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Efficacy of a fungal fermented product as fishmeal replacement in the diet of *Penaeus monodon* Fabricius post-larvae

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ABSTRACT

The efficacy of diets, formulated by inclusion of an *Aspergillus niger* fermented product (AFP) as fish meal substitute, for *Penaeus monodon* post-larvae was determined. AFP was derived by solid state fermentation (SSF) of a mixture of soybean flour, wheat flour, groundnut oil cake and sesame oil cake in the ratio of 4: 3: 2: 1 respectively for 96 h. The feeding trial was conducted in post-larvae stocked @10 animals in circular perspex tanks containing 40 l of water with five dietary treatments each with three replicates for 52 days. The diets containing graded levels of AFP substituting 0, 50, 150, 250 and 350 g kg^{-1} of fishmeal were fed to the post-larvae. Data on the growth performance and nutrient utilization efficiency were recorded from the feeding trials. Results showed that the post-larval shrimp fed diet containing 150 g kg^{-1} AFP exhibited significantly better ($p<0.05$) growth rate (14.79 mg d^{-1} post-$larva^{-1}$), feed conversion ratio (1.62), protein efficiency ratio (1.64) and apparent protein utilization (25.39) than the other diets. The diet with 350 g kg^{-1} AFP showed the best apparent protein digestibility (87.74 %), apparent fat digestibility (97.95 %) and apparent dry matter digestibility coefficient (77.09 %) among the test diets. The results of the present study suggest that AFP can be used to improve the digestibility of nutrients and that partial replacement of fishmeal is possible in *P. monodon* post-larval diets under optimum rearing conditions.

Keywords: *Aspergillus niger,* Fermented product, Fishmeal replacement, *Penaeus monodon*

Introduction

Fishmeal is widely used in marine shrimp feeds because it is rich in protein, highly digestible and an effective attractant (Fox *et al*., 2004). Fish meal with its balanced mixture of essential amino acids and other nutrients, forms the major protein source in aquafeeds constituting about 350-500 g kg^{-1} (Dong *et al*., 2000). The limited supplies and the high price of fish meal have forced aquatic nutritionists to consider alternative sources of protein (Sudaryono *et al*., 1999). Also the perception of over-exploitation of the stock has caused projection of higher future prices (Delgado *et al*., 2002). Watanabe (2002) stated that ideally, fish meal replacements should be less expensive than fish meal and more readily available. Plant protein sources are the only ingredients for which expanded production in future is likely (Crowder, 1990) and certain oilseeds and oilcakes are good protein sources that could be used as main protein sources in shrimp and fish feed when properly supplemented with essential amino acids (Davis *et al.,* 1995; Stickney *et al*., 1996). Ali (1992) identified protein derived from terrestrial animals and plants as being suitable alternative particularly because they were available in sustainable quantities. However, plant derived ingredients may fail to provide adequate amount of some essential amino acids or fatty acids like Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) which are essential for marine organisms and contain anti-nutritional factors that impair the efficiency of digestion (Paulraj, 1993; Lemos *et al*., 2000). Microbial protein is one of the potential feed sources and has long been used in human food and animal feed. *Aspergillus niger* has been used in the fermentation industries to manufacture a variety of enzymes (Pandey, 1999) and other compounds like citrate, oxaloacetate and pyruvate (Wang, 1989) Solid-state fermentation (SSF) is a cheaper and simple technique holding tremendous potential for enzyme production as well as to improve the quality of plant ingredients. The fermented ingredients can be used directly as enzyme source, which in turn will increase the digestibility and availability of nutrients (Tengerdy, 1998; Pandey *et al*., 1999). In the ruminants, SSF of lignocellulosic materials by rumen microbes is being experimented; but in aquaculture there has not been any work on the use of fermented plant ingredients in the diet of shrimp. The present study was conducted to elucidate the suitability of a fungal fermented product of mixed oil cakes, soybean flour and wheat flour as a fishmeal substitute in the diet of the tiger shrimp *P. monodon* post-larvae.

Materials and methods

Source of Aspergillus niger fermentation product

Aspergillus niger NCIM 616 (an amylase producing fungus) was procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. Solid state fermentation (SSF) of a mixture of soybean flour, wheat flour, groundnut oil cake and sesame oil cake ground and mixed in the ratio of 4:3:2:1 respectively, was optimized at 96 h by fermenting for different durations ranging from 12 to 96 h using 10 ml of inoculum containing about 10^6 spores ml^{-1} in 50 g substrate.

