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# EFFECTS OF THERMOSTABLE BACTERIAL α-AMYLASE ON GROWTH AND FEED UTILIZATION IN ROHU, *LABEO ROHITA* (HAMILTON), FINGERLINGS

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Key words: α-amylase, growth performance, *Labeo rohita* fingerlings, nutrient utilization, soil bacteria

**Abstract**

The effects of dietary supplementation of thermostable bacterial α-amylase (produced by *Bacillus stearothermophilus,* a thermophilic soil bacterium) on the growth, feed conversion, body composition and digestive enzyme profile of rohu, *Labeo rohita,* fingerlings were evaluated. Rohu fingerlings (avg wt 0.99±0.01 g) were fed purified isonitrogenous diets (35% crude protein) with crude bacterial α-amylase at 350, 525, 700 and 850 U per 100 g feed for 60 days at 3% of their body weight. Fingerlings fed the enzyme-supplemented diets performed better than the control group which received no enzyme supplementation. The best performance in terms of percent weight gain, SGR, FCR and PER was achieved in fish fed the diet supplemented with 700 U of α-amylase. The bacterial α-amylase led to higher protein accretion and lipid depletion in the carcass up to the 700 U incorporation level. Intestinal protease and α-amylase activity increased in all groups compared to the initial level.

## Introduction

The idea of introducing exogenous enzymes into fish feed is not new but their efficacy in fish feeds must be reinvestigated. Addition of proteolytic enzymes to diets resulted in only small positive effects in common carp (Dabrowski and Glogowski, 1977a,b; Dabrowska et al., 1979), however, these studies were conducted with enzyme extracts from intestinal tissues of fishes.

Enzymes manufactured by synthetic means or derived from plants, animals or microbial sources are increasingly being used as additives in pig and cattle feeds (Anon, 1988; Dvorak, 2000a,b; Lopez-Soto et al.,

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2000; Murillo et al., 2000; Pinos et al., 2000). Production of enzymes by microbial means is usually inexpensive and easy to scale up. In all higher animals, digestion of food material occurs by dual action of the enzyme system of the consumer and inhabitant microorganisms. *In vitro* studies of enzyme production show that bacterial flora in the gastrointestinal tract of rohu are potent producers of proteolytic enzymes and can also moderately produce cellulase (Saha and Ray, 1998; Ghosh et al., 2001). However, there is little information on amylase production by intestinal microflora in fish (Sugita et al., 1996, 1997).

The present study was designed to evaluate the effects of dietary supplementation of thermostable α-amylase produced by a thermophilic soil bacterium, *Bacillus stearothermophilus*, in four isonitrogenous diets for rohu, *Labeo rohita,* fingerlings.

## Materials and Methods

*Isolation of the bacterial strain*. The thermophilic bacterial strain *Bacillus stearothermophilus* is a soil isolate (Chakraborty et al., 1997, 1998). The bacterial strain was isolated according to Chakraborty et al. (1998) as follows: soil samples were collected from domestic kitchen waste depositories in different localities. The surface soil was removed and 20 g of soil from the depth of 2-3 cm were collected in fresh polythene bags. Each soil sample was enriched with 10 ml of 1% starch solution and incubated at 50°C for 24 h. Then 1 ml of the enriched soil suspension was plated with starch-agar medium [$(NH_4)_2SO_4$ - 2 g/l; $KH_2PO_4$ - 4 g/l; $Na_2HPO_4$ - 4 g/l; $MgSO_4,7H_2O$ - 0.2 g/l; $CaCl_2$ - 0.001 g/l; $FeSO_4,7H_2O$ - 0.004 g/l; starch - 1.0 g/l and agar - 15 g/l] and incubated at 50°C. After 24 h of incubation, well-separated colonies were replicated in the same medium and growth conditions. After appearance of the colonies on the replica plates, they were flooded with an iodine solution. Colonies producing clear zones due to starch hydrolysis were marked on the mother plate and transferred to starch agar slants as pure cultures.

*Preparation of the bacterial enzyme*. The organism was maintained on nutrient agar slants and stored at 4°C. The liquid medium used for α-amylase production was $(NH_4)_2SO_4$ - 2 g/l, $KH_2PO_4$ - 4 g/l, $Na_2HPO_4$ - 4 g/l, $MgSO_4,7H_2O$ - 0.2 g/l, $CaCl_2$ - 0.001 g/l, $FeSO_4,7H_2O$ - 0.004 g/l and glucose - 10 g/l (Makula and Finnerty, 1968). The pH of the medium was adjusted to 7. The production medium was inoculated (1% w/v) with the inoculum obtained from 24 h seed culture ($A_{600}$, 0.35) and incubated for 48 h at 50°C. The fermented broth was centrifuged at 5000 rpm for 20 minutes. The supernatant was removed and the precipitate discarded. The enzyme solution was subjected to ammonium sulphate fractionation (60% w/w) kept overnight at 4°C. The precipitate obtained after 24 h of incubation was dissolved in phosphate buffer (0.1 M, pH 7) and used as the crude enzyme. The unit activity (U) of the crude enzyme and optimum pH for enzyme activity were determined (Pantschev et al., 1981).

