Enzymatic method for improving the yield and quality of collagen hydrolysate

Technical bulletin

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The institute's second campus was established on 19th March, 1993 at Abohar, Punjab, India that is primarily responsible for conducting research and development activities on fruits and vegetables, and commercial horticultural crops. ICAR-CIPHET, Ludhiana is also the headquarter for two All India Coordinated Research Projects (AICRPs) viz. AICRP on Post-Harvest Engineering and Technology (PHET) with 31 Centres and AICRP on Plasticulture Engineering in Agriculture Structure and Environmental Management (PEASEM) with 14 Centres across the country.

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लुधियाना, 141004 (पंजाब)

ICAR-Central Institute of Post-Harvest Engineering & Technology
Ludhiana, 141 004 (Punjab)

Published by
Dr. Nachiket Kotwaliwale
Director, ICAR-CIPHET

Editors
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Graphic Designing
Dr. Yogesh Kumar

Citation
Yogesh Kumar, Tanbir Ahmad and AU Muzaddadi (2021). Enzymatic method for improving the yield and quality of collagen hydrolysate. ICAR-Central Institute of Post-harvest Engineering and Technology, Ludhiana (Punjab). Technical Bulletin No.: ICAR-CIPHET/Pub./2021-22/02. pp 1-32

Published by
ICAR-Central Institute of Post-Harvest Engineering & Technology (CIPHET), Ludhiana 141 004 Punjab (India)

Contents

Introduction

Livestock product processing generates a large amount of by-products. These by-products are either in solid or liquid form and can be used to make compost, biogas, animal feed, or other low-value products. Some by-products from animal processing industries contain high-value compounds with various applications in the food and pharmaceutical industries. Thus, the concept of secondary and tertiary processing can be applied to gain more economical benefits by further processing these by-products. By-products of livestock processing are viscera, fat, feet, intestinal contents, bones, blood, low-quality meat, and skin. These by-products amount varies between 33-43% (w/w) of the live weight (Hamilton, 2004). The slaughtering of bovine generates approximately 47-49% (w/w) of by-products of the total live weight (Meeker, 2009).

There is a loss of potential revenues due to the underutilization of these by-products and even an increase in the processing cost due to the higher cost of disposal process of these by-products. The industry has some technological solutions for converting these by-products into compost, animal feed, and low-value products. However, the secondary and tertiary processing of these animal product processing by-products may result in the recovery of high-value compounds. Thus, there is a need to develop suitable technologies for the extraction/recovery of high-value compounds from animal processing by-products.

The most important content of these animal by-products is protein that can be converted into high-value products through various technological interventions. The protein can be hydrolyzed to get bioactive peptides that have food, nutritional, therapeutic, and pharmaceutical applications. These hydrolysates also show potential health benefits hence can be used as nutraceuticals. Collagen is a protein mainly found in fibrous animal tissues, such as skin, bones,

“The present technology relates to a process for production of collagen hydrolysate (CH) from buffalo skin. This enzyme-assisted process results in a higher yield of CH with better functional properties. Collagen hydrolysates are high-value compounds that are being used for various nutritional, therapeutic and pharmaceutical applications. Different quality CHs are available in the market with cost about 2000-8000 Rs/kg. The economics of this process suggests its application at commercial level for sustainable profit.”

blood vessels, tendons, ligaments, etc. It is a long and fibrous protein that is abundant and accounts for approximately 30% of the body's total protein mass. It is a structural protein having three α-chains intertwined to form a very stable right-handed triple helix (Bailey and Light, 1989). The hydrogen bonding between the chains provides the required stability to the triple helical structure (Johnston-Banks, 1990; Te Nijenhuis, 1997). Covalent bonds might join these triple-helical chains into fibril resulting in the formation of fibril forming collagen (Tanbir et al. 2017). The anomalies in the collagen synthesis pathways and abnormal degradation of collagen are correlated to osteoarthritis and joint pain (Bello and Oesser, 2006).

