EXTRACTION OF COLLAGEN FROM FISH WASTES, OPTIMIZATION AND CHARACTERIZATION

By

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Specially dedicated to
my beloved mother and father

ABSTRACT

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Samantha Pang Cheng Fehng

80 million tonnes of fish are harvested in a year and 25 % become waste. This amount of waste is a useful resource for collagen extraction. However, the extraction procedure is yet to be optimized. This study focuses on optimizing collagen extraction conditions from fish waste (fish skin, scales, bones and fins) with the aid of response surface methodology (RSM). The statistical optimization method was designed with a 4-factor, 5-level central composite design (CCD), which the effects of acetic acid concentration (M), pH, extraction temperature (°C) and extraction time (h) in response to the extracted collagen yield (%) were fitted into the CCD. Generally, concentration of acetic acid with pH 2.96 to 3.19 at the range of 0.56 M to 0.67 M, were able to produce high collagen yields. However, the optimum extraction temperature and extraction time varied among fish waste. Optimum extraction temperature for fish skin was slightly lower (13.26 °C), whereas, scales, bones and fins require higher temperatures (16.6 °C to 19.03 °C). In terms of extraction time, fish scales need longer hours (77.51 h), as compared to skin (74 h), bones (73.16 h) and fins (72.36 h). The optimized extraction conditions yielded 2.27 % for skin, 0.13 %, 0.64 % and 0.82 % for scales, bones and fins, respectively. SDS-PAGE pattern confirmed that fish skin, scale, bone and fin collagen were all type I collagen consisting of two α_1 and

α_2 chains. Denaturation temperature (T_d) of collagen from skin, scales, bones and fins were 36 °C, 36.15 °C, 37.80 °C and 32.60 °C, respectively. Besides, fourier transform infrared spectroscopy (FTIR) proved that the collagen of these fish were integrated and native. In addition, reversed-phase high-performance liquid chromatography (RP-HPLC) showed that these collagen had high contents of imino acids, which contributed to their high denaturation temperatures. These findings suggested that fish waste skin, scale, bone and fin collagen possess the potential to be an alternative collagen source for a variety of uses in many fields.

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Samantha Pang Cheng Fehng

APPROVAL SHEET

This dissertation/thesis entitled "**EXTRACTION OF COLLAGEN FROM FISH WASTES, OPTIMIZATION AND CHARACTERIZATION**" was prepared by SAMANTHA PANG CHENG FEHNG and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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It is hereby certified that ***Samantha Pang Cheng Fehng*** (ID No: ***12UEM01365***) has completed this thesis entitled "*Extraction of Collagen from Fish Wastes, Optimization and Characterization*" under the supervision of Dr. Woo Kwan Kit (Supervisor) from the Department of Chemical Engineering, Lee Kong Chian Faculty of Engineering and Science, and Dr. Chang Ying Ping (Co-Supervisor) from the Department of Chemical Science, Faculty of Science.

I understand that the University will upload a softcopy of my thesis in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

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(*Samantha Pang Cheng Fehng*)

DECLARATION

I hereby declare that this project report is based on my original work except for citations and quotations which have been duly acknowledged. I also declare that it has not been previously and concurrently submitted for any other degree or award at UTAR or other institutions.

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(SAMANTHA PANG CHENG FEHNG)

Date 19 September 2016

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g	Force of gravity, RCF
T_d	Denaturation temperature, °C
ε1	Viscosity at 9°C
ε2	Viscosity at measured temperature
ε3	Viscosity at 45°C
AccQ Fluor reagent	6-amino quinolyl-N-hydroxysuccinimide carbamate
ASC	Acid soluble collagen
CCD	Central composite design
EDTA-2NA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
PAGE	Polyacrylamide gel electrophoresis
PSC	Pepsin soluble collagen
SDS	Sodium Dodecyl Sulphate
Std.	Standard
Dev.	Deviation
Adj	Adjusted
Pred	Predicted
Adeq	Adequate
RSM	Response Surface Methodology
VIF	Variance Inflation Factor

CHAPTER 1

INTRODUCTION

1.1 Background

Collagen is the most plentiful animal protein polymer and comprises of about 30 % of the animal body's total protein. In animal skin and bone connective tissue, it is the major structural protein (Gelse et al., 2003; Singh et al., 2011). In the cosmetic, pharmaceutical, biomedical, film and leather industries, collagen has a broad range of applications (Ogawa et al., 2004; Kittiphattanabawon et al., 2010). Collagen's soluble form is useful in a various fields such as food, cosmetics and pharmaceutical due to its unique characteristics i.e biodegradability and weak antigenicity (Kolodziejska et al., 1999). Fish collagen chemical and physical properties are different from those of mammalian collagen (Zhang et al., 2007). As such, collagen from fish waste is not likely to be linked with infections such as transmissible spongiform encephalopathy (TSE), bovine spongiform encephalopathy (BSE) and foot and mouth disease (FMD) which are the major drawback of mammalian collagen. Hence, fish waste may also be an effective alternative source for collagen production (Nagai et al., 2001; Jongjareonrak et al., 2005). Moreover, bovine sources of collagen are prohibited for Hindus and Sikhs. Porcine collagen is strictly not allowed to be consumed by Jews and Muslims, but they can use collagen obtained from other sources. Therefore,

alternative sources of collagen, such as aquatic animals include freshwater and salt fish and mollusks have been obtaining vast attention (Shen et al., 2007). Thus, the aims of this study were to optimize the extraction process of collagen from fish waste obtained from local wet market, and characterize the extracted collagen.

1.2 **Problem Statement**

Fish processing industries dispose huge amounts of fish waste. The fish waste industry's annual discard amount is approximately 25 % of the total production, which is around 20 million tonnes in Malaysia (FAO, 2012). Majority of waste from fish processing industries were bones, skin, scales, and fins, which constitutes over 70 % of fish (Benjakul and Morrisey, 1997; Nurdiyana et al., 2008). These large amounts of waste are discarded as garbage without effort to reduce the harmful effects to the environment and disposal problems that arise. These wastes have almost the same amount of protein content as in fish flesh (Batista, 1999), therefore part of them are utilized as feedstuff. Nevertheless, major parts of these wastes were not fully utilized and most of them were discarded (Arnesen and Gildberg, 2007). This waste load problem does not only occur in Malaysia, as it is a globalized issue. Consequently, studies of fish waste were carried out to produce value added products such as collagen which is feasible and to further diversify the usage of fish waste (Ioannis and Kassaveti, 2008; Singh et al., 2011).

CHAPTER 2

LITERATURE REVIEW

2.1 Collagen

Collagen is the major structural protein in all animal bodies which consists of three polypeptide chains in the triple helix. All collagen differ greatly in terms of their size, function and tissue distribution (Gelse et al., 2003). To date, there are 28 types of collagen, namely type I to XXVIII (Gordon and Hahn, 2010). According to their structure and supramolecular organization, they are categorized into fibril-forming collagens (Collagen type I, II, III, V, and XI), basement membrane collagens (Collagen type IV), microfibrillar collagen (Collagen type VI), anchoring collagens (Collagen type VII), hexagonal network-forming collagens (Collagen type VIII and X), FACIT collagens (Collagen type IX, XII, XIV, XIX, XX and XXI), transmembrane collagens (Collagen type XIII and XVII), multiplexins (Collagen type XV, XVI and XVIII) and others (Collagen type XXVIII) (Gelse et al., 2003; Gordon and Hahn, 2010).

2.1.1 **Collagen Family**

All these types of collagens are characterized by their significant complexity and variety in their structures, splice variants, occurrence of added, non-helical domains and their functions. Fibril-forming collagens are the largest and most extensive family of collagens which comprises 90 % of the total collagen. Types I and V collagen fibrils play a part in the structuring of bone, whereas types II and XI collagen mainly are a part of the fibrillar matrix of cartilage. The tensile strength and torsional stability properties of collagen affect the stability and integrity of these tissues (Mark, 1999; Gelse et al., 2003). Collagen IV forms sheet-like structures that constitute the basement membrane. Collagen VI is a microfibrillar collagen containing disulfide cross-links that contribute to the network of thin-beaded filaments interconnected together with other collagen fibrils and cells (Mark et al., 1984; Rest and Garrone, 1991; Gelse et al., 2003). Collagen of type VII congregates into anchoring fibrils which join epithelial basement membranes and trap collagen fibrils from the underlying stroma to attach the two structures together (Rest and Garrone, 1991). Fibril-associated collagens with interrupted triple helices (FACIT) such as collagen type IX, XII, and XIV connect as single molecules with huge collagen fibrils and may function in controlling the diameter of collagen fibrils (Rest and Garrone, 1991; Gelse et al., 2003). Types VIII and X collagens have supramolecular aggregate forms of hexagonal lattice (Sawada et al., 1990; Kwan et al., 1991; Gordon and

Hahn, 2010). Types XIII, XVII, XXIII, XXV are transmembrane collagens (Franzke et al., 2003; Gelse et al., 2003; Gordon and Hahn, 2010).

2.1.2 **Collagen Structure**

All collagen family members possess a similar right-handed triple helix composing of a three α-chain characteristic as shown in Figure 2.1 (Exposito et al., 2010). Structurally, the right-handed triple helix may be created by three identical polypeptide chains (homotrimers) as in collagens type II, III, VII, VIII, X, and others. However, most abundant collagen types such as I, IV, V, VI, IX and XI are heteromeric species, that is the right handed triple helix which is formed by two or more different chains (heterotrimers). Each three α-chain in the molecule create an extensive left-handed helix and has a pitch of 18 amino acid residues per turn (Fallas et al., 2009). The three α-chains that form the right-handed superhelix have one staggered residue between adjacent chains, and arranged as in a supercoil round a common central axis in a right-handed style to produce the triple helix structure. The triple helical collagenous domain is characterized by a glycine in every third position of the polypeptide chain which creates a $(Gly\text{-}X\text{-}Y)_n$ repeat structure. The α-chains are assembled round a central axis in a manner that all glycine residues are placed in the middle of the triple helix, whilst larger side chains of other amino acids are placed in the outer positions. This permits the peptide chains to pack tightly down the central axis of the molecule whilst avoiding steric clashes in the center of the assembly. The X

and Y positions are frequently filled by proline and hydroxyproline. Relying on the type of collagen, certain proline and lysine residues are altered by post-translational enzymatic hydroxylation. The content of 4-hydroxyproline is vital for the development of intramolecular hydrogen bonds and contribute to the triple helical conformation's stability. The length of the triple helical part differs considerably between different collagen types (Gelse et al., 2003; Exposito et al., 2010; Gordon and Hahn, 2010). In fibril-forming collagens (I, II, III), with the exception of collagens type XXIV and XXVII, the (Gly-X-Y) repeat in the helix is the major triple helical domain. Fibril-forming collagens are about 1000 amino acid residues long and have a perfect Gly-X-Y triplet structure. For non-fibrillar collagens, they all have at least one interruption in the triple helices (Gordon and Hahn, 2010). N-peptide or N-propeptide is the amino end of a fibrillar collagen. It normally consists of at least one small triple helical domain, called the minor helix.

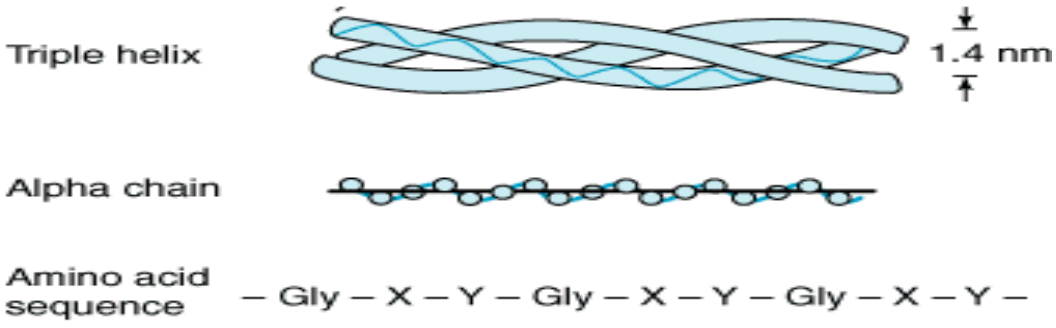


Figure 2.1: Molecular structure of a collagen triple helix in type I collagen molecule (Chhabra, 2013)

After the major triple helix is made, the amino and carboxyl ends are processed. The processed molecules are aligned in an arrangement of a quarter stagger in the growing fibril. Type V and XI collagens respectively nucleate fibrils of types I and II collagens (Kadler et al., 2008; Gordon and Hahn, 2010). The fibril diameter is regulated by the N-peptides of collagen type V and XI which are retained after processing (Birk, 2001; Gordon and Hahn, 2010). Regulating fibril diameter is very important for normal tissue function. For fibrillar collagens, collagen type XXIV and XXVII are special because they have shorter major triple helices and have one or two interruptions compared to other fibrillar collagen members (Koch et al., 2003; Gordon and Hahn, 2010).

2.1.3 **Collagen Source**

Bovine and porcine skins are the main industrial sources for collagen production. Starting from the 1930s, the first raw material to be used for the production of collagen was porcine skin, and to this day, the most significant raw material for large-scale industrial productions is still porcine skin (Gomez-Guillen et al., 2011). Porcine skin collagens have been reported to contain bovine spongiform encephalopathy (BSE), whereas fish collagen has a lower risk of acquiring unknown pathogens such as BSE (Yamauchi, 2002). Besides, religious sentiments for the Jewish and Islamic believers whom prohibit the consumption of pork, whereas Hindus forbid the consumption of cow products, have caused pessimism and concerns among consumers to use mammalian collagen as a

source (Karim and Bhat, 2009). Therefore, a lot of researches have been done to find an alternative source of collagen. So far, fish waste collagen was found to have the most similar characteristics to mammalian collagen. Thus, it is likely to draw the industry's attention as an alternative source to mammalian collagen (Nagai and Suzuki, 2000a; Kim and Mendis, 2006).

Throughout the decade, huge numbers of fish species were investigated as alternatives to the source of collagen. All species studied showed to have different denaturation temperature characteristics. This had raised interest among the research communities in optimizing the extraction conditions and yields, and also characterizing the resultant collagens. These collagens were mainly extracted from fish skin and bone residues. Cod, Atlantic salmon and Alaska pollock are cold-water species that were researched on their physio-chemical and functional properties. Cold-water species were reported to have significantly lower denaturation temperatures (around 4-17 °C) as compared to tropical species (around 18-29 °C). Examples of sub-tropical species were Nile Perch, red Tilapia, channel catfish, yellowfin tuna, skate or grass carp (Karim and Bhat, 2009; Gomez-Guillen et al., 2011).

Fish collagen that were suggested as potential ingredients in the manufacturing of functional food, cosmetics, biomedical and pharmaceutical applications were obtained from trout and hake (Montero and Borderias, 1991), brownstripered snapper (Jongjareonrak et al., 2005), deep-seared fish (Wang, An,

et al., 2008), walleye pollock (Yan et al, 2008), and unicorn leatherjacket (Ahmad and Benjakul, 2010). Fish wastes consist of 5 % of fish scales (Wang and Regenstein, 2009) are major fish industry residues. Red Tilapia and seabream (Ikoma et al., 2003), Sheepshead and black drum (Ogawa et al., 2004), grass carp (Li et al., 2008) and deep-sea red fish (Wang, An, et al., 2008) were widely studied for extraction of collagen from their scales. Fins that were waste products of canned tuna processing were suggested as high-quality source of collagen extracted but with low yields (Aewsiri et al., 2008).

Fish offal from semi-processed products of marinated or salted herring or cold-smoked salmon were researched as a source of collagen where it was found that smoking did not change a collagen's denaturation temperature and had higher denaturation temperatures compared to collagens from marinated or salted skins (Kolodziejska et al., 2008; Gomez-Guillen et al., 2011).

Collagen from alligator bones which were produced as huge waste in China, Thailand and the southern United States were also studied. These collagens were type I collagens that had nearly similar intrinsic characteristics to those of tropical fish species such as black drum and sheepshead (Wood et al., 2008; Gomez-Guillen et al., 2011).

Giant red sea cucumber was also considered a possible collagen source for pharmaceutical applications (Liu et al., 2010) where it was also found to be of

type I collagen but had very low imino acid content as compared to cold-water fishes. Poultry by-products were also used for production of collagen-based, high value-products as collagens type I and type III were extracted (Cliche et al., 2003; Gomez-Guillen et al., 2011).

2.1.4 **Industrial Extraction Process of Collagen**

Collagen in its native insoluble form has to be treated first, only then it can be converted into a form that is appropriate for extraction. This pre-treatment is a step that is completed by heating the collagen in acid or alkali at 45 °C and above (Karim and Bhat, 2009; Gomez-Guillen et al., 2011). Chemical pre-treatment will rupture non-covalent bonds, cleaving intra- and intermolecular covalent cross-links which cause the protein structure to be disordered, therefore creating sufficient swelling and solubility of collagen (Stainsby, 1987; Bailey and Light, 1989; Karim and Bhat, 2009; Gomez-Guillen et al., 2011). Subsequent heat treatment breaks the hydrogen bonds and covalent bonds, causing the triple-helix to be unstable. Thus a transition of helix-to-coil occurs and converts it into soluble gelatin (Djabourov et al., 1993; Gomez-Guillen et al., 2002; Gomez-Guillen et al., 2011). The properties, preservation of the raw material, pre-treatment, extraction process parameters such as temperature, pH and time, affect the polypeptide chain length and the gelatin's functional properties (Karim and Bhat, 2009).

Collagen raw materials include porcine skin, bovine skin and bones are generally used to produce gelatin, which is the hydrolyzed form of collagen on commercial scale. Production of collagen or gelatin differs among these raw materials used. First, all raw materials are cut, washed and cleaned to eliminate non-collagenous materials. Then, the crude collagenous materials undergo further processes to produce either type A or type B gelatin depending on their source.

Type A gelatin (isoelectric point at pH 6 - 9) is produced from an acid treatment and is more appropriate for less covalently cross-linked collagens (or known as young collagen) from porcine and fish skin. Type B gelatin (isoelectric point at approximately pH 5) is extracted from alkaline and acid treatment (Stainsby, 1987; Karim and Bhat, 2009; GMAP, 2013). This method is more suitable for bovine skin and bones, as the collagen molecule is complex which contains more covalently cross-linked (or known as old collagen). Both type A and B collagens are then channeled to the extraction, filtration, concentration, drying, grinding, milling and blending (Karim and Bhat, 2009; GMAP, 2013).

During the extraction process, the raw materials are submerged in warm water, with regulated temperatures, to extract the gelatin. This liquid form is precipitated with NaCl to clean and refine the product which is recovered by filtration. It may also include centrifugation, ultra-filtration and ion exchange to remove the inorganic materials. Then, the liquid is applied to a multi-phase vacuum evaporator to subtract the water content from it, producing a very viscous

solution. A high temperature flash sterilization is applied to prevent the possibility of microbial contamination. Subsequently, the gelatin is made into a gel form by chilling in a votator, mincing and depositing it as a bed onto an open-weave belt that goes through a drying chamber. A filtered dry air stream is flowed into the chamber and through the belt, which dries the gelatin. Following physical and microbiological testing, the gelatin particles are crushed, milled and mixed together to make trade-quality gelatin for specific applications. The gelatin is also examined for approval, packed, stocked and supplied (De Wolf, 2003; Karim and Bhat, 2009; GMAP, 2013).

2.1.5 **Fish Waste as Source of Collagen**

Every year, over 100 million tonnes of fish are being collected globally with 29.5 % of a capture is used as fish feed due to its lack of functional properties (Kristinsson and Rasco, 2000; See et al., 2010). Approximately 70 -85 % of a capture becomes fishery processing waste and 30 % of these wastes are bones, fins, scales, skin which contain high levels of collagen, depending on the process used and the types of products (Shahidi, 1994).

In 2010, fishery captures and aquacultures supplied the world with 148 million tonnes of fish, where 128 million tonnes were used as food. Preliminary data showed that in 2011, an increase of 154 million tonnes of fish was retrieved, and 131 million tonnes of these will be accounted for as food (Table 2.1 and Figure 2.2). For the past 50 years, there was an average 3.20 % per year growth

rate in the period of 1961 - 2009, that was growing faster than the world's population increasing growth rate of 1.7 % per year (Figure 2.3). Out of the 126 million tonnes for human consumption, Africa had the lowest fish consumption (9.1 million tonnes), whilst Asia covered two-thirds of the total consumption with 85.4 million tonnes (FAO, 2012).

Table 2.1: World fisheries and aquaculture production and utilization (FAO, 2012)

	2006	2007	2008	2009	2010	2011
			(Million tonnes)			
Production						
Capture						
Inland	9.80	10.00	10.20	10.40	11.20	11.50
Marine	80.20	80.40	79.50	79.20	77.40	78.90
Total capture	90.00	90.30	89.70	89.60	88.60	90.40
Aquaculture						
Inland	31.30	33.40	36.00	38.10	41.70	44.30
Marine	16.00	16.60	16.90	17.60	18.10	19.30
Total aquaculture	47.30	49.90	52.90	55.70	59.90	63.60
Total world fisheries	137.30	140.20	142.60	145.30	148.50	154.00
Utlization						
Human consumption	114.30	117.30	119.70	12360	128.30	130.80
Non-food uses	23.00	23.00	22.90	21.80	20.20	23.20
Population *(billions)*	6.60	6.70	6.70	6.80	6.90	7.00
Per capita food fish supply *(kg)*	17.40	17.60	17.80	18.10	18.60	18.80

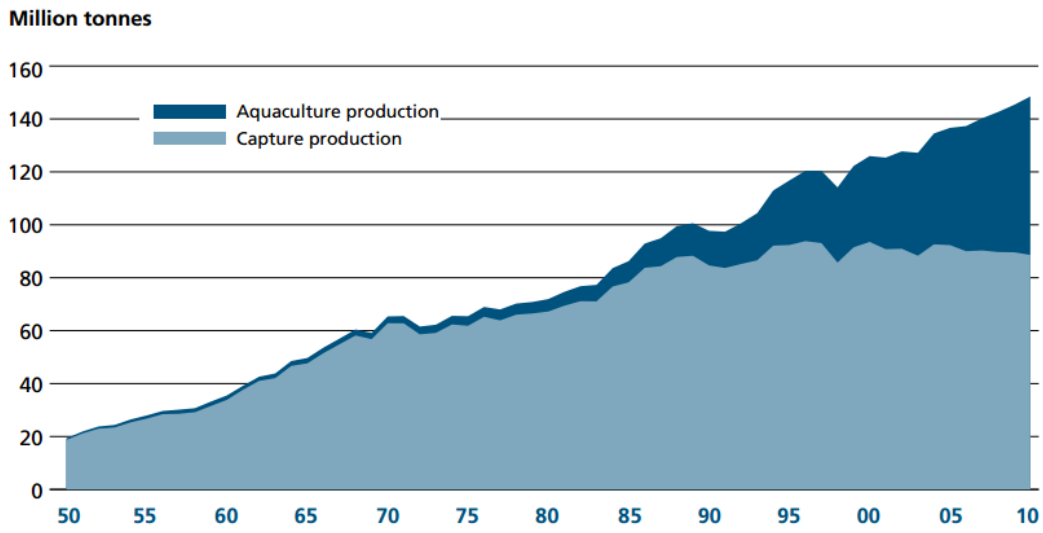


Figure 2.2: World fisheries and aquaculture capture production (FAO, 2012)

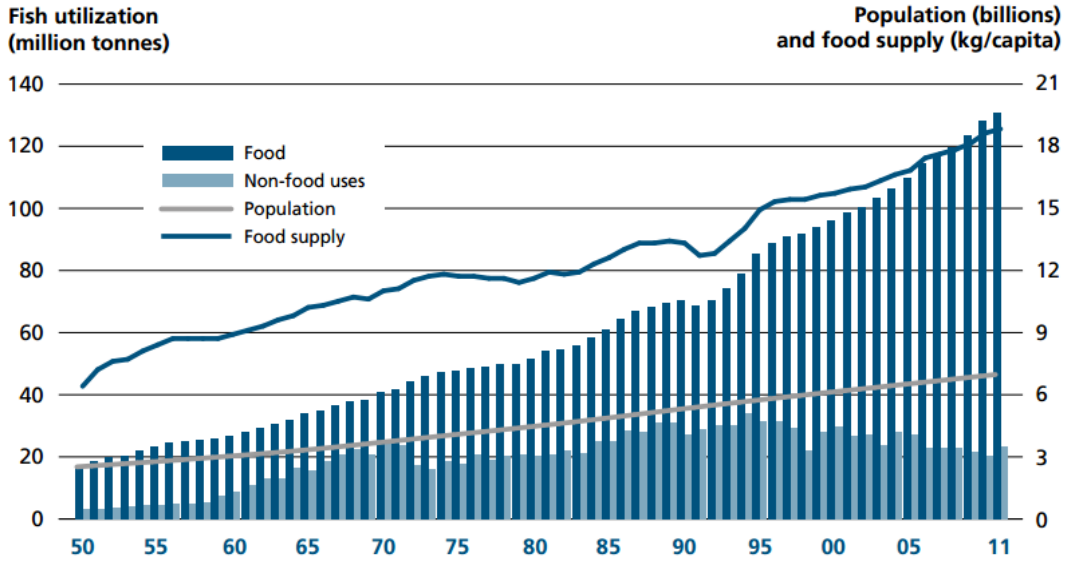


Figure 2.3: World utilization and supply of fish (FAO, 2012)

In year 2010, Malaysia's aquaculture and capture production hit 2 million tonnes compared to 1 million tonnes per year in 1990 as shown in Figure 2.4 (FAO, 2013), where 31 % were accounted as inedible fish waste (FAO, 2012). These discards can serve as good unprocessed materials for manufacturing high protein food such as gelatin. Alteration of these discards into value-added products to produce extra profit has benefits for both economic and waste management of the fish industry (Choi and Regenstein 2000). In Malaysia, 90 % of marine fish are from sea capture, whereas the remaining are of freshwater and aquaculture fish. Approximately 75 % of the fish supply is for human consumption; on top of that, 25 % are utilized for fish oils. 30 % of the fish supplies for human consumption are processed into canned food, fish fillets, chips and protein products. Waste products that are to be discarded are produced at the end of the processing and human consumption. Fish processing itself is a foremost contributor to the overall environmental pollution particularly emitting waste organic odor problems. Various types of efforts have been done to solve this problem of waste discarding which comprise of manufacturing of fish sauce, protein concentrates and hydrolysates protein (Ibrahim et al., 2013).

