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Enzyme-Accelerated Acid Hydrolysis of Untanned Proteinaceous Wastes from Tanning Industry

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ABSTRACT

Traditionally, tanning industry has been producing considerable amounts of solid wastes, which raises serious concerns on account of their environmental impact. Out of these, untanned raw trimmings account for about 5-7% of the total quantity of raw materials processed. This waste could be a value-added cheap source of collagen, which has numerous industrial applications if properly and scientifically utilized. This research work deals with the utilization of raw trimmings of solid waste from tanneries in the process of enzymatic hydrolysis, performed by using acetic acid and protease, in order to obtain protein hydrolysate. The hydrolysis was carried out with varying acid concentrations, acid solutions, temperatures and times. The maximum obtained protein hydrolysate was about 88% at 1.5 M acid concentration, 4% enzyme ratio, and 60 °C.

KEYWORDS

Raw trimmings, Untanned proteinaceous solid wastes, Acid hydrolysis

INTRODUCTION

Tanning raw hides and skins is one of the oldest processes of our ancestors. The search for methods of conserving hides and skins started in the early Stone Age, around 8,000 BCE. Around five thousand years later, the people of Egypt and Mesopotamia are said to have invented plant-based tanning, using the bark or gum of various trees [1]. The modern tanning process involves several steps in converting raw hides and skins into imputrescible substance and also generates large quantities of solid and liquid wastes. On average, the processing of one metric ton of rawhide produces 200 kg of tanned leather, 250 kg of non-tanned waste, 200 kg of tanned waste leather, and 50,000 kg of liquid waste (Table 1). About 50% of leather mass is lost during the tanning process [2].

Table 1. Environmental input of leather processing, adapted from [3]

Raw hide (1 ton)				
Leather	Solid wastes / Byproducts		50^3 liquid effluent	
200 kg	Untanned wastes:		COD	235-250 kg
	Raw trimmings	120 kg	BOD	100 kg
	Fleshing	70-230 kg	Suspended solids	150 kg
	Tanned wastes:		Chromium	5-6 kg
	Tanned splits	115 kg	Sulfide	10 kg
	Trimmings + Shavings	100 kg		
	Dyed / Finished:			
	Buffing	2 kg		
	Trimmings	32 kg		

The tannery sector of Bangladesh is considered as the most polluting of industries, which is well established, and ranked fourth in terms of earning foreign exchange. The first tannery in Bangladesh was established at Narayanganj by R.P. Shaha in 1940. After that, the tannery industry was shifted to Hazaribagh, Dhaka [4]. At present all tanneries of Hazaribagh have been relocated to Savar Tannery Estate in order to protect the environment. As mentioned earlier, tanning processes generate huge amounts of liquid and solid wastes and if these wastes are not properly treated it will degrade the surrounding environment. Although tanneries have been moved to a new location, solid waste management is not properly maintained.

Raw trimmings of hides and skins

Solid waste in a nearby tannery's yard

Figure 1. Solid wastes from tanneries discarded without any treatment

Raw hides and skins are by-products of meat industry. Before pre-tanning operations, the hides and skins are trimmed (for the convenience of machine operations), which generates huge amounts of un-tanned proteinaceous solid wastes. These trimmings account for about 5-7% of the total quantity of raw materials processed. If these proteinaceous materials are not utilized appropriately, that will create hazardous pollution to the environment [5].

Leather solid wastes can be processed into valuable products such as glue, gelatin, artificial fibrous leathers, and collagen for various industrial uses. A number of authors have reported about various way of turning these wastes into valuable products. Enzymes, such as papain and neutrases [6], acids, such as phosphoric acid [7], sulphuric acid [8], propionic acid [9] and hydrochloric acid [10], and alkali, such as sodium hydroxide [11], magnesium oxide, calcium oxide [8] etc. are used as hydrolyzing agents in the treatment of solid waste generated by the tanning industry. Eco-friendly ultrasound technology can also be used to accelerate the

hydrolyzing process [12]. In this research work, acetic acid (CH_3COOH) and protease enzyme are used as hydrolyzing agents in the process of extracting the protein hydrolysate from untanned raw trimmings from tanneries in Bangladesh.

