International Journal of Fisheries and Aquatic Studies 2017; 5(4): 458-462

E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2017; 5(4): 458-462

www.fisheriesjournal.com
Received: 13-05-2017
Accepted: 14-06-2017

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Evaluation of nutritionally enhanced feed through microbial fermentation on growth and survival of fingerlings of *Labeo rohita* (Hamilton)

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Abstract
Increase in fish feed cost leading to non-lucrative fish farming can be counteracted by utilizing inexpensive oil cakes as ingredients. The present study was aimed to use low cost sesame and sunflower oil cakes as ingredients in feeds for *Labeo rohita* fingerlings. Doughs made from oil cake powder with 60% moisture content were fermented for seven days using inoculum with a proportion of probiotics powder (Aqualact/Rhodomax/Uni-Nutrich Plus):jaggery:water: :5g:25g:160ml (per kg of oil cake substrate). The fermented oil cakes were incorporated at a rate 35% to 40% in the test diets. Fingerlings of *Labeo rohita*, fed with test diet containing 24h Aqualact fermented sesame oil cake showed highest weight gain (3.66 ± 0.04 g) with lowest FCR (1.991 ± 0.003) and highest protein value (13.81± 0.02) in the carcass. Fingerlings group fed with the same test diet also showed highest Protein efficiency ratio (1.683 ± 0.003), Apparent protein net utilization (27.45 ± 0.07) and Lipid productive value (29.26± 0.90). These fermented oil cakes are found to be viable alternatives to other costlier fish feed ingredients.

Keywords: solid state fermentation, sesame oil cake, sunflower oil cake, probiotics, *Labeo rohita*

1. Introduction
Aquaculture is a growing industry in India, but freshwater aquaculture production in the country is constrained by inadequate production of manufactured diets [1]. While there is an adequate domestic diet ingredient resource base in the country, the huge gap between requirement and production of manufactured aqua diet is mainly due to poor production of quality fish meal [2] necessitating import of fish meal from abroad or relying on non-conventional sources of plant protein. Recycling nutrients contained in underutilized agricultural by-products through aqua diet formulation is envisaged to be a viable option to produce cost effective eco-friendly diet for fish. Again, the cost of oil cakes used as substitute to fish meal such as: Soya bean meal, ground nut oil cake and mustard oil cake is going high day by day. Other low-cost plant by-products/oil cakes cannot be utilized directly because of uncertainties in their digestibility, palatability, nutritional value and presence of anti-nutritional factors (ANFs). Fermentation is a useful technique to improve nutritional quality of these agricultural byproducts to make them more suitable for incorporation in diet formulation as a protein source [3, 4]. Fermentation considerably decreases the amounts of ANFs, increases crude protein content and reduces crude fiber content of plant materials and therefore increases their nutritional value [5, 6]. Therefore, the present study has been conducted to investigate the possibility of utilizing two low-cost, locally available plant feedstuffs i.e. sesame oil cake (SOC) and sunflower oil cake (SFOC), as substrates for Solid State Fermentation (SSF) and also to evaluate the value addition of these fermented substrates through reduction in the content of ANFs (tannin and phytic acid), crude fibres and increment in the content of crude protein and minerals for their subsequent use as aqua-feed ingredients for *Labeo rohita.*

2. Materials and Methods
In all the previous studies on microbial fermentation of plant materials to enhance its nutritional value, researchers have been used the pure culture lines of fish gut bacteria and/or fungi after procuring it directly from different renowned government or private research

institutes [7, 8, 9, 10, 11]. This practice is quite impossible in the part of an illiterate fish farmer. To make the technology simple and farmers' friendly, in the present study it is novel to use three commercially available powder form feed probiotics (Aqualact/Rhodomax/Uni-Nutrich plus) which contain spores of three or more fish gut bacteria like *Bacillus* spp., *Lactobacillus* spp., *Streptococcus* spp., *Clostridium* spp. and fungi, *Aspergillus niger, A. oryza, Rhizopus* spp., *Saccharomyces cerevisae* (Baker's yeast) as starter or inoculum in SSF of both the oil cakes.

