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Optimization of acid hydrolysis conditions of delimed tannery fleshings by response surface method

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An ecofriendly method, involving mild organic acids to hydrolyse delimed tannery fleshings (TF), has been developed. Combination of formic acid and propionic acid (1:1) was employed to obtain hydrolysates with higher degree of hydrolysis (DH). Better antioxidant properties were obtained by response surface method (RSM) using a factorial design. Effect of pretreatment involving steam cooking (80°C for 15 min) indicated that cooked delimed TF on acid treatment had significantly ($P<0.5$) higher protein extractability than uncooked delimed TF. Optimized levels of factors for obtaining highest DH (>51%) at room temperature for cooked delimed TF were acid mixture (20%) and incubation time (9 days). Liquid hydrolysate from TFs exhibited antioxidant activity (125.56 µg ascorbic acid equivalents /ml extract) and diphenyl picrylhydrazyl (DPPH) radical scavanging activity (46.42%). Chemical score of hydrolysate revealed an excess amount of essential amino acids (arginine, leucine and lysine) as compared to reference protein. This indicates its potential for use in livestock/aquaculture feeds.

Keywords: Antioxidant, Optimization, Organic acids, Protein hydrolysate, Tannery fleshings

Introduction

Indian tanneries produce 150,000 tonnes of solid wastes (raw hide/skin trimmings, limed fleshings, hide splits and chrome shavings), which create disposal problems as well as environmental pollution[1]. A method for de-liming of limed tannery fleshings (TF) is reported for enzymatic hydrolysis and lactic acid fermentation for their utilization[2]. A bacteriocin producing native lactic acid bacteria[3] (*Enterococcus faecium* HAB01) was isolated from TF. Protein hydrolysates from wastes/ by-products of animal processing industry exhibit antioxidative[4,5], antihypertensive[6] and immunomodulatory properties[7,8]. Acid ensilaging, which is widely used to convert wastes from livestock/fish and fruits/vegetable, involves use of mineral acids (H_2SO_4, HCl) and a neutralization step before application of silage[9]. Milder organic acids (propionic and formic acid) are better alternatives to hazardous and strong mineral acids[10]. Optimization of hydrolysis conditions involves varying one factor at a time and keeping other variables constant[11]. On the other hand, response surface method (RSM) can effectively use variables simultaneously and determine optimum conditions required[12].

Present work aims: i) to use organic acids as alternative to mineral acids for ensilaging delimed TF: ii) to optimize hydrolysis condition to produce acid hydrolysate with better degree of hydrolysis and antioxidant activity; and iii) to evaluate amino acid composition protein hydrolysate prepared under optimized conditions to determine its potential for application in animal/aquaculture feeds.

Materials and Methods

Wet TFs from sheep and goat skins were procured from a tannery based at Bangalore, India. Propionic acid, formic acid, hydrogen peroxide (H_2O_2) and hydrochloric acid (HCl) were purchased from M/s Merck Chemicals (Mumbai, India). All other solvents and chemicals were of analytical grade. Proximate composition of wet TF and delimed TF was estimated[13]. Protein measurements were carried out by employing Kjeltec Protein Analyzer (Foss Analytica AB, Sweden). pH measurements were made by directly immersing combined glass calomel electrode of pH meter (Cyberscan 1000, Eutech, Singapore).

Deliming of TF

Deliming of TF was accomplished by standardized method[3]. Minced TF were dispersed in potable water at

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Table 1—Effect of pretreatment on extractability of proteins upon acid hydrolysis of delimed TF at room temperature (30±2°C)

Acid*	1 day		2 days		3 days	
%	C	UC	C	UC	C	UC
5	8.18±0.42	2.96±0.36	10.60±0.42	4.15±0.39	14.30±0.59	6.20±0.39
10	13.45±0.49	8.14±0.4	18.59±0.72	11.17±0.46	24.32±0.72	11.86±0.47
15	16.87±0.69	9.67±0.47	24.70±0.87	11.62±0.57	28.38±0.91	17.48±0.63

*Formic & propionic acid mixture (1:1 v/v); C, cooked delimed TF; UC, uncooked delimed TF

1:10 (w/v) containing H_2O_2 (0.1% v/v of wash water). Material was allowed to stand for 30 min with occasional stirring before draining liquid to collect solids. Collected solids were again subjected to H_2O_2 treatment and water drained after 30 min. Obtained solids were then treated with 0.2N HCl prepared in demineralized (DM) water (1:10 w/v) containing H_2O_2 (0.1% v/v of wash water). Material was allowed to stand for 30 min before draining solution and repeated treatment with water containing HCl and H_2O_2. Solids obtained after draining treatment water were termed as delimed TF.