MATERIALS AND METHODS

Material preparation

Raw trimmings from tanneries were collected from the Savar tannery estate, Savar, Dhaka. These were first washed with an excess amount of tap water in order to remove salt, dirt, dung, blood and other impurities. Hairs were removed by the liming process, using calcium oxide (CaO), and finally unhaired trimmings were delimed by washing them several times with water. After that, they were air dried in the open.
Then, the dried trimmings were cut into small pieces for hydrolysis. Acetic acid (CH_3COOH), lime and protease enzyme were purchased from the local market. Glassware (pipettes, beakers, conical flasks, measuring cylinders, test tubes etc.) that was used was the product of Borosil/Ranken. A magnetic hotplate, a stirrer, Kjeldahl apparatus etc. were used. Acetic acid is commonly used as a souring agent in the process of making vinegar, pickled vegetables and sauce, and as a raw material for spices, in diluted concentrations (4 to 8% by mass). For this reason, acetic acid was chosen as a hydrolysis agent in this study.

Experimental procedure

The study was performed in a batch process in a series of beakers equipped with stirrers by stirring dried raw trimmings and using acetic acid in varying concentrations and at varying temperatures. The detailed experimental methodology is given in Figure 2.

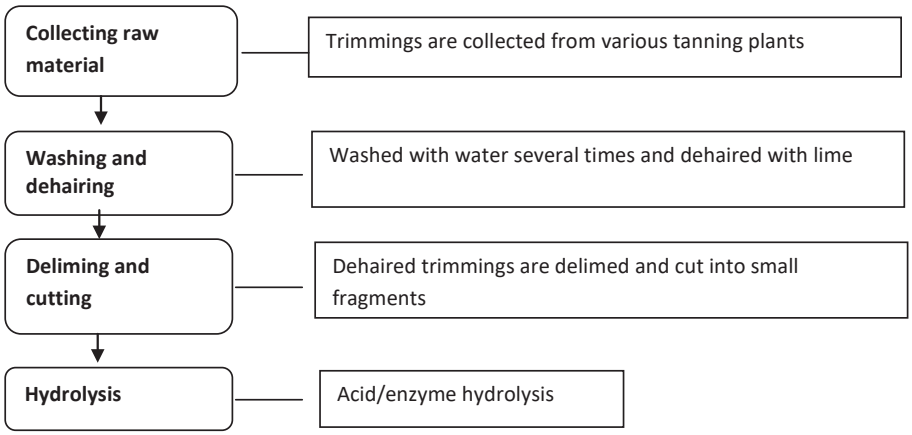


Figure 2. Experimental procedures of hydrolysis

Effect of acid concentration:
The effect of acid concentration on the protein hydrolysis can be examined by varying the concentration of acid in the fixed acid solution at a fixed temperature. 50 gm of cleaned and dried raw trimmings was dissolved in 5 ml of 0.25M, 0.5M, 1.0M and 1.5M acetic acid solution. An extra 400 ml of distilled water was added to each and the temperature was set to 40 °C.

Effect of acid solution:
50 gm of cleaned and dried raw trimmings was put in the beaker with the optimal acid concentration and at a fixed temperature of 40 °C.

Effect of temperature:
The examination of the effect of temperature on the protein hydrolysis was carried out with the optimal acid concentration and its solution.

Effect of hydrolysis time:
The examination of the effect of hydrolysis time (hour) was carried out with the optimal acid concentration, its solution and temperature.

Enzymatic hydrolysis:
The examination of the effect of enzyme concentration was carried out with best the optimal concentration, its solution, time and temperature.

Analysis

The protein of the extracted hydrolysate was identified by the Biuret test and the protein concentration was determined by the Kjeldahl method in a Gerhardt digester (Germany).

a) Biuret test
The Biuret test is based on the ability of Cu(II) ions to form a violet-colored chelate complex with peptide bonds (-CONH- groups) in alkaline conditions. This test confirms the presence of proteins in the sample. In this test, 2 ml of extracted hydrolysate solution was taken in a dry test tube. 3 drops of 10% NaOH and 3-6 drops of 0.5% $CuSO_4$ were added to the sample test tube [13].

b) *Kjeldahl method*
The Kjeldahl method is used to determine the nitrogen content in organic and inorganic substances. For over a hundred years the Kjeldahl method has been used for the determination of nitrogen in a wide range of samples such as foods and drinks, meat, feeds, cereals and forages. It is also used for nitrogen determination in wastewaters, soils and other samples. The Kjeldahl method has three main steps (Figure 3): digestion, distillation, and titration.

