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Combined Effect of Papain and Vitamin-C Levels on Growth Performance of Freshwater Giant Prawn, *Macrobrachium rosenbergii*

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Abstract

Present study was conducted to evaluate the combined effect of proteolytic enzyme, papain and vitamin-C on freshwater giant prawn, *Macrobrachium rosenbergii*. Post-larvae of *Macrobrachium rosenbergii* of average weight 9.5-16.3 mg initial weight were fed with eight different diets supplemented with papain 0.0%, 0.108%, 0.3%, 0.5%, 0.0%, 0.108%, 0.3%, 0.5% and vitamin-C 0.0%, 0.0%, 0.0%, 0.0%, 0.2%, 0.2%, 0.2% and 0.2% for feeds F1 to F8 respectively. Before the start of the experiment, prawns were reared using papain and vitamin-C free diet in order to deplete their vitamin-C stores. The same diet formulation was used in all treatments containing soybean meal, fish meal, shrimp meal and wheat flour as the major ingredients. Feeding level was kept same for all the groups of prawn and fed to satiation. Protease specific activity after one hour of feeding at the end of the experiment was estimated, which have shown a higher value in comparison to the protease specific activity one hour before feeding at the beginning of the experiment. The results obtained after feeding trial demonstrated improved growth ($P<0.05$) at higher concentration (F7 and F8). However, the growth of post-larval stages of prawn fed only vitamin-C or papain showed lesser growth than those of the combined effect. Survival was recorded between 46-56% in all the treatments. No growth reduction or deficiency signs were observed. This finding is the first report of its kind where a combined effect of papain and vitamin-C showing a synergistic effect on the growth performance. Further standardization and commercialization of this finding, through economically viable methods, is needed to improve the growth performance of this premium commodity.

Keywords: *Macrobrachium rosenbergii*, papain, vitamin-C, protease, growth.

Introduction

In intensive prawn culture operations, one of the foremost requirements is the availability of properly formulated practical feeds for different growth stages of prawn. The efficacy of the diet not only depends on the nutrient composition and nutrient balance, but also on the effective utilization of the nutrients by the animal. In the utilization of dietary nutrients, the digestive enzymes play a vital role in catalyzing the hydrolytic reactions splitting the macromolecules into simple absorbable form of molecules. The activity of these biocatalysts is regulated by many physical, chemical and biological factors and thus a shift from these optimum conditions necessary for these enzymes may affect their activity, thereby correspondingly modify the digestibility of nutrients supplied to the animals (Hemambika and Paul Raj, 1993). There are several reports on the use of exogenous proteolytic enzyme in post-larval stages of *Penaeus monodon*. (Hong-Yung and Hsian-Fu, 1990, Fang and Lee, 1992). It is very well reported that proteolytic enzyme of exogenous origin plays an important role in feed digestibility in fishes like utilizing trypsin (Dabrowski and Glogowski, 1977a), papain (Srivastava *et al*., 1994) in common carp diet. Protease activity in the digestive tract is a key determinant of the digestibility and assimilation efficiency of ingested proteins. Dabrowski (1984) suggested that incompletely found digestive tract contain insufficient digestive enzymes to completely digest dry feeds. According to several authors, enzymes from live animals contributed to the digestion process by autolysis or zymogen activation (Janacarik, 1964; Dabrowski and Glogowski, 1977a; Kolkovski *et al.*, 1993; Person-Le Ruyet *et al.*, 1993). In fish and Invertebrates, some mammalian digestive protease-like enzymes have been described (Ikeda *et al.*, 1986; Gildberg, 1998).

Vitamin-C is an indispensable nutrient required

to maintain the physiological processes of different animals (Tolbert, 1979). Fishes and crustaceans are incapable of biosynthesis of ascorbic acid since they do not have the enzyme L-gulonolactone oxidase, which is responsible for synthesis of vitamin-C. (Wilson, 1973). In crustaceans, vitamin-C influences the alkaline phosphatase activity during synthesis of chitin (Paul Raj, 1997).

