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Biostabilization and preparation of protein hydrolysates from delimed leather fleshings

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Wet leather fleshings (LFs) from sheep and goatskins had high alkaline pH (12.1) and ash (18.1%) due to liming of fresh skins for leather production. Treatment of limed fleshings with 0.1% H_2O_2 (1:10 v/v) followed by 0.2 HCl (1:10 w/v) solutions produced very little or no H_2S. Delimed fleshings, mixed with 19.5% (v/w) *Pediococcus acidolactici* culture, 20% (w/w) sugar and 2% (w/w) common salt, on fermentation at 30±2°C yield silage (protein 7%, fat 8% and ash 3.6%), which can be used in animal feeds. Optimized conditions (enzyme, 1.5% v/w of Protex-6L; temp., 55°C; hydrolysis time, 180 min) for hydrolyzing delimed LFs were determined by RSM. LFs yield white hydrolysate (yield, 3.5%; nitrogen, 12.4%; and fat, 1.5% w/w), which can be used in aquaculture and animal feeds to supplement arginine, phenylalamine and tyrosine.

Keywords: Amino acids, Deliming, Leather fleshings, Hydrolysates, Protex-6L

Introduction

During leather processing[1], tanneries in India produce annually 150,000 tonnes of offals as raw hide/skin trimmings, limed fleshings, hide splits and chrome shavings, which are wasted or under-utilised[2]. Deliming of leather fleshings (LFs) with HCl[3] produces toxic H_2S. Biological methods are being used to minimize industrial pollution resulting from tannery effluents[1,4,5]. Collagen, a main protein in LFs[6,7], needs hydrolysis[3] to be more useful as a nutrient in animal feeds. This study presents deliming of LFs with minimal production of H_2S, besides preservation of delimed LFs using fermentation to produce silage product, which can be used in animal feeds. Also, a proteolysis process using response surface methodology (RSM) has been standardized to produce from delimed LFs the protein hydrolysates (PHs), which can also be used in animal feeds.

Materials and Methods

Materials

Wet LFs from sheep and goatskins were procured from a tannery based at Bangalore. Lactic acid bacteria (LAB) cultures (*Lactobacillus plantarum* B4496, *L. lactis* B634, *L. acidophilus* B4495 and *Pediococcus acidolactici* CFR2181) were obtained from institute's culture collection. Dehydrated microbiological media were procured from Hi-Media, Mumbai (India). Sodium dodecyl sulfate (SDS) and acrylamide were obtained from Sigma Chemicals, USA and standard protein markers for molecular weight determination from Genei (M/s Bangalore-Genei, Bangalore, India). Enzyme (Protex-6L), used in preparation of PHs, was supplied in kind by M/s Genencor Inc., USA. All other chemicals and reagents were of analytical grade. Batches of wet LFs used in deliming and biostabilization were different from the ones used in preparation of PHs from delimed LFs.

Methods

pH was measured by directly immersing combined glass calomel electrode into the sample using pH meter (Cyberscan 1000, Eutech, Singapore). Proximate composition of fresh LFs, delimed LFs, fermented LFs and LF hydrolysate was estimated as per AOAC[8]. All proteins in samples were measured by Kjeldahl method using Kjeltec protein analyzer (Foss Analytica AB, Sweden). Total plate count (TPC), *Escherichia coli*

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(*E. coli*) count, *Staphylococci aureus* and yeasts & molds count (YC), were analyzed as per APHA[9]. Tristimulus Hunter color parameters (L^*-lightness, a^*-redness and b^*-yellowness) were measured using Hunter Color measurement system (Labscan XE, USA).

Deliming of LFs

For deliming, 0.2N HCl was optimum although production of H_2S could not be avoided[10]. H_2O_2 was employed to overcome H_2S problem during deliming of tannery fleshings. Minced LFs, in batches of 500g, were dispersed in potable water at 1:10 (w/v) containing varying amounts of H_2O_2 (0.025, 0.5, or 0.1% v/v, of wash water). Material was allowed to stand for 30 min with occasional stirring before draining the liquid. H_2O_2 treatment was repeated again and water drained after 30 min of standing time. H_2O_2 treated minced LFs were then treated with 0.2 N HCl prepared in potable water (1:10 w/v) containing different concentrations of H_2O_2. Material was allowed to stand for 30 min before draining solution to collect the solids. The treatment with water containing HCl and H_2O_2 was repeated again. Release of H_2S, if any, during treatment was assessed by smell.

