

DEVELOPMENT OF FOOD PACKAGING FILM USING GELATIN EXTRACTED FROM SURIMI REFINER DISCHARGE

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of the requirements
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by:

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SEPTEMBER, 2018

Dedicated to my guide and mother

DECLARATION

I hereby declare that the thesis entitled “**DEVELOPMENT OF FOOD PACKAGING FILM USING GELATIN EXTRACTED FROM SURIMI REFINER DISCHARGE**” is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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SYMBOLS AND ABBREVIATIONS

cm^{-1}	Per centimetre
cm^2	Square centimeter
C-N	Nitrile group
C=O	Carbonyl group
$\text{Ca}(\text{OH})_2$	Calcium hydroxide
COO	Carboxylic group
CuSO_4	Copper sulphate
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g/g	Gram per gram
h	Hour
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
H_3PO_4	Phosphoric acid
J/g	Joule per gram
J/cm^2	Joule per square centimetre
K_2SO_4	Potassium sulphate
Kg	Kilogram
Kgf	Kilogram force
kDa	Kilodalton
kGy	Kilogray
kgcm^{-2}	Kilogram per square centimetre
l	Litre
M	Molar

mm/s	Millimetre per second
mm/min	Millimetre per minute
mg	Milligram
mA/gel	Milliampere per gel
mg/ml	Milligram per millilitre
min	Minute
ml	Millilitre
mm	Millimetre
NaOH	Sodium hydroxide
NaCl	Sodium chloride
N-H	Amine
NH ₃	Ammonia
nm	Nanometre
O ₂	Oxygen
UV	Ultra violet radiation
pH	Potential of hydrogen
ppm	Parts per million
SDS-PAGE	Sodium dodecyl sulphate polyacrylic gel electrophoresis
sec	Second
SPSS	Statistical package for the social sciences
vol	Volume
α	Alpha
β	Beta
γ	Gamma
μs	Microsecond
μm	Micrometre

μl	Microliter
μg	Microgram
%	Percentage
°	Degree
°C	Degree Celsius
<	Less than
w/w	Weight by weight
w/v	Weight by volume
v/w	Volume by weight

ANNEXURE 1

Buffer composition

Citric acid monohydrate	50 g
Glacial acetic acid	12 ml
Sodium acetate trihydrate	120 g
NaOH	34 g
Total vol.	1 l

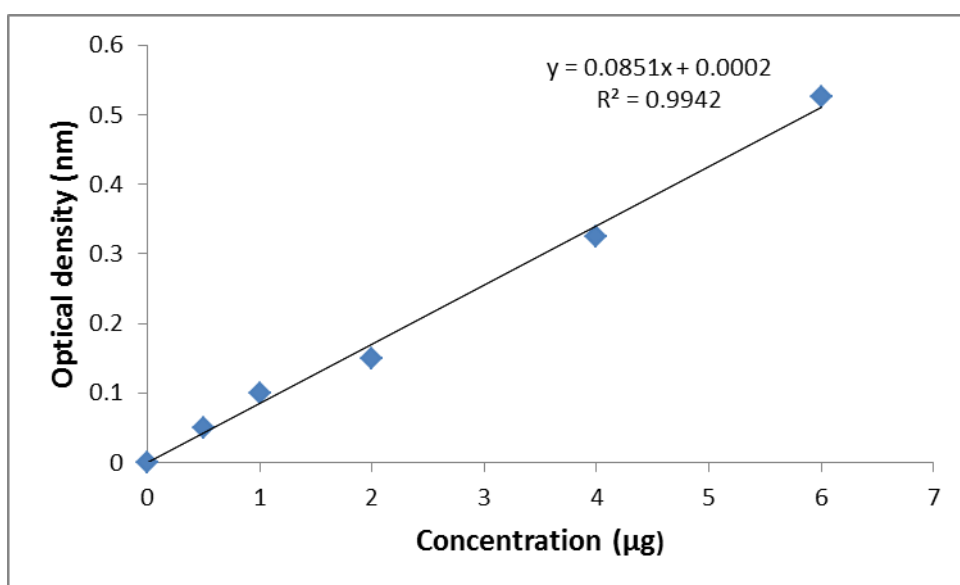


Figure: Standard graph of hydroxyproline

ANNEXURE 2

Protein estimation by Biuret method

Reagents:

- **Bovine serum albumin:** Prepared by dissolving 200 mg protein in 10 ml distilled water (20 mg/ml).
- **Biuret solution:** Prepared by dissolving 3.0 g copper sulphate pentahydrate in 500 ml distilled water, and add 9 g sodium potassium tartarate and dissolved with constant stirring. Latter 5 g potassium iodide was added and dissolved. To the above solution, 8 g of sodium hydroxide pellets was added and dissolve with constant swirling. The solution was diluted to 1 l with distilled water and store in a polyethylene bottle.

Procedure

1. Pipette a known amount of protein standard and distilled water as shown in Table into a test tube.
2. Add 4.0 ml of Biuret solution in all the tube and allow standing 30 min at room temperature. Tube without standard was used for blank reading
3. Record optical density at 546 nm on a spectrophotometer (see Table)
4. Plot absorbance at 550 nm against the concentration of the protein standard

Table: Concentration and Optical Density of Bovine serum albumin

Tube	Volume Standard (ml)	Volume water (ml)	Volume Biuret reagent (ml)	Concentration standard (mg/ml)	Optical Density 546 nm
1	0.00	1.00	4	0.0	Set at 0
2	0.10	0.90	4	2.0	0.116
3	0.20	0.80	4	4.0	0.211
4	0.40	0.60	4	8.0	0.421
5	0.60	0.40	4	12.0	0.619
6	0.80	0.20	4	16.0	0.884
7	1.00	0.00	4	20.0	1.012

5. The protein content of the unknown sample was determined by using the equation established from the standard graph (figure)

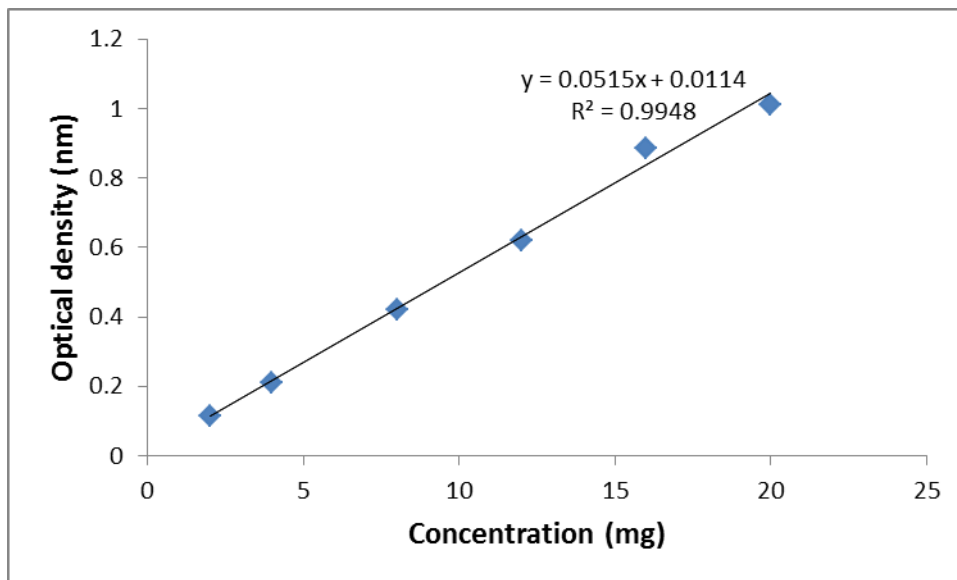


Figure: Standard graph of BSA

ABSTRACT

The overall objectives of the present study were to extract collagen and gelatin from waste like refiner discharge of the surimi processing industries and to explore the possibility of making packaging films from extracted gelatin. Collagen was extracted from the pink perch (*Nemipterus japonicus*) surimi refiner discharge and characterized. The average extract yield of collagen was 11.47 %. The denaturation temperature of the extracted collagen recorded from the Differential Scanning Calorimetry Thermogram was 23.07°C. The amino acids composition showed that the extracted collagen was rich in glycine, proline and alanine. The extracted collagen was identified as Type I collagen as SDS-PAGE revealed chain compositions of α_1 and α_2 at 125 kDa and 113 kDa respectively with high molecular β chain at 176 kDa. In order to establish the optimum gelatin extraction conditions from surimi refiner discharge, Response Surface Methodology with a 3 factor, 3 level, Box Behnken Design was conducted. The second order regression equation was established with regards to three independent variables namely NaOH concentration (M, A), extraction temperature (°C, B) and extraction time (h, C) on yield (% , Y) as dependent variable. The optimum extraction conditions to obtain the highest yield of 15.60 % are 0.34 M NaOH, 63.92°C extraction temperature and 4.1 h extraction time. The physical, chemical and functional properties of optimized gelatin were characterized. The gelatin was found to have the bloom value, viscosity and melting point of 143.97 g, 7.44 Cp and 25.5°C respectively. The SDS-PAGE of gelatin revealed to contain α chains and their cross-linked chains (β , γ). Films were developed with different concentrations of gelatin (2-6 %). The gelatin concentration had strong significant ($p < 0.05$) effect on the viscosity of the film forming solution. Later, pulsed light (PL) was employed to modify the physico-chemical and functional properties of the gelatin films by photo-polymerization. The mechanical, structural, molecular aspects and functional properties of the films were then evaluated. PL improved the tensile strength of the film from 66.69 kg cm⁻¹ to 135.81 kg cm⁻¹. There was increase in enthalpy of the denaturation transition with increasing duration of PL treatment. This study demonstrates that refiner discharge can be exploited as a new source of collagen and gelatin production. Packaging films can be successfully prepared from gelatin recovered from refiner discharge and PL can be utilised as one of the quick methods to modify the properties of the gelatin films.

5. DISCUSSION

5.1. Proximate Composition and Constitutional Ratio of Refiner Discharge

The proximate composition data of refiner discharge of pink perch surimi as given in Table 6 shows that, it has average moisture, protein, ash and fat content of 73.95 %, 13.67 %, 2.88 % and 10.61 % respectively. The present crude protein content was similar to refiner discharge from Alaska pollock surimi (13.50 %) (Park *et al.*, 2007). However, it was lower than that of refiner discharge from pacific whiting. Pacific whiting surimi refiner discharge was reported to have protein content of 15.40 % (Kim and Park, 2004). The higher value of ash (10.61 %) could be contributed from higher scale and bone content in the refiner discharge as known from the constitutional ratio study (Table 7). The refiner discharge from pink perch surimi, composed of 61.38 % (muscle and connective tissues) followed by scale (28.70 %) and bone (7.91 %). Kim and Park (2005) reported that surimi refiner discharge from pacific whiting content 95 % muscle followed by skin (2.10 %) and bone (2.90 %). The difference in the composition is due to the difference in fish species and refining processes. Pink perch is a tropical water fish while Alaska pollock and pacific whiting are cold water fishes.

5.2. Extract Yield, Composition and Hydroxyproline Content of Refiner Discharge Collagen

Collagen was extracted from refiner discharge using acetic acid. The extract yield of collagen from refiner discharge was 11.47 % (g of dry collagen per 100 g of wet refiner discharge). The yield in the present investigation was higher than brownstripe red snapper skin collagen (9.0 %), collagen from skin (10.94 %) and bone (1.59 %) of bigeye snapper and collagen from skin of balloon fish (4 %) (Jongjareonrak *et al.*, 2005; Kittiphattanabawon *et al.*, 2005; Huang *et al.*, 2011). However, the yield was lower than the collagen reported from pacific whiting, brown backed toadfish skin (Kim and Park, 2005; Senaratne *et al.*, 2006). The extraction durations of 4 days in 0.5 M acetic acid was selected in view of higher content of bone and scale in the refiner discharge. The yield percentage shows that the employed 4 days duration could extract collagen from the refiner discharge. Collagen

being a rigid structure protein, the yield depends on the type and conditions of extraction method and the conditions used, such as acid soluble, pepsin soluble, homogenization, shaking, mixing, initial acid solubilization and duration (Muralidharan *et al.*, 2013). The yield also varies with collagen composition among different fish species (Rodziewicz-Motowidło *et al.*, 2008).

The proximate composition of collagen powder is given in Table 8. The extracted collagen contained protein as the major component. Other components included moisture (3.03 ± 0.05 %), ash (1.6 ± 0.01 %) and fat (0.51 ± 0.01 %). The protein content of 96.56 ± 1.50 % shows that the purification steps in collagen extraction was good enough to yield good quality collagen. Protein content of collagen ranging from 88.80 % to 91.72 % was reported from skin of shark, rohu and tuna by Hema *et al.* (2013). The ash content (1.6 ± 0.01 %) may be contributed from the minerals of scale or residues of NaCl used for salting out of collagen. The extracted collagen contained negligible amount of fat.

The hydroxyproline content in the refiner discharge collagen was about 7.78 mg/g of collagen (Table 8), lower than that reported for skin (58.50 mg/g) and bone (42.50 mg/g) collagen from bigeye snapper (Kittiphattanabawon *et al.*, 2005). However, it was higher than those found by Sadowska *et al.* (2003) for Baltic cod skin collagen. The imino acid content (proline and hydroxyproline), mainly hydroxyproline plays an important role in stabilizing the triple helix of collagen (Ramachandran, 1956). However, the imino acid content depends on the habitat and body temperature of the fish (Rigby, 1968; Haug *et al.*, 2004). The hydroxyl groups of hydroxyproline and hydroxylysine increases the stability of collagen by interchain hydrogen bonding via a bridging water molecule and by direct hydrogen bonding to a carbonyl group (Wong, 1989). That means higher content of hydroxyproline gave higher thermal stability of collagen. The results suggested that refiner discharge collagen may have higher thermal stability than Baltic cod skin collagen and lower than skin and bone collagen of bigeye snapper. Tylingo *et al.* (2016) reported that collagen from skin of cod, salmon and African catfish content hydroxyproline of 6.50 %, 8.03 % and 8.25 % respectively.

5.3. Physical Characteristics of Refiner Discharge Collagen

5.3.1. Colour

The L^* , a^* and b^* value of the refiner discharge collagen were 94.52 ± 0.23 , 0.05 ± 0.01 , 1.08 ± 0.01 respectively (Table 9). L^* values, which ranged from 0-100, indicate lightness with 100 equal to white and 0 equal to black. The high lightness value of the refiner discharge collagen can give better advantage when used as food additive because it will not lead to any changes in the color of final products. The high lightness of the refiner discharge collagen may be due to the several washing cycles employed during surimi production before subjecting to the screening mechanism of refining. The lightness of the collagen also varies with the source and extraction methods used. In case of skin collagen the pigments in the skin may hinder the whiteness of the collagen (Kim and Park, 2005). Refiner discharge collagen had low and positive values of both a^* and b^* . Positive values of both a^* and b^* values indicates the degree of redness and yellowness respectively. The collagen from red tilapia skins were reported to have average instrumental colour values of L^* (94.63 to 95.62), a^* (-0.65 to -1.10) and b^* (6.38 to 7.24) as affected by extraction conditions (Bakar *et al.*, 2011).

5.3.2. Thermal stability

The study of thermal property of collagen helps us to understand the stability of the collagen as collagen has triple helix structure stabilised by hydrogen bonds. These hydrogen bonds are heat sensitive and converted into helix to coil configuration when heated by the process called thermal depolymerisation (Wang *et al.*, 2008). In the present study, there are two different endothermal peaks in the thermogram of collagen from refiner discharge (Figure 7). The first peak is due to collapse of the triple helix structure of collagen and was recorded at 23.07°C ($\Delta H = 1.1340\text{J/g}$). This result is similar to those reported for collagen from deep-sea redfish scales (17.70°C) (Wang *et al.*, 2008) and carp scales (about 28°C) (Duan *et al.*, 2009). However, the thermal stability of present collagen is higher than Baltic cod skin collagen (15°C), which can be attributed to higher content of hydroxyproline in the present collagen. Collagen from fish sources were reported to have denaturation temperature in the range of $26\text{-}29^\circ\text{C}$ (Burzanadze, 1992). Collagens of high thermal

stability were reported from tilapia scale i.e. 57.90°C to 71.50°C (Huang *et al.*, 2016) and skin of silver carp, 77°C to 88°C (Safandowska and Pietrucha, 2013). The thermal stability of the collagen depends on the imino acids content, environmental temperature of the habitat of the fish, method of preparation of collagen as well as moisture content (hydrated or rehydrated state) of the collagen (Bae *et al.*, 2008, Minh *et al.*, 2014; Rigby, 1968; Huang *et al.*, 2016). Low denaturation temperature of collagen from scales of marine fishes were reported by Minh *et al.* (2014) suggesting such collagen could be an economic advantage for gelatin industry by allowing breakdown of collagen into gelatin at low temperature. The second endothermic peak occurred at 150 °C ($\Delta H = 203.42$ J/g) and is considered due to continued conformational changes of superhelix structure and further destruction of collagen into smaller molecular weights (Safandowska and Pietrucha, 2013).

5.4. Chemical Characteristics of Refiner Discharge Collagen

5.4.1. Amino acid composition

The amino acids composition of the refiner discharge collagen was shown in Table 10. In the present study, glycine was the most abundant amino acid accounted for about 31.65 % of total amino acids present. The glutamic acid, alanine and proline accounted for 10.62 %, 9.98 % and 8.75 % respectively in the present collagen. This result was in accordance with other fish collagen such as collagen from cartilage of amur sturgeon (Liang *et al.*, 2014) and collagen from skin of browstripe red snapper (Jongjareonrak *et al.*, 2005). Glycine content account about 30 % of the total amino acids in collagen as glycine is present on every third residue in collagen chain except for the first 14 residues from the N-terminus and the first 10 residues from the C-terminus (Foegeding *et al.*, 1996; Burghagen, 1999). Collagen from brown backed toadfish skin contained glycine upto 36 % of the total amino acids (Senaratne *et al.*, 2006). The proline content is lesser than collagen from skin of browstripe red snapper, 10.30 %-10.60 % (Jongjareonrak *et al.*, 2005), and similar to amur sturgeon cartilage collagen, 9.30 % (Liang *et al.*, 2014). The amounts of imino acids content determined the stability and denaturation temperature of collagen (Ikoma *et al.*, 2003). The pyrrolidine rings of proline and hydroxyproline contribute to the stability of triple helix of collagen by imposing restrictions on the conformation of the polypeptide chain (Wong, 1989). Hydroxylation of proline and lysine is catalyzed

by enzymes such as proline hydroxylase and lysine hydroxylase respectively (Wong, 1989). The content of amino acids in collagen depends on the source of raw materials. Collagen from bone was found to be higher in alanine, proline, glutamic acid and hydroxyproline than from the skin collagen (Kittiphattanabawon *et al.*, 2005). The hydrophobic amino acids account for around 62 % in the present collagen. The refiner discharge collagen contains 8 out of 9 essential amino acids accounting around 190 residues of the 1000 residues of amino acids. The presence of higher amount of glycine and arginine in the present collagen can be beneficial as protein supplement as these two amino acids are precursors for the biosynthesis of creatine. The hydrophobic and essential amino acids of collagen have close relations to their physical properties and nutritional value.

5.4.2. Structural profile

The peak details of FTIR spectrum of refiner discharge collagen and their corresponding chemical structures are given in Table 10 and Figure 8. Proteins are comprised of amino acids joined together by amide bonds. Amide bands represent different vibrational modes of the peptide bond. Amide bands including Amide A, Amide I, Amide II and Amide III are related to the degree of molecular order and involved with the triple helical structure of collagen (Nikoo *et al.*, 2014). The amide A band peak was detected at 3346 cm^{-1} in the present investigation and it is associated with the N-H stretching vibration. It is established that a free N-H stretching vibration occurs in the range of $3400\text{--}3440\text{ cm}^{-1}$ and when the N-H group of a peptide is involved in a hydrogen bond, the peak position is shifted to the lower frequencies (Doyle *et al.*, 1975). Thus the occurrence of Amide A peak at 3346 cm^{-1} indicates that the sample content hydrogen bonds and more N-H groups of refiner discharge collagen were involved in the hydrogen bonding. In other words, the triple helix structure of collagen is stabilized by hydrogen bonds so more hydrogen bonds indicate more stability of the helix structure. The Amide A bands of skin collagen of *Sebastes mentella* and surf smelt were recorded at 3425 cm^{-1} and 3306 cm^{-1} respectively (Wang *et al.*, 2008; Nagai *et al.*, 2010). The amide B band which is related to the asymmetrical stretch of CH_2 , was found at 2939 cm^{-1} in the extracted collagen of present investigation. The peak of amide I occurred at 1678 cm^{-1} . This peak is associated with the C=O stretching vibration along the polypeptide backbone.

The characteristics frequencies of amide I bands is in the range of 1600 to 1700 cm^{-1} (Payne and Veis, 1988). The shift of this peak to higher frequency indicates that the refiner discharge collagen had a higher degree of molecular order. The amide II and III bands were found at 1551 cm^{-1} and 1239 cm^{-1} , respectively. These bands are indication of N-H bending vibrations and C-H stretching respectively (Payne and Veis, 1988).

5.4.3. Protein pattern

Electrophoretic patterns revealed that the molecular weights (kDa) of the collagen from refiner discharge ranged from 103 to 176 kDa (Figure 9). There are four distinct bands with the molecular weight of around 176, 125, 113 and 103 kDa respectively. The collagen extracted from *C. mrigala* scale also revealed five fractions of protein with molecular weights of 67, 88, 114, 160 and 180 kDa (Mahboob, 2015). In the present collagen the β chain which is due to the intermolecular crosslinking of polypeptides chains appeared at 183 kDa. Acid soluble collagen has more inter and intra cross linked collagen (Kittiphattanabawon *et al.*, 2010). The presence of high molecular weight cross linked collagen depends on the age of the fish, starvation rate (feeding condition), and sources of collagen extraction from fish (Foegeding *et al.*, 1996; Sikorski *et al.*, 1990; Cohen-Solal *et al.*, 1981). The α_1 band was present at 125 kDa while α_2 was seen at 113 kDa. The α_1 band appeared thicker indicating the possibilities of present α_3 chains as α_3 have similar molecular weight with α_1 chain and normal electrophoresis could not separate it (Chi *et al.*, 2014). Molecular weights of the α chains were reported to vary with species and their structural parts. The molecular weight of α_2 chain of ray was lower than α_2 chain of bony fish (Bae *et al.*, 2008). Muyonga *et al.* (2004a) reported the Type I skin collagen of Nile perch consist of two α_1 and one α_2 chains with the molecular weights of 120 kDa and 115 kDa respectively. Skin and bone collagens extracted from the marine fish such as black drum and sheepshead also had similar electrophoretic patterns with molecular weights of α_1 and α_2 fractions as 130 and 110 kDa, respectively (Ogawa *et al.* 2004). Collagen extracted from skin of Baltic cod also had α chains with molecular weight below 116 kDa (Skierka and Sadowska, 2007).

On the basis of protein sub-unit composition, the extracted collagen from refiner discharge is Type I collagen. It has been proven that the major component of type I collagen is composed of two α_1 -chains and one α_2 -chain (Minh *et al.*, 2014; Jongjareonrak *et al.*, 2006). The collagen molecule consists of three polypeptide chains (α chain). The chains are coiled into the so-called triple-helix and stabilized by hydrogen bonds. The chains differ in their amino acid composition and sequence. Each α chain is composed of more than 1000 amino acid residues and has a molecular weight of around 100 kDa, thus for a tropocollagen molecule the total molecular weight is around 300 kDa. Generally collagen molecule has chain composition of two or three non-identical polypeptides chains such as $(\alpha_1)_2(\alpha_2)$ or $\alpha_1 \alpha_2 \alpha_3$. However, the second composition is rarely present and difficult to detect by normal electrophoretic method as it migrates in a similar way to α_1 . The β component is due to the intermolecular crosslinking while that of γ component indicates intramolecular crosslinking (Lewis and Piez, 1964).

Collagen consisting of two α_1 and one α_2 chain as the major component are called Type I collagen (Jongjareonrak *et al.*, 2005). Type V collagen was identified for the first time in fish muscle by Sato *et al.* (1998). Presence of another type of collagen that is Type II collagen with three identical α chain was also reported from scale and bone collagen of carp (Miyauchi and Kimura, 1990). The collagen molecule from cod fish skin also consists of three different chains - α_1 , α_2 , and α_3 (Piez 1965). Presence of chain composition of $(\alpha_1)_2(\alpha_2)$ were reported from the collagen of carp scale (Zhang *et al.*, 2011b), Nile perch (Muyonga *et al.*, 2004a), Baltic cod, grass carp, sardine (Li *et al.*, 2008) etc. Presence of Type I and Type II collagen was also documented by Kimura *et al.* (1991) from the scale and bone of carp which consisted of $(\alpha_1)_2\alpha_2$ as a main component and $\alpha_1\alpha_2\alpha_3$ as a minor component. Also Type I and Type II collagen were reported from brown-banded bamboo shark and black tip sharp (Kittiphattanabawon *et al.*, 2010) and shark cartilage (Rama and Chandrakasan, 1984). The electrophoretic pattern of muscle of leather jacket also showed three distinct of α_1 , α_2 and α_3 subunit and classified as Type V collagen (Muralidharan *et al.*, 2013). It was apparent from the molecular weights obtained for α_1 , and α_2 subunits that the obtained collagen from the refiner is Type I collagen. There are also other fractions of around 103 kDa that appear under the α_2 bands. There is the possibilities of present of two molecular form such as

$(\alpha_1)_2(\alpha_2)$ or $\alpha_1 \alpha_2 \alpha_3$ in the present collagen as muscle form major composition in the refiner discharge (Table 7). Fish muscle collagen were reported to have $\alpha_1 \alpha_2 \alpha_3$ molecular form as major component and $(\alpha_1)_2(\alpha_2)$ as minor component (Sato *et al.*, 1998; Muralidharan *et al.*, 2013). However, further characterization with column chromatography could help to confirm the finding as there are others bands visible in the SDS-PAGE.

5.5. Functional Characteristics of Refiner Discharge Collagen

5.5.1. Water and oil absorption capacities

The water and oil absorption capacities of refiner discharge collagen were 5.76 ml/g and 1.60 ml/g respectively (Table 12). Water absorption capacity is an important protein-water interaction. It is the ability of a protein matrix to absorb and retain water against gravity (Damodaran, 1997). Collagen chains in solution may be covalently cross-linked to form matrices capable of swelling in the presence of aqueous solutions. The values obtained in the present investigation were lower than the collagen extracted from Pacific whiting surimi waste (Kim and Park, 2004). The differences may be due to the different compositions of amino acids. The availability of polar amino groups for hydrogen bonding with water molecules increases water absorption capacity. Water binding capacity of solubilized collagen makes them suitable materials for reducing drip loss and impairing juiciness in frozen fish or meat products when thawed or cooked, and where denatured protein has suffered a partial loss of its water-holding capacity (Gomez-Guillen *et al.*, 2011).

The result of oil absorption capacity of refiner discharge collagen shows that 1 g of collagen could hold 1.6 ml of oil. That means it has low oil absorption capacity even though the amino acids analysis revealed that the refiner discharge collagen had 62.59 residues of hydrophobic amino acids (Table 10). This may be due to the less exposure of side chains as hydrophobic groups are generally buried inside the protein core. The major factors affecting functional properties are protein size, shape, and balance hydrophilic and hydrophobic amino acids, protein denaturation, unfolding and physicochemical environment such as pH, ionic strength and temperature (Sathe and Salunkhe, 1981; Kinsella, 1979).

5.5.2. Emulsion activity and emulsion stability

As collagen finds important applications in food processing industry, emulsifying activity is one of the important functional properties in food processing. Collagen surface properties are based on the presence of charged groups in the protein side chains, and on certain parts of the collagen sequence containing either hydrophilic or hydrophobic amino acids. Both hydrophobic and hydrophilic parts tend to migrate towards surfaces, hence reducing the surface tension of aqueous systems and forming the required identically charged film around the components of the dispersed phase, which can be additionally strengthened by gel formation of proteins (Schrieber and Gareis, 2007). Emulsion capacity and stability depends on the presence of charged groups in the protein side chains, protein concentration and the molecular weight (Dickinson and Lopez, 2001, Aewsiri *et al.*, 2008; Surh *et al.*, 2006).

The collagen from refiner discharge had high emulsifying activity of 50 %. The collagen also had high emulsion stability of 46.15% (Table 12). Similar result were also reported by (Kim and Park, 2004) where the acid-soluble collagen from Pacific whiting surimi refiner discharge was found to present higher emulsifying activity than both acid-soluble collagen from skin and the commercial emulsifier, Tween-80 .

5.5.3. Foaming capacity and foam stability

Foaming capacity is one of the important surface properties of a protein, the other property being emulsifying capacity. The foam must be able to maintain when subject to processing such as heating, mixing and cutting. In food like ice cream, mousses and marshmallow high foam stability is important (Foegeding *et al.*, 2006). The foaming capacity and foaming stability of refiner discharge collagen in the present investigation were 50.55 and 44.79 % respectively (Table 12). In general, proteins exhibiting low foaming capacity have good foam stability and vice versa (Kempka *et al.*, 2015). However, results in the present study shows that both the foaming capacity and foam stability were similar. Presence of higher molecular weight polypeptides (as seen in SDS profile) might help in the formation of stable films around gas bubbles, giving higher foaming property. The present foaming

capacity was higher than gelatin from mackerel bone (38 %) (Khiari *et al.*, 2013). The difference in the result may be due to different composition of amino acids. The Foaming properties of a protein are reported to be influenced by the source, intrinsic properties, the composition and conformations of the protein in solution (Zayas, 1997). The high foam stability of the refiner discharge collagen might be due to presence of balance hydrophilic and hydrophobic amino acids residues in the collagen (Table 12). Foam agent should have amphiphilic property so that at the air-water interface the lipophilic group orient towards the non-polar phase and the hydrophilic group towards the aqueous phase, allowing formation of stable foam (Liceaga-Gesualdo and Li-Chan, 1999).

5.5.4. Solubility

The effects of pH and NaCl on the relative solubility of collagen from refiner discharge are shown in Figure 10 and 11 respectively. From the result, it is revealed that the solubility of the extracted collagen was higher in the pH range of 1-5 with highest at pH 3 (95.75 %). The lowest solubility was observed close in the pH range of 7-10. Similar results were reported from lizard, flying fish and horse mackerel (Minh *et al.*, 2014) and collagen from other fishes (Huang *et al.*, 2011). It is known that, when the pH is lower or higher than isoelectric point, the net negative or positive charge residues of protein molecules increases, thereby increasing the solubility by the repulsive force between chains (Vojdani, 1996; Damodaran, 1997). On the other hand, when the total net charge becomes zero, hydrophobic-hydrophobic interaction increases leading to protein precipitation and aggregation (Jongjareonrak *et al.*, 2005). Foegeding *et al.* (1996) reported that collagen had isoelectric points ranging from pH 6 to 9.

Solubility is the measure of the degree to which a solute is soluble in a solvent. The solubility result of the present study indicates that collagen from refiner discharge is more tolerant to salt. Various authors reported drastic decreased in solubility of fish derived collagen as the NaCl concentration increases (Jeevithan *et al.*, 2014, Minh *et al.*, 2014). Pal *et al.* (2015) reported that collagen from the skins of rohu and catla could achieve relative solubility of 76-100 % upto 0.4 M and below 25 % solubility at 1.4 M NaCl concentration. Collagens from both skin and bone of bigeye snapper were reported to maintain solubility in the presence of NaCl up to 3

%. A marked decrease in solubility was observed with increasing NaCl concentrations above 3 % (Kittiphattanabawon *et al.*, 2005). Thus, collagen from different sources might have different molecular properties, leading to the varied characteristics of solubility. At a high NaCl concentration, the solubility declined by enhancing hydrophobic–hydrophobic interactions and aggregations between protein chains and also competing with the protein for water, leading to protein precipitation (Vojdani, 1996; Damodaran, 1997). The solubility characteristics of collagen with changes in NaCl concentration may play crucial role in understanding its extraction and applications.

5.6. Optimization of Gelatin Yield

Gelatin yield is considered to be one of the most important parameters for the gelatin production industry because of its economic importance. To extract gelatin raw materials were first pretreated with acid to allow the swelling of collagen. Subsequent hot water extraction will hydrolyze and solubilize the gelatin (Nagarajan *et al.*, 2013). So far, the factors that have been shown to affect gelatin extraction efficiency, as well as the functional properties of the resulting gelatin, are mostly related to the pre-treatment and extraction processing steps of the gelatin manufacture process and the different degrees of cross-linkage of the collagen molecules, which appear to be species dependent (Karayannakidis and Zotos, 2016). In the present study the process parameters for gelatin extraction from refiner discharge was optimized using response surface methodology. The concentration of NaOH, extraction temperature and extraction time were taken as independent variables while yield as dependent variable (Table 13). From the optimized response it was found that, the extraction temperature and extraction time were having positive effects on the yield of gelatin from the refiner discharge. The results were in accordance with Arnesen and Gildberg (2007) who reported that gelatin yield increases with increasing extraction time in Atlantic salmon skin. During heating hydrogen bonds that stabilized the triple helix of collagen were destroyed as these hydrogen bonds are heat sensitive. The collapse of hydrogen bonds leads to helix to coil transition, thus converting collagen to soluble gelatin (Benjakul *et al.*, 2009). Hanjabam *et al.* (2015) also reported that extraction time had positive effects on the yield of Unicorn leatherjacket skin gelatin. Swelling of the collagen and its

subsequent solubilization is greatly influenced by the extraction temperature and time (Ahmad *et al.*, 2017). The maximum yield obtained at optimized conditions of 0.34 M NaOH, 63.92 °C and 4.1 h was 16.56 % (Table 16). The yield observed in this study was 16.56 % and is similar to the yield of hoki skin gelatin. Mohtar *et al.* (2010) optimized the gelatin extraction conditions from hoki skin using RSM. However, the yield obtained in the present study is higher than the gelatin from rainbow trout skin. Tabarestani *et al.* (2010) also used RSM to optimize the physicochemical properties of gelatin from rainbow trout skin. Also yields of gelatin may vary mainly due to the differences in collagen content, the compositions of material, degree of denaturation of collagen during extraction etc. (Ninan *et al.*, 2009). Difference in yields have been reported from different sources of fish such as Atlantic cod skin (11-14 %), lizard fish scales (10.60 %), pangasius bone (13.86 %) and blackspotted croaker (17.21 %) (Gudmundsson and Hafsteinsson, 1997; Wangtueai and Noomhorm, 2009; Mahmoodani *et al.*, 2014, Jakhar *et al.*, 2014). Therefore, the yield obtained from the refiner discharge in this study is comparatively at par with the other sources of fish gelatin and can offer scope for alternative source of gelatin extraction. Refiner discharge can have added advantages by omitting the sorting and size reducing stages which are necessary in other materials before extraction process.

5.7. Chemical Composition and Hydroxyproline Content of Refiner Discharge Gelatin

Table 17 represent the proximate composition and hydroxyproline content of refiner discharge gelatin. The gelatin extracted from refiner discharge had 7.09 % moisture and is well below acceptable moisture content for edible gelatin (15%) (GME, 2005). Lower moisture of 4.2 % was obtained in freeze dried gelatin from bigeye snapper (Binsi *et al.*, 2009). Dry gelatin is extremely hygroscopic and under moist ambient conditions it quickly absorbs atmospheric moisture. However, gelatin with a moisture content of around 10 % is in equilibrium with atmospheres of 60 % to 80 % relative humidity and does not gain or loss moisture over storage period (Cole, 2000). The average protein content in the present gelatin was found to be 92.84%. This result is higher in comparison to gelatin reported from skin (72.63%) and bone (69.49 %) of pink perch (Koli *et al.*, 2012); Amur sturgeon skin gelatin (90.4 %) (Nikoo *et al.*, 2013) and Nile tilapia skin gelatin (88.5 %) (Zeng *et al.*, 2010).

However, the present protein content is lower than gelatin from the scales of farmed sea bass (96.0 %) (Dincer *et al.*, 2015). The value for ash contents was 0.84% suggesting EDTA treatment could reduce the ash content. Lower ash content contributes to high quality of gelatin. The pH of the refiner discharge gelatin was 3.69. The lower pH could be due to acid pretreatment by using 0.5 M EDTA for collagen swelling. Wide variations in the pH of gelatin extracted from fishes were reported such as of cod (2.7-3.9) (Gudmundsson and Hafsteinsson 1997), freshwater carps (4.01-4.88) (Ninan *et al.*, 2010) and bigeye snapper (6.44) (Binsi *et al.*, 2009). The pH reported for gelatin from the skin of red tilapia was 3.05 and for black tilapia it was 3.91 (Jamilah and Harvinder, 2002). This was mainly because of the different pretreatments employed during the extraction involving both alkaline and acid treatments.

