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Stimulatory Effect of Probiotic Bacterium *Bacillus firmus* CAS 7 on Growth, Survival and Colour of Tomato Clown *Amphiprion frenatus* (Brevoort, 1856)

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ABSTRACT

In the present study, stimulatory effect through dietary administration of probiotic bacteria, *Bacillus firmus* CAS 7 on growth, survival and skin colour of the tomato clown *Amphiprion frenatus* was investigated. A total of four different experiments with different concentrations of probiotic *B. firmus* with with basal diet (25, 50, 100 and 150 mg kg^{-1}) and control (without probiotic) were planned and the fishes were fed for 120 days. The results obtained from the present study suggested that the fishes fed with the diets supplemented with probiotic at 100 and 150 mg kg^{-1} exhibited similar weight gain (21.06 ± 1.4 and 21.09 ± 1.5), specific growth (0.176), survival rate (100%) and feed conversion ratio (0.109), but higher than the control and other experiments. The carotenoid content in fish skin was comparatively higher in experiment III and IV (6.79 mg/g) than other experiments and control (4.48 mg/g). Although the stimulatory effect in terms of growth, survival and colour of fishes fed with diets containing 100 and 150 mg kg^{-1} of probiotic was higher than control and lower concentration, there was no significant difference between them and hence, it is recommended to use at a concentration of 100 mg kg–1 for the enhanced growth and color of the tomato clown *Amphiprion frenatus* which has significant importance in ornamental fish industry. Further study on immune stimulatory effect of this probiotic is under progress for its application in large scale to prevent any undesired effects.

KEY WORDS: *AMPHIPRION FRENATUS*, PROBIOTIC, *BACILLUS FIRMUS*, GROWTH, SKIN COLOR, CAROTENOIDS..

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INTRODUCTION

The demand for marine ornamental fishes has gained a thrust among aquarium hobbyists due to their multitudinal colour and beauty. The annual worldwide market for ornamental marine reef fish has shown a steady increase over the past few years. Therefore, research on the commercial rearing of these fishes is an imminent necessity to save this fragile ecosystem. However, till date efforts in this direction have been extremely limited to very few coral fishes like damsels, neon gobies etc. and that too only in temperate conditions (Hunziker, 1990; Danillowicz & Brown, 1992; Ignatius, 2001).

Probiotics in aquaculture have been reported to provide beneficial effects and the use of probiotics is an important management tool in ornamental fish culture (Balcazar et al., 2006). In general, probiotic administration during early developmental stages is most effective, usually resulting in greater than an order of magnitude increases in survivorship (Gatesoupe, 2007). It has also been reported that in captive rearing, higher mortality occurs frequently (Benetti et al., 2008), and growth abnormalities lead to higher incidence of skeletal deformities (Fernandez et al., 2008). Probiotics as feed supplements benefit the host by improving the feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, growth promoting factors and increasing immune response (Harikrishnan et al., 2010).

Several reports suggested that the probiotics supplementation can reduce the cost of culture by improving the growth and feed utilization efficiency of fish (Mazurkiewicz et al., 2007). The genus, *Bacillus* as putative probiotics has been used extensively as aquaculture feed additives, because of its resistance to high temperature and high pressure (Rengpipat et al., 2000). Dietary supplementation of *Bacillus spp*. improved the growth performance, immunity and disease resistance of fish fish (Ai et al., 2011; Geng et al., 2012). In rainbow trout (*Onchorhynchus mykiss*), significant improvement of feed conversation ration (FCR), specific growth rate (SGR) and protein efficiency ratio (PER) was observed when the fish was fed with diets containing Bacillus spp (Bagheri et al., 2008; Merrifield et al., 2010).

