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Research Paper

# Isolation, Identification, Enzyme Productivity, Antibacterial Activity and Molecular Characterization of Intestinal Bacteria of Ornamental Fish Koi Carp (Cyprinus Carpio Var Koi.) and It's Role on Growth

Aquaculture

**KEYWORDS :** Isolation, enzymatic, antibacterial, molecular, intestinal bacteria, koi carp, growth.

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## ABSTRACT

*The present study deals with the isolation, identification, enzymatic, antibacterial and molecular characterization of intestinal bacteria of Koi carp and its role on growth for a period of 45 days. Five distinct colonies were isolated from the intestine of Koi carp based on biochemical tests, enzymatic productivity and molecular characterization.The bacterium was identified as Bacillus sp and mass multiplied. Six different feeds having different concentration of Bacillus sp. such as feed I (Control), Feed II (1ml), Feed III (2ml), Feed IV (3ml), Feed V (4ml), and Feed VI (1ml yeast) were prepared by using fish meal, groundnut oilcake, wheat flour and tapioca flour and given to koi carp for a period of 45 days. The feed consumption, feed conversion ratio, feed conversion efficiency, growth, percentage growth, relative growth rate, assimilation, metabolism, gross growth efficiency and net growth efficiency was higher in feed V (4 ml of Bacillus sp.).*

## INTRODUCTION

In India, six hundred species have been identified as potential fishes with ornamental value. The share of India in global ornamental fish market continues to be poor with just 0.007% despite rich faunistic resource. It is estimated that the share can go up to 0.1% in the next fifteen years. (NABARD, 2001). To achieve a significant growth in ornamental fish industry it is necessary to adopt a scientific approach towards breeding, rearing and nutritional management of ornamental fishes (Douillet, 1993). The success of ornamental fish culture depends on the health status of the candidate species (Hossen, 2009). Persistent disease problem, aquatic pollution, indiscriminate use of different chemicals and antibiotics, constitute the major obstacles in successful ornamental fish culture(Witte *et al.,* 2000).Pathogenic microorganisms generally enter the fish through the gills, skin or gastrointestinal tract (Birkbeck and Ringo, 2005). Poor water quality and inadequate nutrition, many predispose an ornamental fish to bacterial disease. Antibiotics can affect the normal intestinal bacteria of the digestive tract which is beneficial to host (Aly *et al.,* 2008). However, the use of antibiotics as a preventive measure has been questioned because it can alter the gut macrobiotic and induce resistant bacteria population (Verschuere *et al.,* 2000). Alternative methods to disease control include immunostimulants and vaccines. Though vaccines are being developed and marketed and cannot be used as a universal disease control measures in ornamental fish culture (Abdul Kader Mohiden *et al.,* 2010). In this context, use of probiotic bacteria is a new approach to control potential pathogens (Lara-Flores, 2011).The work related to the isolation, identification, enzyme productivity, antibacterial activity and molecular characterization of intestinal bacteria of koi carp and its role on growth is totally wanting. Hence the present study is carried out.

## MATERIALS AND METHODS

For the present study, Koi carp *Cyprinus carpio var.koi* were collected from Angel Aquarium, Dindigul, Tamil Nadu, India and transported to the laboratory in Polythene bags filled with aerated water. Intestinal content of Koi carp was collected, serially diluted and plated over sterilized nutrient agar medium and incubated at 37$^0$C for 24 hours. (Bergy's manual of Determinative Bacteriology, 1994). After incubation, bacterial colonies were invalid at random from each plate and examined for gram reaction, spore formation, cellular morphology, motility and identified at the genus and species level. The intestinal bacteria of Koi carp was examined for the productivity of digestive enzymes like Amylase, Cellulase, Lipase and Protease using selective media. (Muge Aliye Hekimoglu *et al .,* 2014).Selected intestinal bacteria was examined for Double layer Screening Antibacterial activity using selective media. (Jawahar Abraham 2008).The isolated *Bacillus species* (10-6 Cells) was mass multiplied by inoculating in the nutrient broth.

For growth studies one control (without bacteria ), four experimental feeds by using different quantity (1,2,3,& 4 ml) of isolated bacteria and one feed by using commercially available probiont (yeast) was prepared according to square method (Ali,1980). Composition of different ingredients in experimental feeds is given in Table 1.

