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Application of Microbial Carotenoids as a Source of Colouration and Growth of Ornamental Fish *Xiphophorus helleri*

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Abstract: Carotenoids are the pigments which fishes cannot synthesize; they rely on dietary supply to achieve their natural skin pigmentation, which is one of the most important quality criteria determining the market value of ornamental fishes such as koi carp, sword tail and goldfish. In the present study an attempt was made to recover carotenoids in the skin of red swordtail fish by supplementing microbial carotenoid-rich feed. In control fishes the carotenoids content was 4.83 µg/g of integument, whereas the fishes exposed to stress showed reduction of 0.49 µg/g or complete loss. Supplementation of microbial carotenoids incorporated feed resulted in the recovery of 2.53 and 1.14 µg/g of carotenoids in the stressed fishes and growth, respectively. On feeding the stressed fishes with control feed, 1.84 and 0.59 µg/g of carotenoids was obtained, respectively. The results indicated that the fishes fed with carotenoid enriched feed showed faster recovery of carotenoids in the skin of the fishes when compared to the control feed supplemented fishes and the results were found to be significant ($p<0.001$). After 28 days of feeding trials, the growth parameters were found to be statistically significant ($p<0.01$) when different diets were compared. The study proved that microbial carotenoids not only improve the pigmentation, but also promotes the growth of the ornamental fishes effectively.

Key words: Microbial carotenoids % Fish feed % *Xiphophorus helleri* % Carotenoids recovery

INTRODUCTION

Ornamental fishes have traditionally been fed with live feed, which were often nutritionally deficient and can act as the transmitter of diseases (parasitic, bacterial and viral) if not stored properly [1]. However, large commercial producers of aquarium fishes emphasise the importance of regular supplementation of formulated feeds with live feed, as the inclusion of live feed improves growth [2]. The development of manufactured feed could be considered as one of the contributing factors to the tremendous growth of this hobby's widespread popularity over the past 50 years [3]. The increased acceptability and reliance upon manufactured feed for ornamental fishes have been focussed the attention on the nutritional requirements of these animals. Most information on the quantitative and qualitative nutrient requirements of ornamental fishes in public and home aquaria were derived principally from research carried out by the aquaculture industry since the 1970s. These results do have limitations in their applicability to ornamental fishes, because it is based on a small number of fish species raised for food, which are often kept under totally different conditions from those kept as ornamentals in public or home aquaria [4].

Pigments are responsible for the wide spectrum of colours in fishes which is an essential prerequisite for the quality as they fetch higher price in the commercial market. As fishes cannot synthesize their own colouring pigments *de novo*, the colouring agents which are synthesized by some plants, algae and microorganisms, need to be incorporated in their diet [5]. Varieties of colouring agents are used in aqua industry to impart colour for the muscle and skin of fishes. Thus pigmentation is an important criterion for fishes, since their colour affect commercial acceptability. Fish use oxygenated carotenoids which are one of the most important groups of natural pigments used for pigmentation of skin and flesh [6]. Natural carotenoids are categorised into three groups as plant, animal and microbial based carotenoids [7]. Fishes contain various kinds of carotenoids, the dominant of which is peculiar

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to the species concerned. Carotenoids commonly occurring in fishes with their colours are tunaxanthein (yellow), lutein (greenish yellow), $-carotene (orange), ", $ doradexanthins (yellow), zeaxanthin (yellow orange), canthaxanthin (orange red), astaxanthin (red), eichinenone (red) and taraxanthin (yellow) [8]. Plant based carotenoids sources include marigold (petal meal), seaweeds, *Alfa alfa* and animal based carotenoids sources like Antarctic krill (*Euphausia superba*), crayfish meal, shrimp meal and crab meal [7]. Microbial carotenoids are present in anoxygenic and oxygenic photosynthetic bacteria, microalgae (*Haematococcus pluvialis*, *Chlorella vulgaris*, *Dunaliella salina*, *Arthospira maxima*) and in many fungi [9,10].