Carotenoids, which are lipid soluble pigments, are responsible for skin colour of ornamental fishes, and can determine their commercial value (Paripatanamont et al., 1999). The ornamental fishes are unable to perform de novo synthesis of carotenoids (CD) like other animals and therefore rely on dietary supply to achieve their natural pigmentation (Goodwin, 1984). Under intensive farming conditions and aquarium rearing, ornamental fish are fed exclusively with compound feeds, which must therefore be supplemented with carotenoids Tropical marine species are beautiful as some marine species, such as members of Pomacentridae, are important in the world trade for ornamental fish (Wilkerson, 2001), and a popular subject of research (Arvedlund et al., 2000). Of which, clown fishes are considered to be most attractions of aquarists, and they are important in the aquarium trade in view of their bright colour, interesting display behavior and their ability adopt in captive conditions (Wilkerson, 1998). The tomato clownfish or, after its scientific name, *Amphiprion frenatus* (Brevoort, 1856), also known as black back anemonefish, fire clown, one band anemonefish, or red clown are under Order Perciformes (Jung, 2006). The tomato clown fish is considered to be major candidate species in ornamental fish industry and it plays a major role in world aquarium trade. Although the utility of probiotics has been recognized in aquaculture by several researchers worldwide, no attempts were made to improve its growth, survival and color through probiotic administration till date. In the present study, an attempt was made to investigate stimulatory effect of probiotic bacteria *B. firmus* CAS 7 on growth, survival and skin color of tomato clown, *Amphiprion frenatus* (Brevoort, 1856).

Table 1. Ingredients used for feed formulation and proximate composition of the prepared basal diet

Ingredients	(g kg^{-1})	Proximate composition of the basal diet	(g kg^{-1})
Fish meal	600	Dry matter	941
Shrimp meal	160	Crude protein	491
Soybean meal	20	Crude lipids	98
Wheat flour	140	Ash	96
Fish oil	40		
Soybean phospholipids	20		
Vitamin mineral mix	10		

Vitamin mineral mix (EMIX PLUS, Mumbai, India) (Quantity per kg)

Vitamin A: 22 00 000 IU; Vitamin D3: 4 40 000 IU; Vitamin B2: 800 mg; Vitamin E: 300 mg; Vitamin K: 400 mg; Vitamin B6: 400 mg; Vitamin B12: 2.4 mg; Calcium Pantothenate: 1000 mg; Nicotinamide: 4 g; Choline Chloride: 60 g; Mn: 10 800 mg; I: 400 mg; Fe: 3000 mg; Zn: 2000 mg; Cu: 800 mg; Co: 180 mg; Ca: 200 g; P: 120 g; L L-lysine: 4 g; DL-Methionine: 4 g; Selenium: 20 ppm.

MATERIAL AND METHODS

Isolation and culture of probiotic bacteria B. firmus CAS 7: The probiotic bacteria, *B. firmus* CAS 7 was isolated from marine environment and identified by both conventional (morphology, physiology and biochemical) and molecular approaches (16S rRNA gene sequence). The 16S rRNA gene sequences of the probiotic strain

CAS 7 obtained from the present study was deposited in NCBI with accession number HQ116811. Further, the probiotic strain *B. firmus* CAS 7 was cultured and prepared as described by (Sun et al., 2010). 500 mL of fresh nutrient broth was seeded with 1% inoculum (1.50 x 106CFU mL-1) and kept in a shaker incubator (200 rpm) at pH 7.5, temperature 28 °C, and salinity 30 PSU for 48 h. After incubation period, the cells were harvested by centrifugation at 5000 xg for 10 min, washed twice with phosphate-buered saline (pH 7.5) and re-suspended in same PBS buffer. The growth of probiotic bacteria was estimated by measuring optical density at 600 nm from the aliquots withdrawn at every 6 h intervals.

Table 2. Morphological, physiological and biochemical characteristics of the probiotic strain CAS 7

Characteristics	Results
Shape	Rod
Gram stain	Positive
Spore formation	+
Motility	+
Glucose	+
Mannitol	+
Xylose	+
Starch Hydrolysis	+
Gelatin Hydrolysis	+
Fat Hydrolysis	+
Casein Hydrolysis	+
Catalase activity	-
Nitrate reduction	+
Indole	+
Citrate	+

Preparation of control and probiotic feed: The control basal diet was formulated using the ingredients such as fish meal, shrimp meal, soya bean meal, wheat flour, fish oil and vitamin mineral mix (Table1) (Sun, Y.Z., et al., 2010). All the ingredients were dried overnight at 80° C in a hot air oven and powdered. The powdered ingredients were sieved through a fine-meshed screen (0.5 mm diameter) and mixed well. The dough was prepared by adding required amount of water with the ingredients, sterilized (autoclave at 121° C for 15 min) and incorporated with 3% (v/w) commercial vitamin mineral mix (EMIX PLUS, Mumbai, India) and pelletized using hand pelletizer to obtain 1 mm pellets. The pellets were initially sun dried and then oven dried at 60 ± 5°C for 12 hours to get moisture content. Further, they were manually broken into smaller bits and stored at room temperature in an air tightened sterile polypropylene containers.