Collagen peptides (CP) or collagen hydrolysates (CH) are obtained by hydrolysis of collagen through various methods. These CHs have been shown to possess many bioactive, functional, as well as therapeutic properties. Hence, these are used as dietary supplements as well as components in cosmetics, nutritious products, and pharmaceuticals. Due to their health benefits, the global market of CH is projected at a higher pace with a compound annual growth rate (CAGR) of 7.1% and to exceed $1200 million by 2025.

The quality of CH depends on the source of animal processing by-products. Even the quality of CH is dependent on different organ/by-products from the same species. The extraction of CH is done from different sources such as bovine, porcine, marine, and poultry species. Bovine species is a preferred source for the extraction of CH. Various researchers have shown the extraction of CH from bovine species and reported their biological and functional effects in different in vitro/in vivo systems. Blood, tendons, bone, ligaments have been studied by researchers to prove their therapeutic potential. CH is generally prepared by acidic and alkali hydrolysis. The quality of CH obtained from the acid/alkali hydrolysis process is inferior because of various reasons.

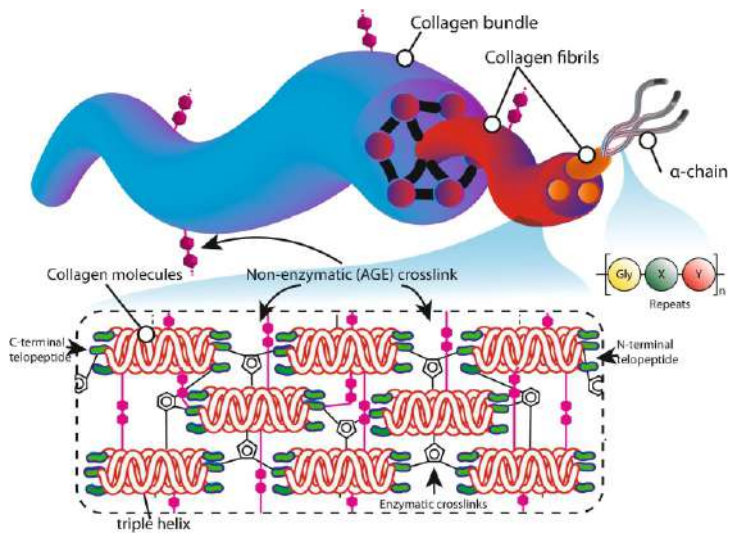


Figure 1. The higher order structure of mature collagen. The X and Y are frequently proline or hydroxyproline, respectively (Hong et al. 2019).

The protein has been hydrolyzed by acids which is an effective conventional method of producing peptides and amino acids. Inorganic (hydrochloric acid) or organic (acetic acid, citric acid, lactic acid, formic acid) acids can be used for this purpose. Hydrochloric acid (HCl) is commonly used to obtain protein hydrolysates. However, a higher concentration of HCl at elevated temperatures results in the destruction of amino acids like tryptophan, tyrosine, serine, and threonine (Fountoulakis and Lahm, 1998). Alkali hydrolysis is advantageous over the acid method due to lesser negative effects on tryptophan amino acid. However, both alkali and acid treatment at higher concentrations and at harsh conditions are corrosive to processing equipment and result in higher salt content in the final product.

Enzyme hydrolysis has many advantages over chemical hydrolysis; hence, it is now preferred for the preparation of bioactive peptides. Enzymes gently hydrolyze the proteins at a relatively lower temperature than acid/alkali. Enzymes are specific in nature and cleave specific peptide bonds to produce predicted bioactive peptides with a higher probability (Marcet et al. 2016). Due to the higher efficacy at a lower concentration and temperature, the degradation of amino acid is lower. Moreover, the salt contents are also lower in the final product. Hence, there is a need to develop suitable enzyme-assisted technology for obtaining CH from bovine skin.

“The demand for collagen is rising at approximately 20% annually and global collagen-based bio-materials market is predicted to reach US$5 billion by 2025 (Noorzai and Verbeek, 2021).”

