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Characterisation of Pigment Producing Bacteria Isolated from Bio Floc Ponds and Its Colour Enhancement Effect in *Xiphophorus helleri*

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ABSTRACT

Background: Biofloc technology is a minimal or zero water exchange technology which exerts beneficial effects on water quality, improves feed conversion ratio by recycling microbial protein in the culture of commercially important finfishes and shellfishes. This culture technique also improves the pigmentation in the ornamental fish culture. The presence of pigment producing bacteria and the absorption of the pigments by the ornamental fishes under biofloc technology would pave a long way to boost the production and export market of the high value fishes. Therefore, the present study evaluated the use of bacterial pigments isolated from the biofloc culture in the diets of Swordtail ornamental fish for its colouration and pigment absorption ability.

Methods: A 30 days trial was conducted to evaluate the colour enhancement in swordtail through the supplementation of pigment produced from the bacteria in their diets. Swordtail fishes (1.2±0.01 g) were stocked in 100 litres tanks (20 nos/tank) in triplicates. Fish fed with normal diet served as control and diet supplemented with pigment (50 mg/kg) isolated from the bacteria *Exiguobacterium profundum* (T1), *Chryseobacterium joostei* (T2), *Staphylococcus pasteuri* (T3), *Staphylococcus arlettae* (T4) served as treatments. The characteristic features of the pigments isolated from the four different bacteria were checked and showed enhanced antibacterial, total antioxidant activity and the reductive ability.

Result: Significant difference was observed in water quality parameters except temperature between the experimental groups. Growth parameters showed significant difference between control and treatment. Highest carotenoid concentration was found in fishes fed with pigments extracted from *Staphylococcus pasteuri* compared to other experimental groups. The present study proved the incorporation of bacterial pigments in the diets of Swordtail enhanced the total carotenoid concentration.

Key words: Carotenoids, Colouration, Pigments, Sword tail.

INTRODUCTION

Aquaculture is a globally fast-growing sector consisting of different freshwater and marine water species including finfish, shellfish, mollusks and ornamental fish. The ornamental fishes are characterized by a wide diversity of colours and the increasing demand and success in the economical trade is dependent on the vibrant colour of the fish (Gouveia and Rema, 2005). The ornamental fishes use their colour mainly for signaling during courtship, mating or sexual communication and also used as a strategy to protect from predators (Endler, 1991). Among the ornamental fishes Sword tail has a wide abundance and because of its diverse habitats it can adjust to a wide range of environment in captivity (Gamble *et al*., 2003). The fish skin often shows faded colouration under captive culture conditions due to poor availability or absence of dietary pigments (Halten *et al*., 1997). In most ornamental fishes, the optimum coloration can be achieved by intake of biological pigment in their diet. The pigment supplemented in the diets of fishes not only impart colour but also be easily absorbed and utilized by the body. Certain fishes have no ability to convert beta-carotene to impart colour in such cases they should be supplied with commercially available extracts which induces coloration (Gupta *et al*., 2007). The absorption of supplemented pigments depends on the acid base balance of the feed applied. The addition of pigment concentrations obtained from natural resources (plants and microbes) or synthetic origin were in the range of 60 mg/kg to 700 mg/kg of dry feed (Duffose, 2006). Microorganisms produce different types of pigments including carotenoids, melanin's, flavins, monascins, violacein and indigo based on the environmental factors (Duffose, 2006). Among them carotenoids are most commonly observed which arenothing but the lipid soluble molecules that are highly sensitive to oxygen, heat and light (Ciapara *et al*., 2004). Pigments extracted from microbes acts as protective agents against

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oxidative damage and reported that they have antimicrobial, antioxidant and antibacterial activities (Scolnik and Bartley, 1995). Biofloc technology has been adopted as a novel technique for the intensification of aquaculture. Biofloc involves the biotic and abiotic factors for the improvement of water quality limiting their exchange, growth and survival with improved animal performance against the pathogenic infections. The beneficial effects of biofloc technology in terms of maintenance of optimum water quality parameters, growth and survival has been recorded in many commercially important finfishes and shellfishes (Crab *et al*., 2012). However, adoption of this advanced culture technique for rearing ornamental fishes have not been reported much. Biofloc technology would uplift the breeding and seed production of ornamental fishes and export market of the sector in India. The microbial consortium present in the floc would contain pigment producing bacteria that enhance pigmentation in ornamental fishes and thus it can be applied for rearing high value ornamental fishes. A clear evidence of carotenoid enhancement of *Cyprinis carpio* (Antonyraj, 2016) by rearing them in the biofloc technology was observed. Therefore, the present study evaluated the use of bacterial pigments isolated from the biofloc culture in the diets of sword tail for its colouration and pigment absorption ability.

