Evaluation of Probiotic Characterization of Marine Bacteria and its Growth Performance in Zebra Fish

Bharathi K\textsuperscript{1}, Akila M\textsuperscript{1} & Selvakumar D\textsuperscript{1,2,*}

\textsuperscript{1}PG and Research Department of Biotechnology, Mohamed Sathak College of Art and Science, Sholinganallur 600116, Tamil Nadu, India
\textsuperscript{2}PG Department of Biotechnology, Kumararani Meena Muthiah College of Arts and Science, Adayar, Chennai - 600020, Tamil Nadu, India

Abstract: Nearly three probiotic strains (PBR1, PBR2 and PBR3) were isolated from the gut of marine Grouper, Epinephelus longispinnis and they were identified as Bacillus sp. (PBR1), Streptobacillus sp. (PBR2), Streptococcus sp. (PBR3) by conventional biochemical characterisations. Three tests (Acid Tolerance, Bile salt concentration and auto-aggregation assay) were carried out for probiotic characterization. The strains were acid tolerant up to a low pH of 3, had the ability of resistant to 0.3% bile salt concentration and auto-aggregation capacity even long time interval of six hours. Later the potential of these three probiotic strains for the growth of Zebra fish was investigated. Three probiotic feed were prepared and their effects were compared with those of control feed. After 15 days of feeding trials the growth parameters like Food conversion Efficiency and Food Conversion Ratio were assessed. The food conversion efficiency were found to be significantly (p<0.05) higher in groups that received Probiotic feed than in the control, whereas food conversion ratio was lower. The fishes fed with probiotic bacterial PBR2 mixed feed exhibited better growth efficiency when compared to rest of feed fed fishes as well as control feed fed fishes. Thus it was found that the strains not only exhibit the characters of probionts, could also promote the growth of ornamental fish effectively and thus marine gut isolate play an important role as a probiotics in aquaculture nutrition.

Key words: Probiotics, Marine Fishes, Fish gut, Zebra fish, Aquaculture

1. Introduction

Probiotics are commonly defined as ‘Live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance’ [1]. The use of probiotics in farm animals is based on the concept that the balance of intestinal microorganisms in healthy animals increases resistance to diseases and is necessary for efficient digestion and maximum absorption of nutrients [2]. Probiotics most used in aquaculture are those belonging to the genus Bacillus sp. (B. subtilis, B. licheniformis, B. circulans), Bifidobacterium sp. (B. bifidum, B. lactis, B. thermophillum), lactic acid bacteria (Lactobacillus sp., Carnobacterium spp.) and the yeast Saccharomyces cerevisiae [3].

Fishes are quite different from humans and land animals in the nature that men and terrestrial livestock undergo embryonic development within an amnion, whereas on the other hand the larval forms of most fish and shellfish are released in external environment where vaccination cannot be used. These young ones are highly exposed to gastrointestinal associated disorders because they start feeding even though their digestive tract is not yet fully developed [4, 5] and thus probiotics, specially isolated from the gut of the mature fishes, are more desirable for early stages of fishes as the same can improve the immune response system, reduce mortality significantly and increase fish yield considerably [6, 7]. Probiotics can also improve the microflora apart from increasing digestibility, growth and nutrient utilization in the fishes.

The research on probiotics for aquatic animals is thus increasing with the demand for environment-friendly aquaculture and it is expected that it can assure the nutritional security in the next millennium [8, 9]. The beneficial effect of probiotics have been attributed to their ability to promote the immunological and non-immunological defense barriers in the gut, normalization of increase in intestinal permeability, exogenous production of digestive enzymes and altered gut microflora. They have also been shown to enhance humoral response and consequently to promote intestine’s barriers. Probiotics have also been shown to stimulate non-specific host resistance to microbial pathogen and thereby, aid in immune elimination of pathogen [10].