As a procedure of SSF, first of all an 'oil cake substrate' has to be processed and prepared. For this, Intended powdered oil cake was first sieved properly through 400 micron mesh. Then 5kg of finely sieved oil cake was mixed thoroughly with 3 litres of tap water *i.e.* upto 60% moisture content to make a dough. The dough was autoclaved at 121^0C for 15 minutes in 15lb pressure by keeping it in a cylindrical stainless steel vessel. Then the heat processed dough was allowed to cool down to tepid warm stage (approximately to 37^0C), transferred to a plastic trash can and was kept ready for inoculation with 'Microbial suspension'.

By following the procedure coined by [7], approximately 800ml of 'Aquaguard' filtered tap water was collected in one litre capacity glass beaker. Then 25g intended probiotics powder and 125g of jaggery were added to the water (at a rate of 5g probiotics and 25g jaggery per kg of prepared substrate respectively). It was mixed properly by stirring with a sterilized glass rod for 5 minutes and kept static for another 5 minutes to allow the fibres to settle down. The upper part 800 ml of supernatant or the microbial suspension was then added to the above said luke-warm oil cake substrate and mixed thoroughly with a sterilized melamine spatula.

After being substrate was inoculated with the microbial starter, the SSF process was carried out in an open fermentation chamber (plastic trash can, top was only covered with mosquito net) under ambient temperature (27-30^0C) in a static condition for 7 days with periodical stirring once in 24 hours just prior to daily collection of sample. At every 24 hours interval (everyday by 8 pm), approximately 200g samples in triplicate was taken out into aluminum casseroles by means of a sterilized melamine spatula, kept in an oven at 60 ± 5^0C for drying. The samples were dried up to constant moisture level and then packed in air-tight plastic containers, brought to laboratory for proximate nutrients and ANFs analysis.

Both the sunflower oil cake (SFOC) and sesame oil cake (SOC) were subjected to SSF process by using separately the three probiotics as microbial inoculums. For the said purpose six fermentation chambers were used. Periodically, in every 24 hours interval samples were collected from each fermentation chamber for 7 days, oven-dried and analyzed for its proximate nutrients and ANFs composition. In each case, optimization of fermentation process was adjudged by keeping in view the maximum enrichment of crude protein content and maximum reduction of crude fibre content in the sample. After ascertaining the optimum stage, the experiment was repeated so as to accomplish mass production of six types of fermented feed ingredients with maximum crude protein content. The six types fermented feed ingredients were:- 24 hours Aqualact fermented SOC, 24 hours Rhodomax fermented SOC, 24 hours Uni-Nutrich plus fermented SOC, 24 hours Aqualact fermented SFOC, 168 hours Rhodomax fermented SFOC and 48 hours Uni-Nutrich plus fermented SFOC. The ANFs like tannin and phytic acid content in raw sesame oil cake were 0.96% and 0.47% respectively. Similarly, the tannin and phytic acid content in raw sunflower oil cake were 1.02% and 0.63% respectively. But, none of these two ANFs were detected in the fermented oil cake samples (at a detectable limit of 0.05%).

Eight sets of isonitrogenous (Crude protein approximately- 30 %) and isocaloric (Gross energy approximately- 3.7 kcal/g) experimental diets were formulated using raw SOC and SFOC meal (control diets, D1 and D2) and fermented SOC and SFOC meal (D3-D8). Proximate composition and proximate analysis of the experimental diets have been presented in Table 1 and Table 2 respectively. Dietary ingredients were finely powdered, sieved to obtain uniform particle size (<400 m in diameter) and mixed thoroughly. The mixture was added with 'Vitapet oil' (cod liver oil: soya oil: 50:50) at 3% level and was made to dough using lukewarm water and wheat flour or *Maida* (2%) as binder. A vitamin - mineral premix (Agrimin Forte, Virbac Animal Health India Private Limited, Mumbai-66) was added at 1% to the diets before pelletization. The dough was passed through a hand pelletizer (pellet size: 1.5 mm in diameter). The pellets thus obtained were initially sun dried and further in a hot air oven at 60 ± 5^0C for 96 hrs. The dried pellets were crumbled and packed in airtight plastic containers to store in a refrigerator until use for the growth study of *Labeo rohita*. The proportion of different feed ingredients was determined by using Pearson's square method for making diet isonitrogenous.