Diets

After proximate composition analysis of all the ingredients, the nutrient composition (including crude protein) in diets were formulated using Excel Programme by considering the final protein level @380 g kg^{-1} and crude fat @70 g kg^{-1}. Diet formulations and compositions are presented in Table 1. The diets incorporated with AFP at 50, 150, 250 and 350 g kg^{-1} by replacing fishmeal in the same proportion were designated as F1, F2, F3 and F4 along with one control diet (CF) containing 350 g kg^{-1} fishmeal and devoid of AFP. Groundnut oil cake, sesame oil cake and shrimp meal were increased or decreased as required to maintain the dietary protein and lipid levels.

Table 1. Composition of experimental diets F1 to F4 and CF (% dry matter basis)

Ingredient	F1	F2	F3	F4	CF
AFP[a]	5	15	25	35	-
Fish Meal	30	20	10	0	35
Shrimp meal	16	16	18	22	15
Wheat flour	27.5	22	17	16	26.5
Peanut cake	17.5	15.0	8	5	19.3
Sesame oil cake	0.5	7.3	15.5	20	0.2
Fish oil	2.0	2.5	3.0	3.5	2.0
Mineral mix[b]	0.5	1.2	3.0	3.5	1.0
Vitamin Mix[b]	1.0	1.0	1.0	1.0	1.0
Proximate analysis (% dry matter basis)					
Crude protein	38.51	38.60	38.68	38.73	38.46
Crude fat	6.92	6.97	7.03	6.93	6.98
Crude Ash	12.34	12.3	12.32	12.12	12.21
Crude Fibre	2.19	2.34	2.59	2.86	2.85
NFE[c]	39.81	39.48	38.61	38.84	38.06

[a]*Aspergillus* fermented product
[b]Commercial grade (supplevit)
[c]Nitrogen-free extract (calculated by difference)

The ingredients including AFP were dried, ground in a laboratory pulverizer, sieved (<0.4 mm) and used at varying levels in the diets. Chromic oxide was added at the rate of 1% in all the diets as an inert marker for digestibility studies. The diets were pelleted using 2 mm die in a hand pelletizer and dried overnight at 55 °C, cooled to room temperature, packed in plastic bottles and stored in a freezer at - 20 °C until further use.

The amino acid profiles of the diets are given in Table 2. Amino acids were determined using HPLC system (Waters, India) after hydrolysis in 6 mol l^{-1} HCl (Finn *et al*., 1995). Since tryptophan is destroyed by acid hydrolysis, separate set of samples were used for alkaline hydrolysis and analyzed for tryptophan content (AOAC, 1990).

Table 2. Amino acid profile of diets incorporated with AFP at different levels (g 100 g $protein^{-1}$)

Amino Acids	Diets				
	CF	F1	F2	F3	F4
Aspartic acid	10.02	8.60	8.34	6.43	7.53
Glutamic acid	19.64	18.47	19.28	22.92	25.88
Serine	5.15	4.59	4.96	5.27	6.36
Glycine	7.24	6.04	6.82	6.10	7.08
Histidine	2.64	3.18	2.72	2.79	2.76
Arginine	6.49	6.74	7.44	7.68	8.85
Threonine	3.66	3.85	3.82	3.77	4.71
Alanine	6.19	5.79	5.97	6.02	7.14
Proline	5.77	5.61	6.73	6.42	6.93
Tyrosine	2.45	2.93	2.49	2.73	1.71
Valine	4.10	4.22	3.99	4.23	2.87
Methionine	3.15	3.69	2.93	2.67	1.68
Cystine	0.52	1.67	0.96	0.96	0.55
Isoleucine	2.76	3.58	3.45	3.56	2.43
Leucine	7.93	8.11	8.30	8.29	6.01
Phenyl alanine	5.21	5.22	5.43	5.28	3.74
Lysine	7.10	7.25	6.29	4.87	3.73
Tryptophan	2.65	1.54	1.81	1.87	1.98

The water stability of the diets was evaluated by estimating the loss of total dry matter of pellets at 1 h, 2 h, 3 h and 4 h intervals in 15 ppt seawater (Obaldo *et al*., 2002).

Experiment design and feeding trial

Feeding trials were conducted for 52 days in 50 l circular perspex tanks in an indoor facility in the marine hatchery. The four experimental diets and the control diet constituting the five treatments and control were arranged randomly with three replications (tanks) per treatment.

