ISSN 1330-9862
(FTB-2543)

original scientific paper

Evaluation of *Streptomyces* as a Probiotic Feed for the Growth of Ornamental Fish *Xiphophorus helleri*

Selvakumar Dharmaraj and Kandasamy Dhevendaran*

Department of Aquatic Biology and Fisheries, University of Kerala, Kariavattom Campus, Trivandrum 695 581, Kerala, India

Received: February 15, 2010
Accepted: May 3, 2010

Summary

The potential of *Streptomyces* as a probiotic feed for the growth of ornamental fish *Xiphophorus helleri* has been investigated. The *Streptomyces* strains used as probiotics were isolated from the marine sponges, namely *Callyspongia diffusa*, *Mycale mytilorum*, *Tedania anhelans* and *Dysidea fragilis*. Seven probiotic feeds were prepared and their effects were compared with those of control diet containing no probiotics. After 50 days of feeding trials, the growth parameters, namely absolute growth rate, specific growth rate, relative growth rate and feed conversion efficiency were found to be significantly ($p<0.05$) higher in groups that received probiotic feed additive than in the control, whereas feed conversion ratio was lower. The fish fed with probiotic feed showed significant improvement in length than the fish fed with control feed. Thus it was found that in addition to being effective antibiotic agents against harmful pathogens, *Streptomyces* could also promote the growth of fish effectively. Marine Actinobacteria, particularly *Streptomyces*, could thus be a promising probiotic in aquaculture.

Key words: probiotics, Actinobacteria, *Streptomyces*, ornamental fish *Xiphophorus helleri*

Introduction

Probiotics, which are beneficial microorganisms, or their products that provide health benefits to the hosts have been used in aquaculture as disease control agents, as supplements to improve growth and in some cases as a means of replacing antimicrobial compounds (*1*). The concept of using probiotics in animal feed, particularly poultry and aquaculture, is slowly becoming popular (*2*). Common organisms in probiotic products are *Aspergillus oryzae*, *Lactobacillus acidophilus*, *L. bulgaricus*, *L. plantarum*, *Bacillus* sp., *Bifidobacterium bifidium*, *Streptococcus lactis* and *Saccharomyces cerevisiae* (*3*). These products can be administered through water or incorporated in the feed. Despite being the source of several novel antibiotics, marine Actinobacteria have been given less attention as probiotics in aquaculture and poultry farming. These have many advantages like: (*i*) the degradation of macromolecules, such as starch and protein, in the pond culture, (*ii*) the production of antimicrobial agents, and (*iii*) the formation of heat- and desiccation-resistant spores (*4*).

There are few works reported on the usage of marine Actinobacteria, particularly *Streptomyces*, as probiotics in shrimp aquaculture. The application of *Streptomyces* as a probiotic in the laboratory culture of black tiger shrimp that resulted in the improved growth, survival and disease resistance of the prawn has been reported (*5*). About twenty-five antibiotic extracts from marine *Streptomyces* were incorporated in the feed to observe their *in vivo* effect on white spot syndrome virus (WSSV) in black tiger shrimp. Six probiotic feeds showed positive effect against WSSV-infected penaeid shrimps (*6*). The application of marine *Streptomyces* as a potential organism against biofilms produced by *Vibrio* sp. was studied and the use of marine *Streptomyces* to prevent the disease caused by *Vibrio* sp. was recommended (*7*). There have been reports on the usage of *Streptomyces*-incor-

*Corresponding author; Phone: ++91 96269 49380; E-mail: biochem_selva@yahoo.com
Special issue: Probiotics, Prebiotics and Synbiotics

porated feed as a probiotic source for the growth of juvenile prawn *Macrobrachium idella* (*8*). By considering the above facts, an attempt has been made for the first time to use *Streptomyces* as probiotic feed for the ornamental fish *Xiphophorus helleri* and to evaluate its effects on the growth of the fish.

Materials and Methods

Isolation of Streptomyces from marine sponges

The marine sponges, namely *Callyspongia diffusa*, *Mycale mytilorum*, *Tedania anhelans* and *Dysidea fragilis*, were collected at a depth of 5 to 10 m by scuba diving from Vizhinjam port, situated about 16 km to the south of Trivandrum at 8°22'30" N latitude and 76°59'16" E longitude on the South-West coast of India. The marine sponges were transported to the laboratory by storing in an ice box within the minimum possible time to avoid external microbial contamination and excessive proliferation. The sponge extract was obtained by squeezing the sponges gently with a glass stick. Aliquots (1 mL) of each sponge extract were diluted with sterilized seawater. A volume of 1 mL of the dilutions was mixed with 20 mL of sterile glycerol asparagine agar medium (ISP-5) and incubated at room temperature ((28±2) °C) for seven days. Rifampicin (2.5 μg/mL) and amphotericin B (75 μg/mL) were added to ISP-5 medium to inhibit bacterial and fungal contamination, respectively. The total number of *Streptomyces* in the plates was counted (*9*).

Maintenance of isolates

The isolated strains were maintained as ISP-5 medium slant cultures at (28±2) °C (*10*). The inoculum used in all the experiments was a seven-day-old culture, unless otherwise stated.