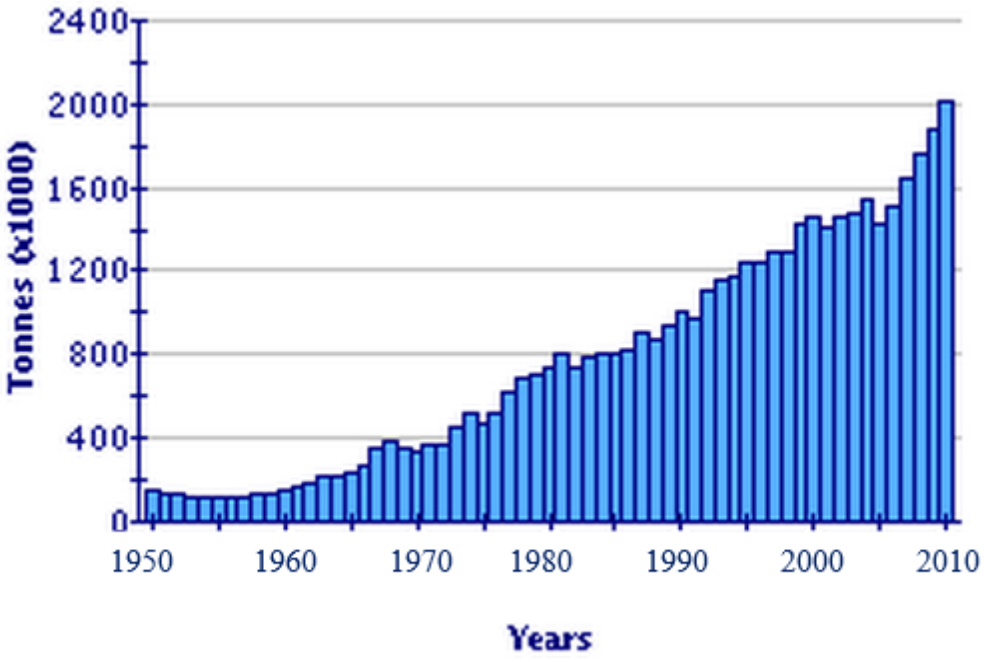


Figure 2.4: Malaysia's aquaculture and capture production from year 1950 to 2010 (FAO, 2012)

2.2 Physico-chemical Properties of Fish Collagen

Amino acid composition and thermal stability are the major factors that contribute to the physico-chemical properties of collagen. These characteristics vary among fish species. Amino acid composition in collagen plays a crucial role in thermal stability. Collagen triple helix consist of repeating sequences of X-Y-Gly, in which X and Y are proline and hydroxyproline, Gly represent glylcines. Presents of imino acids (proline and hydroxyproline) promote the formation of hydrogen bonds between hydroxyproline residues belonging to the X and the Y positions of adjacent chains (Berisio et al., 2004). Therefore, a higher amount of

imino acids in a collagen would contribute to higher thermal stability, for example in brownstripered snapper skin contains 212 residues per 1000 residues of imino acids, would have a high denaturation temperature of 31.50 °C (Jongjareonrak et al., 2005) as compared to grass carp skin collagen with imino acid contents of 186 residues/1000 residues shows the denaturation temperature of 24.60 °C (Duan et al., 2009). Besides, high amino acid compositions resulted in high denaturation temperatures although both animals live in different habitats. For example, sub-tropical fish species such as the *Oreochromic niloticus* Tilapia (Sujithra et al., 2013) had nearly similar characteristics (amino acid compositions and thermal stability of 32 °C) to mammalian collagen (porcine skin collagen with thermal stability of 37.80 °C). Therefore, fish collagen with similar characteristics to mammalian collagen would show that fish collagen is suitable to be an alternative source to mammalian collagen in various applicable fields.

2.2.1 **Amino Acid Composition**

The amino acid composition between fish and mammalian collagen are slightly different, as amino acid composition affects the collagen molecule structure, while the structure and conformation of the collagen in solutions affect the physical properties of the collagen such as rheological properties (Ramachandran, 1988; Zhang, M. et al., 2010).

As a guide for an approximate amino acid composition of fish waste collagen extracted from fish, Table 2.2 lists the amino acid composition of acid-soluble collagen of carp skin, scale and bone in comparison with calf skin collagen (Duan et al., 2009). Fish collagen has been proven to differ widely in their amino acid composition. In particular, the amount of imino acids which are hydroxyproline and proline vary significantly among fishes. About 34 % of glycine was present in all fish skins, scales, bones and calf skins collagen (Table 2.2). Reports from Jongjareonrak et al. (2005), Liu et al. (2007), and Nagai et al. (2010) revealed that fish skin had approximately 23 % of glycine as it is the most abundant amino acid in collagen. However, Nile Tilapia skin had 35.6 % of glycine (Zeng et al., 2009). Generally, glycine appears evenly, at every third residue all through most of the collagen molecules, except for the first 10 from the C-terminus and the first 14 amino acids from the N-terminus (Foegeding et al., 1996; Jongjareonrak et al., 2005; Zeng et al., 2009). No traces of cysteine, and tryptophan, and very low amounts of methoinine, histidine and tyrosine are found in most fishes such as in carp skin, scale, bone collagen (Duan et al., 2009) and Nile Perch skin (Muyonga et al., 2004). High quantities of glycine (Gly), proline (Pro) and alanine (Ala) are the characteristics of all collagens (Ogawa et al., 2004), as clearly seen in carp skin, scale and bone, and calf skin collagen, these three amino acids are in high amounts which is more than 100 residues/1000 residues. Methionine and phenylalanine distribution in calf skin collagen is the lowest amount compared to the other amino acid contents in calf as depicted in

Table 2.2. There are no reported evidence to relate these amino acids and denaturation temperatures of collagen (Table 2.2).

Table 2.2: Amino acid composition of calf skin, carp skin, scale and bone collagen (Duan et al., 2009)

Amino acid (residues/1000 residues)	Calf skin collagen	Carp skin collagen	Carp scale collagen	Carp bone collagen
Aspartic acid	45	49	48	47
Threonine	18	24	23	25
Serine	33	35	35	33
Glutamic acid	75	76	76	76
Glycine	330	332	336	334
Alanine	119	118	119	122
Cysteine	-	-	-	-
Valine	21	19	19	19
Methionine	6	14	14	13
Isoleucine	11	10	9	10
Leucine	23	22	21	21
Tyrosine	3	3	2	3
Phenylalanine	3	13	12	13
Hydrolysine		7	8	8
Lysine	26	28	26	26
Histidine	5	5	5	4
Arginine	50	55	55	54
Tryptophan	-	-	-	-
Hydroxyproline	94	76	77	80
Proline	121	114	115	112
Imino acid	215	190	192	192

Imino acids such as hydroxyproline and proline in carp skin, scale and bone were 190, 192, and 192 residues/1000 residues, respectively but skins of calf consists of 215 residues/1000 residues, which are about 19% of the collagen (Table 2.2) (Giraud-Guille et al., 2000; Duan et al., 2009). This shows that mammalian collagen have higher amounts of imino acids.

Collagen extracted from many fish species has similar imino acid profiles. The imino acid content in collagen extracted from various fish skin are within the range of 180 – 210 residues/1000 residues. For example, the skins of Nile Tilapia (210 residues/1000 resides), brown banded bamboo shark skin (207 residues/1000 residues), cod skin (154 residues/1000 residues) (Duan et al., 2009), bigeye snapper skin (193 residues/1000 residues) (Kittiphattanabawon et al., 2005), and surf smelt skin (172 residues/1000 residues) (Nagai et al., 2010). Imino acid contents are around 17.0 - 19.7 % for fish scale collagen of ocellate puffer fish (Nagai et al., 2002), bone collagen of *Pagrus major* and *Oreochromis niloticus* (163 residues/1000 residues) (Ikoma et al., 2003) and 19.3 % in Japanese sea bass caudal fin collagen (Nagai, 2004).

Proline and hydroxyproline amounts in various fish collagen would differ among their species and living environment (Foegeding et al., 1996). The amount of imino acids, especially hydroxyproline affects collagen's thermal stability. This is due to the presence of hydroxyproline in the inter-chain hydrogen bonding that stabilizes the collagen's triple helical structure (Gurry et al., 2010). If fish collagen's thermal stability can be improved, the fish skin, scale, bone and fin collagen's industrial use would be extended to numerous fields (Nagai and Suzuki, 2000a) especially in medical or food applications. For example, in medical usage (such as implants and scaffolds), the denaturation temperature which is higher than normal body temperature (37 °C) will be sufficient to withstand changes of physical properties (David et al., 2015).

Fish collagen generally has lower imino acid contents than that of mammalian collagen, as the imino acid content is related to their habitats (Foegeding et al., 1996; Kittiphattanabawon et al., 2005). Hydroxylation is derived from proline by post-translational hydroxylation which is mediated by prolyl hydroxylase, and the amount of hydroxylation is related to high denaturation temperature of collagen (Nagai et al., 2010).

The degree of hydroxylation of proline for carp skin, scale and bones was 40 %, 40.1 % and 41.7 %, respectively (Duan et al., 2009). Almost the same degree of hydroxylation of proline residue was observed in other collagens i.e 33.1 % for cod skin (Duan et al., 2009), 37.2 % for surf smelt skin (Nagai et al., 2010), 39.9 % for bigeye snapper skin, and 41.1 % for bigeye snapper bone. For fish scales, it was 43.65 % for sardine, 44.39 % for red seabream, 44.04 % for Japanese sea bass (Nagai et al., 2004). These reports showed that fish bones generally consist of a higher degree of hydroxylated proline as bones have more complicated structures when compared to its skins and scales of a fish (Duan et al., 2009). Hydroxyproline content's different distribution in different parts of the fish might be due to the different distribution of proline hydroxylase. This is because proline hydroxylase catalyses the oxidation of proline to its hydroxylated residue (Pearson and Young, 1989; Wong, 1989; Foegeding et al., 1996; Burghagen, 1999; Kittiphattanabawon et al., 2005).

2.2.2 **Thermal Stability**

Thermal stability is the ability of molecules to be stable at high temperatures. Molecules with a higher stability have a higher resistance to decompose at high temperatures. For collagen, the thermal stability is determined by its denaturation temperature (T_d). The T_d is determined by analyzing the viscosity of the collagen, as T_d is calculated as the temperature when fractional viscosity is 0.5 (Nagai and Suzuki, 2000a; Nagai et al., 2010). A promising feature of fish collagen is that it has a close T_d to mammalian collagen, as a high thermal stability in collagen would boost its applicability for food and pharmaceutical industrial use as it can replace mammalian collagen which are used in food and pharmaceutical industry (Nakagawa and Tagawa, 2000; Lee et al., 2001; Rodziewicz-Motowidlo et al., 2008; Singh et al., 2011). All fish species and mammalians have different T_d which is related to their environment and body temperature (Rigby, 1968; Kittiphattanabawon et al., 2005; Singh et al., 2011). Moreover, it is also suggested to be correlated with their imino acid content, and the complex interactions determined by these imino acids such as cross-linking of the imino acids, and the degree of hydroxylation of proline (Kittiphattanabawon et al., 2005; Rodziewicz-Motowidlo et al., 2008; Singh et al., 2011).

In many studies, tropical fishes were found to have higher T_d than cold-water fishes. However, the T_d of most fish collagen is lower than mammalians such as porcine collagen (37 °C). Cold-water fish's skins such as Hake (10 °C)

(Ciarlo et al., 1997), Alaska Pollack (16.8 °C) (Kimura and Ohno, 1987), and cod skin (15 °C) (Rigby, 1968) have relatively low T_d. For subtropical fishes, T_d was 26.5 °C (Japanese sea bass), 25.6 °C (chub mackerel), and 25.0 °C (bullhead shark) (Nagai and Suzuki, 2000a). Tropical fish's collagen, such as bigeye snapper (30.4 °C), brown stripered snapper (31.5 °C) (Jongjareonrak et al., 2005; Zhang et al., 2007), and striped catfish skin (39.3 °C) (Singh et al., 2011), revealed to have high T_d. Besides, T_d of surf smelt skin (32.5 °C) (Nagai et al., 2010) was also high and were just about 4.5 °C lower than porcine skin collagen (Liu et al., 2007). Fish bones showed to have high T_d with the examples of 30 °C for Japanese sea bass, 29.7 °C for skipjack tuna and ayu, 29.5 °C for yellow seabream and horse mackerel (Nagai and Suzuki, 2000a), and 30.8 °C for bigeye snapper (Jongjareonrak et al., 2005). Whereas, T_d of fish scales of sardine at 28.5 °C, red seabream at 28.0 °C and Japanese sea bass at 28.0 °C were lower than these fish bone's T_d respectively (Nagai et al., 2004). Also, Japanese sea bass caudal fin collagen was 28 °C, which showed to be 9 °C lower than porcine collagen (Nagai and Suzuki, 2000a). Nevertheless, T_d of tropical fish skins, bones and fins are higher than cold-water fish species because T_d is correlated with the environment and body temperature of the fish.

When comparing collagen's imino acids, that stabilize the collagen molecule thermally, among tropical and cold-water fish species, a high imino acid (hydroxyproline and proline) content of a tropical fish skin, which is the striped catfish (206 residues/1000 residues) (Singh et al., 2011) was obvious when

compared to cold-water fish species collagens that is the cod skin (154 residues/1000 residues) (Duan et al., 2009), carp (186 residues/1000 residues) (Duan et al., 2009), surf smelt (172 residues/1000 residues) (Nagai et al., 2010) and bigeye snapper (193 residues/1000 residues) (Kittiphattanabawon et al., 2005).

Hydroxyproline is vital in sustaining the collagen's trimers to be stabilized. Denaturation temperature (T_d) would be predicted to increase with increasing of hydroxyproline content in the collagen (Liu et al., 2007). By comparing the hydroxyproline content striped catfish's skin has a higher amount of hydroxyproline content (86-91 residues/1000 residues) (Singh et al., 2011) than red snapper's skin (81 and 86 residues/1000 residues, respectively) (Jongjareonrak et al., 2005), but had a lower hydroxyproline content when compared with collagen of calf skin (94 residues/1000 residues) (Ogawa et al., 2003; Jongjareonrak et al., 2005). Moreover, striped catfish's skin collagen was suggested to have higher T_d as it has a higher cross-linkage when compared to other fishes such as red snapper skin. Cross-linkage occurs because of the presence of imino acid content of the collagen. Therefore, as the imino acid content increases, the T_d or thermal stability of the collagen would subsequently increase as well (Sikorski et al., 1984; Wong, 1989; Kittiphattanabawon et al., 2005; Nagai et al., 2010).

2.3 Types of Extraction

Generally, there are two types of extraction methods widely used to extract collagen from fish skin, scales, bones and fins. These two types of extraction methods are extraction of acid solubilized collagen and extraction of pepsin solubilized collagen. The difference between these extraction methods is that the latter involves the use of porcine pepsin to cleave telopeptide cross-linked regions without breaking the integrity of the triple helix (Zhang et al., 2007). However, the yield, chemical composition and characteristic of the extracted collagen, using these two methods would differ from one another.

2.3.1 Extraction of Acid Solubilized Collagen (ASC)

Extraction of acid solubilized collagen (ASC) is generally adapted from the methods reported by Nagai et al. (2000) and Kittiphattanabawon et al. (2010). The whole extraction process was conducted at 4 °C. Fish skin (Appendix A), bones (Appendix B), scales (Appendix C) and fins (Appendix D) first go through an alkali treatment, using 0.1 M NaOH in order to eradicate non-collagenous proteins, fat from skin and insoluble matters. Next, it was thoroughly rinsed with distilled water until neutralized pH, followed by alcohol treatment on the skin to dissolve various odoriferous substances. After that, acid treatment was carried out with 0.5 M acetic acid to extract the collagen followed by salting out. The pellet obtained from centrifugation would be dialyzed and lyophilized. Fish bone is

decalcified and defatted with 0.5 M EDTA solution before the alkali and alcohol treatments to extract the collagen, later subjected to salting out and lyophilization (Nagai and Suzuki, 2000a; Wang, An, et al., 2008; Duan et al., 2009). Scales are first treated with NaOH and later are washed with distilled water, before they are decalcified with EDTA. Then the residues would be extracted with acid treatment, salting out and lyophilization (Duan et al., 2009). For extraction of fin collagen, after the alcohol treatment, acid soluble collagen and acid insoluble collagen are produced. The acid soluble collagen undergo the alkali and alcohol treatments whereas the acid insoluble collagen is first decalcified and defatted before proceed to the steps of alkali and alcohol treatments, salting out and lyophilization (Nagai et al., 2000).

For the past few years, many studies were accomplished to extract collagen from aquatic organisms in order to use in food, cosmetics as well as medical industries. Collagen extracted from fish skins, bones, scales and fins resulted in different amounts of yield attained which is due to the different hydroxyproline content and the degree of cross-linking in the collagen. According to Table 2.3, extraction of collagen from different types of fish skin ranged up to 50.00 %. An amount of 10.94 % collagen was extracted from skins of bigeye snapper (Kittiphattanabawon et al., 2005), Channel catfish skins and surf smelt yielded 24.00 % on dry weight (Liu et al., 2007; Nagai et al., 2010), carp skin yielded 41.30 % on a dry weight basis (Duan et al., 2009), 49.80 % from chub

mackerel, 50.10 % from bullhead shark and 51.40 % from Japanese sea bass, (Nagai and Suzuki, 2000a).

Bone collagen was 42.30 % (skipjack tuna), 40.70 % (Japanese sea bass), 53.60 % (ayu), 40.10 % (yellow seabream) and 43.50 % (horse mackerel) (Nagai and Suzuki, 2000a), but the bone collagen from carp fish contained 1.06 % collagen on dry weight basis (Duan et al., 2009), and 1.60 % from bones of bigeye snapper on a dry weight basis (Kittiphattanabawon et al., 2005). Whereas fish scales (carp fish) yielded 1.35 % (on a dry weight basis) (Duan et al., 2009), scales of sardine yielded 50.90 %, red seabream yielded 37.50 % and Japanese sea bass yielded 41.00 % (Nagai et al., 2004). For fish fin collagen, a 5.20 % (Japanese sea bass) on the basis of dry weight basis, of collagen can be obtained (Nagai and Suzuki, 2000a).

The yield of skin collagen from bigeye snapper was 10.90 %, which is a higher yield than the bone collagen of bigeye snapper (1.60 %). It was because of the higher hydroxyproline content in the skin than bone (Kittiphattanabawon et al., 2005). The different amount of yield obtained from different parts of the fish such as the skin and bone of bigeye snapper are suggested to be due to the difference in hydroxyproline content in each part (Kittiphattanabawon et al., 2005). Besides, collagen yield also differs among fish species. Different collagen yields extracted among different species could be due to the collagen is constructed differently amongst dissimilar species. Each fish species has been

reported to have different amino acid compositions, such as imino acid (hydroxyproline) contents, affecting the structure of the collagen. Therefore, bone collagen is suggested to have more complex structures than skin with the higher degree of hydroxylation of the imino acids (Kittiphattanabawon et al., 2005).

Some fish species yielded very low amounts of collagen such as the bones and scales of carp fish which were only around 1.00 %, as compared to the other fish and marine species which can ranged up to 50.00 % due to the presence low degree of cross-linked collagen. The two terminal ends of a collagen molecule are non-helical, whereas the other parts are helical. These terminal ends engage in a significant function in the cross-linking of the structure. The solubility of the collagen in acid solutions would increase when the collagen molecules are weakly cross-linked among each other at the telopeptide region, (Foegeding et al., 1996). Thus, fish species with low amounts of collagen extracted are suggested to have a lower degree of cross-linking among the collagen molecules, when compared to other marine fishes that yield higher amounts of acid solubilized collagen (ASC) collagen after extraction (Duan et al., 2009).

Table 2.3: Types of fish and their extracted collagen yield

Types of fish	Yield extracted (%)	Reference
Skin		
Bigeye snapper	50.00	Kittiphattanabawon et al., 2005
Japanese sea bass	51.40	Nagai and Suzuki, 2000a
Bullhead shark	50.10	Nagai and Suzuki, 2000a
Chub mackerel	49.80	Nagai and Suzuki, 2000a
Carp	41.30	Duan et al., 2009
Channel catfish	24.00	Liu et al., 2007
Surf smelt	24.00	Nagai et al., 2010
Bones		
Skipjack tuna	42.30	Nagai and Suzuki, 2000a
Japanese sea bass	40.70	Nagai and Suzuki, 2000a
Yellow seabream	40.10	Nagai and Suzuki, 2000a
Horse mackerel	43.50	
Carp	1.06	Duan et al., 2009
Bigeye snapper	1.60	Kittiphattanabawon et al., 2005
Scales		
Sardine	50.90	Nagai et al., 2004
Japanese sea bass	41.00	Nagai et al., 2004
Red seabream	37.50	Nagai et al., 2004
Carp	1.350	Duan et al., 2009
Fin		
Japanese sea bass	5.20	Nagai and Suzuki, 2000a

2.3.2 **Extraction of Pepsin Solubilized Collagen (PSC)**

Collagen extractions with the aid of pepsin are similar in general, minor modifications are sometimes applied in certain conditions. Jongjareonrak et al. (2005) described the pepsin extraction method where it was applied to the undissolved residues after acid extraction collagen was recovered with NaCl precipitation.

The amount of yield obtained from different types of fish ranged from as low as 4.00 % to 50.00 % were found among many researches, as this is due to the difference in fish species. A few examples of pepsin extracted collagen yields

include the brownstripe red snapper skin with a yield of 4.70 % (Jongjareonrak et al., 2005), striped catfish skin with a yield of 7.70 % (Singh et al., 2011), largefin longbarbel catfish with a yield of 28 % (Zhang, M., et al., 2009), and Nile Tilapia skin with a yield of 48.21 % (Potaros et al., 2009).

In the extraction of pepsin-solubilized collagen (PSC), the enzyme (pepsin) is added in the process of extraction, where pepsin cleaves the collagen by cleaving off its non-helical ends. Consequently, the collagen's physico-chemical properties are modified but non-collagenous proteins are hydrolyzed and an augmentation of collagen solubility is achieved (Cheng et al., 2009).

It was stated that when pepsin was applied, the cross-linked regions at the telopeptide of collagen were broken without interrupting the integrity of the triple helix (Zhang et al., 2007). Some studies reported that the extraction of pepsin-solubilized collagen would produce a two-fold yield of collagen with similar properties. For example, 85.50 % of PSC was produced from flatfish skin as compared with 57.30 % of acid solubilized collagen (ASC). Both PSC and ASC of flatfish skin had similar denaturation temperatures of 26.70 °C and 26.60 °C respectively (Heu et al., 2010).

