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Sukhanandi SM
Department of Biosciences, Bakrol-Vadtal road, Sardar Patel Maidan, Sardar Patel University, Vallabh Vidyanagar 388120, Gujarat, India.

Bhatt SS
Department of Biosciences, Bakrol-Vadtal road, Sardar Patel Maidan, Sardar Patel University, Vallabh Vidyanagar 388120, Gujarat, India.

Correspondence
Bhatt SS
Department of Biosciences, Bakrol-Vadtal road, Sardar Patel Maidan, Sardar Patel University, Vallabh Vidyanagar 388120, Gujarat, India.

Corn steep liquor with lysine and chelated minerals as supplementary feed for *Labeo rohita* fingerlings

Sukhanandi SM, Bhatt SS

Abstract
Corn steep liquor (CSL) as protein source with lysine and chelated minerals is evaluated as dietary supplement for *Labeo rohita* (rohu) fingerlings, with weight ranges between 1.48 - 1.65 g. Experimental diets were formulated with CSL at 50% to 100% level with lysine and chelated minerals (D1-D5); and control diet contained fishmeal as a protein source (CD). Experimental diets hampered the growth performance, protein content and amylase activity; except D1. CSL based diets were found to be as digestible as control diet. Significant increase in hexokinase (D3 to D5), lactate dehydrogenase (D1 to D5), glucose-6-phosphatase (D3 to D5) and alanine amino transferase (D1 to D3, D5) activities indicates increased glycolysis and gluconeogenesis in fishes. Reduction in alkaline and acid phosphatases; and increase in lysozyme, superoxide dismutase and acetylcholine esterase activities suggest role of CSL in lowering nutrient transportation and reducing stress respectively. The test diet is not suitable for rohu early fingerlings.

Keywords: Corn Steep Liquor, Fish Feed, Growth performance, key metabolic enzyme, *Labeo rohita*

1. Introduction
The fast growing aquaculture industry is responsible for increasing demand and price for fish meal; and therefore a partial or complete substitution of fish meal with protein rich agro materials has become a focal point in aqua feed research (Kaushik 1995) [22]. The carp is a dominant group of fishes in pond culture practice in Indian subcontinent; and the use of plant proteins as a partial replacement of fish meal in their diet has been considerably attempted (Hossain *et al*. 2001) [20]. However use of agro-based protein sources in fish feed is limited due to the presence of antinutritional factors, poor protein quality and low digestibility (Oliva-Teles and Goncalves 2001) [36]. The use of agro-industrial by-products such as brewery waste and corn starch industry by-products have shown promising results (Pereira and Teles 2003; Chovatiya *et al*., 2011) [39, 10].

Corn steep liquor is a corn by-product from starch milling plant and it contains condensed steep water with solubles. It is rich in crude proteins, minerals, vitamins, reducing sugars, organic acids (mainly lactic acid) and enzymes; it is also rich in amino acids including sulphur amino acids, though deficient in lysine; and it is free from fibres and antinutritional factors (Chovatiya *et al*., 2011) [10]. All these make it an excellent potential nutritive source for livestock and fishes as reported in the literature (Talpada *et al*., 1987; Chovatiya *et al*., 2011) [47, 10].

The requirement of lysine and sulphur containing amino acids are considered rate limiting for carps and other cultivable fishes; and the supplementation of deficient essential amino acids in the agro-based feed is known to improve the fish performance (Mukhopadhyay and Ray 2001; Cheng *et al*., 2011) [34, 9]. The use of chelated minerals, especially with amino acids and low molecular weight peptides, in animal feed is a recent trend; and the use of chelated minerals in poultry and piggery feed has already been established (Paik *et al*., 2000; Salim *et al*., 2012) [37, 43]. However this approach does not seem to have been thoroughly assessed in aquafeed.

In an earlier work, Chovatiya *et al.* (2011) [10] have reported that partial replacement of fish meal with CSL does not hamper the growth of rohu in advance fingerling stage. In the present study, we report on suitability of CSL as sole protein source with lysine and minerals chelated with metheonine for rohu early fingerlings. We focus not only on growth performance but also on metabolism and stress level in the fishes.