MATERIALS AND METHODS

Biofloc production

The biofloc was developed in the fresh water pond for GIFT Tilapia culture for a period of four months and maintained. The use of inorganic fertilizers for the faster development of floc with the predominant heterotrophic bacterial community was followed as suggested by (Menaga *et al*., 2020). To maintain the C: N ratio at 10:1, distillery spent wash obtained from M/s. Rajshree BioSolutions, Coimbatore has been used as a carbon source.

Isolation and biochemical characterisation of pigment producing bacteria from biofloc culture ponds

The biofloc culture water from the GIFT Tilapia ponds were serially diluted and spread plated on nutrient agar. The plates were kept for incubation at 37°C. Different morphological and coloured colonies were streaked and the pigment producing bacteria was isolated and morphological characterization was carried out according to Bergey's Manual of Determinative Bacteriology (Brown, 1939).

Genotypic identification and phylogenetic tree construction for pigment producing bacteria

The genomic DNA isolation from pigment producing bacteria was carried out using Phenol-Chloroform method. Then the isolated DNA was amplified using the Forward primer-AGAGTTTGATCCTGGCTCAG and Reverse primer-CGGTTACCTTGTTACGACTT for its 16s rRNA region. Sequencing was done for the amplified DNA and then subjected to BLAST analysis. In order to obtain the accession number, the aligned sequences were submitted in the Genbank database. The phylogenetic tree construction was done to identify the relationship among the Pigment producing bacterial isolates using mega 6.0 software (Kiran *et al.,* 2014).

Experimental site

A 30 days trial was conducted in Advanced Research Farm Facility, Madhavaram at Chennai. Sword tail fish used for the experimental trial were collected from ornamental fish hatchery of Aquatic Rainbow Technology Park (ARTP), located at Madhavaram. Fishes weighing (1±0.05 g) were stocked in culture tanks at the rate of 20 nos/tank (100 litres) for 30 days in triplicates.

Pigment extraction

The extraction of pigment from the bacterial isolates was carried according to (Wei *et al.,* 2005). 24 hrs old culture broths were centrifuged and the pellet was suspended in equal volume of methanol (W/V) and then dried in hot air oven to obtain the pigment.

Diets used during the culture trial

The experimental group includes Control (C) (Commercial diet containing 30% Crude protein), Treatment-1 (T1) - Control diet supplemented with pigment extracted from the bacteria at the rate of 50 mg/kg as per Rashidian *et al*. (2020) from *Exiguobacterium profundum*, Treatment- 2 (T2) - Control diet supplemented with the pigment extracted from the bacteria *Chryseobacterium joostei*, Treatment- 3 (T3) - Control diet supplemented with pigment extracted from the bacteria *Staphylococcus pasteuri*, Treatment-4 (T4) - Control diet supplemented with pigment extracted from the bacteria *Staphylococcus arlettae.* The fishes were fed twice a day at 5% of body weight throughout the trial.

Pigment characterisation

Reductive ability

Reductive ability was carried out with a slight modification using the protocol of (Yildirim *et al*., 2001). The pigment extract (cell pellet suspended in 1 ml of methanol) was mixed with 2.5 ml of phosphate buffer along with 1% potassium ferricyanide and incubated for 30 minutes at 50°C. After incubation 2.5 ml of 10% trichloroacetic acid was added and centrifuged for 10 minutes at 3000 rpm. 2.5 ml of supernatant was diluted with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride was added to it and the absorbance was measured at 700 nm.

Total antioxidant activity

Total antioxidant activity of the pigment extract was evaluated by Prieto *et al.* (1999). To 2 ml of the sample, 1 ml of the reaction mixture (0.6 M of sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added. Control was prepared using methanol along with the reaction mixture and the reaction solutions were incubated at 95°C for 90 minutes. The absorbance was measured at 635 nm using spectrophotometer.