Delimed TF in both cooked (80°C, 15 min) and uncooked form was treated with a mixture of propionic and formic acid (1:1 v/v) at different levels (5, 10 or 15% v/w), in order to establish effect of heat treatment prior to acid hydrolysis. Delimed TF was mixed with salt (2%) and acid mixture. Mixture was incubated for 3 days at room temperature (30±°2C). Extractability (%) of hydrolysed protein was evaluated [Eq. (1)] daily to select best start material for acid hydrolysis. Since, cooking prior to acid hydrolysis was better than uncooked delimed TF (Table 1), all further experiments were carried out with cooked TF. Hydrolysed protein was extracted three times with DM water (1:2 w/v) to collect filtrate and residue. Extractability, degradation efficiency and degree of hydrolysis[14] are calculated as

Extractability (%) = (Water soluble protein on hydrolysis ÷ total protein in delimed TF) × 100 …(1)

DE (%) = { – (Protein content in residue ÷ total protein)} …(2)

DH % = (10 % TCA soluble N_2 in sample ÷ Total N in sample) × 100 …(3)

In-vitro Antioxidant Properties

Antioxidant Activity

Acid hydrolysed mass was extracted with DM water (1:2 w/v) to collect hydrolyzed filtrate (FL). FL was further centrifuged at 5000×g for 20 min to obtain a sediment free supernatant, centrifuged FL (CFL), used for all *in-vitro* antioxidant activity assays. Total antioxidant activity (TAO) of CFL was determined[3] by mixing sample (0.3 ml) with reagent solution [3.0 ml; 0.6 M sulfuric acid: 28 m M sodium phosphate: 4 m M ammonium molybdate (1:1:1 v/v/v)]. Reaction mixture was incubated in a water bath at 95°C for 90 min. Absorbance of all sample mixtures was measured at 695 nm. Total antioxidant activity (µg / g) was expressed as of ascorbic acid equivalents (AAE) in acid hydrolysed sample.

DPPH Radical Scavenging Activity

DPPH radical scavenging effect (%) of CFL was determined[3] by adding 0.16 mM DPPH solution (made 2.0 ml in methanol) to test tube containing CFL (0.2 ml of CFL made up to 2 ml with distilled water), vortexed for 1 min and kept at room temperature (30±2°C) for 30 min in dark. Absorbance of all sample solutions was measured at 517 nm. Sample blank and control samples were performed and scavenging effect (%) was calculated[15].

Optimization of Acid Hydrolysis Conditions

Two independent factors [acid mixture (10, 15, 20 % v/w) and hydrolysis time (3, 6, 9 days)] were optimized (Table 2) by RSM using a factorial design involving two factors (X1 and X2) at three equidistant levels (-1, 0 and +1) in one block encompassing 9 runs. Response variables were pH and DH. Each run comprised mixing of cooked (delimed) TF (100 g) with salt (2% w/w) and pre-determined levels (v/w) of acid mixture in bottles (250 ml) followed by incubation at room temperature (RT) (30±2°C) for pre-determined time. Designed model was further validated using random combinations of independent variables.

