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IMPACT OF PROBIOTICS ON GROWTH PERFORMANCE OF ZEBRA FISH (DANIO RERIO)

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Abstract:

The present study deals with isolation, identification, enzymatic, antibacterial and molecular characterization of intestinal bacteria of Zebra fish and its growth performance. Isolated five different intestinal bacteria of zebra fish (*Aeromonas* sp., *Pseudomonas* sp., *Micrococcus* sp., *Escherichia coli sp*., and *Bacillus* sp.,) and were further identified through biochemical tests and enzymatic activity (Amylase, lipase and protease). Antibacterial activities (Klebsiella pneumonia, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus and E. coli) of selected five different bacteria were studied through Agar well diffusion method. Among five different bacteria, enzymatic (amylase and lipase) and *in-vitro* antibacterial activity of *Pseudomonas sp.* was higher and its molecular characterization also examined, new bacterium was found. Selected new bacterium was mass cultured. Six feeds were prepared with six different quantity (F1 - Control, F2 - 1ml, F3 - 2ml, F4 - 3 ml, F5 - 4ml of *Pseudomonas sp.* and F6 - 1ml of Yeast). Feed were given for Zebra fish and growth studies were carried out for a period of 45 days. The feed consumption, feed conversion ratio, feed conversion efficiency, weight gain, percentage growth, relative growth rate, assimilation, metabolism, gross and net growth efficiency were higher in F5 containing 4 ml of *Pseudomonas sp*. (07.67 ± 0.56, 04.09 ± 0.52, 0.03 ± 0.01, 0.17 ± 0.04, 33.52 ± 11.84, 0.08 ± 0.02, 6.61 ± 0.55, 5.98 ± 0.58, 2.90 ± 0.97 and 2.32 ± 0.74). ANOVA was studied for FC, WG, GGE and NGE. These results indicate that probiotic (intestinal bacteria) feed enhance the growth of Zebra fish.

Key Words: Isolation, Enzymatic and Molecular Characterization, Antibacterial Activity & Growth Performance

Introduction:

Ornamental fish culture in India has shown a rapid progress during past few years but some major problems are hindering the progress path and disease being one of them. Pathogens that may cause disease in trade animals themselves or to other susceptible host encountered in supply chains, at pet shops or end destination aquaria (Katherin F.Smith et. al., 2012). Persistent disease problem, aquatic pollution due to various anthropogenic activities, indiscriminate use of different chemicals and antibiotics constitute the major obstacle in successful aqua farming. Ornamental fishes are susceptible to bacterial, viral, fungal and protozoan infections (Krishna Prema and Ksturi, 2013). It makes the biggest constraint in ornamental fish culture, because water makes very suitable medium for the proliferation of several diseases like dropsy, bacterial kidney disease, bacterial gill disease, pop eye, vibriosis, epizootic ulcerative syndrome, ulcerative disease and fin and tail rot (Gahlawat *et. al.*, 2006). Even though vaccines are being developed and marketed and cannot be used as a universal disease control measures in aquaculture (Abdul Kader Mohiden *et.al.*, 2010). The gut bacteria have a role in nutrition, growth, disease susceptibility in fish as it has been established for homoeothermic species. (Floch et.al., 1970) An understanding of the indigenous micro biota in fish may help to improve feeding and other condition for the intensive rearing of fishes (Kar *et.al.*, 2008). Fish receive bacteria in the digestive tract from the aquatic environment through water and food. Being rich in nutrient the environment of digestive tract of fish confers a favorable culture environment for the microorganisms.