The gastrointestinal microbiota of fish and shellfish are peculiarly dependent on the external...
environment due to the continuous water flow passing through their digestive tract. Most bacterial cells in the gut are transient due to continuous intrusions of microbes coming from water and food. The use of probiotics is successful when the administered microbes survive in the gastrointestinal tract. Three main characteristics identified in microbes, to be used as probiotics, which aid in improving the health of their host are: (i) The antagonism to pathogens; (ii) The colonization potential and (iii) Adhesion potential. Many other beneficial effects may also be expected from probiotics such as competition with pathogens for nutrients or for adhesion sites [11], enzymatic activity against pathogenic strains, improvement of the immune system [16] and augmentation of the beneficial effects may also be expected from probiotics such as competition with pathogens for nutrients or for adhesion sites [11], enzymatic activity against pathogenic strains, improvement of the immune system [16] and augmentation of the immune system [16] and augmentation of the immune system [16]. Several bacterial strains which are common member of the non-pathogenic microflora are capable of inhabiting fish pathogenic in -vitro bacteria in in -vitro assay. This has been demonstrated for lactic acid bacteria [17], Bacillus sp. [18], Vibrio sp. [19] etc. But probiotics for aquaculture are generally selected only by their ability to produce antimicrobial metabolites; however, attachment to intestinal mucosa is important in order to remain within the gut of the host. The selection of suitable strain of a microorganism is a primary requirement for the use of probiotics. The selection criteria for a probiotics demands that the strain should be of the same species origin, be safe in same species use, resistant to acid and bile, and that it adheres to the intestinal mucosa and produce antimicrobial components [20].

### Isolation of Probiotic bacteria:

The dissected gut was dissected by using sterile knife. The surface of the fish was washed by using sterile water and the gut was dissected by using sterile knife.

### Sample Collection:

Marine Fish grouper (Epinephelus longispinis) were collected from Nagapattinam Beach, Tamil Nadu, India. They were brought live to laboratory. The surface of the fish was washed by using sterile water and the gut was dissected by using sterile knife.

### Antagonistic activity:

Nearly six strains were isolated at first phase which were designated as PBR1, PBR2, PBR3, PBR4, PBR5, PBR6. The isolates (PBR1, PBR2, PBR3) were morphologically characterized by Gram’s staining and motility test.

### Biochemical characterization

#### Indole test:

A loop full of culture was inoculated into the Tryptone broth (Tryptone 10 g, NaCl 0.5 g, distilled water 100 ml, pH 7.5) and incubated at 37°C for 12 hrs. After incubation 5 drops of Kovac’s reagent was added to the surface of the each culture and observed for ring formation. Appearance of Cherry red color layer ring at the top of the broth in a test tube indicates positive result. No color change of the media indicates negative result.

#### Methyl red - Voges Proskauer test:

A loop full of culture was inoculated into the MR-VP broth (7g peptone, 5g dextrose, 5g Dipotassium phosphate, 5g NaCl, 1000 ml distilled water) and incubated at 37°C for 12 hrs. After incubation 5 drops of methyl red indicator for methyl red and 5 drops of Barritt’s reagent A and B for Voges Proskauer were added. Appearance of red color indicates positive results and no color change indicates negative result.

#### Citrate utilization test:

It was done by inoculating overnight culture into the Simmon’s citrate agar slants (0.2g (NH₄)₂SO₄, 1.0 g (NH₄)₂HPO₄, 1.0 g K₂HPO₄, 0.1% NH₄H₂PO₄, 2.0 g sodium citrate, 5.0 g NaCl, 15g Agar, 1000ml distilled water) and incubated at 37°C for 12 hrs. If the reaction was positive, it would give a blue color otherwise it would give the yellowish green color and also there would be no growth in the slant if it is a negative result.

### Catalase test:

A loop full of culture was inoculated into the slide containing a drop of hydrogen peroxide solution. Production of gas bubbles indicates positives result and their absence indicates negative.