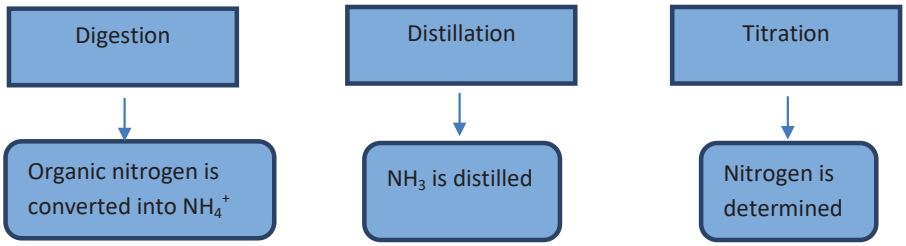


Figure 3. Main steps of Kjeldhal method

Digestion
The aim of the digestion procedure is to break all nitrogen bonds in the sample and convert all of the organically bonded nitrogen into ammonium ions (NH_4^+), carbon dioxide and water (equation 1). During digestion, the organic material carbonizes, which can be visualized by the transformation of the sample into black foam. After that, the foam decomposes and finally a clear liquid indicates the completion of the chemical reaction. For this purpose, the sample is mixed with sulfuric acid at temperatures between 350 and 380 °C. The higher the temperature, the faster the digestion can be obtained.

The speed of the digestion can be greatly enhanced by the addition of salt and catalysts. Potassium sulfate (K_2SO_4) is added in order to increase the boiling point of sulfuric acid, and catalysts (e.g. $CuSO_4$) are added in order to increase the speed and the efficiency of the digestion procedure [14].

$$\text{Protein (-N)} + H_2SO_4 = (NH_4)_2SO_4 + CO_2 + H_20 \quad (1)$$

Distillation

In the distillation step, the ammonium ions (NH_4^+) are converted into ammonia (NH_3) by introducing alkali (NaOH), as showed in equation (ii).

$$(NH_4)_2SO_4 + 2NaOH = 2\ NH_3 + Na_2SO_4 + 2\ H_20 \quad (2)$$

The ammonia (NH_3) is captured in absorbing solution like boric acid, sulfuric acid or hydrochloric acid into the receiver vessel by means of steam distillation. Boric acid (H_3BO_3) of 2-4 % concentration is commonly used for capturing the ammonia, forming solvated ammonium ion (equation iii).

$$H_3BO_3 + NH_3 + H_20 = NH_4^+ + B(OH)_4^- \quad (3)$$

Titration

The concentration of captured ammonium ions is determined by either direct titration or back titration. Both methods indicate the ammonia is present in the distillate with a color change and allow for calculation of unknown protein concentrations.

In direct titration, boric acid is used for capturing ammonia by forming ammonia-borate (NH_4^+:$H_2BO_3^-$) complex, which is neutralized by the addition of sulfuric acid, producing a change in color.

In back titration, sulfuric acid is used for capturing ammonia. The residual sulfuric acid (the excess that did not react with NH_3) is titrated with sodium hydroxide standard solution and the amount of ammonia is calculated by difference.

Working procedure

In this work, 0.5 g of hydrolysate sample was taken in a Kjeldahl flask and digested with 15 ml of concentrated sulfuric acid in the presence of a mixture of K_2SO_4 and $CuSO_4$ in the ratio of 5:1 and distilled into a 4% boric acid solution. The formed borate anions were titrated with 0.05M H_2SO_4 by a direct titration procedure which was converted to nitrogen in the sample [13]. The nitrogen content and the crude protein of the raw trimmings were determined by the following formulae [15].

Calculation of nitrogen content (w_n):

$$w_n = \frac{2(v_1 - v_0)c\ M}{m} \quad (4)$$

where W_n is the nitrogen content, in grams per kilogram, of the test sample; V_1 is the volume, in milliliters, of the sulfuric acid required for the determination; V_0 is the volume, in milliliters, of the sulfuric acid required for the blank test; c is the concentration, in moles per liter, of the sulfuric acid used for the titrations; M is the molar mass, in grams per mole, of nitrogen (M 14 g/mol); m is the mass, in grams, of the test portion.

Calculation of crude protein (w_p):

$$w_p = 6.25\ w_n\ g/kg \quad (5)$$

or

$$w_p = 0.625\ w_n\% \tag{6}$$

where W_p is the crude protein content, expressed in grams per kilogram or in percentage.

Yield

The percentage of yield can be derived from the following equation [16]:

$$Percentage\ yield = \frac{Actual\ yield}{Theoretical\ yield} \text{ X } 100 \tag{7}$$

where actual yield is the amount of product obtained from hydrolysis and the theoretical yield is 68% [2].

RESULTS AND DISCUSSION

FTIR analysis

The FTIR spectrum of the protein hydrolysate obtained from untanned raw trimmings of solid waste from tanneries in the region 500-4000 cm^{-1} is shown in Figure 4. Wavelength is in the 3600–3200 cm^{-1} region, resulting from superimposed OH and NH^{3+} stretching bands. Signals at 1645 cm^{-1} and 1541 cm^{-1} correspond to the carbonyl group (C=O) and N–H, which is the evidence of protein structure [17].