The test feeds for the experiments were prepared by gently spraying the required amount of bacterial suspension on the control diet and mixing it part-by-part in a drum mixer to obtain a final concentration of 25, 50, 100 and 150 mg kg-1 in experiments I, II, III and IV respectively. The probiotic cell suspensions were added to the control diet after the dosage had been autoclaved and subsequently cooled, before pelletizing. The proximate composition (moisture, protein, ash, lipid and fibre) of all probiotic feeds and control feed were determined by the standard procedures of AOAC (1990). The probiotic strain-incorporated feeds were packed in sterile polypropylene containers and stored at 4°C for viability studies.

Experimental setup: The juveniles of tomato clown Amphiprion frenatus (Brevoort, 1856) were obtained from MAV Breeders (Mandabam, Tamilnadu, India) and acclimatized for 4 weeks before the trial at Aquaculture breeding center, CAS in Marine Biology, Annamalai University, India. The feeding experiment was conducted in (20 L) rectangular fibreglass tanks, with temperature ranging from 26 - 30°C, salinity 28 -30 PSU, pH 7.4 - 7.8; and Dissolved oxygen 4.2 to 5.6 mg L^{-1}. A total of 30 fish seeds were maintained in each tank throughout the experiment and each treatment was conducted in triplicate. A total of five trials were made during the study (control and four as experiments).

The fishes were feed with prepared pellet feed alone in control and feed contains probiotic at a concentration 25, 50, 100 and 150 mg kg-1 in experiments I, II, III and IV respectively. The feeding rate was about 3% of biomass per day provided in equal rations at 8.00 AM, 1.00 PM, 6.00 PM for 120 days and the excess diet was collected and dried at 60 °C, put in room temperature for 3 days to restore the natural moisture and then weighed. Daily feed was adjusted every 30 days by batch weighing of fish in each tank after a 24 h period of starvation. Experimental tanks were cleaned and water exchange was done once a week.

Growth indices: The growth parameters such as weight gain, specific growth rate (SGR), survival rate and feed conversion ratio (FCR) were assessed at 30, 60, 90 and 120 days. The weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) was evaluated based on standard formula as follows.

Weight gain = (Final weight – Initial weight)
Specific Growth Rate (SGR) = 100 (ln W2 - lnW1)/T

where, W1 and W2 are the initial and final weight, respectively, and T is the number of days in the feeding.

Feed conversion ratio FCR= total feed quantity given (g)/total weight gain (g).

Colour enhancement and carotenoid content estimation: The color enhancement was monitored by visual examination and estimation of carotenoid content in the skin of experimental fishes. The carotenoid content of the experimental fish skin was extracted according to the method of Torrissen and Naevdal (1984). The fishes were randomly sampled from each experiment per sampling period (30, 60, 90 and 120 days) and used for carotenoid content analysis in triplicate. Briefly, 2 mg of skin were

collected from both sides between the abdominal and dorsal regions of the fish and then transferred to 10 mL of pre - weighed glass tubes after the fat layer had been removed from the skin and ground well with acetone containing anhydrous sodium sulphate and made up to 10 mL with acetone. The samples were stored for 3 days at 4oC in a refrigerator, and then extracted three times till no further colour could be obtained and centrifuged at 5000 xg for 5 min. The total carotenoid content of the samples was determined using spectrophotometer (Shimadzu, UV mini 1240) using extinction coefficients (E1%, 1 cm) of 2000 for astaxanthin (Hata & Hata, 1971) at 475 nm, and 2500 for carotenoids from alfalfa at 450 nm (Schiedt & Jensen, 1995).

Table 3. Growth and survival of tomato clown Amphiprion frenatus (Brevoort, 1856) fed with control (without probiotic) and experiments [basal diet supplemented with probiotic *B. firmus* CAS 7- 25 mg/kg (Experiment - I), 50 mg/kg (Experiment - II), 100 mg/kg (Experiment - III) and 150 mg/kg (Experiment - IV). The values are presented in mean ± SD, n = 3, FCR - feed conversion ratio; SGR - specific growth rate.