*Stability profile of α-amylase*. The thermostability profile of the enzyme was studied by assaying the residual activity after thermoinactivation by exposing the enzyme to different temperatures (25-100°C) for 60 minutes (Chakraborty et al., 1997).

*Diet preparation*. Five purified diets (D1, D2, D3, D4 and D5) with casein, gelatin and potato starch were prepared. The experimental diets (D2 to D5) were supplemented with bacterial α-amylase at 350 U (D2), 525 U (D3), 700 U (D4) and 850 U (D5) per 100 g feed (one unit of α-amylase is defined as the amount of enzyme needed to hydrolyze 1 mg potato starch per minute). The reference diet (D1) was not supplemented with bacterial α-amylase. Proportionately equal amounts of cod liver oil and sunflower oil were used as sources of lipids to supply the essential fatty acid ($18_{n-3}$ and $18_{n-6}$) requirements of the fish (Bromley, 1980).

Requisite quantities of casein, gelatin and potato starch (SRL Pvt. Ltd., Mumbai, India) were mixed thoroughly with lukewarm water to form a thick dough using carboxymethylcellulose as a binder. A ready-made vitamin-mineral mixture (Vitaminetes Forte, Roche India Ltd., Mumbai, India) was added to the diets before pelletization. Chromic oxide (1% w/w) was added to each diet as an external digestibility marker. The dough was steam-cooked and

then passed through an electrically operated semiautomatic pelletizer (pellet size 1.5 mm diameter). The pellets were dried at 40°C in a BOD incubator, packed in airtight plastic bags and stored in a refrigerator until used. The composition of the diets is presented in Table 1.

*Experimental design*. Rohu (*Labeo rohita)* fingerlings were obtained from a local fish seed dealer and acclimatized to laboratory conditions for 15 days prior to the start of the experiment. The fingerlings (avg wt 0.99±0.01 g) were randomly distributed into 90 l glass aquaria at a stocking density of 15 fish per aquarium with three replicates of each dietary treatment. The feeding trial continued 60 days under laboratory conditions with continuous aeration. Temperature, pH and dissolved oxygen ranged 26-30°C, 6.8-7.6 and 5.7-8.0 mg/l, respectively.

Fish were fed daily at 08:00 and 12:00 at a feeding rate of 3% of the total body weight per day. The daily ration was adjusted every tenth day according to the weight increment. Uneaten feed was removed and stored sepa-

Table 1. Ingredients (% dry weight) and proximate composition (on dry matter basis) of the experimental diets, n=3.

| | Diets | | | | |
|---|---|---|---|---|---|
| | *D1* | *D2* | *D3* | *D4* | *D5* |
| *Ingredients* | | | | | |
| Casein | 36 | 36 | 36 | 36 | 36 |
| Gelatin | 12 | 12 | 12 | 12 | 12 |
| Potato starch | 35 | 35 | 35 | 35 | 35 |
| Sunflower oil | 2 | 2 | 2 | 2 | 2 |
| Cod liver oil | 2 | 2 | 2 | 2 | 2 |
| Vitamin premix[a] | 2 | 2 | 2 | 2 | 2 |
| $Cr_2O_3$[b] | 1 | 1 | 1 | 1 | 1 |
| Cellulose | 10 | 10 | 10 | 10 | 10 |
| α-Amylase (U) | 0 | 350 | 525 | 700 | 850 |
| *Proximate composition* | | | | | |
| Moisture | 7.39 | 7.44 | 7.4 | 7.43 | 7.48 |
| Dry matter | 92.61 | 92.56 | 92.6 | 92.57 | 92.52 |
| Crude protein | 34.35 | 35.23 | 34.36 | 35.45 | 35.41 |
| Crude lipid | 4.46 | 4.45 | 4.46 | 4.48 | 4.49 |
| Ash | 5.89 | 6.39 | 6.45 | 6.36 | 6.28 |
| Crude fiber | 4.93 | 4.68 | 4.71 | 4.56 | 4.82 |

[a] Vitamin and mineral mixture (Vitaminetes Forte, Roche Products Ltd., 24/28 Pt. M. M. Road, Mumbai 400 034, India)

[b] Chromic oxide, external digestibility marker

rately for calculating the feed conversion ratio. Fecal samples were collected separately from each aquarium by pipette (Spyridakis et al., 1989). The samples were oven-dried (60°C) and analyzed for digestibility estimations. At the end of the experiment, fish from all dietary treatments were weighed and analyzed for carcass composition.