The use of beneficial bacteria to display pathogens by competitive process is being used in the animal industry as a better remedy than administering antibiotics and is now gaining acceptance for the control of pathogens in aquaculture (Suganya *et al*., 2014). Probiotics are live microorganisms, which contributed significantly to increase the animal growth and improve health condition by increasing resistance to aquatic disease such as *Tetraselmis, Bacillus, Lactobacillus, Streptcoccus, Vibrio, Aeromonas* and *Saccharomyces* (Fuller, 1987). Among probiotics, *Saccharomyces cerevisiae* (yeast) is a resistant fungus to antibiotics such as sulfatides and other antibacterial agents. In fish species, probiotics have been shown to be effective in a wide range of growth promoter, immune system enhancement, survival rate and stress resistance (Forough Mohammadi *et al*., 2016). Among the ornamental fishes, zebra fish is an animal model for laboratory studies and also it have genetic similarities with human being. These similarities helpful to study the human diseases and the nutritional value related studies. The study related to the intestinal bacteria and its role on growth and survival rate of zebra fish is totally wanting. Hence, the aim of the present study is the isolation, identification,

enzymatic, antibacterial and molecular characterization of intestinal bacteria of zebra fish and its role on growth.

Materials and Methods:

Collection and Isolation of Fish Intestinal Bacteria:

The study was conducted at Department of Biology, The Gandhigram Rural Institute – Deemed University, Gandhigram, Tamilnadu, India. Experimental fish was collected from A.M. Aqua farm, Madurai, Tamil Nadu, India and transported to the laboratory in polythene bags filled with oxygenated water. Fishes were maintained in the glass tanks fed with control feed. Intestinal sample of healthy fish was collected by dissecting the abdomen of the Zebra fish. The sample was serially diluted and the appropriate dilutions 10^{-6} were selected for the isolation of bacteria. The serially diluted sample was sterilized, plated in the Nutrient agar medium and incubated at 37^{o}C for 24 hrs. After incubation the colonies were identified and five different colonies were selected for further tests.

Biochemical Characterization:

The predominant colony were selected and identified based on morphological, microscopic and biochemical characteristics like Gram staining, Indole, Methyl red, Voges-proskauer, Citrate, Catalase, Starch, Gelatin hydrolysis, Oxidase, Lactose and Sucrose test.

Enzymatic Activity:

Based on the biochemical results, selected bacterial isolates were employed for amylase, lipase and protease tests.

Antibacterial Activity:

Five different bacterial aquatic pathogens (*Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus* and *E. coli*) were selected for the antibacterial activity (Agar well diffusion method) of the selected five different bacterial species isolates from the intestinal content of zebra fish.

Mass Multiplication and Feed Preparations:

Based on higher enzymatic and inhibition activity, *Pseudomonas* sp., was selected and mass multiplied by inoculating in nutrient broth. For growth studies, fishes were acclimated in glass aquaria (60×45×45 cm) for a period of 15 days at 28 ± 20^{0} C. During acclimation, fishes were fed with trainee feed containing fish meal, groundnut oil cake, wheat flour and rice bran in the form of dry pellets. One control (without bacteria), four experimental feeds by using different quantity (F2 - 1ml, F3 - 2ml, F4 - 3 ml, F5 - 4ml) of isolated bacteria and one feed by using commercially available probiont (yeast) was prepared according to square method (Ali, 1980).

Molecular Characterization:

The intestinal bacteria *Pseudomonas sp.*, were examined for molecular characterization using 16S rRNA.

Statistical Analysis:

The experimental results are presented in the form of tables and graphs using Microsoft Excel (Version 2007). Mean, Standard deviation and T- test were also calculated with the help of the same tool, One-way ANOVA was used for the analysis using Duncan multiple range test (1955) according to Obe, *et al.*, (2015). The data was input manually and computed. The output results obtained from the software indicate whether the difference is between the treatments and days. Sum of square variations (SS), Degree of freedom (dF), Variability of sample means (MS), Critical probability value (F) and Probability (Prob.) were also obtained.