Days of culture	Growth Parameters	Control	Experiment I	Experiment II	Experiment III	Experiment IV
			25 mg kg–1	50 mg kg–1	100 mg kg–1	150 mg kg–1
0-30	Initial weight (g)	7.33±0.38	7.61±0.31	7.57±0.29	7.63±0.32	7.89±0.41
	Final weight(g)	10.27±1.6	11.89±1.1	13.49±1.2	15.53±1.4	15.94±1.1
	Weight gain (g)	2.94±1.6	4.28±1.3	5.92±0.29	7.9±0.30	8.05.24±1.2
	SGR	0.098	0.143	0.197	0.263	0.268
	FCR	0.061	0.089	0.123	0.164	0.167
0-60	Survival rate (%)	60	60	85	90	90
	Initial weight (g)	7.33±0.38	7.61±0.31	7.57±0.29	7.63±0.32	7.89±0.41
	Final weight(g)	12.83±1.5	15.13±1.3	16.92±1.6	20.57±1.1	21.56±1.3
	Weight gain (g)	5.5±1.5	7.52	9.35±1.2	12.94±1.4	13.67±1.4
	SGR	0.092	0.125	0.156	0.216	0.228
	FCR	0.057	0.078	0.097	0.134	0.142
0-90	Survival rate (%)	60	60	85	90	90
	Initial weight (g)	7.33±0.38	7.61±0.31	7.57±0.29	7.63±0.32	7.89±0.41
	Final weight(g)	15.24±0.8	17.78±1.3	19.32 ±1.5	24.02 ±1.3	24.56 ± 0.9
	Weight gain (g)	7.91±1.6	10.17	11.75 ±1.5	16.39±1.3	16.67±0.9
	SGR	0.008	0.113	0.131	0.182	0.185
	FCR	0.55	0.070	0.081	0.113	0.115
0-120	Survival rate (%)	60	60	85	100	100
	Initial weight (g)	7.33±0.38	7.61±0.31	7.57±0.29	7.63±0.32	7.89±0.41
	Final weight(g)	18.14±1.3	21.39±1.1	25.94±1.2	28.69±0.9	28.98±1.4
	Weight gain (g)	10.81±1.4	13.78±1.2	18.37±1.3	21.06±1.4	21.09±1.5
	SGR	0.090	0.115	0.153	0.176	0.176
	FCR	0.056	0.071	0.095	0.109	0.109
	Survival rate (%)	60	60	85	100	100

RESULTS

Isolation and growth of probiotic bacteria B. firmus CAS 7: The bacterial strain was isolated from marine sediments of Parangipettai, Tamil Nadu, India and identified as B. firmus based on the morphology, physiological and 16S rRNA analysis (GenBank accession no. HQ116805) and was designated as *B. firmus* CAS 7. The growth of the bacteria was started from the stationary phase itself and it reached the maximum during logarithmic phase (24 - 30 h) (Fig.1). After incubation, cells were harvested from the culture broth by centrifugation (6000 xg), washed and resuspended with PBS buffer after the incubation and added to the basal diet at desired concentration and used for further study.

Growth analysis: In the present study, the fishes were fed with basal diet supplemented with probiotic at four different experiment and control (without probiotic) for 120 days and the growth parameters such as weight gain, SGR and FCR were determined and the results are presented in Table 3. The results suggested that the weight of experimental fishes was increased with the increasing days of culture in control and experiments. It was reported that there was significant weight gained in all experiments where fishes were fed with probiotic supplemented basal feed than control. The weight gain was found to be higher in experiment IV (21.09±1.5) followed by III (21.06±1.4), II (18.37±1.3), I (13.78±1.2) and control (10.81±1.4). Similarly, the fishes in experiment IV and III had significantly higher SGR

(0.176) when compared to the fishes in experiment II (0.153), I (0.115) and control (0.090).

Figure 1: Time course and cell growth of probiotic bacteria B. firmus CAS 7.