Characterisation of the Streptomyces isolates

The isolates were characterised by the methods of nutritional, morphological, physiological and biochemical properties which are detailed below (*11*). The microorganisms were characterised by acid-fast staining and Gram staining techniques. In the nutritional uptake experiments given below, the culture was inoculated into the basal medium with nutrient and incubated at 28 °C for seven days. The biomass thus obtained was separated from the broth, dried and weighed. The mass of the biomass was expressed in grams.

Pigmentation of mycelia

The cultures were inoculated on starch casein agar, incubated at 30 °C for seven days and were examined for aerial and substrate mycelium colour. The cultures were classified into white, grey, yellow, red, blue, green or violet series, depending on the mycelial colour.

Spore morphology

The cultures were grown on a Petri dish containing casein-starch-peptone-yeast extract (CSPY) agar medium with a cover slip inserted at an angle of 45°. The cover slip was removed after seven days of incubation, air dried and observed under scanning electron microscope for visualisation of spore morphology.

Melanoid production

The cultures were streaked onto peptone-yeast extract-iron agar slants and incubated at 28 °C for 48 h. Cultures forming greenish-brown to brown and then to black diffusible pigments were recorded as positive melanoid production while the absence of these colours signified the lack of melanoid production.

Utilisation of carbon sources

The cultures were inoculated to test tubes containing 10 mL of basal mineral salt medium to which sterilized carbon sources (xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol, sucrose and glucose) were added to a final concentration of 1 %. The tubes were incubated at 28 °C and after seven days the growth of the cultures was observed. Glucose was used as positive control.

Influence of amino acids

Various amino acids, namely glycine, cystine, alanine, tryptophan and valine, were added at a concentration of 0.1 % each to 5 mL of basal mineral salt medium in order to examine their effect on the growth of *Streptomyces*. The medium was inoculated with the cultures and incubated at 28 °C for seven days. The biomass thus obtained was separated from the broth, dried and weighed. The mass of the biomass was expressed in grams.

Sodium chloride tolerance

To evaluate the tolerance of *Streptomyces* strains to sodium chloride, various concentrations (1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 %) were added to 5 mL of the basal medium. The medium was inoculated with the cultures and incubated at 28 °C for seven days. The biomass thus obtained was separated from the broth, dried and weighed. The mass of the biomass was expressed in grams.

Physiological and biochemical characteristics

These were studied according to the previously described methods (*12*,*13*). The methods are detailed below.

Starch hydrolysis – The cultures were streaked on starch agar plates and incubated at 37 °C for seven days. After incubation the plates were treated with Gram's iodine solution. Hydrolysis of starch is indicated by a clear zone around the colony, whereas the unhydrolysed starch will appear black/blue.

Production of hydrogen sulphide – The cultures were inoculated in 0.3 % agar medium containing ferrous sulphate and incubated at 37 °C for seven days. Evolution of hydrogen sulphide will combine with the iron salt to form a visible black ferric sulphide in the tubes.

Degradation of cellulose – *Streptomyces* cells were point inoculated on basal Gauze agar medium (K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, KNO_3 1 g, $FeSO_4{\cdot}2H_2O$ 0.01 g, NaCl 0.5 g, carboxymethyl cellulose 10 g, agar 25 g, distilled water 1 L) in Petri dishes and were incubated for seven days at 28 °C. The cellulose-degrading strains show a clear hydrolysis zone around the colonies when compared with the rest of the opaque media.

Liquefaction of gelatine – The nutrient gelatine medium (enzymatic digest of gelatine 5 g, beef extract 3 g, gelatine 120 g, distilled water 1 L, pH=(6.8±0.2)) was pre-

pared, sterilized and poured into presterilized tubes. The tubes were inoculated with cultures and incubated for 24 h. The tubes were placed on ice for 30 min and observed for liquefaction of gelatine.

Coagulation of milk – Litmus milk broth (skim milk powder 100 g, litmus 0.5 g, sodium sulphite 0.5 g, distilled water 1 L, pH=(6.8±0.2)) was prepared and sterilized. The broth was inoculated with cultures and incubated at 37 °C for seven days. Soft, gelatinous clot formed in the tubes, due to coagulation of milk protein.

Peptonisation of milk – Litmus milk medium (skim milk powder 100 g, litmus 0.75 g, distilled water 1 L, pH=(6.8±0.2)) was sterilized, inoculated with cultures and incubated at 37 °C for 14 days. Peptonisation of milk turns the medium brown due to the digestion of casein in the skim milk by proteolytic enzymes.

Degradation of urea – Urea broth (peptone 1 g, glucose 1 g, NaCl 5 g, monosodium phosphate 2 g, phenol red 0.012 g, urea 20 g, distilled water 1 L, pH=(6.8±0.1)) was sterilized, inoculated with cultures and incubated at 37 °C for seven days. Production of ammonia changes the colour of the broth from pink to bright orange; the colour change being brought about by the indicator, phenol red.

Citrate utilisation – Citrate broth ($NH_4H_2PO_4$ 1 g, K_2HPO_4 1 g, NaCl 5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, trisodium citrate 2 g, bromothymol blue 0.08 g, distilled water 1 L) was prepared and sterilized. The tubes were inoculated with the cultures and incubated at 37 °C for seven days. Citrate utilisation changes the colour of the medium from green to blue due to an increase in alkalinity of the medium, the change being brought about by the indicator, bromothymol blue.