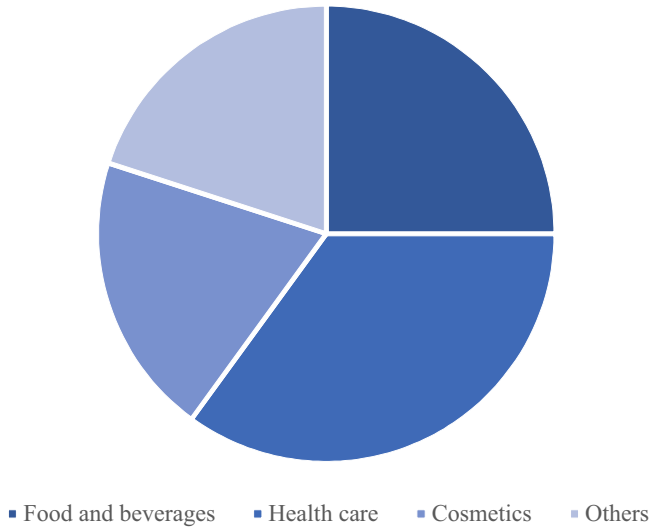


Figure 2. Collagen hydrolysate market share (%) by application, 2019.

Factors that affect the quality of collagen hydrolysate

There are various factors that affect the yield and quality of CH. These factors are related to the type and quality of raw material (species or tissue) as well as on processing conditions during extraction and hydrolysis of collagen. Collagen is a long-lived protein and naturally resistant to degradation; hence in general, it is hard to hydrolyze collagen. The resistance to hydrolysis is due to the presence of cross-links and hydroxyproline. The composition and degree of hydrolysis of collagen are factors that increase functional properties such as antioxidant capacity, antimicrobial activity, and bioavailability. These properties are mainly dependent on the molecular weight of the peptides.

Cross-linking
The collagen of animal skin is hard to hydrolyze due to the presence of strong and tough cross-links. This cross-linking lowers the solubility and reduces the hydrolysis process of collagen. Hence, bovine skin needs a combination of strategies to extract and hydrolyze collagen for the production of quality CH.

Hydroxyproline (Hyp) content
Hydroxyproline (Hyp) is responsible for collagen stability (Shoulders and Raines, 2009). The presence of a higher amount of proline and hydroxyproline results in resistance to hydrolysis.

It has been demonstrated that the Hyp-containing peptides successfully survive through the digestive system and transport into the bloodstream after oral ingestion of collagen hydrolysates.

Processing variables
Various factors and their combination affect the quality of collagen and CH. The main factors are pH, temperature, time, medium (acid, alkali, etc.) during pre-treatment, extraction, and hydrolysis process (Table 1). One of the major properties is the length of the polypeptide chain which affects the functional properties of collagen/gelatin/CH (Kołodziejska et al. 2008). The pre-treatment process factors are decided by the degree of cross-linking present in the raw material. The chemical pre-treatment is necessary to cleave non-covalent bonds of the protein structure which results in swelling and collagen solubilization (Tanbir et al. 2017). Swelling properties and solubilization of collagen fibre are dependent on the type and concentration of acid used; and the amount of cleavage of the cross-links between collagen chains.
Afterward, a suitable heat treatment destabilizes the triple helix of collagen fibre which results in helix-to-coil transition and conversion into soluble gelatin by breaking the hydrogen and covalent bonds. Covalent and non-covalent bonds are broken down in sufficient numbers to release free α chains and oligomers (Johnston-Banks, 1990). Additionally, some amide bonds are cleaved in

the native collagen structure during hydrolysis. Therefore, the resultant collagen contains a combination of lower molecular weight polypeptides (Asghar and Henrickson, 1982). In general, low-molecular weight CHs are advantageous in terms of bioavailability (Yamamoto et al. 2016).

Basic steps for obtaining collagen hydrolysate

There are three basic steps for production of CH: 1) pre-treatment of raw by-products; 2) extraction of collagen (gelatin); and hydrolysis of collagen.

Pre-treatment of raw by-products

This step removes non-collagenous material from the raw material. The process also mildly disrupts the triple-helix structure and cross-linking of collagen. Mostly, sodium hydroxide (NaOH) is used to remove non-collagenous protein. The optimization of NaOH concentration is necessary because at harsh conditions the yield of collagen and CH suffers. Lipids are partially removed by NaOH treatment. Some studies use organic solvents for the removal of lipid. Some studies suggest higher yield using an acid pre-treatment followed by an alkaline neutralization (Zhou and Regenstein, 2006).