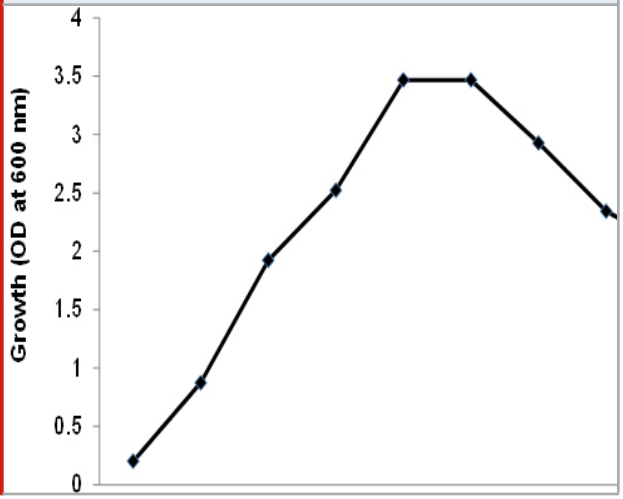
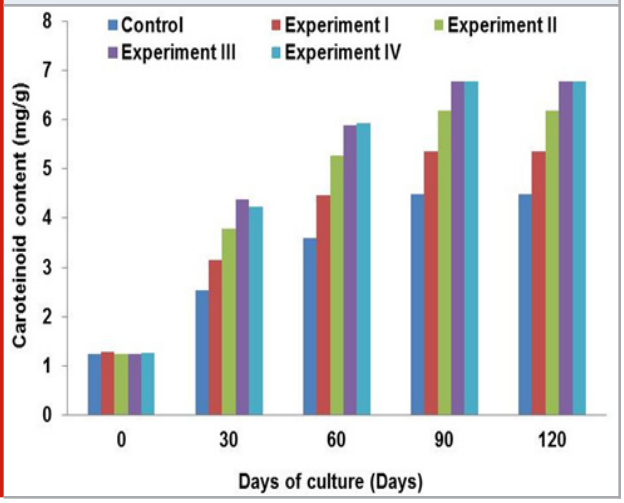


Figure 2: Carotenoid content of skin of tomato clown *Amphiprion frenatus* (Brevoort, 1856) in control and experiments.



Furthermore, feed conversion ratio (FCR) was similar in experiment IV and III (0.29) which comparatively lower than that of experiment II (0.33), I (0.44) and control (0.56). Likewise, survival rate in experiment IV, III, II, I and control was about 100, 100, 85,60 and 60% respectively. It seems that there was no mortality in experiment III and IV, whereas it was higher in experiment I, II and control setup. It seems that the probiotic supplemented in feed significantly stimulated the growth and survival of fishes in experiment III and IV. Although weight gain, SGR, FCR and survival rate was significantly higher in experiment III and IV than control and other experiments, no significant difference was reported when the feed supplemented with 100 and 150 mg kg-1 of probiotic.

Carotenoid content analysis: The results of the carotenoid content of skin suggested that the initial carotenoid content of the fish skin varied between 1.24 and 1.28 mg g-1 in experiments and control and it was increased gradually with increasing days of culture. Further, carotenoid content in experiments I, II, III, IV and control were about 5.3, 6.18, 6.79, 6.80 and 4.48 mg g-1 respectively (Fig. 2) at the end of experiment. It seems that the carotenoid content of fish skin in experiment III and IV where the feed was supplemented with 100 and 150 mg kg^{-1} of probiotic was comparatively higher than control and other experiments.

DISCUSSION

In the past decade of years, there are few reports on successful larval rearing of marine ornamental fishes such as *Amphiprion clarkii*, *A. percula* (Alava & Gomes, 1989; Malpass, 1996; Allen, 1998), *Dascyllus albisella* and *D. aruanus* (Danillowicz & Brown, 1992) in temperate waters. The benefits of probiotics in fish farming are improvements of growth performances, immunity and pathogen exclusions (Qi et al., 2009; Sun et al., 2010). *Bacillus* species significantly improved the growth in tilapia (Aly et al., 2008), *Catla catla* (Bandyopadhyay & Mohapatra, 2009), *Labeo rohita* (Ghosh et al., 2003), *Macrobrachium rosenbergii* (Keysami, M. A. et al., 2007) and *Penaeus monodon* (Rahiman, K. M. M. et al., 2010).

