; Charteris *et al.,* 1997).

## Materials and Methods

### Isolation of bacteria

Soil samples were collected from the river banks of Krishna, Andhra Pradesh, India. The soil samples were collected from the upper layers where most of bacteria are concentrated; approximately 3 g of soil sample was collected in a clean dry and sterile polythene bag using sterilized spatula. From this 1 g of soil sample was subjected to serial dilution and dilution factor of $10^{-4}$ to $10^{-8}$ used to streak on nutrient agar plates. Single colony was isolated and further sub cultured to isolate the pure cultures (Musliu Abdulkadir and Salawudeen Waliyu, 2012).

### Biochemical characterization of isolated bacteria

Different biochemical and molecular studies were performed to characterize and identify bacteria at Genus and species level.

### Biochemical tests

### Grams staining

The culture after 24 hours growth was taken on a slide and heat fixed. The smear on slide was flooded with crystal violet and incubated for 1 min. The slide was then washed in a gentle and direct stream of tap water for 2 seconds. The slide was again flooded with iodine mordant and incubated for 1 min. the slide was washed again in a gentle and direct stream of tap water for 2 seconds. Later on counter stain safranin was added and the slide was then washed in 95% ethanol and observed under microscope (Bergey *et al.,* 1994).

### Catalase test

Catalase production can be determined by adding the substrate hydrogen peroxide to an approximately incubated nutrient agar slant. If catalase is present bubbles of free oxygen gas is formed. The enzyme catalase is present in the mist cytochrome containing aerobic and facultative aerobic bacteria. Organism that lack cytochrome also lack catalase and they are not able to break hydrogen peroxide.

The nutrient broth slant inoculated with isolated bacterial culture and after incubation at 37ºC for 24 hours, the slant was added with 1ml of hydrogen peroxide and observed for evolution of bubbles.

### Indole production test

Indole was a nitrogen containing compound formed from the degradation of the amino acid tryptophan by some bacteria. Tryptophan present in the culture media was degraded by the enzyme tryptophanase and converted in to indole, skatol and indole acetic acid. Tryptone broth was prepared and the pH was adjusted to 7.2, from this 5 ml of broth was dispensed into the tubes and sterilized at 121 ºC for 15 minutes at 15 lbs. After cooling, the tubes were inoculated with the isolate. Control tube was also maintained which is without addition of isolated bacteria and incubated at 37ºC for 48 hours. After well grown of isolated bacteria 1 ml of Kovac's reagent was added to each tube and mixed thoroughly. The results were observed after incubating for five minutes.

### Voges Proskauer (vp) test

Voges Proskauer (VP) test determines the capability of an organism to ferment glucose and production of non-acidic or neutral end products such as acetyl methyl carbinol. MR-VP broth was prepared and sterilized in tubes. The tubes were then inoculated with the culture and incubated at 37 °C for 24 hours. After incubation two drops of Barrit's

reagent was added. After 10 minutes of incubation the results were noted.

**Molecular level characterization: 16s rRNA studies**

The DNA was isolated from the overnight incubated culture. The culture was centrifuged and pellet was collected, suspended in Extraction buffer and kept in water bath for 20 minutes at 60 °C, again centrifuged. The supernatant was collected and mixed with equal volume of isoproponal, centrifuged again and DNA pellet was collected, dissolved in TE buffer for further analysis. Quality was analyzed both spectrophotometrically (Sambrook *et al.,* 1989) and by running agarose gel, stored in TE buffer.

**PCR amplification of 16s rRNA gene**

Extracted DNA was used to amplify the 16s r RNA gene. Universal primers for 16s r RNA encoding genes were used to carry out the reaction. The primers used for amplification are 16SF5´-AGAGTTTGATC CTGGCTCAG-3´ and 16SR5´-GGTTA CCTT GTTACGACTT-3´ (Lie zhong Chen *et al.,* 2014).

**PCR reaction mixtures**

The PCR cycle used was 95°C for 5 minutes, 95°C for 30 sec, 55°C for 30 sec, 72°C for 10 minutes and 10°C hold. The 1 µl of forward primer, 1 µl of reverse primer, 30 µl of PCR master mix and 2 µl of Isolated DNA was taken to run 30 cycles of PCR. The PCR parameters are in house developed and optimized for isolated DNA sample.