2. Materials and Methods

2.1. Diet Formulation

Six iso-nitrgenous (≈37% crude protein) and iso-caloric (≈18 kJ g^{-1}) diets were formulated containing CSL respectively at 50%, 75% and 100% replacement of fish meal (D1 to D3). The 100% CSL based diet groups were further supplemented with lysine (D4) and lysine+ minerals chelated with metheonine (D5) separately. The control diet contained fish meal as the main protein source (CD). The corn gluten was used to adjust nitrogen level to make the diets iso-nitrogenous (Table 1). Chromic oxide was used as an external marker for the nutrient digestibility study; and bentonite was used as a binder. The feed ingredients were mixed and made into moist pellets of 3 mm diameter with hand pelletizer, oven dried at 50 ˚C for 24 h and stored at 4 ˚C.

Tables 1: Formulation and proximate composition of the experimental diets replacing fishmeal with various levels of CSL as percentage of total dry matter

DIETS (% of fish meal protein replaced)	Diet groups					
	CD 0%	D1 50%	D2 75%	D3 100%	D4 100% + Lysine	D5 100% +Chelated minerals
Ingredients (g)						
Fish meal	45	22.5	11.25	-	-	-
CSL	-	30.0	45.0	57	57	57
Corn gluten	8.0	12.0	12.0	15	15	15
Rice bran	38.4	26.9	23.15	19.4	17.18	17.18
Premix [a]	2.5	2.5	2.5	2.5	2.5	-
Oil pre mix [b]	5.0	5.0	5.0	5.0	5.0	5.0
Bentonite [c]	0.1	0.1	0.1	0.1	0.1	0.1
Cr2O3 [d]	1.0	1.0	1.0	1.0	1.0	1.0
Lysine [a]	-	-	-	-	2.22	2.22
Chelated minerals [e]	-	-	-	-	-	2.5
Proximate composition (% dry matter)						
Crude protein	36.41	37.35	36.72	36.76	36.53	36.53
Lipid	15.50	11.76	10.12	8.57	8.229	8.229
Ash	10.95	9.64	9.42	8.04	8.57	8.57
Gross energy (KJ/g)	20.766	19.598	17.580	16.684	16.269	16.269

[a] Vitamin mineral mixture and Lysine (Vitaminetes Forte, Roche Products Ltd., Mumbai, India)
[b] Oil Pre mi,x (2 Corn oil: 1 Cod liver oil); Corn oil: Tirupati active, N.K. Proteins Co., Mehsana, Gujarat, India; Cod liver oil: Seacod, Universal Medicare Pvt. Ltd., Mumbai, India
[c] Bentonite purchased from Gujarat Minechem, Bhavnagar, Gujarat, India
[d] Chromic oxide (Cr2O3): Qualigens R, Mumbai, India
[e] Supplied by Charotar animal feed, Vallabh Vidyanagar, Gujarat, India

2.2. Fish and Experimental Design

Labeo rohita fingerlings were obtained from Gujarat Govt. Fish Seed Centre, Navali, Anand, Gujarat (India), acclimatized to laboratory conditions for 15 days and fed with a 1:1 mixture of finely powdered rice bran and groundnut oil cake. The feeding trial was conducted in 150 L glass aquaria (0.91 × 0.38 × 0.45 m^3). Fingerlings (mean weight 1.57± 0.05 g) were stocked at a density of 25 fishes per aquarium with three replicates for each treatment group. Un-chlorinated tube well water was used for the experiment. The fishes were fed with the formulated feed twice a day at 9:00 and 15:00 h at the rate of 5% of the body weight per day for 60 days. The fishes were weighed every week to adjust feeding ration. During experimentation, continuous aeration was provided, and the temperature was maintained constant at 28 ˚C. The water pH, dissolved oxygen and alkalinity were monitored weekly following the methods of American Public Health Association (APHA 1980) [2] and they were found to be 7.56-8.59, 7.15-7.25 mg L^{-1} and 103.95-104.65 mg L^{-1} respectively.

2.3. Chemical Analysis and Data Collection

Formulated Diet, faecal samples and fish carcass were analysed for their proximate compositions following the method of Association of Official Analytical Chemists (AOAC 1990) [1] as follows: moisture by oven drying at 105 ˚C for 24 h, protein (N×6.25) by Micro Kjeldahl method, ash by ignition at 550 ˚C in a muffle furnace to constant weight and lipid by gravimetric method. The energy content was calculated using the average caloric conversion factors as 9.45, 4.10 and 5.65 kcal g^{-1} for lipid, carbohydrate and protein respectively (Henken *et al*., 1986) [18]. Chromic oxide was estimated following the method of Furukawa and Tsukahara (1966) [17]. Faeces were collected once in morning during the last 2 weeks of experimentation before feeding. Uneaten feed was siphoned out from the aquaria after the last feeding in the evening for the calculation of feed utilization parameters. Faeces collected from triplicate treatment groups were pooled, oven dried at 60 ˚C and stored for digestibility study. Pooled faecal samples for each treatment were analysed separately. At the end of the feeding trial, fishes were starved for 24 h prior to sampling. Fishes in each tank were weighed individually and counted for information on growth, feed efficiency, and survival. Percent body weight gain (% weight gain), specific growth rate (SGR, % day^{-1}), feed intake (mg feed/fish/day), feed conversion ratio (FCR), protein efficiency ratio (PER), apparent protein digestibility (APD, %), apparent lipid digestibility (ALD, %) and thermal growth co-efficient (TGC) were calculated using standard formula as below:

SGR specific growth rate (% day^{-1}) = (log W_t - log W_0)/t × 100

FCR feed conversion ratio = feed intake (g)/live weight gain (g)

PER protein efficiency ratio = weight gain (g)/crude protein intake (g)

APD = 100 – 100 × ((% Cr2O3 in diet/% Cr2O3 in faeces) ×

(% protein in faeces/% protein in diet))
ALD = 100 -100 × ((% Cr2O3 in diet/% Cr2O3 in faeces) × (% lipid in faeces/% lipid in diet))
TGC thermal growth coefficient = $[(W_t)^{1/3} - (W_0)^{1/3} \times (T - t)] \times 1000$
Where W_0 is initial weight in g, W_t is final weight in g, T is the temperature in C, and t is the time in days.

2.4. Sampling and Sample Preparation for Enzyme Assay
At the end of the feeding trial, fishes were weighed and three fishes from each replicate from each treatment group were randomly collected; the muscle, gut, liver, brain and kidney tissues were dissected out and used for enzyme studies; and muscles were also used for the analysis of proximate composition. The gut was rinsed with chilled distilled water, homogenized in tissue homogenizer in cold (4 ˚C) phosphate buffer with pH 7.2 (1: 10 w/v), centrifuged (10,000 g 10 min at 4 ˚C) and supernatant was stored at -20 ˚C for estimating digestive enzyme activities. Liver, muscle, kidney and brain tissues were homogenized (1: 10 w v^{-1}) with chilled sucrose solution (0.25 M), centrifuged (5,000 g, 4 ˚C, 10 min) and the supernatant was stored at -20 ˚C for the enzymatic studies.

2.5 Enzyme Assay
The α-amylase, protease and lipase activities were assayed using, soluble starch, casein and olive oil as substrates respectively (Bernfeld 1955; Kunitz 1974; Rathelot *et al*., 1975) [7, 28, 41]. The hexokinase (HK) and Glucose-6-phosphatase (G6Pase) activities were measured by the method of Brandstrup *et al*., (1957) [6] and Baginsky *et al*., (1974) [4] respectively. The activity of lactate dehydrogenase (LDH), alkaline phosphatase (ALP), acid phosphatase (ACP), alanine amino transferase (ALT) and aspartate amino transferase (AST) were measured by the method of Scand (1974) [45], Kind and King (1954) [24], King and Jagatheesan (1959) [25] and Henry (1974) [19] respectively. The lysozyme (LSZ) activity was estimated by the method of Ellis (1990) [14]. The superoxide dismutase (SOD) activity was estimated by the method of Kakkar *et al*., (1984) [21]. The acetylcholine esterase activity was studied by the Ellsman's method (Ellsman *et al*., 1961) [15].

2.6 Statistical Analysis
The data were subjected to a one-way analysis of variance (ANOVA), and the significance of the difference between means was determined by Tukey's multiple range test ($P<0.05$) using the SPSS Version 16. Values are expressed as mean ± SE.

3. Results
3.1. Growth Performance
Data on growth performance and feed utilization by fishes in terms of percentage weight gain, specific growth rate (SGR), thermal growth co-efficient (TGC), feed intake, feed conversion ratio (FCR), protein efficiency ratio (PER), apparent protein digestibility (APD) and apparent lipid digestibility (ALD) are presented in Table 2. The percent weight gain, SGR, TGC and PER of the fishes fed with experimental diets were observed to be lower ($p<0.05$) in comparison to control (CD). The FCR was lower ($p<0.05$) in control group (CD) when compared to experimental groups (D1 to D5); and these values have increased with increasing the levels of CSL.