Table 2—Actual levels of independent factors (X1, X2) and observed response (Y1, Y2, DE, E, DPPH, TAX) variables determined during experimental runs

nRun #	X1	X2	Y1	Y2	DE	E	DPPH	TAX
1	10	3	3.63	18.8	36.61	26.21	9.51	47.38
2	10	6	3.78	21.13	44.53	28.72	12.75	83.26
3	10	9	3.87	30.11	46.38	30.41	16.60	93.15
4	15	3	3.22	20.6	43.27	34.21	25.08	52.85
5	15	6	3.36	28.43	45.68	39.34	32.52	103.28
6	15	9	3.48	39.37	52.85	43.59	36.41	105.91
7	20	3	3.05	23.6	48.73	35.39	25.41	56.28
8	20	6	3.18	33.64	54.96	44.56	37.56	118.25
9	20	9	3.28	51.64	65.80	59.57	46.41	125.62

X1, acid level, % v/w; X2, hydrolysis time, days; Y1, pH; Y2, degree of hydrolysis, %; DE, degradation efficiency, %; E, extractability, %; TAX, total antioxidant activity; DPPH, diphenyl picrylhydrazyl radical scavenging activity

Acid Hydrolysis under Optimized Conditions and Amino Acid Composition of Hydrolysate

Four independent batches (1.25 kg each) of cooked delimed TF were hydrolyzed optimally (RT; acid mixture, 20% v/w; incubation time, 9 days). In post hydrolysis, mixture was extracted thrice with DM water (1:2 w/v, 3X). Extractants were pooled and concentrated to minimum volume and spray dried by a spray drier (Labplant SD05, LP Technologies, UK) with an inlet temperature (150°C), outlet temperature (90°C) and a flow rate (70 ml/min). Product yield based on material as well as nitrogen recovery was calculated.

Amino acid composition was determined using phenyl isothiocyanate (PITC) pre-column derivatization[16] by employing Water's PicoTag column and workstation. Chemical score of hydrolysate was computed[17] as per Eq. (4) considering essential amino acids (EAA) requirement of common carp according to reference protein[18].

$$\text{Chemical score} = [\text{EAA in test protein (g 100 g}^{-1}) \div \text{EAA in standard protein (g 100 g}^{-1})] \times 100 \quad \ldots(4)$$

Statistical Analysis

Differences in treatments were determined by analyzing data using analysis of variance (ANOVA) technique and mean separation was accomplished by Duncan's multiple range test in case of significant difference. RSM data was analyzed to obtain response surfaces and desirable levels of independent factors to obtain optimum DH by least square method. Statistical software, STATISTICA[19], was used.

Results and Discussion

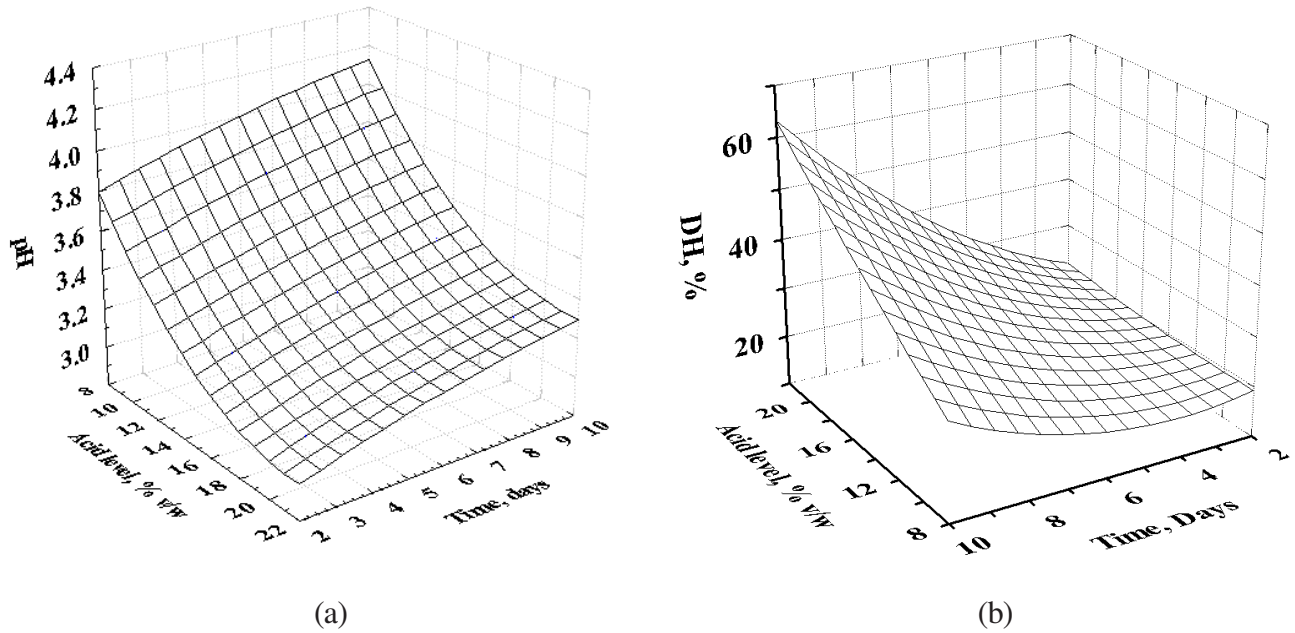
Effect of Cooking on Protein Extractability