A dietary requirement for vitamin-C has been reported for several species of crustaceans (Tacon, 1987). Crustaceans fed diets deficient in vitamin-C develop melanized lesions distributed throughout the collagenous tissue underlying the exoskeleton, decolourization and abnormal colourization and mortality (Deshimaru and Kuroki, 1979; Lightner *et al.*, 1979, Heinen, 1984; Shiguein and Itoh, 1988). Most aquatic animals including shrimps-prawn require a dietary source of vitamin-C to prevent the development of deficiency symptoms; such as melanized lesions throughout the collagenous tissue underlying the exoskeleton, reduced growth, poor wound healing capacity and eventually, mortality (Hunter *et al.*, 1979; Margarelli *et al.*, 1979; He and Lawrence, 1993; Shiau and Hsu, 1994). Till date, the ascorbic acid requirements of farmed species of shrimps have been studied only from juvenile stage onwards (Merchie *et al.*, 1995). Quantitative estimates of vitamin-C requirement reported for crustaceans include 5000-10000 $mg.kg^{-1}$ of diet for Penaeus japonicus (Guary *et al*., 1976) 1000-2000 $mg.kg^{-1}$ diet for *P. californensis and P. stylirosris* (Lightner *et al.*, 1979) and 215 to 430 $mg.kg^{-1}$ diet for Penaeus japonicus (Shiguein and Itoh, 1988). Recommended dietary ascorbic acid (AA) levels for shrimp using ascorbic acid-polyphosphate and crystalline ascorbic acid are 20 and 120-130 mg $AA.kg^{-1}$ for the post-larvae of tiger shrimp, *Penaeus monodon* and white shrimp, *Penaeus vannamei* respectively (He and Lawrence, 1993; Lavens *et al.*, 1998, 1999). Reported vitamin-C requirements for crustaceans are generally at least ten fold higher than those reported for various species of fish (Tacon, 1987). The estimated dietary requirement for *Macrobrachium rosenbergii* falls with in the range (60 -150 mg) vitamin-C activity per kg of diet reported for several species of fish, and found in tissue of clams and adult brine shrimp as calculated by Conklin (1998).

Considerable work has been done on the utilization of exogenous enzymes in fish and shellfish diet (Janacarik, 1964; Dabrowski and Glogowski, 1977a,b; Kolkovski *et al.*, 1993; Person-Le Ruyet *et al.*, 1993) and also on dietary supplementation of vitamin-C, which are evident from various reports. (Merchie *et al.*, 1997a; Blom *et al.*, 1999; Adham *et al.*, 2000), but none of the reports are available on the combined effect of supplemented proteolytic enzyme, papain and vitamin-C, neither in fish nor in crustaceans.

Thus, the present study has been undertaken to evaluate both the individual and combined effect of dietary incorporation of papain and vitamin-C in post-larval stage of *Macrobrachium rosenbergii* in terms of growth, deposition of nutrients in terms of flesh and survival. Here, selected digestive enzymes activities in post-larvae before and after feeding the diets with and without dietary proteolytic enzyme, were assessed.

Materials and Methods

Experimental Setup

The experiment was carried out using freshwater giant prawn post-larvae with average size between 9.4±0.92 and 16.3±5.43 mg over a period of 40 days at Aquafeed laboratory, Department of Fish Nutrition and Biochemistry, Central Institute of Fisheries Education, Mumbai, India. It was set up in 8 distinct groups each with 3 replicates. Uniform sized plastic pools of 50 liters capacity were used for the experiment. All the pools were cleaned and filled with bore-well water up to 30 liters. Each of the pools was stocked with 25 prawns at 26 + 1°C for a period of 40 days. Aeration was provided throughout the period with 2HP air blower. Experimental pools were supplied with chlorine-free bore-well water with a flow rate 1 $liter.min^{-1}$ throughout the experimental period. Each of the pools was covered with perforated cover to prevent the animals from jumping out. Physico-chemical parameters were monitored and recorded. Poly-Vinyl-Chloride pipes were used as hideout for prawns. The post-larvae were starved for a day before taking the initial body weight. Weighing of prawns was carried out in an interval of 20 days to assess the growth during the experimental period of 40 days. Prawns were anesthesized with Tricaine methane- sulfonate (MS-222) @ 50 mg/L.