Extraction of Collagen

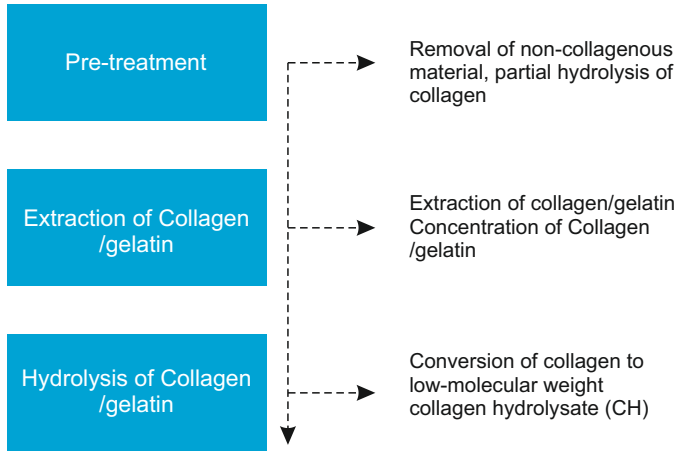


Figure 3. Basic steps for production of collagen hydrolysate.

After removal of non-collagenous substances extraction of collagen is carried out using different hydrolysis methods. The most common conventional method for collagen extraction is acid hydrolysis at elevated temperatures. After acid treatment, the collagen fibres swell and dissolve. This process is mediated by the higher H+ concentration provided by the acid. Various organic, as well as inorganic acids, have been studied for the extraction of collagen from animal industry by-products.
However, the acid treatment has certain disadvantages of amino acid degradation, accumulation of salt, and corrosive effect on the equipment. Hence, enzymes are now under investigation for extraction as well as hydrolysis of collagen. Enzymes have reaction selectivity and specificity as well as have no destructive effect on amino acids. Pepsin is the most studied enzyme for the extraction of collagen (Yu et al. 2018) (Roy et al. 2017). Alkali treatment is more efficient for the extraction of highly cross-linked collagen. However, it has disadvantages of deamination process as well as longer treatment duration than the acid treatment (Keenan, 1994).

Hydrolysis of collagen
There are acid and alkali treatment processes for the hydrolysis of collagen into relatively low-molecular weight compounds with higher hydrophilicity. The disadvantages of using acid and alkali treatment are similar to that of collagen extraction i.e. destruction of amino acids. Hence, enzymatic hydrolysis is now given preference for the production of bioactive peptides from food industry by-products. Enzymes show no negative effects on the quality of CH. Enzymes are gentle and hydrolyze the collagen at a lower temperature than acid or alkali methods. Since collagen cross-links are comparatively resistant to thermal and acid treatment (Galea et al. 2000), normally a low yield is obtained (Nalinanon et al. 2008). Previously, some proteases capable of breaking the collagen cross-links have been used to increase the extractability (Nalinanon et al. 2008). Chomarat et al (1994) used the pepsin and protease (isolated from Aspergillus niger) to extract the collagen from bovine skin but the relatively low amount was recovered, its gel strengths and viscosities were also low.

Enzymatic method for improving the yield and quality of collagen hydrolysate

The non-collagenous materials were removed by treating the skin with NaOH solution. After this, the hairs on the skin were removed. Distilled water was used to wash the skin thoroughly till neutral pH was achieved from the wash water.

The skin was soaked in HCl with discontinuous stirring at room temperature for swelling. The samples were washed thoroughly with distilled water until neutral wash water was obtained. The sample was kept in water bath at elevated temperature for certain duration of time. After this treatment, filtration was done through suitable medium.

The filtrate was were incubated with enzyme papain/bromelain at optimum temperature and pH. The material was centrifuged and the supernatant was freeze dried and termed as collagen hydrolysate (CH).