Initial experiments on effect of pretreatment involving cooking indicated that cooked delimed TF had increased extractability at different levels of acid ($p<0.05$) as compared to uncooked delimed TF (Table 1). Collagen, main protein in TF[20,21], needs to be hydrolyzed to be as a more useful nutrient in animal feed. Hydrolysis of protein involves major structural changes where protein molecule is gradually cleaved into smaller peptide units. Solubility of hydrolyzed protein also increases with increase in DH[22]. Cooking of delimed TF prior to fermentation improves fermentation efficiency and results in increased protein hydrolysis[3]. Thus all experiments were conducted by employing cooked delimed TF.

Optimization of Acid Hydrolysis Conditions by RSM

Influence of acid level (X1; % w/w) and time (X2; h) on hydrolysis of delimed TF protein (initial pH 7.2 ± 0.2) was determined using a factorial design. Among observed values for response variables [pH (Y1) and DH (Y2; %)], only DH was found to be more reliable (Table 2). ANOVA table for pH (Table 3A) and DH (Table 3B) indicated that both independent factors and their interactions had significant effect ($p<0.01$) when DH is considered, but significant effect (and not their interaction) only when pH is considered.

Response surface graph for pH, as a function of acid level and hydrolysis time (Fig. 1A), clearly indicates that with increase in acid levels, pH decreased (up to 20 % v/w), beyond which it does not show much decrease. TF is a collagen rich material and peptides released upon hydrolysis have a buffering[2,3]. Hence, pH may not be an



(a) (b)

Fig. 1—Response surface graph as a function of levels of acid mixture (% v/w) and hydrolysis time (days) during hydrolysis of delimed TF at room temperature (30±2°C) of: A) pH; and B) DH, %

Table 3—Analysis of variance (ANOVA) of response variables as affected by independent variables and their interactions based on experimental runs

	SS	df	MS	F	p
(A) pH					
Independent variables					
1. Acid level, % v/w (L)	1.0000		0.5222	4440.3	0.00000007**
Acid level, % v/w (Q)	0.0249	1	0.02494	212.1	0.000702104**
2. Time, days (L)	0.0888	1	0.08882	755.3	0.000105738**
Time, days (Q)	0.0006	1	0.0007	5.7	0.09666055
Interactions					
1 * 2	0.000025	1	0.000025	0.2	0.676111129
Error	0.0003	3	0.0001		
Total SS	0.6369	8			
(B) DH, %					
Independent variables					
1. Acid level, % v/w (L)	251.4243	1	251.4243	318.077	0.0003**
Acid level, % v/w (Q)	0.249689	1	0.249689	0.315882	0.6133
2. Time, days (L)	562.9891	1	562.9891	712.2378	0.0001**
Time, days (Q)	17.44436	1	17.44436	22.06887	0.0182**
Interactions					
1 * 2	69.97323	1	69.97323	88.52317	0.0025**
Error	2.371353	3	0.790451		
Total SS	904.452	8			

SS, sum of squares; df, degrees of freedom; MS, mean sum of squares; F, F ratio; p, significance;
*very significant, p<0.05; **highly significant, p<0.01

appropriate factor to rely upon as a response variable. However, response surface graph for DH, as a function of acid level and hydrolysis time (Fig. 1B) clearly indicates that DH increased significantly (p<0.05) with an increase in both factors individually as well as their intreaction.