Experimental Diet

Papain (extracted from Carica papaya), Betaine Hydrochloride and L (+) Ascorbic acid were procured from E-MERCK Darmstadt, Germany. Nachini (local name) was used as natural feed attractant. Details of the diet components were given in Table 1. Diet F1 was used as control. All the ingredients were dried in hot air oven at 80°C for 8 hours, powdered and sieved through a 60 µm mesh size nylon netting to get a powder. All the ground ingredients used for basal diet were analyzed for their crude protein content before preparing the feed. Accordingly, eight isonitrogenous diets were prepared to keep approximately 38% protein content and approximately 8% fat. All the ingredients were mixed thoroughly with required amount of water to make dough, which were passed through a twin-screw extruder having a die of 2 mm size (Basic Technology Pvt. Ltd, Kolkata, W. B.). Pellets obtained were dried at 60°C in a hot air oven overnight and stored in a cool place till their use. Feeding were done up to satiation and was adjusted

Table 1. Composition of experimental diets (% DM basis)

Ingredients	Inclusion rate (%)							
	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8
Soybean meal	30.0	30.0	29.9	29.9	29.9	29.9	29.9	29.8
Fish meal	29.0	29.0	28.9	28.9	30.0	28.9	28.9	28.8
Acetes	15.0	15.0	15.0	14.8	16.0	15.0	14.8	14.8
Wheat flour	10.4	10.28	10.3	10.3	10.3	10.28	10.3	10.28
Cod liver oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Carrot powder	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Soya lecithin	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin mixture*	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Nachini	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sucrose	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Betaine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Papain	-	0.108	0.3	0.5	-	0.108	0.3	0.5
Vitamin- C	-	-	-	-	0.2	0.2	0.2	0.2
Protein	38.3	37.9	39.1	38.2	37.8	38.5	40.1	39.2
Fat	8.21	8.88	7.11	6.14	7.94	7.76	6.96	7.04
Fibre	10.94	8.04	9.16	8.95	8.15	11.07	7.85	10.84
Moisture	8.3	7.8	7.6	7.1	8.0	8.3	7.5	8.0
Ash	15.34	15.25	13.73	12.33	15.75	13.32	12.56	14.72

*Composition of vitamin mineral mixture: Vitamin A: 20,00,000 I. U. , Vitamin D_3:4,00,000 I.U., Vitamin B_2: 0.8 g, Vitamin E: 300 I.U., Vitamin K: 0.4 g , Calcium Pantothenate: 1.0 g, Nicotinamide: 4.0 g, Vitamin B_{12}: 2.4 g, Choline chloride: 64.0 g, Calcium: 300.0 g, Manganese: 11.0 g , Iodine: 0.4 g , Iron: 3.0 g, Zinc: 6.0 g , Copper: 0.8 g , Cobalt: 0.16 g (Source: Sarabhai Chemicals, Baroda, INDIA)

on daily observation of feed intake of post-larvae by visual estimation.

Crude fat in the feed was estimated by Soxtec system (Model HT2, 1045 extraction unit Foss Tecator, Sweden) using diethyl ether as a solvent (boiling point 55°C), dry weight by drying at 1050 C for 6 hours, ash by burning for 6 hours at 600°C, crude fibre by the method suggested by Bennik (1994), using Fibretec system (Model 1017 Hot Extraction, Foss Tecator, Sweden). Carcass protein was estimated by the method of Lowry *et al.* (1951). Bovine serum albumin was used as the standard. Nitrogen in the feed was estimated by Micro Kjeldahl method using Foss Tecator 2200 Kjeltec system and the crude protein is calculated as N x 6.25, as suggested by Chang (1994).

Digestibility of dietary protein by post-larvae was assessed by protease assay developed by Peterson (1977). Tyrosine calibration curve was used to evaluate the data. The results of the specific activity are given in µg of liberated tyrosine mg^{-1} of dissolved protein.$minute^{-1}$. Lipase activity was found out by Cherry and Crandall (1932), using olive oil emulsion.

Data were processed for Analysis of Variance (ANOVA) and significance were tested for various growth parameters and survival by the method described by Snedocor and Cochran (1961).

Results and Discussion

Composition of Diet

The proximate composition of different experimental diets is given in the Table 1. The estimated crude protein of different experimental diets varied from 37.8-40.1%. It is reported that required level of protein in post-larvae is a little higher than the grow-out stages. However, the optimum requirement of crude protein in the diet for *Macrobrachium rosenbergii* is found to be in the range 23.8-38.5% (Corbin *et al.*, 1983), 30-35% (D'Abramo and Reed, 1988; Freuchtenicht *et al.*, 1988). The ether extract in the experiment was found to be in the range of 6.14% to 8.88%, which is in the optimum range for *Macrobrachium rosenbergii* PL as suggested by D' Abramo and Sheen (1989) and Sebastian (1996). In the present study, the fibre content was in the range from 7.85% to 11.07% as suggested by Sebastian (1996). Fair *et al.* (1980) reported that the incorporation of crude fibre in to the diet up to 30 % was showing good result in terms of growth performance. The soluble carbohydrates of different experimental diets were in the range of 18.91% to 27.28%. For better growth performance in *Macrobrachium rosenbergii*, optimum range of soluble carbohydrate in the feed was found to be up to 40% (Briggs, 1991).