Post-larval *P. monodon* were procured from a commercial shrimp hatchery at Kochi, Kerala, India and transported in oxygen filled polythene bags to the Marine Research Hatchery at Central Marine Fisheries Research Institute, Kochi (15 km). The post-larvae were acclimated to hatchery conditions in 500 l fiberglass tank containing 15 ppt water for two days prior to the initiation of the experiment. Before stocking into the experimental tanks,

the post-larvae were weighed individually. Each tank was stocked with 10 post-larvae 40 l^{-1} (mean weight: 33 mg ± 0.7).

One-third of the water was exchanged daily before feeding in all the experimental tanks and complete exchange was done once a week. Water temperature, pH and dissolved oxygen during the 52 day period ranged between 28 and 31 °C, 7.5 and 8.2 and 4.0 and 4.5 mg l^{-1}, respectively. The total ammonia level was <0.01 mg l^{-1} in the experimental tanks throughout the period. Each tank was covered with a black cloth to prevent the shrimp from jumping out. The shrimp were fed at the rate of 15 % of the body weight throughout the experimental period twice a day at 0830 and 1800 h (40 and 60 % ration respectively) (Fox, 1993). The ration was adjusted after each sampling at 10 d intervals.

Every 10th day, shrimp numbers were counted for survival estimates from each tank and sampled for growth record. The biological parameters used to evaluate the quality of diets were calculated by equations as follows (Sudaryono *et al*., 1999):

Weight gain (g)	-	(Final weight-Initial weight)/ No. of days
Mean growth rate (GR, %)	-	[Final weight-Initial weight/ Time (day)] x 100
Feed conversion ratio (FCR)	-	Dry feed intake/Wet weight gain
Protein efficiency ratio (PER)	-	Wet weight gain/Dry protein intake
Apparent protein utilization (APU, %)	-	[Protein gain in the body/Dry protein intake] x 100

Digestibility studies

For the digestibility studies, faecal matter strands were collected daily afternoon, after 5 h of first feeding, by siphoning the bottom of the tank into a collection sieve made of bolting silk (40 µ) without trapping the uneaten feed (Sudaryono *et al*., 1996). The faecal matter from each of the treatment tanks were pooled together and stored at -20 °C and after the termination of the feeding trial after 52 days, the frozen faecal matter was dried at 50±1 °C for 24 h, ground and stored at -20 °C for further nutrient analyses for dry matter, protein and fat digestibility studies. After proximate composition analysis of all the ingredients, the nutrient compositions (including crude protein) in diets were formulated using Excel Programme by considering the final protein level @380 g kg^{-1}. Then the diets and faecal samples were analysed for dry matter, crude protein and crude fat contents by adopting approved methods (AOAC, 1990). Chromic oxide in samples of feeds and faecal matter was estimated by the method described by Furukawa and Tsukahara (1966) and the apparent nutrient digestibility calculated as follows:

Apparent nutrient digestibility (%)

$$= 100 - 100 \left\{ \frac{\% \ Cr_2O_3 \text{ in feed}}{\% \ Cr_2O_3 \text{ in faeces}} \times \frac{\% \text{ nutrient in faeces}}{\% \text{ nutrient in feed}} \right\}$$

Apparent digestibility coefficient (ADC)

$$= \left\{ \frac{\text{Feed intake (g) - Faecal output (g)}}{\text{Feed intake (g)}} \right\} 100$$

At the start of the experiment, three groups of post-larval shrimp (150 no. each) were weighed and dried at 55°C for 24 h to a constant weight and the dry matter content was determined. The dried samples were analyzed for crude protein, crude fat and crude ash. After the final weights of the shrimps from different treatments and the control were recorded on day 52, the shrimp from each treatment were pooled, dried and crude protein by Kjeldahl method, crude fat by soxhlet extraction and crude ash at 550 °C using muffle furnace were determined (AOAC, 1990).

Data analyses

The data were analyzed for weight gain, mean growth rate, feed conversion ratio (FCR) protein efficiency ratio (PER), apparent protein digestibility, apparent fat digestibility, apparent dry matter digestibility, apparent protein utilization (APU), shrimp body composition and pellet water stability. All the data were statistically analyzed using one-way analysis of variance (Gomez and Gomez, 1976). New Duncan multiple range tests were used to determine significant differences ($p<0.05$) among treatment means (Duncan, 1955). All statistical analyses were conducted using SPSS for Windows (Statistical Package for Social Sciences, Windows Version, Chicago, IL, USA).