Other variables observed during optimization of condition were extractability, DE and antioxidant activities (DPPH radical scavenging activity and total antioxidant activity), which corroborated well with DH (Table 2). Increase in extractability resulting from higher DH is

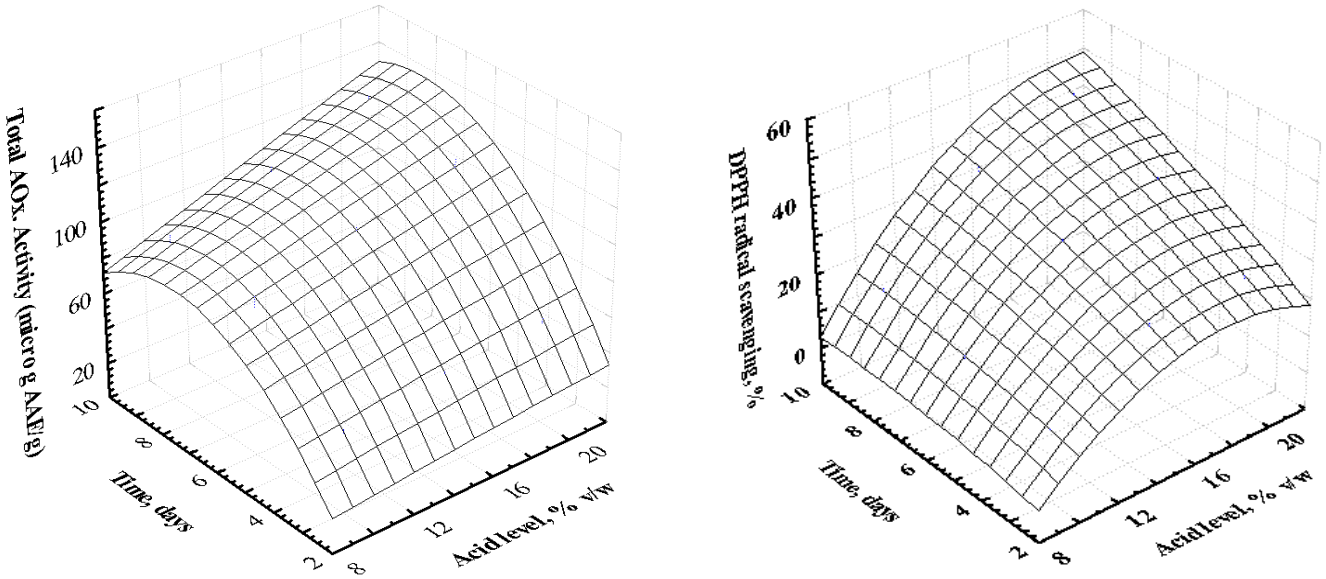


Fig. 2—Response surface graph as a function of level of acid concentration (% v/w) and incubation time (days) during acid hydrolysis (Incubation temperature - 30±2°C) for: A) total antioxidant activity (µg AAE/ml of extract); and B) DPPH radical scavenging activity (%)