**TABLE 1: COMPOSITION OF DIFFERENT INGREADIENTS IN EXPERIMENTAL FEEDS (g/100gm)**

| S.No | INGREDIENTS | EXPERIMENTAL FEEDS | | | | | |
|---|---|---|---|---|---|---|---|
| | | Feed I control | Feed II | Feed III | Feed IV | Feed V | Feed VI |
| 1 | Fishmeal | 33.75 | 33.75 | 33.75 | 33.75 | 33.75 | 33.75 |
| 2 | GNOC | 33.75 | 33.75 | 33.75 | 33.75 | 33.75 | 33.75 |
| 3 | Wheat flour | 11.25 | 11.25 | 11.25 | 11.25 | 11.25 | 11.25 |
| 4 | Topioca | 11.25 | 11.25 | 11.25 | 11.25 | 11.25 | 11.25 |
| 5 | Fish oil | 2 | 2 | 2 | 2 | 2 | 2 |
| 6 | Sunflower oil | 2 | 2 | 2 | 2 | 2 | 2 |
| 7 | Suppelvite mix | 4 | 4 | 4 | 4 | 4 | 4 |
| 8 | Sodium chloride | 1 | 1 | 1 | 1 | 1 | 1 |
| 9 | Sodium benzoate | 1 | 1 | 1 | 1 | 1 | 1 |
| 10 | Microbes ($10^5$ Cells) | - | 1ml | 2ml | 3ml | 4ml | 1ml Yeast |

GNOC – Groundnut Oil Cake.

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For rearing studies uniform size of Koi carp *(Cyprinus carpio var. koi)* (3.66 ± 0.37 g) were selected and introduced in the rectangular glass tanks (45 cm L × 22 cm B × 22cm H) having a capacity of 18 liters. Five fishes were introduced in each tank. For each treatment triplicates were maintained and reared for a period of 45 days. On the 46th day length and weight of the fishes were measured in live condition and different feed utilization parameters were calculated.

The experimental results are presented in the form of tables and graphs using MS EXCEL (Version 2007). Mean, Standard deviation and T- test were also calculated with the help of the same tool, One-way ANOVA method was used for the analysis using MS EXCEL (Version 2007). 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**

The selected intestinal bacteria was *Bacillus* sp., based on the Biochemical tests and Enzyme Production (Amylase, Cellulase, Lipase and Protease) (Table 2 & 3). The antibacterial activity of intestinal bacteria of koi carp is presented in Table 4. Molecular characterization is presented in Fig.1.

**TABLE 2: BIOCHEMICAL CHARACTERIZATION OF INTESTINAL BACTERIA OF KOI CARP**

| Test | K1 | K2 | K3 | K4 | K5 |
|---|---|---|---|---|---|
| **Simple staining** | Rods | Rods | Rods | Rods | Rods |
| **Gram's staining** | Negative | Negative | Negative | Positive | Negative |
| **Motility Test** | Motile | Non - motile | Non- Motile | Motile | Motile |
| **Indole test** | Positive | Positive | Positive | Negative | Positive |
| **Methyl Red** | Positive | Positive | Positive | Negative | Positive |
| **Voges Prokauser** | Positive | Positive | Positive | Negative | Positive |
| **Citrate test** | Positive | Positive | Negative | Negative | Positive |
| **Catalase test** | Positive | Positive | Positive | Positive | Positive |
| **Starch test** | Positive | Positive | Negative | Positive | Positive |
| **Gelatin hydrolysis** | Positive | Positive | Negative | Positive | Negative |
| **Oxidase Test** | Positive | Not Performed | Negative | Negative | Negative |
| **Lactose Test** | Positive | Positive | Not Performed | Positive | Positive |
| **Sucrose Test** | Positive | Positive | Not Performed | Not Performed | Positive |
| **Lipid Test** | Positive | Positive | Positive | Positive | Negative |
| **Identification result** | ***Aeromonas* sp.,** | ***Vibrio* sp.,** | ***Enterobacter* sp.,** | ***Bacillus* sp.,** | ***Escherichia* sp.,** |