However, some findings show a reversed trend where ASC had a higher yield and similar denaturation temperature value when compared to PSC. This can be observed in the extraction of collagen from brown red snapper conducted by

Jongjareonrak et al. (2005). Collagen skin yields of ASC and PSC brownstripe red snapper were 9.00 % and 4.70 %, respectively (on a wet weight basis) had denaturation temperatures of 30.52 °C and 30.46 °C respectively.

On the other hand, another study showed that the extraction of PSC had a slightly higher yield and also a similar denaturation temperature compared to extracting ASC. This is proven by the extraction of collagen from Nile Tilapia's skin, as the acid extracted collagen (ASC) yielded 38.84 %, whilst pepsin extracted collagen (PSC) resulted 48.21 % yield. Nonetheless, both collagens have the denaturation temperatures of 34.29 °C and 34.32 °C, respectively (Potaros et al., 2009). The study above had contradictory findings, although these collagen extractions used the method of Nagai and Suzuki (2000a). However, PSC of brownstripered snapper skin was produced using 10 % pepsin for 48 h (Jongjareonrak et al., 2005), and PSC of Nile Tilapia skin was produced using 0.10 % pepsin for 6 h (Potaros et al., 2009). When higher percentage of pepsin was used at a longer duration, PSC yield was lower. This was due to when high amount of pepsin used, the collagen was eventually cleaved, impairing the triple helix's integrity. Consequently, a moderate amount of pepsin should be used in order to obtain a higher yield.

2.4 Applications of Collagen

During the last two decades, there has been a worldwide trend of rising alertness on the financial, community and ecological aspects of the most favorable application of fishery by-products, and of its significance in decreasing waste. The global gelatin market size was 412.7 kilo tonnes in 2015. Food and beverage is the biggest application consisting of 29.0 % of the global gelatin market. Pig skin accounted for 40.6 % of that market, with Europe as the largest market, consisting of 41.2 % of the global revenue. Asia Pacific is predicted to have the fastest growth at compound annual growth rate (CAGR) of 6.0 % from 2016 to 2024. A positive outlook in the healthcare sector for Thailand and Malaysia is seen as the sales volume of pharmaceuticals is expected to increase the usage of gelatin in the future (Grand View Research, 2016). The use of fishery by-products developed into an important industry in many countries, with a rising awareness in treating by-products in a secure and hygienic way. Collagen and gelatin are utilized in various fields which include food, biomedical and cosmetic industries (Gomez-Guillen et al., 2002; Kim and Mendis, 2006).

2.4.1 Potential as an Alternative Source of Collagen and its Applications

Recently, there has been an increase in demand for collagen and gelatin in food and pharmaceutical industries worldwide. The most widely used source of collagen, especially on a commercial scale is from mammalian collagens, which are from porcine and bovine skins and bones (GMAP, 2013). Nonetheless, these

sources have been facing major restraints and skepticism among consumers because of religious beliefs. Besides, there are strong concerns about the possibility of animal tissue-derived collagens may transmit pathogenic vectors such as prions that cause bovine spongiform encephalopathy (BSE) outbreaks (Wilesmith et al., 1991). Fish collagen, especially warm-water fish collagen, has been stated to have characteristics that are similar to porcine collagen. As a result, fish collagen could be deemed as an alternative to mammalian collagen. The manufacturing of fish collagen not only fulfils the needs of consumers but also helps to decrease the amount of fish waste from a capture (Karim and Bhat, 2009). Fish collagen can be extracted from waste such as fish skin, bone and fin of fish processing by-products. Consequently, fish waste is considered a potential source of collagen.

2.4.2 **Household**

Fishery by-product collagen are applied for many functions, which include the manufacturing of cosmetics, as direct feeding for livestock and aquaculture, added into pet feed or incorporated into feed for animals that are kept for fur production, ensiling, landfill and fertilizer. For example, in the field of cosmetics, “Fish collagen +HA” (Neocell, 2014) is a product consisting of fish collagen, which is consumed to promote a healthy, strong and hydrated skin. Besides, collagen from fish waste were also used in food packaging (Ioannis and Kassaveti, 2008; Singh et al., 2011).

2.4.3 **Medical**

Collagen in the body is involved in entrapment, storage and delivery of growth factors and cytokines, thus they play significant roles in organ development, tissue repairing and wound healing (Yamaguchi et al., 1990, Gelse et al., 2003). Therefore, in the pharmaceutical industry, collagen is generally utilized for the production of wound dressing, vitreous implant, as carriers for drugs, proteins and gene delivery (Lee et al., 2001). Neuskin-F is a collagen dressing product made from fish waste collagen, which is an effective epidermal substitute that supports epidermal cell attachment and migration for donor sites or non-healing donor sites, superficial to partial thickness burns, abrasions and skin tears (Medira, 2016). Besides, collagen type I in the form of pellet was used specifically for gene transfer. It is used as a gene delivery carrier promoting bone and cartilage formation (Nakagawa and Tagawa, 2000; Lee et al., 2001; Kim and Mendis, 2006).

2.4.4 **Functional Foods**

There have been many studies claiming functional foods contain important health benefits. Food researchers have studied peptides that are obtained from food protein hydrolysates as potential pharmaceuticals (Hyun and Shin, 2000) and as an ingredient in functional foods (Babji et al., 2011). These studies claimed that

gelatin and gelatin hydrolysate possess beneficial biological functions. Therefore, it has a wide use in food supplements and pharmaceutical preparations.

Some fish gelatins have been discovered to hold noticeable antioxidant, anti-hypertensive, anticancer, and antimicrobial, as well as immuno-modulatory and cholesterol-lowering effects (See et al., 2010). Studies have claimed that peptides derived from a range of fish gelatin function as potential antioxidants. Moreover, skin gelatin of Hoki fish (Mendis et al., 2005) and Alaska pollock (Kim et al., 2001) contain antioxidant peptides.

Cold-water fish skin produce gelatin that does not gel at room temperature as the temperature to gel is below 8 - 10 °C (Norland, 1990). Thus, cold-water fish gelatin may be employed in applications that do not need high gelling temperatures such as prevention of syneresis and texturization, as it can be used in frozen or chilled products (Karim and Bhat, 2009).

Cold-water fish gelatin may also be utilized in frozen products. It can be used for microencapsulation in dry products, especially microencapsulation of vitamins, other pharmaceutical additives or microencapsulation of colorants. Only a least amount is needed to produce soft-gel capsules, which are generally found in nutrition supplements (Soper, 1999; Karim and Bhat, 2009). Gelatin of warm water fish also can be used as food flavors encapsulators such as lemon oil, black pepper or garlic flavour (Soper, 1999; Karim and Bhat, 2009).

Regarding to the use of gelatin used in gel desserts, fish gelatin that has low melting temperatures are able to improve emission of smell and provide a strong flavor in gel desserts (Choi and Regenstein, 2000; Karim and Bhat, 2009). By intensifying fish gelatin concentration or by using gelatin mixtures in making desserts, the resulted dessert would resemble desserts prepared from gelatin of pork skin (Zhou and Regenstein, 2007; Karim and Bhat, 2009).

Mixtures of fish gelatin together with other hydrocolloids would expand its application in food ingredients. It was reported that fish gelatin mixed with pectin produced low-fat spreads (Cheng et al., 2008). Therefore, there are numerous suppliers of food grade fish collagen as food additives sold over the internet to be used to make healthy foods, as food additives, frozen food, beverages, dairy products, candy, cakes, or as food packaging materials (Alibaba.com, 2016; SeaSource Collagen, 2016).

2.5 **Optimization**

Optimization studies the effects of various factors on the process of an experiment to determine the optimized conditions for optimal results. An appropriate experimental design is therefore required for complex processes where the targeted response is influenced by many factors (Li and Fang, 2007). The efficiency of the collagen extraction process from fish waste is affected by several process factors such as acid concentration, extraction time, temperature and so on. Thus, a suitable experimental design to analyze multiple factors is

required. In an experimental design, once the region of optimal response is detected by preliminary studies, it is often required to characterize the response in that region. Box-Behnken design and central composite design (CCD) are mainly used in experiments for response surface methodology to approximate a second-order polynomial estimation to a response in that region (Wang and Wan, 2009). These designs were used to investigate optimum collagen extraction conditions of skins of grass carp (Wang, Yang, et al., 2008) and yellowfin tuna scales (Han et al., 2010).

2.5.1 **Response Surface Methodology (RSM)**

Response surface methodology (RSM) is a technique using mathematics and statistics to model problems where variables have an effect on a response of interest, and the main goal is to obtain an optimized response (Montgomery, 2005). When the independent variables has a linear function that affects a response, then the first-order model is the approximate function of it. When a response surface has a curvature, a higher degree polynomial is used, which is a second-order model. The response surface design is a type of design for fitting response surfaces. There is a one-factor-at-a-time design and factorial design. When two or more levels are studied, the factorial design is used. The factorial category includes full factorial and fractional factorial designs. Whenever a number of runs for a full factorial design are too huge to be predicted, a fractional

factorial design is the alternative. Box-Behnken design and CCD are one of these fractional factorial designs widely used for optimization (Wang and Wan, 2009).

The advantages of using RSM are reduced number of experimental attempts to assess multiple parameters and the capability of the statistical tool to recognize interactions. It also produces a mathematical model that depicts the whole process (Batista, 1999; Nurdiyana, et al., 2008). Using conventional techniques such as a one-factor-at-a-time method, is very laborious and time-consuming for these operations to be performed (Wang and Lu, 2005; Pan et al., 2008). Moreover, this does not depict the interactive effects among the variables and guarantee the determination of optimal conditions. On the contrary, the statistically based experimental design is a time-saving method, which minimizes the error in verifying the effect of the parameters (Abdel-Fattah and Olama, 2002; Pan et al., 2008). Therefore, RSM is the most suitable experimental design to be used in this study. Widely used statistical methods in RSM are CCD (Rai et al., 2009) or the Box-Behnken design (Wang, Yang, et al., 2008) which have been successfully employed for optimization in collagen extractions.

2.5.2 **Central Composite Design (CCD)**

Box and Wilson built the central composite design, which consist of a five-level fractional factorial design (Box and Wilson, 1951; Wang and Wan, 2009). This design is made up of a 2^n full factorial design, $2 \times n$ axial designs and *m* central designs. Besides, the axial design is similar to the central design apart

from one factor that would be on levels either lower than the low levels or higher than the high levels of the 2^n full factorial design (Kuehl, 2000).

In a RSM, second-order polynomial model is generally suggested to explain the effects of a variety of factors on a response based on the CCD test results (Equation 2.1) (Wang and Wan, 2008; Wang and Wan, 2009).

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j \qquad \text{(Eq. 2.1)}$$

(where, y = response, β_0 = constant, β_i = linear coefficient, β_{ii}= quadratic coefficient, $\beta_{i\,j}$= interactive coefficient, x_i = coded factor level)

The estimated second-order polynomial model may be presented as a surface plot or a contour plot, by adjusting only two factor levels, whilst keeping other factor levels constant.

The surface and contour plots would graphically illustrate the response in that range of the factor levels. Moreover, this would point out the preciseness of the response to every varying factor level and the extent of the factors interaction as they influence the response.

According to the analysis of variance (ANOVA) of the predicted model, terms with substantial effects on the response can be clarified. Also, with the

assistance of the regression model, the optimal response can be determined through calculating the derivatives of the model (Wang and Wan, 2009). For example, See et al. (2011) investigated the effects of temperature, pH and enzyme to substrate level on the degree of hydrolysis, using a three-factor, five levels CCD for RSM. The CCD consisted of 20 experimental runs including six axial points (α = 1.68), 2^3 factorial points and six replicates of the central point. The effects of the three factors on the enzymatic hydrolysis were described by a second-order polynomial equation (Equation 2.2).

$$Y= \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2+ \beta_{22}X_2^2+ \beta_{33}X_3^2+ \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{23}X_2X_3 \quad \text{(Eq. 2.2)}$$

(Where, Y = estimated dependent variable, β_0 = constant term; β_i, β_{ii} and β_{ij} = linear, quadratic and interactive terms (I = 1-4; j = 1-4) respectively)

The estimation of the R^2 value, the multi-factor ANOVA, lack of fit value, and the second-order model prediction determinants the desirability function approach is used to attain the optimal conditions. The response surface plots (surface plots and contour plots) were plotted to illustrate the effects of the three factors on the degree of enzymatic hydrolysis. According to the ANOVA of the estimated model, it was concluded that effects of enzyme to substrate level, temperature and pH had interactive effects on the degree of hydrolysis. Therefore, with the use of the regression model, the optimum conditions achieved were an enzyme to substrate level of 2.5 % (v/w), temperature of 55.3 °C and pH of 8.39

to attain the highest degree of hydrolysis (77.03 %) using Alcalase (See et al., 2011).

2.5.3 **Box-Behnken Design**

Box-Behnken design is a three-level fractional factorial design built by Box and Behnken (1960). The design may be considered as a mixture of a two-level factorial design with a partial block design. In every block, a definite number of variables set for all combinations for the factorial design, whereas other factors are maintained at the central levels. Box-Behnken design is slightly similar to the CCD but has fewer factor levels and does not have extremely high or extremely low levels (Wang and Wan, 2009).

Many statistical optimization designs on collagen extraction have been reported. In an instance of optimizing protein extraction, Nurdiyana et al. (2008) used RSM to examine the effects of independent variables, which include pH, time, rotation speed and NaOH: substrate ratio on extraction of protein from freeze dried fish waste of sardine (*Sardina pilchardus*). The experimental design and statistical analysis were carried out using Design Expert Software. The experimentation was based on a Box-Behnken design using a quadratic model to examine the combined effects of the four independent variables (pH, time, rotation speed and level of sodium hydroxide to sample). Each of the four independent variables is signified as X_1, X_2, X_3 and X_4 respectively. These independent variables had 3 levels which were -1, 0 and +1. The dependent

variable is known as response function. After the experimental conditions of the independent variables and the corresponding response values from the experimental design were obtained, there are analyzed to attain a regression equation that could predict the response within the range that was given. The protein extraction's regression equation is as follows:

$$\text{Protein extracted, mg/ml} = 61.68 - 1.50\ X_2 - 1.16\ X_3 + 4.68\ X_4 + 2.41\ X_2X_3 - 9.47\ X_2X_4 - 0.73\ X_3X_4 + 0.97\ X_1^2 + 8.93\ X_2^2 + 1.70\ X_3^2 + 4.74\ X_4^2 \quad \text{(Eq. 2.3)}$$

(where, X_1 = level of NaOH: sample, X_2 = the speed rotation, X_3 = the time and X_4 = pH)

Extracted protein (mg/ml) experimental values versus those calculated from the equation would be plotted and showed a good fit. Results of ANOVA produced a coefficient of determination (R^2) of 0.8980 indicated the applied model's adequacy. The R-value of the model and the probability (P) of the regression model significance that is less than 0.05 would both indicate that the model is significant. Thus, the developed model could represent the actual relationships among the parameters chosen. After analyzing from the response surface graphs, the optimal conditions of speed rotation (104.77), pH (10.56), time (48.61) and level of NaOH: sample (1.54), were achieved with the highest predicted protein value of 85.02 mg/ml.

As compared to CCD, Box-Behnken is an economical alternative to CCD because of the less numbers of factor levels used and it does not include extremely high and low levels. However CCD is a more reliable technique to be used when more factor levels are needed to be used and extremely high and low levels are used to confirm the character of the response is in that region. Therefore, it will be an advantage to use CCD for optimization (Wang and Wan, 2009).

2.6 **Factors Affecting Fish Collagen Extraction**

2.6.1 **Temperature**

Collagen is a protein that is termed thermo-instable as it easily denatures at room temperature. The susceptibility to temperature is related to its chemical structure as there is a difference in the amino acid composition, particularly the varying imino acid (proline and hydroxyproline) content among fish species (Gudmundsson and Hafsteinsson, 1997; Wang, An et al., 2008). Fish species with lower levels of hydroxyproline have lower denaturation temperatures as compared to those with higher levels of hydroxyproline (Muyonga et al., 2004). This is due to the hydroxyproline in the inter-chain is able to form hydrogen bonding within the collagen molecule creating a more stable molecular structure. Denaturation temperatures of collagen from cold water fish such as cod, ayu and chum salmon have very low denaturation temperatures, which are 15 °C, 29.7 °C and 19.4 °C,

respectively (Rigby, 1968; Kimura et al., 1988, Nagai and Suzuki, 2000a). Therefore, the manipulation of the temperature throughout the extraction of collagen is vital in order to maintain the collagen's native structure. Higher temperatures to a definite value can cause the denaturation of collagen into gelatin that would raise the yield of collagen extracted. However, appropriately high temperatures are able to modify the conformation of collagen that will reduce its solubility. This is why there is a drastic decrease of collagen yield when temperatures reach 30 °C and above for grass carp (Wang, An et al., 2008). For grass carp, the optimum temperature for extraction is 24.7 °C, as the denaturation temperature is 28.4 °C.

2.6.2 **pH on Collagen Solubility**

In general, collagens are soluble in acidic pH range from 1 to 4 (Jongjareonrak et al., 2005). The effect of pH on the solubility of skin collagen showed that the highest solubility is at pH 2 for striped catfish and bigeye snapper (Kittiphattanabawon et al., 2005; Singh et al., 2011;) and pH 3 for brownstripered snapper skin (Jongjareonrak et al., 2005) however bone collagen of bigeye snapper is soluble at pH 5 (Kittiphattanabawon et al., 2005). The dissimilarity in pH maxima solubility between skin and bone collagens is due to the dissimilarity in molecular properties and conformations. Collagen from skin has higher solubility than bone collagen which is at pH > 6. This suggests skin collagen contains lower degree of cross-linking and prevalence of weaker bonds. Marked

decreases in solubility would occur in neutral and alkaline pH ranges. Brownstripered snapper has drastic declines in solubility when pH is increased to 7, whereas for striped catfish collagen, solubility drastically declines when pH is increased to 4. When the pH is above or below its pI value, the net charge of the protein molecules are higher, thus the solubility is enhanced by the repulsion forces between the chains (Vojdani, 1996). In comparison, as the sum of the net charge of protein molecules is zero, the hydrophobic-hydrophobic interaction intensifies. This causes precipitation and aggregation to occur at pI. The isoelectric points of collagen is ranging between pH 6 and 9 (Foegeding et al., 1996). Striped catfish (Singh et al., 2011), trout muscle collagen (Montero et al., 1991) and brownstripe red snapper skin (Jongjareonrak et al., 2005) have the lowest solubility which is at pH 7.

2.6.3 **Acid Extraction Duration**

In a study on the isolation of collagen from the backbone of Baltic cod (*Gadus morhua*), it showed that by lengthening the extraction time from 48 hour to 72 hours, the collagen solubility increased by 5 to 10 % (Zelechowska et al., 2010). Extraction of collagen from skin and bone of bigeye snapper *(Priacanthus tayenus),* in a repeated process of 24 hour extraction in 0.5 M acetic acid solution, showed that only about 1.9 % of collagen was dissolved (Kittiphattanabawon et al., 2005). In another study, the optimal extraction time was longer (32.1 hours) to extract a higher amount of collagen from grass carp (Wang et al., 2008).

Skin collagens of Japanese sea bass, chub mackerel and bullhead shark were revealed to be not completely solubilized in 0.5 M acetic acid for 3 days. Therefore, the residues were extracted again in the same solution for another 2 days and highly viscous solutions were attained, and yielded about 50 % of collagen. Besides, more collagen was found to be solubilized when extraction was done twice in 0.5 M acetic acid solution for 72 hours, followed by another 48 hours to decalcify the bones of Japanese sea bass (*Lateolabrax japonicas*), and yellow seabream (*Dentex tumifrons*) (Nagai and Suzuki, 2000a). For fish fins, such as the Japanese sea bass caudal fin, similar extraction solution was used after decalcification using 0.5 M EDTA and able to obtain a relatively high content which is 36.4 % yield (Nagai and Suzuki, 2000a). However, low pH solution such as 1.0 M HCl solutions would decrease the ability of collagen to absorb water, thus the solubility is reduced (Gustavson, 1956; Zelechowska et al., 2010).

It is generally known that mass transfer rate of analytes from the matrix is a significant factor in the extraction efficiency (Bartle et al., 1991; Wang, An et al., 2008). In conventional extraction, the diffusion process controls the mass transfer rate which is dependent on the extraction time. The amount of analytes retrieved amplifies with the lengthening of time in the diffusion controlled process. Therefore, it is suggested that the extraction time affects the amount of collagen yield (Wang, An et al., 2008).

2.6.4 **Acid Concentration**

The solubility of collagen in acid solutions is a significant factor in the extraction effectiveness (Kiew and Don, 2013). When collagen is in an extracting medium with sufficient amounts of proton (H^+), its solubility is increased. Sufficient quantities of H^+ assist the entry of water into the collagen fibers. Water is detained in the collagen by electrostatic swelling that is electrostatic forces among charged polar groups. As a result, the collagen solubility in the extracting medium is significantly affected by the acid concentration used due to the effect of swelling properties (Kiew and Don, 2013).

Partial solubility of fish skins indicates the presence of inter-molecular cross-links in collagen molecules. The first phase of solubilization of collagen is the continuation of hydration of the fibrous collagen due to its contact with acids (Skierka and Sadowska, 2007). The structure of protein and its electrostatic interactions would change when the acid concentration changes, this is due to the pH value affects the charge of the protein's charge density of protein (Wang, An et al., 2008).

In an experiment on the extraction of collagen from Hybrid Clarias skin, various concentrations of acetic acid were used, which was ranging from 0.1 M to 0.9 M. When an increase of the acetic acid's concentration from 0.1 M to 0.7 M happened, the yield was increased to a maximum of 26.7 %. However when the

acetic acid concentration increased to 0.9 M, an opposite trend was observed and the yield was decreased to 20.4 % (Kiew and Don, 2013). Wang et al. (2009) reported a similar trend of up to 0.5 M acetic acid was used, and the yield was found decreasing thereafter. Other studies showed that the optimal acetic acid concentration was 0.54 M for the extraction of grass carp collagen (Wang, An et al., 2008).

When the extracting medium has a low pH for example 0.9 M acetic acid (pH of 2.39) it will cause a decrease in the water absorption ability of collagen. A low pH would trigger the positively charged amine groups of proteins to form bonding with anions (CH_3COO^- in acetic acid aqueous solutions), creating more fragile electrostatic repulsive forces among the one-nominal charged group. Consequently, loosening of the collagen fibers structure and decrease in the ability to bond with water causes a reduction of the collagen solubility in the medium (Skierka and Sadowska, 2007). Besides, collagen denatures at extremely low pH values which is below 2.0 causes collagen fibers reduce in size, makes the hydration of protein become unachievable (Carvalho et al., 2003; Wang, An et al., 2008).

2.6.5 **Effective Acid Solvents for Collagen**

Collagen extraction is frequently undergone by directly extracting collagen by means of organic acids such as acetic acid, chloroacetic acid, citric acid or lactic acid (Skierka and Sakowska, 2007). Inorganic acids were also used for extraction, such as hydrochloric acid, but it was ineffective compared to organic acids (Wang et al., 2009; Cheng et al., 2009; Kiew and Don, 2013). It was reported that the best solvent for extracting native collagen are acetic acid and lactic acid compared to HCl and citric acid or enzymatic treatment. Extraction of cod skin collagen was tested with these acids, coupled with enzymatic treatment (which is called pepsin solubilized collagen, PSC), and the collagen was entirely dissolved. A total of 0.5 M acetic acid or 0.5 M lactic acid is suitable solvents for extracting collagen in their native structures. Collagen fibrils solubilized in acetic acid or lactic acid did not endure enzymatic degradation, therefore, α1, α2 and β-chains were present in SDS-PAGE analysis (Skierka and Sadowska, 2007). This was in accordance with Ogawa et al. (2004), was reported ASC of bone and scale of subtropical fish black drum and sheepshead seabream contained high quantities of cross-linked components to its PSC. In other words, the β and γ components which are the intra- and/or inter-molecular cross-linking of collagens were richer in ASC than in PSC.

CHAPTER 3

METHODOLOGY

3.1 Raw Materials

Fish waste (consists of the fish species Tilapia, catfish, pomfret and mackerel) such as skins, scales, bones and fins were collected from a local wet market at Genting Klang, Setapak, Kuala Lumpur. The wastes were sorted according to skins, scales, bones and fins. These wastes were immediately washed with tap water and frozen until further used. Generally, fish waste samples were thawed at room temperature prior to the extraction procedure. The respective collagen samples from different parts of the fish (skins, scales, bones and fins) were extracted according to the extraction conditions designed by the central composite design (CCD) software. Extraction procedures for each type of fish waste generated from the CCD consist of 30 combinations of the tested parameters (acetic acid concentration, pH, temperature and extraction time) (Nagai and Suzuki, 2000a).