Indole production – The cultures were inoculated in test tubes containing peptone broth and incubated at 37 °C for 48 h. After incubation, 0.5 mL of Kovacs' reagent (isoamyl alcohol 75 mL, *p*-dimethylaminobenzaldehyde 5 g, concentrated HCl 25 mL) was added to the culture broth. Positive result is indicated by the presence of a red or red-violet colour on the surface of the broth, while negative result remains yellow.

Catalase test – The cultures were cultivated on glycerol asparagine agar at 28 °C for seven days. A few drops of 3 % hydrogen peroxide were allowed to flow slowly over the cultures. Appearance of bubbles over the culture indicates the production of the enzyme catalase, which splits the hydrogen peroxide to hydrogen and oxygen.

Production and estimation of indole acetic acid

A loopful of 7-day-old culture was inoculated in 50 mL of indoleacetic acid (IAA) production medium (tryptone 10 g, yeast extract 5 g, NaCl 5 g, tryptophan 0.5 g, distilled water 1 L) in a 150-mL flask with various pH levels (5, 6, 7, 8 and 9) and incubated on a rotary shaker (180 rpm) at 30 °C for 7 days. After incubation, the production medium was centrifuged at 10 000 rpm for 30 min and the clear supernatant was acidified with 4 M HCl to pH=2.8. Then, 0.5 g of activated charcoal was added to the supernatant and shaken for 2 h. Charcoal was separated by centrifugation at 2000 rpm for 10 min. IAA was further extracted with 16 mL of aqueous acetone (95 % by volume). The acetone fraction was air-dried and reconstituted in methanol (2 mL). Then 1 mL of Salkowski reagent (12 g of $FeCl_3$ in 1 L of 7.9 M H_2SO_4) was added to 1 mL of the extract, mixed well and incubated in the dark at 30 °C for 30 min. Absorbance was determined by using UV/VIS spectrophotometer 2101 (Systronics, Ahmedabad, India) at 535 nm against the reagent blank (*14, 15*). IAA production was expressed in μg/mL. The concentration of IAA was calculated from a standard plot ranging from 0.1 to 10 μg/mL prepared with pure IAA (Sigma-Aldrich, St. Louis, MO, USA).

Experimental fish

Red swordtails (*Xiphophorus helleri*, Cyprinidae) weighing about 0.6 g were stocked in eight 20-litre plastic troughs. Each trough had twenty fish. One trough contained the fish fed with control feed, whereas the rest of the fish were supplemented with probiotic feed. The experiment was conducted for 50 days and repeated in triplicate.

Streptomyces as probiotics

Single colonies of *Streptomyces* strains isolated from marine sponges were purified by successive streaking. The strains were inoculated in 500 mL of starch casein broth in a 1-litre Erlenmeyer flask and incubated at room temperature for seven days. The *Streptomyces* grew as a mat on the surface of the broth (non-motile form). The mat was harvested and the cell mass was lyophilised and mixed with the formulated feed ingredients (*5*).

Feed preparation

Eight different feeds were prepared, namely the probiotic feed and control feed (without probiotic). The fish were fed with these feeds for 50 days. Formulated diets were prepared according to the least square method (*16*). The protein content in the ingredients was estimated by Kjeldahl method (*17*). The compositions of the ingredients used for preparing formulated diets and their protein mass fractions are shown in Tables 1 and 2.

Table 1. Mass fraction of proteins in the ingredients added to the formulated feeds

Ingredient	*w*(protein)/%
rice bran	10.5
chickpea flour	35.5
groundnut oil cake	40.0
tapioca flour	2.5
fish meal	55.0
Streptomyces sp. AQBCD03 (F1)	55.1
Streptomyces sp. AQBCD11 (F2)	56.7
Streptomyces sp. AQBCD24 (F3)	53.4
Streptomyces sp. AQBMM35 (F4)	55.3
Streptomyces sp. AQBMM49 (F5)	56.2
Streptomyces sp. AQBTA66 (F6)	55.1
Streptomyces sp. AQBDF81 (F7)	55.9

Table 2. Composition of ingredients in the formulated feeds

Ingredient	*w*/%	
	Control feed	Probiotic feed
rice bran	16.5	16.5
chickpea flour	18.5	18.5
groundnut oil cake	23.5	23.5
tapioca flour	16.0	16.0
fish meal	25.5	15.5
Streptomyces cell mass	–	10.0

Control feed

The ingredients used for control feed preparation consisted of fish meal, rice bran, groundnut oil cake and chickpea flour. The binder used was tapioca flour. The ingredients were ground to a fine powder and mixed thoroughly with sufficient water to obtain smooth dough. The dough thus prepared was steam cooked for 30 min and allowed to cool. This was extruded through a pelletiser. The pellets were dried and then stored in dry airtight containers at 28 °C.

Probiotic feed

The steam cooked dough was prepared and cooled as given above. To this dough, a known quantity of probiotic, namely cell mass of *Streptomyces*, was added and mixed well. This was extruded through a pelletiser, the pellets were freeze dried and kept in dry airtight containers at 4 °C.