Antibacterial activity

The antibacterial activity of the extracted pigment was checked by well diffusion method (Ahmad *et al.,* 2012). In order to determine, the activity *Aeromonas hydrophila* was streaked on Muller Hinton agar using a sterile swab and 50 µL of the pigment extract was seeded in the wells. After 24 hours of incubation at 37°C, the inhibition zone was calculated.

Physico-chemical parameters of water

Temperature, pH, dissolved oxygen, Alkalinity, Ammonia (NH_4-N), Calcium and Magnesium ion concentrations and Nitrite (NO_2-N) were analysed on weekly basis according to APHA (Olsen, 1979).

Growth parameters

The various growth parameters like Weight gain, Specific growth rate and survival were determined based on the following formula as suggested by (Olvera-Novoa *et al*., 1990).

$$\text{Weight gain (WG in g)} = W_t - W_0$$

$$\text{Specific growth rate (SGR, \% day}^{-1}) = \frac{[\ln W_t - \ln W_0]}{t} \times 100$$

Where,

W_0 and W_t = Initial weight (g) and final weight (g) of fish.

t = Culture time in days.

$$\text{Survival rate (\%)} = \frac{\text{Total number of fish harvested}}{\text{Total number of fish stocked}} \times 100$$

Estimation of total carotenoid concentration

Total carotenoid concentration (TCC) in the fish skin was estimated on weekly basis using the method suggested by (Olson, 1979).

Statistical analysis

One-way ANOVA was performed to find the significant difference between the treatments and control using SPSS version 20.0 for the water quality and growth parameters. The post hoc analysis was done by using Duncan Multiple range test at 5% level of significance.

RESULTS AND DISCUSSION

Out of 42 bacterial isolates identified from biofloc culture system, only four bacteria were identified as pigment producing bacteria. The morphological and biochemical characterization of four bacterial isolates were shown in the Table 1. From Genbank database the accession numbers obtained for the pigment producing bacteria were as follows *Exiguobacterium profundum*- MK256351 (494 bp), *Chryseobacterium joostei*- MK346045 (675 bp), *Staphylococcus pasteuri*- MK629234 (668 bp), *Staphylococcus arlettae*-MK574873 (961 bp). From the phylogenetic tree (Fig 1) constructed for four pigment producing bacterial isolates it is inferred that there is a close relationship found between *Chryseobacterium joostei* and *Staphylococcus pasteuri.* A close relationship is also seen

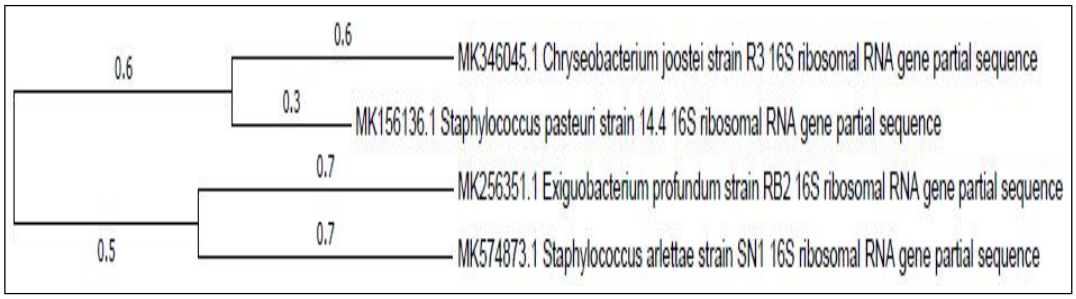

Fig 1: Neighbor joining phylogenetic tree constructed for pigment producing bacterial isolates.

Table 1: Morphological and biochemical characterisation of four pigment producing bacterial isolates.