### Carbohydrate fermentation test:

It was performed by inoculating the culture into the tubes containing respective sugar containing the medium (Casein enzyme digest 1g, NaCl 0.5 g, KCl 0.05 g, MgSO₄·7H₂O 0.08 g, Na₂HPO₄ 0.03 g, KH₂PO₄ 0.025 g, distilled water 100 ml, pH 7.5) and incubated at 37°C for 12 hrs. If the reaction was positive, it would indicate the production of gas bubbles and acid. No gas bubbles and color change indicates negative result.
Later, they are incubated at 37°C for different times and resuspended with PBS to make the volume of 1 ml. Then the cell pellets were washed twice with PBS and cultured at 750 rpm for 10 minutes. After incubation the plates were flooded with iodine solution and allowed to stand for 5 minutes.

**Starch hydrolysis test:** It was done by spot inoculating the culture on the starch agar plates (Nutrient agar + 1% starch) and incubated at 37°C for 12 hrs. After incubation the plates were flooded with iodine solution and allowed to stand for 5 minutes.

**Nitrate reduction test:** Inoculate the Nitrate broth with culture and incubate at 37°C, for 12 hrs. Add 6-8 drops of Nitrite reagent A. Add the same number of drops of Nitrite reagent B. The broth will turn a deep red within 5 minutes. If there is no color change, which indicate a negative result.

**Screening for Probiotic Properties**

**Acid tolerance test:** To detect the acid tolerance the isolates were grown overnight at 37°C for 12 hrs and were centrifuged at 7000 rpm for 10 minutes. The cell pellets were washed twice with PBS (phosphate buffer saline) of pH 7.3 and resuspended in PBS to reach the final volume 1 ml. The strains were diluted in PBS 1:100 dilutions at various pH 1, 2, 3, and 4. The mixture of each strain was then incubated at 37°C for 24 hrs. Optical density values were taken in ELISA reader at 595 nm intervals (0, 2, 6, 12, 24 hrs), the viability of bacterial cell were determined by plating the cells in Nutrient agar plates at different time interval (0, 1, 2, 3, 6, 12 and 24 hrs). Growth of the bacteria was expressed in colony forming units/ml (log10 CFU/ml) and the survival percentages of the three isolates to different pH values were calculated.

**Bile salt concentration:** The ability of the isolates to grow in the presence of bile salts was determined using the method described previously [21], with minor modifications. The probiotic cultures were grown at 37°C for 12 hrs in nutrient broth without bile salts. Then 1 ml of the culture broth was poured onto the nutrient broth with varying bile salt concentration (0.15, 0.3, 0.5, 0.75, 1.0). The ability of the isolates was measured at 595 nm using ELISA reader after different periods of incubation (0, 2, 4 and 6 hrs).

**Auto aggregation assay:** The strains were grown in LB broth and incubated at 37°C for 24 hrs. The culture was centrifuged at 5000 rpm for 10 minutes. Then the cell pellet were washed twice with PBS and re-suspended with PBS to make the volume of 1 ml. Later, they are incubated at 37°C for different time intervals (0, 2 hrs, 6 hrs, 12 hrs, 24 hrs, 48 hrs) and values was observed in ELISA reader at 595 nm.

**Protein Estimation:** The protein content in all the three isolates was determined [22].

**Evaluation of the strains as Probiotic Feed**

**Experimental fish:** Zebra fish (Danio rerio, Cyprinidae) weighing about 2.4 g were stocked in four 500 ml plastic troughs. Each trough had five fishes. One trough contained the fish fed with control feed (Tetra commercial feed), whereas the rest of the fish were supplemented with probiotics mixed commercial feed. The strains were mass cultured in nutrient broth and 24 hours cultures were centrifuged in a cooling centrifuge at 5000 rpm for 20 minutes. The cell pellets were collected and mixed with the 10 g of commercial feed and stored in dry airtight containers in a freezer. The experiment was conducted for 15 days and repeated in triplicate. The protein content for probiotic strain PBR1 40 mg/ml, PBR2 35 mg/ml PBR3 37.5 mg/ml.