Many of the naturally bright coloured fishes may show fading in colouration due to various factors like constant exposure to sunlight, chlorine, starvation, pH, hardness of water, turbidity of water, stress, toxicants and pollutants in water, low quality of water, poor husbandry techniques, poor quality feed, nutrient-deficient live feeds, etc [11, 12]. Excess exposure to sunlight may even lead to melanoma and other types of skin cancers in fish. Global warming is air pollution and has impact on fading in colouration of fishes. These bleached fishes have a decreased market value. A method of improvisation and measurement of skin colour to the bleached fish was possible only when carotenoids were included as a dietary component. In the present investigation the red swordtail fish, *Xiphophorus helleri* showed reduced content of carotenoids in their integument on exposure to various stresses and its recovery was achieved by supplementing the fishes with microbial carotenoids enriched feed.

MATERIALS AND METHODS

Exposure to Stress: The female red swordtail fish (*Xiphophorus helleri*) of about 1.5 grams were stocked in five 20 litre plastic troughs. Each trough had twenty fishes. One trough was the control, whereas other four troughs were for the stress experiment. Two of the troughs were exposed to sunlight for six hours everyday, while the other two was subjected to chlorine stress by using 0.01 g calcium hypochlorite. The experiment was conducted for four days. After four days of treatment, the faded fishes were collected and their integuments were removed for qualitative and quantitative analysis of carotenoids.

Extraction and Quantification of Carotenoids from Fish Skin Carotenoid Extraction: The method used for carotenoid extraction was described earlier [13]. The skin of each of the fishes were pre-weighed and homogenised separately by addition of acetone. The homogenate was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected. The supernatant was allowed to condense to a minimum volume. The condensed samples were taken for qualitative and quantitative analysis.

Qualitative Estimation of Carotenoids by TLC: The condensed samples were spotted on the silica gel plate and dried at room temperature. The plates were placed in a TLC chamber pre-saturated with the mobile phase solvents consisting of methanol (5 %): toluene (95:5). The chromatogram was developed by exposing the plates to iodine vapour. The R_f values were calculated.

Quantification of Carotenoids: To estimate total carotenoids, the extracted samples were added to a separating flask. These were overlaid with methanol and hexane in the ratio 2:3. The contents in the flask were shaken for two hours. Two distinct layers were obtained, in which upper organic phase was collected. Small aliquots of the collected samples were read at 350-500 nm using spectrophotometer. Total carotenoids in the sample were calculated as:

$$\text{Total carotenoid} = \frac{\text{Optical density (nm) x Volume of sample x Dilution factor (10 ml)}}{\text{Mean absorption coefficient}}$$

Production of Microbial Carotenoids: Submerged fermentation of *Streptomyces* sp. AQBWWS1 strain yielded carotenoids which are reported earlier [14] and after fermentation the broth was lyophilized.

Microbial Feed Supplementation: Two types of feed were prepared, namely control feed and carotenoid enriched feed. Formulated diets were prepared according to the square method [15]. The concentration of protein and the compositions of the ingredients used for preparing formulated diets were shown in Table 1.

Control Feed: The ingredients used for control feed preparation consisted of fishmeal, rice bran, chickpea flour and groundnut oil cake. The binder used was tapioca flour. The ingredients were ground to a fine powder and

Table 1: Composition and proximate composition of the formulated diets - control feed and carotenoid enriched feed and protein content of the ingredients

	Formulated feeds (g/ 100 g)		
Feed ingredients	Control feed	Carotenoids enriched feed	Protein (%)
Rice bran	16.50	16.50	10.522
Chickpea flour	18.50	18.50	35.540
Groundnut oil cake	23.50	23.50	40.010
Tapioca flour	16.00	16.00	2.520
Fish meal	25.50	15.50	55.004
Streptomyces sp.	-	10.00	56.672
Proximate composition (%)			
Moisture	10.87	11.90	
Crude protein	35.04	45.20	
Crude fat	12.02	14.20	
Ash	13.80	13.34	
Crude fiber	2.45	2.85	
Nitrogen-free extract (NFE)	25.82	12.51	

mixed thoroughly with sufficient water to obtain smooth dough. The dough thus prepared was steam cooked for 30 minutes and allowed to cool. This was extruded through a pelletiser. The pellets were dried and then stored in dry air-tight containers at 28°C.

Carotenoid Enriched Feed: The steam cooked dough was prepared and cooled as given above. To this dough, the lyophilized broth of *Streptomyces* sp. (AQBCD11) was added (10 g) and mixed well. This was extruded through a pelletiser, the pellets freeze dried and kept in dry air-tight containers at 4°C.

Determination of absolute growth rate, specific growth rate, food conversion efficiency and food conversion ratio.