*Chemical analysis and data collection*. Experimental diets and fecal samples were analyzed for proximate composition (AOAC, 1990) as follows: moisture content by oven-drying at 105°C for 24 h, protein content (N $\times$ 6.25) by semimicro Kjeldhal digestion and distillation after acid digestion, lipid content by extracting the residue with 40-60°C petroleum ether for 8 h in a Soxhlet apparatus, crude fiber as loss of dried lipid-free residues after digestion with $H_2SO_4$ (1.25%), and NaOH (1.25%) by ignition at 550°C in a Muffle furnace to a constant weight. Nitrogen free extract (NFE) was computed by combining the values for crude protein, crude lipid, ash, crude fiber and moisture and subtracting the sum from 100 (Maynard et al., 1979). Chromic oxide in the diets and in the fecal samples was estimated spectrophotometrically (Bolin et al., 1952). Proximate analyses of the carcasses were made before the start and after the termination of the experiment following the same procedures used to analyze the diets and fecal samples. Water quality parameters were monitored following methods outlined by the APHA (1985).

Fish from each experimental set were dissected on an ice tray prior to the start and at the end of the experiment. The intestine and liver were removed to determine digestive enzyme activity. $\alpha$-Amylase was quantitatively determined following the method described by Bernfeld (1955). Protease activity was measured according to the method of Moore and Stein (1948) using bovine serum albumin as the substrate.

The average live weight gain (%), specific growth rate (SGR; %/day), feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated using standard formula outlined by Steffens (1989).

The apparent digestibility (AD) of nutrients was calculated according to De Silva and Anderson (1995), using the following formula: AD(%) = 100-100 (% $Cr_2O_3$ in diet/% $Cr_2O_3$ in feces)(% nutrient in feces/% nutrient in diet).

*Statistical analysis*. Analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan, 1955) were used to determine significant differences between groups with respect to growth, muscle composition, nutrient digestibility, digestive enzyme profiles and general performance of the fish.

## Results and Discussion

The major problem of using exogenous enzymes in fish feeds is that most enzymes are thermolabile. Therefore, attempts were made to prepare feed with a thermotolerant enzyme produced by *Bacillus stearothermophilus* which would withstand processing during feed formulation. The crude enzyme produced by the *B. stearothermophilus* showed optimum activity at pH 7. The optimum activity of the enzyme preparation was 35 U per ml. The thermostability profile of the enzyme showed 100% residual activity within the temperature range 30-80°C. The diets were isonitrogenous (35% crude protein, Table 1).

The growth performance, feed utilization efficiency and apparent nutrient digestibility of the *L. rohita* fingerlings are presented in Table 2. The highest weight gain was obtained in fish fed diet D4 (700 U $\alpha$-amylase), followed by diets D5 and D3. Diet D4 also resulted in the highest percent weight gain, which was not significantly different ($p>0.05$) from that of diet D5 (850 U $\alpha$-amylase). The poor growth was possibly due to the fact that the feed was not optimal for fish in this early stage of growth. In addition, the feeding rate (3% of the total body weight per day) was lower than the rate normally given to fish of this size. The feed conversion ratio (FCR) and protein efficiency ratio (PER) were best with diet D4. The performance was poorest with diet D1 which had no enzyme supplementation. The results suggest that, for rohu fingerlings, due to the thermostable nature of the amylase obtained from the soil bacterium, the enzyme remained stable and viable even after processing during feed manufacture and within the gut of the fish.

Table 2. Growth performance and apparent nutrient digestibility of *Labeo rohita* fingerlings fed experimental diets for 60 days. The results are means±SD of three determinations.