Determination of absolute growth rate (AGR), specific growth rate (SGR), relative growth rate (RGR), feed conversion efficiency (FCE) and feed conversion ratio (FCR)

The fish were fed with prepared feeds at the rate of 5 % body mass once a day. The unconsumed feed was siphoned out 6 h after feeding. The following morning, the faecal matter was collected from each trough. The unconsumed feed and faecal matter were dried in an oven at 60 °C and their mass was recorded. About 75 % of water from each trough was changed every day with minimum disturbances to the fish. The initial mass was measured before the experiment and the final mass on the 50th day after the feed supplementation. The experiment was repeated three times and analysed statistically. The growth parameters were calculated by the method described earlier (*18*). The formulae for calculation of AGR, SGR, RGR, FCE and FCR are given by the Eqs. 1–5, respectively:

$$\text{AGR}=(\text{final mean mass}-\text{initial mean mass})/g \quad /1/$$

$$\text{SGR}=[(\ln \text{final mass}-\ln \text{initial mass})/\text{rearing period}]\times 100 \quad /2/$$

$$\text{RGR}=[(\text{final mean mass}-\text{initial mean mass})/(\text{initial mean mass})]\times 100 \quad /3/$$

$$\text{FCE}=[(\text{final mass}-\text{initial mass})/(\text{feed given}-\text{unconsumed feed})]\times 100 \quad /4/$$

$$\text{FCR}=[(\text{feed given}-\text{unconsumed feed})/(\text{final mass}-\text{initial mass})] \quad /5/$$

Chemical analysis

The proximate composition of feed and tissue of the fish were analysed for moisture, crude protein, crude fat, crude fibre and ash and nitrogen-free extract according to the standard methods of AOAC (*19*). The proximate compositions of the formulated feeds are displayed in Table 3.

Statistical analysis

One way analysis of variance (ANOVA; SigmaStat v. 3.5, Systat Software Inc, San Jose, CA, USA) was used to determine whether significant variation between the treatments existed. Difference between means was determined and compared by Duncan's new multiple range test (*20*). All tests used a significance level of $p<0.05$. Data are reported as mean values±standard errors.

Results and Discussion

In the present investigation, the application of *Streptomyces* as probiotic feed for the growth of ornamental fish *Xiphophorus helleri* was assessed. Seven isolates of *Streptomyces* from marine sponges were used as probiotics (*9*). The preliminary characterisations of these *Streptomyces* isolates were carried out by the methods recommended by International *Streptomyces* Project (*10*). The colonies were slow growing, chalky, folded and aerobic. The strains were acid-fast negative and found to be Gram-

Table 3. Proximate composition of different feeds

Formulated feed	Proximate composition/%					
	Moisture	Crude protein	Crude fat	Ash	Crude fibre	Nitrogen-free extract (NFE)
Control	13.87	35.04	14.02	14.80	1.45	20.82
F1	10.89	45.12	12.31	12.24	3.75	15.69
F2	9.97	46.28	11.34	11.34	4.05	17.02
F3	10.64	45.11	12.26	12.95	3.72	15.32
F4	10.91	45.22	12.21	12.66	3.78	15.22
F5	10.95	46.15	12.31	12.33	3.94	14.32
F6	10.48	45.08	12.28	12.58	3.70	15.88
F7	10.56	45.56	12.26	12.28	3.81	15.53

-positive. The aerial mycelial colour patterns of the strains were found to be white, grey and yellow series; the vegetative mycelial colour differed for all the strains. The strains did not produce melanoid pigments. The morphological characteristics of the aerial mycelia, vegetative mycelium, sporophore, and the spores of the strains could be observed clearly. In an observation using the scanning electron microscope, spore morphology showed smooth spore surface and rectiflexibiles (RF) hyphae (Table 4). The strains showed the typical morphology of Streptomycetes when analysing the shape and spore chains under scanning electron microscope, as reported earlier (*21*).

The identification of *Streptomyces* is a very complex process. The *Streptomyces* classification system is mainly dependent on characteristics like the form of spores, melanoid production and utilisation of carbon. The nutritional characteristics of the strains were studied using criteria like carbon utilisation, the influence of amino acids and sodium chloride tolerance. The utilisation of carbon sources is shown in Table 4. The strains grew well in media containing glucose and xylose but they did not assimilate inositol. The growth of the strains was weak or absent from the medium containing sucrose. The amino acids glycine and tryptophan seemed to positively influence the growth of the strains except the strain AQBCD11, which showed better growth in media containing cystine (Fig. 1). At a sodium chloride concentration of 6 and 7 %, the strains showed profuse growth. Strains AQBCD11, AQBMM35 and AQBMM49 exhibited maximal biomass production at a concentration of 6 %, whereas the rest of the strains showed maximal biomass production at 7 % (Table 5). The results of physiological and biochemical characteristics of the strains are shown in Table 6. All the strains were able to grow at 22–45 °C and pH=4–10. All strains except AQBMM49 and AQBTA66 were able to liquefy gelatine. AQBCD24 was the only strain that could neither coagulate nor peptonise milk. Strains AQBMM35 and AQBMM49 were able to hydrolyse starch and cellulose, but unable to produce hydrogen sulphide. Urea was effectively degraded by strains AQBCD11 and AQBMM35. Positive utilisation of citrate was confirmed in strains AQBCD03, AQBCD11, AQBMM35, AQBMM49 and AQBTA66. Strains AQBMM35 and AQBMM49 did not produce indole, while the rest did. Catalase activity was not seen only in strains AQBCD11 and AQBMM35. The nutritional uptake, physiological and biochemical characteristics clearly proved that the marine isolates