The stress fishes were fed with prepared feed at the rate of 5 % body weight per day. The unconsumed feed was siphoned out six hours after feeding. The following morning, the faecal matters were collected from each trough. The unconsumed feed and faecal matter were dried in an oven at 60°C and their weights were recorded. About 75 % of water from each trough was changed daily with minimum disturbances to the fishes. The final weights were taken on the 28^{th} day after the feed supplementation and the initial weights before the experiment. The experiment were repeated three times and statistically analysed. The growth parameters were calculated by the method described earlier [16].

Chemical Analysis: Analyses of the experimental feeds (moisture, crude protein, crude fat, crude fiber and ash and nitrogen free extract) were determined according to the standard methods [17]. The proximate compositions of the formulated feeds are displayed in Table 1.

Estimation of Carotenoids in the Fishes Supplemented with Formulated Feeds: After 28 days of supplementing carotenoid enriched feed and control feed to the stress fishes, the skin of fishes were removed, pre-weighed and homogenised by addition of acetone. Then it was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected. The supernatant was allowed to condense to minimum volume. The condensed samples were analysed for carotenoids as described earlier.

Statistical Analysis: All experiments were performed in triplicate and the results are expressed as mean values with ± Standard Deviation. Error bars shown in figures are Standard Deviation of the experimental data from the mean values. Data were subjected to analysis of variance (ANOVA; SigmaStat v. 3.5, Systat Software Inc, San Jose, CA, USA) and means were separated by least significant difference (LSD) test at 1 % probability level.

RESULTS

Exposure to stresses resulted in the reduction and loss of carotenoids in the integuments of red swordtail fishes. This was deduced by spectral analysis of the carotenoids extract from the integument and is shown in Table 2 and Figure 1. The control fishes, which were maintained under normal condition throughout the experiment, exhibited a carotenoids content of 4.83 µg/g of integument, whereas in the stress fishes this was reduced to 0.49 µg/g and completely lost in some others. Thin layer chromatographic analysis of the carotenoid extracts from the integument of control fishes showed the spot with R_f values of 0.75. In stress fishes, absence of spot was resulted from the carotenoid fraction.

Table 2: Spectral analysis, TLC values and total volume of carotenoids present in control and stress fishes

Integument of fishes	Spectral analysis Maximum (nm)/ absorbance values	TLC R_f values	Total volume of carotenoid (µg/g)
Control	448 - 2.41445	0.75	4.83
Light exposed	448 - 0.24377	0.75	0.49
Chlorine stressed	-	-	-

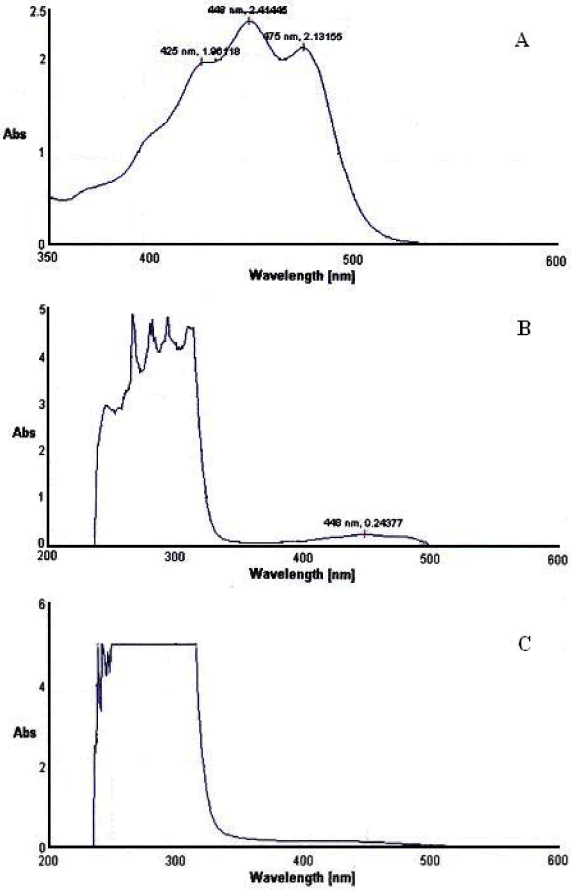

Fig. 1: Spectral analysis of carotenoids in the integuments of A) Control, B) Light exposed and C) Chlorine stressed fishes