Fermentation of Delimed LFs

Delimed LFs were stabilized using lactic fermentation. LAB cultures were evaluated for fermentation efficiency. Based on experiments on shrimp waste fermentation[11], culture (19.5% v/w) and sugar (20% w/w) levels were fixed. Common salt (2% w/w) was added to check growth of spoilage microbes[12]. Fermentation mixture was held at ambient temperature (30 ± 2°C) for 60 days with occasional mixing daily. Four separate batches of fermented LFs were prepared, and proximate composition, pH and microbiological characteristics were evaluated.

RSM for Optimizing Hydrolysis Conditions

Optimization of hydrolysis conditions was achieved by employing RSM by Box-Behneken design (3 factors, 3 levels). Experimental design had a total of 15 combinations of three variables. The design involved 3 independent factors (time – X1, temperature – X2 and enzyme concentration – X3) each at 3 equidistant levels (-1, 0, +1), and the response variable (Y) was degree of hydrolysis (DH, %). The model developed was further validated independently through random combination of experimental variables. DH was estimated[13] as

$$\% \text{ DH} = \frac{\text{10 \% TCA soluble } N_2 \text{ in sample}}{\text{Total } N_2 \text{ in sample}} \times 100$$

Protein Hydrolysate (PH) Preparation using Optimized Conditions

Delimed minced LFs (500 g) were mixed with distilled water at 1:0.5 (w/v) and hydrolyzed at 55±1°C (optimum temperature for Protex-6L) for 180 min with an enzyme to substrate ratio of 1.5 ml: 100 g. After hydrolysis, enzyme was inactivated by heating for 15 min at 85±1°C. PH was then cooled to room temperature, allowed to stand in refrigerator (4-8°C) and centrifuged in a refrigerated centrifuge (M/s Remi Engineering, Mumbai, India) at 6700×g, for 20 min at 10°C. PH was separated into three layers (bottom unhydrolysed sediment, a middle layer containing PH and top layer mostly of fat). Both the top and bottom layers were discarded and middle layer containing PH was spray dried using a bench top spray drier (Labplant SD05, LP Technologies, UK) at 160-180°C to collect a white powder. Four separate batches of PHs were prepared under optimized conditions.

Amino Acid Analysis, Electrophoresis and Color Determinations

Amino acid composition was determined using phenyl isothiocyanate (PITC) pre-column derivatization[14] by employing Water's PicoTag Column and Workstation. Chemical score of PH was computed[15] considering essential amino acids (EAA) in standard protein as per FAO/WHO[16].

$$\text{Chemical score} = \frac{\text{EAA in test protein (g 100 g}^{-1})}{\text{EAA in standard protein (g 100 g}^{-1})}$$

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out[17] on 12% gels of 0.75 mm thickness. Tristimulus Hunter color parameters were measured.

Statistical Analysis

Design and data obtained through RSM, to optimize hydrolysis conditions as well as data obtained from validation runs were analyzed by STATISTICA[18]. Desirability function to get optimum DH was fitted by the least square method using same software. Differences in treatments were determined by analyzing the data using analysis of variance (ANOVA) technique. Wherever differences were significant, mean separation was accomplished by Duncan's multiple range test.

Table 1—Effect of varying concentrations of H_2O_2 on H_2S production and pH of leather fleshings (n = 4)

No. of washes	H_2O_2[#] concentration (% v/v of wash water)			pH[$]
	0.025	0.05	0.1	
1*	-	-	-	11.50 ± 0.14
2*	-	-	-	11.25 ± 0.20
3**	+ + +	+ +	+	8.35 ± 0.30
4**	+ +	+	-	6.58 ± 0.23

+ + +: Strong H_2S smell; + +: Moderate H_2S smell; +: Mild H_2S smell; -: No H_2S smell

[#]Commercial H_2O_2 50% (v/v) strength; *Wash with 0.1% (v/v of wash water) of H_2O_2 only; **Wash with 0.1% (v/v of wash water) of H_2O_2 and 0.2 N HCl (1:10 w/v); [$]Refers to treatment with 0.1%(v/v) H_2O_2.