Results

The AFP obtained after 96 h fermentation used in this study as dietary ingredient for *P. monodon* post-larvae contained higher levels of crude protein (39.95±0.38 g 100 g^{-1}) and crude fat (3.41±0.39 g 100 g^{-1}) than the unfermented mix (35.04 and 2.52 g 100 g^{-1} respectively). The NFE content of the ingredient mix ranged from 52.16±0.55 in the control to 43.18±0.62 in the AFP.

At the conclusion of the 52 day feeding trial, survival of the animals were cent percent in all the treatments as well as in the control. The diet F2 containing 150 g kg^{-1} AFP produced significantly higher ($p<0.05$) weight gain (7.69 g ±0.93) and mean growth rate (14.97 mg d^{-1} post-$larva^{-1}$) (Table 3). The feed conversion ratios (FCR) ranged

from 2.09 to 1.62 for the test diets and it was 3.30 for the control diet**.** The diet F2 provided the best FCR (1.61), highest PER (1.63) and APU (25.27) (Table 3).

Table 3. Results of the 52 day feeding trial for *P. monodon* post-larvae using AFP incorporated diets

Index	CF	F1	F2	F3	F4
Mean weight gain (%)	0.11	0.23	0.40	0.31	0.28
Mean growth rate (mg d^{-1} post-$larva^{-1}$)	7.87	7.48	14.78	9.84	9.35
Feed conversion ratio	3.30	2.09	1.61	2.00	1.81
Protein efficiency ratio	0.78	1.26	1.63	1.30	1.44
Apparent protein digestibility	58.04	79.50	84.05	87.11	87.79
Apparent protein utilization	10.20	16.49	25.27	19.15	20.37
Apparent fat digestibility	79.34	88.59	93.65	97.92	97.95
Apparent digestibility coefficient	60.84	71.30	74.92	74.86	77.09

Significantly higher ($p<0.05$) apparent protein digestibility (87.74 %) and fat digestibility (97.95 %) were observed for shrimp fed diet F4 containing 350 g kg^{-1} AFP, as compared to all the other diets (Table 3).

Carcass composition analysis revealed that the shrimp fed diet F2 had significantly higher ($p<0.05$) dry matter and body protein (65.45 %) contents than the other treatments and the control (Table 4). Differences in the ash contents among the treatments were not significant. There was an increase in dry matter content in all the treatment groups, which ranged from 21.72 to 23.68 %, when compared to the control (21.71 %).

Table 4. Body composition of *P. monodon* post-larvae from different treatments and control (dry matter basis)

Initial & Treatments	Moisture	Dry matter	Crude protein	Crude fat	Crude ash
Initial	82.08	17.92	59.27	2.29	21.96
CF	78.29	21.71	60.48	3.51	20.46
F1	78.44	21.56	60.51	3.61	18.79
F2	76.32	23.68	65.45	4.04	18.01
F3	76.68	23.32	63.01	3.81	18.02
F4	77.10	22.9	61.80	3.75	17.51

Pellet water stability, tested for 1, 2, 3 and 4 h for diets varied markedly for each diet (Table 5). The dry matter loss was the highest for diet F2 at all durations and F1 had the lowest dry matter loss after 4 h.

Table 5. Dry matter weight loss (%) of experimental diets in 15 ppt water

Diet	1h	2h	3h	4h
CF	17.82	20.39	22.18	21.87
F1	14.82	18.19	19.35	20.15
F2	17.98	28.10	29.86	29.94
F3	14.38	14.73	17.89	20.61
F4	13.66	16.85	19.40	22.80