| | Diets | | | | |
|---|---|---|---|---|---|
| | *D1* | *D2* | *D3* | *D4* | *D5* |
| *Performance* | | | | | |
| Initial weight (g) | 0.99±0.01 | 0.99±0.01 | 0.99±0.01 | 0.99±0.01 | 0.99±0.01 |
| Final weight (g) | $1.78^{d}$±0.05 | $1.92^{c}$±0.06 | $2.21^{b}$±0.10 | $2.37^{a}$±0.08 | $2.36^{a}$±0.07 |
| Avg live weight gain (%) | $79.79^{d}$±3.08 | $93.94^{c}$±4.16 | $123.23^{b}$±5.57 | $139.39^{a}$±5.26 | $138.38^{a}$±5.41 |
| SGR (%/d) | $0.98^{d}$±0.03 | $1.10^{c}$±0.04 | $1.36^{b}$±0.05 | $1.45^{a}$±0.05 | $1.44^{a}$±0.05 |
| PER | $0.86^{e}$±0.04 | $1.02^{d}$±0.04 | $1.32^{c}$±0.05 | $1.69^{a}$±0.07 | $1.48^{b}$±0.06 |
| FCR | $2.25^{a}$±0.10 | $1.92^{b}$±1.09 | $1.43^{c}$±0.06 | $1.29^{d}$±0.04 | $1.30^{d}$±0.05 |
| Survival (%) | 100 | 100 | 100 | 100 | 100 |
| *Apparent digestibility(%)* | | | | | |
| Dry matter | $45.89^{c}$±2.11 | $48.21^{c}$±2.19 | $56.18^{b}$±2.33 | $61.67^{a}$±2.75 | $57.81^{ab}$±2.62 |
| Protein | $78.64^{b}$±3.03 | $82.17^{b}$±2.71 | $87.18^{ab}$±3.01 | $89.38^{a}$±2.26 | $88.06^{a}$±3.15 |
| Lipid | $76.71^{a}$±3.21 | $66.83^{b}$±2.78 | $60.69^{c}$±2.42 | $54.99^{d}$±1.87 | $55.50^{d}$±2.13 |

Values with same superscript in the same row are not significantly different (p>0.05).

Seeto et al. (1996) documented the role of bacterial enzymes in fermentation of carbohydrates in the herbivorous marine fishes, *Odax exanomelas* and *Crinodus lophodon*. Tang et al. (1994) reported that there are stable microflora in the intestine of common carp, *Cyprinus carpio,* and that bacterial amylases play a promoting effect on starch digestion by the host. In the present study, potato starch was a source of carbohydrates in the diets. It appears, therefore, that the bacterial α-amylase played an important role in hydrolysis of the starch and proper utilization of the carbohydrates. The increased utilization of carbohydrates might have spared protein because less protein was used for energy. Earlier results of feeding trials with fish revealed the contrary – enzyme addition generally led to little or no improvement in either weight gain or efficiency of feed utilization in fish fed supplemented diets, so that the advantages of adding exogenous proteases and amylases to fish feeds may be minimal (Campbell and Bedford, 1992). Carter et al. (1992) also were unable to establish statistically significant effects on the consumption-growth relationship or feed utilization efficiency in *Salmo salar* parr fed three rations of a control diet and one ration of a diet containing the supplementing enzyme, α-amylase.

In the present study, the apparent dry matter and protein digestibility values progressively increased with the increasing level of α-amylase supplementation (Table 2). On the other hand, the apparent lipid digestibility value decreased with the increasing level of enzyme supplementation, being lowest for diet D4 (700 U α-amylase). The apparent protein digestibility for all the diets was high, ranging 78.64-89.38%. The apparent digestibility of protein for diet D4 was highest and did not significantly differ from diets D3 and D5. The lowest dry matter and protein digestibilities were recorded for diet D1 which was not supplemented with the enzyme. The apparent nutrient digestibility values indicate a linear correlation with growth performance of rohu fingerlings. The addition of the exogenous α-amylase to the diets might have augmented the enzyme activity of the fish which, in turn, helped to improve nutrient digestibility (Jobling, 1994).

The gut enzyme profile is the indicator of nutrient digestibility and utilization. In the present study, the activity of both protease and α-amylase in the intestines of the fingerlings fed the experimental diets was higher than the initial values. The activity of both enzymes was highest in the fish fed diet D4 (700 U α-amylase; Fig. 1). The results clearly indicate a correlation between formulated diet intake and digestive enzyme activity, resulting in diet-related growth differences. Similar diet-related differences in growth and digestive enzyme activity have been reported with rainbow trout (Kawai and Ikeda, 1973).

Data on the carcass composition of the *L. rohita* fingerlings are presented in Fig. 2. There was significantly higher body protein and lower body lipid accretion in groups fed α-amylase-supplemented feed than in the control. Among the groups fed the supplemented feed, the highest amount of body protein was recorded in group D4 (700 U α-amylase) and the lowest in D2 (350 U α-amylase). The body lipid content was higher in the D2 group and lowest in the D4 group. α-Amylase supplementation did not significantly affect the carcass ash content. The carcass compositions indicate that α-amylase-induced suppression of lipid deposition possibly resulted in a concomitant elevation of the amount of dietary nutrients directed towards muscle growth, expressed as higher protein accretion.