Table 2—Fermentation efficiency of different lactic acid bacteria as measured by pH reduction (n=4)

Cultures	Fermentation time, days		
	1	2	3
Lactobacillus plantarum B4496	6.62±0.00 ^{a,x}	6.00±0.08^{a,x}	5.44±0.16^{a,y}
L. lactis B634	6.65±0.00 ^{a,x}	6.00±0.11^{a,y}	5.35±0.17^{b,z}
L. acidophilus B4495	6.60±0.00 ^{a,x}	5.95±0.11^{a,y}	5.46±0.17^{a,z}
Pediococcus acidolactici CFR 2181	6.58±0.00 ^{a,x}	5.68±0.11^{a,y}	5.26±0.20^{b,z}

Values with different superscripts in columns (a, b, c) and rows (x, y, z) differ significantly (Pd"0.05)

Results and Discussion

Deliming of wet LFs and Biostabilization of delimed LFs

Detection of H_2S

Fresh mince of LFs (pH 12.15) mixed with 0.2 N HCl (1:10 w/v) had strong H_2S smell, as also observed earlier[10]. Washing of fresh LFs once or twice with different levels (0.025-0.1%, v/v) of H_2O_2, however, did not produce perceptible smell of H_2S, possibly due to oxidation of Na_2S to SO_2. This treatment brought down LFs pH to 11.5 and 11.2 after 1st and 2nd wash respectively (Table 1). Further treatment (3rd wash) with 0.2 N HCl (1:10 v/w) produced slight smell of H_2S in the case of treatment with 0.1% (v/v) H_2O_2 and pH was 8.35. Repeating this treatment (4th wash) produced no H_2S and material pH was 6.58 (Table 1), perhaps indicating complete deliming of fleshings waste.

Fermentation with Different LAB Cultures

During fermentation of delimed LFs with different LAB cultures, *P. acidolactici* produced lowest pH (5.26) in 3 days at ambient temperatures (Table 2). Decrease in pH during fermentation has been observed in fish[19], fish viscera[12,20-22], poultry intestine[23-25], silkworm pupae[26] and shrimp waste[11]. Fall in pH during fermentation[21] is associated with decrease in reducing sugar and increase in lactic acid. Fermentation mixture containing *P. acidolactici* culture was stored at ambient temperature (30±2°C) for 60 days, when fall in pH and rise in TTA continued slowly reaching a microbiologically safe pH (4.2)[19] in 15 days (Fig. 1). Fall in pH was faster (pH 4.2 in 3-5 days) in fish viscera[12], poultry intestine[25] and silkworm pupae[26] when fermented with 10% (w/w) molasses. A slow fall in pH in fermentation of LFs could be due to high buffering capacity of proteins and peptides in LFs.

Proximate Composition

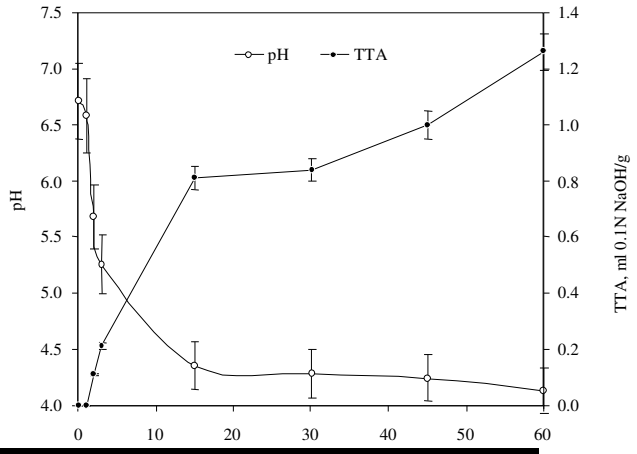
Proximate composition was almost same in delimed fleshings before and after fermentation, besides fresh LFs had high ash content (18.11%) and after deliming with H_2O_2 and HCl, ash content reduced (3.85%) due to solubilisation of minerals in acid (Table 3).