Table 2: Growth performance and feed utilization efficiencies in *Labeo rohita* fingerlings fed experimental diets for 60 days

Feeding group	Initial weight (g)	Final weight (g)	Weight gain (%)	SGR (% day-1)	TGC	Feed intake (mg/fish/day)1	FCR	PER
CD	1.60 ±0.04^{a}	8.37 ±0.13^{b}	497.62^{c}	2.94 ±0.05^{c}	0.54 ±0.00^{c}	143.02	1.25 ±0.02^{a}	2.24 ±0.04^{c}
D1	1.50 ±0.04^{a}	6.13 ±0.18^{a}	329.96^{b}	2.35 ±0.07^{b}	0.40 ±0.01^{b}	122.82	1.70 ±0.05^{b}	1.75 ±0.08^{bc}
D2	1.65 ±0.06^{a}	5.95 ±0.26^{a}	280.92^{ab}	2.13 ±0.07^{ab}	0.37 ±0.01^{ab}	125.91	1.97 ±0.07^{bc}	1.57 ±0.09^{ab}
D3	1.55 ±0.05^{a}	5.06 ±0.32^{a}	236.32^{a}	1.93 ±0.07^{a}	0.33 ±0.01^{a}	114.75	2.24 ±0.08^{c}	1.42 ±0.13^{ab}
D4	1.57 ±0.04^{a}	5.27 ±0.34^{a}	238.65^{a}	1.97 ±0.05^{a}	0.34 ±0.01^{a}	118.93	2.14 ±0.06^{c}	1.43 ±0.12^{ab}
D5	1.48 ±0.06^{a}	5.07 ±0.29^{a}	252.25^{a}	2.02 ±0.06^{a}	0.34 ±0.01^{a}	106.72	2.05 ±0.09^{bc}	1.55 ±0.11^{ab}

Data are mean values ± SE. Values with the same superscript letters in the same column are not significantly different ($P>0.05$) from each other
SGR: specific growth rate, FCR: feed conversion ratio, PER: protein efficiency ratio,
TGC: thermal growth coefficient

3.2. Body Composition, Digestibility and Digestive Enzyme Activities
The proximate composition of carcass and digestive enzyme activities of fishes are shown in Table 3. The carcass lipid, moisture and ash contents of experimental fishes were not significantly different ($p>0.05$) to those of control fishes. Among the experimental groups, only group D1 has shown the protein value quite comparable to that of the control group; and all other experimental groups (D2 to D5) have exhibited lower protein content ($p<0.05$). The protein and lipid digestibility (APD, ALD) values of experimental diets were quite similar to those of control diet, except the 100% CSL group (D3), where some decrease in these values have been detected in comparison to other diet groups. The amylase activity of D1 group was more or less similar to CD, whereas rest of the experimental groups had shown lower ($p<0.05$) amylase activity. Except in D4 group (100% CSL+lysine), in all other experimental groups (D1 to D3 and D5), the protease activity has either increased or it has remained similar in comparison to control group. The lipase activity was found to be higher ($p<0.05$) in D1 and D2 groups; whereas it was lower ($p<0.05$) in D3 to D5 groups as compared to CD.

Table 3: Proximate carcass composition (% wet weight), digestive enzyme activities and apparent digestibility of *Labeo rohita* fingerlings at the end of the 60-day feeding experiment.

Mean values	CD	D1	D2	D3	D4	D5
Moisture	80.18±0.11^{a}	80.14±0.02^{a}	79.40±0.18^{a}	79.64±0.17^{a}	79.47±0.33^{a}	79.53±0.30^{a}
Protein	16.14±0.06^{e}	15.05±0.01^{de}	14.86±0.02^{bc}	13.55±0.01^{a}	14.78±0.00^{b}	14.85±0.02^{bc}
Lipid	3.16±0.16^{a}	3.67±0.16^{a}	4.00±0.50^{a}	3.16±0.44^{a}	3.50±0.57^{a}	3.17±0.16^{a}

Ash	1.63±0.18[a]	1.41±0.08[a]	1.65±0.05[a]	1.42±0.12[a]	1.56±0.06[a]	1.67±0.08[a]
α-Amylase[1]	6.38 ±0.16[f]	5.56±0.11[ef]	5.13±0.02[e]	1.19±0.01[ab]	2.14±0.08[c]	1.11±0.00[a]
Protease[1]	0.33±0.00[b]	0.32±0.02[b]	0.54±0.00[d]	0.31±0.00[b]	0.24±0.00[a]	0.38±0.00[c]
Lipase[1]	5.13±0.12[b]	6.08±0.08[c]	6.43±0.04[c]	4.62±0.21[a]	4.85±0.03[ab]	4.89±0.00[ab]
APD (%)*	88.35	88.92	90.00	78.80	87.19	87.18
ALD (%)*	88.03	89.12	89.45	80.80	87.02	87.01