**TABLE 3 : ENZYME PRODUCTIVITY OF INTESTINAL BACTERIA OF KOI CARP**

| INTESTINAL BACTERIA | AMYLASE | CELLULASE | LIPASE | PROTEASE |
|---|---|---|---|---|
| **K1*(Aeromonas sp.,)*** | ++ | +++ | ++ | ++ |
| **K2*(Vibrio sp.,)*** | + | + | ++ | + |
| **K3*(Enterobacter sp.,)*** | ++ | ++ | + | +++ |
| **K4 *(Bacillus sp.,)*** | +++ | ++ | +++ | +++ |
| **K5 *(Escherichia sp.,)*** | ++ | ++ | ++ | ++ |

\+ - Nil (Absent ) or (Negative) ++ - Low productivity (Positive) +++ - Higher productivity (Positive)

**TABLE 4 : ANTIBACTERIAL ACTIVITY (DOUBLE LAYER SCREENING) OF INTESTINAL BACTERIA OF KOI CARP**

| Intestinal bacteria | Zone of Inhibition in mm | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P1 | CA | P2 | CA | P3 | CA | P4 | CA | P5 | CA |
| **K1 *(Aeromonas sp.,)*** | 13 | 6 | 11 | 5 | 08 | 3 | 15 | 7 | 12 | 5 |
| **K2 *(Vibrio sp.,)*** | 10 | 6 | 07 | 3 | 06 | 3 | 16 | 8 | 07 | 3 |
| **K3*(Enterobacter sp.,)*** | 13 | 7 | 11 | 5 | 07 | 2 | 17 | 10 | 11 | 6 |
| **K4 *(Bacillus sp.,)*** | 08 | 4 | 09 | 3 | 10 | 4 | 18 | 8 | 09 | 4 |
| **K5*(Escherichia sp.,)*** | 12 | 5 | 10 | 4 | 07 | 3 | 14 | 4 | 07 | 3 |

CA – Commercial Antibiotic (Zendamycin). P1 – *Staphylococcus aureus* P2 – *Shigella sonnei*

P3 – *Enterococcus faecalis* P4 – *Pseudomonas aeruginosa* P5 – *Klebsilla pneumonia*

**FIG.1GENETIC CODE (SEQUANCE) OF SELECTED *BACILLUS CEREUS.* FROM INTESTINAL CONTENT OF KOI CARP**

K4 _ CODING _1

TTAGAGTTTGGATCAGGCTCAGGATGAACGCTGGCG-
GCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAA-
GAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAG-
TAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGG-
GAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATG-
GTTCGAAATTGAAAGGCGGCTTCGGCTGCACTTATGGATG-
GACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCAC-
CAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGC-
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG-
GCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACG-
GAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCG-
TAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAA-
GCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAAC-
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT-
TATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCT-
TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGT-
CATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTG-
GAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAG-
GAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACT-
GACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTA-
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT-
GTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCAT-
TAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACT-
CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT-
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT-
GACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGG-
GAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT-
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC-
CTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGT-
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT-
CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGC-
TACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTG-

GAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCT-GCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCG-GATCAGCATGCCCGGTGGAATACCTTTCCCGGGGCCTTG-TACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAA-GTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGG-GACAGATGATTGGGGTGAATC

Condition factor (K) of Koi carp *Cyprinus carpio var koi.* was estimated for comparative purposes to assess the feed. The average initial condition factor is 2.36 ± 0.27 and the final condition factor increased in feed V (3.6 ± 0.35) and in all others the final condition factor was decreased(Table 5). Suganya et al ((2014) also reported same result in gold fish.