Traditional CH was extracted without any enzymatic treatment from the HCl treated skin. In could be concluded based on the yield and quality that papain, particularly at level 20 unit/g of skin, and bromelain at level 30 unit/g of skin, were found to be better with better functional properties of CH.

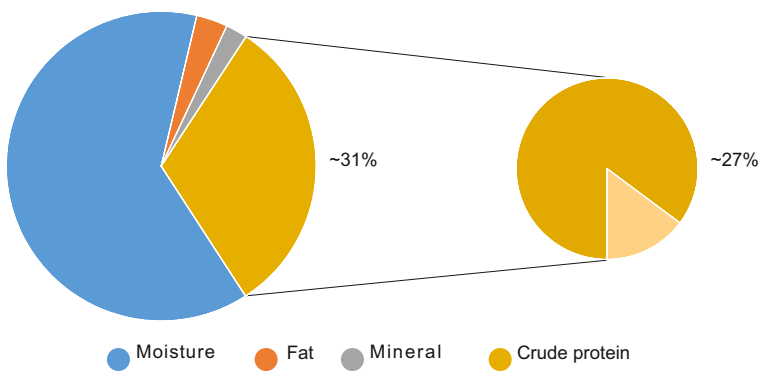


Figure 4. Conversion (Yield) of skin protein to collagen hydrolysate.

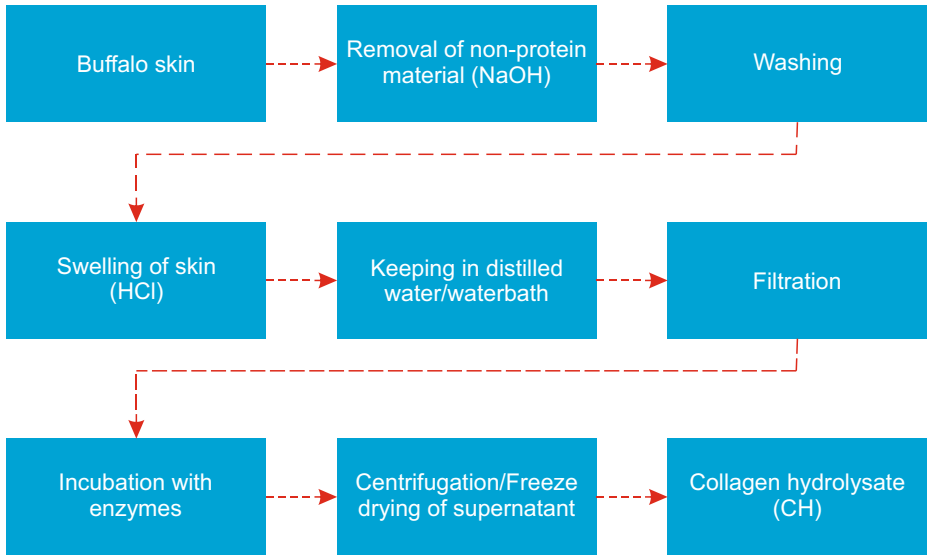


Figure 5. Enzymatic method for production of collagen hydrolysate.

Traditional CH was extracted without any enzymatic treatment from the HCl treated skin. In could be concluded based on the yield and quality that papain, particularly at level 20 unit/g of skin, and bromelain at level 30 unit/g of skin, were found to be better with better functional properties of CH.

> This process is available for licensing at ICAR-Central Institute of Post-harvest Engineering & Technology, Ludhiana. The process parameters (pH, temperature, dilution, incubation time etc.) and the strength and concentration of various chemicals will be provided during licensing of this technology at ICAR-CIPHET.

Quality of collagen hydrolysate developed by enzymatic method

Yield

The results on the yield of CH are shown in Table 1. In papain enzyme treatment, a higher yield is obtained in P20 group. In the case of bromelain, a higher yield is obtained in B30 group. These results suggest that papain at 20 units or bromelain at 30 units is effective for a maximum yield of CH.