The hydroxyproline content in the refiner discharge gelatin was 6.53 mg/g gelatin. The present value is lesser than the parent collagen (7.78 mg/g). The difference might be due to variation in protein content of collagen (96.56 %) and gelatin (92.84 %). Hydroxyproline content increase with an increase in protein content (Kittiphattanabawon *et al.*, 2005). The hydroxyproline content of refiner discharge gelatin is lesser than cod skin gelatin, 8.30 mg/g, African catfish gelatin, 8.42 mg/g and rohu skin gelatin, 8.90 mg/g (Gome-Guillen *et al.*, 2002; Alfaro *et al.*, 2014 and Ninan *et al.*, 2011). The hydroxyproline content of fish gelatin is lesser than mammalian gelatin (Eastoe and Leach, 1977). Among the fishes gelatin, warm water fishes have reported to content higher hydroxyproline than cold water fish gelatin (Gudmundsson and Hafsteinsson, 1997).

5.8. Physical Characteristics of Refiner Discharge Gelatin

All the determined physical properties of the extracted gelatin from refiner discharge were tabulated in Table 18.

5.8.1. Colour and gel clarity

Color did not affect functional properties of gelatin (Ockeman and Hansen, 1988). However, both colour and clarity of a gelatin gel are important aesthetic properties. Degree of clarity of a solution is very important in food applications because addition of gelatin should not impart any strong colour attribute to the product. Instrumental colour measurement of gelatin extracted from Pink perch surimi refiner discharge was expressed in terms of L^* , a^* , and b^* (Table 18). The average value of L^* , a^* , and b^* are 68.01, 3.40 and 15.32 respectively. The lightness value in the present study was lower than gelatin extracted from ribbon fish surimi processing waste (Norziah *et al.*, 2014). The color of gelatin depends on the source of raw materials, gelatin processing method and number of extractions performed (Poppe, 1997). The extracted gelatin showed good transmittance (61.26 %) showing that the extraction method employed was efficient to remove much of inorganic contaminants. The skin and bone gelatin of pink perch were reported to have poor percentage transmittance of 44.30 and 40.13 respectively (Koli *et al.*, 2012). The turbidity and dark colour of gelatin is commonly caused by inorganic, protein and muco-substance contaminants, introduced or not removed during its extraction (Eastoe and Leach, 1977). Gelatin colour has no correlation with molecular composition however as the collagen subunits decreases due to ageing the colour become darker (Cole and Roberts 1996). A stable cross-link collagen gives pale colour gelatin.

5.8.2. Bloom value

Bloom value is the most significant physical properties of gelatin. It determines the quality of produced gelatin. The average bloom value of the refiner discharge gelatin was 147.97g. The application of gelatin is determined by the range of bloom values. The bloom value found in the present study is low when compared to that of gelatin from the skins of warm water fish such as grass carp (267 g) (Kasankala *et al.*, 2007), tilapia (328 g) (Songchotikunpan *et al.*, 2008). Bloom value of fish gelatin ranges from as low as zero to 426 g, compared to 200–300 g for

bovine or porcine gelatin (Karim and Bhat, 2009). But the result obtained is higher than the values obtained from cold-water fish species such as salmon (108 g) (Arnesen and Gildberg, 2007), cod 70–90 g (Gomez-Guillen *et al.*, 2002), rohu (124 g) (Shyni *et al.*, 2014). Bloom value not only depends on average molecular weight of gelatin but also is dependent on other factors such as the chemical treatment of raw collagen material, type and concentration of the gelatin and the time/temperature history of the sample (Babin and Dickinson, 2001; Kolodziejska *et al.*, 2004). Bloom value also depends on the hydrogen bonds between water molecules and free hydroxyl groups of amino acids in gelatin (Arnesen and Gildberg, 2002). Gelatin is compatible with a wide variety of foods and ingredients. Gelatin of different bloom strength from the same source can be produced by merely varying the extraction method (Jamilah *et al.*, 2011). Gelatin is sold with a wide range of special properties such as bloom value and viscosity to suit particular applications. Usually, the gel strength of commercial gelatins ranges from 100-300 g, but gelatins with gel strength of 250-260 g are the most desirable and suitable for a wide range of applications in the food industry especially in the processing of jellies, canned meat, marshmallows and yoghurts (Holzer, 1996). The present gelatin extracted from surimi refiner discharge with lower bloom strength as compared to commercial bovine gelatin may find application in microencapsulation, light sensitive coatings, low-set time glues, as shampoo ingredient. Fish gelatin with low bloom value and low melting point can accelerate the flavour release than high bloom value gelatin because of its melt-in-mouth property (Rustard, 2003; Zhou and Regenstein, 2007).

5.8.3. Viscosity

Viscosity is the second most important rheological properties of gelatin solutions after bloom value (Ward and Courts, 1977). The viscosity of refiner discharge gelatin was 7.44 Cp. The viscosity of commercial gelatin ranges from 1.5 cP to 7 cP (Johnston-Banks, 1990). The higher value of the viscosity found in the present gelatin may be due to intact high molecular weight β and γ chains in the gelatin as seen in the SDS- profile of the gelatin (Figure 15). Niu *et al.* (2013) observed a strong correlation between molecular weight distribution and viscosity of gelatin. pH is known to influence the viscosity and minimum viscosity for gelatin has been reported in the pH range of 6-8 (Stainsby, 1987). In the present study the pH of

the refiner discharge was found to be 3.69 and this might enhance the viscosity. This is supported by Jamilah and Harvinder (2002), who stated that the effect of pH on viscosity is minimum at the isoionic point of gelatin and maximum at pH 3 and 10.5. The viscosities of Pink perch skin and bone gelatin are 8.47cP and 6.80 cP respectively (Koli *et al.*, 2012). Jamilah and Harvinder (2002) reported the viscosity values of 3.2 cP and 7.12 cP for red and black tilapia skin gelatin, respectively, whereas for channel catfish skin gelatin, it was 3.23 cP (Yang *et al.*, 2007). Higher viscosity of gelatin were reported from grouper bone gelatin and red snapper bone gelatin of 18.5 cP and 15.30 cP respectively (Shakila *et al.*, 2012b).

5.8.4. Melting point

The melting point of extracted gelatin was 25.50 °C. The present gelatin exhibit higher gel melting point than the results of Unicorn leatherjacket skin (22.61 °C) (Hanjabam *et al.*, 2015) and other gelatin extracted from cold water fishes (Gudmundsson and Hafsteinsson, 1997; Gomez-Guillen *et al.*, 2002). The melting point of gelatin is the point at which the gelatin gel starts melting when the temperature increases above a certain point. Typical melting points for fish gelatins range from 11 to 28 °C while for porcine and bovine gelatins, the melting points range from 20 to 25 and 28 to 31°C, respectively (Karim and Bhat, 2009). Viscosity is partially controlled by molecular weight and molecular size distribution (Sperling, 1985). Also fish gelatin with low bloom value and low melting point can accelerate the flavour release than high bloom value and higher melting point gelatin (Zhou and Regenstien, 2007).

5.8.5. Gel setting point and setting time

Gel setting point is defined by the temperature at which gelling process begins by transforming from random coil to triple helical structure of gelatin (Haug *et al.*, 2004). Gelatin solution from refiner discharge gelled at 18.50 °C. Gelatins from skins of salted and marinated herrings formed a gel at 5 °C and from cod skins at about 5.5 °C. According to Gomez-Guillen *et al.* (2002) gelling of cod gelatin occurred at 12-13 °C. Gelling temperature depends on the imino acid composition, ionic strength and pH of the gelatin. The imino acids were found to stabilize the ordered conformation when gelatin forms the gel network during gelling and with the

increase in ionic strength of >0.5 mol/l decreased gelling temperature, which was probably due to the reduced electrostatic interaction preventing attractive ionic inter-chain bridging and gelation of fish gelatin (Muyonga *et al.*, 2004b and Haug *et al.*, 2004).

The Setting time is defined as a time required for a gelatin solution to become a gel (Janus *et al.*, 1965). Setting time required for extracted gelatin was 120.66 seconds. Similarly, Ninan *et al.* (2011) also observed higher gelling times in rohu (106 s) and common carp (103 s) skin gelatins. The difference may be due to different source of gelatin. The setting time of gelatin gel is a function of bloom value and intrinsic viscosity (Janus *et al.*, 1965). According to Sinthusamran *et al.* (2015) setting time is also affected by size of fish used as raw material for gelatin extraction.

5.8.6. Thermal stability

DSC measures the amount of heat energy absorbed or released by a sample, as it is heated, cooled or held at a constant temperature. Figure 13 shows the DSC heating thermographs of gelatin obtained from pink perch surimi refiner discharge. There is a broad endothermic peak at 77.20 °C having transition enthalpy (ΔH) of 277.72 J/g. Besides this endothermic peak, there is no evidence of other thermal events. The maximum of the endothermic temperature peak in DSC thermogram is taken as the melting/transition temperature of the gelatin. The transition temperature found in the present study is lower than tilapia skins gelatin which was reported as 191.3 °C by Hani *et al.* (2016) and 210 °C by Chen *et al.* (2015). This transition is due to structural relaxation or other structural changes of macromolecules such as melting of crystalline structure of gelatin. The gelatin behaves as a molecularly dispersed molten plastic due to disruption of the crystalline region (Sobral and Habitante, 2001). Rahman *et al.* (2008) reported that the thermal transitions of gelatin differ with different sources and moisture content or water activity of the gelatin. The heat flow detected by DSC corresponds to the energy necessary to melt the junction zones (Cheow *et al.*, 2007). Gelatin solution with water shows lower transition temperature as water enhanced the mobility of gelatin polymer chains (Norziah *et al.*, 2009; Uriarte-Montoya *et al.*, 2011).

5.9. Chemical characteristics of refiner discharge gelatin

5.9.1. Amino acids composition

The amino acids profile of extracted gelatin is given in Table 19. The extracted gelatin is rich in glycine (33.78 %) followed by glutamic acid (11.83 %), proline (9.76 %) and alanine (7.08 %). There are differences in the amino acids composition of refiner discharge collagen and gelatin (Table 10). The changes in the amino acids compositions may be due to different isolation process and protein recovery. During acid extraction of gelatin some of the side-chain amides like glutamine and Asparagine gets converted to their respective acidic forms (Jamilah and Harvinder, 2002). In general glycine is the most predominant amino acid in any gelatin as glycine occurs every third position in the chain and represents nearly one third of total amino acids residues (Benjakul *et al.*, 2009). However, glycine content in the extracted gelatin was slightly lowered than Shark skin, rohu skin and tuna skin gelatin (Shyni *et al.*, 2014). Proline and hydroxyproline are important for rigidity and stability of the triple helix of collagen which affects the functionality of the gelatin (Gomez-Guillen *et al.*, 2002). Among the essential amino acids phenylalanine was found at highest amount (3.08 residues/100 residues) and isoleucine (0.70 residues/100 residues) at least. The present gelatin is also a good source of essential amino acids called lysine (2.46 residues/100 residues). Lysine is absent or found in low proportions in most plant foods. The proportion of hydrophobic amino acid in the collagen might affect the physical properties of the gelatin. Badii and Howell, 2006 reported that the higher gel strength found in tilapia and horse mackerel gelatin when compared with non-gelling cod gelatin was due to higher amounts of hydrophobic amino acids in the former gelatin. However, the above relation between total hydrophobic amino acid and gel strength was not established in work reported by Nikoo *et al.* (2013).

5.9.2. Structural profile

FTIR spectroscopy has been used to study the secondary structure and functional groups of gelatin from refiner discharge (Table 20 and Figure 14). FTIR spectroscopy is based on the vibrational excitation of molecular bonds by absorption of infrared light energy. The sum of vibrational spectra for a cell macromolecule (nucleic acids, proteins, lipids, polysaccharides, etc.) can produce an infrared absorption spectrum that looks like a molecular “fingerprint” for such biological material. This spectrum can be used in typing or identification applications through clustering (Essendoubi *et al.*, 2007). Proteins are comprised of amino acids joined together by amide bonds. Amide bands represent different vibrational modes of the peptide bond. Amide bands including Amide A, Amide I, Amide II and Amide III are related to the degree of molecular order and involved with the triple helical structure of collagen (Nikoo *et al.*, 2014). Amide-I represents C=O stretching/hydrogen bonding couple with COO, Amide-II represents bending vibration of N-H groups and stretching vibrations of C-N groups, Amide-III is related to the vibrations in plane of C-N and N-H groups of bound amide (Muyonga *et al.*, 2004c; Benjakul *et al.*, 2009, Sai-Ut *et al.*, 2012).

The corresponding wavenumbers (cm^{-1}) of the characteristics infrared bands of refiner discharge gelatin is given in Table 20. The Amide I band of the gelatin from refiner discharge was found at $1654.47\text{ (cm}^{-1}\text{)}$ which is in agreement with data reported by Zarai *et al.*, (2012) of gelatin from marine snail (*Hexaplex trunculus*) and Liu *et al.* (2009) of gelatin from Channel catfish (*Ictalurus punctatus*) heads and bones. However, Amide I band of gelatin extracted from the skin of giant squid (*Dosidicus gigas*) was observed at 1635 cm^{-1} (Uriarte-Montoya *et al.*, 2011). The differences in the bands may be due to the fact that the amide I band shows several components or shoulders due to mixed secondary structures of protein. The extent of these changes also influenced by the gelatin extraction conditions, the number of native crosslinks in the collagen structure, and the amount of collagenous tissues from which gelatin is extracted (Muyonga *et al.*, 2004b; Benjakul *et al.*, 2009; Uriarte-Montoya *et al.*, 2011). Nagarajan *et al.* (2012) reported that secondary structure and functional group of gelatins obtained from the skin of splendid squid was affected by extraction temperature. Amide I absorption band gives the information of secondary

structure of proteins (Surewicz and Mantsch, 1988; Yakimets *et al.*, 2005; Liu *et al.*, 2009). The exact absorption peak of the Amide I depends on the hydrogen bonding and conformation of the protein structure (Uriarte-Montoya *et al.*, 2011).

The gelatin obtained from refiner discharge exhibited the amide II bands at the wavenumbers of 1578.53 cm^{-1} . The result was in accordance with the report of Kittiphattanabawon *et al.* (2016) and Kaewruang *et al.* (2013). The amide III band of the gelatin samples was observed at wavenumbers of 1232.79 cm^{-1} . The Amide III helps in identifying the helix to coil structure of gelatin. So Amide III generally indicates disorder in the gelatin molecules due to loss of triple helix state of collagen. Gelatin is the partially hydrolyzed form of collagen (Friess and Lee, 1996). The amide III band represents the combination peaks between CN stretching vibrations and NH deformation from the amide linkages as well as the absorptions arising from wagging vibrations of CH_2 groups in the glycine backbone and proline side-chains (Jackson *et al.*, 1995; Sinthusamran *et al.*, 2014).

Generally Amide A bands which represent free N-H stretching vibration occurs in the range of $3400\text{--}3440\text{ cm}^{-1}$. The Amide A band of gelatin obtained from refiner discharge was seen in 3315.95 cm^{-1} . This shift to the lower frequency may be due to degradation of gelatin and involvement of short peptide fragments in hydrogen bonding with other reactive groups (Benjakul *et al.*, 2009, Ahmad *et al.*, 2012). Similar shifting of Amide A region into lower wavenumber was reported by Nikoo *et al.* (2014) for Amur sturgeon (*Acipenser schrenckii*) skin gelatin extracted with different acid concentration. Lower wavenumbers of the amide A band ($3289\text{--}3304\text{ cm}^{-1}$) of gelatin from seabass skin with different extraction conditions was also reported (Sinthusamran *et al.*, 2014). The primary structure (α -chain), secondary structures (triple-helical structure) and functional group of gelatins were affected by extraction conditions (Kittiphattanabawon *et al.*, 2016).

5.9.3. Protein pattern

The gelatin extracted was analyzed using SDS-PAGE (Figure 15). The gelatin has high intensity of β - and α -chains (α_1 and α_2) as the major components though a faint γ - chain is also visible. Bands of low peptides were not noticeable. Some authors have observed lower molecular weight fractions of gelatin, particularly due to cleavages of protein chains at high temperature extraction process (Muyonga *et al.*, 2004b; Shyni *et al.*, 2014). According to Gomez-Guillen *et al.*, (2002), damage or partial losses of α chains occur due to the heat extraction and gel preparation resulting in the lower molecular weight fractions.

5.9.4. Mineral composition

The mineral composition of refiner discharge gelatin (ppm) is given in Table 21. Among the mineral analyzed calcium was present in high amount (1577 ppm) followed by sodium (708 ppm), magnesium (94.23) and potassium (72 ppm). The mineral content found in the present gelatin was lower than the gelatin reported by Benjakul *et al.*, (2009). This may be due to the demineralizing effect of EDTA, which was used as pretreatment agent in extraction process. The ash content of the refiner discharge was 10.61 % as it contained scale at 29.70 % and bone at 7.90 % (Table 7). This have contributed to the mineral content of gelatin as the minerals could be leached out into the extraction medium during gelatin extraction to varying degrees. Gelatin extracted from the skins of bigeye snapper were reported to be rich in minerals like calcium, sodium, potassium, and magnesium and phosphorous (Benjakul *et al.*, 2009). The available minerals in the gelatin could give added advantage in enhancing the functional properties or could be of health benefits. Koli *et al.* (2011) has used MgSO_4 as co-enhancers for improving the gel strength of fish gelatin.

5.10. Functional characteristics of refiner discharge gelatin

All the determined functional properties of the extracted gelatin were tabulated in Table 22.

5.10.1. Water and oil absorption capacities

Water and oil absorption capacities are important functional properties. They are related to the ability of protein to imbibe water, oil and retain it against a gravitational force within protein matrix. The water and oil absorption capacities of refiner discharge gelatin were found to be 2.20 ml/g and 3.06 ml/g respectively. These values were different from the parent collagen. This may be due to different composition of amino acids in the collagen and gelatin extracted from refiner discharge. The water absorption capacity of present gelatin is lower than croaker skin (4.50 ml/g) and bone (3.00 ml/g) gelatin but higher than bone gelatin (1.50 ml/g) of pink perch (Koli *et al.*, 2012). Fat binding capacity is related to the degree of exposure of the hydrophobic residues and the high amount of tyrosine in the gelatin (Ninan *et al.* 2011). The tyrosine content in the gelatin (6.77 residues/100 residues) was more than the parent collagen (5.71 residues/100 residues) from refiner discharge. This might be the cause of higher oil absorption capacity of gelatin than collagen (1.6 ml/g). Also due to partial hydrolysis of collagen the embedded hydrophobic amino groups might get exposed to the surface, thus enhancing the oil absorption capacity of gelatin.

5.10.2. Emulsion activity and Emulsion stability

Emulsifiers are surface active materials that adsorb to interfaces and facilitate the production of small droplets by lowering the interfacial tension during homogenization (Walstra, 2003). Proteins are widely used as emulsifiers in food products because of their ability to improve the stability of oil-in-water emulsions (McClements, 2004). Gelatin is surface-active and is capable of acting as an emulsifier in oil-in-water emulsions (Aewsiri *et al.*, 2009). The characteristics used to describe emulsifying properties of protein are emulsion capacity and emulsion stability. Emulsifying capacity and emulsion stability of refiner discharge gelatin were 50.77% and 50.44% respectively. The present result shows that the gelatin had strong emulsion stability. According to Gimenez *et al.* (2009), a higher content of

hydrophobic amino acid residues results in an effective distribution of hydrophilic/hydrophobic amino acids which leads to an improvement of the emulsifying properties of gelatines. Hence the higher proportions of hydrophobic amino residues (59.65 residues/100 residues) than hydrophilic amino groups (40.34 residues/100 residues) in the gelatin may have resulted better emulsion stability.

The emulsifying capacity and emulsion stability found in the present study was higher than the gelatin from skin and bone of pink perch gelatin (Koli *et al.*, 2012). Surh *et al.* (2006) found that the oil-in-water emulsion prepared with high molecular weight fish gelatin (~120 kDa) was more stable than that prepared with low molecular weight fish gelatin (~50 kDa). Also EC and ES depend on the properties of protein, its concentration, pH, ionic strength and viscosity. Synthetic emulsifiers are being replaced with natural emulsifier in the food industry due to consumer likeness towards the latter one. Such gelatin may find place in food industry. Proteins from natural sources can be used as emulsifiers in foods because of their ability to facilitate the formation, improve the stability, and produce desirable physico-chemical properties (Dickinson, 2003; McClements, 2004).

5.10.3. Foaming capacity and foaming stability

Foaming capacity is one of the functional properties that determine the application of gelatin as products like marsh-mallows required high foaming agent (Zuniga and Aguilera, 2009). The foaming ability of proteins is related to their film-forming ability at the air-water interface. The foam capacity of gelatin extracted from refiner discharge was 38.45 %. Like other functional properties, foam capacity and foam stability also depends on the amino acids compositions of the protein. Higher amount of hydrophobic amino acids residues gives higher foam capacity (Khiari *et al.*, 2013). A protein must be capable of migrating rapidly to the air-water interface, unfolding and rearranging at the interface to express good foaming properties (Halling, 1981).The foaming stability of gelatin was found to be 24.80 % after 30 min. Mutilangi *et al.* 1996 reported that gelatin foam stability depends on the nature of the film and the extent of protein interaction within the matrix. Higher content of negatively charged amino acids prevented the neutralisation of charge in gelatine molecules and enhanced the foam stability (Khiari *et al.*, 2013).

5.11. Viscosity of film forming solution of refiner discharge gelatin

Table 23 shows the viscosity of refiner discharge gelatin film forming solution. The rheological property of film forming solutions affects spreadability, thickness and film performance (Garcia *et al.*, 2009). The result shows that the concentrations of gelatin of the film forming solutions influenced solution viscosity. Viscosity of the film forming solution increases with increasing gelatin concentrations. This is due to the higher energy required to translate or rotate the molecules in solution. However, in a given solutions, viscosity is affected by several factors. Viscosity is partially controlled by molecular weight and molecular size distribution (Sperling, 1985). Also viscosity of the film forming solution is depending on the pH of the solution and the protein content. (Cho *et al.*, 2006). In this study the pH of the film forming solution was kept constant. The viscosity of the film forming solutions is an important parameter as it affects the film formation and drying process. Film forming solution with low viscosity is preferred. A lower viscous solution accelerates coacervation of the film forming solution from the film casting surface causing uneven coating to the surface. Higher viscosity reduces the phase separation between film forming solution and the surface and makes the peeling process of the film easy. However, highly viscous film forming solution may create uncontrollable film thickness.

5.12. Characterization of films from refiner discharge gelatin

Films were able to form with all the concentration of gelatin in the film forming solutions. The films were easy to peel from the plate and have smooth surface appearance. However, film formed from 2% gelatin was tacky and shrink easily after peeling off from the plate.

5.12.1. Color

Among the important optical properties of the films for use in packaging, color is an important factor to evaluate the quality of the films. Color is an important parameter in the characterization of the films; it is related to the raw material used to make the products (da Rocha *et al.*, 2013). Higher L* (87.92 to 88.94) values were observed in all the films and there were no significant differences among the gelatin concentrations. The a* values in all the films were negative indicating a shift of colour

towards greenness. The films shows slight yellowness as indicated by the negative values of the b^* (Table 24). Similar results were reported for commercial tilapia skin, pork skin and beef skin gelatin films where, L^* values are above 90.00 with negative a^* and positive b^* values. (Nur Hanani *et al.*, 2012). The colour of gelatin films is affected by type of gelatin used for films and concentrations of the gelatin (Nur Hanani *et al.*, 2012).

5.12.2. Mechanical properties

The mechanical properties of refiner discharge gelatin film at different gelatin concentrations were expressed in terms of tensile strength (TS) and elongation at break (EAB) (Table 25).

TS of the film increased with increasing film concentration, but the increase was not significant. Similar observation was also found by Kaewprachu *et al.* (2016) in myofibrillar protein based film. The result was also in accordance with those reported on gelatin films (Nur Hanani *et al.*, 2012) in which there were no significant different in TS when the concentrations of 4 % and 6 % were used in film forming solutions. However, at 8 % there was significant difference in TS from 4 % and 6 % films. In general, TS of a film increased with increasing protein concentration because as the protein increases the chances of number of potential intermolecular interactions also increases (Cuq *et al.*, 1996). TS of the films also depend on the gel strength of the original gelatin. Ninan *et al.* (2010) compared the films properties of carp skin gelatin with porcine skin gelatin and bovine skin gelatin and found that the higher the gel strength of the gelatin source, the better will be the TS.

There was a significant difference between EAB of refiner discharge gelatin films at different gelatin concentrations. EAB increases from 129.1 % to 203.6 % with increasing gelatin concentration from 3 to 6 % in the film forming solutions. The result was in accordance with those reported by Jongjareonrak *et al.*, (2006) in which the EAB increased with increasing protein concentrations. Usually, the higher the polymer content in the film forming solution the higher the resistance of the films (Ku *et al.*, 2008). Another possibility for higher EAB is that higher protein content might result in a higher intermolecular aggregation of protein, compared with the lower amount, resulting in improve flexibility of the films (Kaewprachu *et al.*, 2016).

However, the intended applications of the films will be the deciding factor for optimum values of TS and EAB of the films. Because film with high extensibility is required for food wrap application and high TS and low EAB values film may required to tolerate normal stress during transport or stress during handling to maintain its integrity (Kaewprachu *et al.*, 2016).

5.12.3. Swelling index

Swelling index indicates the amount of water or liquid that can be absorbed by film. The result reported in Table 26 indicates that swelling reduces on increasing the protein content of the films. Generally, biopolymer films produced from carbohydrates or proteins initially swell when they absorb water, which results in changes of their structure. Hence, details relevant to swelling characteristics are essential for successful application of biopolymer films.

Further increase in the amount of gelatin beyond 4 % leads to significant decrease in swelling index of the film. Swelling took place following a chain relaxation mechanism. During the swelling process, as the solvent moves inside the gel phase, the network chains become increasingly mobile. As a result, the probability that one network chain meets another chain increases as the gel swells, so that the rate of crosslinking reactions increases (Durmaz *et al.*, 2002). Swelling of gelatin in water or salt solutions is an osmotic phenomenon, and that gelatin consists of a network of an insoluble substance enclosing a solution of a soluble constituent (Northrop and Kunitz, 1926).

5.13.3. Determination of Solubility in water

Films developed from refiner discharge gelatin have good solubility in water (Table 26). The solubility was similar to the film made with cod-skin gelatin which has water solubility values as high as 90–100% (Perez-Mateos *et al.*, 2009). Other work with tuna skin gelatin film has reported to have water solubility of 39.9 % (Gomez-Estaca *et al.*, 2009) compared with values of around 34.3 % for bovine gelatin film. Solubility in water is an important property of edible films. Water solubility is the measure of tolerance to water, and higher solubility of a film indicates lower water resistance (Handa *et al.*, 1999). Potential applications may require water insolubility to enhance product integrity and water resistance. However, in some

cases film water solubility before consumption of the product might be beneficial (Perez-Gago *et al.*, 1999). Films with high solubility may be beneficial for using as edible wrapper for which they can easily dissolved in the mouth (Sothornvit and Krochta, 2000). The solubility ability of the film depends on presence of very high molecular weight fractions and also the remain of heat stable crosslinked fractions after drying of the film (de Carvalho *et al.*, 2008; Gomez-Estaca *et al.*, 2009).

The solubility of a hydrophilic polymer involves the penetration of water to the polymer bulk and swelling. This is followed by disruption of hydrogen and Van der Waals forces between polymer chains. Chemical degradation leading to the creation of oligomers and monomers occurs via hydrolysis or enzyme-catalysed hydrolysis (Turhan and Sahbaz, 2004). Increase in gelatin concentration from 4% to 6 % significantly ($P < 0.05$) increased the solubility of beef-derived gelatin films, but showed no significant differences when the concentration increased from 6% to 8%. The water solubility of the films is greatly influence by the plasticizer used in the film forming solution. Glycerol helps in increasing the solubility of the films due to its hydrophilic property (Vieria *et al.*, 2011). The amount of heat treatment of the film forming solution also affects the solubility of the film. The increase in heat treatment leads to hydrolysis of monomers and peptides of low molecular weight materials resulting in more solubility (da Rocha *et al.*, 2013).

5.13. Effect of pulsed light treatment on the properties of refiner discharge gelatin films

5.13.1. Mechanical properties

The mechanical properties of the films (TS and EAB) were significantly affected by the pulsed light treatment (Figure 16). TS values increased from 66.69 kg cm⁻² for control film to 135.81 kg cm⁻² for films receiving 1min duration of pulsed light treatment. This increase of film TS over the range of pulsed light duration might be due to cross-linking effects of the proteins by the pulsed light. In contrast to TS, EAB of film decreased ($P < 0.05$) with increasing pulsed light treatment durations. The decrease in EAB while increasing TS was reported to be due to formation of a denser and tighter film structure (Gennadios *et al.*, 1998). The formation of cross links in the protein matrices makes the films stronger but less extendible. Similar findings were

also reported in heat cured soy protein films (Gennadios *et al.*, 1996). The higher cross linking effect found in the study could also be due to presence of higher amount of tyrosine and phenylalanine in the refiner discharge gelatin as aromatic amino acids can absorb UV radiation and recombine to form covalent cross-links in proteins (Fujimori, 1965; Forbes and Sullivan, 1966; Tomihata *et al.*, 1992).

5.13.2. Protein pattern

SDS-PAGE technique was used to detect any changes in the protein profiles of the gelatin films due to pulsed light treatment by separating those protein samples into different bands (Figure 17). Prominent bands of β and α chain are visible in control and 15 sec PL treated gelatin films. The intensity of the bands decreases with the PL treatment time in gelatin films. Similar observations were made in UV treated tilapia gelatin where intensity of high molecular weight proteins (α -, β -and γ -chains) appeared slightly weaker with increasing radiation doses (Otoni *et al.*, 2012). It may be due to crosslinking via non-disulphide covalent bond as cysteine was absent in the gelatin. There is visible formation of higher molecular weight aggregates at the top of the gel too. Pulsed light can cause intra- or inter-molecular cross-linkage, or fragmentation or denaturation of protein (Shriver *et al.*, 2011). Due to cross-linking molecular weight increases leading to constrained in protein mobility. The same phenomena were also reported by Norziah *et al.* (2009) when transglutaminase at more than 0.5mg/g induced cross linking in fish gelatin. Those cross-linked proteins that are much larger than the native proteins can be held on top of the gel, which means that they cannot run down the gel during gel electrophoresis. On the other hand fragmented protein sections that are smaller than the limit of the SDS-PAGE gel may travel through the acrylamide pores more quickly than other protein molecules. Gutierrez *et al.* (2017) reported dual phenomenon of pulsed light treatment on starch films. It produced starch retrogradation in higher amylose content starch films but on the other hand created crosslinks between the starch chains in less amylose content starch films. Few studies reported the underlying principles of mechanisms of cross-linking in gelatin films by radiation. One of the hypotheses is by that of Rhim *et al.* (1999). Proteins undergo conformational changes with radiation treatment. UV radiation is absorbed by double bonds and aromatic rings, which causes free radical formation in amino acyls (such as tyrosine

and phenylalanine). These instable products can react together and lead to the formation of intermolecular covalent bonds, which can further enhance physical properties of protein films (Rhim *et al.*, 1999). Benbettaieb *et al.* (2016) also reported the generation of free radicals in the form of unpaired electrons due to electron beam irradiation in fish gelatin film.

5.13.3. Swelling index and solubility

A known mass of film sample were immersed in water for 2 min to study the effect of PL on swelling index of the gelatin films. Water diffuses into the network chains of the films, thus causing the film to swell. The swelling index of the films decreases significantly from 463 to 159 with increased in pulsed light treatment from 0 to 3 min (Figure 18). The high swelling index of the control film might be due to the availability of active hydroxyl groups on the surface of the film. Gelatin is a highly hygroscopic molecule and could absorb water quickly. The decrease in swelling index of the films with PL treatment shows that crosslinking effect of PL may influence the swelling ability of the films. Cross-linking reduces the interaction between water molecules and the intermolecular hydrophilic functional groups of gelatin molecules, leading to less swelling index. Tiwary and Rana (2010) had reported that cross-linking has strong impact on swelling capability of chitosan film. Swelling index reduces with higher cross-linking density in the chitosan film.

Solubility of films in water is a gel property which demonstrates tolerance to water. During film formation there involves the transformation of film forming solution into a gel-like state. In general, films with more tolerance to water are required, however, for some food products like premeasured dry food, packaging films water solubility before consumption might be beneficial (Perez-Gago and Krochta, 2001; Yin *et al.*, 2007; Aguirre *et al.*, 2013). The films treated with PL showed lower solubility in water than the untreated film. The untreated film showed higher solubility of 93.5 %. The bloom value of the gelatin influenced the film solubility. With higher bloom values of the gelatin, a decrease in the solubility of the film matrix in water occurred (Kempka *et al.*, 2014). The treated film showed 70.27 %, 54.7 %, 48.52 %, 38.73 %, 35.91 % and 35.15 % solubility when treated with PL for 15 sec, 30 sec, 45 sec, 1 min, 2 min and 3 min respectively (Figure 19). Reduction in the solubility of protein films might be due to crosslinking effect of PL. Cross-linking of

the proteins has been reported to decrease the films solubility. In a study reported by Ustunol and Mert (2004), it was found that unlike other chemical cross-linkers, UV treatment do not enhance the tolerance of whey protein-isolate films in water. Thus, the results showed that the crosslinking effect of PL is more effective than UV, considering major component in PL is UV light. Film solubility is affected by the degree and nature of protein-protein interactions (Perez-Gago and Krochta, 2001).

5.13.4. Structural characteristics

FTIR spectra measurements were performed to examine the changes on secondary structure content of gelatin films as affected by the PL treatments (Figure 20). Generally, protein structural units has a characteristics set of absorption regions known as amide modes in the infrared spectrum. These amide modes namely Amide A, B and Amide I-VII, arise from the amide bonds that linked the amino acids. Among them, Amide I and Amide II bands represent the vibrational bands of the protein backbone and are sensitive to secondary structure of a protein. The absorption associated with the Amide I ($1600\text{-}1700\text{ cm}^{-1}$) represent the stretching vibrations of the C=O bond of the amide, while absorption associated with the Amide II bands represent bending vibrations of the N-H bond. However, Amide II band has been reported as not as good as Amide I band for predicting secondary structure of proteins. Thus Amide I ($1600\text{-}1700\text{ cm}^{-1}$) is the most useful band for investigating the secondary structure of protein. In particular the Amide I band consists of α helix ($1650\text{-}1660\text{ cm}^{-1}$), β sheet which contains intermolecular β sheet of protein aggregation ($1605\text{-}1615\text{ cm}^{-1}$), intramolecular β sheet ($1615\text{-}1640\text{ cm}^{-1}$), anti-parallel β sheet ($1690\text{-}1700\text{ cm}^{-1}$), β -turn ($1675\text{-}1690\text{ cm}^{-1}$) and random coil ($1640\text{-}1650\text{ cm}^{-1}$) (Haris and Severcan, 1999; Severcan *et al.*, 2004; Liao *et al.*, 2013). Figure 20 shows the FTIR spectra of control and PL treated refiner discharge gelatin films in the $2000\text{-}400\text{ cm}^{-1}$ region. Variations in the peak positions were observed in the control film and PL treated films. Siddique *et al.* (2016) reported that PL treatment induce partial unfolding of protein structure while forming aggregates. Irradiation treatments to the polymers are known to produce better crosslinked but might also cause degradation of polymers (Kume *et al.*, 2002). The degradation effects of PL treatment might depends on the type of polymer and the dose of PL. In this study PL treatment upto 3 min did not cause major degradation to the films. In another study

conducted by Bhat and Karim, (2014) it was found that the UV irradiation does not cause degradation of fish gelatin films. However, edible films made from cassava and taro starch treated with PL suggest the modification of films by crosslinking. But the photo-degradation reaction was found to be more dominated than the photo-polymerization reaction (crosslinking) (Gutierrez and Gonzalez, 2016).