**Determination of Growth performance:** The experiment was conducted for 15 days to study the effect of probiotic feed on growth of the fishes. They were carried out in four 500 ml capacity plastic troughs. Fishes of same brood having approximately 2.4 g weight were selected. Five fishes were stocked in each trough. The total weight of fish in troughs was ascertained. Fishes were given one week for acclimatization to the experimental diet and starved for 24 hours prior to the initiation of the experiment. The fishes were fed at the rate of 5% body weight once daily. The unconsumed feed was siphoned out six hours after feeding. The next day morning, the faecal matter was collected from each trough. The unconsumed feed and faecal matter were dried in an oven at 60°C and weights were recorded. About 75% of water from each trough was changed daily with minimum disturbances to the fishes. The final weight were taken on the 15th day after the feed supplementation and the initial weight before the experiment is given. The growth parameters like Feed conversion efficiency and Feed conversion ratio were calculated by the method described previously [23].

\[
\text{Feed conversion efficiency} = \frac{\text{(Final weight} - \text{Initial weight)}}{\text{(Feed given} - \text{Unconsumed feed})} \times 100
\]

\[
\text{Feed conversion ratio} = \frac{\text{(Feed given} - \text{Unconsumed feed})}{\text{(Final weight} - \text{Initial weight})}
\]
3. Results

Nearly six different isolates from marine fish gut were obtained on nutrient agar plates. Of the six isolates, only three showed better antibacterial activities against fish pathogens. The size of zone of inhibition was measured and found to be between 5 to 10 mm which was shown in Figure 1.

Further these three isolates were studied for their morphological and biochemical characterization. Morphological identification indicates that the strains were motile and Gram’s positive. Biochemical characterizations were done for all the three strains which are shown in Table 1.

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th>Isolated bacterial strains</th>
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<tr>
<td></td>
<td>PBR1</td>
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<tr>
<td>Indole</td>
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<td>Methyl red</td>
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<td>Voges proskauer</td>
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<td>Citrate utilization</td>
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<td>Catalase</td>
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<td>Glucose</td>
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<td>Sucrose</td>
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<td>Fructose</td>
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<td>Maltose</td>
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<td>Starch hydrolysis</td>
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Table 1: Biochemical characterization of isolated strains

Nirate reduction: -ve -ve -ve
Gram’s staining: +ve +ve +ve
Motility test: +ve +ve +ve
Antagonistic test: Zone of inhibition

Aeromonos hydrophilia: 2.5mm 3.2mm 5mm
Vibrio harveyi: 3mm 5mm 7mm

All the three isolates exhibited Indole test, Catalase test, Voges proskauer test, Urease test as negative, whereas Methyl red, Nitrate reduction, Starch hydrolysis test as positive. For citrate utilization test the strains PBR1 and PBR2 resulted in negative and PBR3 exhibited positive. All the three strains exhibited acid positive, gas negative for all sugar test (Sucrose, Glucose, Maltose, Fructose). The cultures were tentatively identified as Bacillus sp. (PBR1), Streptobacillus sp. (PBR2) and Streptococcus sp. (PBR3) by using Bergey’s Manual of Determinative Bacteriology.

Based on their antibacterial activity, the three strains were further studied for possession of probiotic characters. Three tests namely Acid tolerance, Bile salts concentrations and Auto aggregation were carried out for probiotic characterizations. Acid tolerance test showed that all the three bacterial strains were resistance up to pH 3 and 4. The strains were screened for their ability to tolerate the bile salts at different concentrations. Strains were detected in 0.3% during 4 to 5 hours which are shown in Figure 2.

According to the results, the strains exhibited resistance up to 0.3% bile salts. The strains showed auto-aggregation capacity even at long interval of time up to six hours which were shown in Figure 3.