Enzyme Activity

The specific protease activity at the beginning of the experiment was found to be 0.279 IU.μg^{-1} protein (F4) to 0.4316 IU.μg^{-1} protein (F8) and 0.4279 IU.μg^{-1} protein (F1) to 0.7036 IU.μg^{-1} protein (F7) at the end of the experiment as shown in the Figure 1. The lipase activity at the beginning of experiment was found to be 0.005 IU.μg^{-1} lipid (F2 and F3) to 0 0.015 IU.μg^{-1} lipid (F6) and that at the end was found to be 0.0.15 IU.μg^{-1} lipid (F1and F5) 0 0.015 IU μg^{-1} lipid (F6 and F8) as shown in Figure 2. The effect of exogenous

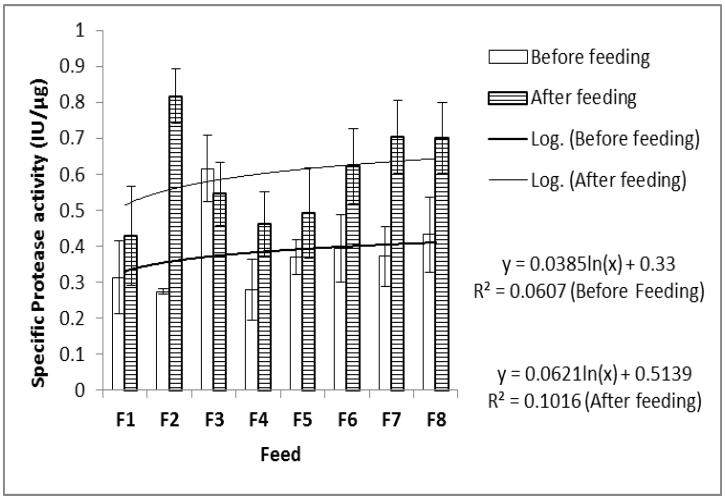

Figure.1. Protease specific activity of the *M. rosenbergii* PL before and after feeding trial.

digestive enzymes on the endogenous digestive enzyme activities of the shrimp, however, has been less clear and quantitative correlation between digestive enzyme activities and shrimp growth is also not conclusive (Dabrowski, 1979; Maugle *et al.*, 1983 b). It is not clear whether shrimp growth could be positively correlated to the digestive enzyme positively. Many studies, however, have failed to support the correlation (Maugle *et al.*, 1983b; Lee *et al.*, 1984). The use of compounded diets to replace Artemia nauplii and other live food organisms for the larval and post-larval Penaeus monodon rearing has become popular. However, the quantity of exogenous enzymes received through them is decidedly lower. Maugle *et al.* (1983a,b) have reported that studies of diets with micro encapsulated amylase and bovine trypsin have shown increased growth in Penaeus japonicus juveniles. The present study concerns to evaluate the effect of incorporation of proteolytic enzyme, papain in *Macrobrachium rosenbergii* post-larval stage through diet on growth and survival and the effect of these feed supplements on the digestive enzyme activities of the post-larval prawn. Results reveal that there is an increase in growth performance as well as elevation in protease activity. Total protease activities, however, reflected differences associated with enzyme supplements, while the difference in lipase activity could not be correlated. The concentration of protease in the total tissue homogenate was elevated. This result has similarity with findings of Janacarik (1964); Dabrowski and Glogowski (1977a); Kolkovski *et al.* (1993) and Person-Le Ruyet *et al.* (1993) gives concurrence to the present findings. Contrary to this, (Kolkovski, *et al.*, 2000) reported reduced growth while supplementing exogenous source of enzyme. The lipase activity in the tissue homogenate was found increased but it has no correlation in the body weight gain. It is known that dietary compositions have obvious influence on digestive enzyme activities. Lee *et al.* (1984) concluded that the protein levels influenced enzyme activity in Penaeus vannamei of all sizes, while the protein source had a greater influence on the enzyme activities in shrimp less than 10 g.