5.13.5 Thermal stability

Thermal properties of the pulsed light (PL) treated films were studied using DSC (Figure 21). The transition temperatures and respective enthalpies are given in Table 27. Two major transitions were found in the films. Similar transitions were also reported from fish gelatin film incorporated with seaweed extract (Rattaya *et al.*, 2009). Transition temperatures of all gelatin films were in the range of 46.64°C-60.96 °C and 114.11 °C-136.77 °C for first transition (T_1) and second transition (T_2) respectively. Transition temperature indicates the destruction of protein network due to disassociation of intramolecular bonds formed during films preparation. The first small transition peaks was taken as transition due to the breakdown of gelatin-glycerol complex, formed in the film matrix. Of the two endothermic peaks, the second peak is of interest. The second transition was postulated to be related with the glass transition of film matrix. Since the gelatin film was dried at temperature above gelation temperature, it is assumed that there is no helix-coil transition of gelatin in the film. Studies of gelatin films have revealed that in gelatin films prepared by evaporating the solvent at temperature above 35 °C, the gelatin macromolecules have mainly coiled structure (Kozlov, 1983). ΔH or the area under endothermic peak represents the energy used for breaking those bonding. In the second transition, initially the transition temperature decreases and afterwards increases at higher PL treatment time. However, the enthalpy of transition was found to be higher after PL treatment in the film. This may be due to the effective cross linking by pulsed light. Similar finding were reported by Gutierrez *et al.* (2017) where cassava starch films treated with pulsed light have higher enthalpy (ΔH) values associated with glass transition as PL favours intermolecular interactions between the starch chains. The decrease enthalpy may be due to a decrease in the inter- and intra-molecular interactions. Cross-linking induced by pulsed light might results in lower molecular mobility thereby increasing the polypeptides chain rigidity. The increase in transition

enthalpy may be also due to higher intensity of both inter- and intramolecular interactions, including hindrance to internal rotation along the macromolecular chain (Rattaya *et al.*, 2009). A reduction in the number of hydrogen bonds with a simultaneous increase in the extent of covalent cross-linking resulted in the increase in thermal stability (de Carvalho and Grosso, 2004). However, there are chances of protein fragmentation caused by photo-degradation enabling the protein chains to become more mobile in addition to cross-linking by pulse light treatment.

5.14. Conclusion

Byproducts like refiner discharge have added advantage of avoiding the segregation problem of solid waste into skins, scale and bones before the extraction of gelatin. Surimi refiner discharge contained Type 1 collagen. RSM can be successfully employed to optimise the extraction conditions of gelatin. Gelatin extraction process was significantly influenced by extraction temperature and time. Refiner discharge gelatin has high L* value. Gelatin films with high solubility and higher EAB may be beneficial as edible wrappers. Pulsed light treatment induces changes in conformational and functional properties of the films. Refiner discharge of surimi processing industries can be explored as a collagen/ gelatin source and for packaging films development and PL can be applied for modifications of films properties.

सारांश

प्रस्तुत शोध का मुख्य उद्देश्य, सुरीमी प्रसंस्कृत उद्योगों के रिफाइनर स्राव से कोलाजन एवं जेलाटिन का निष्कर्षण तथा निष्कर्षित जेलाटिन से पैकेजिंग फिल्मों निर्माण की संभावनाओं अन्वेषण करना था। इस अध्ययन में पिक पर्च मछली (नेमिटेरस जपोनिकस) की सुरीमी के रिफाइनर स्राव से कोलाजन का निष्कर्षण तथा उसके गुणों का चित्रण किया गया। विभेदक स्कैनिंग उष्मामापी के द्वारा निष्कर्षित कोलाजन का विकृति-करण ताप 23.07°C पाया गया। निष्कर्षित कोलाजन में ग्लाइसिन, प्रोलीन तथा एलेनिन नमक अमीनो अम्ल अधिक संख्या में थे। एसडीएस-पेज की जांच में निष्कर्षित कोलाजन 'टाइप - 1' प्रकार का पाया गया। कोलाजन में α_1 , α_2 और उच्च आणविक द्रव्यमान की β पेप्टाइड शृंखलाएँ पाई गयीं। इनके द्रव्यमान क्रमशः इस प्रकार हैं- 125, 113 और 176 केडीए। सुरीमी रिफाइनर स्राव से जेलाटिन निष्कर्षण की अनुकूलतम परिस्थितियाँ अनुक्रिया-तल प्रणाली के बॉक्स-बेहकन अभिकल्प द्वारा ज्ञात की गई। निर्भर चर के रूप में कोलाजन प्राप्ति (%) का स्वतंत्र चरों (NaOH सांद्रता, निष्कर्षण ताप और समय) से अनुमान लगाने के लिए द्वितीय-आर्डर की प्रतिगमन समीकरण स्थापित की गई। कोलाजन की उच्चतम प्राप्ति (15.60 %) के लिए अनुकूलतम परिस्थितियाँ हैं : NaOH- 0.34M, निष्कर्षण ताप- 63.92°C तथा समय- 4.1h. इस कोलाजन के भौतिक, रासायनिक तथा कार्यात्मक गुणों का चित्रण किया गया। प्राप्त जेलाटिन की ब्लूम बिंदु, श्यानता और गलनांक बिंदु क्रमशः इस प्रकार हैं -143.97 g , 7.44 cP और 25.5°C । एसडीएस-पेज से ज्ञात हुआ की जेलाटिन में α शृंखलाएँ तथा क्रॉस-लिंकड (β , γ) शृंखलाएँ मौजूद हैं । फिल्मों को जेलाटिन के विभिन्न सांद्रणों (2-6%) के साथ विकसित किया गया। फिल्म बनाने के लिए प्रयुक्त विलयन की श्यानता, जेलाटिन के सांद्रणों द्वारा अर्थपूर्ण ($p < 0.05$) तरीके से प्रभावित हुई। पश्चात, स्पंदित प्रकाश के द्वारा बहुलकीकरण से जेलाटिन फिल्मों के भौतिक-रासायनिक एवं कार्यात्मक गुणों को संशोधित किया गया। पश्चात, फिल्मों के यांत्रिक, संरचनात्मक, कार्यात्मक एवं आणविक गुणधर्मों को ज्ञात किया गया। स्पंदित प्रकाश की बढ़ती अवधि के साथ विकृतिकरण ट्रांजीशन की तापीय धारिता में वृद्धि पाई गई। प्रस्तुत शोध से यह प्रदर्शित हुआ की रिफाइनर स्राव का जेलाटिन और कोलाजन के उत्पादन के लिए प्रयोग किया जा सकता है। प्राप्त जेलाटिन के प्रयोग से संवेष्टन के लिए प्रयोग में लायी जाने वाली फिल्मों का विकास किया जा सकता है एवं स्पंदित प्रकाश के माध्यम से फिल्मों के गुणधर्मों को संशोधित किया जा सकता है।

1. INTRODUCTION

Surimi is stabilized mince made from deboned and washed fish meat. Surimi processing involves processing whole or gutted fish into mince, repeated washing of the mince and refining. Refining is a screening mechanism, where the remaining scale, connective tissues and bones are separated from the mince. The waste generated in the form of head, viscera, skin and bone during fish surimi processing constitute 50-70 % of the raw material (Morrissey *et al.*, 2000). The surimi solid by-products does not find used as a human food. Most surimi solid by-products are conventionally used to produce fish meal and fertilizer or are directly discharged into estuaries, resulting in eutrophication (Ciarlo *et al.*, 1997). Refining mechanism in surimi manufacturing produces 15-22 % refiner discharge which constitutes 4-8 % connective tissue of the whole fish (Wendel, 1999). Such waste can form an ideal raw material for collagen/gelatin production. Fish collagens are of interest in the food-processing industry for their use in the production of gelatin from the extracted collagen (Kim and Park, 2004). Of the 19 different types of collagen identified (Bailey *et al.*, 1998), Type I collagen is the most widely occurring collagen in connective tissue (Ikoma *et al.*, 2003). In India there are 17 surimi plants concentrated mainly in Gujarat and Maharashtra (www.fao.org). One potential source of fish collagen/gelatin might include pink perch (*Nemipterus japonicus*) by-products generated from the surimi industry. These by-products include substantial quantities of skin, scale, bones which are good source of collagen/gelatin.

So far the main source of collagen/gelatin is limited to those of land origin such as bovine or porcine skin and bone. However, gelatin obtained from porcine skins or bones cannot be applied for some foods due to esthetic and religious objections (Sadowska *et al.*, 2003). Therefore, alternative sources, especially fish processing wastes have been paid increasing attention for its extraction (Pranoto *et al.*, 2007). Search for new alternative to replace mammalian collagen/gelatin led to patents for fish collagen/gelatin production (Grossman and Bergman, 1992; Holzer, 1996; Shimizu *et al.*, 2001).

The collagen unit structure is a triple helix of 3 polypeptide chains coiled around a common axis to form a rigid tropocollagen molecule. The tropocollagen molecules are joined end to end and aggregate to form fibrils. These fibrils line up alongside each other, each one shifted by one quarter of its length along its neighbouring molecule to form fibers. These fibers are stabilized by intermolecular cross linking between lysine or hydroxyproline residue and a lysine (or hydroxylysine) derived aldehyde, forming a labile aldimine bond, which becomes stable as the collagen ages (Balian and Bowes, 1977; Asghar and Henrickson, 1982). One-third of collagen residues are made up of glycine while one-fourth was mainly proline and hydroxyproline (Harrington and Hippel, 1962). Collagen from aquatic by-products is generally obtained by following acid-alkali or acid-enzyme extraction methods (Kittiphattanabawon *et al.*, 2010; Ogawa *et al.*, 2004).

Gelatin is soluble protein compound obtained by controlled hydrolysis from the fibrous insoluble protein, collagen. Warm-water extraction process is generally used for hydrolysis of collagen into gelatin. Heat treatment cleaves the hydrogen and covalent bonds to destabilize the triple-helix, resulting in helix-to-coil transition and conversion into soluble gelatin (Djabourov *et al.*, 1993; Gomez-Guillen *et al.*, 2002). There are two main types of gelatin based on the chemical used during pre-treatment. Type A gelatin with isoionic point of 7 to 9, is derived from collagen with exclusively acid pretreatment. Type B, with isoionic point of 4.8 to 5.2, is the result of an alkaline pretreatment of the collagen (Veis, 1964; Ward and Courts, 1977). Gelatin yield from raw material is important and it depends on the biochemical characteristics of the raw materials, processing conditions and the experimental settings. Thus to optimized yield, statistical tools like response surface methodology (RSM) has been used by many authors. Gudmundsson and Hafsteinsson (1997) have used RSM to study the effect of chemical treatment on gelatin yield from cod skin. Wang *et al.* (2008) also employed RSM for optimization of conditions for extraction of collagen from grass carp. While, Mohtar *et al.* (2010) have used RSM for process optimization of gelatin from hoki skin.

Gelatin finds enormous application in food industry due to its specific “melt-in-mouth” texture. It has also wide applications in pharmaceutical and photographic industry (Karim and Bhat, 2009). Gelatin is also known to have good

film forming property. Such property can be utilized for developing edible packaging films or coatings. Main important properties of packaging films are good mechanical strength and barrier properties. Synthetic packaging films satisfied these properties. However, synthetic packagings are non-degradable which can cause serious problems to the environment. Trends are to develop edible packaging films from biodegradable source.

An edible film is defined as a thin layer of material which is edible and can provide barrier to moisture, oxygen and solute movement of foods (Guilbert, 1986). The materials are mainly the renewable sources such as proteins, carbohydrates and lipids (Tharanathan, 2003). Because of their good film-forming abilities, fish gelatins may be a good alternative to synthetic films. Protein-based films generally exhibit the poor films properties. Therefore, to improve the physical and chemical properties of such films, chemical cross linking agents such as gossypol, formaldehyde, glutaraldehyde (de Carvalho and Grosso, 2004; de Carvalho *et al.*, 2008), and transglutaminase (Mariniello *et al.*, 2003) are used. Nevertheless, some cross-linking agents have the toxicity and high cost (Bigi *et al.*, 2002; Cao *et al.*, 2007a). Therefore, a natural cross-linking agent with no toxicity is of great interest for improving the properties of protein based film films. Collagen and gelatin films have been used for sausage casing production (Johnston-Banks, 1990). Also edible wrappings based on blends of gelatin with other constituents have been marketed (Torres, 1994). Edible films can be formed by two process i.e., casting and extrusion (Hernandez-Izquierdo and Krochta, 2008). Gelatins from the skin of unicorn leatherjacket (Ahmad *et al.*, 2012), bigeye snapper (Rattaya *et al.*, 2009), tilapia (Pranoto *et al.*, 2007), squid (Nagarajan *et al.*, 2013) have been used for film preparation. Fish gelatins have been reported to exhibit transparent, colourless, and elastic films (Jongjareonrak *et al.*, 2006). Gelatin can also co-blend with other polymers to form composite edible films (Pranoto *et al.*, 2007; Shakila *et al.*, 2012a; Guerrero *et al.*, 2011). Additionally, it can be used as smart packaging, in which antioxidants or antimicrobials can be incorporated (Jongjareonrak *et al.*, 2008; Tongnuanchan *et al.*, 2012).

Based on the above information the present study was taken with the following objectives:

- Isolation and partial characterization of collagen from the surimi refiner discharge.
- Optimization of gelatin extraction from surimi refiner discharge using Response Surface Methodology.
- Preparation of Gelatin based packaging film.

2. REVIEW OF LITERATURE

The global collagen market is anticipated to reach USD 6.63 billion by 2025. Gelatin and hydrolyzed collagen are the key product segments in this market. The market is growing due to increasing demand for collagen-based products in healthcare applications (wound healing, tissue engineering and bone reconstruction), food and beverages and cosmetics industries (www.grandviewresearch.com). Collagen is the major structural protein and partial hydrolysis of collagen gives another soluble protein called gelatin (Djagny *et al.*, 2001). Commercially, the key sources for extraction of collagen include bovine, porcine and poultry. However, after outbreaks of bovine spongiform encephalopathy, there were restrictions on collagen from these sources. Therefore by-products from fish processing industries have been used for collagen and gelatin extraction (Liu *et al.*, 2007; Parenteau-Bareil *et al.*, 2010). Thus, the future growth of collagen market is expected to be driven by the marine segment due to increasing use of marine-based products in cosmetics and healthcare applications (www.grandviewresearch.com; Hashim *et al.*, 2015). Several studies were reported on collagen extraction from different body parts of fishes such as skin (Skierka and Sadowska, 2007), fins, scales, bones and swim bladders (Liu *et al.*, 2012). The waste from surimi processing such as refiner discharge could also be the primary material for extracting collagen. Such sources are worth investigating considering that collagen properties differ from one source to another (Lin and Liu, 2006).

2.1. Fish Processing Wastes

Fish processing waste is defined as fish material left over after primary processing during the fish manufacturing process (He *et al.*, 2012). The wastes in the form of scales, skin, head, viscera, bones, frames etc. comprised of around 50-80 % of the whole fish. About 30% of the wastes consists of skin and bone with a high collagen content (Wasswa *et al.*, 2007; Kim and Mendis, 2006). Table 1 shows the solid waste generated from different methods of processing of fish. An important waste reduction strategy for the industry is the recovery of marketable byproducts from such fish wastes as fish waste is a great source of minerals, proteins and fat.

Common methods of utilization of fishery waste are production of fish meal or fish oil, fish silage and also manure. Other possible utilization of processing waste would be manufacturing of valuable biomolecules such as collagen and gelatin.

Table 1: Solid waste generated in fish processing

Process	Solid waste (Kg/1000Kg)
White fish filleting	440-690
Scaling of white fish	Scales: 20-40
Deheading of white fish	Head and debris: 270-320
Filleting of deheaded white fish	Frame and off cuts: 200-300
Skinning of white fish	Skin: 40
Oily fish filleting	400-450

Source: Arvanitoyannis and Kassaveti, 2008.

2.1.1. Surimi processing waste

Surimi processing generates 75% of total fish as waste. These by-products are rich source of connective tissues. Surimi is stabilized fish mince that is made from deboned, washed fish meat mixed with cryoprotectants, such as sugar and sorbitol. After filleting and mincing, 32 - 36% yield can be achieved from whole fish to mince. When the mince goes through the following washing, refining and de-watering steps, an additional 33-50% of the mince weight is lost, mainly in the form of water soluble proteins and connective tissue. The final surimi processing yield of whole fish weight is therefore 17-24% (Wendel, 1999). In Figure 1, a general processing flow chart for surimi with mass balance is presented.

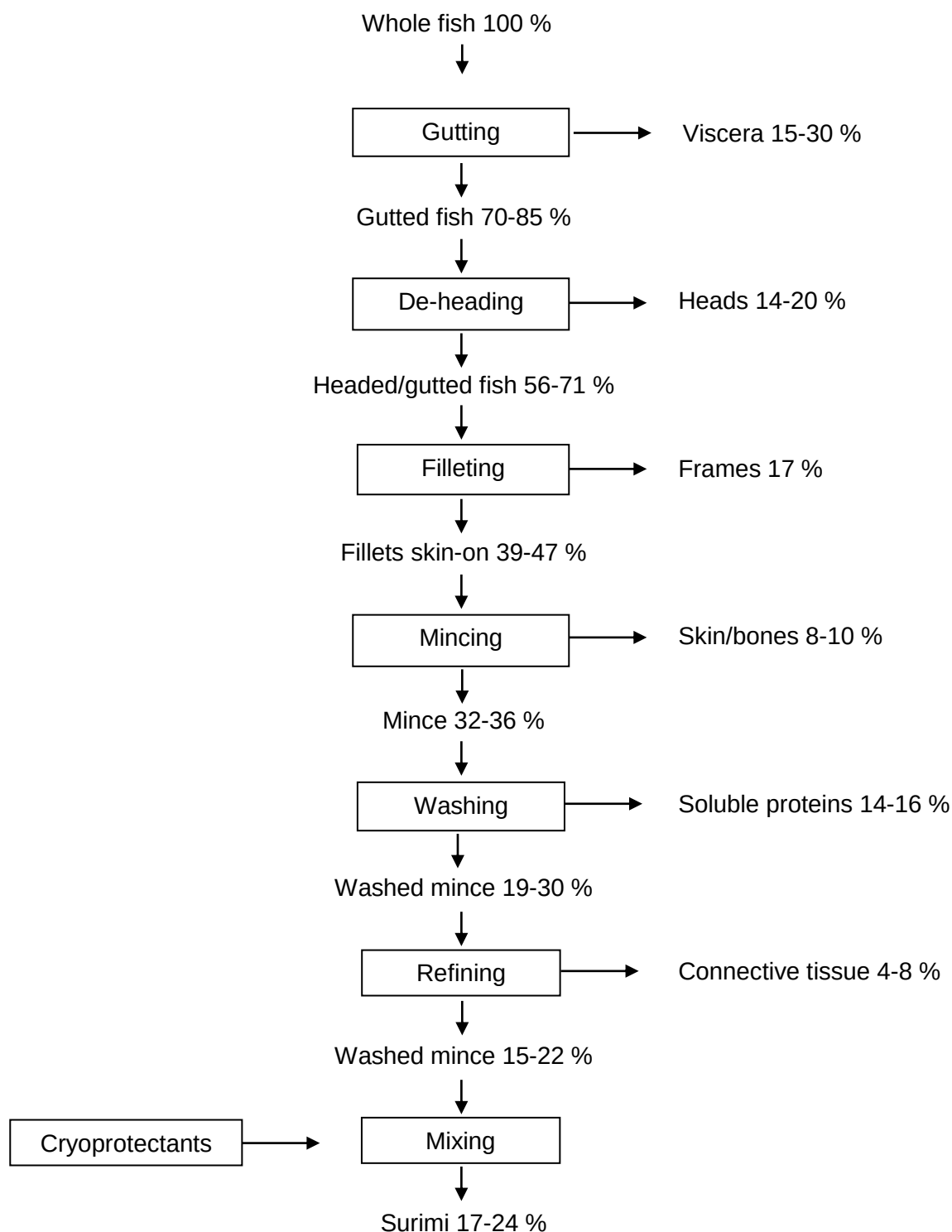


Figure 1: Surimi processing flow chart with mass balance. (Source: Wendel, 1999)

The recovery of chemical components from seafood waste materials, which can be used in other segments of the food industry, is a promising area of research and development for the utilization of seafood by-products. The history of Indian surimi dates back to 1990 when Marine Products Export Development Authority of India introduced the concept of surimi. Hindustan Lever Limited was shortlisted for surimi production. As of 2012, there are seven surimi factories in Gujarat, five in Mumbai, two in Ratnagiri, one in Goa, one in Udupi, and one in Vizag on the East coast. With almost no domestic consumption, the majority was exported to Europe and the United States. Threadfin bream is the main species (70 %) used for surimi production and various other species include lizard fish, croaker, big eye snapper, goat fish and ribbon fish (Guenneugues and Morrissey, 2005).

2.1.2. Refiner Discharge

In surimi processing steps, there is an important step called refining. It is screening mechanism where the washed mince were passed through a perforated screen with perforation size of 1.5-1.7 mm to remove the remaining connective tissues and bones, skin, scales or other unwanted materials (Park and Lin, 2005). A typical refiner consists of a cylindrical perforated screen and a screw shaped rotor. When the washed and dewater mince is fed into the refiner, the soft meat emerge from the front part of the refiner. Towards the rear part of the refiner, hard materials which could not pass through the screen such as connective tissues, bone, scale, fish, skin, etc. are discharged. This waste is called refiner discharge. Majority of the protein in the refiner discharge is stroma protein derived from connective tissue (Kim and Park, 2005).

2.2. Value Added Products from Fish Processing Wastes

Processing of fish in any form leads to the generation of a large amount of waste. Such waste is generally dumped leading to environmental problem. Thus, a useful waste reduction strategy for the industry will be the recovery of marketable value added products from fish wastes (Laufenberg *et al.*, 2003). The use of such processing waste as animal feed has been the most common methods for utilisation of such waste by converting into fishmeal (AWARNET, 2004; Zynudheen, 2010).

However, recently fish processing wastes has been explored as a source of high added value compounds like poly-unsaturated fatty acids, free amino acids, chitin and chitosan, calcium or hydroxyapatite, anti-freeze proteins, asthaxanthin, collagen and gelatin. These high added value compounds find application as nutraceutical, functional food formulation and pharmaceutical (Ferraro *et al.*, 2010).

2.2.1. Collagen and gelatin

The name “collagen” is used as a generic term for proteins having a characteristic triple helix structure and is the most abundant protein found in animal body. It is the major constituent of skin, bones and connective tissues, comprising nearly 30 % of the total protein in the animal protein. 19 genetically distinct collagen types have been described. The different collagen types are characterized by considerable complexity and diversity in their structure, their splice variants, and the presence of additional, non-helical domains, their assembly and their function (Gelse *et al.*, 2003). Despite the rather high structural diversity among the different collagen types, all members of the collagen family have one characteristic feature i.e each molecule composed of three α -chains (Okuyama *et al.*, 2006). Collagen is mainly sought for the production of gelatin, a high value functional protein due to its unique gel-forming capacity; of all hydrocolloids in use today, none is as popular as gelatin.

Gelatin is produced by partial hydrolysis of fibrous insoluble collagen. Thus, gelatin is a heterogeneous mixture of water soluble single or multi-stranded polypeptides, each with extended left-handed proline helix conformations. It is a proteins of high molecular weight, in the range between 80 and 250 kDa as its parent protein, collagen. Gelatin in solution at low temperature undergoes coil-helix transition followed by aggregation of the helices by the formation of collagen-like right-handed triple-helical proline/hydroxyproline rich junction zones. There are two types of gelatin depending on the solvent used in the production process. Preparation involving acid treatment is called Type A gelatin and those involving alkali treatment are called Type B gelatin (Wasswa *et al.*, 2007).

Mammalian collagen/gelatin (porcine and bovine) is the most popular and widely used. However, due to risk of transmissible diseases like bovine spongiform encephalopathy (mad cow disease), ovine and caprine scrapie, and other

zoonoses (Lynn *et al.*, 2004; Pati *et al.*, 2012; Bhagwat *et al.*, 2016) marine sources have been looked into for collagen and gelatin extraction. Different parts of fish such as skin, bone, scale and swim bladder have been tried for collagen and gelatin extraction by several authors (Muyonga *et al.*, 2004a,b; Kim and Park, 2004; Ikoma *et al.*, 2003; Sripriya and Kumar, 2015). In recent years fish collagen and gelatin are also receiving a great deal of attention due to their diverse applications, including in the food, pharmaceuticals, cosmetics and biomedical industries. Hence, gelatin is being utilized as ingredient to improve elasticity, consistency and stability of food, as well as for encapsulation and film formation in the pharmaceutical, cosmetic and photographic industries (Montero and Gomez-Guillen *et al.*, 2000).

2.2.1.1. Structure and Composition of Collagen/Gelatin

The collagen molecule consists of three polypeptide chains (α chain). These might be three identical chains (homotrimers) or two or more different chains (heterotrimers). Ramachandran (1956) first proposed the triple-helical structure of the collagen molecule.

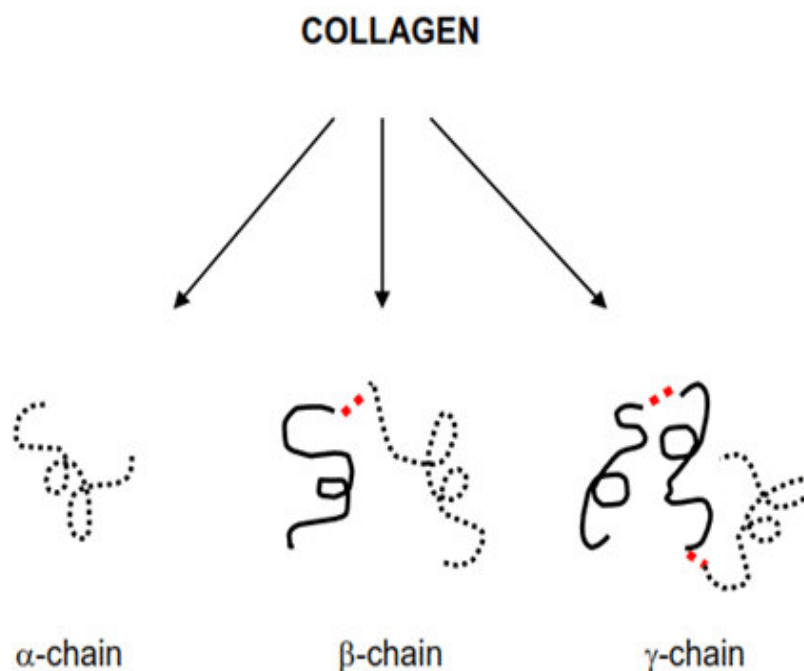


Figure 2: Typical polypeptide strands of collagen. (Source: Mahmood *et al.*, 2016)

Each of the three α -chains within the molecule forms an extended left-handed helix with a pitch of 18 amino acids per turn. The three chains, staggered by one residue relative to each other, are supercoiled around a central axis in a right-handed manner to form the triple helix. The triple-helix is approximately 300 nm in length, and the chain has a molecular weight of approximately 105 kDa (Papon *et al.*, 2002). The triple helices are stabilized by inter-chain hydrogen bonds. β dimmers and γ trimers are also found in collagen. The β component is due to the intermolecular crosslinking while that of γ component indicates intramolecular crosslinking (Figure 2) (Lewis and Piez, 1964).

The fibril-forming collagen molecules have an uninterrupted triple helix of approximately 300 nm length and 1.5 nm in diameter, followed by short extra helical telopeptides. The telopeptides do not adopt triple-helical conformations due to the absence of repeating Gly-X-Y units where Gly is glycine, Y positions are mostly occupied by the imino acid proline and hydroxyproline and X is any of the other amino acids account for 2% of the molecule and plays a vital role in fibril formation (Kadler *et al.*, 1996).

Collagen is rich in nonpolar amino acids such as glycine, valine, proline, alanine and hydroxyproline (Gomez-Guillen *et al.*, 2002; Kim and Mendis, 2006). Glycine occurs at every third position and makes the collagen structure compact as this allows the very close association of the collagen fibers within the molecule, facilitating hydrogen bonding and the formation of intermolecular cross-links. The stability of the collagen triple helix is related to the total content of proline and hydroxyproline, which together make up about 20% of the total amino acids in human fibrillar collagens (Hulmes, 2008). Collagen exists in many different forms but gelatin is only derived from sources rich in Type I collagen which contains no cysteine; however, hide or skin contains some Type III collagen which can be the source of cysteine (Prockop, 1998).

The primary structure and composition of gelatin also resembles the parent collagen. Upon heating, at about 40°C, the hydrogen bonds that stabilize the triple helical structure of collagen rupture (Balian and Bowes, 1977), leading to the formation of warm-water soluble collagen i.e., gelatin (Schrieber and Gareis, 2007). Many studies in this field have shown that the loss of the triple helical conformation of

collagen leads to the formation of two main components, the α -chains (α_1 , α_2 , and α_3), with molecular weights ranging from 91–95 kDa, and the β -chain, with a molecular weight approximately twice that of the α -chain (Balian and Bowes, 1977; Bama *et al.*, 2010; Nagai *et al.*, 2004; Senaratne *et al.*, 2006). Furthermore, another component, namely the γ -chain, with a higher molecular weight (300 kDa) than the β -chain, has been isolated and is composed of three covalently crosslinked α -chains (Balian and Bowes, 1977).

2.2.1.2. Sources of Collagen/Gelatin

Mammalian collagens (porcine and bovine) are the most popular and widely used but are facing problems due to their allergic reactions as well risk of transmissible diseases like bovine spongiform encephalopathy (mad cow disease), ovine and caprine scrapie, and other zoonoses (Lynn *et al.*, 2004; Pati *et al.*, 2012, Bhagwat and Dhandge, 2016). Different parts of fish such as skin, bone, scale and swim bladder have been tried for collagen extraction by several authors. Collagens of fish studied in recent years were: Carp (*Cyprinus carpio*) scales (Bhagwat and Dhandge, 2016), tilapia fish scale (Ikoma *et al.*, 2003; Huang *et al.*, 2016), northern pike (*Esox lucius*) scales (Kozłowska *et al.*, 2015), seabass (*Lates calcarifer*) scale (Chuaychan *et al.*, 2015), Amur sturgeon cartilage (Liang *et al.*, 2014), Amur sturgeon muscle (Wang *et al.*, 2014), silver carp skin (Zhang *et al.*, 2009; Rodziewicz-Motowidło *et al.*, 2008; Tang *et al.*, 2015), balloon fish skin (Huang *et al.*, 2011), spotted golden goatfish scale (Matmaroh *et al.*, 2011), scale of *Pagrus major* (Ikoma *et al.*, 2003), skin of brown backed toadfish (Senaratne *et al.*, 2006), skin, scale and bone of deep-sea redfish (Wang *et al.*, 2008), scale of pacific saury (Mori *et al.*, 2013), skin of brown banded bamboo shark (Kittiphattanabawon *et al.*, 2010), backbone of Baltic cod (Zełechowska *et al.*, 2010), skeleton and head bone of silvertip shark (Jeevithan *et al.*, 2014), skins of several underutilised fishes, namely dusky spinefoot (*Siganus fuscescens*), sea chub (*Kyphosus bigibbus*), eagle ray (*Myliobatis tobijei*), red stingray (*Dasyatis akajei*) and yantai stingray (*Dasyatis laevigata*) (Bae *et al.*, 2008), skin of skate (*Raja kenojei*) (Hwang *et al.*, 2007), skin of Brownstripe red snapper (*Lutjanus vitta*) (Jongjareonrak *et al.*, 2005), skins of young and adult Nile perch (*Lates niloticus*) (Muyonga *et al.*, 2004a), skin of ocellate puffer

fish (*Takifugu rubripes*) (Nagai *et al.*, 2002), skin of cuttlefish (*Sepia lycidas*) (Nagai *et al.*, 2001).

Refiner discharge from pacific whiting, a cold water fish, surimi processing plant have been explored as a new source of collagen extraction and found that collagen from refiner discharge have highest functional properties compared with the other byproducts such as skin and frame of pacific whiting (Kim and Park, 2004). To our knowledge no studies have been reported on refiner discharge of surimi from warm water fish.

2.2.1.3. Extraction Methods for Collagen/Gelatin

Collagen from aquatic by-products is generally extracted by treatments with acid or alkali, enzyme, and microorganisms. Chemical extraction is more commonly used as enzymatic processes are more expensive though it reduces processing time and generate less waste. Generally, there are two steps followed in the extraction process. The first step involved is help in removal of non-collagenous substances. Depending on the nature of the raw materials, acid or alkali pre-treatment have been employed. The acidic process is more suitable for more fragile raw materials with less intertwined collagen fibers, such as porcine and fish skins (Almeida, 2012). NaOH and Ca(OH)₂ are often used for pre-treatment of thicker materials that require a more aggressive penetration by the treatment agents, such as bovine ossein or shavings (Ledward, 2000). General steps for isolation of collagen and gelatin is shown in Figures 3 and 4 respectively.