Data are mean values ± SE (n = 5). Values with the same superscript in the same row are not significantly different (P>0.05) from each other
*Statistical analysis was not possible as determinations were performed on pooled samples
[1] Enzyme activities are expressed as follows: a-Amylase = mg maltose liberated h^{-1} mg^{-1} protein; Protease = mg tyrosine liberated h^{-1} mg^{-1} protein; Lipase = U mg^{-1} protein
APD: apparent protein digestibility, ALD: apparent lipid digestibility

3.3. Key Metabolic Enzyme Activities

The effect of CSL based feed on key metabolic enzymes is shown in Table 4. In liver, the HK and G6Pase activities were found to be higher ($p<0.05$) in 100% CSL groups (D3 to D5); and the activities were lower ($p<0.05$) in 50% as well as 75% CSL groups (D1, D2 respectively) as compared to control group (CD), except G6Pase activity in D1 group which was found to be similar to that of control. It is also evident that the test groups supplemented with Lysine (D4) as well as with lysine+minerals chelated with mitheonine (D5) have shown lower ($p<0.05$) G6Pase activity in comparison to only CSL group (D3). The LDH activity in control group is found to be highest in liver (6.4 U mg^{-1} of protein) followed by kidney (5.2 U mg^{-1} of protein) and muscles (3.8 U mg^{-1} of protein). Both liver and muscle LDH activities increased ($p<0.05$) in all the experimental groups in dose dependent manner with increasing CSL level; however in kidney, increase ($p<0.05$) in the activity was detected only in D4 and D5 groups when compared to CD. In liver and muscles in control fishes, the AST activities (16.9 U mg^{-1} of protein; 34.5 U mg^{-1} of protein) were found to be higher than ALT activities (11.9 U mg^{-1} of protein and 25.1 U mg^{-1} of protein); and both the enzymes were found to be higher in muscles in comparison to liver. The CSL in diet resulted in significant decrease ($p<0.05$) in AST activity and significant increase ($p<0.05$) in ALT activity in both liver and muscles in comparison to control group, except in ALT activity in liver in D4, which is found to be similar to that of control.

Table 4: Enzymes of glycolytic pathway, gluconeogenic pathway and protein metabolism (HK, hexokinase; LDH, lactate dehydrogenase; G6Pase, glucose-6-phosphatase; AST, aspartate amino transferase and ALT, alanine amino transferase) in *Labeo rohita* fingerlings fed different experimental diet.

Feeding group	HK[2] Liver	LDH[2]			G6Pase[2]	AST[2]		ALT[2]	
		Muscles	Liver	Kidney	Liver	Liver	Muscle	Liver	Muscle
CD	1.63 ±0.00[c]	3.80 ±0.00[a]	6.40 ±0.01[a]	5.24 ±0.03[ab]	1.39 ±0.00[b]	16.90 ±1.16[d]	34.48 ±0.87[b]	11.99 ±0.78[a]	25.14 ±1.20[a]
D1	1.38 ±0.01[a]	5.79 ±0.08[b]	6.67 ±0.09 [a]	5.23 ±0.01[ab]	1.41 ±0.02[bc]	11.21 ±0.57[ab]	30.85 ±0.04[a]	26.45 ±0.52[d]	47.41 ±0.18[d]
D2	1.57 ±0.00[b]	7.82 ±0.01[c]	7.68 ±0.01[b]	5.47 ±0.01[b]	1.09 ±0.00[a]	10.79 ±0.01[a]	29.33 ±0.33[a]	25.44 ±1.89 [d]	43.83 ±1.66[cd]
D3	1.72 ±0.02[d]	10.21 ±0.07[d]	9.22 ±0.12[c]	4.82 ±0.01[a]	1.84 ±0.07[e]	12.93 ±0.17[abc]	30.74 ±0.01[a]	19.74 ±0.81[c]	44.25 ±0.51[cd]
D4	1.79 ±0.00[e]	14.00 ±0.01[f]	11.56 ±0.06[e]	8.94 ±0.01[c]	1.54 ±0.00[cd]	14.33 ±0.31[cd]	30.17 ±0.00[a]	11.02 ±0.49[a]	47.98 ±0.32[d]
D5	1.78 ±0.01[de]	13.37 ±0.07[e]	10.28 ±0.12[d]	11.60 ±0.31[d]	1.55 ±0.01[d]	13.91 ±0.53[bc]	30.14 ±0.00[a]	16.73 ±0.38[bc]	40.43 ±0.76[bc]