Microbial Profile

Fresh mince of LFs had TPC of 1.7 log cfu/g and LAB, *S. aureus, E.coli* and YC, could not be detected,

Table 3—Proximate composition (wet weight basis) of leather fleshings used in biostabilization experiments (n=4)

Parameter	Fresh leather fleshings, %	De-limed leather fleshings, %	Delimed and fermented leather fleshings, %
Moisture	70.4 ± 2.50	82.6 ± 1.96	81.5 ± 1.22
Protein	5.1 ± 1.73	6.9 ± 1.46	7.0 ± 1.37
Fat	7.3 ± 0.65	7.5 ± 0.93	7.8 ± 0.68
Ash	18.1 ± 2.75	3.9 ± 1.22	3.7 ± 1.47



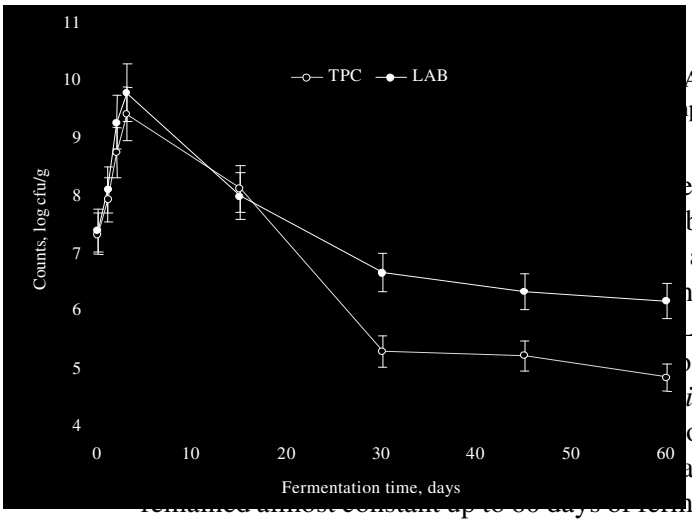


Fig. 2—Changes in total plate counts (TPC) and lactic acid bacteria (LAB) counts in the delimed leather fleshings during fermentation at ambient temperature (30±2°C).

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(Fig. 2). Similarly, LAB counts increased to 9 log cfu/g up to 3 days, decreased gradually to 6 log cfu/g in 35 days and then remained at 6 log cfu/g for 60 days of fermentation. *S. aureus* and *E. coli* were not detected in fermentation mixture after 1-2 days of fermentation whereas yeasts and mould counts were only about 1 log cfu/g throughout the fermentation period of 60 days. Similar observations on microbial profile during fermentation in the first few days for fish[19,27] fish waste[22,28] and poultry waste[25] are reported.

Optimization of Conditions for Hydrolyzing Delimed LFs

Raw Material Characteristics

Proximate composition of fresh and delimed LFs along with pH changes due to deliming (Table 4) were similar to those observed during present fermentation experiments as well. Yield of LFs after deliming was 54.4% (w/w) and recovered 73% nitrogen as compared to the original.

RSM for Optimization of Variables and Prediction Model

Actual and coded values of different independent variables are presented in Table 5. Analysis of observed DH values of PH from 15 different combinations of experiments (Table 6) by ANOVA indicated that all three variables (X1, X2 and X3) and their interaction (except interaction between time and enzyme concentration) had a significant effect on DH of PH (Table 7). RSM graphs depicting effect of two independent variables on dependent variable, when the third variable is kept at the center of its level (Fig. 3) indicate temperature and

Table 4 — Proximate composition of leather fleshings at various stages of processing and hydrolysis (n=4)

	Moisture	Fat	Protein	Ash	pH	Yield	
	%	%	%	%		A	B
Fresh leather fleshings	70.4±2.5	7.2±0.7	5.4±1.7	17.1±2.8	12.2±0.2	-	-
Delimed leather fleshings	80.6±1.9	9.4±0.9	6.8±1.5	2.8±1.2	6.6±0.2	54.4±2.5	73%
Protein hydrolysate	4.2±0.6	1.5±0.3	12.4±1.6[@]	0.98±0.2	6.1±0.2	3.5±0.8	49%

A: as percentage (w/w) of the fresh leather fleshings; B: Nitrogen recovery as percentage of nitrogen content of the fresh leather fleshings; [@]: Nitrogen content

Table 5—Independent factors, their coded and actual levels, and combination of independent factors for optimization experiment