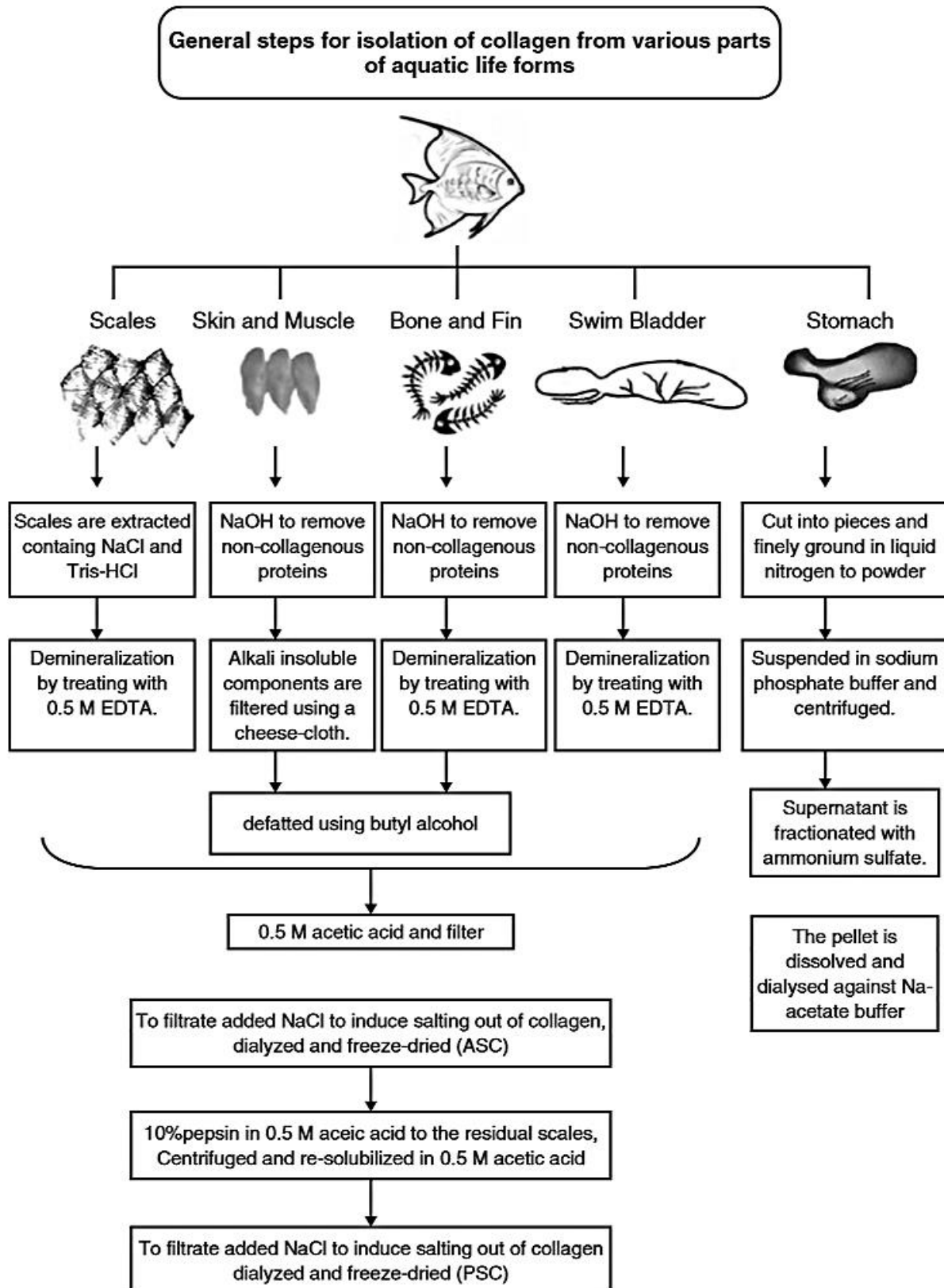


Figure 3: General steps for isolation of collagen from different fish components. (Source: Dhara *et al.*, 2013)

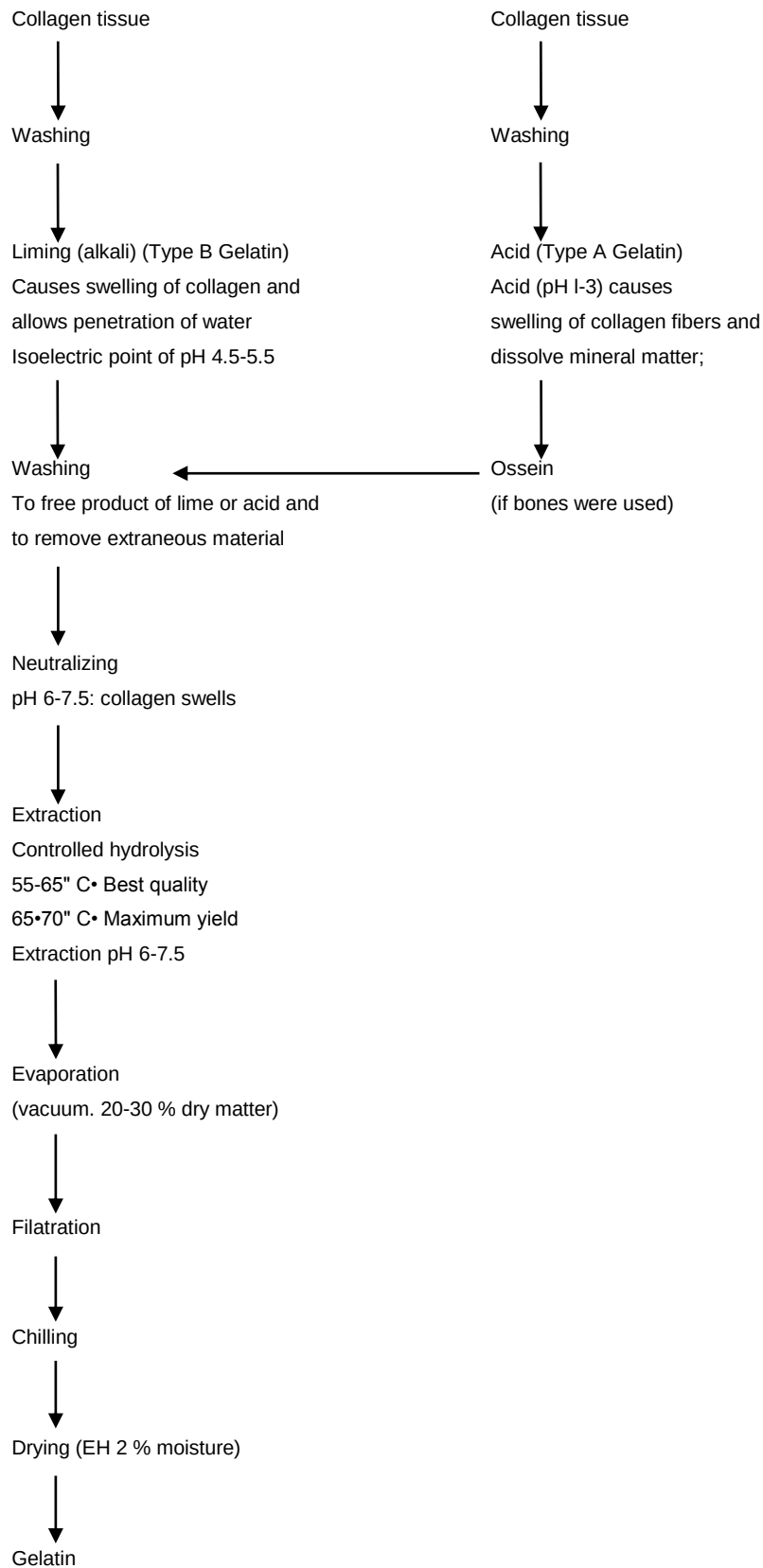


Figure 4: General flowchart of gelatin extraction.

During conversion of collagen to gelatin, the inter- and intra-molecular bonds linking collagen chains as well as some peptide bonds are broken. The more severe the extraction process, the greater the extent of hydrolysis of peptide bonds and therefore the higher the proportion of peptides with lesser molecular weight. The age of the source animal may influence the ease with which gelatin can be extracted and the extent of peptide hydrolysis during the extraction (Cole and McGill, 1988; Reich *et al.*, 1962). Older animal collagen is more cross linked and a more severe process is required to denature it to form gelatin (Reich *et al.*, 1962). There are differences in the extent and type of cross linking found in bones and skins (Sims and Bailey, 1992). This may also affect the ease with which collagen may be solubilised and transformed to gelatin and may result in differences between the properties of gelatins extracted from the two tissues.

During the hydrolysis of collagen into gelatin the following hydrolytic products may be obtained (Mariod and Adam, 2013):

- Formation of three randomly coiled independent α chains.
- Formation of a β chain (two α chains linked by one or more covalent bonds) and an independent α chain.
- Formation of a γ chain (three chains linked by covalent bonds).

The method of extraction can influence the length of the polypeptide chains and the functional properties of collagen, such as viscosity, solubility, as well as water retention and emulsification capacity. This varies according to the processing parameters (enzyme, temperature, time and pH), the pretreatment, method of storage and the properties of the initial raw material (Karim and Bhat, 2009).

2.2.1.3.1. Chemical Process

2.2.1.3.1.1 Acid

A study by Liu *et al.* (2015) evaluated the effect of alkaline pre-treatment on the extraction of acid soluble collagen (ASC) from the skin of grass carp (*Ctenopharyngodon idella*). Concentrations of NaOH from 0.05 to 0.1 M were effective in removing non-collagenous proteins without losing the ASC and structural modifications at temperatures of 4, 10, 15 and 20°C. However, 0.2 and 0.5 M NaOH caused a significant loss of ASC, and 0.5 M NaOH resulted in structural modification

in the collagen at 15 and 20°C. This is followed by demineralisation in case of scale and bone raw materials. Decalcification is essential for dissolution of collagen from the mineral particles (Pati *et al.*, 2010). Demineralization of the scales is accomplished by treatment with 0.5 M EDTA solution at pH 7.4. Demineralization is followed by extraction procedure. Acid hydrolysis can be performed by using organic acids such as acetic acid, citric acid and lactic acid, and inorganic acids such as hydrochloric acid. However, organic acids are more efficient than inorganic acids (Skierka and Sadowska, 2007; Wang *et al.*, 2008). Organic acids are capable of solubilizing non-crosslinked collagens and also of breaking some of the inter-strand cross-links in collagen, which leads to a higher solubility of collagen during the extraction process (Liu *et al.*, 2015). Therefore, acidic solutions, especially acetic acid, are commonly used to extract collagen. For the extraction of acid-soluble collagen, the pre-treated material is added to the acid solution, usually 0.5 M acetic acid, and maintained for 24 to 72 h under constant stirring at 4°C, depending on the raw material (Wang *et al.*, 2014; Nagai, 2015; Kaewdang *et al.*, 2014). After the extraction stage, a filtering is performed to separate the supernatant (residue) from the collagen, which is in the liquid phase. To obtain collagen powder, the filtrate is usually subjected to precipitation with NaCl. The precipitate is then collected by centrifugation and subsequently redissolved in a minimum volume of 0.5 M acetic acid and then dialyzed in 0.1 M acetic acid for 2 days, and distilled water for 2 days, with replacement of the solution on an average for every 12 h. Several authors followed the described methods for collagen isolation from fish (Ogawa *et al.*, 2004; Wang *et al.*, 2008; Duan *et al.*, 2009). Collagen isolated by acid hydrolysis is called “Acid Soluble” collagen.

Collagen can be converted into soluble gelatin by using acid. In this process, collagen is acidified to about pH 4 and heated to denature the collagen, defatted, filtered and then dried to obtain gelatin. The resulting gelatin has an isotonic point of 7 to 9 based on the severity and duration of the acid treatment. This processing method causes limited hydrolysis of the asparagine and glutamine amino acids side chains (Cole, 2000). The gelatin obtained by using acid hydrolysis is called ‘Type A’ gelatin. Type A gelatin (dry and ash free) contains 18.5 % nitrogen (Eastoe and Leach, 1958). Jamilah and Harvinder (2002) extracted gelatin from red and black tilapia skins (*Oreochromis nilotica* and *O. mossambicus*). The fish skin was washed

under running tap water for around 1 h to remove the superfluous materials. Then the cleaned raw material was soaked in dilute alkali solution (0.2 % w/v) for 40 min. Later, the material was soaked in H₂SO₄ of the same concentration and followed by 1.0 % citric acid treatment. After the given time of treatment, the solution was neutralized. Finally, the extraction was carried out with distilled water at 45°C for 12 h. The extracted material was filtered through double-layered cheese cloth. The filtrate was dried at 50°C in a hot air oven.

2.2.1.3.1.2. Alkali

Alkali process is generally for extraction of gelatin and not applied for collagen extraction. The alkali process is a lengthy process as compared to acid process as it involved treatment of collagen with caustic soda prior to extraction. After the alkali treatment the material is treated with acid and heated and followed similar procedure as the acid treatment. Gelatin obtained by alkali hydrolysis is called 'Type B' gelatin. Type B gelatin contains only about 18 % nitrogen (Eastoe and Leach, 1958). The resulting gelatin has an isotonic point of 4.8 to 5.2. The alkali hydrolyses the asparagine and glutamine side chains of the protein into aspartic and glutamic acids (Veis, 1964).

Tavakolipour (2011) had reported the extraction of gelatin from skins and fins of silver carp fish (*Hypophthalmichthys molitrix*) at room temperature using lime solution. The raw material was cleaned in running water and soaked in lime solution (8.25 %, pH 12) after reducing the size to 10-30 cm at 15-20°C for 4 weeks. After incubation time, the material was washed with water keeping the pH around 10. Later, the solution was filtered and mixed with 5 % solution of HCl to lower the pH and then freeze dried.

2.2.1.3.1.3. Enzymatic Process

For the extraction of collagen by enzymatic hydrolysis, the raw material, which can be the residue of acidic extraction, is added to 0.5 M acetic acid solution containing selected enzymes such as pepsin, alcalase or flavourzyme. The mixture is continuously stirred for about 48 h at 4°C followed by filtration (Li *et al.*, 2013; Wang *et al.*, 2014). The filtrate is subjected to precipitation and dialysis under the same conditions as for obtaining acid soluble collagen.

A patented enzymatic process for the extraction of gelatin was proposed by Rowlands and Burrows (2000). These methods have claimed to yield gelatin of high gel strength and lack of residual chemicals in gelatin due to non-usage of chemical compounds in the pretreatment step. Another enzymatic process for the production of gelatin from fish skins was patented by Kawahara and Tanihata (2005). The resulting fish gelatin has been reported to be devoid of fishy odor (after treating the gelatin liquor with activated carbon), have a white appearance, and give a clear and colorless solution when dissolved in water.

2.2.1.3.1.4. Advanced/Modified Process

A novel extrusion–hydro-extraction process for extraction of collagen was developed by Huang *et al.*, 2016. Extrusion cooking has been used for many years to produce several types of animal feed and human food. It is characterized by high temperature short time and high shear force processes. The authors found that extruded scale samples had a 2–3 times higher protein extraction yield than that of non-extruded scale samples. Extrusion cooking offers numerous advantages, such as easy operation, continuous production, high yield, limited waste, low labor cost, and multiplicity of products. The extrusion process helps to decompose the intimate linkage between collagen and hydroxyapatite, and facilitates the release of collagen from fish scale extrudates by water extraction. Moreover, the extrusion process may also alleviate the unpleasant smell of fish scale and obtain a weaker collagen odor.

Ultrasound has been used successfully in collagen extraction by reducing the processing time and increasing the yield (Kim *et al.*, 2012; Kim *et al.*, 2013; Ran and Wang, 2014; Tu *et al.*, 2015). According to Kim *et al.* (2013) the use of ultrasound in the extraction of collagen generated a higher rate of yield than the conventional extraction method with 0.5 M acetic acid, even when using a low concentration of acid (0.01 M). In addition, the yield of collagen from the skin of Japanese sea bass (*Lateolabrax japonicus*) increased greatly with increased treatment time and amplitude of ultrasound.

2.3. Characteristics of Collagen and Gelatin

2.3.1. Amino acids composition

Several studies have demonstrated that the amino acid composition of fish collagen is similar to that of mammalian collagen. Glycine is the most abundant amino acid, accounting for more than 30 % of all amino acids. In addition, the degree of hydroxylation of proline has been calculated to be 35–48 %, similar to the level observed in mammalian tissues (approximately 45 %). Thus fish collagens have lower imino acid contents to a lesser extent than mammalian collagen. Furthermore, a linear relationship between the stability of collagen and the hydroxyproline content has generally been recognized (Yamada *et al.*, 2014).

The source and type of collagen will influence the properties of the resulting gelatins. Generally warm water fish gelatins have a higher imino acid (proline and hydroxyproline) content than cold-water fish gelatin (Eastoe and Leach, 1977). The proline and hydroxyproline contents are approximately 22-25 % for warm-water fish gelatins (tilapia and Nile perch), and 17 % for cold-water fish gelatin (Muyonga *et al.*, 2004b). The lower content of proline and hydroxyproline gives fish gelatin a low gel modulus, and low gelling and melting temperatures. It should be kept in mind that the super-helix structure of the gelatin gel, which is critical for the gel properties, is stabilized by steric restrictions. These restrictions are imposed by both the pyrrolidine rings of the imino acids in addition to the hydrogen bonds formed between amino acid residues (Te Nijenhuis, 1997).

2.3.2. Molecular weight distribution

The collagen molecule consists of three polypeptide chains (α chain). These might be three identical chains (homotrimers) or two or more different chains (heterotrimers). Each of the three α -chains within the molecule forms an extended left-handed helix with a pitch of 18 amino acids per turn. The three chains, staggered by one residue relative to each other, are supercoiled around a central axis in a right-handed manner to form the triple helix. The triple helices are stabilized by inter-chain hydrogen bonds. β dimers and γ trimers are also found in collagen. The β component is due to the intermolecular crosslinking while that of γ component indicates intramolecular crosslinking (Lewis and Piez, 1964). The γ chain had high

molecular weight of approximately 200 kDa (Mathew, 2002). While the β and α chains had molecular weights below 200 kDa. Varying molecular weights of different chains were reported by several authors. Molecular weight of α chains of collagen isolated from the skin of bigeye snapper was reported as 120 kDa for α_1 and 112 kDa for α_2 respectively (Nalinanon *et al.*, 2007). However, collagen extracted from Baltic cod skin had α chains below 116 kDa (Skierka and Sadowska, 2007). The conversion of collagen to gelatin yields molecules of varying mass, due to the cleavage of inter-chain covalent cross links and the unfavorable breakage of some intra-chain peptide linkages (Zhou *et al.*, 2006). As a result, the gelatin obtained has a lower molecular weight than native collagen, and consists of a mixture of fragments with molecular weights in the range of 80-250 kDa (Poppe, 1997).

2.3.3. Structural analysis

Fourier transform infrared (FTIR) spectroscopy has been used to study the changes in the secondary structure of gelatin (Aewsiri *et al.*, 2008; Muyonga *et al.*, 2004c). FTIR spectroscopy has been used to study collagen crosslinking (Paschalis *et al.*, 2001), denaturation (Freiss and Lee, 1996), thermal self-assembly (Jakobsen *et al.*, 1983; George and Veis, 1991) and gelatin melting. The spectral changes indicate the changes in collagen secondary structure and have been shown to include changes in the amide A (Milch, 1964), amide I ($1636\text{--}1661\text{ cm}^{-1}$), amide II ($1549\text{--}1558\text{ cm}^{-1}$) (Renugopalakrishnan *et al.*, 1989) and the amide III ($1200\text{--}1300\text{ cm}^{-1}$) regions (Freiss and Lee, 1996). The amide I is the most useful peak for infrared analysis of the secondary structure of protein including gelatin (Surewicz and Mantsch, 1988). Yakimets *et al.* (2005) reported that the absorption peak at 1633 cm^{-1} was characteristic for the coil structure of gelatin. Denaturation of collagen to gelatin has been found to lead to a reduction in the intensity of amide A, I, II and III peaks, narrowing the amide bands (Freiss and Lee, 1996; Prystupa and Donald, 1996).

2.3.4. Thermal properties

Differential Scanning Calorimetry (DSC) has been used to study the changes in the collagen due to changes in temperature. The study of thermal property of collagen helps us to understand the stability of the collagen as collagen

has triple helix structure stabilised by hydrogen bonds. These hydrogen bonds are heat sensitive and converted into helix to coil configuration (gelatin) when heated by the process called thermal depolymerisation (Wang *et al.*, 2008). The thermal stability of collagen depends on the imino acids content, environmental temperature of the habitat of the fish, method of preparation of collagen as well as moisture content (hydrated or rehydrated state) of the collagen (Bae *et al.*, 2008, Minh *et al.*, 2014; Rigby, 1968; Huang *et al.*, 2016).

High denaturation temperature of silver carp collagen has been reported by Safandowska and Pietrucha, 2013. This relatively high denaturation temperature of silver carp collagen may be also attributed to the presence of a certain amount of elastin in the sample. As silver carp skin has been reported to content about 8 % of elastin (Rodziewicz-Motowidło *et al.*, 2008).

2.3.5. Functional Properties

2.3.5.1. Gel strength/Bloom value

Although collagen and gelatin are different forms of the same macromolecule, their gel forming mechanisms are different. The gelation process for both collagen and gelatin is thermo-reversible, but in opposite directions: collagen gels melt by lowering the temperature, while gelatin gels melt by raising the temperature (Gomez-Guillen *et al.*, 2011). During collagen gelation the process of collagen molecule aggregation and fibril formation takes place, induced by changes in ionic strength, pH and temperature. During the collagen gelation process, there is a lag phase where the primary aggregates (dimers and trimers of collagen molecules) are nucleated. Then, microfibrillar aggregation starts with the lateral aggregation of sub-units until equilibrium is reached. The self-assembly process of type I collagen from vertebrates occurs when the gelation temperature is raised from 20 to 28°C. In contrast, the basic mechanism of gelatin is related to the reverse coil-to-helix transition triggered by cooling solutions below 30°C, during which the helices that are created are similar to the collagen triple-helix, but in this case, no equilibrium is reached (Djabourov *et al.*, 1993).

The principal gelling property that determines the value of commercial gelatins is gel strength (Wainwright, 1977). During cooling of gelatin solution gel

nuclei are formed from helical interactions within and between polymer molecules, whereas the rate of nucleation is affected by the concentration of polymers and the degree of cooling below the maximum gelation temperature. The formation of a gel network is achieved by involvement of polymer chains in more than one junction zone, where triple helices are forming the junction zones of the network. Following the formation of triple helices, a packing into fibrils, similar to but not as organized as native tropocollagen fibrils, is observed, which constitutes a dynamic process where bonds are continually breaking and reforming within the gel due to weak polymer–polymer interactions (Tosh *et al.*, 2003; Karim and Bhat, 2009). The skin of warm-water fish such as tilapia, yellowfin tuna, sole and megrim is a potential material for gelatin production, and gelatin obtained from these sources can be used in products requiring very high gel strength. On the other hand, cold-water fish, such as cod and Alaska pollock, have very low gelling and melting points (Karim and Bhat, 2009).

2.3.5.2. Gelling and melting point

The setting and melting points of gelatin are also considered important indices of the quality of gelatin preparations. Being thermo-reversible, gelatin gels will start melting when the temperature is increased above a specific point, the melting point, which is usually lower than the temperature of the human body. For gelatins from fish species, setting temperatures are in the range of 8–25°C, while the range of melting temperatures is 11–28°C. The melting and gelling temperatures of gelatin have been shown to correlate with the proportion of Proline and Hydroxyproline in the original collagen (Haug *et al.*, 2004).

2.3.5.3. Water binding properties

Water binding capacities are functional properties that are closely related to the ability to hold its own and added water during the applications of forces, pressing, centrifugation or heating (Hermansson, 1986). Most important functional properties of proteins are related to their interaction with water. Gelatin or collagen chains in solution may be covalently crosslinked to form matrices capable of swelling in the presence of aqueous solutions, creating what are commonly known as gelatin hydrogels. Hydrogels, which are characterized by their hydrophilicity and insolubility in water, have the ability to swell to an equilibrium volume while preserving their

shape (Gomez-Guillen *et al.*, 2011). Borderias *et al.* (1994) has used freeze-dried plaice skin collagen to improve the water-holding ability and the sensory properties of cod mince during frozen storage. They found the improvements in sensory characteristics were not only due to the prevention of drip loss, but also because upon heating the fish mince portion, the collagenous material gelatinized, giving it a much more desirable texture than portions without this material. The gel-forming capability of threadfin bream (*Nemipterus japonicus*) mince was substantially increased by adding gelatin (0.5 %) from the skin of bigeye snapper (Binsi *et al.*, 2009).

2.3.5.4. Solubility

Collagen is not soluble in water. However, fish type I collagen is unique in its extremely high solubility in dilute acid compared to avian and mammalian collagen (Yamaguchi *et al.*, 1976). Gelatin is only partially soluble in cold water; however dry gelatin swells or hydrates when stirred in water. On warming to about 40°C gelatin that has been allowed to hydrate for 30 mins melts to give a uniform solution. Alternatively, dry gelatin can be dissolved by stirring in hot water, but stirring must be continued until dissolution is complete (Cole, 2000). The solubility of collagen is affected by the pH and NaCl concentration of the solution. Jongjareonrak *et al.* (2005) reported that maximum solubility of brownstripe red snapper was observed at pH 3 for acid soluble collagen. While no changes in solubility was observed in the presence of NaCl upto 3 %, thereafter a sharp decrease in the solubility was observed.

2.3.5.5. Surface properties

The surface properties of collagen and gelatin are based on the charged groups in the protein side chains and on some parts of the collagen sequence concluding either hydrophilic or hydrophobic amino acids. Both hydrophobic and hydrophilic parts tend to migrate towards surfaces, such that weakening the surface tension of aqueous systems and forming the required identically charged film around the dispersed phase, which can additionally strengthen by gel formation (Schreiber and Gareis, 2007).

Type A gelatins, with a relatively high isoelectric point (pI-7.0), are suitable for creating oil-in-water emulsions with a positive charge over a wider range of pH values than is possible with conventional protein emulsifiers, such as soy, casein or whey proteins (Dickinson and Lopez, 2001). Early studies by Kim *et al.* (1996) reported that cod bone gelatin had emulsifying properties similar to those of a commercial emulsifier, such as Tween-80.

The emulsifying capacity of acid-soluble collagen from hake skin was found to be higher than that for trout skin, in both cases being lower than in the corresponding muscle connective tissues (Montero and Borderias, 1991). The acid-soluble collagen from Pacific whiting surimi refiner discharge was found to have higher emulsifying activity than both acid-soluble collagen from skin and the commercial emulsifier, Tween-80 (Kim and Park, 2004).

Gelatin and soluble collagen also exhibit suitable foaming properties. For adsorption at the air-water interface, molecules should contain hydrophobic regions which become more exposed upon protein unfolding, thus facilitating foam formation and stabilisation (Townsend and Nakai, 1983). The foam capacity of gelatin from farmed giant catfish was found to be higher than that from calf skin, due to the higher content of hydrophobic amino acid residues in the former gelatin (Jongjareonrak *et al.*, 2010).

2.4. Optimization of Process Parameters for Gelatin Extraction using Response Surface Methodology

2.4.1. Optimization of variables for improvement of yield value

The yield of gelatin depends on the complete hydrolysis of parent collagen. The success of hydrolysis process depends both the pretreatment and extraction variables such pretreatment time, concentration of acid or alkali, enzyme/substrate ratio, pH and extraction temperature, time. When more than two factors influence the process, with different combinations of factors, optimization is vital in order to discover the best alternative or combination among all the possible combinations of factors or variables in the process. It reduces the overall cost, time, and effort, as statistically derived experimental designs are framed to develop the models (Gudipati and Kannuchamy, 2014).

2.4.2. Response Surface Methodology

Response Surface Methodology (RSM) is a collection of mathematical techniques and statistical applications for formulating, improving and optimizing processes (Myers and Montgomery, 1995). This tool has been used by many researchers to optimize gelatin extraction from fish. RSM uses quantitative data in an experimental design to determine, and simultaneously solve multivariate equations, to optimize processes and products (Giovanni, 1983). It has important application in the design, development and formulation of new products. The RSM also helps us to find out the specific levels of the important variables which can produce the optimum responses. Table 2 shows some of the studies on extraction and optimization of fish gelatin using RSM.

Different authors have worked on the extraction of fish gelatin by optimizing the process parameters and observed significant improvements in the response variables, such as gelatin yield, gel strength, melting point, etc. Gudmundsson and Hafsteinsson (1997) have used RSM to study the effect of chemical treatment on gelatin yield from cod skin and improved the yield from 11 % to 14 %. Similarly, Mohtar *et al.* (2010) has employed RSM to optimise gelatin yield from Hoki skin and found that the optimum conditions for extraction were 0.75 M NaCl for 9 min of pre-treatment time and hot water extraction at 49.30°C for 60 min having predicted yield of 17.40 %.

Sanaei *et al.* (2013) used a central composite design, comprising 30 treatments including 24 factorial points, six replicates of the central point, and eight axial points, for extracting catfish bone gelatin. The four independent variables were the concentration of HCl, treatment time, extraction time, and extraction temperature. The catfish bone gelatin yield was improved from 17.52 to 60.54 %, and the gel strength was improved to 230.25 g.

Jakhar *et al.* (2014) has used RSM to extract gelatin from Blackspotted croaker (*Protonibea diacanthus*) skin by following central composite design to determine the optimal conditions of four independent variables namely concentration of NaOH (X_1), soaking time (X_2), extraction temperature (X_3) and extraction time (X_4) for three response variables namely yield, gel strength and melting point. Based on

these models, the optimum conditions to achieve the predicted maximum values were: yield of 17.21 % at $X_1 = 0.23$ %, $X_2 = 46.19$ min, $X_3 = 55.29$ °C and $X_4 = 17.29$ h.

Lizard fish scales were treated with 2 vol (v/w) of alkali solution (0.1-0.9 % NaOH) at room temperature (30°C) for 1-5 h to remove the non-collagen protein and subcutaneous tissue after they were swollen. After the alkali treatment, the scales were neutralized by washing under running tap water until they had a pH of about 7. The scales were then subjected to a final wash with distilled water to remove any residual matter. The extractions were carried out in distilled water at control temperatures within the range of 70-90°C for 1-5 h (Wangtueai and Noomhorm, 2009).

Tabarestani *et al.* (2010) used a central composite rotatable design for the extraction of rainbow trout skin gelatin. In this study, depending on the pretreatment, gelatin recoveries were in the range of 19.80-99.50 % and the optimum pretreatment conditions were found to be 0.148 N concentration of NaOH, 0.181 N concentrations of acetic acid, and 2.71 h pretreatment time, which resulted in a gelatin recovery of 84.30 %.

Table 2: Optimization of variables for gelatin yield by employing Response surface methodology

Gelatin source	Pretreatment /extraction variables	Optimized yield	References
Atlantic cod skin	Pretreatment: NaOH and H ₂ SO ₄ concentration (0.1-0.2%), Citric acid concentration (0.7%)	11-14%	Gudmundsson and Hafsteinsson, (1997)
Lizardfish (<i>Saurida</i> spp.) scales	Pretreatment: NaOH concentration (0.1-0.9%), Soaking time (1-5 h) Extraction: Extraction temperature: (70-90°C), Extraction time: (1-5 h)	10.60%	Wangtueai and Noomhorm, (2009)
Rainbow trout (<i>Onchorhynchus mykiss</i>) skin	Pretreatment: NaOH concentration (0.01-0.21 N), acetic acid concentration (0.01-0.21 N), soaking time (1-3 h)	9.36%	Tabarestani et al. (2010)
Pangasius catfish (<i>Pangasius sutchi</i>) bone	Pretreatment: HCl concentration (1-5%), soaking time (0-32 h) Extraction: extraction temperature (30-90°C), extraction time (2-8 h)	13.86% (w/w).	Mahmoodani et al. (2014)
Catfish (<i>Clarias gariepinus</i>) bone	Pretreatment: HCl (1-5%) pretreatment time (0-32 h) Extraction: Extraction temperatures (30-90°C), Extraction time (2-8 h)	60.54%* (w/w).	Sanaei et al. (2013)
Catfish (<i>Clarias gariepinus</i>) Skin	Pretreatment: NaOH of varying OH ⁻ concentrations (0.07-0.23 mol/l), acetic acid concentrations (0.04-0.14 mol/l), Extraction: Extraction temperatures (40-80°C)	21.79 g/100g	Ardekani et al. (2013)
Blackspotted croaker (<i>Protonibea diacanthus</i>) skin	Pretreatment: NaOH concentration (0.1-0.3 N), soaking time (15-75 min) Extraction: Extraction temperature (35-75°C), Extraction time (8-24 h)	17.21%	Jakhar et al. (2014)
Ribbon fish (<i>Lepturacanthus savala</i>) surimi processing wastes	Pretreatment: citric acid solution (sample: solvent ratio of 1:6 (w/v)) containing bromelain enzyme concentration (0.006-0.124 g/l), soaking time (0.65-4.85 h) Extraction: Extraction temperatures (30-80 °C), Extraction time (1.3-19.7 h)	18.3%	Norziah et al. (2014)
Unicorn leatherjacket (<i>Aluterus monoceros</i>) skin	phosphoric acid (H ₃ PO ₄) concentration (0.15-0.25 M), extraction temperature (40-50°C) and extraction time (4-12 h)	10.58%	Hanjabam et al. (2015)

*based on hydroxyproline content

2.5. Applications of Collagen and Gelatin

Collagen is very important biomolecule. Enormous health benefits of collagen have led to the establishment of collagen supplements industry. The collagen may advance the function of skin dermis and epidermis by increasing the water absorption ability of the outermost skin layer. Collagen supplement can boost up lean muscle gain, decrease recovery time, reconstruct damaged joint structure and improve cardiovascular performances. Among various types, Type I collagen has been extensively used as biomaterial for the development of tissue engineering constructs and wound dressing systems due to its low antigenic and high direct cell adhesion properties (Bhagwat and Dandge, 2016). Gelatin, the product of partial hydrolysis of collagen, finds a variety of applications in the food, photographic and pharmaceutical industries. There are reports of commercial fish collagen products available in the market. Malaysia-based snack food manufacturer, Munchy has introduced fish-derived collagen crackers called “Wheat Krunch Collagen” (Hashim *et al.*, 2015). Also, collagen-infused drinks are on trend in global market. New york-based company Avon has formulated a drink made from salmon skin collagen peptide along with vitamin C and fructo-oligosaccharides (Najumudeen, 2011). Nestle had also released new product, collagen coffee that contained collagen from fish source (Hashim *et al.*, 2015).

2.5.1. Food Applications

The unique hydrocolloid nature of gelatin find extensively applications in food industry, especially in confectionery, low-fat spreads, dairy products, bakery products, meat products, beverages, desserts and ice cream etc. Food-grade fish gelatin is reported to have no or reduced risk to fish-allergic patients (Hansen *et al.*, 2004), demonstrating its potential wide application in the food industry. Furthermore, gelatin has been reported to be one of the first materials used as a carrier of bioactive components (Gomez-Guillen *et al.*, 2011). An example of utilization of functional properties of the gelatin in different food systems is given in Table 3.

Table 3: Functional properties of gelatin and their utility in different products

Functional property	Products
Gelation	Water-gel desserts Jelly confectionery Dairy products Desserts
Emulsifying property	Toffees Low-fat margarines Salad dressings Whipped cream Baked products Cheese spreads Fruit chews
Texturizing property	Flavoring syrups Canned syrups Dairy products
Film-forming property	Hard and soft capsules Edible packaging materials Bioactive coatings
Water-holding capacity	Meat products Canned hams
Stabilizing property	Frozen desserts Icings and glazes Ice creams
Clarifying property	Beverages
Whipping property	Marshmallow Aerated confectionery
Foaming property	Gelatin patch for wound Bandage

Source: Gudipati, 2013.

2.5.1.1. Gelling agent in food products

The most common use of gelatin in food products is in water gel desserts, due to its unique melt-in-the-mouth property. It is a unique protein, having solubility in water and the ability to form a thermoreversible gel. During heating of gelatin solutions, the molecules assume a random coil conformation. Subsequent cooling below a critical temperature causes the native structure to begin to reform, namely the random coils undergo a coil to helix transition (Tosh *et al.*, 2003). Certain other hydrocolloids also have thermoreversible characteristics, but they generally

melt at higher temperatures. Gelatin desserts made from various gelatins may provide variety in texture and gel melting behavior, offering new product development opportunities. Unlike the chemical gels, where the bonds responsible for linking the subunits are covalent chemical bonds, gelatin gels are physical gels formed by physical interactions. Such interactions include van der Waals forces and hydrogen bonds (Jones, 2002; Karim and Bhat, 2009). During aging, the gel gradually assumes a more stable conformation, the final structure of the gel being highly dependent on the gel setting temperature (Djabourov, 1988).

The gelling characteristic of the gelatin is determined by measuring the gel strength also known as bloom value. The bloom value of a gelatin is defined as the weight in grams required to push a 12.5 mm-diameter plunger to a depth of 4mm into a gel surface of 6.67 % (w/w) concentration matured at 10°C for 16-18 h. The higher the bloom value, the stronger the gel. Based on its gel strength or bloom value, gelatin is categorized in terms of low (<150), medium (150-220) and high (220-300) bloom (Johnston-Banks, 1983).

Gelling is also regarded as the 'water-absorbing property' of a given product. Gelatin swells when placed in water, absorbing 5-10 times its own volume. It is highly prized for its ability to gel with water during the production of table jellies. An interesting application of this gelation property is the addition of gelatin to canned ham before cooking. On cooking, the exudates from the meat are absorbed by the gelatin and appear as a gel when the can is opened (Cole, 2000).

Fish gelatin has been evaluated as an additive in surimi, in order to improve the mechanical and functional properties of gels. The results indicate that fish gelatin may be used as a functional additive to produce Alaska pollock surimi gels, but it is not recommended for FA-grade surimi (Hernandez-Briones *et al.*, 2009).

2.5.1.2. Edible films and coatings

Gelatin is known to have good film forming property. Such property can be utilized for developing edible films or coatings. Natural biopolymers have an advantage over synthetic polymers in that they are biodegradable and renewable as well as edible. Gelatin-based film coating or packaging can maintain the quality of foods during storage, due to its barrier to oxygen and light and its prevention of

dehydration and lipid oxidation (Jongjareonrak *et al.*, 2010). Natural biopolymers films have certain limitations due to its poor mechanical and low moisture barriers properties. Many studies have been focused on the modification of natural biopolymer-based films to improve their mechanical and water-vapor barrier properties. Different compounds have been used as active additives in gelatin-based films and coating for enhancement of its properties (Ramos *et al.*, 2016). The film-forming ability of protein can be influenced by amino acid composition, distribution and polarity, ionic cross-links between amino and carboxyl groups, hydrogen bonding, and intra- and intermolecular disulfide bonds (Gennadios and Weller, 1991).

Gelatin films and coating not only protect the foods; it can also be used as a carrier of some active substances such as antioxidants, antifungal agents, antimicrobials, colors and other nutrients (Wong *et al.*, 1994). Essential oils have been used widely in film formation solutions as additives for improvement of films performance.

Martucci *et al.* (2015) developed gelatin-based films by using lavender or oregano essential oils and a mixture of them (50:50) at concentrations ranging between 0 and 6000 ppm. *Escherichia coli* as Gram-negative and *Staphylococcus aureus* as Gram-positive bacteria were selected to evaluate the antimicrobial activity of the new films. The results showed that both microorganisms exhibited sensitivity to all active films, showing lower values of inhibition zone for *S. aureus* compared to *E. coli*, being 10.6 ± 1.5 mm and 13.7 ± 0.5 mm, respectively, at 4000 ppm. Essential oil-based antimicrobial packaging has high potential for food applications (Emiroglu *et al.*, 2010). Applications of gelatin film or coating in different food system have been summarized in Table 4.