Table 4. Characterisation of and carbon utilisation by *Streptomyces* strains

Streptomyces strains	Colour of aerial mycelium	Colour of vegetative mycelium	Melanoid pigments	Spore surface	Spore chain	No carbon source (negative control)	D-xylose	L-arabinose	Rhamnose	D-fructose	D-galactose	Raffinose	D-mannitol	Inositol	Sucrose	D-glucose (positive control)
AQBCD03	grey	yellow	–	RF	S	–	+	+	+	+	–	–	+	–	–	+
AQBCD11	white	white	–	RF	S	–	+	+	–	+	–	+	–	–	–	+
AQBCD24	white	orange	–	RF	S	–	+	–	+	+	+	–	–	–	±	+
AQBMM35	white	pale yellow	–	RF	S	–	+	+	–	–	+	+	–	–	±	+
AQBMM49	yellow	white	–	RF	S	–	+	+	–	–	+	–	+	–	–	+
AQBTA66	white	red	–	RF	S	–	+	+	–	+	+	–	+	–	±	+
AQBDF81	grey white	yellow	–	RF	S	–	+	–	–	+	+	–	+	–	±	+

+ positive results, – negative results, ± doubtful results; RF – rectiflexibilis; S – smooth

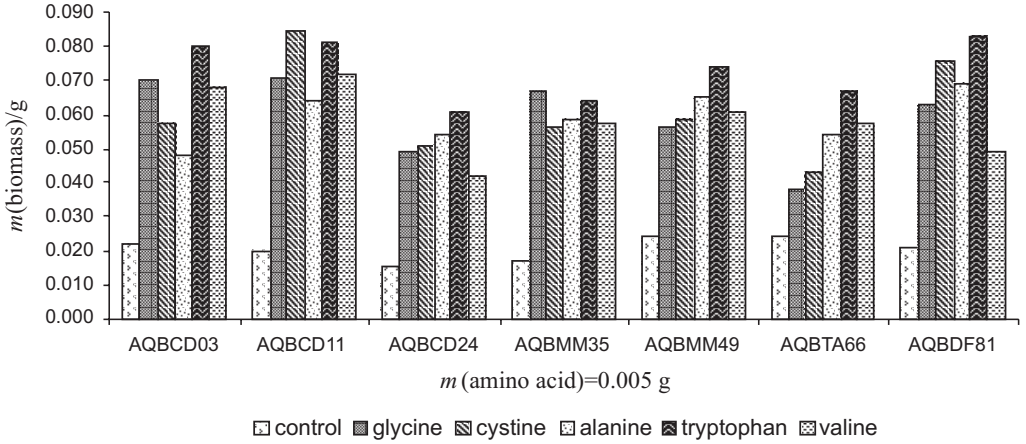

Fig. 1. Influence of amino acids on the growth of the seven strains

Table 5. Sodium chloride tolerance of the strains at various mass fractions

Strain	*m*(dry biomass)/g												
	w(NaCl)/%												
	0	1	1.5	2	2.5	3	4	5	6	7	8	9	10
AQBCD03	0.034	0.052	0.058	0.064	0.077	0.086	0.094	0.107	0.122	0.127	0.105	0.094	0.076
AQBCD11	0.033	0.043	0.055	0.062	0.069	0.071	0.083	0.097	0.119	0.102	0.091	0.082	0.077
AQBCD24	0.032	0.047	0.049	0.065	0.088	0.093	0.105	0.113	0.125	0.132	0.114	0.099	0.082
AQBMM35	0.047	0.057	0.065	0.069	0.072	0.076	0.079	0.084	0.101	0.095	0.08	0.074	0.067
AQBMM49	0.038	0.053	0.057	0.067	0.090	0.095	0.112	0.124	0.132	0.115	0.098	0.082	0.072
AQBTA66	0.044	0.049	0.056	0.062	0.085	0.094	0.115	0.121	0.129	0.136	0.122	0.109	0.097
AQBDF81	0.037	0.043	0.049	0.055	0.062	0.077	0.092	0.104	0.114	0.128	0.117	0.101	0.092

Table 6. Physiological and biochemical characteristics of *Streptomyces* strains

Parameter	AQBCD03	AQBCD11	AQBCD24	AQBMM35	AQBMM49	AQBTA66	AQBDF81
starch hydrolysis	–	–	–	+	+	–	–
production of H_2S	+	+	+	–	–	+	+
degradation of cellulose	–	–	–	+	+	–	–
liquefaction of gelatin	+	+	+	+	–	–	+
coagulation of milk	+	+	–	+	+	+	+
peptonisation of milk	+	+	–	+	+	+	+
degradation of urea	–	+	–	+	–	–	–
citrate utilisation	+	+	–	+	+	+	–
indole production	+	+	+	–	–	+	+
catalase	+	–	+	–	+	+	+

+ positive results, – negative results

could be classified under the genus *Streptomyces*, as reported earlier (*10–12*).