Biochemical characteristics	*Staphylococcus arlettae*	*S. pasteuri*	*Chryseobacterium joostei*	*Exiguobacterium profundum*
Pigment colour	Yellow	Yellow	Yellowish orange	Orange
Colony morphology	+vecocci	+vecocci	-ve rods	+ve rods
Indole	-ve	-ve	-ve	-ve
Methyl red	-ve	-ve	-ve	-ve
Voges proskauer's	-ve	-ve	-ve	-ve
Citrate utilization	-ve	-ve	-ve	+ve
Glucose	+ve	+ve	+ve	+ve
Adonitol	+ve	+ve	+ve	+ve
Arabinose	+ve	+ve	+ve	+ve
Lactose	+ve	+ve	+ve	+ve
Sorbitol	-ve	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve	+ve
Sucrose	+ve	+ve	+ve	+ve

between *Exiguobacterium profundum* and *Staphylococcus arlettae*. The reductive ability for the pigment producing bacterial isolates was shown in Table 2. The pigment extracted from the bacteria *Exiguobacterium profundum* showed higher reduction ability (0.131) and the lowest reduction was found for *Staphylococcus pasteuri* (0.041). The total antioxidant activity for the pigment producing bacterial isolates was shown in Table 3. The antioxidant activity was found to be higher for the pigment extracted from the bacteria *Exiguobacterium profundum* (406.5 µg) and the lowest activity was found at 250.05 µg for *Staphylococcus arlettae*. The antibacterial activity for the pigment producing bacterial isolates were shown in Table 4. The zone of inhibition was found to be 20 mm for the bacterial isolates such as *Staphylococcus pasteuri* and *Exiguobacterium profundum*. A 30 mm inhibition zone was measured in

Table 2: Reductive ability of the pigment producing bacterial isolates.

Isolates	Absorbance (700 nm)
Staphylococcus pasteuri	0.041
S. arlettae	0.062
Chryseobacterium joostei	0.054
Exiguobacterium profundum	0.131

Table 3: Total antioxidant activity of the pigment producing bacterial isolates.

Isolates	µg
Staphylococcus pasteuri	304.5
S. arlettae	250.05
Chryseobacterium joostei	271.38
Exiguobacterium profundum	406.5

Table 4: Antibacterial activity for the pigment producing bacteria.

Isolates	Zone of inhibition (mm)
Staphylococcus pasteuri	20
S. arlettae	30
Chryseobacterium joostei	30
Exiguobacterium profundum	20

Staphylococcus arlettae and *Chryseobacterium joostei*. The various water quality parameters along with the statistical data were shown in Table 5. Significant difference in the water quality parameters such as pH, alkalinity, nitrite, dissolved oxygen, calcium and magnesium were found between control and treatments. Ammonia concentrations were found to be present in negligible traces throughout the experimental trial in control as well as treatment groups. Growth parameters for the experimental groups were shown in Table 6. Final weight, weight gain and specific growth rate were found to be significantly different between control and treatment. Total carotenoid concentration for the experimental group was shown in Fig 2. The highest Carotenoid concentration was found in the animals supplemented with the pigments extracted from *Staphylococcus pasteuri* compared to other experimental groups.

Pigments obtained from natural sources are usually plant and microbial origin. Microorganisms are potential biopigment producers than plants considering their stability, economic feasibility, availability, degradability, production and purification (Joshi *et al*., 2003; Radha, 2015). Microorganisms are ideal source of variety of biopigments such as carotenoids, quinines, monascins, melanin's, flavins, violancein (Duffose, 2006) as they are produced as a metabolite for survival against microbial community (Manon Mani *et al*., 2015). The present study revolves around the isolation of pigment producing bacteria from the biofloc culture water meeting the increased demand for the color enhancement in ornamental aquaculture. Not, all the bacteria isolated from biofloc were pigment producing yet four strains belonging to cocci and rods were found to produce orange- and yellow-colored pigments in the nutrient agar medium. From the identified strains, *Exiguobacterium profundum* was previously reported to produce the carotenoids (Fatima *et al.,* 2013).