Table 4: Utilization of gelatin as films and coating of different food system.

Product	Film/coating Matrix	Processing Method	Final Product	References
Rainbow trout	Cold water fish skin gelatin, chitosan and glycerol	Casting and dipping into matrix solution	Film and coating	Gomez-Estaca <i>et al.</i> (2010)
Cod fillets	Bovine hide gelatin, chitosan, sorbitol and glycerol with clove essential oil	Casting	Film	Nowzari <i>et al.</i> (2013)
Minced trout fillets	Cold water fish skin gelatin, chitosan, glycerol, red grape seed extract and <i>Ziziphora clinopodioides</i> essential oil	Casting	Film	Kakaei <i>et al.</i> (2016)
Rainbow trout fillets	Food grade gelatin, glycerol, sorbitol, Tween 20 and laurel leaf essential oil	Casting	Film	Alparslan <i>et al.</i> (2014)
Tuna meat	Gelatin, red pepper seed meal protein and several plasticizers (glycerol, sorbitol, fructose and sucrose)	Casting	Film	Lee <i>et al.</i> (2016)
Fish sausages	Warm-water fish gelatin, chitosan, shrimp concentrate, Tween 80 and glycerol	Casting	Film	Aleman <i>et al.</i> 2016
Atlantic Salmon	Warm-water fish gelatin, lignin, sorbitol and glycerol	Casting	Film	Ojagh <i>et al.</i> (2011)
Salmon	Porcine skin gelatin, barley bran protein, sorbitol and grapefruit seed extract	Casting	Film	Song <i>et al.</i> (2012)
Cold smoked Salmon	Pork gelatin, chicken feather protein, sorbitol and clove oil	Casting	Film	Song <i>et al.</i> (2014)
Shrimps	Gelatin, glycerol, sorbitol, Tween 20 and orange leaf extract	Dipping into matrix solution	Coating	Alparslan <i>et al.</i> (2016)
Indian salmon fillet	Gelatin, chitosan, lime juice/garlic extract	Dipping into matrix solution	Coating	Thaker <i>et al.</i> (2015)

2.5.2. Pharmaceutical applications

Collagen is widely used for soft tissue (e.g. skin), cartilage, and bone repair. Its intrinsically high water retention capacity has been taken advantage of in creams for treating rough skin. Natural collagen triggers low immune responses, so it is a good substrate for cell adhesion, with good capacity to interact with host tissues (Salgado *et al.*, 2004). Collagen is used in the manufacture of biocompatible glues (which consist of a mixture of collagen with citric acid as a cross-linking agent) that are particularly powerful at binding tissue–tissue interfaces and yield less toxicity than such semi-synthetic and synthetic formulations as gelatin–aldehyde and cyanoacrylate-based glues (Taguchi *et al.*, 2006).

Gelatin also finds medical applications for the preparation of hard and soft capsules, for encapsulation of drugs and bioactive molecules, as an antioxidative hydrolysate, as a carrier in controlled drug delivery, as a component in dietary health supplements and in wound care. Other pharmaceutical applications include its use in tablets, emulsions, ointments, medical glue, salves, granulation and syrups (Gudipati, 2013). Gelatin is also utilized as a matrix for implants, in injectable drug-delivery microspheres and in intravenous infusions (Pollack, 1990; Saddler and Horsey, 1987). It has also been reported that live attenuated viral vaccines used for immunization against measles, mumps, rubella, Japanese encephalitis, rabies, diphtheria and tetanus contain gelatin as a stabilizer (Burke *et al.*, 1999).

Recent trend is to use marine derived collagen and gelatin as wound healing agent. A study reported by Wang *et al.* (2015) revealed that oral administration of chum salmon skin collagen peptides improves wound healing. In another study oral administration of fish skin gelatin was found beneficial for treating wound disorders associated with diabetes (Zhang *et al.*, 2011a).

2.6. Development of Edible Films from Gelatin

Gelatin possesses excellent film forming and barrier properties against oxygen and light (Ahmad *et al.*, 2012). Edible film is defined as a thin, continuous layer of edible material, which can extend the shelf-life and improve the quality of almost any

food system by serving as a barrier to mass transfer or a mechanical damage (Guilbert, 1986; Torres, 1994). Surface films formation method involved two-step, involving the heat denaturation of the proteins in film solutions followed by surface dehydration of the solutions. Film matrix is formed due to intra and inter-molecular interaction between the films forming protein. Heating changes the three-dimensional structure of proteins and the existing functional groups. These, such as CO and NH of peptidic bonds, side chain amine groups and hydrophobic groups, engage in intra-molecular hydrogen bonding and electrostatic interaction (Wang and Damodaran, 1991). During drying, the unfolded proteins approach each other and get linked through intermolecular interactions (disulfide and hydrophobic interaction). This yields the formation of a protein network that acts as the matrix for entrapping film components such as plasticizing agents (Gennadios and Weller, 1991). When the formation of film occurs in denaturated conditions, it is assumed that the protein remains in the fully denaturated state in the film. However, it is quite possible that the denatured protein may undergo partial refolding, thus regaining some secondary structure during the film process. It is conceivable that the extent of such refolding affects the number of functional groups available for intermolecular interactions and thus the formation and stability of the film network (Subirade *et al.*, 1998).

Protein-based edible films have received considerable attention in recent years because of their advantages, including their biodegradability and possibility to use as edible packaging materials, over the synthetic films (Leceta *et al.*, 2014). Protein-based edible films are generally formed from solutions or dispersions of the protein as the solvent/carrier evaporates. The solvent/carrier is generally limited to water, ethanol or ethanol-water mixtures. Generally, proteins must be denatured by heat, acid, bases, and/or solvents in order to form the more extended structures that are required for film formation. Once extended, protein chains can associate through hydrogen, ionic, hydrophobic and covalent bonding. The chain-to-chain interaction that produces cohesive films is affected by the degree of chain extension and the nature and sequence of amino acid residues. The promotion of polymer chain-to-chain interaction results in films that are stronger but less flexible and less permeable to gases, vapors and liquids. Polymers containing groups that can associate through hydrogen or ionic bonding result in films that are excellent oxygen barriers but susceptible to moisture. Thus, protein films are expected to be

good oxygen barriers at low relative humidity. Because of the poor water vapor resistance of protein-based edible films and their lower mechanical strength in comparison with synthetic polymers their application is limited in food packaging. The improvement of protein-based edible films properties has been investigated to seek suitable applications (Wittaya, 2012).

Guilbert (1986) studied that gelatin films could be formed from 20-30 % gelatin, 10-30 % plasticizer (glycerol or sorbitol) and 40-70 % water followed by drying the gelatin gel. There are two main processes i.e. casting and extrusion (Hernandez-Izquierdo and Krochta, 2008). The film-formation process most often reported in the scientific literature is the casting method. Briefly, it involves dissolving the biopolymer and blending it with plasticizers and/or additives to obtain a film-forming solution, which is cast onto plates and then dried by driving off the solvent. The extrusion method relies on the thermoplastic behavior of proteins at low moisture levels. Films can be produced by extrusion followed by heat-pressing at temperatures that are ordinarily higher than 80°C. This process may affect film properties, but its use would enhance the commercial potential of films by affording a number of advantages over solution-casting, e.g., working in a continuous system with ready control of such process variables as temperature, moisture, size/shape, etc. (Hernandez-Izquierdo and Krochta, 2008).

Gontard *et al.* (1992) suggested that an edible film must withstand the normal stresses encountered during its application, subsequent shipping and handling of the food, to maintain its integrity and also barrier properties. Tensile Strength is a measure of integrity and heavy-duty use potential of films, and Elongation at Break is a quantitative representative of the film's ability to stretch (Gennadios *et al.*, 1993). Guilbert *et al.* (1996) reported that films composed primarily of proteins or polysaccharides have suitable overall mechanical and optical properties but are highly sensitive to moisture and exhibit poor water vapour barrier properties. Andreuccetti *et al.* (2009) studied the effect of hydrophobic plasticizer on functional properties of gelatin-based films.

The main function of food packaging is to protect the food product from the surrounding environment and to maintain the quality of the packaged food throughout its shelf-life. For packaging materials that come in direct contact and/or

interact with the packaged food, additional requirements need to be fulfilled. These include acceptable sensory properties, adequate biochemical, physicochemical, and microbial stability, absence of toxics and safety (Debeaufort *et al.*, 1998).

2.6.1. Methods to enhance the properties of gelatin films

The interconnection of protein molecules during the drying process leads to the formation of film matrix and the extension or unfolding of protein molecule may favor the interaction among the molecules (Hoque *et al.*, 2010). Though proteins are promising biomaterials for films formation, it lacks mechanical strength and has poor water vapor barrier property because of their hydrophilic nature (McHugh and Krochta, 1994). Generally, gelatin films get swollen when comes in contact with the surface of foodstuff with high moisture content, due to its highly hygroscopic nature. In this context, several authors have worked on to improve the properties of gelatin films by adding different substances such as crosslinkers, strengthening agents and other additives with antimicrobial or antioxidant properties (Ramos *et al.*, 2016).

The presence of reactive functional groups in the amino acid side chain of protein makes this cross-linking process possible through chemical, enzymatic or physical treatments. Chemical agents used for covalent cross-linking of protein have included glutaraldehyde, glyceraldehyde, formaldehyde, glyoxal and others (Hernandez-Munoz *et al.*, 2004; Orliac *et al.*, 2002). Most of these agents, not being edible, impose some limits on their use for edible packaging. Physical cross-linking such as UV- and γ -irradiation (Ouattara *et al.*, 2002; Rhim *et al.*, 1999) are increasingly becoming accepted in the food industry (Pszczola, 1997). Among enzymes, transglutaminase has received extensive attention recently for its ability to cross-link proteins.

2.6.1.1. Chemical cross-linking

Chemical agents used for the covalent cross-linking of proteins have included aldehydes such as glutaraldehyde, glyoxal or formaldehyde and glyceraldehyde (Bourtoom, 2009). Among the chemical cross-linking agents used in the protein films, formaldehyde is the most effective, and has the widest reaction specificity, because of its low molecular weight it can easily migrate between the

protein chains and create covalent linkages with the cysteine, histidine and lysine side groups of proteins (Galiotta *et al.*, 1998). Protein cross-linking by glyoxal involves lysine and arginine side chain groups (Marquie, 2001) at alkaline pH.

de Carvalho and Grosso (2006) studied the effect of cross-linking using formaldehyde and glyoxal on properties of gelatin films. Films reticulated with formaldehyde and glyoxal had higher strengths, lower water vapor permeabilities and higher opacities than the untreated films. Formaldehyde-based films showed a significant increase in strength as a function of the increase in concentration of the reticulate agent in comparison with glyoxal-modified films. In addition to amines, formaldehyde reacts with sulphhydryl, phenolic, imidazolyl, indolyl and guanidinyl groups. Some researchers have also reported the formation of methylene bridges between lysine and tyrosine in formaldehyde-treated protein films (Hernandez-Munoz *et al.*, 2004). However, although highly reactive such chemical crosslinkers, also have a major disadvantage i.e their toxicity (Bigi *et al.*, 2002).

2.6.1.2. Natural cross-linking

Because of the toxicity of aldehydes, many researchers have been trying to use the natural cross linking agents to improve the protein film properties. Recent research on biopolymer-based films, have investigated the incorporation of plant extracts to improve the biological activity of films (Kadam *et al.*, 2015). Plant extracts have a wide range of biological activities and possess generally recognized as safe (GRAS) status (Wang *et al.*, 2012).

Araghi *et al.* (2015) investigated the properties of cold water fish gelatin films modified by natural cross-linker such as ferulic acid and caffeic acid. Results showed that solubility, O₂ permeability and water permeability have been reduced in gelatin film containing caffeic acid at concentration of 5 %. In film containing ferulic acid, solubility was decreased by increase concentration but no significant change was observed in O₂ permeability and water vapor permeability. With respect to importance of barrier and physicochemical properties for packaging products, caffeic acid is a more effective phenolic compound rather than ferulic acid.

Fish skin gelatin films incorporated with 6 % oxygenated seaweed (*Turbinaria ornata*) extract (based on protein content) with pH of 9 or 10 were

prepared. Films incorporated with seaweed extract at both pH exhibited the higher elongation at break than the control film. However, no differences in tensile strength and transparency between films without and with seaweed extract were observed. Water vapor permeability, and film solubility decreased as seaweed extract was incorporated, regardless of pH. The addition of seaweed extract had the impact on the property of fish skin gelatin film, most likely due to the enhanced formation of protein cross-links (Rattaya *et al.*, 2009).

2.6.1.3. Enzymatic cross-linking

The improvement of barrier properties, mechanical strength and other attributes of protein based edible films can also be achieved by enzymatic cross linking. Some enzymes that have been used for cross linking proteins include transglutaminase, lipoxygenase, lysyl oxidase, polyphenol oxidase and peroxidase. Among the said enzymes, transglutaminase is widely used (Gomez-Guillen *et al.*, 2011).

Transglutaminase is considered as safe and effective cross-linking agent (GomezGuillen *et al.*, 2011). Transglutaminase can catalyze the formation of ϵ -(γ -glutamyl)-lysine crosslinks in proteins via an acyl transfer reaction, in which the γ -carboxyamide group of glutamine serves as the acyl donor and the ϵ -amino group of lysine serves as the acyl acceptor. As a result, intra- and inter-molecular covalent cross-linkages within the protein molecules are formed (Motoki and Seguro, 1998).

A study by Yi *et al.* (2006) revealed that incorporation of transglutaminase in cold water fish gelatin films plasticized with sorbitol increases tensile strength and oxygen permeability and decreases elongation of the films. The authors explained that triple-helix structures present in gelatin molecules makes the gelatin matrix compact, which results in low oxygen permeability. Cross-linking with transglutaminase resulted in intra and intermolecular covalent bonds, which caused the increase in film strength; however, the cross-links hindered the helical structure formation, hence the decrease in the flexibility of the gelatin matrix.

Kolodziejska and Piotrowska (2007) also reported that cod gelatin-chitosan films cross-linked by TG had increased film brittleness. Adding up to 30 % glycerol decreased tensile strength and increased elongation but did not change the

solubility or water vapour permeability of transglutaminase-modified cod gelatin-chitosan films. The authors described that the hydrophilic nature of the added plasticizer could mask the effect of the cross-linking induced by the enzyme.

In a study conducted by Wangtueai *et al.* (2010), the films of lizard scale gelatin with 0.5 % microbial transglutaminase had higher tensile strength than the control films. The authors explained the possible effect of cross linking by the enzyme.

Jiang and Tang (2013) study the effects of transglutaminase on sorption characteristics including adsorption kinetics and sorption isotherm, mechanical properties, moisture content, total soluble matter and surface hydrophobicity of gelatin films. Transglutaminase treatment significantly decreased moisture adsorption rate and balance moisture content of gelatin films. Transglutaminase treatment increased the tensile strength value and the mean contact angle by 8.4-25.6 % and 2.1-2.3°, respectively; while decreasing the moisture content value by 2.6-9 %. The authors suggested that transglutaminase treatment could be an effective and practical method to modify moisture sorption characteristics, moisture content and surface hydrophobicity of gelatin films, thereby improving the water-barrier ability of gelatin films.

Recently, Weng and Zheng (2015) investigated the effect of transglutaminase on the properties of tilapia scale gelatin films in the presence of soy protein isolate. When 3 % transglutaminase was added into gelatin films, the total soluble matter and protein solubility of films were decreased from 89.36 % and 92.78 % to 35.83 % and 40.05 %, respectively, and the decline was promoted by adding 5 % soy protein isolate. The strength of the films was increased by adding 1 % transglutaminase irrespective of soy protein isolate addition, but decreased when the transglutaminase concentration was further raised.

2.6.1.4. Irradiation cross-linking

Inducing cross linking through irradiation for the improvement of both barrier and mechanical properties of protein-based edible films has been tried. Proteins can be converted to higher molecular weight aggregates through the generation of inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and the formation of disulfide bonds (Davies and Delsignore, 1987). For example, the hydroxyl and superoxide anion radicals that are generated by the radiation of film-forming solutions can modify the molecular properties of proteins. This can result in the alteration of the protein films by covalent cross linkages formed in the protein solution after irradiation (Garrison, 1987).

Few studies have used irradiation technology in investigating the release kinetics of antioxidant incorporated in film forming solutions. The influence of irradiation dose (40 and 60 kGy) on the retention of quercetin in the chitosan-gelatin edible films revealed that irradiation induced a reduction of the release rate for quercetin, revealing that cross-linking probably occurred during irradiation between biopolymers and the quercetin leading to an entrapment of a significant amount of the antioxidant (Benbettaieb *et al.*, 2016). A study on effect of electron beam accelerator doses on properties of plasticized fish gelatin film shows that free radical formed during irradiation induce intermolecular cross-linking. The tensile strength for gelatin film significantly increases after irradiation (improved by 30 % for 60 kGy). Also irradiation enhances the thermal stability of the gelatin film, by increasing the glass transition temperature and the degradation temperature (Benbettaieb *et al.*, 2016).

UV light is also being used as cross linker of protein films. UV irradiation generates radicals at the aromatic residues of collagen and gelatin amino acids such as tyrosine and phenylalanine. The binding of these radicals will react to each other, resulting in cross-linking (Fujimori, 1965; Ozeki and Tabata, 2005). Ozeki and Tabata (2005) also reported that cross-linking density of gelatin largely changed depending on UV irradiation time. When the irradiation time was short, UV irradiation would enable gelatin to cross-link intermolecularly. However, it is possible that irradiation for longer time preferably acts on the chain scission of gelatin molecules.

A study conducted by Otoni *et al.* (2012) on the effect of UV radiation on cold-water and warm-water fish gelatin reported that the cold-water fish gelatin samples made from irradiated gelatin granules showed greater tensile strength in comparison to the warm-water gelatin films made from irradiated gelatin granules but better water vapor barrier properties. This might be due to the UV induced cross-linking in warm-water gelatin that disrupted helical structures. The authors also reported that cold-water fish gelatin films exhibited greater tensile strength at higher UV doses greater than 14.9 Jcm^{-2} .

Recently, pulsed light have been used to induce dissociation and partial unfolding of whey protein isolate (Siddique *et al.*, 2016). Pulsed light is a non-thermal technique to decontaminate surfaces by killing microorganisms using short pulses of an intense broad spectrum, rich in UV light (Gomez-Lopez *et al.*, 2007). Pulse light technology has been successfully applied to inactivate a number of pathogenic and spoilage microorganisms *in vitro* or in food products (Oms-Oliu *et al.*, 2010). Only few studies have been published discussing the cross-linking effect of pulsed light on carbohydrate films (Gutierrez, 2017). Till date, no previous studies have characterized the effect of pulse light treatment on fish gelatin films.

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3. MATERIALS AND METHODS

3.1. Raw Material

Pink perch (*Nemipterus japonicas*) surimi refiner discharge was collected from a commercial surimi processing plant (Ulka Seafoods Pvt. Ltd., Maharashtra, India) and transported in ice to the laboratory where it was kept frozen at -30°C until used for study.

3.2. Chemicals

All the chemicals used were of analytical grade and purchased from various companies such as Sigma, Merck, Nice, SRL and Qualigens.

3.3. Composition Study

Proximate composition of refiner discharge was performed as per AOAC (2005) method.

3.3.1. Moisture

Moisture content in the refiner discharge was determined by oven drying at 100±5°C. Moisture percentage was calculated from the weight difference.

3.3.2. Crude protein

Crude protein content in the refiner discharge was determined by using Micro-Kjeldahl unit. Briefly, 0.5 g of refiner discharge was taken in a clean and dry Kjeldahl's digestion flask and digested with 1.6 g of digestion mixture (K₂SO₄ and CuSO₄ in 5:1 ratio) in 20 ml concentrated H₂SO₄ till the solution become clear. Digested sample was diluted to 250 ml and 5 ml of it was added in the distillation chamber and the liberated NH₃ was collected in a conical flask containing 10 ml of 2 % Boric acid and Toshiro's indicator. The amount of NH₃ liberated was determined by titrating with 0.1 N H₂SO₄. Crude protein content was calculated by multiplying the total nitrogen content with conversion factor of 6.25 and expressed as percentage.

3.3.3. Crude fat

The crude fat content in the refiner discharge was determined by Soxhlet extraction method. About 1 g of moisture free refiner discharge was taken in a Whatman thimble. The thimble was plugged with cotton loosely and placed in a Soxhlet extraction unit. Petroleum ether (40-60°C boiling points) was used as solvent. Extraction was continued for 16 h. After the extraction, the pre-weighed receiver flask containing the extracted fat was dried initially on a water bath at $98\pm 2^{\circ}\text{C}$. After complete drying, the receiver flask was cooled in desiccators and the weight was obtained. The difference in the initial and final weight of receiver flask was determined as crude fat and expressed as percentage.

3.3.4. Ash

The ash content in the refiner discharge was determined by using Muffle furnace (Phoenix CEM Corporation, USA). Briefly, 5 g of the sample was taken in a previously ignited and weighed silica crucible. It was then transferred to a muffle furnace and the temperature was raised to 600°C and kept for 6 h until white ash was obtained. Weight was taken after cooling and the percentage of ash was calculated from the weight difference.

3.3.5. Constitution Ratio of Refiner Discharge

The constitution ratio of refiner discharge was measured following the method described by Kim and Park, (2005) with slight modifications. Thawed refiner discharge was stirred in 20 ml (v/w) of 0.2 M NaOH for 1 day at 4°C and the solution was changed every day to remove the non-collagenous proteins. The mixture was centrifuged at 20,000 g for 20 min. The residue was thoroughly washed with cold distilled water and soaked in filter paper to remove water. The objectionable matter like skin, bone and scale were removed by hand picking and dried at $100\pm 5^{\circ}\text{C}$ for 2 h. The constitution ratio of each objectionable matter was calculated by the following equation:

(Weight of dried objectionable matter) / (Weight of total refiner discharge based on dry wet) x 100 %

3.4. Extraction of Collagen from Refiner Discharge

Collagen was extracted from refiner discharge following the procedure described by Duan *et al.* (2009) (Figure 5). Refiner discharge was stirred in 0.2 M NaOH for 24 h at a sample/alkali solution ratio of 1:10 (w/v) to remove non collagenous proteins. The alkali solution was changed every 12 h. Then the refiner discharge was washed with cold potable water until neutral pH of wash water was obtained. The refiner discharge was then decalcified with 0.5 M EDTA at sample/acid solution ratio of 1:10 (w/v) for 48 h, and then washed with cold potable water. The residue was extracted with 0.5 M acetic acid at sample/acid ratio of 1:5 (w/v) for 4 days. The extract was centrifuged at 20,000 g for 1 h. The supernatant was salted out by adding NaCl to a final concentration of 2.4 M. The resultant precipitate was redissolved in 0.5 M acetic acid and dialysed against distilled water for 2 days (Plate 1). All the procedures were performed at temperature of 4°C. The dialyzed solution was lyophilized and referred to as collagen. The extract yield was calculated from the percentage of dry weight of collagen extracted in comparison with the wet weight of the initial refiner discharge.



Plate 1: Collagen precipitate

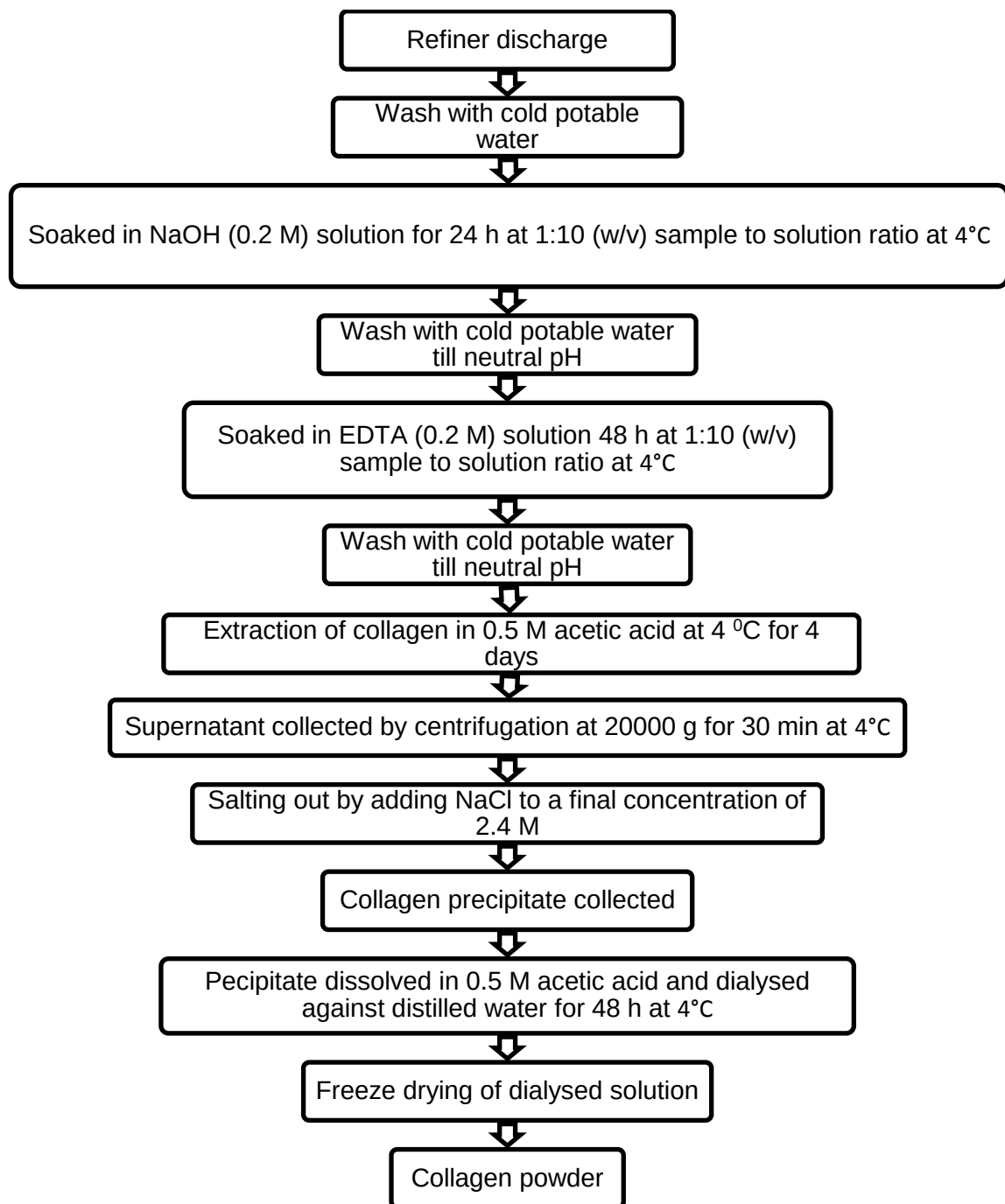


Figure 5: Flow chart of the experimental process for extraction of collagen from refiner discharge

3.5. Characterization of Refiner Discharge Collagen

3.5.1 Proximate composition and hydroxyproline content

The proximate composition of collagen from refiner discharge was carried out as per section 3.3. However, the conversion factor of 5.95 was used for crude protein estimation of collagen.

Hydroxyproline content of collagen was determined according to the method of Bergman and Loxley (1963) with slight modifications. Defatted collagen powder was hydrolyzed at 110°C for 24 h in a test tube with 6 N HCl (10 mg collagen/ml). The residue was then dissolved and made up to 10 ml with 0.05 M HCl and then filtered through a membrane filter (Thermo Scientific, MK, England) with a nominal pore size of 0.45 µm. 50 µl of the filtrate was diluted with 2.25 ml of distilled water. To this 0.5 ml of 0.1 M buffer (pH 8.7) was added and mixed well. 2 ml of freshly prepared 0.2 M chloramine T solution was added and allowed to oxidize for 25 min. Upon completion Ehrlich's reagent was added and incubates at 60°C for 25 min. Absorbance was measured against water at 560 nm. A hydroxproline (SRL, Mumbai) standard solution ranging from 0.75 µg to 6.0 µg was also prepared in the same manner except hydrolysis step (Annexure 1). Hydroxyproline content was calculated and expressed as mg/g collagen.

3.5.2. Physical Characterization of Refiner Discharge Collagen

3.5.2.1. Determination of collagen colour

Colour measurement was made by using a Lab Scan XE spectrophotometer (Hunter Lab scan XE, USA) according to the method of CIE (1986) and it was calibrated to white and black standard sites of sample. The tristimulus $L^*a^*b^*$ measurement mode was used as it relates to the human eye response to colour. The L^* variable represents lightness ($L^*=0$ for black, $L^*=100$ for white), a^* scale represents the red/green ($+a^*$ intensity in red and $-a^*$ intensity in green) and b^* scale represents the yellow/blue ($+b^*$ intensity in yellow and $-b^*$ intensity in blue). The collagen was filled into the glass sample cup and kept in the port and readings were then taken.

3.5.2.2. Thermal Stability Analysis of Collagen

The study of the thermal property of the collagen was performed using a Differential scanning calorimeter (DSC) (TA DSC 250), USA (Plate 2) according to the method of Liu *et al.* (2012). The collagen sample was weighed into aluminium pans, and hermetically sealed and scanned from 20 to 200°C with ramp of 5 °C/min. The instrument was calibrated for temperature and enthalpy using indium as the standard. An empty hermetically sealed aluminum pan was used as the reference and the measurements were done while samples were constantly purged with ultrahigh-purity nitrogen at 50 ml/min. The transition temperatures were recorded by the software as the peak temperature of each endothermic peak and the total transition enthalphy (ΔH) for each peak was determined by measuring the corresponding area under each endothermic peak.



Plate 2: Differential Scanning Calorimeter

3.5.3. Chemical Characterization of Refiner Discharge Collagen

3.5.3.1. Amino acid composition of collagen

Amino acid composition of collagen was performed according to Ishida *et al.* (1981). 100 mg of defatted collagen was hydrolyzed at 110°C for 24 h in a test tube with 10 ml of 6 N HCl. After the hydrolysis, the filtrate was flash evaporated to dryness to remove traces of HCl. The residue was then dissolved and made up to 10 ml with 0.05 M HCl and then filtered through a membrane filter (Thermo Scientific, MK, England) with a nominal pore size of 0.45 μm . The

sample (10 μ l) was injected into the amino acid analyzer (HPLC–LC 10 AS, Hitachi L-2130 Elite La Chrome, Japan). The identification of amino acids in the sample is done using D-2000 Chromatography Data Station Software.

3.5.3.2. Structural Analysis of Collagen

Fourier transform infrared spectroscopy (FTIR) was used to characterize the presence of amide bonds by scanning over the range of 4000-400 cm^{-1} by using a Thermo Fisher Scientific FT-IR spectrometer (Model Nicolet™ iS™ 10, Thermo Fisher Scientific, Waltham, MA). A background spectrum was collected by keeping the resolution at 4 cm^{-1} .

3.5.3.3. Protein Pattern of Collagen

SDS-PAGE of the extracted gelatin was done according to the method of Laemmli (1970). Gelatin was dissolved in 5 % SDS and heated at 80°C for 30 min followed by centrifugation (HERAEUS multifuge X1R, ThermoScientific,) at 3500 x g for 5 min. The protein was diluted to 1:1 (v/v) with sample buffer (0.5 M Tris HCL, pH 6.8 containing 10 % SDS, 10 % glycerol and β -mercaptoethanol) in microcentrifuge tube and boiled for 3 min at 100°C. 5 μ l of the solution containing 15 μ g of protein (protein content determined by Biuret method, Annexure 2) was loaded onto the polyacrylamide gels (7.5 % separating gel and 4 % stacking gel) and electrophoresis was done at a constant current of 15 mA/gel using a Mini Protein II unit (Bio-Rad Laboratories, USA) (Plate 3). After electrophoresis, the gel was stained with 0.05 % (w/v) Coomassie blue R-250 in 15 % (v/v) methanol and 5 % (v/v) acetic acid for 30 min and destained with 30 % (v/v) methanol and 10 % (v/v) acetic acid for 2 h. Wide range molecular weight protein marker (Sigma Chemical Co., St. Louis, MO, USA) with a molecular weight range of 6500-200,000 Da was used to estimate the molecular weight of proteins.



Plate 3: Mini Protein II Unit

3.5.4. Functional Characterization of Refiner Discharge Collagen

3.5.4.1. Water and oil absorption capacities of collagen

Water and oil absorption capacities were determined by the methods of Kim and Park (2004). Freeze dried collagen (0.3 g) was mixed with 10 ml distilled water for water absorption measurement (Plate 4). Mixing was done at fast speed using a vortex mixer for 15 sec and allowed to stand at room temperature for 30 min before centrifuging at 10,000 g for 30 min. The volume of supernatant was measured in a graduated cylinder, and value was expressed on a dry weight basis.

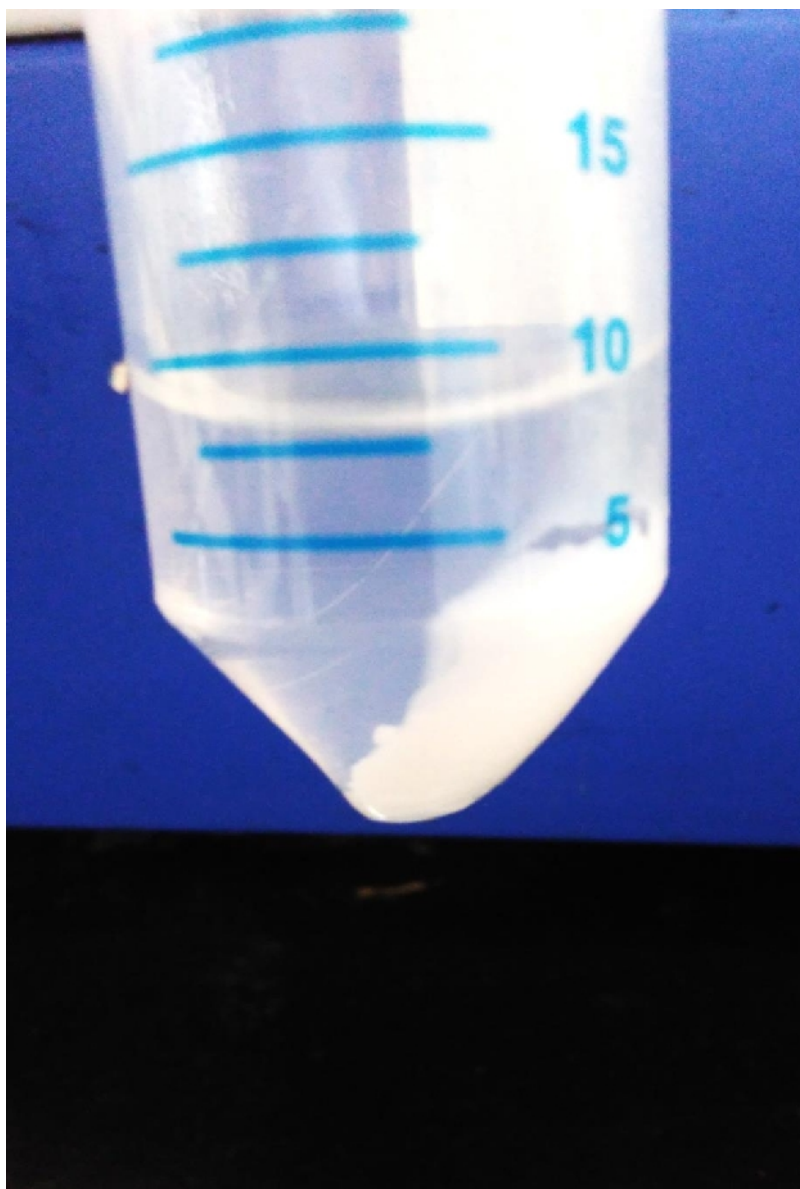


Plate 4: Measurement of water absorption capacity of collagen

For oil absorption capacity freeze dried collagen (0.3 g) was mixed with 10 ml vegetable oil for oil absorption measurement. Mixing was done at fast speed using a vortex mixer for 15 sec and allowed to stand at room temperature for 30 min before centrifuging at 10,000 g for 30 min. The volume of supernatant was measured in a graduated cylinder, and value was expressed on a dry weight basis.