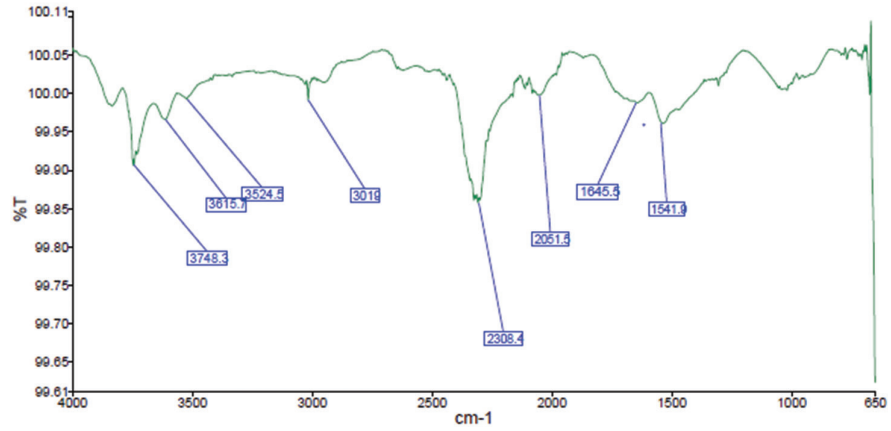
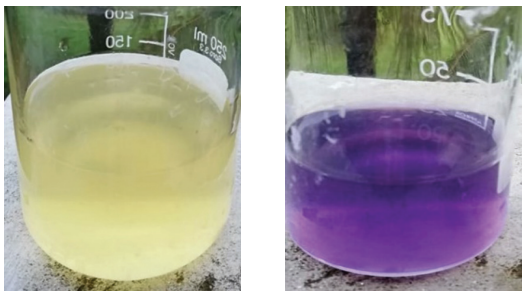


Figure 4. Effect of acetic concentration on protein hydrolysis

Biuret and Kjeldahl methods

Figure 5 shows the change in color of the extracted hydrolysate from grey (a) to purple (b) after the addition of biuret reagent. This change in color represents the presence of proteins in the hydrolysate extracted from raw trimmings.



(a) (b)

Figure 5. Biuret test (a) before addition of biuret reagent, (b) after addition of biuret reagent

Protein hydrolysis of raw trimmings from tanneries by acetic acid was performed with the parameters of acid concentration, acid concentration solution, temperature, and hydrolysis time. Protein concentrations (crude protein) of the extracted hydrolysates were determined by the Kjeldahl method.

Effect of acetic acid concentration on protein hydrolysate

The effect of acetic acid concentration (M) on protein hydrolysis of raw trimmings from tanneries was carried out by hydrolyzing 50 gm of raw trimmings with various acid concentrations (M) with fixed acid solution (5ml) and at a fixed temperature (40 °C). It is shown in Figure 6 that the percentage of protein was increasing with the increase in the concentration of acetic acid from 0.25 to 1.5 molar (M). Since acetic acid is a weak acid and used as a souring agent in vinegar, pickled vegetables etc. in diluted concentrations, it is taken as a hydrolyzing agent in this study. As analytical grade acetic was used in this research, the concentration of acid was not more than 1.5M. The maximum of about 32% protein hydrolysate was extracted from 1.5M acid concentration and the yield was about 23%.

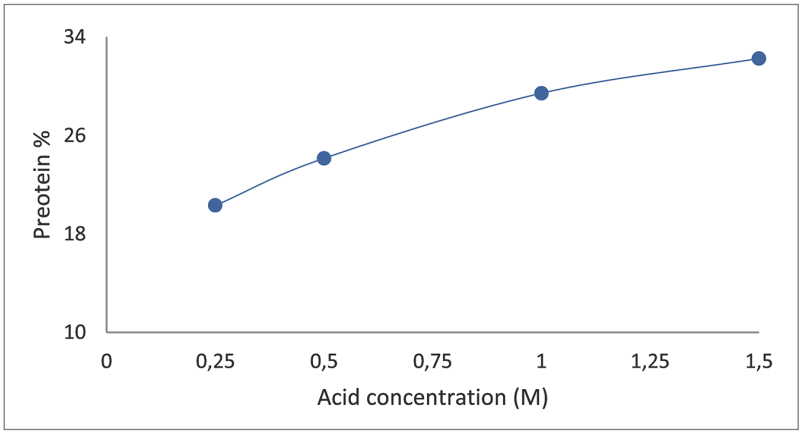


Figure 6. Effect of acetic concentration on protein hydrolysis

Crude protein calculation

Table 2. Volume of H_2SO_4 (ml) required for crude protein determination by titration and yield for effect of acetic acid concentration on hydrolysis