Results and Discussion:

Five distinct bacterial colonies were isolated from the intestinal content of Zebrafish. The isolated colonies are ZB1 - *Aeromonas* sp., ZB2 - *Pseudomonas* sp., ZB3- *Micrococcus* sp., ZB4- *Escherichia coli* sp., and ZB5 - *Bacillus* sp., Based on biochemical, enzymatic and antibacterial activity (Table 1, 2 & Figure 1), *Pseudomonas* sp., was selected for the probiotic feed preparation. Commonly *Pseudomonas* sp., is present in aquatic system and usually these organisms are beneficial and non – beneficial applications. The other organisms isolated are *Aeromonas* sp., *Micrococcus* sp., *Escherichia coli sp*., and Bacillus sp., which are act as a causative agent and also they have low biochemical, enzymatic and antibacterial activity when compared to *Pseudomonas* sp., (Sivakumar and Rajan, 2015). 16s rRNA gene sequence was carried out for isolated intestinal bacteria of *Pseudomonas* sp., Phylogenic tree shows that the isolated bacteria was identified as (Fig.2) *Pseudomonas maltophilia* and it is renamed as *Stenotrophomonas maltophilia*. Somerita *et al*., (2013) reported the characterization of *Pseudomonas aeruginosa* isolated from *Labeo bata* by 16s rRNA gene sequence analysis

The condition factor of Zebrafish (*Danio rerio*) reared in different feeds are indicated in Figure 3. The initial condition factor of Zebra fish was 0.84, 1.00, 1.25, 1.12, 0.78 and 0.65 for feed I (Control), II, III, IV, V, and VI (1 ml of Yeast) respectively. The final condition factor was increased. Shankar and Kulkarni (2005) reported that the condition factor provide information on the suitability of environment for survival, growth and reproduction of fish (*Notopterus notopterus*) and the values are (0.794± 0.027) highest during pre spawning phase and lowest during post spawing phase. Sivakumar et *al*., (2016) reported that the average initial condition

factor of yellow molly was 1.84 and the final condition factor increased in feed V (2.65) when fed with *Escherichia fergusonii.*

The results of feed utilization and ANOVA (analysis of variance) of Growth parameters (Feed Consumption, Weight Gain, Gross Growth Efficiency and Net Growth Efficiency) are presented in Table 3 & 4. Feed consumption of Zebra fish was higher in feed V (7.67 ± 0.56) containing 4 ml of *Pseudomonas* sp. and lower in feed I (control) (6.41 ± 0.92). Bisht *et al*., (2012) reported that the feed consumption in common carp (*Cyprinus carpio* Linnaeus) was higher (95%) in diet D3 (100g of *Bacillus subtilis*) and lower (85%) in diet D1 (Control feed). Chandra and Rajan, (2009) also reported that the feed consumption of Koi carp was higher in feed V containing 4ml of *Lactobacillus.* Feed Conversion Efficiency of Zebra fish was higher in feed V (4.09 ± 0.52) containing 4 ml of *Pseudomonas* sp. In feed I, II, III, IV and feed VI the feed conversion efficiency were gradually decreased. Asma Chaudhary and Javed Iqbal Qazi (2007) reported that the feed conversion efficiency of Rohu *Labeo rohita* was higher in SSF2 (without live bacteria) (44.09 ± 4.25) lower in control (35.97 ± 4.06). But in the case of gold fish, the feed conversion efficiency gradually decreased from lower to higher quantity of *Pseudomonas* sp. (Rajan and Jeyachristina Arokia Selvi, 2014). Feed Conversion Ratio (FCR) of Zebra fish was lower in feed V (0.03 ± 0.01) and higher in feed I (0.13 ± 0.02). Birol Baki *et al*., (2012) reported that the feed conversion ratio was higher in Feed I (Control) and gradually increased in remaining three treatments of European *seabass*. Akeem (2010) also reported that the feed conversion ratio of *Clarias gariepinus* was higher in feed I (1.28± 0.06) and lower in feed V (1.27± 0.03). Seenivasan *et al*., (2012) also reported that the feed Conversion Ratio (FCR) in *Macrobacterium rosenbergii* was higher in feed I (5.55± 0.50) and lower in feed V (0.065± 0.018)