**TABLE 5 : CONDITION FACTOR (K) OF KOI CARP**

| FEEDS | INTIAL | FINAL |
|---|---|---|
| EX. Feed I (Control) | 2.36 ± 0.27 | 2.41 ± 0.34 |
| EX. Feed II (1 ml) | 2.21 ± 0.09 | 2.84 ± 0.36 |
| Ex. Feed III (2 ml) | 2.34 ± 0.07 | 2.59 ± 0.35 |
| Ex. Feed IV (3ml) | 2.68 ± 0.15 | 3.05 ± 0.31 |
| Ex. Feed V (4ml ) | 2.81 ± 0.25 | 3.6 ± 0.35 |
| Ex. Feed VI (1ml Yeast) | 2.57 ± 0.12 | 2.96 ± 0.03 |

The different feed utilization and growth parameters are presented in Table 6. The ANOVA (Analysis of variance) of Growth parameters such as Feed Consumption, Growth, Gross Growth Efficiency, Net Growth Efficiency were presented in Table 7. Feed consumption of Koi carp was higher in feed V (7.9 ± 0.30) containing 4 ml of *Bacillus* sp. and lower in feed I (control) (6.3 ± 0.83). Bisht *et al.,* (2012) reported that the feed consumption in common carp (*Cyprinus carpio* Linnaeus) was higher (95%) in diet D3 and lower (85%) in diet D1. Feed Conversion Efficiency of Koi carp was higher in feed V (0.13 ± 0.05) containing 4 ml of *Bacillus 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 *Labeo rohita* was higher in SSF2 (44.09 ± 4.25) lower in control (35.97 ± 4.06). Feed Conversion Ratio (FCR) of Koi carp was lower in feed V (6.63 ± 0.68) and higher in feed I (16.81 ± 5.61). Parthasarathy and Ravi (2011) reported that the feed conversion ratio of *Catla catla* was higher in T1 control (47.6) and lower in T4 (14.52). Growth of Koi carp was higher in feed V (1.11 ± 0.02) containing 4 ml of *Bacillus* sp. and in feed I, II, III, IV and VI was decreased. Dhanraj *et al.,* (2010) reported that the growth of Koi carp (*Cyprinus carpio var koi*) was higher in diet 3 (SCD) (0.32 ± 0.07) lower in control (0.19 ± 0.02).Like the growth, the percentage growth of koi carp was higher in feed V (23.33 ± 0.95) and feed I, II, III, IV and VI was decreased. The Percentage growth was higher in koi carp fed with 1ml of *Lactobacillus sp.* (Chandra and Rajan 2009). Sivakumar *et al.,* (2014) reported that the percentage growth of Common carp was higher in feed V (51.12 ± 22.30) and lower in feed I control (16.11 ± 9.53). The relative growth rate of koi carp was higher in feed V (0.55 ± 0.15) containing 4ml *Bacillus sp.* and lower in feed I (0.19 ± 0.06). Suganya *et al.,* (2014) reported that the relative growth rate of Gold fish was higher in feed V (0.46 ± 0.06) and lower in feed I (0.30 ± 0.01). Seenivasan *et.al.,* (2012) reported that the relative growth rate of fresh water prawn *Macrobrachium rosenbergi* was increased when fed with *Bacillus substilis.* Assimilation of Koi carp was higher in feed V (4.43 ± 0.15) lower in feed I (2.43 ± 1.06). Rajan and Revathi (2011) reported similar results for assimilation in Platy. Same result was reported in gold fish (Rajan and Jayachristina Arockia Selvi, 2014).Metabolism of Koi carp was higher in feed V (3.32 ± 0.13) lower in feed I (1.86 ± 0.82). Same result was also reported by Chandra and Rajan (2009) in koi carp.Gross and Net growth efficiency of Koi carp was higher in feed V and lower in feed I. Rajan and Revathi (2011) also reported higher gross and net growth efficiency when Platy was fed with higher levels of *Bacillus substilis* in the feed. From the results, it is inferred that the some of the feed utilization parameters such as Feed Consumption, Feed Conversion Efficiency, Growth, Percentage Growth, Relative Growth Rate, Gross growth efficiency and Net growth efficiency were higher in feed V containing 4ml of *Bacillus sp.*