It is concluded from the present study that thermostable α-amylase produced by the soil bacterium *B. stearothermophilus* enhanced growth and improved body composition (higher protein and lower lipid accretion) and nutrient utilization at the 700 U dietary incorporation level. It, thus, has potential in formulated feeds for the Indian major carp, rohu, under culture conditions.

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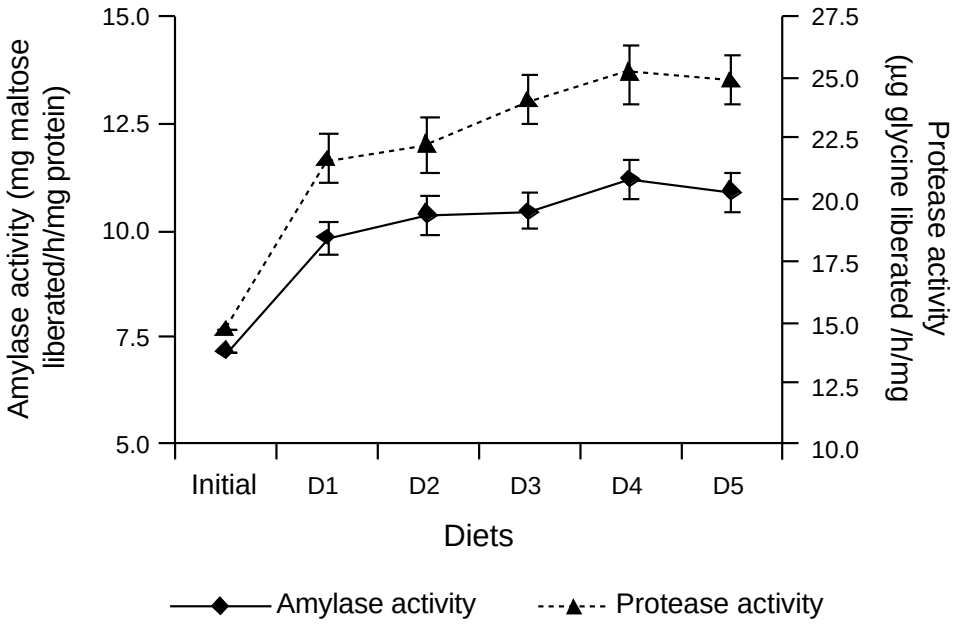


Fig. 1. Intestinal α-amylase and protease activity in rohu fingerlings fed experimental diets for 60 days.

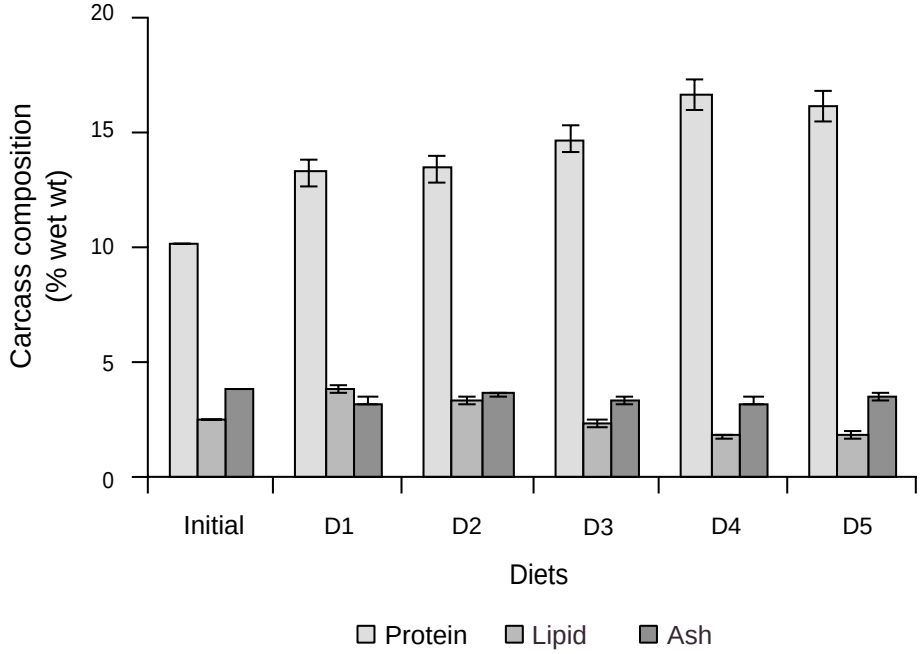


Fig. 2. Proximate carcass composition (% wet weight) in rohu fingerlings fed experimental diets for 60 days.

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