Sample	Volume of 0.05M H_2SO_4 (ml)			Average (ml)	N content (g/kg), Equation (iv)	Crude protein (%), Equation (vi)	Crude protein (g/50gm)	Yield (%), Equation (vii)
	Initial	Final	Diff.					
Blank	0	0	0	0	-	-	-	-
0.25 a	0	11.8	11.8	11.6	32.5	20.3	10.2	14.9
0.25 b	12	23.4	11.4					
0.5 a	30	44	14	13.8	38.6	24.2	12.1	17.8
0.5 b	0	13.6	13.6					
1.0 a	20	36.6	16.6	16.8	47	29.4	14.7	21.6
1.0 b	0	17	17					
1.5 a	25	43.6	18.6	18.4	51.5	32.2	16.1	23.7
1.5 b	0	18.2	18.2					

Effect of concentration solution on protein hydrolysate

The effect of acetic acid concentration solution (ml) on protein hydrolysis of raw trimmings from tanneries is shown in Figure 7. Solution of 5 ml, 10 ml, 15 ml and 20 ml of 1.5M acetic acid were taken for each 50 gm of raw trimmings. From Figure 7, it can be observed that the percentage of yield was increasing with the increase in acid solution and the maximum crude protein percentage was found with 20 ml solution of 1.5M acetic acid and its yield was about 34%.

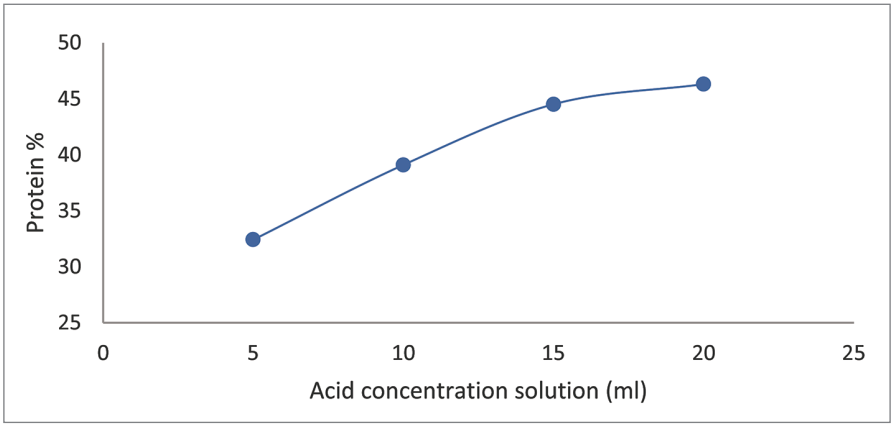


Figure 7. Effect of acetic acid solution on protein hydrolysis

Effect of temperature on protein hydrolysate

Figure 8 shows the effect of temperature on hydrolysis of tannery raw trimmings using best acid concentration (1.5M) and concentration solution (20ml) with temperature variation from 40 to 110^0 C. On heating, trimmings, mainly composed of collagen, are disintegrated and dissolved very quickly. The maximum percentage of protein hydrolysate was obtained about 58 at temperature 80^0 C and yield was about 43%. Temperature above this, the denaturation of protein was occurred and hence yield declined. As raw trimmings are biological materials, the temperature should not increase to high due to avoiding denature of protein.

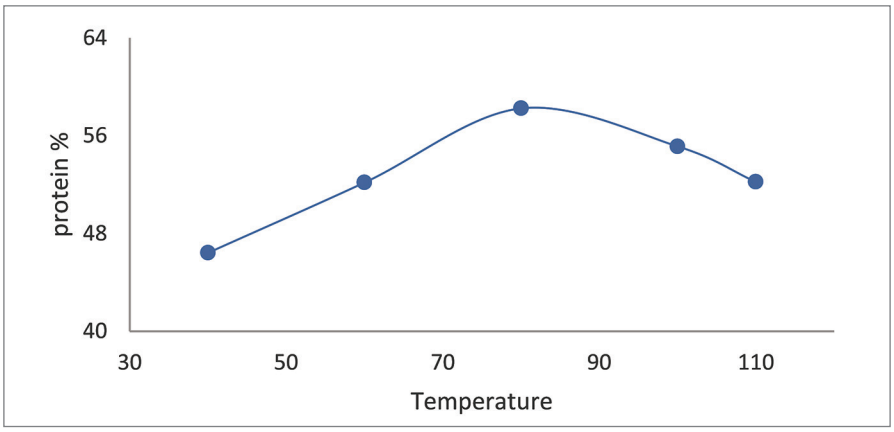


Figure 8. Effect of temperature on protein hydrolysis

Effect of hydrolysis time on protein hydrolysate

Figure 9 shows the effect of hydrolysis time (hour) on hydrolysis with 1.5M acetic concentration, 20 ml acid solution and at 80 °C temperature. About 71% of protein hydrolysate was obtained after 14 hours of hydrolysis and the yield was about 52%. After that the trend of increase was negligible.