Discussion

Growth of the shrimp was significantly affected by incorporation of AFP *in lieu* of fishmeal and the diet F2 containing 150 g kg^{-1} AFP showed the best performance. The protein and lipid level in the present diets were well within the recommended range for commercial shrimp diets (Alava and Lim, 1983; Pascual *et al.,* 1983; Akiyama *et al.,* 1992; Bautista and Subosa, 1999) (Table 1). The crude protein content of all the feeds was relatively high (@380 g kg^{-1}), so the small differences in protein content and amino acid composition among the feeds are unlikely to account for the difference in growth rate among the diets (Guillaume, 1997; Chuntappa *et al*., 1999). The amino acid profiles in the present diets were well above the requirement for shrimp and the growth repression with higher inclusion of AFP may be resulted from growth depression due to excess of some amino acids like arginine (Chen *et al*., 1992; Millamena *et al.,* 1998). The amino acid profile has shown a dominance of certain essential amino acids (histidine, arginine, threonine, isoleucine, leucine and phenylalanine) in F2 than in the control diet (Table 2). The feeding rate was fixed at 15 % of the body weight and there were no indications of feed wastage even at the highest levels of AFP suggesting good acceptance of the ration offered. Delayed acclimation to those diets with increased incorporation of AFP by the shrimp in the beginning of the trial might have resulted in the reduced growth rate of shrimp in these treatments. Reduced feed intake in the absence of feeding effectors have been reported by Smith *et al*. (2005) who have demonstrated that the shrimp were capable of discriminating between a feed containing low levels of ingredients recognized for their stimulatory characteristics. Paripatananont *et al*. (2001) reported that the feed intake by shrimps was not different at 0, 25 or 50 % fishmeal (FM) substitution by soy protein concentrate (SPC), but was significantly decreased at 75 and 100 % of substitution levels. They have also reported severe negative effect on the body weight gain by 100 % FM substitution in the diet. Since all the diets contained similar ingredients other than different levels of AFP and fishmeal and that the diets were isoproteic, the results indicate that the level of alternate protein source (AFP) was the major factor that influenced the growth rates.

Even though it was not experimentally quantified in the present study, there is a possibility of reduction in the level of anti-nutrients in the AFP due to the fungal transformation of the substrate into better digestible and bio-available product resulting in improved performance of shrimp fed diet F2. The process of SSF itself would have modified the ingredient mix to easily digestible components with the release of enzymes that remain in the

AFP as reported by earlier workers (Pandey, 1992; Kamini *et al*., 1997; Romero-Gomez *et al*., 2000). Singh *et al*. (1990) reported increased digestibility of complex polysaccharide by SSF due to increased lignocellulolytic activity of fungus *Coprinus femetarius*. Brito *et al*. (2001) had observed increased amylase activity when post-larvae were fed artificial diet, which was apparently related more to the origin of the starch than to the total carbohydrate level of the diet. They found no obvious relationship between enzyme activity and growth in any feed combination.

The mean values observed in FCR and PER of the present study are superior to those reported by other authors who used alternate protein sources in diets for shrimp (Alava and Lim, 1983; Shiau and Chou, 1991; Shiau and Peng, 1992; Sudaryono *et al*., 1995). The control diet with no AFP recorded the poorest FCR (3.30), which was significantly lower ($p<0.05$) than the other treatments. Paripatananont *et al*. (2001) observed severe negative impact in the FCR, PER and protein as well as fat gain in the *P. monodon* post-larvae with increasing substitution of fishmeal (75 % and above) by soy protein concentrate (SPC). The net protein utilization was the highest for F2, which suggests better availability of protein in the diet to the shrimp.

Chitin, a polymer of glucosamine is found in the cell walls of fungi and it also forms 50–80 % of organic compounds in crustaceans shells (Muzzarelli, 1977). In the present study, the incorporation of AFP up to 150 g kg^{-1} in the diet would have contributed some level of chitin in the diet which enhanced shrimp growth. Three significant ($p<0.05$) peaks of glucosamine content ranging from 9-11 mg $gdfs^{-1}$ for *A. niger* in SSF was reported by Asha-Augustine *et al.* (2006). Fox (1993) reported that shrimp-head meal is a good source of chitin, which can be safely used up to 44% of the diet of juvenile *Penaeus monodon*. Also, the addition of chitin either from shrimp head meal (natural source) or in purified form enhanced the growth rate of *M. rosenbergii* PL significantly ($p<0.05$) over the control group (Kumar *et al*., 2006). Shiau and Peng (1997) had observed that dietary chitin level enhanced the growth of *P. monodon* and its level should not exceed 5 % in diets.

The APD, AFD and ADC were the highest for *P. monodon* fed diet F4 but the APU was the highest for shrimp fed diet F2, which clearly indicates the necessity of some quantity of animal protein in the diet. The increased protein content and lipid in the shrimp when fed with diet F2 may be correlated with the enhanced protein and lipid synthesis due to improved protein and fat digestibility.

The lowest water stability for F2 cannot be attributed to the incorporation of AFP in the diet. The possibility may be due to inadequate gelatinization of wheat flour, which was not observed during feed preparation. The diet F4 showed the least dry matter loss during four hours of trial.

The results of the present experimental study indicate that a combination of 150 g kg^{-1} AFP along with 200 g kg^{-1} fish meal in diets can support normal growth of post-larval *P. monodon.*

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