3.5.4.2. Emulsifying Activity and Stability of Collagen

Emulsifying activity was determined by the method described by Kim and Park (2004) with slight modifications. Freeze dried collagen (0.2 g) was added to 20 ml of 0.5 M acetic acid and homogenized for 2 min. 20 ml vegetable oil was added to the mixture and homogenize again by a homogenizer at room temperature for 3 min. The blended sample was divided equally into 50 ml centrifuge tubes. One centrifuge tube was directly centrifuged at 1300 g for 15 min while the other was centrifuged under the same conditions after heating in a water bath at 80°C for 30 min and cooling to room temperature. Emulsifying activity was expressed as the following equation:

$$(\text{Height of emulsified layer} / \text{Height of total contents in the tube}) \times 100 \%$$

Emulsion stability was expressed as the following equation:

$$(\text{Height of emulsified layer after heating} / \text{Height of total contents in the tube}) \times 100 \%$$

3.5.4.3. Foaming Capacity and Stability

Foaming capacity and foaming stability of collagen were determined using the Balti *et al.* (2010) method with slight modifications. 20 ml of (0.2 g) collagen solution in 0.5 M acetic acid were homogenized for 1 min at room temperature in a 50 ml graduated cylinder and the total volume of whipped foam was measured at 0 and 30 min. Foaming capacity at 0 min was calculated according to the following equation.

$$FC(\%) = \frac{V_T - V_0}{V_0} \times 100$$

Foaming stability was calculated as the volume of foam remaining after 30 min.

$$FS(\%) = \frac{V_t - V_0}{V_0} \times 100$$

Where, V_T is the total volume after whipping (ml); V_0 is the volume before whipping; V_t is the total volume after leaving at room temperature for 30 min.

3.5.4.4. Solubility of Collagen

Collagen solubility was determined with the method of Singh *et al.* (2011) with a slight modification. The collagen was dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/ml and the mixture was stirred at 4°C for 1 h until collagen was solubilized.

3.5.4.4.1. Effect of pH

pH of collagen solutions (8 ml) of 3 mg/ml were adjusted to give the final pH of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 either with 6 N NaOH or 6 N HCl. The volume of the solution was made up to 10 ml by distilled water previously adjusted to same pH. The solution was kept at 4°C for 30 min followed by centrifuge at 10,000 g for 30 min at 4°C. Protein content in the supernatant was determined by the Biuret method (Annexure 2) using bovine serum albumin as a standard.

3.5.4.4.2. Effect of NaCl

Collagen solutions (5 ml) of 3 mg/ml were mixed with 5 ml of NaCl in 0.5 M acetic acid at various concentrations [0, 2, 4, 6, 8, 10 and 12 % (w/v)] to give the final concentration of 0, 1, 2, 3, 4, 5 and 6 %. The mixture was kept at 4°C for 30 min followed by centrifugation at 10,000 g for 30 min at 4°C. Protein content in the supernatant was determined by the Biuret method using bovine serum albumin as a standard (Plate 5).

Protein solubility was calculated using the following equation:

$$\text{Solubility} = \frac{\text{Protein content of the supernatant}}{\text{Total protein content in the sample}}$$

$$\text{Relative solubility} = \frac{\text{Solubility at given pH or NaCl concentration}}{\text{Highest solubility in the various pH or NaCl range}}$$

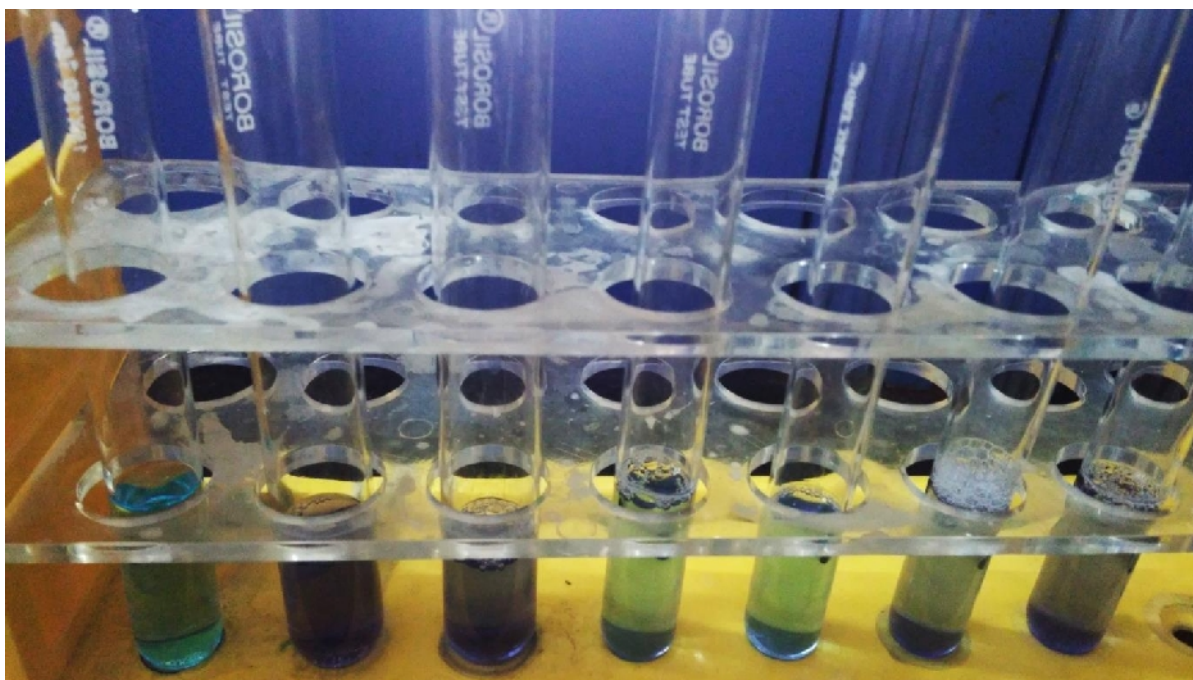


Plate 5: Estimation of protein content in collagen solutions with different NaCl concentration

3.6. Gelatin Extraction and Process Optimization for its Yield

3.6.1. Gelatin extraction from refiner discharge

Gelatin was extracted from refiner discharge following the methods described by Wang and Regenstien (2009) with slight modifications (Figure 6). Refiner discharge was soaked in 0.20-0.60 M NaOH with a sample/solution ratio of 1:10 (w/v). The mixture was kept for 2 h at room temperature with occasional stirring and the alkaline solution was changed every 1 h interval in order to remove non collagenous proteins and pigments. The treated sample was then washed with potable water until neutral or faintly basic pH of wash water was obtained. The sample was then soaked in 0.20 M EDTA with a sample/solution ratio of 1:10 (w/v) for 2 h under same conditions as described to swell the collagenous material. Acid pre-treated sample was washed thoroughly with potable water until wash water became neutral or faintly basic. To extract gelatin, the swollen sample was soaked in distilled water with a sample/water ratio of 1:3 (w/v) in a temperature-controlled water bath at 50–70°C for 3–5 h with an occasional stirring. The mixture was then filtered using cheese cloth. The filtrate was further filtered using a Whatman No. 4 filter paper and the filtrate was

concentrated in Rotary evaporator at 45°C until the volume become half followed by drying the solution in vacuum dryer for 12 h. The yield of gelatin was calculated based on the weight of dry gelatin in comparison with that of wet refiner discharge weight.

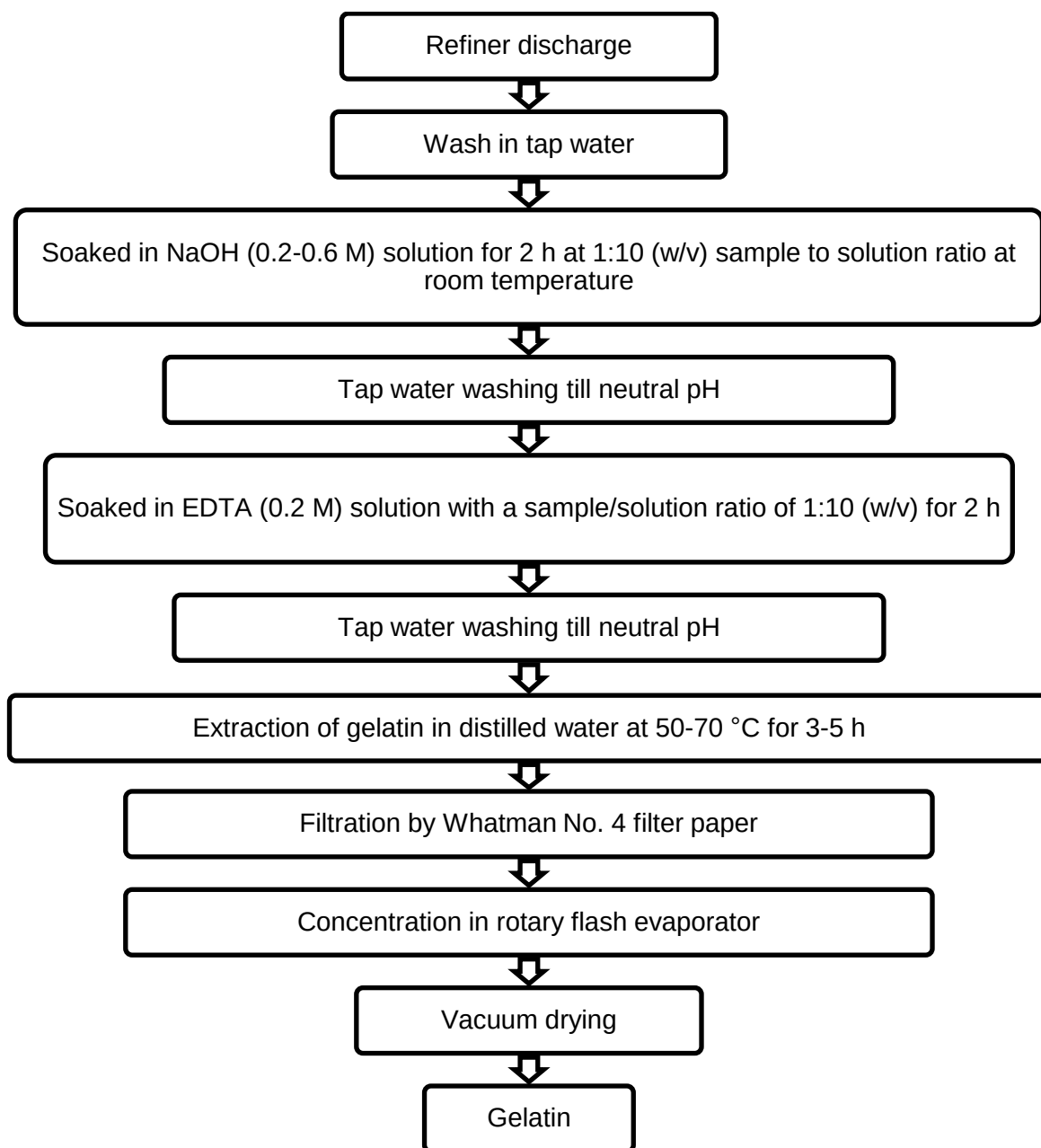


Figure 6: Flow chart of the experimental process for extraction of gelatin from refiner discharge

3.6.2. Experimental Design

To optimize gelatin extraction from refiner discharge, Response Surface Methodology (RSM) was adopted. A Box-Behnken Design was employed to determine the effects of the independent variables on the yield of gelatin extraction. The three independent variables i.e molar mass of NaOH (A), hot water extraction temperature in °C (B) and extraction time in h (C) were coded to three different ranges of -1, 0, +1. The design provides 15 groups of conditions during the optimization with 3 replicates for the central point. RSM software helps to understand the linear, square and interactive effects of the independent variables on the response variable to generate the optimal conditions for achieving maximum response. The experimental design is shown in Table 5. Gelatin yield is the dependent variable.

Table 5: Experimental design of independent variables in the Box-Behnken design for gelatin extraction from refiner discharge

Process	Independent variables	Symbol	Range and levels		
			-1	0	+1
Pre-treatment washing	NaOH concentration (M)	A	0.02	0.04	0.06
Hot-water extraction	Extraction temperature (°C)	B	55	65	75
	Extraction time (h)	C	3	4	5

3.7. Characterization of Refiner Discharge Gelatin

3.7.1. Proximate composition and hydroxyproline content of gelatin

Proximate composition such as moisture content, crude protein, fat and ash content of gelatin were estimated as per the procedure described in section 3.3. The conversion factor of 5.95 was used for protein estimation of gelatin.

The hydroxyproline content was determined as per procedure described in section 3.5.1.

The pH of 1 % (w/v) gelatin solution was measured using a digital pH meter (pH tester, HANNA, USA). The pH was measured after calibrating the electrode of pH Meter with 4 and 7 pH Standard buffers.

3.7.2. Physical Characterization of Refiner Discharge

3.7.2.1. Determination of gelatin colour and gel clarity

The colour measurement of the gelatin powder was performed as per the procedure described in section 3.5.2.1.

Gel clarity was determined by following the method described by Avena-Bustillos *et al.* (2006). A 6.67 % (w/v) gelatin solution was prepared and determined the gel clarity by measuring transmittance (%T) at 620 nm in spectrophotometer (Spectronic Instruments Inc., Rochester, N.Y., USA).

3.7.2.2. Bloom value of gelatin

Bloom strength was measured according to the method described by Gelatin Manufacturers Institute of America (GMIA, 1986). A 6.67 % gelatin solution was prepared by dissolving 7.50 g gelatin powder in 105.0 ml distilled water and heated in a Bloom (Schott Duran, Germany) (150 ml capacity) jar at 60°C for 30 min in a water bath (Plate 6). The solution was kept at 10°C for 16 h for gel maturation. The bloom strength of the gel at 10°C was determined using a texture analyser (Lloyd Instruments, Model LRX Plus, Sussex, U.K.) with 5 kg load cell and 1.27 cm diameter cylindrical delrin probe at 1 mm/s test speed. The

gel strength was expressed as maximum force (in g), required for the plunger to penetrate by 4 mm into the centre of gelatin gels.



Plate 6: Measurement of bloom value

3.7.2.3. Viscosity of gelatin

The viscosity of the gelatin (6.67 % concentration at 60°C) was measured using a Brookfield digital viscometer (model DV-E, Brookfield Engineering, Middleboro, MA, USA) equipped with a No. 1 spindle at 30±0.5 °C. The measured values were obtained directly in centipoise (cP) from the instrument.

3.7.2.4. Melting Point of Gelatin

Determination of melting point was based on the JIS K6503 (JSA, 1996) method with slight modification. A 6.67 % (w/v) gelatin solution was prepared in thin walled screw cap test tubes. The test tubes were held in a refrigerator (7°C) for 16 h, after which they were transferred into water bath (10°C). One or two drops of mixture of 75 % chloroform and 25 % reddish brown dye (food colour) were added in all test tubes to observe the melting point. The

temperature of water bath (Haake D3) was increased gradually at the rate of 1°C per min from its initial temperature of 10°C. The temperature was read using an electronic digital thermometer (Fischer Scientific, Germany). The water of bath was constantly stirred manually using a spatula in order to maintain uniform temperature of bath. The temperature at which the drops began to move freely down the gel was taken as the melting point.

3.7.2.5. Setting point and setting time of gelatin

Setting point and setting time of gelatin was performed by following the method of Muyonga *et al.* (2004b). Gelatin solutions of 10 % (w/v) were prepared by dissolving dry gelatin in distilled water at 60°C for 30 min. The dissolved gelatin solution is transferred in thin wall (12×75 mm) test tubes. For setting point determination, the test tubes were transferred to a circulating water bath held at 40°C (Haake D3). The bath was then cooled at the rate of 2°C/min. A thermometer was inserted into the test tube and lifted out at 15 s intervals. The temperature at which the gelatin solution no longer dripped from the tip of the thermometer was recorded as the setting temperature.

For setting time determination another test tube with dissolved gelatin solution was transferred to the water bath maintained at 10°C (Haake D3). A glass rod was inserted into the gelatin solution and raised at intervals of 15 sec. The time at which the glass rod could not detach from the gelatin solution was recorded as the setting time.

3.7.2.6. Thermal Analysis of Gelatin

The study of the thermal characteristics of the gelatin was performed as per the procedure described in section 3.5.2.2.

3.7.3. Chemical Characterization of Refiner Discharge Gelatin

3.7.3.1. Amino acid composition of gelatin

The amino acid composition of gelatin was performed as per the procedure described in section 3.5.3.1.

3.7.3.2. Structural Analysis of Gelatin

The study of the thermal property of the gelatin was performed as per the procedure described in section 3.5.3.2.

3.7.3.3. Protein Pattern of Gelatin

Molecular weight distribution of gelatin was performed as per the procedure described in section 3.5.3.3.

3.7.3.4. Mineral Composition of Gelatin

For minerals analysis, 1 g of the gelatin powder was digested with 8 ml of concentrated nitric acid (Trace-Metal™ Grade, Fisher Scientific) and 2 ml of hydrogen peroxide (30–32 %, Optima, Fisher Scientific) in microwave (Milestone START D, Italy) digestion chamber for 40 min. The digested residue was then made up to 50 ml solution by Milli-Q water and the metal concentrations were determined by using inductively coupled plasma optical emission spectroscopy (iCAP 6300 Duo, Thermo fisher Scientific, Cambridge, England) with dual configuration (axial and radial) and iTEVA (version 2.8.0.97) operational software. Multielement standard solution (CertiPUR, Merck) was used for preparation of calibration solutions and Yttrium as internal standard.

3.7.4. Functional Characterization of Gelatin

3.7.4.1. Water and Oil absorption capacities

The water and oil absorption capacities of the gelatin were determined as per the procedure described in section 3.5.4.1.

3.7.4.2. Emulsifying capacity and emulsifying stability

The emulsifying capacity and emulsifying stability of gelatin was determined as per procedure described in section 3.5.4.2.

3.7.4.3. Foaming capacity and foaming stability

The foaming capacity and foaming stability of gelatin was determined as per procedure described in section 3.5.4.3.

3.8. Development of Films from Refiner Discharge Gelatin

3.8.1. Film forming solution

Film forming solution (FFS) was prepared with the gelatin concentration of 2-6 % (w/v). Gelatin powder was first allowed to swell in distilled water for 30 min. Then the solution was incubated at 60°C for 30 min. After complete solubilization of gelatin powder, glycerol was added as plasticizer at 0.25 g/g of gelatin and incubated for another 30 min with occasional stirring. The solution was cast on acrylic plate (40 ml in 12x12 cm dimension) and dried in vacuum dryer for 24 h. The resulting films were peeled off and kept in a desiccator (silica gel) in room temperature until analysis.

3.8.2. Viscosity of film forming solution

The viscosity of the film forming solution was determined as per the procedure described in section 3.7.2.3.

3.9. Characterization of Gelatin Films

3.9.1. Colour of the films

The colour of the gelatin films was determined as per the procedure described in 3.5.2.1.

3.9.2. Mechanical properties of the films

Tensile strength (TS) and elongation at break (EAB) of film samples were determined as per ASTM D 882-91 (ASTM, 2002) standard method using the Lloyds Texture Analyzer (Lloyd Instruments, Model LRX Plus, and U.K). For testing, rectangular film strips (10 x 1.5 cm) were prepared (Plate 7) and the film samples were lapped with the initial grip length of 5 cm and deformed under tensile loading using a 100 N load cell with the cross head speed of 500 mm/min until the samples were broken (Plate 8).

The maximum load and the final extension at break were used for calculation of TS, and EAB respectively.

$TS = F_{\max}/A$, where $F_{\max}$ is the maximum load (kgf) needed to pull the sample apart

A is the cross-sectional area (cm^2) of the samples

$EAB = E/5 \times 100$, where E is the film elongation (cm) at the moment of rupture

5 = initial grip length (cm) of the samples



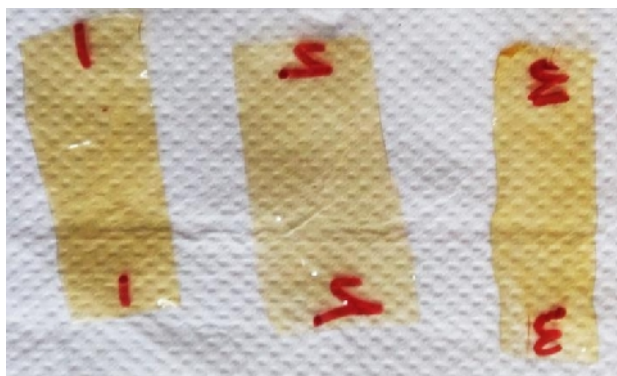
Plate 7: Film strips for mechanical properties study



Plate 8: Measurement of mechanical properties of films

3.9.3. Determination of swelling index of the films

The swelling index of films was determined using the modified method described by Cao *et al.* (2007b). The films were cut into pieces of 20 mm X 20 mm, oven-dried ($100\pm5^{\circ}\text{C}$) for 6 h (to a constant weight) and weighed. They were then immersed in 25 ml distilled water for 2 min at room temperature (Plate 9). Wet samples were wiped with filter paper to remove excess liquid and weighed. The amount of absorbed water was calculated by subtracting the initial mass of air-dried films from the mass of the wet samples and expressed in percentage.



(a)



(b)

Plate 9: Measurement of swelling index of the films (a) gelatin film strips before water immersion; (b) gelatin film strips after water immersion

3.9.4. Determination of solubility of the films in water

Film solubility in water was measured according to the method of Wang *et al.* (2007) by trimming the samples into small strips. Small films were dried in oven ($100\pm5^{\circ}\text{C}$) for 6 h (to a constant weight). Each dried sample was immersed in 100 ml of distilled water for 24 h (Plate 10). Film samples were then removed from the solutions and re-dried at 100°C for 24 h. Final weights were recorded and solubility was calculated as:

$$\text{Solubility} = (\text{Initial Weight} - \text{Final Weight}) / \text{Initial Weight} \times 100 \%$$

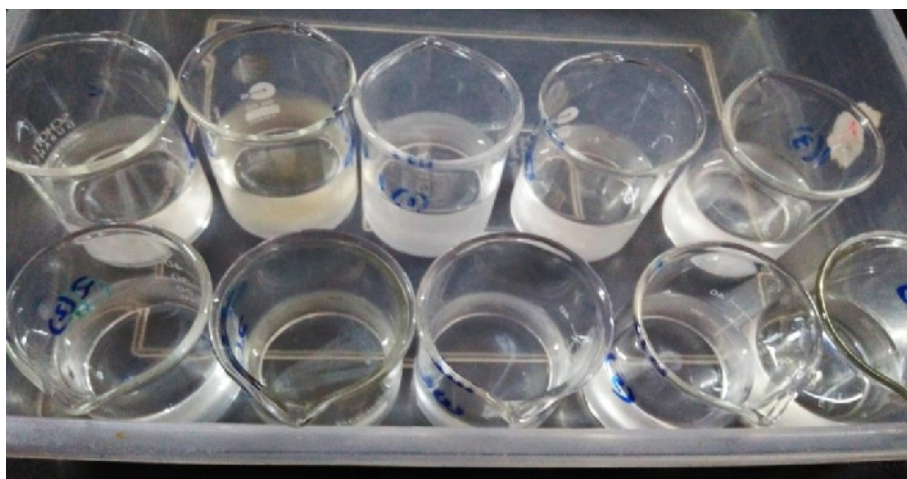


Plate 10: Solubility study of gelatin film strips

3.10. Effect of Pulsed Light (PL) Treatment on the Properties of Films

3.10.1. Pulsed light (PL) treatment

Films samples were treated with different duration of PL (0, 15 sec, 30 sec, 45 sec, 1 min, 2 min and 3 min) using Xenon (Xenon-steripulse, Model:RC-847, Xenon corporation, Wilmington, USA) to examine the effect of PL on the properties of the films (Plate 11). Pulses were emitted at a rate of 3 per sec with a pulse width of 360 μ s, under these conditions, the maximum energy level of the emitted radiation was 1.27 J/cm² per pulse as per the factory calibration. The film without pulsed treatment (0) was kept as control.

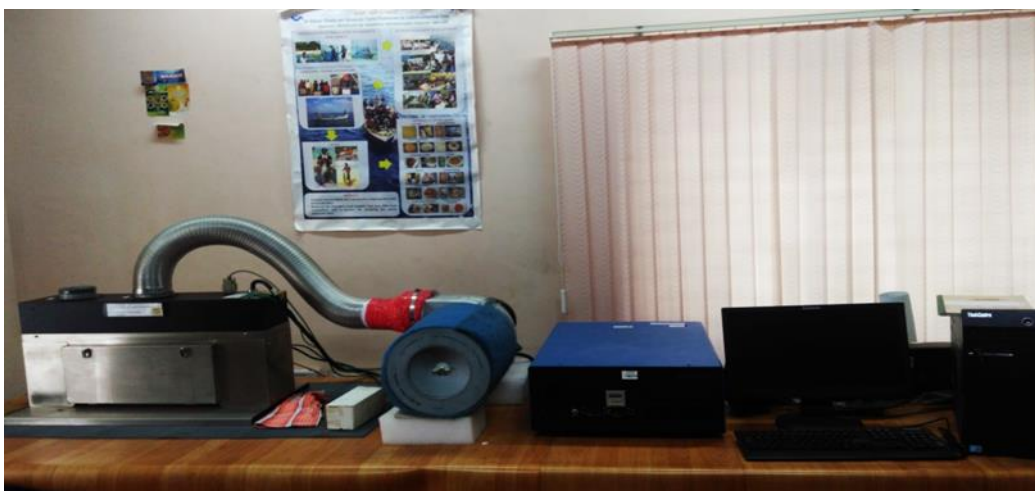


Plate 11: Pulse light equipment (Xenon-steripulse, Model: RC-847, Xenon corporation, USA)

3.10.2. Characterization of Pulsed Light Treated Films

3.10.2.1. Mechanical properties of the films

TS and EAB of film samples were determined as per the procedure described in section 3.9.2.

3.10.2.2. Swelling index and water solubility of the films

The swelling index and water solubility of the films were determined as per the procedure described in section in 3.9.3 and 3.9.4. respectively.

3.10.2.3. Thermal stability analysis of the films

The thermal stability of the films was determined as per the procedure described in section 3.5.2.2.

3.10.2.4. Structural analysis of the films

The structural analysis of the films was determined as per the procedure described in section 3.5.3.2.

3.10.2.5. Protein pattern of the films

SDS-PAGE of the films was determined as per procedure described in 3.5.3.3.

3.11. Statistical Analysis

Collagen was extracted and all analyses were conducted in triplicate, and results were presented as means $\pm$ standard deviations (SD).

The effect of design variables on gelatin yield was studied and experimental data were statistically analyzed by Design-Expert (6.0.11, State-Ease, Inc., Minneapolis MN, USA). A second order polynomial equation was chosen to represent the linear, quadratic and interactive effects of the response.

$$Y = \beta_0 \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j$$

Where, Y is the dependent variable, β_0 is a constant, β_i , β_{ii} , β_{ij} are regression coefficients and X_i , X_j are levels of independent variables ($i = 1-3$; and $j = 1-3$). From the ANOVA table, less significant terms were ignored to improve the model adequacy. The response surface plots for each response were generated as a function of two variables, while keeping other two variables at the optimal conditions.

Films were developed and all analyses were repeated three times unless otherwise specified. The mean values and standard deviations of the experimental data were calculated. Analysis of variance (1-Way ANOVA) was performed and significant differences between mean values were determined by Duncan Test, at a level of significance of $p < 0.05$. The above statistical analyses were conducted using SPSS 20.

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4. RESULTS

4.1. Proximate Composition and Constitutional Ratio of Refiner Discharge

The proximate composition of refiner discharge is depicted in Table 6. The moisture level in the refiner discharge was 73.95 ± 0.65 %. The average crude protein and ash content of the refiner discharge was 13.67 % and 10.61 % respectively. Crude lipid content was found to be more than 2.0 %.

The study of constitutional ratio of the refiner discharge shows that muscle and connective tissues constituted the major component (61.38 %) and minute scale and bones account about 35 % of the refiner discharge (Table 7).

Table 6: Proximate composition of refiner discharge

Source	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Refiner discharge	73.95 ± 0.65	13.67 ± 1.49	2.88 ± 0.02	10.61 ± 2.73

Values (n=3) expressed as mean $\pm$ S.D

Table 7: Constitutional ratio of refiner discharge

Components	Percentage
Muscle and connective tissues	61.38 ± 1.09
Scale	28.70 ± 1.89
Bone	7.91 ± 0.05

Values (n=3) expressed as mean $\pm$ S.D

4.2. Extract Yield, Proximate Composition and Hydroxyproline Content of Refiner Discharge Collagen

Table 8 shows the collagen yield, its hydroxyproline content and proximate composition. The extract yield of collagen from refiner discharge was 11.47 ± 1.36 % (Plate 12). The average hydroxyproline content in the refiner discharge collagen was 7.78 mg/g of collagen. The proximate composition of refiner discharge collagen showed that it has 96.56 % protein, 3.03 % moisture, 1.6 % ash and 0.51% fat.

Table 8: Collagen yield, proximate composition and hydroxyproline content of refiner discharge collagen

Components	Value
Collagen yield	11.47 ± 1.36 %
Moisture	3.03 ± 0.05 %
Protein	96.56 ± 1.50 %
Ash	1.6 ± 0.01 %
Fat	0.51 ± 0.01 %
Hydroxyproline content	7.78 ± 0.05 mg/g

Values (n=3) expressed as mean $\pm$ S.D

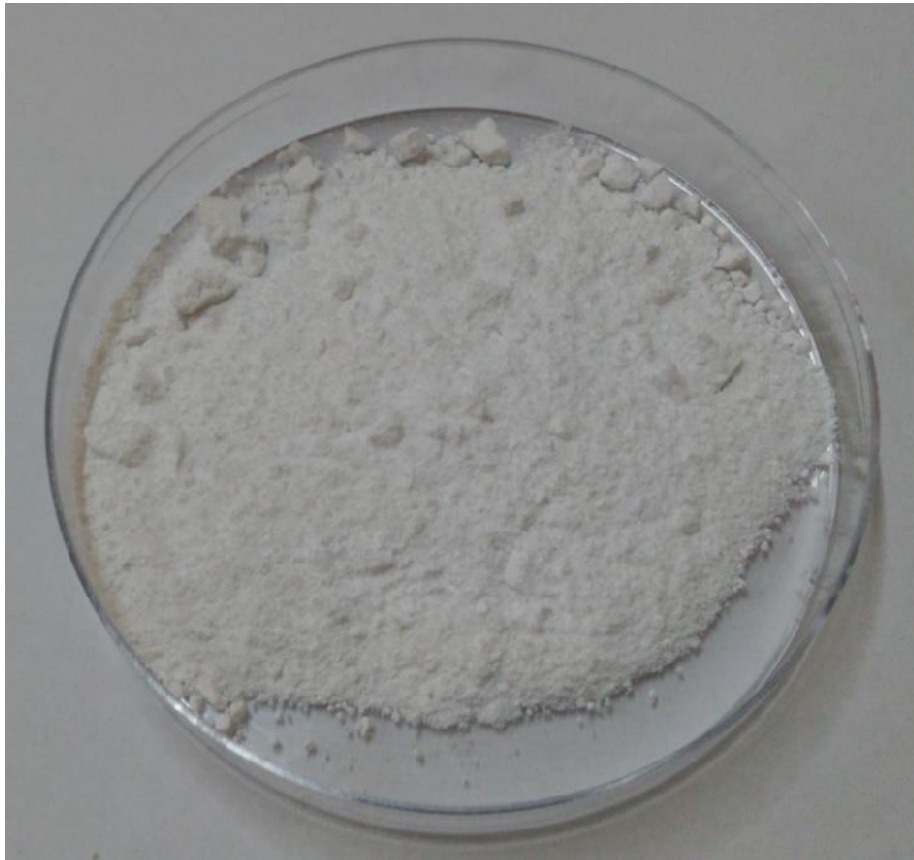


Plate 12: Freeze dried refiner discharge collagen

4.3. Physical Characteristics of Refiner Discharge Collagen

4.3.1. Colour of refiner discharge collagen

The hunter colour values of refiner discharge collagen are given in Table 9. The lightness of the collagen powder as indicated by the L* (94.52) value is very high. The a* (redness) and b* (yellowness) of the collagen was 0.05 and 1.08 % respectively.

Table 9: Hunter Colour value of refiner discharge collagen

Colour	Refiner discharge Collagen
L*	94.52±0.23
a*	0.05±0.01
b*	1.08±0.01

Values (n=3) expressed as mean ± S.D

4.3.2. Thermal stability of refiner discharge collagen

The DSC thermogram of collagen from the refiner discharge is given in Figure 7. There are two different endothermal peaks in the thermogram of collagen from refiner discharge. The first peak was recorded at 23.07°C ($\Delta H = 1.1340\text{J/g}$). While the second peak occurred at 150 °C ($\Delta H = 203.42\text{J/g}$).

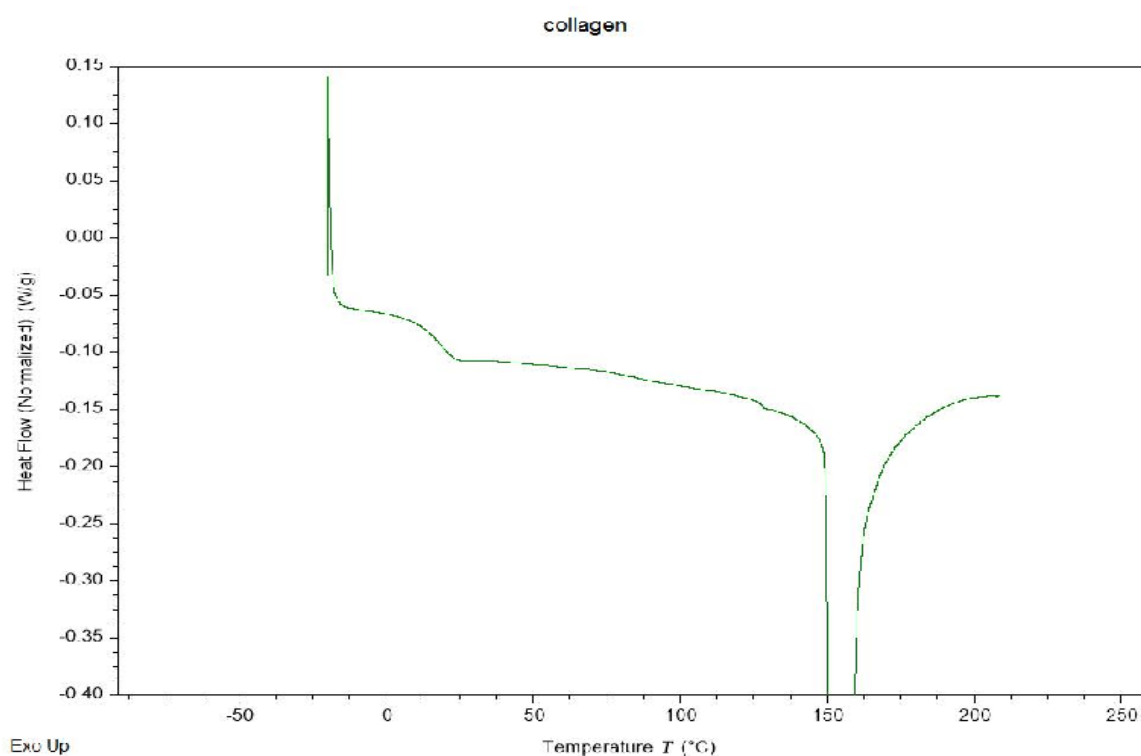


Figure 7: DSC thermogram of refiner discharge collagen. (zoom image)

4.4. Chemical Characteristics of Refiner Discharge Collagen

4.4.1. Amino acid composition of refiner discharge collagen

The amino acid composition of collagen from refiner discharge is given in Table 10. Glycine (316.5 residues/1000 residues) was found to be the most abundant amino acid followed by glutamic acid (106.2 residues/1000 residues), alanine (99.8 residues/1000 residues) and proline (87.5 residues/1000 residues). Others amino acids like threonine, valine, methionine and isoleucine were found at lower concentrations. Cysteine was not detected in the extracted collagen (so in the table cysteine column was omitted). The hydrophobic amino acid (summation of proline, alanine, valine, methionine, glycine, isoleucine, leucine and phenylalanine) account for 625.9 residues/1000 residues in the refiner discharge collagen. The refiner discharge collagen contains 8 out of 9 essential amino acids. The summation of isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine and histidine in the extracted collagen was 193.7 residues/1000 residues.