3.2 Methods

3.2.1 Preparation of Collagen

The collagens were prepared using the methods described by Nagai and Suzuki (2000a) with slight modifications. All preparation procedures were carried out at temperatures not higher than 4 °C.

3.2.2 Extraction of Skin Collagen

The fish skins were first cut with a pair of scissors into small pieces of about 0.5 cm in length. They were then extracted with 0.1 M NaOH at a sample to alkali ratio of 1:8 (w/v) to remove non-collagenous proteins. The mixture was stirred for 6 hours. Then for every 3 hours, the alkaline solution was changed. Next the samples were washed with distilled water, until neutral pH was achieved.

The deproteinized skins were soaked overnight in 10 % butyl alcohol at sample to alcohol ratio of 1:10 (w/v) to remove the fat from the skin. Then the samples were washed with distilled water repeatedly and thoroughly, subsequently the skins were extracted with a variety acetic acid concentrations ranging from 0.1 - 1.0 M for one to five days according to the experiment layout as stated in Section 3.3. The residue was filtered using a cotton cloth and proteins were recovered with a salting-out procedure using NaCl to a final concentration of

2.5 M in the presence of 0.05 M tris(hydroxymethyl)aminomethane, at pH 7.0. Precipitated proteins were collected after centrifugation at 20,000×*g* for 30 min. The pellet was dissolved in 0.5 M acetic acid, then dialyzed against 0.1 M acetic acid and distilled water, respectively. Lastly, this acid-soluble collagen was lyophilized (Duan et al., 2009).

The process was repeated for optimization with different acetic acid concentrations, acid extraction time, acetic acid pH and temperature.

3.2.3 **Extraction of Bone Collagen**

The fish head was hammered, and washed with distilled water for 5 hours. Next, they were immersed in 0.1 M NaOH at a sample to alkali ratio of 1:5 (w/v) to remove non-collagenous proteins for 24 hours. The alkali solution was changed every 6 hours. Then the residue was washed with distilled water until a neutral pH value was reached.

Bones were decalcified in 0.5 M EDTA-2Na solution of pH 7.5 for 5 days, subsequently defatted with 10 % butyl alcohol at a sample to alcohol ratio of 1:10 (w/v) overnight. The bones were washed with distilled water thoroughly, prior extraction with a variety of acetic acid concentrations ranging from 0.1 to 1.0M for the duration of one to five days according to the experiment layout stated in Section 3.3. The insoluble matter was re-extracted with the same solution at

sample to acid ratio of about 1:2.5 (w/v) for 2 days. Each extract was filtered by cotton cloth. Both solutions were combined and salted out with NaCl to a final concentration of 2.5 M in the presence of 0.05 M tris(hydroxymethyl)aminomethane. Precipitated proteins were centrifuged at 20,000×*g* for 30 min, and dissolved in 0.5 M acetic acid. Dissolved proteins were then dialysed against 0.1 M acetic acid and distilled water respectively. After that, the extracted collagen was lyophilized (Duan et al., 2009).

The steps were conducted repeatedly with different acetic acid concentrations, acid extraction time, acetic acid pH and temperature for optimization.

3.2.4 **Extraction of Scale Collagen**

Non-collagenous proteins were removed by treating the fish scales with 0.1 M NaOH for 6 hours at a sample to alkali solution ratio of 1:8 (w/v). Decalcification was carried out for 24 hours by immersing the scales in 0.5 M EDTA-2Na of pH 7.5 at a sample to EDTA solution ratio of 1:10 (w/v). After that, the scales were washed with distilled water. The residue was extracted with a variety acetic acid concentrations ranging from 0.1 to 1.0 M at sample to acid ratio of 1:2.5 (w/v) for duration of one to five days according to the experiment planned for optimization. It was then filtered using a cotton cloth. The supernatants were salted-out by incorporating NaCl to a final concentration of 2.5

M with the presence of 0.05 M tris(hydroxymethyl)aminomethane. Centrifuging at 20,000×*g* for 30 min was carried out to obtain the resultant pellet. The pellet was then dissolved in 0.5 M acetic acid, and dialyzed against 0.1 M acetic acid and distilled water respectively. This acid-soluble collagen from fish scales was then lyophilized (Duan et al., 2009).

These methods were carried out repeatedly with varying acetic acid concentrations, acid extraction time, acetic acid pH and temperature for optimization of collagen extraction.

3.2.5 **Extraction of Fin Collagen**

Fins were immersed in 0.1 M NaOH to remove non-collagenous proteins. After that, they were washed thoroughly with distilled water and lyophilized. The insoluble fraction was then extracted with acetic acid with concentrations ranging from 0.1 M to 1.0 M at sample to acid ratio of 1:2.5 (w/v) for one to five days according to the experiment layout in Section 3.3. The extract was centrifuged for 1 h at 20,000×*g* to two fractions, namely acid-soluble and acid-insoluble fractions. The acid soluble fractions were salted out with NaCl to a final concentration of 2.5 M (at neutral pH) in 0.05 M Tris-HCl (pH 7.5). Precipitated proteins were centrifuged again at 20,000×*g* for 1 h, and then dissolved in 0.5 M acetic acid before dialyzed against 0.1 M acetic acid. Acid-soluble collagen was obtained after it was lyophilized.

On the other hand, the acid-insoluble fraction was washed with distilled water and decalcified with 0.5 M EDTA (pH 7.4) for 5 days. The EDTA solution was changed once a day. The decalcified bones were washed in 10 % butyl alcohol for 1 day. Extracted collagen was removed by centrifugation at 20,000×g for 1 h, and subsequently washed with distilled water and lyophilized. Finally, it was dissolved in 0.5 M acetic acid and dialyzed against 0.1 M acetic acid. The acid-insoluble collagen was obtained after lyophilization (Duan et al., 2009).

The process was repeated with different acetic acid concentrations, acid extraction time, acetic acid pH and temperature for optimization of the extraction of collagen.

3.2.6 **Calculation of Extracted Yield**

Collagen yield was estimated by measuring the percentage of the weight of collagen extracted from the weight of the fish skins, scales, bones and fins. Collagen yield was expressed as percentage of dry weight of collagen yield based on the freeze dried weight of the fish waste raw materials, i.e fish skins, scales, bones or fins. Equation 3.1 was used for the estimation of collagen.

$$\text{Collagen (\%)} = \frac{\text{Dry weight of collagen (g)}}{\text{Dry weight of fish waste (g)}} x\ 100\ \% \qquad \text{(Eq. 3.1)}$$

3.3 **Optimization of Collagen Extraction**

Response Surface Methodology was used to create an experimental model, evaluate the data, and design construction with the assistance of the software Design Expert ©Version 7.1.3 (Stat-Ease, Inc., USA). The central composite design (CCD) with four independent variables was used to determine the response design and to create a model.

3.3.1 **Response Surface Methodology (RSM)**

The optimum conditions for collagen extraction from fish skins, scales, bones and fins were revealed by RSM. The four independent variables were acetic acid concentration (M), acetic acid pH (pH), extraction temperature (°C) and extraction time (h). The range of each independent variable was fixed according to the outcome of preliminary studies (data not shown). The collagen yield (%) was set as the dependent variable. The study was optimized by using a four-factor, five-level CCD. There are 5 levels where each numeric factor was varied over, which were the plus and minus alpha (axial points), plus and minus 1 (factorial points) and the center point (Tables 3.1 and 3.2). The CCD consists of 30 runs which include 6 center points and 24 non-center points (Tables 3.3, 3.4, 3.5 and 3.6). The design of the experiments and dependent variables values are presented in the tables 3.1, 3.2, 3.3, 3.4, 3.5 and 3.6.

Table 3.1: A 5-level CCD of fish skin independent variables

Name	Unit	-1 Level	+ 1 Level	-alpha	+alpha
Acetic Acid concentration	M	0.44	0.81	0.25	1
Extraction Temperature	°C	-7.50	17.50	-20.00	30.00
Extraction Time	h	45.00	111.00	12.00	144.00
pH of Acetic Acid	pH	2.87	3.62	2.50	4.00

Table 3.2: A CCD of fish scales, bones and fins independent variables

Name	Unit	-1 Level	+ 1 Level	-alpha	+alpha
Acetic Acid concentration	M	0.43	0.81	0.25	1.00
Extraction Temperature	°C	15.50	38.50	4.00	50.00
Extraction Time	h	45.00	111.00	12.00	144.00
pH of Acetic Acid	pH	2.87	3.62	2.50	4.00

Table 3.3: CCD of fish skin independent variables

	Variables			
Run	A Concentration (M)	B Temperature (°C)	C Time (h)	D pH (pH)
1	0.44	-7.50	45.00	3.63
2	0.81	-7.50	45.00	3.63
3	1.00	5.00	78.00	3.25
4	0.63	5.00	12.00	3.25
5	0.44	-7.50	111.00	3.63
6	0.63	5.00	78.00	4.00
7	0.25	5.00	78.00	3.25
8	0.44	17.50	45.00	2.88
9	0.63	5.00	78.00	3.25
10	0.44	-7.50	45.00	2.88
11	0.63	5.00	78.00	3.25
12	0.63	5.00	144.00	3.25
13	0.44	17.50	111.00	2.88
14	0.63	5.00	78.00	3.25
15	0.63	30.00	78.00	3.25
16	0.44	-7.50	111.00	2.88
17	0.44	17.50	111.00	3.63
18	0.63	5.00	78.00	3.25
19	0.81	17.50	111.00	3.63
20	0.63	5.00	78.00	3.25
21	0.81	-7.50	111.00	3.63
22	0.63	5.00	78.00	2.50
23	0.81	17.50	111.00	2.88
24	0.81	-7.50	45.00	2.88
25	0.63	-20.00	78.00	3.25
26	0.44	17.50	45.00	3.63
27	0.81	17.50	45.00	2.88
28	0.81	17.50	45.00	3.63
29	0.81	-7.50	111.00	2.88
30	0.63	5.00	78.00	3.25

Table 3.4: CCD of fish scales independent variables

	Variables			
Run	A Temperature (°C)	B Concentration (M)	C Time (h)	D pH (pH)
1	27.00	0.63	78.00	3.25
2	27.00	0.63	78.00	3.25
3	27.00	0.63	12.00	3.25
4	27.00	1.00	78.00	3.25
5	38.50	0.81	111.00	2.88
6	15.50	0.44	111.00	2.88
7	27.00	0.63	78.00	3.25
8	38.50	0.44	111.00	2.88
9	15.50	0.81	111.00	3.63
10	15.50	0.81	45.00	3.63
11	38.50	0.81	111.0	3.63
12	27.00	0.63	78.00	4.00
13	27.00	0.25	78.00	3.25
14	27.00	0.63	144.00	3.25
15	15.50	0.81	111.00	2.88
16	50.00	0.63	78.00	3.25
17	38.50	0.44	4500	2.88
18	27.00	0.63	78.00	3.25
19	4.00	0.63	78.00	3.25
20	38.50	0.44	45.00	3.63
21	38.50	0.44	111.00	3.63
22	27.00	0.63	78.00	2.50
23	15.50	0.44	45.00	2.88
24	27.00	0.63	78.00	3.25
25	38.50	0.81	45.00	2.88
26	15.50	0.44	45.00	3.63
27	27.00	0.63	78.00	3.25
28	15.50	0.81	45.00	2.88
29	15.50	0.44	111.00	3.63
30	15.50	0.44	111.00	3.63

Table 3.5: CCD of fish bones independent variables

	Variables			
Run	**A Concentration (M)**	**B Temperature (°C)**	**C Time (h)**	**D pH (pH)**
1	0.44	15.50	111.00	2.88
2	0.63	27.00	78.00	3.25
3	0.44	15.50	45.00	2.88
4	0.44	38.50	111.0	2.88
5	0.81	38.50	111.0	2.88
6	0.81	15.50	45.00	2.88
7	0.44	38.50	45.00	2.88
8	0.44	15.50	45.00	3.63
9	0.63	27.00	78.00	3.25
10	0.63	4.00	78.00	3.25
11	0.63	50.00	78.00	3.25
12	0.44	15.50	111.00	3.63
13	1.00	27.0	78.00	3.25
14	0.44	38.50	45.00	3.63
15	0.81	15.50	45.00	3.63
16	0.25	27.00	78.00	3.25
17	0.63	27.00	78.00	3.25
18	0.63	27.00	78.00	2.50
19	0.63	27.00	78.00	3.25
20	0.81	38.50	111.00	3.63
21	0.63	27.00	12.00	3.25
22	0.63	27.00	78.00	3.25
23	0.63	27.00	78.00	3.25
24	0.63	27.00	78.00	4.00
25	0.81	15.50	111.00	2.88
26	0.44	38.50	111.00	3.63
27	0.81	38.50	45.00	2.88
28	0.63	27.00	144.00	3.25
29	0.81	38.50	45.00	3.63
30	0.81	15.50	111.00	3.63

Table 3.6: CCD of fish fin independent variables

	Variables			
Run	**A Temperature (°C)**	**B Concentration (M)**	**C Time (h)**	**D pH (pH)**
1	27.00	0.63	78.00	3.25
2	27.00	0.63	78.00	3.25
3	27.00	0.63	12.00	3.25
4	27.00	1.00	78.00	3.25
5	38.50	0.81	111.00	2.88
6	15.50	0.44	111.00	2.88
7	27.00	0.63	78.00	3.25
8	38.50	0.44	111.00	2.88
9	15.50	0.81	111.00	3.63
10	15.50	0.81	45.00	3.63
11	38.50	0.81	111.0	3.63
12	27.00	0.63	78.00	4.00
13	27.00	0.25	78.00	3.25
14	27.00	0.63	144.00	3.25
15	15.50	0.81	111.00	2.88
16	50.00	0.63	78.00	3.25
17	38.50	0.44	45.00	2.88
18	27.00	0.63	78.00	3.25
19	4.00	0.63	78.00	3.25
20	38.50	0.44	45.00	3.63
21	38.50	0.44	111.00	3.63
22	27.00	0.63	78.00	2.50
23	15.50	0.44	45.00	2.88
24	27.00	0.63	78.00	3.25
25	38.50	0.81	45.00	2.88
26	15.50	0.44	45.00	3.63
27	27.00	0.63	78.00	3.25
28	15.50	0.81	45.00	2.88
29	15.50	0.44	111.00	3.63
30	38.50	0.81	45.00	3.63

3.3.2 **Statistical Analysis**

Experimental data was investigated by using multiple regressions to ensure the quadratic equation to all independent variables fit. The suitability of the model created was assessed by the ANOVA. Interactions between the responses and independent variables were illustrated by surface response plots and contour plots generated by the Design Expert.

3.4 **Characterization of Fish Waste Collagen**

3.4.1 **Electrophoresis**

Protein patterns of collagen samples were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method as described by Laemmli (1970), using 5 % stacking gel and 7.5 % resolving gel. Collagen samples were dissolved in 0.1 M acetic acid. The solubilized collagen samples were mixed with 5 times sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4 % (w/v) SDS, 20 % (v/v) glycerol) with 10 % (v/v) mercaptoethanol (βME), at a sample buffer to sample ratio of 1:4 (v/v). Next, the samples were heated to 93 °C - 95 °C for 4 minutes prior loaded on to the PAGEL-Compact precast gel (7.5 % gel) and applied to electrophoresis at a constant current of 70 V/gel utilizing a Compact-PAGE apparatus (Atto Co., Japan). Following electrophoresis, 0.05 % (w/v) Coomassie blue R-250 in 15 % (v/v) methanol and

5 % (v/v) acetic acid were used to stain the gel. Then 30 % (v/v) methanol and 10 % (v/v) acetic acid were used to distain it. High-molecular-weight marker (Sigma Chemical co., St. Loius, Mo, USA) was used to estimate the molecular weights of proteins ranging from (66.4 - 212 kDa). Bornstein and Traub Type I human collagen (Sigma Chemical, USA) was used as a control.

3.4.2 **Fourier Transform Infrared Spectroscopy (FTIR)**

All spectra were obtained using a FTIR (Omnic spectra) spectrophotometer (Perkin Elmer, USA). A total of 0.5 g collagen was fixed onto the crystal and knob of the Nicole i50 and placed on the FTIR apparatus. At an interval of 1.0 $cm^{-1,}$ each sample was subjected to 32 scans from 4000 to 400 cm^{-1} at a resolution of 4.0 cm^{-1}. Samples of skin, bones and scales and fin collagen were analyzed in triplicates, respectively. For secondary structure analysis of each sample, all amide I band number and location of sub-bands was achieved by Fourier self-deconvolution (Duan et al., 2009).

3.4.3 **Denaturation Temperature (T_d)**

The denaturation temperature (T_d) was measured according to the method described by Zhu and Kimura (1991). A total of 2 ml of 2.5 % collagen solution in 0.1 M acetic acid was used for viscosity measurements using a rheometer (Anton Paar, USA). The sample was heated from 9 °C to 50 °C at a rate of 1

°C/min. The denaturation temperature can be determined by fractional viscosity. The temperature, where the fractional viscosity is 0.5, is considered as the denaturation temperature (Muyonga et al., 2004). The fractional viscosity is determined by measuring the viscosity of the sample and applying it to the equation below.

$$\text{Fractional viscosity} = [(\varepsilon 2/C)-(\varepsilon 3/C)]/\ [(\varepsilon 1/C)-(\varepsilon 3/C)] \qquad \text{(Eq. 3.2)}$$

(Where, C = concentration of collagen (mg/ml), ε1 = the viscosity at 9 °C, ε2 = the viscosity at measured temperature (°C), and ε3 = the viscosity at 50 °C.)

3.4.4 **Amino Acid Composition**

Dry collagen (100 - 200 mg) from skins, scales, bones or fins of fish waste were mixed with 6 N HCl to a ratio of 1:10 (w/v) was prepared for hydrolysis prior to amino acid analysis at 110 °C for 24 h. The hydrolysate was then added with 2.5 mM of internal standard and distilled water. The mixture was then filtered through a 0.4 µm membrane prior loading to the HPLC system. A total of 10 µl of sample was derivatized by adding 20 µl AccQ-Fluor (Waters, USA) reagent and 70 µl of borate buffer. The sample was left to stand for 1 minute at room temperature (20 – 25 °C and 5 µl was loaded to a AccQ Tag Column (3.9 x 150 mm) in the HPLC system (Waters, 2790), with a Waters column heater and a fluorescence detector (Waters, 2475) at E_λ = 250 nm and E_m = 395 nm.

The amount of amino acids in the sample was estimated based on the area under the HPLC chromatogram. An internal standard was used to estimate the concentration of each amino acid (Eq. 3.3), as the internal standard method was used to improve the precision and accuracy of the results where volume errors are difficult to predict and control. Amino acid composition revealed from the HPLC analysis is reported as unit residues per 1000 residues (Eq. 3.4). On the other hand, hydroxylation ratio (%) of proline (Pro) was calculated on the based on Equation 3.5 (Nagarajan et al., 2013).

$$\%\ \text{Amino acid} = \frac{\text{sample peak area}}{\text{standard peak area}} \text{x}100 \qquad \text{(Eq. 3.3)}$$

$$\text{Amino acid residues per 1000 residues} = \frac{\%\ \text{amino acid}}{\text{Total amino acid}} \text{x}\,1000 \qquad \text{(Eq. 3.4)}$$

$$\text{Hydroxylation ratio of Pro}\,(\%) = \frac{\text{Hydroxyproline}}{\text{Proline} + \text{Hydroxyproline}} \text{x}100 \qquad \text{(Eq. 3.5)}$$

CHAPTER 4

RESULTS

4.1 Optimization of Collagen Extraction

Preliminary studies were carried out to identify the suitable extraction parameters of fish waste collagen. The results obtained (data not shown) were used as a guideline to determine the input range of each independent variable for the RSM analysis. Optimization of collagen extraction parameters was carried out with the aid of RSM, the parameters tested were acetic acid concentration, pH, temperature and extraction duration, based on the experimental design. The optimum extraction conditions for maximum yield were revealed.

4.1.1 Optimization of fish skin collagen extraction conditions

The effect of acetic acid concentration (A), extraction temperature (B), extraction time (C), pH of acetic acid (D) on the yield of extracted collagen (Y) was ascertain by using RSM. Desirability profile was used to prove the optimum level of each condition. Acetic acid concentration (A), extraction temperature (B), extraction time (C) and pH of acetic acid (D) were set in arranged ranges. On the other hand, collagen yield (%) was set to be maximized.

The impact of the independent variables on the collagen yield can be evaluated through the three-dimensional views of the response surface plots and their respective contour plots (Figure 4.1). The plots stand as a function of two factors at a time, keeping other factors at a fixed level (optimal level).

Figure 4.1 depicts the graph of the effects of pH, concentration, time and temperature of acetic acid on the collagen yield. When pH was depicted against concentration, the temperature and time were set constant at 5 °C for 78 h. whereas when time was depicted against temperature, the concentration and pH were set constant at 0.63 M and pH 3.25. Red regions on both surface plots and contour plots represent high collagen yield regions. Optimum extraction conditions with the highest yield obtained in the experiment are indicated as a red dot on the plots, showing the temperature, extraction time, pH and concentration of acetic acid as 5 °C, 78 h, pH 3.25 and 0.63 M, respectively.

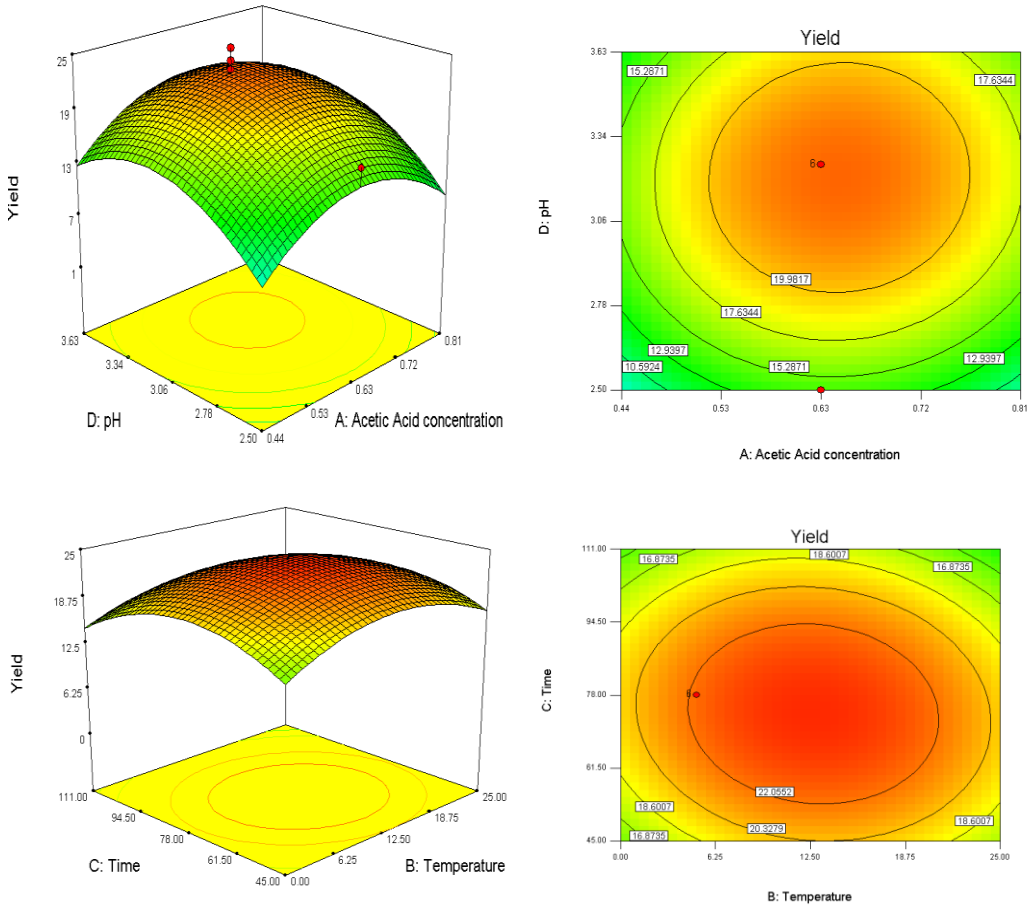


Figure 4.1: Surface plot (left) and contour plot (right) for fish skin collagen yield in response to pH, acetic acid concentration, extraction time and temperature

4.1.2 **Fish Skin Model fitting and Optimization**

Based on the RSM, an ANOVA for the RSM quadratic model was generated. The ANOVA confirms the adequacy of the quadratic model where the Model F value of 31.05 implies that the model is significant, and that only a 0.01 % chance the model took place due to noise. Terms A, B, AB, A^2, B^2, C^2, D^2 are significant terms as the Prob > F is less than 0.05. The F-value of the "Lack of Fit" is 2.16 which indicate that the lack of fit is not significant. This shows it has

only a 20.5 % chance the “Lack of Fit” happened due to noise. Table 4.1 shows the details of the analysis.