Strain AQBCD11 showed maximum IAA production of 2.7 μg/mL at pH=8.0. The minimal IAA production was recorded at pH=5.0 (Fig. 2). The better growth rate of the fish fed with the probiotic feed may be due to the production of the growth-promoting hormone indoleacetic acid by the *Streptomyces* strains (*22*). The *Streptomyces* isolates used as probiotics in the present study have already been proved to produce antibacterial and antifungal substances against human pathogens *Bacillus subtilis*, *Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli*, *Aspergillus niger*, *Saccharomyces cerevisiae* and *Candida albicans*. The inhibition zones occurred in the range of 15 to 20 mm (*9*).

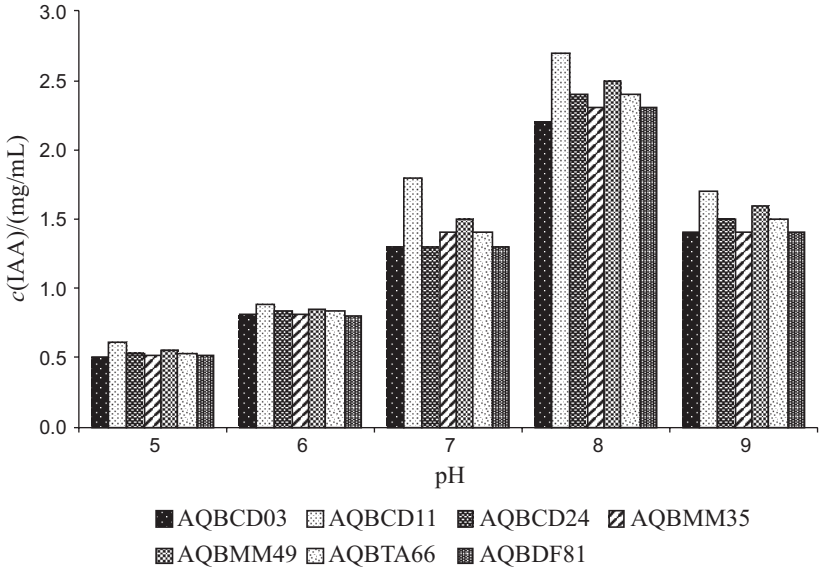

Fig. 2. Indoleacetic acid (IAA) production by the strains at different pH levels

After 50 days of feeding trials with probiotic and control feeds, the growth parameters and length of the fish were analysed. All the fish fed with probiotic feed showed significantly ($p<0.05$) higher growth performance of absolute growth rate (AGR), specific growth rate (SGR) and relative growth rate (RGR) than the fish fed with the control feed. Of the eight diets, the AGR (1.37± 0.01) g, SGR (1.57±0.06) % and RGR (101.53±0.96) % of the fish fed with probiotic feed F2 exhibited increased growth compared to the fish fed with control feed, which showed AGR (0.79±0.02) g, SGR (1.28±0.10) % and RGR (41.67±1.67) %. The results also show that the FCE values were increased and FCR values were significantly decreased ($p<0.05$) when compared to control fish. All the fish fed with probiotic feed showed a marginal increase in length compared to the control feed-fed fish. The fish fed with probiotic feed F2 showed higher increment in length of 41.15 mm when compared to the control feed-fed fish (30.99 mm). The proximate compositions of red swordtail fish fed on different diets are shown in Table 7. Statistical analysis by Duncan's new multiple range test showed significant differences of $p<0.05$ between the fish fed with probiotic feed and fish fed with control feed, which are shown in Table 8.

There are reports in support of the *Streptomyces* as probionts in aquaculture. The application of marine *Streptomyces* incorporated in the formulated feed and supplemented to juvenile prawns weighing about 0.130–0.160 g was studied. After 50 days of feeding trials, the prawns fed with *Streptomyces*-incorporated feed showed improved growth (140.54 %) and feed conversion efficiency (45 %), and higher protein content (54.72 %), whereas the prawns fed with control feed showed less growth of 89.52 %, lower feed conversion efficiency (20 %) and protein content of 35.02 %. The feed conversion ratio was less (2.217) than the control (5.015) (*8*). Preliminary report on the growth increment of tiger shrimp on supplementation of *Streptomyces*-incorporated feed was reported (*5*). Cells of *Streptomyces* were incorporated at different concentrations (0, 2.5, 5.0, 7.5, and 10.0 g/kg feed) in the formulated feeds. At a high concentration of 10 g of *Streptomyces* cells in 1 kg of formulated feed, the fish showed increased growth in terms of length (15.79 %) and mass (57.97 %) when compared to the control (length 4.08 % and mass 32.77 %).