Table 1: Proximate ingredient composition (% dry matter basis) of the experimental diets

Ingredients	Control-1 (D1)	Control-2 (D2)	(D3)	(D4)	(D5)	(D6)	(D7)	(D8)
Fish meal	10	10	10	10	10	10	10	10
Sesame oilcake	30	-	-	-	-	-	-	-
Sunflower oil cake	-	30	-	-	-	-	-	-
Sesame oil cake+ Aqualact	-	-	35	-	-	-	-	-
Sesame oil cake +Rhodomax	-	-	-	38	-	-	-	-
Sesame oilc ake + Nutrich plus	-	-	-	-	37	-	-	-
Sunflower oil cake + Aqualact	-	-	-	-	-	39	-	-
Sunflower oil cake + Rhodomax	-	-	-	-	-	-	40	-
Sunflower oil cake + Nutrich plus	-	-	-	-	-	-	-	39
Rice bran	30	28	28	29	27	26	26	27
Soybean meal	23	25	20	16	19	18	17	18
Oil	3	3	3	3	3	3	3	3
Mineral & Vitamin premix	2	2	2	2	2	2	2	2
Binder (Wheat flour)	2	2	2	2	2	2	2	2

Table 2: Proximate analysis (% dry matter basis) of the experimental diets

Parameters	Control-1 (D1)	Control-2 (D2)	(D3)	(D4)	(D5)	(D6)	(D7)	(D8)
Crude protein	29.78	29.53	29.88	29.90	30.04	30.39	29.86	30.31
Ether extract	9.05	10.30	7.62	7.54	8.72	10.83	11.65	10.97
Gross energy (kcal/g)	3.77	3.79	3.56	3.60	3.63	3.81	3.86	3.86

Experimental design

Based on eight types of experimental diets (D1 to D8), eight groups of rohu fingerlings each in triplicates kept in 24 number of aquaria. Each aquarium stocked with randomly chosen 15 number of test species rohu fingerlings of ABW 4.4 ± 0.16 g in 90 litres of water. The aquaria were laid out in a completely randomized block design [12] with three replicates for each of the eight diet treatments. They were fed daily at a rate of 5% of total biomass. Fortnightly, weights of the fishes were recorded and accordingly the daily rations for the next fortnight were scheduled. Fortnightly the water quality parameters were determined during the feeding trial by the procedures of [13] (Table 4).

After the end of 60 days experiment, the FCR, growth performances, protein and lipid utilization efficiencies, proximate carcass composition of fingerlings were estimated. Proximate analysis of feed ingredients, experimental diets and the carcass were performed following the [14].

Statistical analysis

Statistical analysis of data was performed by analysis of variance (ANOVA) using Microsoft software SPSS followed by Duncan's Multiple Range Test (DMRT).

3. Results and Discussion

From the Table 3, it is evident that the average weight gain was found to be remarkably highest (3.66 ± 0.04 g) and the FCR was significantly lowest (1.991 ± 0.003) for the group of fishes fed with test diet D3 which constituted of 'Aqualact' fermented sesame oil cake. The highest weight gain (%), mean growth rate (% per day) and specific growth rate (% per day) were recorded as 81.27±0.06, 6.09±0.07 and 0.991 ± 0.001 respectively for the group of rohu fingerlings fed with test diet D3 which contained 'Aqualact' fermented sesame oil cake. Protein utilization efficiencies can be assessed by calculating PER (Protein Efficiency Ratio) and APNU (Apparent Protein Net Utilization, in %) or PPV (Protein Productive Value, in %). The highest PER and APNU / PPV were recorded as 1.683 ± 0.003 and 27.45 ± 0.07 respectively for the group of rohu fingerlings fed with test diet D3 which composed of 'Aqualact' fermented sesame oil cake. Lipid utilization efficiency can be determined by calculating LPV (Lipid Productive Value in %). The highest LPV was recorded as 29.26± 0.90 for the group of rohu fingerlings fed with test diet D3 which made up of 'Aqualact' fermented sesame oil cake. The proximate carcass composition of the experimental fish at the end of the feeding trial showed significant increase in protein content in comparison to the initial values in all of the dietary treatments. The highest carcass protein was recorded as 13.81± 0.02 for the group of rohu fingerlings fed with test diet D3 which contained 'Aqualact' fermented sunflower oil cake.