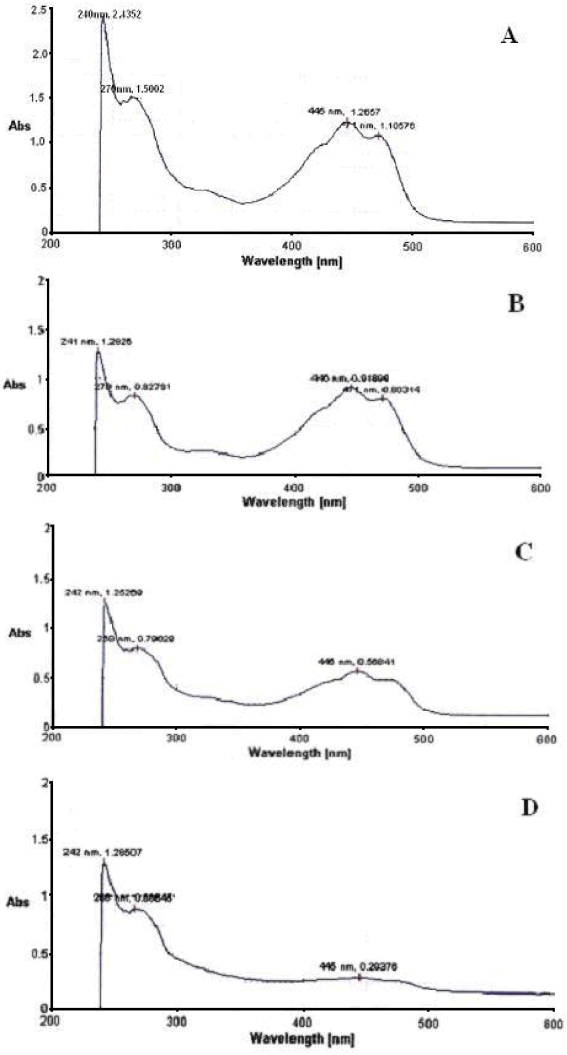

Fig. 2: Carotenoids recovery in the stress fishes by feeding the formulated diets [A and C with the carotenoid enriched feed given to light exposed and chlorine stressed fishes; B and D are the control feed given to light exposed and chlorine stressed fishes]

The fishes that were exposed to stress were given the formulated diets. After 28 days, the fish integuments were collected and analysed for carotenoids. The fishes that were supplemented with microbial carotenoid enriched feed led to the recovery of carotenoids. Nearly 2.53 µg/g of carotenoids recovered in sunlight exposed and 1.14 µg/g in chlorine stressed fishes. When the stress fishes supplemented with control feed resulted with carotenoid content of 1.84 and 0.59 µg/g, respectively. The above results indicated that the fishes that were fed with carotenoid enriched feed showed higher and faster recovery of carotenoids when compared to the control feed (Table 3, Figure 2). The carotenoid content were found to be significant ($P<0.001$) by least significant

Table 3: Spectral analysis and carotenoid content in fishes fed with control feed and carotenoid enriched feed

	Spectral analysis	
Formulated feeds	Maximum (nm)/ absorbance values	Carotenoid content (µg/g)
Carotenoid enriched feed given to light exposed fishes	445-1.2657	2.53±0.02[d]
Control feed given to light exposed fishes	446-0.9189	1.84±0.03[c]
Carotenoid enriched feed given to chlorine stressed fishes	446-0.56841	1.14±0.01[b]
Control feed given to chlorine stressed fishes	445-0.29376	0.59±0.02[a]

Mean followed by the different letter do differ significantly (P<0.001)

Table 4: One way ANOVA for carotenoid content of *Xiphophorus helleri* fed with different experimental diets

Source of Variation	Degrees of freedom	Sum of squares	Mean square	F
Between Groups	3	6.39500	2.132000	4737.111***
Within Groups	8	0.00360	0.000450	
Total	11	6.39900		

*** Significant (p<0.001)

Table 5: Shows AGR, FCE, FCR and SGR in *Xiphophorus helleri* fed with control feed and carotenoid enriched feed. [F1 – Control fishes fed with control feed, F2, F4 - Carotenoid enriched feed given to light exposed and chlorine stress fishes, F3, F5 - Control feed given to light exposed and chlorine stress fishes]