Weight gain of Zebra fish was higher in feed V (0.17 ± 0.04) containing 4 ml of *Pseudomonas* sp. and in feed I, II, III, IV and VI the growth was decreased. Suganya *et al*., (2014) and Sivakumar *et al.,* (2014) reported that the weight gain was higher in feed V (0.548) and lower in feed I (0.2502) by mixing of *Pseudomonas sp.* The relative growth rate of Zebra fish was higher in feed V (0.08 ± 0.02) containing 4ml *Pseudomonas sp.* and lower in feed I (0.03 ± 0.01). Dhanaraj *et al*., (2013) reported that the *S. cervisiae* (diet 3) produced the highest growth rate (2.46) and lowest in diet 1 (2.36). Assimilation of Zebra fish was higher in feed V (6.61 ± 0.55) and lower in feed I (5.26 ± 0.93). Assimilation of Platy was higher in Feed V containing of 104 cells of *Bacillus subtilis*. (Rajan and Revathi, 2011). Metabolism of Zebra fish was higher in feed V (5.98 ± 0.58) lower in feed I (5.18 ± 0.94). Gross and Net growth efficiency of Zebra fish was higher in feed V and lower in feed I. Similar result was reported in clown fish fed with *Lacto bacillus*. Pushparaj *et al*., (2012) reported higher gross and net growth efficiency when Platy was fed with higher levels of *Bacillus subtilis* in the feed.

Feed consumption, weight gain, gross growth efficiency and net growth efficiency values were significant when compared to control group ($p < 0.0.5$) (Table 4). Ananth et *al*., (2015) reported similar result of feed consumption, growth, gross growth efficiency and net growth efficiency ($p < 0.0.5$) in Koi carp.

Conclusion:

From this study, it was concluded that the isolated bacteria *Pseudomonas sp.,* can improve the growth of Zebra fish and act as potential probiotic feed additive. In addition, yeast cells has proven too effective growth promoter on Zebra fish. There was no negative effect during the experiment on Zebra fish. So, it is suggested to improve the health of Zebra fish and isolated bacterium was also suggested for testing as probiont's in the medical field for future.

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Appendix:

Table 1: Biochemical Characteristics of Intestinal Bacteria of Zebra Fish

Test	ZB1	ZB2	ZB3	ZB4	ZB5
Simple Staining	Rods	Cocci	Rods	Rods	Rods
Gram's Staining	Gram positive	Gram negative	Gram positive	Gram negative	Gram negative
Indole Test	Positive	Negative	Positive	Positive	Negative
Methyl Red	Positive	Positive	Positive	Positive	Positive

Voges Prokauser	Positive	Negative	Positive	Negative	Negative
Citrate Test	Negative	Negative	Positive	Positive	Positive
Catalase Test	Positive	Positive	Positive	Positive	Positive
Starch Test	Positive	Negative	Positive	Positive	Positive
Gelatin Hydrolysis	Positive	Negative	Positive	Not Performed	Positive
Oxidase Test	Negative	Positive	Negative	Negative	positive
Lactose Test	Positive	Negative	Not Performed	Positive	Positive
Sucrose Test	Positive	Negative	Not Performed	Positive	Positive
Identification Result	*Aeromonas* sp.,	*Pseudomonas* sp.,	*Micrococcus* sp.,	*Escherichia coli* sp.,	*Bacillus sp.,*

Table 2: Enzyme Productivity of Intestinal Bacteria of Zebra Fish

S.No	Intestinal Bacteria	Amylase	Cellulase	Lipase	Protease
1	ZB1*(Aeromonas sp.,)*	++	++	+++	++
2	ZB2*(Pseudomonas sp.,)*	+++	+++	+++	++
3	ZB3*(Micro coccus sp.,)*	+++	++	++	+
4	ZB4 *(Bacillus sp.,)*	NP	++	+++	++
5	ZB5 *(Escherichia sp.,)*	++	++	++	+