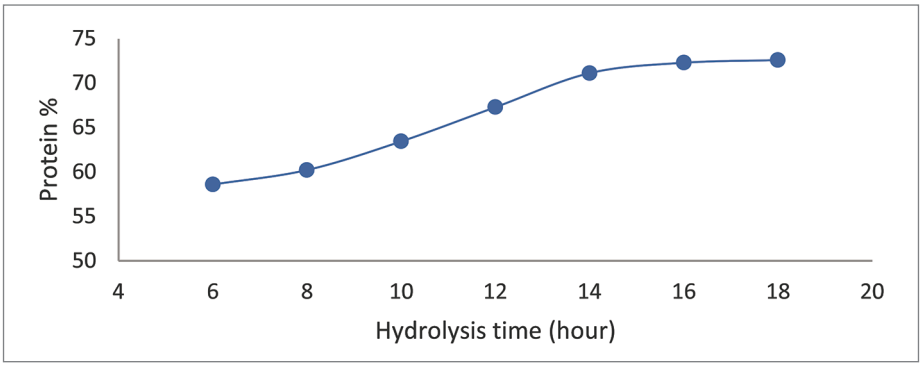


Figure 9. Effect of hydrolysis time on protein hydrolysate

Effect of enzyme concentration (%) on hydrolysis

10 ml solution of 1 to 6% enzyme on the basis of sample weight was prepared for hydrolysis at 1.5 M acetic acid concentration as shown in Figure 10. Other operating parameters, temperature and hydrolysis time, were 80 °C and 4 hours respectively. Among the various enzyme ratios, about 55% protein was extracted from 4% ratio of enzyme and the yield was about 41%. After that, the percentages were negligible.

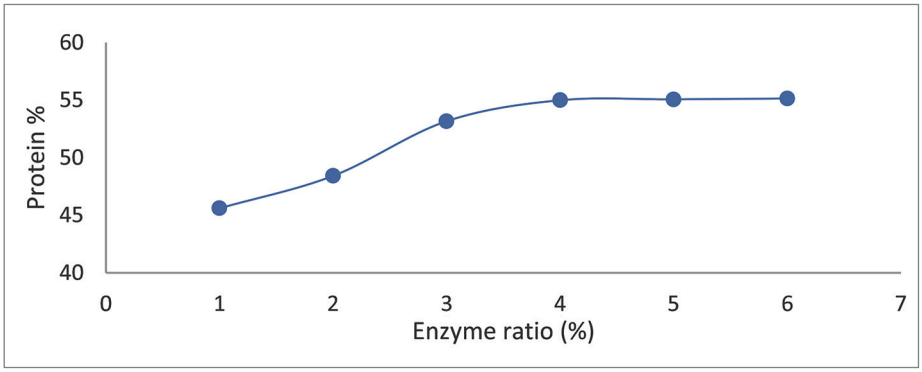


Figure 10. Effect of enzyme concentration (%) on hydrolysis

Effect of enzyme solution (ml) on hydrolysis

Figure 11 shows the effect of enzyme solution on hydrolysis. After the addition of 20 ml enzyme solution, the percentage of protein, which was about 63%, was not increased significantly and the yield was about 46%.

