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Research Article

ISOLATION AND GENOMIC ANALYSIS OF PROBIOTIC ISOLATE AND ROLE OF AMYLASE ENZYME ACTIVITY IN FISH GROWTH

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ABSTRACT

Probiotics are beneficial microbial population, which enhance the growth and immunity of fish under stressful environmental conditions by modulating the gut colonization of the probiotic bacterial strains. The present investigation was designed to isolate fish gut bacterial samples. Isolated strains were screened and four strains were assessed with probiotic activity towards the growth of fish in the particular habitat. The experiments were conducted by admixing the strain with water and fortified with the commercial feed for the growth of *Labeo rohita*. The result clearly revealed that only two strains showed better activity. These isolated probiotic microorganism's genomic DNA was analysed using Universal 16S primers and electrophoresed to confirm the taxonomic identity. The 16S rRNA sequence of the isolates was amplified using primers using PCR. Blast analysis of 16S rRNA sequence was carried out with the sequences available in the NCBI database. The 16S rRNA sequences of the isolates were aligned with reference sequences obtained from the GenBank databases. Identical 16S rRNA sequence was recognized by Phylogenetic trees and manual comparisons. These sequences were found to be associated with enzyme amylase and peptidases. Amylase enzyme, protease and lipase enzymes are markedly increase the activity of the probiotics by improving the water quality parameters, food absorption and growth of *L. rohita*. The identified strain was examined for its effect on fish growth and considered for improving *L. rohita* fish growth.

Keywords: 16S rRNA sequence, Blast, GenBank databases, *Labeo rohita*, PCR primers.

INTRODUCTION

Probiotics are live microorganisms introduced into the body for its beneficial qualities. The use of probiotics for aquatic animals is increasing with the demand for ecofriendly sustainable aquaculture practices (Gatesoupe, 1997). The benefits of probiotic dietary supplements include improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, growth promoting factors and increased immune response (Verschuere *et al.*, 2000). Probiotics play a very positive effect on the digestive processes as well as the assimilation of food components (Irianto and Austin, 2002) and it increases the nutrient digestibility because of better availability of the exoenzymes produced by probiotics. Indeed, the use of antimicrobials as animal growth promoters became a political issue and banned to counteract the emergence of antibiotic-resistant bacteria (Heuer *et al.*, 2006). The use of probiotics for aquaculture is reported earlier by Mohanty *et al.* (1996), Wang *et al.* (2005) and Wang and Xu (2006). The screening of probiotic bacteria from the aquaculture environment was reported in 1980s by (Dopazo *et al.* (1988).

Fish digestive enzymes emphasizing the mechanisms and best use of nutrients are of unquestionable importance as a background for the optimization of fish feeding procedures (Suarez *et al*., 1995). Amylase is a digestive enzyme that aids in the breakdown of carbohydrates by breaking the bonds between sugar molecules in polysaccharides through a hydrolysis reaction. It is important in the digestion of starch into sugars to make available energy sources for the body. Probiotics are supplementary microbes which have a positive effect on the host by increasing the feed nutritional value and improving the host immune response towards disease (Verschuere *et al*, 2000). The industrial demand of highly active preparations of proteolytic enzymes with appropriate

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specificity and stability to pH, temperature, metal ions, surfactants and organic solvents continues to stimulate the search for new enzyme sources. Proteases with high activity and stability in high alkaline range and high temperatures are required for bioengineering and biotechnological applications (Mach, 2006). Proteases are the most important industrial enzymes accounting for about 50% of the total industrial enzyme market. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. The proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

Large amounts of bacilli consistently enter the gastrointestinal and respiratory tracts of healthy people through air, water, and food and exist in the gut microflora. Some researchers showed that *Bacillus* organisms are a normal component of human intestinal microflora. *Aeromonas veronii* is a Gram-negative, rod-shaped bacterium, commonly isolated from clinical, environmental, and food samples (Ashbolt, 2004). Thus, the understanding of these intestinal microfloras could be useful for the mass-scale rearing and developing effective probiotic feed to get better growth would be remarkable in the field of aquaculture. They increase host fitness by balancing the intestinal micro flora, inhibiting the growth of pathogenic bacteria, promoting good digestion, and boosting immune function. Bacteria of the *Bacillus* genus are omnipotent in nature, predominant in soil and these bacteria have also been isolated frequently from water and air. Large amounts of bacilli consistently enter the gastrointestinal and respiratory tracts of healthy people through air, water, and food and exist in the gut microflora. Some researchers showed that *Bacillus* organisms are normal component of human intestinal microflora. Proteases are the most important industrial enzymes accounting for about 50% of the total industrial enzyme market.

Identification and characterization of the microorganisms are essential to determine their specific functions in fish nutrition, effect on gut enzyme activity and their role as probiotics in enhancement of food digestibility. Studies reported some diverse microbial communities in the GI tract of carnivorous, herbivorous and omnivorous fish species (Nayak, 2010). Surprisingly, the genetic composition information of bacteria associated with the gastrointestinal tract is to understand intestinal microfloras could be useful for the mass-scale rearing and developing effective probiotic feed to get better growth would be remarkable in the field of aquaculture. Bacteria of the *Bacillus* genus are omnipotent in nature, predominant in soil, and these bacteria have also been isolated frequently from water and air.