Table 10: Amino acid composition of refiner discharge collagen (residues per 1000 residues)

Amino acids	Refiner discharge collagen (residues per 1000 residues)
Aspartic acid	043.4
Theronine	019.5
Serine	038.1
Glutamic acid	106.2
Proline	087.5
Glycine	316.5
Alanine	099.8
Valine	016.7
Methionine	010.3
Isoleucine	008.3
Leucine	037.1
Tyrosine	057.1
Phenylalanine	049.7
Histidine	031.2
Lysine	020.9
Arginine	060.4
Total	1000.0
Hydrophobic amino acid	625.9
Essential amino acids	193.7

Hydrophobic amino acid

= Σ proline+alanine+valine+methionine+glycine+isoleucine+leucine+phenylalanine

Essential amino acid

= Σ isoleucine+leucine+lysine+methionine+phenylalanine+threonine+valine+histidine

4.4.2. Structural profile of refiner discharge collagen

The frequencies at which major peaks occurred for collagen from refiner discharge are summarized in Table 11 and Figure 8. The Amide-A peak which is associated with N–H stretching coupled with the hydrogen bond of a carbonyl group in a peptide chain, appeared at 3346.63 cm^{-1} . While the Amide-B peak was occurred at 2939.22 cm^{-1} . This peak represents the asymmetric stretching vibration of alkenyl C–H, as well as NH_3^+ . The Amide-I, II and III peak were observed at 1678.52, 1551.87 and 1239.87 cm^{-1} respectively. The amide-I vibration mode is primarily due to the C=O stretching vibration of the peptide linkages. The amide-II vibration modes are attributed to the N–H in-plane bend. The amide-II peak is due to the N-H bend coupled with C-N stretch.

Table 11: Infrared band of refiner discharge collagen and general peak assignments for the bands

Designation	Normal Wavenumbers range (cm^{-1})	Wavenumbers of Refiner discharge collagen (cm^{-1})	Assignment ¹⁾
Amide A	3400-3440	3346.63	NH stretch
Amide B	2850-2950	2939.22	CH_2 asymmetrical stretch
Amide I	1600-1700	1678.52	C=O stretch
Amide II	1550-1600	1551.87	NH bend
Amide III	1320-1220	1239.87	NH bend coupled with CN stretch

¹⁾wang *et al.* (2014)

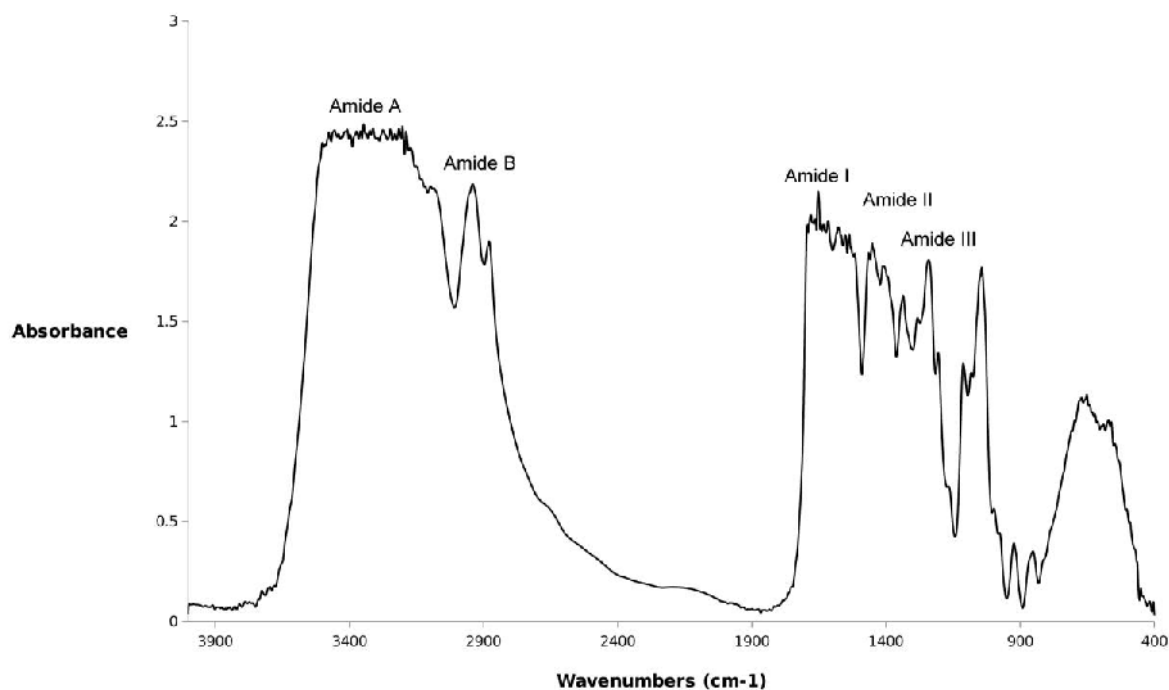


Figure 8: FTIR spectra of collagen from refiner discharge

4.4.3. Protein pattern of refiner discharge collagen

Electrophoretic pattern of collagen from refiner discharge is given in Figure 9. From the figure, it can be seen that there are four distinct bands with the molecular weight of around 176, 125, 113 and 103 kDa respectively. Thus the refiner discharge collagen content α chains and high molecular weight β chain. The β chain appeared at 176 kDa. The α_1 band was present at 125 kDa while α_2 was seen at 113 kDa. There are also other fractions of around 103 kDa that appear under the α_2 band.

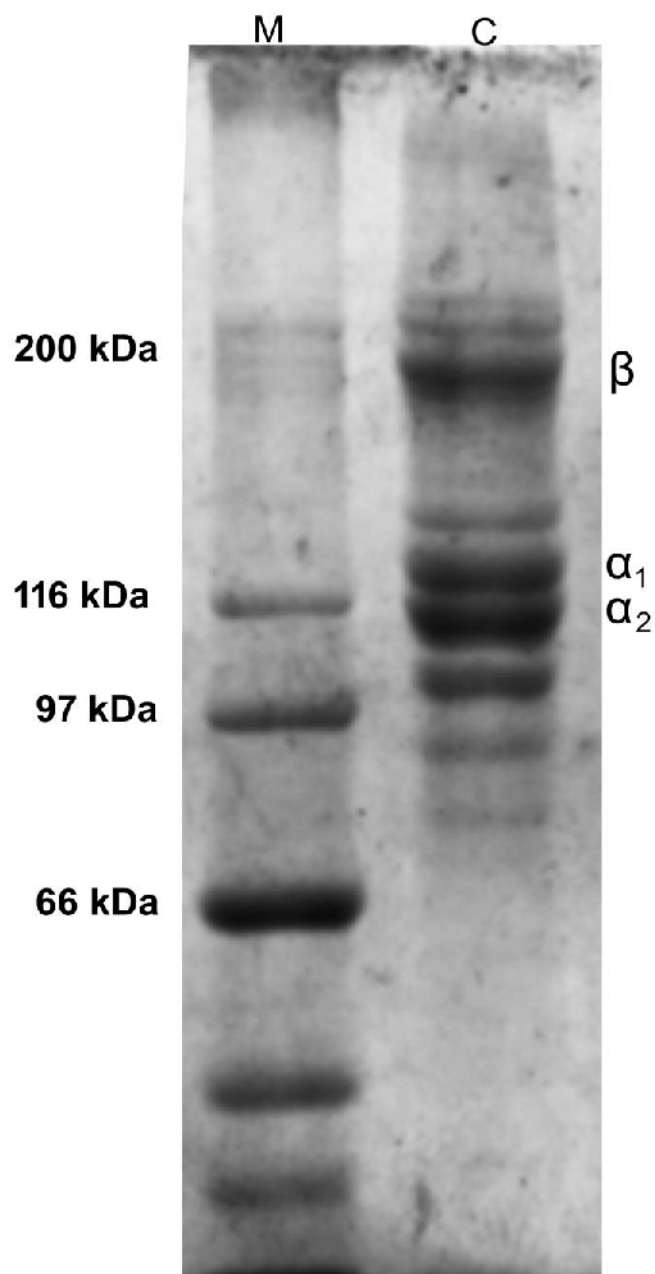


Figure 9: SDS-PAGE pattern of collagen from refiner discharge: M indicate standard and C indicate refiner discharge collagen

4.5. Functional Characteristics of Refiner Discharge Collagen

The functional properties of refiner discharge collagen such as water absorption capacity, oil absorption capacity, emulsifying activity, emulsion stability, foaming capacity and foaming stability are depicted in Table 12. From the results, it was observed that the average water and oil absorption capacities of refiner discharge collagen were 5.76% and 1.6% respectively. It had the average emulsifying activity of 50 %. The average emulsion stability of refiner discharge collagen was 46.15%. The collagen was found to have foaming capacity and foaming stability of 50.55 and 44.79 % respectively.

Table 12: Functional properties of refiner discharge collagen

Functional properties	Collagen
Water absorption capacity (ml/g)	5.76±0.83
Oil absorption capacity (ml/g)	1.6±0.05
Emulsion activity (%)	50±0.38
Emulsion stability (%)	46.15±0.12
Foaming capacity (%)	50.55±0.92
Foaming stability (%)	44.79±0.76

Values (n=3) expressed as mean ± S.D.

4.6. Solubility of Refiner Discharge Collagen

The effect of the pH and NaCl concentrations on the solubility of refiner discharge collagen are given in Figure 10 and 11 respectively. From the result, it is revealed that the solubility of the extracted collagen was higher in the pH range of 1-5. The solubility was 79.92 % at pH 1 and maximum was found at pH 3 (95.75 %). It decreases drastically to 55.67% beyond pH 5 and latter to 25.89 % at pH 10.

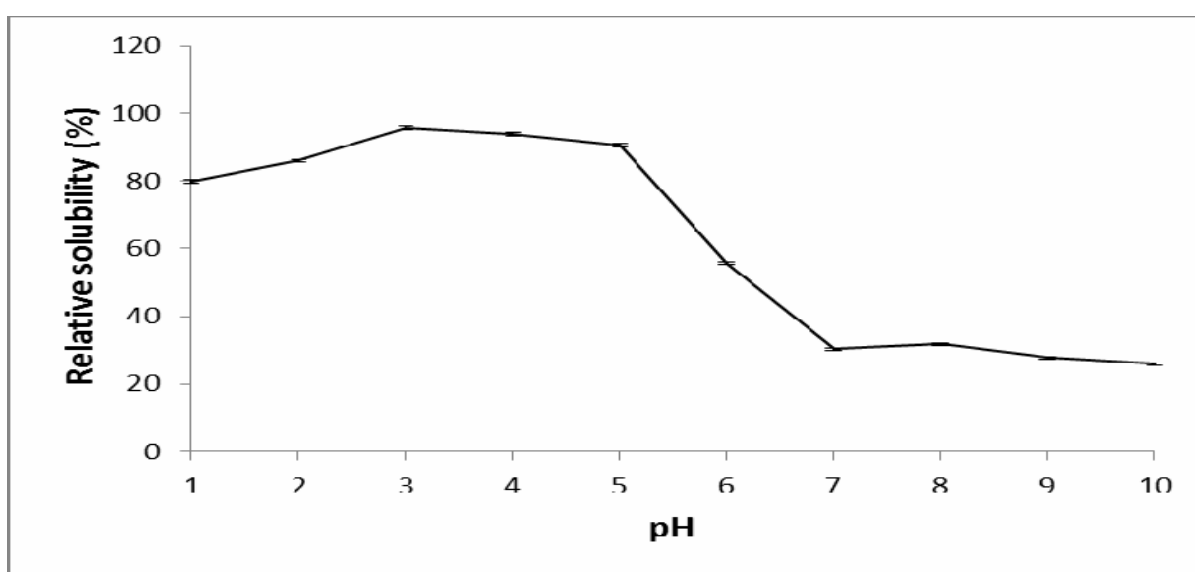


Figure 10: Relative solubility of collagen from refiner discharge at different pH.

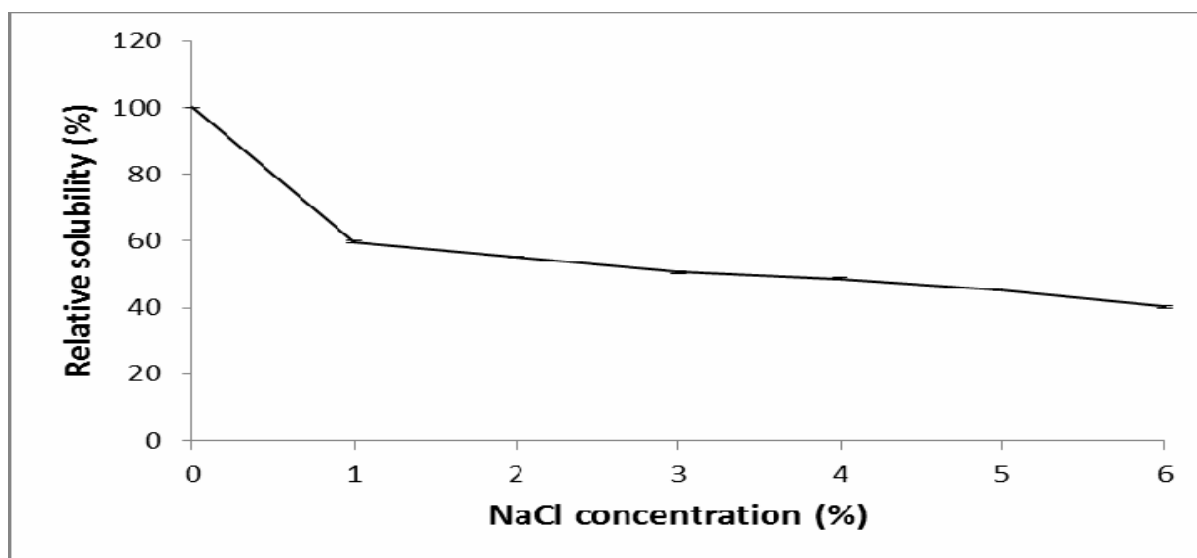


Figure 11: Relative solubility of collagen from refiner discharge at different NaCl concentrations.

In case of NaCl, maximum solubility of the collagen (100%) was observed at zero NaCl concentration. A drastic decrease in the solubility of collagen from 100 % to 59.70 % was observed when NaCl concentration was increased from 0 to 1 %. However, with increased concentration of NaCl from 1 % onwards not much change in the solubility was observed.

4.7. Optimization of Process Parameters for the Gelatin Yield

4.7.1. Development of response surface model and data analysis

A total of 15 runs including three center points were carried out to study the effect of the NaOH Concentration (A), extraction temperature (B) and extraction time (C) on the gelatin yield. The results of the response obtained from the extraction under different combinations of independent variables are given in Table 13. The significance of the quadratic polynomial model equation was estimated by the analysis of variance (ANOVA) (Table 14). Any term in the modal, which has a small p-value and a large F-value would imply more significant effect on the relevant response variable. The P-value of 0.0002 implies that the model is significant. Thus, the Quadratic model ($P < 0.0002$) was found best model to explain the variability in gelatin yield (Y) as a function of process parameters. The lack of fit of model was not significant ($P > 0.05$) which indicates that the calculated response surface represents

the true surface. The values of “R-squared”, “Adjusted R-squared”, “Predicted R-squared” and “Adequate Precision” are 0.9889, 0.9689, 0.8289 and 20.665 respectively. The high value for “R-squared” implies that the model can explain the high percentage of variability in the observed data. The “Predicted R-squared” and the “Adjusted R-squared” are in reasonable agreement. “Adequate Precision” value reflects the signal to noise ratio. A value greater than 4 is desirable for this ratio. The ratio in this study was 20.665, which indicates an adequate signal and implies that the model is suitable to navigate the design space. To develop the fitted response surface, all significant terms are retained in the model to predict the value of the gelatin yield and as to optimize the process parameters (Table 15). The fitted model with regression coefficients is given below:

$$\text{Yield} = -171.0 + 3.90B + 24.56C + 0.52 AB - 0.08 BC - 96.23 A^2 - 0.02 B^2 - 0.28C^2$$

Extraction temperature was the most influencing factors on yield. All the square effect of the independent variables had negative effect on the yield. The response surface plots of gelatin yield as a function of two independent variables while keeping other variable at the optimal conditions are given in Figure 12 (a) and (b).

Table 13: Responses of dependent variables for the yield (y, %) of gelatin to changes in the independent variables

Experimental design runs	Variables levels			Response (Y, %)
	A (M)	B (°C)	C (h)	
1	0.6	60	5	10.01
2	0.4	70	5	13.09
3	0.4	60	4	15.43
4	0.2	60	5	10.17
5	0.4	60	4	15.61
6	0.4	50	5	10.00
7	0.4	50	3	5.67
8	0.2	70	4	10.42
9	0.2	60	3	8.31
10	0.4	70	3	12.06
11	0.2	50	4	6.28
12	0.6	70	4	13.11
13	0.4	60	4	15.21
14	0.6	50	4	4.76
15	0.6	60	3	8.63

A (concentration of NaOH, M), B (extraction temperature, °C), C (extraction time, h)

Table 14: Analysis of variance (ANOVA) for response surface quadratic model

Source	Sum of squares	DF	Mean Square	F Value	p-value	Remarks
Model	169.53	9	18.84	49.52	0.0002	significant
A-NaOH	0.22	1	0.22	0.58	0.4802	
B-Temp	60.34	1	60.34	158.60	< 0.0001	significant
C-Time	9.25	1	9.25	24.30	0.0044	significant
AB	4.43	1	4.43	11.65	0.0190	significant
AC	0.058	1	0.058	0.15	0.7132	
BC	2.72	1	2.72	7.16	0.0441	significant
A ²	54.72	1	54.72	143.83	< 0.0001	significant
B ²	31.58	1	31.58	83.02	0.0003	significant
C ²	19.31	1	19.31	50.77	0.0008	significant
Residual	1.90	5	0.38			
Lack of Fit	1.82	3	0.61	15.13	0.0626	not significant
Pure Error	0.080	2	0.040			
Cor Total	171.43	14				

A (concentration of NaOH, M), B (extraction temperature, °C), C (extraction time, h)

Table 15: Regression coefficients for 2nd order response surface model in terms of actual factors

Parameter	Term	Coefficient
β_0	Intercept	-171.0
β_1	A	48.64
β_2	B	3.90
β_3	C	24.56
β_{12}	AB	0.52
β_{13}	AC	-0.60
β_{23}	BC	-0.08
β_{11}	A^2	-96.23
β_{22}	B^2	-0.02
β_{33}	C^2	-2.28

A (concentration of NaOH, M), B (extraction temperature, °C), C (extraction time, h)

Design-Expert® Software

Factor Coding: Actual

yield (%)

• Design points above predicted value

• Design points below predicted value

15.61

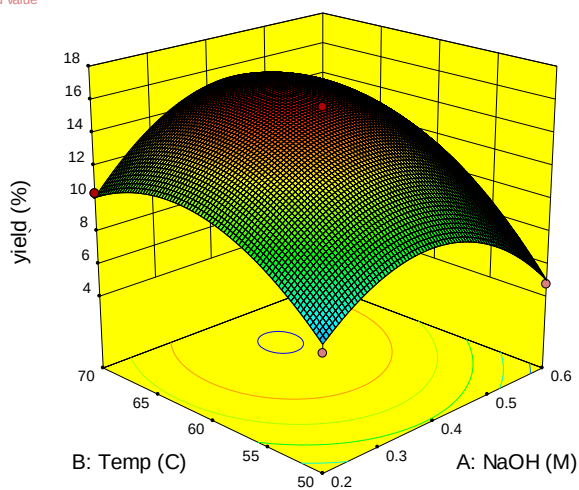
4.76

X1 = A: NaOH

X2 = B: Temp

Actual Factor

C: Time = 4



(a)

Design-Expert® Software

Factor Coding: Actual

yield (%)

• Design points above predicted value

• Design points below predicted value

15.61

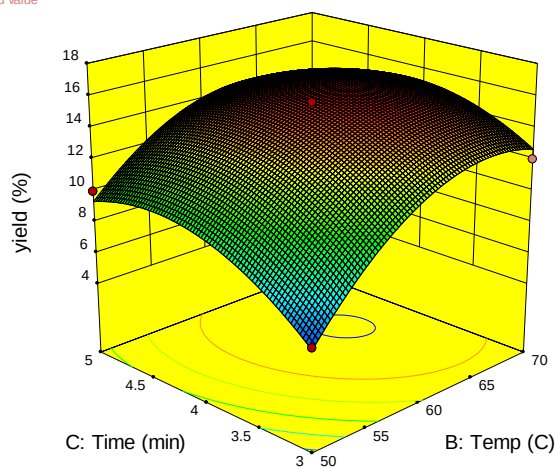
4.76

X1 = B: Temp

X2 = C: Time

Actual Factor

A: NaOH = 0.4



(b)

Figure 12: Response surface plots of yield: a) showing the effect of NaOH concentration, M and temperature, B; b) showing the effect of time, C and temperature, B.

Figure 12a shows that yield increased rapidly to an optimum value with increased concentration of NaOH and temperature. Also as indicated in Figure 12b, the dependent variable rose by increasing time and temperature.

4.7.2. Conditions for process parameters for optimum response of yield

The effect of concentration of NaOH (A), extraction temperature (B) and extraction time (C) on the gelatin yield was determined using response surface methodology. Desirability profile was employed to establish the optimum value of each condition. Concentration of NaOH (A) was fixed at minimum, extraction temperature (B) and extraction time (C) were set in ranges, while the dependent variable, yield was fixed at maximum to obtain optimized solution. The resulting optimum conditions were 0.34 M NaOH, 63.92°C and 4.1 h and the predicted value of yield was 15.60 % with desirability of 0.897 (Table 16). While the actual value of yield was recorded as 16.56%.

Table 16: Optimum values of independent variables for the extraction of gelatin

Independent Variable	Optimum values
NaOH (M)	0.34
Extraction temperature (°C)	63.92
Extraction time(h)	4.10

4.8. Proximate Composition of Refiner Discharge Gelatin

The proximate composition of the gelatin extracted from refiner discharge is given in Table 17. The pH of the gelatin was 3.69. The moisture, protein, fat and ash values for gelatin were 7.09, 92.84, 0.39 and 0.84 % respectively. The hydroxyproline content in the refiner discharge gelatin was 6.53 mg/g of gelatin.

Table 17: Proximate composition and hydroxyproline content of refiner discharge gelatin

Source	Moisture (%)	Protein (%)	Crude fat (%)	Ash (%)	Hydroxyproline content
Gelatin	7.09± 0.20	92.84± 1.47	0.39 ± 0.03	0.84±0.05	6.53±mg/g

Values (n=3) expressed as mean ± S.D.

4.9. Physical Characteristics of Refiner Discharge Gelatin

The physical properties of the refiner discharge are given in Table 18.

4.9.1. Colour and gel clarity

The hunter colour measurement of the refiner discharge gelatin is shown in Table 18. The refiner discharge gelatin had whitish yellow colour. The L*, a* and b* values of the extracted gelatin were 68.01±0.40, 3.40±0.12 and 15.32±0.32 respectively. In case of the gelatin gel, it has transmittance of 61.26%.

Table 18: Physical properties of refiner discharge gelatin

Physical properties		Gelatin
Bloom value (g)		147.97 ± 1.78
Viscosity (cP)		7.44 ± 0.14
Melting point (°C)		25.50 ± 0.50
Setting point (°C)		18.50 ± 0.50
Setting time (s)		120.66 ± 2.08
Colour (powder):		
	L*	68.01 ± 0.40
	a*	03.40 ± 0.12
	b*	15.32 ± 0.32
Gel clarity:		
Transmittance (%)		61.26 ± 1.55

Values (n=3) expressed as mean ± S.D.

4.9.2. Bloom value

Bloom value is the most significant physical properties of gelatin. The average bloom value of the refiner discharge gelatin was 147.97g.

4.9.3. Viscosity

Viscosity is the second most important rheological properties of gelatin solutions. The average viscosity of refiner discharge gelatin was 7.44 Cp.

4.9.4. Melting point

The melting point of gelatin is the point at which the gelatin gel starts melting when the temperature increases above a certain point. The melting point of the refiner discharge gelatin was 25.50°C.

4.9.5. Gel setting point and setting time

Gelatin solution from refiner discharge gelled at 18.50 °C and the gelling time required for refiner discharge gelatin was 120.66 s.

4.9.6. Thermal stability

Figure 13 shows the DSC heating thermograph of refiner discharge gelatin. There is a broad endothermic peak at 77.20°C having transition enthalpy (ΔH) of 277.72 J/g. Besides this endothermic peak, there is no evidence of other thermal event.

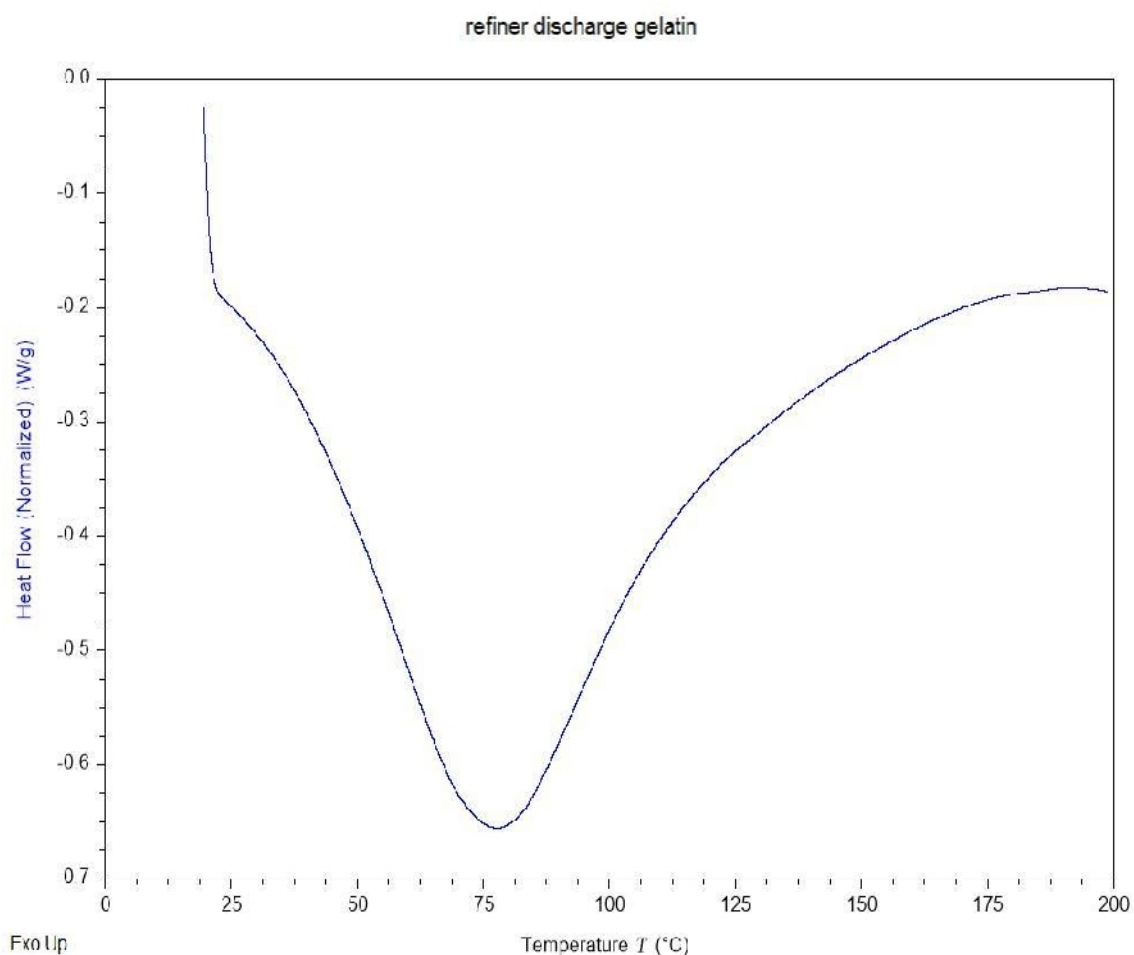


Figure 13: DSC thermograph of refiner discharge gelatin

4.10. Chemical Characteristics of Refiner Discharge Gelatin

4.10.1. Amino acids composition

The amino acids profile of extracted gelatin from pink perch refiner discharge is given in Table 19. The results showed that the refiner discharge gelatin contains 16 different types of amino acids. Among these the extracted gelatin is rich in glycine followed by glutamic acid, proline and alanine. The residues of abundant amino acids i.e glycine, glutamic acid, proline and alanine are 337.8, 118.3, 97.6 and 70.8 per 1000 residues respectively. Cysteine was not detected in the gelatin. Among the amino acids, the hydrophobic groups contribute 596.5 residues per 1000 residues. On the other hand the essential amino acids contribute 160.2 residues per 1000 residues.

Table 19: Amino acids composition of refiner discharge gelatin (residues per 1000 residues)

Amino acids	Refiner discharge gelatin (residues per 1000 residues)
Aspartic acid	042.2
Threonine	018.8
Serine	037.4
Glutamic acid	118.3
Proline	097.6
Glycine	337.8
Alanine	070.8
Valine	018.5
Methionine	013.3
Isoleucine	007.0
Leucine	020.7
Tyrosine	024.6
Phenylalanine	030.8
Histidine	026.5
Lysine	024.6
Arginine	067.9
Total	999.9
Hydrophobic amino acids	596.5
Essential amino acids	160.2

Hydrophobic amino acid

= Σ proline+alanine+valine+methionine+glycine+isoleucine+leucine+phenylalanine

Essential amino acid

= Σ isoleucine+leucine+lysine+methionine+phenylalanine+threonine+valine+histidine

4.10.2. Structural profile

The corresponding wavenumbers (cm^{-1}) of the characteristics infrared bands of refiner discharge gelatin is given in Table 20 and the FTIR spectra is given in Figure 14. The gelatin obtained from refiner discharge exhibited the amide-A and B bands at 3315.95 cm^{-1} and 2967.03 cm^{-1} respectively. Amide-I band was found at 1654.47 cm^{-1} while that of amide-II and amide-III bands occurred at the wavenumbers of 1578.53 cm^{-1} and 1232.79 cm^{-1} respectively.

Table 20: Infrared band of refiner discharge gelatin given in wavenumbers (cm^{-1}) and general peaks assignment

Designation	Refiner discharge gelatin	Assignment ¹⁾
Amide A	3315.95	NH stretch
Amide B	2967.03	CH_2 asymmetrical stretch
Amide I	1654.47	C=O stretch
Amide II	1578.53	NH bend
Amide III	1232.79	NH bend coupled with CN stretch

¹⁾wang *et al.* (2014)

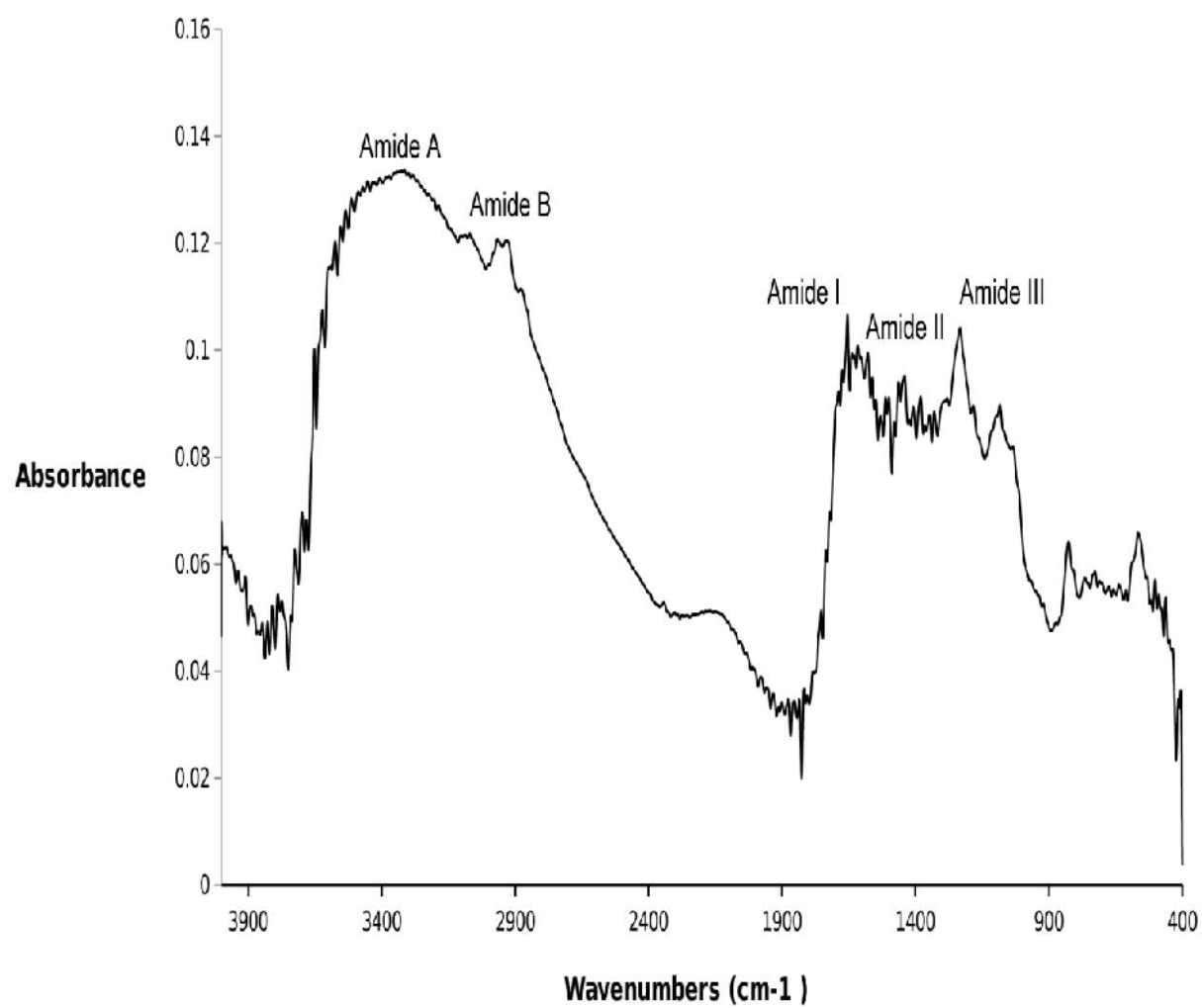


Figure 14: FTIR spectra of gelatin from refiner discharge

4.10.3. Protein pattern

The refiner discharge gelatin was analyzed using SDS-PAGE (Figure 15). The gelatin has high intensity of β - and α -chains (α_1 and α_2) as the major components though a faint γ - chain is also visible. Bands of low peptides were not noticeable. The molecular weight of β , α_1 and α_2 are 18.36, 134.87 and 124.10 kDa respectively. The γ - chain was found at 188.10 kDa.

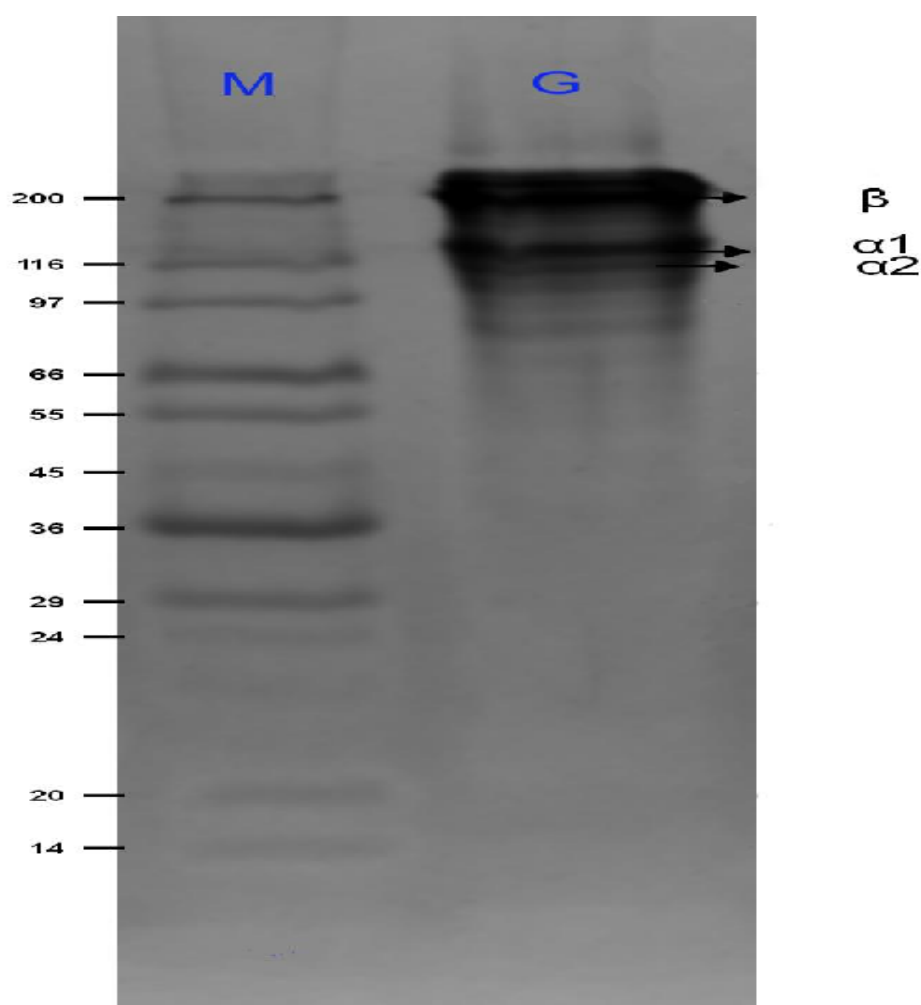


Figure 15: SDS-PAGE pattern of extracted gelatin (M: marker; G: refiner discharge gelatin)

4.10.5. Mineral composition

The mineral composition of pink perch surimi refiner discharge gelatin (in ppm) is given in Table 21. Among the mineral analyst the extracted gelatin is rich in calcium (1577 ppm) followed by sodium (708.58ppm), potassium (72.10 ppm) and magnesium (94.23ppm).