Run No.	Time, min (X1)		Temp., °C (X2)		Enzyme conc., v/w (X3)	
	Coded level	Actual level	Coded level	Actual level	Coded level	Actual level
1	-1	60	-1	35	0	1.0
2	+1	180	-1	35	0	1.0
3	-1	60	+1	55	0	1.0
4	+1	180	+1	55	0	1.0
5	-1	60	0	45	-1	0.5
6	+1	180	0	45	-1	0.5
7	-1	60	0	45	+1	1.5
8	+1	180	0	45	+1	1.5
9	0	120	-1	35	-1	0.5
10	0	120	+1	55	-1	0.5
11	0	120	-1	35	+1	1.5
12	0	120	+1	55	+1	1.5
13	0	120	0	45	0	1.0
14	0	120	0	45	0	1.0
15	0	120	0	45	0	1.0

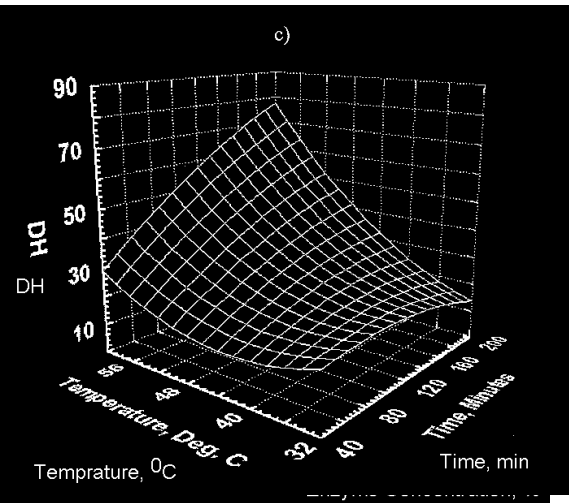
enzyme concentration have a profound effect on DH, both independently and in combination. Regression model developed using regression coefficients (Table 8) to predict dependent variable (DH, %) is

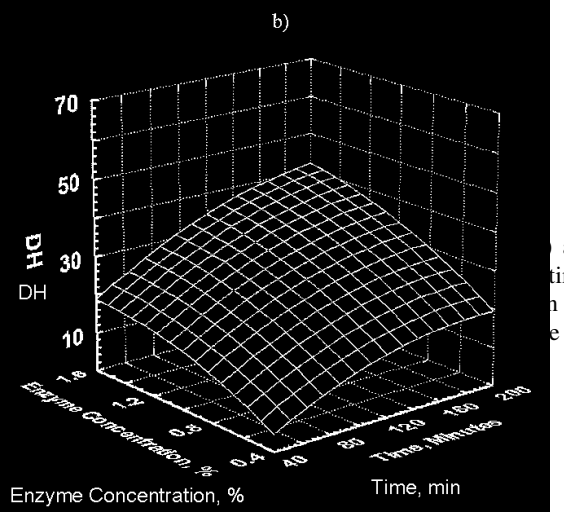
$$DH = 135.8897 + (-320840*X1) + (-0.000650*X1^2) + (-11.65080*X2) + (-9.18833*X2^2) + (-5.51462*X3) + (0.048204*X3^2) + (0.04125*X1*X2) + (0.012342*X1*X3) + (0.897*X2*X3)$$

Optimized levels of factors were determined using the profiles for predicted values for DH and desirability function (Fig. 4). Optimized conditions based on the highest desirability were 180 min at 55°C with an enzyme to substrate concentration of 1.5% (v/w). Usefulness of the model was further validated using different random combinations (other than used for optimization) of three independent variables (Table 9). Predictability of the model was assessed by comparisons of observed and predicted values for the independent variable DH, as observed during both experimental (Table 6) as well as validation runs (Table 9). Regression coefficient and slope for DH were 0.9766 and 0.9766 respectively in experimental runs, as compared to 0.9751 and 0.9893 respectively in validation experiments,

Table 6—Degree of hydrolysis (DH) and pH of hydrolysate observed during RSM experiments

Run #.	Time min	Enzyme conc., %	Temp., °C	DH, %
1	60	0.5	45.0	13.89
2	180	0.5	45.0	22.93
3	60	1.5	45.0	22.56
4	180	1.5	45.0	36.55
5	60	1.0	35.0	18.76
6	180	1.0	35.0	21.23
7	60	1.0	55.0	26.16
8	180	1.0	55.0	58.25
9	120	0.5	35.0	12.98
10	120	1.5	35.0	23.44
11	120	0.5	55.0	29.87
12	120	1.5	55.0	58.27
13	120	1.0	45.0	27.62
14	120	1.0	45.0	28.67
15	120	1.0	45.0	29.56





) as a function of: a) time of hydrolysis, 120 n (temp. of hydrolysis, e conc., 1.0%)

indicating usefulness of the model for prediction. High values of regression coefficients (close to 1.0) in validation experiments indicate validity of prediction model.