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. Proteases and amylases are used together in many industries such as food industry, pharmaceuticals, detergent industries, etc. Detergent formulations are fortified with both proteases (Hmidet *et al*., 2009) and amylases (Hadj-Ali *et al* 2007).

Identification and characterization of the microorganisms are essential to determine their specific functions in fish nutrition, effect on gut enzyme activity and their role as probiotics in enhancement of food digestibility. However, surprisingly, no information on the genetic composition of bacteria associated with the gastrointestinal tract is available. In the present study, the genomic DNA of probiotic isolates from the gut and the gene sequence responsible for enzyme activity of *Labeo rohita* were analyzed

MATERIALS AND METHODS

The amylase activity was determined by Bernfeld (1951) method. Quantitative activity of protease was measured by Garcia-Carreno (1992) method. Quantitative estimation of lipase was assayed based on Borlongan (1990) method. Soluble protein in the crude enzyme was measured by the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as a standard. The extraction of total bacterial genomic DNA was performed according to procedures described by Hesham *et al.* (2006).

The genomic DNA was isolated by the method of Byun *et al.* (1986). Gram's staining method following modification of Wirtz method (Bartholomew and Mitwer, 1950) was performed for morphological testing. All biochemical tests were determined by following the Society of American Bacteriologists (1957). The biochemical characterization such as Gram staining motility, starch hydrolysis, nitrate reduction, oxidase catalase, indole, H2S production and carbohydrate fermentation were carried out according to the guidelines outlined by Griffin (1992). Isolates were grown in broth cultures and studied after 24 hours of incubation at 37°C for the physiological characteristics.

The bacterial population, Colony Forming Units (CFU) per ml of sample and identification of bacterial isolates were done by 16S rRNA analysis to confirm the taxonomic identity. The idea behind the usage of universal primers is that the 16S rRNA gene is unique for different species as well as for different strains of the same species but the flanking regions of the 16S rRNA gene remains highly conserved across different species.

Therefore the primers can be designed for a novel species also since it would have the same flanking regions of its 16S rRNA gene, and so the primer will attach to these flank-ing regions and facilitate the extension of the gene by the respective DNA polymerase enzyme. The bacterial isolates the 16S rRNA sequence of the isolates was amplified using primers (16SF 5'-AGAG TTTGATCCTG GCTCAG-3' and 16SR 5'- ACGGCTACC TTGTTACGA CTT-3') under the following PCR condition: 94 ºC for 3 min, 30 cycles of (94 ºC for 1 min, 52ºC for 1 min, 72 ºC

for 2 min), 72 ºC for 10 min. The PCR products were cloned into pGEM-T vector (Promega, Germany) and then sequenced by the dideoxy chain termination method at Macrogen, South Korea.Blast analysis of the 16S rRNA sequence was carried out with the sequences available in the NCBI database.

The 16S rRNA sequences of the isolates were aligned with reference sequences obtained from the GenBank databases (NCBI, Bethesda, MD, USA).Identical 16S rRNA sequence was recognized by analysis of Phylogenetic trees and manual comparisons, in which sequence with more than 90% similarity was defined as identical, and these sequences were used for further Phylogenetic analysis as an operational taxonomic unit (OTU). All sequences were submitted to Genbank for preliminary analysis using the program BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to identify putative close Phylogenetic relatives. Distance based evolutionary trees were constructed by using the neighbour joining algorithm.

The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program G blocks 0.91b. The G blocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007).

Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 1983 was used for tree rendering (Dereeper *et al.*, 2008).

RESULT AND DISCUSSION

Genomic DNA isolation and 16S ribosomal RNA gene partial sequence of strain from Fish Gut is identified as *Exiguobacterium aurantiacum* (Genbank Accession: KX458117.1 GI:1041522627).Genomic DNA isolation and 16S ribosomal RNA gene partial sequence of strain MA18 (Figures 1-4) from Fish Gut is identified as *Aeromonas veronii* (Genbank Accession: KX458116.1 GI:1041522625).

The isolated probiotic microorganism's genomic DNA was analysed using Universal 16S primers and electrophoresed to confirm the taxonomic identity. The 16S rRNA sequence of the isolates was amplified using primers using PCR. Blast analysis of 16S rRNA sequence was carried out with the sequences available in the NCBI database. The 16S rRNA sequences of the isolates were aligned with those reference sequences obtained from the GenBank databases. Identical 16S rRNA sequence was recognized by Phylogenetic trees and manual comparisons and these sequences were found associated for enzyme amylase and peptidases.