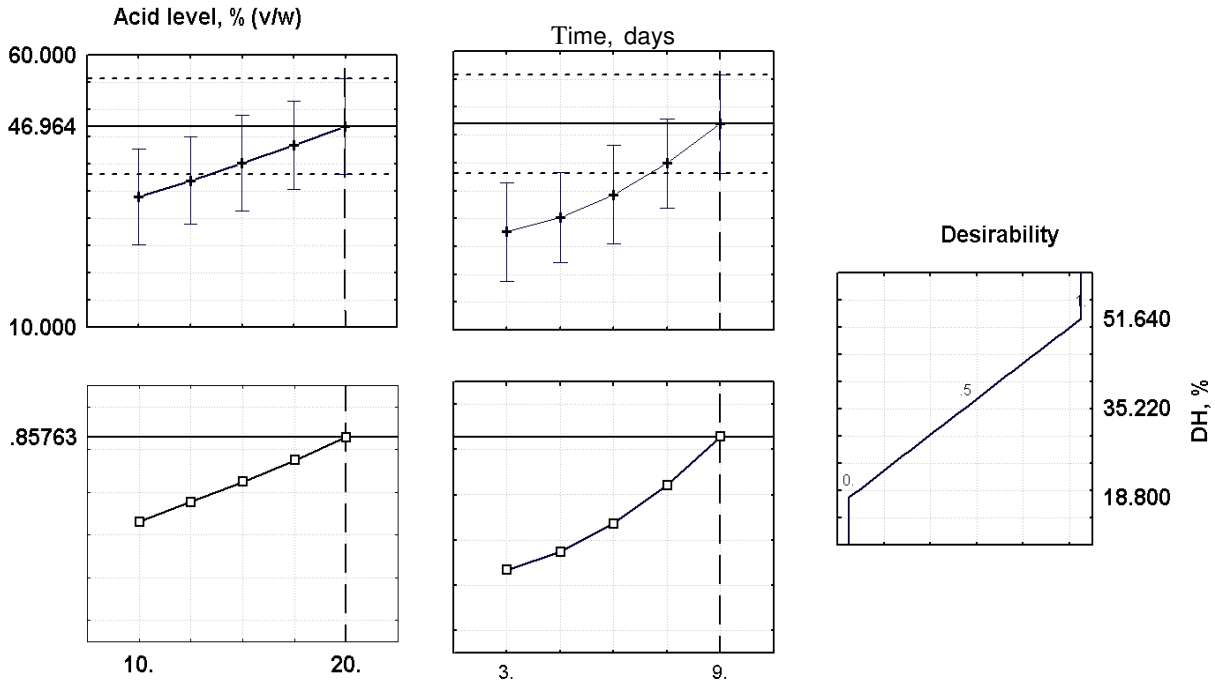


Fig. 3—Desirability profiles for DH at optimized levels of independent factors

probably due to smaller peptides resulting from protein hydrolysis that result in increased hydrophilicity[23,24] of hydrolysate as compared to intact protein in unhydrolysed mass. Higher DH also points to potential increase in digestibility of these proteins.

Protein in TF gets hydrolyzed on acid treatment. Hydrolyzed liquor is rich in peptides and amino acid. Protein hydrolysates from shrimp exhibit antioxidant activity[25]. Antioxidant activity of amino acid on protection of H_2O_2 induced tissue oxidative stress in human epithelial cells[26]. Total antioxidant activity, which increased gradually with increasing hydrolyzing time, was found highest (125.56µg ascorbic acid equivalents/ml extract) for hydrolysate treated with acid concentration (20%)

Table 5—pH and proximate composition (% wet weight basis[#]) of wet, delimed and hydrolysed TF along with acid hydrolysate obtained

Material	pH	Moisture	Protein	Fat	Yield	
					A	B
Wet TF	12.2±0.2	70.4±2.5	5.8±1.7	7.2±0.7	-	-
Delimed TF	7.2±0.2	80.9±2.1	7.1±0.6	9.1±0.8	63.9	83.6
Acid hydrolysate	-	3.38±0.4	11.7±2.1*[@]	1.8±1.3[*]	5.6	45.2

A, as percentage (w/w) of wet TF; B, Nitrogen recovery (% of N_2 content of wet TF)

[#]All values are on wet wt basis except those marked with asterisk

*Values are on dry wt basis; [@]nitrogen content of the material

Table 4—DH (%) values observed during validation runs and respective predicted values predicted by model [Eq. (5)]

Run #	Acid level (X1, % w/w)	Time (X2, days)	DH, % Observed	DH, % Predicted
1	12	5	22.14	21.70
2	17	7	33.56	34.26
3	11	6	23.14	22.54
4	14	8	32.95	33.43
5	18	2	19.89	20.50
6	16	4	22.65	23.10
7	9	7	21.98	22.12

on 9th day (Fig. 2A), contributed by antioxidant peptides produced on hydrolysis.