Free amino group contents

A higher free amino group contents (mmol/g of skin) are produced at 20 and 30 units of papain. Similarly, higher amino group contents are produced at 30 and 50 units of bromelain enzyme treatment. A lower content at higher enzyme concentrations may be due to the formation of cross-

Table 1. Yield of collagen hydrolysate extracted from buffalo skin using papain and bromelain.

Sample	Yield (%)
P20	27.38±0.14[a]
P30	26.32±0.21[a]
B30	20.71±0.32[b]
B50	16.19±0.26[c]

Means with different superscripts indicate significant difference at $p<0.05$. P20, P30 & B30/B50 refer to collagen hydrolysate (CH) extracted using enzymes papain (P) and bromelain (B) pretreatment at corresponding level of 20, 30 or 50 units of enzyme/g of skin, respectively.

Figure 6. Extracted collagen hydrolysate from buffalo skin using papain and bromelain

linking between low-molecular weight peptides.

Molecular weight characterization

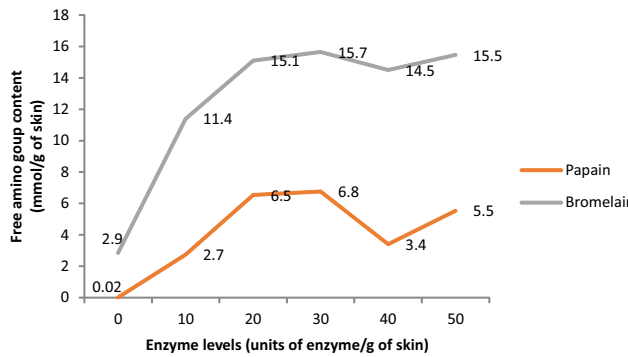
SDS-PAGE analysis shows a higher degradation or hydrolysis at 20 and 30 units of papain enzyme treatment. The results show complete degradation of the b-chain (Figure 3).

Based on free amino group estimation and SDS-PAGE images, it is established that papain enzyme degrades the proteins most effectively at levels 20 and 30 units of enzyme per gram of skin, and bromelain is most effective at level 30 and 50 units of enzyme/gram of skin to hydrolyze the skin collagen. Thus, the properties of these CH samples are provided below.

Enzyme levels (units of enzyme/g of skin)	Free amino group content (mmol/g of skin) of buffalo skin sample treated with	
	papain	bromelain
0	0.02	2.85
10	2.74	11.39
20	6.54	15.10
30	6.76	15.65
40	3.40	14.50
50	5.53	15.46

Table 2. Free amino group content (mmol/g of skin) in the degraded skin sample incubated with various levels of enzymes papain and bromelain.

Figure 7. Free amino group content (mmol/g of skin) in the degraded skin sample incubated with various levels of enzymes papain and bromelain.



Proximate analysis and hydroxyproline content

The results of the proximate analysis are shown in Table 1. All samples show similar protein contents and the values are non-significant between the four groups. Higher hydroxyproline contents are observed in CH samples obtained at 30 units of papain or 50 units of bromelain enzyme treatment.

Antioxidant properties

DPPH radical scavenging activity (%), reducing power, and hydroxyl radical scavenging activities are shown in Table 1-1. Results suggest significantly lower IC50 values in CH samples obtained at 30 units of papain or bromelain enzyme treatment. Lower IC50 values depict the higher antioxidant potential of the samples. All CH samples show higher reducing power.