Growth Parameters

The body weight of experimental treatments recorded at 20 days intervals have been given in Figure 3. A trend of higher body weight gain was observed in F7 treatments compared to other treatments at the end of the experiment (60.75±1.93 mg). Comparative growth parameters of different experimental groups are as shown in Table 2. The absolute growth rate of different experimental treatments was found to be in the range of 9.8±0.3 mg and 48.8±5.15 mg. The highest and the lowest values were recoded in F7 and F2 respectively. The difference was found to be significant ($P<0.05$). Lobao *et al.* (1995) reported that the experiments in *Macrobrachium rosenbergii* with the diets containing vitamin-C @ 3 $g.Kg^{-1}$ have shown best growth performance. It is suggested that, on adding microbial enzyme in prawn diet (1 mg.g-1), the growth was found to increase by 13.87% (Zhong-Jhun, *et al.*, 1994). The relation of feed intake and weight gain, i.e. FCR of different treatments were recorded in the range of 2.07 (F7 treatment) to 3.5 (F2 treatment), the difference was not statistically significant ($P>0.05$). The highest value of protein efficiency ratio of different experimental treatments (PER) was recorded in F2 treatment (0.45±0.04) while the lowest was observed in F3 treatment (0.15±0.02). The difference between the treatments was found to be significantly different ($P<0.05$). The survival (%) of the post-larvae of different treatments has been presented in Table.2 and Figure 4. The highest survival was recorded in F4 treatment (56%) whereas the lowest was recorded in F3, F7 and F8 treatment (46%), however, the survival of different groups did not differ significantly ($P>0.05$). Low survival reported in the experimental trial can be due to the stress during handling and weight measurement.

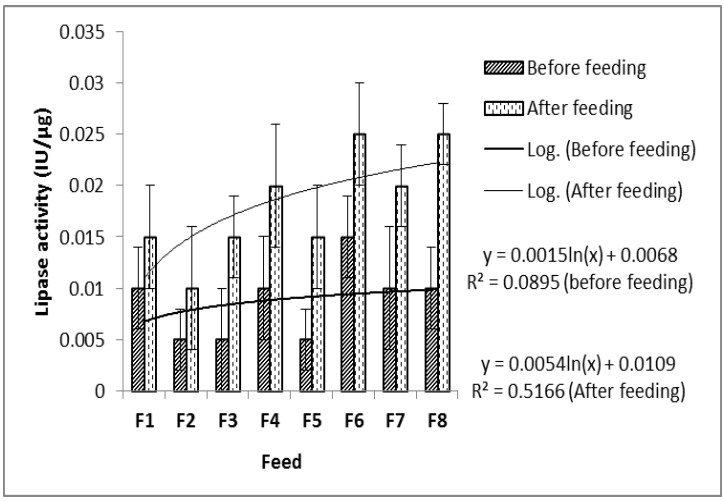

Figure 2. Lipase activity of the *Macrobrachium rosenbergii* PL before and after feeding trial.

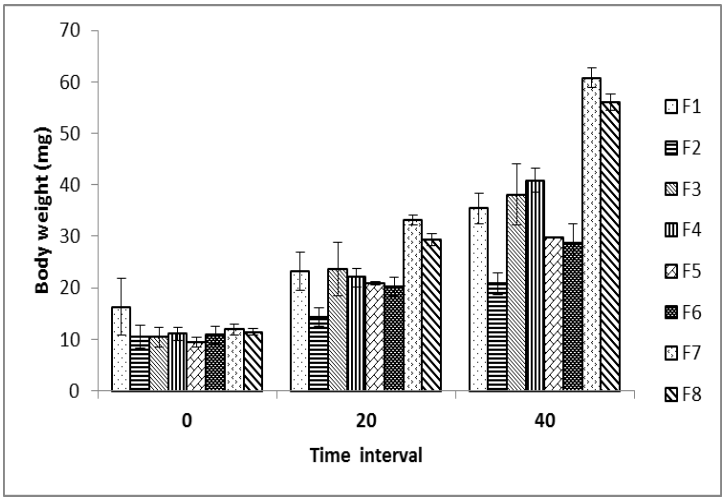

Figure 3. Body weight gain(mg) of different experimental groups.