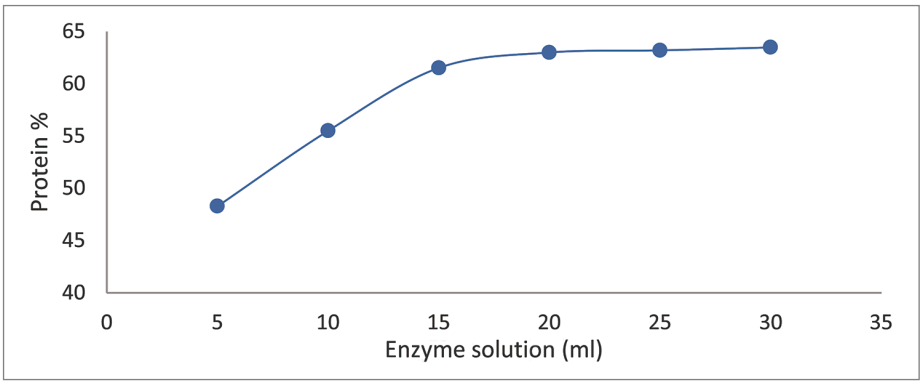


Figure 11. Effect of enzyme solution (ml) on hydrolysis

Effect of heating time on enzymatic hydrolysis

Heating time in enzymatic hydrolysis significantly increases the protein extraction, as shown in Figure 12. The hydrolysis was conducted with the temperature not above 50-60 °C due to enzyme activity in the 1st stage and the final hour used for solidification of the digested sample at 80 °C in the 2nd stage. About 22% of protein hydrolysate was obtained by increasing hydrolysis duration from 4 hours to 6 hours with 1.5 M acetic acid concentration and 20 ml of its solution. The maximum of 88% of protein hydrolysate was obtained after 12 hours of hydrolysis and the yield was about 65%.

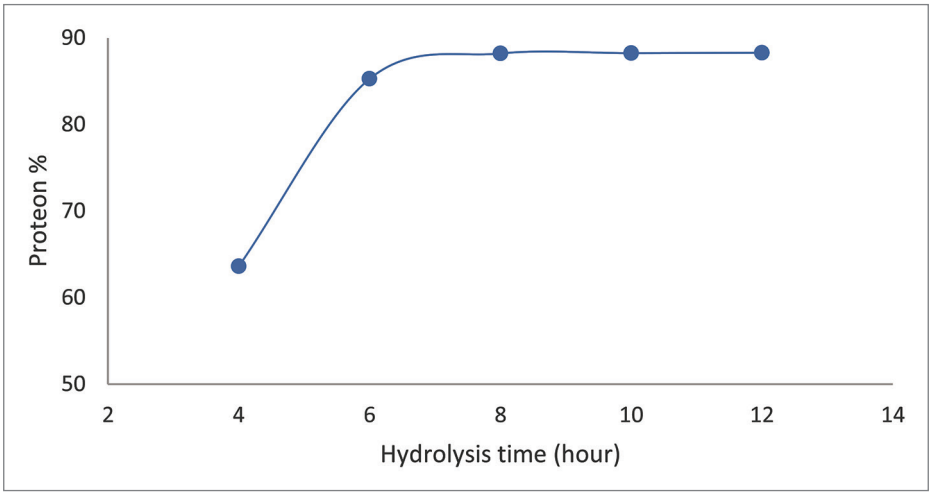


Figure 12. Effect of heating time on enzymatic hydrolysis

Table 3. Crude protein analysis by Kjeldhal method (acetic acid hydrolysis), sample weight 50 gm

Conc. (M)	Solution (ml)	Temp (°C)	Time (h)	Crude protein (%)	Crude protein (g/50gm)	Yield (%)
0.25	5	40	6	20.3	10.2	14.9
0.5	5	40	6	24.2	12.1	17.8
1.0	5	40	6	29.4	14.7	21.6
1.5	5	40	6	32.2	16.1	23.7
1.5	5	40	6	32.4	16.2	23.8
1.5	10	40	6	39.2	19.6	28.8
1.5	15	40	6	44.5	22.2	32.7
1.5	20	40	6	46.2	23.1	33.9
1.5	20	40	6	46.4	23.2	34.1
1.5	20	60	6	52.2	26.1	38.4
1.5	20	80	6	58.3	29.1	42.9
1.5	20	100	6	55.1	27.6	40.5
1.5	20	110	6	52.2	26.1	38.4
1.5	20	80	6	58.6	29.3	43.1
1.5	20	80	8	60.2	30.1	44.3
1.5	20	80	10	63.5	31.8	46.7
1.5	20	80	12	67.2	33.6	49.4
1.5	20	80	14	71.2	35.6	52.4
1.5	20	80	16	72.3	36.1	53.2
1.5	20	80	18	72.5	36.2	53.3