**Sequencing the 16s rRNA gene**

The product thus amplified is gel eluted and the product is sequenced on ABI 3730xl Sequencer by Sanger's method using internal primers specific for whole 16S region. The sequence of the insert was determined using the automated DNA sequencing service provided at Bioserve Laboratory (Pvt.) Ltd., Hyderabad, India.

**Test for resistance to low pH**

The isolated four bacterial cultures were tested for their potentiality to be used as probiotics in aquaculture. Resistance and surveillance of bacteria at low pH is essential for any probiotic bacteria as they need to colonize at gut of the aquatic animals where pH is low. Acid tolerance test was performed to test the isolated bacteria resistance to low pH. The bacterial cultures were incubated at pH 2, 3 and 6 for one hour, two hours and six hours each to test their low pH tolerance.

**Results and Discussion**

**Isolation of Bacteria:** The bacterial colonies were observed on nutrient agar media. Colonies were well grown in nutrient agar medium, the morphology of colonies was identified and single colony was picked up and subcultured. The obtained cultures were named as ProBtIs-1, ProBtIs-2, ProBtIs-3 and ProBt Is-4. Out of the isolated four different bacteria, ProBtIs-1was selected for further in detail biochemical and molecular level characterization and evaluated potentiality to be as probiotic in aquaculture by the methodology of resistance to low pH.TheProBtIs-1isolatedpureculture of bacteria was represented in figure 1. The Bacterial isolate formed the round, opaque, light cream coloured colonies and microscopic observations revealed *Bacilli* form bacteria.

**Acid tolerance test**

Survival under low pH is the most important character of any probiotic bacterium, especially gut probiotics. All the isolates

were subjected for acid tolerance test under different incubation periods. The results have shown that all the isolates tolerated low at pH2 and 3 for 1 hr and 6 hrs. All the isolates were resistant to low pH, the percentage of survival rates of isolates at pH 2 and pH 3 for 1 hr and 6 hrs were given in table 1.

Isolate ProBt Is -1 showed highest survival rate at both pH 2 and 3 for 1hr that is 90 ± 1 and 89 ± 2 and 86 ±1.7. ProBt IS -4 showed least survival rate 40±6.5, 64±3.7 and 54± 3.5 (Table 1). Among these four strains ProBt IS -1 showed highest survival rate at pH 2 for 1 hour incubation period which is 90 ± 1 while ProBt Is – 4 showed least survival rate at pH 3, for 6 hours of incubation period which is 40±6.5.

Summary and conclusion: In the present study the bacteria was isolated from the natural habitat, tested its potentiality to be used in aquaculture as probiotic, identified the isolated strain at Genus and species level and different biochemical parameters studied to identify the biochemical efficacy of isolated bacteria.

Based on overall results the isolated microorganism can be concluded as *Bacillus pumilus*.

Domain: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: *Bacillus*
Species: *pumilus*

**Table.1** Percentage of Survival rate of the isolated bacteria 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 | 90 ± 1 | 89 ±2.2 | 86 ±1.7 |
| Pro Bt IS-2 | 76 ± 2.2 | 74±2.1 | 70 ±2.0 |
| Pro Bt IS-3 | 83 ±3.2 | 82 ±2.1 | 63 ±4.1 |
| Pro Bt IS-4 | 64±3.7 | 54±3.5 | 40±6.5 |