+ - Present, ++ - Good, +++ - Very good, NP – Not performed

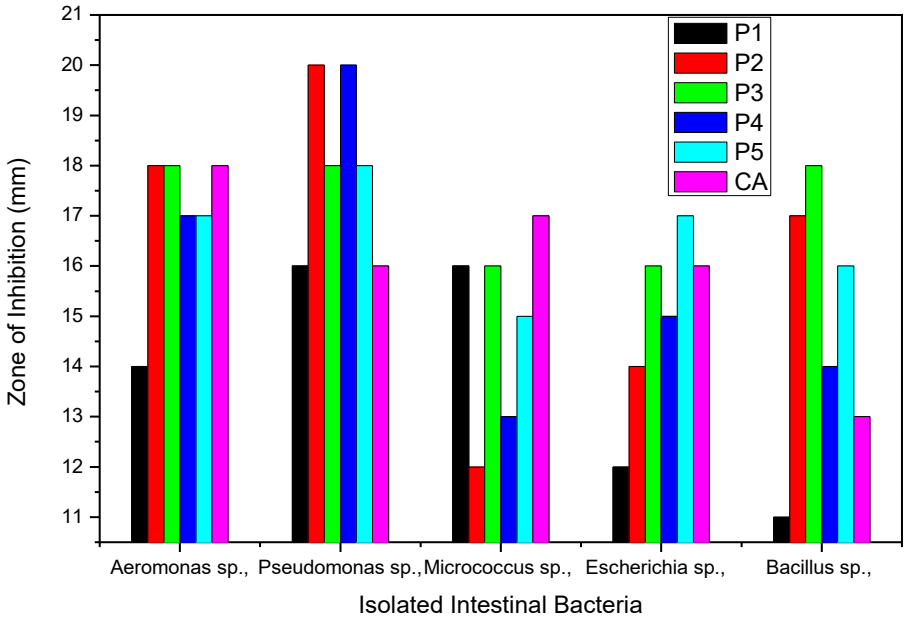

Figure 1: Antibacterial activity of intestinal bacteria of Zebra fish. P1- Klebsiella pneumonia, P2- Pseudomonas aeruginosa,P3- Bacillus cereus, P4- Staphylococcus aureus and P5- E. coli and CA- Commercial Antibiotic (Streptomycin)

>Contig_ZF2 Stenotrophomonas maltophilia

AGTCGAACGGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGA
ATCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGC
AGGGGACCTTCGGGCCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCC
ACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGT
GAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCTGGTTGGGA
TGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGC
GTTACTTGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCT
CAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAATGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGC
AGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACATTGACACTGA
GGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGG
ATGTTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCG
CAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCA
ACGCGAAGAACCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACT
CGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTACAATGGTAGGG
ACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGC

AACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCGGGA

Figure 2: Genetic Code (Sequence) of Isolated Stenotrophomonas Maltophilia from Intestinal Content of Zebra Fish