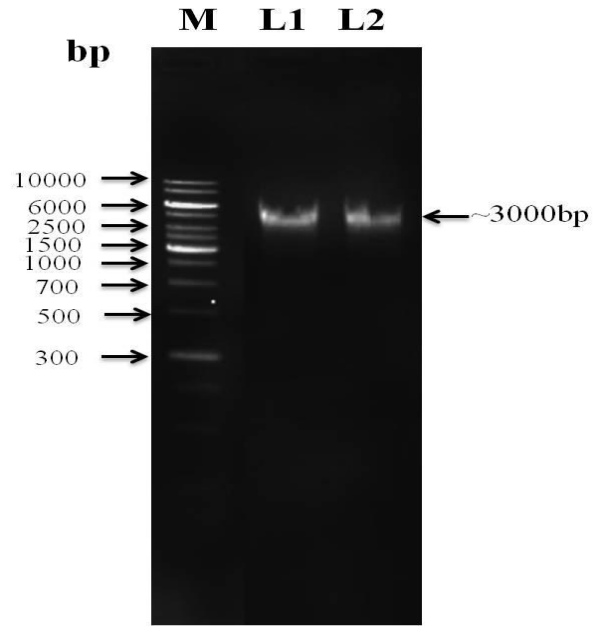

Figure 1. Genomic DNA of isolate MA2 (L1) and MA18 (L2).

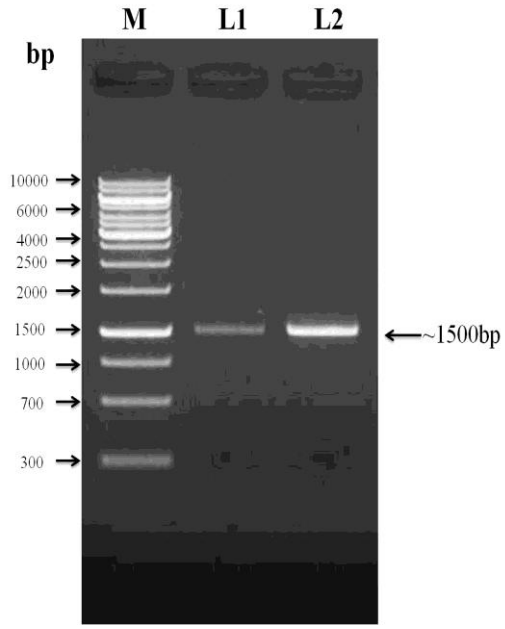

Figure 2. PCR amplification of 16S rRNA, Isolate MA2(L1); MA18 (L2).

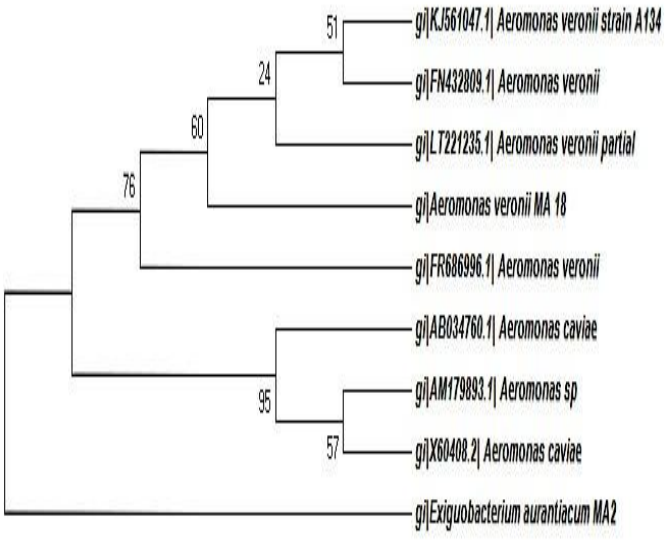

Figure 3. Phylogenetic Tree of isolated bacteria MA2.

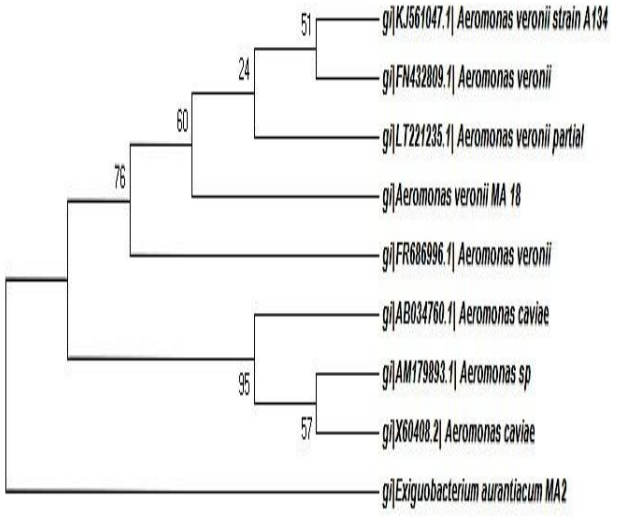

Figure 4. Phylogenetic Tree of isolated bacteria MA18.

CONCLUSION

Amylase enzyme, protease and lipase enzymes were markedly increased the activity of Probiotics and improved the water quality parameters, food absorption and growth of *Labeo rohita*. Identical 16S rRNA sequence was recognized by Phylogenetic trees and manual comparisons proved that these sequences were associated for enzyme amylase and peptidases synthesis which proved beyond doubt that the identified strain improved the growth of L. *rohita.*

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