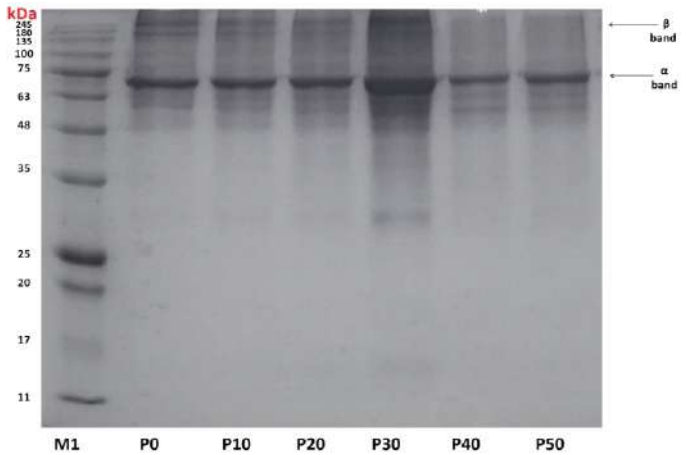
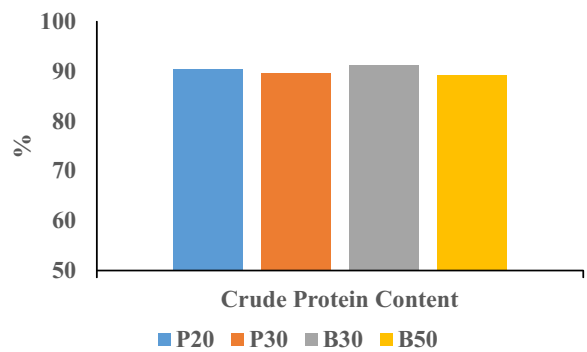


Figure 8. SDS-PAGE image of samples incubated with papain enzyme at various levels of 0, 10, 20, 30, 40 & 50 units of papain (designated as P0, P10, P20, P30, P40 & P50, respectively). M1 denotes the marker.

Proximate analysis (%)	Collagen hydrolysate (CH) samples			
	P20	P30	B30	B50
Moisture	2.66	2.89	2.51	2.63
Fat	0.65	0.74	0.54	0.59
Minerals	2.39	2.56	2.26	2.68
Crude Protein	90.47	89.65	91.2	89.14

Table 3. Proximate analysis of Collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.

Figure 9. Crude protein content in the collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.



Values Non-significant

Collagen hydrolysate (CH) samples	Hydroxyproline content (%)
P20	8.23
P30	9.75
B30	9.12
B50	10.89

Table 4. Hydroxyproline content of Collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.

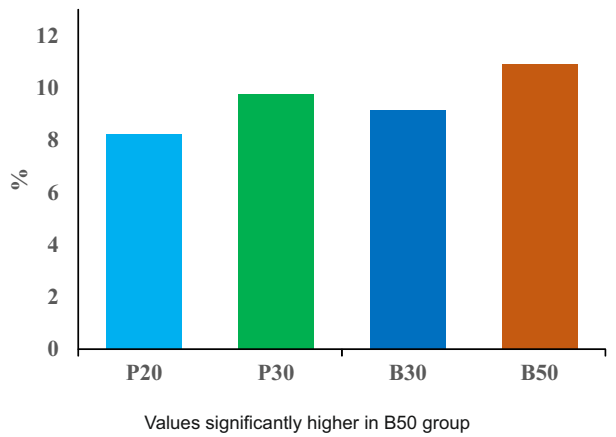


Figure 10. Hydroxyproline content of Collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.

> **"The effects of enzyme treatment on other functional properties have also been evaluated. The data will be shared during licensing of technology"**

Table 5. DPPH radical scavenging activity of Collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.

Concentration of CH sample (mg/ml)	DPPH activity (%)			
	P20	P30	B30	B50
0.2	35.09	32.32	35.59	31.63
0.4	46.56	38.20	46.27	41.22
1	53.82	63.28	59.25	55.27
2	62.46	70.46	65.15	66.96
5	70.27	79.89	74.29	89.24
10	78.23	90.59	82.76	94.51

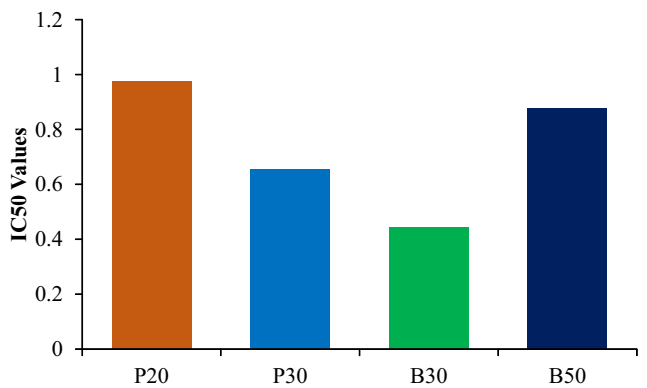


Figure 11. IC50 values (DPPH radical scavenging activity) of collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.