Formulated feeds	Initial weight (g)	Final weight (g)	AGR (g)	FCE (%)	FCR(g)	SGR (%)
F1	1.530±0.020	1.840±0.030	0.310±0.010[a]	26.940±1.875[a]	3.724±0.256[e]	0.659±0.012[a]
F2	1.533±0.025	2.540±0.020	1.007±0.006[e]	78.732±0.946[e]	1.270±0.015[a]	1.803±0.031[e]
F3	1.520±0.010	2.050±0.010	0.530±0.010[c]	46.167±0.871[c]	2.167±0.041[c]	1.068±0.030[c]
F4	1.520±0.020	2.243±0.020	0.723±0.003[d]	63.014±0.812[d]	1.587±0.020[d]	1.390±0.016[d]
F5	1.530±0.010	1.993±0.015	0.463±0.006[b]	39.881±0.947[b]	2.508±0.059[b]	0.945±0.006[b]

Values in the same column sharing a common superscript are not significant (p<0.01)

Table 6: One way ANOVA for weight gain of *Xiphophorus helleri* fed with control feed and carotenoid enriched feed

Source of Variation	Degrees of freedom	Sum of squares	Mean square	F
Between Groups	4	0.864	0.216	3927.879**
Within Groups	10	0.000550	0.0000550	
Total		14	0.865	

** Significant (p<0.01)

Table 7: Proximate composition of Red Swordtail fish fed on formulated feeds

	Proximate composition (%)					
Formulated feeds	Moisture	Crude Protein	Crude Fat	Ash	Crude fiber	Nitrogen - free extract (NFE)
Control feed	77.00	15.00	3.00	3.20	1.00	0.80
Carotenoid enriched feed	78.00	15.25	3.15	2.65	0.75	0.20

difference (LSD) test, when comparing control feed and carotenoid enriched feed given fishes which are displayed in Table 4.

Microbial carotenoid enriched feed resulted in a marked increase in the weight of the fishes when compared to control feed given fishes. When comparing the FCR, the control feed given fishes showing the higher value of 3.724 ± 0.256 than the others. The FCE values of F1 (78.732 ± 0.946), F3 (63.014 ± 0.812) are higher than the control feed given fishes (26.940 ± 1.875). There was significant increase in food conversion efficiency when fed with carotenoid enriched feed and decreased values of food conversion ratio. The microbial carotenoid enriched feed was rich in protein and this may be the reason for the better growth rate. The carotenoid enriched feed given fishes were found to be significant than the control fishes with respect to AGR and SGR (Table 5). The weight gain showed significant differences at the 1% level by least significant difference (LSD) test, when comparing the control feed and carotenoid enriched feed given fishes (Table 6). The proximate compositions of Red Swordtail fish fed on formulated diets are displayed in Table 7.

DISCUSSION

The colouration of ornamental fishes gives the market value for the fish. Many of the brightly coloured fishes showed fading in colouration due to various factors like exposure to sunlight, chlorine, starvation, pH, hardness of water, turbidity of water, stress, toxicants and pollutants in water, low quality of water, poor husbandry techniques, poor quality feeds, nutrient-deficient live feeds, etc. The resultant ornamental fishes have decreased market rates when compared to the brightly coloured ones. An attempt was made on supplementation of microbial cell mass (rich in carotenoids) incorporated feed to the fishes which were affected either biologically or chemically and the recoveries of colouration of the fishes were studied.

The results proved that fishes fed with carotenoid enriched feed showed faster recovery of carotenoids when compared to those provided with only the control feed. The main reason for the colour recovery in stressed fishes could be attributed to the presence of the carotenoids in the lyophilized fermented broth [14]. The carotenoids used as a feed additive contributes to the colouration of the fishes, which normally fishes cannot synthesise. Fishes usually acquire carotenoids from the external environment which are then stored and/or metabolised. The conversions of carotenoids of different forms are initiated by enzymes [18]. The predominant carotenoids in the skin of red sword tail fish are zeaxanthin, lutein and â-carotene which are reduced or lost on exposure to stress [19]. Abundant supply of lycopene in fish feed, recovered the pigments on metabolic conversion by cyclization and hydroxylation of lycopene to zeaxanthin, lutein and $-carotene [20].