Table 2. Comparative growth parameters for different experimental groups

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8
Per day increment (mg)	0.47±0.3^a	0.24±0.009^a	0.69±0.3^a	0.74±0.15^a	0.50±0.04^a	0.44±0.08^a	1.22±0.1^b	1.1±0.09^b
Specific growth rate (%)	2.34±1.8^a	1.7±0.43^a	3.26±1.2^a	3.29±0.6^a	2.8±0.3^a	2.4±0.1^a	4.09±0.4^a	3.9±0.2^a
Absolute growth (mg)	19.1±5.2^a	9.8±0.3^a	27.7±13.5^a	29.8±6.2^a	20.2±1.6^a	17.8±3.3^a	48.8±5.15^b	44.6±3.8^b
FCR	2.8±0.4^a	3.5±0.1^a	2.12±0.01^a	2.34±0.1^a	2.97±0.4^a	3.1±0.6^a	2.07±0.08^a	2.77±0.1^a
FER	0.36±0.0^a	0.28±0.01^a	0.47±0.002^b	0.43±0.02^b	0.35±0.04^{ab}	0.33±0.06^a	0.48±0.02^b	0.36±0.01^b
PER	0.23±0.09^a	0.45±0.04^b	0.15±0.02^a	0.22±0.07^a	0.24±0.05^a	0.26±0.04	0.18±0.03^a	0.26±0.0^a
Survival	48±4.16^a	52±2.13^a	46±1.15^a	56±2.3^a	48±2.3^a	48±1.7^a	46±3.05^a	46±1.15^a

Superscripts in a row with different alphabets indicate significant difference (P<0.05).

Requirement of Ascorbic Acid

According to Merchie *et al.* (1997b), higher levels of ascorbic acid levels are required in post-larvae during metamorphosis than in larval stages. However, vitamin-C requirement of juvenile freshwater prawns is much lower than previously reported for other crustaceans. The lower requirement is more likely attributed to the source of vitamin-C used rather than interspecific differences. Previous reports of vitamins-C requirements of crustacean species were based upon the studies that used sources that are less stable and more subject to leaching, circumstances likely leading to over estimates of the requirement. Moreover, the estimated dietary quantitative requirement for *Macrobrachium rosenbergii* falls in the range (60-150 mg Vitamin-C activity.kg^{-1} diet) reported for several species of fish,

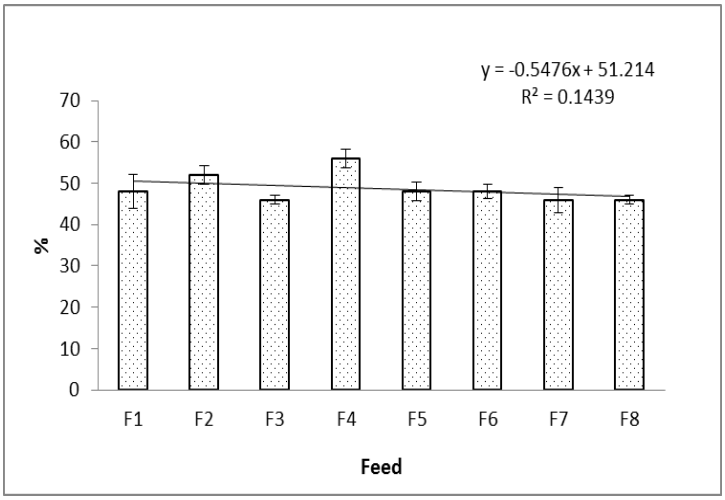

Figure. 4. Survival rate in percentage of different experimental groups during the feeding trial.

and found in tissue of clams and adult brine shrimp as calculated by Conklin (1998) from published values. In the present feeding trial, we have incorporated high dose of vitamin C to make sure the availability of ascorbic acid to the animal. D' Abramo *et al.* (1994) suggested that by adding high level of L- crystalline ascorbic acid in the diet would be sufficient enough to compensate for the assumed rapid loss due to high rates of leaching. The low requirement in comparison with the supplement suggest that even if 98% of the crystalline Vitamin-C were lost due to leaching prior to the time before consumption, a sufficient amount would remain to satisfy the requirement.

Field trials on this study in pond conditions are required for further standardization of this work. Later, this enzyme application in crude form in combination with vitamin-C can be suggested to the farmers for better feed conversion ratio of feeds used.

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