Table 21: Mineral composition refiner discharge gelatin (ppm)

Minerals	Refiner discharge gelatin
Calcium	1577.00±37.37
Sodium	708.58±16.96
Potassium	72.10±0.88
Magnesium	94.23±2.01

Values are given as mean±S.D.

4.11. Functional Characteristics of Refiner Discharge Gelatin

The functional properties of the refiner discharge gelatin are given in Table 22. The water holding capacity and oil holding capacity of refiner discharge gelatin were found to be 2.20 ml/g and 3.06 ml/g respectively. Emulsifying activity and emulsion stability of gelatin were 50.77% and 50.44%. The extracted gelatin was found to have foaming capacity of 38.45 %. The foaming stability of gelatin was 20.16% at 30 min.

Table 22: Functional properties of refiner discharge gelatin

Water holding capacity (ml/g)	Oil holding capacity (ml/g)	Emulsion activity (%)	Emulsion stability (%)	Foaming capacity (%)	Foaming stability (%)	
					30 min	60 Min
2.20±0.26	3.06±0.15	50.77±0.58	50.44±0.66	38.45±1.63	24.80±1.67	20.16±0.76

Values are given as mean± standard deviation of triplicate.

4.12. Development of Films from Refiner Discharge Gelatin

4.12.1. Viscosity of film forming solution

Viscosity of film forming solutions is given in Table 23. The concentrations of gelatin of the film forming solutions influenced solution viscosity. Viscosity of the film forming solution increases from 2.26 Cp to 7.83 Cp with increasing gelatin concentrations from 2 to 6 %.

Table 23: Viscosity of film forming solutions with different concentrations of gelatin

Gelatin (%)	Viscosity (Cp)
2	2.27±0.15 ^a
3	3.62±0.13 ^b
4	4.15±0.12 ^c
5	5.09±0.10 ^d
6	7.83±0.13 ^e

Values are given as mean± standard deviation of triplicate. Different superscripts in the same column indicate significant differences ($p < 0.05$).

4.13. Characterization of Film from Refiner Discharge Gelatin

4.13.1. Colour of the films

Color of the gelatin films with different concentrations of gelatin is presented in Table 24. The films were quite transparent visually. From the result, the developed films had higher L^* (lightness) values irrespective of gelatin concentrations. All the films had negative a^* ($-a^*$ =greenness; $+a^*$ =redness) value and positive b^* ($-b^*$ =blueness; $+b^*$ =yellowness) values.

Table 24: Colour of the films as affected by gelatin concentrations

Gelatin (%)	Colour parameters		
	L^*	a^*	b^*
3	87.92±1.50 ^a	-1.30±0.02 ^b	10.91±0.57 ^b
4	87.30±1.52 ^a	-1.29±0.02 ^b	10.67±0.52 ^b
5	87.83±1.50 ^a	-1.13±0.01 ^a	9.59±0.25 ^a
6	88.94±0.70 ^a	-1.14±0.02 ^a	9.63±0.50 ^a

Values are given as mean± standard deviation of triplicate. Different superscripts in the same column indicate significant differences ($p<0.05$).

4.13.2. Mechanical properties of the films

Tensile strength (TS) and Elongation at break (EAB) of the gelatin films with varying protein concentrations are shown in Table 25. The film prepared from film-forming solution (FFS) with 2% protein content was too thin to peel off. The films obtained with 3-6 % gelatin concentration were homogeneous, flexible and easy to handle. TS of the film increase with increasing gelatin concentration, however the increase was not linear. TS increase from 66.69 kg/cm² to 71.33 kg/cm² as the protein increase from 3% to 6%. Higher EAB values were observed in all the films. Higher EAB indicate that the films made from refiner discharge gelatin are highly flexible and elastic. The general phenomenon of decreasing EAB with increasing TS was not observed in the present study. EAB increases from 129.1 % to 203.6 % with increasing gelatin concentration from 3 to 6 % in the film forming solutions.

Table 25: Effect of gelatin concentrations on the tensile strength and elongation at break of films

Gelatin (%)	Tensile strength (kg/cm ²)	Elongation at break (%)
3	66.69 ±4.05 ^a	129.1± 7.87 ^a
4	73.50±7.05 ^b	190.8±8.40 ^b
5	69.50±5.38 ^a	186.6±7.26 ^b
6	71.33±7.27 ^{ab}	203.6±9.90 ^c

Values are given as mean± standard deviation of triplicate. Different superscripts in the same column indicate significant differences (p<0.05).

4.13.3. Swelling index and solubility of the film

Swelling index of the gelatin films reduces from 251.30 to 160.89 on increasing the gelatin content of the films from 3 to 6 %. The refiner discharge gelatin films had 94.97 to 70.12 % solubility in water. The values of swelling index and solubility in water are shown in Table 26. The maximum solubility of the films was observed in films made with 3 % gelatin.

Table 26: Swelling index and solubility of refiner discharge gelatin films

Gelatin (%)	Swelling index	Solubility in water (%)
3	251.31± 10.05 ^c	94.97±1.70 ^c
4	281.06±7.10 ^c	81.69±1.20 ^b
5	172.03±9.00 ^b	85.89±2.00 ^b
6	160.89±5.80 ^a	70.12±2.70 ^a

Values are given as mean± standard deviation of triplicate. Different superscripts in the same column indicate significant differences (p<0.05).

4.14. Effect of Pulsed Light Treatment on the Properties of Refiner Discharge Gelatin Films

4.14.1. Mechanical properties of the films

TS and EAB of film were affected by pulsed light treatment (Figure 16). TS value of the control films was 66.69 kgcm^{-2} . There was a significant change in TS due to pulsed light treatment of the films. Among the treatments, the highest TS value of 135.81 kgcm^{-2} was attained when treated for 1 min with pulsed light.

In case of EAB, pulsed light treatment reduces EAB from 129.07 % to 52.51 % as the treatment increases from 0 to 3 min. However, there were no significant differences between control film and treated films till 45 sec treatment. Thereafter, the EAB of the films decreased significantly.

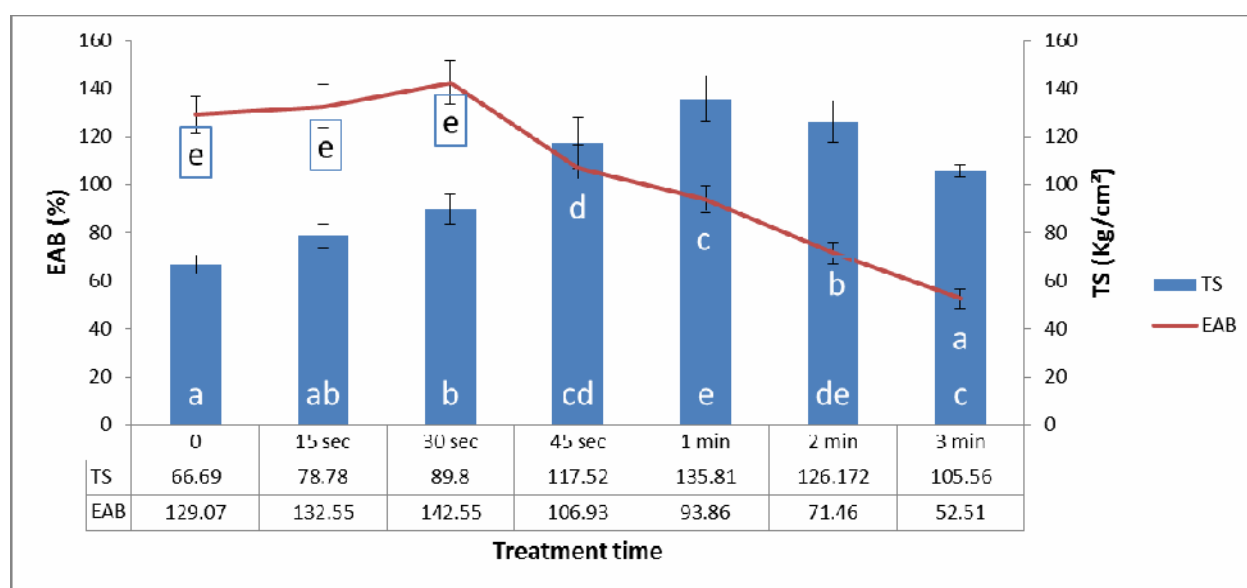


Figure 16: Mechanical properties of refiner discharge gelatin film with different pulse light treatment duration. 0: control; 15 sec; 30 sec; 45 sec; 1min; 2 min and 3 min are PL treatment durations of the films. Values (n=3) expressed as mean±S.D. Different superscripts in the same column indicates statistical differences (p<0.05).

4.14.2. Protein pattern of the films

The SDS-PAGE (Figure 17) shows the protein bands of the control and PL treated gelatin films along with the protein marker. Prominent protein bands such as β and α were observed in control film. Faint bands of β and α were observed in 15, 30 and 45 sec PL treated gelatin films. However low intensity bands were appeared in the molecular weight of 116 kDa to 97 kDa in 1, 2 and 3 min PL treated films.

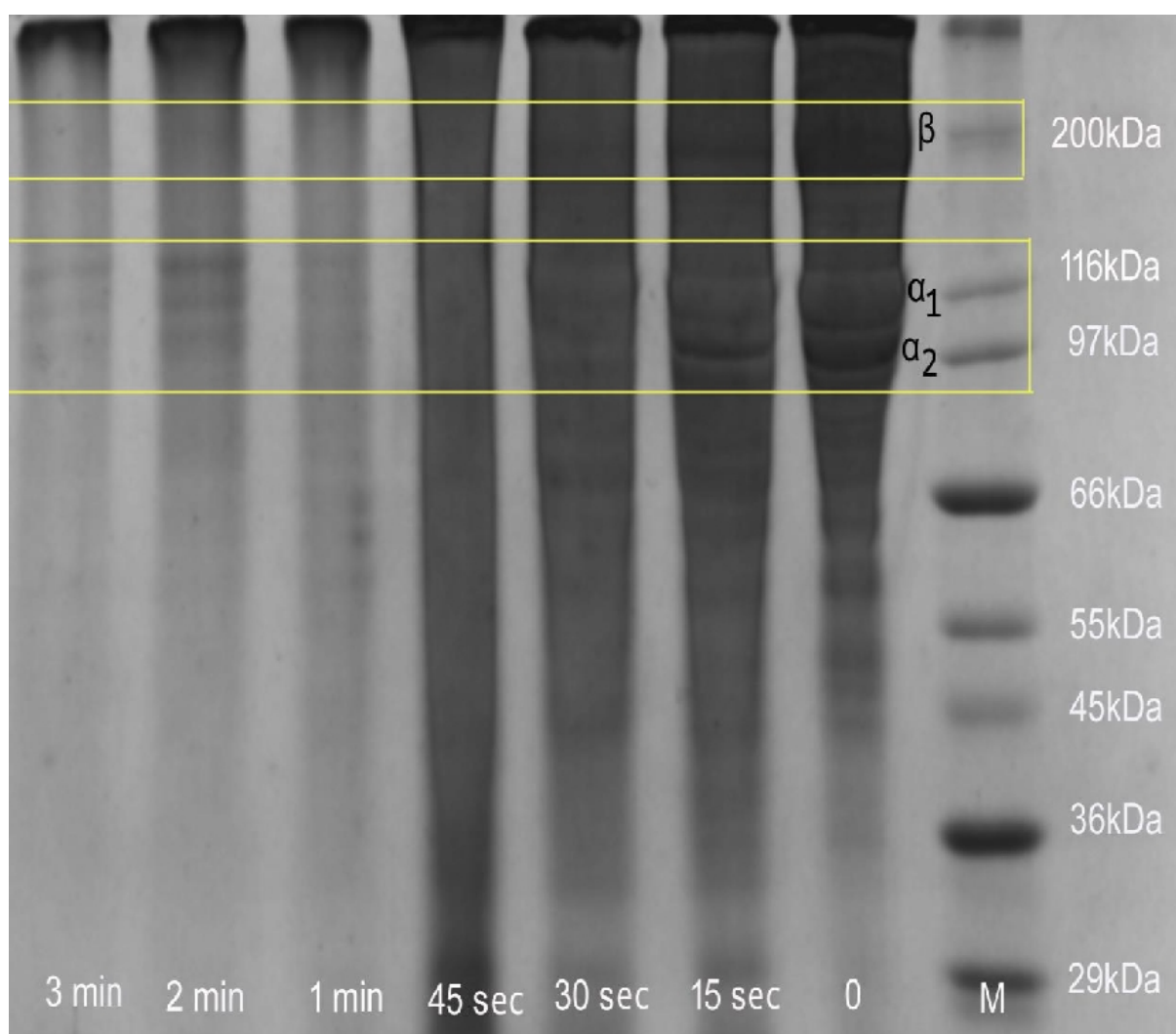


Figure 17: SDS-PAGE patterns for refiner discharge gelatin films treated with different PL durations (M: marker; 0: control; 15 sec; 30 sec; 45 sec; 1m min; 2 min and 3 min are PL treatment durations of the films)

4.14.3. Swelling index and solubility of the films

Figure 18 depicts the swelling index of the gelatin films in water. The swelling index of the films decreases significantly from 463 to 159 after pulsed light treatment. Likewise, the water solubility of the gelatin film also decreases significantly with increase in pulsed light treatment time (Figure 19). The control film was highly soluble (93.5 %) in water at room temperature. Films solubility decreases from 93 % to 35 % with increasing PL treatment time. However, the decrease was not significant after 1 min of PL treatment.

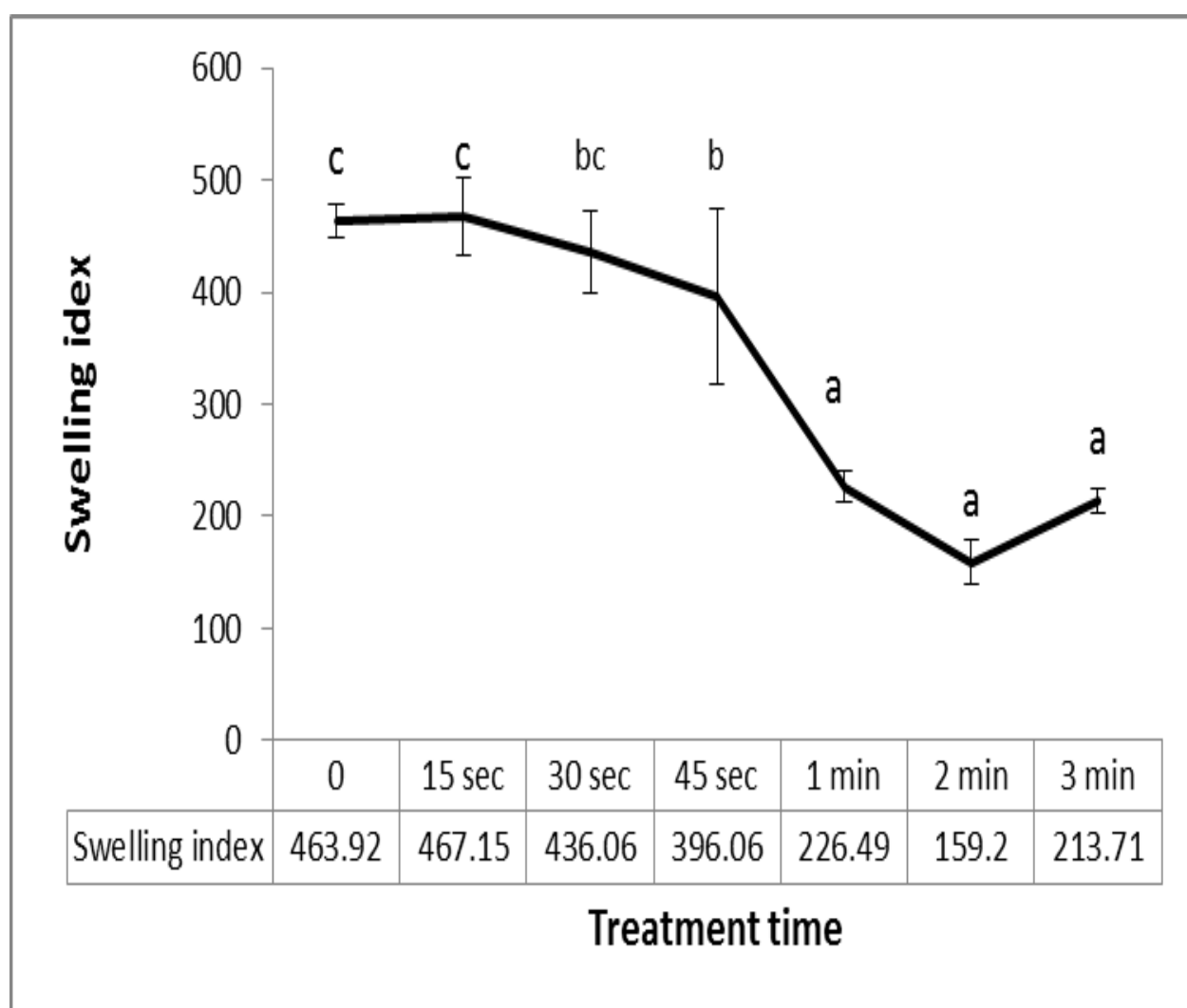


Figure 18: Swelling index for refiner discharge gelatin films treated with different PL duration (15 sec, 30 sec, 45 sec, 1min, 2 min and 3 min). Different letters above the line indicate significant differences between mean values of the treatment ($p < 0.05$).

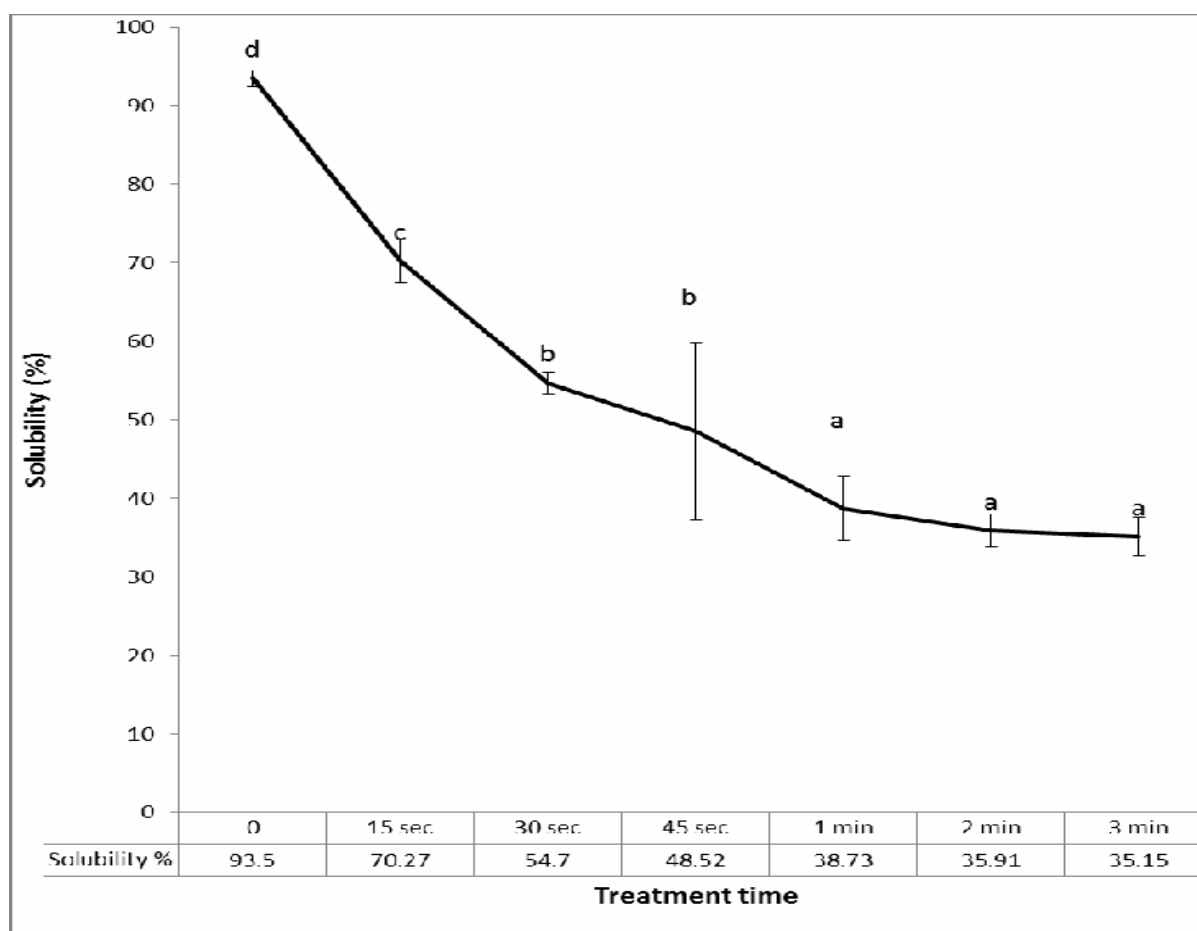


Figure 19: Solubility for refiner discharge gelatin films treated with different PL duration (15 sec, 30 sec, 45 sec, 1min, 2 min and 3 min). Different letters above the line indicate significant differences between mean values of the treatment ($p < 0.05$).

4.14.4. Structural characteristics of the films

Figure 20 shows the FTIR spectra of control and PL treated refiner discharge gelatin films in the wavenumbers range of 2000-400 cm^{-1} . Variations in the peak positions were observed in PL treated films as compared with the control film. In the control film, prominent absorption peaks in the Amide I and Amide II region are seen. However, the intensity of the peaks of Amide I and Amide II region decreases with the increased in PL treatment time from 15 sec to 3 min.

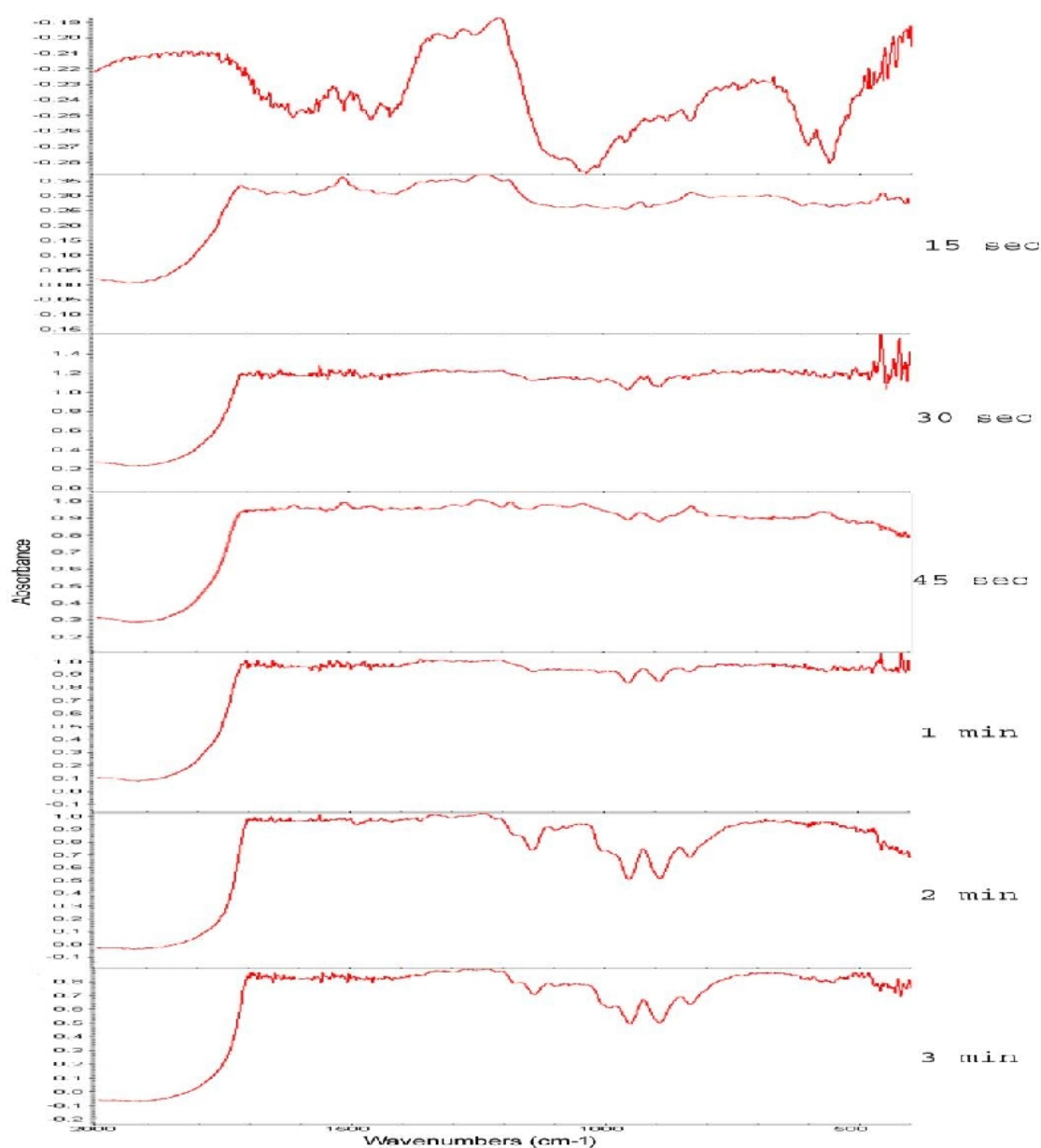


Figure 20: FTIR spectra of refiner discharge gelatin film at different PL treatment (0:control film without PL treatment;15 sec, 30 sec,45 sec, 1 min, 2 min, 3 min: different duration of PL treated films).

4.14.5 Thermal stability of the films

The DSC profiles revealed that pulsed light treatment altered the thermal behavior of the gelatin films (Figure 21). The transition temperatures and respective enthalpies are given in Table 27. There are two endothermic peaks, small and broad respectively in all the films. The first small endothermic peak is taken as first transition temperature (T_1) while the second broad endothermic peak is taken as second transition temperature (T_2) of the films. The second endothermic peaks of the pulsed light treated films are deeper than the second endothermic peak of the control films. The T_2 of PL treated film was spread between 114.11 °C to 136.77 °C with no correlation with PL treatment time. The T_2 of control film occurred at 127.29 °C. The enthalpies of T_2 of PL treated films are higher than the control films.

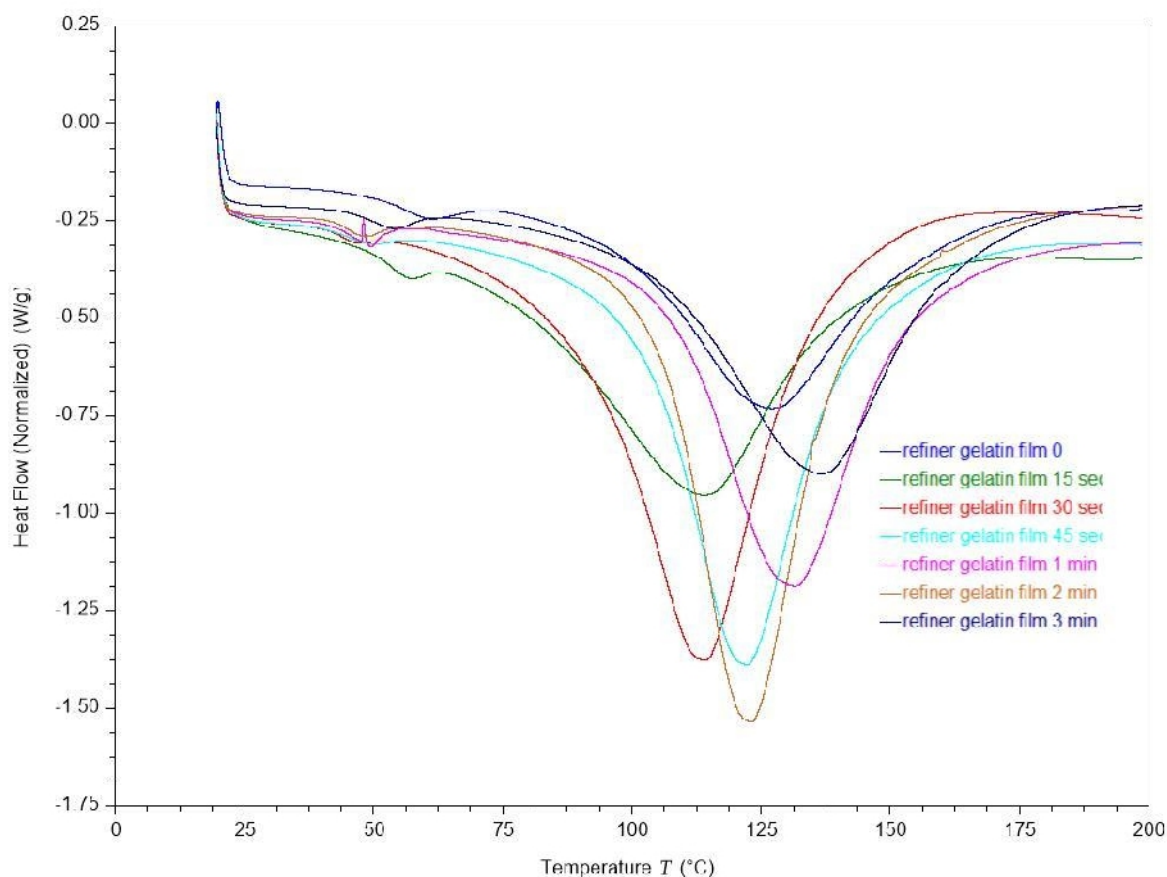


Figure 21: DSC thermogram of refiner discharge gelatin films at different PL treatment (15 sec, 30 sec, 45 sec, 1 min, 2 min and 3 min different duration of PL treated films; 0 indicate control film)

Table 27: Transition temperatures and respective enthalpies of the pulsed light treated gelatin films

Treatment	First transition temperature (T ₁)	Enthalpy ΔH_1 (j/g)	Second transition temperature (T ₂)	Enthalpy ΔH_2 (j/g)
0	60.96	3.62	127.29	201.08
15 sec	56.87	1.97	114.11	271.04
30sec	46.82	1.23	114.34	346.13
45sec	49.47	2.14	122.35	296.61
1 min	49.46	3.42	131.48	253.64
2 min	48.32	2.39	123.13	330.24
3 min	46.64	3.26	136.77	277.23

SUMMARY

Waste management resulting from the fishing industries has been of vital concern to environmental pollution. The waste generated such as skin, bone, scale can be utilized for collagen and gelatin extraction. For a long time gelatin has been produced from mammalian sources. Nowadays, there has been focus on potential substitute to mammalian gelatin. Of the fish processing wastes, skin, scale and bones are well documented for collagen and gelatin extraction. Waste generated in fish surimi plant such as refiner discharge can be new potential raw materials for protein extraction.

The proximate composition of refiner discharge from pink perch surimi revealed that it contained fairly high amount of protein (13.67 %) making it suitable for the preparation of value added compound such as collagen and gelatin. Because, collagen is the main protein in connective tissue, the study of constitutional study also support that refiner discharge can be a good source of collagen/ gelatin extraction as muscle and connective tissues constituted the major component (61.38 %) of it.

Type I collagen was successfully extracted from refiner discharge with extract yield of 11.47 %. The collagen have higher L* value (94.52). The high lightness value of the refiner discharge collagen can give better advantage when used as food additive because it will not lead to any changes in the color of final products. The thermal stability of the extracted collagen (23.07 °C) was lower than the body temperature. The collagen is rich glycine, alanine, glutamic acid and proline. The collagen also has higher emulsion activity and stability. The collagen has high tolerance to salt i.e it has higher relative solubility of about 59.70 % at 1 % NaCl concentration and not much change in the solubility was observed on further increase of NaCl concentrations. Except tryptophan the refiner discharge collagen contains all the essential amino acids accounting around 19.30 % of the total amino acids.

The FTIR analysis of the refiner collagen shows that Amide-A peak appeared at 3346.63 cm^{-1} . While the Amide-B peak occurred at 2939.22 cm^{-1} . The Amide-I, II and III peak were observed at 1678.52 , 1551.87 and 1239.87 cm^{-1} respectively. Electrophoretic studies on refiner discharge collagen revealed the occurrence of non-crosslinked α monomers (α_1 at 125 kDa while α_2 at 113 kDa), along with cross-linked component β dimers at 176 kDa. There are also other fractions of around 103 kDa that appear under the α_2 band showing the possibilities of presence of α_3 chain.

RSM was successfully employed to optimise the extraction conditions of gelatin and the optimised parameters are 0.2 M NaOH pretreatment concentration, 70°C extraction temperature and 3.2 h extraction time. Extraction procedure of refiner discharge gelatin was significantly influenced by alkali concentration, extraction temperature and extraction time. The bloom value, viscosity and melting point of the refiner discharge gelatin were found to be 147.97 g, 7.44 Cp and 25.50°C respectively. The essential amino acids in the refiner discharge gelatin contributed 16.02 % of the total amino acids.

The SDS-PAGE profile revealed that the optimised conditions do not causes protein break down as it has high intensity of β - and α -chains as the major components. No visible low molecular weight bands were found. The gelatin obtained from refiner discharge exhibited the amide-A and B bands at 3315.95 cm^{-1} and 2967.03 cm^{-1} respectively. Amide-I band was found at 1654.47 cm^{-1} while that of amide-II and amide-III bands occurred at the wavenumbers of 1578.53 cm^{-1} and 1232.79 cm^{-1} respectively.

During the last decades several biopolymers were used to produce films and for this purpose fish gelatin can also be used as it have good film forming properties. At the same time physical modifications can be easily employed to improve the properties of the film.

Gelatin films were successfully developed from refiner discharge gelatin and minimum gelatin requirement to form film was found to be 3 %. The developed films have high solubility in water and higher EAB which may be beneficial as edible wrappers. Pulsed light treatments (15 sec, 30 sec, 45 sec, 1 min, 2 min and 3 min) greatly influence the mechanical, functional and conformational properties of the gelatin films. Crosslinking effect of pulse light is visible in all the treatment durations of pulse light. Crosslinking effect of pulsed light on gelatin film was supported by the electrophoresis, FTIR and DSC data. Prominent protein bands such as β and α were observed in control film. However, low intensity bands appeared in the PL treated films. There was visible formation of higher molecular weight aggregates at the top of the gel in SDS-PAGE. Variations in the peak positions of the Amide I were observed in the control film and PL treated films. The DSC profiles revealed that the enthalpies of the transition temperature of the PL treated films were higher than the control film indicating the formation of higher molecular weight protein aggregates.

This study demonstrates that refiner discharge can be exploited as a new source of collagen and gelatin production. Refiner discharge has added advantage of avoiding the segregation problem of solid waste into skins, scale and bones before the extraction of gelatin. Packaging films can be successfully prepared from gelatin recovered from refiner discharge. These gelatin films present a good potential for their utilization in food packaging as an alternative to synthetic films and pulsed light can be employed for easy modifications of the gelatin films properties.