Water quality of each rohu experimental tanks was monitored every 15th day for temperature, pH, dissolved oxygen, total alkalinity, nitrite nitrogen and ammonia nitrogen. Temperature range of the water was found to be 28.8 -30.1 ^{0}C. Up on studying the pH the range was found to be 7.53-8.16. Total hardness and total alkalinity were found to be 103.74 -107.53 and 110 -120 mg / l respectively. Dissolved oxygen, nitrite nitrogen and ammonia nitrogen range were 6.6-7.1, 0.06-0.08 and 0.07-0.09 mg/l respectively. (Table 4).

The present results indicate that the high amount of crude protein present in the SOC and SFOC can be utilized in carp diet formulation if it is fermented with suitable microorganisms. These two fermented oil cakes could be incorporated in rohu diets up to 35% to 40%. Increased level of crude protein and decreased level of crude fibre content was achieved in fermented plant matters in comparison to the raw substrates is consistent to the findings of past researchers [15, 16,].

SSF significantly increased the crude protein contents and decreased the crude fibre contents of both the plant matters (SOC and SFOC). These findings are in agreement with the previous works where crude protein content increased and crude fibre content decreased due to SSF. Compared to the unfermented Rape Seed Cake (RSC), in 72h *Aspergillus niger* fermented RSC at incubation temperature 34°C resulted in a steep increase ($P < 0.05$) in the trichloroacetic acid soluble protein (TCA-SP), crude protein and ether extract contents to 103.71%, 23.02% and 23.54%, respectively [17]. Significant increase in crude protein and decrease in crude fibre levels of locust bean, *Parkia biglobosa* [18] ; finger millet, *Eleusine coracanagrass* [19] ; grass pea, *Lathyrus sativus* [16] ; black gram, *Phaseolus mungo* [20] ; grass pea, *Lathyrus sativus* [21] and water hyacinth, *Eichhornia crassipes* [9] due to SSF have been reported.

Raw SOC and SFOC were used as substrates which contain good quantities of protein, lipid and minerals and the presence of certain ANFs may hinder their frequent nutritional utilization. Tannin, phytic acids and trypsin inhibitors are some of the common antinutritional substances in the plant feedstuffs [22]. Results of the present study suggest that after 24h of fermentation no tannin or phytic acid were found (with a detectable limit up to 0.05%) in the fermented samples of sesame and sunflower oil cake.

Regarding water quality parameters many similar and diverse results were observed by various scientists. According to one earlier report water quality for temperature, pH, dissolved oxygen and total alkalinity were 29-32^{0}C, 6.5-7.3, 4.9-7.2 mg/l and 160-182 mg/l respectively [9]. The range of dissolved oxygen was found to be higher and contradictory result in pH was observed in the current study. It is revealed that the range of dissolved oxygen is in a similar range in both the experiments which may be due to artificial aeration through air compressor.

Table 3: Growth performance and feed utilization efficiencies in *Labeo rohita* stunted fingerlings fed experimental diets for 60 days