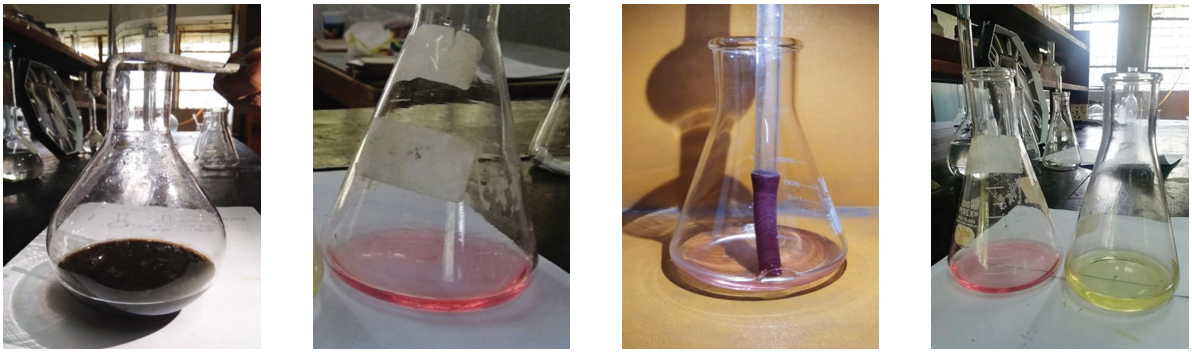
Table 4. Crude protein analysis by Kjeldhal method (acid-enzyme hydrolysis), sample weight 50 gm

Acid conc. (M)	Enzyme ratio (%)	Enzyme Solution (ml)	1st stage Temp (°C)	1st stage time (h)	2nd stage Temp (°C)	2nd stage time (h)	Crude protein (%)	Crude protein (g/50gm)	Yield (%)
1.5	1	10	50-60	3	80	1	45.7	22.8	33.6
1.5	2	10	50-60	3	80	1	48.3	24.2	35.5
1.5	3	10	50-60	3	80	1	53.2	26.6	39.1
1.5	4	10	50-60	3	80	1	55.3	27.7	40.7
1.5	5	10	50-60	3	80	1	55.1	27.6	40.5
1.5	6	10	50-60	3	80	1	55.1	27.6	40.5
1.5	4	5	50-60	3	80	1	48.3	24.2	35.5
1.5	4	10	50-60	3	80	1	55.3	27.7	40.7
1.5	4	15	50-60	3	80	1	61.6	30.8	45.3
1.5	4	20	50-60	3	80	1	63	31.5	46.3
1.5	4	25	50-60	3	80	1	63.2	31.6	46.5
1.5	4	30	50-60	3	80	1	63.4	31.7	46.6
1.5	4	20	50-60	3	80	1	63.5	31.8	46.7
1.5	4	20	50-60	5	80	1	85.2	42.6	62.7
1.5	4	20	50-60	7	80	1	88.2	44.1	64.9
1.5	4	20	50-60	9	80	1	88.2	44.1	64.9
1.5	4	20	50-60	11	80	1	88.4	44.2	64.9

Some pictures of protein determination by Kjeldhal method are shown in Figure 13 and 14.

(a) (b)

Figure 13. Digestion of raw trimmings (a) at the start of digestion and (b) at the end of digestion



(a) (b) (c) (d)

Figure 14. Distillation of the digested sample: (a) adding NaOH to the digested sample, (b) boric acid with the methyl red indicator, (c) capturing the NH_3 in boric acid (yellowish color), (d) boric acid solution before and after capturing NH_3

Raw trimmings hydrolysis and the final product are shown in Figure 15.

(a) (b)

Figure 15. (a) raw trimmings hydrolysis and (b) final product

APPLICATIONS OF RAW TRIMMIMGS

The collagen / gelatin extracted from untanned raw trimmings of hides and skins has huge application potential in the field of packaging, biomedicine and cosmetics according to recent studies. The gelatin extracted by acetic acid from raw trimmings was blended with polyvinyl alcohol (PVA) and used to prepare a kind of biodegradable packing material. Here, PVA was used to reduce high water-solubility of gelatin. Collagen burn-healing membrane was prepared from the collagen extracted from pig skin by enzymatic hydrolysis. Hybrid films can be prepared by using collagen extracted from the trimmed skin by acetic acid and then blending with starch/soy protein [18].

Collagen can bind large fat quantities as an emulsifier in meat products. Gelatin is playing a major role in stabilizing ice-cream and other frozen foods. The most common use of gelatin in pharmaceutical industry is covering the outer layer of capsules [19]. Tissue adhesive, vascular grafts, aortic heart valves, drug delivery matrices, wound dressing, and tissue engineering scaffold can be made from collagenous materials derived from leather trimmings [3].

CONCLUSION

Solid wastes generated by the tanning industry pose a great threat to the environment if not properly managed. Untanned raw trimmings do not get any treatment in the tanning process and they can be used as a cheap source of collagen, which has various industrial applications. In this study, the addition of enzyme to the process of hydrolysis with acetic acid accelerated the extraction of protein hydrolysate when compared to hydrolysis with acetic acid alone. About 88% of protein hydrolysate was obtained from enzyme-assisted acid hydrolysis of raw trimmings from tanneries.

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