Values represent mean ± SE
Mean values in a column (organ wise) under each parameter bearing same superscripts did not vary significantly (P>0.05)
[2]Enzyme activities are expressed as follows:
HK- Activities expressed as U min-1 mg-1 protein, LDH- Activities expressed as Δ 0.01 OD min-1 mg-1 protein, G6Pase- mg phosphorus released min-1 mg-1 protein, ALT- Activities expressed as nanomoles sodium pyruvate released min-1 mg-1 protein
AST- Activities expressed as nanomoles oxaloacetate formed min-1 mg-1 protein

3.4. Activities of Enzymes related to First Line Defence and Stress

The effect of experimental feed on the marker enzymes associated with nutrient transport, defence and stress is shown in Table 5. Both ALP and ACP activities were found to be higher in liver in comparison to gut in control fishes. The ALP activity in both liver and gut was found to be lower ($p<0.05$) in experimental groups as compared to control, except D1 group in which it was observed to be higher ($p<0.05$) in liver. In liver, the ACP activity was observed to be higher ($p<0.05$) in 50% as well as 75% CSL groups and it was lower ($p<0.05$) in all the 100% CSL groups in comparison to control. In gut in all the CSL groups (D1 to D3), the ACP activity was either found to be higher or it has not changed as compared to CD; and supplementation of lysine (D4) as well as that of lysine+chelated minerals (D5) in the test feed lowered ($p<0.05$) this activity when compared to only CSL groups (D1 to D3) as well as CD. The activities of lysozyme (kidney), SOD (liver) and AchE (brain) were found to be significantly higher ($p<0.05$) in D1 to D3 groups as compared to CD; however, in D4 and D5 groups these activities were found to be lower ($p<0.05$) in comparison to D3 group.

Table 5: Effects of formulated feed on key markers for nutrient transport, defence and stress (ALP, Alkaline Phosphatase; ACP, Acid phosphatase; LSZ, Lysozyme; SOD, Superoxide dismutase; AchE, Acetyl cholin Esterase) in *Labeo rohita* fingerlings.

Feeding groups	ALP[3]		ACP[3]		LSZ[3]	SOD[3]	AchE[3]
	Liver	Gut	Liver	Gut	kidney	Liver	Brain
CD	4.47 ±0.47^{d}	1.32 ±0.09^{d}	0.55 ±0.03^{bc}	0.23 ±0.01^{c}	0.11 ±0.00^{a}	64.66 ±0.33^{b}	4.60 ±0.01^{b}
D1	6.04 ±0.35^{e}	0.72 ±0.01^{bc}	0.75 ±0.02^{d}	0.26 ±0.00^{cd}	0.13 ±0.00^{b}	70.00 ±0.57^{c}	5.35 ±0.07^{d}
D2	1.44 ±0.09^{ab}	0.72 ±0.09^{bc}	0.69 ±0.00^{d}	0.29 ±0.00^{d}	0.13 ±0.00^{b}	70.33 ±0.66^{c}	5.44 ±0.00^{d}
D3	0.98 ±0.05^{a}	0.47 ±0.01^{ab}	0.33 ±0.03^{a}	0.25 ±0.00^{c}	0.67 ±0.02^{e}	71.00 ±0.00^{c}	5.44 ±0.00^{d}
D4	2.88 ±0.13^{c}	0.54 ±0.07^{b}	0.43 ±0.01^{ab}	0.16 ±0.00^{b}	0.22 ±0.00^{c}	60.00 ±0.57^{a}	4.72 ±0.01^{b}
D5	2.58 ±0.23^{bc}	0.19 ±0.02^{a}	0.45 ±0.01^{b}	0.08 ±0.00^{a}	0.24 ±0.00^{d}	60.33 ±0.33^{a}	4.91 ±0.02^{c}

Data are mean values ± SE. Values with the same superscript letters in the same column are not significantly different (P>0.05) from each other.
[3]Enzyme activities are expressed as follows: ALP: IU/ mg of tissue, ACP: IU/ mg of tissue, LSZ: U/ mg of tissue where one enzyme unit= 0.01 absorbance/min, SOD: mM H2O2 decompose/ gm of tissue, AchE: moles of acetylecholin hydrolyzed /min/gm of tissue