Table 4.1: Analysis of variance (ANOVA) of fish skin model

[1]Source	Sum of Squares	Df	Mean Square	F value	p-value Prob>F
Model	2113.11	14	150.94	31.05	$<0.1 \times 10^{-3}$
A	25.15	1	25.15	5.17	0.0380
B	557.29	1	557.29	114.65	$<0.1 \times 10^{-3}$
C	15.09	1	15.09	3.10	0.0985
D	6.73	1	6.73	1.38	0.2576
AB	44.79	1	44.79	9.21	0.0083
AC	2.05	1	2.05	0.42	0.5257
AD	0.81	1	0.81	0.17	0.6881
BC	6.04	1	6.04	1.24	0.2825
BD	1.71	1	1.71	0.35	0.5620
CD	0.45	1	0.45	0.092	0.7662
A^2	825.79	1	825.79	169.88	$<0.1 \times 10^{-3}$
B^2	434.91	1	434.91	89.47	$<0.1 \times 10^{-3}$
C^2	573.97	1	573.97	118.08	$<0.1 \times 10^{-3}$
D^2	139.10	1	139.10	28.62	$<0.1 \times 10^{-3}$
Residual	72.91	15	4.86		
Lack of Fit	59.19	10	5.92	2.16	0.2050
Pure Error	13.72	5	2.74		
Cor Total	2186.03	29			

[1] A,(Concentration); B, (Temperature); C, (Time); and D, (pH)

From the quadratic model, the standard deviation was calculated as 2.2 (Table 4.2), with a mean of 9.2. For each run in this CCD, it will generate a set of replicated samples. After these repeated samples in every run were collected, both the mean and standard deviation are modeled. Through this, certain strategies for the optimization process are enabled, such as the factor settings that meet the targeted response to be found from the mean response. Besides, the operating conditions that are robust to uncontrolled variables are achieved from using the

statistics on variation. The pooled standard deviation, is used as a measure to support statistical testing. The R^2, adjusted R^2 and predicted R^2 were 0.97, 0.94 and 0.84, respectively. The R^2 value of 0.97 indicates that 96.66 % of the variability in the response can be explained by the final equation (Eq 4.1). Moreover, the "Adj R^2" and the "Pred R^2" are in reasonable agreement. Furthermore, the "Adeq Precision" of 16.40 is more than 4, which indicates that there is an adequate signal (Walia et al., 2014).

Table 4.2: Statistics of fish skin model

Statistics	Number
Std. Dev.	2.20
Mean	9.22
R^2	0.97
Adj R^2	0.94
Pred R^2	0.84
Adeq Precision	16.40

The model equation is generated in terms of coded factors. These terms are able to be used to rebuild the results of this experiment, but are not able to be employed for modeling beyond the responses tested. The final equation in terms of coded factors is:

$$\text{Yield} = 22.25 + 1.02\,A + 4.82\,B - 0.79C - 0.53\,D + 1.67\,AB + 0.36\,AC + 0.23\,AD - 0.61\,BC - 0.33\,BD - 0.17\,CD - 5.49\,A^2 - 3.98\,B^2 - 4.57\,C^2 - 2.25\,D^2 \quad \text{(Eq. 4.1)}$$

(Coded factors: A -Temperature, B - Concentration, C - Time, D - pH)

The Variance Inflation Factor (VIF) and R-squared (R^2) were calculated for each term. The VIF values for all the terms were 1.00 except A^2, B^2, C^2, D^2 which were 1.05, but all the VIF less than 10, indicated these terms are significant. R^2 value was ideal for all terms and slightly higher in value for the terms A^2, B^2, C^2, D^2 (Table 4.3).

Table 4.3: VIF and R^2-squared of fish skin model

Term	VIF	R^2-Squared
A	1.00	0.00
B	1.00	0.00
C	1.00	0.00
D	1.00	0.00
AB	1.00	0.00
AC	1.00	0.00
AD	1.00	0.00
BC	1.00	0.00
BD	1.00	0.00
CD	1.00	0.00
A^2	1.05	$4.67x10^{-2}$
B^2	1.05	$4.67x10^{-2}$
C^2	1.05	$4.67x10^{-2}$
D^2	1.05	$4.67x10^{-2}$

Based on the quadratic equation, experiments were carried out to further verify the equation and optimum extraction conditions. The experiment conditions were conducted according to Table 4.4. The optimum extraction conditions are 0.66 M of acetic acid at pH 3.19, at 13.26 °C, for 74 h (Table 4.4). The actual yield obtained were approximately 22.77 % of collagen as compared to the predicted yield (24.03 %) with 1.26 % difference. The percentage of error calculated was found to be 5.24 % (Table 4.4). Therefore, the quadratic equation

is suitable for predicting collagen yield with the parameters tested.

Table 4.4: Suggested optimum conditions for fish skin

Set	Concentration (M)	Temperature (°C)	Time (h)	pH (pH)	Actual Yield (%)	Expected yield (%)	Percentage of error (%)
1	0.66	13.26	74	3.19	22.77	24.03	5.24

4.1.3 **Fish Scales Optimization**

The CCD was used to determine the combined effects of the four factors which were extraction temperature (A), acetic acid concentration (B), extraction time (C), extraction pH (D) on the responses of collagen yield. A total of 30 experimental runs were assessed and the extracted collagen yield was attained according to the design generated. As a result, the effects of the four factors on the yield of collagen as well as their interactions are shown in Figure 4.2.

The results of extraction temperature and time on collagen yield from fish scale waste are shown in Fig. 4.2. High yields are obtained in the range of 50 h to 90 h and between temperatures of 7 °C to 22 °C, with the highest yield obtained exactly at 27 °C for 78 h. Figure 4.2 also shows the influence of acetic acid concentration and pH on the yield of collagen. High yields were obtained in the pH range of pH 2.5 to 3.5 and in an acetic acid concentration with the range of

0.53 M and 0.72 M. The highest yield was obtained where 0.63 M acetic acid was used at 3.25 pH.

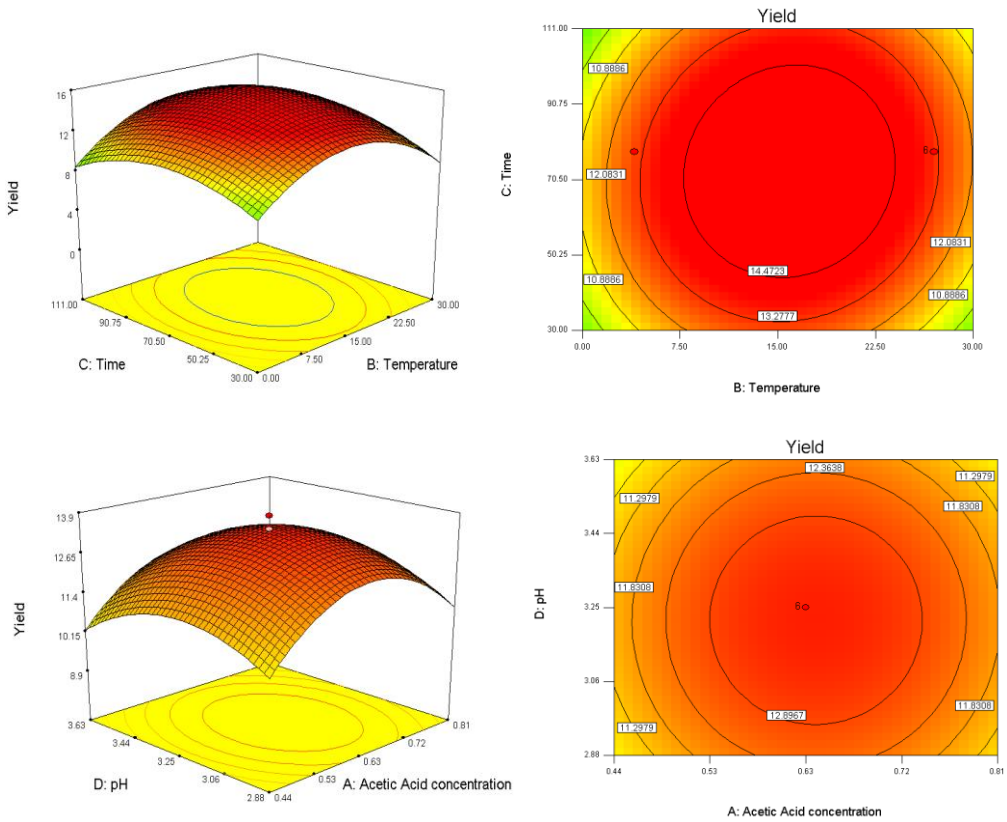


Figure 4.2: Surface plot (left) and contour plot (right) for collagen yield of fish scales in response to time, temperature, pH and concentration

4.1.4 Fish Scale Model fitting and optimization

According to the experimental design and response value, the mathematical model was generated, the adequacy of the model was examined. An ANOVA was generated for this response. The analysis shows that the model is significant as the model F-value was 8.44. It was calculated that only a 0.01 %

chance that the model occurred due to noise. The terms B, A^2, B^2, C^2, D^2 showed to be significant as the "Prob>F" was less than 0.05. Table 4.5 presents the details of the analysis.

Table 4.5: Analysis of variance (ANOVA) of fish scale model

[1]Source	Sum of Squares	df	Mean Square	F value	p-value Prob>F
Model	649.88	14	46.42	8.44	$<0.1 \times 10^{-3}$
A	1.24	1	1.24	0.23	0.64
B	386.57	1	386.57	70.26	$<0.1 \times 10^{-3}$
C	0.82	1	0.82	0.15	0.71
D	1.51	1	1.51	0.27	0.61
AB	1.63	1	1.63	0.30	0.59
AC	0.027	1	0.027	4.9×10^{-3}	0.94
AD	0.40	1	0.40	0.072	0.79
BC	0.73	1	0.73	0.13	0.72
BD	0.94	1	0.94	0.17	0.68
CD	0.07	1	0.07	0.01	0.90
A^2	80.50	1	80.50	14.63	1.70×10^{-3}
B^2	157.70	1	157.70	28.66	$<0.1 \times 10^{-3}$
C^2	71.44	1	71.44	12.99	0.0026
D^2	43.57	1	43.57	7.92	0.0131
Residual	82.52	15	5.50		
Lack of Fit	78.87	10	7.89	10.80	8.5×10^{-3}
Pure Error	3.65	5	0.73		
Cor Total	732.40	29			

[1] A,(Concentration); B, (Temperature);C, (Time); and D, (pH)

A reassessment was carried out after performing the ANOVA to check the significance of the runs on each response. The calculated standard deviation and mean based on the quadratic model were 2.35 and 7.17, respectively. The R^2, adjusted R^2" and predicted R^2 were 0.89, 0.77 and 0.37, respectively as shown in Table 4.6. A satisfactory value of the determination coefficient (R^2) indicates that 89 % of the variability in the response can be explained by the second-order

model equation. The "Adj R^2" and the "Pred R^2" were not as close to each other as expected. This proves that there are outliers in the model. Nevertheless, Cook's distance showed that all the data points were < 1 (Fig 4.3), that proved these outliers are not worth to validate. Therefore, a minor number of outliers are involved, but do not have a high enough impact to affect the accuracy of the regression model (Cook, 1977). Besides, the "Adeq Precision" used to measure the signal to noise ratio was more than 4 in this present study, (10.62) indicates the signal is adequate.

Table 4.6: Statistics of fish scale model

Statistics	Number
Std. Dev.	2.35
Mean	7.17
R^2	0.89
Adj R^2	0.78
Pred R^2	0.37
Adeq Precision	10.62

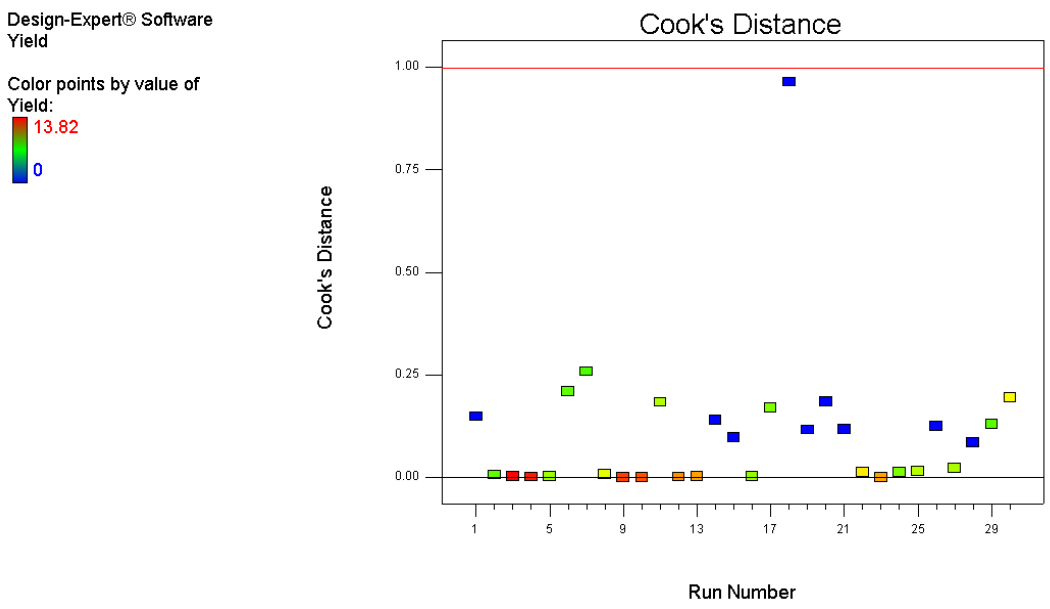


Figure 4.3: Cook's Distance for Fish Scale Collagen Model

The VIF values of this study of all terms were achieved as 1.00 as coefficients were properly estimated (Table 4.7). Slightly higher VIF of 1.05 for the terms A^2, B^2, C^2 and D^2 showed that these terms were inter-related. However the effect will not be significant if the changes of the parameters are less than the power of two.

Table 4.7: VIF and R^2-squared of fish scale model

Term	VIF	R^2
A	1.00	0.00
B	1.00	0.00
C	1.00	0.00
D	1.00	0.00
AB	1.00	0.00
AC	1.00	0.00
AD	1.00	0.00
BC	1.00	0.00
BD	1.00	0.00
CD	1.00	0.00
$\mathbf{A^2}$	1.05	4.67×10^{-2}
$\mathbf{B^2}$	1.05	4.67×10^{-2}
$\mathbf{C^2}$	1.05	4.67×10^{-2}
$\mathbf{D^2}$	1.05	4.67×10^{-2}

A final second-order model equation was successfully created for the factors to predict the yield at specific conditions of the factors. The final equation in terms of coded factors is:

$$\text{Yield} = 12.75 + 0.23A - 4.01B - 0.18C - 0.25D - 0.32AB - 0.041AC - 0.16AD + 0.21BC + 0.24BD + 0.068CD - 1.71A^2 - 2.40B^2 - 1.61C^2 - 1.26D^2$$

(Eq. 4.2)

(where A - Temperature, B - Concentration, C - Time, D - pH)

The quadratic model suggested a few optimum conditions for the maximum yield of collagen which were in the range of 0.62 M acetic acid, pH 2.96, 19.03 °C, for a duration of 77.51 h. Three sets were chosen and conducted in Table 4.8. The expected yield from the combinations was approximately 13.94 %,

whereas the actual yield obtained was 12.88 %. The difference between the predicted and the actual yield showed a percentage error of 7.60 %.

Table 4.8: Suggested optimum conditions for fish scale

Set	Concentration (M)	Temperature (°C)	Time (h)	pH (pH)	Actual Yield (%)	Expected yield (%)	Percentage of error (%)
1	0.62	19.03	77.51	2.96	12.88	13.94	7.60
2	0.63	19.78	75.78	3.00	13.00	14.05	7.47
3	0.59	16.95	85.78	3.29	13.20	14.06	6.11

4.1.5 **Fish Bone Optimization**

The effects of the tested parameters (temperature, extraction time, pH and acetic acid concentration) are shown in Figure 4.4. Based on the surface plot and contour plot, 12 °C to 30 °C of extraction temperature and 50 h to 95 h extraction time demonstrated the highest yields. While, high collagen yield was found in the range of pH 2.5 to 3.25 with 0.5 M to 0.7 M of acetic acid. Optimum yield was obtained with the combination of 0.63 M acetic acid, pH 3.15, 78 h of extraction at 27 °C.

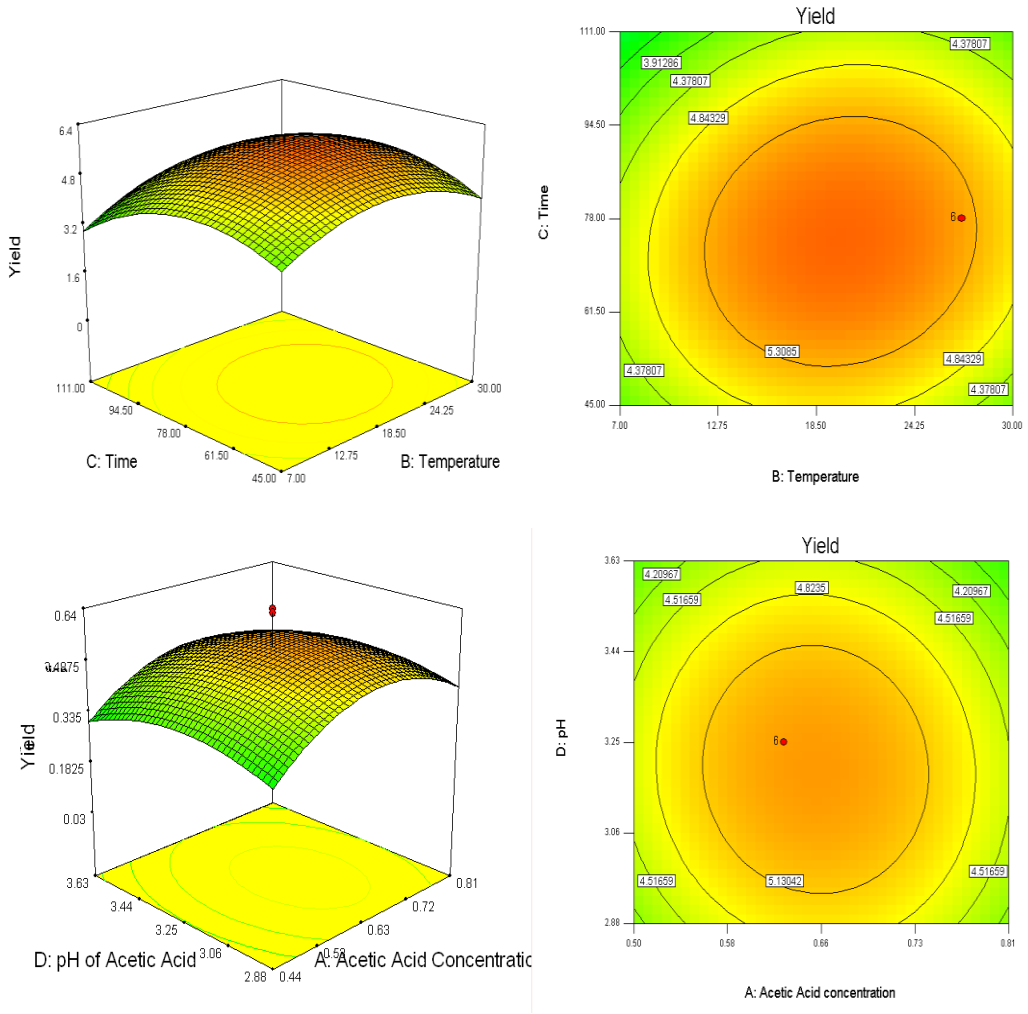


Figure 4.4: Surface plot (left) and contour plot (right) for collagen yield of fish bones in response to time, temperature, pH and concentration

4.1.6 **Fish Bone Model fitting and optimization**

Corresponding to the experimental runs and the outcome of the results, a quadratic model was produced. The suitability of the model was assessed using the ANOVA. ANOVA generated a Model F value of 22.61 that proved the model generated was significant and there was a 0.01 % chance that the model happened due to noise (Table 4.9). A, B, AB, A^2, B^2, C^2, D^2 were shown to be significant model terms because the Prob > F values were less than 0.05. Moreover, the F-value of the "Lack of Fit" was shown to be insignificant with a value of 0.53. Therefore, further proving the model produced was suitable.

Table 4.9: Analysis of variance (ANOVA) of fish bone model

[1]Source	Sum of Squares	df	Mean Square	F value	p-value Prob>F
Model	120.27	14	8.59	22.61	$<0.1 \times 10^{-3}$
A	2.68	1	2.68	7.05	0.01
B	33.84	1	33.84	89.08	$<0.1 \times 10^{-3}$
C	0.46	1	0.46	1.21	0.28
D	0.84	1	0.84	2.20	0.15
AB	2.40	1	2.40	6.32	0.02
AC	3.60×10^{-3}	1	3.60×10^{-3}	9.47×10^{-3}	0.92
AD	0.09	1	0.09	0.24	0.63
BC	0.57	1	0.57	1.50	0.23
BD	0.51	1	0.51	1.35	0.26
CD	0.28	1	0.28	0.73	0.40
A^2	40.80	1	40.80	107.38	$<0.1 \times 10^{-3}$
B^2	26.39	1	26.39	69.45	$<0.1 \times 10^{-3}$
C^2	30.22	1	30.22	79.53	$<0.1 \times 10^{-3}$
D^2	11.84	1	11.84	31.17	$<0.1 \times 10^{-3}$
Residual	5.7	15	0.38		
Lack of Fit	2.93	10	0.29	0.53	0.81
Pure Error	2.76	5	0.55		
Cor Total	125.97	29			

[1] A,(Concentration); B, (Temperature); C, (Time); and D, (pH)

All values in the ANOVA were shown to be sufficient to confirm the model. The standard deviation and mean were calculated as 0.62 and 2.27 respectively (Table 4.10). The determination coefficient R^2 is 0.95, suggesting 95 % of the variability in the response fits in the final equation in Eq. 4.3. Additionally, both Adj R^2 (0.91) and Pred R^2 (0.83) showing the experiment results are in close agreement with the predicted value from the RSM system. The "Adeq R^2" which determined the signal to noise ratio was 14.45, a value greater than 4.00 indicating the error from noise can be ignored.

Table 4.10: Statistics of fish bone model

Statistics	Number
Std. Dev.	0.62
Mean	2.27
R^2	0.95
Adj R^2	0.91
Pred R^2	0.83
Adeq Precision	14.45

All terms showed to be significant as ideal VIF values of 1.00 were achieved by all terms except for all quadratic terms, A^2, B^2, C^2, D^2 that resulted in slightly higher VIF or 1.05 (Table 4.11). The R^2 values of all terms were 0.0 which is an ideal value, except for the quadratic terms that had values of 4.76 x 10^{-2}, which were only slightly higher than ideal values. Nevertheless, all terms had significant VIF and R^2 values which showed the model has no multicollinearity.

Table 4.11: VIF and R^2 of fish bone model

Term	VIF	R^2
A	1.00	0.00
B	1.00	0.00
C	1.00	0.00
D	1.00	0.00
AB	1.00	0.00
AC	1.00	0.00
AD	1.00	0.00
BC	1.00	0.00
BD	1.00	0.00
CD	1.00	0.00
$\mathbf{A^2}$	1.05	4.76×10^{-2}
$\mathbf{B^2}$	1.05	4.76×10^{-2}
$\mathbf{C^2}$	1.05	4.76×10^{-2}
$\mathbf{D^2}$	1.05	4.76×10^{-2}

A final equation was generated for the yield of fish scale collagen. The final equation in terms of coded factors is:

$$\text{Yield} = 0.54 - 0.33A - 1.19B - 0.14C - 0.19D - 0.39AB - 0.015AC - 0.075AD + 0.19BC + 0.18BD + 0.13CD - 1.22A^2 - 0.98B^2 - 1.05C^2 - 0.66D^2$$

(Eq. 4.3)

(Coded factors: A - Temperature, B - Concentration, C - Time, D - pH)

An optimum condition was generated for the maximum yield of collagen extracted from fish bones. The optimum condition was at 0.67 M acetic acid, pH 3.15, at a temperature of 19.01 °C, for a duration of 73.16 h, and an actual yield of 5.42 was achieved. Compared to the expected yield that was calculated by the model of 5.89 %, a percentage error of 7.97 % was calculated (Table 4.12).