The probiotic bacterium, *B. firmus* CAS 7 isolated from marine environment was used as feed supplement to evaluate the stimulatory effect on growth, survival and skin colour of tomato clown *Amphiprion frenatus* (Brevoort, 1856). The growth studies suggested that the maximum cell growth was achieved at logarithmic phase (24th h). Similarly (Elayaraja, S. et al., 2011), studied the effect of B. cereus on the growth of polycheate and maximum cell growth as well as enzyme production at late logarithmic phase (36th h). Thus, maximum growth of this bacterium at shorter incubation period makes this a potential probiotic candidate species which could be used in the aquaculture industry.

In the present study, the fishes were fed basal diet supplemented with probiotic *B. firmus* CAS 7 at 25, 50, 100 and 150 mg kg^{-1} and the growth performance such as weight gain, survival rate and color enhancement were evaluated for 120 days. The weight of fishes in experiment III (28.98±1.4) and IV (28.69 ± 0.9 g) was significantly higher than experiment II (7.57 ± 0.29 to 25.94 ± 1.2 g), I (7.61 ± 0.31 to 21.39 ± 1.1 g) and control (7.33 ± 0.38 to 10.81 ± 1.4 g). Moreover, the weight gain (21.09 ± 1.5 g) and survival rate (100%) were also significantly higher in experiment III and IV where fishes were fed with 100 and 150 mg kg-1 of probiotic mixed with basal diet respectively when compared other experiments and control. The specific growth rate (SGR) were significantly higher in fishes reared in experiment III and IV (0.176) followed by II (0.153), I (0.115) and control (0.090).

Likewise, the FCR rate was in the range of 0.83 – 0.56, 0.57 -0.44, 0.41 – 0.33, 0.31-0.29 and 0.30 - 0.29 respectively in control, experiment I, II, III and IV. (Jafaryan et al., 2008), also reported that the probiotic (*Bacillus sp*.) supplemented diet significantly increased the weight, length and SGR of fish when compared to the control diet. (Giri, S.S. et al., 2013), suggested that the probiotic supplementation needs to be done for 60 days to obtain a significant improvement in SGR and FCR. Several studies suggested have that the probiotic *Bacillus sp*. supplementation has significantly increased the weight gain and SGR in *Rachycentron canadum* (Geng, X. et al., 2012), *Labeo rohita* (Giri, S.S. et al., 2013), *Oreochromis niloticus* (Aly et al., 2008), *Epinephelus coioides* (Sun, Y.Z. et al., 2010) and *Larimichthys crocea* (Ai et al., 2011). Thus, the reduction in FCR of fishes in experimental groups revealed dietary nutrients were utilized more efficiently when the diet was supplemented with probiotics.

Carotenoids are commonly found in pigmented bacteria which are known to have a positive role in the intermediary metabolism of fish that could enhance nutrient utilization and may ultimately result in improved growth (Steiger, S. et al., 2012). The results of stimulatory effect on skin color study suggested that the carotenoid content in the skin of fishes fed with probiotic maintained coloration during periods of social interaction, suggesting that the probiotics may play important roles in maintaining fish skin coloration. After 30 days, the carotenoid content in the skin of fishes fed with probiotic supplemented feed exhibited increasing pigment levels and started to differ from that of control diet. The carotenoid content of fish skin was about 6.80 mg g-1 in experiment III and IV, whereas it was 4.48 mg g-1 in control. Thus, the results confirmed that the color enhancement in terms of total carotenoid content in the fish skin increased significantly with increasing probiotic bacteria concentration in the basal diet. Support to the above fact, (Hong, 2008), reported that the yellow/orange carotenoids formed by *B. indicus* HU36 significantly increased the pigmentation of the experimental fishes.

The result obtained from the present study suggested that the fishes fed with basal diet supplemented 100 and 150 mg kg^{-1} of probiotic *B. firmus* exhibited better stimulatory effect on growth, survival and skin colour of *Amphiprion frenatus*. Although the fishes fed with diets containing 100 and 150 mg kg^{-1} of probiotic exhibited higher effect than control and lower dosage, no significant difference reported between 100 and 150 mg kg^{-1} and hence, it is recommended to use at a concentration of 100 mg kg^{-1} for the enhanced growth and color of the tomato clown *Amphiprion frenatus* which has significant importance in ornamental fish industry.

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