LF Protein Hydrolysate (PH) and its Characteristics

Yield of PH and its nitrogen content (3.5%, 49%) from delimed fleshings (Table 4) in present investigation are lower, probably due to high collagen content in LFs, as compared to PH and its nitrogen content from sheep viscera[29] (6%, 63%) and catla fish viscera[30] (5.8%, 64.6%). High nitrogen recoveries (60-70%) from fish wastes are also reported[31-33]. Fat content in PHs from LFs in present study was low (1.5%) similar to that for beef hydrolysates (1.23%)[34], catla fish viscera hydrolysates (1.94%)[30] and sheep viscera hydrolysates (1.23%)[29]. Spray dried PH was almost white as evidenced by high Hunter colour L^* value (87.4) and low a^* (0.86) and b^* (14.3) values. These colour values are similar to those observed for hydrolysates from sheep viscera[29] and catla fish viscera[30]. Electrophoresis study revealed that molecular weights (8-19 kDa) of peptides from PHs of LFs (Fig. 5) are similar to that of sheep viscera

Table 7—ANOVA table for DH as a function of time, enzyme and temperature levels

	SS	df	MS	F
Factors				
Time L+Q	434.7432	2	217.3716	230.5026**
Enzyme L+Q	486.8981	2	243.4491	258.1553**
Temp. L+Q	1241.1585	2	620.5792	658.0671**
Interactions				
1*2	6.1256	1	6.1256	6.4957
1*3	219.3361	1	219.3361	232.5857**
2*3	80.4609	1	80.4609	85.3214*
Lack of fit	57.5995	3	19.1998	20.3596*
Pure error	1.8861	2	0.9430	

*P d•0.05; **P d•0.01

Table 8 — Regression coefficients for main factors and their interactions

	Factor/Interaction	Regression coefficient
	Mean/Interaction (β_0)	135.8897**
1 (X1)	Time (L) (β_i)	-0.3208*
	Time (Q) (β_{ii})	-0.0006*
2 (X2)	Temperature (L) (β_i)	-5.5146
	Temperature (Q) (β_{ii})	0.0482*
3 (X3)	Enzyme concentration (L) (β_i)	7.6249**
	Enzyme concentration (Q) (β_{ii})	-11.6508*
	1L x 2L (β_{ij})	0.0123
	1L x 3L (β_{ij})	0.0142**
	2L x 3L (β_{ij})	0.8970*

Table 9 — Degree of hydrolysis (DH) observed during validation experiments and corresponding predicted values

Run #	Time, min	Enzyme conc, %	Temp., ºC	DH-observed, %	DH-predicted, %
1	60	1.5	35.0	17.81	18.91
2	120	1.0	45.0	27.78	28.62
3	180	0.5	55.0	41.39	42.06
4	90	1.0	45.0	23.70	24.43
5	45	1.5	45.0	18.44	19.77
6	60	1.5	55.0	35.11	37.11
7	90	1.0	45.0	23.36	24.43
8	120	1.5	35.0	23.32	22.28

Table 10 — Amino acid composition of protein hydrolysate from leather fleshings

Amino acids	Reference protein[16], %	Protein hydrolysate (PH) Amino acids, %	Chemical score[15]
Essential amino acids			
Histidine	2.00	0.77	0.385
Isoleucine	4.00	2.09	0.522
Leucine	7.00	4.65	0.664
Lysine	5.50	4.63	0.842
Methionine	3.5	0.54	0.154
Phenyl alanine	-4.29	2.69	-0.930
Tyrosine		1.30	
Threonine	4.00	2.14	0.535
Tryptophan	1.21	-	-
Arginine	5.00	8.41	1.682
Valine	5.42	3.60	0.664
Non essential amino acids			
Aspargine / aspartate	-	5.63	-
Glutamine / glutamate	-	12.65	-
Serine	-	3.61	-
Glycine	-	24.70	-
Alanine	-	10.93	-
Proline / hydroxyproline	-	12.41	-
Cystine	-	0.23	-