**Figure.1** Bacterial isolate ProBt Is-1

**Figure.2** Voges proskauer (vp) test

**Figure.3** Indole production test: Test tube 1 - Control, Test tube 2 - indole positive, Test tube 3 - isolate

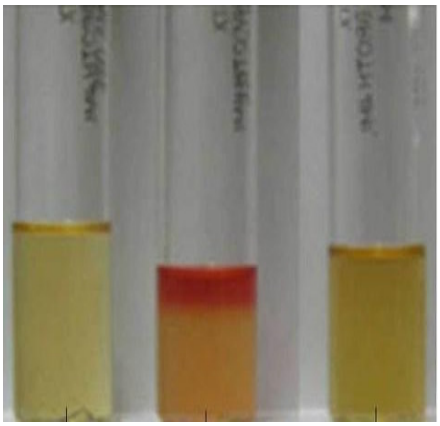
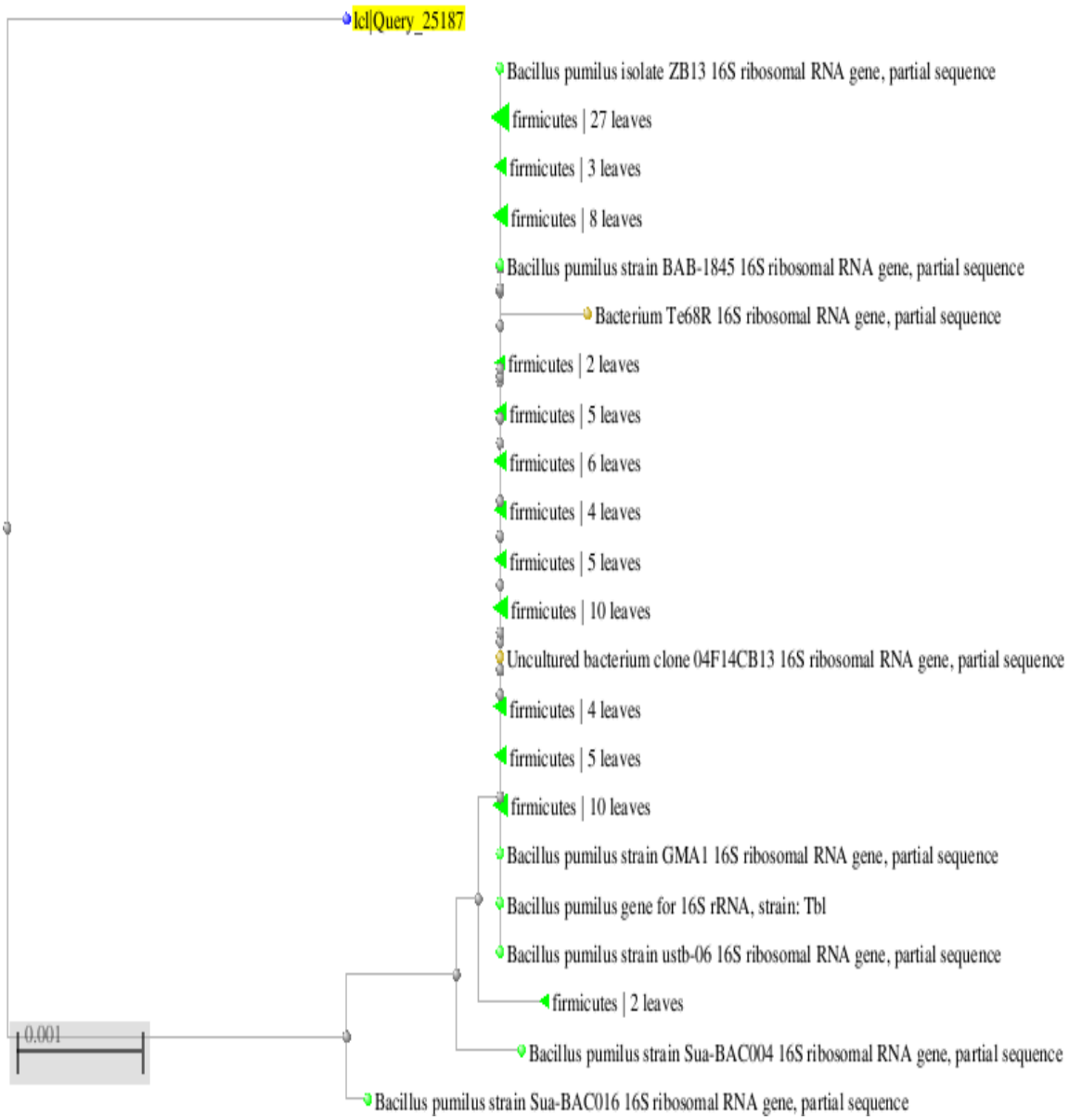


**Figure.4** Catalase test

**Figure.5** Phylogenetic tree




*Bacillus pumilus* is a spore-forming bacterium that is rod-shaped, Gram-positive, and aerobic. It resides in soils and some colonize in the root area of some plants where *B. pumilus* has antibacterial and antifungal activity. Several biochemical assays found in the analytical profile index (API) have been used in pursuit of its classification. *B. pumilus* is amylase, lipase, and protease-positive. It has a variety of mechanisms of nitrate reduction, gas production from glucose, and acid production from a variety of carbon sources, namely arabinose, mannitol, xylose, glucose, and lactose. Notably, *B. pumilus* can generate acetyl butanediol (ABD) from acetoin, as seen by a positive result for the Voges-Proskauer test. Its ability to colonize in the gut of aquatic animals and resistance to low pH, makes this bacteria as indispensable strain to be used as probiotic in aquaculture.

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