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 toyoi* and other *Bacillus* sp. when used as feed additive increased the growth rate of yellow tail, *Seriola quinquiradiata* (*23*); turbot, *Scophthalmus maximus* (*24*); common snook, *Centropomus undecimalis* (*3*) and giant tiger prawn, *Penaeus monodon* (*25*). The commercial preparations of *Streptococcus faecium* and a mixture of bacteria and yeast improved the growth and food conversion efficiency of *Cyprinus carpio* (*26*) and *Catla catla* (*27*), respectively. The usage of two probiotic bacteria and the yeast *Saccharomyces cerevisiae* as growth promoters in the Nile tilapia (*Oreochromis niloticus*) fry was studied (*28*). The results indicate that the fry subjected to diets with a probiotic supplement exhibited greater growth than those fed with

Table 7. Proximate composition of *Xiphophorus helleri* fed on different diets

Formulated feed	Proximate composition/%					
	Moisture	Crude protein	Crude fat	Ash	Crude fibre	Nitrogen–free extract (NFE)
Control	78.40	10.41	5.04	4.15	1.00	1.00
F1	75.50	15.40	3.00	3.03	2.50	0.57
F2	74.09	16.50	3.05	2.99	2.97	0.40
F3	76.02	15.40	3.02	3.12	2.09	0.35
F4	75.97	15.47	3.02	3.11	1.95	0.48
F5	76.01	15.46	3.04	3.12	2.06	0.31
F6	76.05	15.48	3.02	3.11	2.03	0.31
F7	76.01	15.47	3.04	3.12	2.00	0.36

Table 8. FCR, FCE, SGR, AGR, RGR and increase in length of *Xiphophorus helleri* fed with different experimental diets

Formulated diet	Initial mass / g	Final mass / g	FCR	FCE / %	AGR / g	SGR / %	RGR / %	Initial length / mm	Final length / mm	Net increase in length / mm
Control	0.63±0.04	1.42±0.02	1.03±0.08	97.02±6.98	$(0.79±0.02)^a$	$(1.28±0.10)^a$	$(41.67±1.67)^a$	21.08±0.03	30.99±0.02	$(9.91±0.01)^a$
F1	0.66±0.02	1.80±0.01	0.62±0.01	162.46±1.80	$(1.14±0.01)^c$	$(1.42±0.06)^b$	$(79.72±0.96)^e$	20.57±0.02	39.37±0.01	$(18.80±0.01)^c$
F2	0.65±0.01	2.02±0.01	0.45±0.01	221.65±5.19	$(1.37±0.01)^e$	$(1.57±0.06)^d$	$(101.53±0.96)^g$	20.57±0.005	41.15±0.01	$(20.57±0.01)^d$
F3	0.63±0.02	1.72±0.01	0.62±0.01	161.83±2.26	$(1.09±0.01)^b$	$(1.47±0.06)^c$	$(71.39±0.96)^b$	21.08±0.01	39.37±0.02	$(18.29±0.01)^b$
F4	0.65±0.03	1.73±0.01	0.58±0.01	173.20±3.50	$(1.08±0.03)^b$	$(1.41±0.09)^b$	$(73.20±0.87)^c$	21.08±0.01	38.86±0.02	$(17.78±0.01)^b$
F5	0.66±0.01	1.83±0.01	0.53±0.01	188.09±1.93	$(1.17±0.01)^d$	$(1.45±0.02)^c$	$(83.06±1.27)^f$	20.83±0.01	38.35±0.01	$(17.53±0.02)^b$
F6	0.64±0.01	1.78±0.01	0.69±0.02	145.70±4.07	$(1.14±0.01)^c$	$(1.47±0.01)^c$	$(77.78±1.27)^d$	20.83±0.02	39.88±0.01	$(19.05±0.03)^c$
F7	0.65±0.02	1.73±0.01	0.63±0.01	160.29±1.76	$(1.08±0.02)^b$	$(1.41±0.06)^b$	$(73.20±0.87)^c$	20.57±0.01	38.61±0.03	$(18.03±0.02)^b$

Values in the same column sharing a common superscript are not significant ($p<0.05$)

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 (*29*,*30*).

In the present work the probiotic bacteria replaced nearly 30–40 % of the fish meal incorporated in the feed. In aquaculture operations, essential and expensive components of the feed are proteins, especially the fish meal. Since the supply of fish meal has become uncertain and the prices have increased rapidly, the need for cheaper alternative protein sources has increased. Among unconventional protein sources, microbial origin appears to be a promising substitute for fish meal, replacing up to 25–50 %. The above-mentioned researches indicate the importance of marine actinomycetes, particularly the application of *Streptomyces* in aquaculture.

Conclusions

Probiotic microorganisms synthesise macromolecules and vitamins which benefit animal nutrition. There are very limited reports on the application of marine actinomycetes, particularly *Streptomyces* as probiotics in aquaculture. This is the first report on the application of *Streptomyces* as probiotic feed for the growth of ornamental fish and this study proved that there is a significant increment in the growth as well as the length of the ornamental fish *Xiphophorus helleri* fed with probiotics. Therefore, in the near future the applications of *Streptomyces* as probionts will play an important role in aquaculture nutrition.

Acknowledgements

The authors wish to thank the Department of Science and Technology (DST), New Delhi, India for financial support.