4. Discussion

4.1. Growth Performance

The replacement of fish meal by plant proteins in aqua feed is considered to be one of the important aspects in formulating cost effective nutritionally balanced diet for cultivable fishes; however the partial or complete replacement of fish meal by plant proteins in aqua feed is reported to be suitable in cultivable fishes (Ramachandran and Ray 2007; Desai *et al*., 2012) [40, 13]. The partial and complete replacement of fish meal by CSL in the present experiment negatively influenced growth performance and feed efficiency of early fingerlings, possibly due to fish meal replacement. The lower feed intake, SGR, TGC and PER, and higher FCR with experimental diets seem to be responsible for reduced growth performance. Several reports have shown depressed growth performance of fishes when fish meal is replaced by plant products; and the low metabolic adaptation by fishes with plant based feed is considered to be responsible for lower feed efficiency (Kaushik *et al*., 2004; Wacyk *et al*. 2012) [23, 49]. Among the plant proteins, the use of corn by products appear to be quite satisfactory in aqua feed; the corn gluten meal has been shown to be used successfully up to 60% level (Men *et al*., 2014; Pereira and Oliva-Teles 2003) [30, 39]; and the corn steep liquor (CSL) can replace fish meal up to 75% level for rohu fingerlings in the size range of 3 g or more (Chovatiya *et al*., 2011) [10]. The presently observed lower growth performance of rohu at 50% to 100% inclusion level of CSL indicates that CSL is probably not suitable for presently used early fingerlings (1.57± 0.05 g), since the age of the fishes is suggestively one of the factors influencing the growth of the fishes (Men *et al*., 2014) [30]. This suggests further studies to determine suitable level of incorporation of CSL for rohu in different age groups.

The supplementations of lysine alone or with chelated minerals to CSL based diets (D4, D5) have not shown any improvement in growth parameters in comparison to only CSL group (D3). Earlier reports have also stated that the supplementation of any deficient amino acid alone to the plant based diet has not improved the growth performance of fishes (Mukhopadhyay and Ray 2001; NRC 2011) [34, 35]. The use of chelates in fish diet has not been adequately tried out; though contradictory effects of use of chelated minerals on the growth performance of *Oncorhynchus mykiss* have been reported (Satoh *et al*., 2001; Apines-Amar *et al*., 2004) [44, 3].

4.2. Muscle Proximate Composition, Digestive Enzymes and Digestibility

The CSL as protein source with lysine and with lysine+chelated minerals were adversely affect carcass protein content of fishes (Table 3). The reduction in carcass protein during the replacement of fish meal with plant proteins is known to be one of the major changes due to the feeding of low quality protein; and the type and level of plant protein is suggested to influence carcass protein and lipid contents (Robaina *et al*. 1995) [42]. Increase in the carcass lipid due to the replacement of fish meal with plant proteins has been reported in several fish feeding experiments (Wacyk *et al*. 2012; Pereira and Oliva-Teles 2003) [49, 39]. However, no significant changes in lipid deposition have been detected with CSL based diets in present study. The CSL has not inhibited protease activity; and even lipase activity remains quite similar to that of control group. These results are relatively comparable to our previous findings (Chovatiya *et al*., 2011) [10]. One of the major reasons for this could be the absence of ANFs in CSL, since ANFs are known to block the action of digestive enzyme activities (Song *et al*., 2014) [46]. The low amylase activity in rohu early fingerlings at higher CSL incorporation level appears to be due to the carbohydrate composition of CSL; as the type of carbohydrate is known to influence the amylase activity. The digestive enzyme activity is an indicator of nutrient digestibility and nutrient utilization; and dietary amino acids play an important role in augmentation of digestive enzymes (Mitra *et al*., 2008) [31]. The supplementation of lysine to test diet fails to improve the digestive enzyme activities of early fingerlings. The use of minerals chelated with metheonine along with lysine in CSL based diet does not seem to be very effective in enhancing the digestive enzyme activities except protease activity, in which a marginal increase has been detected. This indicates that increase in mineral availability does not seem to be an appropriate measure to improve the digestive enzyme activities for rohu (Satoh *et al*., 2001) [44].

Quite comparable APD and ALD values of experimental diets to that of control (Table 3) indicate that CSL is not hampering the digestibility in rohu; and these findings are quite consistent with that of earlier report on CSL as alternative protein source for rohu advance fingerlings (Chovatiya *et al*., 2011) [10]. The digestibility values of diet with CSL at 100% inclusion level is observed to be somewhat lower as compared to other groups; however, the APD values of this group is found to be better in comparison to protein digestibility of corn gluten meal at more than 60% level in the diet of Japanese sea bass (Men *et al*., 2014) [30].