Table 6. Reducing power of collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.

Concentration of CH sample (mg/ml)	Spectrophotometer reading (700 nm)			
	P20	P30	B30	B50
0.2	0.189	0.196	0.146	0.215
0.4	0.211	0.229	0.202	0.252
1	0.242	0.256	0.236	0.296
2	0.276	0.289	0.265	0.307
5	0.447	0.478	0.423	0.496
10	0.661	0.706	0.643	0.723

Table 7. HO^- scavenging (HRSA) activity (%) of collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.

Concentration of CH sample (mg/ml)	HO^- scavenging (HRSA) activity (%)			
	P20	P30	B30	B50
0.2	28.60	30.25	32.44	36.26
0.4	36.93	42.33	40.05	43.35
1	45.62	52.12	50.74	53.90
2	55.19	60.76	60.53	66.92
5	61.57	71.85	72.08	75.42
10	68.48	78.32	80.36	84.64

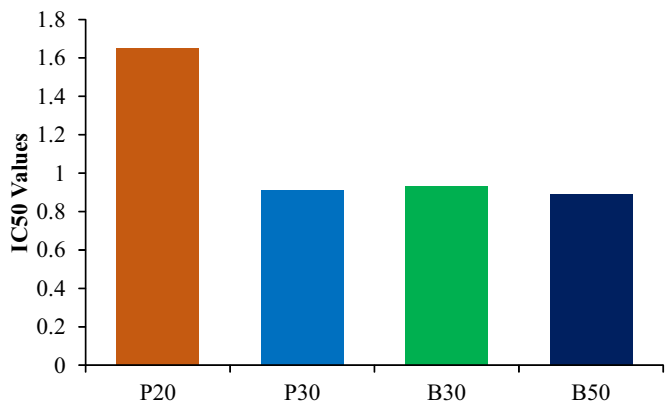


Figure 12. IC50 values (HO^- scavenging (HRSA) activity) of collagen hydrolysate samples incubated with selected levels of enzymes papain and bromelain.

Economics of production of collagen hydrolysate

Points considered while calculating the economics of collagen hydrolysate production

1. Processing of 1 kg of buffalo skin yields 270 g of collagen hydrolysate (CH) taking 27% CH yield using papain at the rate of 20 units of enzyme/g of buffalo wet skin.
2. Papain has activity of 30000 units/mg. For 1 kg of buffalo skin, 0.66 mg of papain is required. This costs less than Rs. 1.
3. The price of various commercial collagen hydrolysate (from bovine and marine sources) products available in the market varies from Rs. 2000 to Rs. 4000 per kg. A premium product costs at Rs. 8000 per kg.

The cost of establishment of processing plant (batch process) is approximately 15-20 lakhs. The plant has components such as metallic tanks, concentrators, heating unit, centrifugal machines,

Table 8. Economics of processing one kg buffalo skin for collagen hydrolysate production.

Sr. No.	Name of the chemicals	Rate (INR)	Quantity required to process 1 kg of buffalo skin	Cost (INR)
1.	Sodium hydroxide	640/kg	20 g	13
2.	Hydrogen chloride (35%)	370/L	37 ml	14
3.	Papain	2500/25 g	0.66 mg	1
4.	Buffalo skin	500 for 20 kg skin	-	25
5.	Overhead charge (20% of all other cost) (incubation, filtration, drying)	Addition of S. No. 1 to 4 is 53 (20% of 53)	-	11
	Total	-	-	**64**

Quantities of chemicals are calculated based on working strength of solutions.
The rate of buffalo skin is for an average quality by-product.
The quantity of enzyme is calculated based on the concentration at which highest hydrolysis occurs.

filtration unit, dehydrator/freeze dryer

Conclusion

Thus, 270 g of collagen hydrolysate is obtained by expenditure of Rs. 64/-.
Hence, one kg of collagen hydrolysate is obtained by expenditure of Rs. 237/-.

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