After 15 days of feeding trials with probiotic mixed commercial feed and commercial feed as control feed, the growth parameters of the zebra fish were analysed. All the fish fed with probiotic mixed feed showed significant increase in growth performance. The results showed that the FCE values were increased and FCR values were significantly decreased when compared to control fish shown in Table 2 and Figure 4. The fishes fed
with probiotic bacteria PBR2 mixed feed exhibited better growth efficiency when compared to rest of feed as well as control feed.

| Table 2: Food conversion ratio and Food conversion efficiency of zebra fish |
|-----------------|----|----|----|----|
| P               | C  | S1 | S2 | S3 |
| WF              | 0.3±0.02 | 0.3±0.01 | 0.3±0.02 | 0.3±0.01 |
| WU              | 0.2±0.01 | 0.15±0.02 | 0.18±0.02 | 0.17±0.01 |
| WI              | 0.13±0.01 | 0.15±0.01 | 0.12±0.01 | 0.13±0.02 |
| IW              | 2.4±0.01 | 2.4±0.4 | 2.4±0.1 | 2.4±0.2 |
| FW              | 2.5±0.01 | 2.8±0.4 | 2.8±0.1 | 2.8±0.2 |
| WG              | 0.1 | 0.4 | 0.4 | 0.4 |
| FC              | 2.03±0.15 | 0.89±0.22 | 0.47±0.03 | 0.25±0.16 |
| FCE             | 100±10.1 | 266.6±17.8 | 333.3±2.3 | 307.07±48.6 |
| WC              | 2 | 7 | 4 | 8 |

Values are significant when compared with control (p<0.05); P – Parameters; C- Control, S1- sample 1, S 2- sample 2, S 3- sample 3; WF - Weight of feed given; WU- Weight of unfed; WI- Weight of intake; IW - Initial Weight of fish; FW- Final Weight of fish; WG - Weight gain of fish; FCR - Feed Conversion Ratio; FCE - Food Conversion Efficiency

4. Discussion

Aquaculture is one of the most viable and fast growing systems in the world in which availability of adequate nutritionally balanced diet is one of the most important aspect. Studies pertaining to nutrition in aquaculture had resulted in the development of new feed formulations for aqua-culturally important fish species. The present study was undertaken to isolate probiotic bacterial strains from marine fish intestine and to evaluate for probiotic effect on ornamental fish. In intensive aquaculture system diseases especially bacterial infections remain primary constraints to its continued expansion [24, 25]. Probiotics have an important role in disease control strategies for aquaculture, and may provide an alternative to the use of antimicrobial compounds [26]. Thus, there is an increasing research effort to evaluate the effect of probiotics on the fish health and immune system.

Three isolates from grouper fish gut of were characterized morphologically and biochemically. The fish intestine is a favorable ecological niche for microorganisms, which reach much higher numbers than in the surrounding water [27]. Earlier in a study nearly 50 strains were isolated from the fish intestine of black porgy fish samples, of which one isolate was considered to be probiotic bacteria according to the morphological, biochemical characteristics and metabolic products. The strain and to e valuate for probiotic effect on intestine of black porgy fish samples, of which one study nearly 50 strains were isolated from the fish intestine and to e valuate for probiotic effect on

The three strains in the present study exhibited auto aggregation for longer time. Autoaggregation of probiotic strains appeared to be related to adhesion to intestinal epithelial cells [36]. The autoaggregation percentage was measured by the sedimentation rate. The percentage of autoaggregation of Bifidobacteria animalis sub sp. lactic BB12 and Lactobacillus casei Shirota were respectively 36.7% and 17.9% [37]. The abilities of

Probiotic properties of the three strains were also evaluated in the present study. A similar frequency of inhibitory bacteria was observed for isolates from halibut larvae [29], rainbow trout [30], turbot [31], and shrimp [32]. The inhibition was caused by the release of chemical substances with bactericidal or bacteriostatic effects. The reduction of pathogen growth and cell density indicate that extracellular bacteriolytic products produced by probiotic bacteria were responsible for this inhibition. Bacillus subtilis P 33 and 72 isolated from the gut of giant freshwater prawns were found to have high inhibition activities against the growth of A. hydrophila by two assay methods: paper disc and well diffusion [33]. Being resistant to low pH is one of the major selection criteria for probiotic strains [34]. Since, to reach the small intestine they have to pass through from the stressful conditions of stomach. Although in the stomach, pH can be as low as 1.0, in most in vitro assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below [35].