DPPH radical was widely used in model system to investigate free-radical-scavenging ability of various compounds[15]. It is a type of stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[27]. Response surface graph for DPPH radical scavenging activity, as a function of acid level and hydrolysis time, found to be highest (46.42 ± 1.45) in case of TF hydrolyzed in optimized condition (Fig. 3B). Radical scavanging activity has increased gradually with increasing hydrolyzing time and acid concentration. Antioxidant activities correlated with extractability of protein and degree of hydrolysis as smaller peptides are responsible for antioxidant activities.

From experimental runs, DH is found more reliable and better response variable than pH and other response variables. Hence, prediction model was deduced with only DH as response variable. Desirability functions were fit by least square method by assigning a desirability level of 1.0 to higher DH and 0 to lowest level of DH. Optimized levels of factors were determined using desirability profiles (Fig. 3) and desirable levels of factors for obtaining highest DH were acid mixture (20% v/w) and hydrolysis time (9 days) at RT (30±2°C). Regression equation for DH of hydrolysed delimed TFs, as a function of independent variables (X1 and X2) and their interactions, using the constant, linear and quadratic regression coefficients was derived as

$$DH\ (\%) = 28.79278 + (-0.80233*X1) + (0.014133*X1^2) + (-4.89139*X2) + (0.328148*X2^2) + (0.278833*X1*X2) \quad \ldots(5)$$

Table 6—Amino acid composition (g $100g^{-1}$) of acid hydrolysate of delimed TF along with its chemical score in comparison to reference requirements of juvenile carps

Amino acid	TFH	RP	CS
Essential			
Isoleucine	1.96	2.5	0.784
Leucine	4.24	3.3	1.28
Lysine	5.91	5.7	1.04
Methionine	0.54	3.1	0.17
Phenyl alanine	1.96	6.5[$]	0.3[$]
Tyrosine	0.95		
Threonine	1.65	3.9	0.42
Arginine	11.45	1.31	8.74
Valine	3.20	3.6	0.89
Non-essential			
Aspargine / aspartate	0.92	-	-
Glutamine / glutamate	4	-	-
Serine	3.08	-	-
Glycine	31.12	-	-
Alanine	12.74	-	-
Proline / hydroxyproline	16.26	-	-
Cystine			

TFH, acid hydrolysate prepared from delimed TF; RP, reference protein, amino acid requirements of juvenile carps as per NRC 1993; CS, chemical score for TF hydrolysate in comparison to RP; [$]sum of phenyl alanine and tyrosine

Usefulness of model was further validated using different random combinations of two independent variables (Table 4) and comparing observed and predicted values. Coefficient of determination and slope of regression equation for DH was 0.9927 and 1.0089, which clearly indicate usefulness of the model.

Yield and Composition of Acid Hydrolysate Prepared under Optimized Conditions

Yield of hydrolysate (5.6%) and its nitrogen content (45.2%) based on delimed TFs (Table 5) are lower as compared to other studies, probably due to high collagen content in TF, as compared to other hydrolysates from distantly related materials like sheep viscera[28] and fish viscera[29]. Protein content of hydrolyzed powder is around 73% (11.7 X 6.25) (Table 5), which is lower as compared to other studies. Although TF have considerable fat content upon acid hydrolysis, fat got separated on silage surface and it was removed physically before spray drying; lower amount of fat was observed in hydrolysate.

Evaluation of chemical score, by comparing levels of EAAs between test and standard protein sources, is an accepted method to determine nutritive value of an ingredient[30]. Hydrolysate (TFH) had higher content of arginine, leucine and lysine as compared to reference protein (Table 6). However, methionine, phenylalanine and threonine were most limiting followed by isoleucine and valine. Even though there are minor deficiencies in certain EAAs, protein hydrolysate has potential to be an ingredient in balanced livestock/aquaculture diets, especially as a good source of arginine, leucine and lysine.

Conclusions

Acid hydrolysis of delimed TF is a possible low cost method for effective utilization of solid wastes from tannery. Acid hydrolysate is a good source of arginine, leucine and lysine in livestock/aquaculture feeds. Acid hydrolysates also provide an ecofriendly way of treating TF due to their GRAS status.

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