The reductive ability can be directly correlated with the potential antioxidant activity. The reduction of Fe^{3+} to Fe^{2+} was measured using colored metabolite extract. The reductive ability of the pigments was found to be higher in *Exiguobacterium profundum* and this can be attributed due

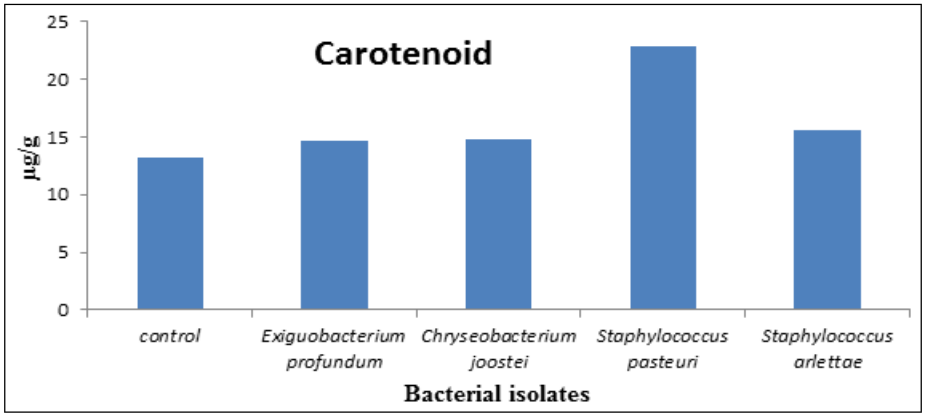

Fig 2: Total carotenoid concentration of different treatments.

to the presence of hydrophilic polyphenolic compounds (Manon Mani *et al*., 2015). Total antioxidant capacity of the colored pigment extract was evaluated routinely using phosphomolybdate method by the reduction of MO (IV) to MO (V). The pigment extracted from *Exiguobacterium profundum* possesses higher antioxidant activity. Antioxidant activity of the pigment can be linked with its effectiveness in interception, prevention and repair mechanisms against injury. Bacterial pigments are also known to inhibit photodynamic lipid peroxidation in liposome thereby protecting against photodamage (Tuli *et al*., 2013; Rajagopal *et al*., 1997). Microbial pigment also stimulates the defence mechanism thereby aiding in protection against oxidative damage (Antonisamy and Ignacimuthu, 2010; De Azevedo *et al*., 2000; Dura'n *et al*., 2003). The antibacterial activity of all the tested pigments showed the zone of inhibition which may be attributed to the presence of antibacterial agents and difference in composition level of the pigments. Highest antibacterial activity was found in the bacterial strains *S. arlettae* and *C. joosti* against *Aeromonas hydrophila,* the predominant pathogen in fishes. Antimicrobial activity of the pigment extracted from *E. profundum* against the growth of *S. typhi, E. coli* and *P. aureginosa* with the inhibition zone of 2.1 cm, 2.5 cm and 1.3 cm has been reported (Manon Mani *et al*., 2015). The findings of the present study can be correlated with the study conducted by (Manonmani *et al*., 2015) who also identified the reductive ability, antioxidant and antagonistic activity of the pigment extracted from *E. profundum*.

Water quality parameters were maintained at optimum levels throughout the culture trial. Temperature acts as an important factor that controls the growth of fishes, as it directly affects metabolism, oxygen consumption and survival. Ideal temperature for the fish growth of 30°C was found in all the experimental groups. Higher levels of alkalinity, calcium and magnesium were found in T2 which may be due to the fact that alkalinity is always in line with levels of calcium and magnesium. T4 has the higher level of pH (7.86) which can also be indirectly correlated with the increased levels of nitrite (0.045 mg/L). In the current study the pigments supplemented in the diets of swordtail showed enhanced growth performance with good survival compared to other treatments except T3 and T4. The negative effect of pigment supplementation on survival in T3 and T4 than control was in line with the study conducted by (Arulvasu *et al*., 2013) who observed *Rosa rubiginosa* when supplemented in the diets of *Xiphophorus helleri* has a negative influence in the survival.

Pigments extracted from *Staphylococcus pasteuri* showed the highest carotenoid enhancement followed by *S. arlettae, C. joostei* and *E. profundum* and it may be due to the highest amount of pigment producing ability of the bacteria among the other bacterial isolates. The pigment producing ability of the bacteria varied based on the metabolites which are produced naturally, under unfavourable conditions and stimulated by the addition of carbon and nitrogen amendments (Ramesh *et al*., 2019). The highest pigment production by the bacteria can also be

Table 5: Water quality parameters of experimental groups for the 30 days culture trial.