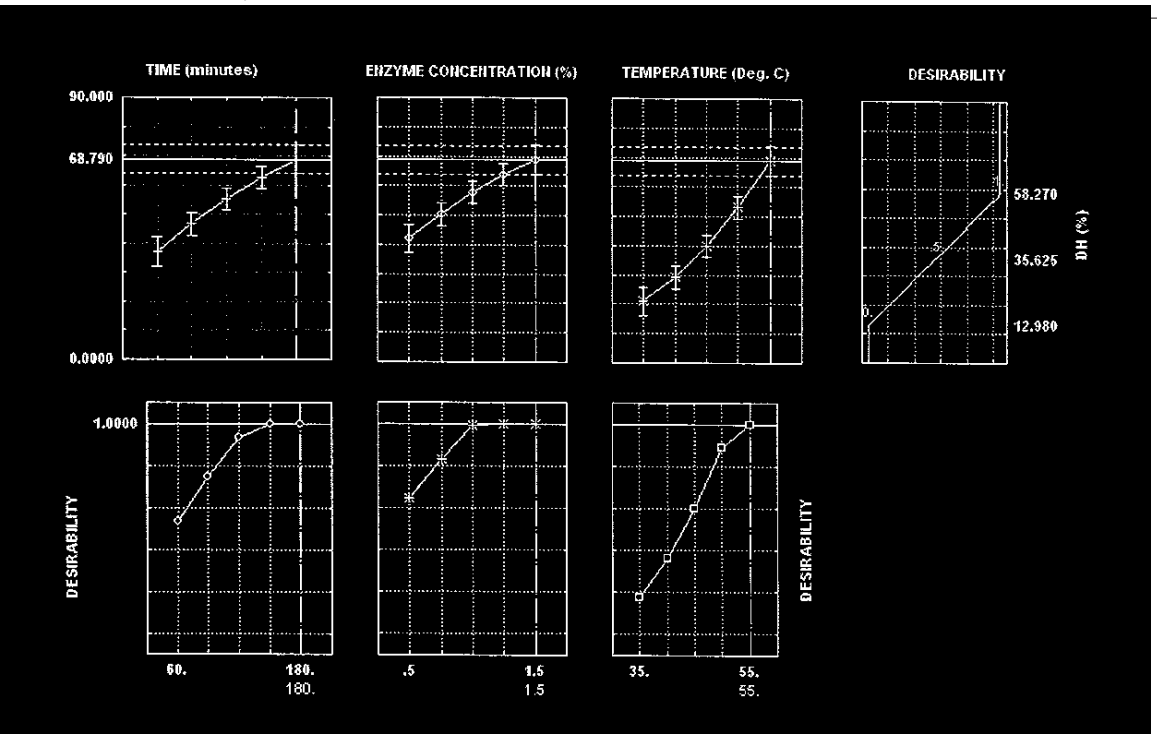


Fig. 4—Profiles for predicted degree of hydrolysis (DH%) and the desirability level for different factors for optimum DH.

Fig. 5—Electrophoretic pattern of hydrolysate from delimed leather fleshings (M: Marker proteins; S1 and S2: Hydrolysates)

(>10 kDa)[29] and catla viscera (8 kDa)[30], but higher than that of beef liver hydrolysates (0.3-3 kDa)[35].

Protein quality as EAAs and chemical scores determines usefulness of a product as an ingredient in food or feed. In present study, PHs from LFs had poor EAA compared to reference protein except arginine, phenylalanine and tyrosine (Table 10). Being low in many EAAs, PHs from LFs can be used in aquaculture and animal feeds to supplement arginine, phenylalamine and tyrosine.

Conclusions

Deliming can be achieved without the production of H_2S and delimed LFs are amenable for fermentation, which indicates the potential for its use in animal feeds. Delimed LFs can also be converted into PHs using commercial proteases and such PHs are rich in arginine, phenylalanine and tyrosine. Both fermented (delimed) LFs and PHs of LFs have potential for use in aquaculture and animal feeds. Further studies on effect of such products on growth performance of animals are needed. Conversion of wet LFs into such valuable products also minimizes disposal problems associated with these solid wastes originating from tanneries.

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