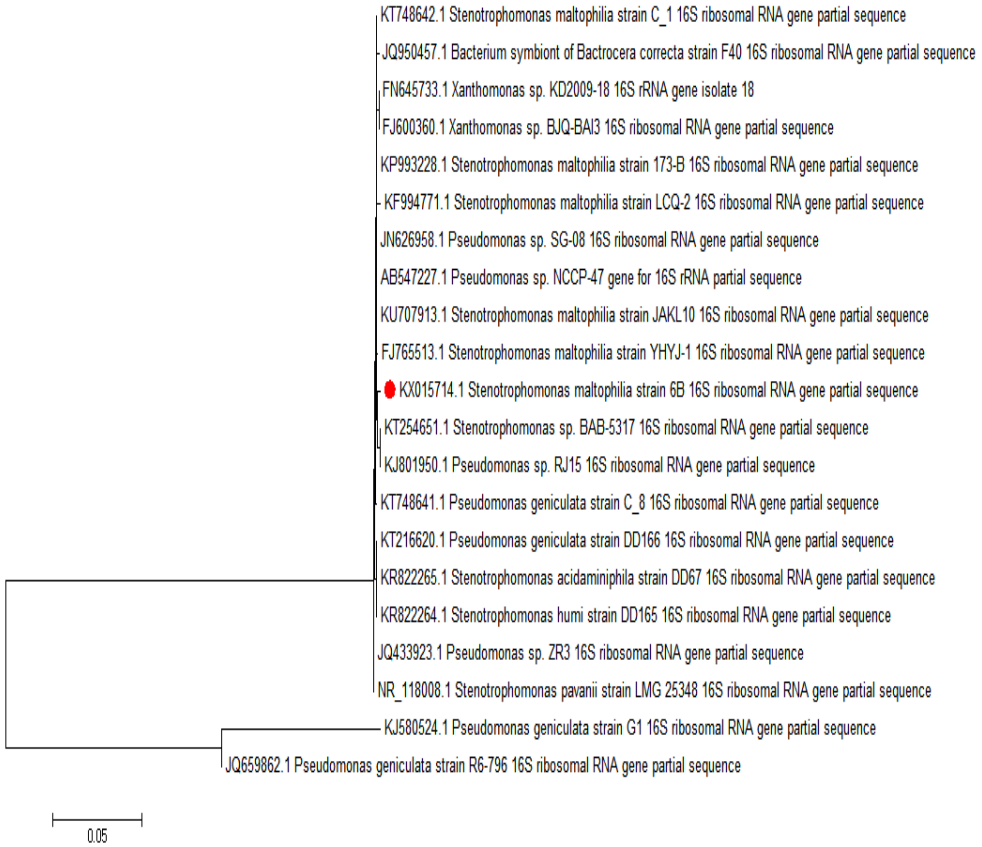

Figure 3: Phylogenetic tree of isolated Stenotrophomonas maltophilia from intestinal content of Zebra fish

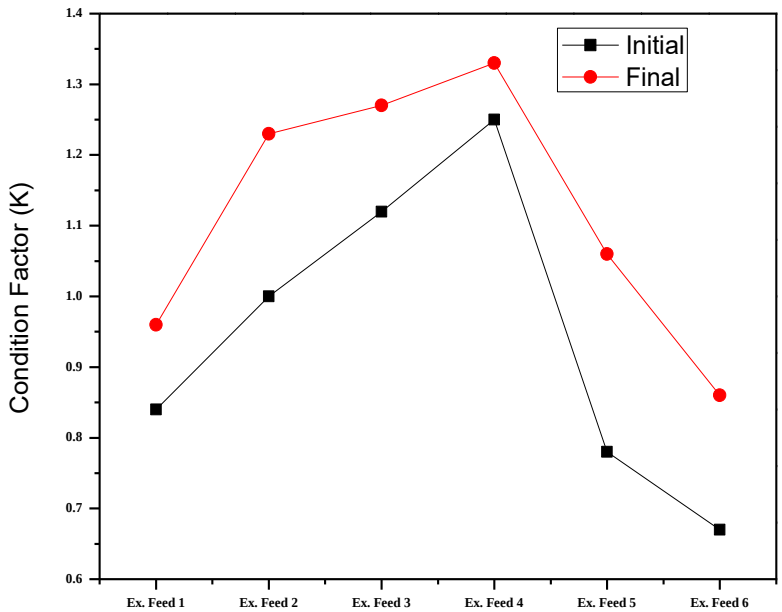

Figure 4: Condition factor (K) of Zebra fish

Table 3: Anova (Analysis of Variance) of Growth Parameters (Feed Consumption, Growth, Gross Growth Efficiency, Net Growth Efficiency) of Zebra Fish