Colour enhancement through the use of carotenoids in feed has been confirmed by a number of authors. Reports on intense colouration of freshwater red velvet swordtails (*Xiphophorus helleri*), rainbow fish (*Pseudomugil furcatus*) and topaz cichlids (*Cichlasoma myrnae*) when fed diet containing carotenoid rich strain of *Spirulina platensis* and *Haematococcus pluvialis* [21]. Use of six different commercial diets resulted with different colouration of Red oranda variety of gold fish (*Carassius auratus*) was reported [22]. Reports on application of China rose *(Hebiscus rosasinensis*) petal as a potent natural carotenoid source for goldfish (*Carassius auratus*) to enhance its colour [23]. Encouraging results on colouration of red swordtails (*Xiphophorus helleri*) fed with diet containing marigold petal meal. In addition to enhancing colouration of the fish, the different pigments used in the diets are also reported to give better results of growth and accelerate gonadal development in the ornamental fish [24]. Red colouration is imparted in goldfish and common carp by astaxanthin, a carotenoid that is readily metabolised from the yellow pigment zeaxanthin are reported earlier [25]. It has been previously demonstrated that under certain well-defined culture conditions (nitrogen depletion, high salinity and light intensity) the algae *Chlorella vulgaris, Haematococcus pluvialis* and *Arthrospira maxima* (Spirulina) will accumulate carotenoids and this might be used to replace costly synthetic colourings in ornamental fish feed [26]. The *Streptomyces mediolani* on fermentation yielded a carotenoid, 3-hydroxy isorenieratene which showed an interesting property of improving the colour of egg yolk when administered as a feed additive as reported earlier [27]. Earlier an investigation on the absorption and distribution of canthaxanthin in rainbow trout showed the skin, muscle, liver and kidney to have the highest proportion in the tissues examined, other than the gastrointestinal tract [28]. During active growth, high concentrations of carotenoids were deposited in the skin. During sexual maturation, carotenoids were redistributed from the flesh to skin and eggs [29]. Literature review indicated that ornamental fish require between 50 and 400 mg/L of synthetic or natural carotenoids (e.g., red pepper and marigold extracts) in their diet to develop color similar to those of fish eating live foods [30-32].

In aquaculture operations, essential and expensive components of the feed were proteins, especially fishmeal. Since the supply of fishmeal has become uncertain, it is of great importance to replace the fishmeal to a minimum possible extent in fish rations. Among unconventional protein sources, single cell protein (SCP) of microbial origin appear to be a promising substitute for fishmeal, which can replace up to 25-50 %. In this experiment it had been observed that nearly 30-40 % replacement of fish meal by microbial carotenoid enriched feed increased the growth of stressed fishes when compared to control feed. Similarly, marine streptomycetes incorporated into the diets increased the growth, enhanced the food conversion efficiency and protein increment of the juvenile prawn, Macrobrachium idella as reported previously [33]. In the fish, Oreochromus mossombicus fed with single cell protein (SCP) better growth and conversion efficiencies were reported [34]. Earlier it has been demonstrated that by daily supplementation of *Daphnia* sp. as live feed to swordtail (*Xiphophorus helleri*) brood stock maintained on an artificial flake diet resulted in a significant increase in fecundity as a result of more rapid growth, a higher

number of embryos and an improved feed conversion ratio [35]. It has also been reported that on supplementation of diets with *Artemia* there was increased growth of juvenile angelfish (*Pterophylum scalare*) [36]. Some species, such as the ruby barb (*Puntius nigrofasciatus*), a voracious and indiscriminate feeder, prefers live feed to artificial feeds [37]. A preliminary report on the use of *Streptomyces* as probiotic for the growth of black tiger shrimp was studied [38]. Antibiotic product extracted from marine *Streptomyces* was incorporated in the feed to study the *in vivo* effect on white spot syndrome virus in black tiger shrimp [39]. Reports on the activity of marine *Streptomyces* as a potential organism against biofilms produced by *Vibrio* spp. and recommended the use of *Streptomyces* to prevent the disease caused by *Vibrio* spp [40]. Recently the potential application *Streptomyces* as a probiotic feed for the growth of the ornamental fish, *Xiphophorus helleri* were studied [16]. There has been significant ($P<0.05$) increase in the growth parameters and length of the fishes that received probiotic feeds than control were resulted.

The formulated diets which carry the microbial carotenoids in the feed resulted in recovery of skin carotenoids to the fishes subjected to stress. Hence it can be concluded that microbial carotenoids could be used as a food colourant and also as a feed additive for the growth and colouration of other ornamental fishes which may interest the ornamental fish feed manufacturers.

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