4.3. Key Metabolic Enzymes

The significant increase ($p<0.05$) in the activity of Liver HK at higher inclusion level of CSL and the liver and muscles LDH activities in all the experimental groups indicate the stimulation of glycolytic pathway. The presence of high level of sucrose and lactic acid in CSL appears to be responsible for

elevation of glycolytic pathway, since the quality and quantity of dietary carbohydrates are known to influence the glycolytic enzymes like hexokinases in fishes (Panserat *et al*., 2000) [38]. The increased LDH activity also suggests the inter-conversion of pyruvate to lactate and vice-a-versa along with increased stress level in experimental fishes (Kumar *et al*., 2011; Moon and Foster 1995) [27, 33]. The increased liver HK activity also indicates the possibility of increase in glycogenesis and in the activation of pentose phosphate pathway along with increase in glycolysis, presumably a response to the increased blood glucose level with CSL in diet (Enes *et al*., 2009). At the same time, higher level of pyruvate due to high lactic acid content of CSL is likely to be responsible for significantly higher G6Pase activity in 100% CSL groups, indicating the possibility of gluconeogenesis and glucose homeostasis (Kumar *et al*., 2009; Cox and Nelson 2008) [26, 11]. The increase in the ALT activity in experimental fishes may be due to high level of alanine production from pyruvate and lactate because of the CSL based diet. In the present study increase in ALT could simply be due to high level of alanine rather than higher protein metabolism; and the lower AST activity supports this interpretation (Cox and Nelson 2008) [11]. The presently observed effect of CSL on protein metabolism is quite similar to the effect of corn gluten meal supplementation in the diet of Japanese sea bass (Song *et al*., 2014; Men *et al*., 2014) [46, 30]. Several fish feeding experiments have shown the effect of diet composition and its protein content on the level of ALT activity (Mohapatra *et al*., 2012) [32].

The decrease in both of alkaline phosphatase and acid phosphatase activities in liver as well as in gut when CSL is used at higher level indicates the inhibition of nutrient transportation in fishes, because these enzymes are considered to be indicators of nutrient transportation (Debnath *et al*., 2007) [12]. Even supplementation of lysine and minerals chelated with amino acid has failed to improve these activities. It also appears that troubled nutrient transportation is responsible for presently observed decrease in the growth performance of experimental fishes, since growth rate of fishes is positively correlated with the activity of ALP (Lemieux *et al*., 1999) [29].

4.4. Enzymes related to First Line Defence and Stress

The significant increase in the level of stress markers mainly lysozyme, SOD and AchE with increasing level of CSL indicates enhanced defence response possibly due to generation of ROS, a protective mechanism and also due to enhanced immune response as a result of incorporation of plant protein (Song *et al*., 2014) [46]. Increased serum lysozyme activity has been reported in juvenile starry flounder when fed with soy protein hydrolysates (Song *et al*., 2014) [46]. The increased SOD activity indicates its role in reducing the ROS induced by stress due to CSL based diet. The nutritional factors are known to affect the activity of antioxidant enzymes in fishes (Brownlee 2001) [8]. Several fish feeding experiments have shown enhanced expression of antioxidant enzymes when plant proteins are used in diet, possibly due to the presence of antinutritional factors (Zheng *et al*., 2012; Song *et al*., 2014) [50, 46]. Presently observed increased SOD activity with CSL, which is devoid of ANFs, indicates a role of carbohydrate fraction of CSL in increasing ROS level. The AchE activity in fishes is widely considered as a biomarker for environmental stress (Vani *et al*., 2012) [48]. However not many studies have been undertaken to understand the effect of plant based fish feed on this enzyme. The increased AchE activity indicates that the fishes are more competent to combat stress with experimental feed. Increase in AChE activity has also been reported by Mohapatra *et al*. (2012) [32] with probiotic supplemented diet in fishes. The presently observed higher stress in experimental fishes may be due to high glucose led hyper glycolysis and electron transport chain (ETC) operated at its peak, producing ROS (Brownlee 2001) [8]. The significant decrease in stress markers in lysine and chelated mineral supplemented groups indicates that increase in the availability of EAAs as well as minerals with CSL as principle protein source decreased the stress and ROS level in early fingerling.

5. Conclusion

The present findings indicate that CSL is not suitable as alternative protein source even with lysine and chelates for rohu early fingerling stage; though it has exhibited promising results for rohu advance fingerlings (Chovatiya *et al*., 2011) [10]. At the same time it has enhance glucose metabolic pathways and enhanced defence response in fishes. Thus its suitability for different developmental stages requires further investigations.

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