S.No	Parameters	Source	SS	Df	MS	F	PROB
1		Columns	17.018	5	3.40361	3	0.055
			13.6001				
		Errors	30.6182	12	1.13334		
		Totals		17			S
2	Growth	Columns					
			0.02118	5	0.00424	8.67	0.0011
		Errors	0.00587				
				12	0.00049		
		Totals	0.02705				
				17			S
3	Gross growth efficiency	Columns					
			4.82196	5	0.96439	3.45	0.0365
		Errors					
			3.35067	12	0.27922		
		Totals					
			8.17263	17			S
4	Net growth efficiency	Columns					
			3.77313	5	0.75463	2.93	0.0588
		Errors					
			3.08727	12	0.25727		
		Totals					
			6.8604	17			S

Table 4: Feed Utilization and Growth Parameters of Goldfish Carassius Auratus in Relation to Different Concentration of Pseudomonas. Sp.(Cells) Each Value is the Average (± Sd) Performance of 5 Individuals in Tripilicates Reared for 45 Days

S.No	Parameters	Experimental Feeds					
		FEED I (Control)	FEED II (1 ml)	FEED III (2ml)	FEED IV (3ml)	FEED V (4ml)	FEED VI (1ml Yeast)
1	Feed Consumption(FC) (g/g live wt/45days)	06.41 ± 0.92[a]	06.68 ± 0.09[b]	07.02 ±0.73[c]	07.31 ± 0.77[d]	07.67 ± 0.56 [e]	07.37 ±0.51[f]
2	Feed Conversion Efficiency (FCE)	02.32 ± 0.49	02.53 ± 0.51	03.12 ± 0.08	04.09 ± 0.52	04.09 ± 0.52	03.19 ± 0.51
3	Feed Conversion Ratio (FCR)	0.13 ±0.02	0.09 ± 0.03	0.06 ± 0.01	0.04 ± 0.02	0.03 ±0.01	0.04 ± 0.02
4	Growth (G) (g/g live wt/ 45 days)	0.07 ± 0.01[a]	0.08 ± 0.01[b]	0.11 ± 0.01 [c]	0.13 ± 0.02 [d]	0.17 ± 0.04[e]	0.10 ± 0.01 [f]
5	Percentage Growth (PG) (%)	17.17 ± 0.95	16.74 ± 3.36	23.39 ± 4.64	25.42 ± 3.42	33.52 ±11.84	22.05 ± 2.73
6	Relative Growth Rate (RGR)	0.30 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.08 ± 0.02	0.05 ± 0.01
7	Assimilation (A)	5.26 ±0.93	5.59 ± 0.52	5.61 ± 0.81[c]	5.63 ± 0.93[d]	6.61 ± 0.55[e]	5.77 ± 0.63[f]
8	Metabolism (M)	5.18 ± 0.94	5.51 ± 0.80	5.50 ± 0.80	5.48 ±0.95	5.98 ±0.58	5.62 ±0.66
9	Gross Growth Efficiency (GGE) (%)	1.43 ±0.33[a]	1.51 ± 0.32[b]	1.96 ± 0.10[c]	2.48 ± 0.69[d]	2.90 ± 0.97[e]	1.91 ± 0.17[f]
10	Net Growth Efficiency (NGE) (%)	0.88 ± 0.73[a]	1.28 ± 0.25[b]	1.56 ± 0.03[c]	1.89 ± 0.48[d]	2.32 ± 0.74[e]	1.50 ± 0.45[f]

Feed consumption	Growth	Gross growth efficiency	Net growth efficiency
a vs b (P>0.05) S	a vs b (P>0.05) S	a vs b (P>0.05) S	a vs b (P>0.05)
a vs c (P>0.05) S	a vs c (P>0.05) S	a vs c (P>0.05) S	a vs c (P>0.05) S
a vs d (P>0.05) S	a vs d (P>0.05)S	a vs d (P>0.05) S	a vs d (P>0.05) S
a vs e (P>0.05) S	a vs e (P>0.05) S	a vs e (P>0.05) S	a vs e (P>0.05) S
a vs f (P>0.05) S	a vs f (P>0.05) S	a vs f (P>0.05) S	a vs f (P>0.05) S