Table 4.12: Suggested optimum conditions for fish bone

Set	Concentration (M)	Temperature (°C)	Time (h)	pH (pH)	Actual Yield (%)	Expected yield (%)	Percentage of error (%)
1	0.67	19.01	73.16	3.15	5.42	5.89	7.97

4.1.7 **Fish Fin Optimization**

The results of temperature and concentration of acetic acid on the yield of collagen from fish waste fin are shown in Figure 4.5. High yields are obtained at an acetic acid concentration ranging from 0.48 to 0.82 M, and in a temperature range of 5 to 30 °C. The condition with the highest yield obtained was at 0.63 M and 27 °C. Figure 4.5 also shows the results of pH and time on collagen yield from fish fin. High yields are obtained at a pH ranging from pH 2.88 to 3.44, and with a duration of 61.5 to 94.5 h extraction time. Consequently, the optimum condition was pH 3.25 and 78 h.

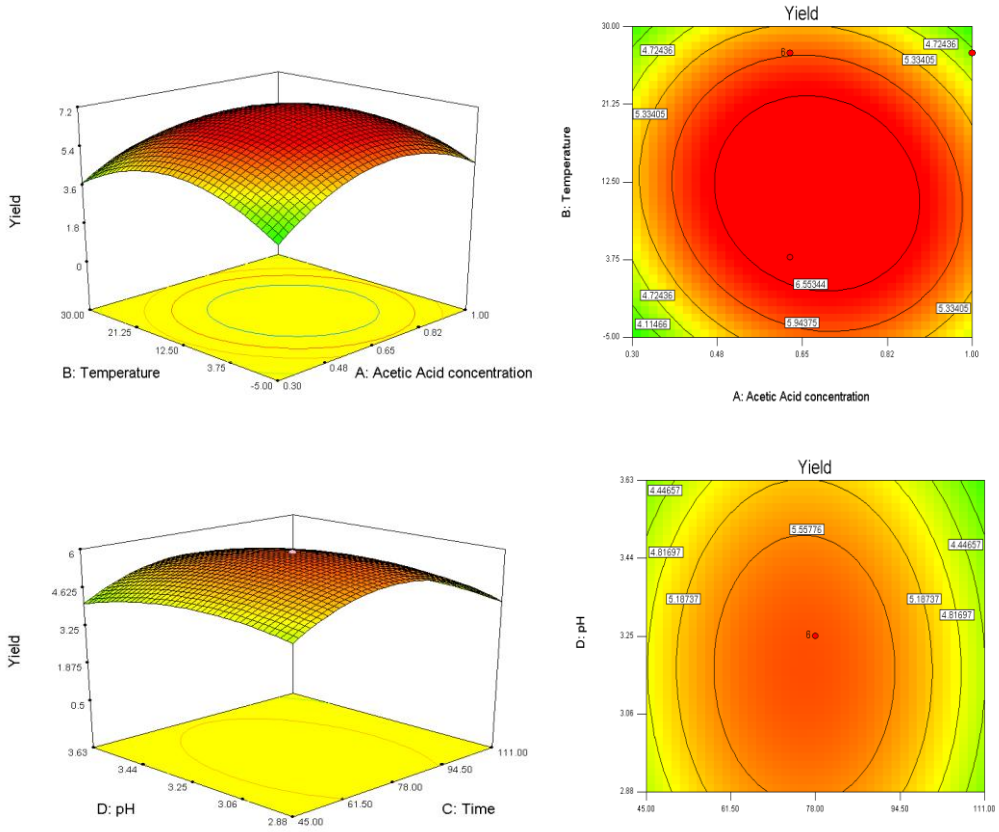


Figure 4.5: Surface plot (left) and contour plot (right) for collagen yield of fish fins in response to concentration, temperature, time and pH

4.1.8 **Fish Fin Model fitting and Optimization**

Analysis of variance (ANOVA) on the results revealed that the model is significant with a F-value of this model of 25.15. Thus, there is a 0.01 % chance the F-value of the model happened due to noise. B, A^2, B^2, C^2, D^2 terms have values of "Prob > F" less than 0.05, therefore, these terms are significant terms (Table 4.13).

Table 4.13: Analysis of variance (ANOVA) of fish fin model

Source[1]	Sum of Squares	df	Mean Square	F value	p-value Prob>F
Model	137.50	14	9.82	25.15	$<0.1 \times 10^{-3}$
A	0.43	1	0.43	1.09	0.31
B	79.04	1	79.04	202.42	$<0.1 \times 10^{-3}$
C	0.74	1	0.74	1.88	0.19
D	1.09	1	1.09	2.79	0.12
AB	0.20	1	0.20	0.52	0.49
AC	0.049	1	0.049	0.12	0.73
AD	0.062	1	0.062	0.16	0.67
BC	0.022	1	0.022	0.057	0.81
BD	6.28×10^{-3}	1	6.28×10^{-3}	0.016	0.90
CD	2.46×10^{-3}	1	2.46×10^{-3}	6.21×10^{-3}	0.93
A^2	6.43	1	6.43	16.47	1.0×10^{-3}
B^2	12.45	1	12.45	31.89	$<0.1 \times 10^{-3}$
C^2	46.13	1	46.13	118.13	$<0.1 \times 10^{-3}$
D^2	6.77	1	6.77	17.34	0.8×10^{-3}
Residual	5.86	15	0.39		
Lack of Fit	5.86	10	0.59		
Pure Error	0.000	5	0.000		
Cor Total	143.36	29			

[1] A, (Concentration); B, (Temperature); C, (Time); and D, (pH)

The mean and standard deviation were estimated to be 3.54 and 0.62. Hence, the R^2 was 0.96. The R^2 specifies that 95.91 % of the variability in the response is accounted by the second-order model equation in Eq. 4.4. The "Adj R^2" (0.92) and "Pred R^2" (0.76) values are in reasonable agreement as they were close to each other. Besides, the "Adeq R^2" presented a value of 6.61, which was greater than 4.00, points out that there is a suitable signal to noise ratio in the model.

Table 4.14: Statistics of fish fin model

Statistics	Number
Std. Dev.	0.62
Mean	3.54
R^2	0.96
Adj R^2	0.92
Pred R^2	0.76
Adeq Precision	16.43

A final equation of a second-order model was produced for the yield of collagen from fish fins. The final equation in terms of coded factors is:

$$\text{Yield} = 5.90 + 0.13A - 1.81B - 0.18C - 0.21D - 0.11AB + 0.055AC + 0.062AD + 0.037BC - 0.02BD - 0.012CD - 0.48A^2 - 0.67B^2 - 1.30C^2 - 0.50D^2 \quad \text{(Eq. 4.4)}$$

(Coded factors: A - Temperature, B - Concentration, C - Time, D - pH)

The VIF and R^2 for all terms were significant (Table 4.14). VIF values were 1.00 for all terms except the quadratic terms (A^2, B^2, C^2, D^2), which had a value of 1.05. The R^2 for all values were ideal values of 0.0, except for the quadratic terms (A^2, B^2, C^2, D^2), that were 4.67×10^{-2}. The model is proven to be adequate as the VIF's show the coefficients do not have multicollinearity, and the R^2 shows that the terms are not highly correlated with each other (Table 4.15).

Table 4.15: VIF and R^2 of fish fin model

Term	VIF	R^2
A	1.00	0.00
B	1.00	0.00
C	1.00	0.00
D	1.00	0.00
AB	1.00	0.00
AC	1.00	0.00
AD	1.00	0.00
BC	1.00	0.00
BD	1.00	0.00
CD	1.00	0.00
$\mathbf{A^2}$	1.05	4.67×10^{-2}
$\mathbf{B^2}$	1.05	4.67×10^{-2}
$\mathbf{C^2}$	1.05	4.67×10^{-2}
$\mathbf{D^2}$	1.05	4.67×10^{-2}

Several optimum conditions were suggested for the achievement of maximum collagen yield from fish fins. These optimum conditions are acetic acid concentrations of 0.5 M, with a pH of 3.05, at temperatures of 18.76 °C, for the duration of 71.65 h. The actual yield obtained was in a range of 5.94 % to 6.18 %. An expected yield calculated was around 6.48 % to 6.77 %. Thus, a percentage of error of around 8.33 % to 8.98 % was attained (Table 4.16).

Table 4.16: Suggested optimum conditions for fish fin

Set	Temperature (°C)	Concentration (M)	pH (pH)	Time (h)	Actual Yield (%)	Expected yield (%)	Percentage of error (%)
1	18.76	0.50	3.05	71.65	5.94	6.48	8.33
2	16.60	0.56	3.00	72.36	6.18	6.77	8.71
3	18.11	0.66	2.89	67.14	5.98	6.57	8.98

4.2 **Electrophoresis**

The protein profile of the extracted collagen was observed with SDS-PAGE with the commercially available Type I human collagen as control. The SDS-PAGE pattern showed that fish skin, scale and fin collagen had a doublet pattern for α_1 and α_2 chains at corresponding to 132.04 kDa, and 120.06 kDa, respectively, and a β chain (220.67 kDa), whereas the molecular weight of fish bone $\alpha_{1,}$ α_2 and β chains were slightly higher which were 139.79 kDa, 124.72 kDa and 229.22 kDa, respectively. The density for α_1 was twice as much as α_2, (Figure 4.6). Similar results were obtained for collagen of skin, scales, bones and fins of fish waste which matches the band patterns of the human skin collagen. Based on the patterns of α_1 and α_2, it suggested that the fish collagen has a composition of $(\alpha 1)_2\alpha 2$ heterotrimer, a type I collagen.

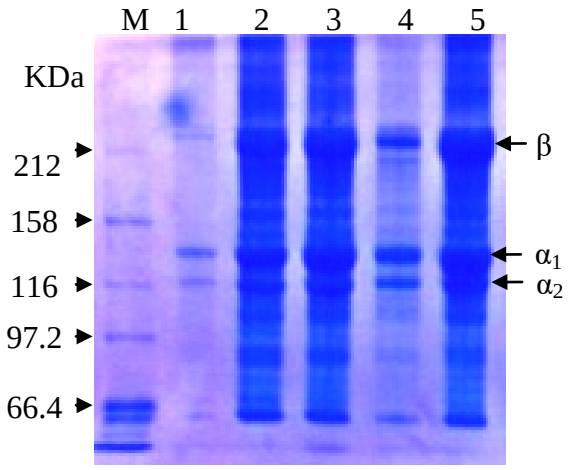
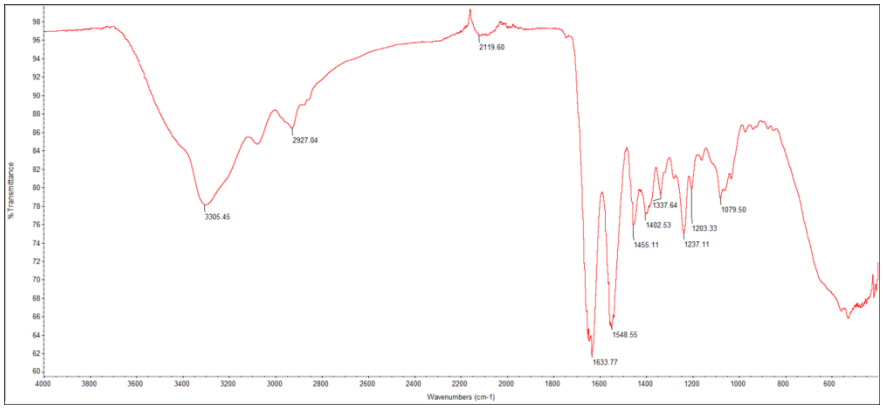


Figure 4.6: SDS-PAGE pattern of collagen extracted from different parts of the fish (skin, bone, scale and fin) on 7.50% gels. Lane M, molecular weight marker; lane 1, human collagen; lane 2, fish skin collagen; lane 3, fish scale collagen; lane 4, fish bone collagen; lane 5, fish fin collagen

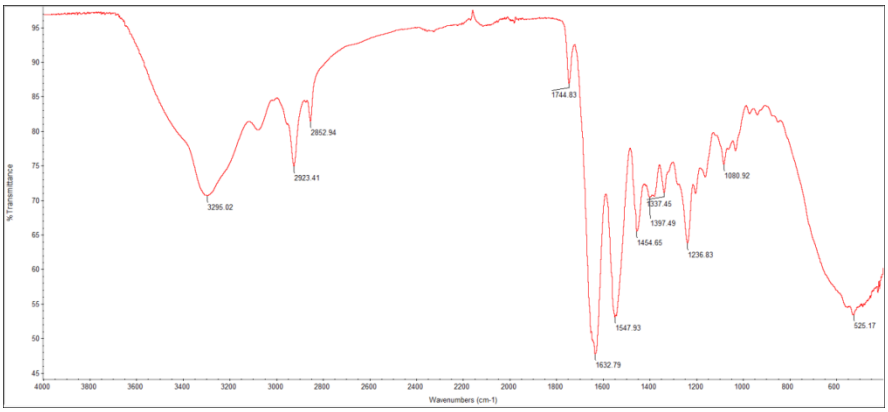
4.3 Fourier Transform Infrared Spectroscopy (FTIR)

The structure of collagen from fish wastes were analyzed using FTIR. The FTIR spectra of fish wastes were compared with human skin collagen. Amide bands A, B, I, II and III were revealed in the fish waste and also in human skin collagen (Figure 4.7). The Amide A, B, I, II and III bands were identified and listed in Table 4.17.

(a)



(b)



(c)

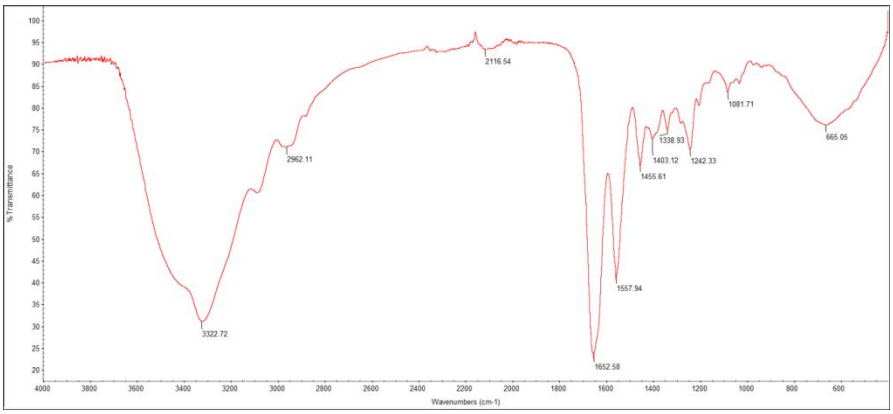
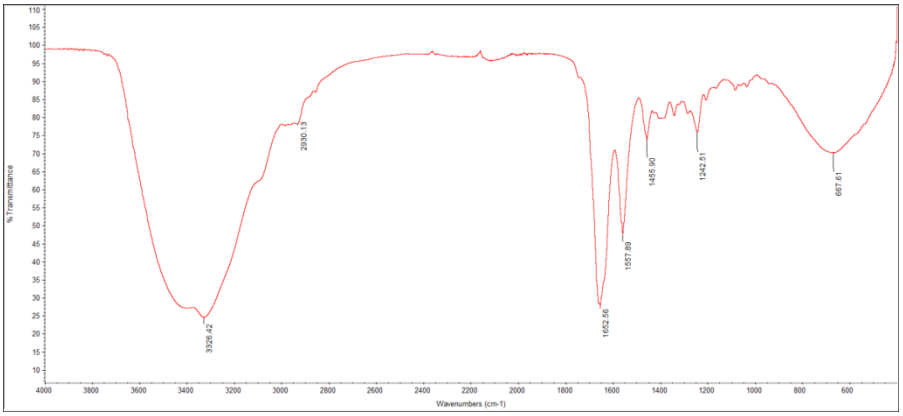


Figure 4.7: FTIR spectra of human and fish waste collagen (a) Human collagen, (b) Fish Skin collagen, (c) Fish Scale collagen, (d) Fish Bone collagen, (e) Fish Fin collagen.

(d)



(e)

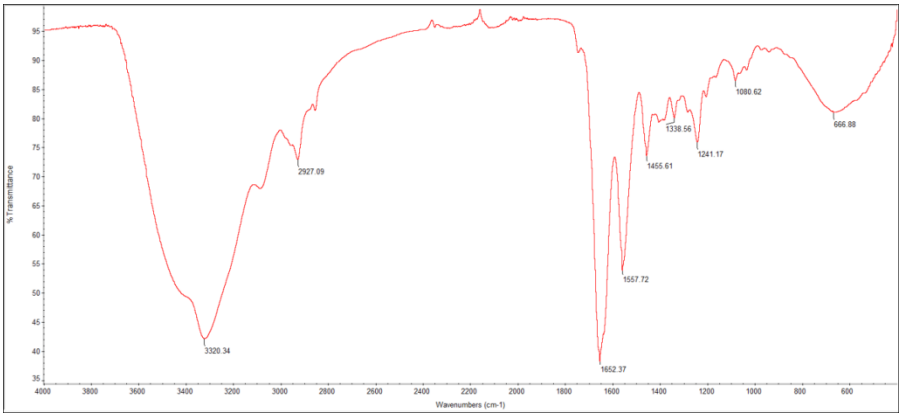


Figure 4.7 (cont'): FTIR spectra of human and fish waste collagen (a) human collagen, (b) fish skin, (c) fish scale, (d) fish bone, (e) fish fin collagen.

Table 4.17: Amide band wavenumbers for human skin, fish skin, fish bone, fish scale and fish fin collagen

Amide Band	Human	Skin	Bone	Scale	Fin
A	3305.45	3295.02	3326.42	3322.72	3320.34
B	2927.04	2923.41	2930.13	2962.11	2927.09
I	1633.77	1632.79	1652.56	1652.58	1652.37
II	1548.55	1547.93	1557.89	1557.94	1557.72
III	1237.11	1236.83	1242.51	1242.33	1241.17

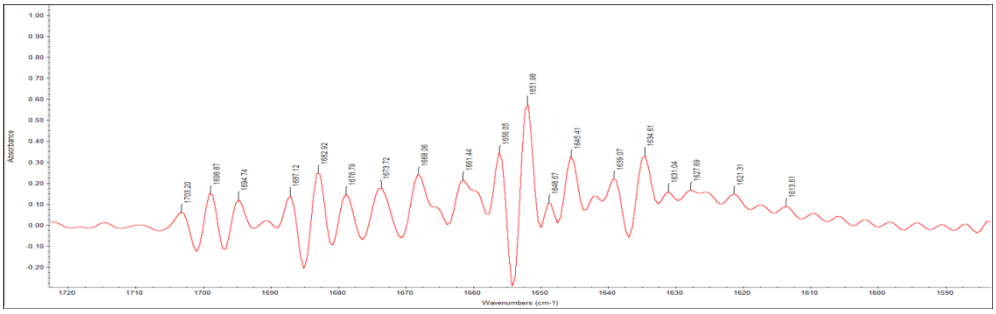
The IR ratios for amide III bands and the peak at 1450 cm^{-1} region were calculated for fish skins, scales, bones and fins, as well as the human skin collagen and are depicted in Table 4.18.

Table 4.18: IR ratios for human skin, fish skin, fish bone, fish scale and fish fin collagen

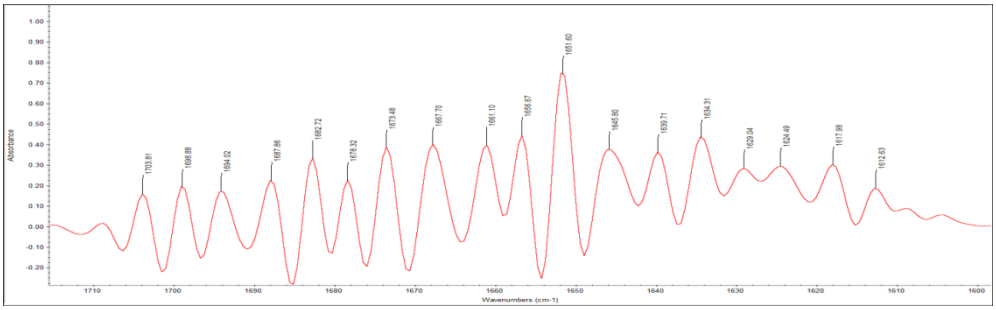
Collagen	Amide III (cm^{-1})	1450 peak (cm^{-1})	Ratio
Fish Skin	1236.83	1454.65	1.17
Fish Scales	1242.33	1455.61	1.17
Fish Bones	1242.51	1455.98	1.17
Fish Fins	1241.17	1455.81	1.17
Human skin	1237.11	1455.11	1.17

Fourier self-deconvolution of Amide I bands of the human skin collagen, fish skin, scale, bone and fin collagen were obtained and are depicted in Figure 4.8. The peaks revealed from the spectra were summarized in Table 4.19. Based on the peak number obtained, the secondary structure of the fish waste collagen contained β-sheets, β-turns, α-helices and random coils. The distribution of the secondary structures is shown in Table 4.20. The results showed that the distribution of the secondary structures in fish skins, scales, bones and fins collagens are almost similar to that of the human skin collagen. The percentage of α-helices, β-sheets and random coils are relatively higher in fish scales collagen as compared to the other fish waste samples collagen. However, β-turns contributed almost half (45.81 %) of the protein secondary structure in bone collagen.

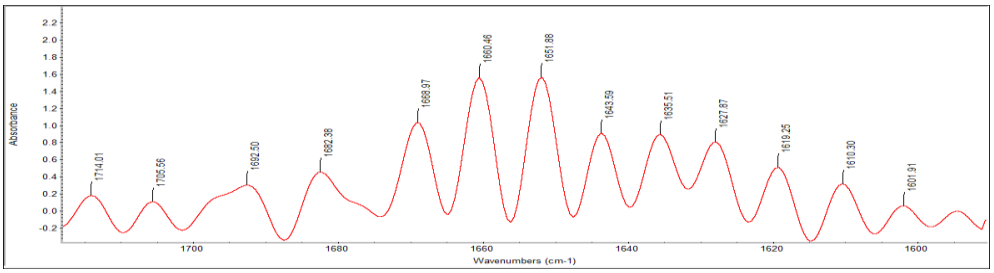
a



b



c



d

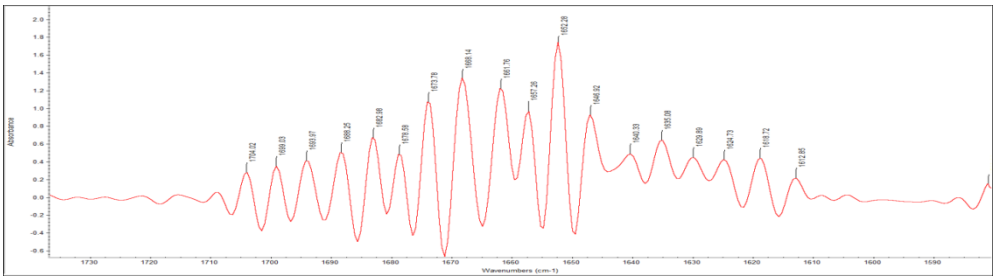


Figure 4.8: Fourier self-deconvolution of Amide I (a) human skin collagen, (b) fish skin collagen, (c) fish scale collagen, (d) fish bone collagen and (e) fish fin collagen.

e

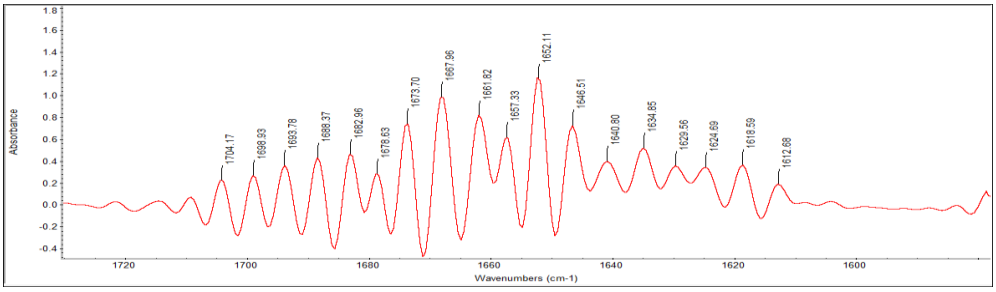


Figure 4.8 (cont'): Fourier self-deconvolution of Amide I (a) human skin collagen, (b) fish skin collagen, (c) fish scale collagen, (d) fish bone collagen and (e) fish fin collagen.