**TABLE 6: FEED UTILIZATION AND GROWTH PARAMETERS OF KOI CARP *Cyprinus carpio var koi* IN RELATION TO DIFFERENT CONCENTRATION OF *Bacillus Sp.,* (CELLS). EACH VALUE IS THE AVERAGE (± SD) PERFORMANCE OF 5 INDIVIDUALS IN TRIPILICATES REARED FOR 45 DAYS**

| PARAMETERS | EXPERIMENTAL FEEDS | | | | | |
|---|---|---|---|---|---|---|
| | FEED I (CONTROL) | FEED II (1 ml) | FEED III (2ml) | FEED IV (3ml) | FEED V (4ml) | FEED VI ( 1ml Yeast) |
| Feed Consumption(FC) (g/g live wt/45days) | 6.3 ± 0.83[a] | 6.6 ± 0.75[b] | 6.9 ± 0.30[c] | 7.4 ± 0.21[d] | 7.9 ± 0.30[e] | 6.73 ±1.33[f] |
| Feed Conversion Efficiency ( FCE) | 0.06 ± 0.02 | 0.10 ± 0.05 | 0.11 ± 0.02 | 0.12 ± 0.01 | 0.13 ± 0.05 | 0.10 ± 0.03 |
| Feed Conversion Ratio (FCR) | 16.81 ±5.61 | 9.65 ± 0.65 | 8.8 ± 1.50 | 7.92 ± 0.92 | 6.63 ±0.68 | 9.87 ± 3.14 |
| Growth (G) (g/g live wt/ 45 days) | 0.4 ± 0.12[a] | 0.63 ± 0.11[b] | 0.8 ± 0.10 [c] | 0.94 ± 0.08 [d] | 1.11 ± 0.02[e] | 0.70 ± 0.16[f] |
| Percentage Growth (PG)(%) | 9.94 ± 2.51 | 16.25 ± 3.14 | 20.53 ± 2.73 | 20.54 ± 1.41 | 23.33 ± 0.95 | 17.43 ± 4.25 |
| Relative Growth Rate(RGR) | 0.19 ± 0.06 | 0.31 ± 0.05 | 0.39 ± 0.05 | 0.46 ± 0.40 | 0.55 ± 0.15 | 0.35 ± 0.08 |
| Assimilation (A) (g/g live wt/45days) | 2.43 ± 1.06 | 2.69 ± 0.35 | 3.63 ± 0.70 | 3.83 ±0.51 | 4.43 ± 0.15 | 3.53 ± 0.83 |
| Metabolism (M)(g/g live wt/45days) | 1.86 ± 0.82 | 2.21 ± 0.24 | 2.83 ± 0.73 | 2.89 ± 0.59 | 3.32 ± 0.13 | 2.82 ± 0.79 |
| Gross Growth Efficiency (GGE) (%) | 6.51 ± 2.58[a] | 10.37 ± 0.67[b] | 11.59 ± 2.09[c] | 12.72 ± 1.50[d] | 14.11 ± 0.22[e] | 10.75 ± 2.99[f] |
| Net Growth Efficiency (NGE) (%) | 19.54 ± 1.69[a] | 21.5 ± 1.32[b] | 22.64 ± 5.37[c] | 24.98 ± 5.37[d] | 25.04 ± 0.46[e] | 20.62 ± 6.07[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) S |
| 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 |

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**TABLE 7 : ANOVA (ANALYSIS OF VARIANCE ) OF GROWTH PARAMETERS (FEED CONSUMPTION, GROWTH, GROSS GROWTH EFFICIENCY,NET GROWTH EFFICIENCY) OF KOI CARP (*Cyprinus carpio var koi)***

| Parameter | Source | SS | df | MS | F | P value | F crit |
|---|---|---|---|---|---|---|---|
| Feed consumption | Between groups<br>Within groups<br>Total | 0.07111<br>13.38<br>13.45111 | 2<br>15<br>17 | 0.035556<br>0.892 | 0.03986 | 0.951025<br>NS | 3.68232 |
| Growth | Between groups<br>Within groups<br>Total | 0.914517<br>0.153133<br>1.06765 | 5<br>12<br>17 | 0.182903<br>0.012761 | 14.33287 | 0.000105<br>S | 3.105875 |
| Gross growth efficiency | Between groups<br>Within groups<br>Total | 100.882<br>45.67433<br>146.5563 | 5<br>12<br>17 | 20.1764<br>3.806194 | 5.300937 | 0.008438<br>S | 3.105875 |
| Net growth efficiency | Between groups<br>Within groups<br>Total | 74.87709<br>573.2093<br>648.0864 | 5<br>12<br>17 | 14.97542<br>47.76744 | 0.313507 | 0.895438<br>NS | 3.105875 |

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