The strains, resistant to low pH, were screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h. Tolerance to bile is important for the probiotic strains to grow and survive in the digestive tract. In another report Compared to the control (0 g/L), the viable bacteria decreased from 6.3x10^6 to 1.7 x 10^6 cfu/mL when the bile concentration was 4 g/L. The isolate could tolerate bile, but high bile concentration could affect the survival of the isolate.

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bacterial autoaggregation and coaggregation were beneficial properties, which were respectively related to cell adherence and inhibition against pathogen, and played an important role in intestinal tract. The isolate could have the ability of aggregating itself and coaggregating other bacteria, which could be helpful for the health of fish in aquaculture.

The strains of probiotic bacteria that incorporated may be rich in protein. The protein percent in each strain was assessed it was found to be PBR1-40, PBR2-37, and PBR3-35. This shows that protein content is rich in these strains and they may synthesize the lower weight precursors like macromolecules and vitamins. This may be the reason for the better growth rate. The results corroborated with the previous studies [38], in which the probiotic fed fish provided better growth and conversion efficiencies. The supply of fishmeal has become increasingly uncertain and the price has been raised rapidly. Thus the increase in the cost necessitated to look for cheaper alternate source with efficient growth promoters. It is better and cost efficient to supplement microbial incorporated feed.

Many workers have used other probiotics (Lactobacillus sporogens, L. acidophilus, Bacillus sp., Streptococcus faecium, Saccharomyces cerevisiae) successfully to improve the growth performance of fish. The spores of Bacillus toyoii and other Bacillus sp. when used as feed additive increased the growth rate of yellow tail, Seriola quinguiradiata [39]; turbot, Scophthalmus maximus [40]; common snook, Centropomus undecimalis [41] and giant tiger prawn, Penaeus monodon [42]. The commercial preparations of Streptococcus faeciam and a mixture of bacteria and yeast improved the growth and food conversion efficiency of Cyprinus carpio [43] and Catla catla [44], respectively. The usage of two probiotic bacteria and the yeast Saccharomyces cerevisiae as growth promoters in the Nile tilapia (Oreochromis niloticus) fry was studied [45]. The results indicate that the fry subjected to diets with a probiotic supplement exhibited greater growth than those fed with the control diet. Recent reports on the use of Lactobacillus spp. and Bacillus spp. have also demonstrated beneficial effects of stimulating the gut immune system and the growth improvements in the fish larvae [46, 47].

5. Conclusion
Marine diversity is nutrient rich diversity provided with all required habitats. Here we studied isolation of probiotic bacteria found to be associated with the gut of Marine Grouper fish. The results of morphological, biochemical and probiotic characterization studies clearly indicated that the three strains (PBR1, PBR2 and PBR3) selected belong to one of the group of probiotic bacteria. Probiotic microorganisms synthesise macromolecules and vitamins which benefit animal nutrition. There are very limited reports on the application of marine fish gut associated probiotic bacteria, as probionts in aquaculture.

As far as the microbial feed supplementation to the ornamental fish Zebra fish showed good results for the growth of the fish, which were increased, and the fish remained healthy. The feed prepared with the strains isolated from gut of marine fish clearly proved that these strains can be used as probiotic feed source and which has vast biotechnological application. The supply of fishmeal has become increasingly uncertain and the price has been raised rapidly. Thus increase in the cost it is necessitated to look for cheaper alternate source with efficient growth promoters. It is better and cost efficient to supplement microbial incorporated feed or probiotic feed as reported. Further by using molecular tools to identify the strains were under process for completion of the work.

6. References


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