Table 4.19: Amide I peak wavenumber of human skin, fish skin, fish scale, fish bone and fish fin collagen

Peak Wavenumber	Assignment	Human (wavenumber)	Skin (wavenumber)	Scale (wavenumber)	Bone (wavenumber)	Fin (wavenumber)
1605-1615	(Tyr) side chains/aggregated strands	1613.61	1612.63	1610.29	1612.85	1612.68
1616-1621	Beta-sheets	1621.31	1617.98	1619.26	1618.72	1618.59
1622-1627		1627.60	1624.49	1627.85	1624.73	1624.69
1628-1637		1631.04	1629.04	1635.43	1629.89	1629.56
		1634.61	1634.31		1635.08	1634.85
1638-1646	Random coils	1639.07	1639.71	1643.50	1640.33	1640.80
		1645.41			1646.92	1646.51
1647-1655		1648.67	1645.80	1651.84	1652.28	1652.11
		1651.98	1651.60			
1656-1662	Alpha-helices	1656.05	1656.67	1660.49	1657.26	1657.33
		1661.44	1661.10		1661.76	1661.82
1663-1670	Turns	1668.06	1667.70	1669.10	1668.14	1667.96
1671-1685		1673.72	1673.48	1682.22	1673.78	1673.70
		1678.79	1678.32		1678.58	1678.63
		1682.92	1682.72		1682.98	1682.96
1686-1696		1687.12	1687.86	1692.89	1688.25	1688.37
		1694.74	1694.02		1693.97	1693.78
1697-1703	Beta-sheets (weak)	1698.87	1698.88		1699.03	1698.93
		1703.20	1703.81			

Table 4.20: Percentages of human skin, fish skin, fish scale, fish bone and fish fin collagen secondary structures

Structure	Human (%)	Skin (%)	Scales (%)	Bones (%)	Fin (%)
α-helix	10.21	11.68	16.00	13.93	12.70
β-sheets	27.18	20.76	21.08	15.75	16.59
β-turns	35.75	38.92	32.39	45.81	44.51
Others (random coils and Tyr side chains)	26.88	28.63	30.52	24.49	26.19
Total	100.02	99.99	99.99	99.98	99.99

4.4 Denaturation temperature (T_d)

Denaturation temperature of fish waste collagen was determined with a rheometer in which the fractional viscosity was calculated. The temperature, upon which the fractional viscosity was 0.50, is considered as the denaturation temperature, T_d. The fractional viscosity was calculated using equation 3.2 in Section 3.4.3. Generally, all samples tested (fish skins, scales, bones and fins) were above 24 °C. The results of each sample was shown in Table 4.21, and the denaturation temperature curves are depicted in Appendix E.

Table 4.21: Denaturation temperature (T_d) of fish skin, scales, bones and fins

Samples	Denaturation temperature (°C)
Fish Skin	36.00
Fish Scales	36.15
Fish Bones	37.80
Fish Fins	32.60

4.5 **Amino Acid Composition**

Amino acid composition analysis was determined by acid hydrolysis of fish waste collagen, followed by reversed-phase high-performance liquid chromatography (RP-HPLC). The amino acid composition for fish skin collagen is expressed as residues per 1000 total residues, and is shown in Table 4.22. A total of 18 amino acids were found in all the collagen.

The amino acid compositions of fish skins, scales, bones and fins collagen in Table 4.22, clearly show that the imino acid (proline and hydroxyproline) and glycine contents were the highest among the other amino acids in fish skin, scale, bone and fin collagen. Fish skin, scale, bone and fin collagen had glycine contents of 249.41 residues/1000 residues, 219.83 residues/1000 residues, 230.61 residues/1000 residues, 260.33 residues/1000 residues, respectively. Besides, imino acid contents in fish skin, scale, bone and fin collagen were rather high, which were 205.01 residues/1000 residues, 239.97 residues/1000 residues, 248.68 residues/1000 residues, and 212.03 residues/1000 residues, respectively. Hence, in the RP-HPLC results, the degree of hydroxylation of proline for fish skin, scale, bone and fin were calculated to be 43.64 %, 51.43 %, 48.33 % and 46.03 %, respectively. Scale collagen had the highest degree of hydroxylation of proline, followed by bone, then fins and lastly skin collagen.

Table 4.22: Amino acid composition of fish skin, scale, bone and fin collagen (residues/1000 residues)

Amino acid Results (residues/1000 residues)	Fish skin	Fish scale	Fish bone	Fish fin
Aspartic Acid (Asp)	44.27	37.06	47.18	51.92
Serine (Ser)	34.11	39.19	37.39	28.57
Glutamic Acid (Glu)	95.21	80.86	94.60	105.00
Glycine (Gly)	249.41	219.83	230.61	260.33
Histidine (His)	0.12	0	0.25	0.14
Arginine (Arg)	81.07	122.85	99.37	79.18
Threonine (Thr)	23.48	34.46	28.86	20.44
Alanine (Ala)	85.16	92.30	93.60	92.38
Proline (Pro)	115.53	116.56	128.48	114.43
Tyrosine (Tyr)	3.73	7.70	7.28	7.83
Valine (Val)	19.62	23.93	20.33	26.68
Lysine (Lys)	26.98	27.58	28.86	31.61
Isoleucine (Ile)	10.74	12.15	11.29	15.80
Leucine (Leu)	25.58	24.32	26.35	31.61
Phenylalanine (Phe)	19.62	26.71	25.35	21.17
Cysteine (Cys)	0.12	0.17	0	1.30
Methionine (Met)	75.93	10.83	0	13.92
Hydroxyproline	89.48	123.41	120.20	97.60
Imino acid (Hydroxyproline and proline)	205.01	239.97	248.68	212.03

CHAPTER 5

DISCUSSION

5.1 Yield of extracted collagen from fish waste

In this study, fish skin collagen was extracted with acetic acid under various conditions. The highest fish skin collagen yield was 24.77 % (on a dry weight basis). This was higher as compared with to findings of collagen yields of skins of black rum (15.80 %) but lower than sheepshead seabream (29.30 %) (Ogawa et al., 2003).

For fish scales, the highest yield obtained was 13.82 % (on a dry weight basis) which was relatively low compared to fish skin. It was higher compared to silver carp scale of 1.45 % (on a wet weight basis) (Zhang, J.J. et al., 2010), scales of bighead carp 2.70 % (on a dry weight basis) (Liu et al., 2012), but lower to scales of red seabream, Japanese sea bass and sardine which yielded 37.5 %, 41.0 % and 50.9 %, respectively (Nagai et al., 2004).

Total of 6.4 % of collagen was successfully extracted from fish bones (on a dry weight basis). The content was high as compared to bones of carp fish of 1.06 % (on dry weight basis) (Duan et al., 2009) and bones of Bigeye snapper which was 1.60 % (on a wet weight basis) (Kittiphattanabawon et al.,

2005). It was also relatively high to bones of bighead fish of 2.90 % (on a dry weight basis) and 1.30 % (on a wet weight basis) (Liu, et al., 2012).

Fish fins yielded 5.9 % (on a dry weight basis). This finding is similar to fins of bighead carp which was 5.10 % (on a dry weight basis) (Liu et al., 2012) and 5.20 % for Japanese sea bass (on a dry weight basis) (Nagai and Suzuki, 2000a). Besides, other parts of fish such as the swim bladder of bighead carp yield was 5.10 % (on a dry weight basis) (Liu et al., 2012).

Generally, collagen yields are affected by temperature, extraction time, influence of acetic acid and pH. The effects on the yield of collagen from fish scale are shown in Figure 4.2. For both factors, collagen yield improved with the raise in temperature and time, until it reached the optimum level of 27 °C and 78 h. The result of temperature and time is presented in bell shape pattern with the top of the curve showing the optimum condition of maximum collagen being produced. However, production of yield starts declining after the optimum conditions. This is due to the fact that collagen is a thermo-instable protein that easily denatures at room temperatures owing to its chemical structure. Generally, fish collagens vary in their amino acid composition, in particular to the amounts of the imino acids (proline and hydroxyproline) between species (Gudmundsson and Hafsteinsson, 1997). A lesser amount of hydroxyproline in a collagen exhibits a lesser thermal stability compared to those with a higher amount of hydroxyproline (Muyonga et al., 2004). This is attributed to the assistance of hydroxyproline in the inter-chain linkage by hydrogen bonds that stabilize the collagen's triple-helix

structure. Consequently, manipulating the temperature for a given duration in the collagen extraction process is critical to maintain the collagen's natural structure. Raising the temperature to a definite amount and duration can help disintegrate collagen into gelatin which boosts the yield of fish scale extract. In this study though, temperatures risen to 20 °C for the duration of up to 78 h, would increase the yield of collagen. Nevertheless, higher temperatures at a longer time have the capability to alter the conformation of collagen that could reduce its solubility. Therefore, yield of collagen declines when temperature rises above 20 °C and when the time is lengthened to over 78 h. For that reason, the denaturation temperature of lower than 20 °C were found for collagen from cod, ayu and chum salmon which were reported to be 15 °C, 29.70 °C and 19.40 °C, respectively (Rigby, 1968; Kimura et al., 1988; Nagai and Suzuki, 2000a).

Mass transfer rate of analytes from a matrix takes part in the efficiency of extraction (Bartle et al., 1991). In traditional extraction processes, the mass transfer rate is managed by diffusion process that is affected by time. In the controlled diffusion process, the recovery of analytes increases along the lengthening of time. This justifies the extraction's positive effect with the lengthening of time until a center point of 78 h.

Figure 4.2 also shows the influence of acetic acid concentration and pH the yield of collagen. A positive correlation was established between acetic acid concentration and pH with the yield of collagen. The acetic acid concentration and pH of it are vital variables in influencing the efficiency of

extraction. The yield of collagen would increase as acetic acid concentration would increase up to 0.63 M at a pH of 3.25. After this maximum point of 0.63 M and pH 3.25, the yield of collagen would gradually decrease.

Acetic acid is an organic acid which is frequently applied to extract collagen directly from animal tissues. Inorganic acids, for example, hydrochloric acid, can be used as well, but poorer extraction abilities as compared to organic acids. Acetic acid is generally used to extract collagen as it has high extraction ability and extracts high quality collagen (Skierka and Sakowska, 2007). The collagen's solubility in acetic acid can influence the extraction capacity from fish scale waste.

Besides, pH of the acetic acid also contributed to the extractability of collagen. It is well acknowledged that pH can control the charge density of protein which modifies the electrostatic interaction and structure of proteins (Wang, An et al., 2008). In this research, a comparatively higher amount of collagen was attained around a concentration of 0.63 M at pH 3.25. It could be due to the higher amount of positively charged amine groups of collagen at this concentration and pH. The yield of collagen decreased when the concentration and pH was above or below 0.63 M and pH 3.25. Higher concentrations produced lower yields of collagen as the collagen was denatured at a low pH value.

When a figure of the effects of temperature and acetic acid concentration on collagen yield were also generated using the RSM, a similar effect was witnessed to that of acetic acid concentration, which falls on 20 °C at 0.63 M. Lesser or more than this value would lead to a diminish yield of collagen. As shown in Figures 4.10, 4.11 and 4.12, the optimal values for all the four factors i.e extraction acetic acid concentration, acetic acid time, temperature and pH of acetic acid fall on the same point when compared to one another.

5.2 **Optimization for fish waste for collagen production**

Overall, the optimized extraction conditions for fish waste collagen (skin, scales, bones and fins) were rather similar such as acetic acid concentration (0.5 M to 0.66 M), extraction temperature (16.06 to 19.03 °C), pH (2.96 to 3.09) and extraction time (72.36 to 77.50 h). Except fish skin required lower extraction temperature (13.26 °C). However, the collagen yield obtained from skins, scales, bones and fins were relatively low compared to other reported studies. Optimization and model fitting of collagen extraction procedure was conducted with a statistical approach by using RSM. Based on the parameters tested, RSM revealed that all fish skins, scales, bones and fins fit in the quadratic equation. The ANOVA analysis confirmed all the terms tested are independent, which the terms will show significant effect if the terms are squared. The R^2 value of more than 0.95 of all tested samples indicated the mathematical model is reliable. The mathematical model generated from the RSM analysis was further verified with experimental

results. It was found that the percentage of error for fish skins, scales, bones and fins were 5.24 %, 7.6 %, 7.97 % and 8.71 %, respectively. The value indicated that the experiment data is less than 10 % indifference with the predicted value from the mathematical model. Therefore, the mathematical model can be applied for scale up production of fish waste collagen. However, the mathematical model was generated based on the tested range of parameters. Therefore, other extraction methods and parameters beyond the tested method may not fit the model from the present study.

5.3 **Electrophoresis**

The skin, scales, bones and fins, along with Bornstein and Traub Type I human collagen as a positive control was analyzed by using polyacrylamide gel electrophoresis, in the presence of SDS using 7.50 % gel (Figure 4.5). The SDS-PAGE patterns illustrated that the fish skin, scale and fin collagens had a doublet pattern for α_1 and α_2 chains at (approximately 132.04 kDa, and 120.06 kDa position) and a β chain (220.67 kDa), while the molecular weight of fish bones $\alpha_{1,}$ α_2 and β chains were slightly higher (approximately 139.79 kDa, 124.72 kDa and 229.22 kDa for $\alpha_{1,}$ α_2 and β chains respectively). The density for α_1 is twice as much as α_2, ($\alpha_{1,}$ upper; $\alpha_{2,}$ lower). Similar results were obtained for collagen of skin, scales, bones and fins of fish waste with the band patterns of the human skin Type I collagen. According to the α_1 and α_2 patterns, it implies that the fish collagen might have a composition of $(\alpha 1)_2\alpha 2$ heterotrimer, a type I collagen. The β chains band position on the gel was expressed in rather high densities. A great quantity of β chains appeared for all

the collagen patterns, which showed that the collagens were high in inter- and intra-molecular cross-links. The presence of β chains verifies the occurrence of intermolecular cross-links, as the γ chains show intra-molecular cross-linking (Lewis and Piez, 1964; Kim, 2013).

For fish skins, similar results were observed in skins of surf smelt (Nagai et al., 2010), silver carp skin collagen (Zhang, J., et al., 2009), grass carp skin (Zhang et al., 2007), largefin longbarbel catfish skin (Zhang, M., et al., 2009), channel catfish (Liu et al., 2007) and walleye pollock skin (Yan et al., 2008), skin of baltic cod (Skierka and Sakowska, 2007), Japanese sea bass, bullhead shark (Nagai et al., 2000). β chain components shown in SDS-PAGE analysis verify that collagens have inter molecular cross-links. This type of dimer was also seen in collagen of brownstripered snapper skin (Jongjareonrak et al., 2005) and Nile Perch skin (Muyonga et al., 2004).

For fish scales, results were all in accordance with results observed in scale collagen from black drum and sheepshead seabream fish (Ogawa et al., 2004), scales of red seabream, Japanese sea bass and sardine (Nagai et al., 2004), scales of carp fish (Zhang, J.J. et al., 2010), scales of spotted golden goatfish (Matmaroh et al., 2011) and sheepshead seabream (Ogawa et al., 2004).

For fish bones, these results were in accordance with bones of surf smelt (Nagai et al., 2010), bone collagen from black drum and sheepshead seabream fish (Ogawa et al., 2004), Japanese sea bass, skipjack tuna, and ayu

bone collagen. The collagens of these bones were all also abundant in inter- and intra-molecular cross-linked components (Nagai and Suzuki, 2000a).

The findings of fin collagen were similar to the findings of skins, scales, bones, fins and swim bladders of bighead carp which were of type I collagen, with a chain composition of $(\alpha 1)^2\alpha^2$ (Liu et al., 2012). Even other marine organisms such as purple sea urchin test (Nagai and Suzuki, 2000b) and common minke whale unesu (Nagai et al., 2008) had similar subunit compositions of $[(\alpha 1)_2\ \alpha 2]$.

Fish skin, scale, and fin collagen subunit molecular weights (α_1, α_2 and β chains) revealed to be slightly lower than human collagen. This difference could be due to the dissimilar between aquatic animals and mammalian animals. Surprisingly, fish bone collagen had the same molecular weight as the human collagen.

Generally the molecular weights for α1 and α2 chains are ranging from 132.04 kDa to 139.79 kDa and 120.26 kDa to 124.72 kDa, respectively. The α1 and α2 chains from the present study was higher than other tropical fish species, such as acid soluble collagen of Black drum, which has a α1 chain of 127 kDa, and α2 chain of 116 kDa (Ogawa et al., 2004). Besides, compared to bovine collagen, it has a similar α_1 and α_2 patterns, but the molecular weight is 300 kDa for the chains which is higher than the fish collagen (Kirubanandan and Sehgal, 2010). The difference between the molecular sizes is dependent on the extraction method (acid extraction or pepsin extraction), amino acid

composition, species as well as the habitat of the collagen source (Liu et al., 2008; Karim and Bhat, 2009).

5.4 **Fourier Transform Infrared Spectroscopy (FTIR)**

The FTIR spectra of fish skin, scale, bone and fin (Figure 4.6b, Figure 4.6c, Figure 4.6d, and Figure 4.6e) were similar to that of human (Figure 4.6a). All the FTIR spectra showed the characteristic peaks of Amide I, II, III and Amide A and B. The Amide A band in the samples were mainly associated with N-H stretching vibrations, as a free N-H stretching vibration appeared in the range of 3400-3440 cm^{-1} (Doyle et al., 1975; Li et al., 2004; Duan et al., 2009; Nagai et al., 2010).

The Amide I band is mostly related with the stretching vibrations of the carbonyl group all along the polypeptide backbone (Payne and Veis, 1988; Duan et al., 2009; Nagai et al., 2010). The Amide I band signals in the sample tested indicated the presence of polypeptides, which is the major structure of collagen. This is because Amide I band is a very sensitive marker for the peptide secondary structure (Surewicz and Mantsch, 1988; Nagai et al., 2010).

Amide II bands indicate N-H bending vibrations (Payne and Veis, 1988; Nagai et al., 2010). It was reported that if the Amide II bands position shifts to a lower frequency of 1540 cm^{-1}, it confirms the collagen has the presence of hydrogen bonds in it (Duan et al., 2009). Results from the present

study indicated there are N-H bending vibrations which believe to be the N-H bending of the collagen peptide backbone.

Amide III bands indicate C-H stretching (Payne and Veis, 1988; Nagai et al., 2010). Compared to other studies, the Amide III bands were also nearly the same as the skin of surf smelt is 1235 cm^{-1} (Nagai et al., 2010), and the skin of striped catfish has a Amide III band at 1242 cm^{-1} (Singh et al., 2011). Besides, these collagens also had similar Amide band ranges with bovine collagen (Kirubanandan and Sehgal, 2010).

The IR ratios of all the collagen extracted from fish skins, scales, bones and fins were 1.17. All of these results prove that the helical structures of the collagen were reserved properly in good conditions. The intensity ratio is used to indicate the triple helical structure of collagen. An IR ratio of just about 1 indicates the occurrence of helical structures (Singh et al., 2011). The results revealed that the triple helical structures are similar, as shown by the similar absorption ratio between the peak heights of Amide III and 1450 cm^{-1} (1.17 for all bones, skin, scales, fins and human collagen). The skin of striped catfish also had a similar intensity ratio of 1.17 (Singh et al., 2011). The results also indicated that the extraction condition used in the present study were able to preserve the native conformation of the collagen. The native conformation is important for future application of the collagen.

The Amide B band is correlated to the asymmetrical stretch of CH_2 (Muyonga et al., 2004; Singh et al., 2011). The skins, bones, scales and fins, illustrated a strong C-H stretching vibration at around 2923.41 cm^{-1}, 2930.13 cm^{-1}, 2962.11 cm^{-1} and 2927.09 cm^{-1} for skins, bones, scales and fins, respectively. As for the human skin, same signal was observed at the 2927.04 cm^{-1} region. In comparison, the Amide B band was found at 2964 cm^{-1} for skins of surf smelt (Nagai et al., 2010) and 2926 cm^{-1} for the skins of striped catfish (Singh et al., 2011). The spectrums of fish waste skins, bones, scales and fins, as well as human skin collagen and bovine collagen (Kirubanandan and Sehgal, 2010), reveal to be quite similar, which implies that their structures are nearly the same.

On the other hand, the secondary data analysis (Fourier self-deconvolution) on the Amide I bands of the collagens (Figure 4.7), suggested the structure of the extracted collagen was still in its native form. As the result shown in Table 4.19, fish skin, scales, bones and fins contained 11.68 % 16.00 %, 13.93 % and 12.70 % of α-helix, respectively. Compared to human skin collagen, it had 10.22 % of α-helix structures, which is slightly lower than all the fish waste collagen. Amount of α-helix structures for fishes normally fall in the range of 8 - 11 % based on previous reports (Nagai et al., 2008; Nagai et al., 2010). In contrary, the collagen from porcine skin has 9 % of α-helix. It shows that fish waste scales had the most amount of α-helix, followed by bones, fins, and skins.

For the percentage of β-sheets in the collagen, fish skins, scales, bones and fins had 20.76 %, 21.08 %, 15.75 % and 16.59 %, respectively. However, human collagen contained a higher amount of about 27.18 % of β-sheets structures in it compared to the fish waste. In other studies, the amount of β-sheets was higher. In the surf smelt skin collagen, it had 34 % of β-sheets (Nagai et al., 2010), whereas for the porcine skin collagen, it has 51 % of β-sheets (Nagai et al., 2010), and the common minke whale unesu has 50 % of β-sheets (Nagai et al., 2008).

In terms of β-turns for fish skins, scales, bones and fins were shown to be 38.92 %, 32.39 %, 45.81 % and 44.51 %, respectively. Generally, β-turns from fish waste skin, scales, bones and fins were relatively higher compared to other fishes. Surf smelt skin collagen only had 19 % of β-turns (Nagai et al., 2010), the common minke whale unesu had 15 % β-turns (Nagai et al., 2008), and the porcine skin had only 13 % of β-turns (Nagai et al., 2010).

For other structures such as random coils and (Tyr) side chains or aggregated strands, the fish skin, scales, bones and fins have 28.63 %, 30.52 %, 24.49 % and 26.19 %, respectively. Human collagen have about 26.88 % of these structures in it but only collagen of fish bones had less of these compared to the human collagen. Overall, all these collagens had about the same amount of these components in it. It has been reported that other collagens had lower amounts of other structures in it besides α-helix, β-sheets and β-turns. The surf smelt skin collagen had 21 % (Nagai et al., 2010),

porcine skin collagen had 15 % (Nagai et al., 2008) and common minke whale unesu had the lowest amount which is 17 % (Nagai et al., 2010).

Distribution of secondary structure varies among marine and mammalian source. Surprisingly, secondary structure differs among fish species as well. The diversity of secondary structure among different collagen sources is believed to be due to the amino acid composition of the collagen which plays a major role in the secondary structure distribution.

5.5 Denaturation Temperature (T_d)

One of the collagen's physicochemical characteristics are its high viscosity, as it can be related to high amounts of β and γ chains, causing it to have high molecular weights. Applying high temperatures of heat to the collagen would cause the hydrogen bonds to break down (Wong, 1989). As rising heat is applied to the collagen, the hydrogen bonds progressively break. Resulting in the triple helix structure of the collagen which was held together by the hydrogen bonds was changed into the random coil configuration of gelatin. This was done by the course of thermal depolymerization, which was accompanied by the alteration in physical properties, such as viscosity (Shahiri et al., 2012). If progressive breakage of hydrogen bond continues, collagen will be denatured as the intact trimers (γ) of collagen break into individual chains (α) or dimmers (β) when heated above the denaturation temperature.

The T_d value of each collagen is defined as the temperature where the change in viscosity is half completed. The T_d of fish waste skin, scales, bones and fins (Table 4.20) were 36 ℃, 36.15 ℃, 37.80 ℃ and 32.60 ℃ respectively. The denaturation temperatures (T_d) of these fish wastes were found to be higher than Tilapia (*Oreochromic niloticus*) waste (32 °C) (Sujithra et al., 2013), carp skin, scale and bone (around 28 °C), but higher than cod skin (15 °C) (Duan et al., 2009), Alaska pollack skin (17 °C), swim bladder (18.40 °C) (Kimura and Ohno, 1987), common mackerel (26.10 °C) and Saury (23 °C), chum salmon (19.40 °C) (Kimura et al., 1988).

Collagen from fish waste generally lost its viscosity at approximately 50 °C. A rapid loss of viscosity was observed at 24 °C onwards. For rainbow trout bone collagen, as the temperature increased, the viscosity reduced constantly. However for rainbow trout skin collagen demonstrated a slightly different trend was observed. The rate of reduction was seized in the temperature range of 30 – 40 °C (Shahiri et al., 2012).

Generally, the T_d of collagen of fish living in cold environments is lower than the fish species living in warm environments (Duan et al., 2009). Thus, it is suggested that these collagen from the fish waste skin, scales, bones and fins are more stable than other aquatic species. However, the T_d of the fish waste was lower than calf skin acid solubilised collagen (ASC) (40.80 °C) (Komsa-Penkova et al., 1999) and porcine collagen (37.80 °C) (Nagai and Suzuki, 2000a). The difference in terms of T_d is most likely due to the structure and amino acid composition of the collagens.