Parameters	Control	T1	T2	T3	T4
pH	7.5±0.1^{a}	7.21±0.2^{b}	7.46±0.17^{c}	7.64±0.15^{d}	7.86±0.18^{e}
	(7.2-8.02)	(7.14-8.12)	(7.12-8.21)	(7.38-8.45)	(6.78-8.46)
Temperature (°C)	30.1±0.5^{a}	30.1±0.5^{a}	30.1±0.5^{a}	30.1±0.5^{a}	30.1±0.5^{a}
	(29.9-30.8)	(29.9-30.8)	(29.9-30.8)	(29.9-30.8)	(29.9-30.8)
Alkalinity (mg/L)	75±7^{a}	79±5^{a}	85±9^{b}	81±13^{b}	77±13^{a}
	(70-82)	(70-84)	(76-94)	(64-94)	(72-90)
Nitrite (mg/L)	0.029±0.015^{a}	0.031±0.017^{b}	0.036±0.005^{c}	0.041±0.015^{d}	0.045±0.006^{de}
	(0.024-0.043)	(0.027-0.048)	(0.02-0.041)	(0.016-0.056)	(0.025-0.051)
DO (mg/L)	5.30±0.27^{a}	5.06±0.51^{b}	4.79±0.83^{c}	4.95±0.8^{cd}	5.13±0.32^{abe}
	(5.21-5.57)	(4.98-5.57)	(4.27-5.62)	(4.45-5.75)	(4.99-5.45)
Calcium (mg/L)	75±13^{a}	72±4^{b}	81±11^{c}	78±10^{abd}	80±20^{ce}
	(60-88)	(52-76)	(56-92)	(44-88)	(56-100)
Magnesium (mg/L)	55.5±14^{a}	51.2±23^{be}	65.4±11^{c}	61.8±11^{cde}	58.3±2^{bec}
	(36-69.6)	(40.8-74.4)	(33.6-76.8)	(50.4-72)	(36-60)
Ammonia (mg/L)	-	-	-	-	-

Table 6: Growth parameters with the statistical data for the 30 days culture trial.

Parameters	Control	T1	T2	T3	T4
Initial weight (g)	1.252±0.016^{a}	1.289±0.01^{a}	1.281±0.006^{a}	1.27±0.01^{a}	1.264±0.001^{a}
Final weight (g)	1.30±0.01^{a}	1.751±0.002^{b}	1.796±0.001^{c}	1.616±0.001^{d}	1.399±0.001^{e}
Body weight gain (g)	0.05±0.008^{a}	0.46±0.01^{b}	0.52±0.01^{c}	0.346±0.001^{d}	0.135±0.001^{e}
Specific growth rate	0.125±0.001^{a}	1.02±0.01^{b}	1.13±0.01^{c}	0.623±0.001^{d}	0.338±0.001^{e}
Survival (%)	93.3^{a}	93.3^{a}	90^{b}	86.6^{c}	73.3^{d}

due to the supplementation of distillery spent wash as carbon source in the biofloc systems. However the performance of pigments extracted from *S. arlettae, C. joostei* and *E. profundum* performed well when compared with the control. Previous findings suggest that a diet containing 1.5-2% of a carotenoid rich strain of *Spirulina platensis* and 1% of *Haematococcus pluvialis* significantly improved the intensity of colour in red sword tail (*Xiphophorus helleri*), rainbow fish (*Pseudomugil furcatus*) and topaz cichlids (*Cichlasoma myrnae*). No previous research has been reported in the colour enhancement using bacterial pigments from biofloc systems. The present study spot lighted the new area of research in ornamental aquaculture using bacterial pigments rather than plant pigments.

CONCLUSION

The current study proved the importance of bacterial pigments in the colour enhancement of Sword tail which opened a new avenue in extracting pigments from the microbes. The carotenoid pigment production ability and the performance of isolated bacteria were found in the order of *Staphylococcus pasteuri* > *Staphylococcus arlettae* > *Exiguobacterium profundum* > *Chryseobacterium joostei*. The use of pigments extracted from *Staphylococcus pasteuri* helped in the carotenoid enhancement as well as the growth and survival of Sword tail. Colour enhancement in ornamental fishes through Biofloc technology marked its importance in the use of the bacteria for pigment extraction for commercial aquariculture. The pigments extracted from the bacteria can be an ideal source for the colour enhancement in the ornamental fishes replacing the plant pigments.

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