References

1. D.J.W. Moriarty, The role of microorganisms in aquaculture ponds, *Aquaculture*, *151* (1997) 333–349.
2. F.J. Gatesoupe, The use of probiotics in aquaculture, *Aquaculture*, *180* (1999) 147–165.
3. A. Irianto, B. Austin, Probiotics in aquaculture, *J. Fish. Dis. 25* (2002) 633–642.
4. S. Das, L.R. Ward, C. Burke, Prospects of using marine actinobacteria as probiotics in aquaculture, *Appl. Microbiol. Biotechnol. 81* (2008) 419–429.
5. S. Das, P.S. Lyla, S. Ajmal Khan, Application of *Streptomyces* as a probiotic in the laboratory culture of *Penaeus monodon* (Fabricius), *Isr. J. Aquac. Bamidgeh*, *58* (2006) 198–204.
6. S.S. Kumar, R. Philip, C.T. Achuthankutty, Antiviral property of marine actinomycetes against white spot syndrome virus in penaeid shrimps, *Curr. Sci. 91* (2006) 807–811.
7. J.L. You, X.L. Xue, L.X. Cao, X. Lu, J. Wang, L.X. Zhang, S.N. Zhou, Inhibition of *Vibrio* biofilm formation by a marine actinomycete strain A66, *Appl. Microbiol. Biotechnol. 76* (2007) 1137–1144.
8. K.G. Manju, K. Dhevendaran, Effect of bacteria and actinomycetes as single cell protein feed on the growth of juveniles of *Macrobrachium idella* (Hilgendrof), *Indian J. Exp. Biol. 35* (1997) 53–55.
9. S. Dharmaraj, A. Sumantha, Bioactive potential of *Streptomyces* associated with marine sponges, *World J. Microbiol. Biotechnol. 25* (2009) 1971–1979.
10. E.B. Shirling, D. Gottlieb, Methods for characterization of *Streptomyces* species, *Int. J. Syst. Bacteriol. 16* (1966) 313–340.
11. S. Dharmaraj, B. Ashokkumar, K. Dhevendaran, Fermentative production of carotenoids from marine actinomycetes, *Iran. J. Microbiol. 4* (2009) 36–41.
12. *Bergey's Manual of Determinative Bacteriology*, R.E. Buchanan, N.E. Gibbons (Eds.), Williams and Wilkins Co, Baltimore, MD, USA (1974) pp. 377–378.
13. L.H. Xu, W.J. Lee: *Actinomycetes: Systematic Principle, Methods and Practice*, Science Press, Beijing, PR China (2006) pp. 381–387.
14. E. Glickmann, Y. Dessaux, A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria, *Appl. Environ. Microbiol. 61* (1995) 793–796.
15. C.L. Patten, B.R. Glick, Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system, *Appl. Environ. Microbiol. 68* (2002) 3795–3801.
16. R. Hardy: Fish Feed Formulation. In: *Fish Feed Technology*, UNDP/FAO, ADCP/REP/80/11 (1980) pp. 233–239.
17. R. Belcher, A.L. Godbert: *Semi-Micro Quantitative Organic Analysis*, Longmans, Green and Co, New York, NY, USA (1954) pp. 102–107.
18. *Fish Nutrition*, J.E. Halver (Ed.), Academic Press, London, UK (1972).
19. Official Methods of Analysis, Association of Official Analytical Chemists (AOAC), Gaithersburg, MD, USA (2000).
20. D. Duncan, Multiple range tests and multiple F tests, *Biometrics*, *11* (1955) 1–42.
21. R. Locci: Streptomycetes and Related Genera. In: *Bergey's Manual of Systematic Bacteriology*, S.T. Williams, M.E. Sharpe, J.G. Holt (Eds.), Williams and Wilkins Co, Baltimore, MD, USA (1989) pp. 2451–2493.
22. K. Dhevendaran, M. Sukumaran, M.I. Georgekutty, Growth hormone (IAA) producing bacteria and streptomycetes and their bio-fertilizer effect on mangrove seedling, *Avicennia officinalis*, *J. Mar. Biotechnol. 22* (2004) 209–213.
23. M. Kozasa, Toyocerin (*Bacillus toyoi*) as growth promoter for animal feeding, *Microb. Alim. Nutr. 4* (1986) 121–125.
24. F.J. Gatesoupe, Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic *Vibrio*, *Aquat. Living Res. 7* (1994) 277–282.
25. B. Vaseeharan, P. Ramasamy, Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*, *Lett. Appl. Microbiol. 36* (2003) 83–87.
26. I. Bogut, Z. Milakovic, S. Brkic, R. Zimmer, Influence of probiotic (*Streptococcus faecium* M74) on growth and content of intestinal microflora in carp (*Cyprinus carpio*), *Czech J. Anim. Sci. 43* (1998) 231–235.
27. S.N. Mohanty, S.K. Swain, S.D. Tripathi, Rearing of catla (*Catla catla* Ham.) spawn on formulated diets, *J. Aquat. Trop. 11* (1996) 253–258.
28. M. Lara-Flores, M.A. Olvera-Novoa, B.E. Guzman-Mendez, W. López-Madrid, Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*), *Aquaculture*, *216* (2003) 193–201.
29. I. Salinas, A. Cuesta, M.A. Esteban, J. Meseguer, Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses, *Fish Shellfish Immunol. 19* (2005) 67–77.
30. S.M. Aly, Y.A.G. Ahmed, A.A. Ghareeb, M.F. Mohamed, Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica* (*Oreochromis niloticus*) to challenge infections, *Fish Shellfish Immunol. 25* (2008) 128–136.