Fish species living in cold environments shown to have lower contents of hydroxyproline compared to those fishes living in warm environments (Sadowska et al., 2003). This is because hydroxyproline contributes to the inter-chain hydrogen bonding in the collagen, which stabilizes the collagen's triple helical structure. Therefore, these collagens are more resistant to heat treatment.

Apparently, it appears that the molecular weight of collagen has a relationship with the thermal stability of collagen. Collagen with a higher molecular weight could have a higher thermal stability (Duan et al., 2009). Calf skin collagen was reported to have a molecular weight of 300 kDa, with a high denaturation temperature of 40.8 °C (Kittiphattanabawon et al., 2005). Thus, the molecular weight of fish bone collagen $\alpha_{1,}$ α_2 and β chains which were approximately 139.79 kDa, 124.72 kDa and 229.22 kDa, respectively, had a T_d of 37.80 °C. Therefore, fish skin, scale and fin collagen that showed lower molecular weights of 132.04 kDa, 120.06 kDa and 220.67 kDa for α_1 and α_2 chains and β chain respectively, also had lower denaturation temperatures of 36 °C, 36.15 °C, and 32.60 °C, respectively.

5.6 **Amino Acid Composition**

The amino acid composition of fish waste skin, scale, bone and fin are similar. RP-HPLC results revealed the highest amount was glycine, followed by proline, glutamic acid, hydroxyproline, alanine, arginine, methionine, aspartic acid, serine, lysine, leucine, threonine, phenylalanine, valine,

isoleucine, tyrosine, histidine and cysteine. Calf skin (Gelse et al., 2003) also had a slightly similar amino acid composition. Very little amount of methionine and almost none or slightly detectable amount of cysteine was present in fish waste. This implies that these collagens are of type I collagen. Many studies such as for silver carp collagen (Rodziewicz-motowidlo et al., 2008) and *Pagrus major* and *Oreochromis niloticus* fish scales (Ikoma et al., 2003) also had similar findings. Besides, Owusu-Apenten (2002) stated that there are few or no disulfide bonds in type I collagen, explaining this scarce presence of methionine and cysteine in collagen. This further explains calf skin and fish waste collagen's similar characteristics in structure and thermal stability.

RP-HPLC analysis was carried out to study the amino acid composition of the fish waste collagen. In collagen, the amino acid glycine is the most abundant amino acid, accounting for 30 % of total amino acids. As shown in the Table 4.21, fish skin, scale, bone and fin collagen had very high glycine contents of 249.41 residues/1000 residues, 219.83 residues/1000 residues, 230.61 residues/1000 residues, and 260.33 residues/1000 residues, respectively. In another study, calf skin collagen had higher glycine contents than all these fish collagen (330 residues/1000 residues) (Giraud-Guille et al., 2000). Glycine is the major amino acid in collagen, as the triple helical collagenous domain is characterized by the glycine amino acid in every third position of the polypeptide chains creating a $(Gly\text{-}X\text{-}Y)_n$ repeat structure (Gelse et al., 2003). Tryptophan was not detected in all fish waste collagen, these results were in agreement with collagen from carp skin, scale or bone,

and the contents of tyrosine and phenylalanine were very low (Duan et al., 2009). Referring to the glycine content of calf and fish waste collagen, calf collagen has higher glycine content. The glycine content might be one of the reasons that contribute to the high thermal stability of calf collagen (40.80 °C) (Komsa-Penkova et al., 1999).

Besides glycine, imino acid content played an important role in stabilizing the helical structure of collagen (Ikoma et al., 2003). Fish skin, scale, bone and fin collagen showed to have imino acid contents with approximately of 205 to 250 residue/1000 residues. As compared to calf skin collagen, it was reported to has 215 residues/1000 residues of imino acids (Giraud-Guille et al., 2000) which is lower than fish scale and bone collagen. The fish collagen in this study also showed to have higher imino acid contents than most fish species such as grass carp skin collagen (186 residues/1000 residues) (24.60 °C), brownstripe red snapper skin (212 residues/1000 residues) (31.50 °C) (Jongjareonrak et al., 2005), bigeye snapper skin (193 residues/ 1000 residues) (28.68 °C) (Kittiphattanabawon et al., 2005). Normally, mammalian collagen would have higher quantities of imino acids compared to fish collagen (Foegeding et al., 1996; Jongjareonrak et al., 2005; Duan et al., 2009). Thus, mammalian collagen demonstrated higher T_d (Duan et al., 2009). Due to higher imino acid content, T_d of fish skin, scale, bone and fin collagen were found to be at higher than some fish species such as the grass carp (24.60 °C), brownstripered snapper skin (31.50 °C) and bigeye snapper skin (28.68 °C) (Kittiphattanabawon et al., 2005). The imino acids shape a tropocollagen triple-helix structure and can structure a robust

hydrogen bond with neighboring molecules which cause higher heat stability and a higher T_d (Zhang et al., 2007; Potaros et al., 2009). This explains the reason of higher T_d in collagen with high imino acids contents. The imino acid content of fish collagens is associated with the fish's habitat (Rigby, 1968; Foegeding et al., 1996; Kittiphattanabawon et al., 2005) and varies with species (Love et al., 1976; Foegeding et al., 1996). A high degree of hydroxylation of imino acids was also suggested as the reason to higher T_d (Nagai et al., 2010).

The degree of hydroxylation of proline in decreasing order is 51.43 %, 48.33 %, 46.03 % and 43.64 % in fish scale, bone, fin and skin collagen, respectively. It was surprising that these collagen had higher degrees of hydroxylation of proline than most fish species. Carp fish collagen had a degree of hydroxylation of proline of 40 % in skin, 40.10 % in scales, and 41.70 % in its bones (Duan et al., 2009). 39.90 % and 41.10 % were found in bigeye snapper skin and bones (Kittiphattanabawon et al., 2005), and 37.20 % in skins of surf smelt (Nagai et al., 2010). When compared to calf skin collagen, calf skin has a higher degree of hydroxylation of proline (56.02 %) (Giraud-Guille et al., 2000). This further explains the high thermal stability of calf skin (40.80 °C) (Komsa-Penkova et al., 1999) when compared to fish collagen. Besides having higher imino acid contents to show that the helix is more stable, the degree of hydroxylation of proline residues would also affect the stability of the collagen's helix structure (Ramachandran, 1988). Hydroxylation plays a vital role in stabilizing the triple helix of collagen by interchain hydrogen bonds (Ramachandran, 1988; Ikoma et al., 2003;

Jongjareonrak et al., 2005; Duan et al., 2009). These results show that fish waste skin, scale, bone and fin have relatively stable triple helix collagen.

CHAPTER 6

CONCLUSION

Collagen was successfully extracted from the market fish waste with promising yields from fish skin, compared to fish scales, bones and fins. The highest yields obtained from fish skin, scales, bones and fins were 24.77 %, 13.82 %, 6.4 % and 5.9 % respectively, on a dry weight basis.

Optimized conditions for collagen fish skin extraction are at a concentration of acetic acid of 0.66 M, at temperatures of 13.26 °C, at a pH of pH 3.19 and for a duration of 74 h. Extraction of collagen from fish scales were optimized at 0.62 M, 19.03 °C, pH 2.96 and 77.51 h. Whereas collagen from fish bones were optimized at 0.67 M, 19.01 °C, pH 3.15 and 73.16 h. For fish fin collagen, the optimized conditions are at 0.56 M, 16.60 °C, pH 3 and 72.36 h. Most studies on the optimized conditions for collagen extraction were only experimented on other fish species besides Malaysian fish waste. Most conditions varied slightly from these results which could be due to the differences between fish species and their habitats that influence a fish's collagen molecular structure. Consequently, the results for Malaysian fish waste collagen optimized condition extraction would be different to other studies.

The collagen extracted from fish skins, scales, bones and fins were of type I collagen, which are composed two α_1 and one α_2 chains, as shown by SDS-PAGE patterns. The fish skins, scales and fin collagen $\alpha_{1,}$ α_2 and β chains would have a molecular size of 132.04 kDa, 120.06 kDa and 220.67 kDa, whereas the molecular weight of fish bones collagen $\alpha_{1,}$ α_2 and β chains were slightly higher which are 139.79 kDa, 124.72 kDa and 229.22 kDa. The fish bone collagen was found to have a similar molecular weight to human collagen but lower to bovine collagen of 300 kDa (Kirubanandan and Sehgal, 2010). This explained a higher denaturation temperature found in fish bone collagen to its other counter parts.

The T_d of fish skins, scales, bones and fins collagen were 36.0 °C, 36.15 °C, 37.80 °C and 32.60 °C, respectively. This is in accordance with fish skin, scale, bone and fin collagen's high amount of imino acid contents (hydroxyproline and proline) which were found to be 205.01 residues/1000 residues, 239.97 residues/1000 residues, 248.68 residues/1000 residues, and 212.03 residues/1000 residues, respectively, with high degrees of hydroxylation of proline of 43.64 %, 51.43 %, 48.33 %, and 46.03 % respectively, which were also very high when compared to other fish species. These collagens showed to have an advantage for biomedical application as it has a fairly close T_d to mammalian collagen.

FTIR analysis run on these fish collagen showed that the extracted collagen were in integrated and were in their native forms. All these fish waste had normal amounts of α-helix structures, β-sheets, and random coils, side chains or aggregated strands when compared to human collagen. But, fish bones showed to have an extraordinarily high amount of β-turns compared to its other fish parts and even to the reported bovine collagen.

In conclusion, these results prove that collagen of fish waste skin, scales and fins, especially fish bones, have the capability to be an alternative source of collagen for many uses in numerous fields.

For further studies, it suggests that incorporation of aldehydes or glutaraldehydes may be possible in fish waste collagen to further improve fish collagen's thermal stability, decrease collagen's solubility in water and improve their mechanical properties, for extended uses in the photographic, food, pharmaceutical industry and many more. Due to current technologies and biochemical advancements, cross-linking of collagen with aldehydes is being used to diversify the usage of collagen. In Japan and Brazil, the cross-linking collagen with other proteins by using trans-glutaminase is approved for food use (Heck et al., 2013).

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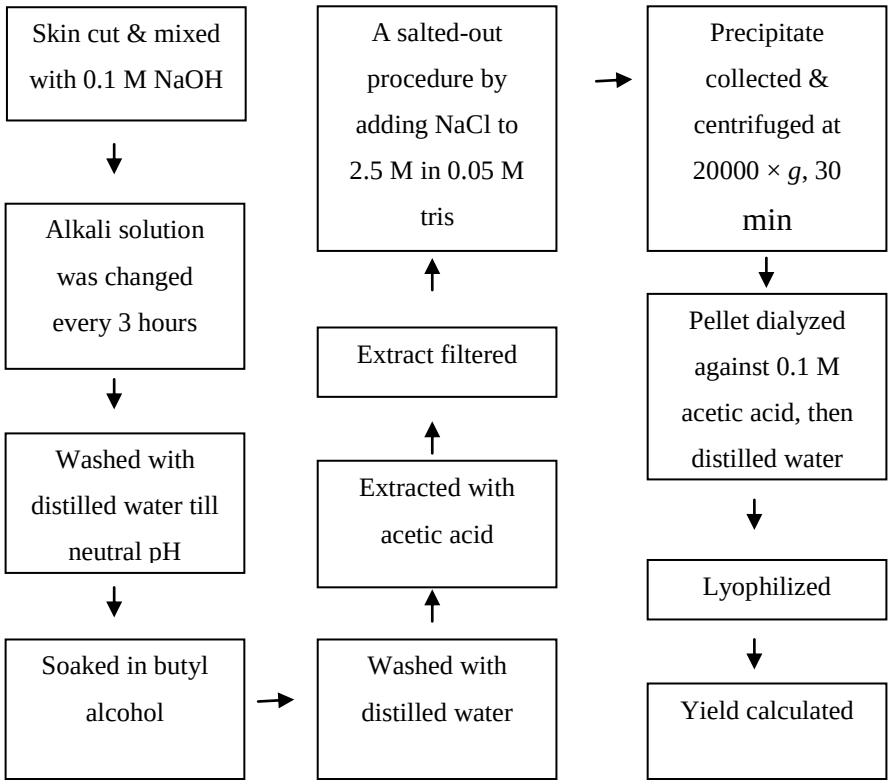
APPENDICES

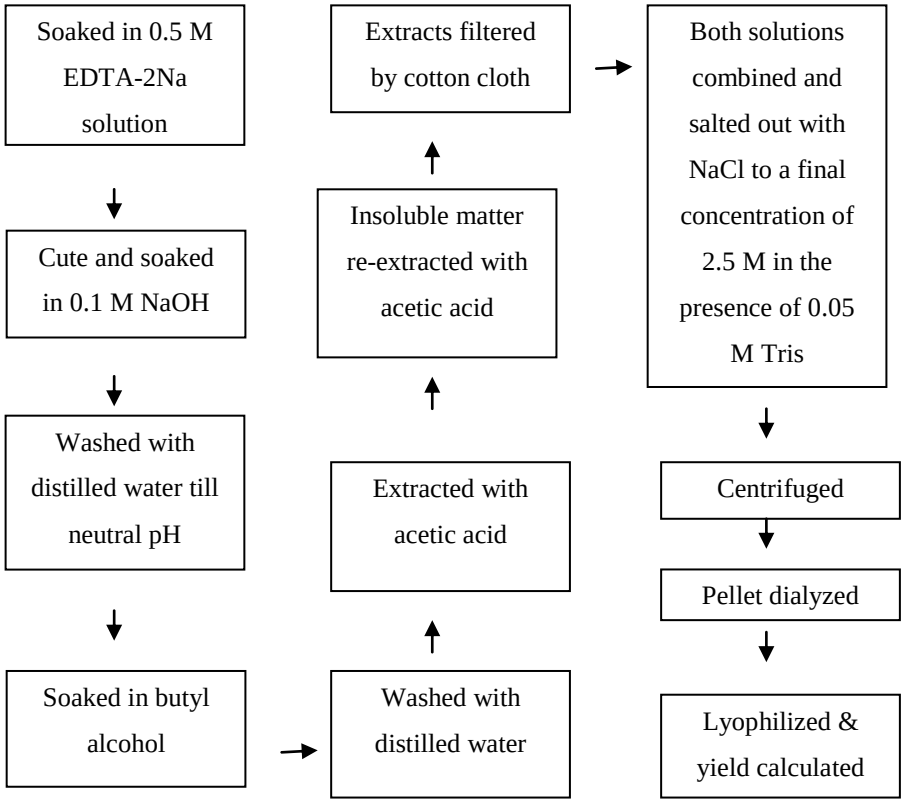
Appendix A

Extraction method of skin collagen's flow chart (Nagai et al., 2000; Kittiphattanabawon et al., 2010)



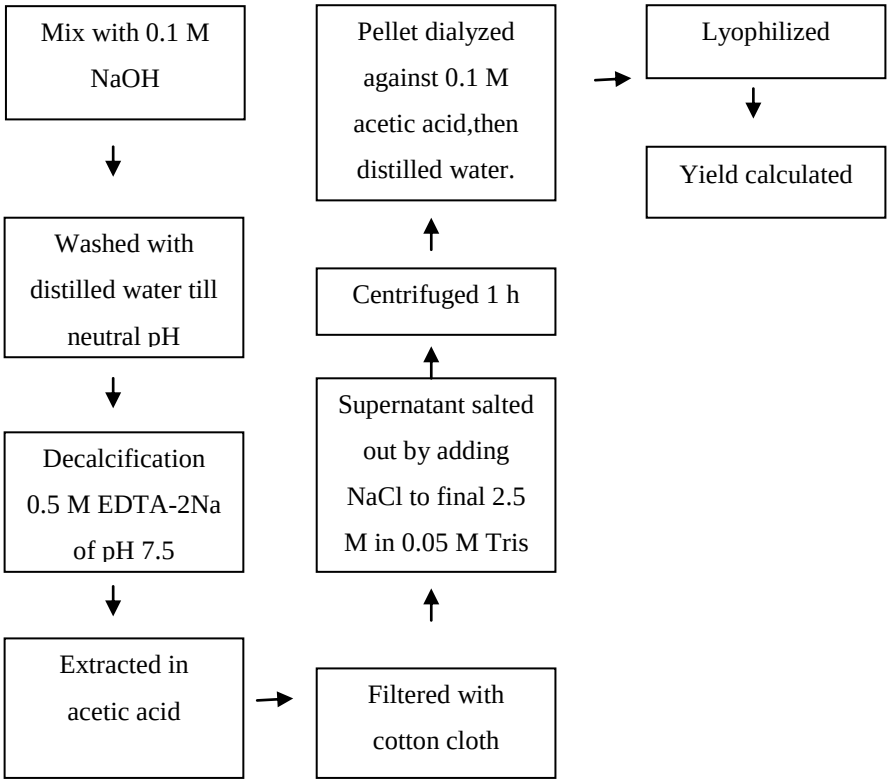
Appendix B

Extraction method of bone collagen's flow chart (Nagai and Suzuki, 2000a; Wang, An, et al., 2008; Duan et al., 2009).



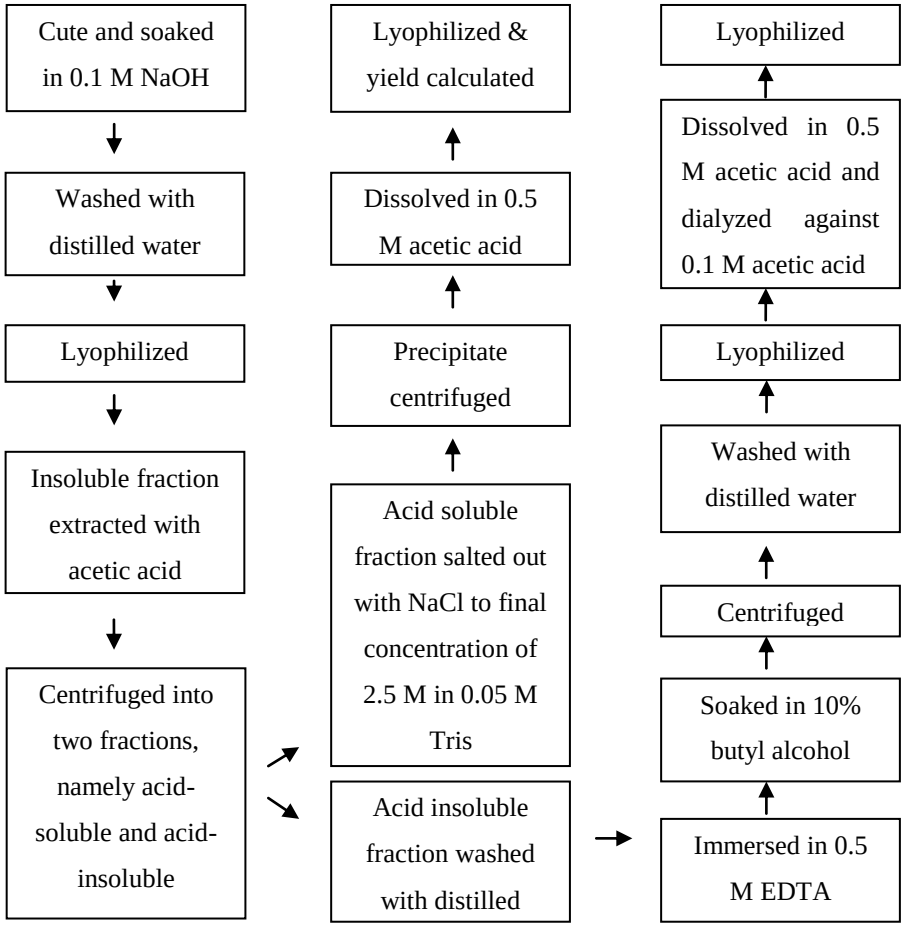
Appendix C

Extraction method of scale collagen's flow chart (Duan et al., 2009)



Appendix D

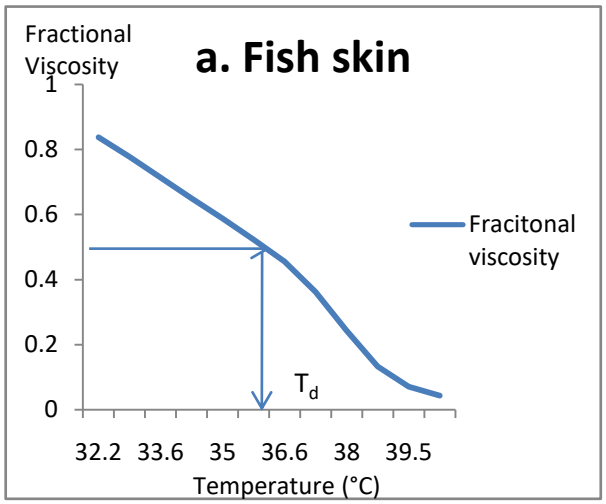
Extraction method of fin collagen's flow chart (Nagai et al., 2000)



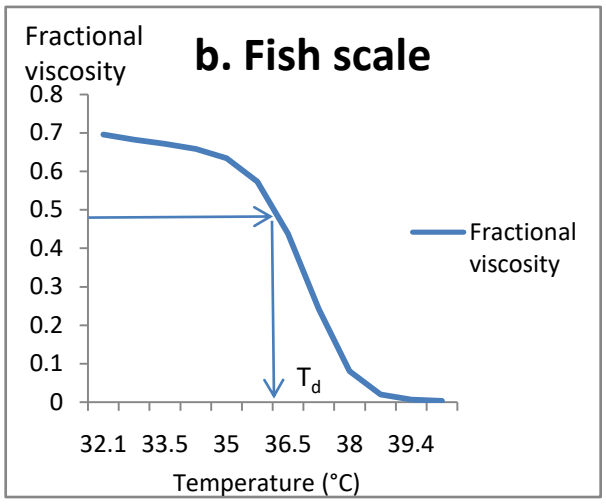
APPENDIX E

Denaturation temperature of fish waste collagen.
(a) Fish skin, (b) Fish scale, (c) Fish bone, (d) Fish Fin.

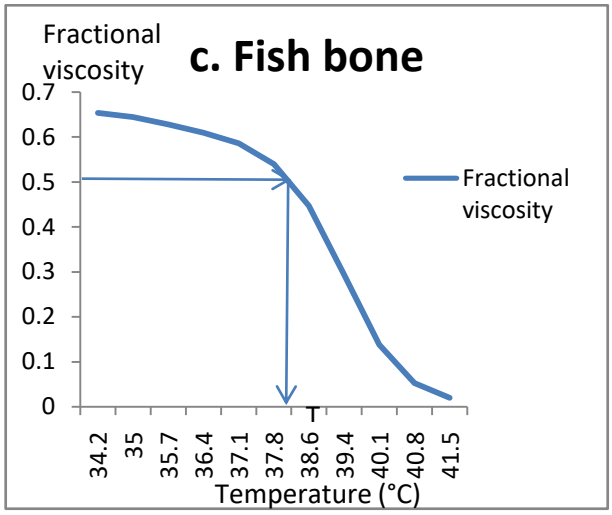
a



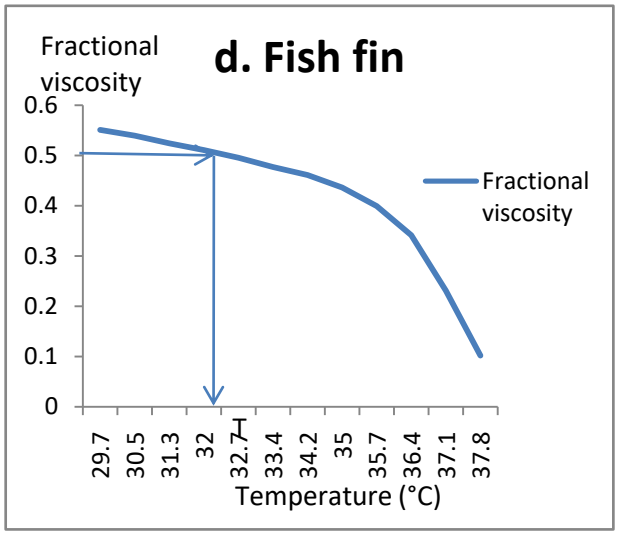
b



c


Fractional viscosity
c. Fish bone
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
T
34.2
35
35.7
36.4
37.1
37.8
38.6
39.4
40.1
40.8
41.5
Temperature (°C)
Fractional viscosity

d


Fractional viscosity
d. Fish fin
0.6
0.5
0.4
0.3
0.2
0.1
0
29.7
30.5
31.3
32
32.7
T
33.4
34.2
35
35.7
36.4
37.1
37.8
Temperature (°C)
Fractional viscosity