Test Diets	Initial Wt. (g)	Final Wt. (g)	Wt. Gain (g)	Feed Consumed (g)	FCR	Protein Consumed (g)	Lipid Consumed (g)	SGR	PER	LPV (%)	ANPU (%) / PPV (%)
D1	4.40 ± 0.03^{a,b}	7.41 ± 0.05^{e,f}	3.01 ± 0.02^{d}	6.56 ± 0.04^{d}	2.181 ± 0.002^{g}	1.95 ± 0.01^{c}	0.593 ± 0.003^{d}	0.868 ± 0.001^{f}	1.537 ± 0.003^{e}	28.30± 1.09^{a}	21.16 ± 0.22^{d}
D2	4.30 ± 0.05^{b}	7.22 ± 0.05^{f}	2.92 ± 0.01^{d}	6.58 ± 0.01^{d}	2.251 ± 0.002^{h}	1.94 ± 0.01^{c}	0.680 ± 0.000^{b}	0.865 ± 0.006^{f}	1.503 ± 0.003^{f}	19.27± 0.95^{c}	23.88 ± 0.28^{c}
D3	4.50 ± 0.05^{a}	8.16 ± 0.09^{a}	3.66 ± 0.04^{a}	7.29 ± 0.09^{a}	1.991 ± 0.003^{a}	2.17 ± 0.03^{a}	0.553 ± 0.007^{e}	0.991 ± 0.001^{a}	1.683 ± 0.003^{a}	29.26± 0.90^{a}	27.45 ± 0.07^{a}
D4	4.50 ± 0.05^{a}	8.09 ± 0.10^{a}	3.59 ± 0.05^{a}	7.25 ± 0.09^{a,b}	2.021 ± 0.002^{b}	2.16 ± 0.03^{a}	0.547 ± 0.009^{e}	0.977 ± 0.001^{b}	1.653 ± 0.003^{b}	18.62± 1.10^{c}	26.96 ±0.21^{a}
D5	4.40 ± 0.04^{a,b}	7.85 ± 0.07^{b,c}	3.45 ± 0.03^{b}	7.15 ± 0.07^{a,b}	2.071 ± 0.001^{d}	2.14 ± 0.02^{a}	0.620 ± 0.006^{c}	0.964 ± 0.001^{c}	1.607 ± 0.003^{c}	10.10± 0.55^{d}	25.35 ± 0.11^{b}
D6	4.50 ± 0.05^{a}	7.97 ± 0.08^{a,b}	3.47 ± 0.03^{b}	7.12 ± 0.07^{a,b}	2.052 ± 0.002^{c}	2.16 ± 0.02^{a}	0.773 ± 0.007^{a}	0.952 ± 0.002^{d}	1.603 ± 0.003^{c}	22.36± 0.86^{b}	25.77 ± 0.20^{b}
D7	4.40 ± 0.05^{a,b}	7.60 ± 0.07^{d,e}	3.20 ± 0.02^{c}	6.78 ± 0.03^{c}	2.119 ± 0.002^{f}	2.02 ± 0.01^{b}	0.790 ± 0.006^{a}	0.910 ± 0.005^{e}	1.577 ± 0.003^{d}	12.01± 1.04^{d}	25.37 ± 0.26^{b}
D8	4.30 ± 0.03^{b}	7.68 ± 0.04^{c,d}	3.38 ± 0.01^{b}	7.06 ± 0.03^{b}	2.090 ± 0.002^{e}	2.14 ± 0.01^{a}	0.773 ± 0.003^{a}	0.966 ± 0.004^{c}	1.577 ± 0.003^{d}	10.64± 0.64^{d}	21.48 ± 0,12^{d}

Mean values with same superscripts in each column are not significant ($p<0.05$). Values are mean of three samples ± SE

Table 4: Water quality parameters in experimental tanks during the growth study of Rohu

Parameters	Range
Temperature (^{0}C)	28.8 - 30.1
pH	7.53-8.16
Dissolved oxygen (mg/l)	6.6 -7.1
Total hardness (mg / l)	103.74 -107.53
Total alkalinity (mg / l)	110 -120
Nitrite nitrogen (NO2-N) (mg/l)	0.06 - 0.08
Ammonia nitrogen (NH3-N) (mg/l)	0.07 - 0.09

4. Conclusion
Fermented SOC and SFOC are both promising alternative raw ingredients for the formulation of Indian Major Carp diets. However, the present wet-lab study will require further field experimentation in earthen ponds with large number of fish and replication. Therefore, it is too early to recommend to the industry to use fermented SOC and SFOC in formulation of aquafeeds. But, the fish farmers can be advised to include 24h probiotics fermented SOC and SFOC in their farm made dough feeds. Moreover, further studies are required to carry out SSF of other low-cost non-conventional feed stuffs with various other commercial feed probiotics available in the market to create more viable options for the fish farmers to use it as fish feed in their ponds.

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